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THE EFFECTS OF GAMMA IRRADIATION ON
PATELLAR TENDON ALLOGRAFTS

presented by

Patrick De Deyne

has been accepted towards fulfillment
of the requirements for

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Roger C. Haut
Major professor

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THE EFFECTS OF GAMMA IRRADIATION ON PATELLAR TENDON ALLOGRAFTS

By

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ABSTRACT

THE EFFECTS OF GAMMA IRRADIATION ON PATELLAR TENDON ALLOGRAFTS

By

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The purpose of this study was to investigate the cause of the altered mechanical properties of patellar tendon allografts due to the sterilization with gamma rays used in tissue banks. A Hydrothermal Isometric Tension device was developed, solubility assays with acetic acid and pepsin were used and electron microscopy was performed. A significant lowering of the shrinkage temperature, unchanged acetic acid fraction, an decreased pepsin released fraction and a decrease in contrast in the banding pattern showed to support the hypothesis that minimal additional cross-linking from gamma irradiation occurs and that the main effect of irradiation is chain scission, possibly at the telopeptide ends of the collagen molecule.

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I. Introduction

Replacement of the anterior cruciate ligament after injury is a relatively common procedure. However, the materials used to replace the anterior cruciate ligament are still a topic of debate. Because no synthetic materials have been able to meet the demands placed on them as ligament substitutes (1). Only autogenous tissues (patellar tendon, semitendinosus tendon, fascia) are widely accepted as suitable substitutes for the anterior cruciate ligament. A potential problem with autogenous tissues, however , is that periarticular tissues must be removed from the knee to function as graft. This loss of tissue may compromise other structures of the knee. Thus the concept of using allografts to replace the injured anterior cruciate ligament is of major clinical significance (2). Current concepts and research pertinent to allograft surgery such as biocompatibility, biomechanical properties, biological fate, procurements and preservation techniques are to be addressed (3).

One of the techniques used by tissue banks that provide tendon allografts is preservation by freeze-drying and/or sterilization using gamma irradiation.

It is of primary importance, in order to guarantee high quality allografts, that the currently used preservation and sterilization procedures are investigated in order to detect any detrimental effects on the strength or physical integrity of the graft. The aim of this thesis is to study

the effect of gamma irradiation on patellar tendon allografts by means of a hydrothermal isometric tension procedure (H.I.T.)

II. Literature survey.

A. Irradiation and collagen.

In the sixties some interest was centered on the possibility of preserving raw meats by subjecting them to a sterilizing dose of ionizing radiation, thereby inhibiting spoilage due to the growth of micro organisms. The process was accompanied by side effects resulting from the interaction of the radiation with the various components of the tissue. One of them was that meat became more tender and investigations were focused on the connective tissue, more specifically collagen.

Various authors have investigated the effects of gamma irradiation on collagen. Person et al (4) used X-ray diffraction patterns to study the phenomenon, and observed a shrinkage of the wet irradiated, not the dry irradiated collagen. J. Cassel (5) noted a decrease in shrinkage temperature and increased water solubility of the collagen. He also concluded the decrease in shrinkage temperature was not influenced by moisture content of tendon during irradiation (5 to 220 Mrad), and that the treatments caused peptide chain scission. Kuntz et al (6) believed that crosslinking was the main effect when the tissue was irradiated wet, and an excess of breaks occurred in the peptide chains when irradiated dry. Bailey et al (7) used the hydrothermal shrinkage temperature to assess irradiation effects, and found a decrease in shrinkage temperature progressively with dose. He discussed the direct effect of

the gamma rays on the collagen molecule. Bowes et al (8) elaborated on the differences between irradiation in the dry state and the wet state of the samples. He contributed the fall in shrinkage temperature and increased solubility to an extensive loss of molecular structure. This was most obvious with dry irradiated samples. The presence of water determines the effects of irradiation on collagen, Bailey et al (9) and (10) discussed the decrease in solubility with increased tensile strength, and contributed that to the formation of new crosslinks.

In the seventies more and more became known about the nature of intramolecular crosslinks and the amino acid sequence of collagen. For a good overview see (11,12) and earlier work (13).

A better understanding of the biology of the collagen molecule gave an impetus to the research to determine the effects of gamma rays on the collagen structure. Grant et al (14) found results consistent with the hypothesis that electron irradiation of collagen in the dry state results in scission of the polypeptide chains and that, in presence of water, this is accompanied by the formation of intermolecular bonds. Their methods consisted of electronmicroscopy, differential enzyme digestion (elastase, collagenase) and the use of glutaraldehyde. Van Caneghem et al (15) deduced that those effects were irreversible and oxygen dependent.

It was still unknown if radiation increased the inter- or intramolecular crosslinks. Earlier work by Dancewicz (16) was inconclusive. J.J.M. Labaut (17) proposed that gamma irradiation in the wet state induced the formation of aldehyde groups, which are of primary importance in the production of intermolecular cross-links. Svojtkova et al (18) studied the effects of gamma irradiation and induced cross-linking in combination with high fat diets and increasing age. M.M. Jelenska et al (19,20,21) studied the effect of gamma irradiation at the level of the tropo collagen molecule and concluded that there was an increase in aldol cross-links and formation of new reducible components of an unknown structure.

The experiments discussed above are in vitro conditions. Data are available on in vivo effects of irradiation on collagen (22,23) and report some changes in its neutral salt solubility (22) are reported to be dose dependent. Pickrell et al (24) notices radiation induced changes in pulmonary collagen metabolism. Ohuchi (27) and De Loecker (28) also reported changes in collagen metabolism. Svojtkova et al (25) and Grant (26) have similar conclusions regarding the effect of gamma irradiation on collagen: depending on the experimental conditions (moisture content) ionizing radiation may cause polymerization or depolymerization of collagen. Irradiated in a wet state the formation of new cross-links is the predominant process, over a wide dose range (50 krad up to 5-10 Mrad),(5-10, 14-

18). The nature of the induced cross-links (intra- or inter molecular) is unknown. There is also a shrinkage of the tissue which is dose dependent (10,14). Irradiated in a dry state depolymerization by peptide chain scission is more predominant (10,14,15). It is also interesting to note that Bailey (7) reported that lowering the temperature below the freezing point during irradiation provides a protection to chain scission.

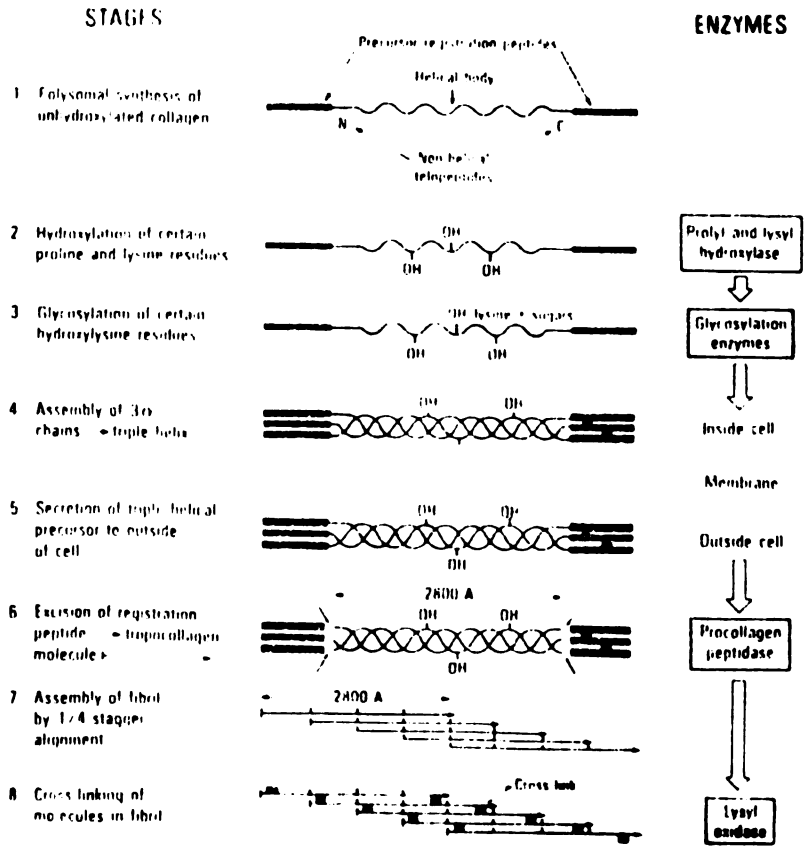
B.Collagen and cross-links.

Cross-linking renders the collagen fibers stable and provides them with an adequate degree of tensile strength and visco-elasticity to perform there structural role. In terms of its mechanical properties other parameters are equally important, such as length and structure of the fiber (29); metabolic interactions between its cells and the extracellular matrix (30,31,32); maturation and development (33) and mechanical testing conditions (34). Viewing the interaction between gamma irradiation and cross-links (see above) and thus mechanical strength (35), I will briefly describe the current state of knowledge of cross-links in collagen. For a general and historical overview of collagen research see Mayne and Burgeson (36).

Structural collagen obtains its stability from its unique molecular coil configuration, the quarter-staggered packing of tropocollagen units (Fig.1), and its ability to form covalent intra-molecular and inter-molecular cross-

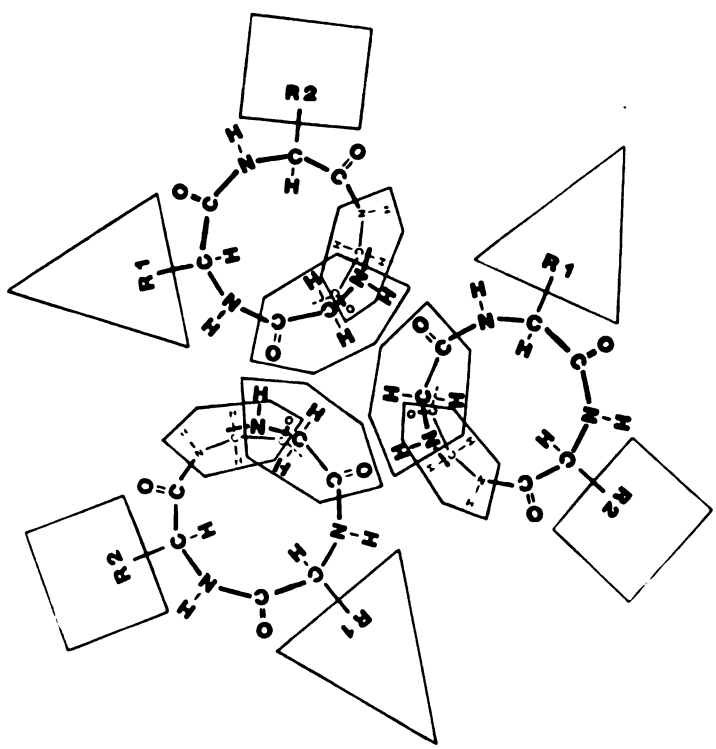
links (37,38). The former links occur between alpha chains of the same tropocollagen molecule, the latter between adjacent tropocollagen molecules (Fig.2). Cross-links are key to both tensile strength characteristics and resistance to chemical or enzymatic breakdown. Their absence causes the collagen fiber to be extremely weak and friable.

Figure 1. Enzymatic stages in maturation of collagen. From M.Nimni (12).



The enzyme of vital importance in the creation of cross-links is lysyl oxidase (38,39). The crosslinks result from enzyme-mediated reactions involving mainly lysine and hydroxylysine. The lysine and hydroxylysine molecules have secondary amine groups on terminal projections extending lateral from alpha chain, which are available for cross-linking reaction.

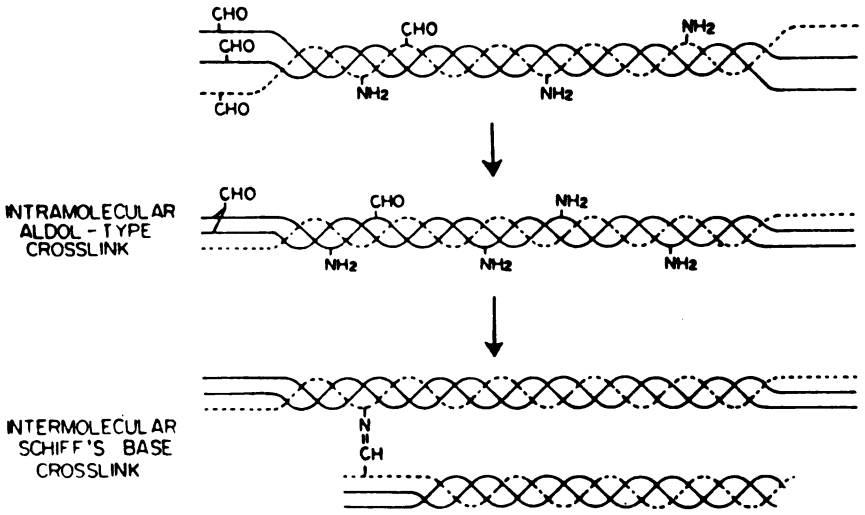
Figure.2. Representation of an end view of the peptide backbone of the collagen helix.(From: Connective tissue disease, molecular pathology of the extracellular matrix, edited by J. Uitto, A.J. Perejda, Marcel dekker, Inc. 1988).



Collagen triple helix

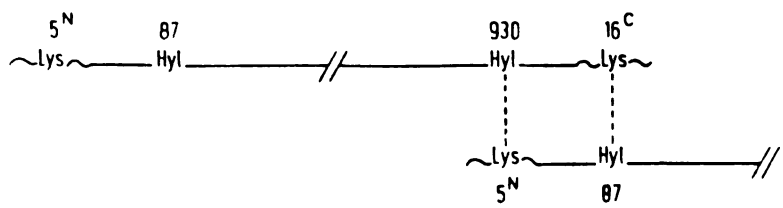
The initial reaction in cross-linking is the oxidative deamination of the terminal to an aldehyde by the enzyme lysyl oxidase. The resulting allysyl residues condense with one another to form an aldol condensation product, characteristic of intramolecular cross-links. Intermolecular cross-links characteristically form in the reaction of allysine with lysine or hydroxylysine to form a Schiff-base (see Fig.3).

Figure.3. formation of intra- and intermolecular cross-links.



For basic mechanisms and pathways see Yameneuchi (40). One must note that the hydroxylation necessary to stabilize cross-links is an intracellular process, while the actual cross-links are formed extracellulary (Fig.1). Collagen intermolecular cross-linking reactions are controlled by the stereospecificity of interacting neighboring molecules (angular orientation and staggered configuration). An important adjunct to the determination of the specific molecular loci of the cross-links is the primary sequence of collagen. In type I collagen, which has 1014 residues in the α 1 (I) chain, cross-linking sites are characterized by low proline and hydroxyproline content and the presence of the sequence Hyl-Gly-His-Arg, which appears to be important as an attachment site for the enzyme lysyl oxidase. Cross-links between residue 5N and the helical residue 930 and between 16C and the helical residue 87 of the α 1 (I) chain have been described (40,41) (Fig.4).

Figure 4. Schematic representation of cross-link sites.



The enzyme mediated reaction mainly involves lysine and hydroxylysine and the Schiff base reaction products are:

-H L N L (hydroxylysinonorleucine)

-D H L N L (dihydroxylysinonorleucine)

-and the more complex H H M D (histidino hydroxy merodesmosine).

Most studies on cross-linking of collagen in the past decade have used tritiated $NaBH_4$ as a marker to the peptides which contain reducible cross-links. For details see Bailey (42,43).

The field of collagen cross-linking has been significantly advanced during the last decade It is that knowledge that will elucidate the geometry of packing and organization of collagen molecules in various tissues.

C.Heat and collagen.

The denaturation of connective tissue, especially collagen, by heat has been employed to assess the structural qualities of its constituents. Native collagen is denatured by heat. The protein loses its highly ordered crystalline structure to a preferred random coil state (59) with higher entropy. A variety of methodologies have been used on different tissues. I will give a brief overview.

Intramuscular connective tissue was tested by Mohr et al (44). Other tissues that were assessed by a form of thermal denaturation were: Achilles tendon by Spadaro et al

(45), Finch et al (46), Jong Jin Lim (47); wrist tendons by Mitchell et al (48); extensor digitorum communis tendons by Goldin et al (49); other tendons by Snowden et al (50) and Mady Le Lous (51). Rat tail tendons, because of their almost exclusive content of collagen, have been a frequent substrate for testing used by: Viidik (52), Ward et al (53), Magy et al (54), Burjanadze (55), Andreassen et al (56), Yue et al (57,58), Andreassen et al (59), and Mitchell et al (48).Thermal stability of skin was assessed by Russell (60), Allain et al (61), Danielsen (62), Gekko et al (63) and Danielsen (64). A variety of other tissues have been under investigation with hydrothermal procedures. Basal membrane and type IV collagen was investigated by Gelman et al (65) and Sank et al (66). Cartilage has been studied by Snowden et al (50,67); scar tissue by Snowden et al (68), type X collagen by Linsenmayer et al (69), and tropocollagen by Berg et al (70).

Besides a diversity of tissues tested there are several heating methodologies, especially with regard to the determination of the initial shrinkage. Two general methods have been employed most often:

-A temperature increase of an isometric tissue and measuring the tissue response as force of contraction, Mohr (44), Finch (46), Mitchell (48), Allain (61), Goldin (49),Andreassen (56) and Le Lous (51).

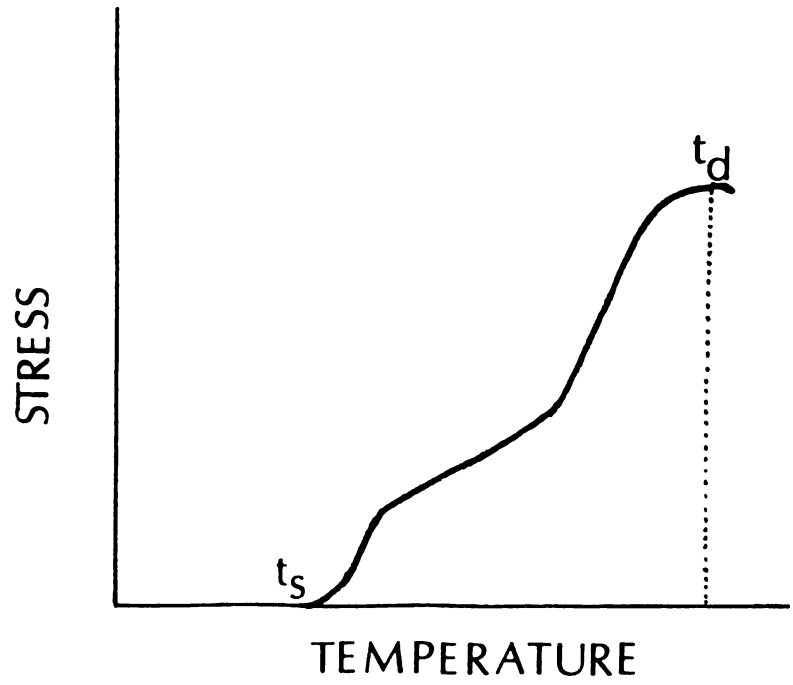
-a constant temperature and observing with specific instrumentation when the changes turbidity of the

macromolecule solution, occur on a time basis. Spadaro (45), Russell (60), Maggy (54).

For our purpose, the study of patellar tendon, the first approach was chosen, this for practical and experimental design reasons (control of heating rate, sensitivity of load cell, the possibility of studying the rate of shrinkage).

The main effect of heat on collagen is a shrinkage and gradual development of tension in an isometrically held tissue. This effect has been applied in surgical therapeutic procedures (71). A generic curve for tension vs. temperature was proposed by Mitchell (48) (Fig.5).

Figure 5. Diagram illustrating the main features of the stress temperature curve, when a tendon is melted under isometric conditions.



The main characteristics are:

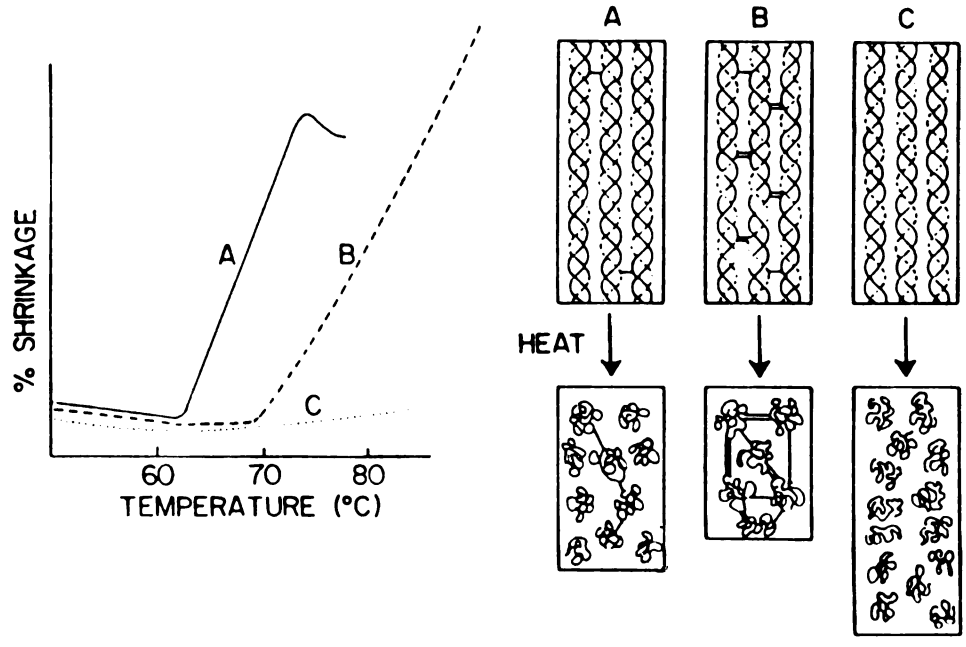
-Ts= shrinkage temperature: temperature at which the tissue starts to shrink.

-The slope of the curve.

These two parameters are characteristic for each tissue, and give an idea of the crystalline organization and stability of the tissue's constituents.

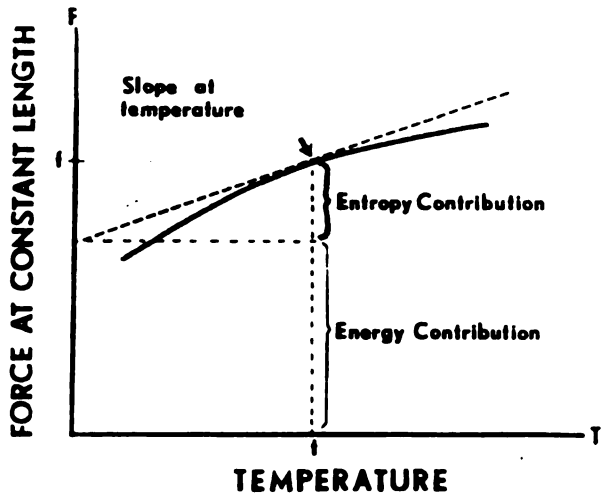
Due to its reproducibility and high sensitivity, hydrothermal procedures have been used to assess the effect of various chemicals on collagen (45,46,60,63);and the effects of induced diabetes on collagen (56,57,58,59). The above mentioned parameters have been correlated with the amount of hydroxyproline incorporated in the collagen molecule (47,53,55,62,70) and the influence of cleavage enzymes (64) on shrinkage temperature has been assessed. The most extensive study (more than 1000 samples) was performed by Le Lous (51).

Figure.6. Thermal shrinkage of collagen and the role of the intermolecular cross-links in providing for the contractile force (as proposed by M.Nimni (39) p.43).



Nimni (12) discusses the effect of heat on collagen and concludes that denaturation by heat can produce a collapse of the helical structure and a shrinkage in the direction of the longitudinal axis of the fiber (fig.6). The isometric tensions developed during thermal shrinkage are used to evaluate the physical properties of the collagen networks. This may quantify the degree of cross-linking of collagen that occur with aging (48,54,57). The authors do not specify the type of cross-links. One could distinguish between the stabilization of the collagen by hydrogen bonds (49,53,55,70), thermo labile vs thermo stabile bonds (51) or reducible cross-links (HLNL, DHLNL, HHMD) (50,56,61). Another perspective is brought by Danielsen (64) who focuses more on structural continuation of the polymeric aspect of collagen and its influence on shrinkage temperature. Karlinski (72) mentions Treloar's model for rubber elasticity (Fig.7) to quantify the change in energy content and increase in entropy with heat.

Figure.7 Force-temperature plot for a pure rubber elastomer.



We can conclude that a hydrothermal isometric tension procedure based on the design by Goldin (49) can be a sensitive, reliable, reproducible method to assess the structural stability of collagen, and in our case any potential damages or changes that occur with gamma irradiation of graft tendons. The value of shrinkage temperature, the slope of tension development with a linear increase in temperature and how these relate to a description of the effects of gamma irradiation on collagen will be treated in the discussion.

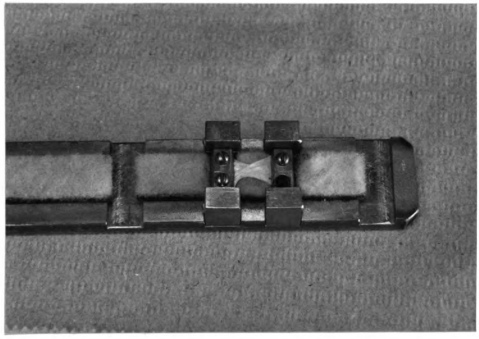
III. Materials and methods.

Testing procedure.

Three patellar tendons, from young males A, B, C, respectively 26, 23, 28 years old, were donated by the Michigan Tissue Bank. They were cut medio-sagitally. One half was fresh frozen and served as a control, the other was frozen at - 70°C and irradiated (2.0 MRad from a cobalt source). The irradiation was performed by the Michigan Tissue Bank using their standard irradiation procedure.The tendons were shipped and stored (maximum 6 weeks) at - 20°C until further experimentation.

Using a dissecting scope small patellar tendons strips were cut out from the medial, central part of the half tendon. Caution was given to dissect, at room temperature, longitudinal intact fibers to have a continuity of fibrils. After dissection the strips of tendon were frozen at -20°C until testing. After being thawed in physiological solution, the samples were mounted in the clamps on a frame to standardize for length (12mm). See picture 1.

Picture 1. Test specimen mounted in clamps, length of the sample is 12 mm.



The samples were placed in the testing device (picture 2) and a custom constructed pyrex cylinder (diameter: 7 cm, length: 20 cm) was slid up so that the sample was completely submerged in a 0.9% saline solution. A pre-tension, applied by a thumb screw mechanism, of 0.16 N (monitored on a digital multi meter) was given prior to the test. 750 ml of 0.9% saline solution was heated from 25^{o}C to 95^{o}C by a 209 W, 1.25 cm wide resistive heating tape (coated with silicone rubber to increase the thermal contact area and to protect from possible electrical shorts from solution spillage). A

variable A.C. transformer was used to drive the heating element and the cylinder was surrounded with fiberglass insulation to improve the linearity of the heating curve. The heating rate was 1.5^{o}C/min.Figure 8 shows the relationship between time and temperature increase.

Figure 8a. Heating rate in the H.I.T. experiment.

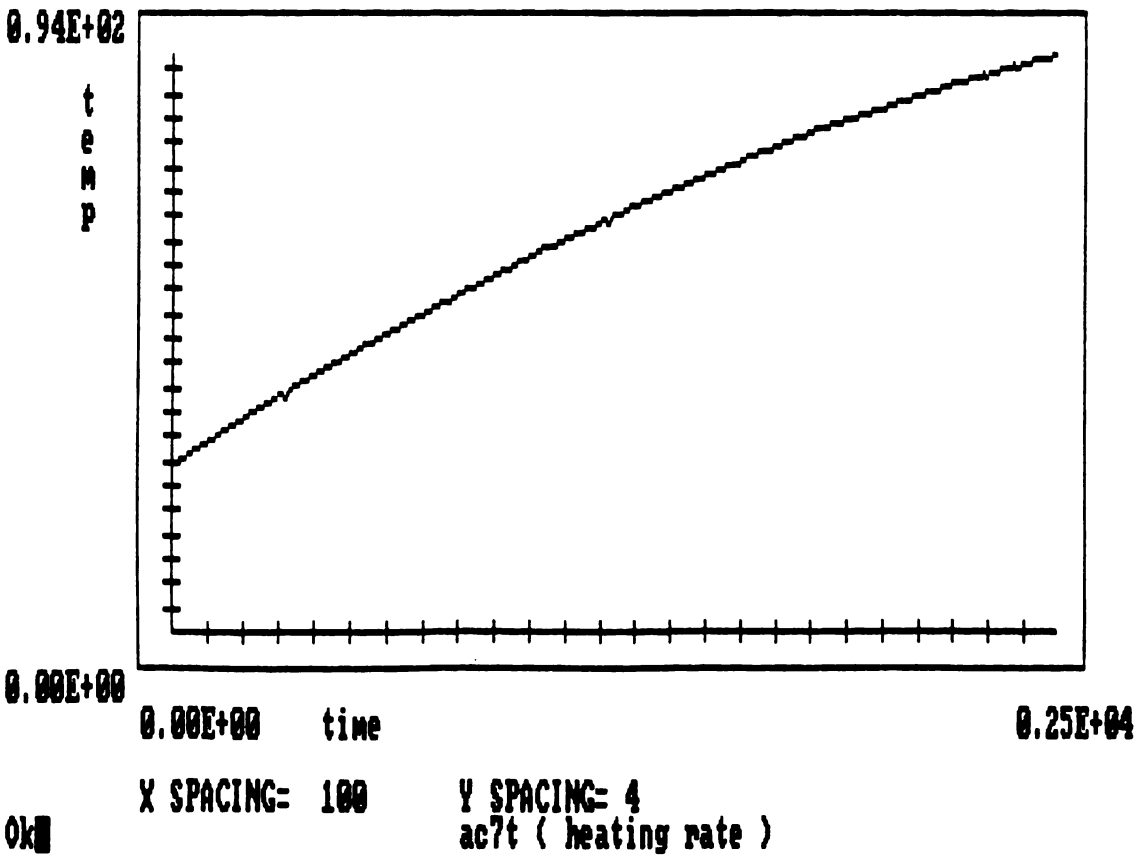
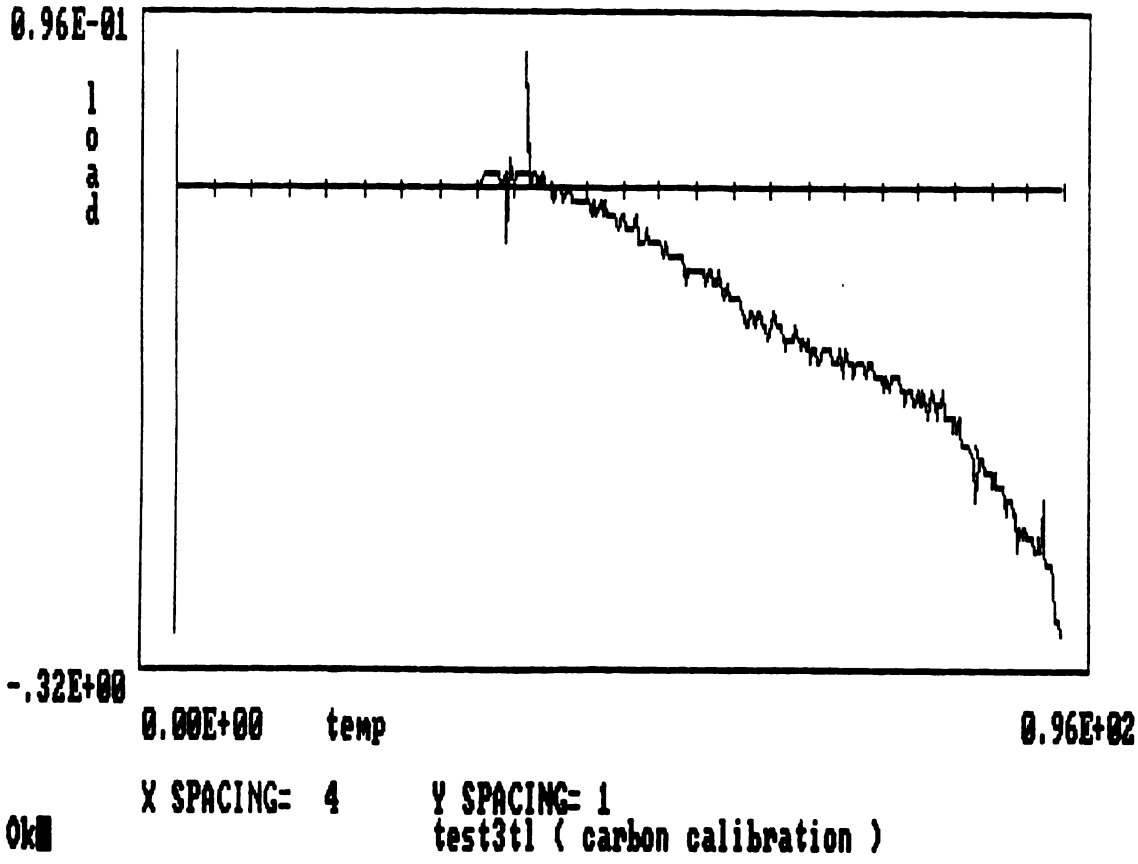


Figure 8b. H.I.T. calibration



The H.I.T. device was calibrated. This was important because of the not fully temperature compensated load cell. The H.I.T. data beyond 80^{o}C were not used in this study due to the decrease in linearity of the heating rate (Fig.8a.) and the sharp increase in negative readings from the load cell (Fig.8b.). One could upgrade the H.I.T. device by using a servo system for controling the heating rate and a temperature compensated load cell (and mounting it away from the heating source) to have valuable data beyond 80^{o}C.

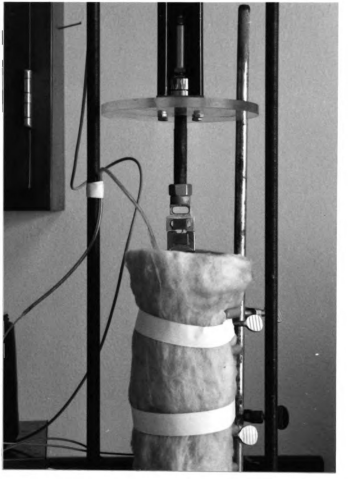
Picture 2. Hydrothermal Isometric Tension (H.I.T.) Test device.

The temperature data were acquired using a type T copper / constantan thermocouple placed in the center of the saline bath. The reference electrode was placed in a Dewar flask filled with ice mush. The signal from the thermocouple was amplified with a Sensotec (model SA-4) strain gauge amplifier. The voltage was then input into an A/D board (METRABYTE model dash 16).

The load cell (Gould-Statham, model UC 3) gathered the tension data. The load cell was not fully temperature compensated. Heat transfer to the load cell was minimized with a plexiglass shield for air current and with a 9 cm

long carbon composite fiber rod (connecting the load cell with the top clamp holding the sample as can be seen on picture 3). The output of the load cell was amplified using a Sensotec (model SA-4) strain gauge amplifier. The signals were then converted to digital data by a Metrabyte (model dash 16) A/D board. The load cell was calibrated using 1.0 N and 2.0 N loads weighed on a laboratory beam balance scale.

Picture 3. Plexiglass shield, carbon composite rod to minimize heat transfer.

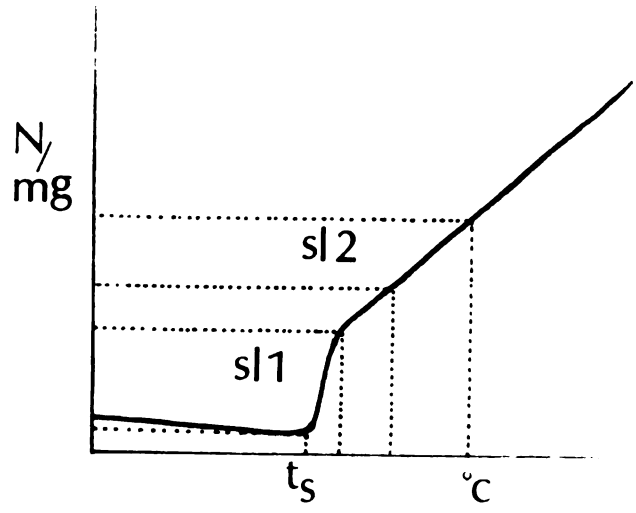


The loads generated in the experiments could reach maximum values of 6.1 N but most were between 3 and 5 N. As it was difficult to determine cross-sectional area for the samples, the load data were normalized per dry weight. Goldin et al (49) shows a good correlation between cross-sectional area and dry weight. In my case where the length of the sample remains constant being tested we can use the same model.

Both signals were monitored during testing with digital multi meters. The data were collected with a custom made computer program at a sampling rate of 0.1 H_z for 2500 sec. After testing the tendon specimen was carefully removed, cut from in between the grips, placed in a test tube and lyophilized. Dry weight of the samples was obtained by using a Mettler (H8) laboratory balance.

Because the load cell was not fully temperature compensated and the decreasing heating rate beyond 80^{o}C (Fig.8a,8b), the secondary slope (SL 2) was calculated from the data available between 70 and 80 degrees C. The slopes (SL 1 and SL 2) were manually calculated. Figure 9 shows the parameters used for this study.

Figure 9.



Ts: The shrinkage temperature was obtained by the projection of the intersection between the horizontal through the deepest point of the curve and the vertical through the point when initial rise occurred.

SL 1: The increase in tension in the segment between Ts and Ts+3°C; the initial slope

SL 2: The secondary slope, the increase in tension between the temperature interval from 70°C - 80°C.

The temperature , slope 1, slope 2 data of the subgroups (A control n=9; B control n=10; C control n=10; A irr.n=10; B irr.n=9; C irr.n=10) were tested with a two - tailed ANOVA test to ascertain if they were from the same population. As the data had a normal distribution (tested for skewness and curtosis), the standard deviations were homogeneous and the subgroup A,B,C were from the same population a two tailed Student t test was used for statistical analysis. There was a control group of 29 test samples and an irradiated group of 29 samples.

Additional tests.

To have a better understanding of the mechanisms involved in gamma irradiation of collagen, solubility studies and electron microscopy were performed.

Solubility data were based on hydroxyproline extraction with 0.5 M acetic acid (referred to as acid soluble fraction) and pepsin digestion (referred to as pepsin released fraction); a method used by Schnider et al (73). Hydroxyproline content was measured by the method of Stegeman (74) and described also by Woessner (75).

1. Tendon samples were pulverized at liquid N_2 temperature and freeze dried for 24 hours.
2. Three aliquots for solubility and two for totals from approximately 30 mg were accurately weighed and lipid removal was accomplished by extracting the sample with 10 volumes of chloroform- methanol (2:1,

v:v). Samples were agitated overnight , spun 1 hour at 2000 g, and supernatant was discarded. A total of three extractions were performed. All samples were lyophilized.

3. Acid soluble fraction was obtained using 0.5 M acetic acid, (for 24 hours at 4^{o}C with agitation then centrifuge 1.5 hour at 2000 g, pool supernatant; a total of three extractions).
4. The pellet was then suspended in 0.5 M acetic acid and digested with pepsin (P-7012, SIGMA, St.Louis, Mo) at a concentration of 1.0 mg/ml for 18 hours with stirring at 4^{o}C. NaCl in Tris HCl buffer (pH 7.4) was added to the viscous digestion mixture, after the incubation was completed, to a salt concentration of 0.17 M. Only one digestion was carried out. Collagen solubilized after pepsin was collected by centrifuging the mixture at 5000 g for 3 hours.
5. The collagen remaining after the acid and pepsin treatment is considered insoluble. Pepsin released fraction is dialyzed for 12 hours against 0.1 M acetic acid.
6. All the samples (pooled supernatants, totals, insoluble fraction) were frozen in dry ice, lyophilized;hydrolyzed with 2 ml of 6 M HCl at 105^{o}C for 16-18 hours; and neutralized with 2.5 M NaOH (indicator was 0.02% methyl red).

7. In a timed manner 1 ml of neutralized sample was added in marked test tube. Then add 1 ml of chloramine-T solution (mix and let stand at room temperature for 20 min.) Add 1 ml of $HClO_4$ solution, mix and let stand at room temperature for 5 min. Finally, add 1 ml of p-DAB solution, mix and let stand at 60°C in a water bath for 15-20 min. Cool tubes to 20°C and read in spectrophotometer at 550 nm. Hydroxyproline content is determined by using a standard curve (from stock hydroxyproline)

Reagents used in assay are:

- 0.03 M chloramine -T (C 9887, SIGMA) solution. Dissolve 0.845 chloramine -T in mixture A. Mixture A= 20 ml H20, 30 ml propanol, 50 ml citrate-acetate buffer at pH 6.
- 3 M perchloric acid (25.5 ml 70% $HClO_4$ H_2O to 100 ml)
- 5 % solution of p- dimethylaminobenzaldehyde(p-DAB), (KODAK Co.) in propanol, dissolve under heating, store covered.
- standards: 0-5 ug Hyp/ml.

Besides acid-soluble, pepsin released fraction, and insoluble fraction, total hydroxyproline content was also determined.

Electron microscopy protocol was based on the procedure by Lendis (76) and the following steps were performed.

1. When excised the samples were placed in cold (4°C)

4% glutaraldehyde in 0.1 M phosphate buffer with pH 7.4. No additives were used.

2. Longitudinal samples of 1 mm were put for the second time in fresh fix for 1.5 hours at $4^{o}C$.
3. The samples were washed 3x in cold 0.1 M phosphate buffer for 20 minutes each wash.
4. Post fixation in 0.1% OsO_4 in the phosphate buffer for 1.5 hours at room temperature.
5. The samples were washed 2x in buffer for 15 minutes, each wash at room temperature.
6. The samples were dehydrated at room temperature in graded ethanol

   25% - 30 minutes
   50% - 30 minutes
   75% - 30 minutes
   100% - 30 minutes gently agitate.
   100% - 30 minutes " "
   100% - 30 minutes " "

7. Samples were embedded into Spur epoxy (Polysciences, Inc. Warrington, Pa.)with graded steps at room temperature.

1:3	resin:ethanol	8 hours	gently agitate
1:1	resin:ethanol	8 hours	" "
100%	resin	8 hours	" "

8. Embedded samples were oriented for longitudinal sectioning. Molds were placed in desiccator 2 hours

before curing at 70°C overnight (000 gelatin capsules).

9. Silver section on 200 mesh Cu grids.
10. Stain with 3% uranyl acetate in ethanol solution for 45 minutes and 3 minutes in Reynolds Pb.

IV Results and discussion.

Collagen shrinks when exposed to heat as a result of the breakdown of the ordered crystalline structure of the collagen to a preferred random coil state (59). In determining the parameters used in this study care must be given to the heating rate. Mitchell and Rigby (48) demonstrated that the shape of the tension curve and the shrinkage temperature are dependent upon the rate of heating during the isometric melting experiment. They conclude that in general, 1) an increase in the heating rate increases the stresses and the shrinkage temperature (Ts), 2) the changes in the heating rate become less marked with in vitro aging.

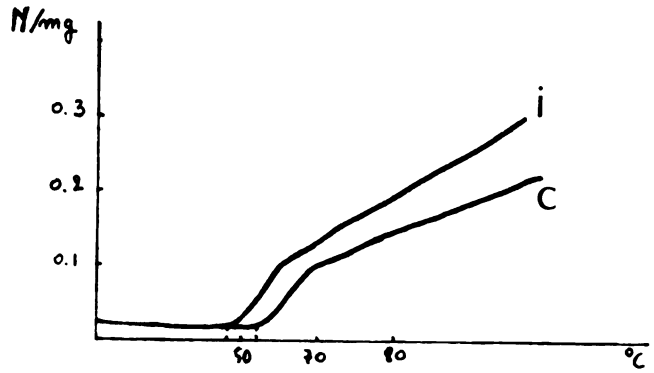
The data collected from the HIT experiment are shown in Table 1. For the control tissue (n=29) the average shrinkage temperature was 62.4^{o}C (S.D. 0.885), the initial slope (SL 1) had a value of 2.806 E-2 N/mg.^{o}C (S.D. 8.67 E-3) and the secondary slope (SL 2) had a value of 1.105 E-2 N/mg.^{o}C (S.D. 3.24 E-3). The irradiated tendon samples (n=29) had a shrinkage temperature of 58·4^{o}C (S.D. 0.96) and an initial slope of 3.038 N/mg.^{o}C (S.D. 6.68 E-3). The secondary slope had a value of 1.644 E-2 N/mg.^{o}C (S.D. 2.81 E-3). The average dry weight was 7.06 mg (S.D. 2.66) for the control tissues and 6.48 mg (S.D. 2.25) for the irradiated samples.

Table 1. Shrinkage temperature, slope 1, slope 2, from HIT experiment.

	CONTROL	IRRADIATED	t-test
Ts (^{o}C)			
$\bar{X}$	62.4	58.4	p< 0.005
S.D.	0.885	0.96	
SL 1 (N/mg.^{o}C)			
$\bar{X}$	2.806 E-2	3.308 E-2	n.s.
S.D.	8.67 E-3	6.68 E-3	
SL 2 (N/mg.^{o}C)			
$\bar{X}$	1.105 E-2	1.644 E-2	p< 0.20
S.D.	3.24 E-3	2.81 E-3	

Figure 10 shows a general view of the H.I.T. curves. One can observe that the irradiated curve is shifted to the left while the secondary slope is steeper, although there was no statistically significant difference from controls.

Figure 10. H.I.T. curves for control (C) tissue and irradiated (I) tissue.



All the samples showed an initial relaxation up to the shrinkage temperature. I did not try to quantify that observation. Rigby et al (77) also reported a thermal expansion prior to the sudden development in tension at the shrinkage temperature. At the shrinkage temperature a rapid increase in tension occurs (SL 1); in the range from Ts to Ts + 3°C. After Ts +3°C the tension levelled off (SL 2) and developed steadily to the end of the test (80°C). In some samples (3) mechanical failure occurred between 90 and 95 degrees C.

The development of the slopes and the shape of the curves are consistent with the data found by Mitchell et al (48), Le Lous et al (51), Goldin et al (49) and Allain et al (61). Andreassen et al (56) uses a different experimental protocol but also reports the particularity of the slopes.

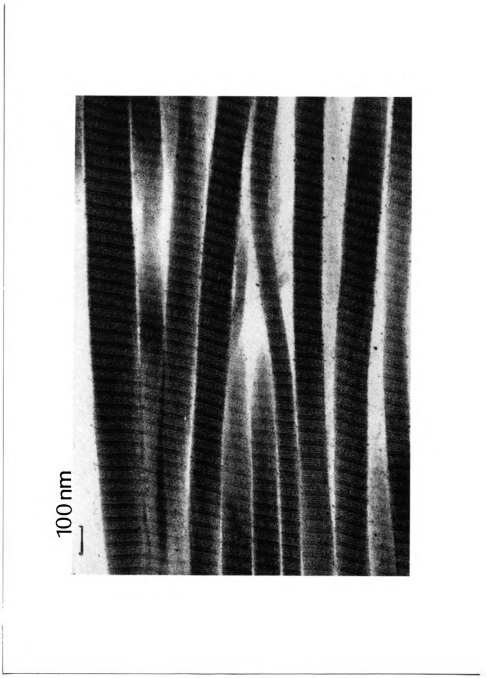
Solubility changes have been observed with increased aging (80) and the increased insolubility has been correlated with increased tensile strength (80,81) and increased cross-linking (9,10). The current solubility studies were performed in addition to the H.I.T. experiment to have a better understanding of the effects of gamma irradiation. The results are shown in Table 2. They show a major increase in insoluble fraction with a concomittant decrease in pepsin released fraction. The total hydroxyproline content for control and irradiated tissue was almost identical (Table 2). As mentioned earlier hydroxyproline content alters the shrinkage temperature (47,53,55,62,70). There is also no change in acid solubility. The main difference is found in the pepsin solubility. It is to be noted that in the experiments some of the pepsin released fraction was lost, most likely in a dialysis step in the assay, and that the actual numbers should be higher. This does not affect the amount of insoluble fraction.

Table 2. Solubility of irradiated and control tendons (in ug Hyp/mg).

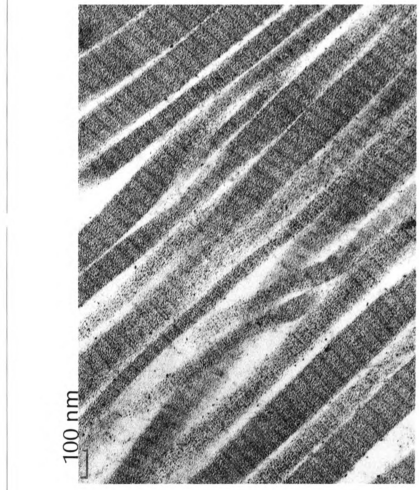
		Control	Irradiated	t-test
Totals				
	$\bar{X}$	84.0	96.0	
	S.D.	13.3	19.8	n.s.
	n	6	6	
Insoluble fraction				
	$\bar{X}$	11.0	76.3	$p < 0.001$
	S.D.	4.82	11.3	
	n	9	7	
Pepsin released fraction				
	$\bar{X}$	37.4	7.86	$p < 0.001$
	S.D.	7.0	1.86	
	n	7	7	
Acid soluble fraction				
	$\bar{X}$	0.343	0.26	n.s.
	S.D.	0.37	0.24	
	n	7	5	

The electronmicroscopy (pictures 4,5) did not show any structural changes in the irradiated collagen, nor was there a change in banding pattern; but a decrease in contrast of the cross-bands was noticable (picture 5). Bailey (9) reports change in structural features with an irradiation dose higher than 10 Mrads.

Picture 4. Collagen from normal patellar tendon (70,000 X).



Picture 5. Irradiated (2 M rad.) collagen from patellar tendon (70,000 X). No change in banding pattern, but some decrease in contrast of the cross-bands.



Discussion

Although the population was small one can observe from Table 2 the obvious changes in solubility. As mentioned earlier a decrease in acid soluble fraction has been correlated with increased cross-linking (9,10), although until now the nature of the cross-links susceptible to acid solubilization has not been determined. Also the shape of the H.I.T. curve (SL 2) has been correlated with cross-linking (48,56,61).

Thermal agitation disrupts these chemical bonds producing fiber shortening due to folding of the collagen chains and a rise in isometric thermic denaturation tension. Isometric denaturation behavior beyond the shrinkage temperature is a reaction to disruption of high energy covalent bonds (49), which progressively form during fibrogenesis. Mitchell et al (48) found an increase in thermal stability (steeper slopes) with aging; they infer two possibilities: 1) an increase in cross-linking density with age or 2) an increase in cross-links that are thermally stable. Allain et al (61) determined the type of cross-link that is responsible for the tension development. The experiments show that with aging there is an increase in the number of HLNL and HHMD cross-links while the number of DHLNL cross-links decrease. They explain the type of slope of the H.I.T. curves in terms of the type of intermolecular cross-link; and contribute the tension development beyond shrinkage temperature (SL 2) to thermally stable DHLNL cross-links and a yet unknown

aldimine cross-link. Although the HHMD cross-link is the major cross-link in the native collagen fiber, Allain et al states that it is thermally labile. Goldin et al (49) draws similar conclusions.

In view of assessing the effects of irradiation on patellar tendon allografts the H.I.T. experiment will assess the amount of induced cross-link formation. As discussed, earlier authors (7,10,26) have reported an increase in cross-linking and an increase in aldehyde content (20) with irradiation, due to the high probability that slope 2 from controls and irradiated samples are from the same population we may infer that there is minimal, induced cross-linking. Although one could report that all the values of slope 2 were consistently higher than the control values for slope 2, but not significantly. Also the major increase in pepsin insolubilty (Table 2) can be explained in function of induced cross-linking from gamma irradiation. But one can ask if a sevenfold increase in pepsin insolubilty should not be more obvious in the H.I.T. curve (SL 2) if it was only caused by new cross-link formation from gamma irradiation.

The unchanged acid soluble fraction also points to the same conclusion that minimal induced cross-linking occurs. The development of tension in an H.I.T. experiment has only been discussed as a function of the quantity and quality of the cross-links (48,56,61), but this does not take into account a gradual straightening of the collagen fibrils and

removal of the waveform (29), nor the slip or glide between the components (fibrils) (12) as a reaction to the tension.

The initial slope, slope 1, which is observed immediately in the interval Ts, Ts+3^{o}C, is not considered by Mitchell et al (48) as being involved in co-valent cross-links. They attribute it to Van Der Waals, hydrogen and electrostatic bonding and hydrophobic bonding (66). One has to note, as recently reported (78) that the α helices that form the tropo collagen molecule are stabilized intra-cellularly by disulfide and hydrogen bonds. The cross-linking process itself is an extra cellular event induced by the enzyme lysyloxidase and the presence of O_2. The hydroxylisation of lysine and proline is important for the stabilization of the triple helix through hydrogen bonding. It is reported (53,55,65,66) that an increase in hydroxyproline content increases the melting temperature (Fig.5).

The significant decrease in shrinkage temperature of the irradiated tissue during the test, relates to some change in structural integrity of the collagen molecule. Danielsen (64) determined the shrinkage temperatures of collagenase treated rat skin and states that the shrinkage temperature of the uncleaved molecule and the N-terminal fragment are independent of the extent of N-terminal cross-linking. He inferred that intra molecular cross-linking had no effect on the shrinkage temperature (62). His data suggest that the shrinkage temperature is a measure of

structural integrity; the shrinkage temperature of the fragments (TCA, TCB) were found to be 4-5^{o}C lower than the uncleaved molecule. If we follow the same analogy this model could explain the decrease in shrinkage temperature as a result of chain scission.

The loss in mechanical strength of irradiated patellar tendon allografts that is reported by Haut et al (35) and Gibbons et al (79) must be due to changes of the factors that determine the tensile strength. The tensile strength of the collagen is due to its unique molecular conformation (33) which is bestowed upon it by the regular repeating units in amino acid sequence, the highly specific alignment and packing of the molecules in the fibrils, and the axial and lateral cohension afforded by the formation of intermolecular covalent cross-links. The changes in mechanical properties could be a result of chain scission. Although formation of new cross-links occurs simultaneously with chain scission (9,10,14) from irradiation, One can presume that chain scission could be predominant with the current sterilization procedures. The unaltered acid solubility and the virtually identical slopes (SL 2) of the H.I.T. data would infer no induced cross-linking, as an effect of gamma irradiation at 2 Mrads; but the rise in pepsin insolubility and the "trend" ($p < 0.20$) that SL 2 is higher for the irradiated tendons leaves to believe that induced cross-linking may not be ruled out. This would infer that the H.I.T. slopes give a global picture of structural

stability rather than only information about the cross-links.

As reported by Danielsen (62,64) the shrinkage temperature can be interpreted as a parameter for structural integrity, independently from covalent cross-linking. The significant decrease (4°C) in shrinkage temperature in irradiated samples could be explained as result of predominant chain scission. The loss of structural integrity or continuity can explain the reported (35,79) mechanical changes. One can refer hereby to the decreased contrast in the electron microscopy preparations (pict.5); while the banding pattern remained unchanged.

Pepsin can only attack collagen when the latter is in a denatured state (random coil); pepsin digestion of collagen will act on the telopeptide ends (first on the NH_2 terminal, the COOH terminal is more slowly digested) of the molecule (82). Pepsin cleaves specifically the peptide bonds indicated (*) in the following aminoacid sequences: Gly-Tyr*-Asp-Glu, Ala-Gly*-Val-Ala. Collagen becomes resistant to pepsin digestion with age and increased cross-linking hinders pepsin digestion reported by Weiss (82).The decreased pepsin released fraction (table 2) can be explained as a result of a decrease in available sites where pepsine can act as proteolytic enzyme. That is consistent with the reported (9,10,14) chain scission from gamma irradiation. This would mean that the chain scission occurs

at the telopeptide end of the collagen molecule, and that it would hinder the proteolitic activity of pepsin.

I cannot conclude that cross-link sites are not affected; as shown on fig.4 the cross-links are between the telopeptide end and the helical region of the adjacent molecule. The loss in mechanical properties can be explained as discontinuity in the polypeptide chain. The cross-links would not be connected in series with the adjacent collagen molecule.

There is reason to believe that the currently used method to preserve and sterilize tendon allografts by gamma irradiation has chain scission for effect. The decreased mechanical qualities should be seen in the light of disruption of the collagen "polymer" at its peptide bonds and more specifically at the telopeptide end of the molecule. With the current procedure there is minimal concomitant cross-linking (which is dependent on the moisture content). Interesting to know is that Bailey et al (7) reports some protective effects against collagen damage by irradiating at lower temperatures (-200^{o}C).This could be directly applicable for tissue banks which are concerned with maintaining the quality of prospective grafts.

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