THE RELAXATION PROPERTIES OF COLLAGEN AND RELATED POLYPEPTIDES

by

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A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1973

This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5/10/73
date

Chairman of Examining Committee

5/10/73
date

Executive Officer

Supervisory Committee

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Abstract

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The dynamic mechanical properties of an enzyme treated steer tendon collagen were studied using a torsion pendulum in the temperature region of 150° to 300° K at 0.3 to 1 cps. The effects of water content, denaturation to gelatin, and crosslinking by formaldehyde were evaluated. Broad-line proton magnetic resonance experiments were also performed on similar samples.

The dynamic mechanical and broad-line proton magnetic resonance experiments were also carried out on poly-L-proline II, which served as a simple model system for the protein.

For collagen in the temperature region studied two relaxation processes were observed: an α transition in the 280°K region, and a β peak, much smaller in magnitude, in the 200°K region. Increasing the water content of these samples has the effect of decreasing the temperature of the α peak substantially while increasing its magnitude. The same qualitative results are seen for the β peak, although the changes are not as pronounced. Although denaturation

of the collagen is shown to leave the temperature position and magnitudes of the two transitions unchanged, the rigidity of the biopolymer increases, as indicated by the level of the storage modulus. Crosslinking increases the rigidity as well as alters the α and β transitions. The β transition is shifted down in temperature to 180°K, while the α transition is diminished in amplitude.

Dynamic mechanical testing of poly-L-proline revealed a relaxation process in the 175°K region accompanied by a seven fold drop in the modulus. The molecular process believed to give rise to this relaxation is a main chain torsional oscillation about the C_α-C=O bond.

The proton magnetic resonance spectra of collagen, gelatin and poly-L-proline contain both broad and narrow components. In collagen and gelatin the narrow line diminished in amplitude with decreasing temperature, and finally was undetectable at approximately 250°K without significant broadening. These characteristics were independent of water content. The narrow line of polyproline broadened on cooling until it was indistinguishable from the broad line. The temperature of broadening was dependent on the water content, decreasing with increasing amounts of water.

The second moments of the wide lines of the dry samples of each of the polymers decreased monotonically as the temperature increased. Added water was observed to sharpen

this drop in certain regions. The regions of steepest decline are the same in gelatin and poly-L-proline II and the region in collagen is not far removed. From temperature-frequency considerations, this drop is correlated with the β mechanical process, while the decline in second moment between 260°K and 300°K is correlated with the α process.

By analogy with poly-L-proline the β relaxation of collagen is assigned to a main chain oscillation in the ordered, apolar regions. From considerations of the results obtained for other polypeptides the α transition is attributable to side chain motion in the polar, disordered regions.

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INTRODUCTION

Collagen has been under study since the early part of this century from the point of view of the classical biochemist. In the 1930's the techniques used by the polymer physical chemist were beginning to find a role in the investigation of the structure and properties of collagen. Since that time wide-angle and low-angle x-ray diffraction, electron microscopy, light scattering and viscosity measurements, and optical rotatory dispersion have yielded a wealth of information which has led to the presently accepted triple helical structure of collagen. This information has also led to a partial understanding of the complicated changes that take place upon aging and denaturation.

We have extended the list of techniques to include the study of the relaxation properties of collagen, gelatin and poly-L-proline II in an attempt to evaluate the structure-function relationship in this protein. One would expect the unique structure of collagen to manifest itself in the relaxation behavior. Also, the effects of aging as related to the degree of crosslinking[1] were studied. The two methods chosen to elucidate the relaxation behavior were nuclear magnetic resonance spectroscopy and dynamic mechanical testing, the latter using a freely oscillating torsion pendulum. Before continuing with a discussion of

these techniques it is necessary that a brief description be given of the structure of collagen, gelatin and poly-L-proline.

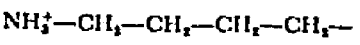
Collagen is the most abundant animal protein, comprising 20 to 25% of the total protein in mammals[2] and roughly 60% of connective tissue[1]. It is found in skin and tendon and is also believed to act as a template for the nucleation of hydroxyapatite to form bones and teeth. The attachment of inorganic crystalline particles at regular spacing on the collagen fibril, called epitaxy, has been amply demonstrated in vitro by x-ray diffraction for various salts[3] and for hydroxyapatite itself[4].

AMINO ACID COMPOSITION

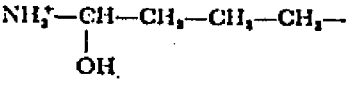
The chemical composition of collagen, given in Table I[1], is unique in that collagen is the only protein containing large amounts of hydroxyproline and having such a high content of glycine and proline. Indeed it is this fact that, to a very large extent determines the secondary and tertiary structure of collagen. The structures of some of the more abundant amino acids are given in Figure 1 for easy reference. Although the actual composition of collagen from different vertebrate and invertebrate sources varies, a general pattern is evident: Very roughly one-third of all residues are glycine; proline and hydroxyproline constitute another two-ninths and alanine another one-ninth. Therefore, together these four amino acids constitute two-thirds of all the residues in collagen, leaving one-third for a distribution

Table I: Amino Acid Composition of Bovine Corium Collagen[1]

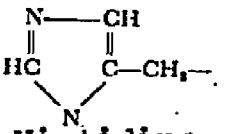
Amino Acid	Residues (per 1000 residues)	Amino Acid	Residues (per 1000 residues)
Lysine	24.8	Glycine	336.5
Hydroxylysine	5.2	Alanine	106.6
Histidine	4.8	Valine	19.5
Arginine	47.9	Methionine	3.9
Aspartic Acid	47.3	Isoleucine	11.3
Glutamic Acid	72.1	Leucine	24.0
Proline	129.0	Tyrosine	4.6
Hydroxy-proline	94.1	Phenyl-alanine	12.6
Serine	39.2	Cysteine	0.0
Threonine	16.6		



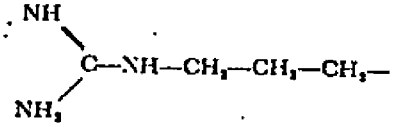
Lysine



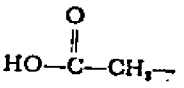
Hydroxylysine



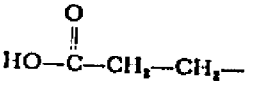
Histidine



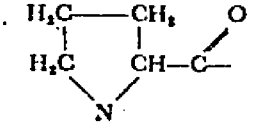
Arginine



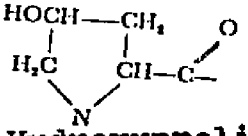
Aspartic acid



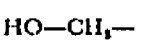
Glutamic acid



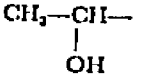
Proline



Hydroxyproline



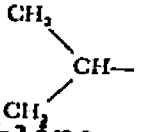
Serine



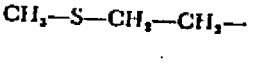
Threonine

Glycine

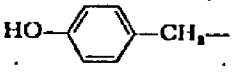
Alanine



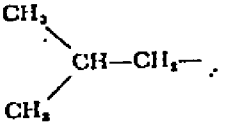
Valine



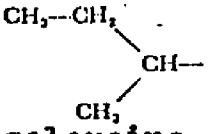
Methionine



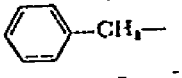
Tyrosine



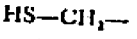
Leucine



Isoleucine



Phenylalanine



Cysteine

Figure 1. Side chains on the α carbon atom of the amino acids.

of the other fourteen amino acids[5].

SECONDARY AND TERTIARY STRUCTURE OF COLLAGEN

The exact manner in which the polypeptide chains with the preceding chemical composition arrange themselves three dimensionally remained a mystery until the 1950's when wide angle x-ray diffraction data were interpreted correctly. The x-ray evidence is as follows: Collagen fibers show a strong meridional reflection of 2.86 °A which increases to 3 °A on stretching of the fiber, an equitorial reflection of 11 °A for dry collagen which increases to 14 °A for wetted fibers, a diffuse, strong blackening on the equator at 4 to 5 °A and a near meridional line at approximately 10 °A and another at 4 °A. An account of earlier proposals offered to explain the data is given by Ramachandran[6]. In this report only the presently accepted structures will be discussed.

On the basis of structural models Ramachandran[7] postulated a modification of the Huggins[8] model whereby three residues giving rise to the 2.86 °A repeat were in three separate, mutually hydrogen bonded, left handed helices arranged parallel and mutually equidistant to each other. Several faults became apparent with this structure, one being that the sequence glycine-proline-hydroxyproline, found to be prevalent in collagen by chemical analysis of the oligopeptides resulting from enzyme degradation, could not be accomodated. Another objection was that the 2.86 °A spacing observed would have to be off meridional; A third objection was that the number of scattering units per turn

would have to be 3.33 and not 3.

To obviate these faults it is only necessary to twist the three coils about a common axis. This manipulation led to the accepted coiled-coil, or superhelical structure.

This structure was shown by Ramachandran[9] to involve a slow right handed twist of the three left handed coils about a common axis such that of the three possible hydrogen bonds only two could be formed per three residues[10]. Rich and Crick[11] postulated another structure on the basis of their structure for polyglycine II. (The polyglycine II structure is a three residue per turn helix arranged with its neighbors hexagonally close packed.) They placed the individual collagen polypeptide chains in the polyglycine lattice, chose three of these strands in an equilateral triangle and coiled them about each other in a right handed helix whose center was the center of the triangle. Actually two structures could be obtained depending on which three strands were chosen. One choice led to hydrogen bonds formed between the N-H of glycine and a carbonyl on the next chain. If one looked down the axis of the superhelix the hydrogen bonds could be seen to spiral up the helix in a right handed manner. The other choice of chains led to hydrogen bonds spiralling up the helix in a left handed manner. These were named the collagen I and II structures. They differed from Ramachandran's two bonded model by allowing only one hydrogen bond per three residues rather than two. It should

be mentioned that in all these helical structures glycine is required to be in every third position along the polypeptide chain.

Some of the obvious differences of the three structures are that the distance of the α carbon atoms in glycine from the central axis of the super-coil is 3.1 ºA in collagen I, 1.6 ºA in collagen II and 1.15 ºA in the two bonded model. There is also a difference in the mode of hydrogen bonding. In collagen I the N-H of glycine is bonded to the carbonyl of glycine on the next chain, in collagen II it is bonded to the carbonyl of residue 2 on the next chain and in the two bonded structure to residue 3 in addition to the two residues in the "two" position being hydrogen bonded to each other[12]. Until recently evidence favored the two bonded structure[5]. However, the most recent evidence of Chapman[13] and Segal[14] favor the collagen II structure of Rich and Crick. Incidently, Rich and Crick themselves preferred the II structure to the I on the basis of steric requirements. Chapman using the dependence of deuterium quadrupole splitting on the orientation of collagen fibers deduced a structure satisfying only the collagen II model. Segal arrived at the same structure using x-ray diffraction methods. However, the question is still far from resolved.

THE TROPOCOLLAGEN MOLECULE

Zachariades in 1900[15] observed that there was a fraction of collagen fibers that would dissolve in cold dilute acetic

acid solutions. Later Nageotte[16] discovered that upon dialysis the dissolved collagen reformed into native fibers that precipitated from solution. The work of Gross et. al.[17,18] led them to postulate the existence of a unit which would explain the diverse results they obtained. This unit was named "tropocollagen"-the collagen former. Using electron microscopy they observed that soluble collagen, when precipitated from solution under carefully controlled conditions, formed not only the native fibril with its 640 °A periodicity, but also "fibrous long spacing" (FLS), and "segmented long spacing" (SLS) segments. These results are outlined schematically in Figure 2, which also shows that each form is completely converted to every other form through the intermediate soluble collagen. The tropocollagen unit having been hypothesized it was now time to characterize it.

Boedtker and Doty[19] using light scattering methods established the molecular weight of soluble collagen as 345,000; osmometry produced a number average molecular weight of 310,000, indicating a relatively monodisperse species. Viscosity measurements indicated a rigid rod, roughly 3000 °A in length and 15 °A in diameter.

Several models have been proposed using this 3000 °A tropocollagen unit to explain the 640 °A periodicity of collagen fibrils observed in both low angle x-ray diffraction[20-22] and electron microscopy[23].

One model is the quarter stagger model in which each tropocollagen unit is laid down on a fiber adjacent to

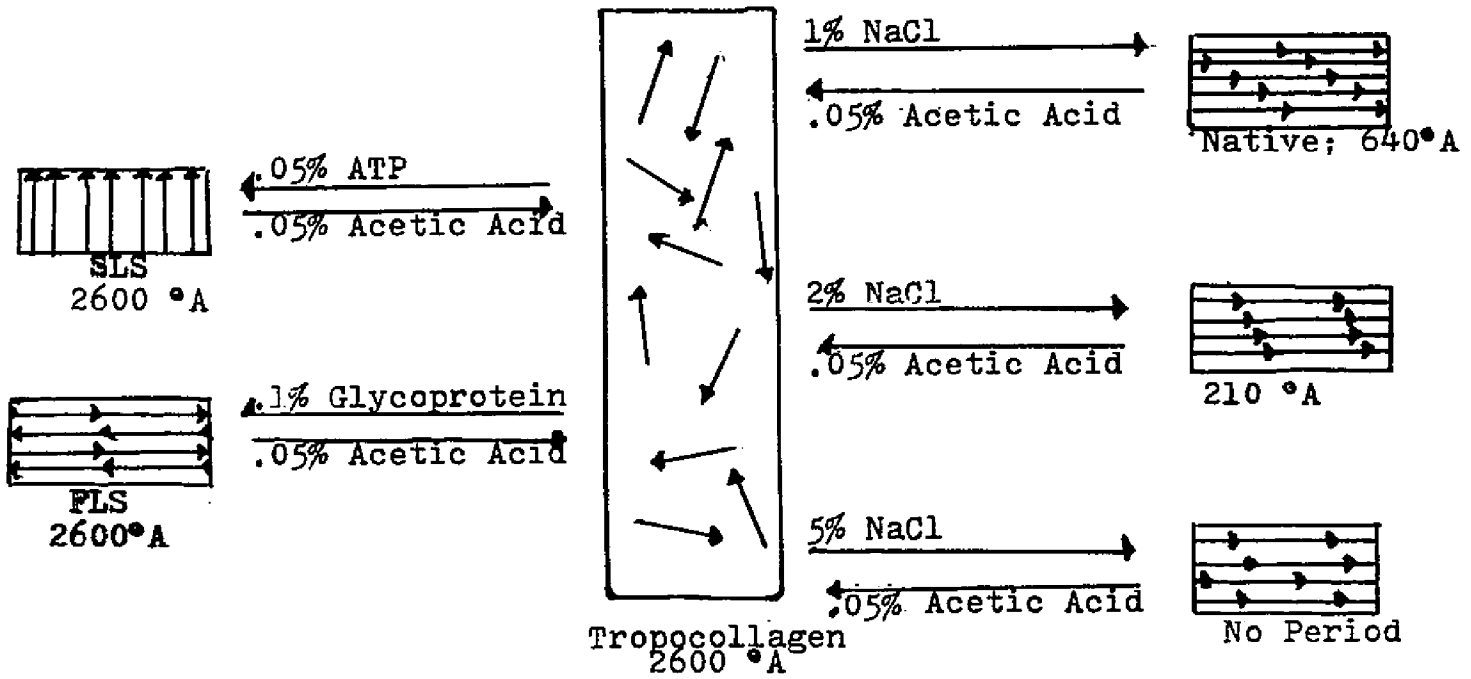


Figure 2: The reconstituted forms of collagen. (from "The Structure of Collagen and Gelatin", by W.F.Harrington & P.H. Von Hippel, in Adv. in Prot. Chem., Vol.16, C.B.Anfinsen,M.L.Anson,K.Bailey and J.T. Edsall Eds., Academic Press, New York & London, 1961, p.62.)

another tropocollagen unit staggered by one quarter of its length. This was proposed by Schmitt[24], Later it was redemonstrated by Hodge and Schmitt[25], who showed that SLS crystallites deposited on native fibrils of collagen with a preferred direction. Also the electron microscope showed that the stained regions on the freshly deposited SLS segments lined up perfectly with the stained regions of the native fiber. Subsequent to a more accurate measurement of the length of the tropocollagen unit[26] Hodge and Petruska were led to modify the quarter stagger model to give a new model which involved a staggered arrangement of tropocollagen units incorporating hole regions with a depleted density of material[27].

In any case once the tropocollagen unit is deposited the aging process begins. Initially, the freshly deposited tropocollagen is solubilized by dilute neutral salt solutions or mildly alkaline solutions. The resulting solution is the individual polypeptide chains comprising the triple helix. Later in time after inter- and intramolecular crosslinking has occurred, the tropocollagen unit itself is solubilized by dilute acetic acid solutions. The neutral salt soluble fraction is no longer obtainable. Still later the crosslinking is so extensive that no form of collagen, as such, is soluble, and only relatively harsh conditions will dissolve the protein[28].

ORDERED AND DISORDERED REGIONS IN COLLAGEN

Until now no mention has been made of the degree of helicity along the tropocollagen molecule. Is it entirely helical and ordered or is it only helical in certain regions? The results of many experiments suggest that the latter is true. A cursory examination of this evidence will lend credibility to any further reference to this type of structure.

The structural models of Ramachandran and Rich and Crick used to interpret the x-ray data available were idealized. They were constructed with glycine in every third position and proline and hydroxyproline in the next positions with only a slight margin for other amino acids. If this were the actual sequence along the entire polypeptide chain, we would expect complete helicity. To strengthen this point there is compelling thermodynamic evidence that the stability of collagen, as indicated by the shrinkage temperature or the melting point, increases with pyrrolidine (proline, hydroxyproline) content[29-32].

First to be established will be the fact that there are alternating regions along the collagen fibril with different properties. Then the chemical composition will be presented to show that glycine, proline and hydroxyproline are not evenly distributed between these regions, and hence the stability of the helix in regions deficient in these amino acids is decreased. Thirdly it will be established that these regions are not chemically negligible.

That there are regions of different properties is clearly seen with the electron microscope using various staining agents. When chromium is used as a stain the native collagen fiber shows regions of intense staining alternating with regions of less staining. These regions are called band and interband regions[23,33,34]. Bear suggested that these were due to periodic alternations of chemical composition along the protofibrillar axis, and that in the laying down of the protofibrils on a fiber these regions lined up[20-22]. Much more detail in the striations is seen when either uranyl nitrate or phosphotungstic acid is used for staining[25]. In fact, evidence for these alternating regions in the tropocollagen unit itself is given by the staining of the segmented long spaced aggregates of the individual tropocollagen units[25]. That these regions of intense staining reflect some chemical difference can be inferred from the fact that the stain PTA is an acid, and will combine with the basic groups present in collagen, and uranyl nitrate will combine with available anionic groups. Since the stain is not evenly distributed, the groups such as the basic side chain of lysine and the anionic glutamyl side chain are not evenly distributed either. Kuhn convincingly demonstrated that the band regions indeed were rich in acidic and basic amino acids and that the interbands were rich in apolar amino acids[35-38].

The actual chemical composition of these polar and non-polar regions comes from chemical analysis of enzymatically digested collagen samples. Conveniently, peptide bonds in the separate regions are attacked selectively by two different enzymes- trypsin and collagenase. Trypsin specifically cleaves the peptide bonds of the residues found in the polar, band regions observed in the electron micrographs, leaving intact the peptide bonds of the residues in the non-polar regions. The opposite is true of collagenase[5]. This information was obtained by actually monitoring the enzyme digestion of stained segmented long spaced forms of reconstituted collagen with the electron microscope. These facts corroborate the non-homogeneous arrangement of polar and non-polar regions, since a direct relation is actually seen between the known activity of the enzymes and the different regions of collagen as seen in the electron microscope. By enzymatic cleavage, separation and analysis it was shown that 97.6% of the interband regions were neutral amino acids. Specifically these were: 85% glycine, proline, hydroxyproline and alanine and 12.6% other neutral amino acids. The remaining 2.4% was composed of 1.6% glutamic acid and 0.8% other polar amino acids[39,40]. By collagenase digestion, the polar regions were found to contain chains of the following composition:

ala-pro-(gly_8,ala_2,glu_2,asp_2,phen,thr)-pro-ala

and

gly-pro-(gly_6,ala_3,val_3,leu_2,lys_2,glu_2,asp,phe,ser)-pro-phe[41].

To generalize, the crystalline, non-polar regions, rich in pyrrolidine residues, contain the sequence gly-pro-X where X is alanine, hydroxyproline, less frequently ser, threo, leu and rarely lys, glu,asp. The amorphous polar regions, are enriched in glutamy, aspartyl,lysyl, histidyl, residues and tyrosine hexoses[28]. The disordered polar regions are unique also in that the γ-glutamyl bond is found incorporated in the polypeptide backbone[28,42], making the possibility of finding stable helical structure even less likely. The regions of polar and non-polar amino acids in collagen fibers necessarily imply a similar alternation in the individual polypeptide strands comprising the triple helix. Although these individual strands were shown to have slightly different amino acid compositions they have identically spaced regions of polar and non-polar character[5]. Sequence studies have shown that the N-terminal region of these polypeptide chains is non-helical[12] and recent evidence shows that the carboxy terminal region is also non-helical[43,44]. Having found that these specific regions are non-helical, of course, does not rule out the possibility that there may be other such regions within the polypeptide chain.

> "...in any case, one no longer thinks of the collagen molecule as assuming a single uninterrupted type of conformation for the entire

length of its structure"[28].

Bear first suggested that chemical attack should occur at the looser more penetrable band regions[20], and inferred that water actually penetrated the disordered regions and smoothed out the corrugated appearance of the collagen protofibril in the optical microscope. Ciferri, in studying the interaction of salt solutions with proteins, was led to the same conclusion since the swelling of collagen on imbibing water did not affect the ordered crystalline regions perceptibly, as indicated in the x-ray diffraction patterns[45]. Bailey and Rhodes[46] used the disordered regions of collagen to explain the results of their irradiation induced crosslinking. They stated that the side chains of the residues in the disordered regions projected radially from the backbone and crosslinks formed first with these side chains. Okamoto and Saiki claim to have studied the mechanical and dielectric relaxations of collagen attributed to motions of the dipoles in the disordered regions[47,48]. It is now apparent these disordered regions should not be neglected when interpreting chemical and mechanical experiments.

DENATURATION OF COLLAGEN

Proteins when subjected to harsh environments, chemical or thermal, undergo drastic alteration in quarternary, tertiary, secondary and in some cases even primary structure. These alterations are combined under the general term denaturation. In the case of collagen, denaturation can lead

to a useful and desired product called gelatin[1]. Depending on the physiological type and age of the collagen and on the denaturation conditions employed a wide variety of gelatins can be produced, differing in the extent to which the denaturation has proceeded. From the point of view of the chemist seeking a means of unraveling the secrets of the collagen superstructure, the one gelatin most desirable is "parent gelatin". This parent gelatin is derived from monodisperse solutions of tropocollagen particles subjected to mild denaturation conditions. An enormous amount of information has been obtained on collagen by studies eventually utilizing this gelatin form[5].

Boedtker and Doty[49], using a variety of solution techniques such as viscometry, ultracentrifugation, osmometry, etc., showed that soluble icthyocol collagen from carp swim bladder tunics was a rigid rod (tropocollagen) and that upon denaturation it yielded three polypeptide chains in solution whose number average molecular weight was 138,000. The configuration of these chains in the solution was most closely random coil as suggested by earlier measurements by the same authors[50] and others[51]. Gallop[52] carried out determinations of the intrinsic viscosity, sedimentation coefficients, partial specific volume and molecular weight by light scattering on parent gelatin. He concluded that the configuration of parent gelatin in solution was either a prolate ellipsoid (20 X 400 °A) or a random coil with an end to end distance of

225 Å.

It was also found, as might be expected, that association can occur[53]. The particles in solution were found to have molecular weights two and three times the molecular weight of the individual polypeptide chain. To summarize these findings briefly: The tropocollagen molecule is comprised of three polypeptide chains referred to as α chains. There are two types differing slightly in hydroxyproline, proline, alanine, valine, isoleucine, leucine, hydroxylysine, lysine and histidine content. To distiguish these, we call one α_1 and the other α_2. In general it seems that collagen contains the two in the ratio of 2:1 (α_1:α_2). With a small degree of crosslinking, natural or artificial, the denatured soluble collagen will give β_1 and β_2 chains in addition to the α chains. Two α_1 chains are used to make the β_2 chain, and β_1 is the equivalent of a one to one mixture of α_1 and α_2. The molecular weight of the β is approximately twice that of the α chains. Upon further crosslinking all three α chains become covalently linked and the solution of denatured collagen will show the γ component with a molecular weight of three times the α chain value (the molecular weight of tropocollagen)[28,53]. This latter form shows a marked tendency to partially renature, giving regions of collagen type structure. This property indicates that the crosslinks have forced the maintenance of a proper longitudinal register such that the appropriate regions of the α chains can reaggregate adjacent to each other and

form the collagen fold.

Having thus far considered the nature of gelatin in solution, what can be said about gelatin in the solid state? As before the answer depends on the conditions used to denature the collagen. However, it is a fact that collagen, swollen or not, shrinks when it is raised above a critical temperature dependent only on the degree of swelling and on the source and age of the collagen. Thermal shrinkage of collagen was shown by Astbury[54], using x-ray diffraction, to involve a transition from a highly ordered crystalline state to an amorphous one. Flory[55,56] argued that the shrinkage phenomenon was a true phase transition characterized by a melting temperature and did not involve formation of a gelatin. The latter part of this argument being based on the fact that no water soluble fraction could be obtained from the melted collagen after it had cooled. This is not a surprising result in itself since the sample of collagen was beef achilles tendon and probably highly crosslinked due to the age of the animal. In the second of the two papers by Flory it was shown that gelatin-glycol systems showed a melting process 2°C removed from that of the collagen-glycol mixtures. By extrapolation to infinite swelling (equivalent to dissolution) the melting temperature becomes coincident with the denaturation temperature observed in soluble collagen as discussed earlier in this paper. The logical conclusion, therefore, is that melting of

swollen solid collagen (shrinkage) involves the same molecular process as the denaturation in solution-that is, unraveling of the collagen superhelix.

It seems now we have a paradox. One the one hand Flory states that swollen collagen melts, shrinks and does not form gelatin; and that gelatin has the same melting point as collagen. Therefore to go one step further, the crystallites in gelatin are identical to those in collagen. On the other hand, we have that in solution denaturation to gelatin involves the same molecular processes as melting. This issue is worth clarifying since the explanation gives insight into the nature of gelatin gels and shrunken collagen.

When in solution collagen, when heated above its denaturation temperature, forms parent gelatin. The parent gelatin is shown to be α chains in random coil form. Upon cooling, the viscosity and optical rotation increase and, depending on the concentration, gelation may or may not occur. In dilute solutions (less than 0.5%) the random coils do not overlap and each coil remains independent of the others; the infinite network required for gelation does not form and the gel does not set. However, as indicated by viscosity and optical rotation, the chains are forming limited regions that are helical as they were in the α-peptide chains in the collagen molecule. In solutions whose concentrations exceed 0.5% gelation does occur since the random coils overlap each other and by various interactions form stable junction points

that act as crosslinks in an infinite network. These regions, involving two or more peptide chains, are the crystallites that exhibit a melting point and x-ray diffraction pattern similar to that of collagen. The process just described is gelation, and should not, by any means be confused with renaturation. The latter is the complete and accurate reformation of the native collagen fibrillar structure. This would involve such a precise alignment of the α chains along their entire length as not to be possible in dilute solutions. However, note that this is, in part, possible for the γ chains as was mentioned previously.

Now according to Flory the melting of collagen in the solid state occurs with shrinkage and upon cooling no gelatin can be found. Obviously, the α chains have not moved far from their proper alignment* and/or the crosslinking of an aged specimen maintains the proper alignment, as it did in the γ chains of parent gelatin. In either case, the melting process is now completely reversible with practically total renaturation. The absoluteness of the renaturation is qualified since even in the samples used by Flory a latent

*That there is initially, in all probability, hardly any randomization of chain alignment in melted polymers, not subjected to any mechanical manipulation is discussed in "Single Crystals" by P.H. Geil, Interscience, New York, 1963 pp. 5-6.

volume change was seen to occur irreversibly on the first melting, thus indicating imperfect reversibility. In line with this reasoning is the work of Wright and Wiederhorn[57], who demonstrated that the spacings obtained by wide angle x-ray diffraction of native collagen and restretched shrunken collagen were not sufficiently different to allow any distinction between the two, but the twenty-one orders of the fundamental 640 °A spacing, obtained in the low angle x-ray diffraction experiment, were not recoverable once the sample had been shrunk. This indicated, of course, that the sample had failed to return to its original long range order.

The results of numerous studies show that gelatin in the solid state, swollen with various fluids obeys the laws derived for unoriented polymers. For example Wiederhorn and Reardon[58] showed that swollen gelatin obeyed rubber elasticity theory. For small elongations a graph of f vs $T(\alpha-\alpha^{-2})v_2^{\frac{1}{2}}$, where f and α are the stress and strain respectively, was shown to be linear as it should be for an ideal unordered network. Another study was undertaken by Preston and Meyer[59] which also established the amorphous nature of solid gelatin. Here the swelling behavior was studied in various buffers and dextran 5000. The results showed that gelatin accurately obeyed the theory of Flory and Rehner[60,61] on the thermodynamics of swelling of amorphous network structures. Hence we may conlude that shrunken collagen is a semi-crystalline structure having a short range similarity to collagen but in general it behaves as an amorphous network

polymer.

CROSSLINKING AND AGING OF COLLAGEN

As mentioned earlier (p. 10) tropocollagen once deposited on a fiber of older collagen begins a process of progressive insolubilization which is believed to be due to covalent intermolecular crosslinks of an ill-defined nature. However, there is evidence that the amino group on the side chain of lysine is involved in the formation of these crosslinks[12]. Many reagents have been used to artificially crosslink collagen. Chromium salts have been used in industry by leather manufacturers, and many studies of chromium tanned cowhides can be found in the journals of this trade and in a review by Gustavson[62]. For research purposes the usual crosslinking agent is an aldehyde. Several studies have concentrated on the nature of the aldehyde itself,i.e. the molecular weight, stereochemical structure and chemical functionality[63,64]. The general conclusions are that for monofunctional and bifunctional aldehydes that crosslink collagen, the sites involved in the collagen were similar although the end products were probably different. As far as chemical functionality is concerned a substance such as glyoxal, although similar to formaldehyde appears to be more efficient in crosslinking collagen. However, somewhat paradoxically it was found that even though the minimum attainable molecular weight between crosslinks was much less for glyoxal and glutaraldehyde as

as compared to formaldehyde, indicating a greater degree of crosslinking, the shrinkage temperature which increases with degree of crosslinking reaches the same value in all three cases[64].

As for the particular chemistry involved, Fraenkel-Conrat and his co-workers[65-68] showed that formaldehyde formed a methylene bridge between the reactive species involved when it reacted with amino acids and proteins.

A) $R'NH_2 + HCHO + R''NH_2 = R'NH-CH_2-NHR''$

B) $R'NH_2 + HCHO + R''CONH_2 = R'NH-CH_2-NHCOR''$

C) $R'NH_2 + HCHO + R''-\underset{\underset{NH}{\|}}{C}-NH_2 = R'NH-CH_2-NH-\underset{\underset{NH}{\|}}{C}-R''$

It is believed that in each case the reaction proceeds via the initial formation of the amino methylol derivative of an uncharged primary amino group followed by a condensation reaction with the remaining reagent involved. The maximum number of crosslinks formed in collagen is probably ten crosslinks per tropocollagen unit[5].

This evidence together with the work of Bornstein et.al.[69,70] gives an indication as to which groups are involved in crosslinking and where they are located. Using cyanogen bromide cleavage under conditions not affecting the helical regions of collagen, it was possible to ascertain the regions of crosslinking. These proved to be the N-terminal regions. They found for the α_1 chains the composition of the N-terminal to be:

H-gly-tyr-asp-glu-lys-ser-ala-gly-val-ser-val-pro-gly-pro-met.

Similarly they found for the α_2 chain:

H-glu-tyr-ser-asp-lys-gly-val-ser-ala-gly-pro-gly-pro-met,

where the cyanogen bromide cleaves the bond after the methionine. Notice that both these chains have a lysyl residue in the fifth position. It is this lysyl group believed to be involved, via the δ-semialdehyde of α-aminoadipic acid, in naturally occurring crosslinks of collagen. Also it is obvious, since this N-terminal region is not expected to be helical, and therefore more easily penetrable, that this same lysyl group may be most subject to artificial formaldehyde crosslinking.

WATER IN COLLAGEN

Reviews exist concerning the role of water in biological systems[71] and in collagen in particular[72]. The interactions between water and the protein are still largely unknown; however it is known that the interaction is specific and indispensible to the structural integrity of fibrillar collagen. This fact is demonstrated in several reports of permanent alteration to the collagen structure by removal of the last traces of water[73-75]. Most convincing were the electron micrographs of collagen reported by Ramanathan,[76] who observed that the fibrous striated structures found in native collagen did not appear in thoroughly dried samples. An interesting note at this point is that Ramanathan found that even prolonged treatment of collagen with P_2O_5, a

strong drying agent, in vacuo, did not remove all the water bound to collagen, but that extended heating at 100°C was necessary. This finding indicates that some fraction of water normally associated with collagen is extremely strongly bound. There is also the possibility that there are several kinds of bound water in collagen. Calorimetric studies performed by Haly and Snaith[77], using the heat of fusion and the temperature of fusion, resolved four types of water in the collagen structure and three in the denatured structure. These are: a) unfreezable water at concentrations less than 0.3 grams of water per gram of dried protein, b) freezable water with an altered heat of fusion in the concentration range of 0.3 to 0.6 grams of water per gram of dried collagen, c) freezable water with the heat of fusion of bulk water but a depressed freezing point, existing in the 0.6 to 0.9 gram range and d) water identical in properties to bulk water. Only the first three were found in gelatin. Hence we may conclude that water plays a more complex role in the collagen structure than is immediately obvious.

POLY-L-PROLINE II

Poly-L-proline II was chosen as a model polypeptide for the collagen system for two reasons. First, together with hydroxyproline, proline residues are involved in the stabilization of the collagen helix[29-32]. Second, the structure of the poly-L-proline helix is very similar to

that of the α-chains in collagen prior to their twisting into the superhelix.

In examining the melting points of collagens derived from a variety of sources, differing in total pyrrolidine content, Josse and Harrington found that the melting point was an increasing function of this content[78]. A theoretical analysis of their results indicated that the required stability of the helical structure was most closely afforded by a two hydrogen bonded structure. The hydrogen bonds contributed to the enthalpic stabilization while the pyrrolidine residues contributed to the entropic term in the free energy expression. This entropic contribution arises from the fact that the configurations of the random coil obtained by melting are increasingly restricted as the content of proline or hydroxyproline is increased in the chain. This in turn arises from the loss of rotation about the C_α-N bond that is now incorporated in the "side chain" five membered ring. Hence we see that a high pyrrolidine content is necessary for the maintenance of the collagen triple helix.

In solution and in the solid state poly-L-proline exists in two forms designated I and II. While in solution the two forms are readily interconverted. By simply adding n-propanol or n-butanol to an acetic acid solution of II the I form is obtained. The change can be monitored by observing any parameter sensitive to chain conformation, such as specific rotation or viscosity[79]. For example, the

I form has a specific rotation of 40° in formic acid, acetic acid or propionic acid and the mutorotated II form has a specific rotation of -540°. The structure of these molecules in solution is not a simple matter to investigate and many hypotheses have been proposed. Whatever the case may be, it is not valid to assume that the structures in solution are identical to those in the solid state. In what follows the structures of the solid forms will be discussed and when form I or form II is mentioned it will refer to the solid obtained from solutions of I or II that is meant. On the basis of x-ray diffraction evidence on low molecular weight poly-L-proline, Cowan and McGavin[80] offerred a structure for poly-L-proline II. They suggested a left handed helix with a rise per residue of 3.12 °A, and three residues per turn. Their sample was prepared by casting a film from a solution in m-cresol, which was shown to be a form II solvent[81]. In similar studies on form I, Traub and Shmueli[82,83] deduce the right handed helix with a rise per residue of 1.90 °A and with three and one-third residues per turn. This helix is a much tighter one than that of form II. It is now commonly believed that the groups involved in the peptide bond, designated "ω" in Figure 3 are planar. The trans configuration is favored but cis can also be found. The two cases correspond to $\omega = 0°$ and 180° (trans and cis respectively). The remaining bonds ϕ and ψ are free to rotate. Specifying these bonds, therefore, suffices to describe the conformation of any helix[84]. However, in

polyproline the ϕ bond can not rotate since the side chain of this polypeptide folds back on the main chain forming the rigid five membered ring. Φ has the value of 120° in the convention set forth in reference 84. Remaining then is the ψ bond. Steinberg demonstrated, using space filling models, that although some rotation was allowed, the ψ bond was also severely restricted in polyproline because on rotation of this bond the carbonyl oxygen interfered with the β carbon's hydrogens[85]. There were two favored conformations called cis' and trans' (using the carbonyl and α carbon's hydrogen as the basis for this nomenclature). With all these restrictions only four possibilities remain for the conformation of the poly-L-proline chain. These are shown in Figure 3.

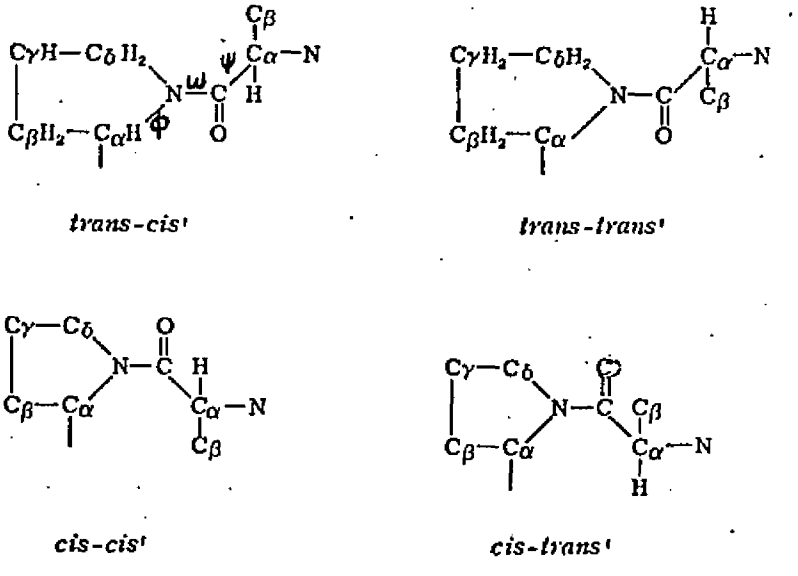


Figure 3. The four possible arrangements of the bonds in the polyproline chain.

The trans-trans' form and the cis-trans' form are the Cowan-McGavin helix and the Traub and Shmueli helix respectively, where the unprimed trans and cis refer to the peptide bond, and the primed are for the $C_{\alpha}CO$ bond. Although the trans' conformation corresponds to an energy minimum, Steinberg estimated there was an allowable rotation to the extent of $\pm 30^{\circ}$ in the Cowan-McGavin helix, DeSantis et.al.[86] performed a calculation to obtain the potential energy of the poly-proline chain as a function of the angle ψ after having fixed ω and ϕ. Minima in the potential energy curves were found only for two values of ψ. One was at 345°, which corresponded to the Traub and Shmueli helix, and the other was a broader curve having one well defined minimum at ψ equal to 335°, corresponding to the value expected for the Cowan-McGavin poly-L-proline II helix. On this same curve was a broad shoulder at ψ equal to 300°. Although the polyproline I and II helices are equally stable when ψ equals approximately 340°, the II form has a greater freedom of rotation, indicated by the range of values for ψ which does not represent too great a loss in energy. DeSantis et.al. arrive at a value of approximately 70° for this rotation as compared to Steinberg's 60°. The angles mentioned correspond to an energy sacrifice of 6-7 kcal. In the polyproline I case a corresponding loss in energy is obtained by rotation of ψ by only 20° (i.e. $\pm 10^{\circ}$). Other workers[87] substantiated the above conclusions using a different approach.

DYNAMIC MECHANICAL TESTING

This method of testing polymers involves measuring the response of the polymer sample to a stress pattern. The force may be a continuously driven type, as in a vibrating reed experiment[88]. The sample is forced to vibrate at a frequency which is continuously changed by a variable frequency oscillator. When a resonant frequency of the polymeric sample is reached the amplitude of the vibration of the free end of the sample goes through a maximum. From this frequency Young's modulus is calculated, and from the width of the peak a damping term is obtained.

In the free oscillation experiment the force is applied at an instant and the sample's response is monitored as it returns to its original state free of the influence of any applied forces. With the torsion pendulum shear force is applied to induce the desired amplitude of oscillation. The rate of decay of the oscillation is related to a damping term, the logarithmic decrement, and the frequency of the oscillations is related to the shear modulus[89]. A third technique is a forced vibration experiment, as was the first, but it is a non-resonance technique similar to the second. Examples are the vibron and the rotating beam apparatus.

In such dynamic experiments the modulus or its reciprocal (compliance) is most conveniently expressed as a complex number[90]. For the shear modulus:

$$G^* = G' + G''$$

where G' is the storage component of the modulus, related to the energy stored and recovered in a cycle of deformation, and G" is the loss modulus, related to the energy dissipated per cycle of deformation[91]. When spring and dashpot models such as the Voigt or Maxwell models are used some insight into the nature of G' and G" is gained. For a Maxwell element one obtains the storage and loss moduli as functions of the frequency of the experiment; thus,

$$E'(\omega) = \frac{E\omega^2\tau^2}{1+(\omega\tau)^2} \qquad \text{and} \qquad E'' = \frac{E\omega\tau}{1+(\omega\tau)^2}$$

where ω is the frequency of the applied stress and τ is the relaxation time. Examination of these expressions shows that at low frequencies and high frequencies E" is small and at the frequency $\omega = 1/\tau$ it passes through a maximum. Hence, when the frequency of an experiment is in the range of the reciprocal of the relaxation time of a molecular process, a maximum in the loss component can be expected. A mechanical damping term $\tan\delta$, where δ is the phase lag between the applied stress and the strain, is shown to be equal to the ratio of the loss to the storage modulus. This damping term is related to the logarithmic decrement obtained in the torsion pendulum experiments[92]. The case in real polymer systems is that E" or $\tan\delta$ has several maxima which can usually be correlated with the onset of a particular kind of molecular motion, either main chain or side chain[93-96]. As indicated by these reviews and the results of many other experiments, dynamic mechanical testing is a sensitive tool

to investigate glass transitions, the effects of molecular weight, crosslinking, crystallinity, copolymerization, molecular aggregation and so forth.

NUCLEAR MAGNETIC RESONANCE

The phenomenon of magnetic resonance was first described by Purcell and co-workers[97] and Bloch[98]. Originally it was only a field of research restricted to the physicist. Eventually its utility as an analytic tool became apparent, and nuclear magnetic resonance spectroscopy of liquids and solutions evolved as a routine method of organic structure determinations. With the development of the capacity to generate larger, homogeneous, stable magnetic fields, the technique was extended to the study of systems too complicated to be observed with weaker fields. As will become apparent later the resolution of the spectrum increases with the strength of the magnetic field. Polymer solutions are just one example of these systems rendered accesssible to n.m.r. investigations[99]. The foregoing aspect of n.m.r. is referred to as high resolution n.m.r. Another aspect is low resolution or broad line n.m.r. Operating in this mode the technique is now applicable to investigating solids and very viscous liquids. It is this aspect for which a detailed discussion follows.

For the purposes of understanding the fundamentals of the relaxation experiment a simplistic quasi-classical approach is suitable and will be outlined here. A more accurate and detailed description of the magnetic resonance

phenomenon is given by a quantum mechanical formulation found in many texts[100,101].

A nucleus possessing a total angular momentum, $\vec{p}$, has associated with it a magnetic dipole, $\vec{\mu}$. The two vectors are parallel and related by:

$$\vec{\mu} = \gamma\vec{p}$$

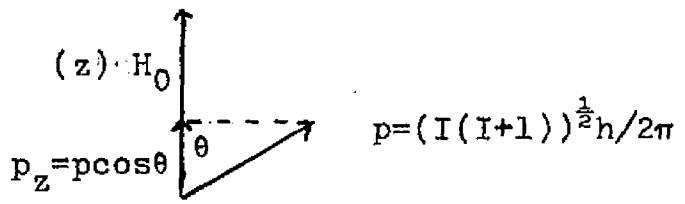
where γ is the gyromagnetic ratio, which is constant for any particular nucleus. The angular momentum is related to a property called the spin of the nucleus and is expressed in terms of this spin by:

$$p = I(I+1)^{\frac{1}{2}}h/2\pi$$

where I is the spin. On both theoretical and experimental grounds "I" can take on only certain values. In addition, the components of this spin vector on a given axis in space are quantized and described by quantum numbers "m". These quantum numbers can have the values ranging from -I to +I in integer steps, giving a total of 2I+1 states. These states are degenerate as long as there is no unique was of defining a direction in the molecular system. More specifically, when a magnetic field is applied to the system the states split into different energy levels according to their components in the magnetic field direction. This field arbitrarily defines the "z" axis by convention. The interaction energy of the magnetic dipoles of the nucleus and this magnetic field is given by:

$$E = -\vec{\mu}\cdot\vec{H}_o$$

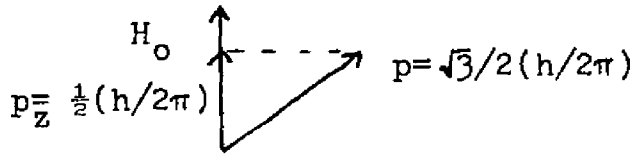
The magnetic dipole has quantized components along the direction of the magnetic field (H_0) by virtue of the fact that it is linearly related to the spin through the angular momentum vector, i.e.



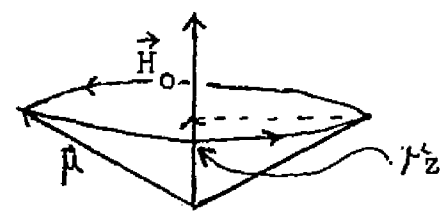
hence $\mu_z=\gamma p_z=\gamma p\cos\theta = \mu\cos\theta$. Therefore,

$$E = -\mu H_o\cos\theta = -\mu_z H_o$$

This point becomes clearer when we look at the specific example of the hydrogen nucleus. For this nucleus $I=\frac{1}{2}$ and therefore $m=\pm\frac{1}{2}$.



The energy of interaction is now $E=\pm(\frac{1}{2}h/2\pi)\gamma H_o$, and we see that the nuclear spins are split into two energy levels, differing in energy by $\gamma(h/2\pi)H_o$. The magnetic dipole may be visualized as precessing about the direction of H_o. This visualization is arrived at by either using a rotating coordinate system or by considering the case of a classical magnetic dipole in a magnetic field.



The precession frequency ω is $-\gamma H_o$ and is the Larmor precession frequency of the latter classical dipole consideration. The x-component of the magnetic dipole describes a circle in the x-y plane with angular velocity, ω. This frequency can also be arrived at by returning to the energy level picture. A radio frequency applied in a direction perpendicular to H_o (in the x-y plane) will cause a transition from one level to another. The energy difference and hence the frequency is given by:

$$E=h\nu = \gamma(h/2\pi)H_o$$

$$2\pi\nu = \gamma H_o = \omega,$$

which is the Larmor precession frequency. So the resonance condition involves matching the frequency of precession of a nuclear dipole with a radio frequency applied in the x-y plane. In a field, H_o, of 10,000 gauss this frequency is 42.6 megacycles/sec.

If this were the entire story all protons would resonate at the same frequency and the technique of n.m.r. would be useless for structural investigations. The magnetic field experienced by a particular hydrogen nucleus is not just the field applied, it is also the sum of any local fields caused by surrounding nuclei and electrons. For high resolution n.m.r. the local fields giving rise to the chemical shift, which is the difference in frequency of resonance of the proton as compared to some standard, are a result of the perturbations of the electrons in bonds near

the resonating protons nucleus, caused by differing chemical environments. For example, a proton near a carbonyl group has a resonance frequency different from that of a proton near an amino group. As might be expected these are extremely small effects and are completely unresolvable in wideline n.m.r. Here the motion of the nuclei in the solids or viscous liquids studied is so slow that the direct interaction of the nuclei through the intervening space is not averaged out and broadening of the resonance condition for any one hydrogen nucleus results. As will be shown later this broadening can be many gauss wide compared to the chemical shifts which are measured in parts per million of the applied field. This averaging out of the dipoles by the random tumbling of the nuclei is the basis for motional narrowing as will be apparent later.

SPIN LATTICE RELAXATION

In the absence of a strong magnetic field the energy states of the nuclear dipoles are degenerate and equally populated. Once thrust into a large magnetic field, the populations change in such a way as to favor the lower energy level. The system can be expected to obey Boltzmann statistics and the relative populations will be given by the Boltzmann factor. Without going into detail, the expression describing the relaxation to equilibrium is given by an equation similar to that describing the charging of a capacitor.

$$n=n_o\ (1-\exp(-t/T_1))$$

where "n" is the excess number of nuclei in the lower energy state, "n_o" is the equilibrium value of n, and T_1 is the spin-lattice relaxation time. The actual interaction causing the relaxation is, as the name implies, one between the nuclear dipole and the surrounding lattice. Motions of the surrounding lattice can be viewed as fluctuating magnetic fields. These fields modulate the local fields at any neighboring nucleus, and can cause a transition between energy states when the motions are near the Larmor precession frequency of the mucleus in question. Bloembergen, Purcell and Pound calculated that it was Brownian type motion that was responsible for the relaxation[102]. Several techniques, both pulsed and non-pulsed, are available for measuring relaxation times[103]. This is a valid way of detecting the onset of motion in polymers as a function of temperature, since the motion will cause a drop in the spin-lattice relaxation time when it becomes fast enough to do so[104-106]. The frequency that is required to effect the relaxation is approximately 10^8 hz, as is the Larmor precession frequency.

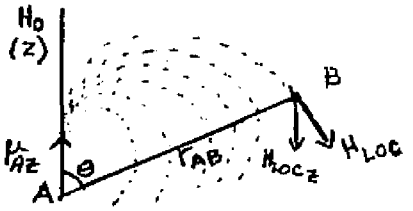
Since in the case of the proton the dipole can be considered to have a component in the direction of the field or anti-parallel to it, T_1 can be related to the decay of the z-component of the magnetization (the z direction being defined by H_o). This provides another useful way of visualizing the molecular process involved, especially when pulse experiments are being interpreted[107].

SPIN-SPIN RELAXATION

The above phenomenon, as stated, was an interaction between a nuclear dipole (spin) with a magnetic field caused by certain lattice movements. Another interaction is possible between two magnetic dipoles. This is called spin-spin interaction, and leads to a relaxation time, T_2, that is inversely related to the line width. This is seen through the following argument. If two hydrogen nuclei are in resonance they are precessing about the applied field direction with the resonance, or Larmor precession frequency. If they are close enough to each other for a long enough time, the component of magnetization in the x-y plane, also precessing at the same frequency, of one nucleus satisfies all the conditions necessary to cause a spin flip in the other nucleus. When the two spins involved are anti-parallel this interaction causes them to exchange spins. This process is conservative in the sense that there is no loss of energy here as there was in the spin-lattice relaxation. A mere spin exchange has occurred which is often called a spin-spin collision. The process just described leads to line broadening through the Heisenberg Uncertainty Principle. The spin exchange serves to limit the lifetime of any one nucleus, and since the uncertainty in energy determination increases with decreasing lifetime, the width of the resonance line increases. Another way of viewing the spin-spin interaction is also useful and will contribute some

numerical approximations to the above arguments.

If one considers that the magnetic field at any one nucleus is the vector sum of the applied field and any local fields arising from other nuclei, the phenomonon of line broadening is easily visualized. Classically, the interaction is computed as follows: Nucleus A exerts a field at B,



The z-component of the local field at B is given by:

$$H_{loc(z)} = \mu_{Az} r_{AB}^{-3} (1-3\cos^2\theta)$$

From the fact that $\mu_{Az} = g\mu_0 m$ and using g equal to 5.6 and μ_0, the nuclear magneton, equal to 5.049×10^{-24} erg/gauss for the hydrogen nucleus we obtain for $\theta = \pi/2$

$$H_{loc(z)} = (5.6)(5.049 \times 10^{-24})\ (\tfrac{1}{2}) r_{AB}^{-3}$$

and at r_{AB} equal to 10^{-8} cm (1 Å),

$$H_{loc} = (5.6)(5.049 \times 10^{-24})\ (\tfrac{1}{2})/(10^{-8})^3 = 10 \text{ gauss}$$

The field spread at a nucleus is then twice H_{loc}, and in frequency units:

$$\Delta\nu = h^{-1}\ \gamma\ H = g\mu_0/h = \frac{(5.6)(5.049 \times 10^{-24})(20)}{6.62 \times 10^{-27}} \simeq 10^4 \text{ sec}^{-1}$$

The reciprocal of this frequency is a time which can be viewed as the time necessary for two nuclei whose x-components are precessing in phase at t = 0 to lose their phase coherence. This arises from the fact that $\Delta\nu$ is actually a spread in the resonance or Larmor precession

frequency. This viewpoint is convenient for pulse experiments. Another viewpoint useful for interpreting line widths is that this time is the time necessary for a nucleus to sample its immediate environment, and if the conditions are right, to undergo a spin-spin collision. In a rigid lattice the atoms or molecules do not move (diffuse or rotate), the nuclei have ample time to detect their neighbors and the conditions are optimal for a spin-spin exchange. T_2 is short, and from the reciprocal nature of T_2 and ΔH, the line width is broad. Obviously the rigid lattice described is an exaggerated extreme. The other extreme is not so exaggerated. For a rapidly diffusing molecule in a low viscosity liquid the nuclei can not sample any one environment, and T_2 is large and hence the narrow line of liquids results. The two points of view are equivalent in that T_2 is the time required for two nuclei initially precessing out of phase to get in phase. Once in phase a spin-spin flip will occur.

Using this T_2 in the Heisenberg uncertainty expression relating energy and time, also leads to the spread in line width derived above.

From the expression $T_2=h/\gamma H$ we see that the line width is proportional to the reciprocal of the spin-spin relaxation time. If the facilities necessary for directly measuring T_2 are not available one may use line widths at the inflection point of the absorption curve as a suitable substitute. In this case one must bear in mind that the absolute value of T_2 is rarely obtainable from these measurements owing to deviations of the

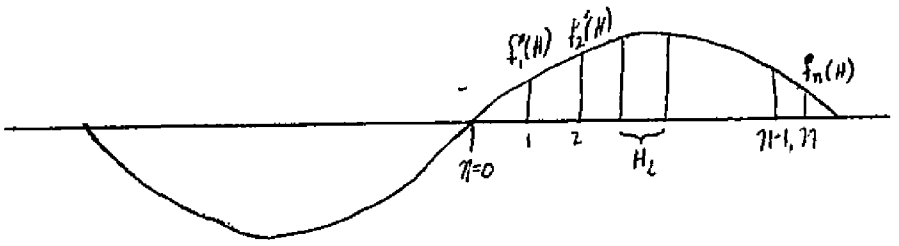
line shapes from ideality, either pure gaussian or lorentzian, and to contributions to the line width from other sources such as applied field inhomogeneities.

Line width measurements are sensitive to motion occurring in the range of 10^{-4}hz. At very low temperatures a situation prevails which is called the rigid lattice. Far from being motionless, the rigid lattice is a state where the motions are ineffective in causing spin-spin relaxation, and the line width is at its theoretical maximum limit. As the temperature is raised certain movements begin to be effective in different ranges. As a particular type of motion begins the width of the line narrows. In effect, the motion is causing a partial time averaging of the local fields at the resonating nuclei.

Because measurement of the line width is subject to some error, especially where there is additional structure besides the broadline, a quantity called the second moment is often used to obtain changes in the line shape. To calculate the second moment from an experimentally obtained curve, the formula

$$\Delta H^2 = \frac{\sum_{n=1}^{n} f_n'(H)(nH_i)^3}{3\sum_{n} f_n'(H)nH_i}$$

is used. This equation is used when the derivative of an absorption curve is obtained. The derivative curve results when modulation techniques are used to facilitate signal detection The following diagram helps clarify some of the variables and the method of calculation of the second moment.



The calculation is carried out for both halves of the curve starting from n=0 and proceeding outward. The signs of H_i and $f_n'(H)$ are such that all terms in the summation are positive.

The advantage in using the second moment is that it can be related to a theoretically calculated rigid lattice value[108] and specific types of motions lead to relatively easily determined decreases in the theoretical second moment [109]. This latter point is misleading since strictly the second moment does not change with motion. However, motion causes satellites to appear at frequencies $\pm 2n\nu_m$, where ν_m is the frequency of the motion; these satellites appear at the expense of the central portion, and since only the central portion is observed, the second moment appears to drop[110].

The Van Vleck rigid lattice value of the second moment is given by:

$$\Delta H^2 = \frac{3}{2}\frac{I(I+1)}{N} g_i^2 \mu_0^2 \sum_{i>j} (3\cos^2\theta_{ij} - 1)^2 r_{ij}^{-6} + \frac{1}{3}\frac{\mu_0^2}{N} \sum_{jf} I_f (I_f+1)(g_f^2)(3\cos^2\theta_{jf} - 1)^2 r_{jf}^{-6}$$

where N is the number of nuclei involved. These will be only those in close proximity since there is a dependence on the reciprocal of the sixth power of the distance. The first term is for all the nuclei in resonance and the second is for non-resonant magnetic nuclei. When an isotropic system is studied, consisting only of protons as the magnetic nuclei, a simpler equation results.

$$\Delta H^2 = (720)\frac{1}{N}\sum_{i>j} r_{ij}^{-6}$$

Here the second term is dropped since there are no non-resonant magnetic nuclei. The expression $(3\cos^2\theta_{ij}-1)^2$ averages out to 4/5 in an isotropic system. All that is needed to calculate a rigid lattice value for the second moment are the interatomic distances r_{ij}. Shown in the appendix is a calculation for poly-L-proline II.

The calculation of the effects of motion on the second moment are only slightly more complicated for simple types of motion. Rotation of a methyl group about its C_3 axis has been done [109,111], as has the case of main chain torsional oscillations[112,113]. Many assignments have also been made on the basis of experimental comparisons[94,114].

As may be obvious at this point correlations may be made between mechanical and magnetic relaxation phenomena[115-117,94]. Remembering that frequency and temperature are dependent on each other, the following generalizations are applicable.

The study of polymeric materials by both n.m.r. and dynamic mechanical testing yields supplementary information.

The two techniques, while often giving the same information are sensitive to different types of motion. For instance, although the two methods are capable of detecting crystalline melting, this transition is usually more easily observed in dynamic mechanical testing except in very highly crystalline materials where n.m.r. may also be used. The reason for this being that by the time the crystalline melting point is reached, the line width in n.m.r. is already so narrow due to other motions that any further changes are not readily discerned. The primary amorphous transitions are detectable in both techniques, good agreement being obtained when the frequency-temperature shift is taken into account. Secondary amorphous transitions involving main chain segmental motion show up more clearly in dynamic mechanical testing. For example, the two low temperature loss peaks in polyamides are only evident in n.m.r. as monotonically decreasing second moments. N.m.r. is more sensitive to side chain reorientations such as methyl group rotations. N.m.r. lends itself to quantitative calculations such as the Van Vleck rigid lattice value, or the expected contribution of a particular motion to the line narrowing process.

STATEMENT OF THE PROBLEM

This investigation was undertaken with three objectives. One was to use the torsion pendulum and nuclear magnetic resonance spectrometer to study the relaxation properties of a unique structure - that of the triple stranded coiled coil, and to see how this structure might be manifested in these relaxation properties.

A second objective was to study the changes in the relaxation behavior of artificially aged samples. Perhaps from this study some insight could be gained into the changes occurring in collagen upon natural aging. How these changes are implicated in the maladies of old age could also be suggested.

Third, by using poly-L-proline II to act as a simplified model for collagen, assignments of molecular processes could be made to the various relaxation phenomena observed in the torsion pendulum and nuclear magnetic resonance spectrum.

EXPERIMENTAL

COLLAGEN

Dispersion preparation- The collagen used was obtained from a 2% aqueous suspension supplied by the Johnson and Johnson Co. The procedure for preparing the suspension is discussed in a thesis by E. R. Lieberman[118]. Because the history of a polymeric sample is often important in interpreting its behavior, the method of preparing the sample will be outlined here.

The flexor tendon of young steers was cleaned according to the procedure set forth in a Canadian patent[119]. Twenty-four hundred grams of the material are frozen, sliced and then treated with 24 liters of pH 6.2 solution of 24 grams of the enzyme ficin and 10 grams of ethylenediamine tetra-sodium acetate. After 17 hours at 25°C the supernatant was decanted and any residual enzyme was deactivated by a 30 minute treatment with 24 liters of an aqueous solution of 80 grams of 30% H_2O_2. The tendon slices were then washed with running water and swelled in a 50:50 methanol-water solution containing 0.58% by weight of cyanoacetic acid. The mass was then stirred for three hours and then circulated through a ½ inch pipe for an hour. This treatment was followed by passage through 1/8 inch jets for an additional half hour. The resulting homogeneous suspension was filtered and de-aerated under vacuum.

Film preparation and properties- Films of the collagen preparation suitable for use on the torsion pendulum were obtained by allowing the suspension to dry for several days at room conditions in a polypropylene mold. These films were then aged for two or more months in the laboratory. Films freshly cast were found to swell in water to a much greater extent than the aged samples. It has been shown by Lin et.al.[120] that the percent crystallinity of rat tail tendon increases with aging, as does the degree of crosslinking.

The films were dried to constant weight under vacuum, at room temperature in the presence of anhydrous $CaSO_4$. These films were used as the dry samples in the experiments to follow. As noted earlier this treatment does not drive off all the tightly bound water. Attempts to dry the samples at high temperatures led to degradation and loss of the sample's shape. Specifically, heating to 160°C in a vacuum oven resulted in immediate shrinkage of the film, and after 15 minutes the sample discolored and puffed up into a ball. Weight loss after this treatment amounted to 25 to30%. A slower, less drastic drying procedure started with a room temperature, vacuum dried sample. The results are summarized in Table 2.

Table 2: Drying of Collagen

Temperature	Time	% Weight Loss From Original Weight	Comments
60°C	24 hrs	1.06%	-
70°C	1 week	2.65%	-
80°C	2 days	4.17%	Edges of film curling
90°C	4 days	4.87%	Curling advancing-yellow color at edges
100°C	1 day	9.36%	Browning
100°C	8 days	17.8 %	Considerable browning

Moisture content adjustment - In order to obtain samples with known water contents, the specimens were placed in a sealed dessicator and allowed to equilibrate over saturated salt solutions whose relative humidities were known[121]. Values reported in the following sections are for water contents after the experiment was completed, rather than before it was started. This procedure was especially necessary for the torsion pendulum where the sample was open to the atmosphere for extended time periods.

Formaldehyde crosslinking - The collagen film was cross-linked by placing it in a fresh 10% aqueous formaldehyde solution with 1% $NaHCO_3$ added. The pH is thus regulated in the range of 8.0-8.2. The sample was treated in this manner for three hours. It was then washed in distilled water for

an hour, then air dried. In order to establish that crosslinking had occurred, a portion of the formaldehyde treated specimen was denatured (shrunk) in hot water. For comparison a control consisting of a similar sample of collagen treated only in 1% aqueous $NaHCO_3$ was also denatured. After denaturation both samples were immersed in ethylene glycol and allowed to swell to their equilibrium values. The extent of swelling is determined by the degree of crosslinking, as pointed out in the Flory-Rehner treatment[60,61]. After 54 hours of immersion, equilibrium was well established. The control had imbibed 1.25 more grams of ethylene glycol per gram of dried protein than did the formaldehyde treated sample.

Denaturation of Collagen- The shrunken collagen used was obtained by two methods. For n.m.r., films were shrunk in boiling water, dried and cut into small pieces. For the torsion pendulum, where uniform shape is required, the above method was innapplicable as it led to quite distorted, non-uniform films. In this case, the dispersion was treated with a 100°C water bath. Loss of viscosity and gelation upon cooling were indicative of denaturation. The hot denatured collagen was poured into a mold and allowed to air dry.

POLY-L-PROLINE II

The poly-L-proline used was supplied by Schwartz Mann Co. The reported molecular weight was 34,000 for the sample used in the n.m.r. experiments, and 49,000 for the sample

used in the torsion pendulum. Optical rotation studies of a formic acid solution of each sample yielded specific rotation, $[\alpha]_D^{25°} = -547 \pm 15°$ indicating in excess of 98% pure II form.

For the n.m.r. experiments the poly-L-proline was used as received. It was simply packed in the n.m.r tube. Films for the torsion pendulum were cast from formic acid solution. The lower molecular weight sample formed films that were too brittle. This fact necessitated the use of the higher molecular weight sample.

DYNAMIC MECHANICAL TESTING

The torsion pendulum designed by Sinnott[122] and modified by Frosini and Woodward[123] was used. Sample dimensions were in the range of 1.0 cm width, 5-7 cm length and 50-200 μ in thickness. Both sample dimensions and tensile load were adjusted to remain within the ASTM recommendations for torsion pendulum testing[124,125]. The equation used to calculate the storage modulus was

$$G' = \frac{4\pi^2 ML}{N} f^2 - \frac{mga^2}{12N} \quad 126$$

This equation arises from that given by Inoue et.al.[127],

$$G' = \frac{4\pi^2 ML}{N} f^2 \left(1 + \frac{\Delta}{4\pi^2}\right) - \frac{mga^2}{12N}$$

where the assumption that the logarithmic decrement (Δ) is small has been made. In these equations G' is the shear modulus, M is the moment in inertia of the system, L is the length of the specimen, f is the frequency, m is the mass of the suspended ring, g is the gravitational constant, a is the

width of the sample and N is a shape factor

$$N = \frac{1}{3} \; ab^3 \left(1 - 63\frac{b}{a}\right)$$

where b is the thickness of the sample. In all the above equations "a" must be greater than 3b.

Although care was always taken to ensure proper alignment, difficulties arose that could not be eliminated completely. As the temperature rose in the sample chamber the specimen was observed to twist about its long axis. A possible reason for this could be variations in thickness of the sample along the length. This twisting phenomenon, if ignored, would give rise to erroneous values of the logarithmic decrement. In the results to be presented here this twisting was compensated for by initially offsetting the specimen. The values of the damping are accurate although there is scatter, especially in the vicinity of a major transition.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Broadline n.m.r. studies were carried out on a Bruker 90Mhz instrument employing a Bruker b-ST100/700 variable temperature unit.

The amplitude of the modulation field was adjusted to avoid modulation broadening of the signal. However, this was not entirely successful in the measurement of the narrow line components. Power requirements were also adjusted to avoid saturation and at the same time provide adequate signal to noise ratio.

RESULTS

The dynamic mechanical properties of a sample of collagen containing 10% water by weight is shown in Figure 4. Two loss processes occur in the temperature region of 110° to 300° K. The higher temperature α peak occurring at 250°K is accompanied by an approximate four-fold drop in the storage modulus (G'). The β peak occurs at approximately 195°K and is six to seven times smaller than the α peak. The effects of a decrease in water content are shown in Figures 5 and 6. The samples contain 3½% and 0.86% water, respectively. The major effect of the removal of water is seen to be a slight increase in the low temperature level of the modulus and an appreciable shift to higher temperatures of the α peak. The α loss process is also shown to be decreasing in magnitude as water is removed. The β process exhibits the same qualitative behavior as the α process, although to a smaller degree.

In Figure 7 are shown the dynamic mechanical properties of a freshly cast film of collagen, not allowed to age in the laboratory as were the samples in Figures 4 to 6. The water content here is 6%. The temperature of the β peak is somewhat higher than might be expected from a comparison with the laboratory aged samples. The magnitude of the α peak appears to be slightly depressed. Otherwise, the results are similar.

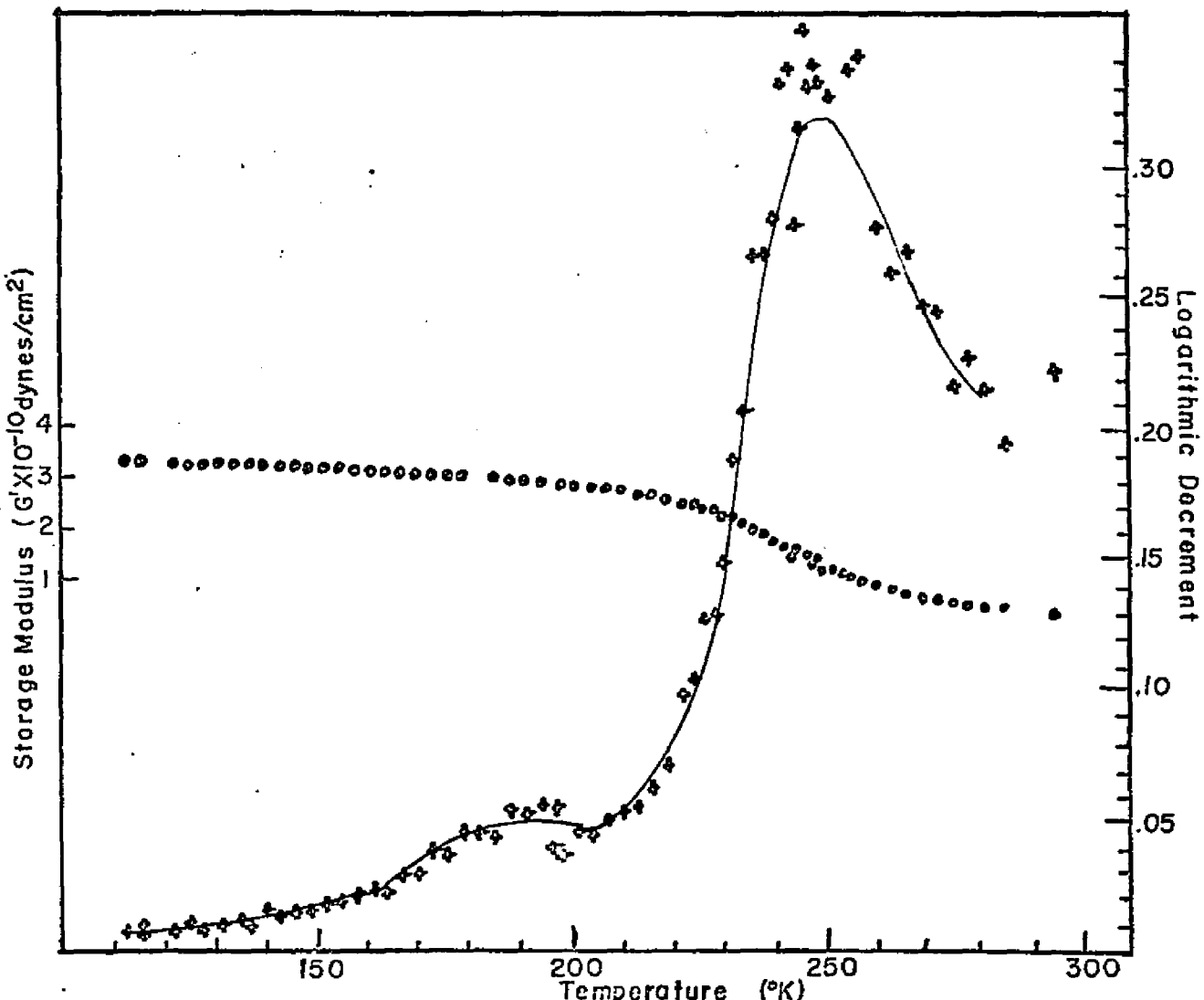


Figure 4. Dynamic mechanical properties (.2-.6 hz) of collagen vs temperature - 10% water

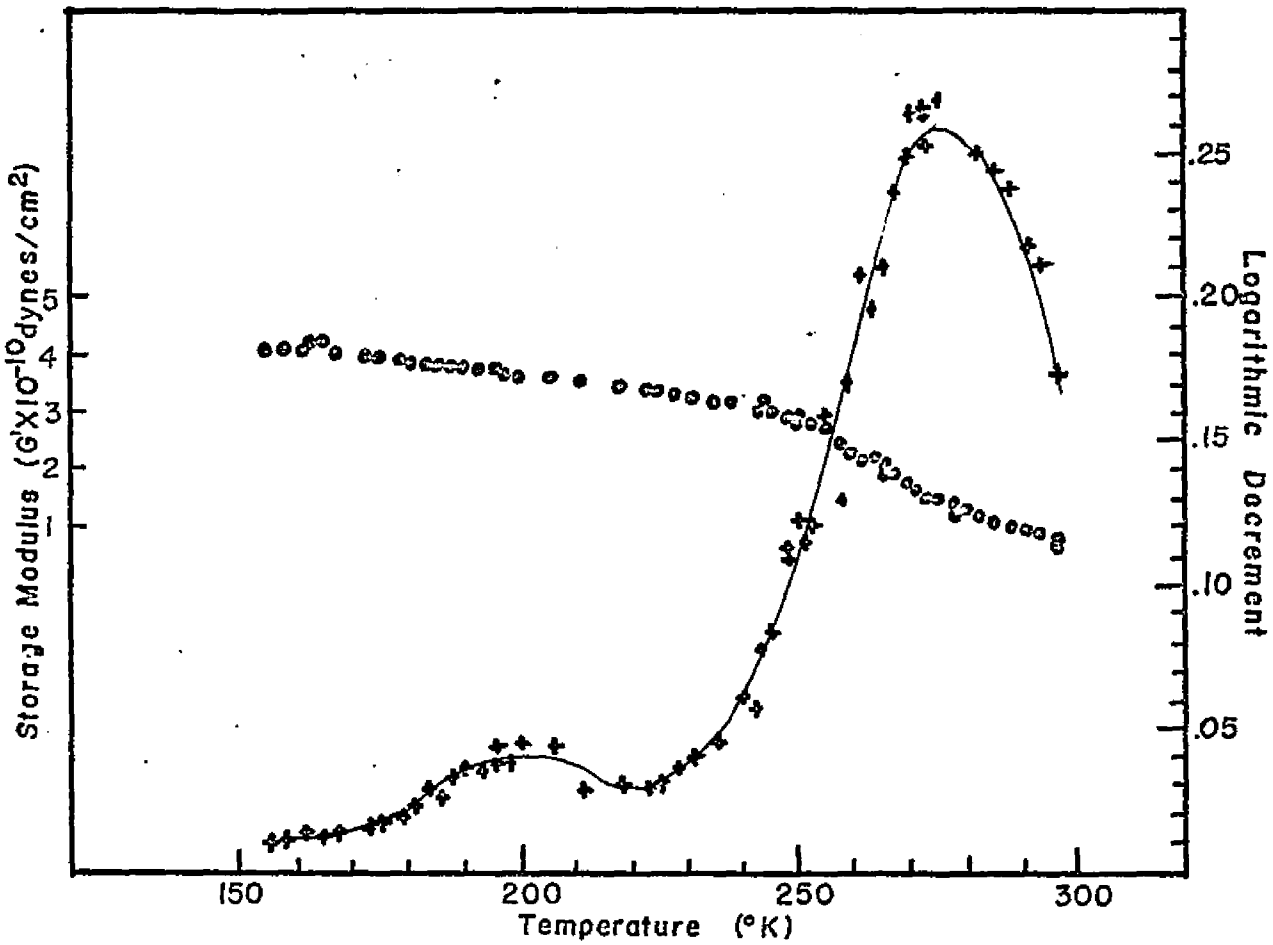


Figure 5. Dynamic mechanical properties (.4-.8hz) of collagen vs temperature – 3.5% water

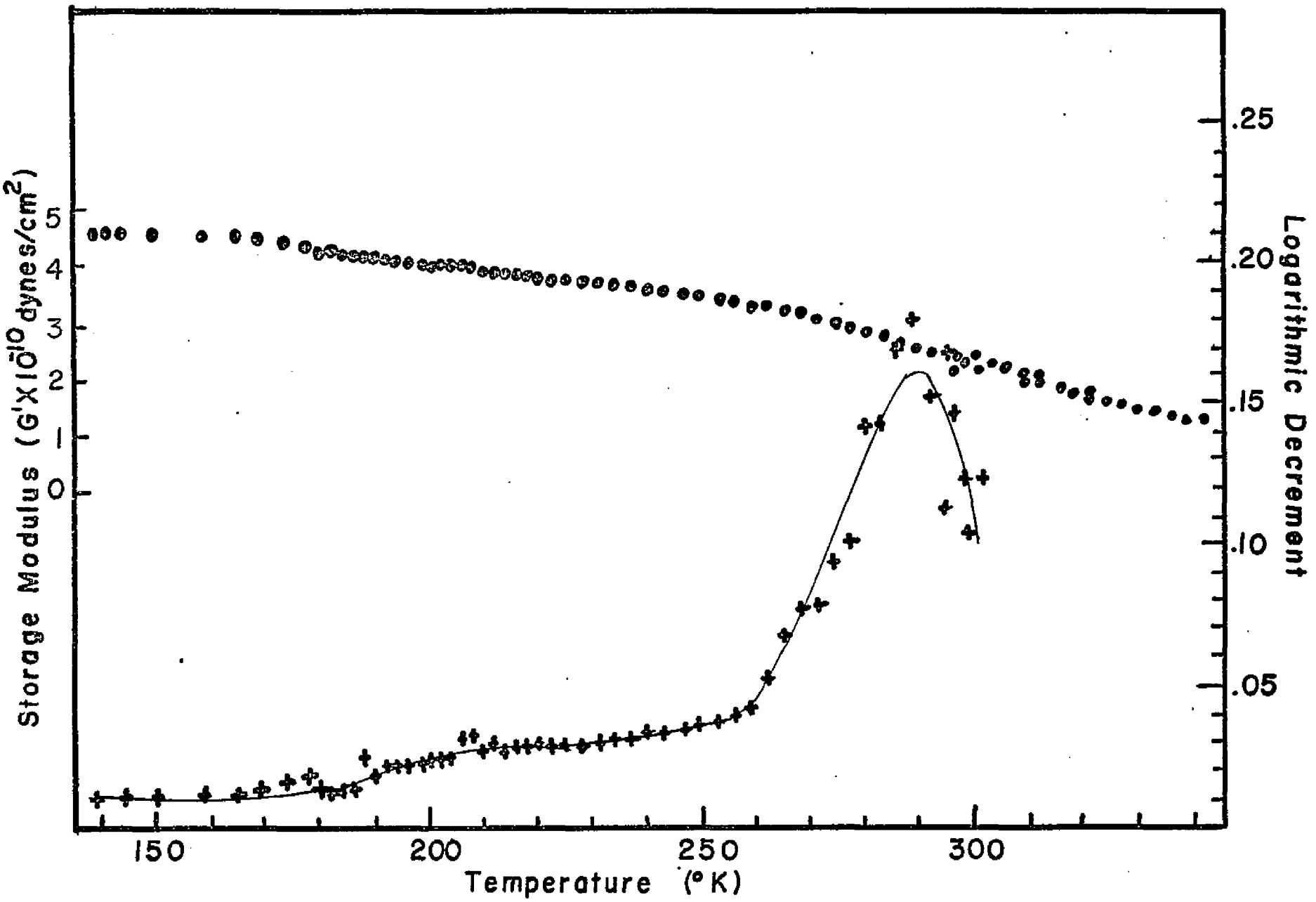


Figure 6. Dynamic mechanical properties (.7-.9hz) of collagen vs temperature-0.86% water

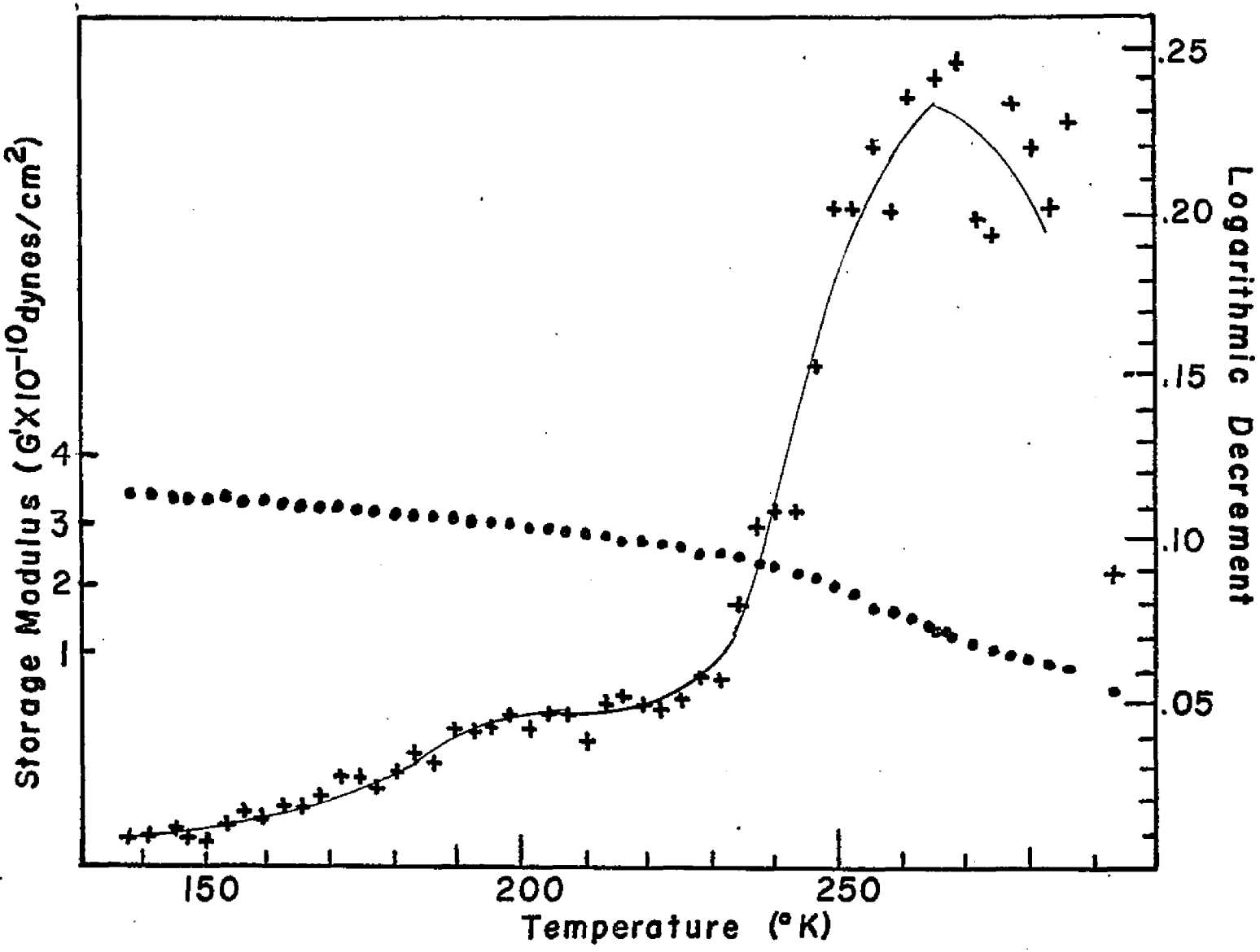


Figure 7. Dynamic mechanical properties (.2-.5 hz) of freshly cast collagen vs temperature - 6% water

Figure 8 shows the results obtained on the formaldehyde crosslinked sample containing 5.5% water. The low temperature value of the modulus is seen to be twice that of the uncrosslinked samples. It drops monotonically as the temperature increases up to 300°K, where a precipitous drop is observed followed by a slow rise above 300°K. This pattern of behavior is reversible upon cooling. Compared to the uncrosslinked samples the magnitude of the α peak is appreciably depressed while that of the β peak is slightly enhanced. The β peak is seen to be shifted down to 180°K, while the α process is shifted to higher temperatures. These facts coupled with the results given in Figure 7, suggest that laboratory aging is in fact mild crosslinking. This is in agreement with the swelling behavior described in the wxperimental section.

Figures 9 and 10 present the results for the shrunken collagen at water contents of 10% and 0% (dried to constant weight at room temperature). These results are analogous to those of collagen in Figures 4 through 6 with the exception that here the low temperature value of the modulus is considerably higher, indicating a more compact structure.

In Figure 11 is shown the dynamic mechanical prpperties of a sample of poly-L-proline II with a water content of 4.8%. The modulus is seen to be smaller than collagen's by a factor of three. It exhibits a sudden decline in the temperature range of 140°K to 250°K reaching a value of 5×10^8 dynes/cm^2 at the higher temperature. The damping

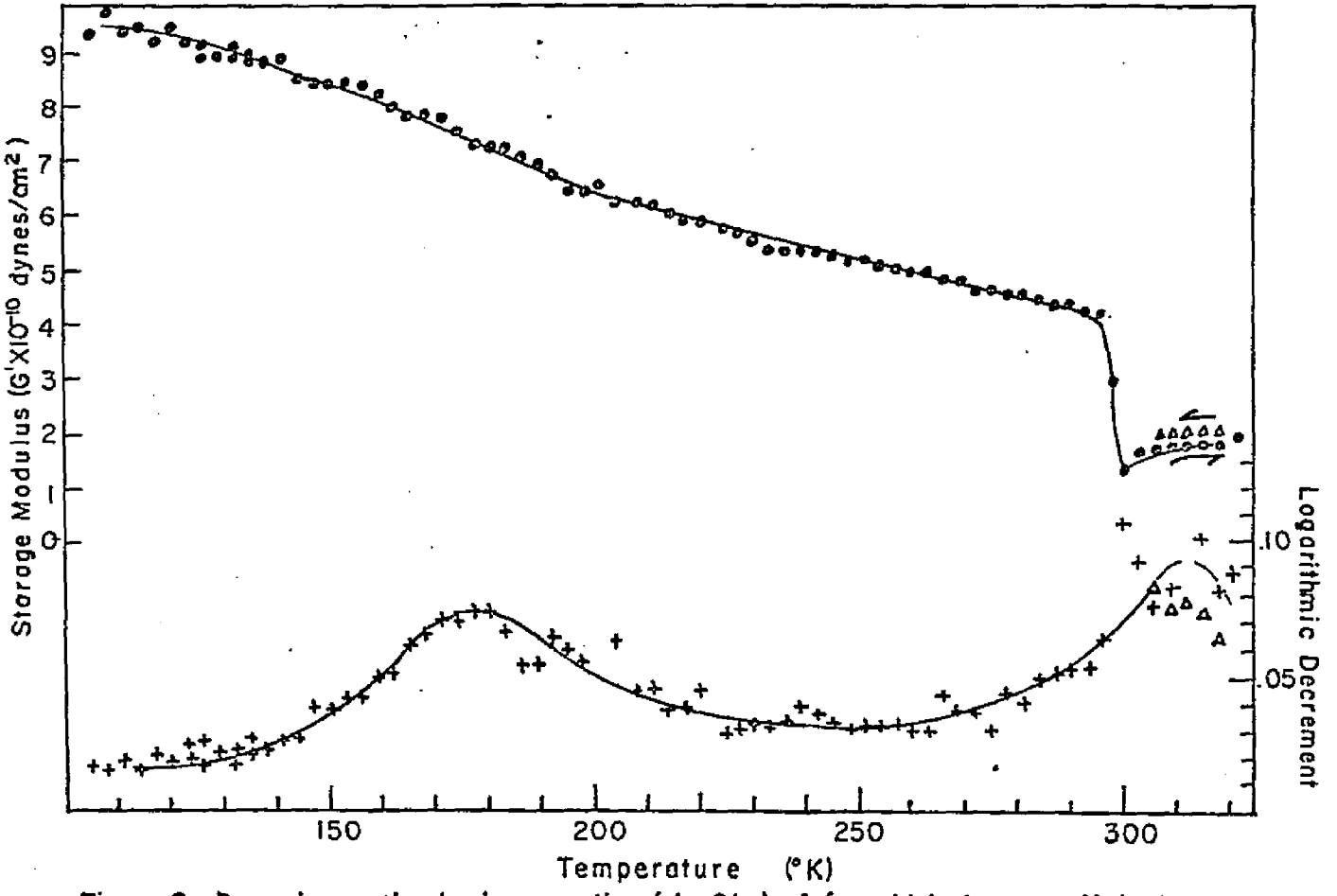


Figure 8. Dynamic mechanical properties (.4-.6hz) of formaldehyde crosslinked collagen vs temperature - 5.5% water

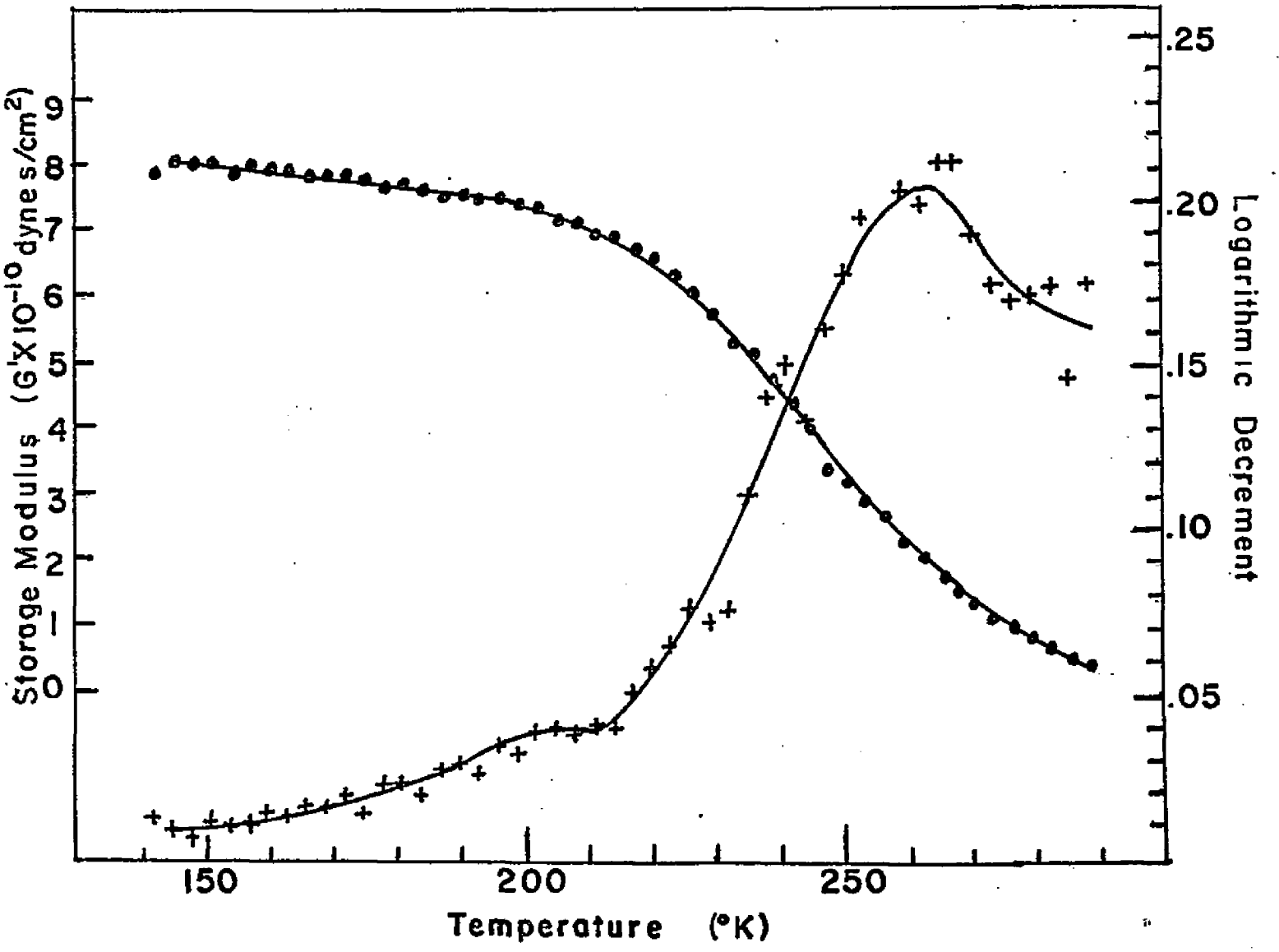


Figure 9. Dynamic mechanical properties (4-.9hz) of shrunken collagen vs temperature - 10% water

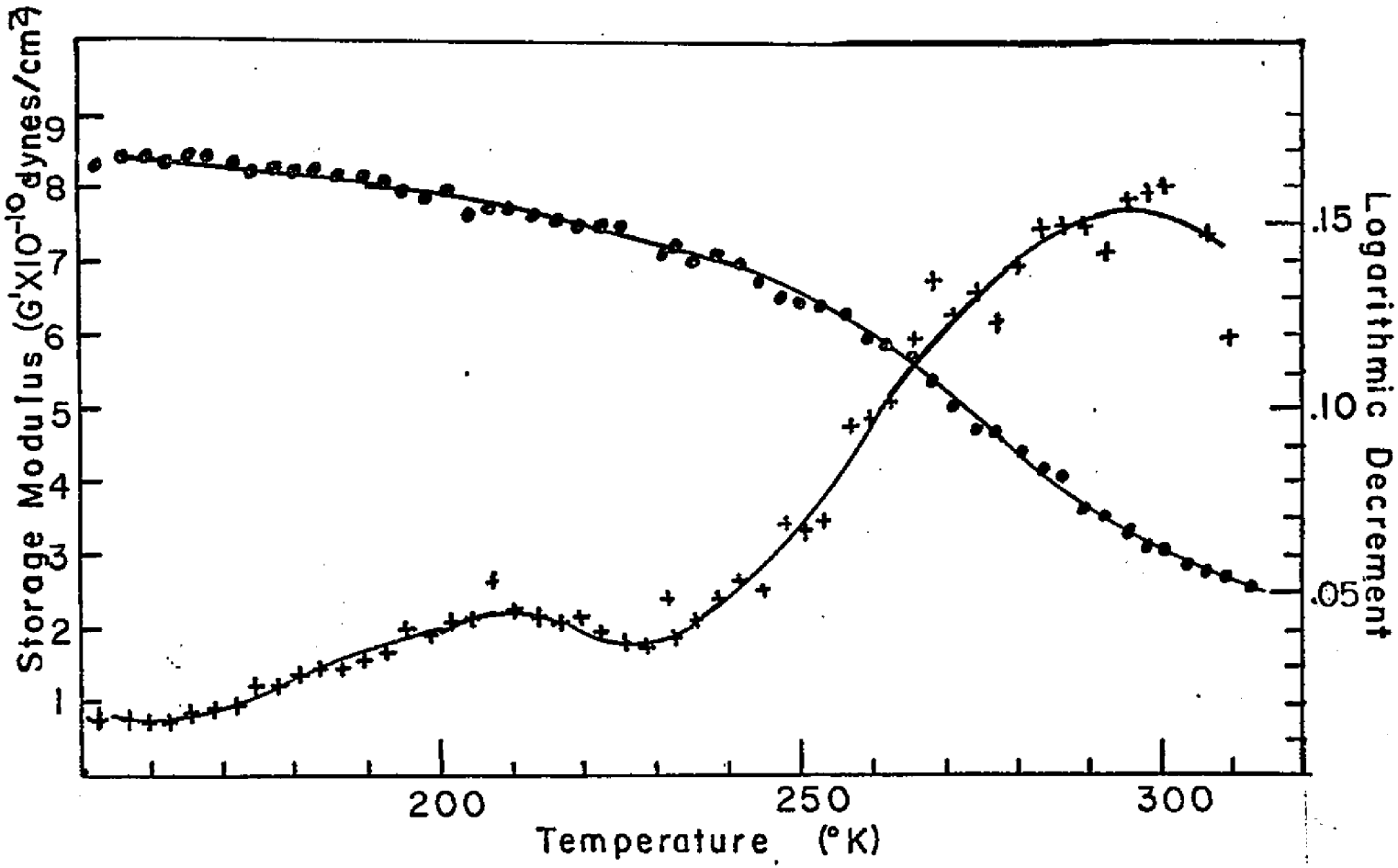


Figure 10. Dynamic mechanical properties (.6-.9hz) of shrunken collagen vs temperature - "0"% water

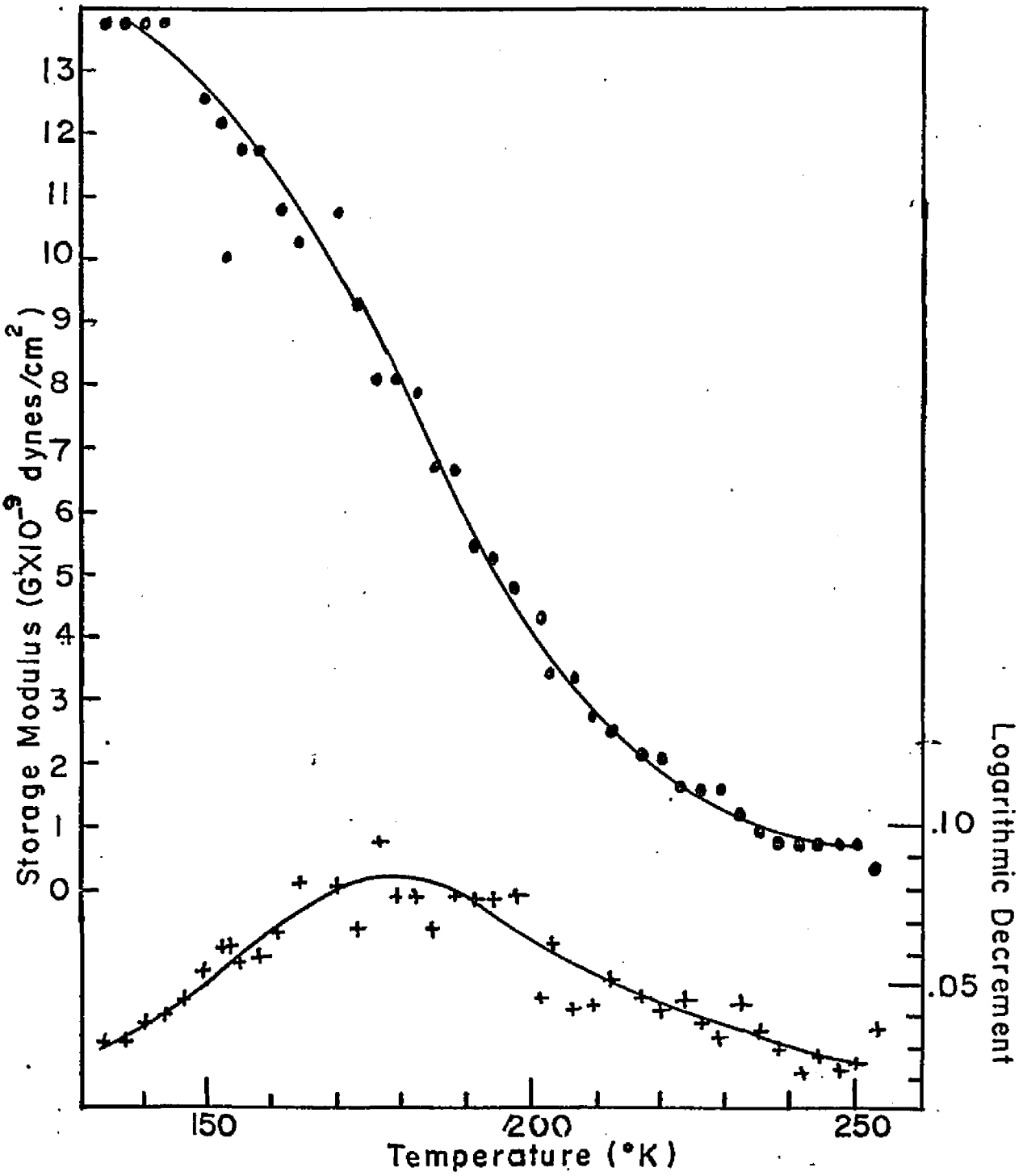


Figure II. Dynamic mechanical properties (.3-4 hz) of poly-L-proline II vs temperature - 4.8 % water

exhibits a maximum of about 0.08 at 175 °K (0.4 cps) quite comparable to that of the crosslinked collagen.

Broad line n.m.r. measurements were obtained in the 180 to 380°K region on a vacuum dried collagen sample and a sample with 6.5% water added. Below about 240°K a single line of 8 to 9 gauss width was evident; around 260°K a very narrow line appeared, its width being less than 0.1 gauss. At the lowest modulation amplitudes possible the narrow line was 0.04 gauss at 305°K. This narrow line appears at temperatures well below those at which the α mechanical process at 1 cps is found, and is independent of water content. Assuming that the mechanical loss peak is due to a relaxation process, any changes in the n.m.r. associated with it should be at higher temperatures, since the frequency for an n.m.r. process is 10^4 cps or greater. Therefore, the initial appearance of the narrow line can not be associated with the mechanical α process. The fact that the temperature of first appearance of the narrow line does not depend on water content is further evidence against the assignment of the narrow line emergence to the α mechanical process.

For vacuum dried collagen samples the n.m.r. absorption shows a steady drop in the second moment with increasing temperature over the total range studied, as can be seen in Figure 12. The presence of 6.5% water causes the second moment drop to become more precipitous in certain temperature regions. The process in the 260 to 300°K region for this

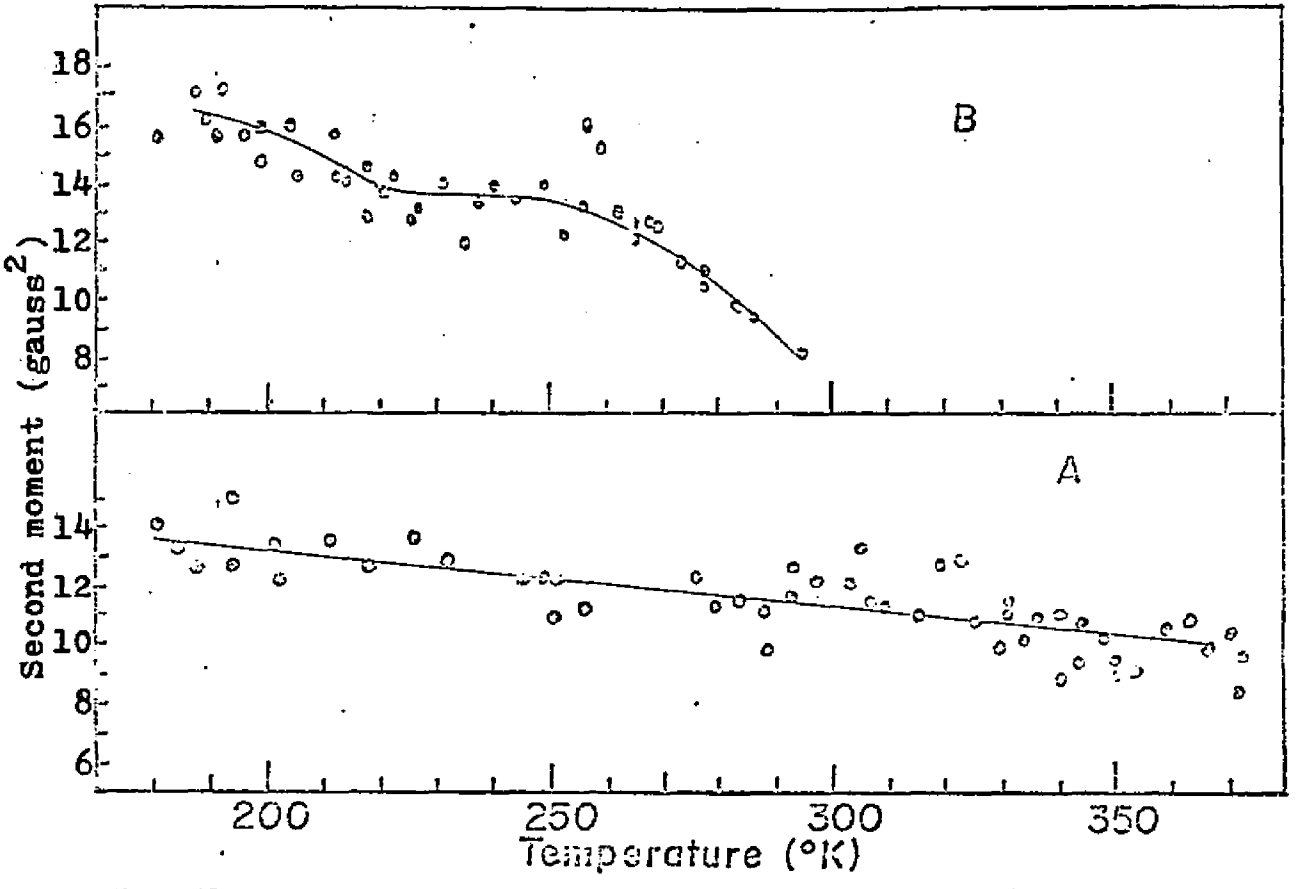


Figure 12. N.m.r. second moment vs temperature for collagen. A) dry B) 6.5 % water

sample can be tentatively correlated with the α loss maximum at 260°k (1 cps) for a sample of the same water content.

Vacuum dried shrunken collagen also displays a narrow line which first becomes evident at about 250°K. Extended drying under vacuum at 373 to 380°K brought about no change iin this line at 300°K. For a sample containing 8.5% water the narrow line width was 0.4 gauss at 253 °K changing to 0.2 gauss at 265°K and above.

The second moment in the 200° to300°K region for a vacuum dried sample, plotted in Figure 13, shows a more precipitous drop than that found for collagen. Theeffect of added water on the n.m.r. second momeam results is to split the gradual drop into a relativel sharp process ar 210° to 260°K and a more gradual one at 260° and higher. This latter process has a smaller slope than that for collagen in the same temperature region. It is doubtful that the 210° to 260°K n.m.r. process of the first appearance of the narrow line are related to the mechanical α process. The gradual n.m.r. change above 260°K could, however, be related to the α process.

Poly-L-proline also exhibits a narrow line in addition to its broader line. For a dry sample the narrow line begins to emerge from the broad line at approximately 330°K; its width at this point is 1.4±.3 gauss. The large uncertainty is due to the difficulty of clearly distinguishing the narrow

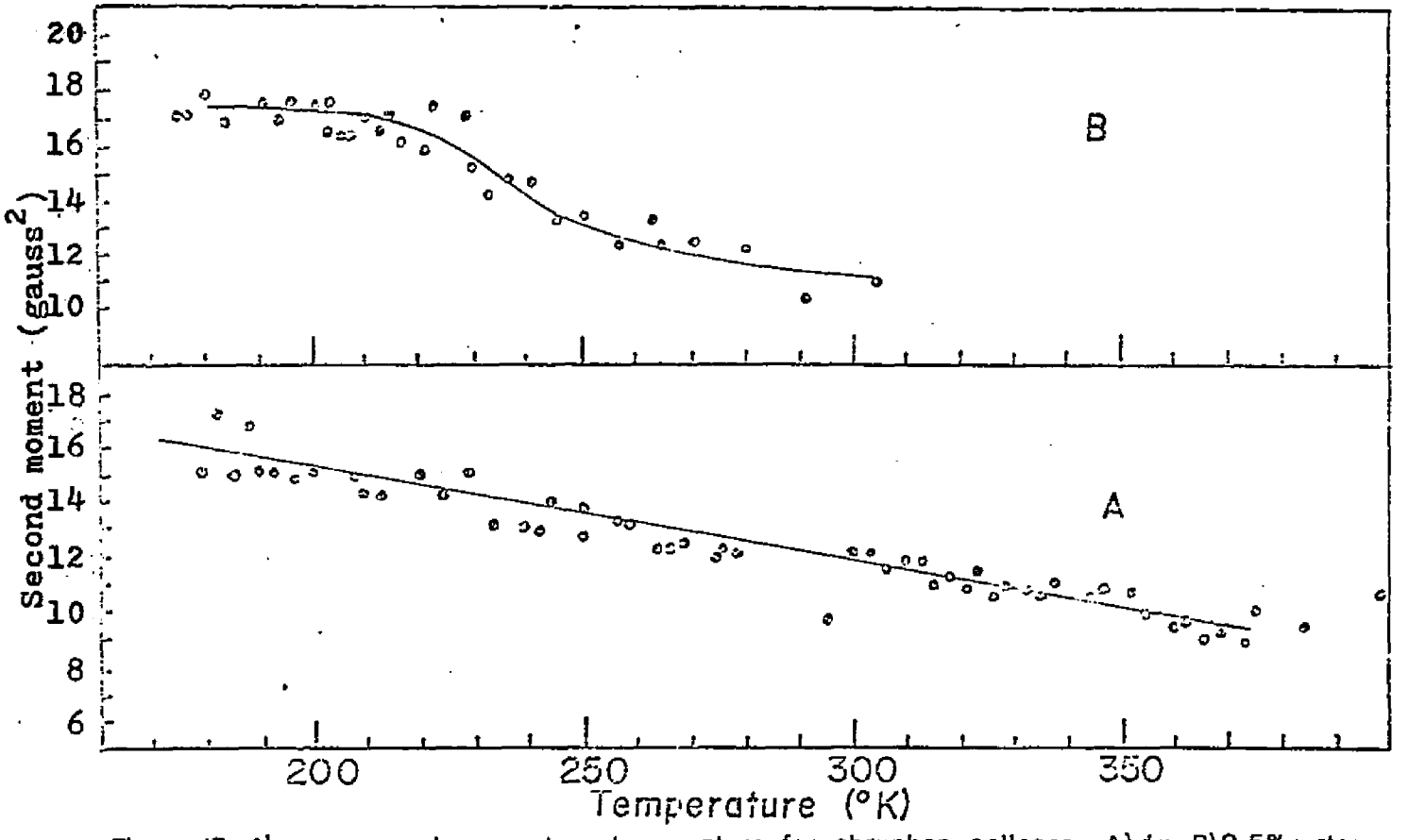


Figure 13. N.m.r. second moment vs temperature for shrunken collagen. A) dry B) 8.5% water

line from the broad line. As the temperature is raised, the narrow line width decreases quite sharply reaching a value of 0.4 to 0.5 gauss at 370°K. This behavior is not comparable to that of the narrow line in collagen samples. In the collagen case the narrow line gradually merged into the background without any noticeable broadening. Also unlike collagen, the temperature of the narrow line appearance in poly-L-proline is dependent on water content. For a sample with 6.5% water, the appearance of the narrow line occurs at 250 °K with a width of 3 gauss. At 300°K the width decreases to 0.3 gauss. When the room temperature dried sample is heated to 400°K or dried at 378°K for 24 hours there is no noticeable change in the characteristics of the narrow line despite an additional weight loss of approximately 2%.

The broad line second moment for poly-L-proline is shown in Figure 14 for a room temperature vacuum dried sample and a sample containing 6.5% water. The dry sample at 160°K has a second moment of 17.5 gauss^2. This decreases to 11 gauss^2 at 380°K. A slightly steeper decline in second moment is evident at about 200°K where the second moment is about 17 gauss^2. This decline ends at 260°K when the value reaches 14 gauss^2. The sole effect of water is to sharpen this transition. The second moment at 200 °K is now 19 gauss^2 (as expected from the addition of more protons from the water), and the value reached at 290°K is now 14

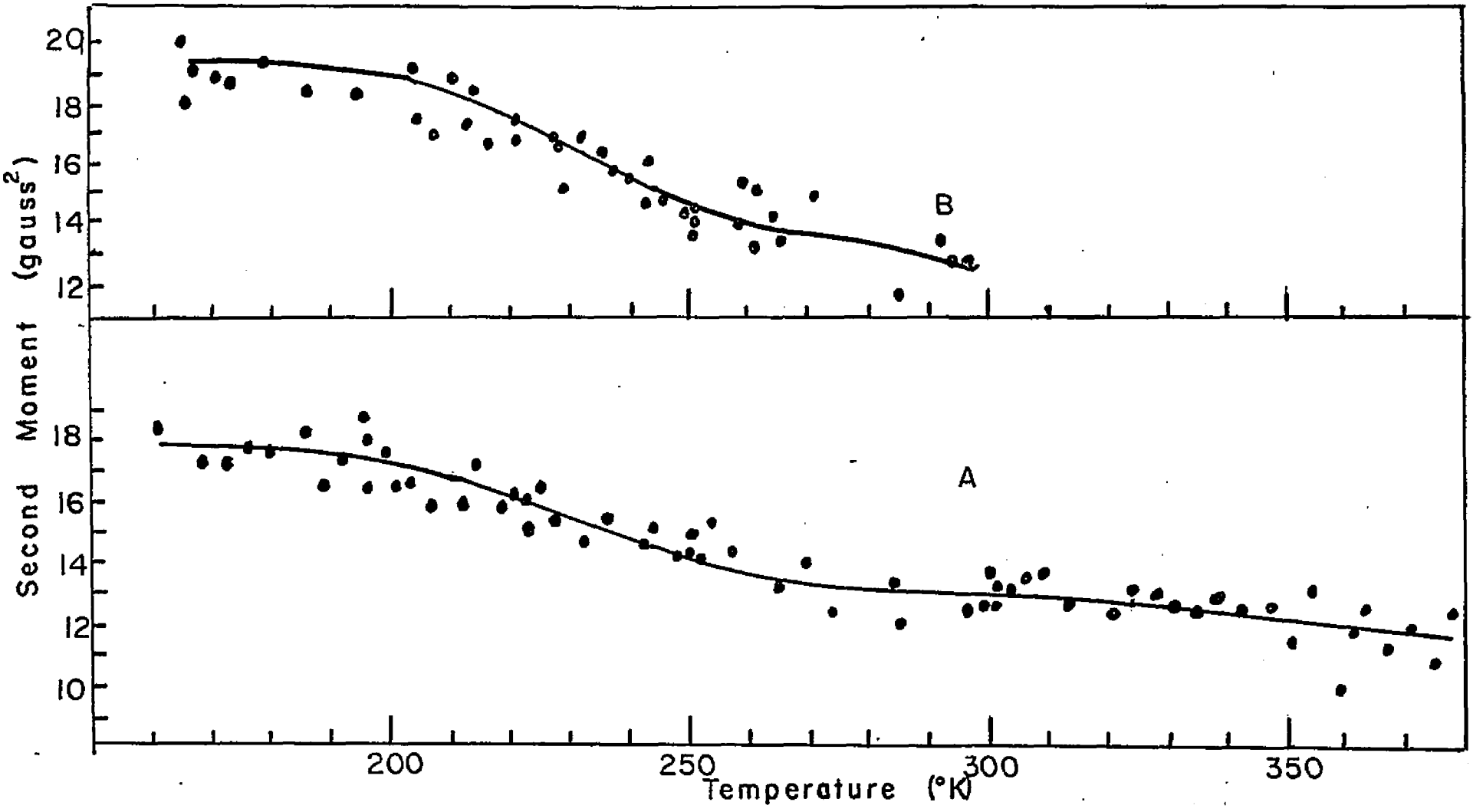


Figure 14. N.m.r. second moment vs temperature for poly-L-proline II. A) dry B) 6.5% water

gauss[2]. The line width also shows a sudden drop in this temperature region from 11.5 to 9.5 gauss.

In Tables 3 and 4 the results just described are summarized for easy reference.

Table 3: Summary of the dynamic mechanical properties.

Sample	α process	β process
Collagen 10% water	250 °K	195°K
Collagen 3.5% Water	275 °K	200 °K
Collagen 0.86% water	288 °K	210 °K
Formaldehyde crosslinked collagen 5.5% water	310 °K	180 °K
Denatured collagen 10% water	260 °K	200 °K
Denatured collagen 0% water	295 °K	210 °K
Poly-L-proline II 4.8% water	--	175 °K

Table 4: Summary of Magnetic Resonance Data

Sample	Second Moment Drop	Narrow line appearance
Collagen 0% water	monotonic	260 °K
Collagen 6.5% Water	200, 260 °K	260 °K
Denatured collagen 0% water	monotonic	250 °K
Denatured collagen 8.5% water	240 °K	250 °K
Poly-L-proline II, 0% water	235 °K	330 °K
Poly-L-proline II, 6.5% water	235 °K	250 °K

DISCUSSION

Investigations of the dielectric properties of collagen and gelatin have been reported[48,128]. Also commercial gelatin samples have been investigated by dynamic mechanical experiments[129,130], and several mechanical tests have been performed on collagen and gelatin[131]. In addition, some experiments of limited scope, concerning the dynamic mechanical properties of collagen, have been reported[47,132].

More recently several reports of the dynamic mechanical properties of collagens from varied sources have appeared. Chien and Chang[133] reported results obtained on a Vibron for rat-tail tendon, enzyme solubilized collagen, and a commercially prepared gelatin sample. For the rat-tail tendon the low temperature value of the modulus is highest, as expected from the high orientation of the sample. Next in magnitude is the gelatin, and thirdly the enzyme solubilized collagen. The modulus of the gelatin and that of the enzyme solubilized collagen showed at least one discrete drop lower in temperature than the main transition at 300°K. Rat-tail tendon exhibited a particularly large drop at this temperature with recovery upon further heating. This large drop was attributed to devitrification of bound water, as suggested in earlier studies on gelatin-water systems[129]. Water contents in the Chien and Chang study were in the 10% region.

The loss tangent provides a more sensitive probe of the

low temperature transitions than does the elastic modulus. The rat-tail tendon examined by Chien and Chang exhibited two loss maxima in addition to the main one at 300°K - a β peak at 223°K and a γ peak at 173°K. The enzyme solubilized sample showed only two peaks, one at approximately 300°K and the other at 193°K. Crosslinking this sample with ultraviolet irradiation shifted these peaks to lower temperatures. This is the same behavior observed for the β peak reported herein. Gelatin has only two loss maxima at or below 300°K very similar to the enzyme solubilized sample. In all samples, the low temperature loss process was assigned, by analogy, to the γ mechanism of polyamides and as mentioned earlier, the transition at 300°K to devitrification. The other transitions remained unassigned.

From a torsion pendulum study of human diaphragm collagen, polyglycine I and nylon-6 Baer et.al.[134] were able to postulate mechanisms for the transitions they observed. Three loss peaks were observed for thirty year old human diaphragm collagen. The α peak at 400°K in a sample with 10% water, shifted down to 320°K in a sample containing 35% water. The β peak, whose position is unaffected by water, occurs at 260°K. The γ peak appeared at 180°K for a sample with 10% water, shifting upwards to 200°K for a sample with 35% water. The effect of crosslinking as determined by comparison of a sample from a 73 year old human is to decrease the magnitude of the loss processes

without changing any temperatures of appearance. By analogy with the nylons the α,β,γ, peaks in collagen were assigned to the processes similar to those responsible for corresponding transitions in these polyamides. The analogies with nylon-6 and nylon-2 (polyglycine) are crude since the structures of these are different from that of collagen. However, a detailed examination of the results will prove useful in understanding the correlation of collagen's transitions with the α,β and γ transitions of nylon. As Baer et.al. admit, the only similarity between the helical structure of collagen and the β-sheet structure of the nylons is the tendency of both to form intermolecular hydrogen bonds. The α-transition of all three materials exhibits the same behavior with changes in water content. Since it was postulated by several investigators that the α process in nylons was due to main chain motion with rupture of the hydrogen bonds in the amorphous regions, the same mechanism is assigned to collagen. For a review of the evidence for this assignment in nylons and for the β and γ processes as well, see reference 93 and 96. This assignment is not unlikely since at 10% water content (in the range of our studies) Baer et.al. observe the α-transition at 400°K. The present measurements were not made to this high temperature since the collagen would denature. Therefore the α transition reported by Baer et.al. may be due to large scale main chain motion occurring in his sample prior to or concurrent with the denaturation process.

The β-process at 260°K observed by Baer et.al. was assigned to a water-polymer complex reorienting under the applied strain. Again this is analagous to the case of the nylons where it is believed by some that the β process is due to motions of the carbonyl or amide group with attached waters. In nylon the β peak is lowered in temperature and increased in magnitude as water is added. Only the latter is apparent in the collagen sample examined by Baer et.al.

More recently[135] it has been stated that the β transition in nylon-6 occurring at 190°K involves the breaking and reformation of the hydrogen bonds between water and the carbonyl groups of the polymer. This conclusion is based on considerations of the geometry of the LI defect in ice, which resembles a picture drawn of water bound to two carbonyls in nylon-6. How this is related to collagen's β peak is not obvious since the latter occurs at 260°K.

The γ transition is attributed to motion of the CH_2 in nylon when the number of such units exceeds three[136]. This clearly cannot be the case with polyglycine I so Baer et.al. have assigned the γ transition at 130°K in nylon-6 to a motion of the carbonyls and amide groups in addition to the CH_2 sequences in the amorphous regions. This explanation now allows the polyglycine results to be rationalized, but it is not clear how the γ process in nylon-6 and polyglycine is different from the β process in collagen since both involve amide or carbonyl groups on the main chain.

In any case the γ process at 200°K in collagen cannot be correlated with any of the processes in the nylons since, as Baer et.al. observes, its temperature position shifts to higher values as the water content is increased. The anomalous behavior of the γ peak remains unexplained.

Several n.m.r. studies on collagen have been reported but they were mainly concerned with the nature of water binding[137-141]. Others used the technique to investigate the structure of collagen[13,144]. Berendsen's two proton splitting signal[137] together with the fact that a repeat distance in the collagen chain fortuitously coincided with with an integral multiple of the repeat distance in the water chain (thus leading to possible hydrogen bond stabilization) led him to postulate a chain structure of bound water adjacent to the collagen chain.

Dehl[140] proposed an alternative explanation for his experiments which essentially refuted Berendsen's chain structure. Dehl's model was one in which all the water molecules were reorienting rapidly and almost randomly. However, a slight bias was induced due to interaction with the fibers of collagen. He also found that some protons contributing to the narrow line in his spectra were not exhangeable with deuterons. It was also found that the narrow line decreased markedly in intensity in the 270°-220°K range as compared to 250°K in our work.

Turning at this time to the results presented for this investigation, several broad generalizations can be made

from the comparison of the results obtained on collagen and gelatin.

First, judging from the level of the modulus in dry collagen (Figure 6) and in dry gelatin (Figure 10) we conclude that gelatin is a more rigid system. This conclusion would be expected if gelatin had a large number of interchain entanglements and hydrogen bonds acting as crosslinks. These results are analogous to Chien's and the explanation parallels his. The low temperature limits of the second moment in each case (Figure 12A and 13A) exhibit the same trend indicating, by the higher second moment, a tighter, more rigid lattice for gelatin.

Second, the positions of the α and β mechanical loss peaks are the same for collagen and gelatin and their water dependence is the same. These facts suggest that the processes responsible for the damping are similar in the two samples and are not affected by the precise conformation of the polypeptide chains.

In previous studies it was shown that poly-γ-benzyl-L-glutamate [146,147] exhibited a mechanical loss peak in the temperature region 260°-300°K (0.2-1cps) attributable to side chain reorientations. A major loss process occurred at 303°K 1cps) in poly (N^{ε} -carbobenzoxy-L-lysine)[147], also was attributable to reorientations of the side chain. Similar motions were employed to explain the relaxation peaks in the 300°K region of several copolymers of L-leucine

and γ-benzyl-L-glutamate[147]. The side chain motions have been observed in the n.m.r. for poly (N^{ε}-carbobenzoxy-L-lysine) as a sharp decline in second moment in the 300°-330°K range[148]. Poly (γ-benzyl-L-glutamate) exhibits a decrease in second moment near 300°K and poly (sodium α-L-glutamate) with trace amounts of water exhibited a liquid-like narrow line in addition to the broad line down to a temperature of 260°K[149]. Poly (β-benzyl-L-asparate) displays a second moment drop attributed to the motion of the entire side chain at 340°K and contributions to the drop from portions of the side chain at lower temperatures[150].

These facts suggest that the α-mechanical loss peak and changes in the n.m.r. occurring in the 260°-300°K region are due to reorientations of the side groups of collagen and gelatin such as those in lysine, and glutamic acid. Consistent with this assignment are the effects of water and crosslinking on the α-process. The temperature of the α process is lower since water can be expected to swell the regions containing the polar side chains, thereby, allowing greater reorientational freedom. As pointed out earlier, these regions will swell with greater ease since they can be expected to be somewhat disordered. Also it can break up charged side chain pairs lowering the barrier to the reorientation (see the example in diagram in Veis[1] p. 31). Since the water presumably would now participate in the motions taking place, the intensity of the α peak increases. Crosslinking with formaldehyde would have the opposite effect. As stated earlier

formaldehyde connects the ends of basic side chains such as lysine, hence tightening the regions containing such side chains. The results are greater rigidity of the sample, as reflected in the level of the modulus, and diminished efficiency of side chain reorientations to contribute to the α process.

If we can relate the observed α mechanical peak to Baer's β peak by virtue of the fact that their temperature of occurrence is similar, we see that, in fact, this process is due to a "polymer-water complex", only we are suggesting that the polymer portion is supplied by the side chains. In this respect our assignment is more specific and is not related to Baer's process in nylon-6 where the polymer-water complex involved the backbone of the nylon chain.

At first it appears reasonable to ascribe the n.m.r. narrow line appearance to side chain motions. However, if this were so, then we would expect a relaxation process in theydynamic mechanical testing. Keeping in mind the temperature-frequency relationship, we would expect this relaxation process to appear at lower temperatures in the lower frequency torsion pendulum experiment. This would be the β peak. However, the temperature of the β peak is too low for side chain motion if the polypeptides discussed in the beginning of this section serve as an accurate model. Dehl[140] proposed that the narrow line was due to non-exchangeable -OH protons of hydroxyproline trapped inside the triple helix. Nothing in the present work either confirms or refutes

this assignment.

The assignment for the β peak at 195°K found in the present work comes from a direct comparison with the low temperature loss process in poly-L-proline II. This comparison is possible since collagen and gelatin contain a large proportion of proline and hydroxyproline. Together with glycine these residues enforce a poly-L-proline II (or poly-glycine II) type of helix structure on the polypeptide chains of the protein. The comparison between collagen and poly-L-proline II made here is more valid than the comparison of collagen and nylon since the three dimensional structure of the polypeptide chains in collagen and polyproline II are similar. The effect of interchain hydrogen bonding, present in collagen but not in polyproline, can be taken into account later. Plausibility is given to the comparison by noticing that the n.m.r. second moment drop for gelatin containing water (Figure 13B) almost coincides exactly in temperature and magnitude of change with that of poly-L-proline II (Figure 14B). The small drop in collagen's second moment at 200°K (Figure 12B) is also not far removed.

Referring back to the introductory discussion of the structure of poly-L-proline II recall that the only bond about which oscillation or rotation can take place is the bond labelled ψ. We now propose that it is this rotation or main chain oscillation that gives rise to the relaxation process observed at 140°-230°K (0.4cps) and 180°-280°K

(10^4 cps). A similar low frequency main chain type motion is then responsible for the β transitions in the collagen and gelatin systems. This is in accord with the generalization made earlier that due to the similarities in the relaxation behavior of both collagen and gelatin the process involved were similar in the two and not markedly influenced by the unique structure of collagen. The small effect of water on the β peak in collagen and gelatin is now understandable, since the water molecules are not expected to penetrate the crystalline regions responsible for the β relaxation process. This latter fact was mentioned earlier in the introduction (p. 15).

If the assignment for the β peak is valid, then it becomes apparent that there is a higher barrier to the chain oscillation in collagen and gelatin as compared to poly-L-proline II. This may be due to interchain interactions such as hydrogen bonds (not possible in poly-L-proline II) or the effect of other amino acids present in the protein. Of the two, collagen and gelatin, the latter most closely resembles poly-L-proline II, since it does not have the triple helical structure imposed on the polypeptide chains. This fact is reflected in the closer correspondence of the n.m.r. second moment drop for gelatin and polyproline.

Crosslinking of collagen with formaldehyde (Figure 8) has the effect of bringing the β mechanical loss process to a lower temperature coincident with the loss process in poly-L-proline II. The explanation for this is probably

related to the fact that even in the apolar, pyrrolidine rich, ordered regions, the regions responsible for our β peak in collagen, there are some long side chains of the type crosslinked by formaldehyde. Under the conditions employed for the crosslinking reaction a certain amount of formaldehyde penetrated these regions and either by cross-linking the aforementioned side chains or by a process of main chain scission decreased the interchain interactions in the crystalline regions sufficiently to allow the shift of the β peak to the lower temperature value.

Also to be taken into account are the reports by Dehl and Hoeve[140] that water reorientationswas taking place at temperatures as low as 220°K (10^4 cps) and Hiltner et.al.[147] who reported small loss maxima in the 175 °K region (1 cps) attributed to water in their synthetic polypeptides. This water reorientation therefore probably contributes to the second moment drop in the 200 °K range for collagen and gelatin.

The calculated Van Vleck rigid lattice second moment for poly-L-proline, neglecting interchain and inter repeat unit interactions is 14.7 gauss^2 (Appendix 1). The low temperature limit obtained in our experiments at 160 °K was 17.5 gauss^2. Obviously there are considerable interactions of the type neglected in our calculations. Another fact to be considered is that our low temperature value of 17.5 gauss^2 may not represent the rigid lattice value, since it can be expected that the methylene units of the five membered ring are moving in and out of the plane of the ring at

this temperature. Going to lower temperatures in the n.m.r. experiment would perhaps freeze this motion also and an increase in linewidth and second moment would be observed.

Although the question of the origin of the narrow lines in collagen and gelatin is far from resolved, there is no evidence whatsoever correlating them with the narrow line of poly-L-proline. In the case of poly-L-proline, as stated in the Results Section, the line gradually narrows as the temperature is raised and its position of first appearance decreases as the water content is increased. In addition, it seems to be unrelated to the second moment drop in the 230 °K range. If the narrow line were due to motions occurring in the amorphous regions of the polypeptide, a mechanical loss process below 250 °K (4.8% water) should be apparent which would exhibit the same behavior with water content change. The only mechanical loss process below 250 °K has been correlated with the second moment drop and hence there appears to be no process for the narrow line corresponding to a T_g for the polymer. It is possible that the narrow line is due to very low molecular weight oligomers in the sample which give rise to the effects seen.

The obvious discrepencies between the results obtained by different investigators are, in all likelihood, due to differences in some poorly understood parameters. Although our results can be compared to some of the features of each investigation, the differences are more apparent and can

probably be attributed to differences in crosslinking (age), water content, chemical and physical history and chemical composition. The relative importance of each of these is hard to evaluate sinde a change in one may well cause changes in one or more of the other parameters. For instance, a change in crosslinking is known to cause a change in the crystallinity and , therefore, also the equilibrium water content. Conversely a change in the water content changes the degree of crystallinity. There is also the possibility that the importance of a change in one parameter is dependent on the extent to which the state of the system is already changed by an earlier value of this parameter. For example, the addition of water to nylons causes relatively larger changes for the first few percent, after this,addition of water does not cause so great a change.

Least important of the parameters mentioned for our system is denaturation since the gelatin systems were the same as collagen in their overall features. No new relations were observed nor were old ones lost.

It is possible that water content will turn out to be extremely important in that there may be a threshold value that may vary with some other parameter, below which a relaxation process is not observed and above which it is apparent. This type of behavior is seen in nylon where the β peak is only seen when there is water present. In collagen, studies have shown that the crystallinity increases

with water content up to approximately 30% water then decreases again[151]. It is conceivable that at high water contents where the crystallinity is decreasing new relaxation phenomena will be observed. For example, the β peak may split into a main chain oscillation in the crystalline regions occurring in the 200 °K range and the same motion involving the same groups but in the 180 °K region as in the crosslinked collagen samples. The difference being the extent to which there are interchain cohesive forces. Needless to say, many questions are raised by these investigations.

SUMMARY

A study of the dynamic mechanical properties of collagen revealed two loss processes occurring in the 150° to 300°K region (1 cps). The same was found to be true for denatured collagen-gelatin. Gelatin was found to be more rigid than collagen and this was ascribed to greater interchain attractions such as hydrogen bonding and entanglements.

The α process in both, occurring at approximately 280 °K, depended strongly on water content, shifting to lower temppratures and increasing in magnitude as the water content increased. By analogy with several synthetic palypeptides containing long side chains, this process was assigned to a side chain motion in the polar disordered regions of collagen and to the regions of the same composition in gelatin.

The β process in collagen and gelatin occurring at 200 °K (180 °K in crosslinked collagen) was attributed to oscillation of the main chain about single bonds. This assignment was based on a comparison with the dynamic mechanical properties of poly-L-proline II, a polypeptide having the same secondary structure as collagen's ordered regions and presumably gelatin's crystalline regions. In fact, it is believed that the high content of proline and hydroxyproline in these regions of collagen and gelatin in part enfordes this secondary structure. Poly-L-proline II was found to exhibit a loss process at 175 °K (0.4 cps).

From an analysis of the types of motional freedom of the polyproline chain it was concluded that rotation about the C_{α}-C=O bond was likely to cause such a relaxation process. This same process is possible in collagen and gelatin although somewhat more restricted due to interchain attractive forces such as hydrogen bonding.

Nuclear magnetic resonance experiments on collagen, gelatin and poly-L-proline tended to confirm and even suggest the correlations between the protein systems and the polypeptide.

In samples containing water, drops in the 200° to 230°K range of the second moment occurred for gelatin and poly-L-proline. Collagen exhibited the decline at a slightly lower temperature. These were correlated, by temperature-frequency considerations, with the β mechanical loss process occurring in each of the samples. For collagen and gelatin the decline of the second moment in the 260° to 300 °K region was assigned to the α process.

SUGGESTIONS FOR FURTHER WORK

The assignment of the main chain torsional oscillation to the mechanical loss process occurring at 175°K (0.4 cps) and the drop in second moment in the 200°K range (10^4 cps) for poly-L-proline II lends itself to further study. Since poly-L-proline I is a tighter helix and its potential energy profile, as discussed earlier, suggests much less possibility for the afore mentioned motion, the transition observed for poly-L-proline II should, in poly-L-proline I, be shifted to higher temperatures and decreased in magnitude if not totally eliminated. This could be investigated simply by observing the second moment of poly-L-proline I as a function of temperature.

In view of the discrepancies in the relaxation properties of collagen reported by the various investigators an obvious but not easily accomplished course of action is suggested. A well characterized sample of collagen should be used as the starting point for a comprehensive evaluation of all possible changes that could take place. Ideally a large quantity of tropocallagen would be used to first study the effects of ageing , both spontaneous and artificial, beginning with a totally uncrosslinked (fresh) film and progressing to a completely crosslinked sample. Concurrently swelling and x-ray properties should be monitored. Next the effect of pyrollidine residues should be investigated in tropocollagen obtained from several animal sources. In this light some of

the synthetic copolymer polypeptides possessing collagen type structure could be used.

The origin of the narrow lines in collagen and gelatin and poly-L-proline are also vague, and investigations employing such techniques as T_1, T_2 measurement using pulsed n.m.r. methods might be useful. Also n.m.r. utilising magic angle rotation could be used to determine if more than one process or "amorphous phase" is contributing to the narrow line[152,153,154].

Another possibility would be to attempt a deuterium exchange on gelatin rather than collagen as performed by Dehl. Since the narrow line in the n.m.r. did not change when deuterons were incorporated in the collagen structure Dehl, as pointed out earlier, suggested protons in the OH group of hydroxyproline as being shielded from exchange by their position in the collagen structure. This would be tested by denaturing the collagen before deuteration.

APPENDIX I

Second Moment Calculation for Poly-L-Proline

The equation used to calculate the second moment was

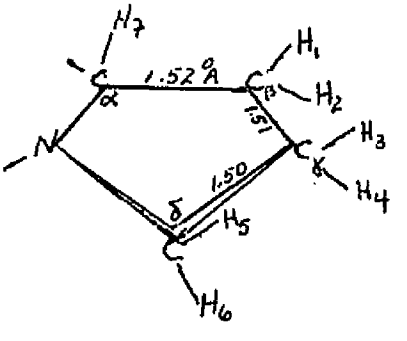
$$\Delta H_2^2 = 720 X \frac{1}{N} \sum_{i>j} r_{ij}^{-6} \qquad (A)$$

It was taken from reference 106 page 233. The assumption that allows us to use this formula rather than a more complex form is that the effect of non-resonant nuclei is negligible.

The assumptions used in our calculation of the second moment are:

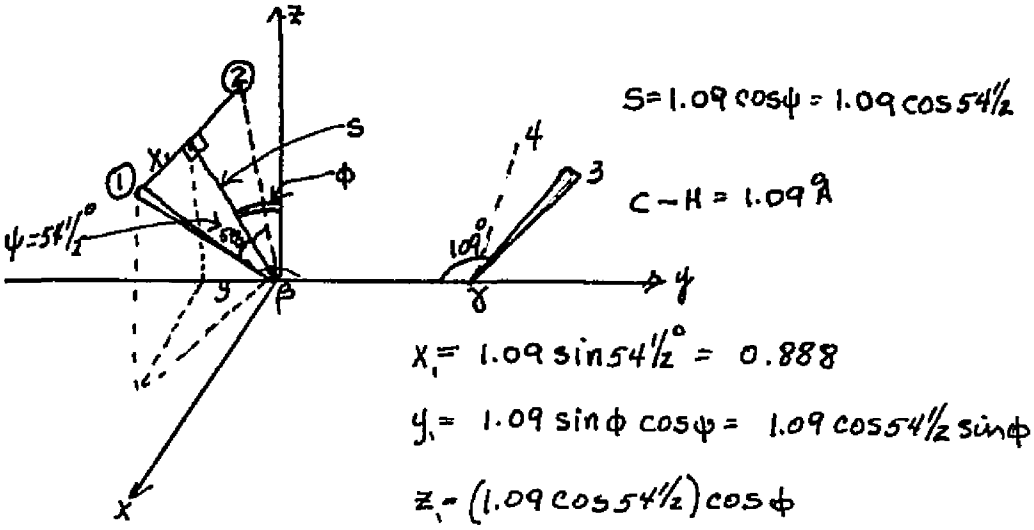
1) interchain interactions are negligible
2) inter-repeat unit interactions are negligible
3) interactions between protons on carbon atoms not adjacent to each other are negligible
4) all sp^3 hybridized carbon atoms have tetrahedral angles.

The protons involved in the calculation are on the five membered ring whose inter-carbon atom distances were taken from reference 5 page 155.



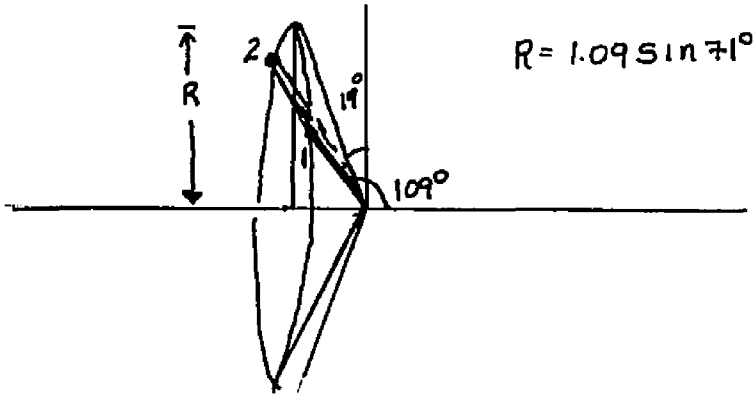
To calculate the interproton distances, r_{ij}, first obtain the cartesian coordinates of the protons then use the equation

$$r_{ij} = [(x_i - x_j)^2 + (y_i - y_j)^2 + (z_i - z_j)^2]^{\frac{1}{2}}. \qquad (B)$$



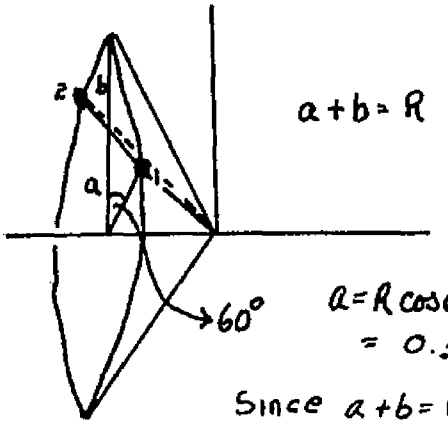
To obtain the angle ϕ:

1) note that the methylene protons when rotated give a cone of basal radius, R



2) However the perpendicular bisector, s, drawn from the apex of the cone to the base of the triangle does not lie on the cone, and hence its angle with the z axis, ϕ, is greater

than 19° ($\phi = 19° + \theta$).



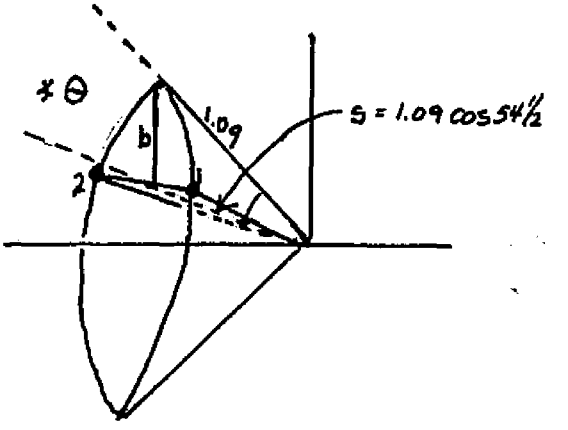
$a = R\cos 60° = (1.03)\frac{1}{2}$
$= 0.515$

Since $a + b = R$

$b + .515 = 1.03$

$b = a = 0.515$

A



use b, s, 1.09 to find θ.

B

from the law of cosines,

$$x^2 = (1.09)^2 + (s)^2 - 2(1.09)(s)\cos\theta$$

$$(0.515)^2 = (1.09)^2 + (.633)^2 - 2(1.09)(.633)\cos\theta$$

$$\cos\theta = .96 \quad \therefore \quad \theta = 17°$$

$$\phi = 19° + \theta = 19° + 17° = 36°$$

Now the y and z coordinates may be evaluated:

$$y_1 = 1.09\sin 36 \cos 54\tfrac{1}{2} = -0.373$$

$$z_1 = 1.09\cos 54\tfrac{1}{2} \cos 36 = 0.513$$

For the coordinates of proton 2:

$$x_2 = -.888, \quad y_2 = -.373, \quad z_2 = .513.$$

The coordinates for proton 3:

$$x_3 = .888, \quad y_3 = 1.51 + .373, \quad z_3 = .513.$$

In general, $x_i = .888$, $y_i = 1 \pm .373$ where 1 is the C-C bond

length, and z = .513 in all cases.

The procedure for calculating the interaction between protons on neighboring carbon atoms is to place the origin on one of the carbon atoms and calculate the proton coordinates involved. This method is good only when assumption 3 is used. It also eliminates the need to consider ring puckering.

Coordinates for the β-γ calculation (origin on β carbon)

proton 1: (.888,-.373,.513)

" 2: (-.888,-.373,.513)

" 3: (.888,1.883,.513)

" 4: (-.888,1.883,.513)

Coordinates for the γ - δ calculation (origin on the γ carbon)

proton 3: same as 1 above

" 4: same as 2 above

" 5: (.888,1.873,.513)

" 6: (-.888,1.883,.513)

Coordinates for the α-β calculation (origin on α carbon)

proton 7: (.888,-.373,.513)

" 1: (.888,1.893,.513)

" 2: (-.888,1.893,.513)

Interaction	r_{ij} (Å)	r_{ij}^{-6}
$r_{12} = r_{34} = r_{56}$	1.776	.0319
$r_{13} = r_{24}$	2.256	.00759
$r_{14} = r_{23}$	2.871	.00179
$r_{35} = r_{46}$	2.246	.00779
$r_{36} = r_{45}$	2.863	.00182
r_{71}	2.266	.00739
r_{72}	2.879	.00176

$$\Delta H_2^2 = 720/7 \left[3(.0319) + 2(.00759) + 2(.00179) + 2(.00779) \right.$$
$$\left. 2(.00182) + .00739 + .00176 \right]$$

$$\Delta H_2^2 = 14.7 \text{ gauss}^2$$

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