Calorimetric studies of the state of water in seed tissues

Christina W. Vertucci

USDA-Agricultural Research Service National Seed Storage Laboratory, Colorado State University Campus, Fort Collins, Colorado 80523 USA

ABSTRACT   To understand the physical state of water in hydrating biological tissues, thermodynamic properties of water in cotyledons of pea and soybean with moisture contents ranging from 0.01 g H_2O/g dw to 1.0 g H_2O/g dw were studied using differential scanning calorimetry. The heat capacity of the tissues increased abruptly at moisture contents above 0.08 and 0.12 g H_2O/g dw for soybean and pea cotyledons, respectively. Melting transitions of water were observed at moisture contents >0.23 and 0.26 g H_2O/g dw for soybean and pea. However, freezing of water was not observed unless moisture contents exceeded 0.33–0.35 g H_2O/g dw. In both seed tissues, the temperatures of the freezing and melting varied with moisture content and showed hysterisis. The energy of the transition also varied with moisture content and was similar to the heats of fusion and crystallization of pure water only at moisture contents >0.54 and 0.58 gH_2O/g dw for soybean and pea seeds, respectively. The thermal properties of water change distinctly as seed moisture content changes: at least five states or water can be identified.

INTRODUCTION

Water affects the structural attributes of macromolecules. Macromolecules also affect the properties of water. This interactive phenomenon is most apparent when solutions or emulsions of macromolecules are concentrated or when biological tissues are dehydrated. However, the extent to which the properties of water change and their relevance to the function of macromolecules is poorly understood. This study was initiated so that comparisons could be made between the properties of water and changes in physiological activity of organisms that survive desiccation.

Most of the data available documenting changes in the thermodynamic and motional properties of water with hydration have utilized protein or phospholipid systems (see reviews 1–4). Based on calorimetric measurements (1, 4–7), nuclear magnetic resonance[1] (NMR) and infrared (IR) spectroscopy (8–10), and ice crystal print sizes (11), researchers have ascribed several hydration levels to protein systems.

Studies of the status of water in biological tissue is complicated by the irreversible changes that occur when desiccation-intolerant organisms are dried. The motional properties of water in desiccation tolerant seeds and *Artemia* cysts have been studied by NMR and dielectric studies (12–17) and at least four types of water have been identified. From these data, models, analogous to those proposed for the hydration of single macromolecules, have been developed to describe hydration of tissues (12, 16–20). Recently, calorimetric techniques have been applied to the study of water in hydrating seeds. Freezable water was observable at 0.23 and 0.26 g H_2O/g dw for soybean and pea, respectively (21). The glass forming capabilities of water between this moisture level and 0.40 g H_2O/g dw (22–23) conferred some resistance to freezing injury (23). Aqueous glasses were also detectable at moisture contents lower than where freezable water was observable (22) suggesting that water with limited mobility may have an amorphous structure.

The purpose of this paper is to further document changes in the physical properties of water in hydrating seeds using differential scanning calorimetry. This information will increase our understanding of water at biological interfaces under ambient as well as subfreezing conditions.

METHODS

Seed material and hydration

Cotyledons of soybean (*Glycine max* cultivar [cv] Williams'82) and pea (*Pisum sativum* cv Alaska) were used to study the relationships between moisture content, heat capacity, and characteristics of phase transitions of water. These two tissues were selected because there were existing data comparing physiological activity with moisture level and because previous studies suggested that the triglycerides present in soybean cotyledons affected the phase behavior of water (21). To achieve moisture contents between 0.01 and 0.23 g/g, whole cotyledons were equilibrated at various relative humidities using saturated salt solutions

---

[1]*Abbreviations used in this paper:* cv, cultivar; DSC, differential scanning calorimetry; g/g, g H_2O/g dw; IR, infrared; NMR, nuclear magnetic resonance; RH, relative humidity.

(18). Moisture levels between 0.14 and 1.0 g/g were obtained by adding known quantities of water to preweighed seed samples. Slices of cotyledons were sealed into aluminum DSC pans and fresh weights were taken before DSC measurements. After DSC measurements, the pans were punctured and dry weights were determined by heating samples at 95°C for 5 d. Moisture contents are expressed on a dry weight basis. The amount of material used in a DSC run varied with the moisture content: ~20–25 mg samples were used when the moisture level was below 0.3 g/g and ~10 mg samples were used when the moisture level was above ~0.3 g/g.

Calorimetry

Freezing and melting transitions of seed cotyledons at various moisture contents were detected using a Perkin-Elmer Corp. (Norwalk, CT) DSC-4 or 7. For heating runs, samples were cooled at 10°C/min to −150°C and then thermograms were recorded during heating at 10°C/min from −150–20°C. Cooling runs were recorded as cotyledon tissues were cooled from 25 to −155°C at 10°C/min. The energy of the transitions were evaluated from the area of peaks above and below the baseline. Deviations from the baseline were detected from changes in the first derivative of the thermogram curve. The onset temperature of the melt was determined as the point of intersection between the baseline and a line drawn from the steepest portion of the initial melt.

The heat capacity of cotyledon tissues hydrated to various water levels was determined using a Perkin Elmer DSC-4. The ordinate displacement was measured from a chart recorder as samples were heated at 2°C/min from −15 to −10°C, −32 to −28°C, and −62 to −58°C. Although this is a relatively rapid scanning rate for heat capacity measurements, the 2°C/min heating rate was chosen to optimize signal-to-noise ratios of dry samples which showed small ordinate displacements at slower scanning rates. Sub-zero temperature ranges were used in the study to eliminate complications from active metabolism, and to verify that water transitions affected heat capacity measurements. Samples were cooled at 10°C/min to the lower temperature, and allowed to equilibrate until a steady baseline was achieved, 1–5 min. For soybean cooled to −32°C, equilibration often required up to 30 min. The power of ordinate displacements was calibrated with a benzoic acid standard treated similarly (24). Differences in weight between the sample and reference pan were corrected for using the heat capacity of aluminum calculated for each temperature range studied.

RESULTS

Analyses of the state of water in pea and soybean seeds were carried out by examining differences in cooling and heating thermograms of cotyledons at various moisture contents and comparing the behavior to that of pure water (Table 1; Figs. 1 and 2). Exotherms from cooling runs of pea cotyledons were observed when the moisture level was ~0.35 g/g or more (Fig. 1 A). These transitions were small and broad at 0.35 g/g (occurring over a temperature range of −25 to −70°C). As the moisture level increased, several freezing exotherms were observable; the size and sharpness of peaks increased with increasing water content (Fig. 1 A). A similar pattern was observed for melting endotherms in pea (Fig. 1 B) except that changes were observable at lower water contents: broad melting endotherms were first noted at 0.25 g/g, and the

peaks become larger and sharper as the cotyledons became more moist. Shifts in power, indicative of glass transitions were noted at moisture contents between 0.3 and 0.4 g/g. A clear example of this is the step-like transition at ~ −60° in the thermogram of pea at 0.35 g/g water content (Fig. 1 B).

In soybean, lipid transitions were always apparent and occurred within broad temperature ranges, often obscur-
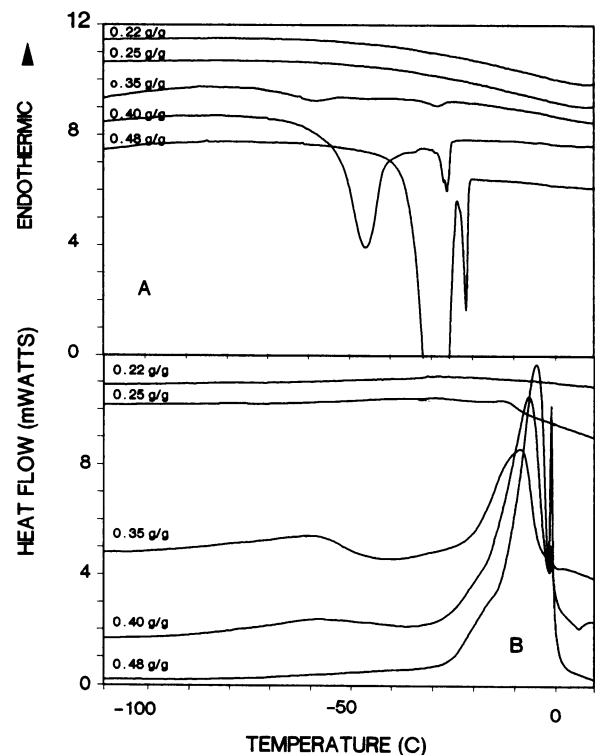

FIGURE 1 DSC cooling (A) and heating (B) thermograms of pea cotyledons at different moisture contents. Samples were scanned at 10°C/min from −150 to 20°C.
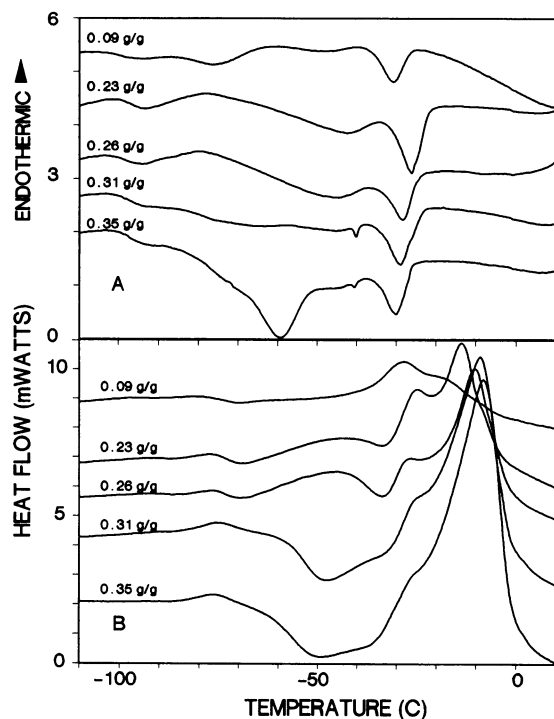
FIGURE 2 DSC cooling (A) and heating (B) thermograms of soybean cotyledons at different moisture contents. Samples were scanned at 10°C/min from −150 to 20°C.
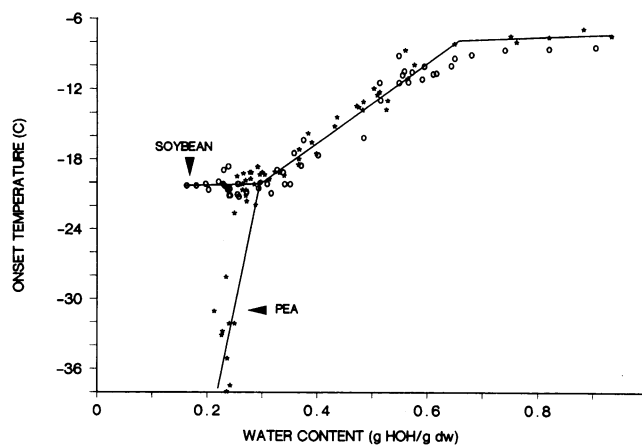

FIGURE 3 The effect of moisture content on the onset temperature of the water melting transitions for pea and soybean cotyledons. Data are taken from thermograms similar to those presented in Figs. 1 B and 2 B. The onset temperature for water transitions in soybeans was determined by assuming the baseline for thermograms of hydrated tissues was the thermogram from dry tissue.

ing the water transitions (Fig. 2). This was most notable during cooling runs, where detection of water freezing was difficult because there were multiple lipid exothermic events (Fig. 2 A). On warming runs, the shape of the endotherms changed with increasing hydration levels in the soybean cotyledon, such that the primary peak with onset at ∼ −37°C became progressively small compared with the peak at temperatures between −17 and −2°C (Fig. 2 B). This secondary peak was interpreted as water melting because the size and temperature increased as water content increased. Shifts in power were also noted in the warming thermograms. The shifts at ∼ −80°C are present in dry tissue and are attributed to the triglycerides (23). Pretransitions at temperatures between −70 and −30°C, observed when the water content was between 0.23 and 0.38 g/g, are probably attributable to aqueous glasses (21, 23).

The onset temperature at which water was observed to melt increased as the water content increased (Fig. 3). For both soybean and pea cotyledons, the onset of the melts defined three distinct moisture regions. In pea, the onset temperature increased sharply from −38 to −20°C as cotyledons were hydrated from 0.22–0.30 g/g (Fig. 3). In contrast, the onset temperature for soybean in this moisture region (0.21–0.30 g/g) was constant at ∼ −20°C.

As hydration proceeded to ∼0.65 g/g, the onset temperature of the water melt increased to ∼ −8°C (Fig. 3). No further changes in the onset temperature were observed with increases in water content above 0.65 g/g (Fig. 3).

To determine the point at which freezable water was present and the enthalpy associated with water freezing or melting, changes in the energy of exotherms and endotherms for pea and soybean were measured as a function of water content (Figs. 4 and 5). Estimates of the energy of the transition(s) were made by calculating the area of the thermogram peaks as in Figs. 1 and 2. Linear regressions of moisture content and enthalpy/dw were performed iteratively to determine the best fit to the data.

Comparisons of enthalpy/dw and water content between 0.33 and 0.67 g/g for cooling runs of pea cotyledons (Fig. 4 A) resulted in a single line ($r^2 = 0.98$) with a slope of 338 J/g H_2O. The x-intercept of this line, representing the point at which water freezing is measurable, is 0.34 g/g. Similar treatment of data from soybean was complicated by the presence of several lipid transitions which occurred in the temperature range of interest (Fig. 2 A). For soybean, the total peak area (exotherms of lipid transitions + exotherms of water transitions) was compared wih water content, and the data was fit to two lines with a point of intersection at 0.32 g/g (Fig. 5 A). The line calculated for moisture contents between 0.06 and 0.30 ($r^2 = 0.50$, slope = 24 J/g H^2O) is interpreted as the energy associated with lipid transitions whereas the line calculated between 0.31 and 0.70 g/g ($r^2 = 0.98$) is interpreted as the sum of the energy of water and lipid freezing. The slope of the latter line is 385 J/g H_2O. Thus,
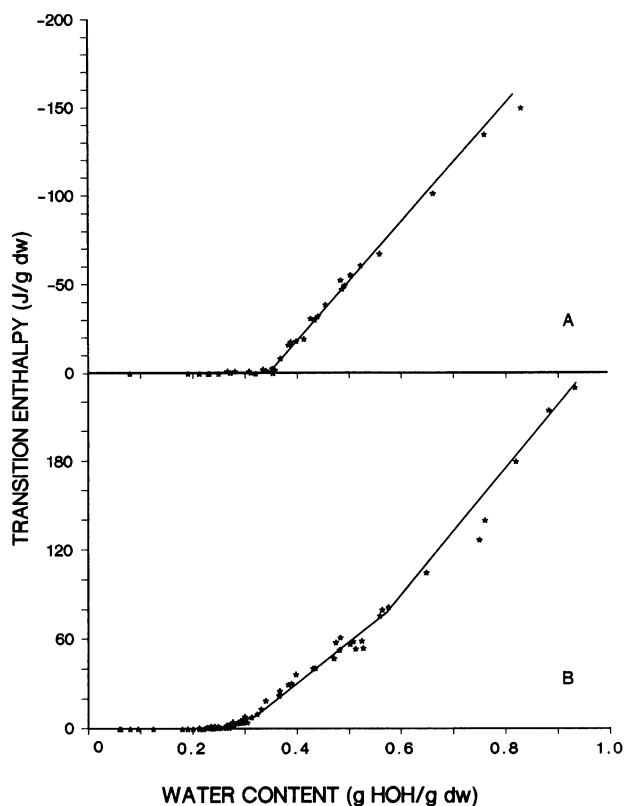
FIGURE 4 The effect of moisture content on the enthalpy of the freezing (A) and melting (B) transitions in pea cotyledons. Data are taken from thermograms similar to those presented in Fig. 1. The lines represent the iterative determination of the least-squares best fit to the data.
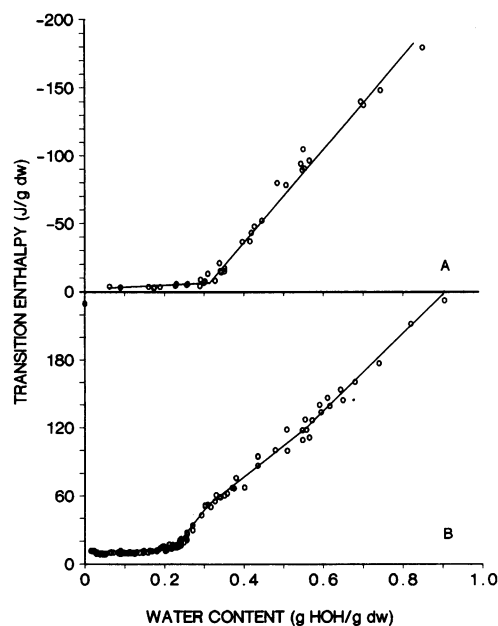

FIGURE 5 The effect of moisture content on the enthalpy of the freezing (A) and melting (B) transitions in soybean cotyledons. Data are taken from thermograms similar to those presented in Fig. 2. The lines represent the iterative determination of the least-squares best fit to the data.

the energy calculated for the freezing of water is 361 J/g H_2O.

Comparisons of the enthalpy/dw as a function of water content for heating runs of pea and soybean cotyledons did not reveal a linear relationship (Figs. 4 B and 5 B). Data for pea described three moisture regions, between 0.23 and 0.33 g/g ($r^2 = 0.78$), 0.33 and 0.58 g/g ($r^2 = 0.95$) and >0.58 g/g ($r^2 = 0.99$) with slopes of 102, 237, and 325 J/g H_2O, respectively (Table 2). Data for soybean were separated into four moisture regions, between 0.05 and 0.22 g/g ($r^2 = 0.70$), 0.22 and 0.32 g/g ($r^2 = 0.95$), 0.32 to 0.56 g/g ($r^2 = 0.93$) and >0.56 g/g ($r^2 = 0.96$) with slopes of 37, 427, 287, and 350 J/g H_2O, respectively (Table 3).

Measurements of the relationship between specific heat and water content were made at $-12.5°$, $-30°$, and $-60°C$ for pea and soybean cotyledons between 0.02 and 0.60 g/g (Figs. 6 and 7). Examination of DSC cooling thermograms of soybean and pea cotyledons in this moisture range (Figs. 1 A and 2 A) demonstrate that freezable water does not freeze at $-15°C$ but the transi-

tion is mostly complete at $-62°C$. Measurements of specific heat at $-30°C$ were taken using two regimes: (a) after the cotyledons had been cooled to $-32°C$ and (b) after they had been exposed to $-62°C$ and then warmed to $-32°C$. In the former case, some ice is present if moisture contents are above 0.35 g/g (see Figs. 1 A and 2 A). In the latter case, ice will be present if moisture contents are above 0.23 g/g.

Evaluation of heat capacity changes as a function of moisture content of both pea and soybean cotyledons suggest a complex relationship. In most cases, four moisture regions can be ascribed (Figs. 6 and 7). For example at $-12.5°C$ (Figs. 6 and 7), heat capacity increases only slightly with increasing moisture content at moisture contents between 0.02 and 0.11 g/g for pea ($r^2 = 0.95$, slope $= 3.26$ J/C/g H_2O) and 0.02 and 0.08 g/g for soybean ($r^2 = 0.96$, slope $= 3.17$ J/C/g H_2O). As moisture content increases to 0.28 g/g for pea and 0.25 for soybean, heat capacity increases abruptly with slopes of 6.36 ($r^2 = 0.99$) and 6.10 J/C/g H_2O ($r^2 = 0.95$), respectively. Within the third moisture region, (0.28–0.42 g/g for pea and 0.25–0.38 g/g for soybean) there was, again, only a slight chance of heat capacity with water content (slope $= 1.94$ and 1.99 J/C/g H_2O, $r^2 = 0.65$ and 0.67 for pea and soybean, respectively). It is in the

TABLE 2 Calorimetric properties of water in pea cotyledons at different water contents

	Hydration level				
	1	2	3	4	5
Moisture range (g/g)	0–>0.12	0.12–>0.24	0.24–>0.35	0.35–>0.58	>0.58
Relative humidity (%)*	0–30	30–80	80–92	nd[‡]	nd
Heat capacity ($J/C/g$ H_2O)					
$-12.5°C$	3.26	6.36	1.94	4.10	nd
$-30°C$	2.81	5.64	1.09	2.66	nd
$-60°C$	2.36	4.10	0.48	1.90	nd
Water melt					
ΔH (J/g H_2O)	0	0	102	237	325
Tonset ($°C$)	na	na	$-38°->-18°$	$-18°->-8°$	$-8°$
Water freeze					
ΔH (J/g H_2O)	0	0	0	338	nd

Enthalpies of transitions and heat capacities are determined from the slopes of curves given in Figs. 4 and 6. Transition temperatures are taken from thermograms similar to those presented in Fig. 1.
*From isotherms measured at 5°C (18).
[‡]"na" and "nd" as described in Table 1.

fourth moisture region (water content >0.42 g/g for pea and 0.38 g/g for soybean) that the slope of the heat capacity vs. water content curve is similar to the specific heat capacity pure liquid water (slope = 4.10 and 4.30 $J/C/g$ H_2O, $r^2 = 0.91$ and 0.94, for pea and soybean, respectively). The slopes of the curves within each moisture region decline as the temperature at which heat capacity was measured was decreased to $-30°C$ and $-60°C$ (Figs. 6 and 7). For soybean, no relationship was discernable between heat capacity and moisture content at $-60°C$ when moisture content was >0.32 g/g. This

may be a reflection of incomplete phase transitions of lipids or water at this temperature (Fig. 2 A). In pea, at moisture contents > 0.35 g/g, there is a >50% drop in the calculated slope for measurements made at $-60°C$ compared with -12.5 °C. This is probably a result of water in this moisture range changing from a liquid at $-12.5°$ to ice at $-60°C$ (Fig. 1).

The observed changes in the phase behavoir and heat capacity of water occur within specific moisture content ranges. These changes, describable by the slopes of the curves drawn in Figs. 4–7, represent changes in the

TABLE 3 Calorimetric properties of water in soybean cotyledons at different water contents

	Hydration level				
	1	2	3	4	5
Moisture content (g/g)	0–>0.08	0.08–>0.21	0.22–>0.33	0.33–>0.55	>0.55
Relative Humdiity (%)*	0–30	30–80	80–92	nd	nd
Heat capacity ($J/C/g$ H_2O)					
$-12.5°C$	3.17	6.10	1.99	4.30	nd
$-30°C$	2.53	6.03	0.97	3.16	nd.
$-60°C$	2.02	3.91	nd	nd	nd
Water melt					
ΔH (J/g H_2O)	0	0	390[‡]	250[‡]	323[‡]
Tonset ($°C$)	na	na	$-20°$	$-20°->-8°$	$-8°$
Water freeze					
ΔH (J/g H_2O)	0	0	0	361[§]	nd

Enthalpies of transitions and heat capacities are determined from the slopes of curves given in Figs. 5 and 7. Transition temperatures are taken from thermograms similar to those presented in Fig. 2.
*From isotherms measured at 5°C (18).
[‡]37 and 24 J/g H_2O, respectively, were subtracted from total slope to account for positive slope associated with lipid transition.
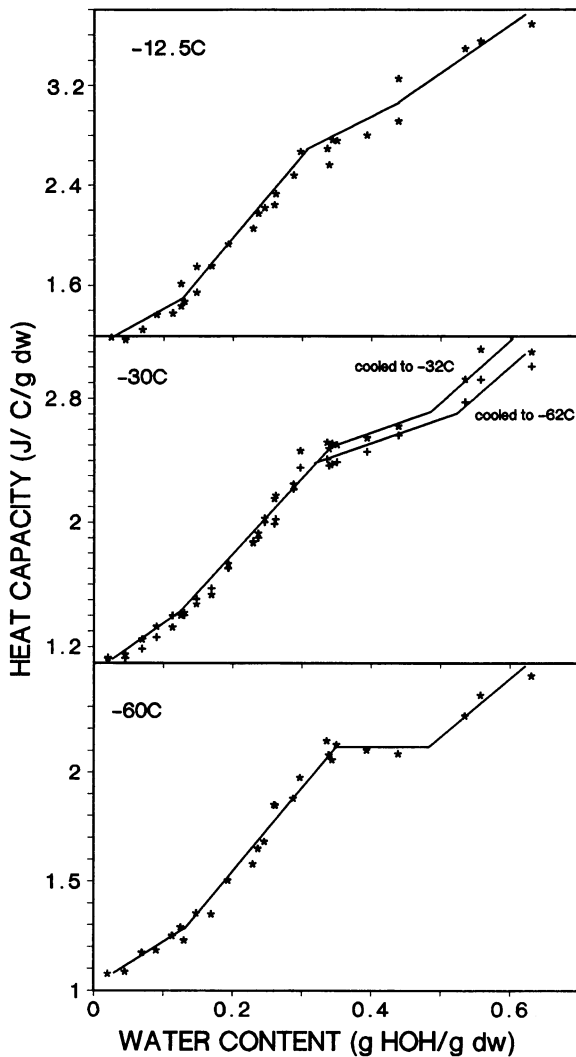
FIGURE 6 The effect of moisture content on the heat capacity of pea cotyledons at −12.5, −30, and −60°C. Heat capacities were determined from the ordinate displacement by samples heated at 2°C/min from −15 to −10°C, −32 to −28°C, and −62 to −58°C. Samples were cooled to the initial temperatures at 10°C/min. The crosses in data presented for −30°C represent samples that were cooled to −62°C and subsequently warmed to −32°C before heat capacity measurements. The lines represent the iterative determination of the least-squares best fit to the data.
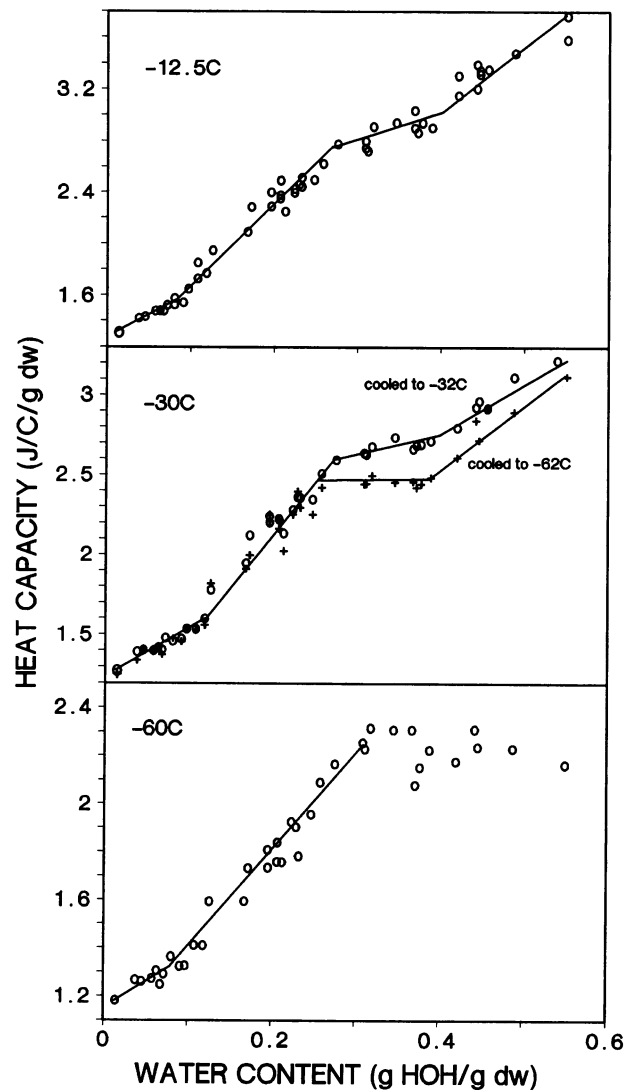
FIGURE 7 The effect of moisture content on the heat capacity of soybean cotyledons at −12.5, −30, and −60°C. Heat capacities were determined from the ordinate displacement by samples heated at 2°C/min from −15 to −10°C, −32 to −28°C, and −62 to −58°C. Samples were cooled to the initial temperatures at 10°C/min. The crosses in data presented for −30°C represent samples that were cooled to −62°C and subsequently warmed to −32°C before heat capacity measurements. The lines represent the iterative determination of the least-squares best fit to the data.

thermodynamic properties of cellular constituents, but can be expressed in terms of the apparent enthalpy of freezing and melting and the apparent specific heat of water (Tables 2 and 3). These values can be compared to the known thermodynamic properties of pure water (Table 1). At low moisture contents (<0.24 and 0.22 for pea and soybean, respectively), water is not observed to freeze or melt. Within this moisture range, two types of water can be identified by heat capacity measurements: below

0.10 g/g, the apparent heat capacity of water is similar to that of ice; between 0.10 and 0.24 g/g the apparent heat capacity of water is ∼50% greater than that of liquid water. As the cotyledon tissue is moistened to levels between 0.21 and 0.35, water is observed to melt, but the freezing exotherm is not observable. The energy associated with the water melt in pea is much less than that of pure water whereas the energy of the melt for soybean is

greater than that of pure water. The apparent heat capacity of water in this hydration range is small. At higher hydration levels, the properties calculated for water begin to resemble those of pure water: the apparent heat capacity is similar to the expected 4.184 J/C/g H_2O of pure water and the energy of the freeze is similar to 333 J/g H_2O. However, at moisture contents between ~0.33 and 0.55 g/g, the onset temperature of the melt changes in a noncolligative way with moisture content (Fig. 3) and the energy of the melt is 25% less than what is expected for pure water. At moisture contents >0.55 g/g the melting properties of water resemble those of water in dilute solutions.

DISCUSSION

The present study demonstrates that at least five hydration levels in seed tissues can be distinguished by the thermodynamic properties of water measured using DSC (Tables 2 and 3). While the presented parameters are described as properties of water, it is more likely that they represent an interaction between water and cellular constituents. Thus, changes observed, and described on a per gram basis of water, might actually reflect changes in nonaqueous components when water is added. The properties measured include the presence of freezing or melting transitions, calculations of energy and temperature of the transitions, and evaluations of the heat capacity of the water before and after the transitions. The moisture ranges at which each of these hydration levels exist are similar for pea and soybean cotyledons, and coincide with moisture ranges presented for model protein systems (1–5, 8, 12). With the exception of water in hydration level 3, the properties of water at each hydration level are similar for the two species (Table 2 and 3).

Hydration levels 1 and 2 are easily distinguished from the other hydration levels because in these regions, water is not observed to freeze or melt (Tables 2 and 3). Water in hydration level 1 has an apparent heat capacity less than that of pure liquid water but greater than that of ice (compare Table 2 and 3 with Table 1). Similar findings have been presented for lysozyme (5). As with ice, the apparent heat capacity of water in hydration level 1 decreases linearly with decreasing temperature, but with a greater slope (27). Calorimetric and dielectric studies of corn embryos at hydration level 1 demonstrate that this water does not exist as a glass at ambient temperatures and has severely restricted mobility (13, 15, 22).

Water in hydration level 2 is also unfreezable, but it can be distinguished from water in hydration level 1 because the apparent heat capacity is greater than that of liquid water (compare Tables 2 and 3 with Table 1) (5, 28). Unlike liquid water before the homogeneous nucleation point (26), the apparent heat capacity of water in hydration level 2 declines linearly with decreasing temperature. It has been suggested that water in hydration level 2 has solvent properties as it has the ability to form glasses (22), and molecular movement is facilitated as evidenced by dielectric and NMR studies (13–15).

Water is first observed to melt in hydration level 3, although no freezing exotherms were observed during cooling runs (Fig. 1 A and 2 A). The thermal behavior, exhibited during heating runs, of water at this hydration level is complicated, and resembles that of water in highly concentrated solutions (29–32). In particular, the endo and exo-thermic transitions before the main first order transition (often referred to as incipient and ante-melting and/or devitrification events) are indications that glasses were formed in the aqueous phase (21, 23, 29). Consequently, this type of water, regarded as slightly perturbed because of "orientational mismatches" according to the Franks and Eagland model (33) of protein hydration, is distinguished from water at other hydration levels because it has sufficient mobility to give rise to detectable glasses and because of it tendencies to melt at lower temperatures (Tables 2 and 3) (29, 32). In spite of the increased mobility, determination of the apparent heat capacity of water in hydration level 3 is much lower than would be expected for liquidous water or ice. This may be a reflection of heat capacity changes in macromolecules within this hydration level. Such changes are necessarily included in the apparent heat capacity determinations and are not corrected for.

Quantitation of the energy of the main melting endotherms reveals that the enthalpy of the melt (ΔH_{melt}) of water in hydration level 3 is quite different than ΔH_{melt} of pure water (compare Tables 2 and 3 with 1). Unusually low apparent ΔH_{melt}'s have been reported for protein systems (6, 7) and blood plasma (29). Recent determinations of the amount of unfreezable water in galactose/water solutions (31) point out the sensitivity of the calculations to the moisture range used in the study as well as the assumed ΔH_{melt} value, and suggest that ΔH_{melt} does indeed change with moisture level. While reductions in ΔH_{melt} are expected when the onset temperature is <0°C (25), the value of the apparent ΔH_{melt} calculated for water in pea cotyledons at hydration level 3 is smaller than can be accounted for by the low temperature of the transition (according to equations by Gekko and Satake [25], ΔH_{melt} of water melting at −35°C is ~260 J/g H_2O). An alternative explanation for the low apparent ΔH_{melt} calculated for water in proteins or pea cotyledons at hydration level 3 is given by the observation that enthalpies determined in the present way contain a heat of solution factor (30). The heat of solution varies with the solute, but is usually exothermic for hydrophilic substances and endothermic for hydrophobic substances

(30). Thus, the low apparent ΔH_{melt} calculated for pea cotyledons may be a reflection of dissolution of hydrophilic substances. The apparent ΔH_{melt} measured for soybean cotyledons in hydration level 3 is greater than that expected for pure water (Table 3). Similarly, measurements of ΔH_{melt} of phosphotidyl-ethanolamine and lecithin mixtures were 363 and 431 J/g H_2O, respectively (1). The large apparent ΔH_{melt} calculated for soybean cotyledons (which are ~20% lipid) and for phospholipids may be a reflection of water interactions with hydrophobic groups. This suggestion is consistent with earlier studies which hypothesized that hydrophobic interactions were initiated at hydration level 3 (4, 18).

In addition to differences in the apparent ΔH_{melt} measured for pea and soybean cotyledons, the temperature of the water melt differs for the two species. In pea, the onset temperature decreases sharply with decreasing water content within this hydration level (Fig. 3). The onset temperature of the water melt does not change for soybean within this hydration level (Fig. 3). Studies of the sensitivity of pea and soybean seeds at this hydration level to subzero temperatures substantiate the hypothesis that the thermal behavior of water differs in the two tissue types (21). When pea seeds with moisture contents below 0.36 g/g were exposed to $-80°C$, there was no reduction in germination. Soybean seeds at the same moisture level were undamaged if exposed to $-30°C$, but killed when exposed to $-50°C$. The reason for the differences in freezing behavior are unknown, but it was suggested that the lipids in the soybean seed played a role in their increased sensitivity to low temperatures (21).

The thermal properties of water in seeds at the fourth hydration level are similar to those of pure water. Water is observed to freeze at this hydration level and the apparent energy of the freeze is simlar to that of pure water (Figs. 1 A and 2 A, Tables 2 and 3). The tendency toward devitrification events is lost at this hydration level (Figs. 1 B and 2 B). This phenomenon has been reported previously (22, 23, 32). At $-12.5°C$, the apparent heat capacity of water in cotyledon tissue at hydration level 4 is similar to liquid water; at $-30°C$, the apparent heat capacity is intermediate between those of liquid water and ice; and at $-60°C$, the apparent heat capacity of water in pea cotyledons is similar to what is expected for ice (compare Tables 2 and 3 with Table 1). However, water in hydration level 4 is still distinguishable from bulk water in that the apparent energy of the melting transition is less than that of pure water, even if the low onset temperature is taken into consideration (compare Tables 2 and 3 with Table 1). The low values of the apparent ΔH_{melt} may be a result of the dilution of hydrophilic solutes since the experimental values probably include a heat of solution factor (30).

The apparent thermal properties measured for water in pea and soybean cotyledons with moisture contents >0.55 g/g are similar to water in dilute solutions. Although cotyledons will continue to take up water, the properties of the water do not change substantially. Thus, at moisture contents >0.55 g/g, hydration level 5, one might consider the seed fully hydrated.

While the data presented in this study provide evidence of at least five levels of hydration they do not reveal whether the structure of water at each hydration level changes or whether the structure of macromolecules associated with the water change. The presented measurements also do not suggest whether all five populations of water/nonaqueous component exist discretely within a fully hydrated seed. Evidence presented in the literature has been conflicting on these points. However, there is a general consensus that at least two populations of water exist around a hydrated macromolecule: one which has severely restricted mobility and one which has properties similar to bulk water. The data presented here is consistent with this finding because there is always a population of water that does not freeze.

The five levels of hydration reported here are based on changes observed in the apparent properties of water when dry tissues are hydrated; however, they correspond well to levels of physiological activity in seeds (19, 20, 34). Few reactions occur in the first hydration level, but progressive removal of this water results in accelerated deterioration of seeds (34, 35). Metabolism becomes increasingly facilitated in the second hydration level and respiration is measurable in the third. Germination of the seed is not evidenced until the seeds have attained moisture contents within the fifth hydration level (36).

It is generally accepted that macromolecular surfaces perturb the mobility and thermodynamic properties of neighboring water molecules. The extent to which these properties change is not well understood. This paper reports a comprehensive study of the changes in the thermal properties of water as a dry biological tissue becomes fully hydrated. Five hydration levels are distinguished in seed tissues. These hydration levels are related to the metabolic status of the seeds.

REFERENCES

1. Ladbrooke, B. D., and D. Chapman. 1969. Thermal analysis of lipids, proteins, and biological membranes. A review and summary of some recent studies. *Chem. Phys. Lipids.* 3:304–356.

2. Drost-Hansen, W. 1971. Structure and properties of water at biological interfaces. *In* Chemistry of the Cell Interface. H. D. Brown, editor. Academic Press, New York. 1–184.

3. Kuntz, I. D., and W. Kauzmann. 1974. Hydration of proteins and polypeptides. *Adv. Protein Chem.* 28:239–345.

4. Rupley, J. A., E. Gratton, and G. Careri. 1983. Water and globular proteins. *Trends Biochem. Sci.* 8:18–22.

5. Yang, P., and J. A. Rupley. 1979. Protein-water interactions. Heat capacity of the lysozyme-water system. *Biochemistry.* 18:2654–2661.

6. Haly, A. R., and J. W. Snaith. 1971. Specific heat of modified wools and the heat of fusion of absorbed water. *Appl. Polymer. Symp.* 18:823–834.

7. Ruegg, M., U. Moor, and B. H. Blanc. 1975. Hydration and thermal denaturation of β-lactoglobulin. Calorimetric study. *Biochem. Biophys. Acta.* 400:334–342.

8. Careri, G., A. Giansanti, and E. Gratton. 1979. Lysozyme film hydration events: and IR and gravimetric study. *Biopolymers.* 18:1187–1203.

9. Pacynko, W. F., J. Yarwood, and G. J. T. Tiddy. 1987. Infrared and far-infrared spectroscopic studies on the structure of water in lyotropic liquid crystals. *Liquid Crystals* 2:201–214.

10. Cameron, I. L., V. A. Ord, and G. D. Fullerton. 1988. Water of hydration in the intra- and extra-cellular environment of human erythrocyte. *Biochem. Cell Biol.* 66:1186–1199.

11. Cameron, I. L., K. E. Hunter, and G. D. Fullerton. 1988. Quench cooled ice crystal imprint size: a micro-method for study of macromolecular hydration. *Scanning Microsc.* 2:885–898.

12. Clegg, J. S. 1986. The physical properties and metabolic status of *Artemia* cysts at low water contents. *In* Membranes, Metabolism and Dry Organisms. A. C. Leopold, editor. Cornell University Press, Ithaca, New York. 169–187.

13. Bruni, F., G. Careri, and J. S. Clegg. 1989. Dielectric properties of *Artemia* cysts at low water contents. *Biophys. J.* 55:331–338.

14. Seewaldt, V., D. A. Priestley, A. C. Leopold, G. W. Feigenson, and F. Goodsaid-Zaluondo. 1981. Membrane organization of soybean seeds during hydration. *Planta.* 152:19–23.

15. Bruni, F., G. Careri, and A. C. Leopold. 1989. Critical exponents of protonic percolation in maize seeds. *Phys. Rev. A.* 40:2803–2805.

16. Askochenskaya, N. A. 1980. The water structure of dormant seeds and its role in storing. *Proc. 19th Intl. Seed Test. Assoc. Congress, Vienna.*

17. Ratkovic, S. 1987. Proton NMR of maize seed water: the relationship between spin-lattice relaxation time and water content. *Seed Sci. Technol.* 15:147–154.

18. Vertucci, C. W., and A. C. Leopold, 1987. Water binding in legume seeds. *Plant Physiol.* 85:224–231.

19. Leopold, A. C., and C. W. Vertucci. 1989. Moisture as a regulator of physiological reaction in seeds. *In* Seed Moisture. P. C. Stanwood and M. B. McDonald, editors. Crop. Sci. Soc. Amer. Special Publication no. 14, Madison, WI. 51–68.

20. Vertucci, C. W. 1989. The effects of low water contents on physiological activities of seeds. *Physiol. Plant.* 77:172–176.

21. Vertucci, C. W. 1989. The relationship between thermal transitions and freezing injury in pea and soybean seeds. *Plant Physiol.* 90:1121–1128.

22. Williams, R. J., and A. C. Leopold. 1989. The glassy state in corn embryos. *Plant Physiol.* 89:977–981.

23. Vertucci, C. W. 1989. Effects of cooling rate on seeds exposed to liquid nitrogen temperatures. *Plant Physiol.* 90:1478–1485.

24. Ginnings, D. C., and G. T. Furukawa. 1953. Heat capacity standards for the range 14 to 1200° K. *J. Am. Chem. Soc.* 75:522–527.

25. Gekko, K., and I. Satake. 1981. Differential scanning calorimetry of unfreezable water in water-protein-polyol systems. *Agric. Biol. Chem.* 45:2209–2217.

26. Wakabayashi, T., and F. Franks. 1986. Heat capacities of aqueous polyvinylpyrrolidone solutions at subzero temperatures. *Cryo Lett.* 7:361–366.

27. Doster, W., A. Bachleitner, R. Danau, M. Hiebl, and E. Luscher. 1986. Thermal properties of water in myoglobin crystals and solutions at subzero temperatures. *Biophys. J.* 50:213–219.

28. Drost-Hansen, W. 1985. Role of vicinal water in cellular evolution. *In* Water and Ions in Biological Systems. A. Pullman, V. Vasilescu, and L. Packer, editors. Plenum Press, New York. 523–534.

29. Simatos, D., M. Faure, E. Bonjour, and M. Couach. 1975. The physical state of water at low temperatures in plasma with different water contents as studied by differential thermal analysis and differential scanning calorimetry. *Cryobiology.* 12:202–208.

30. Franks, F. 1982. The properties of aqueous solutions at subzero temperatures. *In* Water: A Comprehensive Treatise. F. Franks, editor. Plenum Press, New York. 215–338.

31. Blond, G. 1989. Water-galactose system: supplemented state diagram and unfrozen water. *Cryo Lett.* 10:299–308.

32. Wolancyzk, J. P., and J. G. Baust. 1989. The influence of hold temperature on the phenomenon of "time dependency" in hydrated lysozyme glasses. *Cryo Lett.* 10:215–226.

33. Franks, F., and D. Eagland. 1975. The role of solvent interactions in protein conformation. *CRC Crit Rev. Biochem.* 3:165–219.

34. Vertucci, C. W., and E. E. Roos. 1990. Theoretical basis of protocols for seed storage. *Plant Physiol.* In press.

35. Vertucci, C. W., and A. C. Leopold. 1987. The relationship between water binding and desiccation tolerance in tissues. *Plant Physiol.* 85:232–238.

36. McDonald, M. B., C. W. Vertucci, and E. E. Roos. 1988. Soybean seed imbibition: water absorption by seed parts. *Crop Sci.* 28:993–997.