Investigation of Vitrification by Nuclear Magnetic Resonance and Differential Scanning Calorimetry in Honey: A Model Carbohydrate System

Carmel A. Rubin, John M. Wasylyk,[‡] and John G. Baust[*]

Center for Cryobiological Research, State University of New York, Binghamton, New York 13901-6000

The vitrification of honey, a model system, was studied by utilizing nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC). Various dilutions of honey, 100–0%, were analyzed, and heat capacity (C_p) of vitrification was calculated by using DSC. Complementary information was obtained by using NMR for the determination of the degree of proton mobility and magnetic relaxation of aqueous solution protons during DSC-observed events. DSC transitions and thermal events, T_g, T_c, T_d, and T_m, detected heat flow and plots of the first derivative of heat capacity correlated to peak amplitude of the free induction decay (FID) signal observed by NMR. The first derivative of heat capacity, when plotted against temperature, yielded a definitive separation of thermal events. It was observed, on cooling at –5 °C min⁻¹, that 0% honey (100% ddH₂O) crystallized (T_c) at –13 °C and as the concentration of honey increased above 35% the T_c decreased as did the exponential decay of the FID. At concentrations >50% honey there was a loss of the FID signal due to the increase in viscosity which decreased relaxation times. At 50% dilution the FID showed two changes in slope, at –42 and –60 °C, which correlate to two DSC-observed T_g's at the same temperatures following a bulk T_c. It was determined that the second glass was not dependent upon the first as there was no T_d observed in DSC-monitored heat flow or decrease in FID signal between those two temperatures. At concentrations >80% there was a T_g at –77 °C and no ice formation on cooling, but a T_d at a warming rate of +5 °C min⁻¹ or less. In undiluted honey (100%) the T_g was –40 °C and the corresponding change in C_p was 0.81 J °C g⁻¹. An isothermal hold at –62.5 °C for 90 min followed by warming through the T_g showed an overshoot of C_p. This demonstrates the relaxation of the vitreous sample and concomitant loss of entropy. The carbohydrate content of the undiluted sample (referred to as 100% honey) analyzed by high-performance liquid chromatography was 47.2% fructose, 43.2% glucose, 1.6% sucrose, and 8% undetermined component(s), possibly maltose.

INTRODUCTION

The role of vitrification in the storage of carbohydrate–water nonequilibrium systems is of growing interest in the commercial food industry, in low-temperature preservation of biological materials, and in cryopreservation of cells and tissues. Aqueous polyol solutions are known to partially or completely vitrify (Claudy et al., 1988; Luyet, 1939). Recent advances in spectroscopic and thermal analyses of these solutions have provided renewed interest in the quantitative and qualitative aspects of vitrification. The quantity of vitreous water that can be formed from the addition of minimal concentrations of carbohydrates and the temperature limits to which a partial or total vitreous (glassy) state can be maintained are of fundamental interest to the food science industries.

Aqueous carbohydrate solutions demonstrate several thermal events including temperatures of glass transitions (T_g), crystallization (T_c), devitrification (T_d), melt (T_m) and other melt-related events along with their respective changes in enthalpy and heat capacity. Historically, the T_g has been defined as the midpoint temperature in the range over which the transformation from liquid to amorphous state occurs at a given scan rate (Brennan, 1973). A glass is characterized by a decrease in mobility or the loss of translational motion and restriction of rotational motion at the atomic level. At temperatures where $T < T_g$, the viscosity varies from 10^{10} to 10^{14} Pa s, while at $T > T_g$, the molecular motion is restored but partially limited by the high viscosity (Turnbull, 1969).

To provide a model system for the study of vitrification, the quaternary system of natural honey was utilized. This system allows for the characterization of all the above-noted thermal events and complements the current trend in cryopreservation which manifests the vitrifiability of a multicomponent system. Multicomponent systems add to the entropy of the solution, thus depressing the propensity for homogeneous nucleation. By avoidance or reduction of the possibility for homogeneous nucleation, the probability of glassy state formation greatly increases (MacFarlane, 1987).

Nuclear magnetic resonance (NMR) is a noninvasive technique often used for the examination of biological systems and, in this case, the evaluation of solid/liquid states at the atomic level. This technique allows for the determination of relaxation effects of the available protons in samples containing water in various states. From the NMR experiments we determined the spin–spin relaxation (T_2) for all protons in the sample, thus providing insight into the nature of the structure. Protons that are in a rigid crystalline lattice will relax at a much faster rate than those in a solution state. Measurement of this limited proton mobility in vitreous water can be obtained either directly or indirectly by analysis of the free induction decay (FID). The FID is dependent on the T_2 and can easily be monitored due to the short acquisition time required for data accumulation.

Differential scanning calorimetry (DSC) is becoming a

---

* To whom correspondence should be addressed.
‡ Present address: 1 Squibb Drive, P.O. Box 191, Biological Process Development, New Brunswick, NJ 08903-0191.

Vitrification in Honey

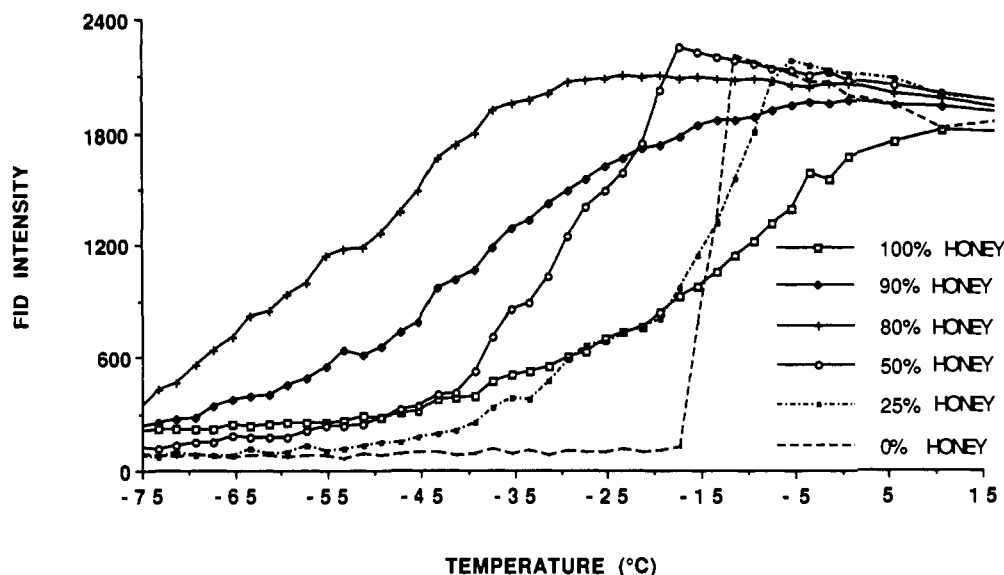*J. Agric. Food Chem.*, Vol. 38, No. 9, 1990 **1825**

Figure 1. NMR evaluation of the cooling of five honey and one control solutions. Data from the experiment were collected from the peak FID intensity and plotted versus temperature. Each data point represents an average of six FID analyses.

widely recognized probe in the analysis of the events thought to be of consequence in bulk samples. As determined by DSC, the T_g is represented as a change in heat capacity, which is observed as the base line shifts on the heat flow curve (thermogram) (Wunderlich, 1981). A common convention is to denote the T_g as the inflection point in the discontinuity of heat capacity on a thermogram, which corresponds to the peak maximum in the first derivative of the heat flow curve (negative derivatives are maximums) (Levine and Slade, 1990). Although T_g is often referred to as a second-order or apparent second-order transition and is characterized by changes in heat capacity, these changes occur over a temperature range and thus are not strictly discontinuous (Brennan, 1973). Continued heating through T_g results in T_m or T_d followed by a T_m, both of which are first-order transitions.

MATERIALS AND METHODS

A NMR spectrometer (Praxis II, San Antonio, TX) equipped with a permanent magnet (2.5 kG) with an operating field strength of 10.7 MHz was used for proton mobility analyses. The 90° pulse width used in the free induction decay (FID) study was applied for 12 s to the 6 mL of honey solution centered in a 25-mm probe. A dead time of 23 s ($T_2 < 23$ s) excluded signals from hydrogen associated with ice ($T_2 < 20$ s). Temperatures were controlled by a programmed nitrogen burst cooler (Cryo-Med Models 1010 and 990C). Samples were allowed to equilibrate for 20 min after each 1–2.5 °C temperature change. Temperatures were recorded by using a digital thermometer (Fluke 2190A) with copper–constantan thermocouples fixed at the center of the sample tubes.

The carbohydrate content of the undiluted honey was analyzed by a Waters high-performance liquid chromatography system (Waters Associates, Milford, MA) with an amine-modified column (Hendrix et al., 1981).

Thermal changes occurring in honey/water dilutions of various concentrations (v/v) were investigated by using power compensated DSC (Perkin-Elmer DSC 7 cooled by liquid nitrogen equipped with a P-E 7700 microcomputer). Samples (16 μL) were placed in weighed 25 μL aluminum volatile pans and sealed, and sample weight was recorded. A standard scan rate of 5 °C min⁻¹ was adopted, and data were collected at the instrument's standard rate.

Isothermal holds of 2–5 min were programmed at the onset and termination of all scans. The varying concentrations of commercially available honey were cooled and warmed between +20 and -150 °C. Heat capacity (C_p) and enthalpy were determined by utilizing Perkin-Elmer DSC software and later

plotted against temperature for identification of slope changes corresponding to subambient thermal events. All thermograms were subtracted from empty pan base lines before being plotted.

RESULTS AND DISCUSSION

The carbohydrate content of the undiluted commercially available honey was determined by high-performance liquid chromatography to be 47.2% fructose, 43.2% glucose, 1.6% sucrose, and 8% unidentified components, possibly maltose.

The honey solutions were analyzed by ¹H NMR and DSC. Analyses of the FID from NMR studies indicated several changes in the relaxation processes of entire solutions at various subambient temperatures. Additional information was provided by analyses of the thermal characteristics of the samples (DSC), including changes in the heat capacity and enthalpy. Rapid establishment of the reproducibility of the thermal events was also accomplished via the calorimetric analyses.

The temperatures were lowered and raised at controlled rates in all experiments. The delay time of 20 s after the radio frequency pulse in the NMR studies allowed for sampling of only the protons in a solution state.

Protons in an ice crystal lattice relax at a faster rate than protons in a liquid state. This relaxation behavior, specifically the T_2 (spin–spin) relaxation process, allows for the differentiation of the liquid and solid states. When sampling was performed after the fixed delay, only protons in a nonsolid state were sampled. Sampling at rates faster than 18 s would represent mostly protons in a solid state (ice in the described experiment).

The onset of crystallization of the distilled–deionized water control was noted (Figure 1) at -13 °C. Continued lowering of the temperature toward liquid nitrogen temperatures (-196 °C) established a base-line control for an almost completely solid state. When the concentration of honey was increased, a shift toward a lower temperature of crystallization was anticipated. This shift was observed for sample solutions >35% honey. The T_c shift in dilute solutions was probably due to the increased propensity for heterogeneous nucleation of the water (with nucleation sites such as organic and inorganic particles) present in honey.

The exponential decay of the FID signal decreased with an increase in the amount of honey present in the sample.
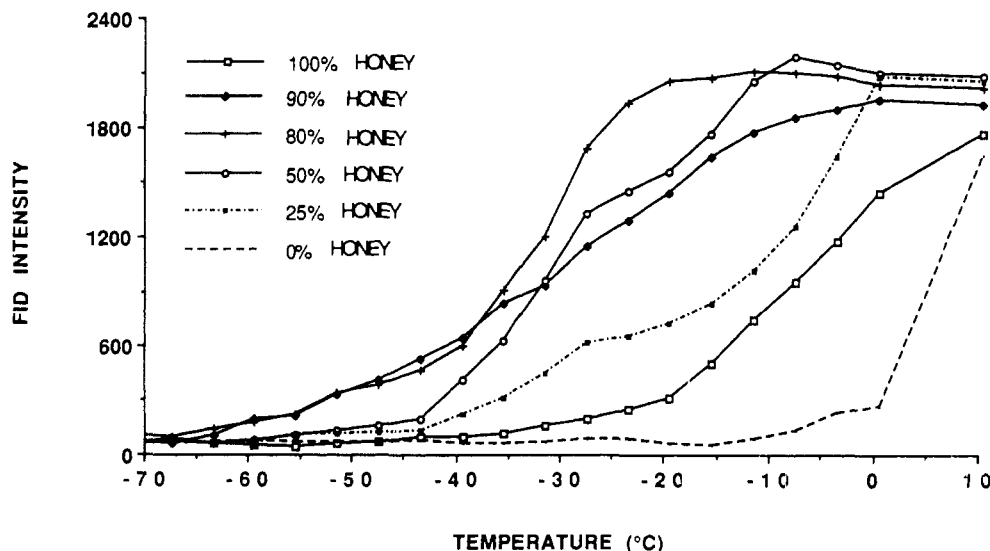
Figure 2. NMR evaluation of the warming of five honey and one control solutions. Data from the experiment were collected from the peak FID intensity and plotted versus temperature. Each data point represents an average of six FID analyses.

With a 15° drop in temperature after the onset of crystallization in the 25% honey solution, the exponential decay in the FID signal decreased approximately 2-fold. The FID signal intensity gradually decreased to near base line levels at approximately -62 °C. Fifty percent honey exhibited behavior similar to that of the 25% solution but with a decrease in decay at -22 °C, 4 °C below its crystallization onset temperature. The FID, upon continued cooling below -22 °C, underwent two distinct changes in slope. One was noted at approximately -42 °C, and a second was at approximately -60 °C (Figure 1).

As the 25% and 50% solutions crystallized, the remaining portion sampled contained the concentrated solution where the composition was independent of the initial honey concentration. A continued decrease in temperature resulted in an increase in viscosity, thereby decreasing the relaxation time due to decreased molecular motion. The two observed changes in the FID signal of the 50% honey solution below -22 °C suggest the possibility that two distinct vitreous domains are present in the sample.

Increasing the concentration of honey above 50%, as shown in Figure 1, resulted in the loss of the exponential FID signal. The gradual loss of FID signal was due to the increase in viscosity with only limited ice crystal formation at concentrations of up to 80%. No ice was observed in the higher concentrations. The NMR sample of undiluted honey became extremely viscous at -5 °C and brittle at -50 °C. Pure honey formed a vitreous state at temperatures below -50 °C as demonstrated by DSC analyses (see below).

When the temperature of the crystalline/vitreous samples was raised, reversal of the cooling behaviors was observed (Figure 2). The aqueous control remained solid with a minimal FID signal. The FID signal of the pure honey began to increase at -54 °C and continued a gradual rise to above 10 °C (Figure 2). The 90% solution maintained a weak, yet steady, rise in FID response until -7 °C. No ice was observed in this sample, and the additional water resulted in greater FID signal as compared to that of pure honey at subambient temperatures. This shift was due to the additional percentage of water molecules being sampled but not undergoing ice crystal formation. It was not until 70% honey (30% water) that the signal shifted toward lower FID intensities due to the formation of ice.

Once again, several changes in the slope of the FID were noted for 50% honey on warming, as on cooling. These changes were at -60 (possible glass transition), -43 (possible second glass transition), -29 (onset of melt), and -10 °C (completion of melt) (Figure 2). The two glass transitions found in this solution may arise from separate distinct vitreous domains. Evidence for this conclusion is presented in DSC arguments below. If the formation of the second glass were dependent on the low-temperature glass, and its formation were a consequence of warming through the low-temperature glass transition, then evidence from the NMR and DSC studies would have been noticed. During the warming scan, at temperatures between -60 and -43 °C, a devitrification would have had to occur with the formation of additional ice. The remaining concentrated honey solution of high viscosity would result in the formation of a second vitreous domain. If this dependent method played a role in the formation of the second glass transition in the solution, then a decrease in FID signal would have been observed between -60 and -43 °C. Because there was no observed decrease in NMR signal in this temperature range, devitrification did not occur to an appreciable level. Therefore, the evidence suggests that the higher temperature glass is independent of the lower temperature glass. The two domains may result from the two dominant carbohydrates found in honey, namely, glucose ($T_g = -43$ °C) and fructose ($T_g = -42$ °C) (approximately 1:1 ratio). Conformational differences of the two carbohydrates in solution at these temperatures probably lead to nonidentical perturbation of their hydration layers.

Analyses of the solutions using DSC provided valuable insight into their thermal behavior. The 100% honey sample vitrified at -49 °C as indicated by the enthalpy and heat capacity data (C_p, see Figure 3). A C_p drop on cooling of 0.81 J °C g^{-1} was noted as well as a proportional rise in C_p on warming (Figure 3). An isothermal hold at -62.5 °C for 90 min, followed by warming through the T_g, exhibited a characteristic "overshoot" in the thermogram and heat capacity data, indicating that the vitreous sample underwent relaxation with a loss of entropy. This relaxation, characteristic of vitreous material, compensates for the loss of entropy.

The 90% honey solution exhibited similar behavior with the exception that the T_g was shifted to -65 °C. A T_g at -77 °C was observed with the 80% honey solution, and

Vitrification in Honey

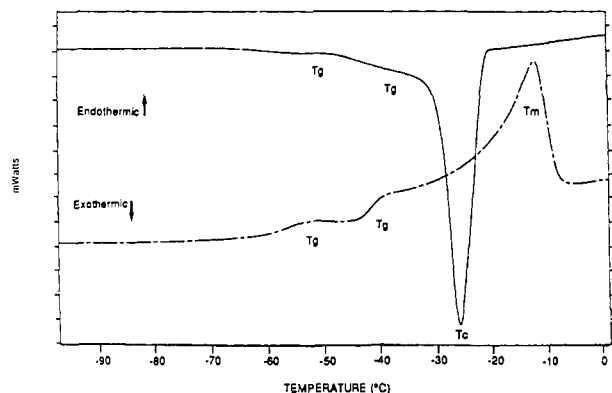*J. Agric. Food Chem.*, Vol. 38, No. 9, 1990 **1827**

Figure 3. DSC thermogram obtained from a sample solution of 50% aqueous honey. The cooling curve is represented by a solid line, and the warming data are noted by a dashed line.
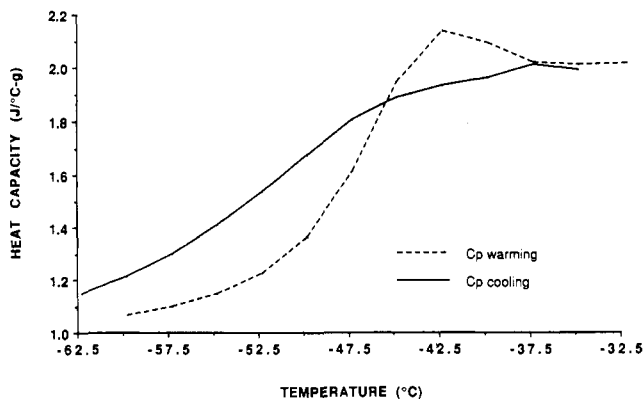

Figure 4. Heat capacity data for both the cooling and warming scans of an undiluted honey solution (100%).

Figure 5. First derivatives of the heat capacity values obtained from two DSC studies. The sample, 50% honey/50% water, was cooled and warmed at a rate of 5 °C min^{-1}. An isothermal hold at -65 °C for 90 min followed by cooling to -80 °C and then warming to 20 °C afforded the heat capacity denoted by a solid line. The dashed line represents data obtained from the same sample without an isothermal hold. Also noted are three transitions: T_g, glass transition; T_d, devitrification; and T_m, the onset of the melt.

at warming rates of 5 °C min^{-1} or less, a devitrification was noted followed by a melt. None of the three honey solutions (100%, 90%, 80%) exhibited ice crystal formation on cooling.

The 50% honey solution demonstrated two changes noted in the thermogram (Figure 3) on cooling after the bulk water crystallization as were two comparable changes in the C_p data (Figure 4). These events suggest the formation of two distinct vitreous states. When the same sample was warmed, two changes in the C_p were noted prior to the melt. Cycling in the region between -75 and -32.5 °C demonstrated the reproducibility of the two T_g's. The first derivative of the C_p data in the "recycling" temperature range (Figure 5) indicated no significant devitrification in the range between the two T_g's. After an isothermal hold at -65 °C for 90 min followed by cooling to -80 °C and then warming through the melt, a T_d at -52.5 °C was observed. During relaxation, a portion of the water molecules may have become less strongly associated with the carbohydrates such that additional crystallization occurred. Isothermal holds at -42.5 °C for up to 2 h allowed for relaxation as noted on warming by the overshoot of the C_p. These experiments suggest the formation of two distinct vitreous states which are most likely dependent on the differences in the hydration layers of the two major carbohydrates.

The remaining sample, 25% honey, demonstrated only one T_g after the crystallization at -62 °C. This suggests that the concentration of the glassy state is independent of the initial concentration. The exothermic crystallization was greatest in this sample, as would be expected.

The application of honey, which is basically a carbohydrate system, to the analyses of DSC and NMR methodologies is simple, yet provides some insight into the
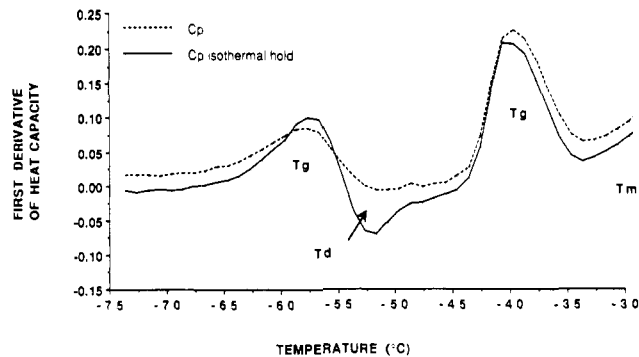
behavior of low-temperature vitrifiable solutions. The NMR experiments required larger samples, which limits comparisons to only those of like volumes. In general, the NMR data are more difficult to interpret due to the complex nature of the protons in solution. External factors, such as volume and temperature equilibration time, also contribute to the complexity. The DSC is generally simplistic in its data acquisition and limits the number of external factors that affect the samples.

ACKNOWLEDGMENT

LITERATURE CITED

Brennan, W. P. What is a T_g? A review of the glass transition. *Perkin-Elmer Therm. Anal. Study* **1973**, No. 7.

Claudy, P.; Commercon, J. C.; Letoffe, J. M. Quasi-static study of the glass transition of glycerol, by DSC. *Thermochim. Acta* **1988**, *128*, 251-260.

Hendrix, D. L.; Lee, R. E.; James, H.; Baust, J. G. Separation of carbohydrates and polyols by a radially compressed high-performance liquid chromatographic silica column modified with tetraethylene pentamine. *J. Chromatogr.* **1981**, *210*, 45-53.

Levine, H.; Slade, L. Cryostabilization technology: thermoanalytical evaluation of food ingredients and systems. In *Thermal Analysis of Foods*; Ma, C. Y., Harwalkar, V. R., Eds.; Elsevier Applied Science: London, 1990 (in press).

Luyet, B. J. The Devitrification Temperatures of Solutions of a Carbohydrate Series. *J. Phys. Chem.* **1939**, *43*, 881-885.

MacFarlane, D. R. Physical aspects of vitrification in aqueous solutions. *Cryobiology* **1987**, *24*, 181-195.

Turnbull, D. Under what conditions can a glass be formed? *Contemp. Phys.* **1969**, *10*, 473-488.

Wunderlich, B. In *Thermal Characterization of Polymeric Materials*; Turi, E. A., Ed.; Academic Press: Orlando, FL, 1981.