# Freezing-Thawing Hysteresis Phenomena of Biological Systems by the New Method of Proton Magnetic Resonance

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An automatic recording system was developed for unfrozen water content and spin-spin relaxation time measurements as continuous functions of temperature, by using a broad-line pulsed NMR spectrometer and a minicomputer. The advantages of this system are that the exact quantitative measurements can be done by calibrating the nonlinearity of the NMR sensitivity, and that for high sensitivity temperature measurement the thermocouple with special device is directly immersed in a sample. Three types of freezing-thawing hysteresis phenomena, (1) recrystallization of solute(hydroxy-L-proline, D-mannitol) and refreezing of released hydrated water molecules in frozen aqueous solutions, and (2) hysteresis as the characteristic feature of gels(gelatin, $\alpha_{s1}$-casein), and (3) supercooling of capillary water in water-insoluble materials(zein, yeast RNA, cellulose) were analysed. The usefulness of this system as an analytical instrument of hydration properties of biological materials is emphasized.

The state of water in biological systems has been one of the most important subjects in life science studies. Proton magnetic resonance gives most useful information in the study of water motions,[1,2] although there are many other methods, for example, dielectric relaxation, thermal analysis, and neutron scattering[3] *etc.* There have been many publications concerning the application of proton magnetic resonance to studies of the state of water in biological systems or food.[4,5] At room temperature, a distinction between the state of water, *i.e.* bound or free, is given by a graphical resolution of the triple exponential decay curve due to the three kinds of spin-spin relaxation times($T_2$) as observed using the Carr-Purcell/Meiboom-Gill method.[6] However this method presents experimental difficulties and is not applicable to homogeneous aqueous solutions, which have both bound and free water but show only the averaged $T_2$. Then the measurements of unfrozen water(UFW) content and its relaxation times as functions of temperature are effective for the investigation of wider range of samples.[2,7] We have developed a convenient system for some purposes; it involves a broad-line pulsed NMR spectrometer and an X-Y analogue recorder.[8] By using this system, only the variation of the free induction decay(FID) amplitude in the voltage value at about 40 μs with temperature change can be measured. This is acceptable as a qualitative method for the measurement of the UFW content as a function of temperature, which is called the freezing curve.

A quantitative method was developed for the freezing curve measurement, by adding a minicomputer system and a special thermocouple device which is directly immersed in a sample. By this means the UFW content and the spin-spin relaxation time as functions of temperature can be recorded automatically.[9] This new freezing curve method is a powerful tool for the investigation of the state of water in biological systems, especially those which show freezing-thawing hysteresis phenomena. The essencial cause of freezing-thawing hysteresis is only supercooling, but it was found by this method that there are three types of supercooling. In this study, water systems of sugar alcohols(D-sorbitol, D-mannitol[9]), and amino acids(glycine, L-alanine, L-methionine, L-proline, hydroxy-L-proline), and proteins(gelatin,[9] $\alpha_{s1}$-casein, glycinin, bovin serum albumin, ovalbumin, zein) and other polymers(cellulose, polyethylene glycols, yeast RNA) were measured. In this paper, some aspects of freezing-thawing hysteresis are described to illustrate the usefulness of our freezing curve method.

## Experimental

*Sample Preparation.* All samples were commercially available, except for $\alpha_{s1}$-casein and glycinin (soybean protein 11s unit), which were obtained by preparative gel filtration chromatography.

*Freezing Curve Measurements.* The block diagram of the system, which consists of a broad-line pulsed NMR spectrometer with proton Larmor frequency of 20 MHz (Bruker, minispec p 20) and a disc based minicomputer system (Yokogawa-Hewlett-Packard, HP 1000) is shown in Fig. 1. Each sample of about 250 mg in a 6.8 mm inner diameter glass tube is cooled or warmed at the usual rate of about three degrees per minute, by using gas and liquid nitrogen, if necessary, whose temperature can be controlled by a variable temperature unit (Bruker B-ST 100/700). The freezing-thawing processes are observed using the NMR spectrometer and the chromel-constantan thermocouple.

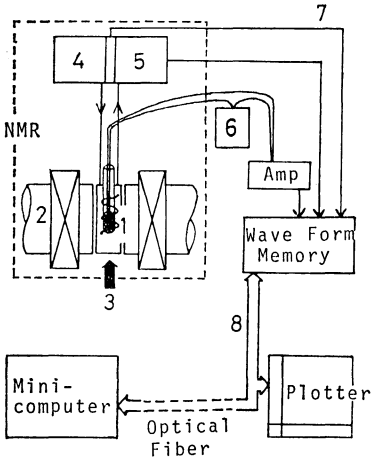


Fig. 1. Block diagram of the system.
1: Sample, 2: parmanent magnet, 3: nitrogen gas flow, 4: transmitter, 5: receiver, 6: cold junction of ice for thermocouple, 7: sampling trigger, 8: HP-IB interface bus with fiber optic link.

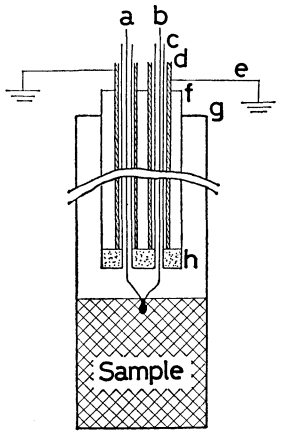


Fig. 2. The measurement of sample temperature with the special thermocouple device.
a,b: Chromel and constantan wires, c,f: teflon tubes, d: aluminum metal, e: ground to the NMR spectrometer body, g: sample tube of glass, h: thermal insulater.

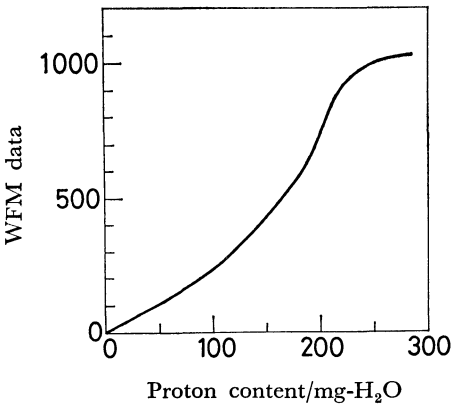


Fig. 3. The sensitivity of the NMR spectrometer. Proton content shows the water amount in the cupric sulfate solution whose sample volume is constant at about 0.27 cm³. A WFM datum is a digital value, which ranges from 0 to 1023.

The signal values at intervals of two μs of free induction decay (FID) curve after a 90° pulse and the sample temperature are A/D converted and memorized at every few seconds by a two channel wave form memory (WFM) apparatus (Kikusui, modified 8702S). The data are transferred to the minicomputer by a fiber optic Hewlett-Packard interface bus(HP-IB) link system (HP, 12050A).

One of the advantages of this system is that the special thermocouple device is directly immersed in a sample as shown in Fig. 2. High sensitivity measurement of the temperature change of a sample can be achieved with this thermocouple. The WFM apparatus and the NMR spectrometer generate the electric clock pulses from quartz oscillators. The noise so generated make it impossible to detect the FID curve; the thermocouple acts like an antenna here. For shielding from this noise, grounded aluminum tape is used. A thermal insulator is necessary to reduce the heat conduction to the sample through the aluminum metal.

Another advantage is that the exact quantitative measurement can be done by calibrating of the FID curve at each 90° pulse. The real FID curves are obtained after calibrating the sensitivity of NMR by using fifteen standards of 0.65 M (1 M=1 mol dm$^{-3}$) cupric sulfate solutions in water–heavy water mixtures which have various proton concentrations and the same sample volume of about 0.27 cm³ at the bottom of a sample tube. This calibration is required because of the nonlinear relationship between the proton content and the signal intensity, as shown in Fig. 3. This calibration curve is stored in our system memory and is used in every measurement of each FID curve.

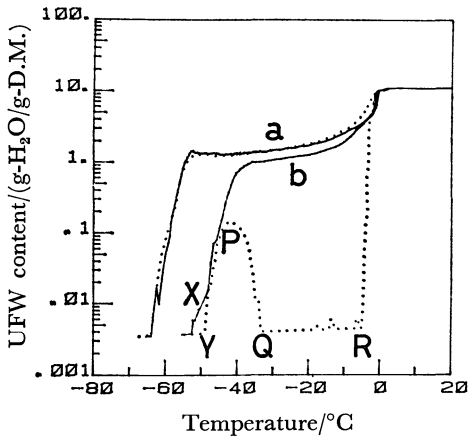


Fig. 4. Freezing (——) and thawing (·····) curves of 9.1% aqueous solutions of L-proline(a) and hydroxy-L-proline(b).
*X* and *Q* are the liquid signal vanishing temperatures, and *Y* and *R* are the liquid signal appearing temperatures, and *P* shows the top of the peak.

On the measurement of FID curves, it generally occurs that a sample exhibits at least two different spin-spin relaxation times $T_{2s}$ and $T_{2l}$, which correspond to solid (short $T_2$) and liquid (long $T_2$) state protons, respectively. The shape of the FID curve of liquid state protons is exponential, but that of solid state protons is Gaussian. The population of liquid state or unfrozen protons (UFP) is obtained by extrapolating the FID curve from 40 to 100 μs back to zero time. Then this point is corrected according to the well-known Curie law; the lower the temperature, the greater the sensitivity. For practical purposes, this UFP value is reduced to a UFW content and converted to a dry basis, *i.e.* gram water per gram dry matter (g-$H_2O$/g-D.M.), using the dry matter value obtained by drying a sample for about 8 h at 115 °C. The UFW content is plotted as a function of temperature on a semilogarithmic graph by a graphic plotter (HP, 9872A). This function was named the freezing curve (liquid content curve). The solid content versus temperature can be obtained in a similar way. Also $T_{2s}$ versus temperature and $T_{2l}$ versus temperature can be obtained.

All of the programs used were written in FORTRAN language of the software package (HP, RTE IVB) of the minicomputer system.

## Results and Discussions

*Recrystallization of Solutes in Frozen Aqueous Solutions.* It has been found that freezing curves of some aqueous solutions show peculiar freezing-thawing hysteresis. The example of D-mannitol aqueous solution has been reported previously.[9] Figure 4(b) shows the remarkably large hysteresis of hydroxy-L-proline aqueous solution, in contrast with the case of L-proline (a). The following mechanism is suggested for this large hysteresis of hydroxy-L-proline. In the cooling process,

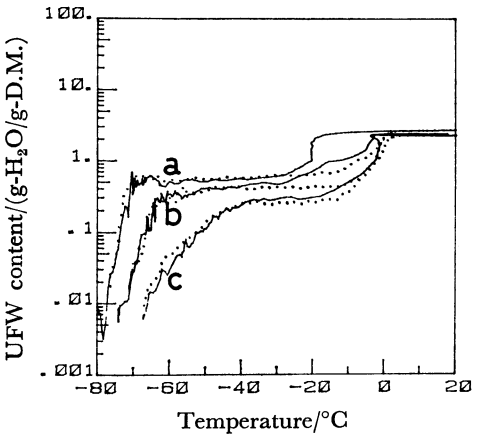


Fig. 5. Freezing (——) and thawing (·····) curves of 32% aqueous colloids of gelatin(a), $\alpha_{s1}$-casein(b), and glycinin(c).

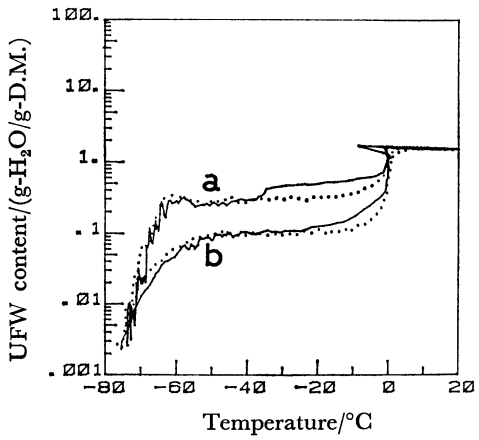


Fig. 6. Freezing (——) and thawing (·····) curves of aqueous suspensions of zein(a, 37%) and cotton cellulose(b, 39%).

free water in aqueous solution is frozen at near 0 °C, and water hydrated(bound) to solute remains as UFW until about −40 °C, and vitrification of the cluster which consists of solute and hydrated water molecules occurs(point *X*). In the warming process, after beginning of thawing(point *Y*), hydrated water molecules are released from the cluster with the beginning of recrystallization of solute, and their refreezing takes place at the point *P*. The difference between D-mannitol and hydroxy-L-proline is that the *X* and *Y* points are not the same in the former case.

Materials such as L-proline and D-sorbitol, which have comparatively high water solubilities, showed no hysteresis loops, at any cooling rates. At the usual cooling rate of about three degrees per minute, materials of low water solubilities such as glycine, L-alanine, L-methionine *etc.* do not show this type of hysteresis loop, but show simple hysteresis without a peak in the thawing curve, due to completion of recrystallization in the cooling process. The solubilities of hydroxy-L-proline and D-mannitol are medium, and these substances also show simple hysteresis loops, at the cooling rate of one degree per minute or more slowly.

The shape of the thawing curves of polyethylene glycols depends upon their molecular weight or crystallinity. In the case of low molecular weight, the shape of the thawing curve is almost the same as that of the freezing curve. But in the high molecular weight(about 6000) case, a remarkably large hysteresis can be observed. This hysteresis loop is similar to that of hydroxy-L-proline, but the rehydration temperature *R* is about −20 °C.

*Characterization of Biopolymer Colloids——Gels and Sols.* Figure 5 shows the freezing and thawing curves of 32% aqueous colloids of three kinds of proteins; $\alpha_{s1}$-casein, glycinin, and gelatin. The UFW content of glycinin, which is known as a "cold-precipated protein,"[10] is much lower than that of gelatin, which forms a viscous aqueous gel. The freezing-thawing curve of gelatin has a remarkably large hysteresis loop, but that of glycinin has a very small hysteresis loop; that of $\alpha_{s1}$-casein is situated between the two.

It is thought that water molecules in gelatin gel are structured in a gel network and supercooled until about −20 °C, and then a phase separation occurs due to freezing, and micro ice crystals are formed, which do not melt until 0 °C during thawing. Hence the freezing-thawing curve of gelatin has large hysteresis, and that of $\alpha_{s1}$-casein has hysteresis of a medium value, due to the high viscosity and comparatively large UFW content of the liquid.

Table 1. UFW contents and signal vanishing temperatures for several proteins

| Proteins | Concentrations/% | UFW[a] g-$H_2O$/g-D.M. | SVT[b]/°C |
|---|---|---|---|
| Glycinin | 32 | 0.24 | −68 |
| Ovalbumin | 31 | 0.32 | −78 |
| Bovine serum albumin | 29 | 0.38 | −80 |
| $\alpha_{s1}$-Casein | 32 | 0.41 | −72 |
| Gelatin | 32 | 0.60 | −77 |

a) At about −30 °C. b) Signal vanishing temperature, which is defined as the temperature at which the apparent UFW content becomes lower than 0.01 g-$H_2O$/g-D.M.

The UFW values at −30 °C, which correspond to tightly-bount water content,[8] and signal vanishing temperatures(SVT) are shown for several proteins in Table 1. The freezing-thawing curve of an aqueous colloid of bovine serum albumin has no hysteresis, although its UFW content at −30 °C is near to that of $\alpha_{s1}$-casein. The viscosity of this colloid is much smaller than that of $\alpha_{s1}$-casein. This suggests that the measurement of weakly-bound water, which is frozen between 0 and −30 °C and does not melt in this case until near 0 °C, is very important to characterize the hydration properties of biopolymers, and that the freezing curve method is very useful for this purpose. The SVT data show the vitrification points of the clusters, which consist of protein and unfrozen water molecules. This data are also useful for the characterization of biopolymers.

*Special Problems for Water-insoluble Biopolymers——Cellulose and Corn Protein etc.* Figure 6(a) shows the freezing-thawing hysteresis phenomenon of aqueous sus-

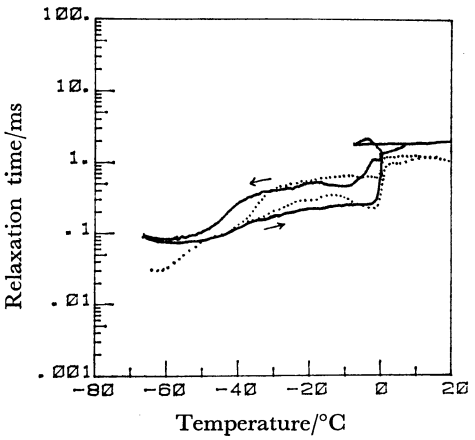


Fig. 7. Temperature dependence of spin-spin relaxation time of aqueous suspensions of zein (·····, 37%) and cotton cellulose (——, 39%).

pension of corn protein(zein). It is shown that a part of the UFW is frozen at about −37 °C in the cooling process. Freeze-drying from the homogeneous solution in 90% ethanol gave fine powder, which does not show such a phenomenon. Hence the cause of this hysteresis is thought to be the freezing of weakly-bound water in capillaries of water-insoluble materials. A similar phenomenon was observed for water-insoluble yeast RNA powder. A related type of freezing-thawing hysteresis is studied in relation to the mechanism of frost avoidance in plant tissues by Burke *et al.*,[11] using NMR and thermal analysis. We think that our method is more convenient and powerful for these studies.

Figure 6(b) shows the freezing-thawing curve of cotton cellulose, which has no obvious hysteresis loop. But the remarkable hysteresis phenomenon of spin-spin relaxation time ($T_{21}$) was observed, which is similar to the case of zein, as shown in Fig. 7. By the thermal analysis for cotton cellulose suspension with a differential scanning calorimeter(DSC; Daini Seikosha, SSC 560U), a broad exothermic peak was observed at about −42 °C. The cause of this phenomenon is not clear but it is obvious from Fig. 6(b) that no water is frozen at about −42 °C. One of the possible speculations is that the mobility of UFW is lowered by the change of surroundings accompanied with the crystal transformation of cotton cellulose at about −42 °C, because cellulose has polymorphism. In general, the freezing curve method would be powerful to elucidate the mechanism of an exothermic or endothermic peak observed in a DSC curve.

## Conclusion

The new freezing curve method is a useful tool for the analysis of hydration properties of biological materials, from the viewpoint of freezing-thawing hysteresis phenomena. Three types of phenomena, (1) recrystallization of solutes and refreezing of released hydrated water in frozen aqueous solutions, (2) hysteresis as the characteristic feature of biopolymer gels, (3) supercooling of capillary water in water-insoluble biopolymers, were discussed and useful information was obtained. Our method can be applied to many practical problems in the food and drug industry *etc.*

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