Bound Water in Soybean Seed and Its Relation to Respiration and Imbibitional Damage

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ABSTRACT
In an attempt to understand the initial stage of seed imbibition—the wetting stage—we have examined water binding in dry soybean cotyledon tissue using water sorption isotherm curves. The sorption isotherms show three levels of water affinity: a region of strongly bound water at moisture contents below 8%, a region of weakly bound water at moisture contents between 8 and 24%, and a region of very loosely bound water at contents greater than 24%. The enthalpies of the water binding for the three sectors were -6 to -12.5, about -2.5, and about -0.5 kilocalories per mole water, respectively.

The degree of physiological activity in the tissue reflects the level of water binding. O₂ consumption is first detectable in the second region of water affinity (8–24% water), and increases dramatically with increasing water content above about 24%. Damage due to imbibing water is greatest when initial seed moisture contents are in the region of strongest water binding. Damage is lessened and finally absent when seed moisture contents are increased to the second and then to the third level of water affinity.

The water content of seeds during storage or prior to imbibition strongly influences the staying quality and the success of subsequent germination. Seeds of low moisture contents, although more stable under storage, are particularly susceptible to stresses during imbibition (17). Hobbs and Obendorf (12) found that imbibitional damage to soybeans was greatest when initial moisture contents were below 13%. Ashworth and Obendorf (3) found that axes could be somewhat protected from imbibitional chilling injury by elevating the initial moisture levels to 17%. Bramlage et al. (4) showed that isolated soybean embryos with moisture contents below 23% were particularly sensitive to cold temperatures upon imbibition. Axes with water contents above 35% water appear to be protected from chilling effects.

The initial entry of water into dry seeds does not follow from Darcy’s Law; it resembles more a wetting phenomenon than a bulk flow through a porous material (23). Thermodynamic studies (24) and nuclear magnetic resonance spectroscopy (10, 22) of biological materials have shown that at low water contents, bulk water does not exist. We have suggested that it is the wetting process that is associated with imbibitional damage to seeds (23).

Pursuing the idea that the initial water entry into tissue is a critical moment in imbibition, we have examined the binding of water in dry soybeans and have attempted to relate the binding of water to the limits of respiratory activity and susceptibility to stress during imbibition.

MATERIALS AND METHODS
Soybean embryos (cv Wayne, 1980) were ground to produce pellets between 0.5 and 1 mm in diameter. The pellets were placed in jars over saturated salt solutions and incubated at 5 or 20°C until a constant weight was achieved. The relative humidities of these constant humidity chambers were taken from Rockland (18), Schneider and Schneider (21), and Anonymous (2). The amount of water taken up by the pellets after equilibration was determined gravimetrically. All water contents are expressed on a dry weight basis. The water content of the tissue at equilibrium with a given RH is used to describe the sorption isotherms in Figure 1. Isotherms were drawn by a computer program which provided best fit for the experimental data.

Using sorption isotherm data, thermodynamic parameters were calculated according to the Clausius-Clapeyron equation for heat of vaporization. Differential quantities are given by

\[
\Delta H = (R \times T_1 \times T_2)/(T_2 - T_1) \times \ln(aw_1/aw_2)
\]

\[
\Delta G = R \times T \times \ln(aw)
\]

\[
\Delta S = (\Delta H - \Delta G)/T
\]

where, at a given tissue water content, aw1 and aw2 are the relative humidities at the lower and higher temperatures, T1 and T2. ΔH is differential enthalpy of hydration; ΔG is differential free energy; and ΔS is differential entropy. R is the gas constant (1.987 cal deg⁻¹ mol⁻¹) and T is expressed in degrees Kelvin.

To examine the effects of initial moisture content on relative leakage during imbibition, soybean cotyledons (with seed coats and axes removed) were incubated in the constant humidity chambers to the desired initial moisture, and then placed in water at room temperature (1 ml/cotyledon). Absorbance at 280 nm was measured every 2.5 min for 25 min to measure the rate of solute leakage from the cotyledons. Thirty cotyledons were used in each trial.

To examine the effects of moisture content on respiration, intact soybean seeds were incubated to the desired moisture content using methods described earlier. O₂ uptake at each moisture content was measured in a Gilson differential respirometer at 25°C. The Warburg flasks contained a wick soaked with 20% KOH in the center well, and no other liquid. At low moisture contents, measurements were taken over a period of 4 d. Ten seeds were used in each sample; the points in Figure 4 each represent individual trials.

RESULTS
Water sorption isotherms were determined for soybean pellets at 5 and 20°C (Fig. 1). The isotherms are roughly similar to those obtained for globular proteins (6), lipids (11), starch (16), and more complex biological systems such as Artemia cysts (9). The sigmoidal curves can be separated into three regions: convex, linear, and concave, which reflect bound, semi-bound, and free water at the liquid-solid interface (8, 19).

The amount of water adsorbed at 5°C is considerably greater than that adsorbed at 20°C, particularly at low RH. This may be due to a weakening of hydrophobic effects and hence a magnification of hydrogen bonding at low temperatures.
BOUND WATER IN SEEDS

Fig. 1. Water sorption isotherm curves for ground soybean embryos at 5°C and 20°C. The water adsorbed at different RH is expressed on a dry weight basis.

Fig. 2. Differential energies and entropies of water sorption at different moisture contents of ground soybean embryos. Thermodynamic values are calculated from the sorption isotherms in Figure 1.

Fig. 3. Early leakage rates of solutes from soybean cotyledons imbibed in water comparing rates from tissues starting at different initial moisture contents. Solutes monitored as A280/min·g. Each point is the average of three replicates.

Fig. 4. The O2 uptake rate of intact soybean seeds at different moisture contents.

Thermodynamic parameters derived from Figure 1 are plotted in Figure 2. Differential enthalpy (ΔH) is the heat of sorption at a given surface coverage in the temperature range being measured. ΔH values rise to a peak at a relatively low water content, and this is generally believed to be due to the increasing affinity of adjacent molecules when the strongest binding is nearly completed (21). In soybean tissues, this peak value is ~−12.5 kcal/mol at a water content of about 7%.

The differential enthalpy curve shows a second region of water binding with a much lesser affinity. This region has ΔH values of ~−1.6 to ~−2.8 kcal/mol at water contents of 8 to 25%. At higher moisture contents, ΔH decreases sharply to ~−0.6 kcal/mol or less.
The differential entropy parameter ($\Delta S$, Fig. 2) reflects the trends in the enthalpy values. There is a large negative $\Delta S$ associated with high negative enthalpy, meaning that over the moisture range at which the dry tissue has a high affinity for water, the water exists in a relatively nonrandom state.

As can be seen from the equations derived from the Clausius-Clapeyron Equation, differential free energy ($\Delta G$) depends on the RH and temperature and is not related to properties of the sorbing surface. Hence, the $\Delta G$ curve in Figure 2 is a smooth exponential curve.

If the wetting process imposes stress on the seed tissue as suggested earlier (23), one might expect tissue damage to reflect the intensity of the energy change during wetting. We have measured the leakage rate (expressed as $A_{250}$/min·g tissue) within the first 25 min of imbibition for soybean cotyledons of different initial moisture contents (Fig. 3). Leakage rates from cotyledons with initial moisture contents below 8% are rapid. Leakage rates from cotyledons with initial moisture contents between 8 and 14% water decline steadily with higher initial moisture contents, and finally level off when the initial moisture content exceeds 24% water.

To determine the extent to which low water content affects respiration—an integrative process requiring functional enzymes and membranes—$O_2$ consumption by soybeans was measured at various moisture contents (Fig. 4). It is evident that $O_2$ uptake rates rise steeply with increases in moisture above 24% water. Below 24% water, there appears to be some $O_2$ consumption, but at very low rates. Although it is difficult to determine the end-point accurately, the shallow-sloped curve appears to intersect zero at about 8% water.

**DISCUSSION**

There are several models of hydration used to explain the sigmoidal shape of sorption isotherm curves. The earliest model, called the BET model (5), assumes that a first shell of water evenly covers the polymer surface and is very tightly bound in a monolayer. Subsequent layers of water have little interaction with the polymer surface, and are assumed to have bulk water properties. More recent evidences from thermodynamic studies (13, 24), neutron diffraction (15), and nuclear magnetic resonance spectroscopy (10) have indicated that low $\Delta H$ (less negative) values are indicative of 'semi-bound' water which is weakly associated with the polymer surface (8, 19).

Studies of the adsorption of water on globular proteins by Rupley et al. (20) have led to the subversion of the idea of successive hydrational shells. For nonhomogeneous materials, they assert that water sorbs according to the strength of attraction at various loci on the macromolecule surface. There appear to be three types of sites for water adsorption on the surface of a protein deriving from three types of amino acid residues: charged, polar, and nonpolar. The charged or dissociable groups 'chemisorb' individual water molecules with a high affinity, giving rise to the convex shape of the sorption isotherm at low water contents. Additional water molecules then cluster around polar groups at a lower affinity. As more moisture is available, water will aggregate over nonpolar residues. The clustered molecules, because of water-water interactions, resemble liquid phase water in contrast to the tightly bound molecules which are in a solid phase.

Thermodynamic parameters can help detect the type of water binding that is occurring at a given moisture content. It should be noted, however, that thermodynamic data derived from sorption isotherms may be complicated by hysteresis effects. The swelling of polymers which results in hysteresis indicates that hydration is not a wholly reversible process in these systems and thus may introduce potential errors in the calculations. The hydration of soybean tissue does exhibit some hysteresis (14); however, at the moisture contents studied here, sorption and desorption curves are not dissimilar, and probably introduce only a slight error in our estimates.

Our data show a large enthalpic peak at about 7% water (Fig. 2), the predicted point calculated for completion of the BET monolayer (data not shown). Our values for the differential $\Delta H$ at the peak (−12.5 kcal/mol) are slightly larger than those observed for erythrocyte membranes (−6.3 kcal/mol) (21) and also for Artemia cysts (−5 kcal/mol) (9) but much lower than those calculated for starch (about −30 kcal/mol) (16). At moisture contents below the enthalpic peak water is presumably chemisorbed. Experiments using differential scanning calorimetry show that this tightly bound water is 'unfreezeable' even at −100°C (13).

In studies of lysozyme hydration, Yang and Rupley (24) proposed that at moisture contents between 7 and 25% water binds principally to polar protein surface groups, and then between 25 and 38% moisture, it aggregates weakly on nonpolar surface groups. At 38%, the protein macromolecule is fully hydrated.

Soybean seed pellets have hydration characteristics quite similar to a globular protein like lysozyme. The strongest negative $\Delta H$ value occurs at about 7% water content, and a small negative $\Delta H$ is observed at moisture contents between 8% and 25% (Fig. 2). Consistent with the model by Rupley et al. (20), the first amounts of liquid water in soybean seed are detectable by H-nuclear magnetic resonance at water contents of about 12% (22). This is within the region where entropy approximates zero (Fig. 2). Clegg (9) and Acker (1) interpret the entropy curve approaching 0 as indicating localized solution effects. The 38% water content determined by Rupley et al. (20) as the point of complete hydration corresponds reasonably well to the 32% water content predicted by us (23) as the approximate limit of the wetting reaction.

The lack of measurable respiration at moisture contents below 8% is consistent with the lack of activity for most enzymes at such dry conditions (1, 9). Acker (1) has attributed the loss of enzyme activity in this range to either the lack of free water to serve as a medium for enzymic reactions, or the decreased mobility of the substrate to reach the reaction site.

The region between 8 and 25% moisture has been termed a region of 'restricted metabolism' (9). This is the moisture range in which liquid water first appears (22), and where the $\Delta S$ values (Fig. 2) indicate the first solution effects. Enthalpy is low but still negative, indicating weak binding to surfaces. Within this region of hydration, there are great changes in the ability of the seed to withstand too rapid imbibition (Fig. 3).

In the final wetting range, at moisture contents between 24 and 32%, respiration begins to expand rapidly in response to moisture, and resistance to leakage and chilling injury is established (3, 4, 12). High angle x-ray diffraction of soybean pellets shows that there was a widening of the membrane bilayer spacing in this moisture range (22), which may indicate an increase in ordering of the membrane bilayer.

We have described the hydration of dry soybean seed in terms of binding energies of water to surfaces and have correlated these with physiological events occurring early on in the hydration or imbibition processes. We suggest that two moisture contents, 8 and 24%, are of special significance to an imbining soybean seed. Eight % moisture content marks the point below which the forces of water binding are very large, respiration is not measurable, and imbibitional damage is very severe. Between 8 and 24% moisture content, water binding forces are moderate, $O_2$ uptake is very restricted, and imbibitional damage is moderate. Above 24% moisture, water binds weakly, respiration expands and imbibitional damage is very limited. We suggest that the interactions of water binding on macromolecule surfaces are reflected
in dynamic characteristics of the imbibing seed.

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LITERATURE CITED

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