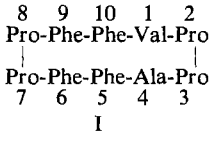
Experimental and Calculated Conformational Characteristics of the Cyclic Decapeptide Antamanide*

Alan E. Tonelli, Dinshaw J. Patel,† Murray Goodman, Fred Naider, H. Faulstich, and Th. Wieland

ABSTRACT: Nuclear magnetic resonance and circular dichroism measurements are coupled with an approximate theoretical treatment to determine the conformational characteristics of the cyclic all L-decapeptide antamanide. Proton nuclear magnetic resonance decoupling studies permitted assignments to the five observable peptide NH doublets with J_{NC^α} = 6.0–8.5 Hz.

Exchange studies with deuterated alcohols for antamanide in $CDCl_3$ solution suggested that the observable peptide NH resonances were neither hydrogen bonded nor in solvent-shielded environments. The circular dichroism spectrum of antamanide exhibits $[\theta]_M > -100{,}000$ in the 190- to 205-nm region of the spectrum suggesting that all peptide residues are in a trans configuration. Solvent effects rather than conformational changes account for the variation in cotton effects in the solvent-dependent circular dichroism spectra of antamanide. The lowest energy cyclic conformation generated in the theoretical portion of the study is consistent with all of the nuclear magnetic resonance and circular dichroism data obtained. This conformation is not stabilized by intramolecular hydrogen bonding and possesses considerable symmetry, *i.e.*, those residues separated by four intervening residues have identical or nearly identical backbone conformations.

Methods are currently available which permit approaches to the elucidation of the solution structures of cyclic biological polypeptides, many of which bind metal ions (see Bovey, 1971, for a recent review of the literature). The amino acid composition and sequence of the cyclic all L-decapeptide antamanide has been deduced by Wieland and his collaborators (1968) and is displayed as I.



I

The peptide grouping is attractive to study by proton nmr spectroscopy in solution. The structures of synthetic cyclic polypeptides have been deduced by Kopple *et al.* (1969a,b), Schwyzer and Ludescher (1969), Torchia *et al.* (1971), and Schwyzer *et al.* (1970). Proton nmr investigations have also deduced the solution structures of gramacidin S (Stern *et al.*, 1968; Conti, 1969; Ovchinnikov *et al.*, 1970), valinomycin (Haynes *et al.*, 1969; Ivanov *et al.*, 1969; Ohnishi and Urry, 1969), nonactin (Prestegard and Chan, 1969, 1970), actinomycin D (Victor *et al.*, 1969), neurohypophyseal peptides (Johnson *et al.*, 1969; Urry *et al.*, 1970), and ferrichromes (Llinás *et al.*, 1970). The above investigations assigned the peptide N and C^α protons and side-chain protons to individual amino acid residues by comparisons to model systems and spin-decoupling experiments. The peptide proton–proton coupling J_{NC^α} was used to obtain the dihedral angle φ' according to the relationship of Barfield and Karplus (1969) and Bystrov *et al.* (1969a,b). Exchange studies with deuterated solvents and temperature coefficients of peptide NH resonances in Me_2SO were analyzed to indicate hydrogen bonding and/or shielding from solvent of NH protons.

CD (Goodman *et al.*, 1971) has also been utilized to determine the conformation of proteins, polypeptides, and oligopeptides. This technique is well suited for the study of peptides containing prolyl residues because of the marked spectral differences between the cis and trans isomers. The presence of both amino and imino residues contained in the cyclic peptide antamanide prevents a definite assessment of its overall conformation. It is possible, however, to draw some conclusions related to the configuration of the prolyl residues in the molecule.

The conformational characteristics of polypeptides in solution have been successfully described by approximate intramolecular potential energy calculations (Brant and Flory, 1965; DeSantis *et al.*, 1965; Ramakrishnan and Ramachandran, 1965; Leach *et al.*, 1966; Scott and Scheraga, 1966; Brant *et al.*, 1967; Schimmel and Flory, 1967, 1968). Residues separated by rigid and planar trans amide or imide bonds (Brant and Flory, 1965; Brant *et al.*, 1967) render the potential energy of rotations φ and ψ, $V(\varphi,\psi)$, about the N–C^α and C^α–C bonds (see Figure 1) in a given residue independent of the corresponding rotations in neighboring residues. Consequently, the total conformational energy of a polypeptide may be estimated by summing the intrinsic threefold torsional potentials about the N–C^α and C^α–C bonds, the nonbonded steric repulsions and London dispersion energies (6–12 potential) and the nonbonded monopole–monopole electrostatic interactions.

Recently it has been shown (Tonelli and Bovey, 1970; Tonelli *et al.*, 1970) that a "Karplus-like relation" connecting the vicinal nmr coupling (Karplus, 1959; Barfield and Karplus, 1969; Bystrov *et al.*, 1969a,b) J_{NC^α} and the dihedral angle φ' between N–H and C^α–H^α in a peptide residue holds for polypeptides in solution. This calculation can be applied to all conformations found to be energetically favorable by the above-mentioned approximate energy calculations. Correct vicinal coupling constants are observed for random coil polypeptides (Tonelli and Bovey, 1970) and dipeptides (Tonelli

* From the Bell Telephone Laboratories, Incorporated, Murray Hill, New Jersey 07974 (A. E. T. and D. J. P.), from the Polytechnic Institute of Brooklyn, Brooklyn, New York 11201 (M. G. and F. N.), and from the Max-Planck-Institut fur Medizinische Forschung, Heidelberg, West Germany (H. F. and Th. W.). *Received March 15, 1971.*

† To whom correspondence should be addressed.

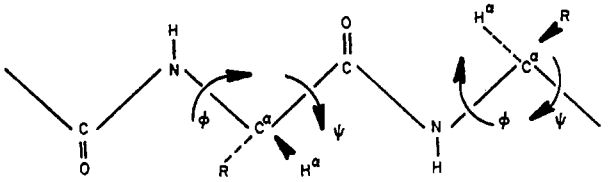


FIGURE 1: A schematic representation of a portion of an L-polypeptide in the all-trans or planar zigzag conformation.

et al., 1970) in solution. This agreement lends further support to the approximate energy calculations and indicates as valid the extension of a Karplus-like relation to the vicinal coupling between amide and α-protons in peptides. A combination of these two approximate theoretical tools may be useful in the conformational analysis of polypeptides or small proteins.

Briefly, the conformations of each residue are restricted by allowing only those values of the rotation angle[1] φ (rotation about the N-C$^\alpha$ bond) which reproduce the measured vicinal coupling according to a Karplus-like relation (Karplus, 1959; Barfield and Karplus, 1969; Bystrov *et al.*, 1969a,b):

$$J_{NC^\alpha} = \begin{bmatrix} 8.5\cos^2\varphi' \ (0° \leq \varphi' \leq 90°) \\ 9.5\cos^2\varphi' \ (90° \leq \varphi' \leq 180°) \end{bmatrix} \quad (1)$$

$$J_{NC^\alpha} = 8.9\cos^2\varphi' - 0.9\cos\varphi' + 0.9\sin^2\varphi' \quad (2)$$

Experimental Section

Nmr spectra were run on a 220-MHz Varian NMR spectrometer equipped with a Varian variable-temperature unit. Ethylene glycol was used to monitor temperatures (±1°). A Fabri-Tek computer of average transients was used to improve the signal-to-noise ratio. A Hewlett-Packard 651B test oscillator was used to carry out spin decoupling.

CD studies were carried out using a Cary 60 spectropolarimeter modified with a Model 6001 CD attachment. The experimental solutions were prepared by weighing antamanide into volumetric flasks and adding the desired solvent. The spectra were obtained using a 0.1-mm path-length cell. Dry prepurified nitrogen was employed to purge the instrument before and during the experiments. Three complete spectral scans were averaged to determine each spectrum.

Results and Discussion

Nmr Studies. The nmr spectrum of antamanide in $CDCl_3$ at 53° is outlined in Figure 2 and the spectral parameters are given in Table I. Correlation of N, C$^\alpha$, and C$^\beta$[2] for each amino acid was carried out from spin-decoupling experiments. Decoupling was undertaken in both directions, *i.e.*, upfield resonances were observed on irradiating the coupled downfield resonances and *vice versa*. The Ala and Val residues were identified readily since Ala has a CH_3 doublet and Val two sets of methyl doublets. Three Phe peptide NH doublets were related by spin decoupling to their C$^\alpha$ and C$^\beta$ proton resonances. Similarly, spin decoupling identified the C$^\alpha$ and C$^\beta$ proton chemical shifts of three proline residues in antamanide. Currently, we are unable to locate the remaining Phe and Pro residues.

TABLE I: Summary of Chemical Shifts and Coupling Constants Obtained from an Analysis of Decoupled Nmr Spectra of Antamanide in $CDCl_3$ at 53°.

	Proton Chemical Shifts (ppm)			Proton–Proton Coupling Constant	Dihedral Angle (Bystrov *et al.*, 1969a,b)
	δ_N	δ_{C^α}	δ_{C^β}	J_{N-C^α}	φ'
Ala	8.01	4.35	1.16	8.5	0,157
Val	7.66	4.49	2.05	6.5	22,143
Phe	7.88	4.67	2.99[a]	6.0	32,138
Phe	7.45	4.64	3.06[a]	8.0	0,153
Phe	7.05	4.11		7.0	18,146
Pro		4.09	2.12[a]		
Pro		3.95	1.83[a]		
Pro		3.85	1.78[a]		

[a] The fragment C$^\alpha$–C$^\beta$ gives an ABX pattern in the nmr spectrum. For Phe as well as Pro, it was possible to decouple only one of the two β-protons on irradiation of α region. The C$^\beta$ chemical shifts reported are for one of two β-protons.

The proton–proton couplings for J_{NC^α} for Val, Ala, and three Phe residues are presented in Table I. From these couplings one determines the dihedral angle φ' (the dihedral angle φ' in a three-bond fragment is defined as zero when protons are eclipsed) of the peptide grouping H–N–C$^\alpha$–H using the correlation of Bystrov *et al.* (1969a,b).

The analysis of the antamanide spectra in Figure 2 and Table I indicate that two Phe residues have similar C$^\alpha$ and C$^\beta$ chemical shifts at 4.65 and 3.00 ppm, respectively, indicating similar magnetic environments. Figure 2 also indicates that the C$^\alpha$ resonances of the two prolines at 4.09 and 3.95 ppm are doublets with J = 8 Hz and with the second vicinal C$^\alpha$–C$^\beta$ coupling too small to be resolved. These couplings correspond to dihedral angles φ' of ~30 and ~90° implying that the puckering of the pyrollidine ring is similar for two of the four prolines.

Exchange rates of peptide NH protons with aqueous–alcoholic solvents have been utilized to indicate the hydrogen-bonded character and/or solvent-shielded environment of these protons (Bovey, 1971). Three (resonances A, B, and C in Figure 3) and five peptide NH doublets are observed at 35 and 55°, respectively, for antamanide in $CDCl_3$. The remaining peptide NH proton(s) are under aromatic phenylalanine protons. Addition of protonated alcohols to antamanide in $CDCl_3$ was followed in peptide NH and C$^\alpha$H regions. Figure 3 outlines chemical shift changes for peptide NH peaks with increasing $(CH_3)_2CHOH$ concentration. On the other hand the C$^\alpha$H region remained unaffected by addition of alcohols.

[1] The angles of rotation φ and ψ (see Figure 1) are taken as zero in the trans or planar zigzag conformation and are measured in a right-handed sense (Edsall *et al.*, 1966a–c). The dihedral angle φ' between N–H and C$^\alpha$–H$^\alpha$ is directly related to the rotation angle φ. Recently a new convention defining the backbone rotation angles in polypeptides has been proposed (Kendrew *et al.*, 1970), where $\varphi = \psi = 180°$ in the planar zigzag conformation. However, we fail to see any improvement in this latest convention, and therefore adopt the older convention discussed above.

[2] The fragment C$^\alpha$–C$^\beta$ gives an ABX pattern in the nmr spectrum for Phe; for Pro, the α-protons give the appearance of the X part of an ABX spectrum, but the β-proton spectrum is more complex than AB. It was possible to decouple only one of the two β-protons on irradiation of the α region. The C$^\beta$ chemical shifts reported are for one of two β-protons.

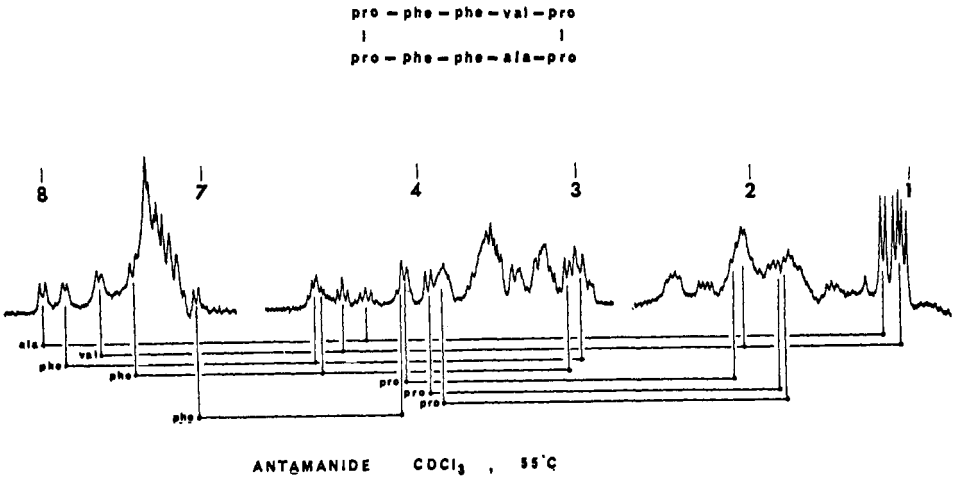


FIGURE 2: 220-MHz porton nmr spectrum of antamanide in $CDCl_3$ at 55°. The lines below spectrum indicate results of decoupling experiments.

Different chemical shift changes were observed on addition of CH_3OH as compared to $(CH_3)_2CHOH$ with better resolution of peptide NH protons in the case of the latter. No differences were observed in the CD spectrum of antamanide in $CHCl_3$ and 15% isopropyl alcohol containing chloroform. The chemical shifts on addition of alcohols are assigned to solvent effects.

Exchange studies were undertaken by addition of 2–10% methanol-d_4, isopropyl alcohol-d_1, and *tert*-butyl alcohol-d_1 to antamanide in $CDCl_3$ from room temperature to 55°. The peptide NH protons exchanged rapidly (<10 min) at 55° and 5–10% deuterated alcohol concentration. The rapid exchange and disappearance of the five observable peptide NH resonances for antamanide in $CDCl_3$ indicates the absence of hydrogen bonds and/or solvent-shielded peptide NH protons. No exchange information is available about the remaining Phe peptide NH proton.

CD Studies. By studying model compounds of proline, knowledge can be gained concerning the contributions that such residues make in the far-ultraviolet region. Examination of the CD of *N*-acetyl-L-proline methyl ester in various polar solvents reveals a molar ellipticity, $[\theta]_M$, for the $\pi \rightarrow \pi$ transition of the amide chromophore of approximately $-15{,}000$. Madison and Schellman (1970a,b) in a comprehensive study of the optical activity of proline derivatives found a similar $[\theta]_M$ in water. In addition using nmr they showed that *N*-acetyl-L-proline methyl ester exists as approximately 80% trans isomer in a variety of organic solvents. The results of these studies suggest that the $[\theta]_M$ for a prolyl residue in the trans configuration should be on the order of $-20{,}000$ to $-30{,}000$ for the $\pi \rightarrow \pi^*$ transition. Since antamanide exhibits a $[\theta]_M$ in the 190- to 205-nm region of well over $-100{,}000$ (Figure 4), it is suggested that all of the residues are in the trans configuration. Further comparison of our spectra to those found for polyproline I and II as well as for other model compounds of proline and (*R*)-thiazolidine-4-carboxylic acid lends strength to our conclusion. The conformational calculations (next section) were carried out assuming the proline residues to be trans.

Madison and Schellman (1970a,b) and Nielsen and Schellman (1967) have also studied the effect of different solvents on the position and intensity of $\pi \rightarrow \pi^*$ and $n_l \rightarrow \pi^*$ Cotton effects of several proline model compounds. In particular they showed that the $n_l \rightarrow \pi^*$ transition for *N*-acetyl-L-prolinamide is especially solvent dependent. In a series of twelve solvents of varying dielectric constant, this cotton effect exhibited a continuous increase in magnitude and a red shift as the polarity of the medium decreased. Similar results were observed for several other proline derivatives.

It is possible that some of the effects reported by Madison and Schellman were due to conformational variations in the different solvent media. Goodman *et al.* (1969), attempted to eliminate such variations by examining the far-ultraviolet CD of two bicyclic camphor lactams. Since the amide chromophore was incorporated in these rigid molecules they were able to discern directly the effect of solvents on the circular dichroism patterns. They found that the high-wavelength Cotton effects ($n_l \rightarrow \pi^*$) for 1,7,7-trimethyl-3-azabicyclo[2.2.1]-heptan-2-one exhibit significant red shifts (10 nm) as the sol-

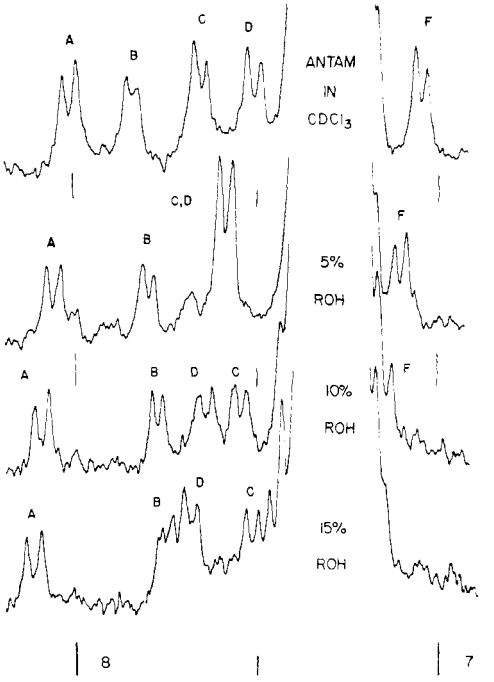


FIGURE 3: The effect of addition of $(CH_3)_2$ CHOH to the 220-MHz proton nmr spectrum of antamanide in $CDCl_3$ at 55°. The spectrum shows the peptide NH region.

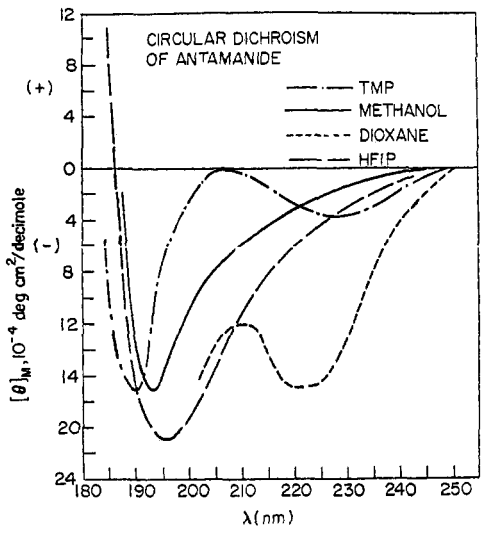


FIGURE 4: The circular dichroism spectra of antamanide in several organic solvents.

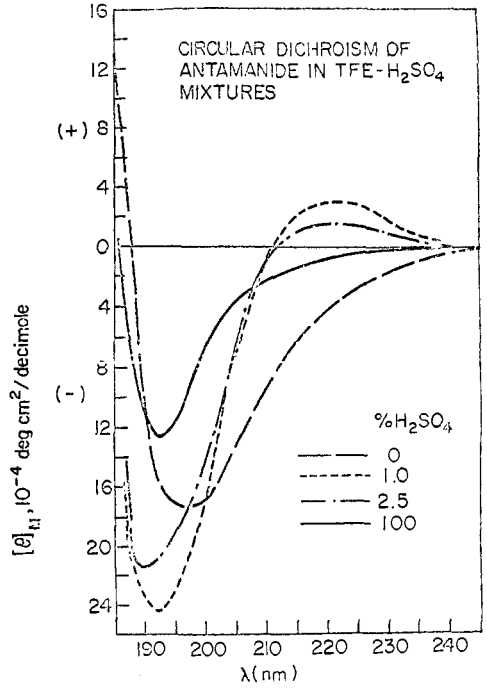


FIGURE 5: The circular dichroism spectra of antamanide in TFE–sulfuric acid mixtures.

vent is changed from water to hexane. The intensity of these extrema changed by a factor of 2. Although the low-wavelength Cotton effect ($\pi \rightarrow \pi^*$) was not as solvent sensitive, both a change in ellipticity and a blue shift were noted as the solvent was changed from water to hexane.

We feel that in light of such findings solvent effects rather than conformational changes explain the variations in the Cotton effects depicted in Figure 4. We believe that the troughs appearing in the 190- to 205-nm region have their origins in $\pi \rightarrow \pi^*$ transitions of both amide and imide residues. The shoulder appearing at approximately 215 nm is strongly solvent dependent, exhibiting a red shift (7 nm) and an increase in ellipticity as one proceeds from hexafluoroisopropyl alcohol to the less polar dioxane. These results suggest that this Cotton effect may have its origin in an $n_1 \rightarrow \pi^*$ transition of the peptide residues.

We also examined the CD of antamanide in several TFE[3]–sulfuric acid mixtures (Figure 5). Our results show that the addition of even small quantities of sulfuric acid has a large effect on the spectral patterns. Addition of 1% sulfuric acid causes a change in sign of the spectral bands in the 210- to 220-nm region and a blue shift (~5 nm) of the Cotton effect associated with the $\pi \rightarrow \pi^*$ transition of the amide chromophore. Higher concentrations of acid lead to nearly complete elimination of the optical activity in the $n \rightarrow \pi^*$ region and a loss of intensity in the $\pi \rightarrow \pi^*$ bands. The molar ellipticity of the $\pi \rightarrow \pi^*$ spectral band in 100% sulfuric acid is $-120{,}000$ or approximately $-12{,}000$ per residue.

Naider *et al.* (1971) conducted a similar investigation on a conformationally restricted camphorolactam. They observed a similar loss of intensity of the $n \rightarrow \pi^*$ Cotton effect accompanied by a blue shift in the $\pi \rightarrow \pi^*$ spectral bands. The molar ellipticity of the resulting $\pi \rightarrow \pi^*$ band in 100% sulfuric acid is about $-13{,}000$/amide residue. Since the camphorolactam could not undergo any major conformational alterations, they attributed the observed spectral changes solely to a solvent effect. They concluded that the sulfuric acid protonates the amide chromophore, resulting in a loss of the $n \rightarrow \pi^*$ transition. We believe that the spectral changes observed for antamanide are mainly due to protonation of the peptide residues. At this time it is not possible to rule out structural variations resulting from such protonation. A closer inspection of Figure 4 shows that addition of 1–2.5% sulfuric acid to TFE substantially alters the $n \rightarrow \pi^*$ spectral region to exhibit small positive ellipticity bands. At the same time the intensity of the $\pi \rightarrow \pi^*$ transitions are enhanced. On the other hand the spectra of antamanide in 10–100% sulfuric acid concentrations do not show $n \rightarrow \pi^*$ transitions, whereas the $\pi \rightarrow \pi^*$ Cotton effects are reduced in intensity. These spectral dependencies imply that antamanide can be altered in conformation as well as protonated when sulfuric acid is present in TFE.

Recently, Goodman and coworkers (1971) found that intramolecular hydrogen bonds in L-alanine oligopeptides are not disrupted by addition of 1% sulfuric acid to TFE. Specifically, the intensity of the $n \rightarrow \pi^*$ Cotton effect is maintained for those oligomers capable of forming helical structures. In light of these findings we believe our acid studies give evidence that strong intramolecular hydrogen bonds are not present in antamanide.

The CD studies in organic and mixed organic acid media suggest that antamanide exists primarily in one conformation in solution. At present we find no evidence for the existence of intramolecular hydrogen bonds. Both of these conclusions support the results of the nmr studies and conformational energy calculations.

Theoretical Studies

Details of Calculation. All amide and imide bonds are assumed to be trans on the basis of the CD measurements previously discussed, and the residue bond lengths and valence angles used in the conformational energy calculations (Brant and Flory, 1965) are adopted. The φ angles in Pro_7 and Pro_2

[3] Abbreviation used is: TFE, trifluoroethanol.

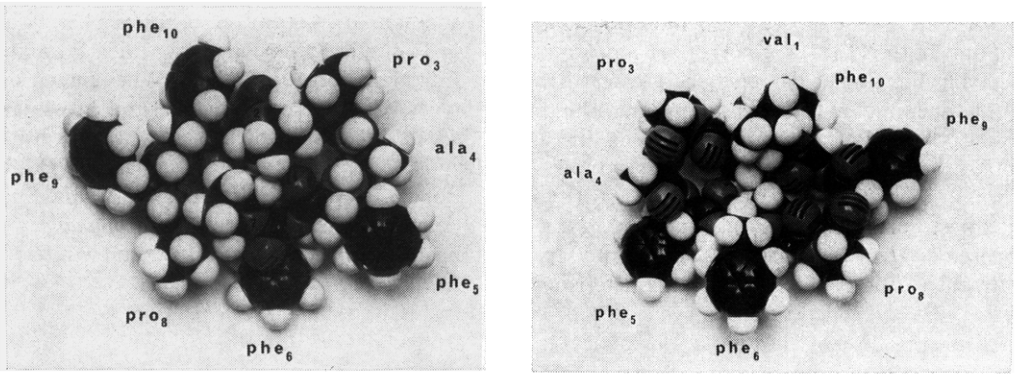


FIGURE 6: Two photographic views of a space-filling molecular model of antamanide in the lowest energy conformation generated: (φ, ψ)Pro_2, Pro_7 = 102°, 310°; (φ, ψ)Pro_3, Pro_8 = 122°, 125°; (φ, ψ)Ala, Phe_9 = 90°, 120°; (φ, ψ)Phe_5, Phe_{10} = 90°, 330°; (φ, ψ)Phe_6 = 90°, 300°; and (φ, ψ)Val = 90°, 270°

are fixed[4] by the pyrrolidine ring at 102°, and φ = 122° in Pro_8 and Pro_3 according to the crystallographic analysis of poly-L-proline (II) (Cowan and McGavin, 1955; Sasisekharan, 1959) and L-leucyl-L-prolylglycine (Leung and Marsh, 1958), respectively. $\psi_{Pro_7} = \psi_{Pro_2} = 310°$ and $\psi_{Pro_8} = \psi_{Pro_3} =$ 125 or 325° are chosen for the prolyl residues. These values of ψ correspond to the calculated energy minima (Schimmel and Flory, 1967, 1968) for an L-prolyl residue succeeded by another L-prolyl residue and for an L-prolyl residue succeeded by any residue other than prolyl, respectively.

Five of the six residues with amide protons have observable vicinal coupling constants: J_{NC^α} = 8.5 Hz (Ala), 6.5 Hz (Val), and 6.0, 7.0, and 8.0 Hz (for three of the four Phe residues). $\varphi_{Val} = \varphi_{Ala} = \varphi_{Phe_9} = \varphi_{Phe_{10}} = \varphi_{Phe_5} = \varphi_{Phe_6} =$ 30, 60, and 90° (φ' = 150, 180, and 150°) are chosen to correspond to the observed couplings of 6.0–8.5 Hz (see eq 1 and 2). φ = 120, 240, and 270° (φ' = 30, 0, and 30°) lead to nearly the same couplings, but they do not correspond to conformations as energetically favorable as those with φ = 30–90° (see the conformational energy maps in Brant and Flory, 1965, Schimmel and Flory, 1958, and Miller and Goebel, 1968).[5] The following sets of ψ rotation angles, which correspond to the lowest energy conformations with φ = 30, 60, or 90° are adopted: $\psi_{Ala} = \psi_{Phe_9} = \psi_{Phe_{10}} = \psi_{Phe_5} =$ 120 and 330° and $\psi_{Val} = \psi_{Phe_6} =$ 270, 300, or 330°.

For each conformation, *i.e.*, for each of the sets of ten pairs of rotation angles φ and ψ, the distance between the α-carbon atoms in Pro_7 and Pro_8 in the corresponding linear decapeptide terminated by these L-prolyl residues is calculated. The transformation of virtual bond vectors method (Brant and Flory, 1965) is employed in the distance calculations. If the calculated distance between C^α Pro_7 and C^α Pro_8 falls in the range 3.7–3.9 Å, then the conformation under consideration is assumed to be cyclic.[6]

The intramolecular energy of a given cyclic conformation is evaluated by summing the individual residue energies obtained from their random coil energy maps (Brant *et al.*, 1967; Schimmel and Flory, 1967, 1968; Miller and Goebel, 1968) which do not include energetic contributions made by intramolecular hydrogen bonds.

Calculated Results. Of the 137 cyclic conformations generated, 24 are found to have intramolecular conformational energies less than 10 kcal/mole of decapeptide relative to the acylcic conformation of minimum energy and exclusive of intramolecular hydrogen bonding. A space-filling model was constructed for each of the 24 lowest energy cyclic conformations in a search for possible long-range steric overlaps and intramolecular hydrogen bonds. No steric overlaps nor any intramolecular hydrogen bonds were observed, and the overall shapes of each conformation were very similar.

The cyclic conformation depicted in the photographs in Figure 6 has an intramolecular energy (5.0 kcal/mole of decapeptide)[7] which is at least 2.0 kcal lower than all of the other cyclic conformations generated. The environments of the α- and β-protons in the Phe_{10} and Phe_5 residues appear to be nearly identical in this lowest energy conformation generated. In addition, the environment of the α-protons in residues Pro_7 and Pro_2 and in residues Pro_8 and Pro_3, respectively, also appear to be very similar. Each of these observations is consistent with the nmr evidence presented earlier.

On the matter of whether the unresolved phenylalanine amide proton is intramolecularly hydrogen bonded or not, it should be noted that none of the cyclic conformations having energies within 5.0 kcal/mole of decapeptide of the lowest

[4] Pro_7 and Pro_2 are succeeded by prolyl residues, and their pyrrolidine rings should, therefore, have the poly-L-proline (II) geometry (Cowan and McGavin, 1955; Sasisekharan, 1959; see Schimmel and Flory, 1967). Pro_8 and Pro_3 are not succeeded by prolyl residues, so their pyrrolidine rings should have the geometry of an isolated prolyl residue (see Schimmel and Flory, 1968).

[5] The energies for the Ala, Phe_9, Phe_{10}, and Phe_5 residues are taken from the conformational energy map appropriate to a residue with a side chain of the type R = CH_2R' succeeded by a residue other than prolyl (see Figure 4 in Brant *et al.*, 1967). The Phe_6 residue energies are taken from the map appropriate to a residue with a side chain of the type R = CH_2R' succeeded by a prolyl residue (see Figure 3 in Schimmel and Flory, 1968). Because the Val residue, which is also succeeded by a prolyl residue, has a side chain of the type R = CHR'R'', only that portion of the map appropriate for a Val residue succeeded by a residue other than prolyl between ψ = 280° and 340° is used for this residue. A similar reduction occurs in the energetically allowed portion of the maps for residues with R = CH_2R' when they are followed by a prolyl residue (compare Figure 3 in Schimmel and Flory, 1968, to Figure 5 in Brant *et al.*, 1967).

[6] In polypeptides with the usual bond lengths and valence angles, the distance between adjacent α-carbon atoms is invariant to conformation (Brant and Flory, 1965; Schimmel and Flory, 1967) and equals 3.8 Å when the amide or imide bonds are trans.

[7] In each of the 24 lowest energy cyclic conformations $\psi_{Pro_8} = \psi_{Pro_3}$ = 125°. Schimmel and Flory (1968) indicate that the energy minimum at ψ = 125° is approximately 1.0 kcal/mole higher than the minimum at ψ = 325° if the electrostatic interactions are considered. Hence, if the electrostatic interactions of the imide groups in Pro_8 and Pro_3 are ignored the lowest energy cyclic conformation generated would have an energy of 3.0 kcal/mole of decapeptide rather than the value of 5.0 kcal mentioned above.

energy cyclic conformation generated possess intramolecular hydrogen bonds. Some of the 113 (137 − 24) cyclic conformations generated having intramolecular energies greater than 10 kcal/mole of decapeptide may have an intramolecular hydrogen bond, but even after inclusion of the favorable energetic contribution (Brant, 1968) made by an intramolecular hydrogen bond, their total energies would still be greater than the lowest energy, nonhydrogen-bonded, cyclic conformation generated. Hence, on the basis of the present approximate treatment of the conformational characteristics of antamanide it may be concluded that this cyclic decapeptide probably does not possess any intramolecular hydrogen bonds in solution.

The lowest energy cyclic conformations generated in this study have a polar face (carbonyl groups) on one side of the plane of the ring and a nonpolar face (side chains) on the opposite side (see Figure 6). The polar face of the lowest energy conformation generated appears capable of complexing metal cations. This conformation also possesses considerable symmetry. The backbone conformations (φ, ψ) of residues Pro_2 and Pro_7, Pro_3 and Pro_8, Ala and Phe_9, and Phe_5 and Phe_{10} are identical, while the conformations of the Val and Phe_6 residues differ from each other by only 30° in the ψ rotational angle (see Figure 6).

It should also be noted that the approximate nature of the potential energies employed in this investigation allow for some small conformational freedom or flexibility away from the conformation depicted in Figure 6. Conformations with similar overall shapes, but with small differences in individual residue conformations, cannot be excluded on this basis. Thus, antamanide may not be totally rigid in solution.

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p*K* Change of Imidazole Groups in Bovine Serum Albumin Due to the Conformational Change at Neutral pH*

B. J. M. Harmsen,† S. H. De Bruin, L. H. M. Janssen, J. F. Rodrigues De Miranda, and G. A. J. Van Os

ABSTRACT: The pH-dependent change in conformation of bovine serum albumin, located between pH 6 and 9 (neutral transition), was studied by means of optical rotation measurements at 313 nm and hydrogen ion titration experiments, both in the presence of KCl or $CaCl_2$. The optical rotatory dispersion measurements revealed that with $CaCl_2$ the transition proceeds at lower pH values and within a smaller pH range than without calcium. From an analysis of the titration curves, combined with the observed influence of calcium ions on the neutral transition, it could be concluded that the transition causes a p*K* shift of certain groups, probably imidazole groups, since as a consequence of the p*K* shift protons are released in the neutral region. That these groups are indeed imidazole groups was further confirmed by measuring the apparent heat of proton dissociation. The highest p*K* was found in the low pH conformation. This suggests that in this conformation several histidyl residues are involved in salt bridges. The effect of calcium on the neutral transition indicates that the affinity of albumin for protons decreases upon calcium binding. The relation between calcium and proton binding to albumin shows much resemblance with the Bohr effect of hemoglobin, *i.e.*, the relation between oxygen and proton binding.

It has been established by several methods that bovine serum albumin (hereafter referred to as the albumin) shows a conformational change between pH 3.5 and 4.5, which is called the normal–fast or NF transition (Aoki and Foster, 1956, 1957; Foster, 1960; Sogami and Foster, 1968) and a change in conformation roughly between pH 6 and 9 (Leonard *et al.*, 1963), the neutral transition. The NF transition can be explained (Foster, 1960) by assuming that the albumin molecule contains four compact parts or "subunits," held together by the peptide backbone itself. Later on evidence has been presented for a three- or four-subunit model (Bloomfield, 1966; Peters and Hawn, 1967; Franglen and Swaniker, 1968; Pederson and Foster, 1969). When the net charge of the molecule increases a rearrangement of the subunits occurs, resulting in an exposure of the interfaces between the compact folded parts to the solvent and consequently in unmasking of certain groups. Only a small rearrangement is supposed to happen at the neutral transition because hydrodynamic parameters remain nearly constant (Tanford and Buzzell, 1956; Leonard *et al.*, 1963). At the NF transition which is accompanied by an appreciable change in these parameters (Sogami and Foster, 1968), presumably a more drastic rearrangement occurs which probably reflects an increase in distance between the subunits.

Experimental data point to the idea that besides tyrosyl residues (Herskovits and Laskowski, 1962; Ohkubo, 1969) also carboxylate groups are involved in the subunit interactions since it has been found by Vijai and Foster (1967) that in the native form of the protein only about 60 of the approximately 100 carboxylate groups are available for protonation. These authors suggest that probably ϵ-amino groups participate as cationic partners of the masked carboxylate groups in the electrostatic interactions between the subunits in the pH region between the NF transition and the neutral transition. This would be in accord with the results of Goldfarb (1966), who found indications for the presence of masked ϵ-amino groups. However, it cannot be ruled out that other cationic groups such as imidazole or guanidinium groups are also involved in this mechanism. Some evidence for the participation of guanidinium groups is presented by Barré and Van Huot (1965). So far, however, the presence of masked imidazole groups has not been shown, although Decker and Foster (1967) had to assume, in order to explain their titration results, the presence of 10 histidines with a fairly high p*K* of about 7.5. This increase in p*K* can be caused by negative charges located near the imidazole groups and therefore could be an indication that these groups are involved in the formation of salt bridges. On the other hand, De Bruin (1969) found a somewhat anomalous temperature dependence of the hydrogen ion titration curve of the albumin in the pH region of the neutral transition, which could not be explained merely by assuming the existence of two classes of histidine residues.

Because this abnormal behavior might be linked to the neutral transition, we made a more detailed study of this transition by means of optical rotation measurements at 313 nm (*cf.* Leonard *et al.*, 1963) and differential hydrogen ion titration curves. Also the influence of the binding of Ca^{2+} ions (Katz and Klotz, 1953; Harmsen, 1970) on the neutral transition was studied. The results strongly indicate the existence of imidazole groups, which show a p*K* shift due to the change in conformation during the neutral transition.

Materials and Methods

Bovine serum albumin was obtained from the Nutritional Biochemicals Corp. (four-times crystallized) and deionized

* From the Laboratory of Biophysical Chemistry, University of Nijmegen, Nijmegen, The Netherlands. *Received April 5, 1971.*
† To whom to address correspondence.