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Test of the Librational Entropy Mechanism of Elasticity of the Polypentapeptide of Elastin

Effect of Introducing a Methyl Group at Residue 5

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The polypentapeptide analogue, $(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Ala_5)_n$, has been synthesized to test the proposed librational entropy mechanism of elasticity which is based on the polypentapeptide of elastin, $(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Gly_5)_n$. The mechanism utilizes the motional capacities of the $Val_4\text{-}Gly_5\text{-}Val_1$ segment of the elastin polypentapeptide in a β-spiral conformation in which the segment is suspended between β-turns. Proton and carbon-13 nuclear magnetic resonance spectroscopies have been used to verify purity and to demonstrate that the $Pro_2\text{-}Gly_3$ β-turn is retained in the Ala_5 analogue. In addition, the temperature profiles for aggregation are found to be similar for both polypentapeptides. Aggregation of the Ala_5 analogue, however, so immobilizes the polypeptide as to give no carbon-13 nuclear magnetic resonance spectrum, whereas a broadened spectrum is observed for the aggregated state of the polypentapeptide of elastin. Scanning electron micrographs are used to compare the aggregated states of the two polymers. On settling, the polypentapeptide of elastin forms a smooth-appearing sheet whereas the addition of a methyl group at residue 5 results in a granular precipitate. In contrast to the elastomeric polypentapeptide of elastin, the Ala_5 analogue, on cross-linking with γ-irradiation, simply fragments when stress/strain studies are attempted. Thus the introduction of a methyl group at residue 5 allows common conformational features to exist but destroys the elasticity in a manner consistent with the proposed librational entropy mechanism.

The effects of this and other analogues of the polypentapeptide of elastin are discussed in terms of accessible configurations, and it is argued that by means of the β-spiral conformation the polypentapeptide of elastin provides a unique configurational entropy which is utilized to produce a new type of entropic elastomer.

Tropoelastin, the precursor protein of fibrous elastin, contains a polypentapeptide sequence, $(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Gly_5)_n$, with $n = 11$ in pig[1, 2a] and $n = 13$ in chick.[2b] High polymers of the polypentapeptide (PPP) have been synthesized with $n \geqslant 40$ and have been found to self-assemble at 37 °C into fibres, observable in the light microscope without any fixative[3] and in the scanning electron microscope with only a Au–Pd coating.[4] On cross-linking, the polypentapeptide is found to be elastomeric and capable of an elastic modulus similar to that of natural fibrous elastin.[4, 5] Thus the PPP of elastin is an anisotropic elastomer. Furthermore, electron microscope studies using negative staining methods have demonstrated parallel aligned filaments with diameters of *ca.* 50 Å in the heat-aggregated states of the polypentapeptide[6] and of tropoelastin[7] and in fibrous elastin.[8, 9] These parallel aligned filaments provide a basis for the self-assembly into fibres.[4, 5]

Studies on the conformation of the PPP using proton and carbon-13 nuclear magnetic resonance spectroscopies[10, 11] have demonstrated the presence of a Type II $Pro_2\text{-}Gly_3$ β-turn utilizing the ten-atom hydrogen-bonded ring, $Val_1C\text{-}O\ldots H\text{-}N\ Val_4$ (see fig. 1). This feature has been confirmed in the crystal structure of the cyclopentadecapeptide, cyclo-$(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Gly_5)_3$,[12] which has been shown to

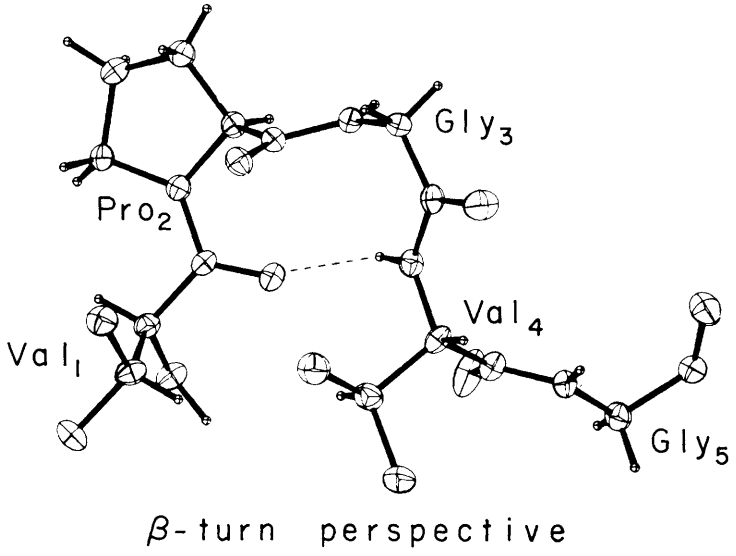
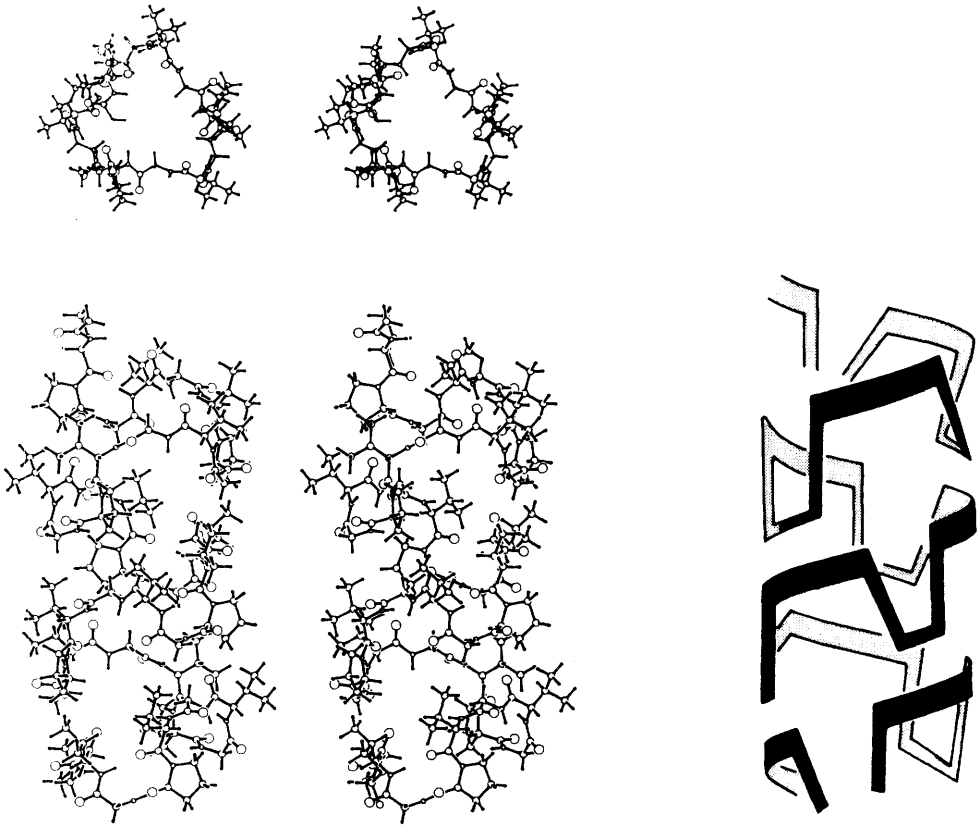


FIG. 1.—Pro_2-Gly_3, type II β-turn of the cyclic conformational correlate of the linear polypentapeptide of elastin as determined from the crystal structure of cyclo$(VPGVG)_3$. Adapted from ref. (12) with permission.

have a nearly identical conformation to that of the linear PPP,[13] and the crystal and solution structures of the cyclopentadecapeptide have been shown to be nearly identical.[14] The linear conformation of the dynamic PPP has been derived from the cyclic structure by means of conformational-energy calculations.[15] The result is a structure which has the same β-turn and a similar number of repeats per turn, n, and helix axis translation per repeat, h, as had been obtained in solution for the most ordered state of the PPP by means of nuclear magnetic resonance methods.[10] The helical structure is called a β-spiral. The term spiral is used for helices in which the repeating unit contains intrarepeat secondary structure in order to differentiate from the classical helices in which hydrogen bonding occurs only between repeating units. The prefix β is used to indicate that a β-turn is the dominant recurring hydrogen-bonded feature.

A schematic representation of the PPP β-spiral is shown in fig. 2(B), where the β-turn is shown functioning as a spacer between the helical turns of the β-spiral. Within the β-spiral is water which can easily exchange with surrounding water through spaces between chains on the surface of the β-spiral. Stereoplots of the dynamic β-spiral of the PPP are shown in fig. 2(A). This is actually one of a class of closely related conformations which can differ slightly in values for n and h and which can differ substantially in terms of the torsion angles, particularly of the $Val_{4,i}$-$Gly_{5,i}$-$Val_{1,i+1}$ segment. This segment, effectively suspended between β-turns, is quite free kinetically with water entirely surrounding it after only a small increase in h. The type of motions of which this segment is capable are librational or rocking motions which can couple to the flexibility of the β-turn to give rise to many states of equivalent energy.[16] Thus the structure is one with substantial librational entropy, particularly on slight extension; however, on further extension, for example on doubling h as occurs on stretching, these motions are damped and the number of states of equivalent energy



(A) (B)

FIG. 2.—(A) Stereo side view of the dynamic β-spiral of the polypentapeptide of elastin. For this particular low energy structure $n = 2.7$ and $h = 3.5$ Å. Reproduced with permission from ref. (15). (B) Schematic representation of PPP β-spiral showing the β-turns as spacers. Reproduced with permission from ref. (3). Note the β-turns which function as spacers between turns of helix with the contacts between turns involving the Val and Pro side chains. Also note the suspended -Val_4-Gly_5-Val_1- segment.

are greatly decreased.[16] We have therefore proposed a librational entropy mechanism for the elasticity of the anisotropic polypentapeptide of elastin.[3, 11, 16]

The simplest librational element is the peptide group, and the peptide groups of greatest librational freedom are those on each side of the Gly_5 α-carbon. Thus a test of the librational entropy mechanism would be to replace a Gly_5 α-proton with a methyl group, *i.e.* by an Ala_5 residue. In terms of the overall repeating unit this is a small change, but in terms of the proposed mechanism of elasticity it would be an important alteration which would significantly decrease the librational freedom of the flanking peptide groups.[16] In the present report the synthesis of $(Val_1\text{-}Pro_2\text{-}Gly_3\text{-}Val_4\text{-}Ala_5)_n$, also abbreviated as $(VPGVA)_n$, is given and the product is verified. This product, referred to as 5-methyl polypentapeptide or 5-Me PPP, is characterized by proton and carbon-13 nuclear magnetic resonance and found to retain the Pro_2-Gly_3

β-turn and generally to exhibit a conformation similar to that of the PPP. The temperature dependence of aggregation is also found to be nearly identical for the two polypentapeptides. At 37 °C in water, however, the 5-methyl PPP is found to be immobile and granular rather than the viscoelastic and glue-like aggregated state of the PPP. On cross-linking the material is found to be without elastic properties.

EXPERIMENTAL

SYNTHESIS

The synthesis is described for the polypentapeptide $(VAVPG)_n$, which in the polymer state can also be written as $VA(VPGVA)_nVPG$. This is an analogue of a sequential polypentapeptide, $(VPGVG)_n$, of elastin in which a hydrogen atom of Gly_5 is replaced by a methyl group. The synthesis was carried out as VAVPG instead of VPGVA for the following reasons: (1) the *C*-terminal glycine will not lead to any racemization during polymerization and (2) since glycine will be least bulky, polymerization should occur to a greater extent with better yields. The synthesis of the peptide was carried out by the classical solution methods. The coupling reactions were mediated by the water-soluble carbodiimide, [1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride] (EDCI).[17] The *N*-protected peptide benzyl ester was converted to the free acid by hydrogenolysis which upon treatment with *p*-nitrophenyltrifluoroacetate[18] was converted to the *p*-nitrophenyl ester. The *N*-terminal protecting group was removed and the peptide *p*-nitrophenyl ester was polymerized in dimethylsulphoxide (Me_2SO).

Elemental analyses were carried out by Mecanal (Tucson, Arizona). Thin-layer chromatography (t.l.c.) was performed on Whatman, Inc. silica-gel plates utilizing the following solvent systems: R_f^1, $CHCl_3$, CH_3OH, CH_3COOH (CMA) (95:5:3); R_f^2, CMA (85:15:3); R_f^3, CMA (75:25:3); R_f^4, CM (5:1). Detection of the peptides on t.l.c. plates was by ninhydrin spray and/or chlorine–toluidine reagent. Boc-amino acids were purchased from Bachem, Inc. (Torrence, California). EDCI was purchased from Aldrich Chemical Co (Milwaukee, Wisconsin). Alanine, valine and proline are of the L-configuration.

Boc-Ala-Val-Pro-Gly-OBzl (I)

Boc-Val-Pro-Gly-OBzl (preparation will be reported elsewhere) (15.0 gm) was treated with 50% TFA/$CHCl_3$ (150 cm³) for 45 min and the solvent removed under reduced pressure. The residue was triturated with petroleum ether, decanted and dried over NaOH and P_2O_5 in a vacuum desiccator, (15 g, 97% yield). To a solution of Boc-Ala-OH (1.79 g, 9.467 mmol) in $CHCl_3$ (20 cm³) and CH_3CN (20 cm³) cooled to −15 °C, EDCI (2.02 g, 10.52 mmol) was added and stirred for 15-20 min. A precooled solution of TFA-H-Val-Pro-Gly-OBzl (5.0 g; 10.52 mmol) in $CHCl_3$ (50 cm³) and Et_3N (1.47 cm³, 10.52 mmol) at −10 °C was added to the above activated Boc-amino-acid solution and stirred at −15 °C for 30 min and at room temperature overnight. The reaction mixture was concentrated under reduced pressure; the residue was taken in $CHCl_3$, extracted with water (3 times), 20% citric acid (3 times), water (3 times), saturated $NaHCO_3$ solution (3 times), water (3 times), and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure (5.0 g, 99% yield). The peptide was further purified by silica-gel column chromatography [2.5 × 86 cm column, Bio-sil A 100-200 mesh, Bio-Rad Laboratories (Richmond, California)] eluting with 1% $CH_3OH/CHCl_3$. All the fractions showing a single spot on t.l.c. were combined and concentrated. R_f^1, 0.43; R_f^4, 0.68. m.p. 63-65 °C. Calculated for $C_{27}H_{40}N_4O_7$: C, 60.88; H, 7.57; N, 10.52. Found: C, 60.42; H, 7.36; N, 10.44.

Boc-Val-Ala-Val-Pro-Gly-OBzl (II)

The peptide I (2.11 g, 3.95 mmol) was treated with TFA (20 cm³) for 30 min. The solvent was removed under reduced pressure and the sample was dried (2.18 g, 100% yield). The TFA salt was coupled with Boc-Val-OH (0.869, 3.95 mmol) using EDCI (0.82 g, 4.32 mmol) and the product was worked up as was done for I, 1.30 g (52.1% yield). R_f^1, 0.43; R_f^2, 0.64. m.p. 99-101 °C. Calculated for $C_{32}H_{49}N_5O_8 \cdot 0.5\ H_2O$; C, 59.98; H, 7.86; N, 10.9. Found: C, 60.15; H, 7.86; N, 10.91.

Boc-Val-Ala-Val-Pro-Gly-OH (III)

II (1.20 g, 1.9 mmol) was taken in gl·HOAc (20 cm^3) and hydrogenated overnight at 40 p.s.i. in the presence of 10% Pd/C catalyst (0.12 g). The catalyst was filtered, washed with $CHCl_3$ and the solvent removed under reduced pressure. The residue was triturated with petroleum ether, decanted and dried (1.03 g, 100% yield). The peptide was precipitated from MeOH–ether–petroleum ether. R_f^2, 0.36; R_f^3, 0.65. m.p. 144-146 °C (decomp.). Calculated for $C_{25}H_{43}N_5O_8 \cdot 0.5\ H_2O$: C, 54.53; H, 8.05; N, 12.72. Found: C, 54.32; H, 7.82; N, 12.53.

Boc-Val-Ala-Val-Pro-Gly-ONp (IV)

To a solution of III (0.80 g, 1.4777 mmol) in pyridine (6 cm^3) was added *p*-nitrophenyltrifluoroacetate (0.529, 2.2155 mmol) and stirred at room temperature. When the reaction was complete as checked by t.l.c., the solvent was removed under reduced pressure; the residue was taken into $CHCl_3$, extracted with water, 20% citric acid, water, saturated $NaHCO_3$ solution, water, and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure. The residue was triturated with petroleum ether, filtered and dried (0.90 g, 92% yield). The peptide was precipitated from EtOAc–petroleum ether. R_f^2, 0.63; R_f^3, 0.83. m.p. 126-129 °C. Calculated for $C_{31}H_{44}N_6O_{10} \cdot H_2O$: C, 54.85; H, 6.83; N, 12.38. Found: C, 55.05; H, 6.92; N, 12.36.

H-(Val-Ala-Val-Pro-Gly)$_n$-Val-OCH_3(V)

The peptide IV (0.81 g, 0.122 mmol) was deblocked with TFA (10 cm^3) and worked up as described earlier. The TFA salt (0.60 g, 0.89 mmol) was taken in Me_2SO (1 cm^3) and Et_3N was added (0.198 cm^3, 1.42 mmol, 1.6 equiv.) while stirring. After 1 h HCl-H-Val-OCH_3 (0.37 mg, 0.0025 equiv.) was added and stirring continued. The reaction mixture turned very viscous within a few minutes and further stirring was not possible. More Me_2SO (2 cm^3) was added and the reaction was allowed to continue for 14 days when HCl-H-Val-OCH_3 (2.97 mg, 0.02 equiv.) was added and allowed to stand for 2 more days. Water was added to the reaction mixture, and the product was dialysed (3500 mol. wt. cut-off) against water for 1 week, changing water twice a day, and then lyophilized to obtain 390 mg of the polypentapeptide. The polymer was then formylated using formic acid and acetic anhydride by the usual procedures.

NUCLEAR MAGNETIC RESONANCE

The 300 MHz ^1H n.m.r. spectra were obtained on a Nicolet NMC-300 wide-bore n.m.r. spectrometer equipped with a multinuclear probe. The Nicolet NMCFT-1280 computer program was used for data collection and processing using 16 K data points. A 6 μs pulse width was used for the 45 ° magnetization vector of the ^1H nucleus, and *ca.* 500 scans were collected for each sample using a 5 s pulse delay. The temperature of the probe was maintained at 32 ± 1 °C.

A JEOL PFT-100 spectrometer operating at 25 MHz was used to obtain the solvent-dependent carbon-13 n.m.r. data in [$^2H_6$]Me_2SO and ^2H_2O. The spectra were accumulated at 30 °C under conditions of complete proton-noise decoupling. Dioxan was used as the internal reference at 67.5 ppm from external hexamethyldisiloxane.

The carbon-13/proton heteronuclear irradiation experiments were performed at 30 °C on a JEOL FX-100 spectrometer utilizing a 10 mm multinuclear probe and a model 1-MI multi-irradiation accessory. A 13 μs pulse width was used for a *ca.* 60 ° tilt of the ^{13}C magnetization with a 3 s pulse recycle time. 20000 accumulations were collected for each spectrum of the sample in [$^2H_6$]Me_2SO, for which the pentamer repeat concentration was 140 mmol dm^{-3}.

The proton temperature-dependence data in [$^2H_6$]Me_2SO were also collected on the FX-100 spectrometer equipped with a 5 mm probe operating at 100 MHz. Probe temperatures were regulated to ± 2 °C by a JEOL VT-3B variable-temperature controller and were measured using an ethylene glycol temperature standard immediately before and after the data accumulation. A 22 μs pulse was used for 90° magnetization along with a 5 s recycle time. Tetramethylsilane (Me_4Si) was the internal reference.

TURBIDITY STUDIES

The temperature profiles of turbidity formation (TPτ) were obtained on a Cary 14 ultraviolet spectrophotometer by monitoring the optical density of the sample solutions at 300 nm in a 1 mm cell undergoing controlled heating (30 °C h^{-1}). Sample temperatures were monitored by a Fluke digital voltmeter utilizing a home-built temperature probe.

SCANNING ELECTRON MICROSCOPY

The scanning electron micrographs were obtained with JEOL JSM-U3 scanning electron microscope at 5 kV accelerating voltage, 0 ° tilt, in the secondary-electron mode with a 200 μm final aperture at an instrument magnification of 150× using a tungsten filament. Prior to examination the samples were dried under vacuum and coated with 100-200 Å of Au–Pd with a JEOL JEE 4C vacuum evaporator.

RESULTS

A most exacting verification of the synthesis and of determination of purity of a polypeptide is obtained by the complete proton (see fig. 3) and carbon-13 (see fig. 4) nuclear magnetic resonance spectra. With the resonances assigned and the absence of extraneous peaks the amino-acid composition and purity are verified. Due to the lack of detection of end-group *H*CO and OC*H*$_3$ resonances, $n \geqslant 40$. By means of the manner in which the assignments are made, it is even possible to verify the sequence

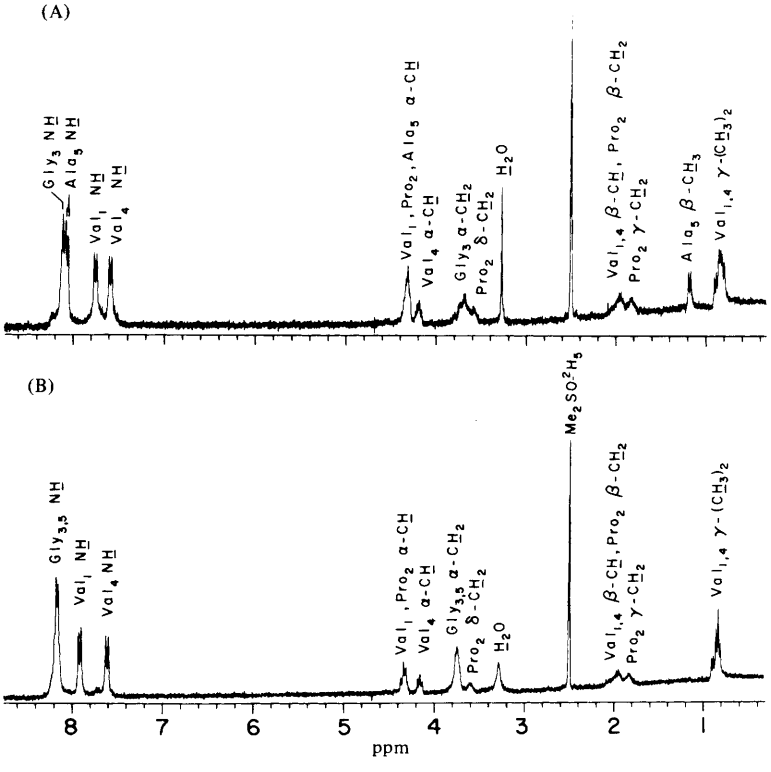


FIG. 3.—^{1}H n.m.r. spectra at 300 MHz. (A) the 5-Me-polypentapeptide, $(VPGVA)_n$, and (B) the polypentapeptide, $(VPGVG)_n$, in [$^2H_6$]Me_2SO at 32 °C.

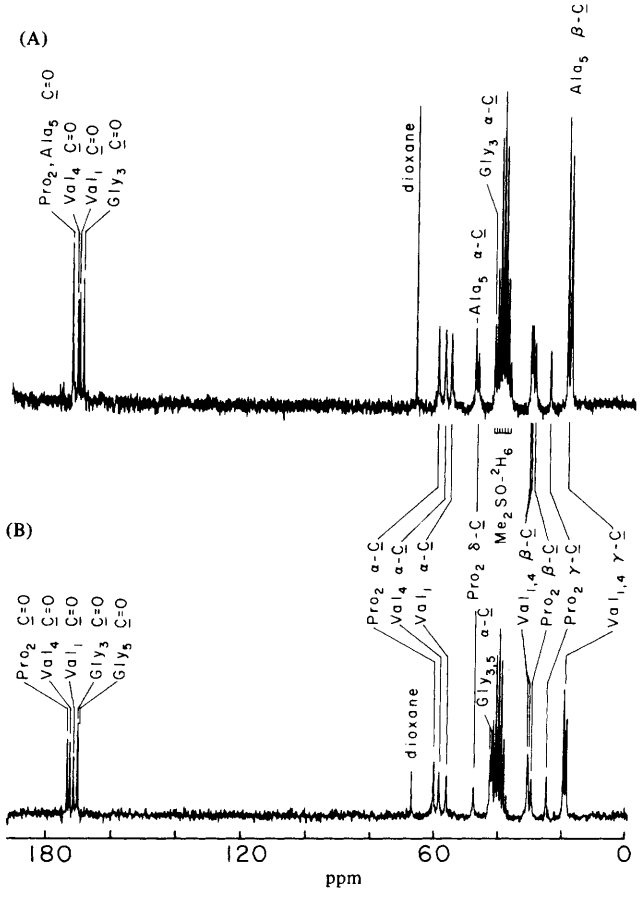


FIG. 4.—^{13}C n.m.r. spectra at 25 MHz. (A) the 5-Me-polypentapeptide, $(VPGVA)_n$, and (B) the polypentapeptide, $(VPGVG)_n$, in $[^2H_6]Me_2SO$ at 30 °C.

of the polypeptide. Some data relevant to this are shown in fig. 5. The carbonyl carbons provide the bridge between residues. For example, the Val_4 N*H* proton and the Gly_3 αCH_2 protons are each ^2J-coupled to the Gly_3 *C*—O nucleus and of such close proximity to result in a nuclear Overhauser enhancement (n.O.e) to the Gly_3 *C*—O resonance. On selective irradiation of these two protons while observing the carbonyl carbon resonances, the Gly_3 *C*—O resonance is found to be selectively intensified. The same Val_4 N*H* is ^3J-coupled to the Val_4 αCH, and the Ala_5 N*H* and the Val_4 αC*H* are ^2J-coupled and n.O.e.-related to the Val_4 *C*—O. In this way both the assignments and sequence are obtained.[19, 20] Thus the correctness of the synthesis is completely verified.

The conformation of the PPP was derived using in large measure the temperature and solvent dependences of the peptide N*H* chemical shifts and the solvent dependence of the peptide *C*—O chemical shifts.[10] A comparison of this type of data for $(VPGVA)_n$ with that of $(VPGVG)_n$ allows ready evaluation of the secondary structure of the former. In fig. 6 is shown the temperature dependence of peptide N*H* chemical shifts

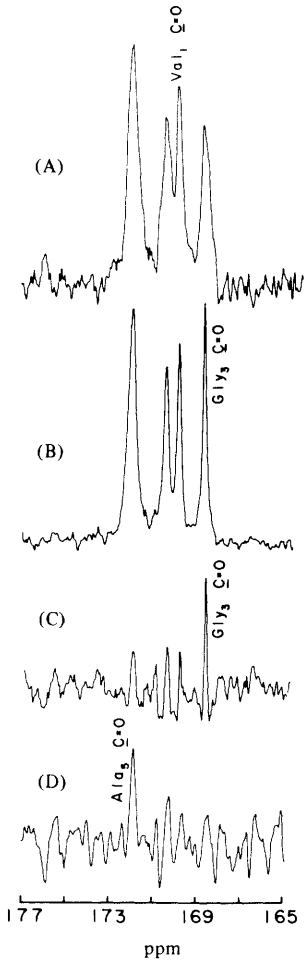


FIG. 5.—^{13}C n.m.r. spectra of the carbonyl carbon resonances of HCO-VA(VPGVA)$_n$-VPGV-OMe in [$^2H_6$]Me_2SO at 30 °C. (A) No proton irradiation (undecoupled). Note the sharp resonance at 169.7 ppm which may be assigned to the Val_1 *C*—O as it has no $^2J_{N-H}$ coupling from the Pro residue. (B) Selective irradiation of the Val_4 N*H* and Gly_3 αC*H* protons showing enhancement of the *C*—O resonance at 168.7 ppm. (C) Difference spectrum obtained by subtracting (A) from (B) showing only the Gly_3 *C*—O resonance. (D) Difference spectrum obtained by subtracting the undecoupled spectrum in (A) from a spectrum in which only the Val_1N*H* was selectively irradiated showing the enhancement of the Ala_5 *C*—O.

for (VPGVA)$_n$ as the solid lines; for comparison that for (VPGVG)$_n$ is shown as the dotted lines. The high-field position of the Val_4 N*H* resonance and its low-temperature dependence is characteristic of a peptide NH intramolecularly hydrogen bonded in the 10-atom ring of a β-turn.[21] The comparison with data of the Val_4 N*H* of (VPGVG)$_n$, which does have the β-turn, is clear. The steeper temperature dependence of the Val_1 N*H* is an indication of exposure of this group to the solvent. At lower field are the Ala_5 N*H* and the Gly_3 N*H* resonances; these have similar slopes and chemical shifts, as do the overlapping Gly_3 N*H* and Gly_5 N*H* resonances of

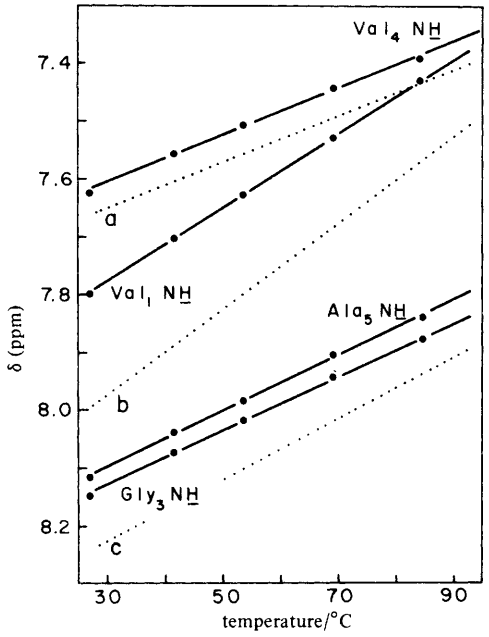


FIG. 6.—Temperature dependence in [$^2H_6$]Me_2SO at 100 MHz for the 5-Me-polypentapeptide, $(VPGVA)_n$. The curves indicated by the dotted lines are for the polypentapeptide $(VPGVG)_n$: (*a*) the Val_4NH, (*b*) the Val_1NH and (*c*) the Gly_3 and Gly_5 NH.

$(VPGVG)_n$. The similar patterns of chemical shift and temperature dependence of chemical shift argue for similar conformations for $(VPGVA)_n$ and $(VPGVG)_n$. As an example of how sensitive such data are to conformation, one need only compare data on cyclo(VPGVG) and cyclo$(VPGVG)_2$ with that on $(VPGVG)_n$; these all have the same sequence, but the cyclopentapeptide and cyclodecapeptide patterns of chemical shift and their temperature dependence are entirely different from each other and from that of $(VPGVG)_n$.[13] These differences are due only to their very different conformations.[22, 23] In spite of the replacement of Gly_5 by Ala_5, the similarity of the patterns in fig. 6 argues for the presence of the β-turn in $(VPGVA)_n$.

The presence of the β-turn is further demonstrated by the peptide *C*—O chemical shifts and their solvent dependences. The chemical shift of a peptide *C*—O resonance can vary by *ca.* 1 ppm due to the magnetic anisotropy of vicinal peptide groups, which changes with conformation, and due to the presence or absence of intramolecular hydrogen bonding.[10] As seen in fig. 1, the three carbonyls that are within the β-turn are the Val_1 C—O, the Pro_2 C—O and the Gly_3 C—O. For the β-turn to be retained and not complicated by the occurrence of other conformational states, these resonances should show little shift. For water in fig. 7 and in column 1 of table 1, it is seen that the chemical shifts of these three resonances are identical in $(VPGVA)_n$ and in $(VPGVG)_n$ within the instrumental digital resolution of 0.05 ppm. Again the comparison of chemical shifts of peptide *C*—O resonances in the three peptides cyclo(VPGVG), cyclo$(VPGVG)_2$ and $(VPGVG)_n$ can be consulted to verify that these chemical shifts are sensitive to conformation.[13] The only reasonable explanation is that of common conformational states for this common peptide segment. The Val_4 *C*—O

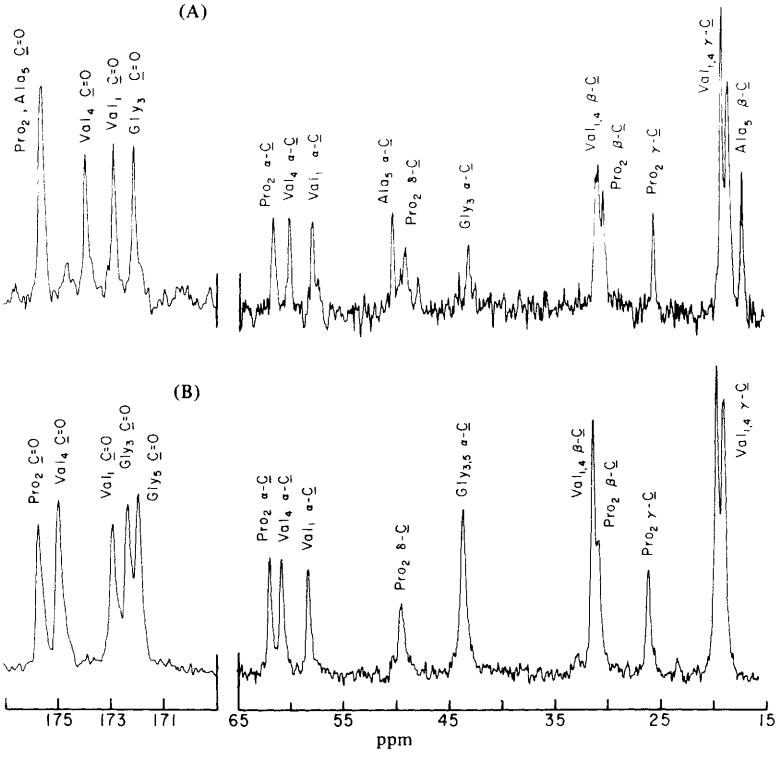


FIG. 7.—^{13}C n.m.r. spectra in ^2H_2O at 25 MHz of (A) the 5-Me-polypentapeptide, $(VPGVA)_n$, (20000 pulses) and (B) the polypentapeptide, $(VPGVG)_n$, at low temperature.

TABLE 1.—[$^2H_6$]Me_2SO–^2H_2O SOLVENT DEPENDENCE OF PEPTIDE CARBONYL CHEMICAL SHIFTS FOR NF-VA(VPGVA)$_n$VPG^a-OMe (5-Me-PPP) AND NF-(VPGVG)$_n$V^a-OMe (PPP) AT 30 °C

peptide residue	δ, D_2O^b (ppm)^c		δ, [$^2H_6$]Me_2SO^b (ppm)^c		$\Delta\delta/\Delta$ solvent (ppm)^c		$\Delta\delta$, [Val_1 (PPP)] (ppm)^c	
	5-Me-PPP	PPP	5-Me-PPP	PPP	5-Me-PPP	PPP	5-Me-PPP	PPP
Val_1 *C*=O	172.60	172.60	170.75	170.94	1.85	1.66	0.19	0
Pro_2 *C*=O	175.41	175.41	172.93	172.88	2.48	2.53	0.82	0.87
Gly_3 *C*=O	171.96	172.01	169.58	169.58	2.38	2.33	0.72	0.67
Val_4 *C*=O	173.61	174.54	171.33	172.06	2.28	2.48	0.62	0.82
Ala_5 *C*=O	175.41	—	172.93	—	2.48	—	0.82	—
Gly_5 *C*=O	—	171.57	—	169.58	—	1.99	—	0.33

[a] V = Val, P = Pro, G = Gly and A = Ala; [b] chemical shifts are given with respect to internal dioxan at 67.4 ppm from external hexamethyldisiloxane; [c] the instrumental digital resolution is 0.05 ppm.

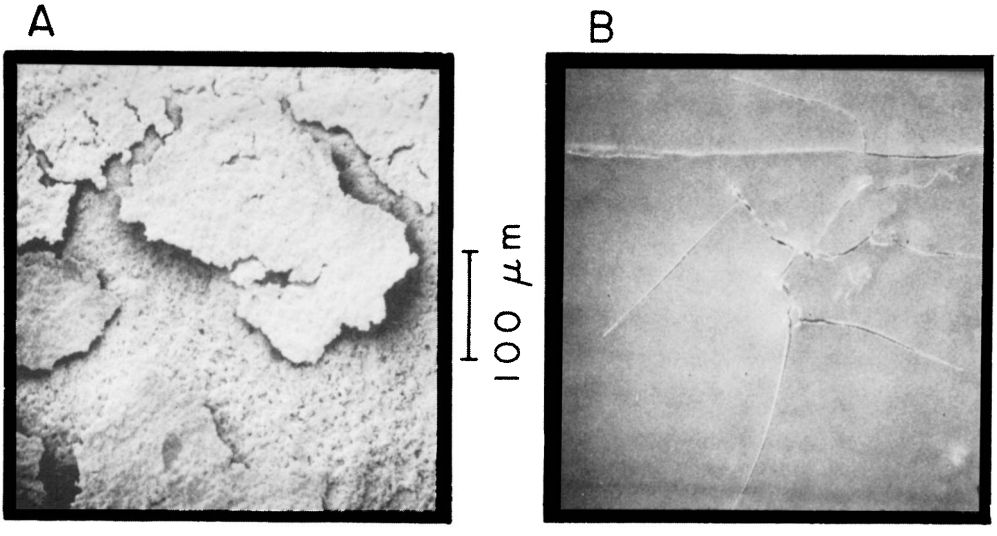


PLATE 1.—Scanning electron micrographs of (A) the heat-aggregated 5-Me-polypentapeptide, $(VPGVA)_n$, which exhibits a very granular nature, and (B) the coacervate of the polypentapeptide, $(VPGVG)_n$, which shows a smooth surface with cracks which occur upon drying.

resonance (as this group is bonded to the Ala_5 NH rather than a Gly_5 NH) on the basis of inductive effects is expected to be shifted, and the Ala_5 *C*—O resonance is expected to differ from that of the Gly_5 *C*—O. The solvent shifts for the $[^2H_6]Me_2SO$–^2H_2O solvent pair are shown in columns 4 and 5 of table 1, where the three carbonyl resonances of the β-turn show similar shifts for $(VPGVA)_n$ and $(VPGVG)_n$, indicated in the table 1 as 5-Me-PPP and PPP, respectively. Thus, particularly in water, the Pro_2-Gly_3 β-turn occurs in $(VPGVA)_n$ much as it does in $(VPGVG)_n$.

As shown in fig. 8, the two polymers exhibit similar temperature dependences of aggregation as followed by turbidity of the solutions, even as a function of

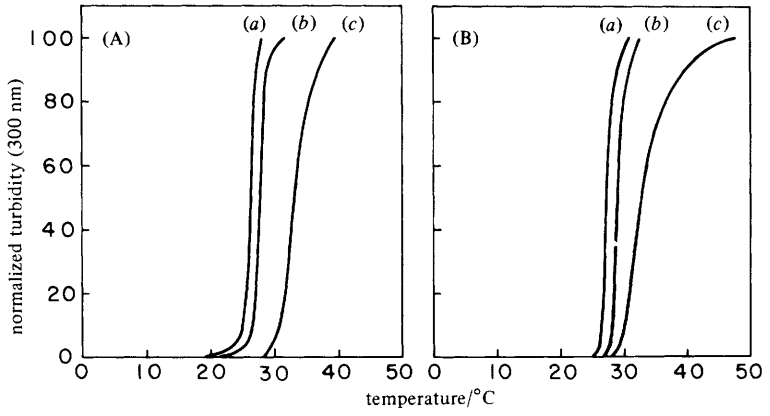


FIG. 8.—Temperature profiles of turbidity formation for (A) the 5-Me-polypentapeptide, $(VPGVA)_n$, and (B) the polypentapeptide, $(VPGVG)_n$. For both polymers curves (*a*) are for a sample with a concentration of 10 (mg peptide) cm^{-3}, curves (*b*) for 5 mg cm^{-3} and curves (*c*) for 1 mg cm^{-3}.

concentration. On raising the temperature of solutions of $(VPGVG)_n$ the solution becomes cloudy, and on standing two phases are formed. The more dense viscoelastic phase is called the coacervate. For $(VPGVG)_n$ this process is readily reversible and is called coacervation. It is a droplet of the cloudy solution of $(VPGVG)_n$ at 40 °C which, when placed on a carbon-coated grid and negatively stained with uranyl acetate and oxalic acid at pH 6.2, shows parallel aligned filaments[7] with a twisted-filament substructure,[24] and it is cross-linking under conditions for coacervation that results in self-assembly into fibres.[4] Thus coacervation has been considered to be a process of fibre formation[24, 25] for the coacervatable elastin peptides; as it is an ordering process it is considered to be an inverse transition in which the dominant intermolecular interactions are hydrophobic. Incidentally, ordering on heating has been notably demonstrated with a cyclic analogue of the elastin repeat hexapeptide sequence. On raising the temperature cyclo$(VAPGVG)_2$ crystallizes and on lowering the temperature it redissolves.[26]

What has been observed up to this stage is the close similarity of conformation and behaviour of $(VPGVA)_n$ and $(VPGVG)_n$. However it is in the properties of the heat-aggregated states that significant differences are found. Heating 20 mg cm^{-3} of the polymer $(VPGVA)_n$ even as high as 30 °C results, on standing or drying, in the irreversible formation of granular aggregates rather than the reversible phase separation of coacervation. The different appearances are shown by means of scanning electron micrographs in plate 1, where a layer of dried $(VPGVG)_n$ coacervate gives a smooth

appearance with cracks due to drying and where similarly treated $(VPGVA)_n$ is seen to be granular.

It is also possible using carbon-13 nuclear magnetic resonance to demonstrate the differences in mobilities of the two heat-aggregated states. In fig. 9(B) is the carbon-13 n.m.r. spectrum of $(VPGVG)_n$ coacervate at 42 °C, which is at a pentapeptide concentration of 1.9 mol dm^{-3}, *i.e. ca.* 50% water. While greatly broadened the resonances are still observed due to the mobility of the chains within the coacervate. On the other hand fig. 9(A) shows that even in a 20 mg cm^{-3} suspension of $(VPGVA)_n$ heated to 42 °C (in which there is no settling of the small aggregates during the accumulation time for 20000 pulses) there are no observable resonances whereas the same sample at 10 °C gave a well-resolved spectrum with 20000 pulses [see fig. 7(A)]. The lack of a spectrum even for the suspended particles of $(VPGVA)_n$ demonstrates a loss of mobility in the heat-aggregated state. The aggregation is not truly irreversible as it can be dissolved on addition of trifluoroethanol, dried and redissolved in water at low temperatures to give the same spectrum as in fig. 7(A). Thus even though there is evidence that there are similarities of conformation and of the thermodynamics of aggregation, the introduction of a methyl group at residue 5 causes a loss of mobility in the heat-aggregated state.

In the same manner as previously carried out for $(VPGVG)_n$,[5] the 5-Me analogue, $(VPGVA)_n$, was cross-linked by γ-irradiation in parallel studies with $(VPGVG)_n$. Whereas cross-linked $(VPGVG)_n$ is elastomeric, as previously reported,[4,5] cross-linked $(VPGVA)_n$ simply fragments on attempting to stretch. $(VPGVA)_n$ when cross-linked is not an elastomer, as can readily be appreciated from its granular nature [see plate 1(A)].

DISCUSSION

For an entropic elastomer there are more polymer configurations of equal low energy accessible to the set of cross-linked chains when the chains are in a relaxed state than when they are stretched. Since the number of states is equivalent to a volume in configuration space, it becomes convenient to speak qualitatively of accessible volumes of equal energy in configuration space. The relationship between entropy, S, and the number of states accessible to a polymer are given by the Boltzmann relation

$$S = \boldsymbol{R} \ln W \tag{1}$$

where $\boldsymbol{R} = \boldsymbol{N}\boldsymbol{k}$ ($= 1.98$ cal mol^{-1} K^{-1}), $\boldsymbol{N}$ being Avogadro's number and $\boldsymbol{k}$ being Boltzmann's constant. W is the number of *a priori* equally probable states accessible to the polymer and is equivalent to a volume in configuration space.

Setting aside for the moment the extensive conformational studies on PPP and related peptides and viewing the relaxed state as random, only a small effect in restricting the accessible volume in configuration space can be expected by addition of a single methyl group to a pentamer, whereas it has just been demonstrated that this modification of the repeat molecular weight from 409 to 423 daltons (a 3% change) causes total loss of elasticity. It is also possible to see the effects of removing methyl groups. The polymer $(APGVG)_n$ has been synthesized and the heat-aggregated state is also granular and without viscoelasticity.[27] An additional well characterized variant of PPP involves the insertion of an Ala residue between Val_1 and Pro_2, *i.e.* $(VAPGVG)_n$. This polymer, which recurs with $n = 5$ in tropoelastin,[1] is also non-elastomeric.[28] These relatively minor modifications, which so completely destroy the elastomeric properties, represent a challenge to the classical description of random-chain elastomers, which awaits explanation from that perspective. On the

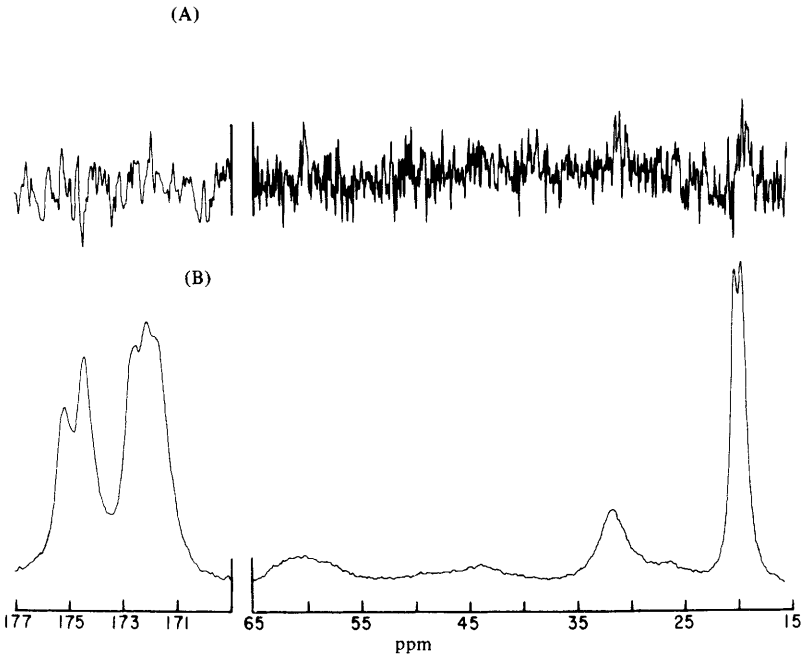


FIG. 9.—^{13}C n.m.r. spectra at 42 °C in $^{2}H_2O$ at 25 MHz of (A) the 5-Me-polypentapeptide, $(VPGVA)_n$ (20000 pulses), and (B) the polypentapeptide, $(VPGVG)_n$. No spectrum is observed for the heat-aggregated state of the 5-Me-PPP, which indicates the lack of sufficient mobility of the peptide at the observation frequency, quite unlike the spectrum obtained for the PPP coacervate which shows broadened, but observable, resonances. Rotational correlation times calculated from the longitudinal relaxation times of the backbone carbon atoms of the PPP are of the order of tens of nanoseconds.

other hand, these effects are explicable in terms of describable conformations and their properties, as presented in this report for 5-Me-PPP and as previously presented for the polyhexapeptide.[10,24]

As outlined in the introduction, 5-Me-PPP was synthesized specifically for the purpose of testing the proposed librational entropy mechanism for elasticity, which centres on the mobility of the conformationally derived suspended segment, $Val_{4,i}$-$Gly_{5,i}$-$Val_{1,i+1}$. The purity of the synthesis has been demonstrated most effectively by the proton and carbon-13 nuclear magnetic resonance spectra (see fig. 3 and 4) and the sequence has been verified by the multiple-irradiation approach for achieving resonance assignments. It has further been demonstrated that the β-turn is retained. This requires that the key effect for the suppression of mobility so apparent in the carbon-13 nuclear magnetic resonance spectra of the aggregate state (see fig. 9) and that the associated loss of elasticity both reside in the peptide segment which connects the β-turns, *i.e.* in the so-called suspended segment. The argument, therefore, is simple and direct. What requires an explanation is how the dynamic β-spiral of the polypentapeptide provides for sufficient entropy change. It has been demonstrated[16] that there is a large decrease in accessible states within a 2 kcal (mol residue)$^{-1}$ energy cut-off on stretching the structure in fig. 2 by 130%. Such a demonstration is fundamental to a proposed mechanism for an entropic elastomer. As will be further discussed below, when this change in entropy of a given pentamer unit is coupled to

the helical recurrence of a dynamic repeating unit, the number of accessible states is not severely reduced on increasing chain length or most significantly on association of chains to form the concentrated state of the fibre. These are taken to be key elements in achieving an adequate accessible volume in configuration space for the relaxed state.

At the outset it is apparent that the retention of a helical form as an initial constraint limits accessible volume in configuration space. However, it is important to realize that much of that limitation is, even in the absence of detailed conformational studies, a simple consequence of the amino-acid composition of the repeating unit. Valyl side-chains greatly restrict the possible ϕ and ψ torsion angles of the backbone, and the Pro_2-Gly_3 sequence, from surveys of the crystal structure of globular proteins,[29] is found to be the most probable sequence for the occurrence of a β-turn (which as a conformational feature is more probable than the β-pleated sheet and almost as probable as the α-helix).[29] Accordingly, even a qualitative consideration of the sequence leads to recognition of very significant limitation in numbers of configurations accessible to the repeating unit, with the Gly_5 residue remaining as the key to the accessible volume.

The contacts between turns of the β-spiral are weak hydrophobic interactions which are not sterically restrictive such that there can be variation in the value of n, the number of repeating units per turn, and h, the translation along the spiral axis per repeat. Even these weak contacts are removed, however, with a small percentage extension of the β-spiral. It is now of interest to consider a specific β-spiral, *e.g.* $n = 2.7$ and $h = 3.5$, and further to consider those states of the ith pentamer which span from the $Val_{1,i}$ α-carbon to the $Val_{1,i+1}$ α-carbon altering in any way the positions of the two Val_1 α-carbons. This enumeration of states is designated as W_i. Since the Val_1 α-carbons are retained in their helical conformation, being related by the same n and h, the same number of states, W_{i+1}, is accessible to the $(i+1)$th pentamer and similarly for all of the n pentamers. For the polypentapeptide in a β-spiral conformation one can then write

$$S = \boldsymbol{R} \ln \prod_{i=1}^{n} W_i = n\boldsymbol{R} \ln W_i. \tag{2}$$

The difference between the relaxed, R, and extended, E, states becomes

$$\Delta S = S^{\mathrm{R}} - S^{\mathrm{E}} = n\boldsymbol{R} \ln W_i^{\mathrm{R}}/W_i^{\mathrm{E}}. \tag{3}$$

As previously shown for a 130% extension to $h = 8$,[16] $W_i^{\mathrm{R}}/W_i^{\mathrm{E}}$ is of the order of 10 for a 2 kcal (mol residue)$^{-1}$ cut-off energy such that ΔS is of the magnitude of 60 entropy units or *ca.* 1 entropy unit per residue. This is approximately one-quarter of the total entropy change which is obtained on complete denaturation of a globular protein.[30] Given the convenient but excessively restrictive constraint of fixing the Val_1 α-carbon positions, the actual cut-off energy for the enumerated states would be below 2 kcal (mol residue)$^{-1}$, which is close to the activation energy of 1.6 kcal mol^{-1} obtained from the temperature dependence of optical anisotropy of single elastin fibres.[31]

The particular advantage of a dynamic β-spiral occurs not so much in the consideration of a single chain but rather in its special properties in the concentrated fibrous state. Whereas the accessible configurations of a single random chain are dramatically decreased when placed in a concentrated state, the effect on a β-spiral is relatively minimal as shown by the following considerations. It has been possible to merge the description of molecular conformation in fig. 2 with the ultrastructure observed in electron micrographs of negatively stained PPP coacervate as well as of elastin where 50 Å diameter twisted filaments are observed. The subfilament diameters

are *ca.* 15-17 Å,[3, 9, 16, 32] which is the diameter of the β-spiral in fig. 2. Three β-spirals can be shown to supercoil into a right-handed 50 Å twisted filament[3, 16] (Venkatachalam and Urry, unpublished data). The perspective of the concentrated fibrous state of the PPP, therefore, is one of supercoiled β-spirals in which the intermolecular contacts are weak hydrophobic interactions. Such interactions do not cause rigid constraints at the contacts between supercoiled chains and, within the twisted filament, only involve about one out of every three pentamer repeating units. The point to be made is that the qualitative discussion above of mobility and entropy of the PPP β-spiral can easily be extended to the fibrous state with little added constraint beyond that already considered for repeating units in helical array.

Thus the librational entropy mechanism for the elasticity of the polypentapeptide of elastin, which is based on a particular class of dynamic β-spiral conformations, does not appear unreasonable at a quantitative level and by the data reported here it is substantiated by the dramatic loss of elasticity resulting from an otherwise seemingly innocuous addition of a methyl group to the Gly_5 residue. More extensive chemical and physical studies of structure, as well as more complete calculations of entropy,[33, 34] are underway to pursue further this proposed new mechanism of elasticity.

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(PAPER 2/1205)