# Synthetic, Cross-Linked Polypentapeptide of Tropoelastin: an Anisotropic, Fibrillar Elastomer†

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ABSTRACT: Physical properties of the cross-linked polypentapeptide of tropoelastin are reported along with chemical characterization of key intermediates in its synthesis. 220 MHz proton magnetic resonance spectra are reported on the constituent pentamers and their respective high polymers which verify structural and conformational integrity. Scanning electron microscopy of the cross-linked material formed without orientation and with flow orientation is reported. The former demonstrates the inherent fibrillar and anisotropic nature of the synthetic product. Stress–strain studies show the cross-linked polypentapeptide to exhibit elastomeric properties that are dependent on the water content of the matrix. At high water contents the elastic modulus is less than that of wet native aortic elastin and becomes greater on drying.

Tropoelastin, the precursor protein of the core of the elastic fiber (Smith et al., 1975), contains repeating peptide sequences (Foster et al., 1973; Gray et al., 1973)—a tetrapeptide ($Val_1$-$Pro_2$-$Gly_3$-$Gly_4$),[1] a pentapeptide ($Val_1$-$Pro_2$-$Gly_3$-$Val_4$-$Gly_5$), and a hexapeptide ($Ala_1$-$Pro_2$-$Gly_3$-$Val_4$-$Gly_5$-$Val_6$). Over the past several years this laboratory has synthesized these peptides and their oligomers and high polymers, and has studied their conformations in solution by proton and carbon-13 magnetic resonance, by circular dichroism, and by ultraviolet absorption spectroscopies (Urry, 1974; Urry et al., 1974a–f; 1975a–d; Urry and Ohnishi, 1974a,b; Long, et al., 1974; Urry and Long, 1976). The peptides all exhibit preferred secondary structure with each containing the $\beta$ turn in Figure 1 as a dominant conformational feature and with each exhibiting additional, more dynamic, preferred secondary structural features. At this stage there are several major questions. Are the solution conformations relevant to the fibrous state? What are the specific functions, if any, of the repeating sequences? Do any of the repeat peptides exhibit elastomeric properties?

In our approach to these questions, we examine the molecular system of interest in three different states—solution, coacervate, and fibrous. The coacervate is the key to bridging from the solution to the fibrous state. Coacervation, a reversible, concentration-dependent phase separation, elicited in this case by temperature, is an uncommon property exhibited by tropoelastin (Sandberg et al., 1969; Smith et al., 1968), by $\alpha$-elastin (a chemical fragmentation product of fibrous elastin) (Partridge et al., 1955; Partridge and Davis, 1955), by the polypentapeptide (Urry et al., 1974b), and by the polyhexapeptide (Urry, et al., in preparation). All of these molecular systems are soluble in water at low temperatures, but, on raising the temperature, the solutions become cloudy and the scattering elements coalesce to form a more dense phase that is about 60% water by volume. Electron microscopy of the coacervates, using the technique of negative staining, shows filamentous structures (Cox et al., 1974, 1973; Volpin et al., 1976a,b; Urry et al., 1974b) with periodicities similar to those of fibrous elastin (Gotte et al., 1974, 1976). The approximate 50 Å filament to filament repeat, observed in the electron micrographs, has been confirmed for fibrous elastin by low-angle x-ray diffraction (Serafini-Fracassini et al., 1976). The coacervate is the stable state at body temperature, contains the same volume percent water (~60%) as fibrous elastin (Partridge, 1967), and is filamentous with periodicities similar to those of fibrous elastin. For these reasons, we take the coacervate to be a model of the relaxed fibrous state and view the process of coacervation as a key step in elastogenesis that concentrates and aligns the subunit prior to covalent cross-linking.

In the present effort we report the synthesis of cross-linked polypentapeptide (X-PPP),[2] the proton magnetic resonance spectra of the component repeating units with cross-linking residues, and of the high polymers prior to cross-linking, scanning electron micrographs of cross-linked products formed without and with flow orientation, and descriptive information on the elastomeric properties of the flow oriented product. It has been argued that the elastomeric properties of elastin are those of an isotropic random network of polypeptide chains (Hoeve and Flory, 1974). The present endeavor is to make cross-linked high polymers of the repeating pentapeptide of elastin and to determine whether the product is isotropic or anisotropic and to assess whether it has elastomeric properties.

## Methods

*Synthesis of Cross-Linked Polypentapeptide.* The initial successful approach has been, in one polymerization, to insert a lysine residue in place of the $Val_4$ residue in one out of about five repeats, in a second polymerization to replace the $Val_4$ residue with a glutamic acid residue in one out of about seven repeats, to combine the two polymers under conditions for coacervation, and to cross-link the glutamyl and lysyl side chains by means of CMCI[2] (see Scheme I) initially without and then with flow orientation of the polymers. The complete synthesis from which the following compound numbers were

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[1] The amino acid residues are of the L configuration.

[2] Abbreviations used are: Boc = *tert*-butyloxycarbonyl, Z = benzyloxycarbonyl, Np = *p*-nitrophenyl, $Bu^t$ = *tert*-butyl, CMCI = 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; ONP, *p*-nitrophenyl ester; X-PPP, cross-linked polypentapeptide; TLC, thin-layer chromatography.

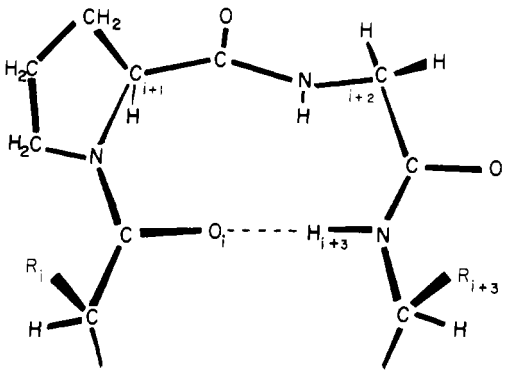
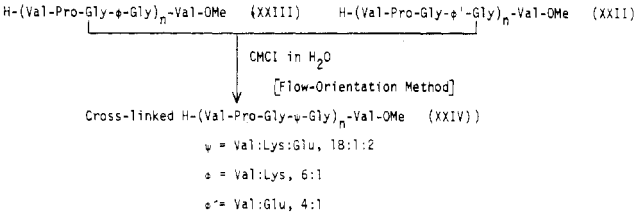


FIGURE 1: β Turn of the repeat peptides of elastin as deduced from proton magnetic resonance studies. In the tetrapeptide $R_i$ = Val and $R_{i+3}$ = Gly. In the pentapeptide $R_i$ = Val and $R_{i+3}$ = Val. In the hexapeptide $R_i$ = Ala and $R_{i+3}$ = Val (From Urry et al., 1975d).

SCHEME I: Synthesis of Cross-Linked H-(Val-Pro-Gly-ψ-Gly)$_n$-Val-OMe (where ψ = Val, Lys, or Glu).




taken will be described in a separate report (Okamoto and Urry, 1976).

Amino acid analyses were carried out on a Beckman 119 H amino acid analyzer, using the ninhydrin method. Thin-layer chromatography (TLC) was performed on silica gel G with the following solvent systems: $R_f^1$, chloroform–methanol–acetic acid (95:5:3, v/v); $R_f^2$, 1-butanol–acetic acid–pyridine–water (15:3:10:12, v/v). Detection on TLC plates was by spraying ninhydrin for a material with free amino group, by spraying 48% HBr and ninhydrin for molecules with a Z or Boc group, or by chlorine/tolidine reaction for those with peptide bonds.

*Boc-Val-Pro-Gly-Lys(ε-Z)-Gly-ONp (X).* Compound X, obtained as a solid, was recrystallized from chloroform–petroleum ether; yield 2.74 g (78% from IX), mp 113–118 °C, $R_f^1$ 0.56, $R_f^2$ 0.92. Anal. calcd for $C_{39}H_{54}N_7O_{12}$: C, 57.62; H, 6.70; N, 12.06. Found: C, 58.00; H, 6.74; N, 11.80.

*Boc-Val-Pro-Gly-Glu(γ-OBu$^t$)-Gly-ONp (XX).* The product containing XX, a solid, was composed of one major component ($R_f^1$ 0.44) and one minor component ($R_f^1$ 0.33). Repeated recrystallizations from chloroform–petroleum ether and further from several organic solvents were attempted, but these attempts were not remarkably effective to fractionate the major component as a pure XX. The component (1.13 g, $R_f^1$ 0.44) containing only traces of the component ($R_f^1$ 0.33) was used in the next step without further purification.

*H-(Val-Pro-Gly-φ'-Gly)$_n$-Val-OMe (XXII).* There was obtained 1.9 g of compound XXII (72% yield and an $R_f^2$ of 0.57), mp 285–290 °C (decomp). Amino acid analysis: Glu, 1.00; Pro, 5.08; Gly, 10.17; Val, 9.14. This polypentapeptide was composed of -Val-Pro-Gly-Val-Gly- sequence (ca. 80%) and -Val-Pro-Gly-Glu-Gly- sequence (ca. 20%). Glutamic acid content of polypentapeptide was ca. 4%.

*H-(Val-Pro-Gly-φ-Gly)$_n$-Val-OMe (XXIII).* There was obtained 171 mg of Compound XXIII (yield ca. 69%), mp 295–300 °C (decomp), $R_f^2$ 0.53. Amino acid analysis: Pro, 7.02; Gly, 13.96; Val, 13.00; Lys, 1.00. This polypentapeptide was composed of -Val-Pro-Gly-Val-Gly- sequence (ca. 86%) and -Val-Pro-Gly-Lys-Gly- sequence (ca. 14%). Lysine content of polypentapeptide was ca. 2.9%.

*Cross-Linked H-(Val-Pro-Gly-ψ-Gly)$_n$-Val-OMe (XXIV).* For flow orientation during cross-linking, a solution of XXII (100 mg) and XXIII (140 mg) in a small amount of water (0.7 ml) was placed in a 45-ml Virtis freeze-drying glass vessel and the vessel was mounted horizontally to a rotary drive. The vessel was slowly rotated and the solution, at a steady orientation, flowed on the surface inside of the rotating vessel for 3 h at 40 °C. The flowing solution became a viscous liquid and then formed a band like a plastic film. To the band was added 3 g of ground 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate with flowing and the mixture flowed at the same temperature. The mixture, after 15 h, was cooled to 35 °C and water (0.2 ml) was added dropwise with flowing. Subsequently, the mixture was gradually cooled to 25 °C and flowed for 2 days. The reaction mixture was washed several times with water by decantation and an insoluble product was obtained. Amino acid analysis. Glu, 2.05; Pro, 21.95; Gly, 43.02; Val, 39.86; Lys, 1.00.

*Proton Magnetic Resonance.* The proton magnetic resonance spectra of the constituent repeats, X and XX, and of the high polymers prior to cross-linking were obtained on a Varian HR-220 MHz spectrometer equipped with a tracking frequency decoupling accessory and an SS-100 computer system with 16K of core for multiscan averaging. Sample temperature was calibrated with methanol or ethylene glycol chemical shifts and controlled to within ±2 °C. Dimethyl-$d_6$ sulfoxide (99.5 and 99.8% $^2$H) was purchased from Columbia Organic Chemical Co., Columbia, S.C. and from Diaprep Corp., Atlanta, Ga.

*Scanning Electron Microscopy.* The insoluble cross-linked polypentapeptide formed without flow orientation and with flow orientation was placed on a glass and Plexiglas substrate, respectively, dried in a vacuum oven, and coated with several hundred Angstroms of Au-Pd evaporated at less than $5 \times 10^{-5}$ mm Hg. The cross-linked polypentapeptide was then examined in a JEOL JSM-U3 scanning electron microscope at a 25 kV accelerating voltage.

*Stress–Strain Apparatus.* The system consists of a moving platform supported by linear motion ball bearings riding on a steel shaft. This moving assembly is driven by a lead screw coupled to an induction motor through a variable speed gear drive. A clamp on the platform holds one end of the specimen. Platform position is recorded on the *x* axis of an x-y recorder using a linear displacement transducer, bridge completion network, and a d-c excitation power supply. The fixed end of the specimen is held by a clamp attached to a load cell. The load cell consists of a Statham Universal transducing cell (UC3) with a UL4-0.5 load cell accessory. Signal conditioning is done by a d-c excitation power supply, balance network, and a signal amplifier. The output signal is an analogue voltage of the applied force that is recorded on the *y* axis of the x-y recorder.

A force–strain curve was recorded by placing one end of the specimen in the load cell clamp that was detached from the load cell. The other end of the specimen was clamped to the platform. The load-cell clamp was then attached to the load cell with an initial length of 1 mm and no initial tensile or compressive force. The drive was turned on and the specimen stretched at a rate of 0.5 mm/s.

## Results

*Proton Magnetic Resonance.* The three pentameric units that are incorporated into the cross-linked high polymers are

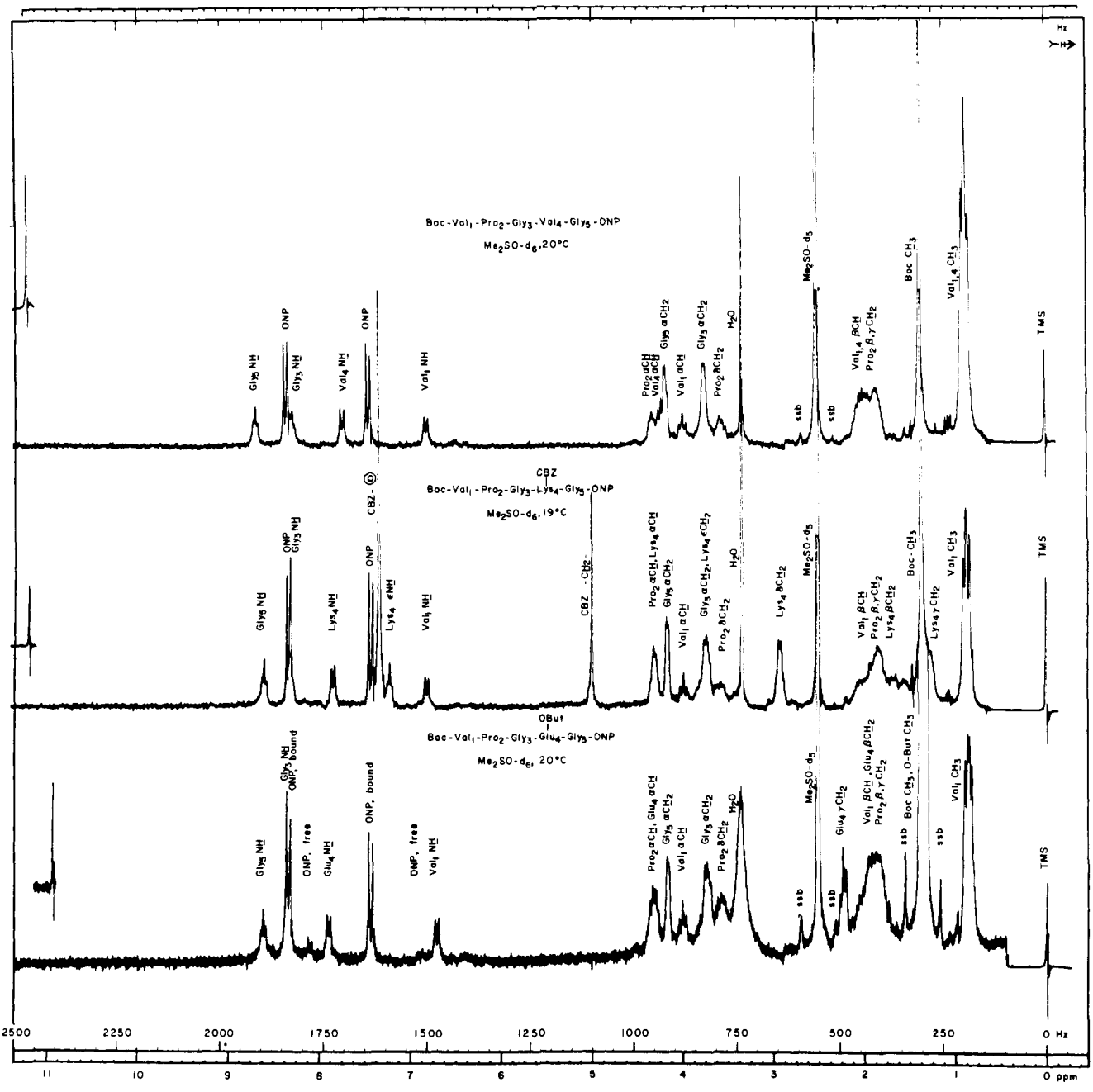



FIGURE 2: 220 MHz Proton magnetic resonance spectra at room temperature in $Me_2SO$-$d_6$ of the Boc–ONP monomeric units used in the synthesis of the cross-linked pentamer high polymer. In the glutamate-containing pentamer, the spinning side bands are evident. When the spectrum was rerun to minimize further the spinning side bands, it was found that the sample had spontaneously cleaved more *p*-nitrophenyl ester (ONP) and that the free *p*-nitrophenol resonances appeared as larger peaks in the downfield region. TMS, tetramethylsilane.

$Val_1$-$Pro_2$-$Gly_3$-$Val_4$-$Gly_5$, $Val_7$-$Pro_2$-$Gly_3$-$Lys_4$-$Gly_5$, and $Val_1$-$Pro_2$-$Gly_3$-$Glu_4$-$Gly_5$. Proton magnetic resonance spectra at 220 MHz of all three as the Boc and Np derivatives, of the second with the $\epsilon$-amino group blocked by benzyloxy carbonyl, and of the third with the $\gamma$ carboxyl blocked by a *tert*-butyl ester are given in Figure 2. In the peptide NH region the highest field doublet near 1500 Hz is the $Val_1$ NH; the lowest field triplet is due to the $Gly_5$ NH, and the pair of intense doublets are due to the *p*-nitrophenyl moiety in all three spectra. In Figure 2A (top spectrum) the remaining doublet is due to the $Val_4$ NH and the resonance just to the high-field side of the lowest field *p*-nitrophenyl doublet is due to the $Gly_3$ NH. In Figure 2B (middle spectrum) the most intense resonance is due to the aromatic protons of the benzyloxy carbonyl moiety and the $\epsilon$-NH triplet is immediately on its high-field side. The $Lys_4$ NH is between the two *p*-nitrophenyl resonances. Otherwise, the resonances are as in spectrum A with the slight shifts which cause the low-field *p*-nitrophenyl doublet to overlap the $Gly_3$ NH resonance. In Figure 2C (bottom spectrum) the $Glu_4$ NH is central between the intense aromatic doublets, and the low-field aromatic doublet and the $Gly_3$ NH resonance overlap. The remainder of the resonances in the peptide NH region of Figure 2C are as in Figure 2A. One can similarly look at the higher field regions and verify that the syntheses are correct and that there are no dramatic differences that would argue for differences in conformation.

The proton magnetic resonance spectra of the high polymers, compounds XXII and XXIII, are given in Figure 3B,C (middle and bottom spectra) and are to be compared with the polypentapeptide, without the Glu and Lys cross-linking residues,

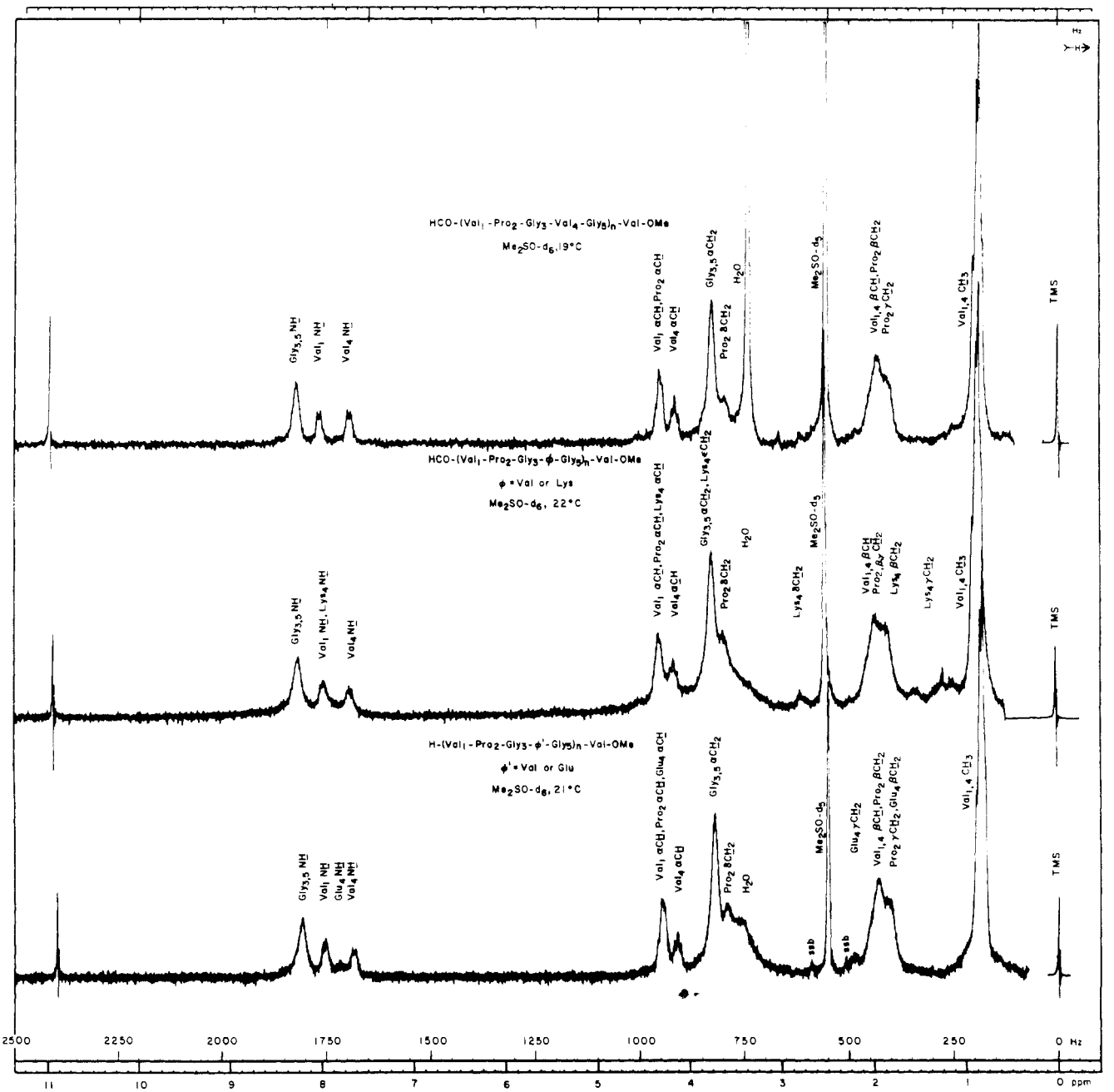



FIGURE 3: 220 MHz Proton magnetic resonance spectra at room temperature in $Me_2SO$-$d_6$ of the pentamer high polymers. The HCO-$(Val_1$-$Pro_2$-$Gly_3$-$\phi$-$Gly_5)_n$-Val-OMe and HCO-$(Val_1$-$Pro_2$-$Gly_3$-$\phi'$-$Gly_5)_n$-Val-OMe high polymers were combined to form the cross-linked pentamer high polymer (X-PPP).

in Figure 3A (top spectrum). The spectra when carefully analyzed, with scale expansion and multiscanning, are entirely consistent with the amino acid analyses of XXII and XXIII. These high polymers coacervate, as does the polypentapeptide, such that cross-linking can be carried out in the coacervate state, and it can be argued that the properties of the cross-linked polypentapeptide are those of the polypentapeptide.

*Scanning Electron Microscopy.* Solutions of the polymers XXIII and XXII, which were cross-linked in a test tube without any movement, exhibited fibers. One such fiber is seen in Figure 4. The fiber in this scanning electron micrograph is seen to splay out at a bend showing the presence of many component fibrils and to recoalesce into a single fiber again. Whereas an isotropic coalescence would simply show spheres, this two-dimensional coalescence clearly demonstrates the fundamental anisotropic nature of the fibers formed from the polypentapeptide of elastin, and it reflects the filamentous substructure observed in the electron micrographs of the polypentapeptide coacervate (Urry et al., 1974b; Volpin et al., 1976b).

When the thick coacervate is formed in a heavy walled flask and rotated slowly with the side horizontal, the coacervate flows to form a coating on the inside of the flask. The cross-linking reagent is then added at about 40 °C and the result is the thick matted matrix seen in Figure 5. On taking this band, after washing and folding it, enough material can be obtained to test its elastomeric nature. The material is very sticky, adhering to almost any surface, and when pulled from its attachment with a pair of tweezers it snaps back like a rubber band. This elastomeric behavior is dependent on the amount of water present as it decreases with increasing water content as shown below.

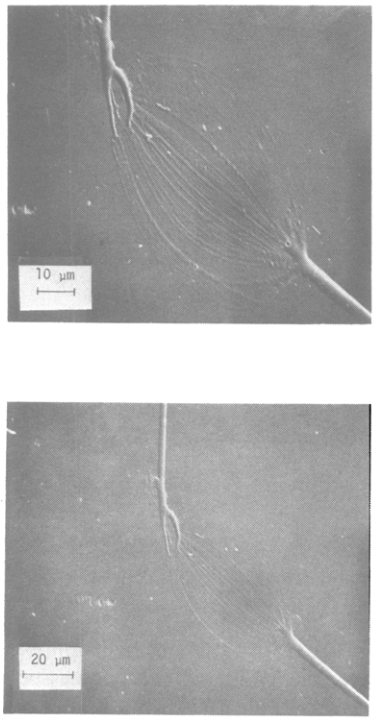



FIGURE 4: Scanning electron micrographs of the cross-linked pentamer high polymer (X-PPP) chemically coupled without flow orientation. The accelerating voltage was 25 kV, with a 45° tilt angle, on a glass substrate. The magnification was 500× and 335× and the marker indicates 10 and 20 μm, respectively. See text for discussion.

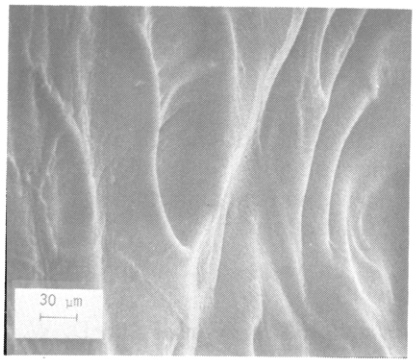



FIGURE 5: Scanning electron micrograph of the X-PPP cross-linked with flow orientation. Conditions were the same as in Figure 4 but the support was Plexiglas.

*Reversible, Temperature Dependent Swelling and Retraction.* The weakly cross-linked polypentapeptide, product XXIV, undergoes a reversible temperature elicited swelling and retraction, the latter of which is directly analogous to coacervation. The temperature profile for this process in Figure 6 is very similar to the temperature profile for coacervation of concentrated solutions of polypentapeptide (Urry et al., 1974b,1975a). The retracted state is simply the cross-linked coacervate state and the swollen-gel-like state would be a solution if it were not for the cross-links.

*Stress–Strain Studies.* The elastomeric properties of the cross-linked polypentapeptide are demonstrated in Figure 7 and compared to those of native aortic elastin. At room temperature the water content of X-PPP can be varied by addition of a given quantity and then by allowing the specimen to dry with time. When X-PPP contains 70% water by weight it exhibits very little elasticity, curve a of Figure 7. On drying with time the initial slopes of the stress–strain curves increase

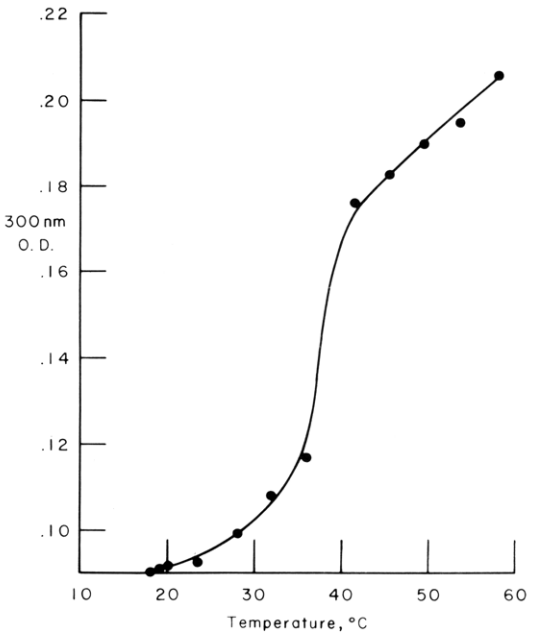



FIGURE 6: Temperature dependence of the swelling and retraction of the cross-linked pentamer high polymer. The synthetic material was immobilized in a 0.1-mm separable cell in a Cary 14 recording spectrophotometer and the sample's absorbance due to light scattering was followed as a function of temperature at 300 nm. The total change observed was 0.150 OD.

dramatically to become greater than those of wetted native aortic elastin, curves a′ and b′ of Figure 7. The elastic modulus of X-PPP increases from 3.6 psi at 70% water to 230 psi where the sample is becoming so dry that it becomes brittle. The undulations in the curves for X-PPP are due to breaking of a composite strand and resultant flow. The abrupt drops in the curves at higher strain are due to complete rupture of the sample. The curves in Figure 7 demonstrate that the elastomeric properties are solvent dependent and comparison with Figure 6 allows correlation to be made between the elastomeric state and the coacervate state.

## Discussion

Deam and Edwards in a recent review (Deam and Edwards, 1976) have noted the assumptions in the classical theories of rubber elasticity to be the occurrence of "phantom chains" that "can pass freely through one another (and themselves)" and that exhibit "no inter or intra chain forces". These assumptions as well as the assumption of an isotropic, non-fibrillar substance do not apply to the elastomeric cross-linked polypentapeptide. In Figure 4 it was shown that filaments or fibrils undergo a two-dimensional coalescence to form larger fibrils or fibers. If the system were truly isotropic and amorphous, spherical droplets would be seen coalescing to form larger spherical droplets. Few would argue with the point that chains cannot pass through one another. This is readily recognized as an assumption for the purpose of simplifying mathematics.

With the extensive studies on the polypentapeptide in solution, with the studies on the process of coacervation, and with the correlation of the coacervate state, retracted state, and low water content state with elastomeric behavior, it becomes possible using this molecular system to consider directly the question of intra- and interchain forces. Solution studies show the presence of the $\beta$ turn (a $Val_4$ NH $\cdots$ OC $Val_1$ hydrogen bond) (see Figure 1) with a probability of occurrence of 70%

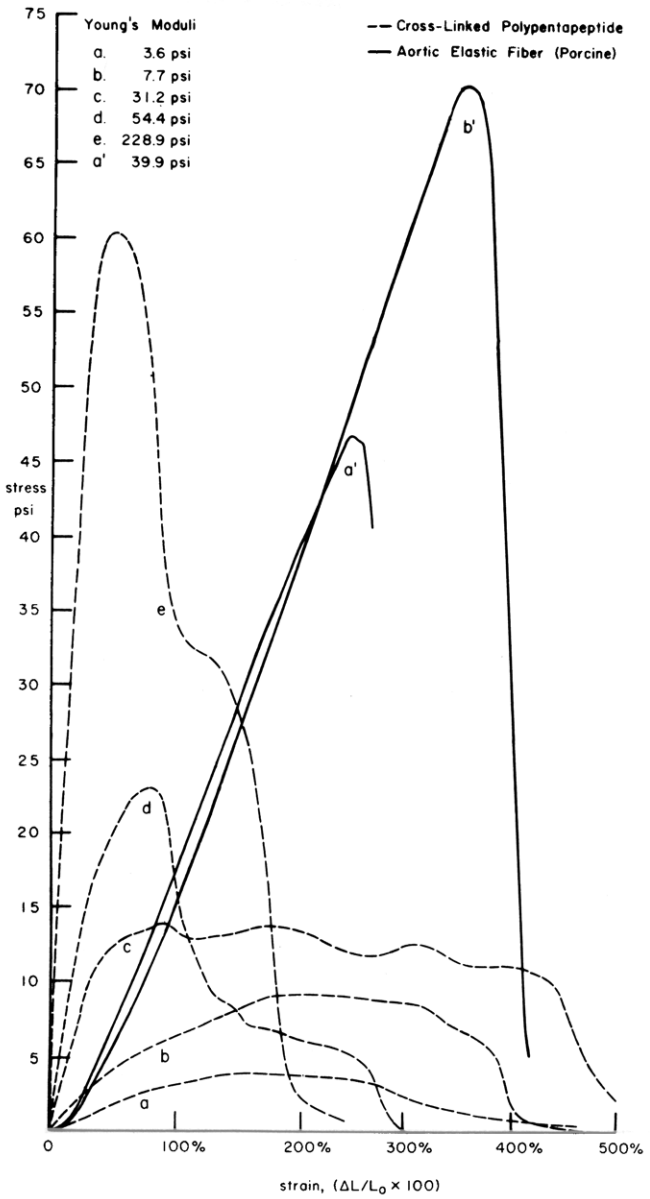



FIGURE 7: Stress–strain curves of the cross-linked pentamer high polymer (- - -) and, for comparison, aortic elastic fibers (—). The water content of the material in curve a was 70% and sequentially lower in curves b through e as the synthetic material dried at room temperature in the apparatus. The aortic fibers were hydrated in a chamber of 100% humidity. The Young's moduli were calculated from the initial slope of each curve and were for a: 3.6 psi, b: 7.7 psi, c: 31.2 psi, d: 54.4 psi, e: 228.9 psi for the X-PPP and for a′: 39.9 psi for the aortic elastic fiber (see text for further discussion).

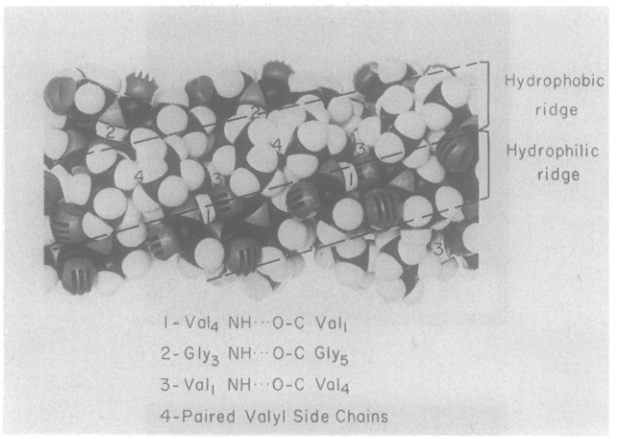



FIGURE 8: Space-filling model of the polypentapeptide, HCO-(Val-Pro-Gly-Val-Gly)$_n$-Val-OMe, in a $\beta$-spiral conformation. Both hydrophobic and hydrophilic ridges are present on the spiral's exterior. The hydrogen bonds, 1, 2, and 3 between the Val$_4$ NH and Val$_1$ C=O, the Gly$_3$ NH and Gly$_5$ C=O, and Val$_1$ NH and Val$_4$ C=O, respectively, are labeled on the figure (adapted from Urry, D. W., and Long, M. M., 1976).

or more in dimethyl sulfoxide, in methanol, and in water at elevated temperature (Urry and Long, 1976). Also, additional intramolecular hydrogen bonding features are considered to occur, i.e., a Val$_1$ NH · · · OC Val$_4$, and a Gly$_3$ NH · · · OC Gly$_5$, with probabilities of occurrence of 50% or more. A working model for the structure with all three hydrogen bonds formed simultaneously is given in Figure 8. Thus, the solution studies argue for significant intrachain interactions. Even so, it should be appreciated that the molecule is a dynamic structure with rotational correlation times for specific segments of the order of 10–100 ns (Urry and Mitchell, in preparation).

With regard to interchain interactions, it has been shown that coacervation is a concentration dependent process (Urry et al., 1975a) and it has been argued that coacervation is an inverse temperature transition with hydrophobic intermolecular interactions as the dominant feature on association to form the coacervate (Urry et al., 1974b). In Figure 6 it is seen that the temperature-elicited retraction to a state of lower water content occurs on raising the temperature in analogy to coacervation, and in Figure 7 it is shown that elasticity increases with decreasing water content as is found for native elastin (Gotte et al., 1968). The strong implication is that hydrophobic interchain interactions are a significant aspect of the elasticity of the cross-linked polypentapeptide.

The implication of these results is that the classical theories of rubber elasticity should not be interpreted literally to relate to the molecular structure of X-PPP and by inference and analogy to the molecular structure of elastin. The evidence indicates that the elastomeric polypentapeptide of tropoelastin is neither random nor isotropic and that it has significant inter- and intrachain interactions.

## Acknowledgment

The authors thank J. A. Barnes and D. W. Mason for technical assistance.

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# Cross-Linked Polypentapeptide of Tropoelastin: an Insoluble, Serum Calcifiable Matrix†

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ABSTRACT: The synthetic, cross-linked polypentapeptide of tropoelastin has been shown to calcify from serum alone even when separated from the serum medium by a dialysis membrane with a low-molecular-weight cut off. By microprobe analysis, it appeared that the only serum elements required for the calcification were calcium and phosphorus. Furthermore, thin sections of the calcified matrix showed the calcification to occur throughout the matrix, and thereby verified that it is a bulk property of the matrix and not an interfacial property. To our knowledge this is the first demonstration of an insoluble, synthetic polypeptide to function as a serum calcifiable matrix and by doing so it opens the door to potential medical applications.

The vascular elastic fiber is a primary site of pathological calcification of the arterial wall (Martin et al., 1963; Urry, 1974b). Tropoelastin, the precursor protein of the fibrous core of the elastic fiber (Smith et al., 1975, 1968; Sandberg et al., 1969), and $\alpha$-elastin, a chemical fragmentation product of fibrous elastin (Partridge et al., 1955; Partridge and Davis, 1955), exhibit an interesting property that has been utilized in studies on calcification. Both molecular systems are soluble in aqueous solutions at lower temperature. However, the solutions become turbid when the temperature is raised to that of the body and settling occurs to form two phases. The more dense, protein-rich phase is called the coacervate. Because the coacervates are the stable state at body temperature, because they contain a similar volume percent of water as fibrous elastin (Partridge, 1967), and because they exhibit filamentous structures in negatively stained electron micrographs (Cox et al., 1973, 1974; Volpin et al., 1976) with periodicities similar to those of native fibrous elastin (Gotte et al., 1974, 1976), coacervation of tropoelastin is viewed as a key step in elastogenesis and the coacervates are considered to be models for relaxed fibrous elastin (Urry, 1976). Coacervates of $\alpha$-elastin, in which all carboxylate groups have been methylated and amino groups formylated, have been shown to calcify from a barbital buffer calcifying medium (Starcher and Urry, 1973) and from a serum medium (Starcher et al., 1974; Cox et al., 1975). Coacervates of tropoelastin, free or chemically blocked by O-methylation and N-formylation, calcify from a serum

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