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BIOMECHANICAL PROPERTIES OF THE CANINE PATELLAR TENDON AS A FUNCTION OF AGE AND DIABETES

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Major professor

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BIOMECHANICAL PROPERTIES OF THE CANINE PATELLAR TENDON AS A FUNCTION OF AGE AND DIABETES

By

Ronald Lynn Lancaster

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ABSTRACT

BIOMECHANICAL PROPERTIES OF THE CANINE PATELLAR TENDON AS A FUNCTION OF AGE AND DIABETES

By

Ronald Lynn Lancaster

Use of the canine patellar tendon in orthopaedic and pathological research continues to grow. The canine model is often used to examine human knee ligament reconstruction techniques. In the present research, age related changes in the biomechanical, biochemical, biophysical, and morphological properties of the canine patellar tendon were investigated. Alterations in these changes caused by naturally occurring diabetes mellitus were also determined. While collagen from control tendon had decreased solubility to pepsin with age, its mechanical properties remained unchanged. Diabetes caused a decreasing trend in strength despite a more rapid decrease in solubility compared to age-matched controls. Thermal stability decreased with age yet increased with diabetes. Further work is needed to better understand both the mechanisms of aging in this connective tissue and alterations caused by diabetes mellitus.

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A	-	Cross-sectional area
ACL	-	Anterior cruciate ligament
ATP	-	Adenosine triphosphate
CHO	-	Carbohydrate
E	-	Tensile modulus
GBM	-	Glomerular basement membrane (kidneys)
HCl	-	Hydrochloric acid
$HClO_4$	-	Perchloric acid
HIT	-	Hydrothermal isometric tension
k	-	Stiffness
L	-	Failure elongation
Lo	-	Initial length
M	-	Molar concentration
NIH	-	National Institute of Health
NaCl	-	Sodium chloride
P	-	Failure load
PT	-	Patellar tendon
PPT	-	Patella-patellar tendon-tibia
S	-	Strain
s.d.	-	Standard deviation
Se	-	Failure strain energy
Sed	-	Failure strain energy density
Sl1	-	Primary slope of HIT curve
Sl2	-	Secondary slope of HIT curve
TEM	-	Transmission electron microscopy
TS	-	Tensile strength
Ts	-	Thermal shrinkage temperature
pH	-	Acid level $pH = -\log[H^+]$
PVC	-	Polyvinyl chloride
(y)	-	Years

I. INTRODUCTION

Use of the canine model in orthopaedic and pathological investigations continues to grow. Orthopaedic studies include evaluation of autogeneic and allogeneic repair procedures in reconstructive joint surgery (7,11,28). The canine patellar tendon (PT) is often used in these investigations because of widespread parallel tissue use in human knee ligament repair. Jones (23) first introduced the use of PT as a graft in 1963. Variations on the amount of graft and location from which it is excised have emerged since that time (2). The canine model in pathology related research is less prevalent (9,35) and yet offers a unique source of genetically transferred disease states common to man.

Changes in the biomechanical, biochemical, and biophysical properties of the canine patellar tendon with both age and diabetes mellitus (often called an age accelerator) is not well documented. In this study, a thorough review of previous investigations into the above changes during aging in connective tissues of several species has been conducted. Additional review of known alterations in these properties with naturally occurring and experimentally induced diabetes mellitus has also been

performed. Concurrently biomechanical, biochemical, hydrothermal isometric tension (HIT), and morphological examinations were conducted on control (non-diseased) and diabetic canine patellar tendons. Variations in measured properties with age and diabetes are documented. This information may help lead the scientific community to a better understanding of both the mechanisms of normal aging and alterations caused by the crippling and sometimes lethal disease, diabetes mellitus.

II. SURVEY OF LITERATURE

CONNECTIVE TISSUE ORIGIN

As a germ cell develops into an embryo it differentiates into three layers. The mesenchyme (middle layer) is the origin of connective tissue. Cells of mesenchymal origin produce an intercellular matrix which contains four major classes of macromolecules: collagen, elastin, proteoglycans, and glycoproteins (lamina and fibronectin) (39).

Tendon primarily consists of the protein collagen (85%) with a small amount of proteoglycans and glycoproteins. These components, in combination, are responsible for the physical properties of tendon. The effects of proteoglycans and glycoproteins on the mechanical function of connective tissues are important but are beyond the scope of this investigation. Because tendon is largely collagen, a basic understanding of the development and alterations of collagen with age is required.

COLLAGEN SYNTHESIS AND GROWTH

Tendon is primarily extracellular tissue with a small number of cells called fibroblasts. These cells produce collagen proteins and the other extracellular components

mentioned previously. The synthesis of collagen is schematically illustrated in Figure 1.

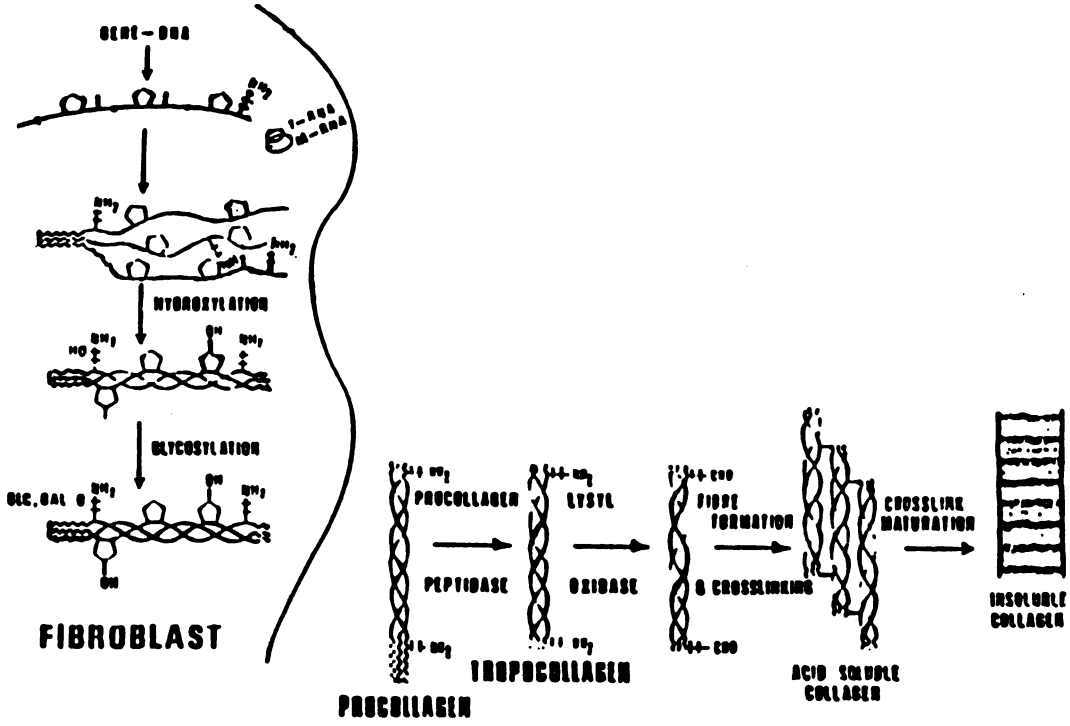


Figure 1. Collagen synthesis (taken from Bailey, 6).

Polypeptide chain synthesis occurs in the fibroblast cell beginning with the DNA (genes). Three linear peptide chains [$\{\alpha 1(I)\}_2\alpha 2(I)$] (type I collagen) combine inside the cell through intramolecular cross-linking and form a triple helix. Hydroxylation (adding of an OH molecule) of proline and lysine residues occurs, along with the enzymatically controlled (prolylhydroxylase and lysylhydroxylase) addition of carbohydrates to specific hydroxylysine residues. Glycosylation of the hydroxylysine molecules then takes place by the addition of galactosyl and glucosylgalactosyl residues (carbohydrates). This process occurs through an

oxygen-glycosidic linkage which is enzymatically catalyzed by collagen galactosyl transferase and collagen glucosyl transferase (39). The procollagen chain is then extruded from the cell by reverse pinocytosis. At this point, a small portion of the chain ends is cleaved by peptidase through hydrolysis leaving a tropocollagen molecule.

The first stage in the formation of cross-links between tropocollagen helices is an oxidative deamination (removal of an amino group - NH_2) of specific lysine or hydroxylysine residues, located at the nonhelical ends (nitrogen- and carbon- terminals), by a copper-dependent enyzyme, lysyloxidase. The resulting aldehydes (monovalent group with CHO) are believed to condense with ε-amino groups of hydroxylysine which are situated in the helical regions of neighboring molecules, to form a network of intermolecular bonds which are assumed to be of the aldimine type. This results in a highly ordered quarter-staggered arrangement of tropocollagen molecules which combine to form fibrils, which later form collagen fibers (6).

Figure 2 illustrates a second form of cross linking which is believed to occur in long-lived proteins throughout the body. These cross-links are formed without the aide of enzymes and begin by the attachment of a carbohydrate molecule to an amino group on a protein to form a Schiff base. The Schiff base formation is unstable and quickly but reversibly rearranges to a more stable Amadori product. At

least twenty such Amadori products have been identified in human beings (8).

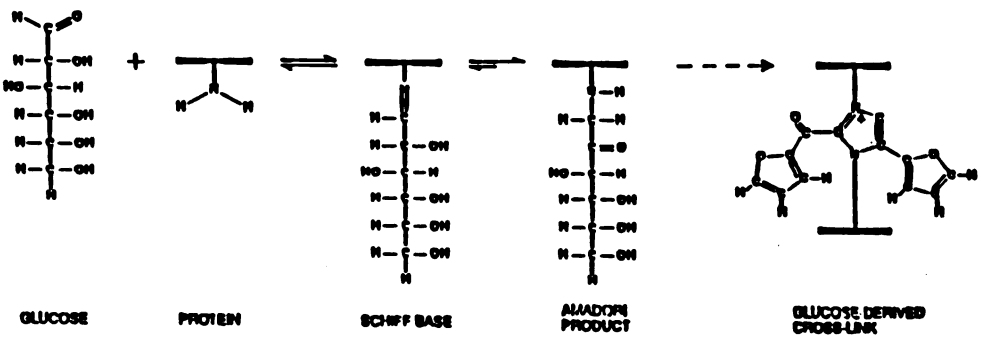


Figure 2. Formation of glucose derived structures (taken from Cerami, et al., 8).

After a long duration (months to years) the Amadori products may slowly dehydrate and rearrange to form a glucose derived structure of several possible types. These structures may combine with certain molecules to form advance glycosylation end products (AGE's). AGE's may have the ability to cross-link adjacent proteins. This form of non-enzymatic cross-linking is called glucosylation (Figure 3).

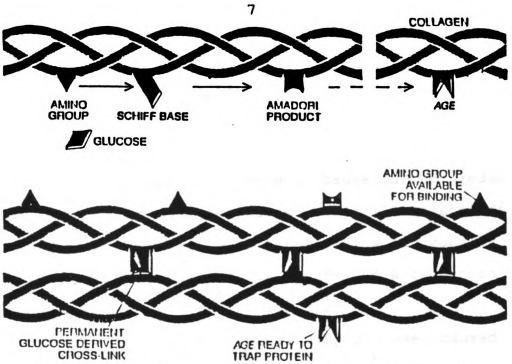


Figure 3. Glucose induced cross-linking (taken from Cerami et al. 8).

Collagen is reported to have a half-life of 2.5 years (41). Degradation of collagen is catalyzed by a collagenase specific to the type of collagen. Phagocytosis also helps to remove degrading collagen. Released hydroxyproline (a major constituent of collagen) is excreted as di- or tripeptides or as free hydroxyproline which is usually resorbed (39).

METABOLIC CHANGES WITH AGE

It is well documented that tolerance to carbohydrates decreases with age (12). Endogenous insulin actually increases during senescence while its effectiveness decreases through a reduction in cellular membrane receptor sites. People over 85 years of age have approximately 3.5 times higher insulin levels two hours after glucose loading

than people 30 years of age. (12). This indicates a decreasing tolerance to insulin by the cells with age. A negative feedback loop is generated first by an age caused reduction of insulin receptors on the cell membrane, followed by a rise in the level of insulin in the blood. Over time, the hyperinsulism will cause further reduction in the number of insulin receptors. This illustrates decreased carbohydrate tolerance with normal aging is probably caused by decreased effectiveness as opposed to a reduction in the amount or production of insulin (12).

The high level of extracellular glucose, caused by the decreased effectiveness of insulin, may cause non-enzymatic cross-linking of collagen and other proteins in the body during normal aging. This leads to pathologies commonly seen in diabetic patients such as stiffened joints, cataracts, etc. Because of this, some authors believe that practically all people over 70 years of age develop diabetic-like features (12).

BIOMECHANICAL CHANGES WITH AGE

Once collagen is arranged and cross-linked it begins to support loads or stresses. It appears that the nature of the loading determines the type of connective tissue formed. How much stress a tissue can support is documented in two ways. Structural properties reflect the strength of the bulk of tissue and include: failure load (P), failure deformation (L), stiffness (k), and failure strain energy

(Se). Material properties are structural properties normalized for geometry and more directly reflect the strength of the specific tissue type and include: tensile strength (TS), failure strain (S), tensile modulus (E), and failure strain energy density (Sed).

How connective tissue responds to loads and stresses is the subject of several investigations. The tensile strength, modulus, and strain energy density to tensile failure of the human anterior cruciate ligament (ACL) significantly decreases with age (15). Hubbard and Little (22), on the other hand, mechanically tested palmaris longus and the extensor hallucis longus tendons from human cadavers and found no statistically significant changes in the tensile strength and modulus with age. The former data compares to that recently reported by Haut, et al., (20), who saw minimal change in the tensile modulus of a limited number of split human patellar tendons between the ages of 18 and 63 years.

The degree of age-related change in the mechanical properties of connective tissues may be tissue specific. Vasseur, et al., (42) studied the extent of tissue degeneration and corresponding changes in the mechanical properties of canine stifle (hind limb knee) joint ligaments with age. The greatest amount of degeneration occurs in the cranial cruciate ligament (ACL), while less is found in the caudel cruciate and collateral ligaments. Only the cranial cruciate was mechanically tested and has a decreasing

modulus, tensile strength, and failure strain energy density with age. Woo, et al., (47) investigated the effects of maturation on the rabbit medial collateral ligament and documents significant increases in the failure load and energy-absorbing capacity of the femur-ligament-tibia complex to maturation. The tensile strength and modulus of the ligament substance increase during maturation, while the rates of these changes decrease with age.

Many studies of age on the mechanical properties of connective tissue have been conducted using the rat model. It is generally agreed that the tensile strength of rat tail tendon increases to twelve months of age (19,40,46). Vogel (46) notes a decrease in tensile strength after 12 months of age, while Haut (19) and Torp (40) find the tensile modulus of rat tail tendon increases throughout life. Galeski (15) documents an increase in the modulus of elasticity with age. Vogel again reports a maximum modulus at twelve months followed by a gradual decline with age.

BIOCHEMICAL CHANGES WITH AGE

Traditional biochemical investigations of collagen includes three separate assays. The first involves the immersion of a specimen in a weak acid solution to determine the most recently synthesized collagen. This is usually followed by digesting the specimen with the enzyme pepsin to quantify the concentration of more mature collagen with newly formed cross-links. The remaining specimen is termed

the amounts of collagen soluble in acid, pepsin, and the insoluble fraction should equal the total collagen concentration (for exact biochemical procedures, refer to section III).

Vogel studied the effects of aging on the biochemical properties of rat tail tendon and dorsal skin and finds a strong correlation between tensile strength and the concentration of collagen insoluble in weak acids (45). Both insoluble and total collagen in rat skin decrease after maturity (4 months of age), while these properties increase in tail tendon until twelve months of age (46). Hamlin, et al., (16) and Schnider, et al., (33) note an increased resistance of collagen to collagenase digestion in human tendon with age. These studies indicate enhancement of cross-linking with age and suggest a corresponding increase in tensile strength and modulus of the tissues.

BIOPHYSICAL CHANGES WITH AGE

The structural integrity of collagen with aging has also been documented from studies of thermal stability (10). It has been noted that collagen held isometrically will generate tension when heated as a result of the breakdown in the ordered crystalline structure to a preferential random coil state (26). The shape of the hydrothermal isometric tension (HIT) curve has been correlated with the character of the cross-linking within collagen.

Allain, et al., (1) measured an increased maximum contraction and a decreased rate of relaxation in rat skin from maturity to 18 months. A corresponding alteration occurs in the chemical bonding within collagen, from the thermally labile dehydro-hydroxylysinonorleucine cross-links in the 1-5 month, to a more thermally stabile cross-link of unknown composition (thought to be an aldimine derivative) in the 5-18 month old rat skin. Despite the increase in cross-link stability, there is no change in the shrinkage temperature of rat skin with age.

Mitchell and Rigby (29) find a small increase in shrinkage temperature of rat tail and human tendon, along with an increase in thermal stability with aging, as measured by the rate of tension development during heating. they infer two possibilities: 1) an increase in cross-link density with age or, 2) an increase in cross-links that are thermally stable with age.

DIABETES MELLITUS

Diabetes affects the islands of Langerhans which are masses of small cells in the pancreas that produce insulin. Insulin is required by all cells in the body, except the brain, to mediate the transport of glucose across the cell membrane. Glucose is used to produce energy (ATP) within the cell. Without insulin, glucose remains in the extracellular fluids of the body and causes a chain reaction of events as illustrated in Figure 4.

extracellular fluids of the body and causes a chain reaction of events as illustrated in Figure 4.

Two forms of insulin dependent diabetes are discussed in this document. Type I diabetes mellitus is called naturally occurring, juvenile onset, and/or spontaneous diabetes. As these names indicate, it is a genetically transferred disease with symptoms that spontaneously emerge at different times during the maturation of many species. The second form of diabetes is experimentally induced in laboratory animals by chemical poisoning (3) or removal of the pancreas (in some animals of the present study) to study the effects of hyperglycemia on several tissues and systems in the animal.

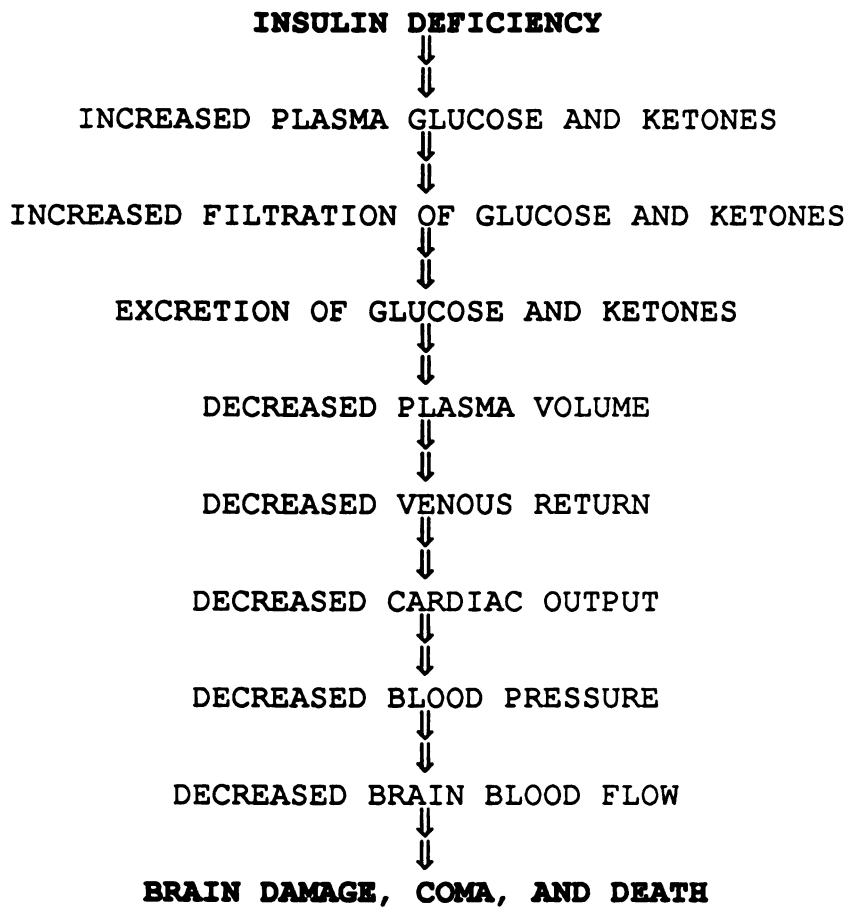


Figure 4. Hyperglycemic chain of events.

Serious complications and sometimes death may result from untreated or poorly controlled diabetes. Alterations in practically all tissues of the body occur as a result of this disease including: peripheral arterial diseases, atherosclerosis, osteoarthritis, osteoporosis, and osteopenia, peridontal diseases, tendonitis, bursitis, pseudo-scleroderma, loss of sensation or feeling in the extremities, limited joint mobility, cataracts, kidney failure, and other pathologies. The degenerative processes which occur in the diabetic individual may be caused or

influenced by genetic abnormalities, metabolic abnormalities, or both (34).

Human skin samples taken from the hands of juvenile diabetics are found to have decreased vascular lumen, accumulation of collagen, and changes in the collagen fibril arrangement (43). Decreased vascular lumen causes tissue hypoxia causing further thickening of the skin tissue and once again illustrates a negative feedback situation in the diabetic metabolism.

Complications from diabetes often simulate pathologies found in senility and lead to the expression, age accelerator. Research was conducted by Hamlin, et al., (17) in which collagenase digestion of human collagen was used to predict donor ages. There is remarkable accuracy in the age prediction of a non-diseased donor while the predicted age of a diabetic patient is much higher than the true chronological age. Many authors have remarked that while the complications of diabetes resemble advancing age, the disease process is very complicated and is probably not true accelerated aging (5,17).

GLYCOSYLATION EFFECTS WITH DIABETES

The differences noted between diabetes (lack of insulin) and normal aging (decreased effectiveness of insulin) is only part of the story. As mentioned previously glucose in the extracellular fluid of the body has the ability to form cross-links between adjacent proteins.

Glycosylation of collagen increases with normal aging (33) and is prematurely present in the connective tissues of diabetic patients (43).

Non-enzymatic glycosylation of collagen is increased in experimentally induced diabetic rat aorta (3), skin (24), and tail tendon (5). It is also increased in human skin and collagen samples from naturally occurring diabetics (32,33). Hemoglobin in blood becomes glycosylated by glucose and its concentration is used to rate the level of metabolic control of diabetes with insulin (43).

BIOMECHANICAL ALTERATIONS WITH DIABETES

The rat is by far the most experimentally studied animal. Experimental diabetes is normally induced by streptozocin injection which poisons the pancreatic cells. Subsequent effects of the induced hyperglycemia on many connective tissues from the rat are well documented.

Andreassen, et al., (5) found a 50% increase in tensile strength of rat tail tendons over controls after 30 days of experimentally induced diabetes. A second group treated with insulin therapy had no changes in the mechanical properties when compared with controls. Galeski, et al., (14), using the same model, reports increased stiffness and modulus after about four months of diabetes. Insulin therapy prevented all documented changes.

Two additional studies were conducted by Andreassen and others (3,4) using the diabetic rat model. The first

involved mechanically testing aortic tissue in which there is an increased tensile strength after three months of hyperglycemia. Insulin therapy again prevented these changes (3). The same investigators measured collagen content and strength in granulation tissue of rat skin formed under hyperglycemic conditions. The results indicate that untreated diabetic rat skin has a decreased failure strain energy density after only twenty days. An insulin treated group showed a decrease in failure strain energy density for the first seven days, which then returned to the level of controls by the tenth day. It is concluded that collagen deposition is decreased during the early phases of healing in experimental diabetes compared with normoglycemic animals (4).

Bones from diabetic rats were tested in torsion by Einhorn, et al., (13). Experimental diabetes decreased the torsional strength, energy absorption, and stiffness of rat femurs. Canine femurs from naturally occurring diabetics were tested in four point bending (9). No statistical changes were found in the material properties of bone from naturally occurring diabetics. There is, however, a decrease in the cross-sectional area caused by osteopenia which leads to a weakening in the structural integrity of the femurs.

BIOCHEMICAL ALTERATIONS WITH DIABETES

Andreassen, et al., (5) used tritiated sodium borohydride to reduce pepsin insoluble collagen and measure the level of glucose attachment to lysine and hydroxylysine residues. While no increase in reducible cross-links is noted, glycosylated collagen increased considerably in the diabetic rat model. There is also reduced collagen content in experimentally induced diabetic rat aorta after three months (3). Glomerular basement membranes (GBM) in induced rats were examined by Romen (30). A decrease in collagen synthesis with an even larger decrease in catabolism leads to an increase in GBM thickness.

Smith, et al., (37) looked at skeletal muscle proteolysis in the diabetic rat model and found a 30% increase in protein degradation along with a 60% increase in myofibrillar catabolism after only one day of diabetes. As with all other experimentally induced diabetic rat tissues, insulin reversed all changes caused by hyperglycemia.

Human dura mater is a lining around the brain which is nearly pure collagen. Schnider, et al., (32) examined human, juvenile diabetic dura mater and found an increased resistance to cyanogen bromide digestion over non-diseased tissues. In discussion of their findings they noted an age-like cross-linking effect caused by the disease.

Human skin from juvenile diabetics has increased cross-linking determined by decreased solubility to collagenase and cyanogen bromide (44). Also seen in the naturally

occurring diabetic is an increased glycation and collagen content (44). Kohn, et al., (25) also reports increased collagen formation in human diabetic skin because procollagen molecules are found to have abnormal reducible cross-links while lacking some normal reducible cross-links.

Galeski, et al., (14) measured an increased collagen fibril diameter of experimentally induced diabetic rat tail tendon.

BIOPHYSICAL ALTERATIONS WITH DIABETES

Experimentally induced diabetic rat tail tendon has a 30% increase in maximum contraction force, a 80% increase in relaxation time after that force is reached, and no changes in these parameters with insulin therapy as compared with controls (5).

III. METHODS AND MATERIALS

SPECIMENS

All tissues were obtained from the Michigan State University Veterinary Clinical Center. Twenty nine canines were used as control animals. Both sexes were used from varied breeds and weights. The ages ranged from six months to fifteen years. Their average weight was 26.3±8.3 kgs. and ranged from 10 to 42 kgs.

Seven naturally occurring and two experimentally induced diabetic canines were also obtained from the MSU Veterinary Clinical Center. Their ages ranged from 2.5 to 9 years old. Both sexes from varied breeds and weights were used in this study. Their weights ranged from 8.2 to 30.8 kgs. with an average weight of 20 ± 7 kgs (Table 1).

Control specimens were acquired at the Veterinary Clinical Center necropsy laboratory from animals that had died acutely or following euthanasia for varied reasons. Examples of acute deaths include: old age, trauma, gastric dilation, and localized tumors. Medical records and necropsy results were screened to ensure that the animals were free from diseases known to cause degradation of connective tissue.

Table 1. Animal age, sex, weight, and breed.

CONTROLS

AGE (YEARS)	SEX	WT. (Kg.)	BREED
0.5	F	22	German Shepard
0.8	F	20	German Shepard
1.5	M	36	German Shepard
1.5	M	37	Schnauzer
2.0	M	27	Samoyed
2.0	M	10	Cocker Spaniel
2.5	M	42	Rottweiler
2.5	M	20	Chow
3.0	M	18	Husky
4.0	F	15	Am. Eskimo
4.0	F	34	Mix
5.0	F	31	Collie
5.5	M	26	Boxer
6.0	F	20	Black Lab.
6.0	M	33	Gld. Retriever
7.0	F	23	Black Lab.
7.0	M	28	Mix
9.0	M	14	Irish Setter
9.0	F	37	German Shepard
10.0	M	18	Mix
10.0	M	33	Shelty
11.0	F	34	Gld. Retriever
11.0	F	33	Airdale
12.0	M	30	Irish Setter
13.0	F	17	Mix
14.0	F	29	Collie
14.0	M	33	Black Lab.
15.0	F	24	Irish Setter

DIABETICS

* 2.5	F	17	Mix
* 2.5	F	8	Beagle
4.0	M	31	German Shepard
5.0	F	19	Gld. Retriever
6.0	F	23	Gld. Retriever
6.0	F	22	Mix
9.0	M	26	Gld. Retriever
9.0	F	14	Gld. Retriever
9.0	M	20	Gld. Retriever

* Indicates experimentally induced diabetic animals.

The diabetic animals were donated to the Veterinary Clinical Center when they became spontaneously diabetic before twelve weeks of age. From the time of diagnosis serum glucose was regulated by one to two daily injections of insulin (NPH or PZI, beef-pork mixture). The dosage was sufficient to prevent ketonuria, however glucosuria and mild hyperglycemia were not uncommon. The dogs were exercised and fed twice daily. Glycosylated hemoglobin values were determined by the method of Standefer and Eaton (38) and ranged from 3.6% to 8.4% with a median of 5.1%. Urinary glucose concentrations determined by the Beckman Dri-STAT Enzymatic Glucose-NK Endpoint Reagent ranged from 0.3 to 162 mg/mg createnine with a median of 28. The dogs were maintained at the Veterinary Clinical Center for life (4 to 9 years). Euthansia was performed following completion of the NIH-supported study (35).

The two experimentally induced diabetics had pancreatectomies at six months of age and subsequently required exocrine pancreatic supplements (Viokase, A.H. Robbins Co., Richmond, VA 23220). They were boarded and cared for in the same manner as the naturally occurring diabetic animals until euthanasia was performed (36).

The hind limbs of each canine were removed and frozen at -20^0 centigrade within 12 hours of death. The limbs were kept frozen for a period no greater than nine months. Prior to mechanical or HIT experiments the limbs were removed and allowed to thaw overnight at room temperature.

Once thawed the patella-patellar tendon-tibia complexes (PPT) were isolated from a randomly chosen limb of each animal for mechanical testing and subsequent biochemical evaluation. Patellar tendons from selected contralateral sides were also isolated for use in the HIT experiments.

MECHANICAL TEST PREPARATION

The patellar tendon measurements were made by a single examiner using vernier calipers. Width and thickness of the tendon were measured at three points along its length and averaged. The initial length was taken from the inferior pole of the patella to the superior-posterior attachment of the tendon on the tibia. The tendon was wrapped in physiological saline (0.9% NaCl) soaked gauze and was kept moistened throughout the testing procedure.

The tibia was cut at the distal end to a length of approximately six inches. A 1.5 inch diameter, 6 inch long polyvinyl chloride (PVC) tube was placed vertically on a paper mat under a ventilation hood (Figure 5). A room curing fiberglass resin (NAPA brand Fiberglass Reinforced Plastic Filler 6371) was mixed with compatible hardener and poured into the PVC tube. The tibia was pushed down into the resin such that the posterior-inferior condyles rested on the top edge of the tube. Care was taken to ensure that the tendon did not come in contact with the resin. Once properly placed the potted configuration was allowed to

harden at room temperature. The tendon remained wrapped in moistened gauze throughout the potting procedure.

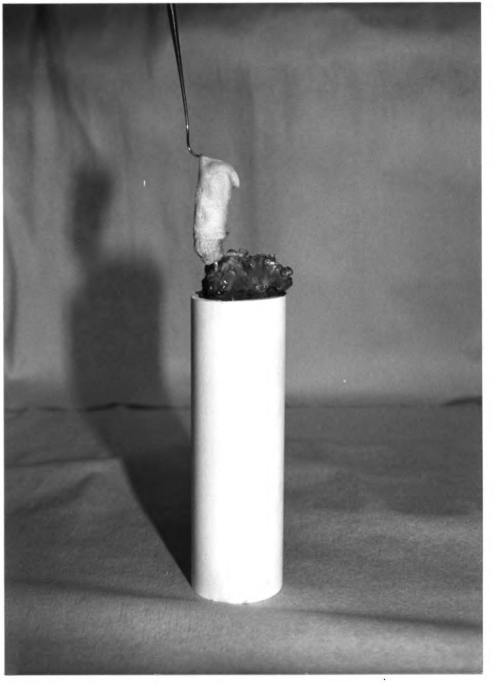


Figure 5. Potted tibia in PVC tube.

Once the tibia was firmly fixed in the PVC tube the patella was potted. A special stainless steel box-like grip (1.5x1.5x1.25 inches) was used to hold the patella (Figure 6). A slot cut in the bottom of the box allowed the tendon to pass through without restricting its motion. The front lid of the box was removed to pot the patella. The inside was sprayed with a silicone based lubricant to allow easy removal of the potted patella once the tensile test was complete. The same fiberglass resin/hardener mix described previously was used to secure the patella. The box was nearly filled with the fiberglass. The patella was pressed into the resin up to the anterior (tendon-covered) surface and covered by the front lid of the box. The apex (inferior pole) of the patella was allowed to rest against the bottom of the box. The tendon was passed through the slot described earlier, the front was secured, and the resin was allowed to harden for ten to fifteen minutes.

A standard servo-hydraulic tensile testing machine (Instron model 1331) was used to mechanically test the complex. A specially designed fixture was built to allow exact alignment of the tendon along the load axis (Figure 7).

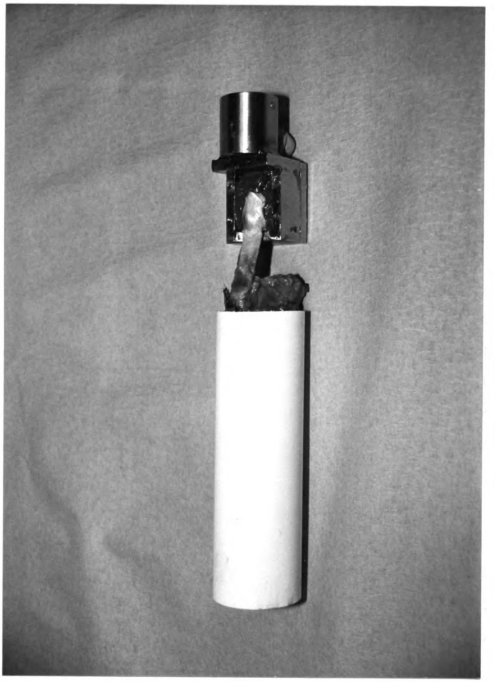


Figure 6. Potted patella in box grip.

The fixture was composed of three horizontal 1/4 in. stainless steel plates and a vertically mounted rotating disc of the same material. The bottom plate was secured to the base of the Instron machine. Screw drives were mounted between each set of plates which permitted direction changes toward, away, and to the right and left of the operator. This x-y table configuration enabled the operator to precisely align the tendon along the load axis.

The rotating disc welded to the top plate simultaneously served two important functions. Its primary function was to provide a secure mounting mechanism for the potted tibia. Its secondary function was to allow for angle changes between the tibia and the load axis. An inclusive angle of 110^{o} was used to simulate the physiological loading configuration and allow pure tensile deformation of the PT without a tearing or cutting action at the tibial tuberosity.

The upper part of the loading mechanism included a 2000 lb. load cell (Instron model 1010-AF) mounted in line with the load axis. Below the load cell was a universal joint used to correct minor offsets in the PPT alignment. The box containing the potted patella was connected to the lowest portion of the universal joint.

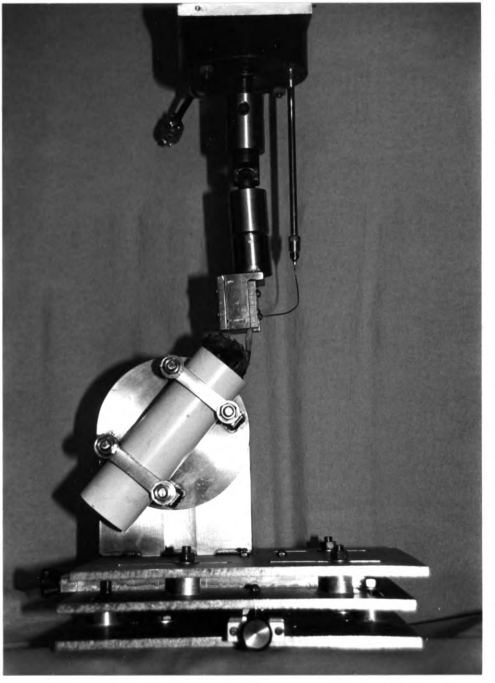


Figure 7. Mounted PPT complex.

Physiological saline was heated to 37° C by a heat exhanger within a tank placed above the crosshead of the Instron. Gravity fed saline was passed through a tube to a drip mechanism which gave the operator control of the saline as it flowed onto the tendon. The drip tube was positioned at the top of the tendon where it first exited the box grip which permitted continuous moistening of the tendon during the mechanical tests.

PRE CONDITIONING

Each PPT complex was cyclically loaded from 90 (N) to 180 (N) at one hertz for 40 cycles. Actual setting was for a frequency of 1 hertz, but inaccuracy in a dial potentiometer indicated the resultant frequency for all tests was slightly more than 1 hertz. A standard linearly variable displacement transducer within the Instron actuator measured the grip-to-grip deformations which occurred during the cyclic tests. A Nicolet oscilloscope and compatible disk drive collected and stored the load and deformation voltage data continuously. Strain versus time curves were generated using the deformation data. Creep in a tendon was defined as the change in strain between the first and fortieth cycle of pre-conditioning. The physiological stiffness was determined by hand from the slope of the loading segment of the fourtieth cycle from the load-deformation data as displayed on the oscilliscopy. Figure 8 illustrates a typical strain time curve and the points at which the creep values were

which the creep values were documented in this study. The physiological modulus was determined using the relationship:

$$E = kLo/A.$$

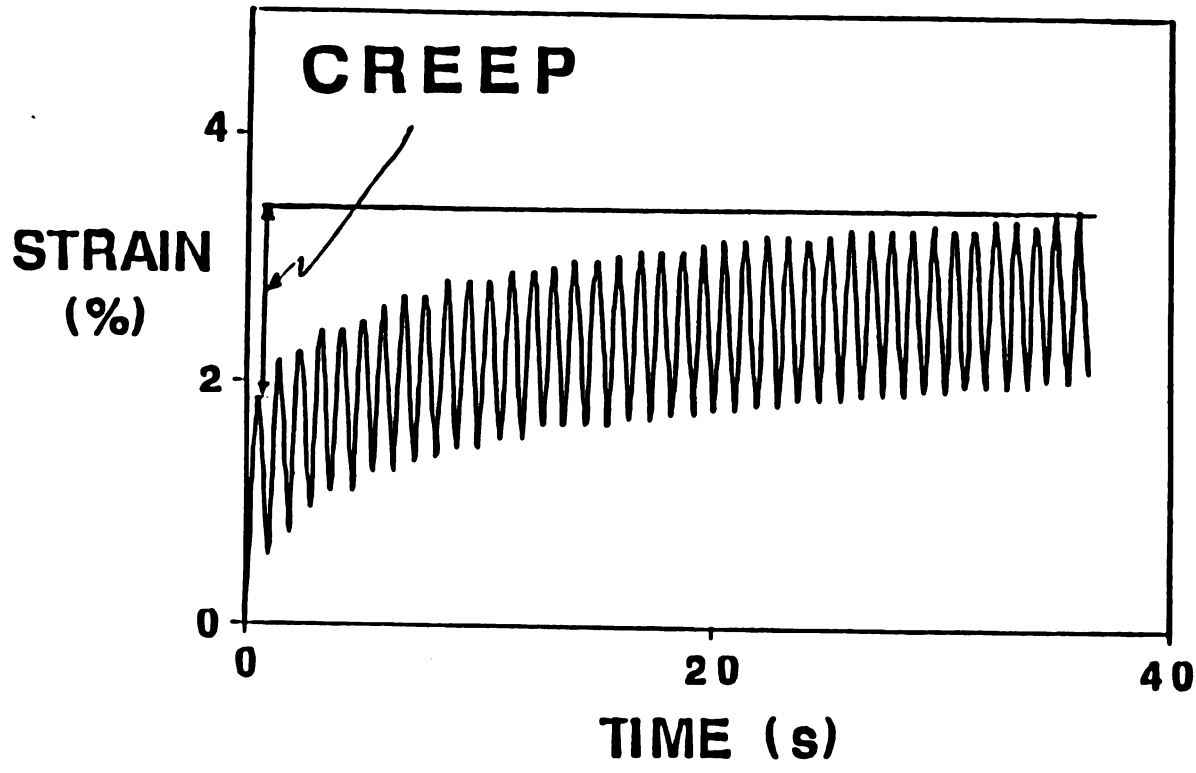


Figure 8. Typical strain-time curve.

TENSILE TESTS

Immediately after pre-conditioning the complex was loaded to failure at a nominal rate of 100 % of the initial length of the tendon per second. Continuous load and deformation data were collected and stored in the same manner as the cyclic tests. The mechanism of failure in the PPT complex was visually determined and documented.

Cross-sectional area and initial length of the tendons were used to normalize the load and deformation data, respectively. Stress versus strain plots were generated using the normalized data, and material properties of the tendon were determined from these plots. The tensile strength was defined as the peak stress achieved in the PPT complex during a midsubstance failure and the corresponding strain value was termed failure strain. The tensile modulus was determined by hand from the slope in the linear portion of the stress-strain curve. Figure 9 illustrates the representation of a typical stress-strain curve and the location at which the above parameters were determined.

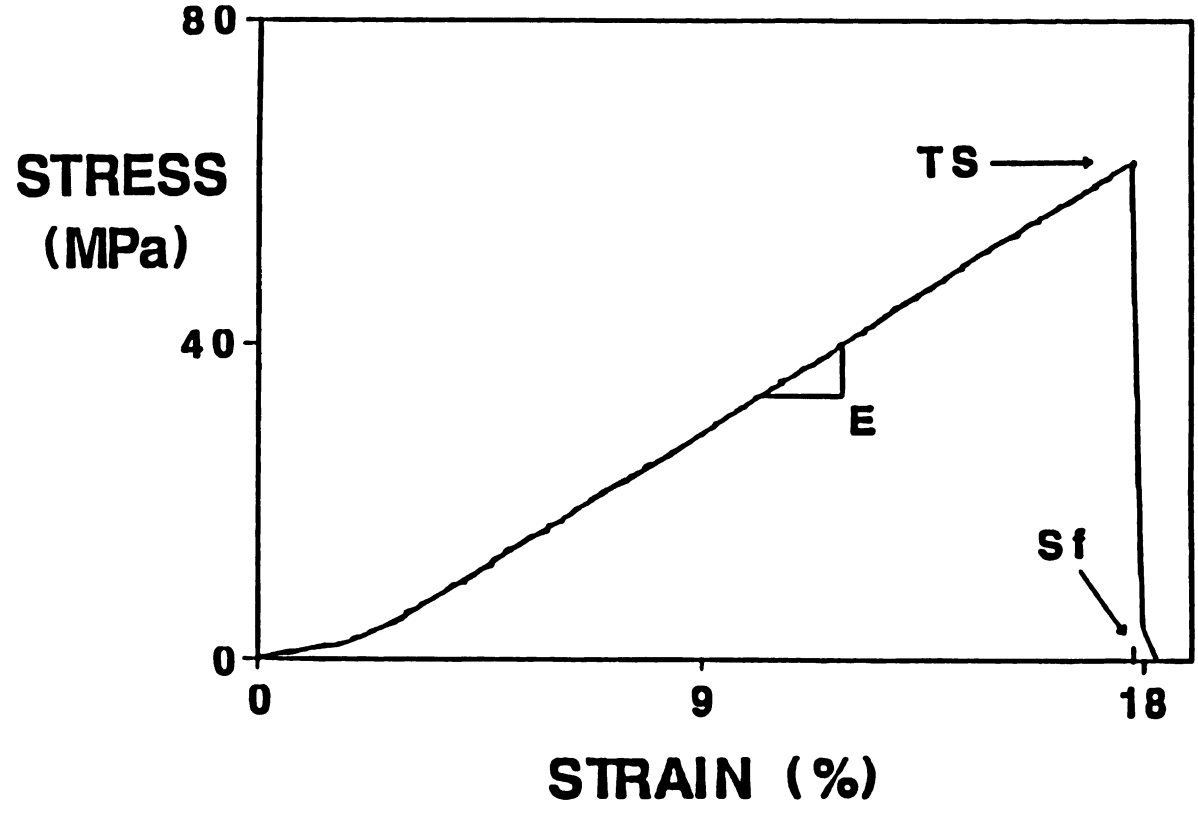


Figure 9. Typical stress-strain curve.

The load-deformation and stress-strain data were loaded into a computer program which calculated the area under each curve using the trapezoidal technique. The failure strain energy was defined as the area under the load-deformation curve and the failure strain energy density was the area under the stress-strain curve to failure.

BIOCHEMISTRY

The collagen content in each tendon was determined through a process of extractions and enzyme digestion. Three aliquots were used in each of two soluble fractions and one insoluble fraction collected and analyzed for their individual collagen content by the method of Schnider and Kohn (33). A separate assay was conducted to determine the total collagen content in each tissue and served as a check for losses during sequential extractions.

For each assay approximately 15-20 mg. of tendon was frozen in liquid nitrogen, mechanically smashed, lyophilized overnight, weighed, and placed into small test tubes. The lipids were removed by agitating the samples overnight in chloroform-methanol (2:1 V:V) at 4^{o} C.

The following day the samples were centrifuged at 2000 rpm for 1.5 hours. The supernatant was removed and discarded. The above procedure was repeated two additional times for a total of 72 hours of extraction.

After removal of the supernatant on the third day, the sample was dried with nitrogen gas. The total collagen samples were immediatly hydrolyzed and neutralized by the procedure to follow.

Once dried the aliquots used for solubility tests were suspended in two ml. of 0.5 M acetic acid, agitated overnight at 4^{o} C, and centrifuged for 1.5 hours on the following day. The supernatants were removed and placed in a clean, dry test tube. The above procedure was repeated

for two additional nights. The supernatants were collected and pooled. After removal of the supernatant on the third day the pellet was suspended in 2 ml. of acetic acid and digested in 2 ml. of pepsin enzyme at a concentration of 1 mg/ml for 18 hours at 4° C. After digestion, a neutral salt solution of 1.0 M NaCl in 0.05 M Tris-HCl buffer (1:1 V:V) with a pH of 7.4 was added to the aliquot. Enough salt solution was added to completely neutralize (pH 7.0) each sample. The samples were centrifuged for one hour at 4000 rpm. The supernatant was removed and transferred to a clean, dry test tube. The pellet was rewashed twice with the salt solution, centrifuged for 20 minutes, and the supernatants were combined. This was the pepsin soluble fraction. The remaining sample was the insoluble fraction.

The pepsin soluble fraction was dialyzed in 0.1 M acetic acid overnight using cellulose membranes (VWR Scientific, m.w. cutoff 12,000-14,000) to remove excess salt. All fractions were frozen and lyophilized overnight and then hydrolyzed.

HYDROLYSIS

2 ml. of 6 M HCl was added to the test tube to dissolve the precipitate. The sample was incubated overnight at 105° C in a sealed test tube.

NEUTRALIZATION

The following day, the sample was neutralized by the following procedure: 2-3 drops of methyl-red indicator was added to the hydrolyzed sample. 2400 µl. of 2.5 M NaOH was then added and vortexed. Additional NaOH was added until a pH of 7.0 was reached. The acid- and pepsin-soluble, and total collagen fractions were diluted to 10 ml. The insoluble fraction was diluted to 25 ml.

An assay for collagen was conducted on all the samples. A set of ten standards (1-10 µg/ml) was prepared from a stock of 1.0 mg hydroxyproline/100 ml. Two water samples were used for zero standards.

The assay was conducted using 1 ml of sample. Three solutions were used during the assay preparation including: 3 M $HClO_4$, 0.03 M chloramine-T solution (0.845 g Chloramine-T in 20 ml H_2O, 30 ml propanol, 50 ml citrate-acetate buffer, pH 6.0), and 5 % solution of p-dimethylaminobenzaldehyde (p-Dab) in propanol (2.0 g/40 ml) dissolved under heat. 1 ml. of the chloramine-T solution was added to each test tube, vortexed, and let to settle for 20 minutes. 1 ml. of 3 M $HClO_4$ was then added to each test tube, vortexed, and let to settle for five minutes. 1 ml. of p-dab solution was then added, vortexed, and incubated at 60° C. for 18 minutes (according to Stegemann, 38).

Once the test tubes had cooled to room temperature, the absorbances of the standards and samples were measured on a Beckman Model DG-spectrophotometer. The water samples were

prepared with reagents as above and served as reference. The absorbances of the samples were compared with the standard curve for the calculation of hydroxyproline per dry weight of tissue.

THERMAL STABILITY

The frozen limbs designated for use in the HIT tests were allowed to thaw at room temperature overnight. The patellar tendon was excised and immediately placed in room temperature physiological saline (0.9% NaCl).

Tendons were laid in a pool of saline on a dissecting table. Through a stereo microscope the strips of tendon were cut along the long axis. The anticipation was for the strips to approximate individual fascicles, at least having continuous fibers of collagen. This, however, was not verified further. Once excised, the tendon strip was mounted between flat plate clamps and held isometrically at 12 mm.

The HIT tests were conducted using a specially designed fixture. The gripped tendon was loaded into the device and an insulated pyrex cylinder (diameter: 7 cm, length: 20 cm) filled with 750 ml. of room temperature saline surrounded the tendon. A thumb screw mechanism was used to apply 0.16 N pre-tension on the tendon. This was measured with a digital multimeter.

The saline was heated at a rate of 1.5^{o} C/min from 24^{o} to 95^{o} C by a 209 watt, 1.25 cm wide resistive heating tape.

The tape was coated with silicone rubber to increase the thermal contact area and to protect from electrical shorts. A variable a.c. transformer was used to supply power to the heating element.

The HIT device was calibrated during a previous set of experiments (11). During the calibration it was discovered that the heating rate was non-linear due to limitations of the transformer, use of a temperature sensitive load cell, and heat loss from the device. For these reasons data collected past 80° C. are not reported in this study.

The temperature of the saline was measured by a T-type copper/constantan thermocouple placed near the center of the bath with the reference electrode in a insulated container of crushed ice. The voltage signal from the thermocouple was amplified by a Sensotec (model SA-4) strain gauge amplifier.

The load cell used to measure tension within the tendon bundle was a Gould-Statham, model UC 3. As mentioned previously, this transducer was not temperature compensated, therefore, a plexiglass shield was placed beneath it to deflect convected heat rising from the saline bath. In addition, a carbon fiber rod was used in line between the top grip and the load cell to minimize heat conduction into the cell. Voltage readings from the load cell were amplified by a matching strain gauge amplifier used for temperature data collection.

Once the test was complete, the tendon between the grips was removed, freeze-dried, and weighed. The dry weight was used to normalize the load data which was plotted against temperature data. Three value were determined from the normalized load-temperature plots as seen in Figure 10. The thermal shrinkage temperature (Ts) was defined as the point at which tendon contraction occurred. The primary (SL1) and secondary (SL2) slopes were also recorded. The primary slope was measured by hand from the initial rise in the load-temperature curve immediately after tendon contraction. The secondary slope was measured between approximately 70° and 80° C.

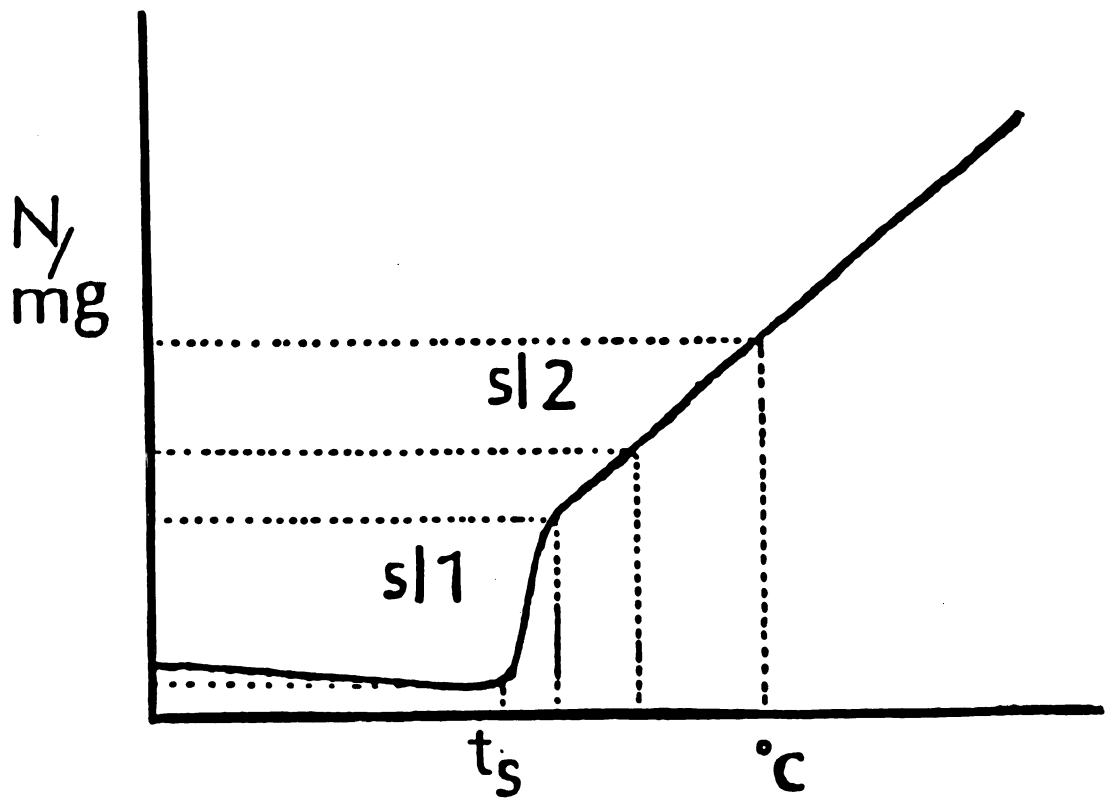


Figure 10. Typical HIT normalized load-temperature response of patellar tendon.

MORPHOLOGY

Transmission electron microscopy (TEM) was used to examine the distribution of collagen of collagen fibril diameters in 2.5, 4, and 9 year old diabetic and control tendons. 1.0 cm samples of patellar tendon fixed in buffered formalin were placed in fresh 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for one hour each at room temperature. Samples were minced further into 0.5 mm sections and then sequentially dehydrated in 70%, 95% and twice in 100% ethyl alcohol. Samples were then embedded in Epon-Arldite epoxy resin in a cross section orientation and polymerized at 65^{o} C for 24 hours. 70-90 nm ultra thin sections of the cross section oriented tendon were cut on a Dupont diamond knife and supported on 200 mesh copper grids for TEM. Thin sections were heavy metal stained with alcoholic uranyl acetate (1% UA in 70% ETOH) and Reynolds lead citrate. A Phillips 201 TEM at 60 kV was used to observe and document cross sectioned samples. A representative random photomicrograph was taken of each diabetic and age-matched control at 20,000X magnification.

A random representative 5 x 5 inch area was selected for each 8 x 10-3 enlarged glossy print prepared from TEM negatives and approximate fibril diameters were digitized in a point to point mode on a Nikon Joyce Loebl Magiscan with a 1.35 enlargement factor. The final scale factor was 81,000X

(20,000-3-1.35X). Statistical analysis of average fibril diameter was carried out with Magiscan software.

STATISTICAL ANALYSIS

Standard linear regression technique was used to determine changes in each property measured in this study as a function of age. In most cases the control data were sufficient in number to adequately perform the analysis. The diabetic data were at the most nine in number and although linear regressions were also performed on this data, a separate analysis of variance was conducted between the diabetic data and the age-matched controls. The diabetic values were plotted onto the linear regression curves of the control data for visual comparison. Changes in a property with age were defined when the slope of the regression line was statistically different from zero at $P<0.05$. Correlation of the data to the line was determined by the regression coefficient r which range from zero (no correlation), to one (perfect correlation). A Students paired t-test was conducted between the diabetic and age-matched controls to determine whether statistically significant differences ($P<0.05$) occurred between the two sample populations.

IV. RESULTS

GEOMETRIC PROPERTIES

There were variations in the geometric features of the patellar tendons due to the diversity of breeds and activity levels of the animals used for mechanical testing (Table 2).

PHYSIOLOGICAL PROPERTIES

Three mechanical properties of the tendons were determined in the estimated physiological range of loading (90 N-180 N). Creep of the tendon was the amount of stretch which occurred during forty cycles of pre-conditioning. Creep in the control tendons had virtually no dependence on age (P=0.96) (Figure 11). The correlation coefficient of control, creep data with age was 0.01. Creep of diabetic tendons was not significantly different than controls at P=0.11 (Figure 11). Linear regression of this data showed almost no correlation to the line (r=0.01), and no significant change with age (P=0.98).

Table 2. Tendon geometric properties.

Controls

Age (Y)	Lo (mmx10^1)	CSA (mmx10^2)	V (mmx10^3)
0.5	3.39	0.33	1.11
0.8	3.90	0.23	0.89
1.5	4.15	0.34	1.41
1.5	4.20	0.37	1.56
2.0	3.20	0.17	0.53
2.0	1.73	0.15	0.26
2.5	4.01	0.29	1.14
2.5	2.28	0.17	0.38
3.0	3.33	0.23	0.77
4.0	2.16	0.11	0.24
4.0	3.39	0.23	0.79
5.0	3.40	0.27	0.92
5.5	3.92	0.32	1.24
6.0	3.20	0.27	0.85
6.0	3.96	0.24	0.93
7.0	3.75	0.40	1.49
7.0	3.81	0.20	0.78
9.0	4.41	0.22	0.95
9.0	3.64	0.26	0.98
10.0	2.59	0.25	0.66
10.0	2.63	0.13	0.42
11.0	3.42	0.22	0.75
11.0	3.27	0.32	0.82
12.0	3.42	0.29	0.77
13.0	3.65	0.21	0.78
13.0	2.70	0.15	0.44
14.0	3.98	0.25	0.98
14.0	3.34	0.27	0.89
15.0	3.51	0.17	0.67
Average ± s.d.	3.39±0.64	0.24±0.07	0.84±0.33

Diabetics

Age (Y)	Lo (mmx10^1)	CSA (mmx10^2)	V (mmx10^3)
2.5	3.21	0.17	0.54
2.5	1.94	0.09	0.26
4.0	3.58	0.32	1.13
5.0	2.76	0.21	0.59
6.0	2.80	0.15	0.41
6.0	3.03	0.19	0.58
9.0	3.09	0.23	0.72
9.0	2.59	0.18	0.45
9.0	3.03	0.24	0.71
Average ± s.d.	2.89±0.46	0.20±0.06	0.60±0.25

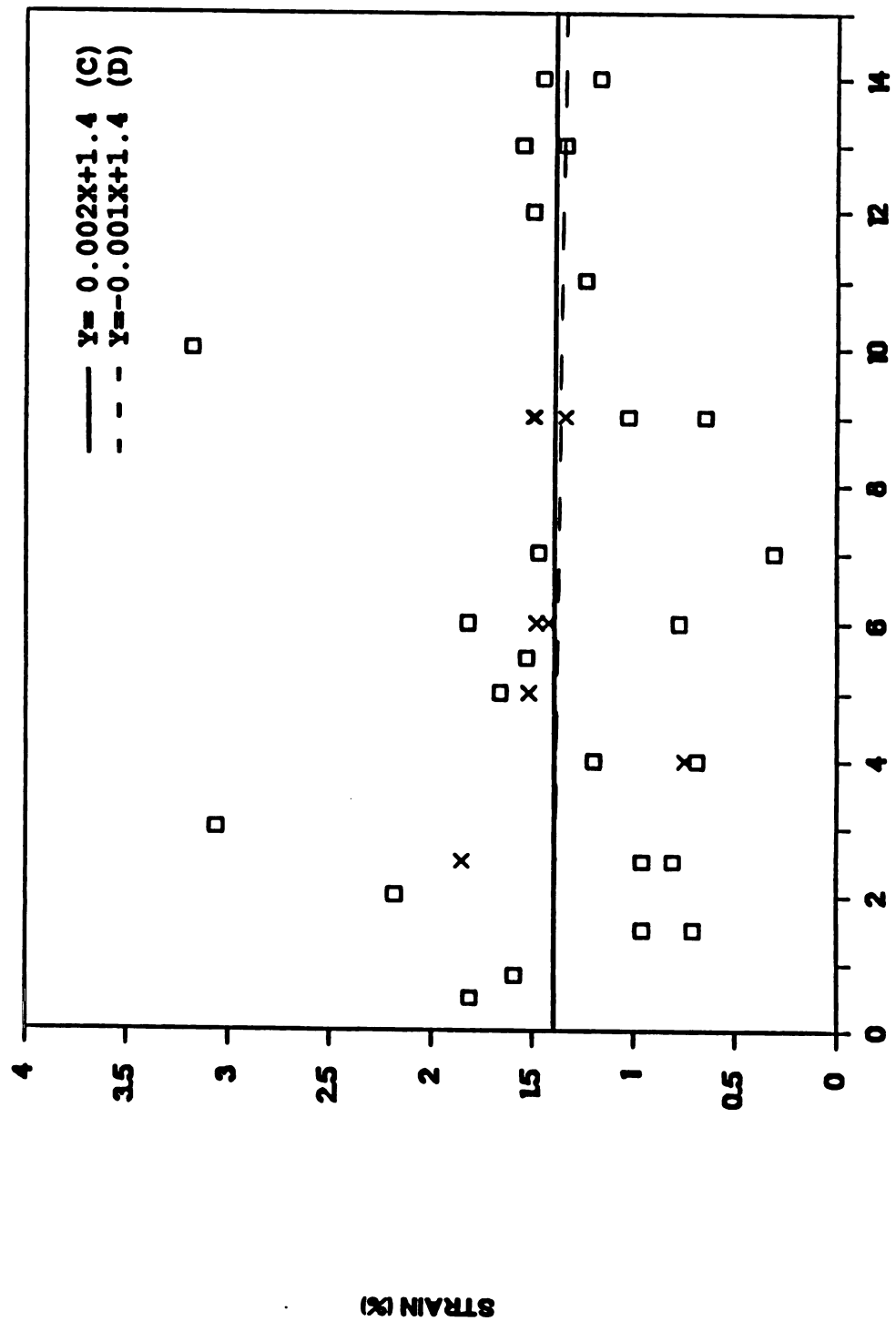


Figure 11. Creep as a function of age.

The second parameter determined from pre-conditioning was the physiological stiffness of the PPT complex. As Figure 12 illustrates, there was a slight downward trend in stiffness versus age, but the slope of the regression line was not statistically different than zero (P=0.65). There was a weak correlation of the stiffness values to the regression line as indicated by a low regression coefficient of r=0.1. While no statistical difference between the diabetics and age-matched controls was found (P=0.37), all the diabetic tendon stiffness values fell above the regression line of the controls, except one experimentally induced. This indicated a tendency for greater stiffness of the long term diabetic tendons than controls in the physiological loading range. There was moderate correlation of the data to the regression line (r=0.65) and a significant increase in the diabetic data with age (P=0.08).

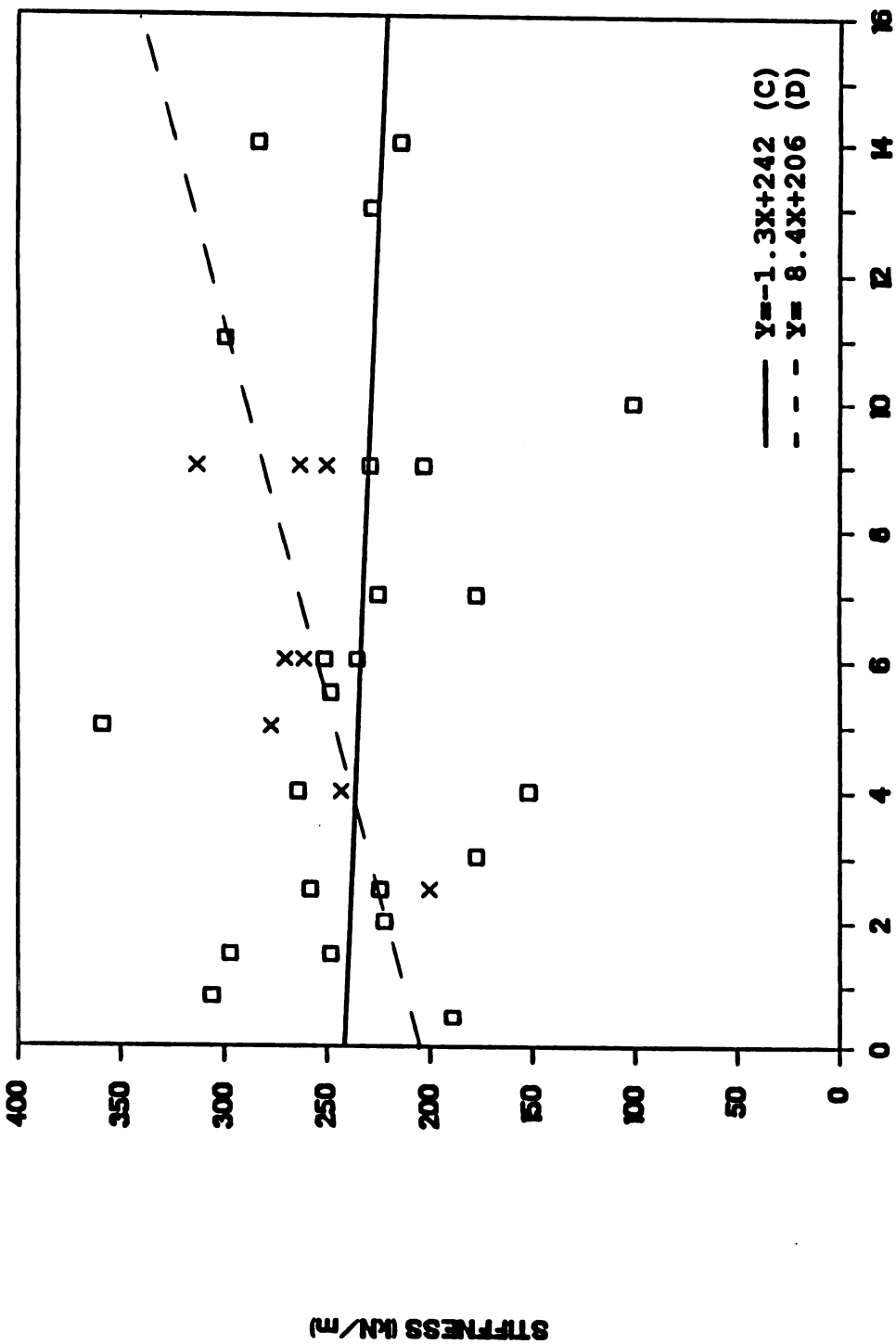


Figure 12. Physiological stiffness as a function of age.

The stiffness data were normalized by geometry to determine age-dependent changes in the physiological modulus (Figure 13). Like stiffness, the moduli had no significant changes with age ($P=0.65$), but a more definite tendency to increase slightly with age. There was weak correlation of the data with the regression line ($r=0.1$). There was no statistical difference between the diabetic tendon moduli and controls ($P=0.57$). Although, once again, the majority of the diabetic tendons had moduli above the regression line for controls, suggesting that diabetic tendon material tended to be stiffer than age-matched controls under physiological loads. There was poor correlation of the diabetic data to the regression line ($r=0.16$) and no significant changes with age ($P=0.7$).

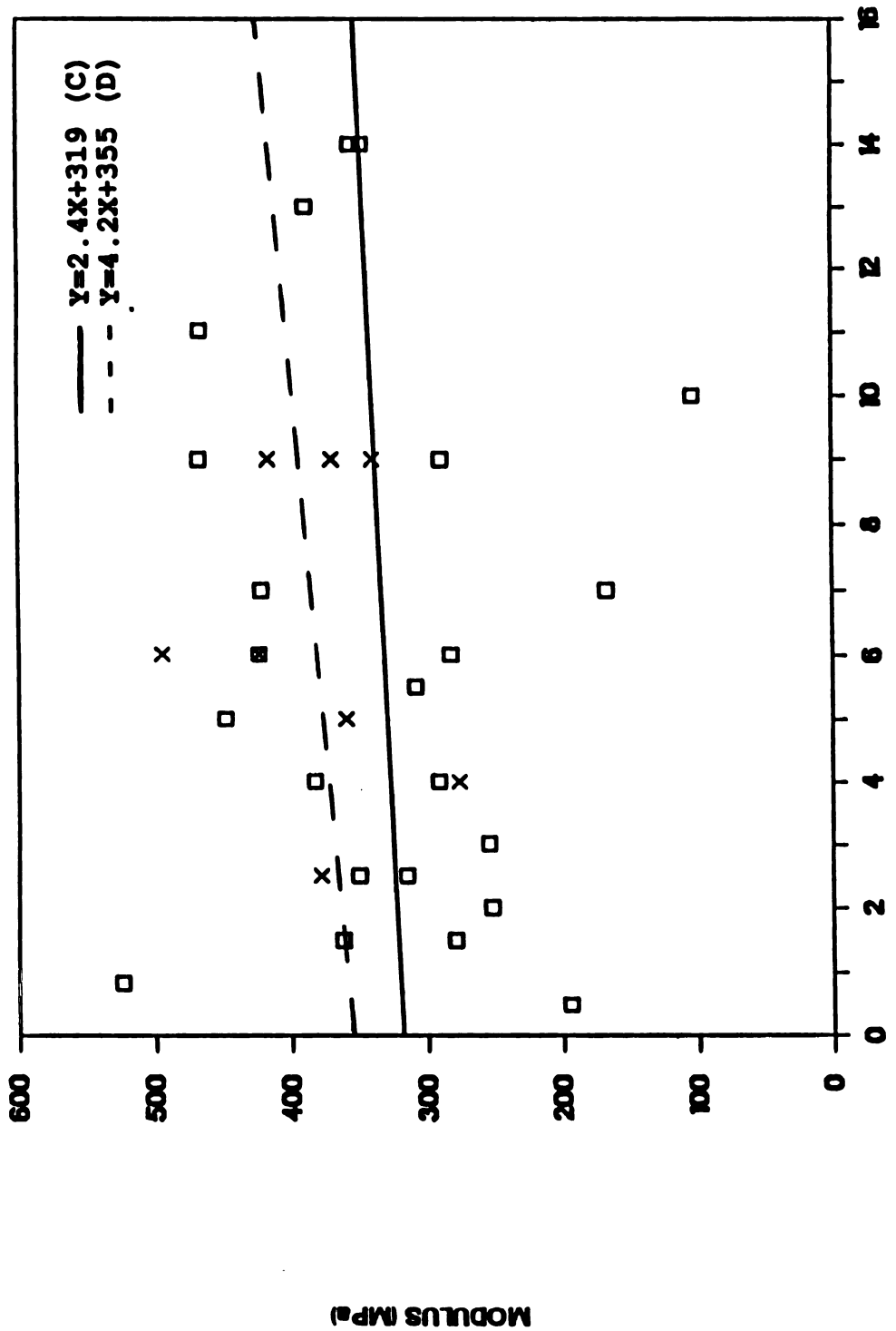


Figure 13. Physiological modulus as a function of age.

STRUCTURAL PROPERTIES

Four types of failure mechanisms occurred in the PPT complexes: tendon midsubstance rupture (A), bony avulsion of the patella (B), shear fracture of the patella parallel to the load axis (C), and tensile fracture of the patella 45^{o} to the load axis (D) (Figure 14). 41% of the control tendons failed midsubstance, while 38% failed by bony avulsion of the patella. While no significant (P=0.3) changes occurred, there was a tendency for a shift from a midsubstance in the young to bony avulsions in the old control PPT complexes. The remaining 21% of the controls failed in a shear fracture of the patella.

No midsubstance tendon failures occurred in the diabetic complexes. 78% of the diabetics failed by fracture of the patella along a plane 45^{o} to the load axis. The remaining 22% failed by bony avulsion of the patella.

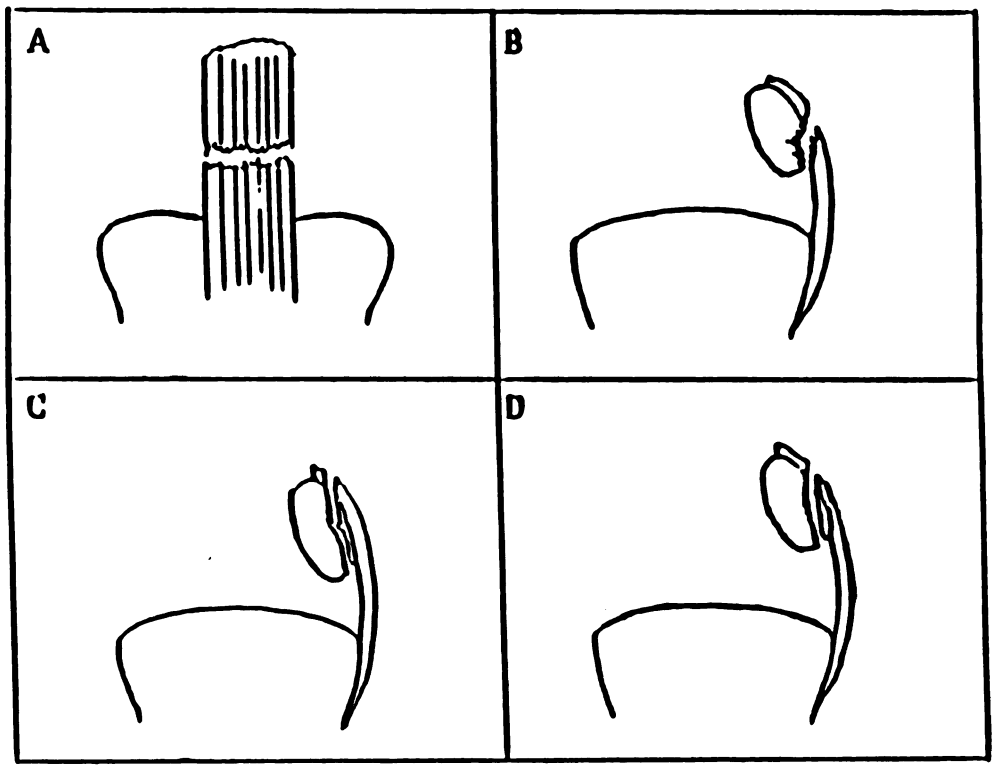


Figure 14. Failure mechanisms.

The structural properties of the canine tendons were plotted as a function of age. The average loads and standard deviations of the control and diabetic complexes were 2.50±0.9 kN and 2.03±0.7 kN respectively. The failure load had a slightly decreasing trend with age (P=0.36) (Figure 15). The correlation of the data with the regression line was weak (r=0.18). When analyzed against the age-matched controls, there was a significant decrease (P=0.03) in the failure load of the diabetic complexes. Regression of the diabetic data had a correlation to the line of r=0.32 and

P=0.41. The trend was for diabetic tissue to increase in failure load with age versus a decreasing trend for diabetic tissue.

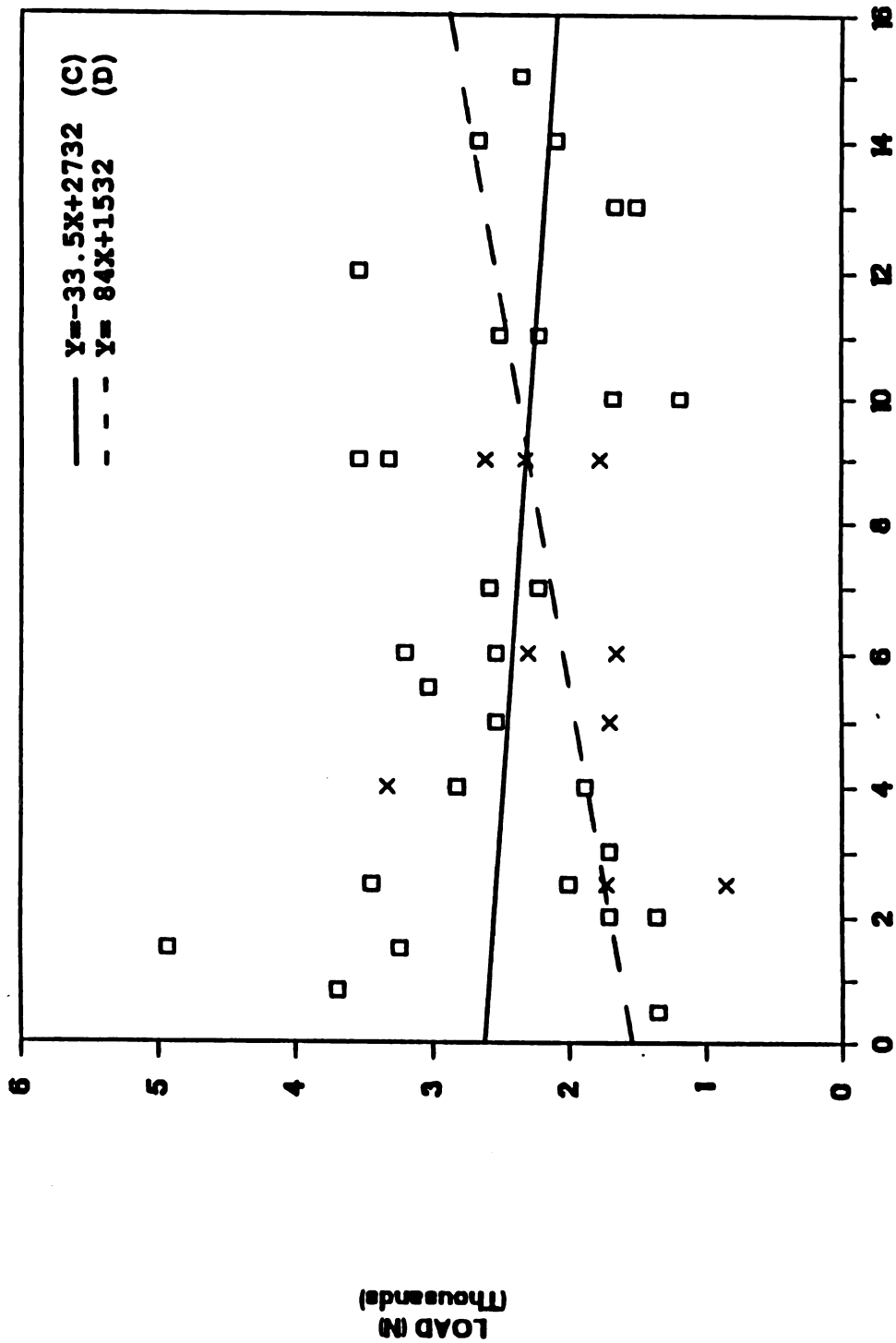


Figure 15. Failure load as a function of age.

As with the physiological stiffness of controls, tendon stiffness prior to failure did not depend on age (P=0.86) (Figure 16). There was also a weak correlation in this data to the regression line (r=0.04). The average and standard deviation values were 308±66 kN/m and 288±45 kN/m for the control and diabetic tendons, respectively. The age-matched controls were stiffer than the diabetic tendons (P=0.05), which differs from a slight increase noted in the physiological stiffness of diabetic tendons versus controls. Correlation of the diabetic data to their regression line was weak (r=0.03) and no changes occurred with age (P=0.93).

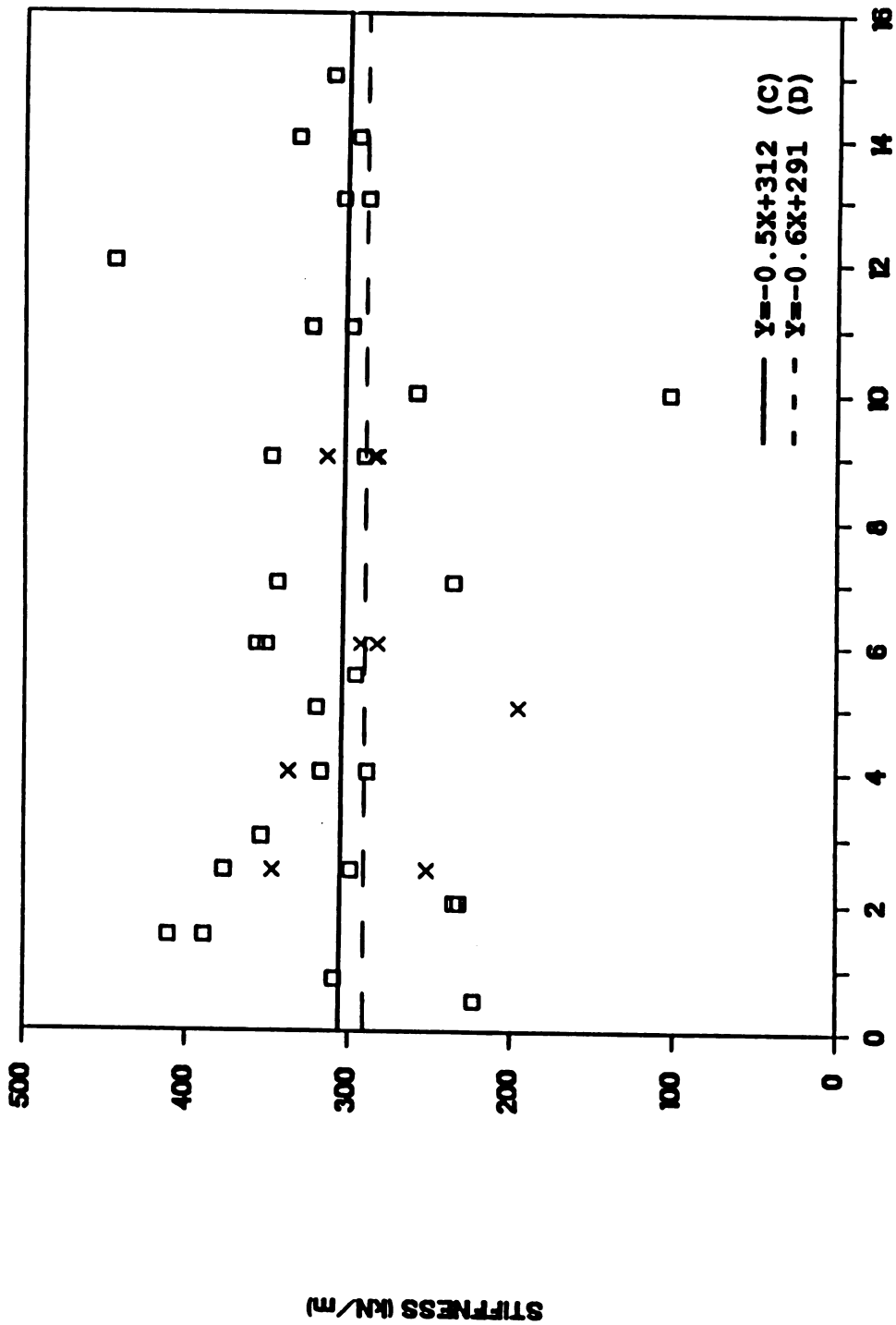


Figure 16. Stiffness as a function of age.

Failure strain energy of controls had a non-significant (P=0.11) decreasing trend with age (Figure 17). There was better correlation of this data with the regression line than parameters discussed previously (r=0.3). The average and standard deviation values of the control and diabetic complexes were 16±12 (J) and 12±7 (J), respectively. When analyzed with the age-matched controls, no difference was found (P=0.15) in the energy of the complexes caused by diabetes. However, most of the diabetic values fell below the regression line for controls. Correlation of the diabetic data to their regression line was poor (r=0.2) and no changes occurred with age (P=0.6).

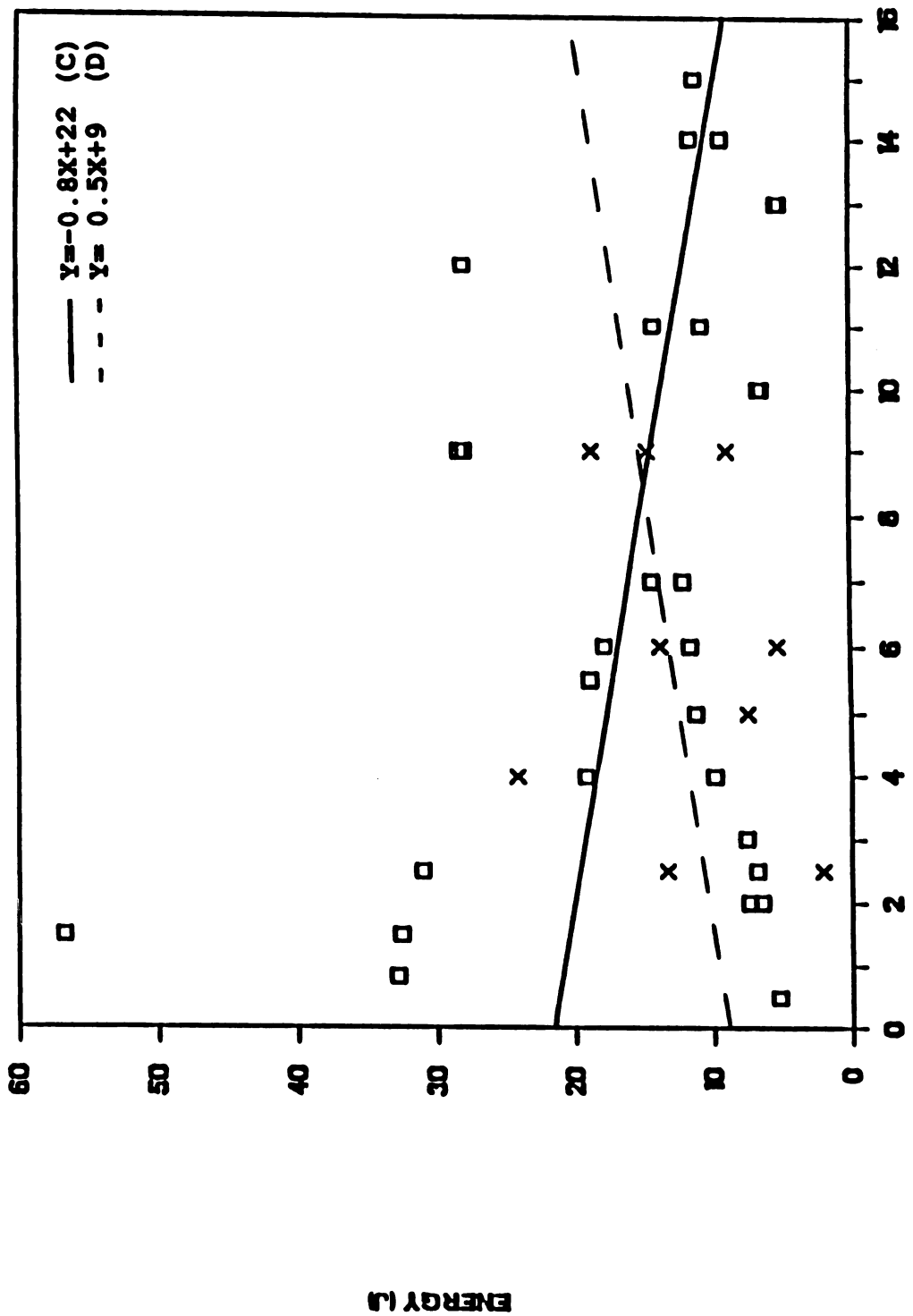


Figure 17. Failure strain energy as a function of age.

MATERIAL PROPERTIES

The strain at failure was one of four material properties evaluated as a function of age. Only midsubstance failures were analyzed to determine material properties of the tendon. A non-significant ($P>0.05$) decrease in failure strain occurred with age (Figure 18). There was very weak correlation of the data with the regression line with $r=0.04$. The average and standard deviation of the failure strain values was 33.4 ± 8.5 (%). No comparison of the control and diabetic strain values was conducted because there were no midsubstance failures in the diabetic tendons.

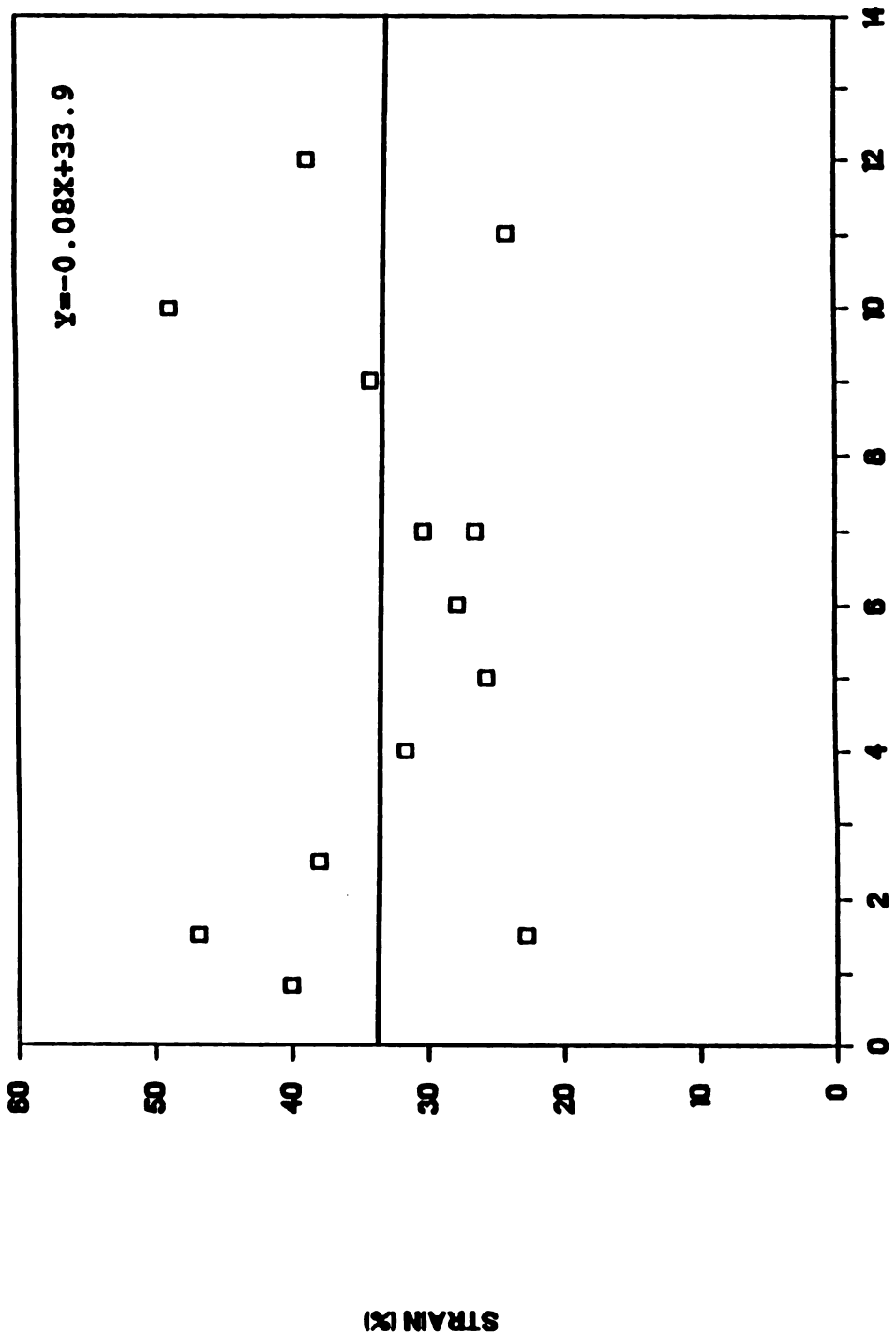


Figure 18. Failure strain as a function of age.

Tensile strength of a complex was defined as the maximum stress achieved before rupture. Like the failure strain, the tensile strength was documented from midsubstance failures only. As illustrated in Figure 19, the tensile strength did not change significantly ($P>0.05$) with increasing animal age, but a downward trend was noted. Correlation of the data to the regression line was moderate ($r=0.37$). The average and standard deviation of the control tendons was 111.2 ± 36.2 (MPa). Once again, no diabetic values were documented due to a lack of midsubstance failures.

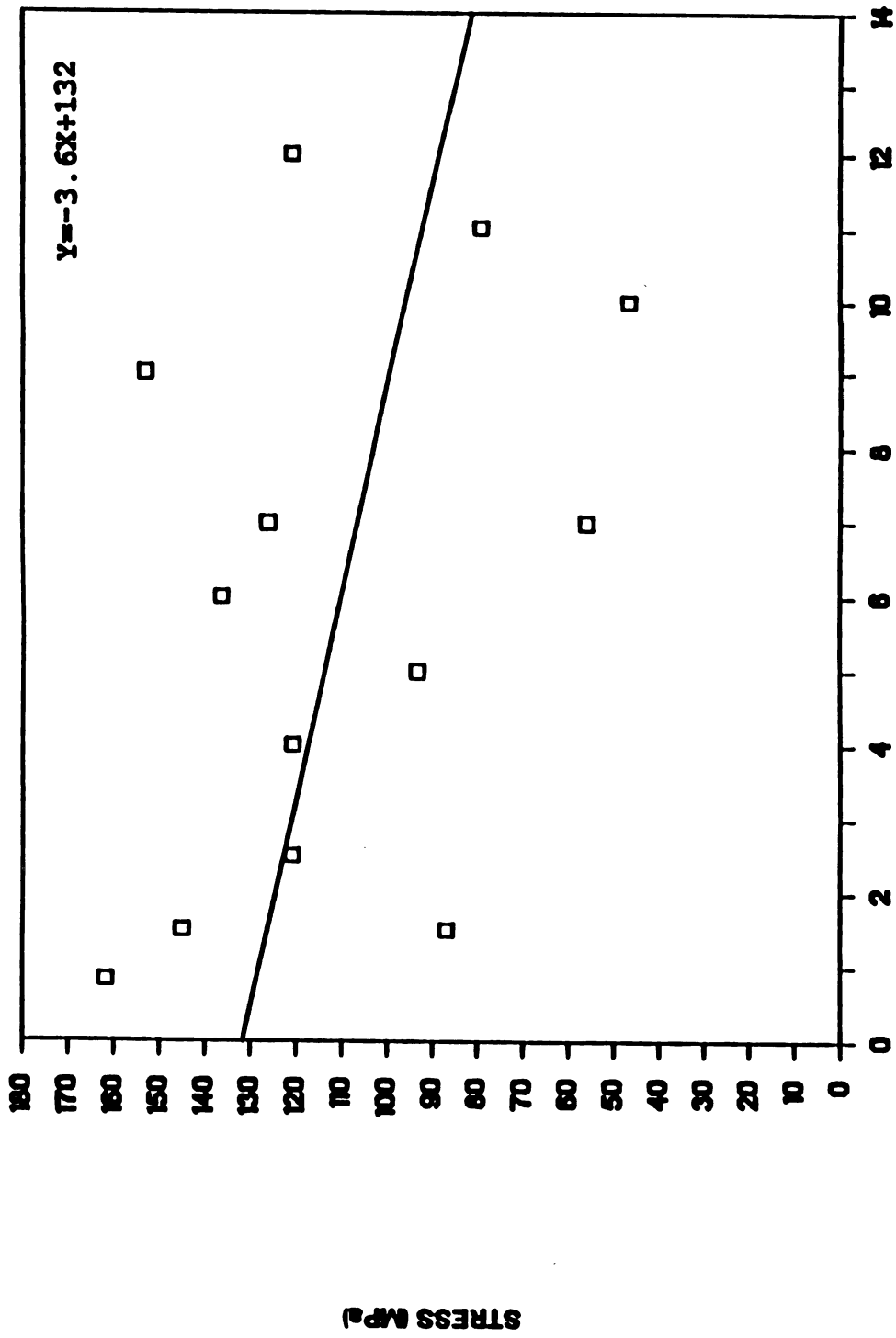


Figure 19. Tensile strength as a function of age.

The slope taken from the linear portion of the stress-strain curve was defined previously as the tensile modulus of the tendon. This was the only material property which had an increasing but non-significant (P=0.36) trend with age (Figure 20). Correlation of the moduli data to the regression line was weak (r=0.18). Diabetic moduli were not statistically different than the age-matched controls (P=0.47). Correlation to the diabetic regression line was moderate (r=0.44) and no statistically significant changes occurred with age (P=0.24). Yet there was a tendency for the diabetic tendon modulus to decrease with age, in contrast to the controls. Average and standard deviation values of the control and diabetic tendons were 453$\pm$126 (MPa) and 454$\pm$144 (MPa), respectively.

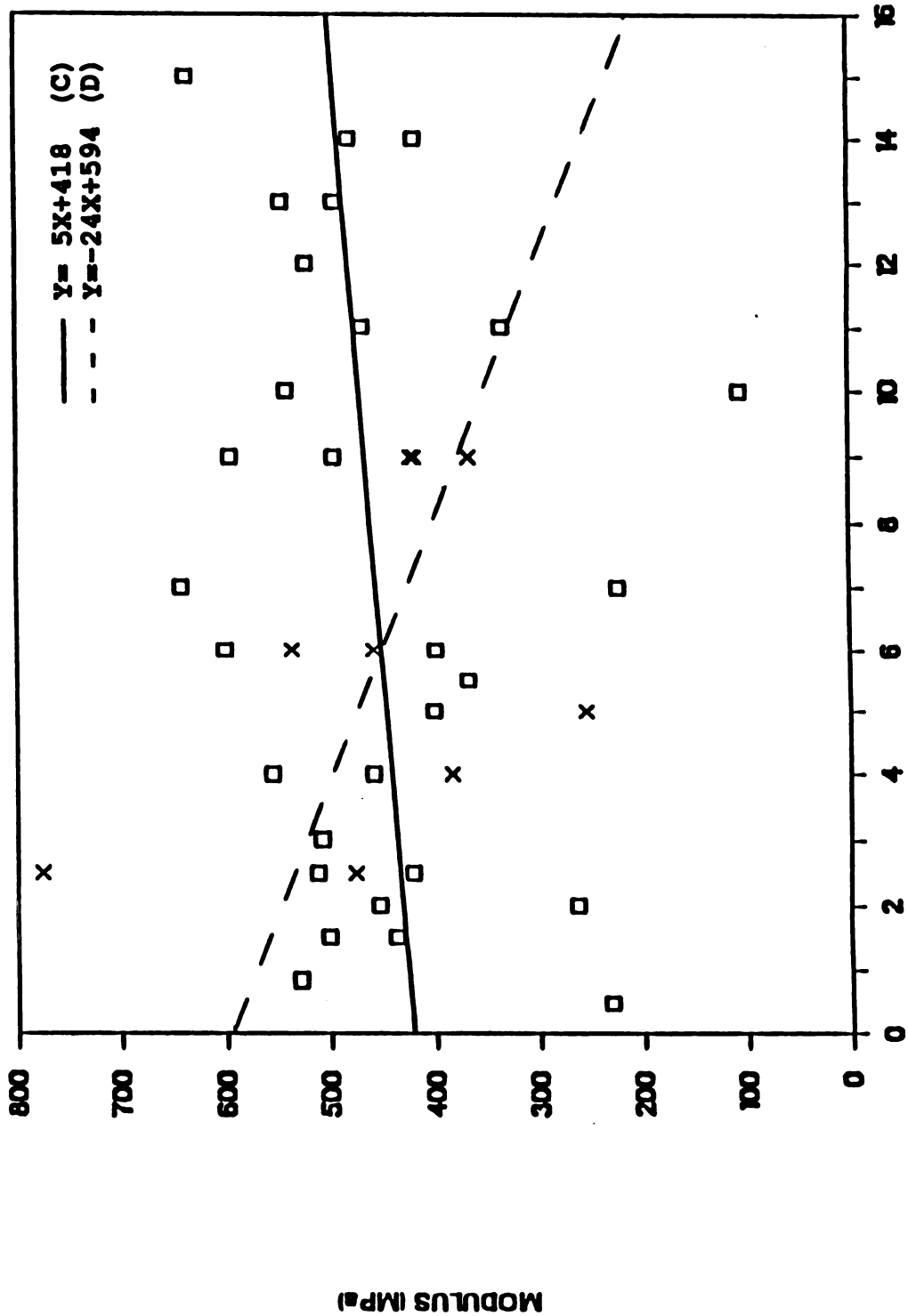


Figure 20. Tensile modulus as a function of age.

The last material property determined was the area under the stress-strain curve called the failure strain energy density. Only values from midsubstance failures were used, and therefore comparison with the diabetic complexes was not conducted. Like the strain energy there was a non-significant ($P>0.05$) decrease with age with a moderate correlation of the data to the regression line ($r=0.31$) (Figure 21). This was expected due to the decreasing trend in both the failure strain and tensile strength with age.

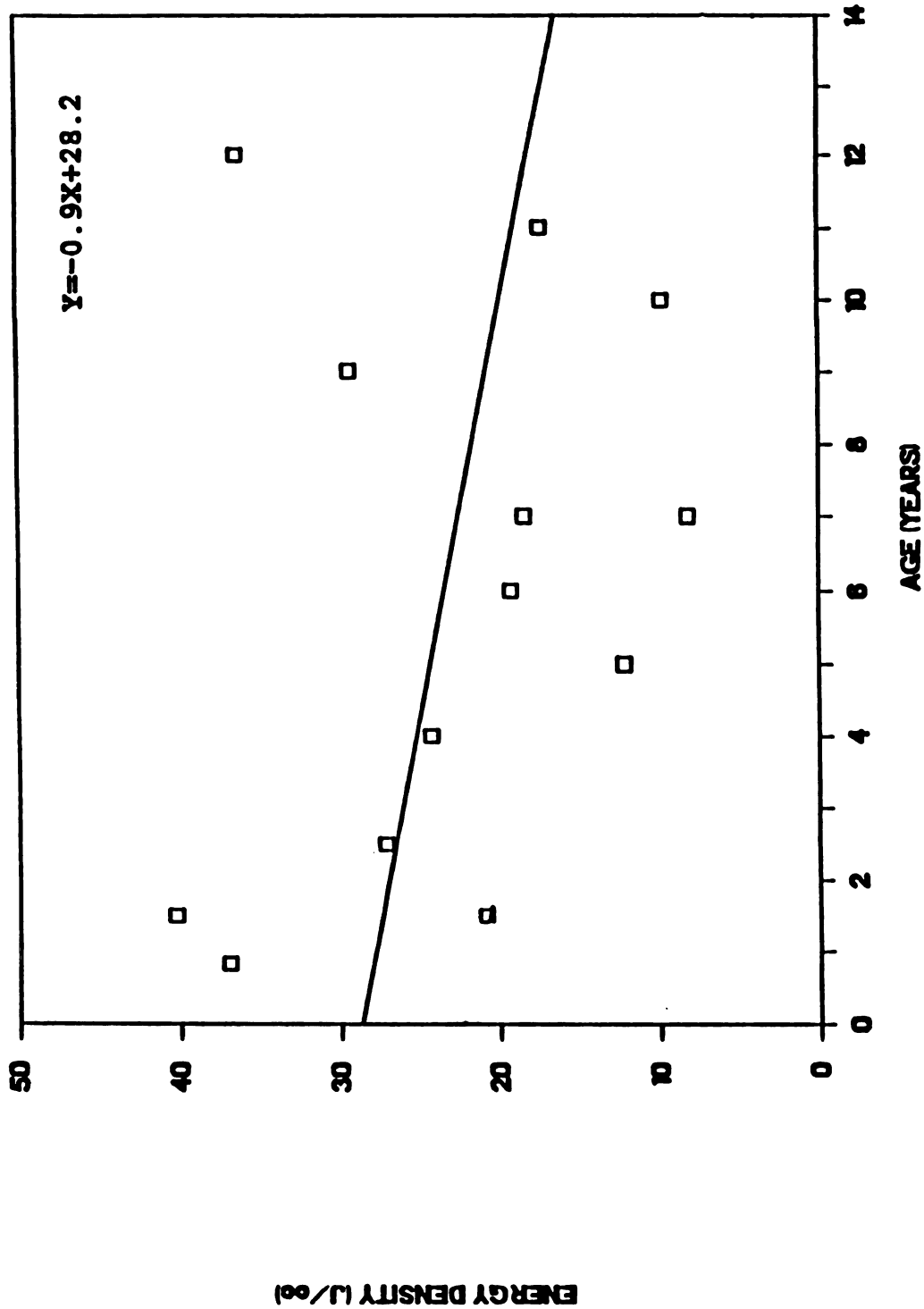


Figure 21. Failure strain energy density as a function of age.

A slight decrease in modulus prior to failure for the diabetic complexes versus aged-matched controls was illustrated in a composite curve of these data (Figure 22). The curve represents the mean data at every 2% strain for controls and diabetics. It is shown here without standard deviations to indicate the qualitative differences between the stress-strain responses of the diabetic and age-matched control complexes.

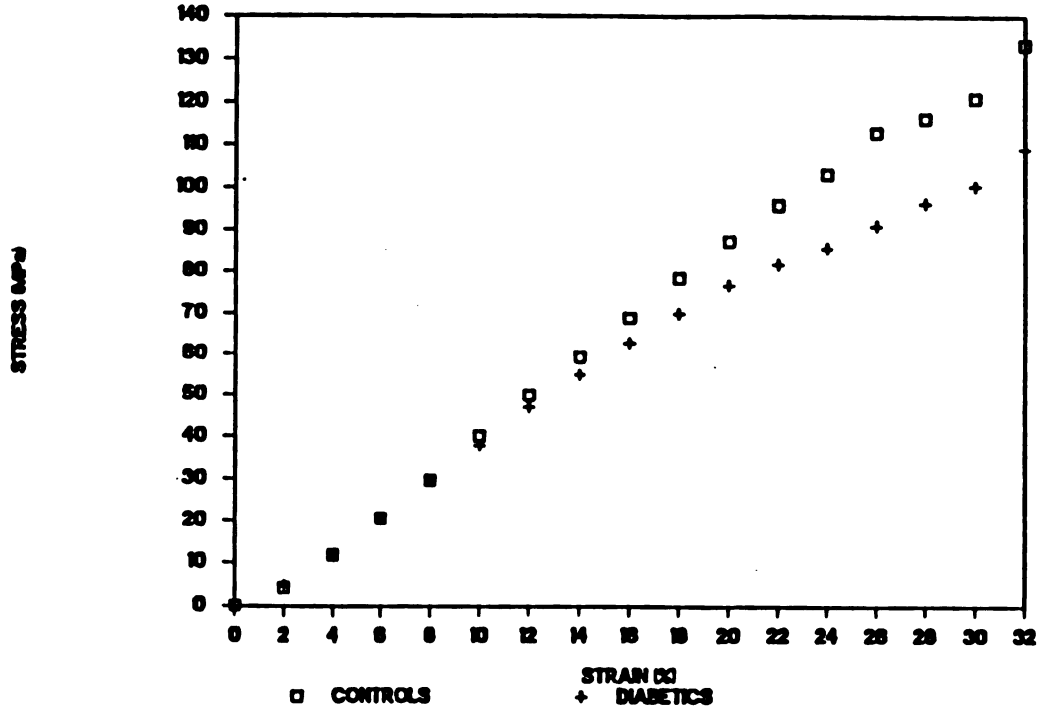


Figure 22. Composite stress-strain curve.

BIOCHEMICAL PROPERTIES

Three fractions were assayed from tissue aliquots of diabetic and control tendon. Total collagen content was also determined in separate aliquots. Small and sometimes non-existent traces of acid soluble fractions were found, indicating that there was very small amounts of newly formed collagen within these tendons. These data were not reported further in this document. Collagen concentrations soluble and insoluble to pepsin, as well as total collagen content, were determined and evaluated as a function of age and diabetes.

The pepsin digestable fraction of collagen was an indicator of the quantity of collagen which was more recently synthesized. It was obvious (as shown in Figure 23) that the solubility of collagen to pepsin decreased significantly ($P<0.05$) with age, and there was a strong correlation of the data to the regression line ($r=.74$). While there was no statistically significant difference ($P=0.5$) between the diabetics and controls, diabetic collagen appeared to become less soluble at a faster rate than controls.

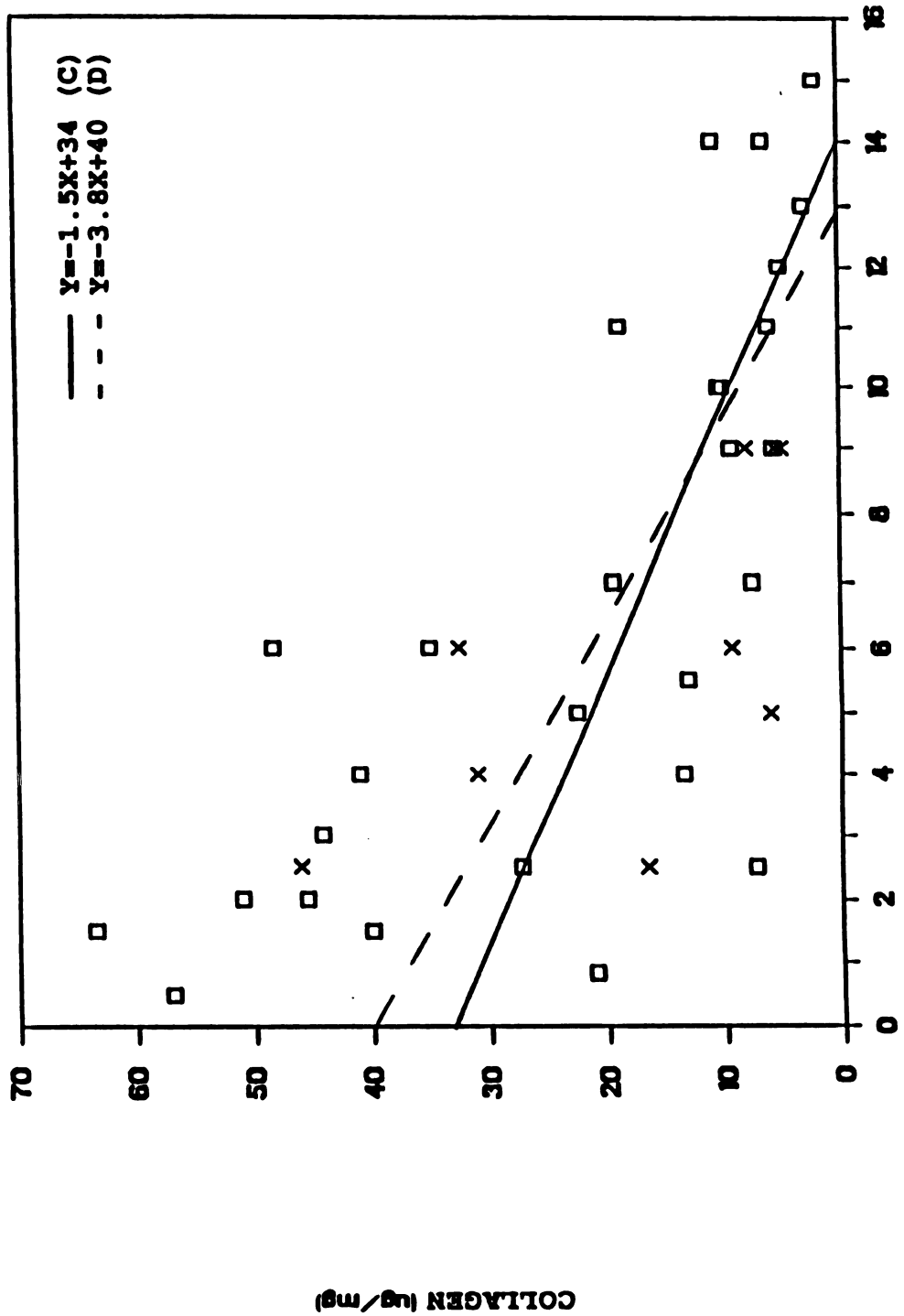


Figure 23. Pepsin soluble fraction of collagen as a function of age.

There was a significant increase in fraction of collagen insoluble to pepsin with age ($P<0.05$) along with good correlation of the data to the regression line ($r=0.62$) (Figure 24). There was no significant difference ($P=0.2$) between the diabetic and control tendons, however, all but one experimental diabetic fell above the regression line of the controls. This suggests that collagen from naturally occurring diabetic tendons may be more cross-linked than controls.

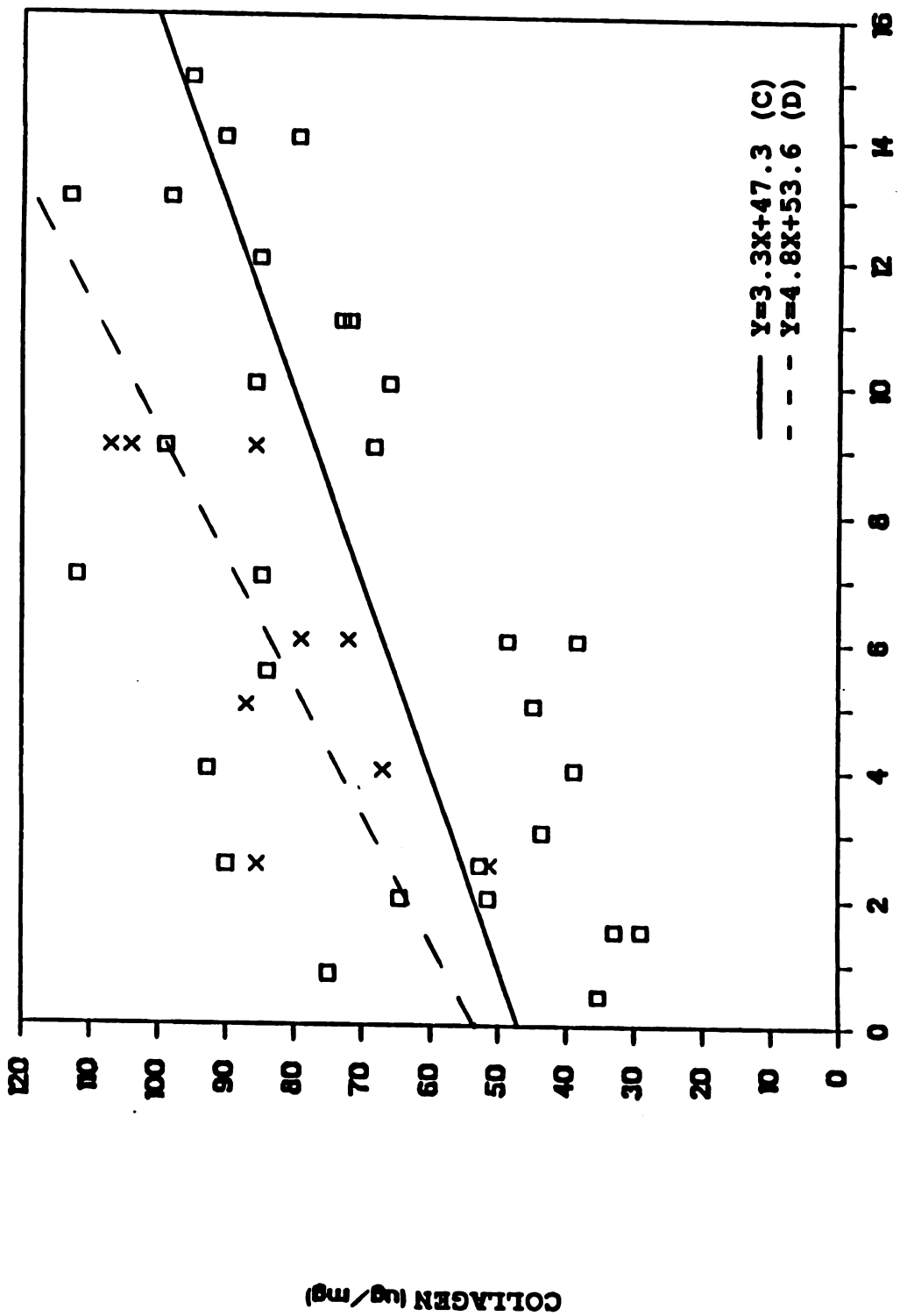


Figure 24. Insoluble fraction of collagen as a function of age

The concentration of total collagen measured in the tendons had no changes with age (P=0.5) or diabetes (P=0.4) (Figure 25). This indicates that the decreased solubility in pepsin of the tendons is compensated by an increased insoluble fraction resulting in little or no change in total collagen content of patellar tendons with increasing age.

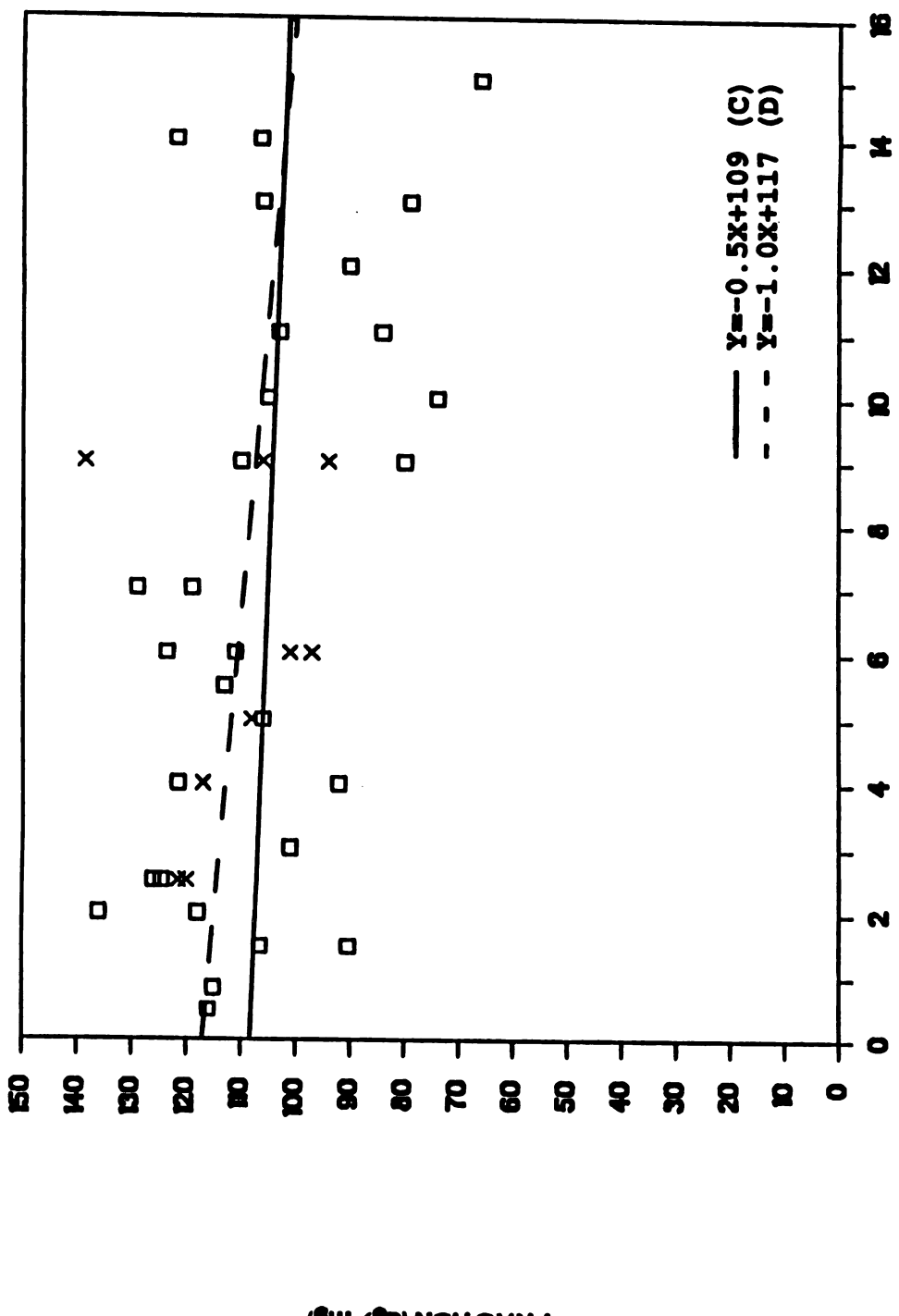


Figure 25. Total collagen content as a function of age.

THERMAL SHRINKAGE PROPERTIES

A limited number of tendon samples were used to obtain information on the thermal stability of collagen. Linear regression analyses were performed on the thermal shrinkage temperatures of control and diabetic tendon (Figures 26 & 27). The analysis of control tendon temperatures showed a significant (P=0.004) decrease occurred with age. Visual inspection of Figure 26 illustrates that there may have been less change in the shrinkage temperature with age than was statistically determined. There was moderate correlation of the data to the regression line (r=0.53). Diabetic tendon, on the other hand, had significantly (P=0.05) increasing shrinkage temperature with age, and also had moderate correlation to the regression line (r=0.50). The above changes with age resulted in a significant difference (P=0.00) between diabetic and age-matched control tendon.

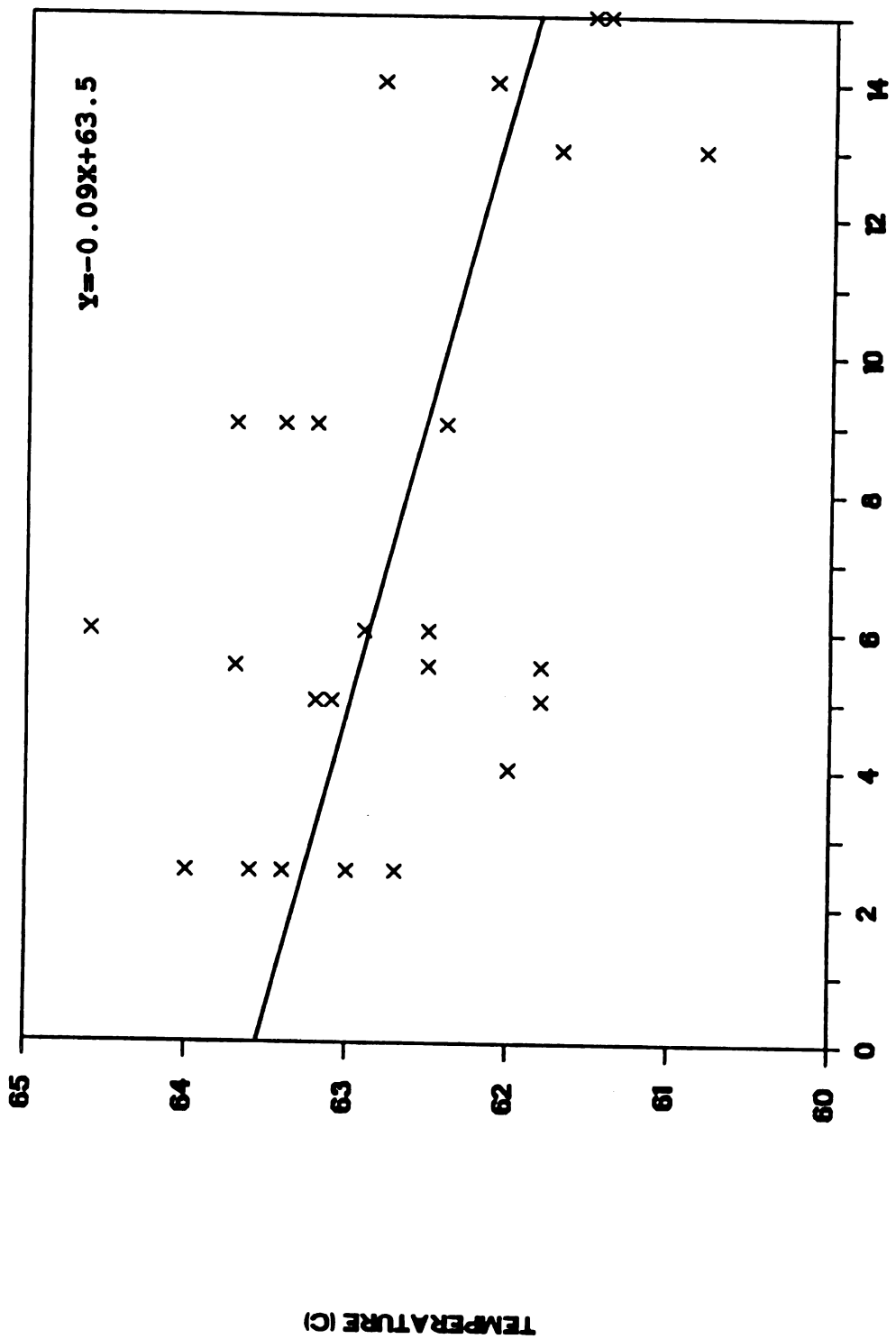


Figure 26. Control tendon Ts as a function of age.

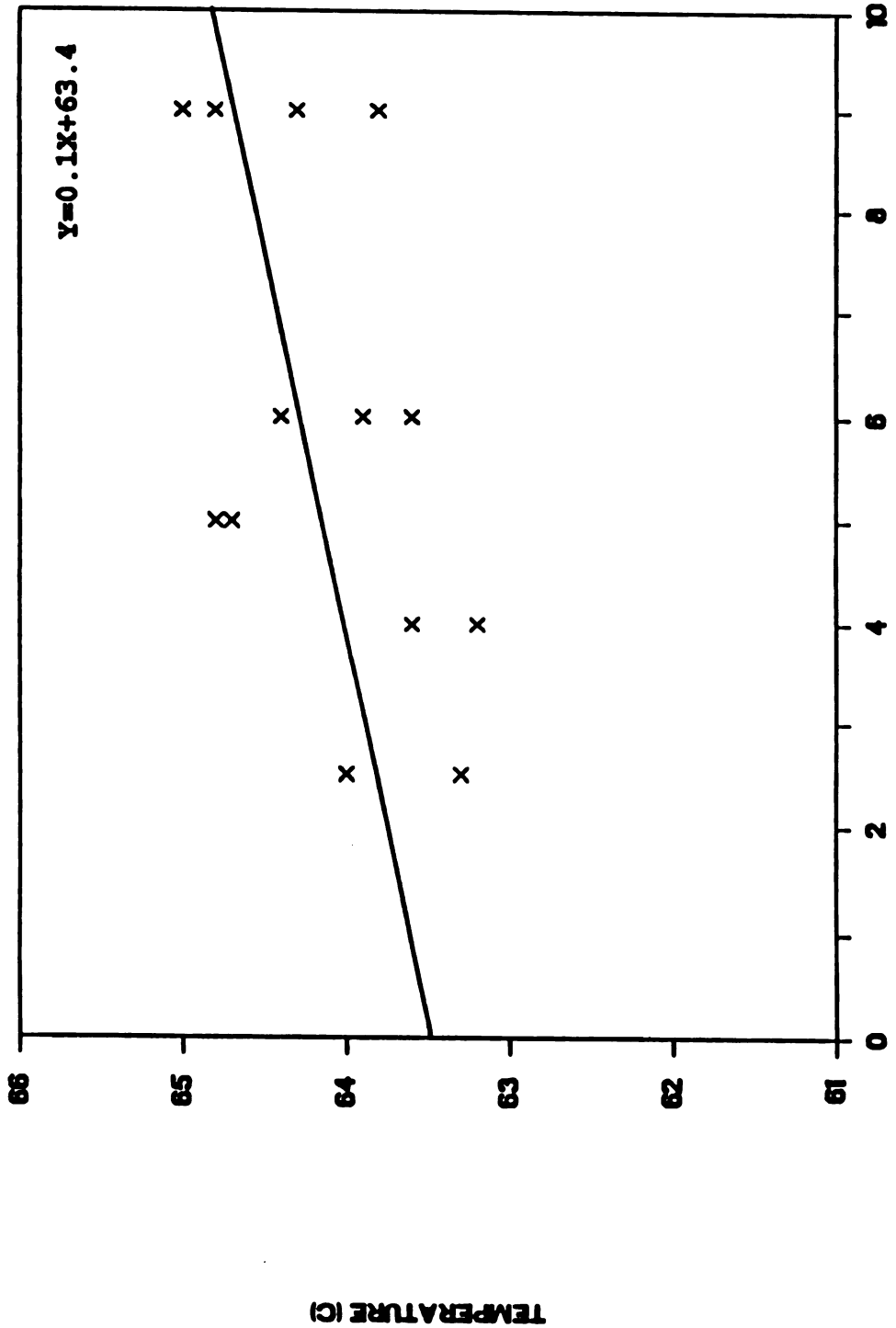


Figure 27. Diabetic tendon Ts as a function of age.

During the HIT preparation it was difficult to separate the tendon into individual fascicles. As a result a larger then desired strip of tissue was often tested. While there was no significant dependence of the primary (Sl1, $P>0.2$) or secondary (Sl2, $P>0.2$) slopes on either diabetes or age (Table 3), some trends were observed in the data. The experimental diabetic (2.5 year old) had lower or more gradual secondary slopes than controls ($P<0.05$). Four and five year old naturally occurring diabetics had nearly equal slopes as controls. Two tailed paired t-tests were used to determine that no significant differences occurred between diabetic and control slopes ($P>0.05$).

Table 3. Thermal shrinkage slope data.

	*CONTROL		*DIABETIC	
Age (y)	S11	S12	S11	S12
2.5	9.12	1.40	2.00	0.96
2.5	4.08	1.17	2.11	0.85
2.5	1.59	1.17		
2.5	2.08	1.61		
2.5	2.44	0.93		
2.5	1.92	1.19		
4.0	1.73	1.50	1.67	1.68
4.0	1.48	1.18	1.54	1.21
5.0	2.10	1.15	1.61	1.20
5.0	2.08	1.32	2.94	1.29
5.0	1.21	1.13		
5.5	2.00	1.52		
5.5	1.80	1.46		
5.5	1.53	1.00		
6.0	2.36	1.14	1.67	1.05
6.0	0.59	0.36	1.85	1.16
6.0	3.23	0.86	2.49	1.49
6.0			1.75	1.21
9.0	3.23	0.96	1.75	1.21
9.0	2.08	0.85	1.97	0.99
9.0	1.43	0.79	1.97	0.91
9.0	3.68	1.14	1.79	1.16
9.0	2.64	0.85		
15.0	2.14	0.99		
15.0	1.85	1.30		

* All slope values are times 10^{-2} (N/mg ^{o}C)

MORPHOLOGY

Photographs from a transmission electron microscope were used to examine collagen fibrils within 2.5, 4, and 9 year old control and diabetic tendons (Figure 28). Computerized digitization of these photographs was utilized to quantify fibril diameters. Frequency histograms illustrated the distribution of fibril diameters within a specimen.

The younger control tendon had a bimodal distribution of large and small diameter fibers which shifted to more uniform and smaller average diameter fibrils in the old tendon

(Figure 29). The 2.5 and 4 year old control tendons had larger average fibril diameters than the 9 year old specimen. While there was no statistically significant ($P>0.05$) difference between the average diameter of the 2.5 year old control and diabetic tendon, there was a clearly defined grouping of smaller diameter fibrils in the diabetic sample. The 4 year old diabetic tendon was significantly ($P<0.05$) smaller than the control and also had an obvious grouping of smaller fibril diameters. There was no significant difference between the average fiber diameter of the 9 year old control and diabetic tendon. It was interesting that the trend for a larger group of small diameter fibrils in diabetics versus controls was not seen at nine years of age.

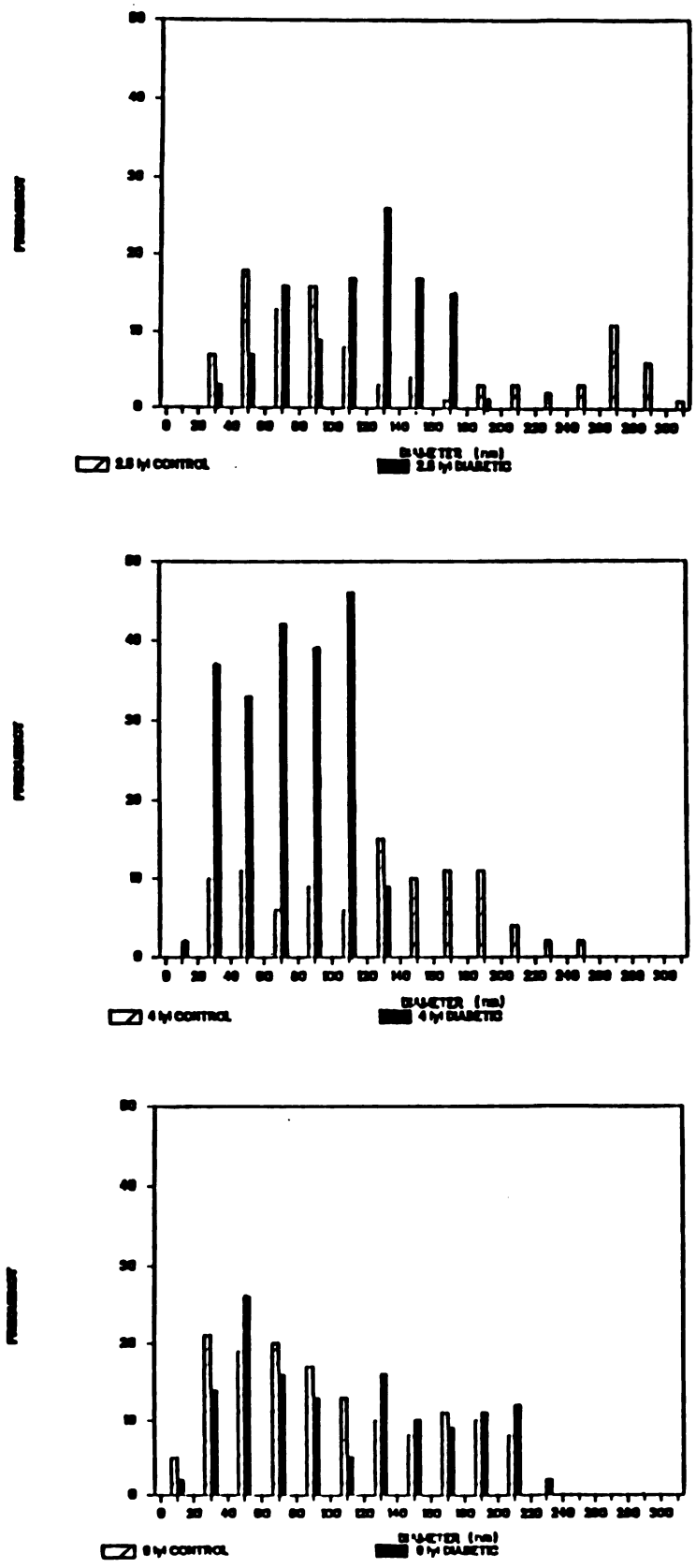
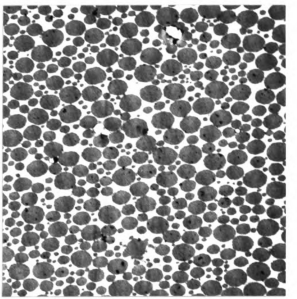
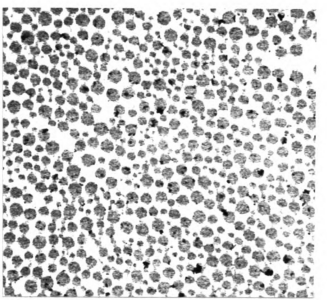


Figure 28. Fibril diameter histograms.



CONTROL



DIABETIC (EXPERIMENTAL)

Figure 29. Fibril diameter TEM - 2.5 year olds.

V. DISCUSSION

The objective of this study was to document alterations in the mechanical, biochemical, and thermal properties of the patellar tendon with age and diabetes using a canine model. Information of this nature is important because of the widespread use of patellar tendon in ligament reconstruction procedures and use of the canine model in diabetes research, as it relates to alterations in the physical properties of musculoskeletal connective tissues.

No particular dependence on age was noted in the geometric properties of the canine PT. This was probably due to the diversity of breeds used in this study. Since these were patients from the Veterinary Clinical Center, activity levels of the dogs were not known and could contribute to wide variations in the dimensions of the patellar tendons.

The cyclic testing was designed to measure properties of the tendon under physiological loading. The inclusive angle in the test fixture of 110^{o} helped simulate the normal configuration in the canine stifle joint. The cyclic load range was estimated to be those in the PT while walking. This resulted in a slight stretch of the tendon. Hubbard et al. (21) examined several different canine tendons and found a rapid change in strain during the first 12 seconds of

cyclic loading which resulted in a measurable amount of hysteresis during that time. Little information is currently available on cyclic or pre-conditioning of tendon as a function of age. Woo et al. (47) collected data on cyclic loading of rabbit knee ligament and found an increase in the area of hysteresis during maturation. Comparable information was not collected during this study. Diabetes and age caused little alteration in the behavior of canine tendon during the initial loading phase as indicated by the constant percentage of creep in both tissue types. The slight increase in physiological stiffness and modulus of the diabetic tendon over controls is consistent with pathologies reported from human diabetic joints. Limited joint mobility is common in diabetes (31).

The large percentage of avulsion failures in this study was different from the numerous midsubstance failures of bone-ACL-bone preparations reported by Vasseur, et al., (42). Assuming that the strength of bone degenerates with age, the slight decrease in failure load may result from a greater occurrence of avulsion fractures at the patella with specimen age. This finding agreed with Grood, et al., (15) in which more avulsion fractures occurred in the human bone-ACL-bone preparation with advancing age. In an earlier study (20), tensile failure tests on patella-PT-tibia preparations exhibited greater occurrence of bone fracture and avulsions for more aged specimens, especially after gamma irradiation sterilization.

In cases of midsubstance failure only, very slight decreases occurred in tensile strength and failure strain energy density of the PT with age. The trend with age compares to Vasseur, et al., (42) for the canine ACL, although to a much lesser extent. In contrast to Vasseur's findings on the ACL, however, the tensile modulus of the PT remains nearly constant or has a slight rise with age. These results are better matched to those of Vasseur for the canine caudate and collateral ligaments. It has been suggested that these ligaments may exhibit essentially no degenerative changes because of a superior blood supply. The extra-articular location of the collateral ligaments may provide significant blood supply from genicular collateral circulation and help provide a mechanism of repair from minor disruptions associated with stresses of normal weight bearing. Canine collateral ligaments may also be preserved because of minimal stress during normal ambulation (27). While actual in vivo loads are not known, physiological forces in the patellar tendon may in fact exceed those within the ACL, yet this results in minimal degeneration of the tendon. The data on age-related changes in the canine patellar tendon follow closely those of Hubbard and Little (22) for human tendons. Interestingly, the data from Vasseur, et al., (42) on the canine ACL parallel those of Grood, et al., (15) for the human ACL.

The tensile fracture of the patella in the majority of diabetic PPT complexes indicates an inherent weakness in the

tensile strength of diabetic bone. This supports earlier studies by Einhorn (13) and Curcione (9) where both experimental and naturally occurring diabetes induced osteopenia and resulted in decreased structural integrity of rat and canine femurs, respectively. It appears that degeneration occurs more rapidly in the more metabolically active bone than tendon. The decreased strength may have resulted from a greater occurrence of bony avulsion failures which in turn caused the decreased failure load in the diabetic PPT complexes.

Alterations different from normal aging have occurred in connective tissue of juvenile diabetic canines. While the material properties of control tendons that failed midsubstance had no changes with age, the tensile modulus of the diabetic tendon just prior to failure appeared to be slightly reduced over age-matched controls. This may, in fact, be a consequence of an incipid failure of underlying bone and a larger percentage of small diameter collagen fibrils in the diabetic tendon. This tends to contradict Andreassen, et al., (5) who reported increased strength in experimentally induced diabetic rat tail tendon after 30 days of disease. Yet, like Andreassen, the current study did show slightly increased physiological stiffness and modulus with diabetes. The current study, however, is in contradiction with Galeski, et al., (14) who reported increased collagen fibril diameters and strength with experimentally induced diabetes.

It is not known exactly what alterations occur in tissues of animals during the onset of diabetes. Perhaps chemical changes occur within the fibroblast cell, such as excessive hydroxylation, that alters the physical make-up of the collagen chains before expulsion into the extracellular medium. Differences noted in the mechanical and thermal stability of experimentally induced diabetic animal models, and the juvenile diabetics examined in this study, may be due to several factors. Studies involving experimentally induced models usually begin with animals which have normal intracellular and extracellular collagen formation. It is not known what alterations in the collagen formation may have already occurred at that point in time, within an age-matched juvenile diabetic animal. In addition, most experimental studies are short term, usually less than six months. Perhaps long term experimental diabetic models would exhibit alterations in tissue properties that more closely match those of the juvenile diabetic. As few differences were noted between the 2.5 year old experimentally induced diabetics and the older juvenile diabetics in the current study, perhaps two years in the hyperglycemic state was enough time to produce comparable degenerative changes.

Biophysical (thermal) and biochemical studies on collagen, the major strength-bearing component of all connective tissues, were performed to help understand mechanisms of age-related change. The documented changes with age of various physical and chemical properties of

collagenous tissues (e.g., decrease in solubility) have been attributed to alterations in the type of covalent cross-linking present in collagen. An interesting result from this study was a moderate decrease in shrinkage temperature of the canine PT with age. Earlier studies on rat tail and human wrist tendon (29), as well as rat skin (1), indicate an increasing thermal stability in the collagen as represented by an increased maximum contraction force and decreased relaxation after that force was reached. This resulted in alterations in the slope of the corresponding isometric tension-temperature curve with age. The shape of the curve did not change significantly with age in the current study. Yet, the slight increase in slope noted in the diabetics with age parallels the decrease in solubility to pepsin, both of which indicates increased mechanical stability over age-matched controls. This is supported by the increased physiological modulus and stiffness of the diabetic tendons over age-matched controls. At the same time there was clearly an increased thermal shrinkage temperature with age for diabetics, in contrast to controls. Controversy exists over the relationship of the amount and nature of covalent cross-linking and the shrinkage temperature of collagen. While the changes in the biochemical nature of human and rat tail tendons (29), rat skin (1), and canine patellar tendons are similar in nature; shrinkage temperatures increased in human tendons and rat tail tendons, remained constant in rat skin, and moderately decreased in canine tendon with age.

Earlier studies have shown that in animals treated with D-penicillamine strips of dorsal skin lose contractile ability (18) due to inhibition in the formation of new intramolecular cross-links and disruption of pre-existing cross-links. In vitro exposure of the skin to the cross-linking agent glutaraldehyde restores shrinkage capacity. Increased concentrations of the fixative lead to an increased shrinkage temperature (18). The slight decrease in shrinkage temperature of the canine patellar tendon with age suggested a decrease in cross-link integrity or density. This may be supported by the decreasing trend in the tensile strength with age. Yet, the amount of collagen insoluble to the enzyme pepsin was increased with specimen age. The results suggest that during normal aging of the canine patellar tendon there was either a decrease in the ratio of thermally stable to labile cross-links that does not parallel changes in pepsin solubility, or alterations occurred in the ordered crystalline structure of the collagen. While increases in the content of insoluble collagen increase with increased tensile strength (45) and probably increased modulus, the modulus of the canine patellar tendons may have remained constant with age because of offsetting changes in integrity of collagen as evidenced by a slight reduction in its biophysical stability.

Biochemically the diabetic tendon resembled advancing age with a more rapid increase in collagen insoluble to pepsin than controls. The small fractions of acid soluble

collagen indicates a lack of newly synthesized collagen in both the diabetic and control tendons. Total collagen concentration in the the diabetics was the same as controls. Biophysically the changes caused by diabetes were somewhat opposite to those of advancing age. A significantly increasing shrinkage temperature in the juvenile diabetic tendon indicated an increasing ratio of thermally stable to labile cross-links, as opposed to the previously mentioned decreasing ratio in controls. Perhaps hyperglycosylation induced by hyperglycemia and continued fluctuations in and out of dehydration during insulin therapy have an as yet unknown influence on the structural integrity of collagen.

One implication of the data from the present study is: since the PT did not degenerate significantly with age, autogeneic and allogeneic grafts from a variety of age groups may provide a acceptably stiff tissue for reconstruction of a torn or otherwise damaged ACL. The assumption, of course, is that age-related changes in the human PT parallel those documented here in the canine PT. The limited data of Haut, et al., (20) would tend to support this statement. On the other hand, these data also suggest relatively less tissue may be needed for reconstruction during advancing age to match changes in the aging ACL. These data also provide information on the mechanical properties of the canine PT to compare with earlier studies on the ACL by Vasseur (42). Differences in the aging properties of these two tissues parallel differences noted by Vasseur et al. for extra versus

intra-articular tissues. The data have also provided new information on correlations of mechanical properties with biophysical and biochemical alterations in collagen. On this issue even more research is suggested.

A second implication from this study is that diabetes mellitus does in fact alter the mechanical, biochemical, and thermal stability of the canine patellar tendon, but maybe not to the extent documented for bone tissue (9,13). This has been likely supported in the current investigation because of the increased incidence of bone fracture in diabetic animals.

Clearly, future research needs to be directed at a better understanding of the characteristics of cross-linking in collagen and correlations with mechanical properties of various connective tissues. Future studies are needed to more fully understand these biophysical, biochemical, and biomechanical properties of collagen in the canine patellar tendon.

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