Thermal Expansion Measurements of Frozen Biological Tissues at Cryogenic Temperatures

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Thermal expansion data are essential for analyses of cryodestruction associated with thermal stresses during cryopreservation protocols as well as during cryosurgery. The present study tests a commonly used hypothesis that the thermal expansion of frozen tissues is similar to that of pure water ice crystals. This study further provides insight into the potential effect of the presence of cryoprotectants on thermal expansion. A new apparatus for thermal strain measurements of frozen biological tissues within a cryogenic temperature range is presented. Results are presented for fresh tissue samples taken from beef muscle, chicken muscle, rabbit muscle, rabbit bone, and pig liver. Pilot studies of the effect of cryoprotectants on thermal expansion are further presented for rabbit muscle immersed in dimethyl sulphoxide (2 mols/l) and glycerol (2 mols/l), and for pig liver perfused with dimethyl sulphoxide (2 mols/l). Thermal expansion of frozen soft biological tissues was found to be similar to that of water ice crystals in the absence of cryoprotectant. Thermal expansion of the rabbit bone was found to be about one half of that of frozen soft tissues. A significant reduction in the thermal expansion at higher temperatures was observed in the presence of cryoprotectants. A rapid change of thermal strain near −100°C was also observed, which is likely to be associated with the glass transition process of the cryoprotectant solutions.

Introduction

The phenomena associated with cryoinjury of biological tissues, due to either cryosurgical or cryopreservation protocols, have been extensively studied during the past few decades (Mazur, 1963; Meryman, 1974; Shlafer, 1977; Fahy et al., 1984; McGrath, 1993). The mechanisms of cryodestruction may generally be separated into two groups depending upon the degree of phase transition. The first group includes destructive mechanisms within the phase transition temperature range, and is related to the dynamics of the freezing/thawing processes. The temperature range of phase transition is dependent on the chemical content of the cryotreated tissue, and on its cooling history (Miller and Mazur, 1976; Gage et al., 1985; Taylor, 1987). When cooling quasi-statically, the phase transition range is typically between 0 to −22°C (assuming that body fluids behave like a $NaCl-H_2O$ mixture). However, the lower boundary of the phase transition decreases as the cooling rate increases due to the departure of the transport phenomena from near-equilibrium conditions; it also decreases when nucleation sources are absent. The lower boundary of phase transition can easily reach −45°C in biological systems (Fahy et al., 1984). Mechanical interaction between ice crystals and cells also enhances the destruction during the phase transition process, causing localized mechanical stresses on cells (Ishiguro and Rubinsky, 1994).

The second group of destruction mechanisms is related to destruction occurring after the phase transition is complete, i.e., in the solid state, and therefore is related to mechanical stress. It has been suggested that elastic deformations resulting from mechanical stresses due to constrained contraction of the frozen tissue (also termed Thermal Stresses) may cause mechanical damage to cell membranes (Rubinsky et al., 1980). It was shown that the thermal stress can easily reach the yield strength of the frozen tissue (Rabin and Steif, 1996), resulting in plastic deformations or fractures. The constrained contraction is driven by temperature gradients, which are developed when the tissue is brought down to the cryogenic temperature range during typical cryosurgical or cryopreservation cooling protocols (Fahy et al., 1990; Hunt et al., 1994; Gao et al., 1995).

In order to gain some insight into the mechanical properties of frozen soft biological tissues, the response of frozen rabbit liver, kidney, and brain to externally applied compressive stresses was investigated (Rabin et al., 1996). It was found that the stiffness of the frozen tissues is of the same order of magnitude as that of sea ice, and that the yield strength of frozen tissues is up to one order of magnitude higher than that of sea ice; where sea ice data are widely available in the literature. Histological changes due to permanent deformations were further studied and it was observed that fractures are often generated, or arrested, in the vicinity of blood vessels or ducts (Rabin et al., 1997).

The driving mechanism for thermal stress is the constrained contraction of the frozen tissue. The free water in fresh tissues usually forms into ice crystals and, therefore, one may assume that frozen biological tissues will have similar characteristics to that of pure water ice crystals during thermal expansion. On the other hand, cryoprotectants, such as dimethyl sulphoxide (DMSO) and glycerol, tend to vitrify, where vitrification is the amorphous solidification of a liquid brought about not by crystallization, but by extreme elevation in viscosity during cooling (Fahy et al., 1984; Angell and Senapati, 1987; Mehl, 1996). Below some critical temperature, the vitrified solution changes phase into a glass, where this temperature is defined

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as the glass transition temperature. This critical temperature is influenced both by the concentration of the dissolved solutes including cryoprotectants and by the rate of cooling. There is experimental evidence that vitrified solutions are more brittle and have a significantly lower thermal expansion value than polycrystalline ice (Archer et al., 1996; Mehl, 1996). The tendency to vitrify increases as the cooling rate and solution concentration increase. Due to the kinetics of phase changes, coexistence of a partly vitrified and a partly crystallized system often exists during attempts to cryopreserve bulky biological systems, such as tissues and organs (MacFarlane, 1987; Fahy et al., 1990).

Together with other physical properties, such as the strength and stiffness, thermal expansion data are required for thermal stress analysis in order to predict the likelihood of fracture formation in frozen tissues during cryopreservation protocol as well as during cryosurgery. It is commonly assumed that the thermal expansion of frozen biological tissues is similar to that of pure water ice crystals (Rubinsky et al., 1980; Rabin and Steif, 1996). The present study tests this hypothesis experimentally and provides some preliminary insight on the effect of the presence of cryoprotectants on thermal expansion. A new apparatus is described for measuring the thermal strain of a frozen biological tissue, in a temperature range between the freezing point and −180°C. Results of expansion tests of fresh tissues from beef muscle, chicken muscle, rabbit muscle, rabbit bone, and pig liver are presented. Results of pilot expansion tests of rabbit muscle permeated by DMSO and glycerol solutions, and pig liver perfused with DMSO solution, are also presented. Results are compared with available data from the literature of the thermal expansion of single ice crystals and polycrystalline ice.

Experimental Setup

The new apparatus for thermal strain measurements is presented schematically in Fig. 1. Tissue sample 9 is hooked by two metallic pins 8 and rings 7 to a telescopic glass tubing, tubes 5 and 6. The hooked tissue sample and the ends of tubes 5 and 6 are placed in a cooling chamber of a temperature-controlled cooling device 10 (Kryo-10, Planer Products, UK). Glass tubes 5 and 6 have an outer diameter of 9 and 4 mm, respectively, and a length of about 800 mm (the reason for the application of such a relative long glass telescopic tubing is discussed below). The tissue sample is placed about 150 mm from the cooling chamber inner wall. Glass tubes 5 and 6 are rigidly connected to a telescopic brass tubing, tubes 3 and 4, respectively, under a pressure fit. The nominal diameter of brass tubes 3 and 4 is 4.76 and 3.97 mm, respectively. Glass tube 6 and brass tube 4 are free to slide axially, inside glass tube 5 and brass tube 3. Analog displacement sensor 1 (Mitutoyo 2358-50) is rigidly connected onto brass tube 3 while its probe, 1a, is brought in contact with plate 2; where the latter is rigidly connected to brass tube 4. The probe of the displacement sensor is spring driven, generating a force of about 70 g, which did not buckle the tissue sample before freezing (the contribution of this load to the measured strain is addressed below). The resolution of the displacement sensor is 1.27×10^{-3} mm, which is about 0.3 percent of the average strain range measured in this study.

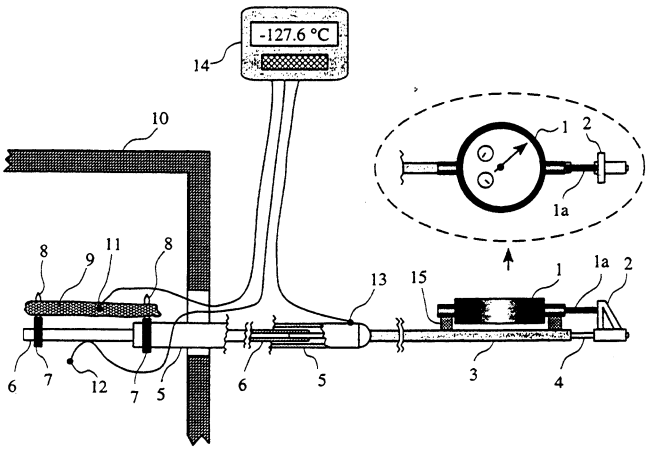

Fig. 1 Schematic description of the experimental apparatus for thermal expansion measurements of frozen biological tissues

The displacement measurement mechanism works as follows: A contraction, or expansion, of the tissue sample 9 causes an axial movement of glass tube 6 with respect to glass tube 5, which in turn, causes an axial movement of brass tube 4 with respect to tube 3. The relative axial movement is transferred via plate 2 and probe 1a to the displacement sensor.

The Kryo-10 device is a temperature-controlled cooler, which allows a precise control of the cooling and the warming rates. The Kryo-10 works in the temperature range between 20°C and −180°C. The chamber temperature is controlled by a balance between an electrical heater and a highly turbulent flow of nitrogen vapors.

Due to the special telescopic configuration, the expansions of tubes 5 and 6 are the same, all along tube 5. Therefore, expansion of the glass tubes affects the displacement reading along the tissue sample length only. Glass was selected as the material for fabrication of tubes 5 and 6 since it has low thermal expansion coefficient and low thermal conductivity. The thermal expansion value of glass is about one order of magnitude smaller than that of biological tissues. Nevertheless, a compensation for the thermal expansion of the glass tubes was taken into account for each experiment, as described in the materials and methods section and discussed in detail in the results and discussion section. The low thermal conductivity of glass and the relative long length of the glass tubes isolates the displacement sensor from the cooling chamber and, therefore, allows it to work in standard conditions (room temperature of about 20°C).

Using copper-constantan thermocouples, temperatures were monitored at 3 points: 11, 12, and 13. Temperature sensor 11 was inserted into the tissue sample, approximately at the cross-sectional center, to represent the sample core temperature. Temperature sensor 12 was located in the free air, adjacent to glass tube 6, to represent the cooling chamber temperature. Temperature sensor 13 was connected to glass tube 5, at its distant end from the cooling chamber, to indicate whether the displacement sensor temperature is affected by the cooling chamber. No deviation from the room temperature was monitored by temperature sensor 13, which confirmed that glass tubes of sufficient length were chosen in order to isolate the temperature sensor thermally.

The effect of friction, in the telescopic tubing and inside the mechanical displacement sensor, on the uncertainty in measurements, was estimated by measuring the friction force in the experimental apparatus. A friction force of 36 g ± 3 g was measured at plate 2 in the axial direction. Taking into account an average sample cross section of 5 × 5 mm and an elasticity modulus of at least 10 GPa (Rabin et al., 1996a), one can estimate the contribution of either the friction force (36 g), or the displacement probe load (70 g), to the sample strain on the order of 10^{-7}. The calculated strains, from the displacement data, are in the order of 10^{-3} and, therefore, both effects of friction and probe loading can be neglected. Off-axis loading and bending may also influence the uncertainty in measurements; however, due to the relatively high stiffness and the large diameter of the glass tubes when compared with the magnitude of the friction force, no significant increase in uncertainty is expected.

For calibration purposes, the thermal strain of the glass tubing was measured as follows: A 99.6 percent pure copper rod was

hooked in the experimental apparatus in a similar manner to that used for the tissue samples. The thermal strain of the copper rod was then measured in a similar way to that described above for the frozen tissues. The thermal strain of the glass tubing was calculated as the difference between the measured data, and data available from the literature for pure copper (Cubberly, 1979).

Materials and Methods

Five animal tissues were selected for the thermal expansion testing: beef muscle, chicken muscle, rabbit muscle, rabbit bone, and pig liver. The effect of cryoprotectants was further studied in the rabbit muscle and the pig liver models. Beef muscle samples and the chicken muscle samples were obtained from unprocessed and fresh meat which were purchased at the food market.

Rabbit muscle samples and rabbit bone samples were obtained from 6 New Zealand White rabbits (2.5–4 kg) by excising the back muscles and hind thigh bones soon after death. These tissues were procured from rabbits that had been sacrificed for other studies in which the heart was excised under general anesthesia of halothane (2.5 L/min) administered via a ventilator.

Five different groups of rabbit muscle samples were prepared as follows: The first group included fresh muscle samples, which were tested experimentally between 1 to 8 h postmortem. The second group included samples that were immersed immediately after excision into phosphate-buffered saline medium (PBS) containing DMSO (2 mols/L) for 24 h at 4°C. The third group was treated in the same way as the second group, but was stored for additional 24 h at room temperature of about 20°C to ensure further permeation of the cryoprotectant. The fourth group included samples that were immersed immediately after excision into medium containing glycerol (2 mols/L) for 24 h at 4°C. The fifth group was processed in the same way as the fourth group, but was kept for an additional 24 h at room temperature of about 20°C. Attempts to equilibrate tissues with 2 molar cryoprotectants were chosen to be representative of a typical initial concentration of cryoprotectant used for the cryopreservation of biological tissues (Mazur, 1984; Taylor, 1984).

Tissue samples for the pig liver model were obtained from two female Yorkshire pigs weighing about 18 kg. Under halothane anesthesia, the portal vein and common bile duct were dissected. The portal vein was catheterized and the common bile duct and hepatic artery were ligated. With the inferior vena cava clamped, the liver was perfused in one case with cold PBS medium, and in the other case, with 1 liter of cryoprotectant solution (2M DMSO in PBS). Fresh liver samples, flushed with PBS, were tested experimentally between 0.5 to 6.5 h postmortem. The liver perfused with cryoprotectant solution was kept immersed in the 2M DMSO/PBS solution for an additional 24 h at 4°C prior to experimental testing.

Soft tissue samples (both muscle and liver) were cut into a prism shape having a typical cross-sectional size of 5 × 5 mm and an average length of 70 mm. All samples were taken from the core of the organ, and special care was taken not to include fat, connective tissues, or the outer most layer of the tissues. In the case of rabbit muscles, tissue samples were first shaped and then immersed in the appropriate cryoprotectant solutions, in order to increase permeation (higher permeation may be expected in thinner samples). For all other tissues, individual samples were prepared immediately prior to testing. Tissues were kept isolated from air at all times, in order to prevent surface drying.

The rabbit bone samples were taken from the hind thigh. The bone sample preparations included a careful removal of all muscles and tendons. Two parallel holes were carefully drilled, one at each end of the bone, at the diameter of pins 8, Fig. 1. The effective length of the sample (the length between the two holes) included the bone shaft only but not the joints region. The bones of the rabbit hind thigh were found to have an elliptical cross section with a typical dimensions in the range of 5 × 8 mm and 6 × 10 mm, and an effective length between 42 and 65 mm (not including the joints regions).

The wet weight fraction for all soft tissues was routinely measured, after harvesting, after 24 h and 48 h of immersion in cryoprotectant solutions, before freezing, and after thawing. Tissue samples having a volume of about 1 cm^3 were weighed before and after drying for more than 72 h at a temperature of 107°C. The proportional hydration was calculated as the complimentary fraction of dry-to-wet weight ratio.

All animal procedures were carried out in accordance with the Guidelines and Standards of the United States Public Health Services for Use and Care of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of the Allegheny University of the Health Sciences.

Experimental testing of thermal strain was performed as follows: The cooling chamber was precooled to 0°C, while the tissue sample was prepared for testing. The cooling chamber was opened, the tissue sample was fixed to pins 8, and thermocouples 11 and 12 were set in place. The controller of the cooling chamber was then programmed to follow a running function of a constant cooling rate of −5°C/min down to −180°C, and then an additional running function of a constant heating rate of 5°C/min, to return the tissue back to the initial temperature. During initial cooling of the fresh tissues, the cooling chamber was held for about 10 min at −10°C in order to allow most of the latent heat of ice crystallization to be released from the tissue and, thereby, reduce the potential temperature difference between the tissue sample and the cooling chamber. The holding period was terminated when both temperature sensors 11 and 12 reached the same readings. The holding temperature was reduced to −20°C for the cryoprotectant cases. An additional holding period of 3 min was set between the cooling and the rewarming stages, in order to allow the tissue sample to reach thermal equilibrium at −180°C. When the tissue reached −40°C during the rewarming stage, the cooling chamber was opened and the effective tissue length was measured (the distance between pins 8).

Data analysis of thermal expansion was performed as follows: The displacement, as indicated by displacement sensor 1, was recorded at 10°C intervals. Experiments were repeated several times in each case (typically five). The thermal strain was calculated by dividing the displacements by the effective length of the tissue sample. The actual thermal strain of the tissue was calculated by adding the thermal strain of the glass tube to the thermal strain of the sample. An average thermal strain was calculated for replicate experiments carried out under each set of conditions. A polynomial approximation was calculated for the average thermal strain, using a least-squares approximation technique. The thermal expansion was calculated as the first power derivative of the thermal strain with respect to the temperature.

It is noted that the main assumption in this study is that the strains in the tissue samples are uniformly distributed. One should bear in mind that because of the heterogeneous structure of biological materials, local loading, and geometric irregularities such as at the grips, a nonuniform strain distribution may exist in the sample. Nevertheless, without this main assumption no practical measurements can be made.

Results and Discussion

Available data from the literature for the thermal expansion of polycrystalline samples (Jakob and Erk, 1928) and of single ice crystals (Powell, 1958; Dantel, 1962) are presented in Fig. 2. Although the thermal expansion of single ice crystals is expected to have different values at different directions, it was

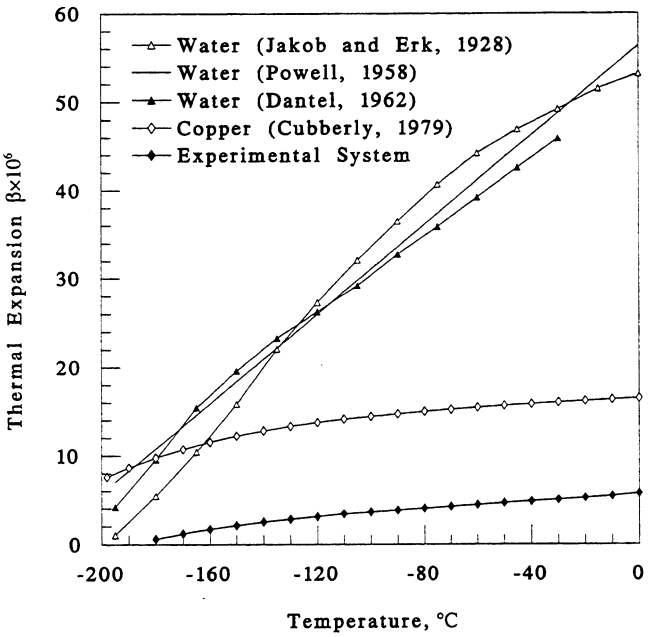

Fig. 2 Thermal expansion of polycrystalline water sample (Jakob and Erk, 1928); single ice crystals (Powell, 1958; Dantel, 1962); pure copper (Cubberly, 1979); and glass tubes of the experimental system

found experimentally that these differences are in the order of 2 percent and, therefore, are insignificant (Fletcher, 1970). Powell (1958) has suggested a linear dependency of the thermal expansion with respect to the temperature, and this is adopted as a reference for the present study.

Figure 2 shows the thermal expansion of pure copper (Cubberly, 1979), which was used for the calibration of the experimental apparatus, and the calculated thermal expansion of the glass tube. It can be seen that the thermal expansion of glass is about one order of magnitude lower than that of water. The thermal expansion of the glass tubes were taken into account when calculating the thermal expansion coefficient of the tissue samples, as described above. The coefficients of the polynomial approximation of the thermal expansion of all materials addressed in this study are listed in Table 1.

The calculated thermal expansion of fresh biological tissues is presented in Fig. 3. It can be seen that all soft biological tissues tested in this study have similar thermal expansion characteristics, which do not deviate significantly from those of water. It can also be seen that the thermal expansion of the rabbit bone is about one half of that of soft tissues. Bone behaves like a composite material, where the core and the outer surface are expected to have different expansion coefficients, and therefore the actual measurements represent the effective property of bone. The soft tissues, however, are expected to have much more homogeneous expansion. The water content of all tissue samples tested in this study were found to fall within the range of 68 to 76 percent, which may in large part explain the similarity between water and soft biological tissues (see Table 2).

Figure 4 shows the change of thermal strain as a function of temperature during a complete cooling/rewarming cycle of a single rabbit muscle sample. A strain offset of less than 2×10^{-4} can be observed between the cooling and the rewarming stages, which is, in part, a consequence of the transition from contraction to expansion in the experimental apparatus, respectively. This offset is extremely small and gives some perspective to the accuracy in measurement. The thermal expansion, however, which is the derivative of the thermal strain, is almost identical in cooling and rewarming.

A highly turbulent flow regime of nitrogen gas is applied during cooling in the chamber of the Kryo-10 device in order to achieve uniform cooling. High turbulent flow can cause surface drying of the tissue sample, especially prior to freezing, and may affect the experimental measurements. Hence, the water content in all cases was measured before freezing and after thawing, as listed in Table 2. No significant differences or systematic differences were observed between the precooled and the thawed tissue samples, which indicates that the surface drying effect was negligible.

The highly turbulent flow resulted in a very high heat transfer coefficient between the cooling fluid and the tissue, which, in turn, resulted in temperature differences in the range of 0 to 3°C between temperature sensors 11 and 12, Fig. 1. This temperature difference may indicate the uncertainty in temperature measurements at which the actual strains were measured, which is of the order of 2 percent of the entire temperature range. However, the thermal expansion coefficient, which is the derivative of the thermal strain, is less sensitive to this temperature uncertainty. A temperature shift of the thermal strain curve is not expected to affect significantly the slope of the thermal strain. This temperature uncertainty can give another partial explanation to the strain shift between the cooling and the rewarming stages, as shown in Fig. 4. The uncertainty in temperature measurements can be decreased by applying a lower cooling/rewarming rate of the tissue sample. Note, the kinetics of freezing of the cryoprotected tissues is affected by the cooling rate, which may affect the thermal expansion.

Although the thermal expansion is typically addressed in the literature, thermal stress modeling is explicitly related with the thermal strain, which is the integral of the thermal expansion with respect to the temperature (Boley and Weiner, 1960). The experimental apparatus presented in this study measures the thermal strain directly, and therefore a polynomial approximation of the data can represent this property adequately. However, it is noted that the thermal expansion, which is calculated by first-order derivative of the thermal strain with respect to temperature, may be sensitive to the degree of polynom chosen for the approximation.

Figures 5 and 6 present the uncertainty in data analysis of thermal strain and thermal expansion, respectively, for fresh pig liver and rabbit bone. It can be seen that the measurements fall within a close range for any specific temperature. Figure 5 shows all the data points, the average strain, and the boundaries of ± one standard deviation (STD) from the average value. It can be further seen that there is wider deviation of data from the mean value at higher temperatures. It is known that phase transition temperature range of fluids within fresh biological tissues is typically in the range between −22 and 0°C, and in some cases in the range between −45 and 0°C (Taylor, 1987). Although thermal expansion occurs at all temperatures, the experimental apparatus can measure strains with high certainty after the beginning of tissue solidification only. This may partially explain the significant increase in measured data distribution at temperatures above −30°C, in the case of pig liver. The fact that bone behaves more like a composite material, in the sense of solid mechanics, may contribute to the observation that the distribution of the data in the case of rabbit bone is higher than that in the pig liver; where the latter is typical to all soft tissues tested in this study. Figure 6 shows that this distribution does not significantly affect the calculated thermal expansion, especially in the temperature range of −180 to −40°C in the pig liver case.

This study of the thermal expansion of frozen biological tissues was extended to include some preliminary measurements in similar tissues following exposure to cryoprotectants. For these pilot experiments the conditions of tissue permeation by the cryoprotectants DMSO and glycerol were chosen empirically.

Tissues frozen with DMSO and glycerol were found to have very different characteristics of thermal expansion compared with those of pure ice crystals (see Fig. 7). The thermal expan-

Table 1 Thermal expansion coefficients of the polynomial approximation: $\beta = C_0 + C_1T + C_2T^2 + C_3T^3$ °C^{-1}; n is the group size and R^2 is the variance of approximation in thermal strain (†) and thermal expansion (‡)

	Temperature Range	C_0	C_1	C_2	C_3	n	R^2
Water (Jakob and Erk, 1928)	-200<T<0°C	$5.264 \cdot 10^{-5}$	$4.207 \cdot 10^{-8}$	$-1.893 \cdot 10^{-9}$	$-3.771 \cdot 10^{-12}$	-	0.9994 ‡
Water (Powell, 1958)	-200<T<0°C	$5.630 \cdot 10^{-5}$	$2.528 \cdot 10^{-7}$	-	-	-	1.0000 ‡
Water (Dantel, 1962)	-200<T<-30°C	$5.548 \cdot 10^{-5}$	$3.603 \cdot 10^{-7}$	$1.788 \cdot 10^{-9}$	$6.614 \cdot 10^{-12}$	-	0.9995 ‡
Beef Muscle	-180<T<-30°C	$7.225 \cdot 10^{-5}$	$3.834 \cdot 10^{-7}$	$2.340 \cdot 10^{-10}$	-	6	0.9988 †
Chicken Muscle	-180<T<-10°C	$6.649 \cdot 10^{-5}$	$3.438 \cdot 10^{-7}$	$2.785 \cdot 10^{-10}$	-	5	0.9994 †
Rabbit Muscle	-180<T<-20°C	$6.224 \cdot 10^{-5}$	$2.744 \cdot 10^{-7}$	$-3.639 \cdot 10^{-11}$	-	5	0.9999 †
Rabbit Muscle Permeated with DMSO	-180<T<-30°C	$5.109 \cdot 10^{-6}$	$-1.749 \cdot 10^{-6}$	$-1.799 \cdot 10^{-8}$	$-4.820 \cdot 10^{-11}$	5	1.0000 †
Rabbit Muscle Permeated with Glycerol	-180<T<-20°C	$3.840 \cdot 10^{-6}$	$-6.774 \cdot 10^{-7}$	$-3.591 \cdot 10^{-9}$	-	2	0.9989 †
Rabbit Bone	-180<T<-10°C	$2.622 \cdot 10^{-5}$	$2.052 \cdot 10^{-7}$	$6.231 \cdot 10^{-10}$	-	5	0.9993 †
Pig Liver	-180<T<-10°C	$7.323 \cdot 10^{-5}$	$4.344 \cdot 10^{-7}$	$6.105 \cdot 10^{-10}$	-	5	1.0000 †
Pig Liver Perfused with DMSO (most perfused lobe)	-95<T<-20°C	$1.668 \cdot 10^{-5}$	$-3.414 \cdot 10^{-8}$	-	-	1	0.9992 †
	-180<T<-100°C	$1.239 \cdot 10^{-4}$	$9.954 \cdot 10^{-7}$	$1.970 \cdot 10^{-9}$	-		0.9944 †
Experimental System	-180<T<0°C	$5.722 \cdot 10^{-6}$	$2.933 \cdot 10^{-8}$	$1.770 \cdot 10^{-10}$	$9.499 \cdot 10^{-13}$	8	0.9989 †
Copper (Cubberly, 1979)	-200<T<0°C	$1.658 \cdot 10^{-5}$	$2.689 \cdot 10^{-8}$	$2.200 \cdot 10^{-10}$	$1.569 \cdot 10^{-12}$	-	0.9995 ‡

sion of "cryoprotected" samples is found to have a peak value in the temperature range of −70 to −100°C. There is previous experimental evidence that the thermal expansion of cryoprotectant solutions at sub-zero temperatures is much lower than that of water (Archer et al., 1996). However, relevant data are available only for a relatively high temperature range of −40 to 0°C, which is well above the temperatures at which the vitrified solution reaches its extremely high viscosity. By extrapolation from the curves presented in Fig. 7 it can be seen that, indeed, the thermal expansion of the cryoprotected tissue decays toward 0°C.

The maxima in thermal expansion shown in Fig. 7 are likely to be related to glass transition in the "cryoprotected" samples. The glass transition temperatures of 2M buffered DMSO and 2M buffered glycerol are about −123°C and −100°C, respectively (Luyet and Rasmussen, 1968; Pegg et al., 1996). The temperature difference, between the known glass transition temperatures of the buffered cryoprotectents and the observed peaks of thermal expansion in the tissue, is probably due to co-existence of partly crystallized and partly vitrified solutions. Pre-

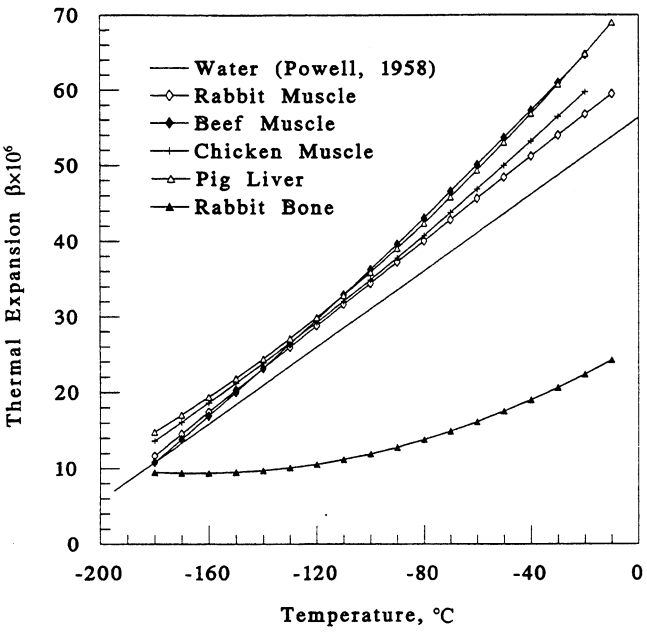

Fig. 3 Thermal expansion of fresh biological tissues and pure water

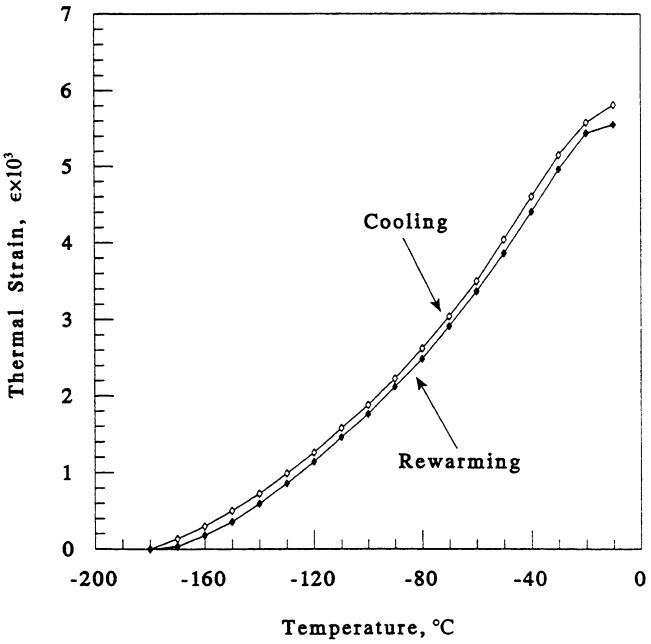

Fig. 4 Typical results of thermal strain versus temperature along the cooling/rewarming cycle of testing, for a single sample fresh rabbit muscle

Table 2 Fluids weight percentage of the various tissues tested in this study, before and after thermal expansion testing. Average values of three samples (n = 3) ± standard error are given.

Tissue Type	Before Freezing	After Thawing
Beef Muscle	72.28 ±4.95	71.91 ±2.00
Beef Fat	40.30 ±10.52	-
Chicken Muscle	79.32 ±3.62	-
Rabbit Muscle	76.41 ±0.16	74.95 ±0.42
Rabbit Muscle Permeated with 2M DMSO*	69.18 ±2.06 after 24 h 75.90 ±3.11 after 48 h	69.18 ±2.77 after 24 h 69.25 ±0.87 after 48 h
Rabbit Muscle Permeated with 2M glycerol*	68.18 ±2.09 after 24 h 74.10 ±2.13 after 48 h	71.36 ±0.39 after 24 h 73.63 ±1.13 after 48 h
Pig Liver	74.43 ±0.47	73.81 ±0.86
Pig Liver Perfused with 2M DMSO**	75.18 ±0.77	75.60 ±1.60

* Tissue samples that were immersed in cryoprotectant for diffusion and kept for 24 h at 4°C and for additional 24 h at 20°C.

** The liver was kept for 24 h at 4°C after perfusion, while immersion in DMSO solution.

existence of other solvents in the tissue sample may also contribute to this temperature difference. Hence, for cryopreservation applications, it is more realistic to measure the actual thermal expansion of the permeated tissue, and not solely that of the cryoprotectants.

In order to decrease tissue degeneration, on the one hand, and to increase the possibility for good permeation of the cryoprotectant, on the other hand, the immersed muscle tissue samples were first kept at 4°C for 24 h. In an attempted to ensure full permeation during a second stage, it was decided to keep some of the tissue samples for an additional 24 h at about 20°C. The rationale for the additional stage was that the cryoprotectant has a significantly higher permeability at higher temperature and, therefore, comparison of the results between the end of the two stages would give an estimation of the full extent of diffusion. For tissue immersed in DMSO solution, the results at the end of the second stage were consistent with those measured at the end of the first stage, which suggests that maximal permeation of the cryoprotectant was achieved. The average data of the first and second stages of permeation were used to calculate the curves shown in Fig. 7. However, significant differences

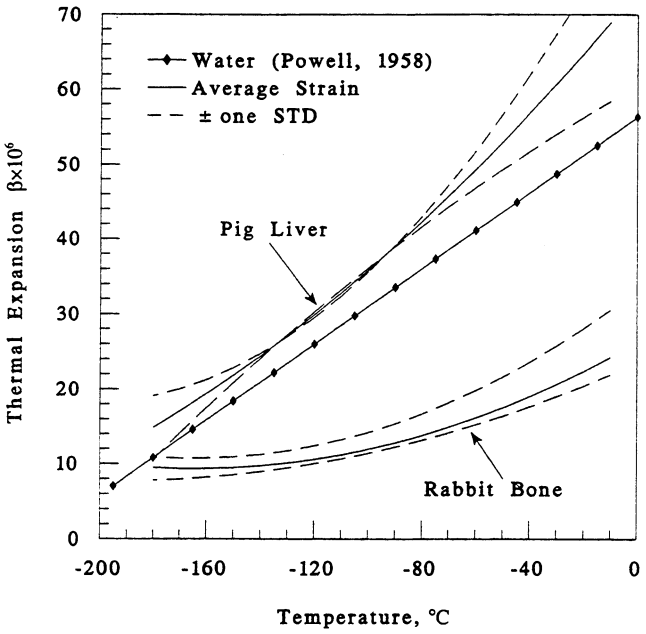

Fig. 6 Calculated thermal expansion for the average strains and the ± one standard deviation presented in Fig. 5

were found between the end of the two stages in tissues exposed to glycerol solution and, thus, results are presented for the end of the second stage only. This was not unexpected since, it is well known that glycerol has lower permeability compared with DMSO (Clark et al., 1984).

Finally, the effect of DMSO on the thermal expansion of a frozen pig liver was studied (Fig. 8). Taking advantage of the high vascularity of the liver, a perfusion technique was used, instead of relying on the immersion–diffusion process described above for the rabbit muscle cases. The DMSO mixture was perfused through the small left lobe of the liver, which is designated as lobe 1; the large left lobe, the large right lobe, and the small right lobe are designated as lobes 2, 3, and 4, respectively. Very different results were obtained from samples

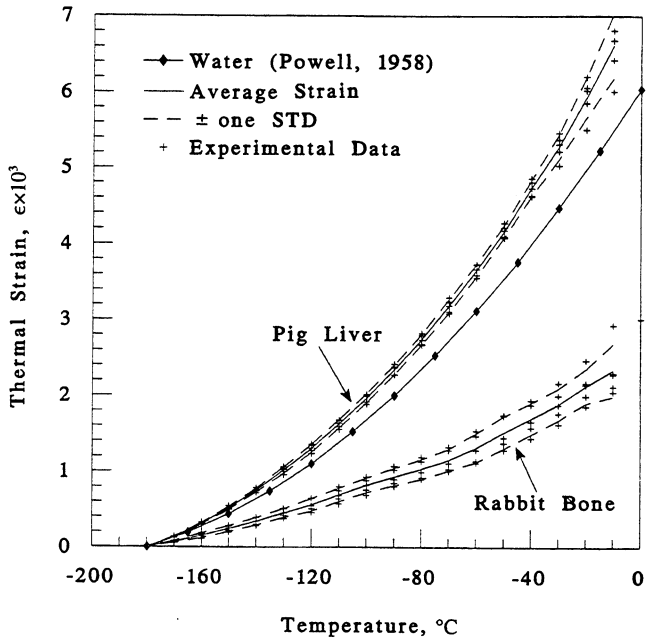

Fig. 5 Experimental data distribution, average curve, and ± one standard deviation error of thermal strain in pig liver and rabbit bone cases

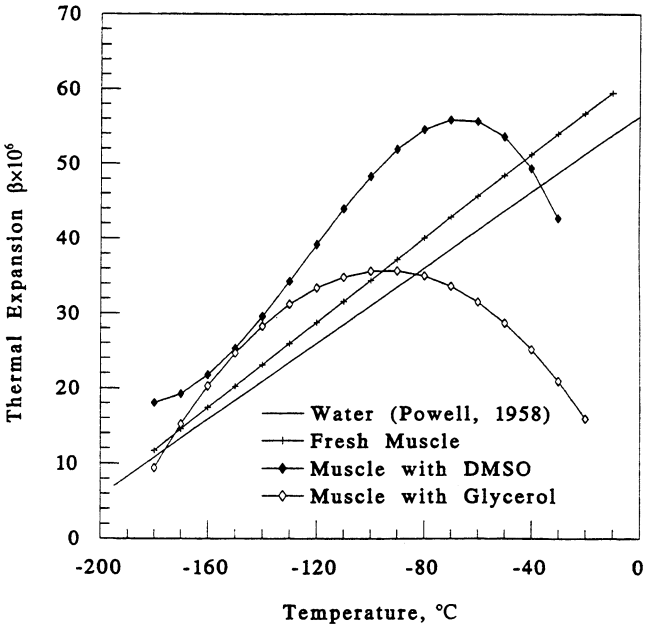

Fig. 7 Thermal expansion of a rabbit muscle following immersion in DMSO and glycerol

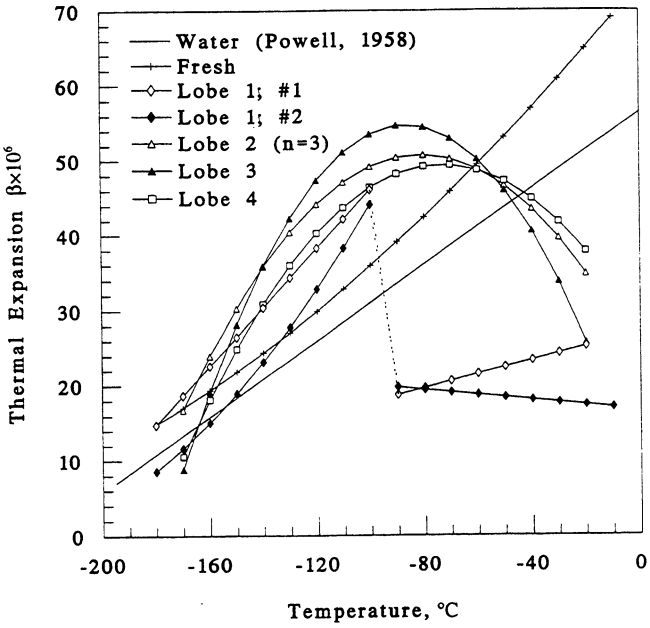

Fig. 8 Thermal expansion of a perfused pig liver with DMSO; lobe designation: (1) small left lobe, (2) large left lobe, (3) large right lobe, (4) small right lobe

taken from lobe 1 compared with those taken from the other lobes (Fig. 8). Measurements from lobe 1 indicate an almost constant thermal expansion down to about −95°C, followed by a rapid change in thermal expansion around that temperature, and a linear dependency of the thermal expansion on temperature thereafter. It seems reasonable to assume that the sudden change in thermal expansion (which caused an extremely rapid movement of the needle of displacement sensor 1) is related to some abrupt change in the physical properties of the material such as eutectic solidification and the glass transition (Steinbrecher, 1975; MacFarlane, 1987). It would appear that only lobe 1 was well perfused with DMSO, and hence the possibility of extensive vitrification and a glass transition. This conclusion is supported by the observation that lobe 1 showed the most uniform and marked change of color by losing its typical reddish color during perfusion. The authors believe that a better permeation can be achieved in the liver by longer perfusion than that used empirically in these pilot experiments.

By comparing the results shown in Figs. 7 and 8, it may be concluded that the rabbit muscle samples were not fully permeated with cryoprotectant since they generated curves that more clearly resemble the results of the liver lobes 2, 3 and 4, and were less like the results of lobe 1 testing. As described above, it was found experimentally that further immersion of the tissue samples would not increase permeation, and it may be concluded that perfusion is required in all cases. It can further be concluded that thinner tissue samples should be used in order to improve permeation; however, this would require significant changes in the experimental apparatus design.

Summary and Conclusions

Thermal expansion is the driving force for cryodestruction associated with thermal stresses during cryopreservation protocols as well as during cryosurgery. Thermal expansion data, together with other physical properties such as the strength and stiffness, is required for thermal stress analysis in order to predict the likelihood of fracture formation in the frozen tissues.

A new apparatus for thermal strain measurements of frozen biological tissues within a range of cryogenic temperatures is presented. High certainty in measurements can be achieved after the initiation of solidification only.

Results of this study support the commonly applied hypothesis that the thermal expansion of frozen soft tissues is similar to that of water ice crystals. Thermal expansion values of the rabbit bone was found to be about one half of that of soft tissues. The water content in all cases was found in a close range of 68 to 76 percent, which would largely explain the similarity in thermal expansion of water ice crystals and fresh tissues. Experimental data distribution increases with temperature. Results were found to be very reproducible; the thermal strain during rewarming follows very closely the original strain during cooling, in cases of fresh tissues.

Data variation increased dramatically when cryoprotectants were involved. The cryoprotectants dramatically reduced the thermal expansion at higher temperatures and created a maximal value of thermal expansion within the temperature range of −70 to −100°C. A significant effect of the DMSO concentration on the thermal expansion of pig liver was found and it appears that the thermal expansion decreases with the increase in DMSO concentration. A rapid change in thermal strain was observed in the lobe suspected of attaining the highest concentration of DMSO, which could be related to a change in physical properties associated with a glass transformation. However, in this pilot study of the response of tissues after equilibration with cryoprotectants, it is suspected that the empirically chosen conditions of perfusion did not permit uniform, or full cryoprotectant permeation. A more complete understanding of the effect of cryoprotectants upon thermal expansion during cooling will require further detailed study in fully equilibrated tissues.

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