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Adsorption inhibition as a mechanism of freezing resistance in polar fishes

(fish antifreeze/crystal growth inhibition/freezing point depression)

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ABSTRACT **Polar fishes are known to have serum proteins and glycoproteins that protect them from freezing, by a noncolligative process. Measurements of antifreeze concentrations in ice and scanning electron micrographs of freeze-dried antifreeze solutions indicate that the antifreezes are incorporated in ice during freezing. The antifreezes also have a pronounced effect on the crystal habit of ice grown in their presence. Each of four antifreezes investigated caused ice to grow in long needles whose axes were parallel to the ice *c* axis. Together these results indicate the antifreezes adsorb to ice surfaces and inhibit their growth. A model in which adsorbed antifreezes raise the curvature of growth steps on the ice surface is proposed to account for the observed depression of the temperature at which freezing occurs and agrees well with experimental observations. The model is similar to one previously proposed for other cases of crystal growth inhibition.**

In many parts of the polar and subpolar seas, water temperatures fall to as much as 1° below the equilibrium freezing point of fishes' body fluids. Avoidance of freezing in many fishes inhabiting these regions has been linked to the presence of unusual serum proteins and glycoproteins (1–3). These "antifreezes" are not found in temperate-water fishes, and they disappear in summer in those polar fishes that experience warmer summer temperatures (1, 4). Some polar fishes do not have an antifreeze, however, and avoid freezing by existing in a supercooled state in ice-free, deeper waters (5). Fishes possessing an antifreeze are generally found in shallow waters where ice particles are abundant.

The glycoprotein antifreeze of the antarctic *Trematomus borchgrevinki* consists of a repeating tripeptide, Ala-Ala-Thr, with the disaccharide galactose-*N*-acetylgalactosamine attached to each threonyl residue, and it is found in eight discrete molecular weights (6). In the smaller fractions (glycoproteins 6–8), an Ala-Ala-Thr unit is occasionally replaced by Pro-Ala-Thr. The antifreeze of the Alaskan saffron cod, *Eleginus gracilis*, appears to be similar to glycoproteins 6–8 except that threonine is occasionally replaced by arginine (3, 7). Carbohydrate-free protein antifreezes from the Nova Scotia winter flounder, *Pseudopleuronectes americanus* (8), and the Alaskan sculpin, *Myoxocephalus verrucosus* (3), like the glycoproteins, consist of roughly 60% alanyl residues. A partial sequence of the flounder antifreeze lacked a repeating tripeptide (9).

The antifreezes make a negligible contribution to the osmotic strength of the fishes' body fluids and thus cause a depression of the "freezing point" by some means other than by a colligative process. This view is supported by the fact that the antifreezes lower the temperature at which ice growth occurs but not the temperature at which ice melts (10). It has been suggested (10–12) that, instead of acting in the liquid phase as do most solutes, the antifreezes adsorb to the ice surface and thereby prevent it from growing. We present here evidence for this adsorption and propose a mechanism by which a freezing point depression would occur.

Abbreviation: BSA, bovine serum albumin.

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MATERIALS AND METHODS

Antifreezes were previously isolated from serum by ion exchange chromatography (3, 8, 13). Poly(Ala-Ala-Lys), molecular weight 13,000, was kindly supplied by A. Yaron.

The ratio of antifreeze concentration in ice to that in the original solution—the distribution coefficient—was determined by a centrifugation method (14) with several modifications. Samples containing 5 mg of antifreeze in 1.0 ml of 0.05 M NaCl were slowly frozen and equilibrated at −2.0° and then centrifuged at 27,000 × *g* for 10 min in a Sorvall centrifuge cooled to −2° ± 0.5° to separate the liquid and solid fractions. The amount of liquid remaining in the ice fraction was determined from the amount of NaCl it contained, because the distribution coefficient of NaCl in ice, α_{NaCl}, in a frozen antifreeze solution is nearly zero (14). Chloride concentration was measured with a Buchler chloridometer, and protein and glycoprotein antifreeze concentrations were determined by the Lowry method (15). In one case, 2.6 mg of bovine serum albumin (BSA) (Sigma Chemical Co.) was included in a solution of *T. borchgrevinki* glycoprotein before freezing. BSA was determined by absorbance at 280 nm (the glycoprotein has negligible absorbance at this wavelength) and the glycoprotein was determined by the phenol–sulfuric acid method (16) which assays for carbohydrate (BSA gave a negligible carbohydrate reaction compared to that of the glycoprotein). The method by which the distribution coefficients were calculated appears elsewhere (7). These calculations accounted for both the residual liquid in the ice fraction and the higher concentration of antifreeze in the liquid phase as freezing progressed.

Molecular weights of the antifreezes of *T. borchgrevinki* (13) and *M. verrucosus* and *E. gracilis* (unpublished data) were measured by sedimentation equilibrium. Because *T. borchgrevinki* glycoproteins 1–5 and glycoproteins 7 and 8 are not easily separated, mixtures (and hence average molecular weights) of these fractions were used. X-ray diffraction patterns of ice needles and antifreeze fibers were made with a Chesley camera (17) with the needle and fiber axes perpendicular to the x-ray beam.

RESULTS

Evidence for adsorption

Concentration Measurements. It was reported earlier on the basis of freezing curves that the antifreezes of *T. borchgrevinki* (11) and *P. americanus* (18) were not concentrated in the liquid phase as are most solutes when a solution is

Table 1. Distribution coefficients (α) and molecular weights of fish antifreezes*

	M_r	α_{AF}	α_{BSA}
Trematomus borchgrevinki (1–5)	15,000†	0.89	0.10
Myoxocephalus verrucosus	5,000	0.44	
Eleginus gracilis	3,300	0.30	
Trematomus borchgrevinki (7, 8)	3,000†	0.17	

* The amount of *P. americanus* antifreeze available was insufficient for analysis. α is the ratio of concentration of a solute in an element of ice to its concentration in that element before it froze. Values for α are calculated in ref 17. M_r = molecular weight; $\alpha_{AF} = \alpha$ for antifreeze.

† Average molecular weight.

frozen. To obtain more quantitative results, solutions containing antifreeze and NaCl and in one case BSA were partially frozen and centrifuged to expel the liquid portion. The ratios of the concentrations of antifreeze and BSA in the ice to their original concentrations before freezing (distribution coefficients) are shown in Table 1. The data show that a significant portion of each of the antifreezes investigated remained in the ice. Ice frozen in the presence of the antifreezes consists of densely packed parallel needles (14, 19), so it is possible that the greater retention of the antifreezes relative to NaCl was due to a lower mobility of the larger molecules, but the fact that BSA was almost entirely expelled makes this unlikely.

The molecular weights of the antifreezes are also shown in Table 1 and indicate that the adsorption of antifreezes to ice may be an increasing function of molecular weight, a relationship that is usually found in the adsorption of macromolecules to solid surfaces (20).

Change of Crystal Habit. Adsorbents are known to cause changes in the habit, or shape, of growing crystals, probably by retarding growth of the crystal faces to which they adsorb (21). At a concentration of 10 mg/ml, each of the four antifreezes, except glycoproteins 7 and 8 of *T. borchgrevinki*, caused ice to grow in long, parallel needles whose diameters were roughly 5–15 μm and whose axes were observed with standard polarizing microscope techniques to be aligned with the ice *c* axis. [Photographs of ice needles grown in the presence of *T. borchgrevinki* glycoproteins 1–5 appear elsewhere (14, 19).] Although others have reported the growth of needle-like ice "whiskers" from the vapor phase (22–24), ice grown from solution normally grows most rapidly in a direction perpendicular to the *c* axis (25) and so inhibition of growth in this direction appeared to be caused by preferential adsorption of antifreeze on crystal faces parallel to the *c* axis. Attempts to detect ice needles in frozen solutions (10 mg/ml) of polyvinylpyrrolidone, dextran, BSA, gelatin, poly(Ala-Ala-Lys), and polylysine were not successful.

Both *a*- and *c*-axis ice needles grew in solutions of the two protein antifreezes. Perturbations on the *a*-axis needles acted as seeds for the *c*-axis needles.

The x-ray diffraction pattern of ice needles grown in the presence of *T. borchgrevinki* glycoproteins 1–5 was similar to that of a single ice crystal rotated 360° about its *c* axis, which indicated that the ice needles were randomly oriented about their *c* axis and that the antifreezes did not alter the crystal structure of ice. No evidence for alignment of the antifreeze molecules could be found; all of the diffraction spots could be attributed to ice.

Freeze-Dried Antifreeze Solutions. Thin films of aqueous solutions (10 mg/ml) of *T. borchgrevinki* and *M. verrucosus* antifreezes were slowly frozen on glass slides so that the ice grew in the usual pattern of long, parallel needles. Microscopic inspection of these samples after freeze-drying showed an intimate relationship between the ice and the antifreeze (Fig. 1). Both antifreezes remained in the form of fibers that were aligned with the ice *c* axis. The simplest explanation for this consistent with the results in Table 1 is that the fibers represent the locations of former ice needles and that the fibers were formerly embedded in the ice. Once the ice had sublimed, a fluffy matrix or skeleton of antifreeze fibrils remained. Sublimation of the *a*-axis needles grown in the presence of the two protein antifreezes left only empty grooves, indicating that the protein antifreezes were incorporated into only the *c*-axis needles. A random orientation of the antifreeze molecules within the fibers was shown by an absence of molecular birefringence and by x-ray diffraction patterns consisting only of weak rings.

When solutions of the low-molecular-weight glycoproteins (*T. borchgrevinki* glycoproteins 7 and 8 and *E. gracilis* glycoprotein) were freeze-dried, they left transparent films rather than fluffy antifreeze matrices. This may be due to their lower distribution coefficients which, besides reducing the amount of antifreeze incorporated into the ice as the solutions are frozen, also results in an increased concentration of the antifreeze in the remaining liquid phase. This increased concentration in the liquid phase may prevent total freezing during freeze-drying so that, as antifreeze fibers buried in the ice are exposed by sublimation, they may be dissolved by the unfrozen liquid portion.

Model of mechanism

The growth of crystals is widely believed to occur by propagation of a step across a crystal face (26). If impurity molecules are adsorbed to a face in the path of a growing step, the step will be forced to grow between the impurities, increasing the curvature and hence the area of the edge. Because of the surface tension, the increased area will affect the equilibrium state of the system. Working from Kelvin's equation (27), Kuhn (28) derived an expression for the lowering of the equilibrium freezing temperature of water due to surface effects:

$$\Delta T = \frac{\sigma T_0 M}{L \rho_i} \cdot \frac{dS}{dV} \qquad [1]$$

in which σ is the surface tension, T_0 is the normal freezing point, L is the molar latent heat, M is the molecular weight of water, ρ_i is the density of ice, and dS/dV is the derivative of the area of an ice crystal with respect to its volume. For the purpose of calculation, it is convenient to approximate the random distribution of impurities on a crystal face by a square array with a spacing l (29). For a step that is forced to grow in a direction perpendicular to a row of impurity molecules in the array, the radius of curvature of the edge between adjacent impurities will decrease from infinity (straight edge) to a minimum of $l/2$ (row of semicircles). At this point, adjacent semicircles are tangent to one another and are thought to fuse, allowing the edge to straighten and proceed to the next row of impurities (29). Evaluating dS/dV for these semicircles gives, from Eq. 1,

$$\Delta T = \frac{2\sigma M T_0}{L \rho_i l}. \qquad [2]$$

This equation says that, as the spacing between the adsorbed impurity molecules gets smaller, the undercooling required to allow the step to propagate through the spacing must be increased.

Ice crystal faces parallel and perpendicular to the *c* axis, in

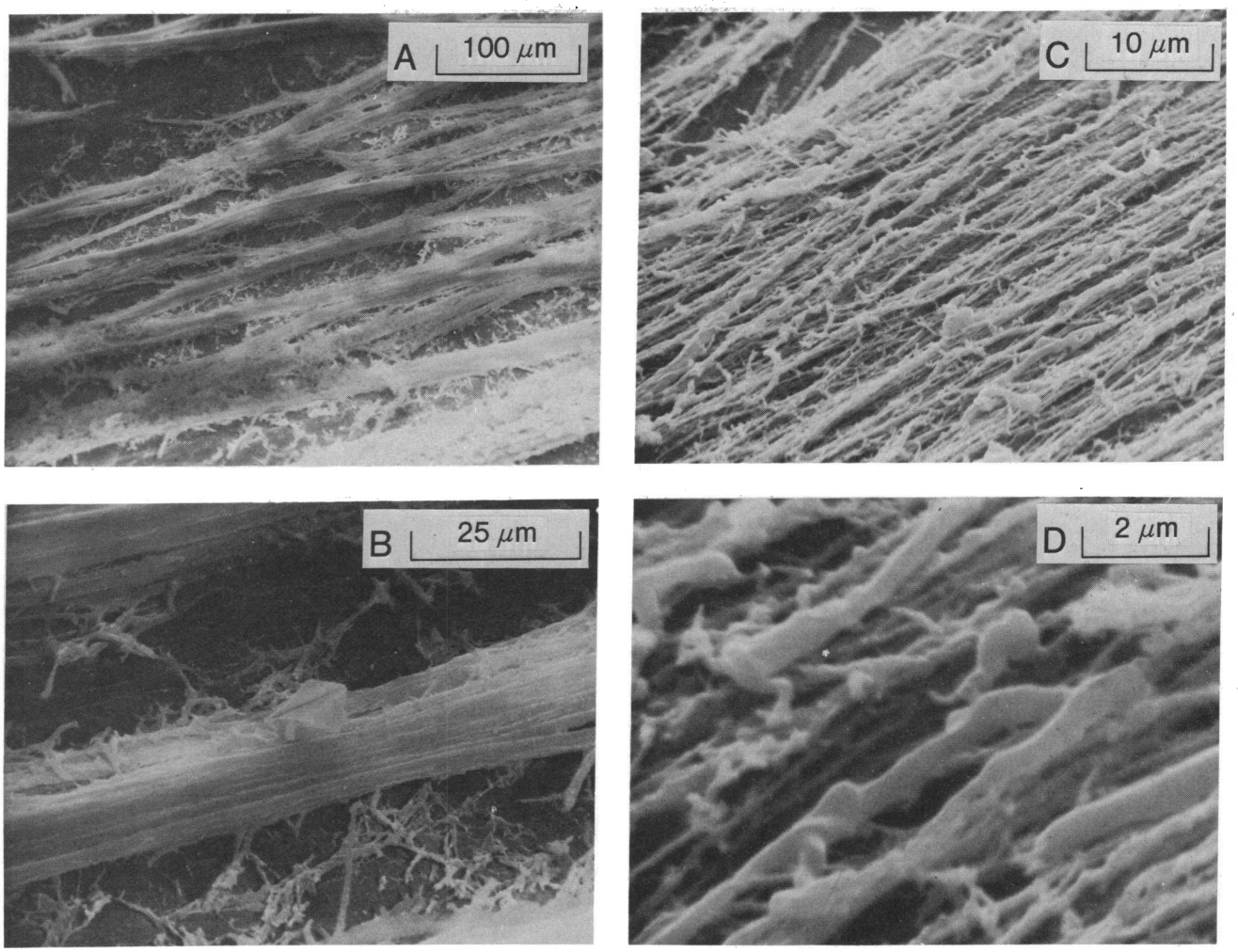

FIG. 1. Scanning electron micrographs of freeze-dried films of solutions of *T. borchgrevinki* glycoproteins 1–5 (*A* and *B*) and *M. verrucosus* protein (*C* and *D*). Direction of ice needles was parallel to that of fibers. Fibers are believed to be "skeletons" of ice needles and probably shrank in diameter during drying.

general, will have different surface densities of antifreeze. Here we make the simplifying assumption that all ice growth inhibition by the antifreezes is governed by a single surface density. The error introduced by this assumption should be small because glycoproteins 7 and 8 do not noticeably retard growth in one crystal direction more than in another, and the other antifreezes cause ice to grow in sharply pointed needles that appear to lack crystal faces perpendicular to the needle axis (*c* axis).

The surface density of antifreeze is not known but, as an approximation, let us assume that only those molecules that are eventually buried in the ice participate in freezing inhibition, so that near the ice–water interface there is an effective antifreeze concentration, αC (α is the distribution coefficient and C is the antifreeze concentration in molecules per cm^3). Viscosity and electrophoretic mobility studies on the glycoprotein antifreezes of *T. borchgrevinki* (13) and Sephadex filtration of the protein antifreeze of *P. americanus* (8) indicate extended rather than globular conformations for these molecules. We thus make a fourth assumption—each of the antifreezes, when adsorbed to an ice surface, lies flat on that surface so that the height of the molecule is equal to twice the molecular chain radius, $2r$. Any molecule whose center lies within a distance r above or below the surface of an ice crystal will intersect it. Therefore, the number of molecules intersecting a plane of area A is $2rA\alpha C$ and the surface density is thus $2r\alpha C$. Equating this density with that on the square array gives $l = (2r\alpha C)^{-1/2}$. If the antifreeze concentration C is expressed in mg/cm^3, $l = (1000\,MW/2r\alpha CN)^{1/2}$ in which N is Avogadro's number and 1000 MW is the molecular weight of the antifreeze in mg. Therefore, Eq. 2 becomes

$$\Delta T = \frac{2\sigma M T_0}{L\rho_i}\left[\frac{2r\alpha CN}{1000\,MW}\right]^{1/2}. \qquad [3]$$

The chain diameters of the glycoprotein and protein antifreezes are unknown and must be approximated. An x-ray diffraction pattern of crystallized glycoprotein 5 of *T. borchgrevinki* showed equatorial and meridional repeat distances of 8.7 and 7.7 Å, respectively (7), suggesting that one of these repeat distances may correspond to the chain diameter. The chain diameters of the protein antifreezes of *M. verrucosus* and *P. americanus* are estimated to be of similar size because these molecules appear from circular dichroism studies to be roughly 80% α-helical (7). We thus estimate the chain diameters of each of the antifreezes to be 8 Å. Using this value for $2r$, and $\sigma = 5.24 \times 10^{-7}$ cal/cm^2 (25), $M = 18$ g, $T_0 = 273°$, $L = 1436$ cal/mol, and $\rho_i = 0.917$ g/cm^3, we get

$$\Delta T = 27.2\,[\alpha C/MW]^{1/2}. \qquad [4]$$

The curve for this function is plotted in Fig. 2 for each of four antifreezes, from values for α and MW given in Table 1. Both the shapes of the curves and the magnitude of the freezing point depressions they predict are in reasonable agreement with the

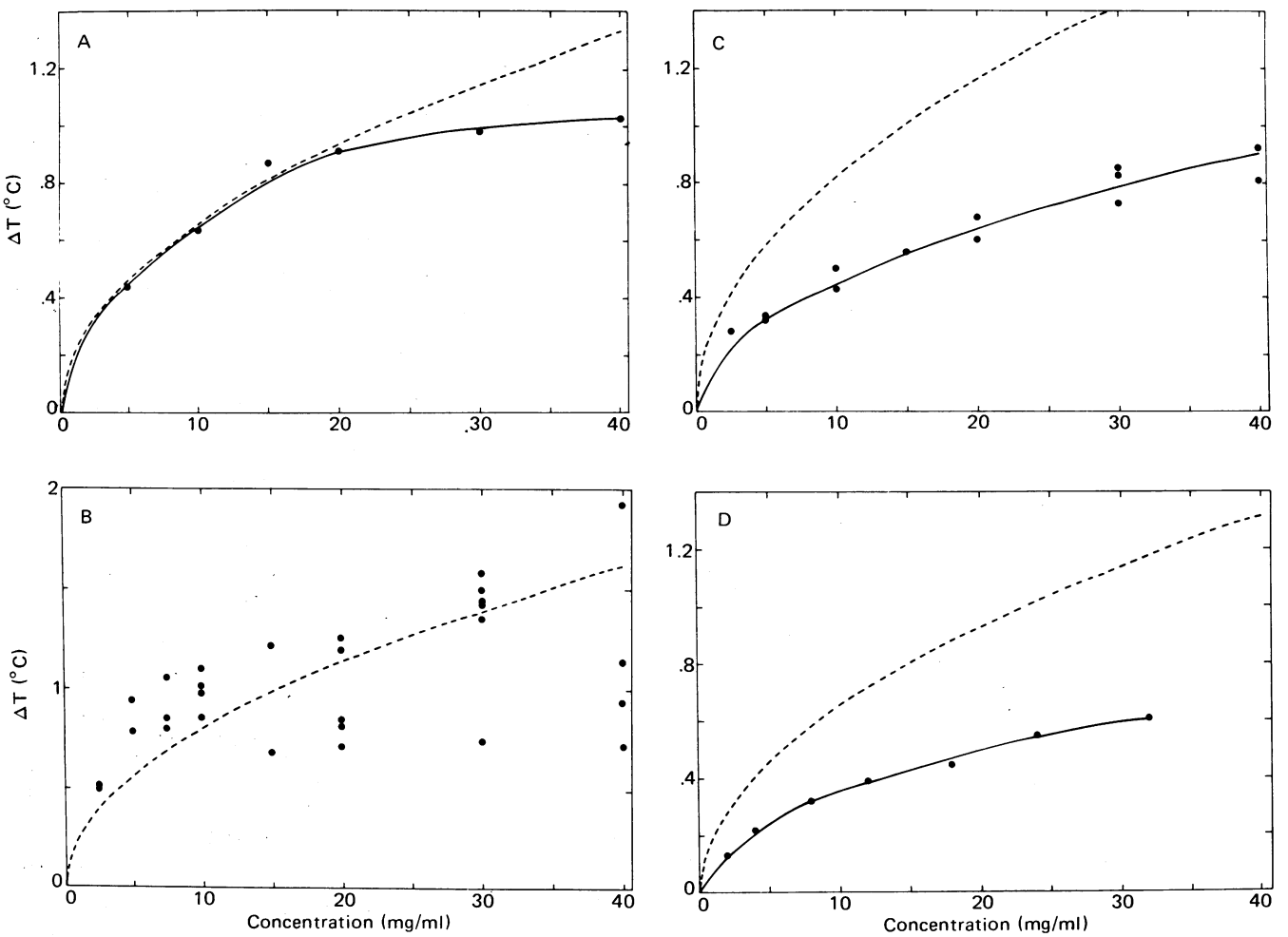

FIG. 2. Freezing point depression of fish antifreezes as a function of concentration. Experimental curves are given by points and solid lines. Broken lines show theoretical values based on Eq. 4. Freezing points were taken as temperature at which ice seed crystal grew rapidly. Freezing points of solutions of protein antifreeze of *M. verrucosus* were less sharp than those of glycoproteins; the seed crystal would usually show a small amount of growth at a lower undercooling (lower points in *B*), and growth would then cease until a greater undercooling was reached (upper points in *B*). (*A*) *T. borchgrevinki* 1–5. (*B*) *M. verrucosus*. (*C*) *E. gracilis*. (*D*) *T. borchgrevinki* 7, 8.

experimentally determined curves. In the worst cases, the magnitudes of the predicted freezing point depressions for the antifreeze of *E. gracilis* and for glycoproteins 7 and 8 of *T. borchgrevinki* are roughly twice the observed values. We are uncertain of the cause of this discrepancy but the answer may be related to the relatively low molecular weights of these molecules. The semicircles along the edge of a step are not actually tangent to one another but are separated by the diameter of the adsorbed molecule. Therefore, the smaller the diameter, the easier it will be for adjacent semicircles to fuse, and the less effective will be the impurity in stopping step growth.

DISCUSSION

There is a wide body of evidence that small amounts of impurities can exert a large influence on the growth of many crystals. Currently it is thought that the impurities adsorb to the crystal surfaces and there slow or stop growth by interfering with the propagation of steps across the surface (30). A table describing 28 examples of impurity-caused inhibition of crystal growth from solution, by electrodeposition and by recrystallization, appears elsewhere (7). The antifreezes provide a fourth type of inhibition in which crystal growth from the melt is inhibited.

Models of Inhibition. Impurities may inhibit the growth rate of a crystal by increasing either the surface tension or the distance between growth kinks, but neither of these effects can presently be experimentally determined (29). They are also unsatisfactory with regard to the antifreeze mechanism (see below) in that they can account only for a decrease in growth rate and not a virtually complete halt to growth.

Sears (31, 32) proposed another mechanism in which crystal growth could be nearly completely inhibited by the complete blocking of a step by a row of impurity molecules, thus forcing growth to occur by two-dimensional nucleation, a much slower process. However, no growth could be detected in ice crystals suspended in solutions of *T. borchgrevinki* glycoproteins 1–5 held at subzero temperatures for several days (7, 33), conditions under which growth of ice by two-dimensional nucleation is easily measurable (34–36). Another objection to the applicability of Sears' mechanism to the antifreezes is that it is based on the behavior of certain low-molecular-weight impurities which appear to have a high specificity for growth steps on a particular crystal. Cabrera and Vermilyea (30) doubt that macromolecular adsorbents possess sufficient specificity to accomplish this and favor instead the view that macromolecules tend to be more evenly distributed over the surface of a crystal.

These workers proposed their own model for the effect of impurities on crystal growth rate (used herein) in which growth

was stopped when the average spacing between adsorbed impurities was equal to or smaller than twice the critical radius of curvature (30). Similar theories have been developed by Price *et al.* (37), Berner and Morse (38), Ohara and Reid (29), and Razumney and Bockris (39).

Others have reported anomalous freezing point depressions of water in cell membranes (40), gels (28, 41, 42), animal tissues (43, 44), and clay (45) in which ice growth inhibition appeared to be due to high curvature on the ice surface caused by the small size of the ice crystals rather than by adsorbents. A necessary property of these matrices appears to be strength because gels crosslinked by only hydrogen bonds failed to show a freezing point depression (46). In view of the antifreezes' high solubility, even after repeated freeze-dryings, it is unlikely that antifreeze matrices such as those shown in Fig. 1 would possess enough strength to create a freezing point depression by a similar process.

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