pooled and evaporated to dryness. The aqueous phase was centrifuged and the mycelium resuspended in 20 ml of water for radiochemical determination. The radioactivity administered was totally recovered (≥ 98%) in the 3 following fractions: chloroform extract 47%, aqueous phase 13% and mycelial cake 40%. The total recovery of the administered radioactivity demonstrates that in the side chain of DA no metabolic degradation occurs up to CO_2 formation. The chloroform extract was dissolved in methanol and chromatographed on several sheets of Whatman paper 3MM buffered with phosphate M/15 pH 5.4. The solvent system used was n-propanol/ethylacetate/water (7:1:2, v/v/v) (system A). A strip from the sheets was scanned for radioactivity and the 2 main peaks detected were at R_f 0.2 and 0.4 corresponding to DX and DA + DDA respectively. The red bands, wich corresponded to the radioactive peaks, were cut and eluted exhaustively with methanol/HCl 0.01 N (4:1, v/v). The neutralized eluates were concentrated in vacuo to about $^1/_{10}$ of the volume, diluted with H_2O, adjusted to pH 8.6 with Tris·HCl buffer 1 M and extracted with chloroform/methanol (4:1, v/v) (acid-base step). The extract containing DA + DDA was chromatographed on analytical silica gel plates in the solvent system chloroform/methanol/acetic acid/water (80:20:7:3, v/v/v/v) (system B) in order to separate DA (R_f 0.2) from DDA (R_f 0.3). Both the products were found to be labeled when scanned for their radioactivity. Also, DX was submitted to chromatography in the solvent system B for further purification. Spots or bands were scraped off and eluted as above for radiochemical analyses. DX, DA and DDA were subsequently chromatographed in TLC on analytical silica gel plates with solvent system chloroform/acetone (4:1, v/v) (system C) in order to reach a constant specific activity. The chemical purity of DX, DA and DDA was checked by HPLC; the radiochemical purity was controlled by scanning the plates run in system C.

Results and discussion. The specific activities of the antibiotics considered are reported in the table, from which it appears that both DX and DDA are significantly labeled. These data confirm that DA is a precursor of DX and DDA. The different values of specific activity of DX and DDA found at the end of the fermentation are the consequence of the different moments at which their production occurs, as the specific activity of DA gradually decreases throughout the fermentation owing to its constant dilution with the unlabeled biosynthesized DA.

Among the schemes proposed for the biosynthesis of antracycline antibiotics, one has been reported[9] for which 10-decarbo-methoxy-ε-rhodomicinone glycoside is considered to be the precursor of DDA by the following 2 different pathways; via DA and via 13-deoxydaunorubicin. Our data confirm the former hypothesis even though the latter cannot be completely ruled out. If this 2nd pathway had been operating in this strain, the specific activity of DDA could also have been lower than the specific activity of DA itself. The value of the specific activity of DDA, which is higher than that of DA, and the lack of evidence for production of 13-deoxydaunorubicin in detectable amounts, makes it unlikely that this 2nd biosynthetic pathway is operating, at least under the conditions and with the strain employed in our study.

Specific activities of doxorubicin (DX), daunorubicin (DA) and 13-dihydrodaunorubicin (DDA), after feeding of daunorubicin-[14-^{14}C] to cultures of *Streptomyces peucetius* strain M76 F.I.

Sample	Purification steps	Specific activities (nCi/μg) DX	DA + DDA	DA	DDA
1	Paper chromatography in system A	1.360	0.680	-	-
	Acid-base step	1.488	0.707	-	-
	TLC in system B	1.494	-	0.637	0.784
	TLC in system C	1.492	-	0.639	0.792
2	Paper chromatography in system A	1.376	0.680	-	-
	Acid-base step	1.490	0.702	-	-
	TLC in system B	1.501	-	0.632	0.778
	TLC in system C	1.504	-	0.636	0.786
	Mean values of final specific activities	1.498	-	0.638	0.789

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Nucleating agents in the haemolymph of an intertidal mollusc tolerant to freezing

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Summary. Nucleating agents are found to be present in the haemolymph of the common mussel *Mytilus edulis*. Such substances are found only during winter, and they cause the haemolymph to freeze at temperatures around −6 °C. In summer supercooling points are around −15 °C.

The mussel *Mytilus edulis* is commonly found in the intertidal zone in arctic and temperate regions. In winter, such sessile invertebrates are exposed to temperatures down to −30 °C for a period of 1-6 h twice daily[2]. *Mytilus edulis* is reported to be freeze-tolerant[3], and ice-formation is shown to take place in the extracellular compartments[4,5]. Insects tolerant to freezing are shown to have nucleating agents in the haemolymph[6,7]. The nucleating agents are

assumed to ensure extracellular freezing at a few degrees below zero, thus preventing an injurious intracellular freezing[8,9]. This paper reports that nucleating agents are present also in the haemolymph of the mussel *Mytilus edulis*. Such substances are found only during winter, when maximum freezing tolerance is acquired. This is the first report of haemolymph nucleators in animals other than insects.

Material and methods. Haemolymph was sampled from *Mytilus edulis* on a location in Trondheimsfjorden, Norway, during 1981. The haemolymph was obtained from the sinus of the posterior adductor muscle by drilling a hole in the shell above the muscle and sucking the haemolymph out with a syringe. All samples were immediately frozen and stored at −25 °C.

To investigate the distribution of nucleating agents during the year, 5-µl samples of haemolymph were used. The samples were sandwiched between 2 layers of paraffin oil in thin glass capillaries, and the capillaries were closed by melting the wide end. This method is described in detail elsewhere[10]. Supercooling points were measured by attaching triplicate samples to a copper-constantan thermocouple which was connected to a temperature recorder.

Results and discussion. The results in figure 1 show that haemolymph samples from *Mytilus edulis* have supercooling points significantly higher in winter than during the summer. In winter the haemolymph freezes at temperatures around −6 °C, indicating the presence of nucleating agents, while in summer, supercooling points vary from −14.6 to −16.6 °C. The nucleating effect obtained in January was constant during changes in the tide. Samples of the mantle fluid obtained from mussels in January supercooled easily to about −16 °C, suggesting that nucleating agents are not present in these compartments.

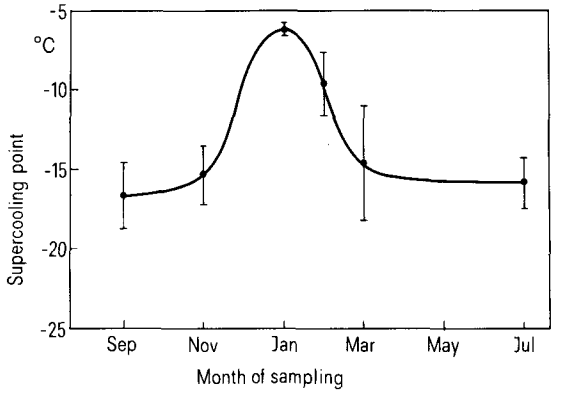

Figure 1. Supercooling points of isolated haemolymph from the mussel *Mytilus edulis* during the year. Each point is the mean of 4 triplicate 5-µl samples, and the bars represent SD.

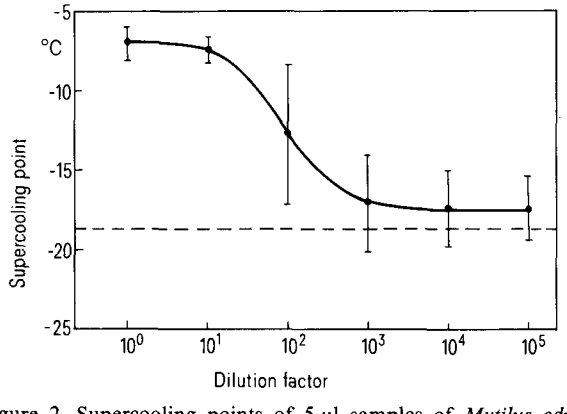

Figure 2. Supercooling points of 5-µl samples of *Mytilus edulis* haemolymph and haemolymph diluted repeatedly by a factor of 10 in 5-µl samples of 0.9% NaCl solution, presented as a function of the dilution. Each point is the mean value of 12 parallel samples, and the bars represent SD. The dashed line indicates the supercooling point of 5-µl samples of 0.9% NaCl solution.

To determine the concentration of nucleating agents in haemolymph sampled in January a method described by Zachariassen, Baust and Lee was used[10]. The haemolymph was diluted repeatedly by a factor of 10 in 5-µl samples of 0.9% NaCl solution, and supercooling points were measured as described above. The supercooling points and corresponding dilutions are shown in figure 2.

After the haemolymph had been heated to +100 °C for 5 min the supercooling points were lowered markedly, indicating that the nucleating agents are temperature-sensitive. Some nucleating effect was still present, since the supercooling points were still above those of samples of 0.9% NaCl solution.

Freezing tolerance in marine invertebrates has been known for several years, but the mechanisms involved are still unclear. Data from different kinds of freeze-tolerant organisms indicate that ice-formation is limited to the extracellular compartments, and that ice protects the organisms against intracellular freezing which is believed to be injurious. According to this model, extracellular freezing causes an osmotic outflux of water from the cells as the freezing proceeds[11]. Intracellular as well as extracellular fluid will be kept at the melting point, and consequently, the probability of an intracellular freezing would be eliminated. The observation of nucleating agents in the haemolymph of *Mytilus edulis* during winter gives further support to this model.

However, nucleating agents do not seem to be an absolute condition for freezing tolerance[12,13]. Intracellular freezing leads to a disruption of the frozen cell due to an osmotic influx of water, following which ice-formation spreads to the extracellular compartments. In this way a protective extracellular freezing is obtained by the sacrifice of one or a few single cells. Intertidal invertebrates undergo freezing and thawing repeatedly twice daily during winter, and thus, in the long run dependence on such a mechanism might lead to extensive tissue damage. It therefore seems to be essential for intertidal invertebrates to ensure freezing in the extracellular compartment at a high subzero temperature. The presence of ice-nucleating agents in the haemolymph is likely to serve this aim.

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