Effect of Cryoprotectants on the Activity of Hemolymph Nucleating Agents in Physical Solutions

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Many insects accumulate polyhydric alcohols, particularly glycerol, sorbitol, and carbohydrates during fall and winter. In the larval hymenopteran Brachon cephi, Salt (11) found glycerol concentrations as high as 5 m and supercooling points as low as -47°C. The production of these substances is correlated with increased cold hardiness in both freezing tolerant and freezing intolerant species. The mode of action of these substances remains partly unclear, but they are believed to promote cold hardiness in a number of ways.

In freezing intolerant insects, which have a high capacity for supercooling, increasing polyol levels have been shown to depress the supercooling points by more than twice the corresponding melting point depression (13), by a mechanism which at least in part must be noncolligative. The low supercooling points of these insects have also been interpreted as a result of a complete removal of all nucleating particles from their body fluid (12, 15). In addition, Baust and Morrissey (1, 2) suggested that the cryoprotectants may serve to "mask" potential ice nucleation loci. Preliminary studies with the freezing intolerant lady beetle Coleomegilla maculata provided support for this hypothesis when it was found that glucose appeared to "mask" nucleators during cyclical temperature fluctuations. For freezing intolerant forms this "masking" would allow for extensive supercooling and the avoidance of freezing, even when nucleators were still present in their body fluids.

In freezing tolerant systems, on the other hand, polyols appear to protect in a purely colligative manner, by reducing the amount of water frozen and thus the concentration of potentially harmful solutes (6, 10, 17). Freezing tolerant insects appear to freeze at remarkably high temperatures, even when they have high concentrations of polyols in their body fluids. These high supercooling points have been ascribed to nucleating agents found to be present in the hemolymph of these insects (5, 9, 14, 18). The fact that the hemolymph nucleators act in the presence of high cryoprotectant concentrations is somewhat anomalous when considered in the light of their proposed role as nucleator "masking" substances.

The present study is an investigation of the ability of various organic compounds in physical solution to "mask" the activity of hemolymph nucleating agents from freeze-tolerant Eleodes blanchardi darkling beetles.

MATERIALS AND METHODS

Hemolymph containing nucleating agents was obtained from Eleodes blanchardi beetles, which were collected in the mountains of Southern California in November 1979. The hemolymph sampling was made by excising a leg of the beetle and withdrawing the exuding hemolymph into a thin glass capillary by means of the capillary force. Five-microliter samples of solutions with various concentrations of organic solutes were sandwiched between two layers of paraffin oil in capillary tubes. Hemolymph
FRC. L Supercooling points of various solutions with and without hemolymph (5% v/v of Eleodes blanchardi). Solutions are represented by the following symbols: glucose (○), glycerol (●), sucrose (△), lactate (x), sorbitol (□), fructose (△), NaCl (☆), trehalose (◇), proline (★), ethylene glycol (○), PVP (p), mannitol (○), and water (●). The upper solid regression line represents solutions containing hemolymph while the lower line represents undiluted solutions. Dashed line indicates theoretical osmolal freezing point depression (−1.86°C per osmol).

was added to the samples by gradual injection into the test solution with the aid of a precisely calibrated micro syringe, so that the concentration of hemolymph was 5% (v/v). For SCP determinations triplicate samples were attached to a 30-gauge copper constantan thermocouple, connected to a multichannel temperature recorder (Leeds and Northrup), and cooled at a rate of 0.3°C min−1. The supercooling points were indicated by the rapid temperature increase due to the release of latent heat of fusion.

The osmolality of the solutions was determined by measuring the melting points as described by Zachariassen and Hammel (19).

RESULTS

Figure 1 summarizes how the supercooling points of the various solutions varied as a function of the osmolality. For all solutes and at all concentrations the addition of 5% (v/v) hemolymph resulted in a marked increase in the supercooling points and a reduction in the variation of the range of the values.

As the solute concentration increased, the supercooling points of both hemolymph diluted and undiluted solutions decreased. For samples containing hemolymph, the supercooling points were strongly correlated with the osmolality (r = −0.97), regardless of the specific solute. Furthermore, the slope of the linear regression line (−1.73°C/osmol) is similar to the theoretical freezing point depression (−1.86°C/osmol). For undiluted samples, the correlation was less pronounced (r = −0.85), and the slope of the linear regression line (−2.3°C/osmol) was higher than the osmolar melting point depression.

In order to assess the effect of solute concentrations on the change in SCP after the addition of hemolymph, the elevation in the SCP versus the solute concentration was plotted. The regression line (y = 1.02x + 9.76) for this plot yielded a low correlation coefficient (r = 0.41) and a slope which did not differ significantly from zero (P > 0.05). This result suggests that the range of cryoprotectant concentrations used did not affect the elevation in the SCP caused by the addition of hemolymph nucleators.

DISCUSSION

Homogeneous and heterogeneous nucleation has been examined in a number of physical systems. Using silver iodide as the seeding agent, Lusena (7) found that solutes (glycerol and NaCl) depress the temperature of heterogeneous nucleation to the same extent that they depress the melting point. In other words, the solutes did not directly affect the activity of the heterogeneous nucleators in water. Block and
Young (3) concluded that glycerol exerts a similar effect on the homogeneous and heterogeneous nucleation of water. In a study of homogeneous nucleation using a variety of solutes, including glycerol, glucose, and sucrose, MacKenzie (8) found that the reduction of the supercooling points was approximately twice the melting point depression.

The observations of the present study suggest that the decrease in the supercooling points with increasing osmotic concentration may be explained solely by the colligative properties of the solutions. This implies that none of the substances tested exhibited any masking effect on the activity of the hemolymph nucleating agents from *E. blanchardi*. Rather, the hemolymph nucleating agents induced nucleation following a constant amount of supercooling (−5.3°C), as was observed by Zachariassen and Hammel (18).

Hemolymph nucleating agents have been found in three insect orders, Coleoptera (4, 15, 16, 18), Hymenoptera (5), and Diptera (14). Despite the diverse phylogeny of these insects, they share the characteristics of freeze tolerance, a relatively high supercooling point and, in most of them, an elevated level of cryoprotectants. The hemolymph nucleating agents appear to be specifically and seasonally produced to induce freezing at relatively high subzero temperatures, thus promoting extracellular freezing and enhancing postfreezing survival. Viewed in this light, it is not surprising that the hemolymph nucleating agents should act in the presence of cryoprotectants of freezing tolerant insects.

The fact that the cryoprotectants exhibited no masking effect on these nucleators does not rule out the possibility that they may function in this manner in freezing intolerant insects. Additional studies are needed to clarify the roles of potential masking agents and nucleating agents in freezing tolerant versus freezing intolerant insects.

**SUMMARY**

The ability of 11 different organic solutes in physical solution to mask the effect of nucleating agents from hemolymph of freezing tolerant insects was tested. The masking effect was tested by measuring the supercooling points of samples with various solute concentrations, with and without hemolymph. Hemolymph was obtained from freeze-tolerant *E. blanchardi* tenebrionid beetles.

The depressive effect of the solutes on the supercooling points was nearly equivalent to the corresponding melting point depression, indicating that the depression was due only to the colligative properties of the solutes. Thus, no ability for nucleator masking was demonstrated.

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