Acta orthop. Scandinav. 37, 141–155, 1966.

From the Department of Human Anatomy (Head: Professor Bo E. Ingelmark, M.D.)
University of Gothenburg, Sweden.

CHANGES IN TENSILE STRENGTH CHARACTERISTICS AND HISTOLOGY OF RABBIT LIGAMENTS INDUCED BY DIFFERENT MODES OF POSTMORTAL STORAGE

By

A. Viidik and T. Lewin

Received 20.viii.65

INTRODUCTION

When carrying out mechanical investigations on components of the locomotor system it is essential to know if and how the postmortal environment affects the tissues.

In a previous paper from this department (*Viidik, Sandqvist & Mägi* 1965) the basic conditions for biomechanical investigations, especially of the tensile strength, of connective tissue were discussed. Storing the intact joint at room temperature for 4 days did not affect in a statistically significant way the tensile strength characteristics of an intra-articular ligament but its histological picture.

Because it is not always possible to use fresh material or to maintain the conditions mentioned above, investigations concerning the postmortal environment of ligamentous material were extended to the following modes of storage of ligaments without protection of surrounding tissues as in the closed joint:

A. A ligament or a tendon obtained at surgery should be put in a saline solution as soon as possible to prevent drying while waiting for testing.

B. If it is not possible to perform the experiments on the same day, the specimens must be stored in saline in a refrigerator.

For longer periods of storage other methods must be employed in order to avoid possible bacterial decomposition and autolysis:

C. A mode of storage used by some investigators is deepfreezing.

D. Another possibility is embalming in formaldehyde which has been done by other investigators.

The present investigations are concerned with the questions as to whether or not these procedures affect the tensile strength and the histological structure of ligaments or tendons.

LITERATURE

A survey of the major literature on the mechanical properties of soft connective tissues has been given by *Viidik et al.* (1965).

Here only some special points are stressed.

Different investigators have arrived at conflicting results when testing the mechanical properties of connective tissue structures treated (tanned) with formaldehyde. *Highberger* (1947) found a decrease amounting to 65 per cent of the original tensile strength. Similar results were obtained by *Compton* (1949) who tanned fiber bundles from kangaroo tail tendons. These findings are contradictory to those of *Mao & Roddy* (1950) and *Roddy* (1952) who found no changes as compared with untreated fibers, in the strength characteristics of single fibres teased from tanned "gross pieces" kangaroo tail tendons.

According to *Gustavson* (1956) these results can be explained by the theory that the interchain crosslinking produced by tanning agents does not affect the cohesive forces between the larger units because of too wide gaps between the molecular chains. The findings of *Highberger* (1947) and *Compton* (1949) can then be explained by the fact that the single fiber bundles in their experiments are more effectively tanned than those in the "gross-piece"-studies and therefore subjected to "case-hardening".

Bearing in mind that aged connective tissue may be characterized by an increased number of chemical cross-linkings, *Mendoza & Milch* (1964) studied the effect of a number of known cross-linking agents on goatskin corium specimens. They found that some of the agents caused a marked decrease in nominal tensile strength (maximal tensile load per unit original cross sectional area) but others, including formaldehyde, caused no significant changes. Their study can be classified as belonging to the "gross-piece"-group. A good review of cross-linkage and aging is given by *Sinex* (1964).

Tensile strength tests of human Achilles tendons stored in formaldehyde have been made by *Stucke* (1950) who found that their stress-strain curves were more uniform than those of fresh tendons. Although

the final rupture of fresh tendons resulted from successive partial ruptures it occurred more suddenly in formaldehyde-fixed tendons.

METHODS

The testing methods are basically the same as used by *Viidik et al.* (1963) and therefore only a brief account will be given here.

An increasing tensile load was applied to the anterior cruciate ligament from the knee joint of the rabbit. The femur and tibia were attached by contour-shaped clamps to the testing machine and all structures except the anterior cruciate ligament were severed. The load was registered by an ink jet recorder (Siemens Oscillomink) from a tensile load pick-up (Philips PR 9226/02) coupled to a Philips PT 1200 direct reading measuring bridge. The elongation occurring in a ligament during a test was registered by Philips PR 9810 strain gauges, cemented to a steel plate which deflected with elongation, and coupled to a GM 5536 direct reading measuring bridge. The load was measured in kiloponds and the elongation in millimeters. The following parameters were studied: (i) the gross shape of the load-elongation curve, (ii) its slope (tan α), (iii) its failure energy, (iv) failure load, (v) elongation at failure, (vi) dips n the load curve and (vi) failure site on the bone-ligament-bone preparation.

The gross shape of the curves was subjected to cursory inspection. The slope of the load-elongation curve in the first linear portion was calculated by the formula for tan α for a straight line. The failure energy was calculated as the area beneath the plotted load-elongation curve which was measured with a planimeter. The remaining values were read from the recordings.

Standard statistical methods were employed. Differences between the control group and the four treated groups were sought by means of Student's t-tests one by one (slope of the load-elongation curve, failure energy, failure load, elongation at rupture and the frequency of dips in the load curve). The rupture site distributions were subjected to Fischer's exact test.

The ligaments and their bony attachments were also subjected to histological investigation, using standard methods, utilizing decalcification in formic acid, paraffin embedding and Ehrlich's haematoxyline stain.

MATERIALS

The material used in this investigation consisted of 32 ligaments from the knees of 16 rabbits. The material was divided into 4 groups of 8 knees each and treated as follows: (1) one group of knees was stored in 0.9 per cent saline solution at +20° C for 5 hours before testing with the joint opened to expose the ligament to the saline solution and to simulate conditions between excision at operation and testing; (2) a second group of knees was stored the same way for 24 hours at +4° C; (3) a third group with the joint intact was stored at —20° C for a week and then rapidly thawed out in water at +37° C; and (4) the fourth group was stored in 10 per cent formaldehyde for 6 days with the ligament exposed to the fluid. The control material consisted of the same 14 ligamentous preparations used by *Viidik et al.* (1965). All rabbits were subjected to X-ray examination and only adult animals, as revealed by fusion of the proximal tibial epiphysis, were used.

Eleven supplementary ligament preparations were weighed immediately after excision and then stored in saline like those in group B. After removal of surface water the preparations were weighed again, and, following vacuum desiccation, determinations were made of the dry weights.

RESULTS OF THE BIOMECHANICAL TESTS

The mean values and standard errors together with statistical analysis of the different parameters are given in Tables 1 and 2. The values of the single observations are given in Figure 1.

All differences in the following account are understood to be statistically significant at the 5 per cent level of confidence and refer to the control group.

*Table 1. Main table for tan α, failure energy, failure load, elongation at failure and body weight. * denotes a significant difference from the controls (N).*

Group	n	Mean values ± standard error				
		tan α	Failure energy kpmm	Failure load kp	Elongation at failure mm	Body weight kg
N	14	1.53 ± 0.07	36.0 ± 5.9	26.9 ± 1.1	1.74 ± 0.07	3.04
A	8	1.73 ± 0.21	70.8 ± 7.3*	40.3 ± 2.8*	2.94 ± 0.31*	3.36
B	8	1.18 ± 0.08*	46.0 ± 4.0	27.4 ± 2.6	2.11 ± 0.22	3.41
C	8	1.81 ± 0.15	53.2 ± 4.9*	33.2 ± 3.0	1.71 ± 0.17	2.75
D	8	1.06 ± 0.12*	23.9 ± 3.1	19.5 ± 1.9*	1.56 ± 0.14	2.91

*Table 2. Frequencey of dips in the curves and their magnitude together with the number of pure ligament ruptures in the different groups(the rest of the preparations are understood to have failed as femoral or tibial tear-off fractures). * denotes a significant difference from the controls (N).*

Group	No. of curves	Dips in the curves					No. of ligament ruptures
		Total no. per group	Frequency per curve	Magnitude in kp			
				min.	mean	max.	
N	14	6	0.4	0.5	1.1	2	3
A	8	23	2.9*	0.5	1.7	9	0
B	8	18	2.3*	0.5	1.7	8	3
C	8	21	2.6*	0.5	1.8	5	5
D	8	15	1.9	0.5	1.4	5	0

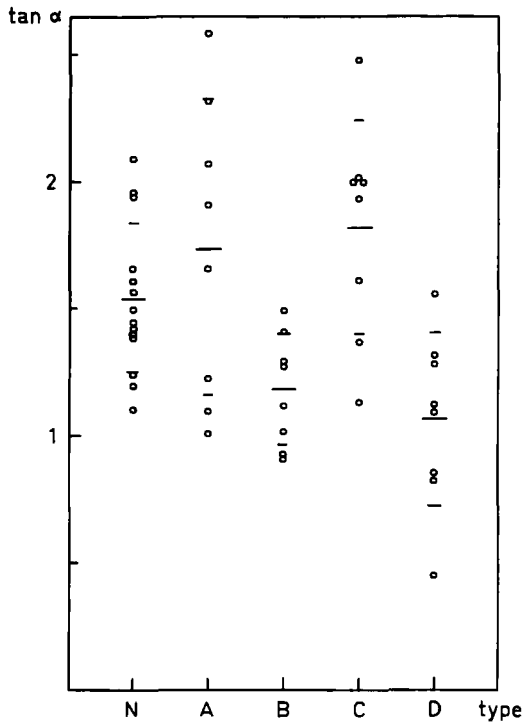

Figure 1 a. Tan α. —— denotes the mean value of a parameter in a time group and – the corresponding S.D. limits.

A. *Storage in 0.9 per Cent Saline at +20° C for 5 Hours*

The gross shape of the load-elongation curves did not differ from those of the control group and the tan α values showed no alteration. The failure energy was higher in this group as was the failure load and the elongation at failure. There was also a higher frequency of dips, but the distribution of the site of rupture was the same.

B. *Storage in 0.9 per Cent Saline at +4° C for 24 Hours*

The gross shape of the load-elongation curves showed no peculiarities, but the tan α values were lower in this group. The failure energy, the failure load and the elongation at rupture did not display any aberrations. There was a higher frequency of dips but the rupture site distribution was unaffected.

In the ligament preparations not subjected to tensile strength tests, the water content expressed in per cent of the dry weights was 146

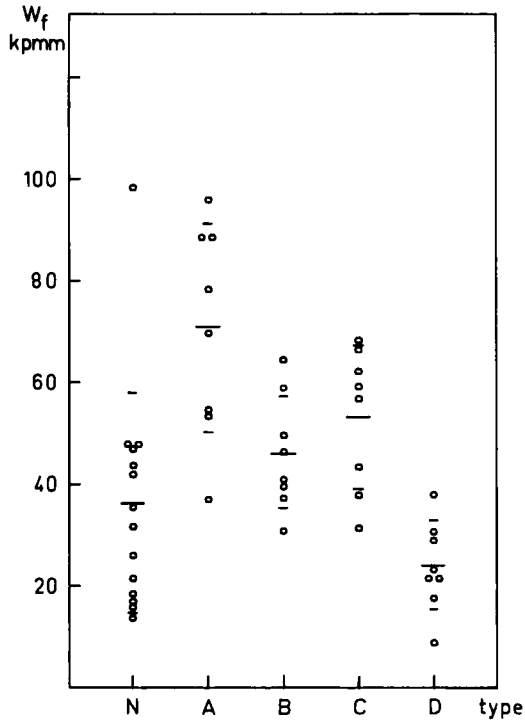

Figure 1 b. Failure energy = W_f. —— denotes the mean value of a parameter in a time group and – the corresponding S.D. limits.

± 12. After storage like the other preparations in this group the water content increased to 298 ± 39 per cent. This is an increase of 60 ± 9 per cent of the weight after excision (t = 6.534) which is significant.

C. *Deep-frozen Storage*

No variation in the gross shape of the load-elongation curves occurred. The tan α, failure load and elongation at rupture values did not differ from those in the control group. The failure energy was higher and there were more dips in the curves. Although there were many ligamentous ruptures in this group, there was no significant difference compared with the control group.

D. *Storage in 10 per Cent Formaldehyde*

The gross shape of the load-elongation curves was unaltered. The tan α and failure load values were lower. The failure energy and the elongation at failure displayed no differences. There were not signifi-

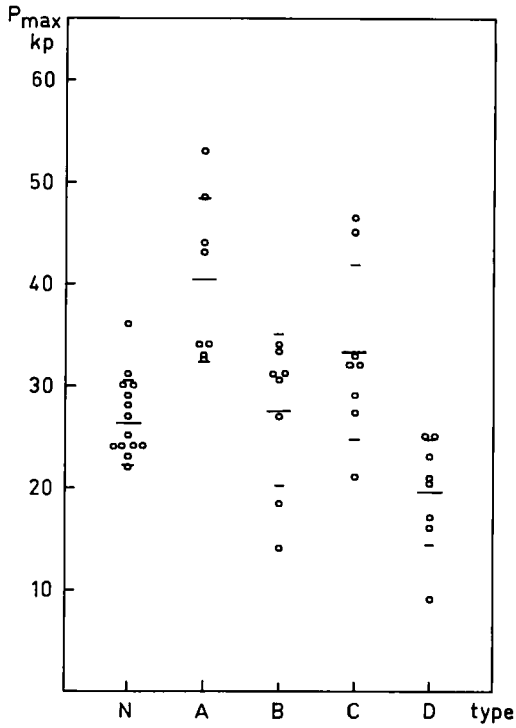

Figure 1 c. Failure load = P_{max}. —— denotes the mean value of a parameter in a time group and – the corresponding S.D. limits.

cantly more dips in the curves and no differences were found in the rupture site distribution.

HISTOLOGICAL INVESTIGATION

The histological part of the investigation was restricted to posterior cruciate ligaments stored in various ways while the anterior cruciate ligaments were subjected to tensile strength tests as well. The following account summarizes the characteristic features in the histological picture as displayed in a majority of sections in the groups.

We were not able to find conclusive differences between the features of the collagenous fibres, the ground substance and the fibrocytes in the different groups.

However, in the majority of slides from preparations stored in formaldehyde the fibrocytes were more heavily stained than in the deep-frozen ones and especially those stored in 0.9 per cent saline. In the latter group the fibrocytes in the more superficial layers of the liga-

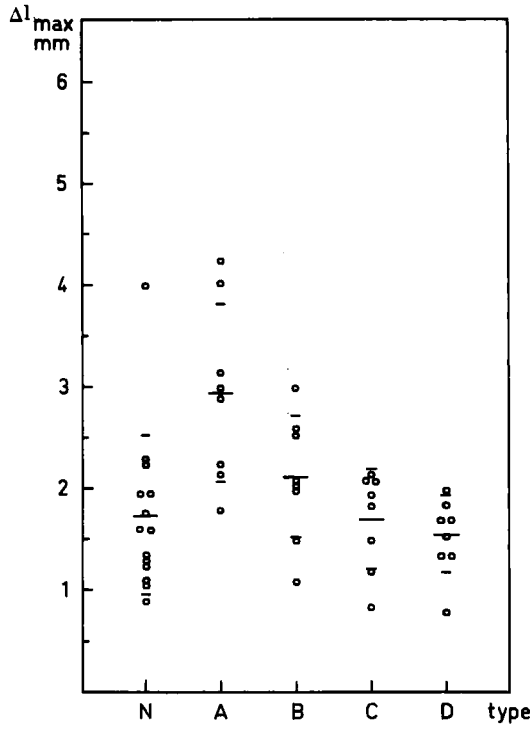

Figure 1 d. Elongation at failure = Δl_{max}. —— denotes the mean value of a parameter in a time group and – the corresponding S.D. limits.

ments had vanished or their loci were represented by darker areas (Figures 2–4).

The collagenous fibres in the formaldehyde-treated group formed easily demonstrable parallel bundles interspersed by rows of ellipsoid fibrocytes. The same was generally true for the deep-frozen ligaments. Ligaments stored in saline, on the other hand, often displayed bundles with no sharp contours. But even here the parallel arrangement of fibres was evident. (Figures 5, 6).

Compared with the preparations in the time factor study by *Viidik et al.* (1965), the features in the group stored in formaldehyde correspond well with the 0-hour group. The deep-frozen ligaments resemble those in the 2-hour group while the ligaments stored in saline display features similar to those in the 2- and 6-hour groups (Figure 7).

The ligaments subjected to tensile strength tests show more frequent and more extensive partial ruptures in the deep-frozen and the saline-treated groups than in the formaldehyde-treated one.

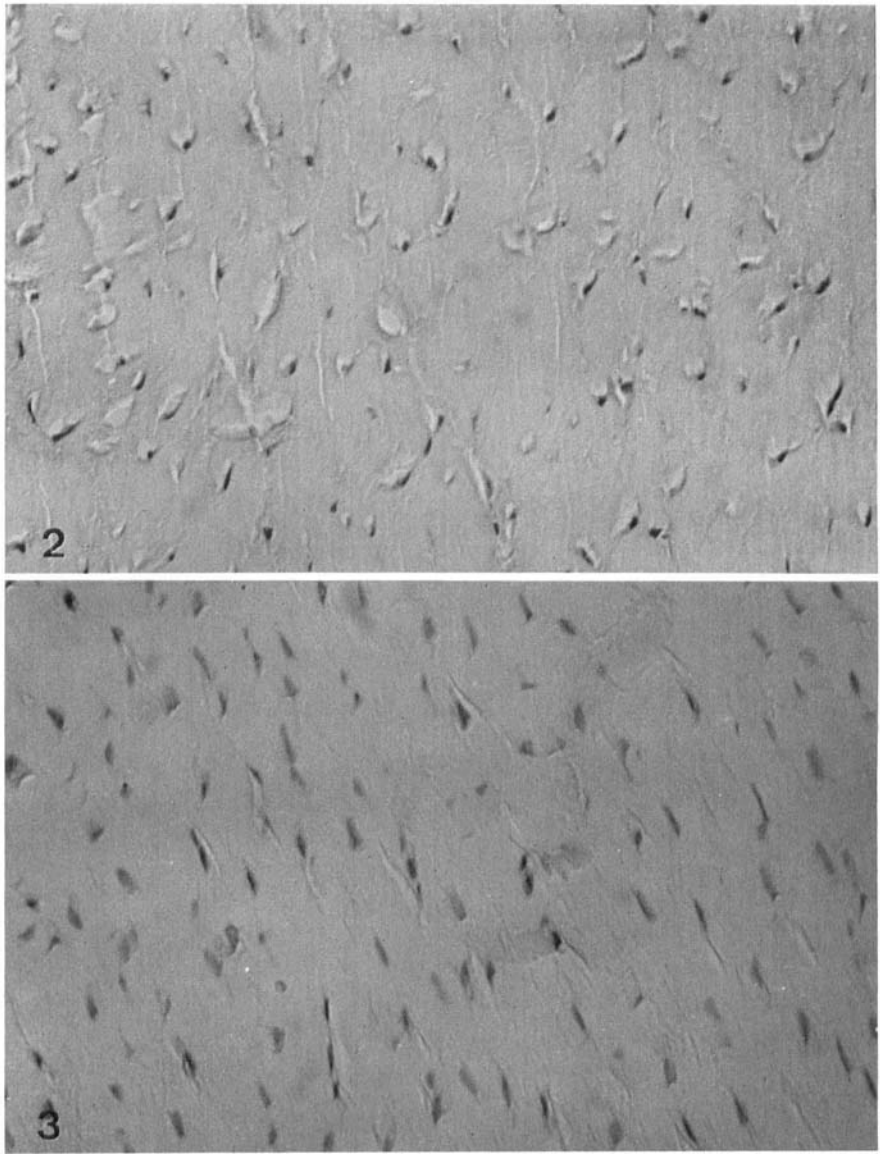

Figure 2. Section from a deep-frozen ligament. The fibrocytes are preserved but are less well stained (363 ×).

Figure 3. Section from ligament stored in formaldehyde. The fibrocytes are well preserved and well stained. The collagen bundles are coherent (363 ×).

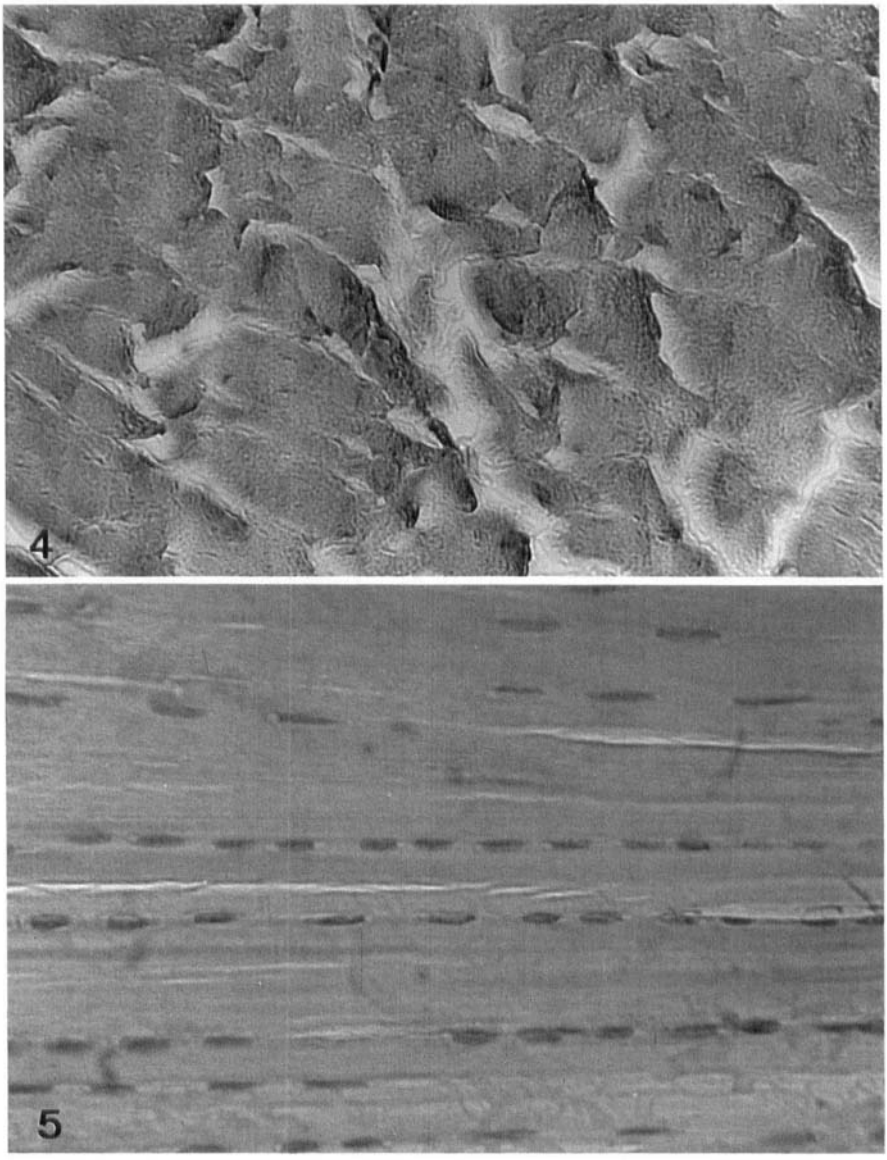

Figure 4. Section from a ligament stored for 24 hours in saline. Most of the fibrocytes have vanished. The collagenous bundles seem swollen and less coherent. 363 ×).

Figure 5. A longitudinal section from a ligament stored in formaldehyde displaying the orderly arrangement and the ellipsoid fibrocytes. (363 ×).

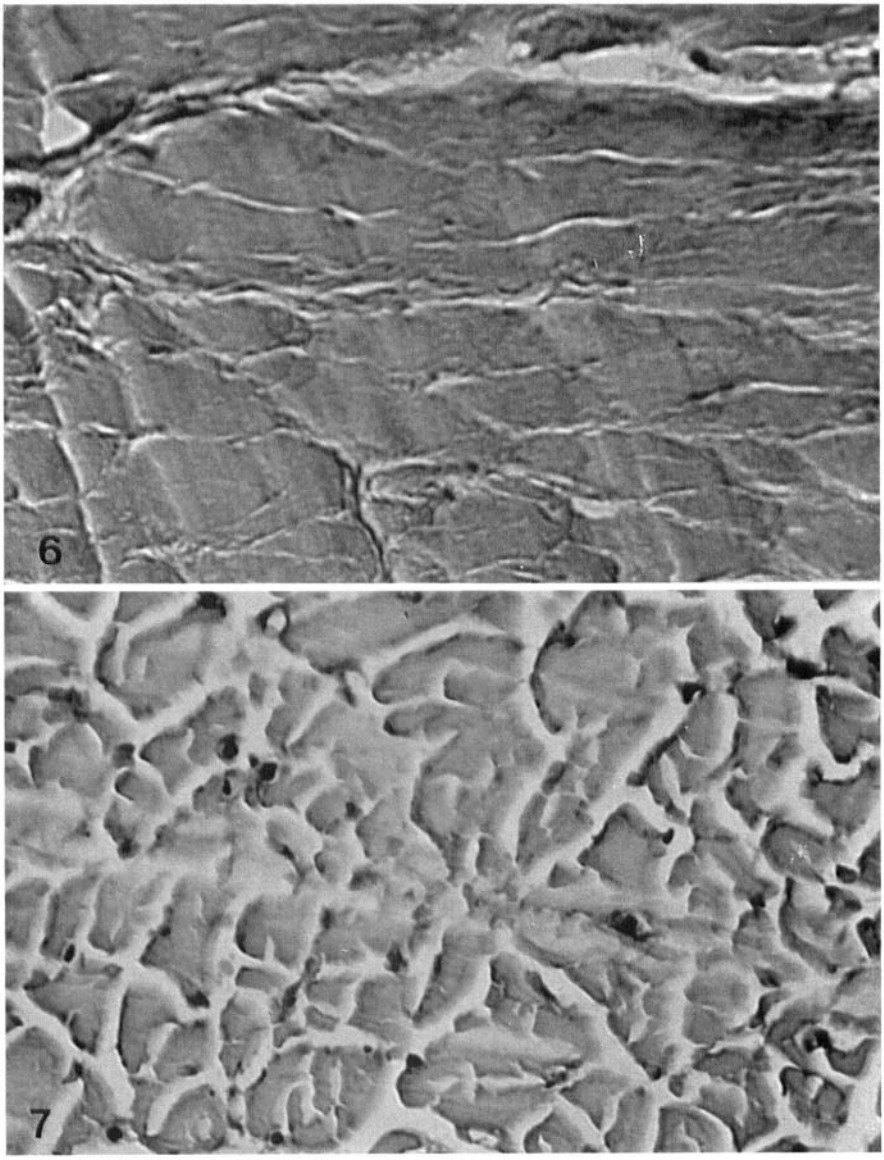

Figure 6. Section from a ligament stored in saline for 24 hours. Cf. Figure 5. (363 ×).

Figure 7. Transverse section from a ligament stored in an intact knee joint for 24 hours (467 ×) [*from* Viidik et al. *1965*].

DISCUSSION

When performing biomechanical experiments it is essential to evaluate basic factors acting on the tissue investigated. In a previous paper from this department *Viidik et al.* (1965) investigated the influence of postmortal changes on the tensile strength characteristics as well as on the histological appearance of intraarticular ligaments in intact rabbit knee joints stored at room temperature. Although changes in the histological picture appeared after 6 hours the tensile strength was not extended this study feeling that it is not always possible to adhere to the conditions mentioned above, especially when using human tissues obtained at surgery and having in mind that earlier investigatiors have utilized different modes of storage. The aim of the present investigation was to search for suitable methods of storage. Storage in saline at room temperature (+20° C) for 5 hours and in a refrigerator (+4° C) for 24 hours, in a deepfrezer (—20° C) and finally in 10 per cent formaldehyde were tested.

The groups stored in 0.9 per cent saline for 5 and 24 hours deviated significantly in important parameters from the control group. As this was not true for the 6- and 24-hours groups in the above-mentioned investigation (*Viidik et al.* 1965), the saline must in some way have altered the tensile strength characteristics of the collagenous ligaments. Investigators of the chemistry of collagen have used different concentrations of sodium chloride to alter the chemical behaviour of collagen but here we refrained from guesses as no chemical studies were included in the present investigation.

In surgical practice saline is considered to be mild to different tissues and is frequently used as a moistener. As judged by the biomechanical tests it affects the tensile strength of collagenous tissue considerably in biopsy and autopsy specimens and it does not prevent imbibition of water by the tissues.

Besides the above-mentioned possibility of solubilisation of collagen the imbibition of water must be considered. According to *Elden et al.* (1962) the mechanical stability of soft connective tissue is dependent on a balance of cohesive and dispersive forces. They consider the swelling to be mainly the result of osmotic pressure, one of the dispersive forces.

Therefore 0.9 per cent saline is not a mild and non-harming agent with regard to the mechanical properties of connective tissue, its water content, its structure and its stainability in histological investigation.

The same is applicable to the deep-frozen group, where the water crystallization and thawing may be the interfering factor.

Storing or rather tanning in formaldehyde certainly affects the molecular structure of collagen as was evident from the significant deviations from the control group in our series.

The conclusion from this investigation is that mechanical tests of collagenous tissue removed at surgery and without protection of the surrounding tissue must be done immediately. If this is not feasible some adequate method of storage must be developed. Possibly some liquid like blood plasma or synovial fluid providing low exchange gradients of different molecules can be used.

When carrying out histological investigations the material must be fixed immediately after removal at surgery if evaluation of finer cellular structures, stainability, etc. is essential.

SUMMARY

The influence of the following modes of postmortal storage on tensile strength characteristics and histology of rabbit ligaments were studied: (A) in saline for 5 hours +20° C; (B) in saline for 24 hours +4° C; (C) deep-freezing; (D) in 10 per cent formaldehyde. In each group 8 knees were included. The fresh control material consisted of 14 knees.

The following parameters were employed: (i) the gross shape of the load-elongation curve, (ii) its slope ($\tan \alpha$), (iii) its failure energy, (iv) failure load, (v) elongation at failure, (vi) dips in the recorder's load curve, and (vii) failure site on the bone-ligament-bone preparation.

Standard histological methods were used.

None of the modes of storage proved satisfactory for the preservation of the tensile strength characteristics of the fresh soft connective tissue.

Only storing in formaldehyde (in fact fixing in it) proved satisfactory for histological studies.

These findings are discussed and it is concluded that biomechanical tests on collagenous tissue removed at surgery must be performed with no time lapse.

RESUME

L'influence des modes suivants de stockage post-mortel sur les caractéristiques de la force de tension et l'histologie des ligaments de lapins a été étudiés: (A) dans l'eau salée pendant 5 heures +20° C; (B) dans l'eau salée pendant 24 heures +4° C; (C) à l'état surgelé; (D) dans 10

pour cent formaldéhyde. Chaque groupe comprenait huit genoux. Le matériel frais de contrôle consistait en 14 genoux.

Les paramètres suivants ont été utilisés: (i) forme approximative de la courbe d'élongation en charge, (ii) son inclinaison (tang. α), (iii) son énergie de rupture, (iv) sa charge de rupture, (v) élongation à la rupture, (vi) chute de la courbe de charge de l'enregistreur et (vii) emplacement de la rupture sur la préparation os-ligament-os.

Des méthodes histologiques ont été utilisées.

Aucune des méthodes de stockage ne s'est montrée satisfaisante pour la conservation des caractéristiques de la force de tension des tissus conjonctifs mous frais.

Seule la conservation dans la formaldéhyde (en fait la fixation dans celle-ci) s'est montrée satisfaisante pour les études histologiques.

Il est discuté de ces trouvailles et il est conclu que le test biomécanique du tissu collagène extirpé chirurgicalement doit être pratiqué sans délai.

ZUSAMMENFASSUNG

Der Einfluss folgender Arten postmortaler Aufbewahrung auf Dehnberkeitsstärke und Histologie von Kaninchenligamenten wurde untersucht: (A) In Salzauflösung für 5 Stunden +20° C, (B) in Salzauflösung für 24 Stunden +4° C, (C) Tieffrierung, (D) in 10 Pro zent Formaldehyd. Jede Gruppe umfasste 8 Kniee. Des frische Kontrollmaterial bestand aus 14 Knieen.

Die folgende Parameter wurden angewendet: (i) Die grobe Form der Belastungs-Verlängerungskurve, (ii) ihr Abfall(Tang. α), (iii) ihre Versagungsenergie, (iv) Versagungsbelastung, (v) Verlängerung bei der Versagung, (vi) Einkerbungen in der Belastungskurve des Rekorders, und (vii) Sitz des Nachgebens am Knochen-Ligament-Knochen Präparat.

Normierte histologische Methoden wurden verwendet.

Diese Befunde werden besprochen und man schliesst, dass biochemische Untersuchungen an kollagenem Gewebe, das anlässlich chirurgischer Eingriffe entfernt wird, ohne Zeitverlust ausgeführt werden müssen.

REFERENCES

Compton, E. D. (1949) Effect of tannage on strength of collagen fibers. *J. Amer. Leath. Chem. Ass.* **44**, 140.

Elden, H. R., Hickey, F. & Boucek, R. J. (1962) Effect of aging on the rate of swelling of rat tail tendons. In: N. W. Shock (ed.): Biological aspects of aging. Columbia University Press,New York, p. 324.

Gustavson, K. H. (1956) The Chemistry and Reactivity of Collagen. Academic Press, New York.

Highberger, J. H. (1947) The structural stability of the collagen fiber in relation to the mechanism of tanning. *J. Amer. Leath. Chem. Ass.* **42**, 493.

Mao, T. J. & Roddy, W. T. (1950) The dry strength of collagen fiber aggregates as influenced by tannery processes. *J. Amer. Leath. Chem. Ass.* **45**, 131.

Mendoza, S. A. & Milch,R. A. (1964) Tensile strength of skin collagen. *Surg. Forum* **15**, 433.

Roddy, W. T. (1952) The dry strength of collagen fiber aggregates. *J. Amer. Leath. Chem. Ass.* **47**, 98.

Sinex, F. M. (1964) Crosslinkage and aging. *Advanc. Geront. Res.* **1**, 165.

Stucke, K. (1950) Über das elastische Verhalten der Achillessehne im Belastungsversuch. *Langenbecks Arch. klin. Chir.* **265**, 579.

Viidik, A., Sandqvist, L. & Mägi, M. (1965) Influence of postmortal storage on tensile strength characteristics and histology of rabbit ligaments. *Acta orthop. scand.*, Suppl. 79.