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A Method for Quantitative Determination of Ice Nucleating Agents in Insect Hemolymph

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The presence of nucleating agents in the hemolymph appears to be a characteristic feature of insects tolerant to freezing (10). Such nucleators have been found in the hemolymph of insects of several phylogenetic orders, i.e., Coleoptera (4, 8, 9, 11), Diptera (7), and Hymenoptera (5). The physiological function of these nucleators is suggested to be to induce a protective extracellular freezing at relatively high subzero temperatures, preventing injurious freezing in intracellular compartments (10).

Several studies indicate that the concentration of nucleators varies seasonally and that they may be absent from the hemolymph during summer (4, 9, 11). Unfortunately, there is no method available which allows a quantitative determination of the level of nucleating agents in the hemolymph of insects. The purpose of the present study has been to develop a method for determining the concentration of nucleating agents in insect hemolymph. The method is developed from experiments with nucleators from hemolymph of freeze-tolerant *Eleodes blanchardi* beetles. The practical applicability of the method is demonstrated by measurements of nucleator concentrations in the hemolymph of the gall fly larvae, *Eurosta solidaginis*, acclimated to different temperatures.

MATERIALS AND METHODS

Freeze-tolerant *Eleodes blanchardi* beetles were collected from their hibernation sites in the mountains of Southern California in November 1979 and kept for a week at 0°C before they were used for hemolymph sampling. Hemolymph samples were obtained by excising one of the legs and drawing the exuding hemolymph into a thin glass capillary by means of the capillary forces (Fig. 1). Larvae of *Eurosta solidaginis* were collected in Minnesota and Texas in September 1979. The larvae were incubated at different temperatures ranging from +10 to −20°C for various periods of time (3). Following laboratory acclimation, the larval cuticle was perforated with a thin needle, and the exuding hemolymph was drawn into the attenuated end of a flame-drawn microcapillary. Paraffin oil was drawn into the capillary to seal the hemolymph from the air, whereafter the capillary was closed by melting the wide end (Fig. 1). The capillary containing hemolymph and paraffin oil was centrifuged at low speed for 2–3 min, leaving the hemolymph at the bottom of the capillary and sealed from the air by a thin layer of the less dense paraffin oil. After cutting the capillary tube just above the paraffin oil, hemolymph was removed with a microsyringe.

The nucleator activity in the hemolymph was investigated by using a modification of a method described by Zachariassen and Hammel (11). Nucleator activity was quantified by diluting the hemolymph repeatedly by a factor of 10 in 5-μl samples of 0.9% NaCl solution, as indicated in Fig. 1. Each sample was prepared by sandwiching the NaCl solution between two layers of paraffin oil in thin glass capillaries. The hemo-

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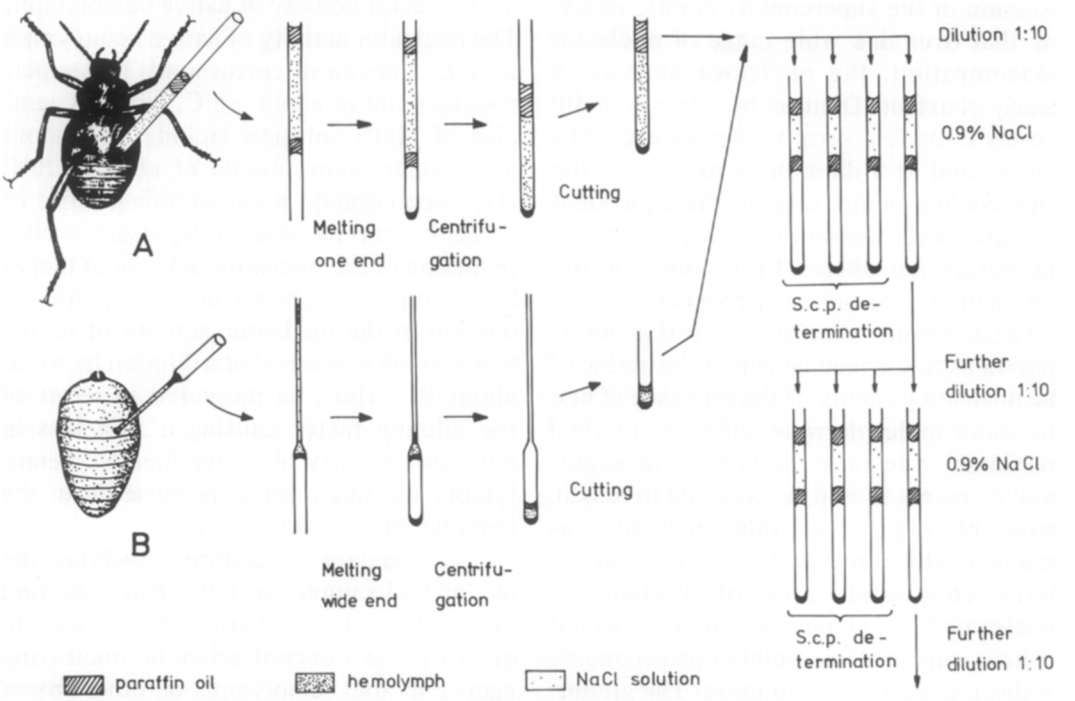

FIG. 1. Procedure for sampling and diluting hemolymph from adult *Eleodes blanchardi* beetles (A) and third-instar larvae of *Eurosta solidaginis* (B).

lymph (or diluted hemolymph) was added by means of a microsyringe through the oil layer.

Supercooling points were measured by attaching triplicate samples to a 28-g thermocouple, which was connected to a potentiometric recorder. The samples were cooled at a rate of about 20°C/hr. The supercooling points were indicated as small upward inflections of the temperature curve due to the release of the heat of fusion of water freezing. Acclimation of *E. solidaginis* was accomplished according to Baust and Lee (3).

RESULTS AND DISCUSSION

The supercooling points of 5-μl samples of 0.9% NaCl solution containing *Eleodes blanchardi* hemolymph diluted repeatedly by factors of 10 are shown in Fig. 2. As the hemolymph is gradually diluted, the supercooling points vary sigmoidally. Dilution by factors of up to 10^3 causes only a slight de-

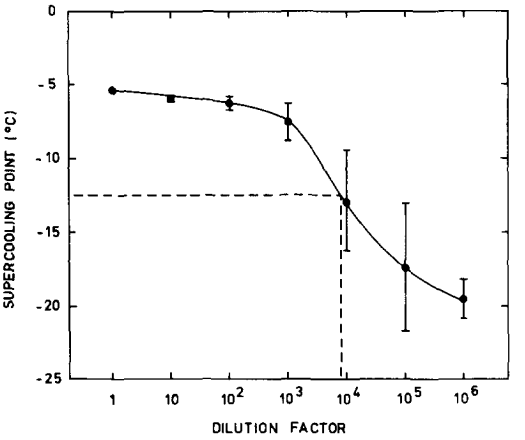

FIG. 2. Supercooling points of 5-μl samples of *Eleodes blanchardi* hemolymph and of hemolymph diluted repeatedly by a factor of 10 in 5-μl samples of 0.9% NaCl solution, presented as a function of the dilution factor. Each point is the mean value of three parallel samples, and the bars represent the standard deviation. The dashed lines indicate the dilution factor corresponding to a 50% reduction of the nucleating activity of native *Eleodes* hemolymph, as described in the text.

pression of the supercooling points, implying that over this wide range of nucleator concentration, the nucleator activity is nearly constant. Dilution by a factor of 10^4 causes a marked drop in the supercooling points, and at a dilution factor of 10^5, the supercooling points drop to the equivalent to pure NaCl solution. Thus, when the nucleators are diluted by a factor of 10^5, their activity appears to be reduced to zero.

These results provide interesting information as to the mechanism of the action of the nucleating agents. If the presence of one nucleator molecule were sufficient to yield maximum nucleator activity, the supercooling points should be expected to remain invariably high until a dilution factor was reached which resulted in some samples being completely free of nucleators. Nucleator-free samples would be expected to have supercooling points corresponding to those of pure NaCl solution. The gradual decrease in supercooling points with increasing dilution of the hemolymph does not fit this pattern, and indicates that high nucleation activity is the result of a quantitative association with nucleator molecules.

The results in Fig. 2 show that as the hemolymph becomes diluted, there is a marked increase in the standard deviation of the supercooling points. As pointed out by Salt (6), ice nucleation is to some extent a random phenomenon, dependent on such factors as solute concentration, sample size, temperature, and time. The low standard deviation at high nucleator concentrations is consistent with results obtained by Zachariassen and Hammel (11), and indicates that at high nucleator concentrations there is a low degree of randomness in the nucleation process.

A relative measure of the nucleator concentration in the hemolymph can be obtained by determining the lowest dilution factor associated with a certain drop in nucleator activity, for example the dilution factor corresponding to a 50% reduction of the nucleator activity of native hemolymph. The nucleator activity of native hemolymph from *E. blanchardi* corresponds to a supercooling point of about −5°C, whereas samples of NaCl solution lacking nucleators have supercooling points of about −20°C (11). Accordingly, a supercooling point of −12.5°C may be said to represent a 50% reduction of the nucleator activity of native hemolymph. As shown in Fig. 2, this reduction in the nucleator activity of native hemolymph is reached at a dilution factor of about 10^4. Thus, as measured in terms of the dilution factor causing a 50% loss in nucleator activity of native *Eleodes* hemolymph, the nucleator concentration in the hemolymph is defined as 10^4.

This standard procedure, involving the use of 5-μl samples of 0.9% NaCl solution kept inside glass capillaries, may be used to quantify the concentration of nucleating agents in the hemolymph of other insect species as well. Since the method is based on the activity of the nucleators in *Eleodes* hemolymph, the concentration unit may conveniently be termed *Eleodes* nucleation equivalents (ENE).

In order to demonstrate the practical applicability of this method, the concentration of nucleating agents was determined in the hemolymph of freeze-tolerant third-instar larvae of the gall fly *Eurosta solidaginis* from Texas and Minnesota. The larvae were incubated at temperatures ranging from +10 to −20°C for various periods of time. A detailed description of the incubation procedure is given by Baust *et al.* (2). The nucleator concentrations, as determined by the standard method described above and expressed as ENE, are shown in Fig. 3.

Figure 3 reveals that in Minnesota larvae, the highest concentrations of nucleators were found following incubation at +5 and −20°C. The concentrations were considerably lower in larvae kept at other temperatures. Larvae from Texas had measurable concentrations only when kept at 0 and

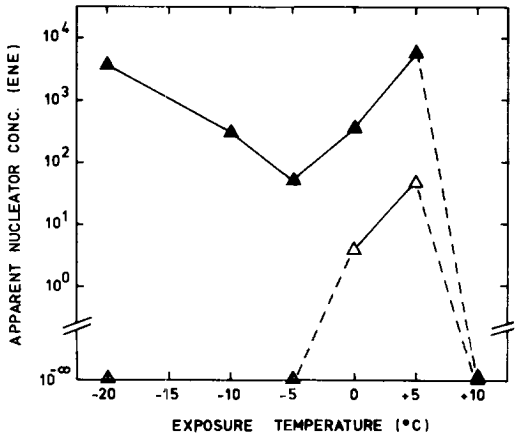

FIG. 3. Concentration of nucleating agents in the hemolymph of *Eurosta solidaginis* larvae from Minnesota (▲) and Texas (△), acclimated to different temperatures, and as determined by the dilution technique.

+5°C. Thus, in both populations, +5°C appears to be the optimal temperature for production of nucleating agents in the hemolymph.

It might appear somewhat surprising that this comparatively high temperature is more effective than lower temperatures in promoting nucleator formation. However, prior to low winter temperatures, insects are likely to pass through a period with gradually declining temperatures, involving exposure to temperatures slightly above zero for weeks or months. It is of vital importance that insects produce nucleators during this peroid, so that optimal nucleator levels are attained before they are exposed to freezing temperatures for the first time. An optimal range of nucleator synthesis at 0−+5°C, may be regarded as a physiological adaptation to satisfy these requirements. These observations are consistent with reported changes in whole body supercooling capacity for this species during similar low temperature exposures (2) with the exception of values reported at −20°C. The biological significance of the variations in nucleator levels found in these populations have been discussed by Baust (1). In addition, Baust and Lee (3) have recently characterized thermal triggers for sorbitol synthesis in Minnesota and Texas populations of the species. This latter triggering response occurred at the same temperature intervals.

It should be emphasized that the present method does not provide a direct measure of the nucleator concentration. Like most other quantitative methods it is based on a quantification of a physical effect of the substance being studied, in this case its ice nucleating activity. Furthermore, even in one individual insect specimen the nucleating activity may be the result of a combined action of several different substances, each having its specific nucleating ability. Thus, the concept nucleator concentration may be difficult to define in a strict sense.

Several studies indicate that the nucleators are peptides or proteins (5, 11). The available methods for quantification of peptides and protein on a molar or weight basis are complicated and time consuming. The present method offers several advantages, in that it provides a simple, practical, and reliable procedure for quantification of nucleating agents.

SUMMARY

The relationship between the concentration of insect hemolymph ice nucleators in samples of 0.9% NaCl solution and the supercooling points of the samples was determined by using a dilution technique. The supercooling points were only moderately reduced following dilution by a factor of up to 10^3, whereas dilution beyond this point caused a marked drop in the supercooling points. The dilution factor corresponding to a 50% reduction in the nucleating activity of native hemolymph is taken as a measure of the concentration of ice nucleators in native hemolymph.

This method was used to determine the concentration of ice nucleators in the hemolymph of *Eurosta solidaginis* larvae from Minnesota and Texas, acclimated to different temperatures. Significant levels of

nucleators were found only in larvae from Minnesota, and +5°C was found to be the optimal temperature for nucleator formation. This comparatively high temperature optimum is interpreted as a physiological adaptation, ensuring sufficient nucleator levels in the hemolymph by the time of the first exposure to freezing temperatures in the winter.

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