DYNAMIC MECHANICAL PROPERTIES OF COLLAGEN AND COLLAGEN-LACTASE SYSTEM

by

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Abstract

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The dynamic mechanical properties of cattle hide collagen film and of a collagen-lactase system were studied using a torsion pendulum at 0.4 to 1 Hz in the temperature region of 120 to 300°K. In the temperature region studied four relaxation processes were observed for collagen. The α_1 and α_2 loss peaks at 290 and 280°K (0.04 g H_2O/g collagen) respectively shift to lower temperatures and increase in magnitude as the water content is increased to 0.13 g H_2O/g collagen. The β_1 loss peak at 185°K (0.19 g H_2O/g collagen) was observed only at high water content. The β_2 loss peak at 200°K (0.03 g H_2O/g collagen) shifts to lower temperatures with increasing water content. The storage modulus of collagen at low temperatures first decreases then increases with increasing water content showing a minimum at 0.13 g/g H_2O.

The α_1 and α_2 processes are assigned to large-scale side chain motion of glutamic acid, lysine, and arginine residues. The β_1 process is attributed to the motion of telopeptides associated with water. The β_2 process is assigned to the small-scale local motion of polar side chains.

The presence of lactase in a collagen film does not change the number of relaxation processes, but affects the characteristics of the dynamic mechanical properties of the collagen. A model for the collagen-lactase system is proposed.

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TABLE OF CONTENTS

	Page
Abstract	i
Acknowledgements	ii
List of Figures	iv
List of Tables	vii
I. Introduction	1
A. Collagen	1
1. Primary Structure	1
2. Molecular Structure	6
3. One-Dimensional Arrangement of Collagen Molecules	7
4. Intermolecular Cross-linking	10
5. Three-Dimensional Molecular Packing in Collagen Fibrils	14
6. Structure Hierarchies in Tendon Collagen	17
B. Water in Collagen	20
C. Mechanical and Dielectric Loss Processes of Collagen	27
D. β-Galactosidase (Lactase)	36
E. Collagen-Lactase System	38
F. Statement of the Problem	41
II. Experimental	43
A. Sample Preparation	43
B. Relative Water Content	44
C. Dynamic Mechanical Testing	46
D. Characterization	48
III. Results	53
IV. Discussion	94
V. Conclusions	114
List of References	116

LIST OF FIGURES

Figure		page
1.	Pertruska-Hodge packing of collagen molecules.....	12
2.	Biosynthesis of the reducible crosslinks present in young collagen.....	13
3.	Scheme of structural hierarchies in tendon collagen	18
4.	Wide-angle x-ray diffraction pattern of aged collagen.....	50
5.	Wide-angle x-ray diffraction pattern of aged C-L...	51
6.	Logarithmic decrement (Δ) vs. temperature (200-300°K) for aged collagen containing various water contents.....	54
7.	Temperature position of the α_1 peak (Δ) vs. water content for aged collagen, C-L, and aged C-L.....	55
8.	Temperature position of the α_2 peak (Δ) vs. water content for aged collagen, C-L, and aged C-L.....	56
9.	Intensity (Δ) of the α_1 peak vs. water content for aged collagen, C-L, and aged C-L.....	58
10.	Intensity (Δ) of the α_2 peak vs. water content for aged collagen, C-L, and aged C-L.....	59
11.	Storage modulus (G') vs. temperature (240-300°K) for aged collagen containing various water contents	60
12.	Storage modulus (G') vs. water content for aged collagen at several temperatures.....	61
13.	Loss modulus (G") vs. temperature (240-300°K) for aged collagen containing various water contents....	63
14.	Logarithmic decrement (Δ) vs. temperature (120-240°K) for aged collagen containing various water contents.....	64
15.	Temperature position of the β_1 and β_2 peaks (Δ) vs water content for aged collagen, C-L, aged C-L.....	65
16.	Temperature position (based on G") and intensity (G") of the β_2 peak vs. water content for aged collagen, C-L, and aged C-L.....	66

17. Intensity (Δ) of the β_1 and β_2 peaks vs. water content for aged collagen, C-L, and aged C-L..... 67

18. Storage modulus (G') and loss modulus (G") vs. temperature (120-240°K) for aged collagen containing various water contents..... 69

19. Logarithmic decrement (Δ) vs. temperature (230-290°K) for C-L containing various water contents..... 71

20. Loss modulus (G") vs. temperature (230-290°K) for C-L containing various water contents..... 72

21. Temperature position (based on G") and intensity (G") of the α_1 peak vs. water content for aged collagen, C-L, and aged C-L..... 74

22. Temperature position (based on G") and intensity (G") of the α_2 peak vs. water content for aged collagen, C-L, and aged C-L..... 75

23. Storage modulus (G') vs. temperature (230-290°K) for C-L containing various water contents..... 76

24. Storage modulus (G') vs. water content for C-L at several temperatures..... 77

25. Logarithmic decrement (Δ) vs. temperature (120-230°K) for C-L containing various water contents..... 79

26. Loss modulus (G") vs. temperature (120-230°K) for C-L containing various water contents..... 81

27. Temperature position (based on G") and intensity (G") of the β_1 peak vs. water content for aged collagen, C-L, and aged C-L..... 82

28. Storage modulus (G') vs. temperature (120-230°K) for C-L containing various water contents..... 83

29. Logarithmic decrement (Δ) vs. temperature (240-300°K) for aged C-L containing various water contents..... 85

30. Loss modulus (G") vs. temperature (240-300°K) for aged C-L containing various water contents..... 86

31. Storage modulus (G') vs. temperature (240-300°K) for aged C-L containing various water contents..... 88

32. Storage modulus (G') vs. water content for aged C-L at several temperatures..... 89

33. Logarithmic decrement (Δ) vs. temperature ($120-240^{o}K$) for aged C-L containing various water contents..... 90

34. Loss modulus (G") vs. temperature ($120-240^{o}K$) for aged C-L containing various water contents..... 92

35. Storage Modulus (G') vs. temperature ($120-240^{o}K$) for aged C-L containing various water contents..... 93

36. Temperature positions (Δ) of the mechanical loss peaks reported by Chien and Chang, Bair et al., Nguyen et al., Stefanou et al., and this study..... 95

37. Temperature position (Δ) of the mechanical loss peaks reported by Nomura et al. and this study.....101

LIST OF TABLES

Table		page
1.	Distribution of amino acids among positions in the collagen triplet Gly-X-Y.....	5
2.	Structure for mucopolysaccharides.....	19
3.	Comparison of mechanical and dielectric relaxation at 0.1 KHz of various collagens.....	29
4.	Dynamic mechanical properties of HDT (30 yr), polyglycine I, and nylon 6 (1 Hz).....	31
5.	Dynamic mechanical properties of collagen and poly-L-proline II.....	33
6.	Broad line proton magnetic resonance data for collagen and poly-L-proline II (90 MHz).....	33
7.	Outline of the dynamic mechanical properties of various collagens from three of the investigations.	35
8.	Amino acid composition of β-galactosidase.....	39
9.	Drying of collagen and collagen-lactase.....	45

I. Introduction

A. Collagen

Collagen is the most abundant animal protein, comprising about 25% of the total protein in mammals.[1] It may be defined as a class of proteins whose composition and structural parameters fall within the following specifications:[2] (1) approximately 33% of the amino acid residues are glycine, and 20% the imino acids proline and hydroxyproline; (2) the x-ray diffraction pattern exhibits a 3 A^{o} meridional arc and 12 A^{o} equatorial spots; (3) an axial periodicity of 668 A^{o} of the fiber is found by electron microscopy; and (4) the fibers shrink and lose their x-ray and electron microscopy patterns on heating. Collagen is highly ordered and has a high tensile strength.[3] Nature has utilized these unique properties throughout the animal body in a variety of ways to overcome mechanical problems. The function of collagen is clearly almost entirely mechanical. Yannas[4] and Chien[5] have thoroughly reviewed the many investigations of the structure and properties of collagen.

Primary Structure

The collagen monomer, termed tropocollagen, is composed of three polypeptide chains. Examination of the subunit composition of the collagen monomer has shown that different types exist. The collagen that has received the most study is type I. Type I collagen is the major one in skin and the only one in bone and tendon. It has the chain composition $[\alpha 1(I)]_2 \alpha 2$ where $\alpha 1(I)$ and $\alpha 2$ are homologous. The collagen specific to hyaline cartilage (type II) has three

identical chains and is designated $[\alpha 1(II)]_3$.[6,7] Similarly, the collagen that is a minor but important constituent in skin and perhaps a major constituent in large blood vessels (type III) is designated $[\alpha 1(III)]_3$.[8] The collagen of basement membrane is referred to as type IV. As reviewed by Kefalides[9], the amino acid composition and limited studies on cyanogen bromide peptides strongly suggest that basement membranes such as lens capsule, Descemet's membrane and glomerulus contain a collagen that is genetically distinct from types I,II, and III. The basement membrane collagen molecule is designated $[\alpha 1(IV)]_3$.[10,11] Recently, Burgeson et. al.[12] reported that human fetal membranes contain 2 new genetically distinct collagen polypeptide chains which are tentatively named αA and αB. Both αA and αB exhibited contents of imino acids and glycine typical of collagens. Comparison with the observed and reported compositions of collagen chains of type I-IV collagens revealed notable differences, particularly in the content of alanine, leucine, isoleucine, and the basic amino acids, lysine, hydroxylysine, and arginine. They suggested a single new species of collagen with a subunit structure $\alpha A(\alpha B)_2$. Current information on the biochemical properties, biosynthesis, and tissue distribution of Type I, II and III collagen has been summarized by Miller.[13]

The collagen monomer is composed of three polypeptide chains each in a left-handed polyproline II type helix and all three twisted into a right-handed super triple helix.

The N- and C- terminal ends of the ∝ chains of type I collagen consist of sequences that do not contain glycine in every third position and therefore cannot be triple helical like the body of the molecule.[14] These non-helical regions (termed telopeptides) are thought to play an important role in film formation,[15] crosslinking[16] and antibody binding.[15,17] The telopeptide segments can be cleaved by proteolytic enzyme, such as trypsin, pepsin, without loss of the triple-helix structure of collagen.[18]

Partial or complete sequences for the N- terminal telopeptide of the ∝1(I) chain and of the ∝2 chain of six species (chick, rat, rabbit, calf, baboon, and human) have been previously summarized by Traub[19] and Gallop.[20] Current information on the sequences of the nonhelical N-terminal regions of type I collagen has been summarized by Piez.[21] The N- terminal nonhelical region of ∝1 (I) chain contains 16 amino acid residues. The lysine residue at position 9, present in all ∝ chains studied, is normally converted to the aldehyde allysine, which is a precursor of crosslinks.[22] In some cases it is first hydroxylated[23] and is then presumably converted to hydroxyallysine (Fig. 2), which is also a precursor of cross-links.

Rauterberg[24] has discussed the C- terminal nonhelical portion of the collagen molecule. The C- terminal sequence for calf collagen ∝1(I) has been determined and was found to contain 25 amino acid residues. The presence of lysine-

containing (residue 1044) and aldehyde-containing forms of the C- terminal telopeptide indicate that this region, like the N- terminal region, is a cross-linking site.

The sequences of the nonhelical N- and C- terminal regions of collagens other than type I have not been determined. Recently the sizes and amino acid compositions of the N- and C- terminal nonhelical regions of type III collagen, extracted from fetal calf skin, have been deduced.[25] The pepsin-sensitive N- and C- terminal regions of type III collagen contain approximately 16 and 56 amino acid residues, respectively. Both N- and C- terminal regions contain lysine and tyrosine residues which are a possible site for intermolecular crosslinking and for antigenicity, respectively.

Data on the primary structures of individual cyanogen bromide peptides and their order in the chain was e . ually used to elucidate the entire primary structure of the collagen α1(I) chain containing 1052 amino acid residues. These data were initially assembled[26] from experiments on rat skin α1(I) which was used to determine the primary structure of the first 418 residues beginning at the NH_2 - terminus of the chain, and from experiments with calf skin α1(I) which was used for the remaining 634 residues of the chain. The amino acid sequences and the literature references to sequences in the helical region of collagen chains are given in Table I and II of reference 21, respectively. To date, information of the primary structure of α1(II) has been accumulated for approximately one-quarter of the

chain.[27] The order of the cyanogen-bromide-derived peptides from α1(III) chains of pepsin-solubilized calf skin collagen has been deduced recently by Fietzek et. al.[28]

The results of these studies clearly demonstrated that the collagen α chain exists throughout its entire length as a linear array of amino acids linked exclusively by α- amino, α-carboxyl bonds. They further showed that the chain is comprised for over 95% of its length by a repeating series of Gly-X-Y triplets, a structural feature which is an absolute requirement for assumption of the triple-chain helical conformation of the native molecule.[29] The distribution of amino acids among positions in the triplet Gly-X-Y in a composite sequence from rat, calf and chick α 1(I) is shown in Table 1.

Table I

Distribution of Amino Acids Among Positions in the Collagen Triplet Gly-X-Y[a]

	Position 1	Position 2	Position 3	Total
4-Hydroxyproline		1[b]	113	114
Aspartic acid		16	15	31
Asparagine		7	5	12
Threonine		3	13	16
Serine		17	18	35
Glutamic acid		41	6	47
Glutamine		8	19	27
Proline		116	3	119
Glycine	337	1		338
Alanine		60	61	121
Valine		9	8	17
Methionine		2	5	7
Isoleucine		3	4	7
Leucine		18	1	19
Phenylalanine		12		12
Hydroxylysine			4	4
Lysine		12	20	32
Histidine		2		2
Arginine		9	42	51
Totals	337	337	337	1,011

a Using a composite sequence from rat, calf and chick α 1(I)
b 3-Hydroxyproline

Molecular Structure

The collagen monomer molecule (tropocollagen) has a rod-like shape of about 3000A° long and 15A° wide. It consists of three similar polypeptide chains wound round each other to form a three-strand rope. A detailed description of this is contained in a review by Ramachandran.[29] The abundance of prolyl and hydroxyprolyl residues in collagen causes the individual chains to resemble the left-handed poly-L-proline II helix. On the other hand, the occurrence of a glycyl residue at every third position (except in the telopeptides) appears also to confer to the individual strands the capacity of poly-glycine II chains for extensive sterically permissible inter-chain hydrogen bonding. X-ray diffraction patterns reveal a helix of pitch 9.5A° with subunits spaced axially by 2.86A°. Molecular model building indicates how the three chains could be arranged on such a helix. Each α chain is itself a helix with approximately three residues per turn and when the chains are wound round each other, the glycine residues are arranged so that they form the core of the three-stranded rope. The three chains are related by the helix of pitch 9.5A° which implies that one α chain is related to another by an azimuthal rotation of 108° and an axial translation of 2.86A°.

Three structures of the collagen molecule with a different number and type of inter-chain hydrogen bonds,

known in the literature as the "two-bonded,"[30,31] the "one-bonded"[32,33] and the "water-bridged two-bonded"[34] structures, have been proposed. Recently Yee et. al.[35] have proved that native collagen has a two-bonded structure. They found that approximately 1.7 NH groups per three residues exchange slowly with tritium when the sample is placed in tritiated water. However, they could not distinguish between the earlier two-bonded model, which contained two direct peptide-to-peptide hydrogen bonds per triplet, and the more recent proposal involving one direct bond and one cross-bridged via a water molecule. The molecular structure of collagen along with the related structures of polypeptide chains having amino acid residues commonly occurring in collagen has been reviewed recently.[36]

One-Dimensional Arrangement of Collagen Molecule

The one-dimensional arrangement means the axial molecular arrangement or the structure as projected onto the fiber axis. The low-angle X-ray diffraction pattern from native rat tail tendon contains a series of meridional reflections which index on a period of 668A° (D or 234 residues)[37]. In the electron microscope this is manifested as a regular set of bands perpendicular to the fibril axis with a period of D. There are two main kinds of banding pattern in the D repeat which depend on the conditions of staining. In one there are about twelve narrow bands in each D repeat while the other has only two broad bands, one heavily the other lightly

stained. The first kind of these are usually interpreted as due to positive staining in which the narrow bands correspond to the location of charged amino-acids,[38] and for the second kind, to negative staining where the stain accumulates in a gap region (dark band) and an overlap region (light band) of the fibril.[39]

A one-dimensional solution to the arrangement of collagen molecules in native fibrils was proposed by Schmitt et. al.[40] in 1955. They suggested that the D repeat could be generated if molecules of length L were staggered axially by a distance D with respect to their neighbors. At that time it was thought that L was equal to 4 D so this was called the "quarter-stagger" model and, because of the integral relation between L and D, the model involved the end-to-end contact of molecules. When the more accurate value of L = 4.4D was determined by Hodge and Petruska,[39] they pointed out that a D stagger between neighboring molecules led to a structure with gaps of some 300A° between the ends of molecules. In this solution, the D period contained an overlap region of 0.4D and a gap region of 0.6 D.

Hodge and Schmitt[41] obtained support for the quarter-stagger model from electron microscopy of a polymorphic form of collagen called segment-long-spacing (SLS). SLS can be obtained by reprecipitation of collagen in the presence of a small concentration of ATP. The molecules line up side by side in parallel register to form a segment of fixed length equal to the molecular length L,

and variable width. Electron micrographs of the SLS form contain 58 closely spaced dark bands under positive staining[42] and it has now been confirmed that these correspond to the location of charged amino acid residues.[38] Recently Ghosh et. al.[44] have demonstrated that densitometric measurements of electron microscopic segment-long-spacing (SLS) sequences in purified renatured rat skin α1-chain collagen are satisfactorily correlated with amino acid clusters in the collagen chain. The dark-staining segments correspond to polar amino acid clusters. Distances between dark bands were determined and correlated with the amino acid sequence from rat skin and calf skin collagen α1 chains. Good correlation was achieved with the exception of 4 (out of 42) bands. The lower quality correlation of these four bands is probably due to incomplete renaturation of the collagen chain. Hodge and Schmitt[41] photographically superimposed the SLS band pattern upon itself staggered by an integral value of the D period and synthesized a pattern of bands with the D repeat which correspond to the positively stained native band pattern from collagen. Recently their work has been confirmed by using the present knowledge of the molecular length and the amino acid sequence.[38,43]

Knowledge of the one-dimensional structure and the amino acid sequence of a complete α1 chain allows one to inquire about the origins of the molecular arrangement.

The first point to test is whether the D repeat can be shown to arise from the primary sequence. This was done[26] by translating the sequence of the 1011 amino acids of the triple-helical region past itself and scoring for favorable interactions between opposing amino acids. It was found that interactions between amino acids of opposite charge and between large hydrophobic amino acids was a maximum when the chains were staggered by OD, 1D, 2D, 3D and 4D, where D is 234 residues. Since molecules in the native fiber are staggered relative to one another in this fashion, the results strongly suggest that alignment of the molecules is controlled by the distribution of charged residues as well as larger hydrophobic residues along the component chains of the molecules. The amino acid sequence of collagen has been analyzed further, and it appears that there may be some significance in the fact that the oppositely ionizable amino acids tend to occur on the molecule in pairs separated by no more than 2 amino acid residues.[45] Interaction curves were calculated on the assumption that these pairs could interact as dipoles and this produced a dominant maximum at an intermolecular stagger of 1D, but not at the other integral multiples of D. Piez and Torchia[46] agreed that the ion pairs were significant for intermolecular interactions.

Intermolecular Cross-linking

The collagen molecules are aligned in parallel in

a quarter-stagger and overlap fashion, directed by the charge profiles of the molecules (Fig. 1). The fibril is subsequently stabilized by the formation of covalent crosslinks between the molecules to prevent slippage of adjacent molecules under tension. This latter step involves oxidation of specific lysine or hydroxylysine residues (residue 9 and 1044) at the terminal non-helical portions (telopeptides) of the molecule and the aldehydes so produced spontaneously condense with hydroxylysine residues (possibly residue 943 and 103) situated in the helical body of a neighouring molecule to form aldimine crosslinks.[21] If the crosslink precursor in the non-helical telopeptide is hydroxylysine-derived aldehyde, the aldimine initially formed undergoes an Amadori rearrangement to a keto form, thus yielding a relatively stable bond.[47] In contrast, the crosslink obtained from the condensation of the lysine-derived aldehyde is chemically and thermally labile since in the absence of a hydroxyl group no such stabilizing rearrangement can take place (Fig. 2). The two histidines (residue 945 and 105) which are the only ones in the helical region have also been implicated in crosslinking.[48] The chemistry of crosslinks, crosslink location and crosslink biology have been reviewed by Tanzer[49] recently. Methods for the separation and analysis of crosslinking components from collagen, prepared by standard means from skin, tendon, demineralized bone and cartilage have been described

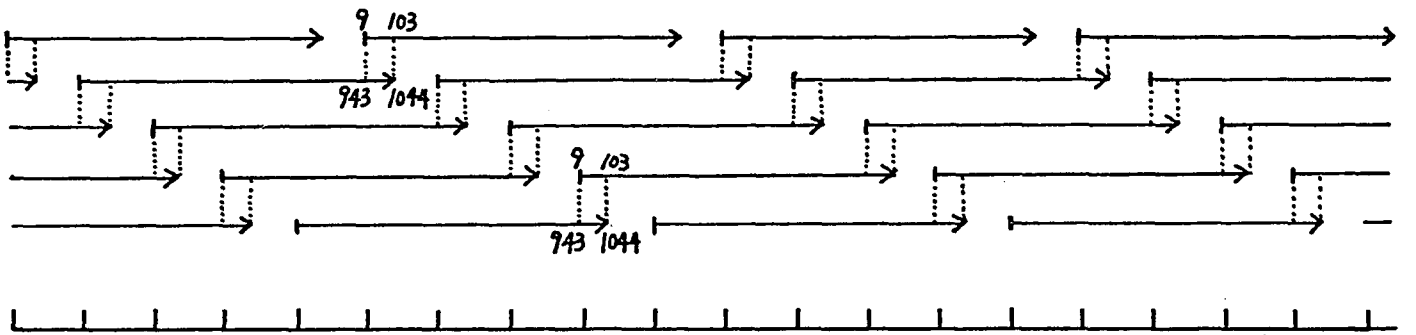


Fig. 1. Pertruska-Hodge[39] packing of collagen molecules (arrows) showing likely positions for intermolecular cross-links (dashed lines). These positions predict the involvement of residue pairs 9-943 and 103-1044 in intermolecular cross-links. The scale shows the native repeat, D = 668 A^{o} = 234 residues.

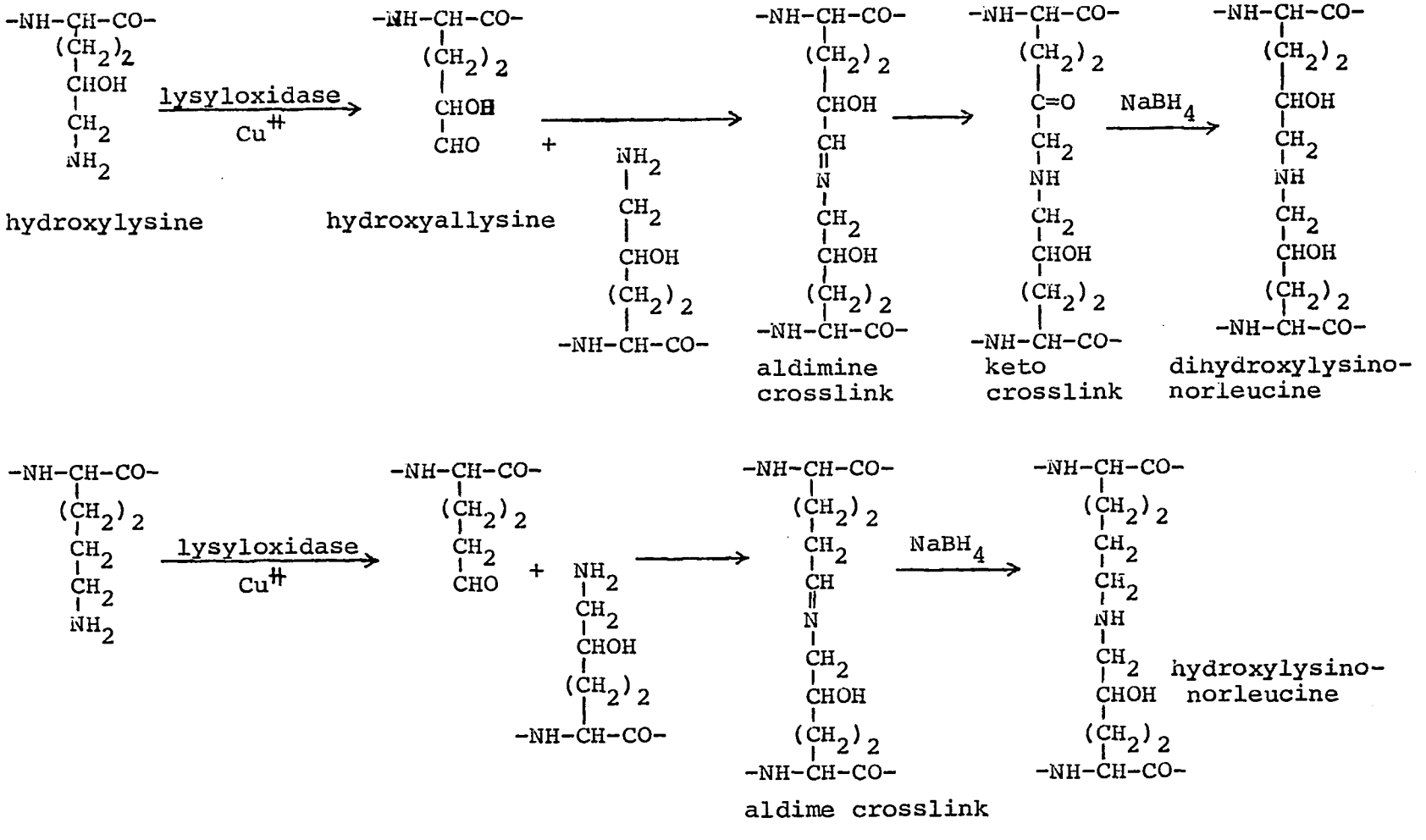


Fig. 2. Biosynthesis of the reducible crosslinks present in young collagen[47]

by Robins[50] recently.

Three-Dimensional Molecular Packing in Collagen Fibrils

The last few years have shown a rapid increase in the number of studies on the three-dimensional arrangement of molecules in collagen fibrils. Considerable progress has been made and well-established findings can be summarized.

1. A collagen fibril contains the elements of crystallinity and thus the molecules are arranged on a regular three-dimensional lattice.[51]

2. The collagen molecules are not parallel to the fibril axis but tilted by about 4^{o}.[51,52]

3. In native tendon at least one lattice vector is not precisely at right angles to the fibril axis but inclined, at about 3^{o}, to a plane perpendicular to the fibril axis.[51]

4. A vector of length $38A^{o}$ is important in the lateral arrangement of molecules.[51,53]

5. There is chemical evidence that collagen molecules shifted axially by 4 D, are covalently linked in the fibril.[54]

6. There is chemical[54] and electron-microscopic[55] evidence that in the fibril molecules are related by axial shifts of OD, ID, and 4D.

7. When projected onto the fibril axis, the molecular arrangement has a periodicity of D ($668A^{o}$)[37].

The complete three-dimensional molecular arrangement

has not yet been determined. Three main models have been proposed. A heuristic model has been described[56] which is preferred since it appears to be consistent with a wide range of types of evidence. This model consists of five-strand microfibrils, supercoiled and packed face to face on a tetragonal lattice. Some possible difficulties with absent low-angle near-equatorial reflections in the x-ray diffraction pattern and a rather low density of molecular packing are criticisms of this model at present. A second model based on a closely similar near-tetragonal cell has been proposed,[52] but this cell contains an octofibril rather than a five-strand microfibril. Criticisms of this model are that it has a high density of molecular packing, that the crystallographic justification for the special type of disorder required is still not explicit, and that so far it has not been related to the triple-helical molecular conformation as have the other models. A third model is based on a hexagonal array of collagen molecules.[57] The specific proposals which have been made would require modification to fit the x-ray diffraction patterns from either native or heavy-metal-stained tendons and to fit the observed axial intermolecular shifts.

Further models have been proposed for the molecular packing. Woodhead-Galloway et. al.[58] accept the tetragonal indexing with slight modification but suggest that

two-stranded microfibrils are supercoiled and arranged in the tetragonal cell. The disadvantage is that it has a high density of molecular packing. Recently Woodhead-Galloway[59] reported that replacing the single molecules by dimers provides a model which gives a fair account of the distribution of the intensity very close to the equator of the diffraction pattern. Veis and Yuan[60] have proposed a model which is based on the five-strand microfibril packed in a tetragonal lattice of side 38 A^{o}, with a superlattice due to the microfibril orientations. This model goes further since it contains details of the relation between the structures of gap and overlap regions. No doubt it will be possible to test such models when relevant experimental observations become available.

The demonstration that the origins of the axial intermolecular stagger lies in the amino acid sequence of the collagen molecule provides an understanding of the self-assembly of the fibril.[26] This success opens the way for analysis of the origins of the various polymorphic forms of collagen.[61] Recently this approach has been extended to three dimensions. From the three-dimensional analysis of electrostatic interactions, Trus and Piez[62] have found that the results are consistent with a 5-fold helical micro-fibril of collagen molecules related by a 1D stagger and a left-handed rotation as the basic substructure of the native collagen fibril.

Structure Hierarchies in Tendon Collagen

Three polypeptide chains form a triple helical unit, the so-called tropocollagen (Fig. 3a). According to the heuristic model five such tropocollagen units form a larger fibrous entity called a microfibril[51] (Fig. 3 b_1). The five tropocollagen units are staggered longitudinally along the micro-fibril axis by a fixed amount somewhat over one-quarter of the molecular length (Fig. 3 b_2). This stagger, combined with a gap between the ends of consecutive tropocollagens, is supposed to be responsible for the characteristic 668A° periodicity manifest both by electron microscopy and low angle X-ray diffraction in collagen in general. The microfibrils are presumably packed in a tetragonal lattice (heuristic model) with a lattice period of 38A° leading up to the collagen fibril[37] (Fig. 3c) itself which with its 668A° banding is the familiar fibrous entity in the electron microscopy of collagen. The diameter of these fibrils at maturity is in the range between a hundred and a few hundred nanometers. The fibrils themselves are surrounded by an extrafibrillar matrix[63], consisting largely of mucopolysaccharides (Table 2) and to a lesser extent structural glycoproteins.[64] It has been thought that this structure extends laterally to form tendon units of diameters of a few hundred micrometers which, together with elastic fibres and fibroblasts, are bundled together to give the macroscopic tendon.[63]

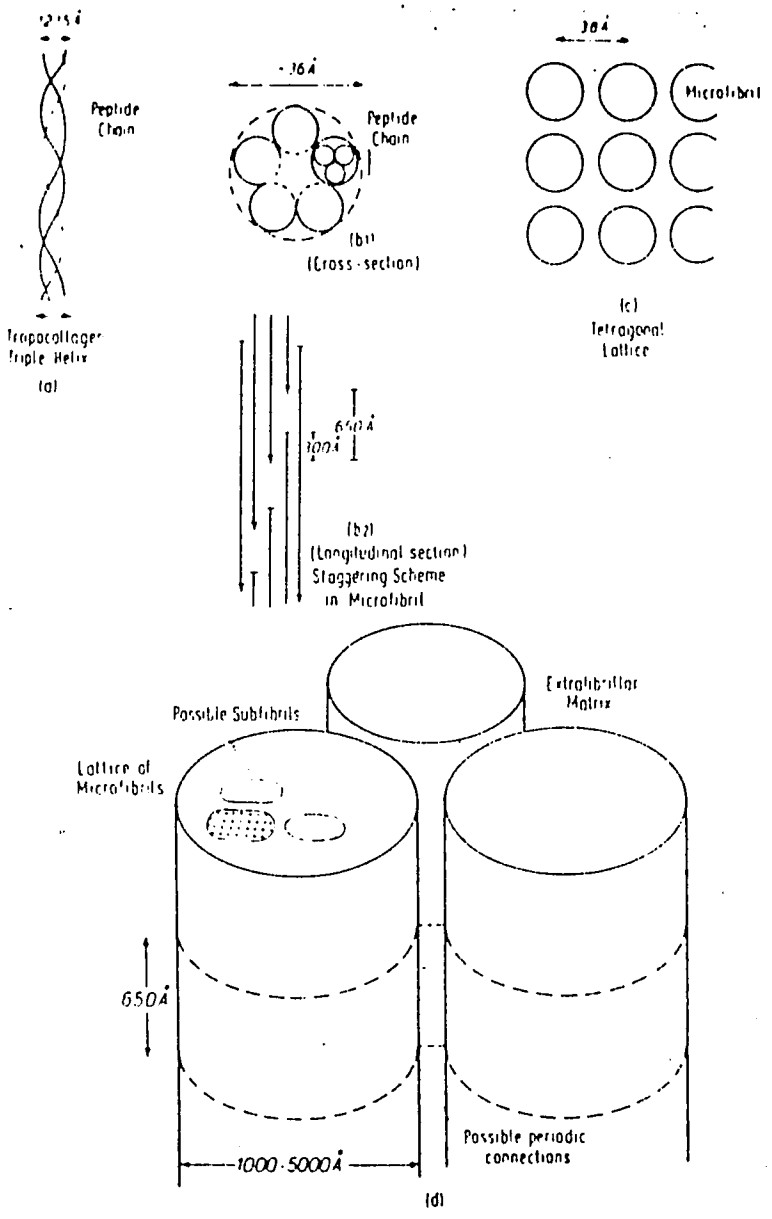
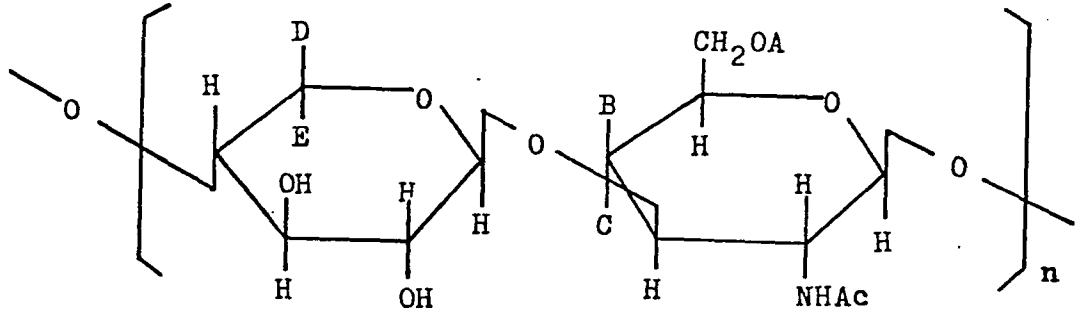


Fig. 3. Scheme of structural hierarchies in tendon collagen.[137]

Table 2. Structure for Mucopolysaccharides[138]



	A	B	C	D	E
Chondroitin sulfate A	H	OSO_3H	H	COOH	H
Chondroitin sulfate B	H	OSO_3H	H	H	COOH
Chondroitin sulfate C	SO_3H	OH	H	COOH	H
Hyaluronic acid	H	H	OH	COOH	H

B. Water in Collagen

Collagen, in its native states, always coexists with water. Upon complete dehydration, collagen becomes insoluble.[65] Major insolubilization occurred below 1% of moisture content. This process is irreversible and has been attributed to cross-linking reactions.[4] That a certain amount of water is essential in the maintenance of tropocollagen (TC) in the native conformation is obvious. Harrington and Von Hippel[66] suggested that the collagen triple-stranded helix is stabilized by water molecules through hydrogen bonds with the peptide groups. Based on a number of X-ray investigations, positions were assigned to water molecules. In a structure similar to that proposed earlier by Rich and Crick,[67] Ramachandran et. al.[34] proposed that the three strands are linked by one hydrogen bond per three peptide units and stressed the stabilizing influence of hydrogen-bonded bridges formed by two water molecules per tripeptide (about 0.12 g H_2O/g TC). Several methods have been used to determine the total amount of water essential for the maintenance of the native collagen conformation which is distinguishable from free or loosely held water. The values in grams of water per gram of tropocollagen are 0.465 by calorimetry,[68] 0.54 by NMR[69], and 0.52 by differential scanning calorimetry.[70] If 0.12 g water of 0.50 g water is of the intramolecular hydrogen bonded type, what is the nature of the remainder? This question has been

examined principally by NMR techniques. However, no agreement has yet been reached by various investigators. Berendsen et. al. [71,72] first reported that the proton magnetic resonance (PMR) spectrum of hydrated oriented collagen fibers consists of a nearly isotropic central line and an angular-dependent doublet caused by direct dipolar coupling of anisotropically rotating water molecules. The doublet lines decrease in separation with increasing water content and merge into a single line with increasing temperature.[72] It was observed by Berendsen[73] and independently by Chapman[74] and by Dehl[75] that the deuteron magnetic resonance (DMR) spectrum of D_2O-hydrated collagen shows a similar angular dependent doublet, caused by quadra-polar interaction. No central line is observed as in the case of H_2O. The distance between the lines remains unchanged at increasing temperature. The conclusion is reached[75,76] that the collapse of the proton doublet at increasing temperature is caused by proton exchange between water molecules. Recently, several two-state models for water absorbed in collagen have been proposed on the basis of wide-line NMR spectra obtained with H_2O and D_2O. In the model of Chapman et. al.[77] one state consists of an ordered chain-like structure of water molecules (originally proposed by Berendsen[70]) which are hydrogen bonded to each other and to peptide groups which are presumably in register with the water molecules. A limited fraction of the water molecules can be absorbed on these sites. When these

sites are filled, additional water molecules enter into a second state, in which they are isotropically rotating. Chapman et. al.[77] deduced from the splitting in the proton NMR spectra that 24% of the water is in the ordered state. Fung and coworkers[78-80] have proposed a different two-state model. They postulated that a small number of water molecules can directly bind to the collagen molecule through hydrogen bonding. Water molecules in the direct neighborhood of both the bound water and collagen form a hydration layer whose structure may not be compatible with that of ice and which cannot be frozen down to 210°K. Additional water molecules would go outside the hydration layer and can be frozen at about 260°K. Only the directly bound water molecules are oriented and show dipolar (for H_2O) or quadrupolar (for D_2O) splitting. The rest of the water molecules, including those in the "unfreezable" hydration layer, have isotropic motions and are in rapid exchange with the bound molecules. The dipolar and quadrupolar splittings in the NMR spectra are weighted average values. A similar model was proposed by Migchelsen and Berendsen.[81] They analyzed the anisotropy of H_2O and D_2O rotation in terms of Saupe's parameters[82,83] and concluded that water molecules are preferentially oriented with their H-H direction perpendicular to the fiber axis. Thus, a chain model of hydration proposed by them earlier [70] can no longer be supported as the

only possible or even the most likely model. They assumed that part of the time water molecules are bound to collagen in the manner proposed by Ramachandran et. al.[34] and that at other times they are rotating freely. Migchelsen and Berendsen found that their NMR data can be reconciled with 12% of bound water. In opposition to the two-state models, a one-state model has been proposed by Dehl and Hoeve.[75] The absence of a central DMR resonance led them to conclude that the central PMR observed by Berendsen may not be due to the H_2O. The maximum separation of the doublet in the DMR is also about 4.6% of its rigid lattice quadrupole splitting value. Dehl and Hoeve postulated that in collagen at moderate relative humidity there is only one kind of water observable by NMR and that the water molecules undergo slightly anisotropic but rapid reorientation. From the study of the effects of salts on the wide-line NMR spectrum of D_2O in oriented collagen fibers Dehl[84] argues that a two-state model cannot satisfactorily account for the D_2O-NMR doublet spectrum, or the effects of salts on it, over the entire range of observed D_2O content.

Arguments supporting the two-state models are provided by other techniques. Tomaselli and Shamos[85] studied the dielectric properties (10^2 - 10^5 Hz) of hydrated native collagen and found that as water is adsorbed, both the dielectric constant and the loss factor increase

simultaneously and rise sharply upward to a hydration level of 0.093 g H_2O/g dry collagen (mean value), which may be associated with the completion of the primary adsorption layer. A maximum in the temperature-dependent curves is found at about +40°C and is explained as the superposition of two processes: (1) the transition of water molecules from bound to free states, and (2) the diffusion of water molecules out of the system. Bardelmeyer[86,87] measured the electrical conductivity of bovine Achilles tendon with various amount of adsorbed water as a function of temperature. A plot of the activation energy versus water content consists of two linear branches, separated by a transition region between 45% and 65% H_2O. The amount of "bound" water in hydrated collagen as deduced from conductivity data is about 50% by weight of water relative to the dry weight. He believes that the "bound" water interacts with adsorptive groups on collagen, whereas the freezable water occurs in both the small "hole zones" within the fibril and larger voids between the fibers. Tanioka et. al.[88] studied the effect of water content on the mechanical properties of proteolytic enzyme treated reconstituted collagen film. The S-shaped sorption isotherm is separated into an adsorption curve C_1 and a solution curve C_2. At 30°C, the storage modulus (G') decreases suddenly, the loss modulus (G") has a peak, and the loss tangent (tan δ) increases in the water content region of 0.05 to 0.1g/g which

corresponds to the region where the C_2 component of sorption becomes detectable. Pulsed DMR was first used by Fung and Siegel[89] to measure the spin-lattice relaxation time of absorbed D_2O. Recently from a more systematic study of pulsed DMR Fung et. al.[90] found that a maximum of about 0.50-0.55 g H_2O/g collagen forms a hydration layer, which cannot be frozen at least down to a temperature of -90°C and which exhibits a distribution of motional correlation times. For collagen samples containing a larger quantity of adsorbed water, the additional water molecules behave like ordinary isotropic water, having a single correlation time and a freezing temperature of about -10°C. More recently Nagamura and Woodward[91] have employed the spin-probe technique to study molecular motion in dispersed cattle hide collagen film in the -160° to +200°C region. The effect of water content in the 0-30 wt. % range on the electron spin resonance (ESR) spectrum of the probe was also investigated. The appearance of a five-component ESR spectrum was assumed to be due to the superposition of two signals, a narrow and a broad triplet. The coexistence of a narrow triplet along with the broad triplet suggests that the substrate is acting like a two-phase system. A possible explanation is that one phase is the polymer and any closely associated water and the other is "mobile" water.

Other evidences against the two-state models are provided by dielectric[92], infrared[93] and heat capacity measurements.[94] Hoeve and Lue[92] investigated the dielectric properties of water in bovine neck tendon collagen for various water (0-14%), NaCl and HCl contents. The absence of a maximum in the loss curves as a function of temperature (from -70° to +23°C) and frequency (from 10^2 to 10^5Hz) indicate an extremely broad spectrum of relaxation times. Master curves for the dielectric constant and the loss factor can be composed for 23°C. They proposed a one-state model in which the water molecules, assumed to be hydrogen bonded to each other in long zig-zag chains can, cooperatively, be oriented in two different directions along the channels between collagen molecules, resulting in large, reversible, dipole moments. These water chains are not rigid, but are flexible liquid-like structures. Diffusion of chains as entities is assumed to be the rate-limiting step for dipole reorientation. Although the rate of diffusion decreases inversely proportional to chain length, the activation energy is independent of chain length. At lower temperatures, diffusion becomes slower, until at the glass point, approximately -100°C, it ceases. Susi et. al. [93] reported infrared measurements on collagen containing various amounts of water. Gradual changes were observed in the frequencies

and intensities of characteristic amide bands over the relative humidity range of 0 to 75%. These changes are particularly pronounced for the amide II band which suggests that the strength of hydrogen bonding of peptide NH groups is gradually increased over a broad range of relative humidity. Since exchange of water molecules between any two states postulated cannot be so rapid that time-averaged infrared spectra result, spectra corresponding to the two states are to be expected. Unless the absorption spectra of both states are assumed to be the same as that of liquid water, the data are difficult to explain. Hoeve and Kakivaya[94] measured the heat capacity contribution of water in kangaroo-tail collagen and found it to be 23 cal deg^{-1} $mole^{-1}$ at 30°C over the entire range from 1 to 100% water. The contribution of water to the heat capacity is approximately 9 cal $deg^{-1}mole^{-1}$ in those compounds in which water is bound and 18 cal $deg^{-1}mole^{-1}$ for liquid water in bulk. The existence of large configurational contributions of water in collagen to the heat capacity indicates that water is in one state, the liquid state, over the entire range of water contents studied.

C. Mechanical and Dielectric Loss Processes of Collagen

The relaxation behavior of collagen has been reported by several investigators. Chien and Chang made dynamic mechanical[95] and dielectric [96] studies of eight-month old rat tail tendon (RTT), enzyme (proctase) solubilized collagen (ESC), UV irradiated ESC (ESC-X), gelatin, acid solubilized

collagen (ASC) and collagen films plasticized with 10% glycerin (AKM). The results are summarized in Table 3. Both the mechanical and the dielectric relaxations found at low temperatures(designated γ) are frequency dependent. The low-temperature process (γ) in all the samples (except AKM 23) have the same dielectric loss transition temperatures at a given frequency. Chien and Chang concluded that they are not specifically associated with either the crystalline or the amorphous region, nor are they related to any particular structural features such as the triple helices or telopeptides. The activation energy for the γ process, calculated from dielectric dispersion data, is 7.3 Kcal $mole^{-1}$ for ASC samples and 7.8 Kcal $mole^{-1}$ for ESC samples. Chien and Chang proposed a mechanism for the γ process as the breaking and reforming of hydrogen bonds between the residues and between the residue and structural water. There is a very prominent mechanical loss process in the vicinity of room temperature. This is attributed to devitrification of water (presumably meaning the transition of bulk water from the glassy state to the liquid state) by Chien and Chang. Much less prominent shoulders can be discerned in the dielectric dispersion data around room temperature. There are only slight changes in dielectric properties, if any at all, as the specimens pass through the temperature region in which the mechanical loss shows a maximum.

Table 3

Comparison of Mechanical and Dielectric Relaxation at 0.1 KHz of Various Collagens[95,96]

Materials	Relaxations	γ Process Transition temp (°K)	γ Process tanδ	Devitrification Transition temp (°K)	Devitrification tanδ	Other transition temp (°K)
ESC (12% H_2O)*	Dielectric	193	0.016	293	0.01	-
	Mechanical	193	0.03	293	0.03	-
ESC-X (12% H_2O)	Dielectric	193	0.022	293	0.016	-
	Mechanical	173	0.03	293	0.034	-
Gelatin (10% H_2O)	Dielectric	193	0.033	283	0.016	-
	Mechanical	173	0.038	283	0.032	-
ASC	Dielectric	193	0.02	293	0.015	-
RTT (Moist)	Mechanical	173	0.03	293	0.078	223
AKM 23 (12% H_2O)	Dielectric	173	0.015	293	0.30	213
	Mechanical	203	0.22	273	0.17	243

* Water content is based on the sample dried in a vacuum desiccator to constant weight.

They explain that motions of intrachain hydrogen-bonded water molecules make the dominant contribution to this process and this motion does not, apparently, enhance the orientability of the tropocollagen molecules by the oscillating electric field. An additional mechanical loss peak is observed only for RTT and AKM 23 samples in the temperature region of 223 to 243°K at 0.1 KHz. All the measurements were made with samples containing about 12% water. The effect of water content on the relaxation behavior of these samples was not studied.

Baer et. al.[97] investigated the dynamic mechanical relaxation behavior of human diaphragm collagen (HDT), of polyglycine I and of nylon 6. The results are summarized in Table 4. The loss maximum of the low-temperature process (designated γ) is located at 150°K for the dehydrated HDT specimen and shifts upward toward 210°K as the water content is increased to 35%. A fourfold increase in the magnitude of this peak was also detected. No mechanism was assigned to this process. Another loss peak below the physiological temperature (designated β) remains constant at 260°K and intensifies in intensity by about tenfold when the water content is increased from 0 to 35%. By a crude comparison with nylon 6, this β process was assigned to the reorientation of polymer-water complex units under the applied stress.

Stefanou et. al.[98,99] made dynamic mechanical and wideline NMR studies of dispersed steer tendon collagen,

Table 4

Dynamic Mechanical Properties* of HDT (30 yr), Polyglycine I, and Nylon 6 [97]

Sample	γ Relaxation		β Relaxation		α Relaxation	
	$T^{\circ}K$	$\Delta max \times 10^2$	$T^{\circ}K$	$\Delta max \times 10^2$	$T^{\circ}K$	$\Delta max \times 10^2$
HDT (Dry)**	150	3.00	260	4.00	>420	-
HDT (100% RH)	210	16.5	260	40.0	315	34.5
Polyglycine I (Dry)	180	2.10	250	1.90	334	2.30
Polyglycine I (100% RH)	145	1.00	220	1.30	285	3.00
Nylon 6 (Dry)	136	13.8	220	8.50	-	-
Nylon 6 (100% RH)	125	6.10	190	8.50	265	35.4

* The measuring frequency is about 1 Hz.

** Apparent dry specimens are accomplished by immersing them in spectro grade acetone for 72 hr. The acetone is removed by placing them overnight in a vacuum oven at 25°C.

denatured collagen, formaldehyde crosslinked collagen, and poly-L-proline II. The results are summarized in Table 5 and 6. At about 1 Hz in the 100-300°K temperature range, there are two mechanical loss processes. In contrast to Baer's results, the low-temperature process (designated β) at 200°K shifts to lower temperatures with increasing water content. The height of the loss maximum (Δ) increases somewhat with water content. The position and height of the β mechanical loss peak 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. From a direct comparison with the low-temperature loss process in poly-L-proline II, the β-process is attributed to oscillations in or of the proline and hydroxyproline groups about the main-chain of collagen. In contrast to Baer's results again, the α-peak (at about 290°K for a vacuum-dried sample) which is a major process shifts to lower temperatures with increasing water content. However, the intensity of the α-peak increases when the water content is increased as Baer et. al. observed. Stefanou et. al. compared the mechanical and NMR relaxation behavior of collagen with that of some poly-α-amino acid derivatives and copolymers of poly-α-amino acids. They ascribe the α-mechanical loss peak and associated changes in the NMR second moment to the reorientation of long-polar side-chain groups of collagen.

Table 5

The Dynamic Mechanical Properties of Collagen[98] and Poly-L-Proline II[99] ($\sim$ 1 Hz)

Sample	β process		α process	
	T^oK	$\Delta_m X 10^2$	T^oK	$\Delta_m X 10^2$
Collagen (10% H_2O)*	195	5.0	250	32.0
Collagen (3.5% H_2O)	200	4.0	275	26.0
Collagen (0.86% H_2O)	210	3.0	288	16.0
Formaldehyde crosslinked Collagen (5.5% H_2O)	180	7.0	310	9.0
Denatured Collagen (10% H_2O)	200	4.2	260	20.0
Denatured Collagen (0% H_2O)	210	4.2	295	15.0
Poly-L-proline II (4.8% H_2O)	175	8.5	-	-

* Water content is based on the sample dried to constant weight under vacuum, at room temperature in the presence of anhydrous $CaSO_4$

Table 6

Broad Line Proton Magnetic Resonance Data For Collagen[98] and Poly-L-Proline II (90MHz)[99]

Sample	Second moment Drop	Narrow Line Appearance
Collagen (0% H_2O)	Monotonic	260°K
Collagen (6.5% H_2O)	200,260°K	260°K
Denatured collagen (0% H_2O)	Monotonic	250°K
Denatured collagen (8.5% H_2O)	240°K	250°K
Poly-L-proline II (0% H_2O)	235°K	330°K
Poly-L-proline II (6.5% H_2O)	235°K	250°K

For easier comparison, the temperature positions, the damping maxima, the storage modulus at low temperature and the possible assignments of mechanisms of the mechanical loss peaks of three investigations described above are outlined in Table 7.

Nguyen et. al.[100] studied acid solublized reconstituted bovine tendon collagen films with several techniques. Two mechanical loss peaks and one dielectric loss peak were observed below the physiological temperature. A broad mechanical loss process at 170-250°K (designated peak 1) is frequency dependent. In the same temperature region a dielectric loss peak (designated β) is well defined and shifts to higher temperatures (fixed frequency) as the moisture content decreases. At 1 KHz it shifts from 180°K for the wettest sample to 290°K for the driest one, a difference of 110°. With the exception of the sample containing relative water content of 25%, the amplitude of the β peak is nearly independent of moisture content. The relative water content is based on the sample dried in vacuum at 140°C for three days. The magnitude of this β peak is still considerable, even for the dry sample. From these facts they believe that the origin of this dielectric loss peak is in the collagen molecule and not due to the water molecules. They also believe this peak to be correlated to a molecular second order transition since there is a distinct frequency dependence which gives the activation energy of 7 ± 1 Kcal $mole^{-1}$. Nguyen et. al. assigned this low-temperature loss mechanism (both mechanical and

Table 7

Chien & Chang[95]	γ-peak		β-peak		α-peak	
Samples[a]	°K	tanδ$_E$X10^2	°K	tanδ$_E$X10^2	°K	tanδ$_E$X10^2
RTT (moist)	173	3.2	223	4.2	291	8.0
ESC (12% H_2O)	193	3.0	—	—	291	3.0
ESC-X (12% H_2O)	173	3.1	—	—	277	3.5
Gelatin (10% H_2O)	173	4.0	—	—	269	3.4
AKM-23 (12% H_2O)	203	20.0	243	13.0	275	12.0
Possible assignment	γ-mechanism of polyamide		none		Devitrification of water	

Baer et. al.[97]	γ-peak		β-peak		α-peak	
Samples[b]	°K	Δ_mX10^2	°K	Δ_mX10^2	°K	Δ_mX10^2
Dehydrated HDT	150	3.0	260	4.0	>420	—
10% H_2O HDT	180	7.0	260	14.0	410	22.0
26% H_2O HDT	190	11.0	260	31.0	350	28.0
35% H_2O HDT	210	16.5	260	40.0	315	34.5
Possible assignment	none		β-mechanism of nylon 6		α-mechanism of nylon 6	

Stefanou et. al.[98]	β-peak		α-peak		G' X10^{-10} dyne/cm^2 (at low temp.)
Samples[c]	°K	Δ_mX10^2	°K	Δ_mX10^2	
0.9% H_2O Collagen	210	3.0	283	16.0	4.6
3.5% H_2O Collagen	200	4.0	275	26.0	4.2
10% H_2O Collagen	195	5.0	250	32.0	3.3
10% H_2O Gelatin	200	4.2	260	20.0	8.0
Possible assignment	Oscillation in or of proline & hydroxyproline groups		Reorientation of long-polar side-chain groups		

a: RTT: rat tail tendons (3-month old rats)
ESC: Enzyme solubilized collagen (proctase treated collagen)
ESC-X: U.V. irradiated ESC
AKM: A commercial membrane. Contains 63% of molecularly dispersed collagen fibril, 27% of ESC and 10% of glycerin as plasticizer.

b: HDT: 30-year-old human diaphragm collagen.

c: Steer tendon collagen.

dielectric) to a motion within the collagen structure that may involve the breaking and reforming of hydrogen bonds since the activation energy is of the right order of magnitude. The dynamic modulus data showed little change in the region of the dielectric β peak and also the tan δ_m peak associated with this same region was small in magnitude. They suggested that the molecular motion associated with the β peak is that of a polar side group such as the hydroxyl proline rather than a backbone motion. They explained that if it were a motion in the main chain, the mechanical properties would have been affected to a greater degree as was the dielectric behavior. The collagen sample containing 15% H_2O shows a sharper mechanical loss peak (designated peak 2) at about 283°K which occurs at the same temperature for all three frequencies (3.5, 11, and 110 Hz) used. The loss modulus of this peak also displays no frequency dependence. The intensity of this loss peak decreases with decreasing moisture content. Nguyen et. al. suggest that this behavior is probably associated with an apparent first order transition, such as "melting" and desorption of water.

D. β-galactosidase (Lactase)

Lactase, or β-galactosidase, a lactose-splitting enzyme of high molecular weight can be isolated from Escherichia coli. Hydrodynamic data for β-galactosidase from E. coli indicate that it has a molecular weight of 518,000.[101]

Its shape can be described as an oblate ellipsoid of rotation with an axial ratio of about three assuming 30% hydration. The length and height of the molecule were calculated to be 150 and 50A° respectively.[102] Craven et. al.[103] studied the molecular weight by low and high-speed equilibrium sedimentation and found values of 489,000 and 538,000 respectively, whereas Colby and Hu[104] by using the same method reported molecular weights of 491,000 and 503,000.

That the native β-galactosidase is composed of four monomers has been demonstrated by several investigators.[105-108] The tetrameric structure of the enzyme was directly demonstrated by electron microscope studies which indicated furthermore that the four subunits are arranged at the corners of a square.[109] The dimensions of the molecule determined by this method are about 120 and 70°A which agrees closely with the values obtained from hydrodynamic data.

Very recently Fowler & Zabin[110] have determined the amino acid sequence of β-galactosidase. The protein contains 1021 amino acid residues in a single polypeptide chain. Physical and chemical studies have shown that the protein is a tetramer of four identical polypeptide chains. From the sequence molecular weights of 116,248 for the monomer and 464,992 for the tetramer were calculated. The amino acid sequence proposed for β-galactosidase is shown in reference 110 and the

amino acid composition is given in Table 8.

E. The Collagen-Lactase System

Immobilized enzymes in which the isolated enzyme is bound to a stable matrix can serve as efficient biological catalysts with many practical applications. Most of the immobilization processes described in the literature[111-113] involve bonding of an enzyme to a synthetic carrier material. The use of reconstituted biological material as the supporting matrix is a relatively new approach to enzyme binding. Collagen has been chosen as a matrix because it has several physical properties which are well suited to enzyme immobilization, such as mechanical stability, hydrophilicity, as well as electro-static charge. The Rutgers Interdisciplinary Enzyme Technology Group has developed and employed several techniques for immobilizing enzymes to collagen by the processes of molecular complexation,[114] impregnation,[115] and electrocodeposition.[116] This group has reported the immobilization of different enzymes to collagen[117] demonstrating the general utility of reconstituted collagen as a carrier for enzyme immobilization. Collagen provides not only good mechanical strength and stability to the enzyme carrier, but also a multiplicity of potential sites for enzyme binding through the formation of noncovalent linkages. Although the individual noncovalent bonds are relatively weak, their cumulative bond energies lead

Table 8

Amino Acid Composition of β-galactosidase[110]

Amino Acid	No. Residue Found	
	Analysis	Sequence
Tryptophan	27	38
Lysine	23	20
Histidine	31	34
Arginine	64	66
Aspartic acid	105	110
Threonine	59	56
Serine	60	61
Glutamic acid	124	121
Proline	62	64
Glycine	72	70
Alanine	81	76
Half-cystine	15	16
Valine	64	63
Methionine	23	23
Isoleucine	38	39
Leucine	96	95
Tyrosine	29	31
Phenylalanine	38	38
Total Residues	1,011	1,021

to stable collagen-enzyme complexes.

Recently Giacin et. al.[118] have immobilized β-galactosidase (E. Coli K_{12}) on collagen membranes and studied the effect of structural modifications on the enzyme-binding capacity of collagen. The enzyme-binding capacity of collagen membrane was evaluated by means of two procedures: (1) by determination of the apparent specific activity of the complex; and (2) by direct determination of the enzymic protein immobilized on collagen by the tryptophan content of the collagen-enzyme complex. In order to impart the required mechanical strength to the membranous collagen as a matrix for enzyme immobilization, collagen films have been modified by cross-linking with the difunctional reagent, glutaraldehyde, or by a natural cross-linking process associated with aging. Such modifications were found to markedly reduce the enzyme (β-galactosidase)-binding capacity of collagen films. The deleterious effect of cross-linking on the binding capacity of collagen was shown to be completely reversed by proteolytic enzyme (pepsin and pronase) treatment of aged films but only partly so for glutaraldehyde-treated films. The tensile strength of a collagen membrane, expressed in terms of molecular weight between cross-links, was recorded as a function of storage time (age of the membrane). A constant molecular weight between cross-links was observed after 21 days of storage,

however the enzyme-binding capacity of collagen membrane decreased continuously with increasing age of the membrane. Giacin et. al. explained that during aging, cross-links occur between helical regions of the tropocollagen molecule as well as through the telopeptide regions. Cross-linkages in the helical regions are of greater importance for tensile properties. Telopeptide-associated cross-links within and between tropocollagen molecules and fibrils may result in the loss of potential binding sites for the enzyme, and/or to the modification of the matrix structure of the collagen membrane. The latter can interfere with the diffusion of enzyme into the membrane matrix during impregnation. The binding capacity regeneration by pepsin or pronase treatment of aged membrane can be attributed to the hydrolysis of peptide linkages located in the telopeptide region of the tropocollagen molecule. Since the formation of cross-linkages during aging has been shown to occur primarily in the telopeptide region,[16,119,120] attack on these appendages is expected to alter the matrix structure of the membrane, leading to an enhanced diffusion of enzyme into the collagen matrix during the immobilization step.

F. Statement of the Problem

This investigation was undertaken with two objectives. One was to use the torsional pendulum to

study the effect of water content on the dynamic mechanical properties of collagen with a wide range of water content. Since controversial results have been reported by several investigators, a more systematic study is necessary and important. A second objective was to study the relaxation behavior of the collagen-lactase system as a function of water content and age. The dynamic mechanical study of collagen immobilized enzymes has never been reported. Since membranous collagen immobilized β-galactosidase has been used to construct biocatalytic reactors such as the capillaric coil modular reactor,[117] it is important to know the mechanical properties and the nature of the binding of lactase to collagen.

II. Experimental

A. Sample Preparation

The collagen used was in the form of films prepared by Professor J.R. Giacin of Rutgers University from cattle hide collagen fibrils obtained from Devro Inc., Sommerville, N.J. The fibrous collagen was washed with 10% sodium chloride solution followed by washing with distilled water. The collagen was then freeze dried and stored for use at room temperature. A collagen dispersion was prepared by suspending 3.5g of freeze-dried collagen in 500 ml of aqueous lactic acid solution at pH 2.8. The dispersion was homogenized with a blender until viscous, and cast onto a Mylar support to form the membrane. The cast film was dried at room temperature for 48 hours. After drying, the film was easily detached from the Mylar sheet. Collagen-lactase complexes were prepared by the interdiffusional penetration procedure[115,121] by Professor J.R. Giacin and coworkers. In this procedure, a collagen membrane was first formed, and it was then impregnated with the enzyme. A solution containing 10 units of enzyme (ONPG, 25°C) per mg of collagen film, in phosphate buffer, pH 7.0 was employed as the impregnation bath. Following the impregnation period of 24 hours, the film was layered on a Mylar sheet and dried at room temperature for 18 hours. The amount of lactase immobilized on the collagen was determined from the

tryptophan content by Professor Giacin and coworkers.

The sample of collagen for which dynamic mechanical properties were obtained was stored at room conditions for 14 months before any tests were carried out. The measurements were made within the next one year period. Two samples of collagen-lactase were used for dynamic mechanical tests. For one, most of the measurements were made within 2 months after receiving the samples. For the other collagen-lactase sample studied, the storage time and measuring period were similar to those for the collagen sample used.

B. Relative Water Content

Samples were dried to constant weight by vacuum drying at room temperature for a week. These samples were used as the apparently dry samples in the calculation of relative water content for a particular sample. As noted earlier this treatment does not drive off all the tightly bound water. There is a small amount of water which cannot be removed without damaging the collagen structure. In order to know how much tightly bound water is present in the apparently dry samples, samples were dried at 105°C under vacuum for 12 days, a procedure which is reported to remove the water completely.[4] The percent weight loss of the samples, relative to apparently dry samples, after vacuum drying at 105°C for several periods of time, is summarized in Table 9. In order to obtain samples with desired water contents, the specimens were placed in a sealed dessicator and allowed to equilibrate

Table 9

Drying of Collagen and Collagen-lactase

Sample	Apparent dry sample vacuum drying room temp., a week		vacuum drying 105°C, 9 hrs.		vacuum drying 105°C 68 hrs.		vacuum drying 105°C, 288hrs. (12 days)	
	wt.	% wt.* loss	wt.	% wt. loss	wt.	% wt. loss	wt.	% wt. loss
Collagen	0.0323 g**	0	0.0328g	0	0.0322g	1.9	0.0319g	3.0
Collagen-lactase	0.0246 g	0	0.0246g	0	0.0242g	1.7	0.0240g	2.7

* Percent weight loss is relative to the apparently dry samples.

** The accuracy of the balance is ±0.0001 g.

over saturated salt solutions whose relative humidities were known.[122] The relative water content of the samples was determined as follows. Two specimens with almost identical dimensions were stored at the same humidity for at least one day. After equilibrium had been reached, one specimen was taken out for the torsional pendulum measurements. The time required for taking the sample out of the dessicator to set up and quench it to low temperature was recorded. The second specimen (reference) was then taken out from the dessicator. After the exact duration time mentioned above, the reference sample was weighed. After all the measurements had been made, the reference sample was dried and the relative water content of the samples was calculated. The relative water content of a sample is defined as ;

$$\frac{\text{(Weight of sample) - (Weight of apparently dry sample)}}{\text{(Weight of apparently dry sample)}}$$

and labelled as a fraction, g H_2O/g dry sample.

C. Dynamic Mechanical Testing

The torsional pendulum designed by Sinnott[123] and modified by Frosini and Woodward[124] was used for the dynamic mechanical measurements. Sample dimensions were in the range of 1.0 cm in width, 3-5 cm in length and 0.006-0.01 cm in thickness. A proper torsion ring of the right weight which provides the moment of inertia was chosen to give a frequency for all the measurements below 1 Hz and to let the tensile

load remain within the ASTM recommendations for torsional pendulum testing.[125,126] In the free oscillation torsional pendulum experiment the force is applied instantaneously to induce the desired amplitude of oscillation and the sample's response is monitored as it returns to its original state free of the influence of any applied forces. The rate of decay of the oscillation is related to a damping term, the logarithmic decrement (Δ), and the frequency of the oscillations is related to the storage modulus (G').[127] The logarithmic decrement, Δ , is obtained from the n-th amplitude, A_n, and the (n + 1)th amplitude, A_{n+1} : $\Delta = \ln\left[\frac{An}{An+1}\right]$. This quantity is related to the loss tangent by $\tan\delta \simeq \frac{\Delta}{\pi}$. The storage modulus (G') is then $G' = \frac{4\pi^2 I L}{N} f^2 - \frac{m g c^2}{12N}$.[128] This equation arises from that given by Inoue et. al.[129], $G' = \frac{4\pi^2 I L}{N} f^2\left(1 + \frac{\Delta}{4\pi^2}\right) - \frac{m g c^2}{12 N}$, where the assumption that the logarithmic decrement (Δ) is small has been made. In these equations I is the moment of inertia of the pendulum, L and C are the length and the width of the specimen, f is the frequency, m is the mass of the suspended ring, g is the gravitational constant, N is a shape factor, $N = \frac{1}{3} CD^3(1 - 0.63 \frac{D}{C})$, where D is the thickness of the sample. In all the above equations, C must be greater than 3D. The equation used to calculate the loss modulus (G") is $G'' = \frac{(\Delta)G'}{\pi}$.[130] The complex shear modulus (G*) is given by $G^* = G' + iG''$, where G' is related to the

energy stored and recovered in a cycle of deformation, and G" is related to the energy dissipated per cycle of deformation.[131]

The water content monitored at the beginning and end of each run could be different by as much as 5% for samples containing high water contents, if the experiment was carried out from 100 to 300°K using a heater. In order to maintain the water content of the samples relatively constant during measurements, the following procedure was followed. The entire temperature region (100-300°K) for the experiment was divided into two parts by using two samples to shorten the experimental time so that a heater would not have to be used. The dividing temperature was chosen as 230°K because no significant damping was observed in the vicinity of this temperature for the samples studied. By using this procedure, the difference in the water content for a particular sample at the beginning and the end of each part was less than 1%.

D. Characterization

Collagen and collagen-lactase films were characterized by using low-angle and high-angle x-ray diffraction, and transmission electron microscopy. Measurements of a rectangular collagen film with the plane normal to the x-ray beam and with the long side at 0,45 and 90 degree relative to the sample holder did not show significant changes in the small-angle x-ray

diffraction data. The fundamental spacing[132-134] of 668A° along the fibrillar axis was not observed for these samples. Samples having a thickness range of 0.001-0.0015 cm were investigated under transmission EM. Both collagen and collagen-lactase films with and without negative staining did not show aligned fibrous structures with an axial periodicity of 600-700A° as native tendon collagen did under EM. Instead, there was a network appearance with some voids. No significant distinction between collagen and collagen-lactase was observed under EM. Collagen and collagen-lactase films with high water contents were studied by wide-angle x-ray diffraction. In order to maintain samples with constant water content during measurement, the experiment was carried out in a sealed plastic bag with a given humidity atmosphere provided by a saturated salt solution. The patterns of collagen and collagen-lactase films obtained with the x-ray beam normal to the plane of the films are shown in Figure 4 and 5. The diffraction pattern of collagen film does not exhibit a 3A° meridional arc and the 12A° equatorial spots as the native collagen does.[29] However, the outer and the inner rings in the pattern (Fig. 4) are in the right order of spacing of 3A° and 12A°, respectively. A spacing of 38A° in the rat tail tendon has been reported[51] and interpreted[37,136] recently as the side of the tetragonal unit-cell.

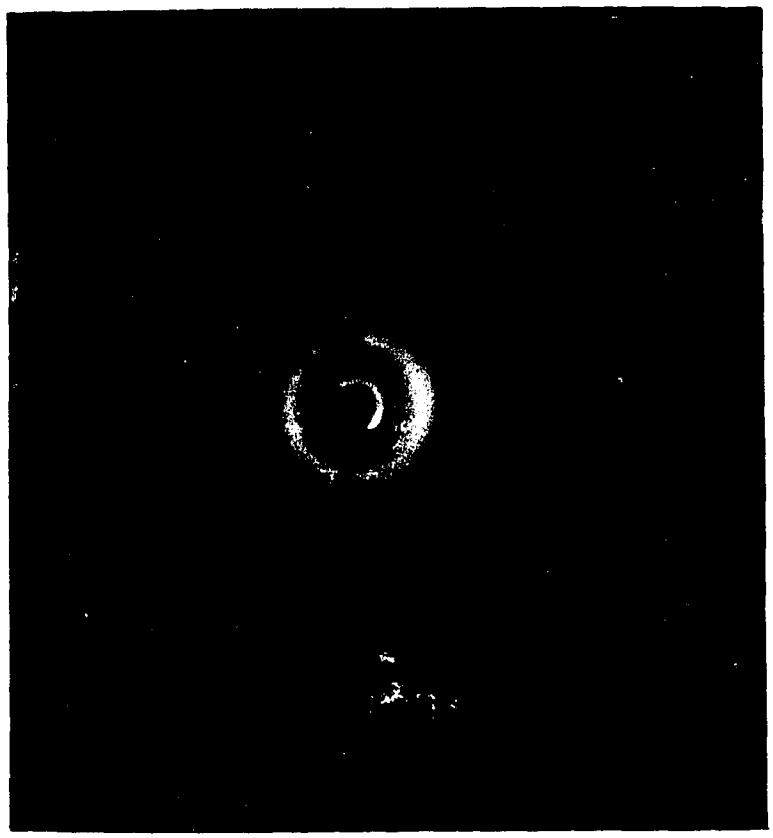


Fig. 4. Wide-angle x-ray diffraction pattern of aged collagen (high water content) obtained with the x-ray beam normal to the plane of the film.

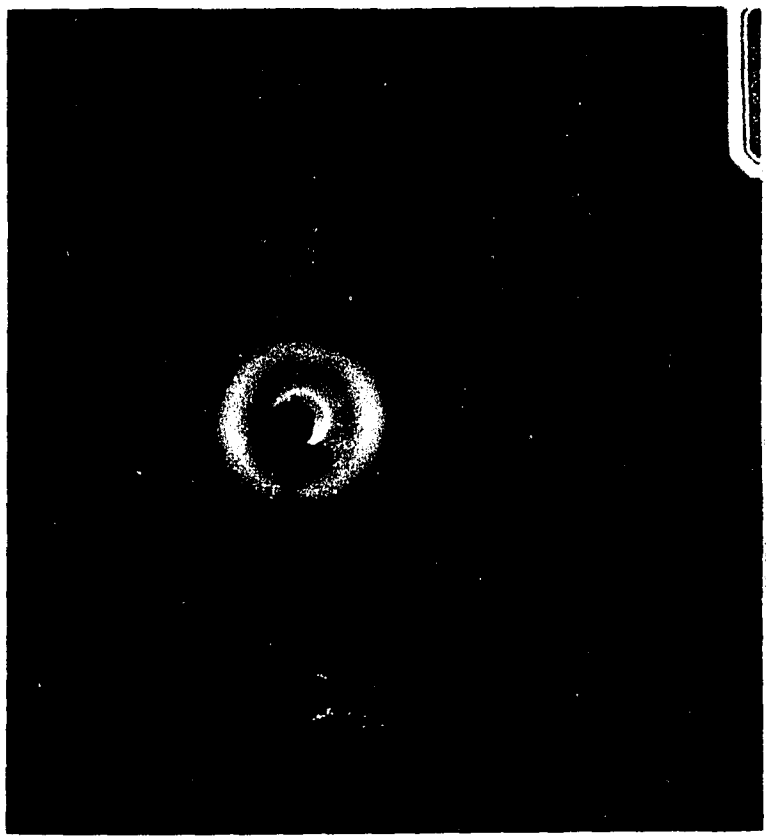


Fig. 5. Wide-angle x-ray diffraction pattern of aged C-L (high water content) obtained with the x-ray beam normal to the plane of the film.

It is not known whether or not the spacing of 38A^{o} is present in the pattern (Fig. 4) because the center area of the pattern is covered by the beam stop. All the evidence shows that the collagen molecules are randomly oriented. The similarity between the patterns of collagen and collagen-lactase films suggests that the lactase does not exist as a separate crystalline phase in or on the collagen film.

III. Results

Aged Collagen

Dispersed cattle hide collagen film shows four mechanical relaxation processes below physiological temperatures. These processes are labeled as α_1, α_2, β_1 and β_2 with descending temperature. The β_1 peak is observed only at higher water content. These peaks undergo significant changes as the water content is altered.

Figure 6 gives the logarithmic decrement (Δ) of samples of aged collagen containing various water contents as a function of temperature in the region of 220°-300°K. As the water content is increased, both α_1 and α_2 peaks shift to lower temperature; the α_1 and α_2 peaks first increase then decrease in intensity (Δ). A plot of peak temperature (based on Δ) versus water content (Fig. 7) shows that the α_1 peak temperature decreases rapidly from 290 to 264°K when the relative water content is increased from 0.04 to 0.12 g/g and then decreases slowly from 264 to 260°K when the relative water content is increased from 0.12 to 0.25 g/g. Figure 8 shows that the α_2 peak temperature (Δ) decreases rapidly from 280 to 240°K when the relative water content is increased from 0.04 to 0.13 g/g and increases slowly from 240 to 248°K when the relative water content is increased from 0.13 to 0.25 g/g. A plot of peak

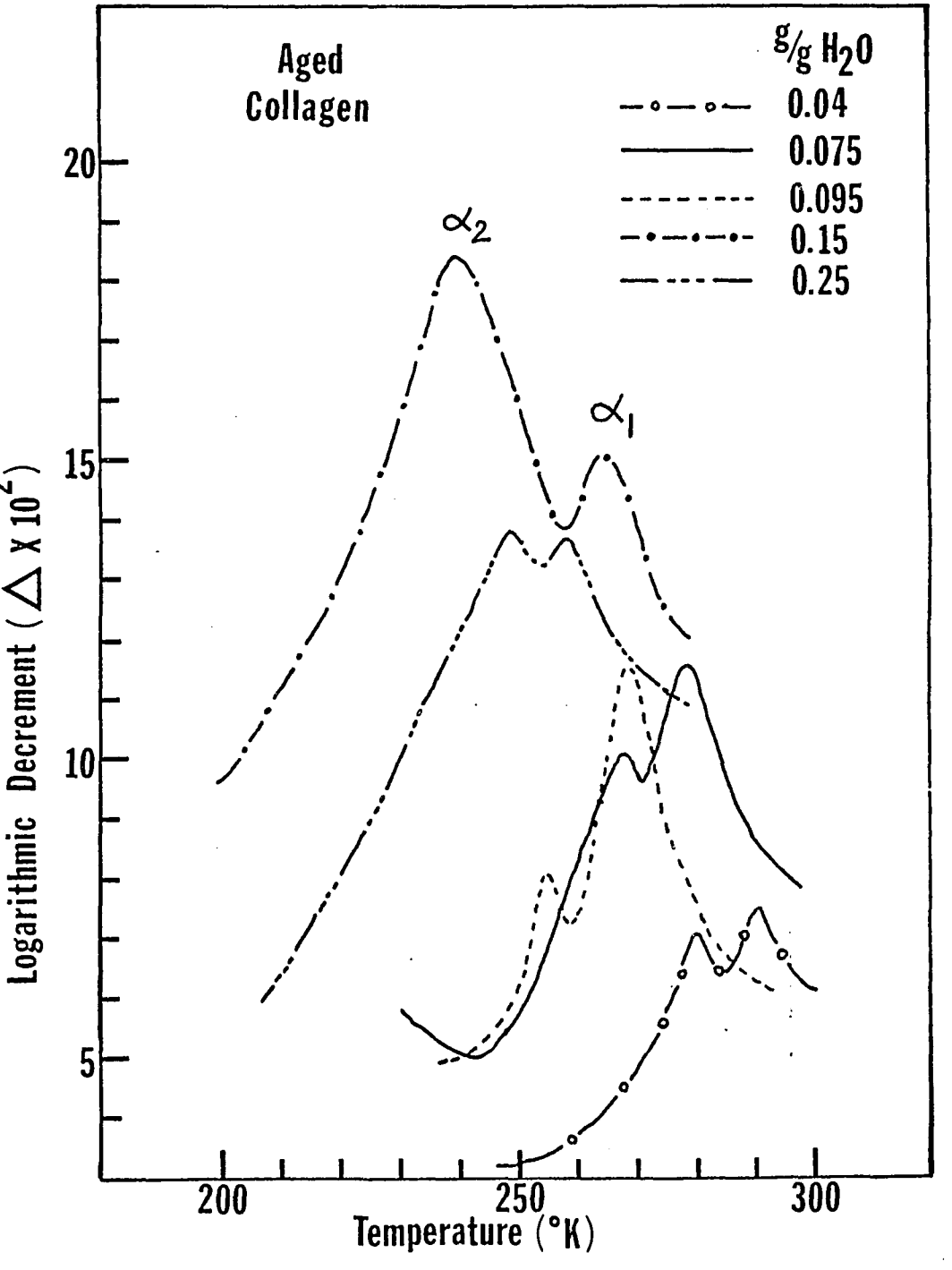


Fig. 6. Logarithmic decrement (Δ) vs. temperature (200 - 300° K) for aged collagen containing various water contents.

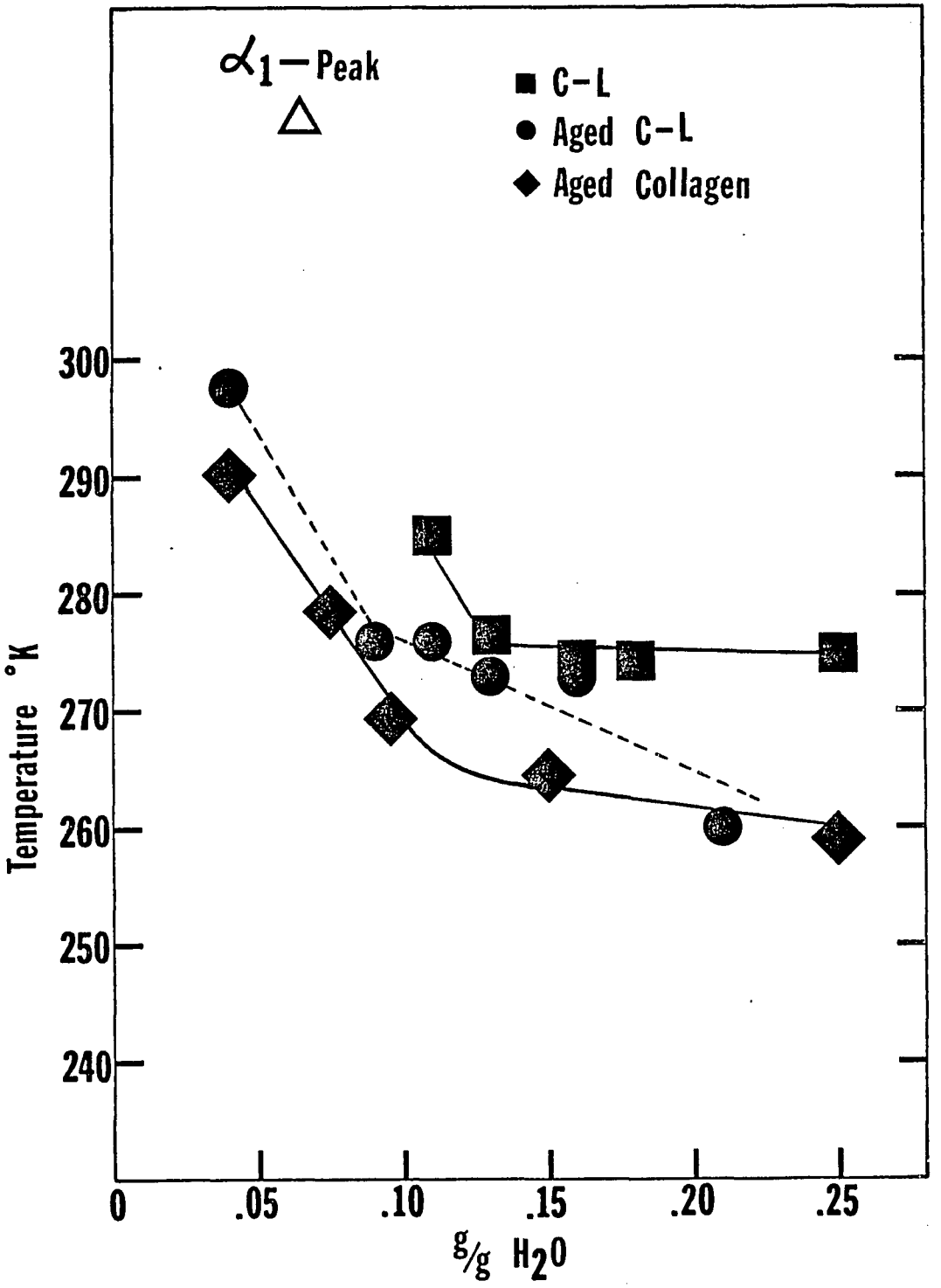


Fig. 7. Temperature position of the α_1 peak (△) vs. water content for aged collagen, C-L, and aged C-L.

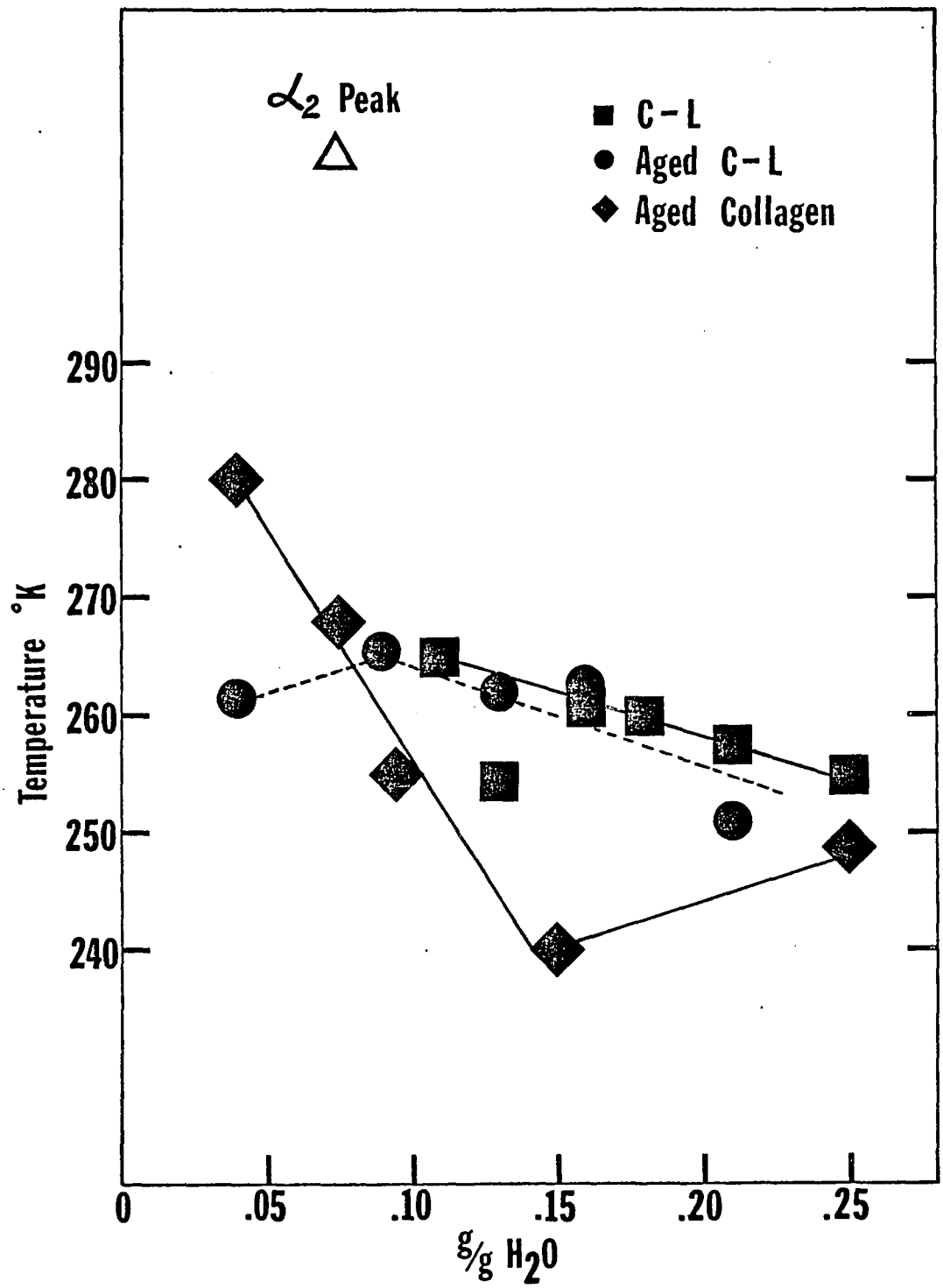


Fig. 8. Temperature position of the α_2 peak (△) vs. water content for aged collagen, C-L, and aged C-L.

intensity (Δ) versus water content (Fig. 9) indicates that the intensity of the α_1 peak increases with increasing water content and reaches a maximum (Δ = 0.15) at about 0.12 g/g water and then decreases. Figure 10 shows that the intensity of the α_2 peak increases and reaches a maximum (Δ = 0.19) at about 0.14 g/g water and then decreases with increasing water content. Figure 11 gives the dependence of storage modulus (G') of samples of aged collagen containing various water contents as a function of temperature in the region of 240-300°K. The storage modulus (G') first decreases with increasing temperature and then increases (except for the sample containing 0.25 g/g water). The decrease in the storage modulus corresponds to the onset of the α relaxation processes. The increase in the storage modulus is not completely due to the loss of water from the sample. The minimum of the storage modulus curves which is in the vicinity of T_{α_1} shifts to lower temperatures with increasing water content. Figure 12 gives the cross plot of the storage modulus (G') as a function of water content for several temperatures. At 245°K and 265°K the storage modulus first decreases slowly between 0.04 and 0.075 g/g water and then decreases rapidly (approximately a five-fold drop) between 0.075 and 0.14 g/g water and reaches a minimum at about 0.14 g/g water and then increases (approximately by a two-fold

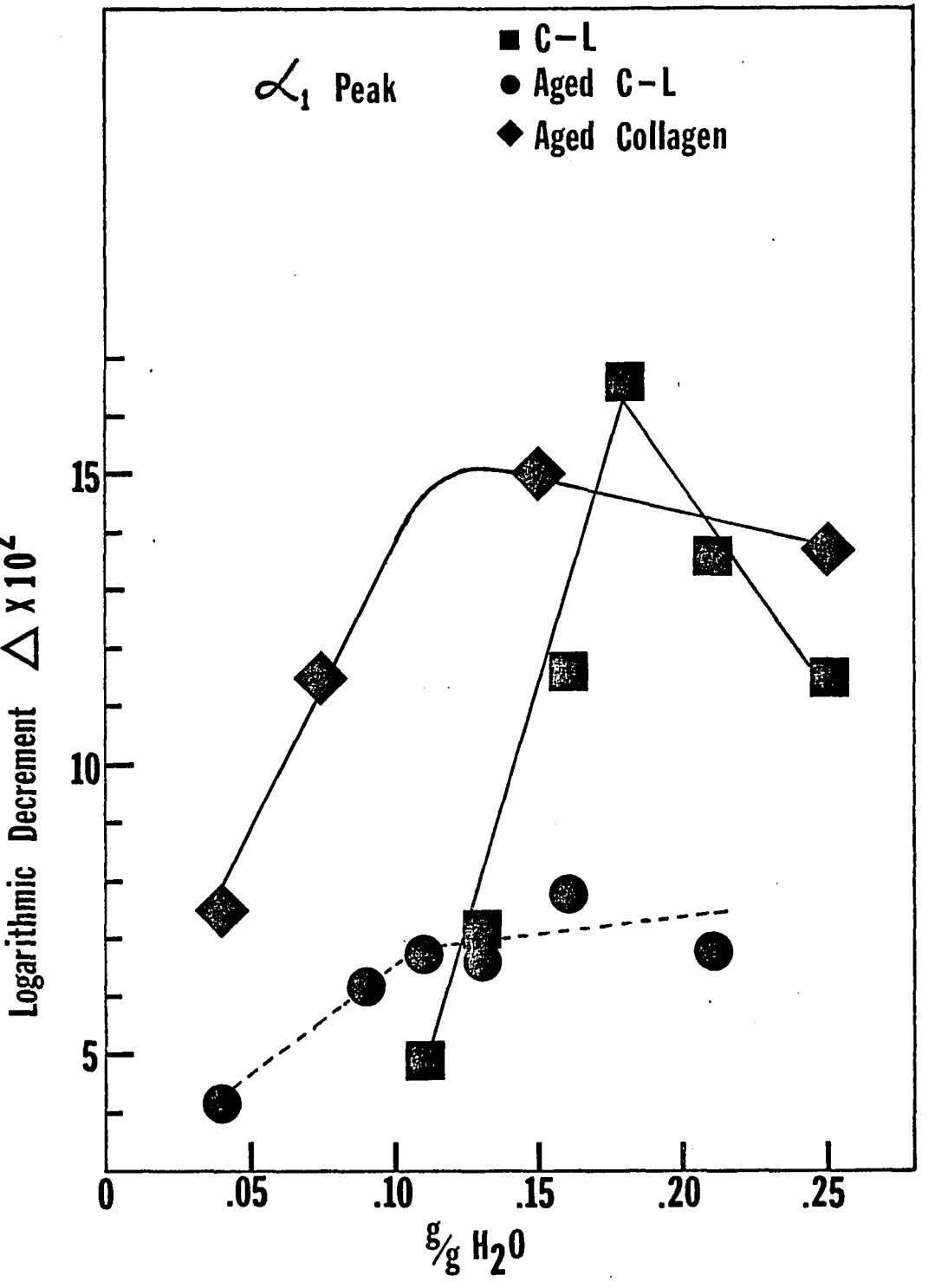


Fig. 9. Intensity (Δ) of the α_1 peak vs. water content for aged collagen, C-L, and aged C-L.

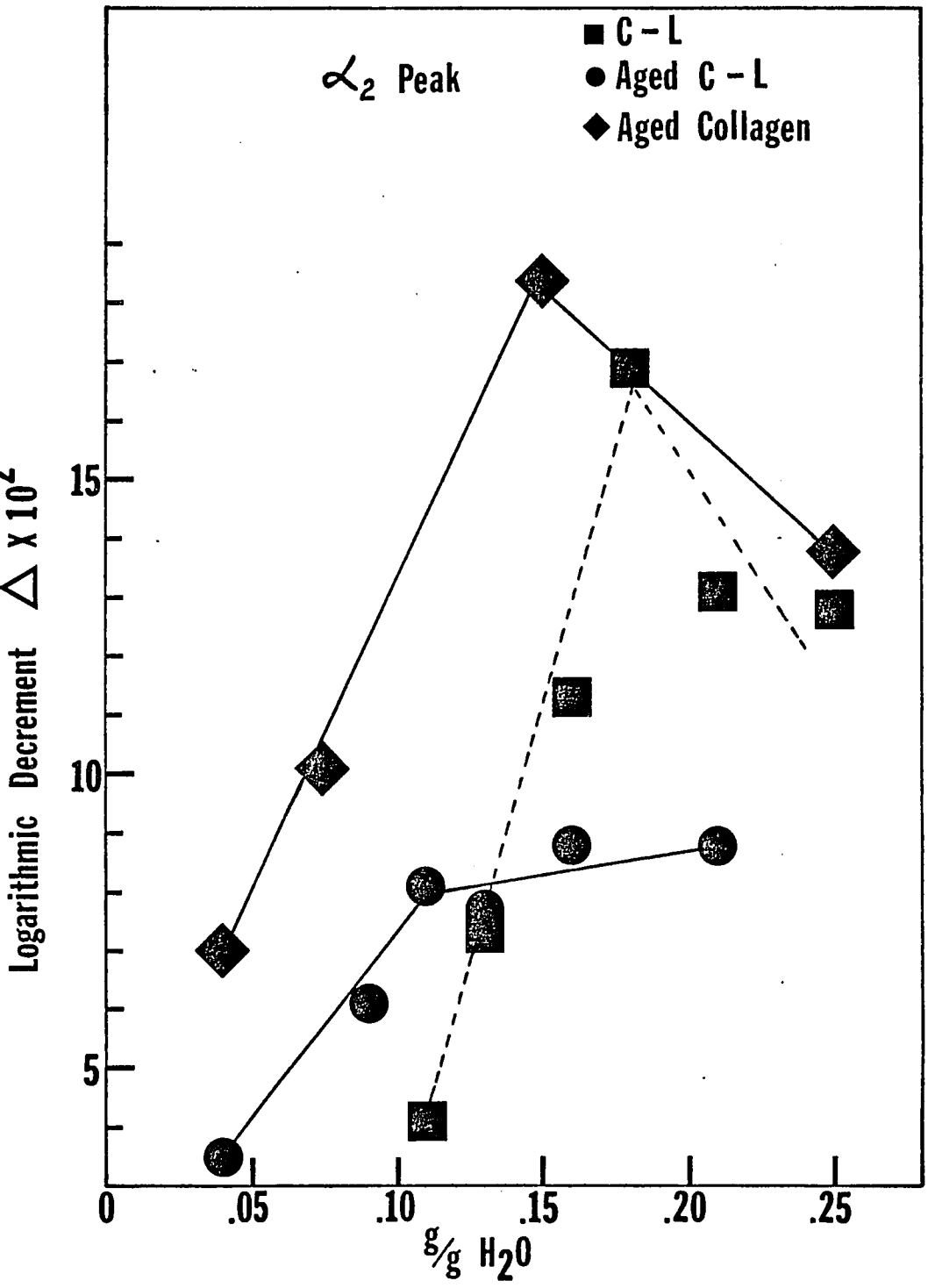


Fig. 10. Intensity (Δ) of the α_2 peak vs. water content for aged collagen, C-L, and aged C-L.

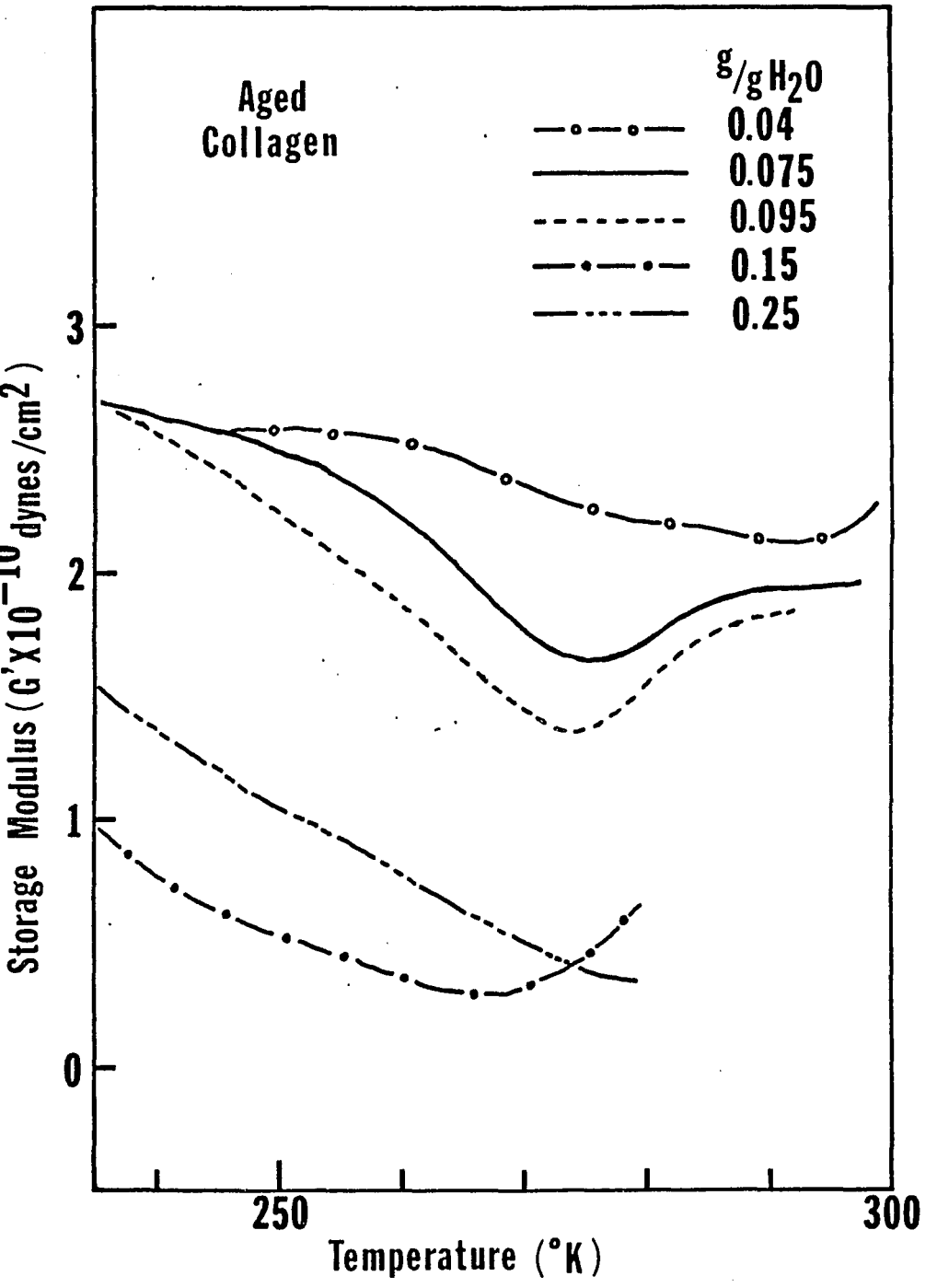


Fig. 11. Storage modulus (G') vs. temperature (240 - 300° K) for aged collagen containing various water contents.

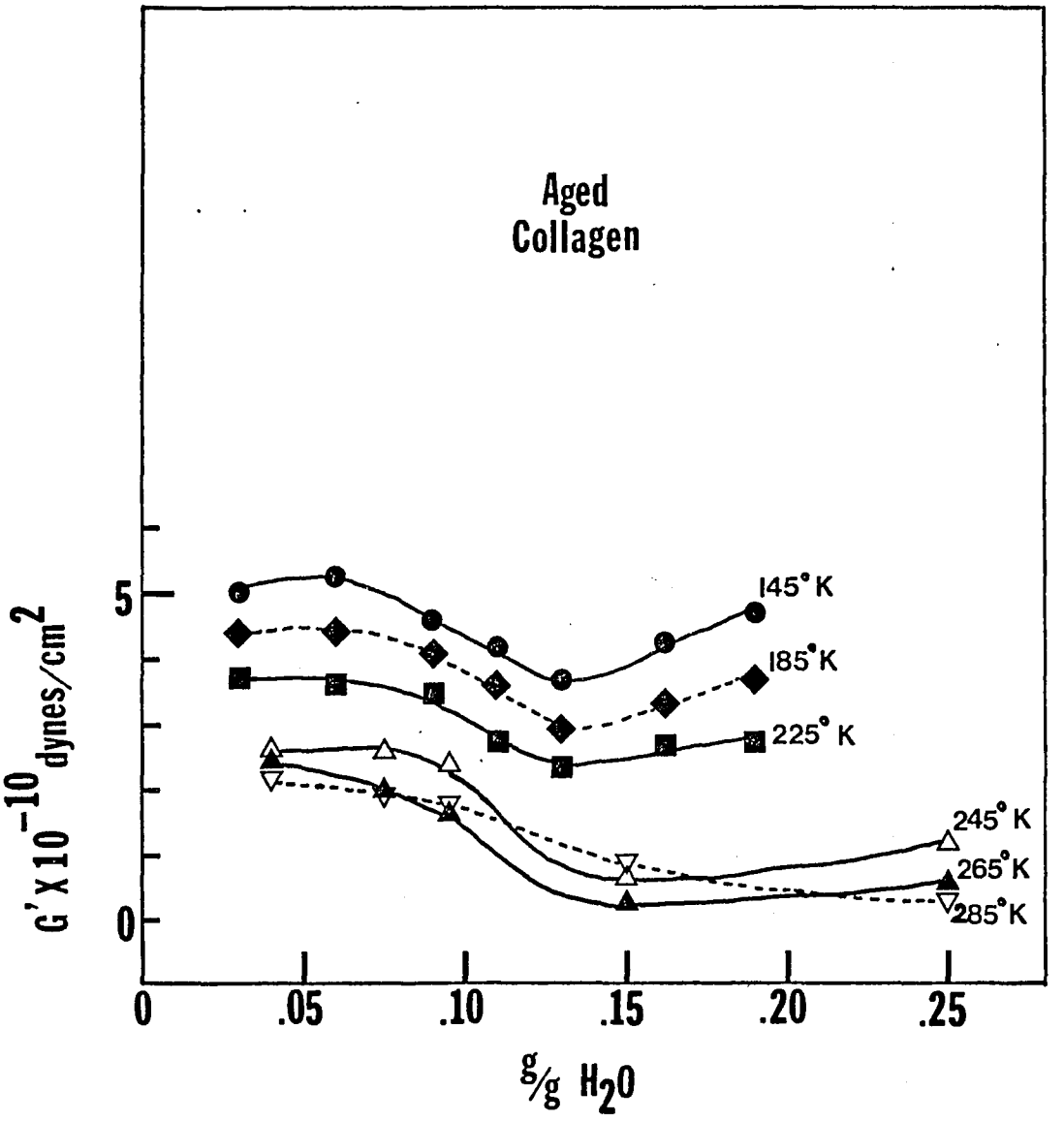


Fig. 12. Storage modulus (G') vs. water content for aged collagen at several temperatures.

increase) between 0.14 and 0.25 g/g water. At 285 ^{o}K, the storage modulus decreases monotonically (approximately a seven-fold drop) when the relative water content is increased from 0.04 to 0.25 g/g. A plot of loss modulus (G") versus temperature in the region of 240-300^{o}K (Fig. 13) shows that both α_1 and α_2 peaks shift to lower temperatures when the relative water content is increased from 0.04 to 0.095 g/g and appear to be a broad dispersion when the water content is higher (0.15 and 0.25 g/g water).

Another water-sensitive peak β_2 is apparent in the temperature region of 150-200^{o}K. Figure 14 gives the logarithmic decrement (Δ) of samples of aged collagen containing various water contents as a function of temperature in the region of 120-240^{o}K. The β_2 peak shifts to lower temperatures as the water content is increased. An additional peak (β_1) which is about 30^{o} higher than the β_2 peak appears at higher water content (0.19 g/g water). A plot of peak temperature (based on Δ) versus water content (Fig. 15) shows that the β_2 peak temperature decreases from 200 to 165^{o}K when the relative water content is increased from 0.03 to 0.13 g/g and then decreases from 165 to 158^{o}K when the relative water content is increased from 0.13 to 0.19 g/g. This behavior is even more obvious when the peak temperature is based on G"(Fig. 16). The intensity (Δ) of the β_2 peak (Fig. 17) versus water content is fluctuating

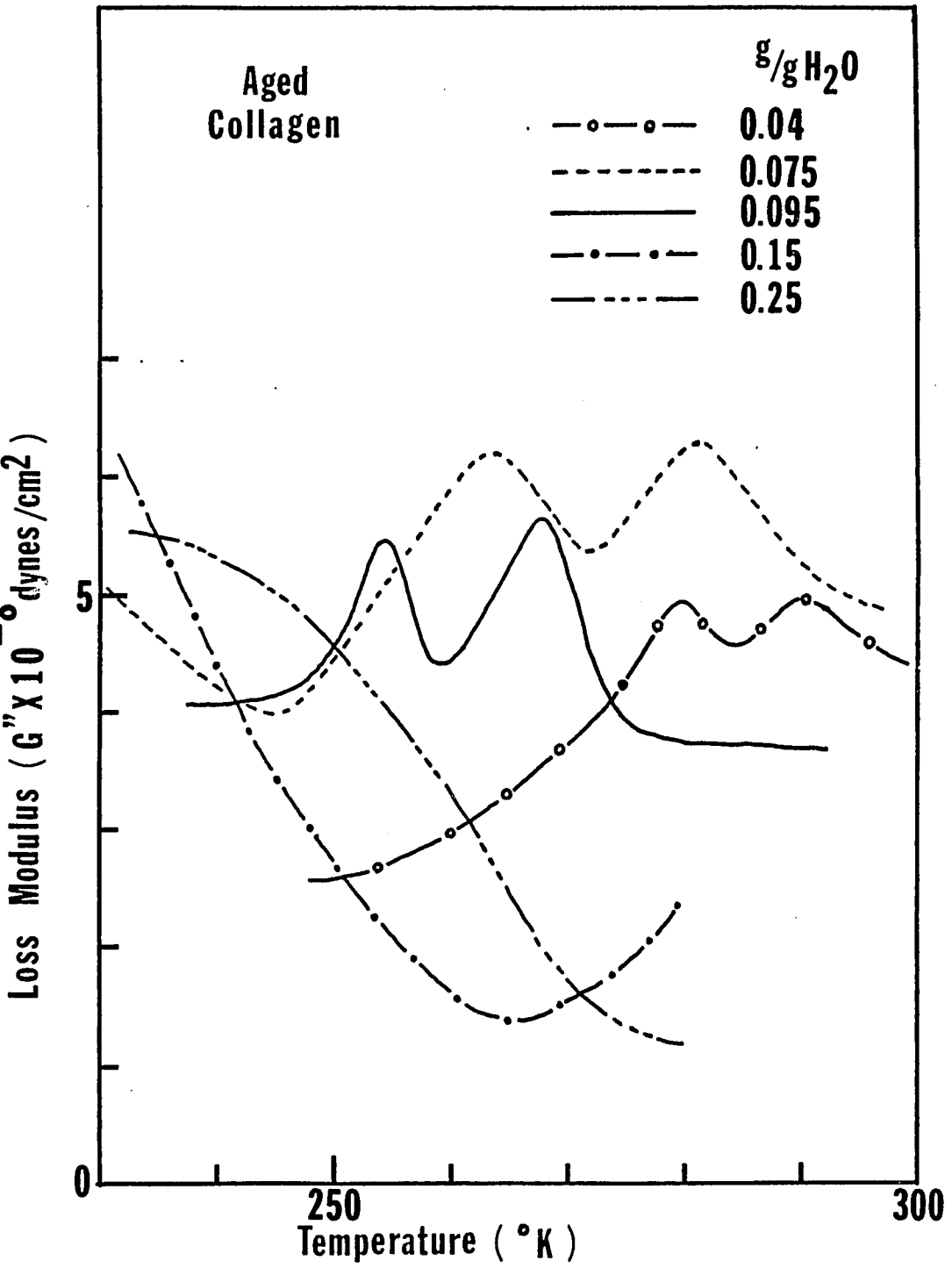


Fig. 13. Loss modulus (G") vs. temperature (240 - 300° K) for aged collagen containing various water contents.

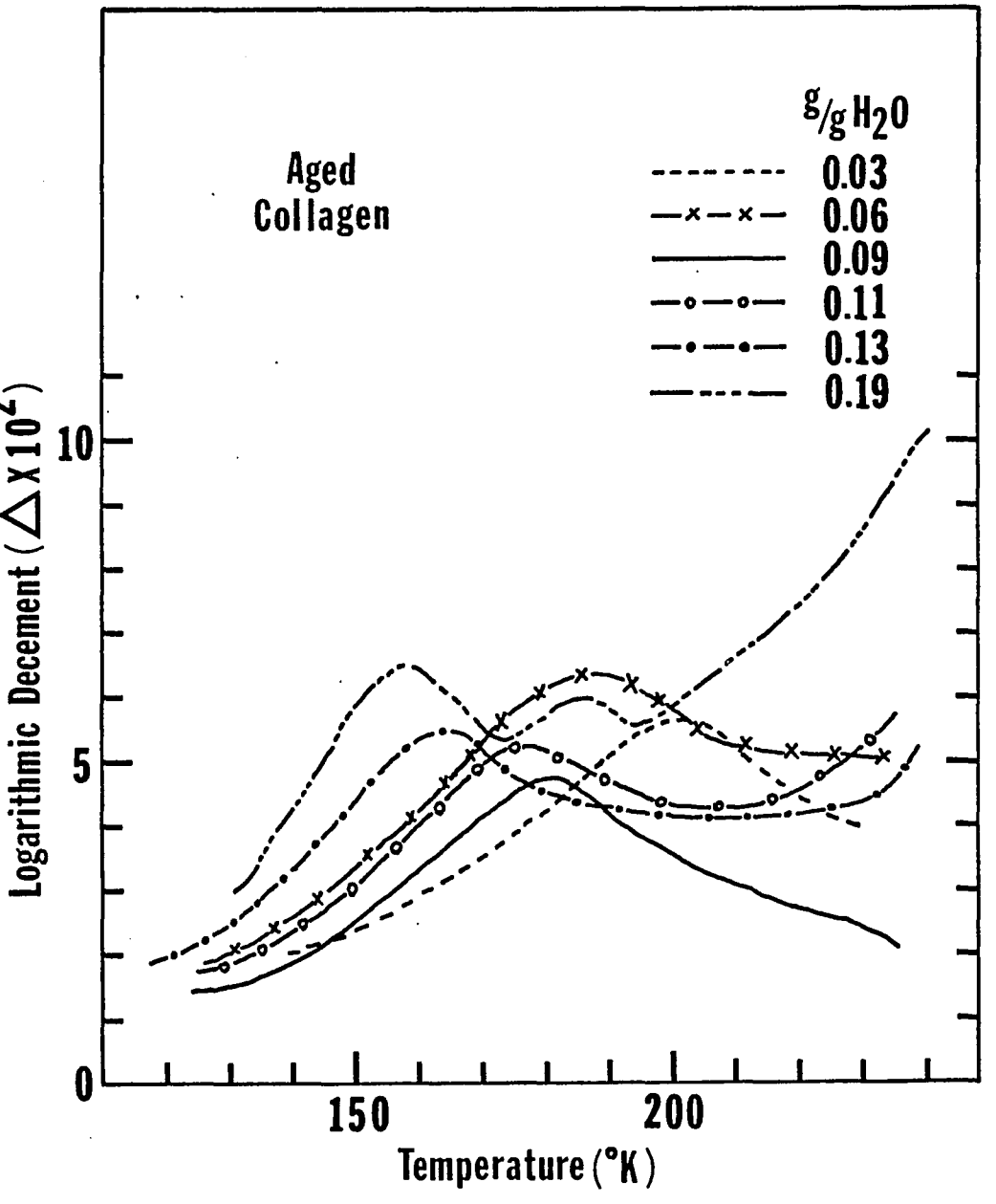


Fig. 14. Logarithmic decrement (Δ) vs. temperature (120 - 240°K) for aged collagen containing various water contents.

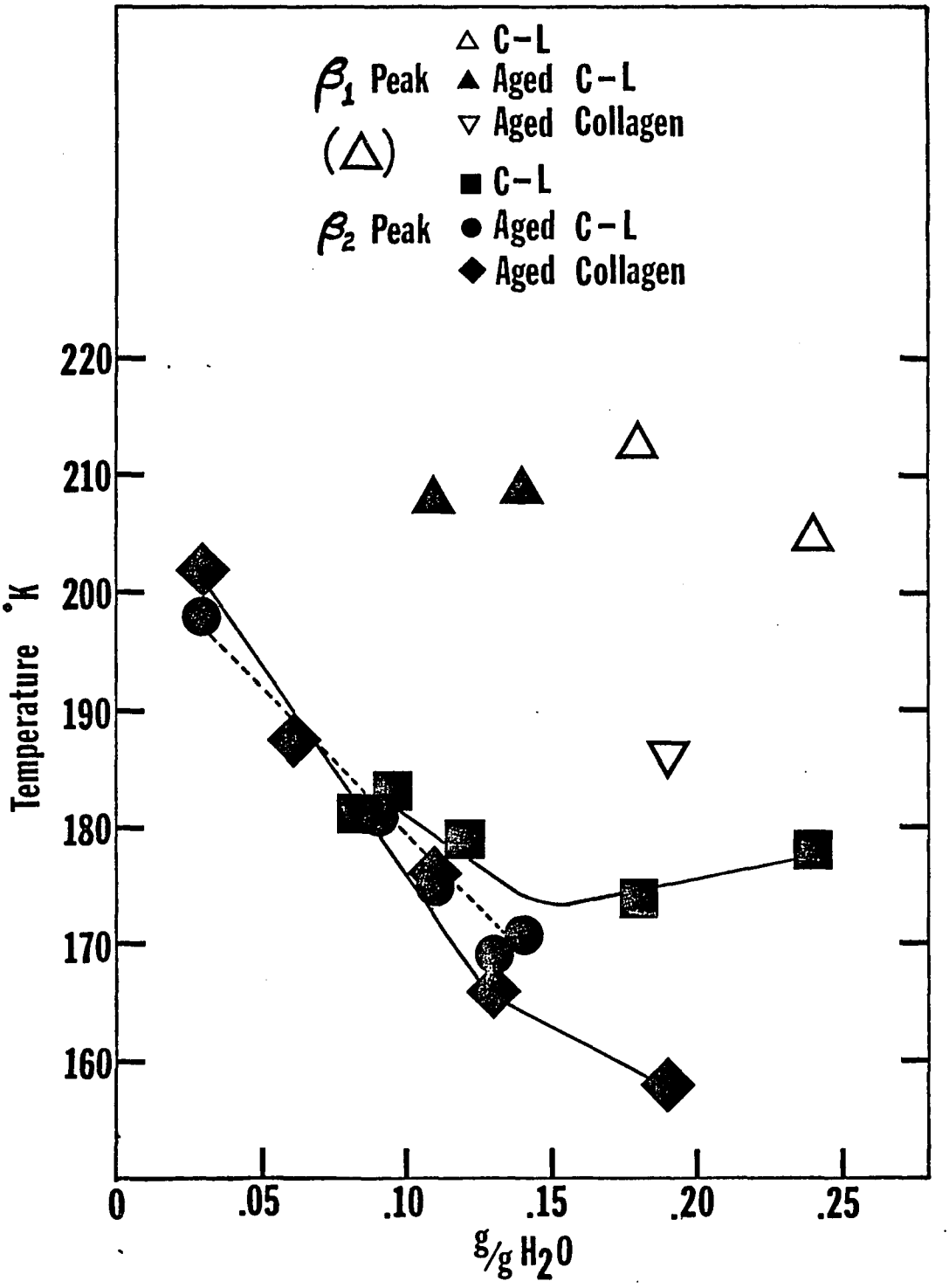


Fig. 15. Temperature position of the β_1 and β_2 peaks (△) vs. water content for aged collagen, C-L, and aged C-L.

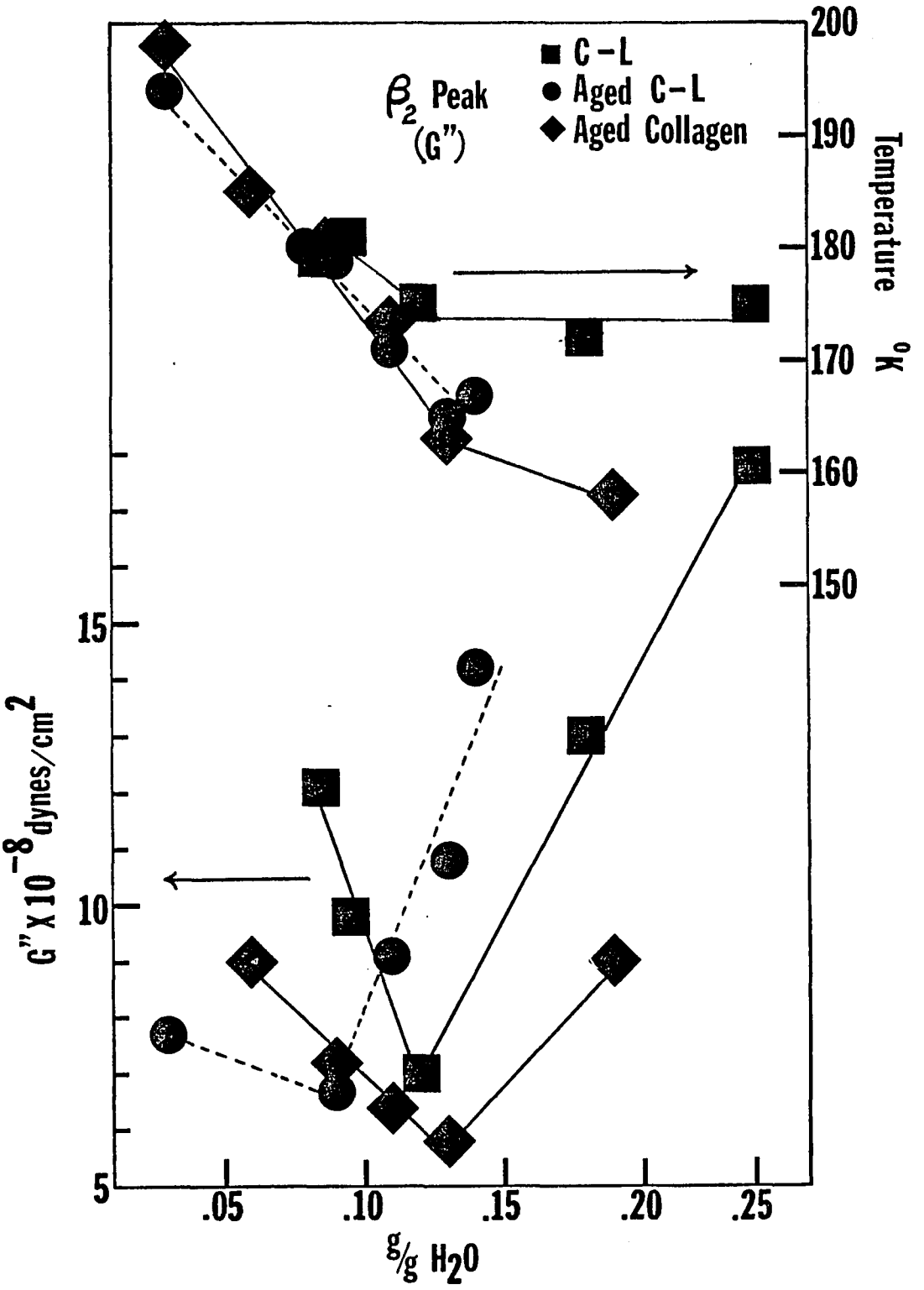


Fig. 16. Temperature position (based on G") and intensity (G") of the β_2 peak vs. water content for aged collagen, C-L, and aged C-L.

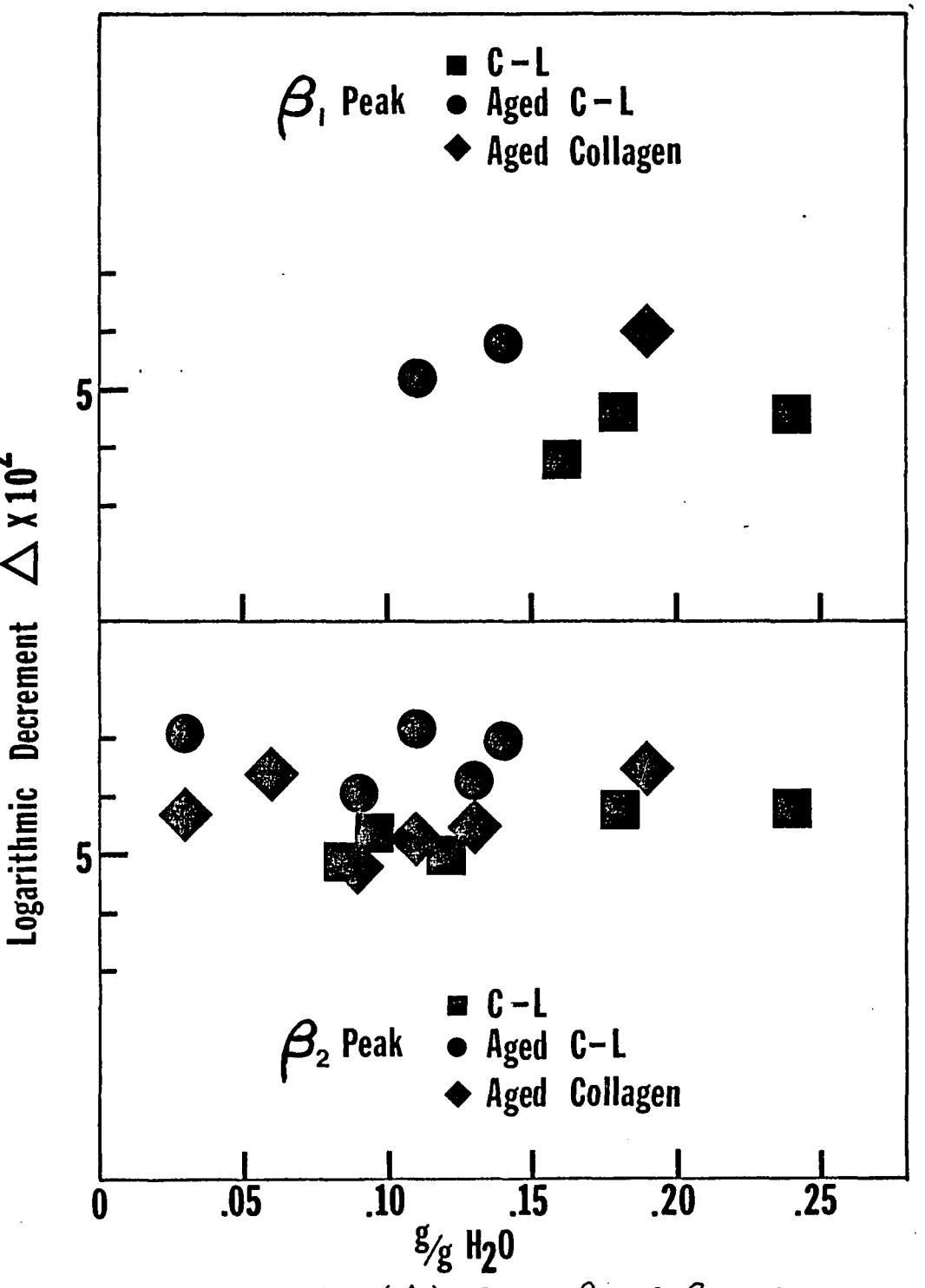


Fig. 17. Intensity (Δ) of the β_1 and β_2 peaks vs. water content for aged collagen, C-L, and aged C-L.

and may be considered as constant within the experimental error. Figure 18 gives the dependence of storage modulus (G') and loss modulus (G"), of samples containing various water contents, on the temperature in the region of 120-240°K. The storage modulus (G') decreases with increasing temperature which corresponds to the occurrence of the β relaxation processes. As the water content is increased, the maximum in the loss molulus (β_2 peak) shifts to lower temperatures and first decreases then increases in intensity (G"). A plot of peak intensity (G") versus water content (Fig. 16) shows that the intensity of the β_2 peak reaches a minimum at about 0.13 g/g water. A cross plot of the storage modulus (G') as a function of water content for temperatures of 145, 185, and 225°K (Fig. 12) indicates that the storage modulus first remains approximately constant between 0.03 and 0.06 g/g water and then decreases (approximately a one and half fold drop) between 0.06 and 0.13 g/g water and then increases slightly with increasing water content.

In summary, the dispersed cattle hide collagen film shows four relaxation processes in the temperature region of 120-300°K. The relaxation behavior of these processes does not change monotonically with water content. A specific water content, (0.13 ± 0.01) g H_2O/g collagen, is associated with abrupt changes in the behavior.

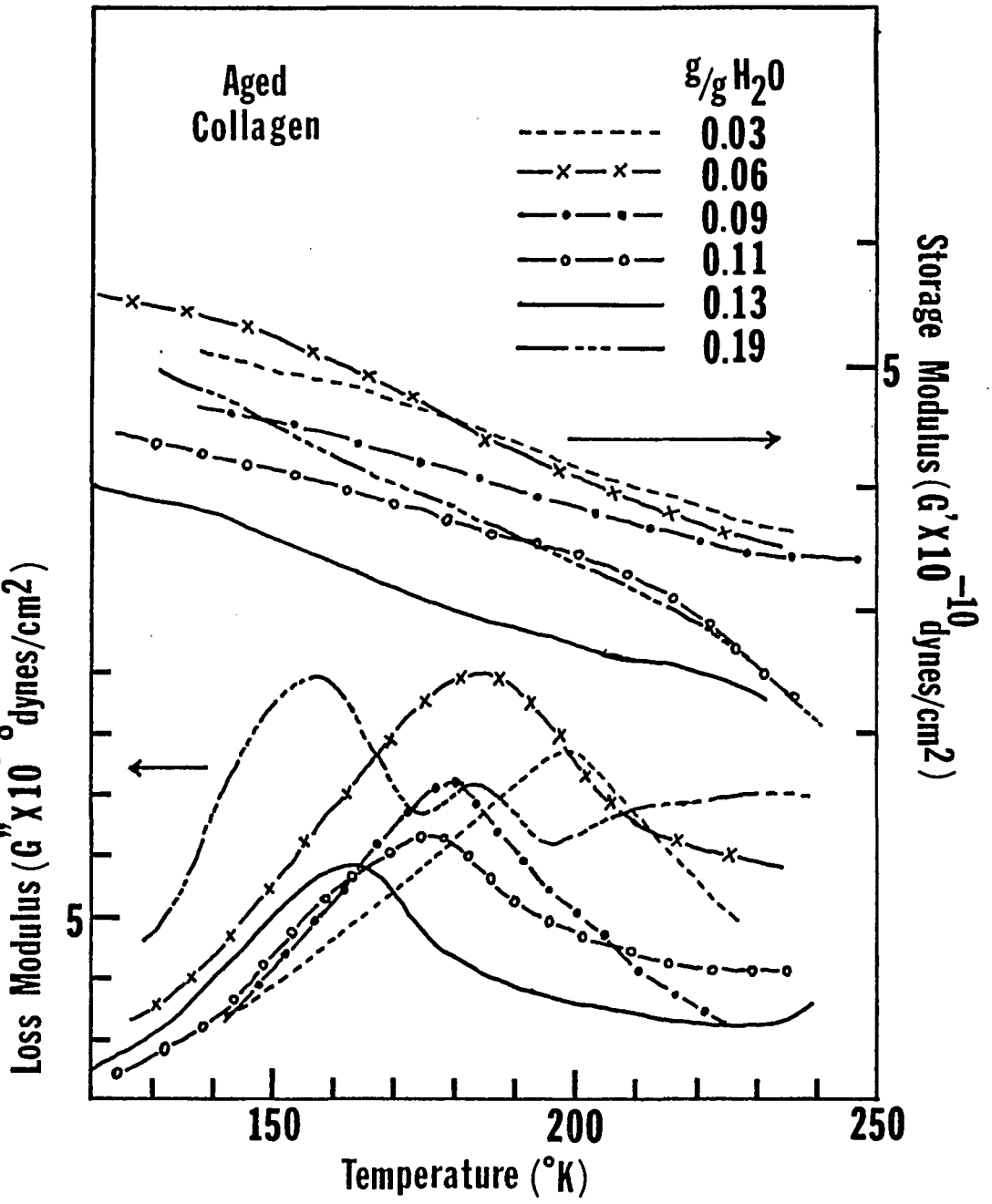


Fig. 18. Storage modulus (G') and loss modulus (G") vs. temperature (120 - 240°K) for aged collagen containing various water contents.

Collagen-lactase

The collagen-lactase system (0 to 2 months) also shows four relaxation processes, labelled in the same way as for collagen (α_1, α_2, β_1, and β_2) in the temperature region of 120-290°K. The β_1 peak is also observed only at higher water content.

Figure 19 gives the logarithmic decrement (Δ) of samples containing various water contents as a function of temperature in the region of 230-290°K. Both the α_1, and α_2 peaks (having approximately the same magnitude) first increase and then decrease in intensity (Δ) with increasing water content (Figs. 9 and 10). Figure 7 shows that the position of the α_1 peak (Δ) shifts by 10° to a lower temperature from 285°K when the relative water content is increased from 0.11 to 0.13 g/g and remains constant at 275°K when the relative water content is increased from 0.13 to 0.25 g/g. The α_2 peak temperature (based on Δ) decreases with increasing water content (Fig. 8). A plot of loss modulus (G") versus temperature (Fig. 20) shows that both α_1 and α_2 peaks do not appear until the relative water content of the sample reaches 0.11 g/g and that both peaks first increase and then decrease in intensity (G") with increasing water content. The α_2 peak has a much higher intensity than the α_1 peak when the relative water content is higher than 0.11 g/g. The sample containing 0.16 g/g relative water content shows a loss process

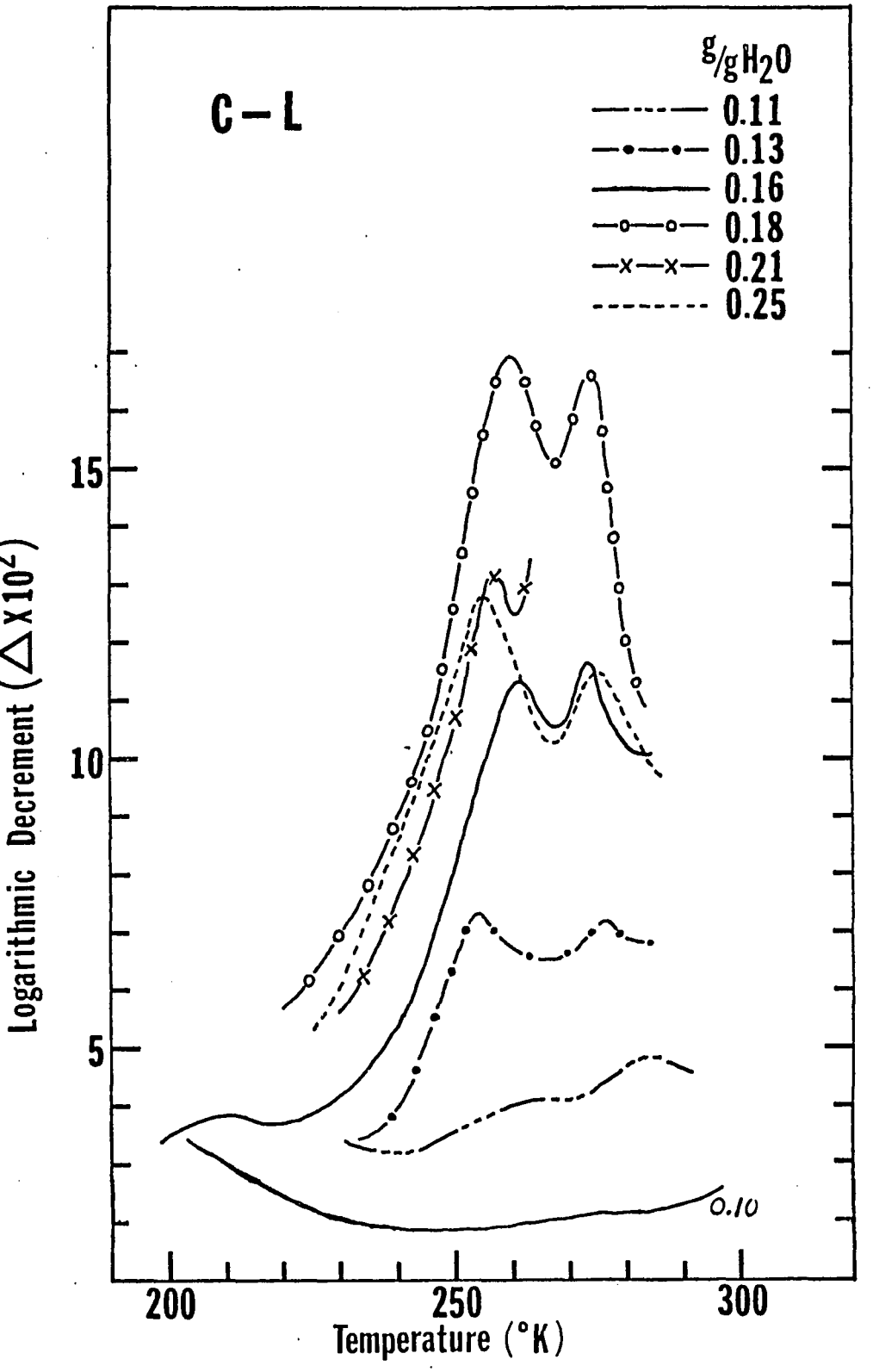


Fig. 19. Logarithmic decrement (Δ) vs. temperature (230 - 290°K) for C-L containing various water contents.

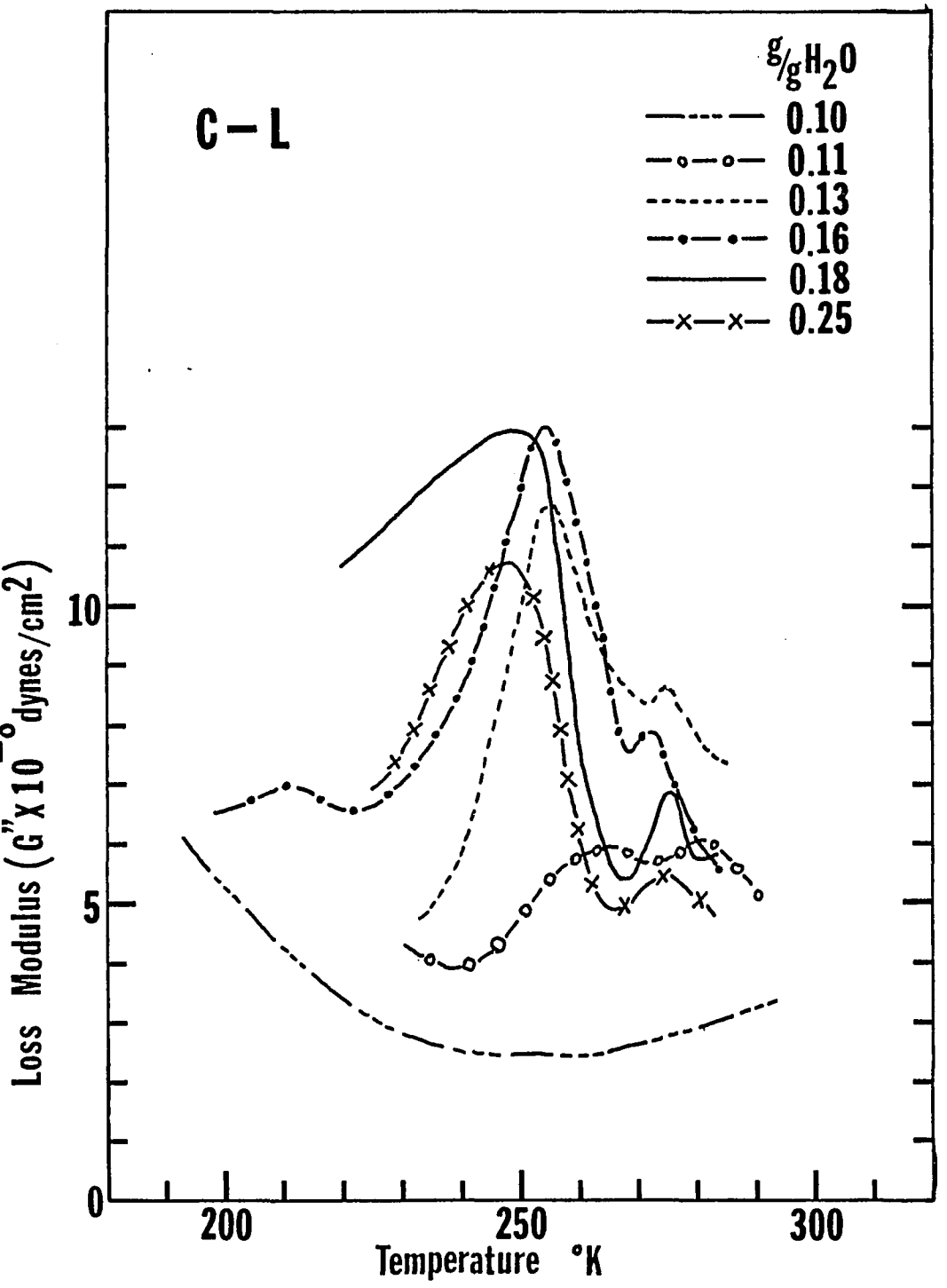


Fig. 20. Loss modulus (G") vs. temperature (230 - 290°K) for C-L containing various water contents.

(β_1 peak) at 212°K. The intensity of the α_1 peak reaches a maximum (G" = 8.5 X 10^8 dyne/cm^2) at about 0.13 g/g water (Fig. 21) and the intensity of the α_2 peak reaches a maximum (G" = 13.5 X 10^8 dyne/cm^2) at about 0.14 g/g water (Fig. 22). Figure 21 shows that the α_1 peak temperature shifts from 281 to 274°K when the relative water content is increased from 0.11 to 0.13 g/g and remains constant at 274°K when the water content is increased from 0.13 to 0.25 g/g. The temperature position of the α_2 peak (Fig. 22) decreases rapidly from 266 to 255°K when the water content is increased from 0.11 to 0.13 g/g and then decreases more slowly from 255 to 248°K when the water content is increased from 0.13 to 0.25 g/g. Figure 23 gives the dependence of the storage modulus (G') of samples containing various water contents on the temperature in the region of 230 to 290°K. The storage modulus (G') of samples containing relative water contents of 0.11 and 0.13 g/g increases with increasing temperature and reaches a maximum which is in the vicinity of T_{α_2} and then decreases. The storage modulus (G') of samples containing water contents from 0.16 to 0.25 g/g drops significantly (at least a three-flod drop) as the temperature increases, which corresponds to the occurrence of the α relaxation processes. Figure 24 gives the cross plot of the storage modulus (G') as a function of water content for several temperatures.

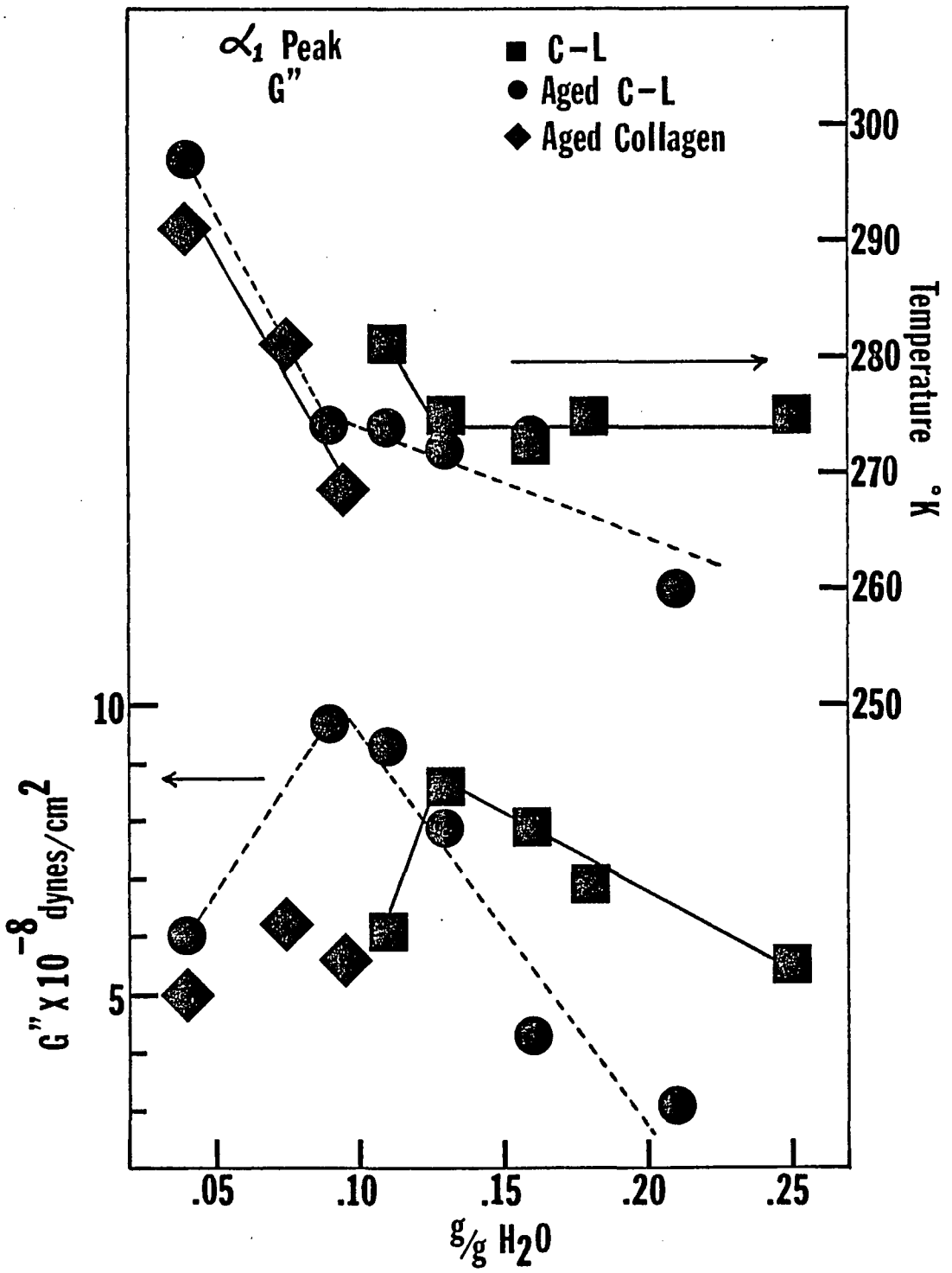


Fig. 21. Temperature position (based on G") and intensity (G") of the α_1 peak vs. water content for aged collagen, C-L, and aged C-L.

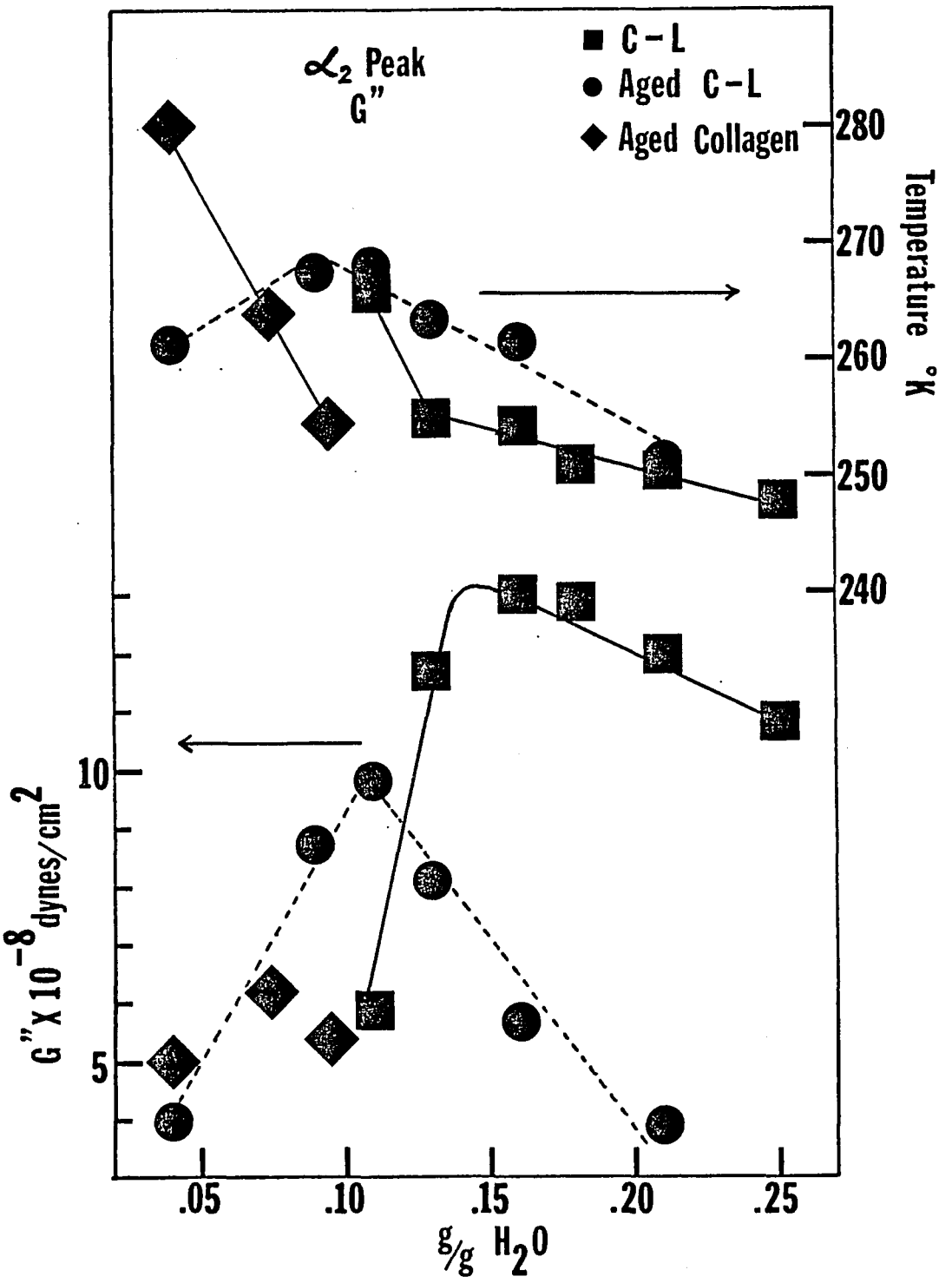


Fig. 22. Temperature position (based on G") and intensity (G") of the α_2 peak vs. water content for aged collagen, C-L, and aged C-L.

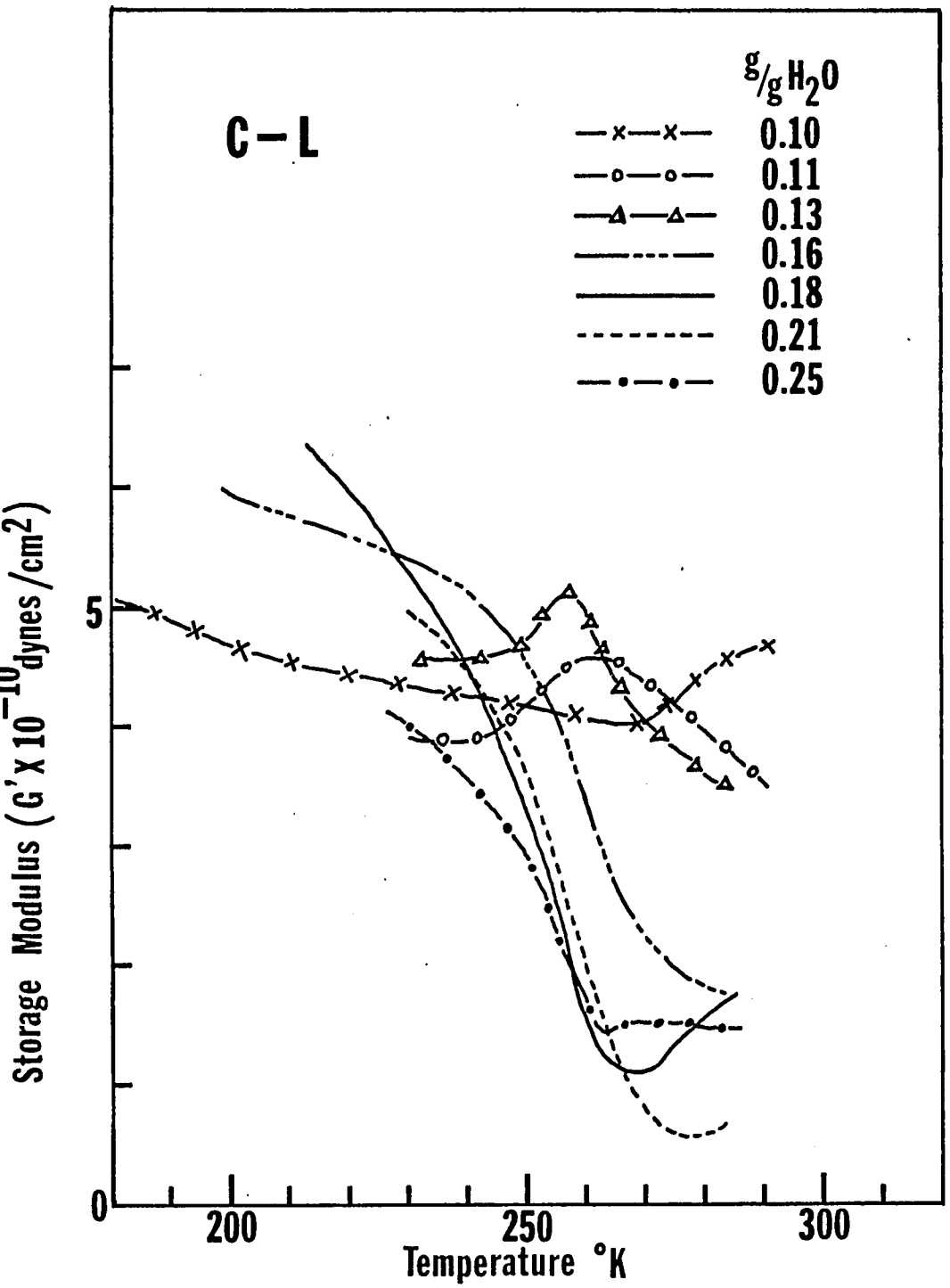


Fig. 23. Storage modulus (G') vs. temperature (230 - 290°K) for C-L containing various water contents.

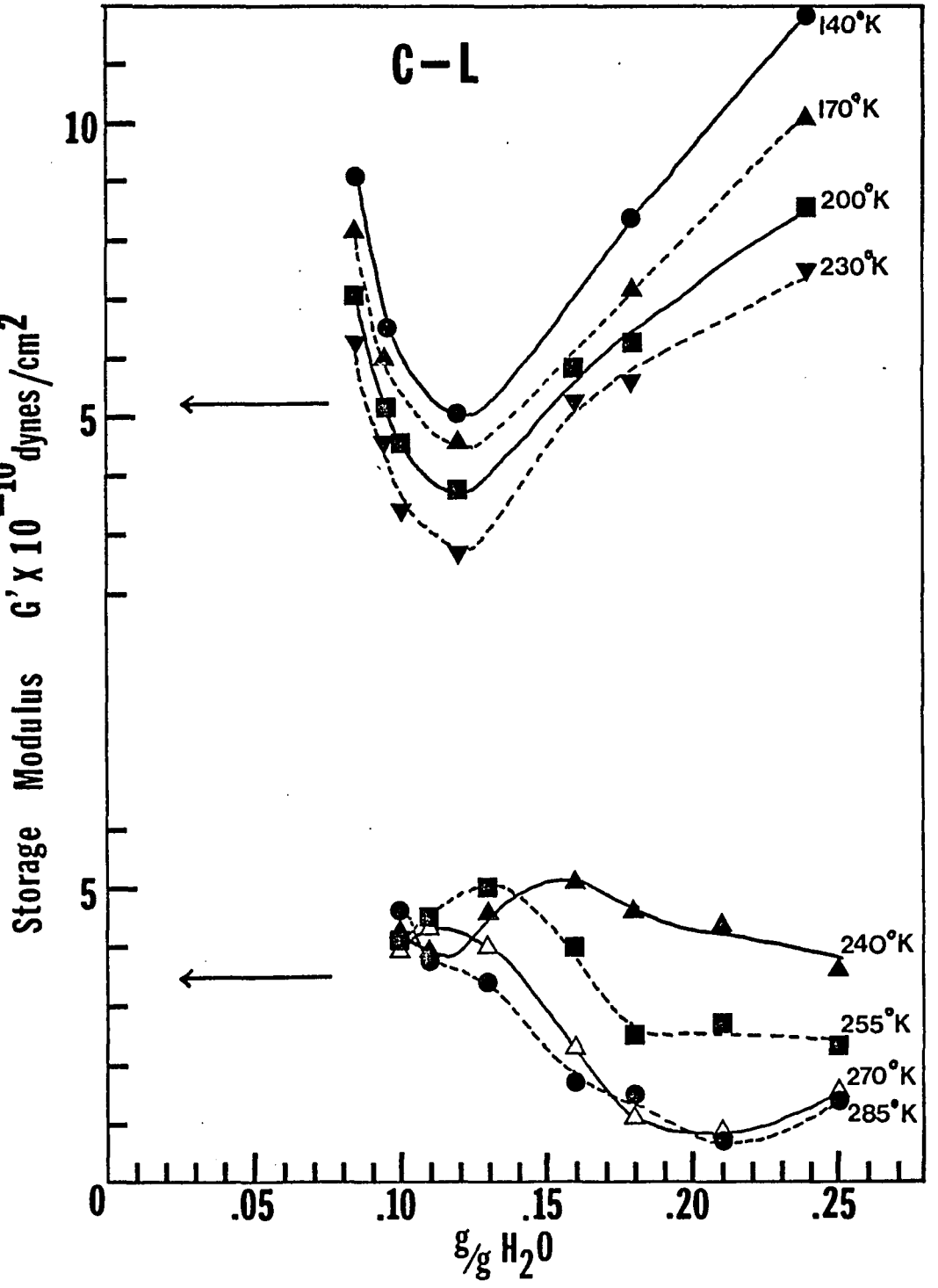


Fig. 24. Storage modulus (G') vs. water content for C-L at several temperatures.

At 240°K, the storage modulus (G') first increases a little and reaches a maximum at about 0.15 g/g water and decreases slowly with increasing water content. At 255°K the storage modulus first increases slightly and reaches a maximum at about 0.13 g/g water and then decreases rapidly (approximately a two-fold drop) between 0.13 and 0.18 g/g water and then remains constant between 0.18 and 0.25 g/g water. At 270 and 285°K, the storage modulus decreases monotonically (approximately a five-fold drop) between 0.11 and 0.21 g/g water and then increases slowly between 0.21 and 0.25 g/g water. The significant drop of the storage modulus between 240 and 270°K indicates that the major relaxation process (α_2) occurs in this temperature region.

Another water-sensitive peak β_2 occurs in the temperature region of 170-190°K. Figure 25 gives the logarithmic decrement (Δ) versus temperature in the region of 120-230°K. At lower water content, only the β_2 process is observed. An additional loss peak (β_1) which appears at higher water content (0.18 and 0.24 g/g water) shifts to lower temperatures and remains constant in intensity (Δ) when water content is increased from 0.18 to 0.24 g/g. Figure 15 shows that the temperature position of the β_2 peak (Δ) decreases from 183 to 175°K when the relative water content is increased from 0.09 to 0.13 g/g and then increases

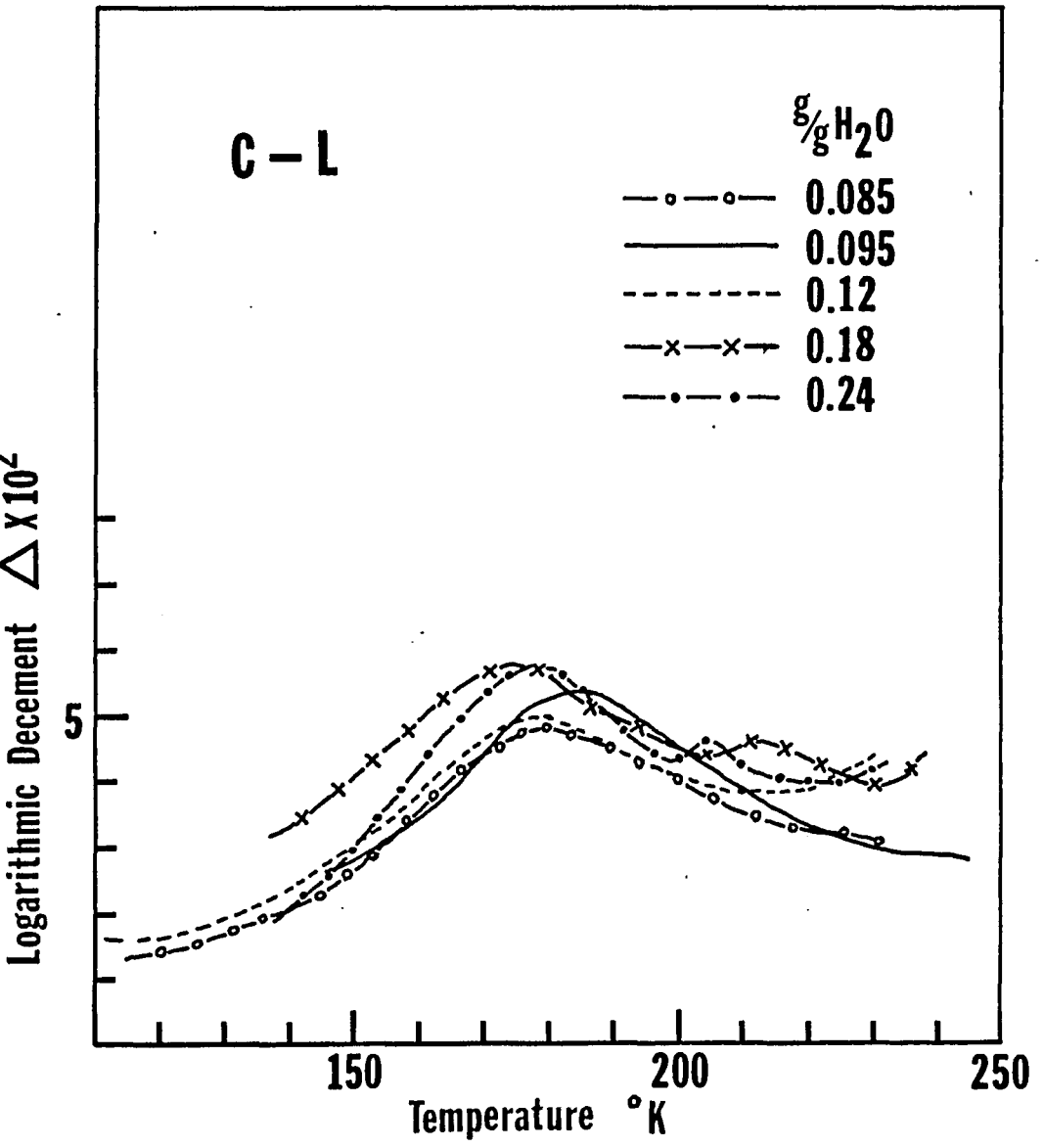


Fig. 25. Logarithmic decrement (Δ) vs. temperature (120 -230°K) for C-L containing various water contents.

slightly between 0.13 and 0.24 g/g water. The intensity (Δ) of the β_2 peak remains constant (Fig. 17) within experimental error. The loss modulus (G") of samples containing various water contents as a function of temperature is given in Figure 26. It is seen that the β_1 peak which appears at higher water content shifts from 212 to 206°K and increases in intensity (G") when the relative water content is increased from 0.16 to 0.24 g/g (Fig. 27). Figure 16 shows that the temperature position of the β_2 peak decreases from 180 to 174°K when the water content is increased from 0.09 to 0.12 g/g and remains constant between 0.12 and 0.24 g/g water. The intensity (G") of the β_2 peak decreases rapidly between 0.08 and 0.12 g/g water and then increases rapidly between 0.12 and 0.24 g/g water. Figure 28 gives the dependence of the storage modulus (G') of samples containing various water contents on the temperature. The decrease in the storage modulus with increasing temperature corresponds to the occurrence of the β relaxation processes. A cross plot of the storage modulus (G') as a function of water content for temperatures of 140,170,200, and 230°K (Fig. 24) indicates that the storage modulus first decreases reaching a minimum at about 0.12 g/g water,and then increases.

In summary, the collagen-lactase system also shows four relaxation processes in the temperature

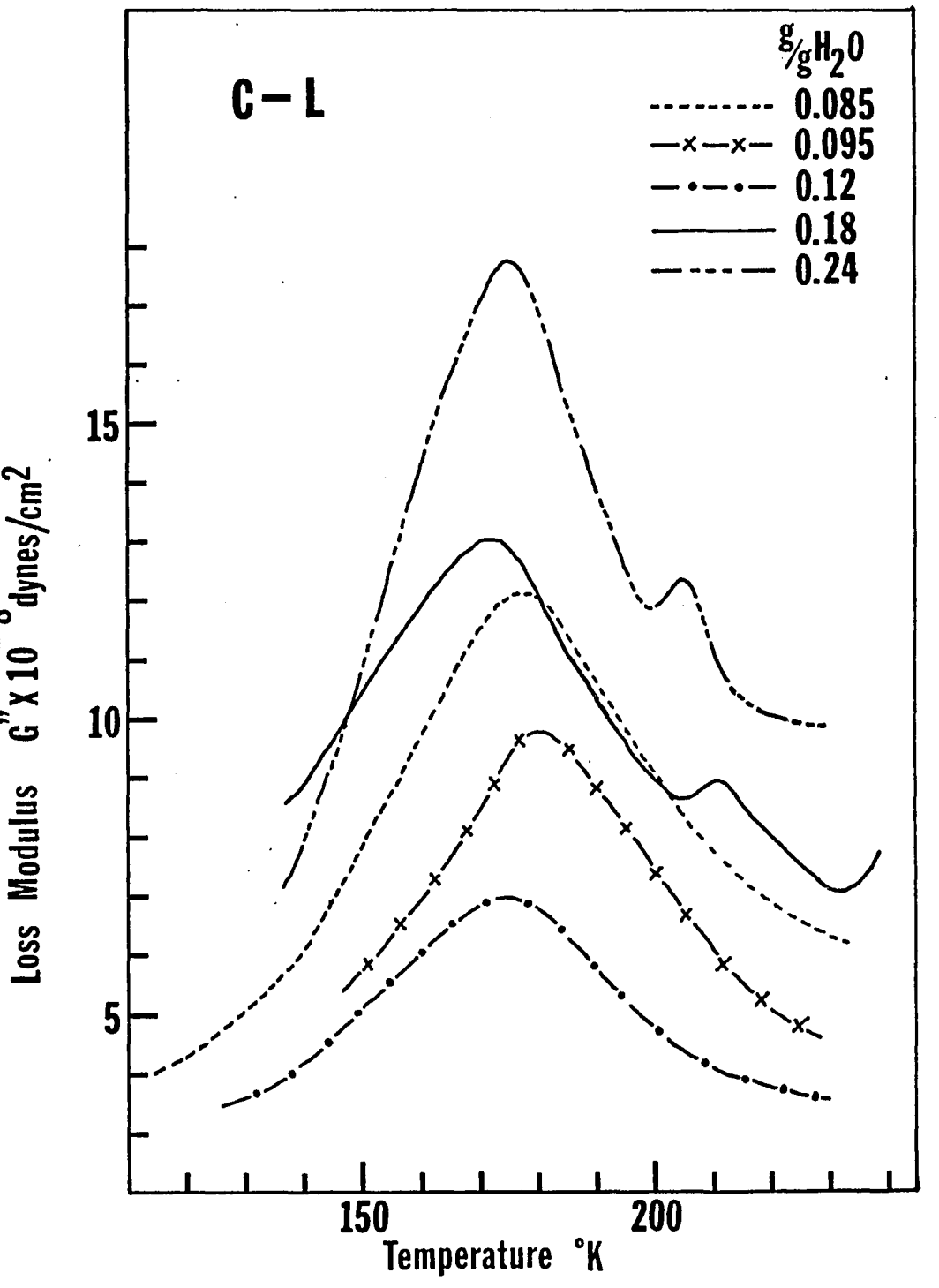


Fig. 26. Loss modulus (G") vs. temperature (120 - 230°K) for C-L containing various water contents.

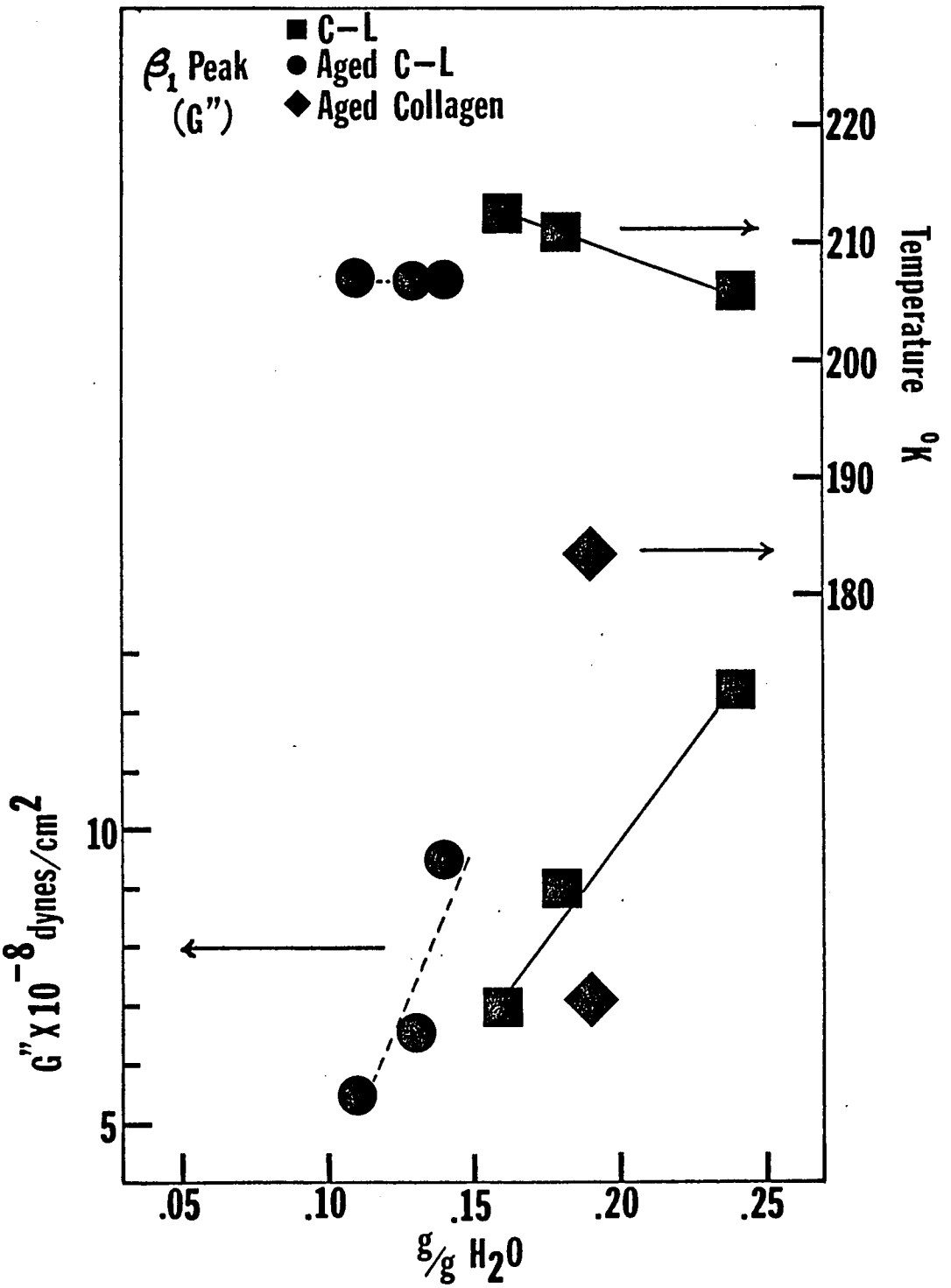


Fig. 27. Temperature position (based on G") and intensity (G") of the β_1 peak vs. water content for aged collagen, C-L, and aged C-L.

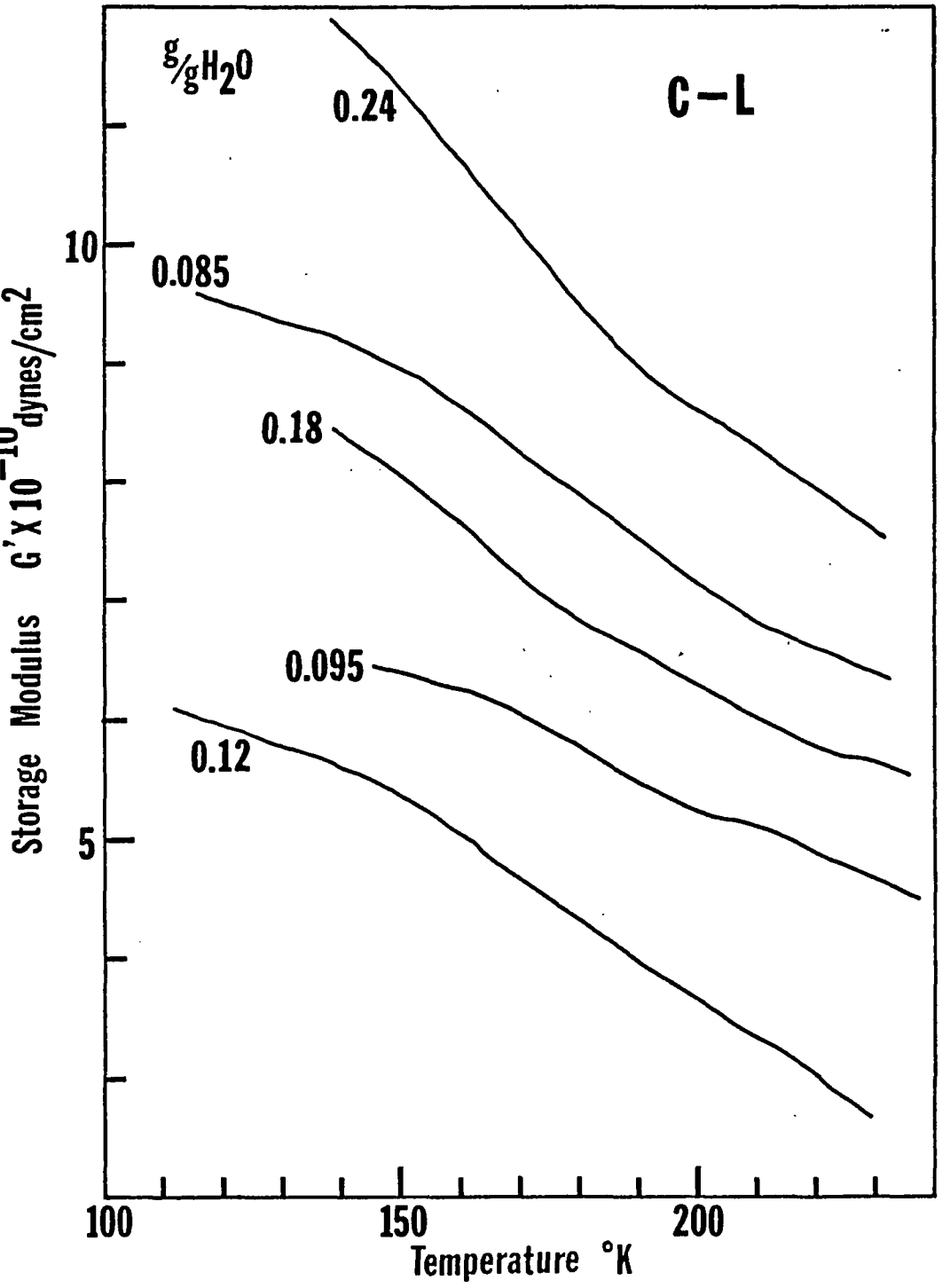


Fig. 28. Storage modulus (G') vs. temperature (120 - 230°K) for C-L containing various water contents.

region of 120-300°K. A specific water content, (0.13 ± 0.01) g/g is associated with abrupt changes in the behavior of these relaxation processes.

Aged Collagen-lactase

Figure 29 gives the logarithmic decrement (Δ) of samples containing various water contents as a function of temperature in the region of 240-300°K. The intensity (Δ) of both α_1 and α_2 peaks increases between 0.04 and 0.11 g/g water (Fig. 9 and 10) and remains constant between 0.11 and 0.21 g/g water. Figure 7 shows that the α_1 peak temperature (based on Δ) decreases rapidly from 297 to 277°K when the relative water content is increased from 0.04 to 0.09 g/g and then decreases more slowly between 0.09 and 0.21 g/g water. The temperature position (Δ) for the α_2 peak (Fig. 8) first increases, reaching a maximum at about 0.09 g/g water, and then decreases with increasing water content. The loss modulus (G") of samples containing various water contents versus temperature is given in Figure 30. It is seen that both α_1 and α_2 peaks increase in intensity (G") with increasing water content and then decrease. The intensity of the α_1 peak reaches a maximum (G" = 10 X 10^8 dyne/cm^2) at about 0.09 g/g water (Fig. 21) and the intensity of the α_2 peak reaches a maximum (G" = 10 X 10^8 dyne/cm^2) at about 0.10 g/g water (Fig. 22). Figure 21 shows that the temperature position (based on G")

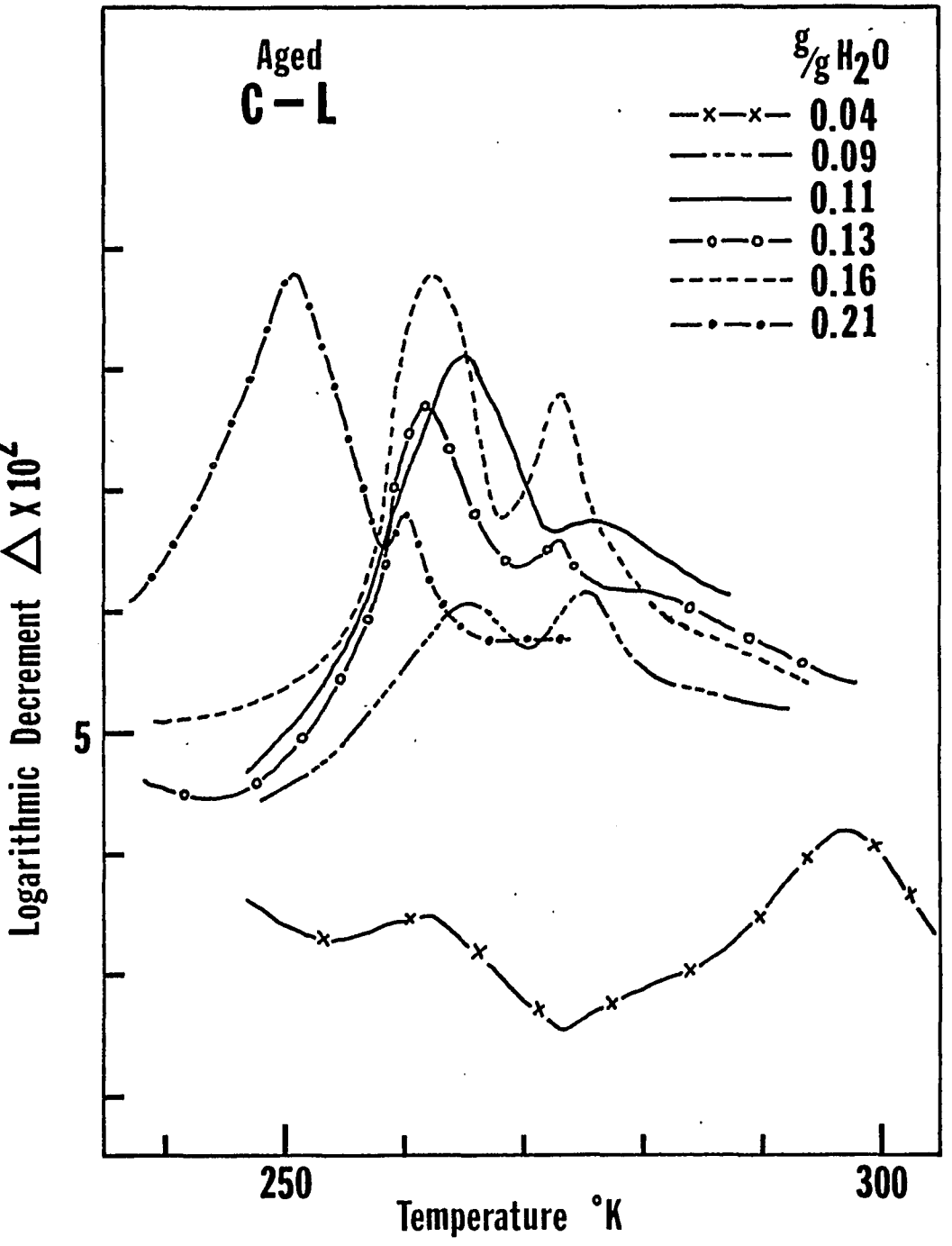


Fig. 29. Logarithmic decrement (Δ) vs. temperature (240 - 300°K) for aged C-L containing various water contents.

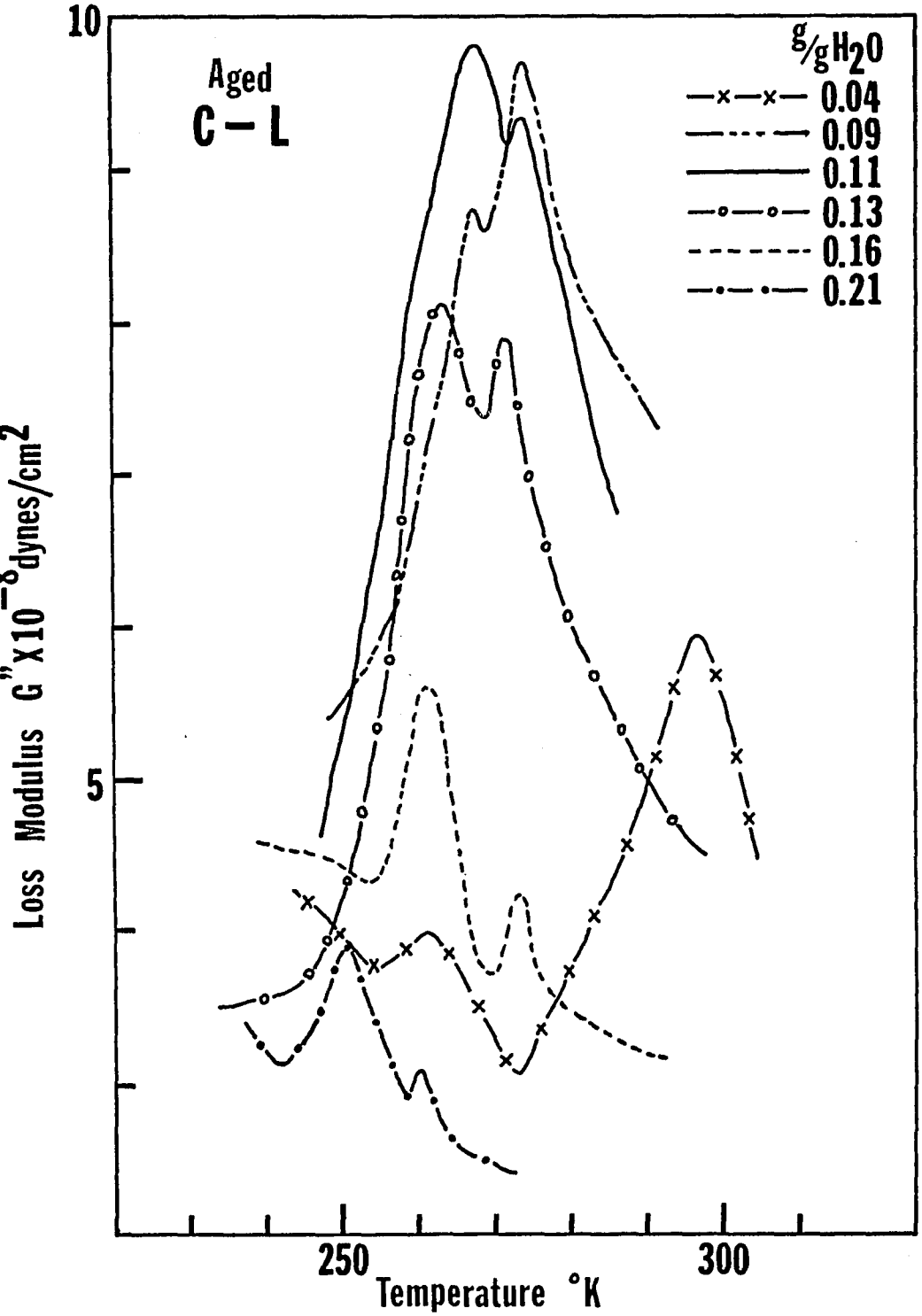


Fig. 30. Loss modulus (G") vs. temperature (240 - 300°K) for aged C-L containing various water contents.

of the α_1 peak decreases rapidly from 297 to 275°K when the relative water content is increased from 0.04 to 0.09 g/g and then decreases more slowly between 0.09 and 0.21 g/g water. The α_2 peak temperature first increases between 0.04 and 0.09 g/g water and then decreases between 0.09 and 0.21 g/g water (Fig. 22). A plot of the storage modulus (G') versus the temperature (Fig. 31) shows that the storage modulus of samples containing relative water contents of 0.04, 0.09, 0.11 and 0.13 g/g increases with increasing temperature and reaches a maximum (in the vicinity of T_{α_1}) and then decreases. The storage modulus of samples containing water content of 0.16 and 0.21 g/g decreases with increasing temperature and then levels off. A cross plot of the storage modulus (G') as a function of water content for several temperatures is given in Figure 32. At 245°K, the storage modulus shows two plateau regions. At 265 and 285°K, the storage modulus slightly increases between 0.04 and 0.09 g/g water and then decreases rapidly (approximately a three-fold drop) between 0.09 and 0.21 g/g water.

Figure 33 gives the logarithmic decrement (Δ) of samples as a function of temperature in the region of 120 to 240°K. At lower water content, only one relaxation process (β_2) is observed. An additional peak (β_1) shows up at higher water content (above 0.11 g/g water). For this β_1 process, the Δ_{max} (Fig. 17) and the peak temperature (Fig. 15) appears to be constant with increasing water content. The β_2 peak shifts monotonically from 198 to 168°K

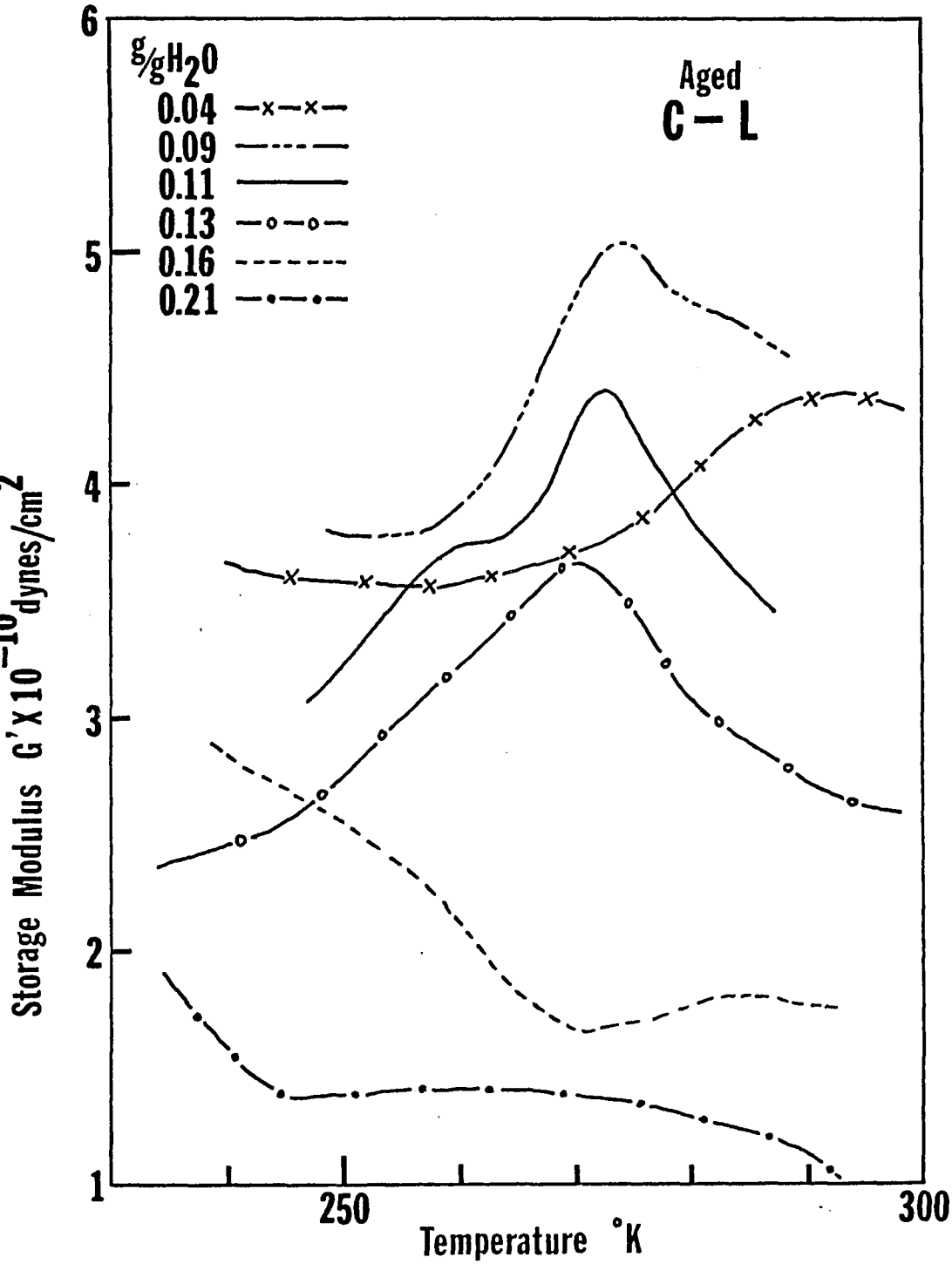


Fig. 31. Storage modulus (G') vs. temperature (240 - 300°K) for aged C-L containing various water contents.

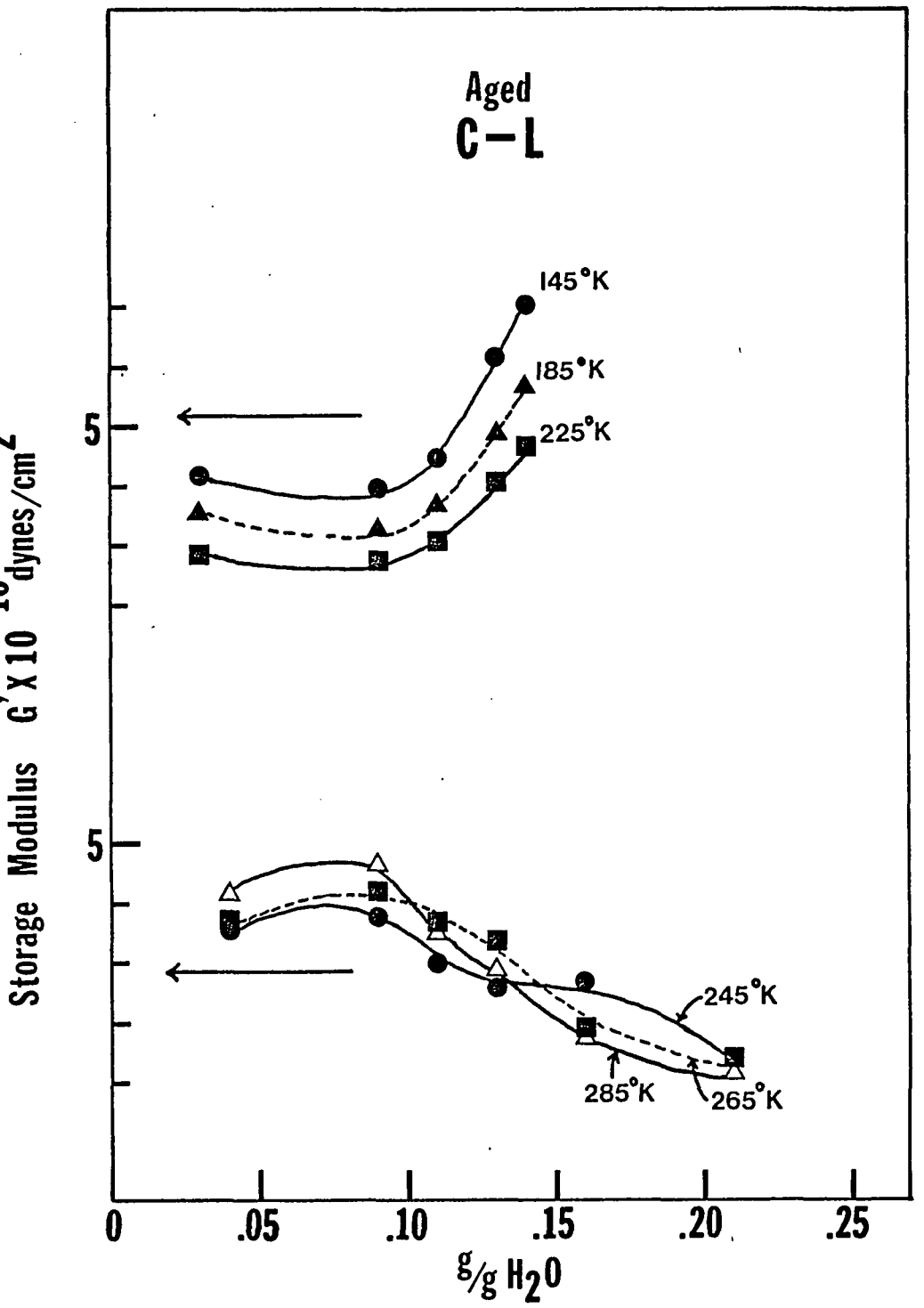


Fig. 32. Storage modulus (G') vs. water content for aged C-L at several temperatures.

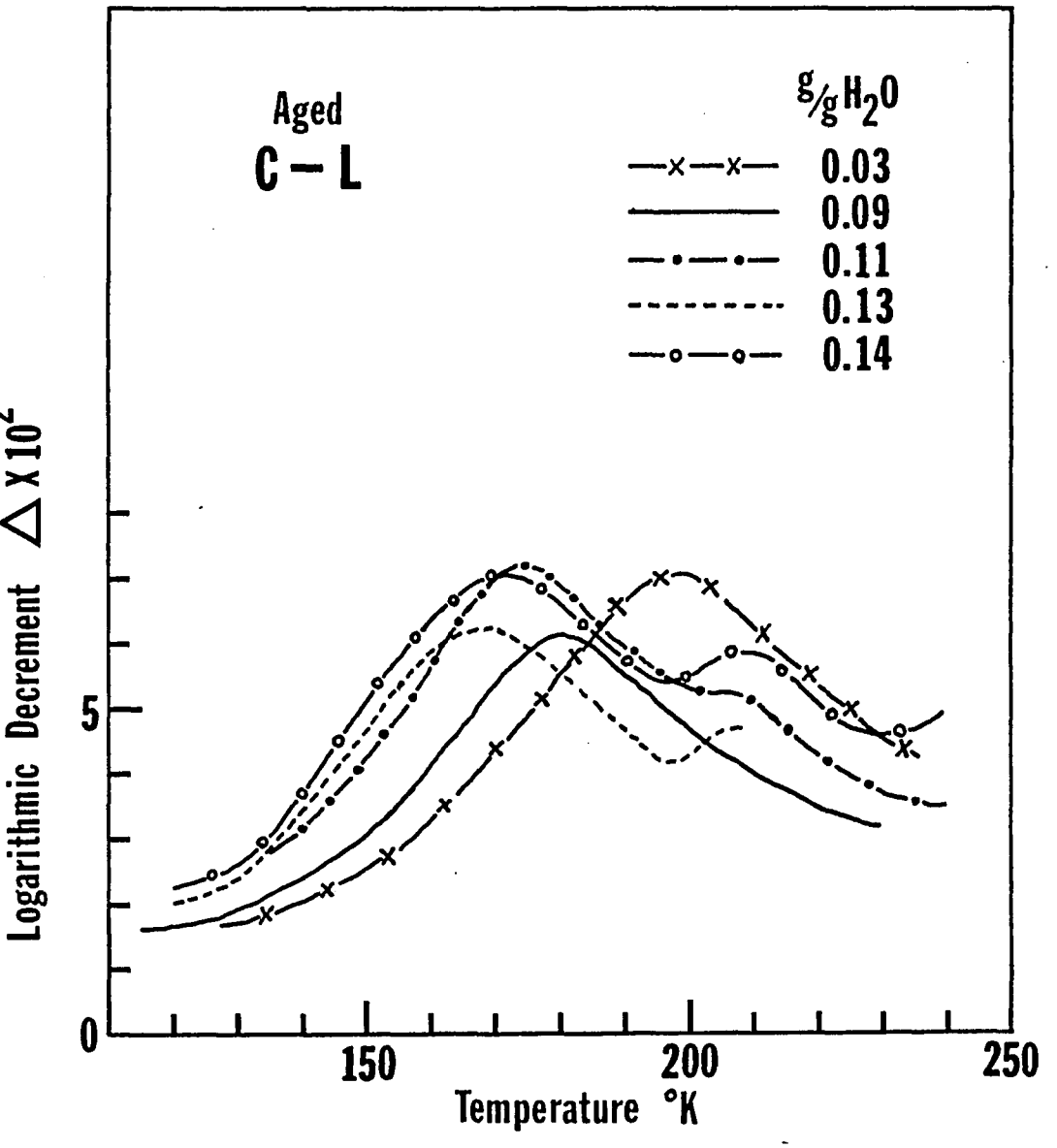


Fig. 33. Logarithmic decrement (Δ) vs. temperature (120 - 240°K) for aged C-L containing various water contents.

(Fig. 15) as the water content is increased from 0.04 to 0.14 g/g. The intensity (Δ) of the β_2 peak is considered constant within the experimental error (Fig. 17). The loss modulus (G") of the various samples as a function of temperature is given in Figure 34. The β_1 peak is not observed until the water content reaches 0.11 g/g. The β_1 peak increases in intensity (G") but its temperature position remains constant with increasing water content (Fig. 27). Figure 16 shows that the β_2 peak shifts from 194 to 165°K when the water content is increased from 0.03 to 0.14 g/g. The intensity (G") of the β_2 peak decreases first and reaches a minimum at about 0.09 g/g water and then increases rapidly between 0.09 and 0.14 g/g water. The dependence of storage modulus (G') on the temperature is given in Figure 35. The decrease in the storage modulus with increasing temperature corresponds to the occurrence of the β processes. A cross plot of the storage modulus versus water content for temperatures of 145, 185, and 225°K (Fig. 32) shows that the storage modulus decreases slightly between 0.03 and 0.09 g/g water and then increases rapidly between 0.09 and 0.14 g/g water.

In summary, the aged collagen-lactase system also shows four relaxation processes in the temperature region of 120-300°K. The specific water content which is associated with abrupt changes in the behavior of these relaxation processes is about 0.09 g/g.

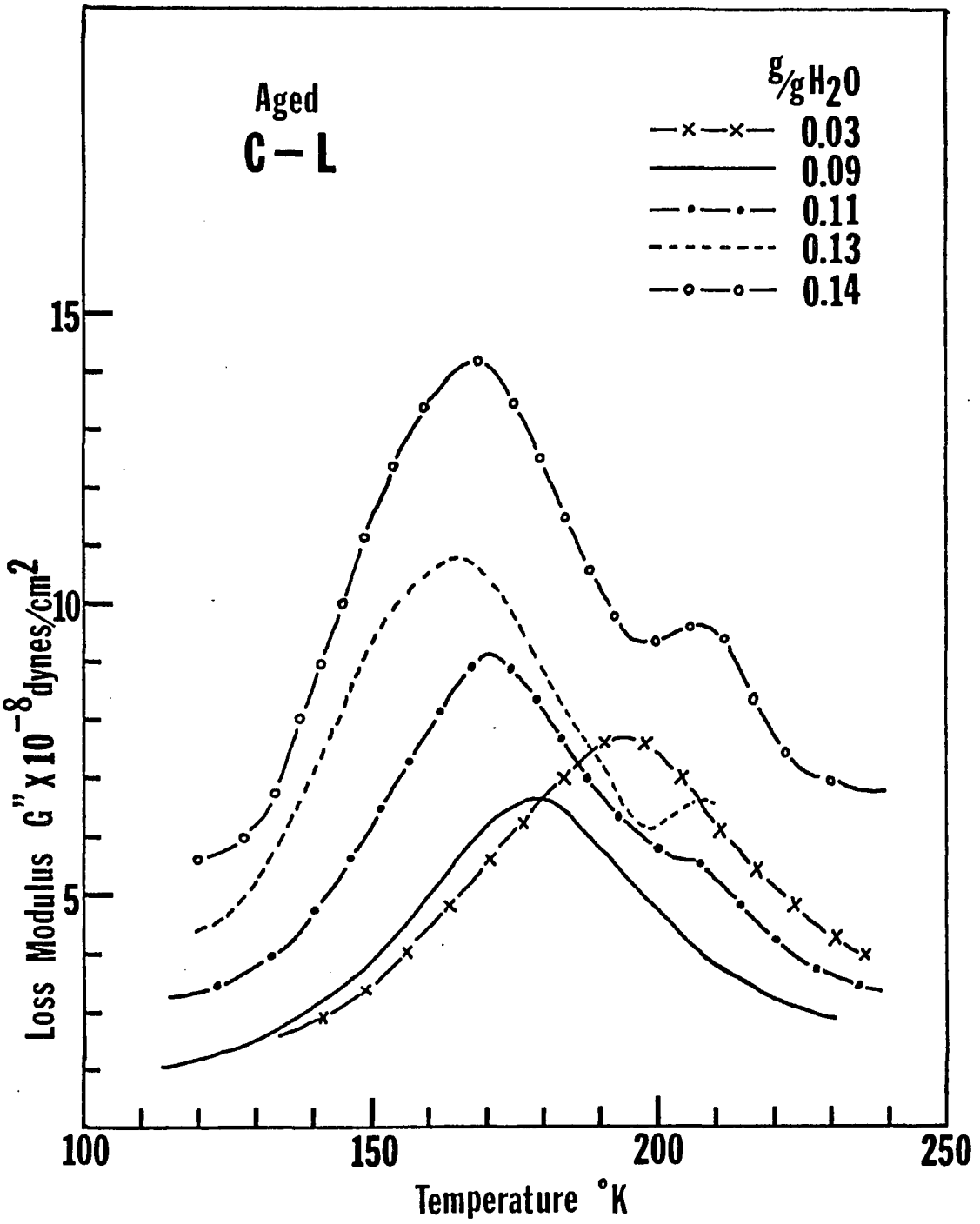


Fig. 34. Loss modulus (G") vs. temperature (120 - 240°K) for aged C-L containing various water contents.

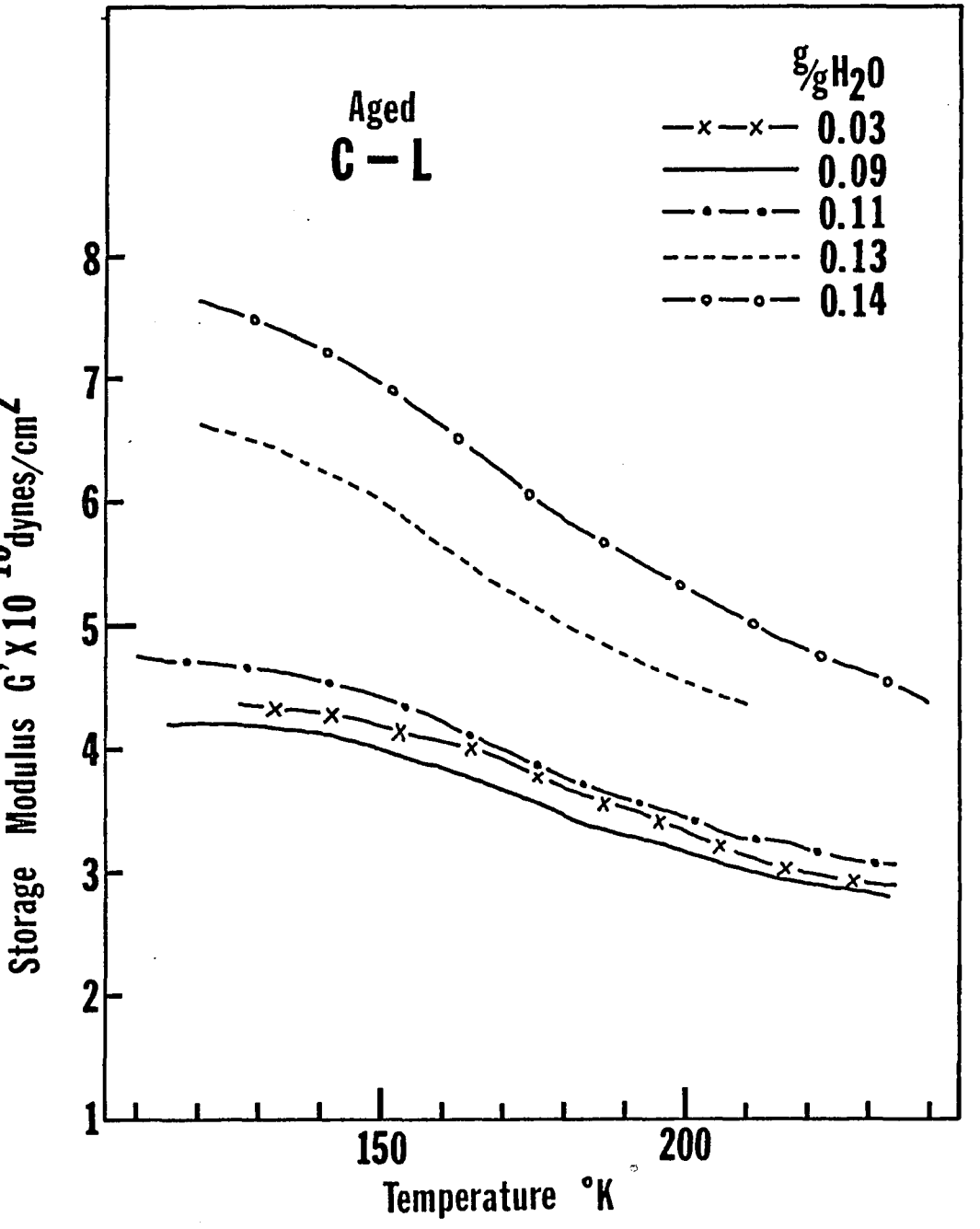


Fig. 35. Storage modulus (G') vs. temperature (120 - 240°K) for aged C-L containing various water contents.

IV. Discussion

Collagen with low water contents investigated by all workers shows two mechanical loss processes below physiological temperatures, one in the room-temperature region and the other having a maximum in the 150-210°K region. Chien and Chang reported an additional loss peak at 220°K (0.1 KHz) in RTT and AKM 23 (about 12% H_2O). In the present study, two loss peaks (α_1 and α_2) are observed in the room temperature region instead of one as reported before and in addition to the low-temperature loss process, a loss peak at about 135°K (<1 Hz) is observed at higher water content (0.19 g/g H_2O). The temperature positions of the mechanical loss peaks of all investigators are plotted together in Figure 36. From Figure 36 it is seen that the γ peak for rat tail tendon presumably containing about 10% water,[95] the γ peak for human diaphram (HDT) collagen containing 10% water[97] and the β peak for steer tendon collagen containing 0.9 and 3.5 % water[98] show a close fit with the data for the β_2 peak in this study. The low temperature peak reported by Nguyen et. al.[100] (peak 1) for a sample containing 15% water is a very broad dispersion. The estimated temperature position of peak 1 is much higher than that for the β peaks found in this study. It is also seen that the β peak for human diaphram collagen containing 10% water and the peak for steer tendon collagen containing 0.9, 3.5,

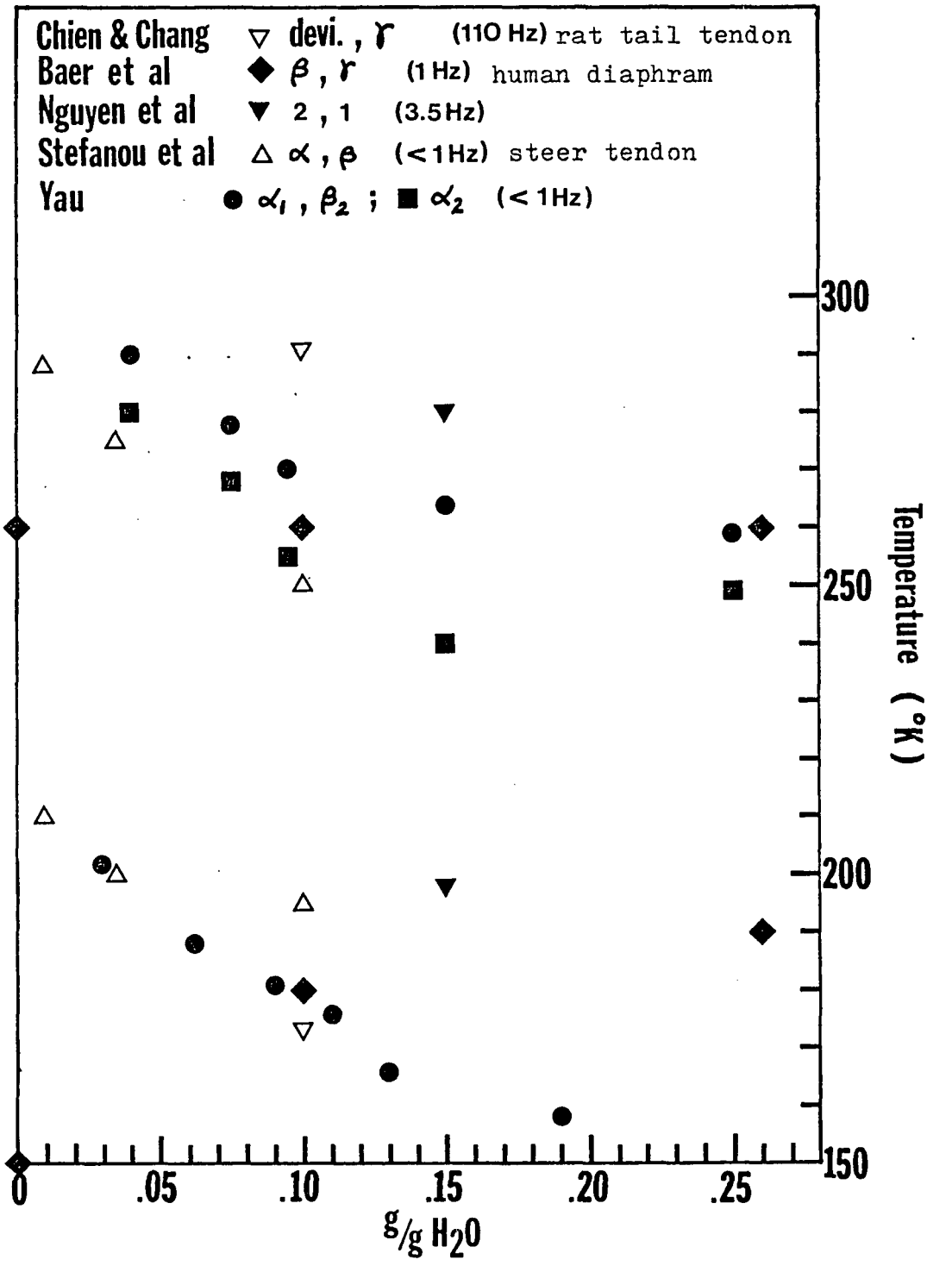


Fig. 36. Temperature positions (△) of the mechanical loss peaks reported by Chien and Chang, Baer et al., Nguyen et al., Stefanou et al., and this study.

and 10% water fit closely with the data of the α_2 peak in this study. The temperature position of the devitrification peak for various collagens presumably containing about 10% water as reported by Chien and Chang and peak 2 from the work of Nguyen et. al. for a sample containing 15% water is at somewhat higher values than that for the α_1 peak in this study. Despite these differences it is believed that these loss processes for different samples are correlated.

The effect of water content on the temperature positions of the α and β loss processes appears to depend on the collagen sample being used. The temperature position of the β peak for HDT collagen[97] increases with increasing water content; the β peak does not show a shift with water content. In contrast, the temperature positions of both the α and β peaks for steer tendon collagen decrease with increasing water contents, which is consistent with the results of the present study.

The maximum intensities of the loss processes from all investigations are difficult to compare with one another on an absolute basis because of the use of different samples, instruments, and parameters. However, a general feature for all investigations is that Δ max for the higher temperature peak is about two to three fold higher than Δ max for the lower temperature one when the water content of the sample

is in the region of 10%. The trend in the effect of water content on the intensity of a loss peak for all investigations is about the same. Baer et. al. and Stefanou et. al. observed that both high- and low-temperature peaks increase in intensity (Δ) with increasing water contents. The two α peaks in this study increase in intensity (Δ) when the water content is increased from 0.04 to 0.15 g/g. However, the intensity of the β_2 peak in this study is constant with water content within the 10% experimental uncertainty although a small increase in intensity is seen between 0.09 and 0.19 g/g H_2O (Fig. 17).

The Young's modulus (E') values reported by Nguyen et. al. at low temperatures, increases between 2 and 10% H_2O and decreases between 10 and 23% H_2O. In contrast, the storage modulus (G') reported by Stefanou et. al. at low temperature, decreases when the relative water content is increased from 0.9 to 10%. The result of Stefanou et. al. is consistent with this study. In this study (Fig. 12), water has little effect on the low-temperature rigidity between 0.03 and 0.06 g/g H_2O; it softens the structure between 0.06 and 0.13 g/g H_2O; and it has a stiffening effect above 0.13 g/g H_2O.

A concurrent investigation of the effect of water content on the dynamic mechanical properties of collagen was reported at the time when the experimental work of this study was in progress. Nomura et. al.[137]

studied the interaction of water with human dura mater (HDM) collagen by using a freely oscillating inverted torsional pendulum at about 1 Hz over the temperature range 80 to 280°K. Native HDM collagen containing 0.75 g/g H_2O, relative to a vacuum dried sample at room temperature, shows three mechanical relaxation maxima below physiological temperature. These occur at 270,200 and 150°K and are designated as the TH_2O, β_1, and β_2 peaks. TH_2O peak is observed only when the water content is above 0.50 g/g and arises from the melting of freezable water. As the water content is increased, the β_2 peak (Δ) increases in intensity and shifts to lower temperatures. The maximum intensity (Δ = 0.09) is observed at about 0.07 g/g H_2O. A plot of peak temperature versus water content indicates that the peak temperature decreases most rapidly between 0 and 0.07 g/g H_2O. It continues to decrease up to 0.25 g/g H_2O with little change in the peak intensity, and above 0.25 g/g H_2O the β_2 peak temperature remains constant at 150°K. The β_1 peak (Δ) which is apparent at water contents above 0.10 g/g shifts to lower temperatures and becomes more intense as the water content increases, reaching a maximum intensity at about 0.45 g/g H_2O. At high water content the peak temperature remains at 200°K while the intensity diminishes. Because of non-uniformity in specimen dimensions, absolute values of G' were not calculated. Instead, the parameter

used for comparison was the relative rigidity defined as $G'rel = (f/f^{o}_{90})^2$ where f is the observed frequency of oscillation and f^{o}_{90} is the frequency for the same specimen in the dry condition measured at 90°K, the relative rigidity goes through first a maximum then a minimum as the water content is increased. From changes in the dynamic mechanical parameters with water content Nomura et. al. have identified four regimes in the hydration of collageneous tissue. The first (0 to 0.07 g/g H_2O) is associated with structural water, the second (0.07 to 0.25 g/g H_2O) with bound water, the third (0.25 to 0.45 g/g H_2O) is a transition region in which both bound and free water are sorbed, and the fourth ($>$ 0.45 g/g H_2O) with free water. They also have suggested that the first water sorbed by collagenous tissue, the structural water, is incorporated into the triple helix, the bound water subsequently imbibed is associated with the polar side chains and is located in the interhelical regions within the collagen fiber, and finally the free or freezable water constitutes, with the mucopolysaccharides, the interfibrillar matrix gel. They have assigned the β_2 process to the rearrangement of water molecules by the breaking and reforming of hydrogen bonds with the macromolecular substrate and the β_1 process to motion of the hydrated side chains of glutamic acid, lysine, and other residues which contain ionic side chains.

The temperature positions of Nomura's β_1 and β_2

peaks in the water content range of this study are plotted together with that of this study in Figure 37. From Figure 37 it is seen that the temperature positions of the β_2 peak reported by Nomura et. al. fit with the data of the β_2 peak in this study perfectly between 0.09 and 0.19 g/g H_2O and are a little higher than that in this study between 0 and 0.09 g/g H_2O. The temperature positions of Nomura's β_1 peak fit with the data for the α peaks in this study very well between 0.10 and 0.18 g/g H_2O and fall a little below those in this study between 0.18 and 0.25 g/g H_2O. The reason that Nomura et. al. did not observe the β_1 peak below 0.10 g/g H_2O nor find an additional peak (β_1 peak in this study) at higher water content is not understood. Obviously, Nomura's β_1 and β_2 peaks are corelated with the α's and β_2 peaks in this study, respectively.

The effect of water content on the temperature positions of low- and high-temperature peaks from both investigations is in complete agreement as shown in Figure 37. The effect of water content on the intensity of the β_2 peak of both investigations is also in agreement. The high-temperature loss processes in both studies first increase then decrease in intensity with increasing water content but the maximum occurs at a different amount in the two studies. The β_1 peak reported by Nomura et. al. starts decreasing in intensity at about 0.50 g/g H_2O and the α peaks in this study at

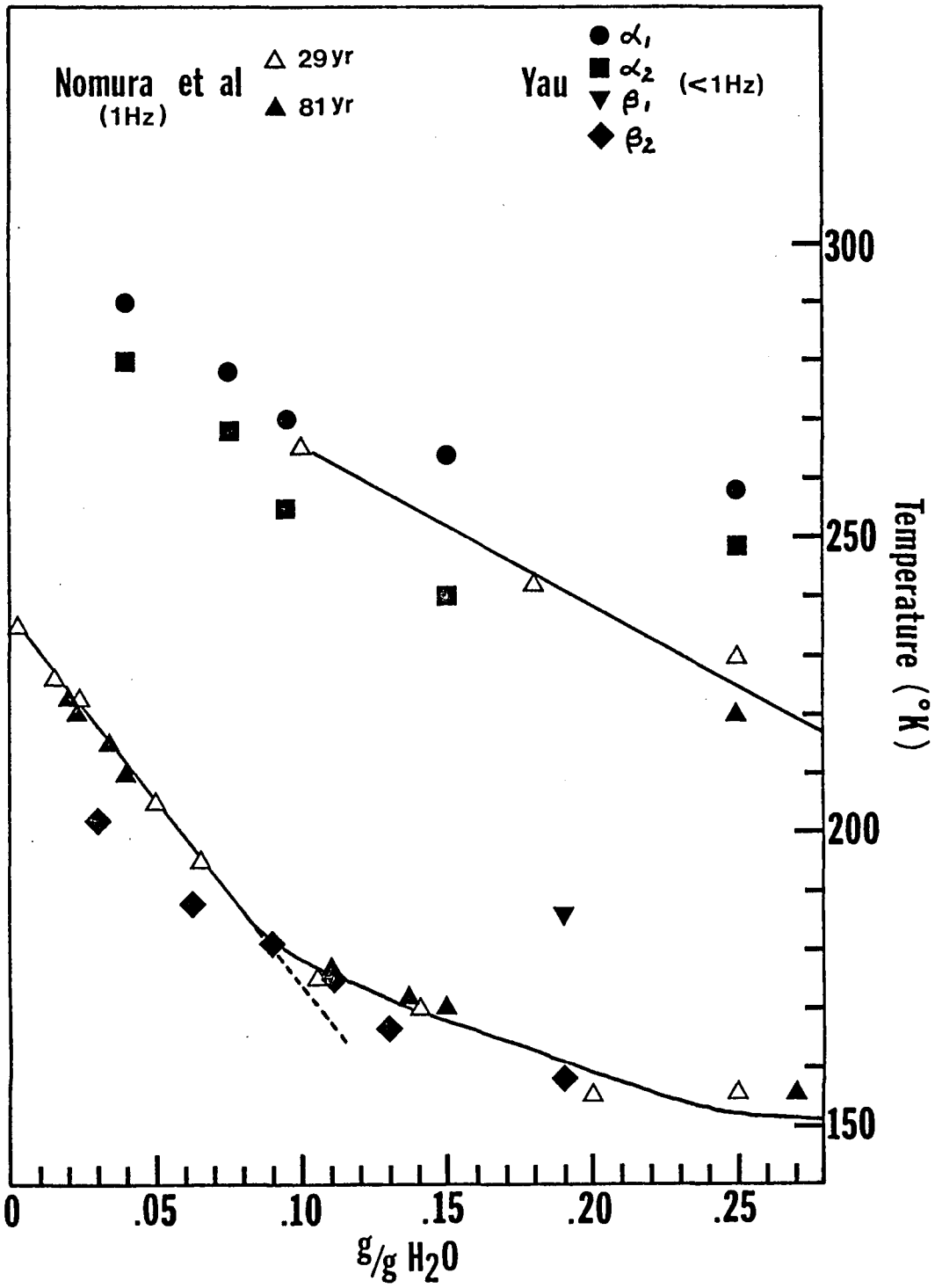


Fig. 37. Temperature positions (△) of the mechanical loss peaks reported by Nomura et al. and this study.

about 0.13 g/g H_2O.

Nomura et. al. observed that the rigidity of the HDM collagen increases with increasing water content between 0 and 0.25 g/g H_2O at low temperatures. In contrast, the rigidity of collagen in this study decreases between 0.06 and 0.13 g/g H_2O and then increases between 0.13 and 0.19 g/g H_2O at low temperatures.

The discrepancies between these two studies may be due to real differences in the samples. Referring back to the introductory section about the ultrastructures of native collagen, the collagen fibrils are surrounded by an extra-fibrillar matrix consisting largely of mucopolysaccharides (Table 2). The sample used in this study does not contain mucopolysaccharides. The presence of mucopolysaccharides may account, at least partially, for the reason that Nomura et. al. are able to get measurements of samples containing such high water contents (e.g. 1.5 g/g H_2O). The highest reliable water content reached in this study is about 0.25 g/g. Although a sample can absorb more than 0.25 g/g H_2O, after about 8 minutes, the minimum time needed to set up a sample and quench it to low temperature for torsion pendulum measurements, the sample loses a significant amount of water.

The presence of two loss peaks in the high temperature region in this study is found to be real and reproducible but was not reported in earlier

work. Close examination of the experimental points in the region of the high-temperature peak in the work of Chien and Chang,[95] of Stefanou et. al.[98] and of Nomura et. al.[139] shows that the data are quite scattered and that the measurements are made in about 5° or larger intervals. In this study, the measurements are made in 2° intervals under conditions in which temperature fluctuation and the loss of water from the sample are minimized. The data in this study show little scatter. Therefore the observation of two loss peaks in this study is attributed to higher resolution and better techniques.

Chien and Chang have assigned the broad high-temperature loss peak (the width at half height is about 70°) to devitrification of water in collagen. Nguyen et. al. assigned this loss process to "melting" and desorption of water. Loss peaks in this temperature region have been reported for samples containing very low water contents, 0.009 and 0.035 g/g by Stefanou et. al. and 0.04 g/g in this study (Fig. 36). Nomura et. al. reported that a water peak (T_{H_2O}) which arises from the melting of freezable water is observed only when the water content is above 0.50 g/g. The water contents of samples studied by Chien and Chang and Nguyen et. al. are about 0.10 and 0.15 g/g, respectively. Therefore, their assignments are not satisfactory. Their mechanisms also can hardly explain the trend of water

effect on the temperature position as well as the presence of two α loss peaks as found in this study.

By a crude comparison with nylon 6, Baer et. al. assigned their β process (high-temperature one) to the reorientation of a polymer-water complex under the applied stress. It is believed by some investigators that the β process of nylon 6 is due to motions of the carbonyl or amide group with attached water molecules. In nylon 6 the β peak is lowered in temperature as water is added. However, Baer's β peak remains constant at 260°K. Therefore, this assignment is not very satisfactory. Stefanou et. al. and Nomura et. al. assigned the high-temperature loss process to motion of the hydrated long-polar side-chain groups of collagen such as glutamic acid and lysine. The evidence from mechanical and NMR relaxation behavior of derivatives and copolymers of some poly-α-amino acids used to support their assignment have been well discussed by these investigators[98,139] and will not be repeated here. However, the dynamic mechanical properties of salts of poly-(L-glutamic acid) and poly(L-lysine) should be pointed out.[139] The sodium salt of poly-(L-glutamic acid) is viscoelastically inactive when dry. In the presence of water, two processes are distinguished: one at about 185°K that does not change in intensity (Δ) but shifts about 15°, from 190° to 175°K, and another that

shifts from above room temperature to about 250°K when water content is increased from 0 to 0.75 g/g. Poly(L-lysine) hydrobromide behaves similarly. A wet specimen shows two peaks at 175° and 240°K, both ten degrees lower than the analogous peaks in sodium poly(L-glutamic acid). The α_1 and α_2 peaks in this study behave similarly to those found for salts of poly(L-glutamic acid) and poly(L-lysine), respectively. These two peaks also behave as a pair and have a temperature difference of about ten degrees at most water contents (Fig. 36). Therefore, the α_1 and α_2 mechanical loss processes are assigned to large-scale motion of the hydrated side-chain groups of glutamic acid and lysine of collagen respectively. However, the possibility of contribution to the α processes from other polar side chain groups of collagen such as arginine and aspartic acid should also be considered. Since the relaxation behavior of poly(arginine) and poly(aspartic acid) have not been reported, a direct comparison between this study and these polyamino acid is not possible. The side chain of aspartic acid is less flexible than that of glutamic acid since the former is one carbon shorter than the latter. The amount of aspartic acid in collagen is less than that of glutamic acid. Therefore, the possible contribution to one of the α processes from the aspartic acid group would not be expected to be as important as that of glutamic acid. On the other

hand, the side chain of arginine is as flexible as that of lysine and the amount of arginine in collagen is higher than that of lysine. Therefore the possibility of a sizeable contribution to one of the α processes from the arginine side chain should not be ignored.

Chien and Chang, Nguyen et. al. and Nomura et. al. have assigned the low-temperature mechanical loss process to the rearrangement of water molecules by the breaking and reforming of hydrogen bonds with the macromolecular substrate. Nomura et. al. argue that this low-temperature loss process is associated with the structural water incorporated in the triple helix of the tropocollagen molecule since the intensity of the peak, which depends on the concentration of relaxing units, reaches a maximum at 0.07 g/g H_2O and remains constant at higher water contents. Since the high-temperature loss peak is not apparent until the water content is above 0.10 g/g, they believe the molecules above the first 0.07 g/g (termed bound water) are located in the interhelical regions within the collagen fiber. However, this assignment for the low-temperature peak does not explain why the temperature of the low-temperature loss peak decreases continuously up to 0.25 g/g water. The observation of Nomura et. al. concerning the high-temperature loss peak is not supported by the results of Stefanou et. al. nor by this study, since this loss peak has been observed at water

content much lower than 0.10 g/g. Therefore, their argument is not fully satisfactory and the mechanism is not well understood. Since both the high- and low-temperature processes are observed at low water contents (Fig. 36) and their temperature behavior versus water content is similar (Fig. 7 & 15), these two processes may involve the same polar side chains but in different modes of motion. A tentative assignment to the low-temperature loss process is small-scale local motion of parts of the long-polar side-chain groups of collagen. Water may reduce polar side-chain-side-chain interactions. Therefore the temperature position of this loss peak shifts to lower temperatures as the water content is increased. This explanation is based on the behavior of Δ with water content. The behavior of the loss modulus (G") with change in water content is not fully understood as yet. However the possibility of some contribution from the oscillations in or of the proline and hydroxyproline groups about the main-chain of collagen to the broad low-temperature process should not be neglected since Stefanou et. al. have observed that poly-L-proline II shows a broad loss process in this temperature region.

An assignment for the β_1 process remains to be made. Chien and Chang are the only previous investigators reporting a similar loss process. They observed an additional mechanical loss peak for RTT and AKM 23

(synthetic membranes produced from intact collagens) at about 220°K (12% H_2O, 110 Hz). No such mechanical loss peak was observed for enzyme (proctase) solubilized collagen (ESC) whose telopeptide segment had been cleaved.[95] Accurate dielectric relaxation measurements[140] show that acid solubilized collagen (ASC) has a loss peak at 228°K and ESC does not. They attribute the 220°K transition to the telopeptides. The water content of the samples measured was not indicated in their report. In this study, the β_1 peak is only apparent at higher water content (0.19 g/g). If this process is associated with the telopeptides, water has to be involved. Another explanation is possible. Fung et. al.[78-80] postulated that water molecules in the direct neighborhood of bound water and collagen form a hydration layer which cannot be frozen down to 210°K. Hoeve and Lue[92] proposed a model involving a water chain which can diffuse as an entity along the channel between collagen molecules. Movement of these chains ceases at the glass point, approximately 173°K. Therefore another possible assignment for the β_1 process is the transition of water from the glassy state to the liquid state.

From this study the following interaction of water with collagen is proposed. The first water, up to (0.13 ± 0.01) g/g sorbed by collagen is associated with the polar side chains and is located in the interhelical regions of collagen. Water can

reduce polar side-chain-side-chain interactions, acting as a plasticizer, which makes the α_1, α_2 and β_2 loss peaks shift to lower temperatures rapidly (Fig. 7 and 8). It also causes the storage modulus at low temperatures to drop (Fig. 12). Since water molecules directly participate in the α_1 and α_2 relaxation processes, the intensity of these two peaks increases with increasing water content. After the polar side chains are saturated with water, additional water (>0.13 g/g H_2O) would be aggregated in the channels between collagen molecules as well as in the telopeptide regions. Water acting as a filler would cause an increase in the storage modulus at low temperatures. Telopeptides associated with water presumably give the β_1 relaxation process.

Assuming the above discussion about collagen is correct, the effect of presence of lactase on the dynamic mechanical properties of collagen can be discussed. The presence of lactase in collagen does not change the number of loss processes. Therefore it is concluded that the four loss-processes observed for the collagen-lactase system (C-L) all are associated with the collagen part of the system. However, the presence of lactase does affect some characteristics of the dynamic mechanical properties of collagen. From Figure 7 and 8, it is seen that the α_1 and α_2 loss peaks of C-L (0 to 2 months) do not appear until the relative water content reaches about 0.10 g/g. Also the temperature positions of the α_1 and α_2 peaks of C-L are higher than that of collagen. After aging

of the C-L the behavior of the α_1 loss peak becomes similar to that of collagen. These facts indicate that the effect of lactase on the behavior of α_1 and α_2 loss processes of collagen changes with storage time of C-L. One possible reason for this is the change of the nature of the binding of lactase to collagen upon aging. From Figure 8, it is seen that the behavior of the α_2 peak of aged C-L is not similar to that of collagen. Figure 22 shows that the α_2 peak of aged C-L has a lower intensity (G") than that of C-L. This is also seen in Figure 10 when the intensity is expressed in terms of Δ. These facts suggest that both the nature and the number of the molecular units causing this loss process are changed after a long storage time. From Figure 15 and 27, it is seen that the β_1 peak of collagen shifts to higher temperatures when the lactase is present. This fact suggests that the presence of lactase on collagen increases the activation energy for the β_1 relaxation process.

In order to discuss these features mentioned above in any detail, a model of the collagen-lactase system must be proposed. The lactase molecule (approximately 120 A^o X 70 A^o) is too big to sit in the channels between collagen molecules (approximately 15 A^o X 15 A^o). It is also difficult for it to reside in the gap regions (approximately 400 A^o X 15 A^o). As mentioned in the experimental section, voids are observed in the sample under the electron microscope. Lactase molecules are probably trapped in these

voids. The collagen molecules in the sample film are randomly oriented. Therefore the lactase molecule is surrounded by helical regions as will as by the non-helical regions of many collagen molecules. The lactase molecule contains many polar and ionic amino acid residues (see Table 8) which are the sites for water binding and protein-protein interaction. For a freshly prepared C-L sample, the lactase molecules are trapped in the voids with most sites available for water binding. The first 0.10 g/g H_2O may be all absorbed by the lactase molecules instead of going into the channels between the collagen molecules. Since the α_1 and α_2 relaxation processes of collagen require water molecules, at water contents below 0.10 g/g no loss peaks would be expected as observed. Since the polar and ionic sites of lactase molecules can form non-covalent linkages, such as hydrogen bonding and salt linkages with the glutamic acid and lysine residues on surrounding collagen molecules, the activation energy for the motion of the side-chain groups of glutamic acid and lysine of collagen is expected to be higher. It is seen that the temperature positions of the α_1 and α_2 peaks of C-L are higher than that of collagen (Fig. 7 and 8). Upon aging the formation of covalent bonds between lactase and collagen molecules have presumably occurred. One possible covalent bond is that due to the lysine- (or hydroxylysine-) derived aldehyde residues present on collagen reacting with the ε-amino group of lysine present on lactase to

form an aldimine cross-link (see Fig. 2). Giacin et. al.[118] have reported that modification of collagen films by a natural cross-linking process associated with aging markedly reduced the lactase-binding capacity of collagen films (see the Introduction section). This may be due to the fact that the majority of lysine- (or hydroxylysine-) derived aldehydes in the telopeptides of collagen have been consumed by intermolecular crosslinking with the adjacent collagen molecules during the aging process and fewer aldehydes are available for covalent bond formation between collagen and lactase. However, the formation of covalent bonds between lactase and the helical region of the collagen molecules is also possible. The chemistry of cross-links[49] applied to two adjacent collagen molecules can also be applied between lactase and collagen. If this argument is correct, the intensity of the α_2 peak of C-L should be expected to decrease after a long storage time since the α_2 process of collagen has been assigned at least partially to the large-scale motion of lysine side-chains. Since most polar and ionic sites of lactase are presumably involved in the formation of covalent bonds after extensive aging, less water would be absorbed by the lactase. Therefore water would likely enter the collagen fibrils and microfibrils and lead to the presence of the α_1 and α_2 peaks as observed at water contents as low as 0.04 g/g for aged C-L. Since lysine residues in both the non-helical and helical regions of the collagen molecules are presumably

involved in the formation of covalent bonds, a dissimilar behavior of the α_2 loss peak for aged C-L is not surprising. Because of formation of covalent bonds in C-L a smaller number of polar side chains of collagen are available for water binding. Therefore the specific water content required to saturate the polar side chains is reduced to 0.09 g/g. Since it is believed that lactase forms protein-protein interactions with telopeptides at the edge of collagen fibrils, the presence of lactase in collagen should increase the activation energy for the motion of telopeptides associated with water. Therefore the presence of lactase makes the β_1 peak shift to a higher temperatures (Fig. 15 and 21).

V. Conclusions

The study of dynamic mechanical properties of collagen film from cattle hide revealed four loss processes occurring in the temperature region of 120-300°K at 1 Hz. The same was found to be true for a collagen-lactase system containing 25 % lactase.

The α_1 and α_2 processes in collagen, occurring at approximately 290 and 280°K respectively, depend strongly on water content, shifting to lower temperatures and increasing in magnitude as the water content is increased to 0.13 g/g (relative to vacuum drying at room temperature). By analogy with synthetic polypeptides containing long side chains, the α_1 and α_2 processes are assigned principally to large-scale side chain motion of glutamic acid, lysine and arginine residues in collagen.

The β_1 process in collagen at 185°K is observed only at high water content (0.19 g/g H_2O). This process is attributed to the motion of telopeptides associated with water.

The β_2 process at 195°K shifts to lower temperatures as water content is increased. This process is assigned principally to the small-scale local motion of polar side chains in collagen.

The storage modulus of collagen at low temperatures first decreases then increases with increasing water content, having a minimum at 0.13 g/g H_2O.

The relaxation behavior of these processes in

collagen does not change monotonically with water content. A specific water content, (0.13 ± 0.01) g/g is associated with change in the behavior. The following interactions of water with collagen is proposed. Water, up to 0.13 g/g, is associated with the polar side chains in collagen. Above 0.13 g/g, water is aggregated in the channels between collagen molecules as well as in the telopeptide regions.

The presence of lactase on collagen does not change the number of relaxation processes, but affects the characteristics of the dynamic mechanical properties of collagen. A model for the collagen-lactase system is proposed to interpret the experimental results. Lactase molecules are trapped in the voids in the collagen film. In freshly prepared samples, lactase forms non-covalent bonds with collagen molecules. After long storage time, the formation of covalent bonds occurs.

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