CRYOBIOLOGY 18, 315–321 (1981)

# Determination of Water "Bound" by Soluble Subcellular Components during Low-Temperature Acclimation in the Gall Fly Larva, *Eurosta solidagensis*

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The third instar larvae of the goldenrod gall fly, *Eurosta solidagensis*, overwinter inside stem galls on goldenrod plants and display a cold hardiness which allows the insects to survive prolonged freezing at temperatures as low as −30°C (17). A number of metabolic adjustments have been linked to the process of freezing tolerance in animals or frost hardiness in plants including: changes in amounts and types of lipids (6, 23), increases in the content of polyhydric alcohols, and/or other sugars with corresponding decreases in glycogen or starch content (6, 14), changes in the amounts and types of amino acids present (6, 14), and increases in the amount of water "bound" by subcellular components (14, 16, 18). In *E. solidagensis*, there is a well-documented increase in the concentrations of the polyols, glycerol and sorbitol, with acclimation to low temperature (3, 17), and changes in the levels of other sugars, glycogen, and amino acids have also been demonstrated (21).

The role of bound water as a mechanism of cold hardening has been investigated in both plants and animals. Bound water is thought to be that water which is "ordered" by cellular components and which, because of ordering, displays altered features as compared to "bulk" water, including (a) a lower partial molar free energy, (b) a lower vapor pressure, and (c) a lower chemical potential (12, 15). Bound water will not be as likely as bulk water to be transferred to growing ice crystals and would effectively result in nonfrozen "shells" (called polarized multilayers) of water surrounding cellular components. Indeed, several theories of cell death due to freezing indicate a crucial role for the presence of this unfrozen, "vital" water to prevent irreversible protein denaturations (11, 14).

Bound water has been measured in plant and animal cells using a variety of techniques which rely on the differences in physical or thermodynamic properties between bound and bulk water (5, 10, 12). The amount of bound water has been found to vary in direct proportion to cold hardiness at least in some studies (6, 16, 18). However, controversy exists as to what importance, if any, bound water has as a potential mechanism involved in overwintering. In some studies, cold hardiness is evident without any demonstrable change in cellular bound water (5, 9, 10).

In the present study, experiments were performed to quantitate the amount of water bound by soluble, subcellular components of *E. solidagensis* larvae acclimated to various temperatures from 22 to −30°C. The amount of water bound by the soluble components of *E. solidagensis* was found to increase directly with decreased acclimation temperature. Both small molecular weight (e.g., polyols, amino

Received July 10, 1980; accepted August 14, 1980.

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0011-2240/81/030315-07$02.00/0

acids, etc.) and high molecular weight (e.g., proteins, glycogen) components were shown to contribute to the total water binding capacity.

## MATERIALS AND METHODS

*Animals.* Third instar larvae of the gall fly, *Eurosta solidagensis*, were collected in western New York state and were acclimated as described by Morrissey and Baust (17). Briefly, ambient temperature was lowered 1°C per day from an initial 22°C until the desired low temperature was reached and then larvae were left at constant low temperature for 1 to 6 weeks. Temperature/time regimes for individual samples are given in Table 1. At the end of acclimation, larvae were quickly dissected out of their galls and stored on dry ice until use.

*Sample preparation:* Frozen larvae were homogenized in 5 vol 5 m*M* Tris–HCl buffer, pH 7.5, containing 5 m*M* $\beta$-mercaptoethanol. The homogenate was centrifuged at 20,000*g* and the supernatant divided into two fractions. One fraction was used directly for the determination of total bound water while the other fraction was dialyzed (in tubing with a molecular weight cutoff of 12,000 daltons) against two changes of homogenization buffer for 2 hr each and then used in determining water bound by the nondialyzable fraction.

*Apparatus and procedure.* Bound water in *E. solidagensis* was measured by a microbalance technique which is based on a continual monitoring of sample weight during controlled drying of the sample (19). Samples of dialyzed or undialyzed supernatant (10 $\mu$l) were placed in a Knudsen cell (Cahn No. 207S), a foil chamber approximately 13 mm in diameter with a 1-mm hole in the top surface through which the sample was loaded and through which evaporation occurred. The Knudsen cell was then placed inside the weighing chamber of a Cahn TGA accessory balance (an RG electrobalance). The weighing chamber was filled with Drierite desiccant, sealed, and evacuated to 90 Torr using an oil pump. The balance was coupled to a Mark II Cahn time derivative computer and to a Texas Instruments Berva/riter II two-channel recorder. Water loss (measured as weight loss with a sensitivity of $\pm 0.1$ $\mu$g) was recorded directly with time on one channel of the recorder to produce desorption curves, $d(H_2O)$ versus $t$. Simultaneously, the weight loss signal was processed through the time derivative computer to produce a derivative plot, $d(H_2O)/dt$ versus $t$, on the second recorder channel. Drying continued until water loss stopped and the final dry weight of each sample was measured.

## RESULTS

*Determination of bound water.* Bound water can be defined as that water, which in the vicinity of subcellular components, shows altered properties to those of bulk water in the same system. In this study, bound versus bulk water is distinguished on the basis of vapor pressure. During the controlled drying of samples in the Cahn microbalance, two distinct phases of water loss from the sample could be distinguished. The two phases, and the sharp transition between them, are seen very clearly on the derivative plot (Fig. 1). In the initial phase, water is lost from the sample at a high and constant rate. The water loss rate (0.16 to 0.20 mg/min) and the duration of the phase varied somewhat between individual samples, partly due to variations in the condition of the desiccant. In the second phase, water was lost at a constantly decreasing rate over 30 sec to 3 min until 0 mg/min was reached. Water lost during the first phase of high and constant rates represents bulk water while water lost during the phase of constantly decreasing rates is considered to be water in association with subcellular soluble components, or bound water. In blank samples, containing only homogenization buffer, the second phase of water loss was over almost instantaneously

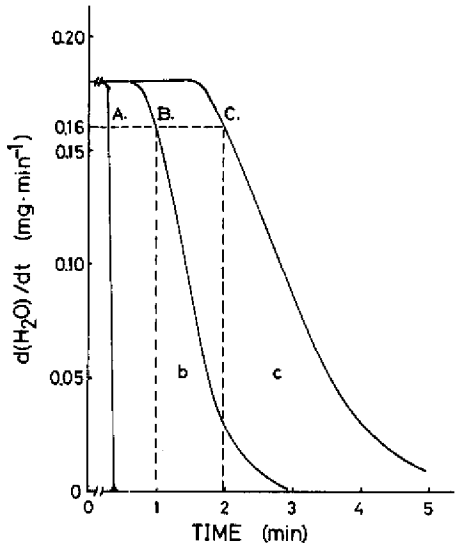


FIG. 1. A time derivative plot showing the rate of water loss from samples of (A) homogenization buffer, (B) a typical dialyzed *Eurosta* supernatant, and (C) a typical undialyzed *Eurosta* supernatant. Samples were prepared and water loss was measured on the Cahn electrobalance as described under Materials and Methods. Using planimetry, the amount of bound water in samples (B) and (C) was measured by determining the areas b and c under the curves.

(Fig. 1) indicating the bulk nature of all but the most minute part of the water in the homogenization buffer. In *E. solidagensis* supernatant samples, however, the length of the second phase of water loss varied considerably, indicating varying amounts of bound water in samples taken from larvae at different acclimation temperatures. In order to calculate the amount of bound water in samples, the arbitrary starting point for the phase of bound water loss was chosen to be a water loss rate of 0.16 mg/min. This rate had some practical significance because when repeats of individual samples were considered, it was found that the water loss curve was highly reproducible (within ±10%) in all cases between the rates of 0.16 and 0.0 mg/min despite a small amount of initial variation between repeats at rates above 0.16 mg/min. Therefore the amount of bound water was calculated for all samples by measuring the area under the water loss curve using planimetry between the rates of 0.16 and 0.0 mg/min. A second method in which actual sample weights were recorded when rates were 0.16, and then 0.0 mg/min, and then subtracted gave comparable results but was not used as the standard procedure.

*Bound water in E. solidagensis.* Total water bound by soluble components in the high-speed supernatant fraction from *E. solidagensis* was increased in larvae acclimated to subzero temperatures (Table 1). The increase was progressive with decreasing temperatures with the amount of water bound in animals acclimated to −30°C being fully three-fold more than that bound in animals acclimated to 22°C. In addition, when held at a constant acclimation temperature (−5 or −10°C), the larval soluble components showed an increase in total water bound with an increase in time at a given temperature. Larvae taken from outdoors, having been exposed to fluctuating environmental temperatures, showed an intermediate amount of water bound, approximately equal to the amount found in animals acclimated to a constant −5°C.

The amount of water bound by the nondialyzable, high-molecular-weight fraction of the samples was measured after dialysis of the supernatants. Water bound by the dialyzable, small-molecular-weight fraction was then determined by subtraction. As Table 1 shows, both the dialyzable and nondialyzable fraction contributed to water binding in *E. solidagensis*. Water bound by the nondialyzable fraction increased 2.5-fold over the 22 to −30°C range while water bound by the dialyzable fraction increased four fold over the same range. In all cases, except for larvae acclimated to 4°C, the nondialyzable fraction accounted for greater than 60% of the total water bound. When animals were held at a constant low temperature (−5 or −10°C) for varying lengths of time, the increase in total water bound over time was found to be due solely

TABLE 1
Effect of Acclimation Temperature on the Amount of Water Bound by Soluble Subcellular Components of *Eurosta solidagensis* Larvae

| Acclimation temperature and time | | Bound water (g $H_2O$/g dry wt of total soluble components) | | |
|---|---|---|---|---|
| °C | Weeks | Total | Nondialyzable fraction | Dialyzable fraction |
| 22 | 3 | 0.193 (0.171–0.209) | 0.153 (0.141–0.165) | 0.037 (0.030–0.044) |
| 4 | 3 | 0.425 (0.353–0.492) | 0.178 (0.138–0.186) | 0.259 (0.215–0.306) |
| 0 | 1 | 0.263 (0.222–0.305) | 0.164 (0.141–0.185) | 0.103 (0.081–0.120) |
| −5 | 1 | 0.298 (0.253–0.339) | 0.162 (0.147–0.195) | 0.122 (0.106–0.144) |
| | 4 | 0.350 (0.302–0.394) | 0.238 (0.220–0.254) | 0.118 (0.082–0.140) |
| −10 | 1 | 0.343 (0.299–0.390) | 0.205 (0.189–0.218) | 0.143 (0.110–0.172) |
| | 4 | 0.433 (0.374–0.492) | 0.302 (0.282–0.320) | 0.143 (0.102–0.172) |
| −17 | 2 | 0.580 (0.522–0.657) | 0.374 (0.350–0.399) | 0.215 (0.172–0.258) |
| −30 | 6 | 0.633 (0.563–0.700) | 0.402 (0.378–0.435) | 0.221 (0.185–0.265) |
| Outdoor larvae | | 0.298 (0.231–0.366) | 0.164 (0.130–0.190) | 0.132 (0.101–0.167) |

*Note.* Results are the average of determinations on four samples from each temperature with the range of values given in parentheses. Total and nondialyzable bound water were measured directly; dialyzable bound water was derived by subtraction. Outdoor larvae were collected at a time when daily maximum and minimum temperatures were 4 and −23°C, respectively.

to an increase in water bound by the nondialyzable fraction while no change in water bound by the dialyzable fraction was seen.

## DISCUSSION

Evidence from a variety of studies now indicates that most, if not all, water in a cell exists in a bound state, i.e., in a state of multilayers, polarized and oriented by cellular macromolecules (11, 15, 22). Still controversial, however, is the role played by bound water in the phenomenon of overwintering hardiness. There is general agreement that a shell of bound water around intracellular macromolecular structures would function to limit intracellular freezing and to protect molecules such as proteins from denaturation (13, 14). Many studies, particularily with plants, show a correlation between winter hardening and increased bound water (16), with increases in the content of soluble, cytoplasmic components (8, 20) presumably accounting for this increase (14). However, other studies find no alteration in the amount of bound water present in tissues despite well-developed cold hardiness (5, 9, 10). The results of the present study show a strong correlation between cold acclimation and water bound by soluble subcellular components in *E. solidagensis* and suggest that alterations in the water binding capabilities of *E. solidagensis* tissues may be an important facet of overwintering cold hardiness in these animals.

Part of the difficulty in obtaining a consensus on the role of bound water in cold hardiness has been the use, by various authors, of many different techniques for quantitating bound water. Each of these techniques measures a different property of water and therefore each has its own intrinsic definition of bound water. In this study, bound versus bulk water is defined by differential rates of water loss during the controlled drying of a sample (19). NMR studies, however, measure the motion of "nonfrozen" water at subzero temperature (10, 16) while the technique of adsorption isotherms measures water adsorbed onto previously freeze-dried samples (5, 12). However, despite differences in the methods used for measuring bound water, the amount of water bound by subcellular com-

ponents of *E. solidagensis* agrees well with the findings of previous studies. The amount of bound water in *E. solidagensis* supernatant ranged from 0.15 to 0.63 g/g dry wt while studies on wheat sap showed bound water ranging between 0.1 and 0.9 g/g dry wt (16). Bound water in protein solutions has been estimated at 0.3 to 0.5 g/g dry wt of protein (12) while for glycoprotein a value of 0.66 g/g carbohydrate has been found (4).

Overwintering *E. solidagensis* larvae exhibit a number of physiological and biochemical adaptations which result in both a cold tolerance and a freezing tolerance in these animals. Freezing and supercooling points are significantly depressed below 0°C with actual freezing of larval fluids initiating at about −8°C (2, 17). Stored glycogen is degraded with high concentrations of the polyhydric alcohols, glycerol, and sorbitol, built up in tissues. There is, however, no increase in total protein content which remains constant at 48 ± 3 mg/g wet wt at all acclimation temperatures (21). In addition, there is no detectable alteration in the amount or type of glycoprotein present during overwintering (21). Water content of the larvae also remains essentially constant during cold acclimation (2). There is, however, as shown by the present data, a significant increase in the amount of water bound by soluble components of *E. solidagensis* and this increase is proportional to acclimation temperature.

Water binding by the dialyzable, low-molecular-weight soluble components of *E. solidagensis* is likely due mostly to the presence of the polyhydric alcohols, glycerol and sorbitol. Total polyol concentration increases linearily with decreased acclimation temperature with glycerol levels reaching a peak of 18.4 mg/g wet wt at 0°C (after which production apparently ceases) and sorbitol synthesis, which is not triggered until 0°C, reaching maximal levels of 18 mg/g wet wt at −30°C (21). Baust (8) lists several probable effects of glycerol including: (a) an inhibition of ice crystal formation at nucleation sites due to glycerol's water binding properties, and (b) a prevention of intracellular dehydration during freezing, thus protecting proteins from denaturation. Both of these effects result from the hydrophillic nature of the polyols, their ability to bind or order water. Other changes in the makeup of the dialyzable, small-molecular-weight fraction of *E. solidagensis* are also found during cold acclimation. The levels of several sugars (glucose, fructose, trehalose) increase and the free amino acid pool doubles in size with an increase in proline concentration from 3.7 to 6.5 mg/g wet wt (between 15 and −30°C) accounting for 84% of the total amino acid increase (21). These compounds may also contribute to the increased water bound by this fraction.

Likely contributors to the increase in bound water during low-temperature acclimation in the nondialyzable, high-molecular-weight soluble fraction of *E. solidagensis* are the major components of this fraction: proteins and glycogen particles. In some systems, cold hardiness can be correlated with an increase in total soluble protein present (14, 20) or with an increase in glycoprotein content (7). However, alterations to the protein content of *E. solidagensis* were not evident when total soluble protein content was analyzed (21) although there is some recent data which indicate the presence of a specialized protein in hemolymph (possibly a nucleator protein) which builds up during cold acclimation (J. Baust, unpublished data). Such a protein might make a significant contribution to the increase in water binding observed during cold acclimation. Another contributor to water binding by the nondialyzable fraction may be glycogen particles. Glycogen is the substrate for polyol synthesis in *E. solidagensis* and soluble glycogen content drops from 39 to 9 mg/g wet wt during the acclimation of *E. solidagensis* from 22 to −30°C. In these lar-

vae, then, water bound by the high-molecular-weight soluble fraction increases during cold acclimation despite an absolute decrease in the dry weight of the fraction due to glycogen conversion to polyols. However, a recent study by Brittain and Geddes (4) may be able to shed some light on this apparent dichotomy. Their experiments indicate that low-molecular-weight glycogen particles bind anomolously large amounts of water compared to high-molecular-weight glycogen particles. This phenomenon could be at work in *E. solidagensis*. When glycogen is degraded for use in synthesizing polyols, the resultant smaller glycogen particles would have characteristics which increase their water binding capabilities.

In summary, this study has shown a direct correlation between low-temperature acclimation and an increase in water bound by the soluble components of *E. solidagensis* larvae. An important role for bound water in overwintering freezing protection in these animals is indicated with contributions to water binding originating from both low-molecular-weight metabolites and high-molecular-weight compounds such as proteins and glycogen.

## SUMMARY

1. The water of hydration, or bound water, associated with soluble subcellular components was estimated using a microbalance drying technique and correlated with acclimation temperature in the overwintering, freezing-tolerant larva of the goldenrod gall fly, *Eurosta solidagensis*.

2. Total water bound by soluble components of *E. solidagensis* increased as the temperature of acclimation decreased from 0.193 g/g dry wt in larvae acclimated to 22°C to a maximum of 0.633 g/g dry wt in larvae acclimated to −30°C.

3. Using dialysis (membrane molecular weight cutoff = 12,000 daltons), low-molecular-weight, "dialyzable" components were separated from high-molecular-weight "nondialyzable" soluble components. Both fractions were found to contribute to the increase in water bound with decreased acclimation temperature. The increase was 2.5-fold for the nondialyzable fraction and four fold for the dialyzable fraction between 22 and −30°C.

4. Total water bound increased as a function of time at a constant acclimation temperature, and this increase was found to be due solely to an increase in water bound by the nondialyzable fraction.

5. The molecular basis of water binding by both the dialyzable and nondialyzable fractions of *E. solidagensis* is discussed and the role of bound water as a mechanism of overwintering freezing-tolerance is evaluated.

## ACKNOWLEDGMENTS

The authors wish to thank the Department of Geology, Duke University for the use of their Cahn electrobalance. Dr. F. MacDowall, Agriculture Canada, Ottawa is also thanked for his helpful discussions. This study was supported by NSF Grants PCM 77-19033 and PCM 78-09589.

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