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LABORATORY CONDITIONS FOR TENSILE TESTS IN ANNULUS FIBROSUS FROM HUMAN INTERVERTEBRAL DISCS

By

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INTRODUCTION

Years ago this laboratory started to construct devices in which parts of the spine were subjected to forces and the relations between vertebrae recorded (1). The deformations of the intervening discs were evaluated and were the subject of further investigations (2, 3).

It was thought that the mechanical response of the disc was closely related to its inner structure. Investigation was then directed towards the measurement of intradiscal pressure both in autopsy material and in living subjects (4, 5).

The correlation between motion and deformation of vertebrae and discs with the forces acting to produce them is at present being studied. Special emphasis is placed on evaluation of mechanical efficiency in lumbar spine fusions (6). An analysis of the factors affecting the mehanical properties of bone has recently been published (7, 8).

This previous work has stimulated our interest in a more detailed study of the individual components of the spine. The annulus fibrosus is now the subject for an investigation of its mechanical response. A number of problems required solution to make this study possible. As samples are removed from the body their surroundings are drastically altered. To the postmortem changes, over which no control is possible in autopsy material, environmental influences are added. The main effect of these, results in alterations in the water content of the samples.

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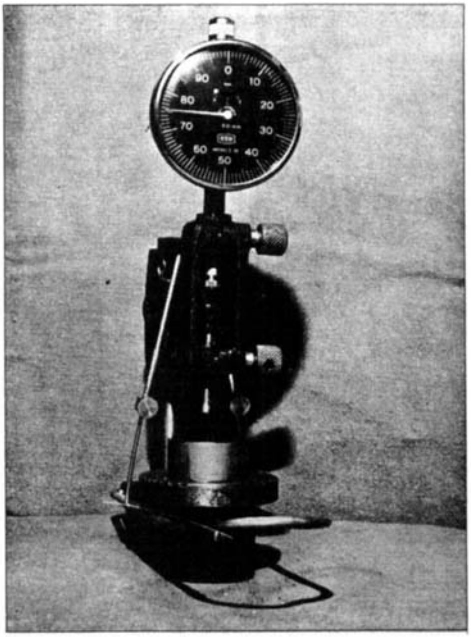

Figure 1. Micrometer used for thickness measurements.

Specimens of reproducible size must be prepared in order to obtain quantitative data from tensile tests. The methods of preparation should not induce changes in the tissue.

The study of two variables (a) methods of preparation and (b) effect of environment on the water content of samples from the annulus fibrosus in regard to tensile behaviour, is the purpose of this presentation.

MATERIAL AND METHODS

The study is based on human material. Autopsy subjects ranging from 20 to 35 years of age with normal intervertebral discs or showing minimal degenerative changes were used. The experiments were performed within 36 hours after death. The lumbar spines with their surrounding muscles and ligaments were placed in polyethylene bags, sealed immediately after removal and stored for short periods at +4° C.

Specimens from the anterior annulus were used. They were cut in samples 1 mm thick in the direction of the concentric sheets of the annulus using a stereo microscope in the early experiments and later with a freezing microtome.

Test samples were stamped out on a specially designed press and die assembly which cut rectangular-shaped specimens 25 × 5 mm. In experiment number 3 a die which cut specimens 25 × 2 mm was used. The long axis of the samples was horizontally placed. These specimens were utilized for comparative, paired ex-

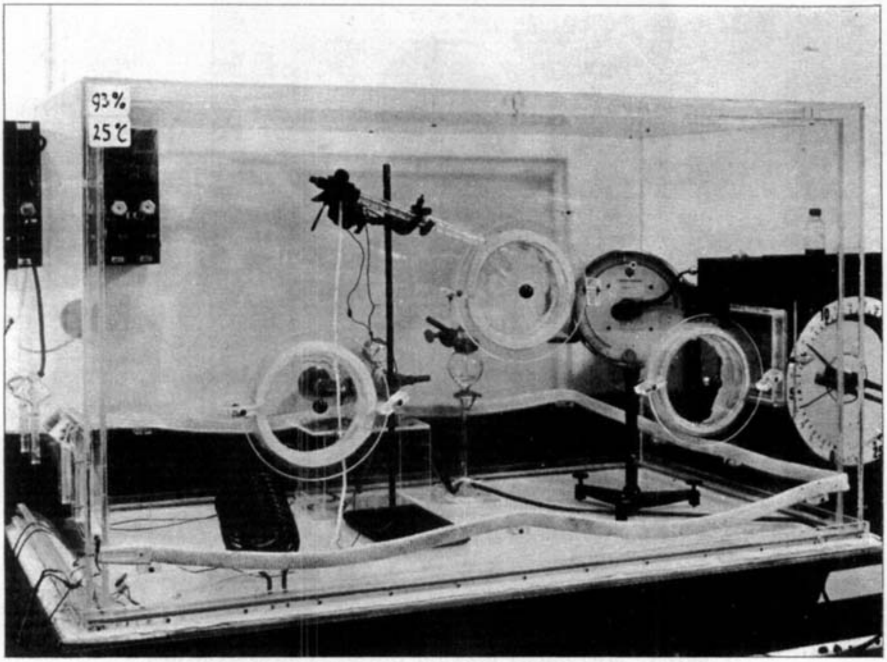

Figure 2. Chamber used in controlled humidity and temperature experiments.

periments to test the effect of variables, where no stress-strain relations were derived.

Thickness measurements were made by means of a specially constructed dial gage designed to measure with a foot pressure of 10 grams distributed over the specimen surface area with an accuracy of ± 0,005 mm (Figure 1).

A. *Swelling experiments.* Specimens were weighed fresh in an analytical balance to an accuracy of ± 0.05 mg and placed in beakers containing the respective solutions at room temperature. They were removed at 5 min., 30 min., 1 hr, 3 hr, 5 hr, 7 hr, 24 hr and 32 hrs and weighed. The surface were blotted for five seconds to remove excess surface liquid before weighing. Dry weights were obtained by oven-drying at 60° C for four days and weighing subsequently.

B. *Air exposure experiments.* Exposure of samples to different atmospheres of controlled temperature and humidity was made in a specially designed box with a double wall to provide dead-space insulation and openings fitted with plastic gloves to allow work inside of the chamber (9).

Humidity was produced by blowing air over a water container and drying accomplished with silica gel. A psychrometer constructed with temperature sensitive diodes was used for humidity control.

Changes in temperature were induced with an electric heater and a cooling coil which circulated tap water. Temperature control was provided by means of a contact thermometer and a relay system.

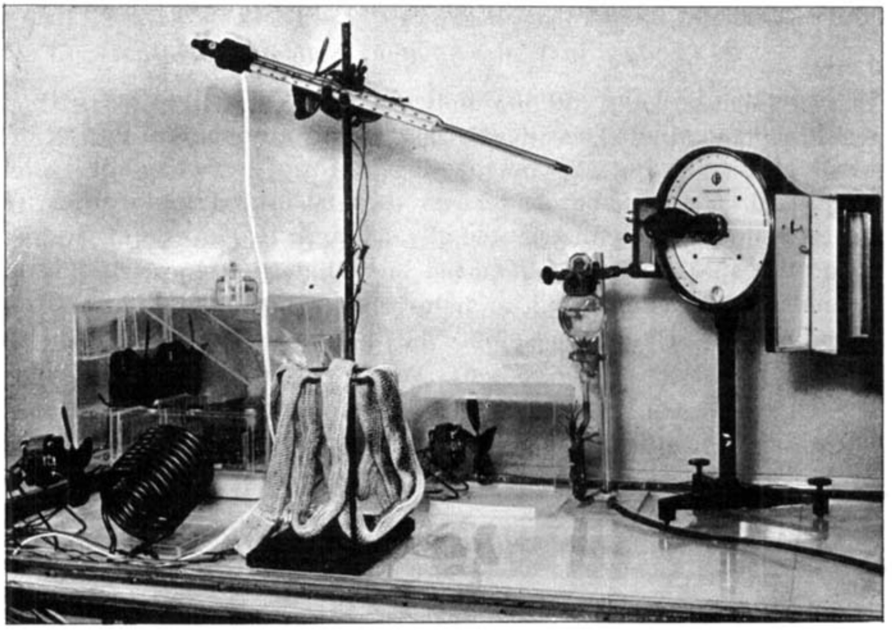

Figure 3. Contents of the chamber.

A torsion balance whose accuracy was ± 0.2 mg was placed inside the box (Figures 2–3).

Samples were tested in groups of five. They were introduced at one minute intervals and weighed initially and every fifteen minutes for one hour.

C. *Tensile test.* Tensile tests were performed on an Instron TTBM, tensile tester, calibrated to an accuracy of ± 0.5 per cent.

Gliding of the specimens from the clamps during application of tension represented a major problem. Special jaw faces were developed with matching serrations at 0.75 mm intervals. These faces were fitted in the Instron model B pneumatic clamps. Tests were performed at 65 per cent relative humidity, 21° C. temperature. Specimens were cycled twice to load of 0.2 kp at rate of elongation of 0.5 cm/m.

From the load elongation diagrams the following values were calculated: Elongation to a 0.2 kp load, residual deformation, and energy dissipation represented by the area under the curves. These data were used only for paired comparisons to test the effects of a variable on the sample. They were not meant for quantitative material evaluation.

STATISTICAL METHODS

Standard statistical methods were used.

The 5 per cent level of significance was applied.

EXPERIMENTS AND RESULTS

1. Changes in Water Content Following Death.

In experiments using autopsy material there is a time lag between death and sampling. To evaluate changes in water content during that period the following experiment was performed. Two adult rabbits were used. Their lumbar and lower thoratic intervertebral discs, ten for each animal, were distributed at random in two groups of ten discs each. Ten specimens were removed immediately after sacrificing the animals. Care was taken not to disturb the ligaments and muscles surrounding the rest of the spine. The incisions were closed and the animals stored at 4° C for 48 hours. At the end of this period the other ten discs were removed. Samples were weighed immediately after removal, vacuum dried for 48 hours and weighed again. Student's test was used to determine the significance of differences in water content between the two groups.

The data is summarized below:

Table 1

Water content of samples	Fresh	48 hours	T test
Wet-dry weight* / Wet weight	0.73675 ± 0.00314	0.73699 ± 0.00633	0.0272 not significant
No. of samples	10	10	

* Wet weight minus dry weight divided by wet weight.

The differences were not significant.

Conclusion: No change was evident in water content of samples within 48 hours after death.

2. Effects of Immersion in Different Solutions.

Prior and during mechanical tests samples from collagenous structures are often immersed in some type of aqueous solution. Connective tissues, however, have a great avidity for water and swelling will occur.

Swelling characteristics of samples from the annulus fibrosus in four different media was investigated: Distilled water, 0.9 per cent sodium chloride solution, human plasma, rheomacrodex.

40 samples from 5 lumbar spines were used. They were divided in

a random fashion in four groups of ten samples each and weighed over a period of 32 hours, after immersion in the respective solutions in room temperature as described previously.

The data is summarized below:

Table 2

Solution	Average Orig. weight gr.	Average swollen weight gr.	No. of samples	% increase
Dist. water	0.10688±0.00596	0.25513±0.02013	10	138.70
0.9 % Na chloride	0.10162±0.00210	0.18710±0.00316	10	80.18
Human plasma	0.09681±0.00210	0.17294±0.00271	10	78.63
Rheomacrodex	0.09200±0.00199	0.19265±0.00663	10	109.40

The difference between original and swollen weights were highly significant in all instances.

In Figure 4 accrued weight has been plotted as a function of accumulated time of swelling.

In all cases the rate of swelling was rapid during the first minutes

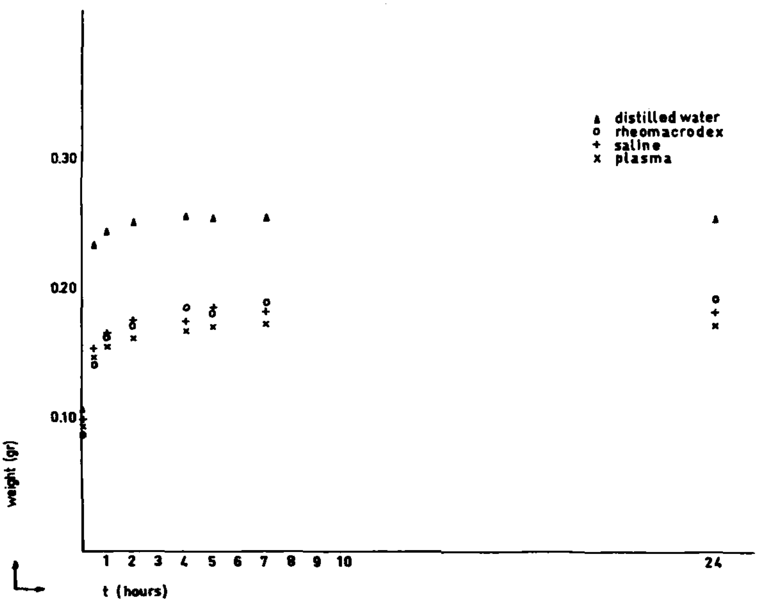

Figure 4. Swelling of samples in different media.

decreasing then progressively until a state of equilibrium was reached. Water uptake was completed within two hours in distilled water and within five hours in the other media investigated.

Conclusions: Considerable swelling was induced in all samples by the different media. Water uptake was higher and faster in distilled water. Concomitant with their gain in weight, the size and shape of the specimens changed so that thickness and width measurements became difficult and inaccurate.

3. Changes in Tensile Characteristics Induced by Swelling.

Eleven samples were prepared from the anterior annulus. Tensile tests were performed in air. Samples were then immersed in 0.9 per cent sodium chloride solution for five hours to allow full swelling and tested again immersed in sodium chloride solution in a plastic receptacle adapted to the clamps of the machine.

The evaluated data is summarized in Table 3.

Table 3

	Elongation mm	Residual deformation mm	Energy dissipation Kp mm	No. of samples
Tested in air	1.132 ± 0.064	0.266 ± 0.022	0.04787 ± 0.00542	11
After swelling in 0.9 % Na. chloride	1.863 ± 0.103	0.560 ± 0.042	0.12091 ± 0.01516	11
T. test	11.147***	6.455***	5.535***	

The differences were significant. The samples in sodium chloride solution exhibited more deformation decreased recoverability and more energy dissipation.

Conclusion: These experiments showed that significant alterations in dimensions and in mechanical characteristics were induced by use of immersion techniques. For this reason they were discarded as a reasonable method of investigation.

4. Exposure of Samples to Controlled Temperature and Humidity.

The purpose of these experiments was to determine if the water content of samples could be kept constant in air.

45 samples from the anterior annulus of discs from three different lumbar spines were removed and distributed at random in nine groups of five samples, each to be tested under one of the following conditions:

Temp. 25° C, rel. humid. 70 per cent, 80 per cent and 90 per cent
Temp. 30° C, rel. humid. 70 per cent, 80 per cent and 90 per cent
Temp. 37° C, rel. humid. 60 per cent, 70 per cent and 80 per cent

For each group, the samples were exposed to the corresponding atmosphere and weighed every 15 minutes during one hour as previously described.

Progressive loss of weight occurred in all cases.

A straight line relation between loss of weight and time was assumed for the 60 minutes interval. A regression coefficient was calculated for each curve and used as a variable in an analysis of variance to test differences between relative humidities at a given temperature.

Table 4

Temp. 25° C	Avge regr. coeff.	± st. err.	% decr. in 60 min.
Rel. humid 70 %	—0.41460	0.01708	11.79
Rel. humid. 80 %	—0.30337	0.00447	5.95
Rel. humid. 90 %	—0.13326	0.00565	2.28

Analysis of Variance for temp. 25° C.

Source of variation	DF	SS	MS	F
Between groups	2	0.20085	0.10042	179.32***
Within groups	12	0.00682	0.00056	
Total	14	0.20767		

The change in values which corresponded to differences of relative humidity at a given temperature were highly significant.

Water was lost at a higher rate in the low humidity sets and at 37° C. Plotting the regression coefficients for each temperature group as a function of relative humidity (Figure 5) it is evident that a zero

Table 5

Temp. 30° C	Avge regr. coeff..	± st. err.	% decr. in 60 min.
Rel. humid. 70 %	—0.41186	0.01319	10.37
Rel. humid. 80 %	—0.30010	0.00199	6.90
Rel. humid. 90 %	—0.13090	0.00565	3.02

Analysis of Variance for temp. 30° C.

Source of variation	DF	SS	MS	F
Between groups	2	0.20009	0.10004	285.85***
Within groups	12	0.00428	0.00035	
Total	14	0.20437		

Temp. 37° C	Avge regr. coeff..	± st. err.	% decr. in 60 min.
Rel. humid. 60 %	—1.04153	0.02502	18.45
Rel. humid. 70 %	—0.61148	0.01922	12.09
Rel. humid. 81 %	—0.32667	0.00623	7.32

Analysis of Variance for temp. 37° C.

Source of variation	DF	SS	MS	F
Between groups	2	1.29813	0.64906	218.53***
Within groups	12	0.03567	0.00297	
Total	14	1.33380		

value, that is to say, no loss of water would be reached at a relative humidity of 100 per cent.

Conclusion: To avoid or minimize the loss of water the preparation and handling of samples must be performed in an atmosphere of 100

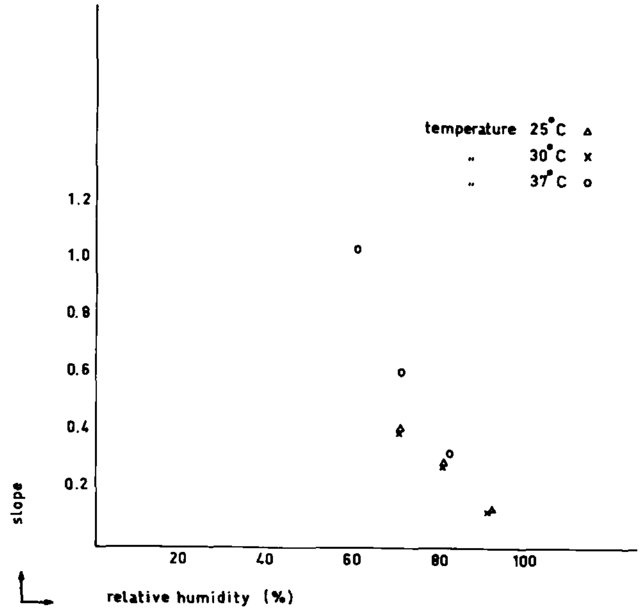

Figure 5. Regression coefficients (slope) of loss of weight vs. time plotted against relative humidity.

per cent relative humidity preferably at a temperature of 25° C or lower.

5. *Effect of Air Drying on Some Tensile Properties.*

For technical reasons it may be difficult to maintain a relative humidity of 100 per cent while performing the tensile tests, or the actual performance of the test may involve a very short period of air exposure and therefore a minimal loss of water.

The following experiment was performed to determine the effect of loss of water on mechanical properties under controlled conditions. Fifteen samples were prepared from the anterior peripheral annulus of four lumbar discs from the same spine.

They were tested in tension in air at a relative humidity of 65 per cent and a temperature of 21° C. The samples were tested within 30 seconds, after ten minutes and after one hour.

The differences were not significant after ten minutes but changes were present in the one hour test.

Conclusion: Samples can be tested at 65 per cent relative humidity at 21° C if the test involves a short period of time. For longer tests, as in stress relaxation, repetitive cycling or creep, a humidity of 100 per cent is necessary.

Table 6

Time of air exposure	Elongation mm	Residual deformation mm	Energy dissipation Kp/mm	No. of samples
30 sec.	1.051 ± 0.071	0.252 ± 0.033	0.0242 ± 0.0018	16
10 min.	1.043 ± 0.058	0.294 ± 0.022	0.0259 ± 0.0012	16
60 min.	0.893 ± 0.114	0.589 ± 0.109	0.0368 ± 0.0030	16
F Test	7.39***	8.73***	16.94***	

The difference between 30 seconds and 10 minutes were not significant; the differences between 30 seconds and 60 minutes were significant.

Table 7

	Elongation mm	Residual deformation mm	Energy dissipation Kp/mm	No. of samples
Fresh	1.352 ± 0.146	0.101 ± 0.012	0.0381 ± 0.0049	10
After freezing	1.217 ± 0.135	0.096 ± 0.010	0.0316 ± 0.0041	10
T test	0.601	0.908	0.900	
	Elongation mm	Residual deformation mm	Energy dissipation Kp/mm	No. of samples
Fresh	1.092 ± 0.090	0.082 ± 0.028	0.0318 ± 0.0029	11
After freezing	1.009 ± 0.094	0.073 ± 0.010	0.0318 ± 0.0029	11
T test	0.619	0.475	–	

6. Effects of Freezing on some Tensile Properties.

To obtain quantitative informations samples of reproducible constant dimensions are essential. It was thought that a freezing microtome could provide specimens of constant thickness.

From two lumbar spines samples were obtained by dissection and divided into two experiments similar in characteristics. The samples were first tested in tension, then frozen under a carbon dioxide snow stream and allowed to thaw in a high humidity atmosphere. They were then retested.

Data is summarized in Table 7.

The differences were not significant.

Conclusion: Freezing under CO_2 snow and thawing at a relative humidity of 100 per cent did not alter the mechanical behaviour of the samples. These experiments justify the use of a freezing microtome for sectioning of the samples.

CONCLUSIONS

In standardizing methods of preparation and testing for the study of mechanical properties of collagenous structures the most difficult problem encountered is to avoid alterations in the water content of the samples.

Water-tissue relations have been postulated as critical in determining deformation characteristics of biological materials (10). Connective tissues have great avidity for water and swelling will result when immersed in aqueous solutions (11, 12). We have shown this process in the annulus fibrosus. The deformation characteristics were significantly altered with swelling. The samples became more extensible, exhibited decreased recovery and increased energy dissipation. Therefore immersion techniques were discarded.

The water content of the annulus fibrosus is high. Pushel reported values between 70 to 78 per cent according to age and degeneration characteristics (13).

Most of this water is loosely bound and for that reason specimens dry rapidly when exposed to air. Their tensile properties are altered under these circumstances (14). The problem is magnified as the sample size decreases. When the sample dimensions are small the environmental factors become critical and must be controlled.

Water vapour diffuses to the specimen surface along a pressure gradient and the slope of this gradient will be a major factor in determining the rate of vapour transfer. This rate is also dependent on temperature. The sample can be coated to retard evaporation, but variables due to the coating material will be introduced. The alternative is to eliminate the pressure gradient providing an atmosphere with a relative humidity of 100 per cent. This investigation has led us to prepare and handle the samples in an atmosphere of 100 per cent relative humidity at a temperature of 21° C.

In this study on human autopsy material no control over postmortal changes was possible. In rabbits knee ligaments, protected from air exposure, Viidik et al found no alterations in mechanical properties up to ninety six hours after death (15). Our study in the intervertebral discs of rabbits showed no alterations in water content forty eight hours following death. These results support the use of autopsy specimens.

Our aim throughout these experiments has been to maintain the water content of the samples at the level present when removed from the body. Although this approach may not resemble the in vivo conditions, where an active water transfer may be present when the tissue deforms, it prevents the highly significant differences induced in the characteristics of the specimens by the immersion methods.

Freezing was used for the preparation of samples. The formation of ice from water is the most important physical change occurring when tissue is carried to low temperature. Injury may then be produced on mechanical or chemical basis and the time element will be of importance (16).

Freezing to the temperature of CO_2 snow (—78° C) and rapid thawing at 100 per cent relative humidity did not alter the tensile characteristics of the samples. Therefore, a freezing microtome was used for sectioning the tissue.

The experimental procedures analysed in this publication provide the background for the methods adopted in the study of mechanical characteristics of the annulus fibrosus.

SUMMARY

A series of experiments is described used to standardize tensile tests in samples from human annulus fibrosus.

The water content of samples in situ remained constant up to 48 hours after death.

Immersion in aqueous solution induced considerable swelling in the samples and significant changes in their tensile behaviour.

Samples exposed to air at different relative humidities and temperatures lost water in all circumstances. Equilibrium was shown to occur at 100 per cent relative humidity.

Mechanical changes induced by air drying at 65 per cent relative humidity temperature 21° C for 10 minutes were not significant.

Freezing under CO_2 snow and rapid thawing at 100 per cent relative humidity under conditions simulating a freezing microtome did not alter the mechanical behaviour of the samples.

RESUME

Il est décrit une série d'expériences tendant à standardiser les tests de résistance à la tension de spécimens d'anneaux fibreux humains du disque intervertèbral. La teneur en eau dans les spécimens in situ reste constante dans les 48 heures qui suivent le décès.

Une immersion dans une solution aqueuse entraîne un gonflement et pas de modifications appréciables du spécimen.

Les spécimens exposés à l'air dans des conditions d'humidité relative et de température différentes perdent en toutes circonstances leur eau. L'équilibre n'est rétabli qu'à 100 pour cent d'humidité relative.

Les modifications mécaniques entraînées par le séchage de l'air dont l'humidité relative est de 65 pour cent et la température 21° C pendant dix minutes sont insignifiantes.

La congélation sous neige CO_2 et le rapide dégel à un taux d'humidité relative de 100 pour cent dans des conditions reproduisant celles d'un microtome congélateur n'ont pas altéré le comportement mécanique des spécimens.

ZUSAMMENFASSUNG

Um eine Standardisierung der Dehnungsuntersuchungen in Proben vom Anulus fibrosus zu erreichen, ist eine Serie von Experimenten gemacht worden.

Der Wassergehalt von Proben, die in situ gelassen worden waren, verblieb während 47 Stunden nach dem Tode konstant.

Eintauchen in Wasserlösungen verursachte eine bedeutende Schwellung der Probestücke mit signifikanten Veränderungen ihrer Stärke.

Probestücke, die in Luft verschiedener Feuchtigkeit und Temperatur exponiert wurden, verloren immer Wasser. Ein Ausgleich wurde bei 100 prozent relativer Feuchtigkeit erreicht.

Mechanische Veränderungen, die während 10 Minuten durch Lufttrocknen bei 65 prozent relativer Feuchtigkeit hervorgerufen worden waren, zeigten keine Signifikanz.

Beim Einfrieren unter CO_2-Schnee und schnellem Auftauen bei 100 prozent relativer Feuchtigkeit—wie es bei Verwendung eines Gefriermikrotoms gemacht wird—wurden die mechanischen Verhältnisse nicht verändert.

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