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(54) Title: ANTIFREEZE GLYCOPEPTIDE COMPOSITIONS TO PROTECT CELLS AND TISSUES DURING FREEZING

(57) Abstract

The present invention relates to aqueous compositions of substances, such as organic molecules, which are useful to protect and preserve viable plant or animal cell membrane and tissue exposed to hypothermal and hyperthermal temperatures or non-physiological chemical conditions, and to modify the freezing process of liquids in biological plant or animal cells or tissue. More specifically, the present invention relates to the use of antifreeze polypeptide or antifreeze glycopeptide which is derived, for example, from the fluid or serum of Artic and Antarctic fish. Preferred antifreeze compounds are related to those polypeptides having multiple alanine-alanine-threonine- or alanine-alanine-alanine- segments. In some embodiments, a pendant sugar group is covalently attached to each threonine moiety. An aqueous solution of the peptide or glycopeptide is contacted with cells ova, sperm, oocytes, embryos, tissue, an organ, or a whole living plant or animal. The cells, tissue, organ or plant or animal is then carefully cooled and/or frozen at 0 °C or below (in some cases to -196 °C or to 4 K) and held at the low freezing (or vitrification) temperatures. The ice forms, if at all primarily along the c-axis with the result that cell membranes are not disrupted and the cells are not dehydrated. The cells, tissue, whole plant or organ or animal are carefully thawed, and all are found to be viable. The preserved organs are particularly useful transplant organs for a human being.

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ANTIFREEZE GLYCOPEPTIDE COMPOSITIONS TO PROTECT CELLS AND TISSUES DURING FREEZING

Field of the Invention

The present invention relates to aqueous compositions of substances, e.g. thermal hysteresis protein, which are useful to modify the freezing process of liquids in biological tissue. More specifically, the present invention relates to the use of antifreeze protein or glycoprotein which is derived, for example, from the fluid or serum of Arctic and Antarctic fish. Preferred antifreeze compounds are related to those obtained from natural animal sources. More preferred are those polypeptides having multiple -alanine-alanine-threonine- or -alanine-alanine-alanine- segments. In some embodiments, a pendant sugar group is covalently attached to the threonine moiety.

A solution of antifreeze protein is perfused through animal tissue or an animal organ. The tissue or organ is then carefully frozen to temperatures below -0.5°C and held at the low temperatures. The ice forms primarily along the c-axis of the ice crystal, and ice formation is inhibited in the direction of the a-axes (faces) of the ice crystal. This spicular ice growth compartmentalizes the concentration of the salts with the result that adjacent cells are not disrupted or completely dehydrated. The tissue or organ is carefully thawed, and the tissue or organ is functioning and viable. The preserved organs are particularly useful in transplantation therapy in a human being.

The present invention also relates to compositions of substances, e.g. thermal hysteresis protein which are useful to improve survival, functionality and/or structural integrity in biological materials, e.g., microorganisms, animal cells, tissues or organs exposed to temperatures and chemical environments different from their normal physiological temperatures and environments, by protecting at least the cell membranes from damage and cell contents from leakage due to exposure to nonphysiological thermal and

chemical conditions.

DESCRIPTION OF RELATED ART

The preservation of viable animal tissue, animal organs and living animals has been the subject of recent intense laboratory and medical research. Human organ transplants of heart, kidney, lung, liver and the like are now possible because of improved surgical techniques, improved anti-rejection drugs, and immediate availability of donated organs. Presently, donor organs are removed from a donor, cooled, stored on wet ice, but not frozen and within a maximum of a few hours are surgically placed in a recipient's body.

The preservation of animal tissue, animal organs and intact viable animals by freezing at lowered temperatures is presently limited to a few hours, because the normal formation of ice in an organ produces localized concentrated salt solutions. Water migrates from the nearby cells irreversibly dehydrating the cell. These events are major problems that disrupt the organ structure and function, and the organ does not reactivate or function upon thawing.

Advances in the development of immunosuppressants, improvements in organ transplantation techniques and the successful use of freezing for long-term preservation of cells have motivated intensive research efforts on methods for long-term preservation of biological organs through freezing. Recently, B. Rubinsky, U.S. Patent 4,531,373 disclosed an experimental technique using a directional solidification stage and low temperature scanning electron microscopy to facilitate the study the process of freezing in biological tissues.

B. Rubinsky et al., (1988) Proceedings of the Royal Society London, B., Vol. 234, pp. 343-358), also describes experimental results and a mathematical model for the freezing process and the mechanism of damage in biological tissue and biological organs.

None of the available literature below disclose a composition or a method to preserve for long times tissue or

organs.

Earlier experimental results show that single, continuous ice crystals normally form along the blood vessels of frozen tissue. B. Rubinsky et al. (1988), Cryo-Letters, Vol. 8, p. 370; B. Rubinsky et al. (1988), Proc. Royal Soc. Lond., B234, 343. The structure of the frozen tissue depends on the cooling rate, (i.e., the temperature variation per unit time) during freezing. When tissue, such as liver, is frozen with low cooling rates (about 1°C/min to about 10°C/min), the smaller blood vessels (sinusoids) expand relative to those of the unfrozen normal liver tissue. In addition, the cells (hepatocytes) adjacent to the expanded sinusoids, are dehydrated without intracellular ice forming. However, at higher cooling rates, intracellular ice forms in the cells (hepatocytes) resulting in a reduced expansion of the sinusoids.

One explanation for the observed formation of continuous ice crystals along the blood vessels, for the expansion of the frozen blood vessels, and for the formation of intracellular ice during freezing with higher cooling rates is that ice formed in the vascular system does not propagate through the cell membranes or the blood vessel wall. Instead, ice forms within and propagates along the blood vessels where there is no barrier to the ice crystal growth process. Water in the cells surrounding the frozen blood vessels, being compartmentalized in small volumes, will, at first, remain supercooled. As the intravascular ice forms, water is removed from the solution in the vascular space, rendering the remaining solution hypertonic (higher in salts concentration). This higher concentration of solutes causes water to migrate irreversibly from the surrounding cells, through the semi-permeable cell membrane, into the blood vessel in order to equilibrate the difference in chemical potential. Consequently, the cells surrounding the blood vessel will dehydrate, and the water that leaves the cell then freezes in the vascular system. Water transport from cells through the cell membrane into the

blood vessel, is a rate-governed process, which depends on the permeability of the cell membrane. Therefore, when larger organs are frozen using higher (i.e. faster) cooling rates, sufficient water remains in the cell for intracellular ice to form prior to the complete dehydration of the cell. A more detailed description of the process of freezing and a mathematical model that supports this description is found in the Rubinsky, et al. (1988) reference above. This result also leads to the conclusion that one of the possible modes of damage to frozen tissue is the observed expansion of the blood vessels which causes the disruption of the structural (mechanical) integrity of the organ. This mode of damage apparently does not affect cells frozen in suspensions, and may explain why organs do not survive freezing under the same conditions in which cells in suspensions survive.

The normal patterns of ice formation, in which the energetically preferred direction of ice growth is also the a-axes (prism face) of the hexagonal prism ice crystal, governs the process of freezing in tissue. Any hexagonal prism facet of the a-axes of the three-dimensional ice crystal has the same energetic preference and, therefore, during freezing of tissue, the ice crystal can continuously follow and grow along the blood vessel. Furthermore, as discussed earlier, the large ice crystals of normal freezing do not incorporate solutes. This rejection of solutes results in more concentrated solutes, a mass transfer process and the irreversible water migration from local cells and tissue into the open vessel. This migration leads to the disruption of the structural integrity of the cells of the tissue or organ.

Additional background information can be found in:

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All references, patents, patent applications, articles, standards, etc. cited in this application are incorporated herein by reference in their entirety.

It would be extremely advantageous to have a composition and a method which would alter the preservation process of biological liquids in animal cell tissue. Thus, when the frozen tissue, organ, plant or animal is carefully thawed, it results in viable cells, tissue, organ, plant or animal. The present invention provides such a preservation composition and method.

SUMMARY OF THE INVENTION

The present invention in one aspect relates to a composition useful in the protection and preservation of viable cells and cell membranes of an animal independently subjected to :

(i) hypothermal temperature conditions from the physiological temperature to about 0°C,

(ii) vitrification temperature conditions from about 0°C to about -190°C,

(iii)freezing temperatures from between about -0.5°C and 4K,

(iv) hyperthermal temperature conditions from the physiological temperature up to about 10°C above the physiological temperature, or

(v) nonphysiological chemical conditions, or

(vi) or combinations thereof which composition comprises:

one or more thermal hysteresis proteins; and

a biologically compatible aqueous preservation solution.

The present invention is related to a composition of biologically compatible substances, e.g., antifreeze peptide, useful to the survival, functionality, stability and structural integrity of biological materials, including proteins, enzymes, lipids, cell membranes, animal or plant cells, microorganisms, tissues, organs, whole animals, or whole plants subjected to nonphysiological temperatures, either higher or lower than the normal physiological temperatures or to nonphysiological chemical environments, by interacting with the proteins, lipids and at least the cell membranes.

In other aspects, the present invention also relates to a composition of antifreeze protein useful in improving survival, functionality, stability and structural integrity of biological materials, including animal or plant proteins, enzymes, lipids, carbohydrates, cell membranes, cells (animal or plant) microorganisms, tissues or organs subjected to temperatures lower physiological and lower than 0°C in the presence of ice crystals:

(a) by modification of the structure of the ice crystals in contact with the proteins, enzymes, lipid or at least the plant or animal cell membranes;

(b) by reducing the number and the size of the ice crystals or by completely eliminating the ice crystals in contact with the proteins, enzymes, lipids or at least the cell membranes; or

(c) by modifying the mode in which solutes are rejected by the ice formation and thereby changing the chemical composition of the solution surrounding the proteins, enzymes, lipids or at least the cell membranes.

The present invention also relates to a composition useful to block ion channels in membranes to retard or prevent ion leakage (in general) and to stabilize cell membranes (in general), or in binding other macromolecules

to proteins, lipids, or at least cell membranes.

The composition whose usefulness discussed above comprises at least one biologically compatible antifreeze substance, and a biologically compatible aqueous solution.

In one aspect the biologically compatible antifreeze substance is a macromolecule (e.g. a polypeptide) from or substantially the same as a macromolecule derived obtained from an animal selected from fish, amphibian, worm, insect or reptile, preferably fish from Arctic, Antarctic, North Temperate or South Temperate Zones. More preferably, the protein is from body fluids (e.g. blood) from Antarctic fish, e.g. from the family Nototheniidae, including the species D. nawsoni and P. borchgrevinki or the Antarctic eel pout Rhigophila dearborni, or the Arctic winter flounder. All these antifreeze proteins are known and have the common property that they modify the structure of ice crystals.

In one embodiment, the biologically acceptable substance is selected from a polypeptide, a glycopeptide, a polypeptide covalently bonded to biologically acceptable carrier, a glycopolypeptide covalently bonded to a carrier or mixtures thereof.

In one embodiment, the aqueous composition further includes additional preserving, protecting or vitrifying compounds selected from glycerol, dimethylsulfoxide, ethylene glycol, polyvinylpyrrolidone, glucose, sucrose, propanediol, propylene, glycol, carboxymethyl cellulose, or mixtures of these compounds which are known to protect cells and biological materials against freezing damage or to promote vitrification.

The ability of the compounds to protect or stabilize membranes are also useful in the preservation of food; in cosmetics to restore, preserve or repair skin tissue; or in therapy for diseases associated with instability of cell membranes.

The ability to block ion channels is used in treating diseases associated with imbalances of the intracellular-extracellular ion transport across cell membranes.

The ability to attach to and interact with cell membrane, is used in attaching various macromolecules to the antifreeze proteins and thereby facilitating their attachment to the cell membrane.

In another aspect the present invention relates to a method for preservation, survival, functionality, stability and structure or integrity of biological materials, at non-physiological temperatures or in nonphysiological chemical compositions, including proteins, enzymes, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, whole animals or whole plants, which method comprises:

(a) bringing the moiety to be preserved in contact with a thermal hysteresis protein in sufficient concentration to interact with the proteins, lipids, cell membranes, cells, microorganisms, tissues or organs;

(b) exposure to the nonphysiological conditions;

(c) optionally first removing the macromolecule;

(d) returning the proteins, lipids, cell membranes, cells (animal or plant), microorganisms, tissues or organs to a physiological temperature and composition, while optionally simultaneously removing the macromolecule; or optionally

(e) subsequently removing the thermal hysteresis protein after returning the biological material to the physiological temperature and composition.

In one embodiment, the temperatures are hypothermic, i.e., close to 0°C or lower and are used for preservation of proteins, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, animals or plants. For example, pig oocytes are preserved in such a way at about 4°C to 24 hr or more. Rat livers are preserved by this method at 4°C for 24 hr and preferably longer 48 hr, 96 hr, etc.

In another aspect the present invention related to a method for preservation of animal proteins, enzymes, lipids, cell membranes, cells, microorganisms, tissues or organs at

temperatures below 0°C to about 4K which method comprises:

(a) bringing the moiety to preserved in contact with the biologically compatible substance (e.g. AFP or AFGP in the presence of only an aqueous solution or with addition the other cryoprotective compounds such as glycerol, propylene glycol, etc.;

(b) cooling preferably to cryogenic temperatures (by such means as liquid nitrogen) and either vitrifying or freezing the system according to the various concentrations and cooling rates using higher concentrations of the additional compounds, such as propylene glycol or glycerol and high er cooling rates which lead to vitrification and to lower freezing temperatures (e.g. with 40% v/v propyleneglycol/water) and with a cooling rate of 1,750°C/min., vitrification is achieved);

(c) maintaining the proteins, lipids, cell membranes, cells, microorganisms, tissues or organs at these temperatures for periods of more than 24 hours, 7 days, 52 weeks or to about 10 years,

(d) warming, by such means as warm fluids or microwave heating, to physiological conditions, and

(e) removing the thermal hysteresis protein e.g. antifreeze glycoproteins and the other compounds, (e.g., by perfusion or flushing) and replacing them with physiological compatible solutions to regenerate the viable biological moiety.

For example, with 12.5% v/v, propylene glycol/water at a cooling rate of 1,200°C/min., ice crystals were formed. In all cases, viable mouse embryos and pig oocytes were obtained after exposure to -130°C for several hours;

Bovine oocytes, pig oocytes, pig embryos and mouse embryos survive this protocol in a aqueous composition of about 10 to 20 mg/ml antifreeze glycoproteins from Antarctic fish from the family Nothotheniidae.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B and 1C are transmission light micrographs of ice crystals (i), in aqueous solution is

frozen with a cooling rate of 4°C/min on a directional solidification stage, see U.S. Patent 4,531,373.

Figures 2A, 2B and 2C are scanning electron micrographs of liver tissue perfused with 40 mg/ml AFGPs (see definitions below) and frozen with a cooling rate of approximately 4000°C/min.

Figures 3A, 3B, 3C, 3D and 3E are scanning electron micrographs of frozen liver tissue.

Fig. 4 (4A to 4D) are photographs of the cryopreservation of immature pig oocytes.

Fig. 5 (5A to 5B) are photographs of the cryopreservation of pig embryos at the two-cell stage.

Fig. 6 (6A, 6B and 6C) are photographs concerned with cryopreservation of mouse embryos at the two-cell stage.

Fig. 7 (7A, 7B and 7C) are photographs showing with the hypothermic preservation of pig oocytes.

Figure 8 is a photographic representation of rat liver tissue (A7) at a magnification of about x 400. This tissue without AFGP treatment was cooled to -35°C at 21.5°C/min.

Figure 9 is a photographic representation of rat liver tissue at a magnification of about x 400. This liver tissue was flushed with a Krebs solution containing 20 mg/ml of AFGP fractions 1-8 (Table 1) at 37°C prior to cooling to -35° at 21.5°C/min.

Figure 10 is a graph of the bile production from whole rat liver treated with Krebs solution and Krebs solution AFGP as a function of time (see Example 7A).

Figure 11 is a graph of the LDH level from whole rat liver treated with Krebs solution and Krebs solution and AFGP as a function of time (see Example 7A).

Figure 12 in a graphic representation of the percentage of oocytes with normal membrane potential after hypothermic exposure for 4 hr at 4°C without and with various concentration AFGPs.

Figure 13 is a graphic representation of the percentage of oocytes with normal membrane potential after hypothermic exposure for 24 hr at 4°C without and with various

concentrations of AFGPs.

Figure 14 shows a graphic representation of the percentage of bovine oocytes with an intact oolemma for morphological microscopic examination (Morph), fluorescein diacetate staining (FDA) and trypan blue exclusion for a control, AFP winter flounder (WF), AFP ocean pout (OP) and AFP sea raven (SR).

Figure 15 shows a graphic representation of the percentage of bovine oocytes matured or fertilized in vitro for control, AFP winter flounder (WF), AFP ocean pout (OP), AFP sea raven (SR) and fresh.

Figure 16 is a graph of current versus time for superimposed raw currents showing the effect of AFP (0.5 mg/ml) on Ca^{+2} currents. Test pulses were given at 20 sec intervals. The currents shown are (a) before infusion (b) after 20 sec and (c) after 40 sec. Complete current suppression is achieved in 40 sec.

Figure 17 is a graph of current versus time for superimposed raw currents showing the effect of AFP (0.5 mg/ml) on K^+ currents. Test pulses were given at 20 sec intervals. To make the figure legible we show currents obtained, before infusion of AFP, curve (a), and then in subsequent 40 sec intervals. Complete current suppression is achieved in 200 sec.

Figure 18 is a graph of current versus time for superimposed raw currents showing the effect of AFP (10 mg/ml) on K^+ currents. Test pulses were given at 20 sec intervals. The currents shown are (a) before infusion of AFP, and (b) 20 sec after the infusion. Complete current suppression is achieved in 20 sec.

Figure 19 is a graphic representation of the effect of Cai concentration (nM) versus time (sec) in parietal cells.

Figure 20 is a graphic representation of the effect of the sequential treatment of parietal cells with 100 micro M carbachol in a control and with AFP versus time (sec).

Figure 21 is a graphic representation of the calcium internal store of Cai concentration (nanoM) for control and

with AFP versus time.

Figure 22 is a graphic representation of Cai concentration with short treatment of glad cells with AFP for a control and AFP treated cells versus time (sec).

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTDS

Definitions:

As used herein:

"Abnormal nonphysiological chemical conditions" refers to conditions different from the normal physiological conditions include, but are not limited to high or lowered temperature, freezing, excess or limited carbon dioxide, excess or limited oxygen, excess or limited inorganic salts, excess or limited organic compounds, different pH values radiation or combinations thereof.

"Antifreeze proteins" or "antifreeze polypeptides" ("AFP") or "antifreeze glycoproteins" or "antifreeze glycopeptides" (AFGP)" are macromolecules found in the body fluids of some animals (e.g. cold blooded) which have the commonly known property that they reduce non-colligatively the phase transition temperature of water by direct interaction with and inhibition of the growth of ice crystal nucleii that form at temperatures below the phase transition temperature.

Antifreeze compounds (from any source) are also known as "thermal hysteresis proteins" because while the phase transition temperature is apparently depressed during freezing by an amount much larger than the colligative effect of the molecule, it is not depressed during melting except to the extent caused by the colligative effect of the molecule. Prior to the present invention, this was the only known property of these antifreeze compounds. (Sources of antifreeze peptide (or protein) are described below).

"Cryogenic temperatures" refers in the area of cryobiology, below 0°C to as low as 4K or lower.

"Freezing" refers to condition (iii) is performed at -0.50°C and below to 4K. The rate of freezing is rapid,

about 1000°C/min or slow about 1°C min, preferably between about 4°C and 200°C/min especially about 4 to 50°C/min.

"Hyperthermic" refers to (condition iv) temperatures higher than the normal physiological temperature of a cell, tissue, organ, plant or animal, e.g. up to 20°C greater than physiological, preferably about 10°C greater, more preferably about 5°C greater.

"Hypothermic" refers to temperatures lower than the normal physiological temperature of a cell, tissue, organ or animal to about 0°C.

"Mammal" refers to any warm blooded mammal as generally defined, including, for example, pig, cow, rabbit, horse, human being, and preferably a human being.

"Non-physiological chemical or environmental conditions" refers to (condition v) excess or reduced oxygen (± 50%), excess or reduced carbon dioxide (± 50%) from physiological concentrations, having different ion concentrations from physiological (± 10% by weight, preferably ± 5% by weight, more preferably ± 1% by weight, pH values ± 3 pH units from physiological or a combination thereof.

"Optional" or "optionally" refers to the situation in which a component may or may not be present, or where a step may or may not be performed, within the scope of the invention.

"Prism planes" refer to another convention to describe the growing ice formation on an ice crystal. There exist secondary prism planes perpendicular to the a-axes and pyramidal planes that project off these planes. Crystallography terminology describes these planes in terms of the following pyramidal Miller-Bravais indices:

Primary prism plane	$(1\ 0\ \bar{1}\ 0)$
Secondary prism plane	$(1\ 1\ \bar{2}\ 0)$
Pyramidal pane from the primary prism plane	$(2\ 0\ \bar{2}\ \bar{1})$
Pyramidal plane from the secondary prism plane	$(1\ 1\ \bar{2}\ 1)$

Ice crystal growth under normal circumstances is along the a-axes. Ice crystal growth using the AFPs or AFGPs of the present invention is altered to be preferred in the direction of the c-axis.

For more information, see Peter V. Hobbs (1974) Ice Physics, Clarendon Press, Oxford, England, Appendix A etc., p. 725 ff.

"Rapid cooling" refers to a technique developed for long term preservation of cells and biological organs at cryogenic temperatures. The rapid cooling is used to produce very small, non-damaging ice crystals, see A. Trounson, (1986) Fertility and Sterility, Vol. 46, 1-12.

"Vitrification" refers to a technique for long term preservation of cells and biological organs at cryogenic temperatures. The technique involves introduction into the biological materials of different cryoprotective compounds such as glycerol, dimethylsulfoxide, propylene glycol, etc. which depress colligatively the phase transition temperature for water and increase its temperature. Next, the whole cell suspension or organ is rapidly cooled in the presence of the cryprotective compounds with the expectation that the water in the biological materials will remain polymorphous in a glass form and that no damaging ice crystals will occur or exist. (See Fahy, G.M. et al., Cryobiology, Vol. 21, 407-426, (1984), W.F., Rall and Rahy, G.M. Nature, Vol. 313, 573-575, 1985)). Rates of cooling for vitrification (condition ii of the invention) for small unorganized cells (e.g. ova, sperm, embryo) and for organs is rapid, about 100 to 2000°C/min, preferably about 200 to 1750°C/min, more preferably about 200 to 1000°C/min, especially about 200°C. The best cooling rate is determined by the objectives of the preservation.

Sources of Antifreeze Proteins

Antifreeze proteins (AP-which includes AFP and AFGP) were found first in the body fluids of marine teleost fish which are hypoosomotic, have a blood serum freezing point of -0.7°C, but inhabit the polar ice-laden waters (Scholander

et al. J. Cell Comp. Physiol., Vol. 49, 5-24, 1957). The first AP's were found by DeVries (Doctoral Thesis, Stanford, 1968) in Antarctic nototheniid fish. Two types of antifreeze proteins have been isolated from polar and temperate fish, glycopeptide and peptides. In studies of fishes with two exceptions, the antifreeze compounds are glycopeptides.

These antifreeze glycopeptides (glycoproteins) are present in eight distinct molecular weight classes ranging from - 2,500 to 34,000. They generally consist of a peptide backbone made-up of repeats of the tripeptide alanyl-alanyl-threonyl (the small glycopeptide may replace some alanines with proline beginning at position 7, with the disaccharide sugar beta-D-galactopyanosyl-(1→3)-2-acetamide-2-deoxy-alpha-D galactopyranose attached via a glycoside linkage to the hydroxyl side chain of each threonine (A. DeVries, Science, Vol. 172, 1152-1155, 1971).

These polypeptide or glycopolypeptides are available from a number of natural sources, e.g.: from body fluids of reptiles (e.g. turtles), invertebrates, insects, amphibians or fish. Preferably, the AFP's are obtained from the serum or body fluids of Arctic, Antarctic, North Temperate or South Temperate fish. More preferably, the serum and fluids of Arctic or Antarctic fish are used, e.g. See Table 1 below.

TABLE 1

MOLECULAR WEIGHTS OF ANTIFREEZE GLYCOPEPTIDES

A. Antifreeze glycopeptide isolated from the Antarctic notothenidae are fish; Pagothenia (Trematomus) borchgrevinki.

Antifreeze Glycopeptide Fraction No.	Molecular Weight (Approx.)
1	33,700
2	28,800
3	21,500
4	17,000
5	10,500
6	7,900
7	3,500
8	2,600

Glycopeptide from Dissostichus mawsoni of structure

Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-
NAGA NAGA
GAL GAL

Thr-Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-
NAGA NAGA NAGA
GAL GAL GAL

The molecular weights of the fractions are essentially identical to those of Table 1 above.

AFGPs isolated from the blood of Antarctic notothenid fish exist in at least 8 sizes depending on the number of repeats of the basic glycotripeptide unit, see Table 1. The molecular weight ranges between 2,600 and 33,700 (DeVries et al. (1970)) The AFGP's make up 3-4% of the blood of the fish and along with the sodium chloride, they lower the fish's freezing points below that of seawater. The AFTPs inhibit the growth of ice crystals by adsorption to the ice crystal (Raymond et al. (1977), DeVries (1984)). Adsorption occurs on specific faces of the ice crystal (primary prism face (1010)) resulting in inhibition of ice growth on these faces (DeVries, 1984), Consequently, in solutions of AFGPs,

ice crystals grow predominantly on the basal plane (parallel to the c-axis), to which the AFGPs do not absorb, and take the form of very small, needle-like ice crystals (Raymond et al., (1977); DeVries, 1988)). Peptide antifreezes can be found in several North Temperate, Arctic or Antarctic fish. The peptides vary in size and composition.

These polypeptide are essentially different lengths of the repeating tripeptide -alanine-alanine-threonine- where substantially each threonine is joined by a glycosidic linkage to the disaccharide B-D-galacto-pyransoyl-(1→3)- 2-acetamido-2-deoxy-alpha-galactopyranose.

The small glycopeptide may also have a small amount of proline located at positions 7, 10 and 13, but are otherwise structurally the same as the large glycopeptide.

Generally the higher the molecular weight, the more effective is the antifreeze glycopeptide in promoting ice crystal growth along the c-axis, for example, fractions 1-5 above individually or as a mixture as obtained by purification. Fractions 6, 7 and 8, having a lower molecular weight, individually or as a group are apparently less effective in preservation.

The antifreeze glycopeptides are essentially similar in all the Antarctic Nototheniidae fish including (Pagothenia borchgrevinki, Trematomis Nicolia, Dissostichus Mawsoni (J.T. Eastman, and A.L.DeVries, A.L. Scientific American, Vol. 254, 106-114 (1986)). The same eight glycopeptides have also been isolated from northern hemisphere gadid, the rock cod (Gadus ogac) and in some other northern cods belonging to the family Gadidae (DeVries, A.L. Comp. Biochem. Physiol., Vol. 90B, No. 3, pp. 611-621 (1988). All the AFGP's isolated so far are similar in structure with relatively small changes such as the position occupied by proline in antifreeze glycopeptide 8 in northern species, or difference in size in northern cod, but essentially the same composition (A.L. DeVries, (1984) Phil. Trans. R. Socl. Lond., Vol. B 304, 575-588).

The other kind of antifreeze proteins found in fish are

polypeptides. While the antifreeze glycopeptide are in general polymers of a glycotripeptide unit Ala-Ala-Thr with disaccharide linked to the Thr side chain, the peptides are quite diverse structurally and vary in size and composition.

The antifreeze protein from the winter flounder, Pseudopleuronectes americanus, although having a specific activity similar to that of the larger molecular weight glycoproteins, lacks sugars entirely and instead has high percentages of hydrophilic amino acids (especially threonine and Asp) while still retaining a large amount (about 60 mol %) of alanine. The flounder protein primary structure has clusters of hydrophilic amino acids separated by sequences of alanine, (Duman and DeVries (1976) Comp. Biochem. Physiol, Vol. 533, 375-380).

Peptides from Winter Flounder

Asp-Thr-Ala-Ser-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Ala-Thr-Ala-Ala.

Origins of the Biologically Compatible Substance.

Peptides from the Antarctic eel pout Rhigophila dearborni

Peptide No.	Molecular Weight
1,2,3 (three components)	6,900

Asn-Lys-Ser-Val-Val-Ala-Asn-Gln-Leu-Ile-Pro-Ile-Asn-Thr-Ala-Leu-Thr-Leu-Ile-Met-Lys-Ala-Glu-Val-Val-Thr-Pro-Met-Gly-Ile-Pro-Ala-Glu-Asp-Ile-Pro-Arg-Ile-Ile-Gly-Met-Gln-Val-Asn-Arg-Ala-Val-Pro-Leu-Gly-Thr-Tyr-Leu-Met-Pro-Asp-Met-Val-Lys-Asn-Tyr-Glu-.

Other fish that produce antifreeze peptides are listed in A.L. DeVries, Phil. Froms, R. Soc. London, Vol. 304, 575-588 (1984) such as the Alaskan plaice, Atlantic sculpins, Grubby Sculpin (Yang, D.S.C. Nature, Vol. 333, 232-237, 1988) and the Antarctic Eelpout (Rhigophila dearborni). Recent reviews of the antifreeze proteins in fishes can be also found in (Feeney and Burchan (1986), Ann.Rev. Biophys, Biophys. Chem., Vol 15, 53-78,) and (Davies et al. (1988)

Canadian J. Zool, Vol. 66, 2611-2617).

V.S. Ananthanarayanan, Life Chemistry Reports, Vol. 7, pp. 1-32 (1989), also describes sources of antifreeze protein, particularly Type I, II and III. See Table 1A below.

TABLE 1A

TYPES OF FISH ANTIFREEZE PROTEIN

Type, composition and size	*Fish species*	*Trivial name*
Antifreeze glycoproteins (AFGPs): contain alanine, threonine and Gal-GalNAc disaccharide; M_r: 2,600–33,700	Antarctic notothenioids:	
	Pagothenia borchgrevinki	
	Trematomus borchgrevinki	Antarctic cod
	Dissostichus mawsoni	
	Northern ocean gadoids:	
	Gadus agac	Greenland cod
	Gadus morhua	Atlantic cod
	Microgadus tomcod	Atlantic tomcod
	Boreogadus saida	Arctic polar cod
	Eligenus gracilis	Saffron cod
Antifreeze Polypeptides (AFPs): Type I, alanine-rich; M_r: 3,300–6,000	Righteye flounders:	Winter flounder
	Pseudopleuronectus americanus	
	Limanda ferruginea	Yellowtail flounder
	Cottids:	
	Myoxycephalus scorpius	Shorthorn sculpin
	Myoxocephalus aenaeus	Grubby sculpin
	Myoxocephalus scorpiodes	Arctin sculpin
Type II, cysteine-rich; M_r: 14,000–16,000	Cottid:	
	Hemitripterus americanus	Sea raven
Type III, no cysteines and are not rich in alanine, M_r: 5,000–6,700	Eel pouts:	
	Macrozoarces americanus	Ocean pout
	Rhigophila dearborni	Antarctic eel pout
	Lycodes polaris	Arctic eel pout

These AFPs, AFGP's (or fractions and mixtures of fractions thereof) and others are available upon request from Dr. Arthur DeVries, Department of Physiology, Burrill Hall, 407 S. Goodwin, University of Illinois, Urbana, IL 61801.

These antifreeze proteins or peptides of the present invention may also be produced by synthetic means. These means include the use of a peptide synthesizer available commercially in the art as Model 430A, Applied Biosystems, Inc., Foster City, California. The operation manuals for this peptide synthesizer are useful. The synthesis procedures of J.J. Nestor, et al., U.S. Patent 4,318,905, and R.B. Merrifield, U.S. Patent 3,531,258 are specifically incorporated herein by reference and are adapted for the preparation of the Ala-Ala-Thr and Ala-Ala-Ala compounds described above. Once the peptide is prepared, the threonine residues are optionally bonded to the disaccharide by conventional methods.

The antifreeze protein of the present invention are independently selected from the protein themselves, or glycoprotein, or the protein or glycoprotein covalently bonded to a carrier such as biologically compatible antibody, gelatin, biocompatible polymer, peptide, sugar, or carbohydrate. Mixtures of these antifreeze materials are contemplated and are part of the present invention. Covalent bonding of a protein to a carrier by methods known to those of ordinary skill in the art are, for example, found in K. Rubenstein, et al., U.S. Patent 3,817,837, or M. Goodman et al., U.S. Patent 4,837,305, which are specifically incorporated herein by reference in their entirety.

Recombinant DNA Production of Antifreeze Polypeptide

It is also contemplated within this present invention to produce compositions wherein the peptides (amino acid sequences) are produced by recombinant DNA technology. The DNA sequences encoding these genes have been elucidated. See, for example, A.L. DeVries et al. (1971), J. Biol.

Chem., Vol. 246, p. 305; Y. Lin, et al. (1972), Biochem. Biophys. Res. Commun., Vol. 46, p. 87; D.S.C. Yang et al. (1988), Nature, Vol. 333, p. 232; Y. Lin (1981), Proc. Natl. Acad. Sci. U.S.A, Vol. 78, p. 2825; P.L. Davies et al. (1982), J. Biol. Chem., Vol. 79, p. 335; B. Gourlie et al. (1984), J. Biol. Chem., Vol. 259, p. 14960; P.L. Davies et al., J. Biol. Chem., p. 9241; G.K. Scott et al. (1986), Can. J. Fish. Aquat. Sci., Vol. 43, p. 1028; G.K. Scott et al. (1988), J. Mol. Evol., Vol. 27, p. 29. Microinjection of the AFP gene into other species has been successful. See for example, Z. Zhu et al. (1985), Angew. Ichtoyol, Vol. 1, p. 31; Kexue Tongbao (1986), Vol. 31, p. 988; D. Chourrout et al. (1986), Aquaculture, Vol. 51, p. 143; R.A. Dunhan et al. (1987), Trans. Am. Fish. Soc., Vol. 116, p. 87; G.L. Fletcher et al. (1988), Can. J. Fish Aquat. Sci., Vol. 45, p. 352; N.D. Maclean et al. (1987), Bio Technology, Vol. 5, p. 257; G.W. Stuart et al. (1988), Development, Vol. 103, p. 403; T. McEvoy et al. (1988), Aquaculture, Vol. 68, p. 27; K. Ozato et al. (1986), Cell Differ., Vol. 19, p. 237; T.T. Chen et al. (1989), UCLA Symposium on Transgenic Animals; T.T. Chen et al. (1989), Aquaculture; P. Zhang et al. (1989), Mol. Reprod. Dev.; D.A. Powers et al. (1989), NIH Symposium on Transgenic Animals. The general formation of the DNA sequences to produce protein is found in the following U.S. Patents 4,237,224; 4,708,948; 4,376,071; 4,350,687; 4,444,760 and 4,722,998. The procedures are adapted to produce AFPs. All of these references are specifically incorporated herein by reference.

Recently antifreeze proteins (thermal hystersis protein) which is useful in the present invention were also found in many invertebrates. A list of these invertebrates is given in Table 2 and 3 with the references, found in the tables following immediately after the tables.

TABLE 2

Thermal Hystersis Protein Producing Invertebrates

A. Insects (minus beetles).

Order	Species	Reference
Collembola	7 spp.	Zettel, 1984
Plecoptera	Arcynopteryx compacta	Gehrken and Somme, 1987
Orthoptera	Parcoblata pennsylvanica	Duman, 1979
Hemiptera	Oncopeltus Fasciatus	Paterson et al. 1981
Mecoptera	Boreus westwoodi	Husby and Zacharissen, 1980
Lepidoptera	Choristoneura fumiferana	Hew et al., 1983

B. Coleoptera (Beetles)

Family	Species	Reference
Tenebrionidae	Tenebrio molitor	Ramsay, 1964 Patterson and Duman, 1978
	Meracantha contracta	Duman, 1977a
	Uloma impressa	Duman, 1979
	Platydema sp	Duman, 1979
Elateridae	Ampedus lineatus	Duman, 1979
	Ampedus sp	Duman, 1979
	Lepidotus discoideus	Duman, 1979
	Melanotus sp	Duman, 1979
Cucjidae	Cucujus clavipes	Duman, 1979
Pyrochridae	Dendroides canadensis	Duman, 1979, 1980

Lampyridae	Photinus sp	Duman et al., 1982
Coccinellidae	Coccinella novemnotata	Duman et al., 1982
Scolytidae	Ips acuminatus	Gehrken, 1984
Cerambycidae	Rhagium inquisitor	Bremdal and Zachariassen, 1988

C. Non-Insect Arthropods

Animal	Species	Reference
Spiders	Philodromus sp	Duman, 1979
	Clubiona sp	Duman, 1979
	Bolyphantes index	Husby and Zachariassen, 1980
Centipede	Lithobius forficatus	Duman et al., 1982 Tursman and Duman, unpublished
Mite	Alaskozetes antarcticus	Block and Duman, 1989

D. Other Invertebrates.

Mussel	Mytilus edulis	Theede et al., 1976

TABLE 3

Amino Acid Compositions of Representative Insect THP's

(Values are in Mol %)

Amino Acid	-1[a]	T-4[b]	T-3[c]	Budworm[d]	Carudensis[d]
Asx	11.3	7.3	5.3	9.5	14.3
Thr	11.0	6.6	2.3	6.0	17.2
Ser	14.8	7.4	11.1	13.0	10.3
Glx	15.3	8.9	12.4	11.0	5.2
Pro	5.9	5.9	0.0	5.0	2.6
Gly	7.6	8.3	11.4	15.0	6.5
Ala	9.6	14.3	5.0	8.0	8.4
1/2Cys	0.0	0.0	28.0	6.0	15.9
Val	7.2	11.5	2.3	3.0	1.7
Met	0.0	4.8	0.0	0.0	0.2
Ile	3.3	7.1	1.0	1.2	1.5
Leu	3.9	0.0	2.2	6.5	1.9
Lys	4.8	6.8	15.4	3.1	3.4
Arg	1.1	2.6	0.0	8.0	4.8
Tyr	1.2	2.3	0.0	1.0	3.9
Phe	1.5	3.9	0.0	2.2	0.0
His	1.5	1.9	3.1	0.0	1.9
%Hydrophilics[f]	58.3	40.0	46.5	50.6	55.2

a Patterson and Duman, 1979

Table 3 continued

b Tomchaney et al., 1982

c Patterson and Duman, 1982

d Hew et al., 1981

e Wu and Duman, unpublished

f The percentage of amino acid residues with hydrophilic side chains (Asx, Glx, Lys, Arg, Ser, Thr), according to groupings of Manavalan and Ponnuswamy (1978).

References pertaining to Tables 2 and 3:

W. Block and J.G. Duman. (1989) J. Exp. Zool. Vol. 250, pp. 229-231.

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D.E. Rancourt, et. al. (987) Mol. Cell Biol., Vol. 7, pp. 2188-2195.

R. Schneppenheim and H. Theede (1980) Comp. Biochem. Physiol. B. Vol. 67, pp. 561-568.

D.G. Slaughter, et. al. (1981) J. Biol. Chem., Vol. 256, pp. 2022-2026.

L. Somme (1978) Norw. J. Ent., Vol. 25, pp. 187-188.

A.P. Tomchaney, et. al. (1982) Biochem., Vol. 21, pp. 716-721.

J. Zettel (1984) Rev. Ecol. Biol. Sol., Vol. 21, pp. 189.

GENERAL

In the present invention using aqueous antifreeze protein compositions, the process of ice freezing in tissue is changed, the viscosity of liquid contacting the membrane is increased, and the structural damage to the tissue reduced or eliminated through modification of the pattern of ice crystal growth. This advance is accomplished by modifying the pattern of ice crystal growth in tissue using novel compositions, e.g., peptides or glycopeptides from Arctic or Antarctic fish or from other sources. The effect of the antifreeze proteins on the freezing pattern in aqueous solutions is documented extensively as cited above. The different antifreeze proteins from different sources adsorb to different crystal faces, however, all antifreeze protein inhibit ice crystal growth parallel to the a-axes, thermodynamically preferred direction of growth. Freezing in the presence of any kind of antifreeze protein invariably

leads to ice crystals forming in the direction of the c-axis. The ice crystals grow in the form of spicules (spikelike structures). These small micron and submicron scale spicular structures are stable and entrap between them the solutes that are rejected during formation of ice.

SOLUTION PREPARATION

The aqueous solution THP (e.g. AFP or APGP) compositions are prepared by any number of methods. Water (usually sterile) is contacted with THP and mixed to produce a solution of between about 0.01 and 100 mg/ml solute in water. Usually the antifreeze protein saturates in water in concentrations greater than about 100 mg/ml. Preferably, a solution of between about 1 and 60 mg AP/ml is produced, especically between about 10 to 40 mg/ml, more preferably about 1 to 20 mg/ml. The aqueous phase may also contain salts, sugars, ions nutrients (e.g. Krebs solution) and mixtures thereof in concentrations known in the art to be useful for preserving biological agents. The aqueous phase may also contain other materials, e.g. glycerol, etc., which are useful in the preservation of tissue, cell membrane, etc.

A number of electrolyte solutions useful as biologically compatible aqueous solutions are known and described in the literature for short term prreservaiton of organs an tissue. R.L. Veech is U.S. Patent 4,663,289 (which is incorporated by reference) discloses a number of processes and compositions for in vitro use in tissue culture media, perfusion media and incubation media. See for Example Tables 4 and 5 below. Balanced salt solutions are disclosed which include but are not limited to selected from sterile normal plasma, normal saline containing 0.9% by weight sodium chloride, normal saline containing 0.95% by weight sodium chloride, Ringers solution, mammalian Ringer UK and Canada solution, lactated Ringer's solution, acetated Ringer's solution, Locke's solution, Tyrode's solution, Krebs solution, Krebs-Henseleit solution, Krebs Ringer phosphate solution, Krebs serum substitute solution, Krebs

Improved Ringer II solution (calcium ion free), or Krebs Improved Ringer III (low bicarbonate, low phosphorus), Schumarach liver solution, Krebs kidney perfusion solution, Bahlman kidney perfusion solution, University of Wisconsin preservation solution, Collins solution, EuroCollins solution, Ross-Marshall solutions purified plasma from the animal from which the organ or tissue is obtained, Fulgaff perfusion solution, and Wikman-Coffelt solution and combinations thereof.

The thermal hysteresis protein is present in these solutions in the concentrations shown immediately above.

TABLE 4

TABLE I

Prior Art Simulated Plasma Electrolyte Solutions for Contacting Living Cells in Vitro

Units mmoles L fluid	Normal Plasma N E J M. 283, 1285 1970	(1) Normal Saline 0.9% NaCl U.S.	(2) Normal Saline 0.95% NaCl U.K.	(3) Ringer's Injection U.S.	(4) Mammalian Ringer's U.K. & Canada	(5) Lactated Ringer's U.S.	(6) Lactated Ringer's (Hartmann)	(7) Acetated Ringer's	(8) Locke's	(9) Tyrode's	(10) Krebs Henseleit	(11) Krebs Ringer Phosphate	(12) Krebs Serum Substitute	(13) Krebs Improved Ringer II Ca^{2+} free	(14) Krebs Improved Ringer III Low HCO_3 Low Pi
Na	136-145	154	162.5	147	156.4	130	129.8	130	157 57	150 1	143	150 76	140.31	147.41	140.86
K	3 5-5.0			4	5.4	4	5.4	4	3 57	5.9	5 9	5.92	5.92	5.92	5.92
Ca	2.1-2.6			2.5	1.15	1.5	0 9	1.5	2.16	1.8	2.5	2.54	2.54		2.54
free $[Ca^{2+}]$	[1.06]														
Mg	0 75-1.25						1.0			0 45	1 2	1.18	1.18	1.18	1.18
free $[Mg^{2+}]$	[0 53]														
Σ mEq Cations	142.7-153.2	154	162.5	156	164.1	137	139	137	163 46	160 5	156 3	164.12	153 7	155.69	154.22
Cl	100-106	154	162.5	156	161.7	109	111 8	109	163 92	147 48	127 8	131.51	104 62	103 06	122.36
HCO_3	26-28				2.4				3 57	11 9	25		24.9	3 56	3 56
Σ Pi	1-1 45									1 22	1 18	17.38	1 18	15 03	3 49
SO_4	0.32-0 94										1 18	1.18	1.18	1.18	1 18
L-lactate	0 6-1.8					28(d.l)	27 2(d.l)								
pyruvate															
Lact/pyr													4 92	4 92	4 92
D-β-OHbutyrate															
acetoacetate															
β-HB/acac															
acetate								28							
Other												fumarate^{2-} glutamate^{-}	5.39	5.39	5.39
Σ mEq anions	128.7-139.4	154	162.5	156	164.1	137	139	137	167.49	161 6	157 3	163.97	4.92	4.92	4 92
Na/Cl	1 28-1.45	1.00	1.00	0.94	0.97	1 23	1.16	1.19	0 96	1 02	1 12	1.15	132.49	156.64	155.17
Glucose	3 9-5.6								5.6-13 9	5 6			1.34	1.43	1.15
or others													11.5	11.5	11.5
CO_2	0 99-1 39										1 24				
pH	7 35-7.45	≈5.5-6.5	≈5.5-6.5	≈5.5-6.5	≈7.0					7 1	7 4	7.4	7 4	≈7 6	≈7 6
Σ mOsm	285-295	308	325	309	324	272.5	276	272 5	336	318 8	308	311.7	309 8	304 1	307 8
Use:															

Footnotes for Table 4:

(1) Usual "physiological saline" in the U.S. is a 0.9% or 154 mM. (Gilman AG, Goodman LS, Gilman A. The Pharmacological Basis of Therapeutics (1980) pp 848-884, Mc Millan, London
(2) "Physiological Saline" in the U.K. is 0.95% NaCl (Diem K ed. Documenta Geigy (1962) pp 333-334, Geigy, Manchester
(3) All "Ringer's solutions" are derived from Ringer S. Physiol 4, 29, & 222, 1883 and 7, 1886 This commercial U.S. version is from Facts and Comparisons, Oct 1981, p. 50, Lippincott, St Louis
(4) From Best and Taylor, Physiological Basis of Medical Practice, 6th edition, Baltimore, 1950
(5) From Facts and Comparisons p. 50, Oct '81, Lippincott, St Louis
(6) Hartmann AF J Am Med Assoc 103: 1349-1354, 1934
(7) Fox CL et al J Am Med Assoc 148: 823-833, 1952
(8) Locke F S Zbl Physiol 8,166, 1894; 14, 670, 1900; 15, 490, 1901
(9) Tyrode MJ. Arch int Pharmacodyn 20, 205, 1910
(10) Krebs HA, Henseleit KA. Hoppe-Seyler's Z Physiol Chem 210, 33-66, 1932
(11) Krebs HA Hoppe-Seyler's Z Physiol Chem 217, 191, 1933
(12) (14) Krebs HA. Biochem Biophys Acta 4, 249-269, 1950

TABLE 5

	"Prior Art Perfusion Fluids"						
Units mmoles L fluid	Normal Plasma N...J.M. 283, 1285 1970	(15) Krebs Liver Perfusion with Bovine serum Albumin and Red Cells	(16), Schimassek Liver Perfusion	(17) Krebs Kidney Perfusion	(18) Hepatocyte Incubation	(19) Bahlman Kidney Perfusion	(20) Fulgraff Kidney Perfusion
Na	136–145	153	151.54	148	153	147	143
K	3.5–5.0	5.9	5.9	5.9	5.9	4.9	4.74
Ca	2.1–2.6	2.5	1.8	2.5	2.5	2.56	1.25
free [Ca^{2+}]	[1.06]						
Mg	0.75–1.25	1.2	0.49	1.2	1.2	1.2	0.59
free [Mg^{2+}]	[0.53]						
Σ mEq Cations	142.7–153.2	166.3	162.02	161.3	166.3	159 4	151.15
Cl	100–106	127.8	147.48	127.8	127.8	127	113.04
HCO_3	26–28	25	11.9	25	25	24.5	25
Σ Pi	1–1.45	1.18	1.22	1.18	1.18	1.18	1.18
SO_4	0.32–0.94	1.18	—	1.2	1.2	1.18	1.18
L-lactate	0.6–1.8	(10Na–1Lac)	1.33	5Na—1Lac	9.09	2.75(d.l)	3.5(?d.l)
pyruvate			0.09		0.91	0.25	0.25
Lact/pyr			14 8		10	10	7 or 14
D-β-OHbutyrate							
acetoacetate							
β-HB/acac							
acetate							5.0
Other							
Σ mEq anions	128.7–139 4	167.0	162.81	162.3	167.0	159 1	151.31
Na/Cl	1.28–1.45	1.12 (1.20)	1.03	1.16	1.20	1.20	1.26
Glucose	3.9–5.6		5.45			6.2	—
or others						6.7 urea	6.7 urea
CO_2	0.99–1.39	1.25	1.24	1.24	1.24	1.24	1.24
pH	7.35–7.45	7.4	7.1	7.4	7.4	7 4	7.4
Σ mOsm	285–295	328	321	318	328	327	307.9
Albumin (g %)	3.5–5	3.9	2.5	5	2.5	5.5	0.05

*Artificial perfusion fluid generally add 1.5 to 8 g % albumin, dialyzed against a medium listed in Table I, that is Krebs-Henseleit (10), Krebs-Ringer Phosphate (11), Tyrode's (9), Locke's (8), or Krebs-Henseleit with a lowered Ca^{++} to the 1 mM range, particularly in heart perfusion. They may or may not contain red cells. Krebs-Henseleit is known to contain about twice the amount of ionized Ca^{++} as serum.

(15) Hems R, Ross BD, Berry MN, Krebs HA. Biochem J 101, 284, 1966. Krebs Henseleit (10) with 3.9 g % bovine albumin

(16) Schimassek H. Biochem Z 334, 440, 1961. Essentially Tyrode's (9) with added lactate and pyruvate

(17) Nishiitsutsuji-Uwo JM, Ross BD, Krebs HA. Biochem J 103, 852–862, 1967. Krebs-Henseleit (10) with 5 g % albumin, dry

(18) Crow KE, Cornell NW, Veech RL. Biochem J 172, 29–36, 1978. Krebs-Henseleit (10) with 2.5 g % dialyzed albumin plus l-lactate plus pyruvate

(19) Bahlman J et al. Am J Physiol 212, 77 1967. Krebs Henseleit (10) with lactate and pyruvate and 5.5 g % bovine albumin

(20) Fulgraff et al. Arch int Pharmacodyn 172, 49, 1972. Krebs-Henseleit (10) with ½ Mg and Ca plus lactate and pyruvate, plus 5 mM acetate, plus 0.05 g % albumin plus 2 g % hemocel

The H.A. Krebs K-Henseleit solution is known as an aqueous solution for viable organ tissue preservation. See Zeit. Physio Chemie, Vol. 210, pp. 33-66 (1932).

The composition in higher mammals is described as follows: Isotonic serum fluid

0.9%	NaCl	(0.15 mol (m))
1.15%	KCl	(0.154m)
	$CaCl_2$	(0.11 m)
2.11%	KH_2PO_4	(0.154 m)
3.82%	$MgSO_4$ $7H_2O$	(0.154 m)
1.3%	$NaHCO_3$	(0.154 m)

with 5 Vol % carbonic acid (H_2CO_3) to obtain a pH of about 7.4.

The Krebs solution of this reference has the following composition:

ION	AMOUNT	(mg-Percent)
Na+	327	mg-percent
K+	23	"
Ca++	10	"
Mg++	2,9	"
Cl-	454	"
PO_4=	11	"
SO_4=	11.4	"
HCO_3-	54	Vol.-percent
CO_2 (38°)	2.5	Vol.-percent
pH		7.4

with 5 Vol % carbonic acid (H_2CO_3) to obtain pH of about 7.4.

The Krebs solution of the present invention has the above composition to within ±0.1 mg-percent, and, preferably between ±0.05 mg-percent. especially ±0.01 mg-percent. Minor deviations are usually not of consequence for effectiveness of this solution.

Of the possible preservation solutions available, the Krebs solution was initially selected because of its weak preservation effect. Therefore, any preservation of cells, membranes and/or functional viability of membranes organs, etc. would be attributed to presence of the THP (e.g., AFP or AFGP).

J. Wikmann-Coffelt teaches additional preservation compositions for mammalian organs and tissue in U.S. Patent

No. 5,066,778, and specifically for mammalian heart in U.S. Patent No. 5,075,210.

For use in organ transplantation and the like, sterile conditions and solutions must be used. The solutions may be prepared using sterile materials and sterile conditions. Also the solutions may be sterilized by methods known in the art, e.g. brief exposure to cobalt-60 radiation.

TISSUE PRESERVATION

To illustrate the effect of AFPs on the ice crystal structure, experimental results of the present invention are presented from earlier research in which the freezing pattern in a physiological saline solution is compared to the freezing pattern in a physiological solution with the addition of between about 1-100 mg/ml preferably about 40 mg/ml of antifreeze glycopeptides from Antarctic nototheniindae fish (Table 1). In this comparison, samples are frozen under controlled thermal conditions on a directional solidification stage. The directional solidification stage, described in greater detail in U.S. Patent 4,531,373 is an apparatus capable of freezing solutions or tissue samples with uniform cooling rates, between predetermined temperatures. The apparatus is used in conjunction with a light microscope to produce results shown in Figures 1A, 1B and 1C which demonstrate the spicular growth in the presence of antifreeze glycoproteins.

One embodiment of the present invention is to perfuse solutions containing antifreeze proteins through the vasculature of an organ. Upon freezing, the ice crystals that form will be small, spicular and will entrap the solutes present. Consequently the cells will not be exposed to high saline concentrations, and the damaging expansion of the blood vessels will be eliminated. This effect will be demonstrated with detailed experimental results using the directional stage and scanning electron microscope in Figures 2A, 2B, 2C and 3A and 3B, 3C, 3D and 3E.

VITRIFICATION

It was formerly observed that cells, tissue or organs

may not survive freezing with rapid cooling or "apparent vitrification". The expression "apparent vitrification" is used here to describe the observation that, at times, a solution is considered to be vitrified if it remains transparent after rapid cooling to cryogenic temperatures. However, the property of transparency is only an indication that the ice crystals are either too small or too few to reflect light and therefore, the vitrification is only apparent. In one aspect of the invention, it was expected that cells (or organs, tissue, animals) preserved by techniques in which the solution containing the cells (or organs, tissue, animals) is frozen by rapid cooling or apparent vitrification may be damaged by the preferential formation of very small ice crystals on the cell membrane, which may serve as a nucleation site. The antifreeze glycopeptides and peptides inhibit the growth of ice crystals and significantly reduce the size of the crystals formed by generating spicular ice structures. Therefore, these biologically compatible substances probably enhance the effectiveness of cryopreservation by preventing the formation of ice crystals on the cell membrane or by reducing the size of these ice crystals.

Pig Oocyte and Embryo Preservation

The effectiveness of the antifreeze proteins (in vitrification) was evaluated on the cryopreservation of immature pig oocytes, two-cell stage pig embryos and mouse embryos, at the two-cell stage frozen by rapid cooling and "apparent vitrification." Pig oocytes and pig embryos at the two-cell stage were chosen because they present a very challenging model for which no successful cryopreservation has been heretofore achieved. In fact, pig oocytes and early-stage pig embryos usually cannot survive exposure to temperatures as high as 10°C for even brief time periods.

The probability of ice crystal nucleation during cooling is an inverse function of viscosity and temperature and a direct function of volume (D. Turnbull, 1969). In cryopreservation by rapid cooling, attempts are made to

reduce the probability for nucleation by increasing the solution viscosity and by reducing the phase transition temperature through an increase in the concentration of various cryoprotectants. However, higher concentrations of cryoprotectants have a damaging effect on biological materials and, therefore, a proper balance must be found between a concentration that is sufficiently high to suppress nucleation and sufficiently low to avoid damaging the fragile cells.

These experiments were performed by exposing droplets of different size and composition to a variety of cooling rates on a special experimental system developed by B. Rubinsky, U.S. Patent 4,531,373. Rapid cooling, as well as rapid warming of samples, was performed using a Leitz Diaplan microscope to which a special directional stage was attached (A. Arav et al., 1990; B. Rubinsky, 1985; B. Rubinsky et al., 1985). The stage allows accurate control of cooling and warming rates between predetermined temperatures particularly as it is applied to vitrification and freezing by rapid cooling. A video camera was used in conjunction with the microscope to evaluate the morphology of the cells and the physical state of the solution.

An "apparent vitrification solution", (AVS) was useful which contains 17.5% propylene glycol, (Fluka Chemicals, Switzerland), 2.5% glycerol (BDH Analar, England), 20% FCS (Fetal Calf Serum) (Gibco, Scotland) and 0.05 M sucrose in PBS (Dulbecco's phosphate buffered saline supplemented with 0.4 m/v BSA (Bovine Serum Albumin), 0.34 mM pyruvate, 5.5 mM glucose and 70 μg/ml kanamycin).

This solution is physiologically compatible with mouse and pig embryos and with pig oocytes. When 0.1 μl droplets of the AVS solution were cooled at the rate of 1,700° C/min (the highest rate possible with the directional solidification stage) to a temperature of -130° C (a temperature lower than the glass formation temperature for this solution) no ice crystals were observed through the microscope at 340x magnification. To illustrate the effect

of volume and solute concentration, ice crystals were observed with all droplets of the AVS solution larger than 0.5 μl, and with all 0.1 μl droplets containing 12.5% propylene glycol and 2.5% glycerol when cooled at 1,700° C/min. No apparent devitrification, (that is, the formation of ice crystals was observed with droplets of the AVS solution larger than 0.5 μl, and with all 0.1 μl droplets containing 12.5% propylene glycol and 2.5% glycerol when cooled at 1,700° C/min.) No apparent devitrification (i.e., ice crystal formation) was observed when the samples were held at -130° C. However, devitrification was observed in some samples during warming to room temperatures even when the rate was as high as 1,700° C/min. The addition of AFGP's or AFP's was the AVS solution did not preclude the seldom and random occurrence of devitrification after "apparent vitrification". The AVS was the basic solution used in the experiment are reported to evaluate the effects of freezing with rapid cooling for used droplets larger than 0.5ml and for "apparent vitrification" droplets of 0.1ml. In the vitrification studies, only results from solutions that did not undergo devitrification were evaluated.

Evaluation of Cryoprotective Properties of AFPG & AFP in Oocytes and Embryos

To evaluate the cryoprotective properties of the AFGP's and the AFP's, immature pig oocytes, two-cell stage pig embryos and two-cell stage mouse embryos were introduced into either 0.1 ml droplets for vitrification, or droplets larger than 0.5 ml for freezing with rapid cooling of AVS with, and without, AFGP or AFP. These droplets were cooled on the directional stage under microscope observation at the rate of 1,700° C/min to -130° C. After 15 minutes at these temperatures, the samples were warmed at the rate of 1,700° C/min to room temperature. The survival of the embryos and oocytes was evaluated by in vitro culture followed by morphological and development analysis. Control experiments were performed by exposing embryos and oocytes to the different solutions in protocols identical to the rapid

cooling experiments, but without cooling and warming, and evaluating their viability. The glycopeptides used in this work were obtained from Antarctic fish belonging to the family Nototheniidae (Dissostichus Mawsoni) (Table 1). A physiological composition was used which consists of one part of fraction 1 to 5 (high molecular weights) and two parts of fraction 7 and 8 (lower molecular weights) as obtained form A. DeVries, University of Illinois. Fractions 1-5 are obtained as a mixture, and fraction 7-8 are obtained as a mixture. Experiments were performed with solution concentrations of 40 mg/ml glycopeptides. PBS is a standard buffered solution. This particular value was chosen because studies have shown that the depression of the freezing point of aqueous solutions of antifreeze glycoproteins is concentration-dependent and at these concentrations, it reaches saturation. A.L. DeVries, (1988).

After the cryoprotective properties of the AFGP's were established, parametric studies were performed with two-cell stage mouse embryos to determine the effect of concentration on the survival of the embryos. This animal model was chosen for parametric experiments because it proved extremely sensitive to the effect of the glycopeptides. While no survival of embryos was achieved without the glycopeptides (0%), very high survival of embryos was obtained with the glycopeptides (82.5%, in vitro development to the blastocyst stage). The details of the parametric studies are listed in Table 6. The experimental procedures for pig oocytes and pig embryos are found in Example 4 below, and for mouse embryos is found in Example 5.

Table 6 lists the results of the experiments of Examples 3 and 4 below, starting with the pig oocytes, followed by pig embryos and mouse embryos. Table 6 also shows the solutions in which the embryos and the oocytes were tested.

TABLE 6

EFFECT OF AFGP ON OOCYTE VIABILITY

Time of Exposure Soln (hr)	PBS	PBS + 0.1 mg/ml AFGP 1-8	PBS + 1 mg/ml AFGP 1-8	PBS + 40 mg/ml AFGP 1-8	PBS + 40 mg/ml AFGP 1-5	PBS + 40 mg/ml AFGP 7,8
4	6/48 (12.5%)	7/25 (24%)	19/27 (70%)	54/70 (77%)	12/43 (28%)	11/47 (23%)
4	9/48 (18.75%)	11/29 (37%)	20/27 (74%)	59/70 (84%)	21/43 (48%)	18/47 (38%)
24	0/17 (0%)	0/14 (0%)	6/14 (42%)	7/17 (41%)	0/14 (0%)	0/13 (0%)
24	0/17 (0%)	0/14 (0%)	9/14 (64%)	9/17 (53%)	0/14 1(0%)	0/13 (0%)

CRITERIA A = $\frac{\text{number of cells with electrical potential} > |u| - |v|}{\text{total number of cells}}$

CRITERIA B = $\frac{\text{number of with electrical potential} > |u| - |2v|}{\text{total number of cells}}$

The protocol to which the cells were exposed is the one described earlier in which the embryos and oocytes were introduced in various solutions with some of the embryos and oocytes exposed to rapid cooling while others which did not undergo cooling, kept as controls for the solution effect. The results are presented, for pig oocytes, as the ratio between the number of oocytes which reached the MI or MII stage after in vitro maturation, and the total number of oocytes exposed to the experimental protocol. For the pig embryos, it is the ratio between the number of embryos that reached the four-cell stage after in vitro development and the total number of embryos exposed to the experimental protocol. For the mouse embryos, it is the ratio between the number of embryos that reached the blastocyst stage over the total number of embryos exposed to the experimental protocol. The numbers in the bracket give the ratio expressed as percentage.

The experiments with pig oocytes, pig embryos, and mouse embryos exposed to the AVS solution, show that this solution does not have a damaging effect. However, when the embryos and the oocytes were cooled rapidly or vitrified to cryogenic temperatures in the AVS solution, not a single embryo or oocyte survived. These results demonstrate that the damage to these cells is a consequence of cooling and exposure to cryogenic temperatures. Microscopic examination revealed that a primary site of damage following rapid cooling in the AVS solution was the oolemma in the case of oocytes and the blastomer membrane for embryos which did not retain integrity as illustrated in the Figures, especially in Figs. 4B, 5A, and 6B. However, in the presence of the glycopeptides the cells that were rapidly frozen or vitrified retained viability as shown in Table 4.

In Figures 12 and 13 are shown the membrane potential for oocytes at 4 and 24 hr at 4°C. The dramatic retained membrane potential viability at concentrations of 1-40 mg/ml of AFGPs is found in Figure 13. Figures 12 and 13 values are mean ± one standard deviation. Each exp. group consists

of 5 oocytes and n represents the number of groups.

In particular, as described in experiment 3, the cell membrane was protected by the glycopeptides.

Bovine Oocyte Preservation

Past research on the properties of "antifreeze" proteins has focused on their ability to modify ice crystal structure on the mechanism by which this is achieved (Refs. 39,40,12,41). On the other hand, these results show that AFGP protects mammalian oocytes at cryogenic temperatures (-130°C) (Ref. 42) and at hypothermic temperatures (4°C) (Ref. 43). Porcine oocytes are normally completely destroyed by cryogenic temperatures (Refs. 42,22,44). However, when they were rapidly cooled to -130°C in a vitrifying solution containing AFGP, approximately 80% of them retain intact oolemmas and 25% undergo in vitro maturation (42). In follow-up experiments exposing porcine intact oocytes to 4°C for 24hr in the presence of AFGP, it was observed that 80% retained an intact oolemma and about 70% a normal membrane potential. In the absence of AFGP the integrity of the oolemma is completely destroyed (43).

The following (Example 9) was conducted to answer two general questions based on the foregoing results. Are the AFGP unique in their ability to protect cell membranes from hypothermic damage, or is this protection a general property of all known antifreeze protein classes? Does the membrane protection afforded by the antifreeze proteins improve the viability of cold-sensitive mammalian cells? To answer these questions, experiments were performed with immature bovine oocytes exposed to hypothermic temperature conditions.

Results and Discussion Regarding Bovine Oocytes

Approximately 1000 oocytes were individually studied in the experiments reported here. The results are summarized in Figures 14 and 15. Figure 14 shows the percentage of oocytes with an intact oolemma after hypothermic exposure as determined by three different tests, morphological examination, fluorescein diacetate (FDA) staining and trypan

blue (TB) exclusion. It is evident that only 10% to 25% of the control oocytes retained an intact oolemma following exposure to 4°C. Similar results were obtained for oocytes incubated at 4°C in PBS solution to which 0.1 M sucrose or 20% v/v fetal calf serum was added. In contrast, when the various AFPs were added to the incubation media, the integrity of the oolemma was retained in 50% to 75% of the oocytes, according to the morphological test used. The level of cell membrane protection from hypothermic exposure afforded by the different AFP was similarly high despite their different primary and secondary structures.

Evidence that the various AFP protected the viability of the oocytes is presented in Figure 15. While only 23.5% of the control oocytes exposed to hypothermic conditions underwent in vitro maturation, 64% to 75% of them matured when exposed to the same conditions in the presence of the various "antifreeze" proteins. This percentage maturation compares well with that observed for fresh oocytes (80%). Heyman et al. (45) reported a 32% maturation rate for bovine oocytes exposed to 0°C for only 30 min. These authors found that the addition of 0.1 M or 0.25 M trehalose, a membrane stabilizing compound, did not significantly increase the maturation rate. Similar negative results were found when 0.1 M sucrose or 20% v/v fetal calf serum was added to the PBS solution.

The results from the in vitro fertilization experiments illustrate clearly the biological significance of the protective effects of the thermal hysteresis protein ("antifreeze" protein). None of the control oocytes exposed to hypothermic temperatures in the absence of "antifreeze" proteins underwent in vitro fertilization, whereas 40-50% of the oocytes incubated in the presence of the antifreeze proteins could be fertilized. This rate of fertilization is comparable to what we normally obtain for fresh immature oocytes (60%).

These results clearly demonstrate that all of the different types of thermal hysteresis proteins ("antifreeze"

protein) currently identified in nature share the ability to protect cells and their membranes from damage during hypothermic exposure. It is evident that the hypothermic protection afforded by the antifreeze proteins extends to the functional viability of the cell. This observation further suggests that hypothermic protection may be a fundamental property and function of all of the "antifreeze" proteins in nature.

Our demonstration that the antifreeze glycoproteins (AFGP) enables cold-sensitive oocytes to maintain a normal membrane potential following exposure to hypothermic temperatures suggests that these proteins can prevent ion leakage. Recent experiments support this by demonstrating, using patch clamp techniques, that antifreeze proteins of different types do control and inhibit ion channels in mammalian cell membranes. These Examples demonstrate that all three of the known antifreeze protein types can protect the integrity of cold-sensitive bovine oocytes to the point where they remain fully functional. Taken together these results support the present invention, that by reducing the leakiness of the cell membranes, cold-sensitive cells become cold tolerant. The practical value of the finding that antifreeze proteins can render cold-sensitive mammalian cells cold-resistive is of importance to the short term preservation of cold-sensitive mammalian cells, particularly in the viable preservation and storage of tissue or organs for transplantation.

These thermal hysteresis proteins are therefore useful in calcium entry therapy for diseases.

UTILITY

It is apparent from the disclosure herein that the thermal hysteresis protein (AFP and AFPG) aqueous composition of the present invention is useful in cell preservation, membrane preservation, tissue preservation or organ preservation.

In general, the antifreeze proteins have the property that they noncolligatively lower the apparent freezing point

of aqueous solution resulting in a freezing temperature that is lower than the melting temperature. They also have the general property that they inhibit or restrict growth on different facets of ice crystals while allowing the growth along the c-axis. Until now it was not know that these proteins can be also use to interact with other proteins, and, in particular, protein on the surface of cell membrane and to protect the structural integrity of the membrane and stop leakage through the membrane and block ion channels. This is the first time that these properties and its applications are observed and are described as part of this invention.

EFFECT OF THE AFGP'S ON THE CELL MEMBRANE

Initially, the effect of the antifreeze proteins on modification of ice crystal growth focused on the use of this property in preservation of cells, tissue, organs and whole animals at temperatures below freezing. However, in studies described above and experiments such as Examples 1 and 4 (also Table 4), in which the morphology of cell membranes was evaluated, it appeared consistently that the antifreeze proteins provide complete protection to the morphology of the membrane and its structural integrity. Therefore, a procedure was developed to determine if the antifreeze proteins protect by interacting directly with cell membranes, and contacting the protein directly to cell membranes.

Pig oocytes were initially chosen as the experimental model in this study because these oocytes are temperature sensitive and cannot survive exposure to hypothermic temperatures as high as 10°C, i.e., temperatures that are higher than the phase transition temperature. Therefore, an experiment was designed in which the effect of the AFGP's on the cell was studied at temperatures higher than the phase transition temperature, but lower than the normal body temperatures. If a protective effect of the AFGP's is found, it is probably not directly related to the ability of the compound to modify ice crystal morphology or inhibit of

ice crystal formation.

The oocytes were introduced in different solutions of standard buffer PBS solution with antifreeze glycoproteins (Fractions 1-5, Table 1) from fish of the family Nototheniidae. They were kept in a constant temperature environment for various periods of time and then the membrane potential was measured. The structural integrity was also determined by microscope evaluation.

To establish a criteria for an intact oolema, preliminary experiments were performed for each batch of oocytes in which the membrane potential of the fresh oocytes was measured at 22°C. The mean value of the electrical potential, u, and the standard deviation, v, were calculated for each batch. The mean and the standard deviation were measured in fresh oocytes in a buffer solution and in a buffer solution with 40 mg/ml antifreeze glycopeptides Fractions 1-8 (Table 1) as obtained from A. DeVries, supra.

Table 6 above summarizes the results derived from measuring the resting potential across the oolemma. Table 4 gives the ratio between the number of oocytes considered to have an intact oolema relative to the number of oocytes used for each experimental condition, (the number in the brackets is the ratio in percentage,) for different concentrations of the AFGP's and different times at 40°C.

The comparison shows that the glycopeptides have little effect on the resting potential of each oocyte. To determine the integrity of the oolema, two statistical criteria were established, one less stringent than the other. The oolema in an oocyte was considered to be intact if the absolute value of the measured resting potential difference, was higher than the absolute value of either $|u|-|v|$ or $|u|-|2v|$.

The results from evaluating the structural integrity of the oolema are consistent with the electrical potential measurement and are illustrated by Figures 7A, 7B, and 7C. The results clearly show that the membrane is preserved morphologically intact in the presence of the glycopeptides.

Furthermore, ion leakage that is probably the most prevalent cause of damage during hypothermic exposure is significantly inhibited in the presence of the antifreeze proteins. This implies that the antifreeze proteins have the ability to protect cell membranes at hypothermic temperatures and to block ion channels. The evidence of the use of this new discovery in hypothermic preservation of cells and organs are given below in Examples 6 and 7, respectively.

It is emphasized that prior to this research it was not known that antifreeze proteins have the useful properties of preserving cell membranes and blocking ion channels.

WHOLE ORGAN PRESERVATION

Cryopreservation of a whole organ, e.g. liver from a mammal, such as a rat, is described in Examples 2, 7 and 7A below. The organ is surgically removed, held in a preservation solution at 20-37°C, preferably 24°C. A major blood vessel is cannulated. The well-known Langendorf perfusion system (with a first bottle containing, for example, Krebs solution and antifreeze polypeptides in a 1mg/ml to 100 mg/ml) is used. See, for example, D.E. Pegg et al. (1986), Cryobiology, Vol. 23, pp. 150-160.

A second bottle of solution contains a physiologically compatible saline solution and appropriate quantities of glycerol, dimethyl sulfoxide, ethylene glycol, polyvinyl pyrrolidone, glucose etc. or mixtures of these substances which are known as protectants for cells of biological origin.

These two bottles of solutions are connected to a mixing valve having known adjustable flow rates (e.g. 0.1 to 10 ml/min, preferably about 5 ml/min) and a computer to accurately vary the flow rate and mixing of each bottle's contents immediately prior to perfusion. The perfusion using the solutions of bottles 1 and 2 is well known in the art as described by D.E. Pegg et al. (1988) above for kidney and G.N. Alink et al. (1976), Cryobiology, Vol. 13, pp. 295-304; (1977) Cryobiology, Vol. 14, pp. 409-417 and 399-408; and (1978) Cryobiology, Vol. 15, pp. 44-58, and K.E.F. Hobbs

et al. (1969), Cryobiology, Vol. 6, pp. 239-245 for heart. The Krebs solution is perfused through the organ held at about 20-37°C at a rate of about 4 ml/min.

The mixing switch provides intermediate amounts of Krebs solution and glycerol solution in pulses of time lengths controlled by the computer, for example, 0.01 sec. to 0.1 sec. The two solutions mix in the delivery tube or in a special mixing chamber.

The antifreeze protein/Krebs solution is initially adjusted so that at the end of the perfusion process a concentration of between 1mg/ml to 40 mg/ml is achieved in blood vessel space in the tissue. The majority of the AFPs are found within the vascular space (bed) of the organ (not within the cells of the liver or the blood vessels). The AFPs ("AFGP's") are usually too high in molecular weight to significantly penetrate the cell membrane. The organ, e.g. liver, is next placed in a cooling stage as described in U.S. Patent 4,531,373, and the temperature of the whole perfused organ is then cooled at a rate of 1°C per minute to -32°C or to -70° or until -150°C is reached. The organ is then cooled as needed using a liquid nitrogen to -196°C or in liquid helium to 4K and held at this temperature for an indefinite time (e.g. 72 hr). The frozen organ is then carefully thawed by immersion in a cold or warm liquid, e.g. water or saline, at a rate of between about 0.1 to 10°C per min. (preferably about 1°C per min.) using known techniques up to 37°C maximum. Alternatively, carefully controlled microwave heating is used to thaw the perfused organ, e.g. liver. When the thawed organ (liver) reaches about 0°C, the nutrient solution of Krebs is perfused through the large cannulated blood vessel. When warmed to about 20 to 37°C, preferably 37°C, the thawed organ recovers not only cell function, but also organ function. Preserved tissue samples are taken as needed.

A systematic study of the effects of the AFGP on rat liver cold-storage was done to compare control storage solutions and solutions containing the AFGP (see Example

7A). The results obtained are compared for three different storage periods, 6, 12 and 24 hr. The functional tests include the production of bile and enzymatic activities of lactic dehydrogenase (LDH). The Krebs solution is selected as the control solution. The reason for this selection is to separate the protective qualities of other storage solutions from the effects of the AFGP.

Useful mammalian organs include liver, kidney, heart, brain, lung, pancreas, spleen, ovary, stomach and the like. The organ of a mammal, such as a human being, is preferred.

PRESERVATION OF CELLS AND TISSUE AT TEMPERATURES BELOW FREEZING

The cryopreservation is demonstrated in cells, e.g. human ooytes, pig oocytes, embryos, human or leucocytes, platelets, e.g. pancreatic islets, hepatocyles, corneas, skin. See examples 4 and 5. Various cryoprotective agents such as glycerol, propylene glycol are introduced in the cell together with the antifreeze proteins essentially as described in Examples 3, 4 and 5. The different solutions of cryoprotective agents are chosen to either produce freezing or vitrification such as 5M propylene glycol. The cells or tissues are then cooled rapidly to either produce freezing or vitrification with cooling rates of e.g. 1750 °C/min or as high as required to temperatures of -130°C to -180°C, or to 4K and held at that temperatures for an indefinite period. The cells or tissue are then carefully thawed. Cell function and tissue function is recovered.

PRESERVATION OF ORGANS BY RAPID FREEZING VITRIFICATION

The procedure is the same as described in the whole organ preservation section except that the concentration of the cryoprotectant is taken to a high level, such as 5M propyleneglycol, and the cooling rates are high enough, such as 1,750°C/min, to produce either rapid freezing or vitrification as desired as opposed to slow freezing in the earlier application. The use of antifreeze proteins is essential for the successful preservation of organ tissue by vitrification.

HYPOTHERMIC PRESERVATION OF CELLS

The procedure of Example 6 is followed except that liver cells are first contacted with aqueous AFGP solution. These cells survive the cooling and are viable upon careful warming to physiological temperatures.

HYPOTHERMIC PRESERVATION OF ORGANS IN COLD STORAGE

The procedure of Example 6 is followed except that an organ, e.g., liver or heart is contacted with the aqueous AFGP solution. This organ survives the cooling and is viable upon careful warming to physiological temperatures.

HYPOTERMIC PRESERVATION OF ORGAN BY CONTINUOUS PERFUSION

Example 7 is repeated except that the blood containing the antifreeze glycoprotein is continuously perfused through the organ.

HYPOTHERMIC PRESERVATION OF CELLS, TISSUE, ORGANS, MAMMALS

Example 6 is repeated with the exception that antifreeze proteins are brought in contact with cells, tissue, organs, mammals where it is desired to protect them from hyperthermic damage.

PRESERVATION OF CELLS, TISSUE (SKIN), ORGANS, MAMMALS FROM A CHEMICAL ENVIRONMENT THAT IS NOT OPTIMAL

Example 6 conditions are repeated with the exception that a non physiological chemical environment such as high carbon dioxide is present.

PRESERVATION OF CELL MEMBRANES

Cell membranes are brought into contact with physiologically capatible solutions with antifreeze proteins.

BLOCKING ION CHANNELS

Cell membranes are brought into contact with physiologically compatible solutions with antifreeze proteins. In channels, for example, sodium and postassium are found to be substantially blocked.

ATTACHMENT THROUGH ANTIFREEZE PROTEINS

Various macromolecules are artificially attached to

antifreeze protein and than introduced in cell suspension, tissue, organs, or whole mammals. The antifreeze protein attach then to cell membranes and thereby bring molecules in the vicinity of the cell membrane.

The preservation of these mammalian organ tissues, etc. is also applicable to the preservation of organs, tissue, etc. in a human being.

Ion Current Determination in Pig Granulosa Cells using Thermal Hysteresis Proteins

Observed hypothermic protection of cells of cell membranes conferred by the thermal hysteresis protein (e.g.,) "antifreeze" proteins) is associated with their ability to inhibit ion leakage. The effects of "antifreeze" proteins from the winter flounder (Pseudopleuronectes americanus), one of the better characterized "antifreeze" proteins was evaluated on known and well characterized ion currents. Pig granulosa cells were used to characterize the potassium and calcium currents using the patch clamp technique in the whole cell configuration (Ref. 46).

The "antifreeze" polypeptides (AFP), from the winter founder (Pseudopleuronectes americanus) consist of a family of seven independently active components containing eight amino acids of which alanine forms about 60 mol. %. Most of the remaining residues are polar: aspartic acid, glutamic acid, lysine, serine and thereonine (Ananthanarayanan, 1989). The compounds range in molecular weight from 3300 Da to 4500 Da. The two major AFP (3300 Da) found in the blood plasma have been sequenced (Picket et al. 1984) and one of them crystalized (Ref. 4). They are amphiphilic alpha-helices in which the majority of the hydrophylic amino acid side chains project along the length of one side of the helix while the opposite side, is predominantly hydrophobic (Ala). Studies on their antifreeze properties show that they lower the freezing point of a solution in a non-colligative way and have no effect on the melting point of the formed ice. This difference between the freezing and melting points of the AFP solution is thermal hysteresis,

and is the major means by which the AFP are identified.

The calcium and potassium currents in pig granulosa cells were characterized in an earlier study (Ref. 46). It was shown that in response to depolarizing pulses from a -80 mV holding potential to -30 MV, a Ca^{2+} current with rapid activation and inactivation phases and characteristics typical of T-type Ca^{2+} channel kinetics can be observed in the granulosa cells. The maximum current amplitude ranges from 20 to 200 Pa with no apparent relation to cell size. The K^+ current has the characteristics typical of a delayed rectifier. The current has a slow, somewhat delayed activation phase but no inactivation during the depolarization pulse. The plateau K^+ current ranges from 100 to 500 Pa at a test potential of 60 Mv with no apparent relation to cell size. Holding the pig cells at -40 Mv has no effect on this current. The experiments reported here determine (Example 10 below) that AFP affects the calcium and potassium currents described above.

Results of Experiments for Ion Currents in Pig Granulosa Cells

Experiments performed with the different concentrations of AFP show that without exception a concentration of 0.1 mg/ml (0.028 Mm) AFP had no effect on the ion currents for they remained unchanged throughout the duration of each individual experiment, (about 5 minutes). However, at concentrations higher than 0.5 mg/ml (0.14 Mm) the winter flounder AFP completely inhibited the ionic currents, in all the experiments. Typical results obtained at an AFP concentration of 0.5 mg/ml are shown in Figures 16 and 17 for the Ca^{2+} and K^+ currents respectively. The figures show the raw currents recorded prior to the injection of the AFP into the droplet and at successive 20 and 40 second time intervals. Figure 16 shows that the Ca^{2+} currents were completely suppressed within 40 seconds after the injection of the AFP in the solution. Figure 17 shows that the amplitude of the potassium currents also decays to zero, albeit much slower than the calcium current, within 200

seconds. The slower decay in the potassium current amplitude was observed in all the experiments. An increase in the AFP concentration resulted in a faster decay of the amplitude of both the calcium and potassium currents to zero. However, at all concentrations the calcium current decayed faster than the potassium current. At a concentration of 1 mg/ml (0.28 Mm) the calcium current was suppressed within 20 seconds, while the potassium current was suppressed within 80 seconds. Only at a concentration of 10 mg/ml (1.4 Mm) was the potassium current suppressed within 20 seconds (Fig. 18). As expected neither bovine serus albumin nor soybean trypsin inhibitor had any effect on the ion currents.

Overall Discussion for Ion Currents in Pig Granulosa Cells

These results (Example 10) demonstrate that (AFP) thermal hysteresis protein (e.g.) has the ability to inhibit and suppress calcium and potassium currents. The non-linear relationship between AFP concentration and ion current suppression, with no effect at a concentration of 0.1 mg/ml. and saturation at a concentration of 0.5 mg/ml, is suggestive of an AFP-protein interaction. In addition, the different rates with which the calcium and the potassium currents are inhibited at different concentrations would indicate that AFP interacts separately with the calcium. While not wanting to be bound by theory, one possible mechanism whereby AFP inhibits ion currents relates to its amphiphilic structure. It is conceivable that the hydrophilic side of the AFP molecule binds with hydrophilic components of ion channel proteins, replacing water molecules and thereby reducing the Gibbs free energy of the membrane. In this orientation the hydrophobic side of the AFP molecule would prevent water molecules from becoming intimately associated with the ion channels, and thus prevent ions from diffusing across the cell membrane. Since calcium ions flow from the exterior to the interior of the cell, and potassium flows in the opposite direction, this may explain why calcium ion flow is inhibited faster than

potassium ion flow.

Ion Transport (Ca Entry) Into Mammalian Cells (Rabbit Gastric Glands)

The AFP used in this example of Ca^{+2} entry into mammalian cells were obtained from the Newfoundland ocean pout (Macrozoarces americanus) and consisted of at least 12 independently active polypeptides with molecular weights ranging from 6000 to 7000 daltons. These are the same AFP used to preserve whole livers during hypothermia (Ref. 49). AFP are present in the blood of the pout throughout the year, and blood levels in winter (20-25 mg/ml) are approximately five times those observed during summer (50).

The effects were studied of AFP on Cai in rabbit gastric glands loaded with the Ca-sensitive fluorescent probe, fura-2, and digital imaging microscopy. Since these cells respond to the cholinergic agonist carbachol with both a rapid release from internal Ca stores and a sustained elevation due to Ca entry across the plasma member (51,52), the effect AFP on both processes could be studied. Also, since the decay phase of the carbachol-stimulated Cai signal is influenced by the activity of Ca pumps, the effects of AFP on Ca pumping could also be evaluated.

Repetitive addition of maximal doses of carbachol to control cells (Figure 19) caused repetitive, biphasic increases of Cai. The rapid initial rise ("spike") in Cai is due to release of intracellular stores (52,53), and 100μM carbachol completely empties this intracellular pool (54). The secondary plateau phase of sustained elevation of Cai, which is stable for up to 50 mins (not shown), is due to the entry of Ca from the extracellular space into the cells. The nature and regulation of the channels that control this entry into parietal and other types of epithelial cells have remained somewhat elusive. The channels appear to be different from those in excitable tissues because they are not blocked by either nifedipine or verapamil (54), through they are, lime other Ca channels, inhibited by 50μM La (54) and low pH (pH<6,7) (54). As shown in Figure 19, the

characteristic profile of each phase is maintained through multiple stimulations, indicating that cells are able to refill their intracellular stores relatively quickly and that desensitization of the response does not occur under control conditions.

Dose-response studies were performed with concentrations of AFP ranging from 0.1 mg/ml to 20 mg/ml (not shown). No effect on Ca transport was observed at 0.1 mg/ml while complete blocking of the carbachol-induced plateau was achieved at concentrations of 1 mg/ml (140μM(and above (Figure 20). At this does, AFP had no effect on the carbachol-induced spike of Cai. Since the spike of Cai (release of the internal store) requires carbachol/receptor binding, phospholipase C activation, and inositol 1,4,5-trisphosphate formation (54), it seems that the AFP had not inhibitory effect on any of these biochemical pathways. In addition, when cells were stimulated with carbachol in Ca-free solutions, the rate of decrease of Cai following spike was the same in both AFP-treated and control cells (now shown), indicating that the AFP also had no effect on the Ca-ATPase which rids the cells of the release Ca. Thus, the action of 1 mg/ml AFP in blocking the secondary plateau phase of the carbachol response suggests that this protein acts selectively to inhibit influx of Ca.

Since influx of extracellular Ca is normally required for refilling of intracellular Ca pools (54), we tested whether AFP blocks influx sufficiently to prevent repetitive carbachol stimulations from causing repetitive increases in Cai. The continued presence AFP prevented a third carbachol stimulus from causing a normal release of Ca from the pool (Figure 21), strongly suggesting that prolonged AFP treatment blocked refilling of the pool, similar to the effect of La. Thus, in the continued presence of 1 mg/ml AFP, only one stimulus is possible. Following washout of AFP, Cai increased in response to carbachol (Figure 22), but the magnitude of the responses was always smaller (by about 40%) than the control. Another interesting feature is that

the Cai plateaus after AFP wash-out were also very small, suggesting some lingering effects of AFP, possibly due to binding of the AFP to cells. Similar lingering blockade of Ca entry has been observed following incubation with the inorganic blocker La (54).

The discovery of an organic substance capable of blocking Ca channels in non-excitable cells is, in itself, important since the best blockers until now were inorganic cations such as H (20μM), La (about 50μM) and/or Ni (about 1mM). With regard to the mechanism of AFP action, it is interesting to note that the effects of AFP on CA entry occurred over the same concentration range as that found to affect ice crystal formation (55). This suggests that one possible mechanism involves the interaction of AFP with water in or around the channel, thus impeding ion flow. These compounds could prove useful in the characterization of Ca channel kinetics in way similar to the use of omega-conotoxin and funnel web spider toxin on N and P type Ca channels, respectively (56,57).

The most important implication of this work, however, relates the emerging concept of how AFP exerts protective effects on cells during hypothermia. Active ion pumps (e.g. Na/K-ATPase and Ca-ATPase) are much more sensitive to ion channels (e.g., Na and Ca channels) to hypothermia, and, as Hochachka (58) has noted, cold-tolerant animals have likely adapted by lowering cell membrane permeability to accommodate the reduced metabolism and activity of ion pumps at low temperatures. We propose that AFP may inhibit hypothermia-induced accumulation of Ca inside cells without interfering with other critical functions of the cell. Further, these findings suggest a mechanism by which AFPs can preserve organs.

DETAILED DESCRIPTION OF FIGURES

The following is a detailed description of some of the Figures:

Figures 1A, 1B and 1C

Figure 1A shows the frozen region 11 (i) and the planar

solid-liquid interface in a physiological saline solution at the onset of the normal freezing process.

Figure 1B shows the final dendritic, finger-like structure of ice 12 (i), during freezing of a physiological saline solution. Figure 1C shows spicular structure of ice crystals 13 (i), during freezing of a physiological saline solution with 40 mg/ml AFPs. The scale bar 14 shown in Figures 1A, 1B and 1C is 50 micrometer.

Figures 1A, 1B and 1C show the ice crystal morphology in aqueous solutions frozen with a cooling rate of 4° C/min on the directional solidification stage. Figures 1A and 1B show a well-known sequence of events during freezing of saline solutions. In saline solutions, ice forms and grows on the prism plane of the ice crystal, forming wide macroscopically smooth surfaces (Fig. 1A). During freezing, the ice rejects the solute which accumulates at the ice-water interface. The increased concentration of salts causes a colligative decrease in the change of phase temperature on the interface and lead, through the well-known phenomenon of constitutive supercooling instability, to the transformation of the ice crystal morphology from a planar structure to a dendritic one as shown in Figure 1B. However, Figure 1C shows that the ice crystal growth pattern in the presence of antifreeze glycoproteins (40mg/ml) is very different. Figure 1C shows spicular (spike-like) ice crystals much smaller in dimensions from the dendritic ice crystals seen in Figure 1B. The antifreeze glycoproteins ice structure is spicular from the onset of the freezing process. Using polarized light, it is shown that the spicular ice crystals grow in the direction of the c-axis. The small spicular ice crystals incorporate between them, the AFPs and the other solutes in the solution. Figures 1A, 1B and 1C show that the incorporation of the solutes stabilizes the spicular ice growth along the c-axis. It is observed that in the presence of antifreeze glycoproteins the ice crystals are very small. The saline solution is incorporated between the submicron size ice spicules. Therefore, the salt solution

does not concentrate significantly to produce a significant change in chemical potential. As a result, in organs water does not migrate from the surrounding cells dehydrating, expanding the blood vessels, and collapsing these cells.

Figures 2A, 2B and 2C

Figures 2A and 2B have black 21 and white arrows 22 which point toward longitudinally and transversely sectioned sinusoids 23 (s), which show spicular ice crystals. All the spicular ice crystals are oriented in the same general direction. Structurally intact hepatocytes surround the sinusoids. The round nucleus is evident in some of the cells, where it is marked with a white dot 24. Rounded ice crystals are observed in all the cells.

Figure 2C shows higher magnification of the spicular ice structures in a large blood vessel 25 (bv). The margin of the blood vessel is shown with black arrows 26. Typical ice crystals in adjacent cells are marked with white circles 27. The scale bar 28 for Figures 2A, 2B and 2C is 10 micrometer.

Figures 3A, 3B, 3C, 3D and 3E

Figure 3A shows normal liver tissue frozen with a cooling rate of 4°C/min on a directional solidification stage, as described hereinabove. Continuous, smooth ice crystals are seen inside expanded sinusoids, (s). The adjacent hepatocytes (h) are dehydrated.

Figures 3B and 3C show liver perfused with antifreeze glycopeptides (40 mg/ml), which are similar to those in Table 1 and frozen with a cooling rate of 4°C/min on a directional solidification stage. The cross-section through a large blood vessel 31 (bv) shows spicular ice crystals 31 confined within the blood vessel 33. (The surface of the blood vessel is marred by debris formed during the fracture.) The outline of box-like hepatocytes 34 (h) fractured along the cell membrane is marked by arrows 35. The dimensions and the shape of the hepatocytes are typical to that of normal hepatocytes.

Figure 3D shows liver tissue perfused with antifreeze

glycopeptides and frozen with a cooling rate of 4°C/min on a directional solidification stage. The fracture is along the cell membrane, with cells removed in a staggered form leaving behind stair-like arranged hepatocytes 36 (h). The outline of box-like, normal size hepatocytes (h) is shown by the black arrows 37.

Figure 3E shows normal liver tissue frozen with a cooling rate of approximately 4000°C/min, and fractured along the cell membrane. The cells are removed in a staggered form leaving behind stair-like arranged, normal size, box-like hepatocytes 38 (h), shown by arrows. The bile duct 39 (bd) (cannaculus) has been preserved intact. The scale bar 40 shown Figure 3A to 3E is 10 micrometer.

Figures 4A, 4B and 4C

Figure 4 includes photographs concerned with the cryo-preservation of immature pig oocytes.

Fig. 4A shows one pig oocyte in a transparent droplet during cooling to -130° C. The dark circular rim of the droplet is shown.

Fig. 4B shows a pig oocyte after rapid cooling to -130° C in the AVS solution following 44 hours in vitro culture and stained. The cytoplasm is completely degenerated and the oolemma is not intact.

Fig. 4B illustrates the appearance of an oocyte that was not considered viable after rapid cooling in the AVS solution. The photograph shows an oocyte in which the membrane (oolemma) is not intact and no nuclear details are visible.

Fig. 4C shows an oocyte that survived rapid cooling to cryogenic temperatures in an AVS solution with AFGP and consequently underwent in culture nuclear maturation to the MII stage. It must be emphasized that this is the first time any method has been developed under any conditions in which pig oocytes survive and develop in vitro after exposure to cryogenic temperatures.

Fig. 4C shows the appearance of an oocyte that reached the MII stage after rapid cooling to -130° C. The nuclear

development stage is evident.

Fig. 4D shows a pig oocyte after rapid cooling to -130° C in the AVS solution with antifreeze glycoproteins like in Table 1 (40g/ml showing a normal morphology but no nuclear maturation (g.v. stage) after 44 hours of incubation: intact oolemma, intact g.v. membrane, normal cytoplasm morphology (the bottom of the photograph shows some cummulus cells). The scale bar for Fig. 4 is 50 μm.

Fig. 4D shows an oocyte cooled rapidly in the AVS solution with 40 mg/ml AFGP. This oocyte was not considered viable because it did not undergo nuclear maturation (remained at the g.v. stage). Nevertheless, it is noted that the cells show a normal morphology with an intact oolemma and an intact g.v. membrane. The intact appearance of the oolemma in Fig. 4D is typical to all oocytes cooled in the presence of the AFGP solution without AFGP, (Fig. 4B) for an illustration of the effect of the AFGP. This set of experiments clearly demonstrates that the AFGP has a cryoprotective effect, which is associated with retaining the integrity of the cell membrane when exposed to severe temperature conditions.

Figures 5A and 5B

Fig 5 includes photographs concerned with cryopreservation of pig embryos at the two cell stage. The scale bar for Fig. 5 is 50 μm.

Fig 5A shows a two cell embryo after rapid cooling to -130° C in the AVS solution and 24 hours in incubation. The complete disintegration of the membrane is evident.

Fig. 5A shows the appearance of a pig embryo that was cultured after rapid cooling in the AVS solution. The cell is obviously not viable and the disintegration of the blastomere membrane is complete.

Fig 5B shows a normal four-cell stage embryo developed from a two-cell stage embryo after rapid cooling to -130°C. The upper right-hand side of the photograph shows an embryo that remained at the two-cell stage. The clear integrity of the membrane is evident even in embryos that failed to

develop. The scale bar for Fig. 5 is 50 μm.

Fig. 5B shows a pig embryo at the four-cell stage after rapid cooling in the AVS solution with AFGP and after in vitro culture. Obviously the embryo shown in Fig. 5B survived the exposure to cryogenic temperatures and developed in a normal way, in vitro. Again, this is the first report of a method for successful cryopreservation of pig embryos at the two-cell state.

Additional important information can be seen in Fig. 5B. The upper right hand side of the photograph shows a pig embryo that remained at the two-cell stage, i.e., it did not survive rapid cooling according to our criteria. Nevertheless, the clear integrity of the blastomere membrane is noted and compared to the appearance of the disintegrated membrane in Fig. 5A.

Figure 6A, 6B and 6C

Fig. 6 includes photographs concerned with cryopreservation of mouse embryos at the two-cell stage. The scale bar for Fig. 6 is 50 μm.

Fig. 6A shows one mouse embryo in a transparent droplet during cooling to -130° C in the AVS solution after 72 hours incubation.

Fig. 6B shows one of the embryos remained at the two-cell stage with shrunken blastomere which implies membrane damage. In the second embryo, the blastomere membranes have disintegrated completely;

Fig. 6B illustrates the appearance of mouse embryos that did not survive rapid cooling in the AVS solution. The blastomere in one of the embryos in Fig. 6B are shrunk which implies loss of membrane integrity. The membrane disintegration is nearly complete in the blastomere of the other embryo shown in the photograph. The survival of rapidly cooled oocytes and embryos did not improve when 50 mg/ml AP were added to the basic AVS solution. The cell membrane integrity did not improve either and, in fact, there was absolutely no difference in survival or morphology between embryos and oocytes cooled in the AVS solution or

the AVS solution with AP.

Fig. 6C shows the typical appearance of a blastocyst following in vitro culture of a two-cell stage mouse embryo cooled to -130° C in the presence of the AFGP.

The typical appearance of normal mouse blastocysts following cooling in the AVS solution with 40 mg/ml AFGP is shown in Fig. 6C. The high rate of survival of mouse embryos in the presence of AFGP's, 82.5% compared to no survival, 0% without AFGP's, provides a clear indication of the cryoprotective properties of the AFGP.

As significant as these results are on survival, equally important are the microscopic observations showing that the integrity of the oolemma in the pig oocytes and of the blastomere membrane in the pig embryos is retained when the cooling occurs in the presence of AFGP. The microscopic evidence shows that the membrane was intact in 35 of the 45 pig oocytes (82.2%) and in the blastomere of 23 of the 23 (100%) pig embryos when the oocytes and embryos were cooled rapidly to cryogenic temperatures in the presence of AFGP. The integrity of the two-cell stage pig embryo was discussed earlier with respect to Fig. 5B.

The results of Figures 5 and 6 show that the addition of 40 mg/ml of AFGP dramatically improved the survival of the embryos and the oocytes, with 24.5% and 26% survival for the pig oocytes and pig embryos, respectively and 82.5% survival for the mouse embryos.

Because the mouse embryo provides such an unambiguous criteria for viability and because the survival in the presence of AFGP is so high, this animal model was particularly useful for the parametric studies. The results of the parametric studies on the effect of AFGP concentration are also listed in Table 3 above, and show a sudden transition between very high survival at concentrations higher than 20 mg/ml AFGP to very low, or no survival at concentrations lower than 10 mg/ml.

The results presented here clearly demonstrate that the AFGP's facilitate the survival of different animal models at

cryogenic temperatures. The results also show that the mechanism of protection is associated with the ability of the AFGP's to maintain the integrity of the cell membrane during exposure to cryogenic temperatures. AFGP's compounds that modify the process of freezing in solutions in a similar form to the AFGP's, sometimes have no effect on maintaining the structural integrity of the membrane.

Figures 7A, 7B and 7C

Figures 7A, 7B and 7C show pig oocytes preserved for 4hr at 4°C without AFGP (7A), and with 40m/ml AFGP (7B and 7C). These figures show that the oolema is damaged without the AFGP(7A). It stays intact with AFGP even in cells that do not survive, and it also facilitates the in vitro development of ooytes to the MII stage Figure (7C). The results in Table 4 suggest that the addition of antifreeze glycoproteins is useful in protecting cell membranes and in blocking ion channels. The observation that antifreeze glycoproteins 1-5 and 7-8 (Table 4, Fractions) separately do not protect the ion flow as well as the 1-5 and 7-8 together suggests that each one of the proteins is active in protecting different proteins and ion channels, i.e., is specific. Therefore it appears that all of the AFGP's are needed for complete protection, while individually, they offer partial protection.

Figures 8 and 9

Figure 8 is a photogrpah of rat liver perfused with Krebs solution only and cooled to -35°C.

Figure 9 is a photograph of rat liver perfused with a identical Krebs solution for Fig. 8 with 20 mg/ml of AFGP fractions 1-8 as obtained fromt he Antarctic fish (see Table 1).

Preservation of Bovine Oocytes

Figure 14 shows the percentage of oocytes with an intact oolemma after 24 hours hypothermic exposure to 4°^C was determined by three different tests; morphological microscopic examination (Morph.), fluorescein diacetate staining (FDA), and trypan blue exclusion (TB). The

experiments were performed with bovine oocytes preserved in: a) PBS (control), b) PBS with 20 mg/ml AFP from the winter flounder (AFP(WF)),c) PBS with 20 mg/ml AFP from the ocean pout (AFP(OP)), and d) PBS with 20 mg/ml AFP from the sea raven (AFP(SR)). Twenty oocytes were used for each of the experiments in the figure.

Figure 15 shows the percentage of oocytes which underwent in vitro maturation and in vitro fertilization. The viability of oocytes after 24 hr hypothermic exposure to 4°C was determined through their ability to undergo in vitro maturation and in vitro fertilization. The figure shows results obtained with controls exposed to hypothermic conditions in PBS (CONTROL), fresh oocytes (FRESH), and oocytes in PBS with 20 mg/ml of the different "antifreeze" proteins; a) winter flounder, (AFP(WF)),b) ocean pout, (AFP(OP)), and c) sea raven (AFP(SR)). Two numbers are given on top of each of the columns. The top number gives the number of oocytes which have successfully undergone either in vitro maturation or in vitro fertilization, and the bottom number gives the number of oocytes used in each of the experiments.

Calcium Ion Migration

Figure 19 shows repeated stimulation of parietal cells with 100μM carbachol causes repeated increases of Cai. As shown previously (52), the initial spike increase of Cai is caused by the release of Ca from internal stores, while the plateau is generated by the entry of Ca from the extracellular space into the cells. The trace represents the average Cai signal from 10 parietal cells in one gastric gland, and is typical of 3 similar experiments (2 animals).

Methods. Isolation of gastric glands. Gastric glands from New Zealand White Rabbits were prepared as described by Berglindh and Obrink (58). Briefly, intact rabbit gastric glands were formed by placing minced gastric mucosa in a digestion medium that contained 0.3 mg/ml type 1A collagenase in an Eagle's minimum essential medium (GIBCO), supplemented with 1 mg/ml bovine serum albumin (Calbiochem),

10-4 M cimetidine (a histaminergic, H2, blocker to insure that glands remain unstimulated) and 20 mM N-2-hydroxyethylpiperzine-N'-2-ethane sulfonic acid (HEPES). The solution was stirred and gassed (100% O_2 at 37°C. Glands were formed within 45 minutes. These glands settled (1g) and were then rinsed several times at room temperature in Eagle's medium containing 40μM cimetidine but without enzymes or albumin.

Dye Loading and Calibration. For Cai measurements, a suspension of isolated gastric glands (5% cytocrit) was loaded with fura-2/AM in Eagle's medium containing 10-4 M cimetidine and 10μM dye for 30 min at 24°C. Following loading, the glands were washed in Eagle's medium and left at room temperature until use.

Cai was calibrated in each cell using the formula derived by Grynkiewicz, Poenie, and Tsien (59) for dual wavelength measurements

$$Cai=K(R-R_{min})/R_{max}-R)$$

where R_{min} is the ratio of fluorescence intensities at 340 and 385 nm obtained at O Ca, R_{max} is the ratio at saturating Ca, and R is the measured ratio. R_{min}, R_{max} and Kd were determined in separate experiments as described earlier (60).

Fluorescence measurements. Fura-2-loaded glands were mounted in a perfusion chamber and placed over the objective (x40) of a Zeiss 1M35 inverted microscope at 37°C as described previously. Fluorescence from single cells within intact gastric glands was measured using digital image processing of video images of the fluorescence of each excitation wavelength as described previously (60). Black and white fluorescence images of whole glands were acquired using a Silicon Intensified Target Camera (Dage 66) and relayed to a Gould FD5000 image processor which was controlled by a DEC computer (PDP 11/73) using software available from Dr. Roger Y. Tsien (Department of Pharmacology and Hughes Institute, University of California at San Diego, La Jolla, CA). After correcting for background and dark current of the camera, the fluorescence

intensity ratio was calculated for each pixel and displayed as one of 32 pseudocolors. These ratios were then calibrated as discussed previously (60). Analysis and plotting of ratio vs time for the individual cells was accomplished using a graphics emulation terminal (Smarterm 240). This imaging system allows the collection of data from individual parietal cells. Acid-secreting parietal cells easily distinguished visually from neighboring enzyme-secreting chief cells. Parietal cells were chosen because they demonstrate larger plateau responses of Cai than do their neighbors the chief cells.

Solutions. All experimental solutions contained the following (in mM): 144 Nacl, 2.5 K_2HPO_4, 2.0 $CaCl_2$, 1.0 $MgSO_4$, 11.0 glucose, 10 HEPES pH 7.45.

Figure 20 shows sequential treatement of parietal cells with 100μM carbachol in control and AFP-treated conditions. Note that in the presence of AFP, stimulation of the cells elicited a large spike increase in Cai, but no plateau. This trace is the average of 10 different cells in one gland, and is typical of 5 different experiments (3 animals).

Isolation of anti-freeze proteins. Blood serum containing antifreeze proteins was chromatographed on a Sephadex G75 in 0.1M NH_4HCO_3. The antifreeze activity (thermal hysteresis) of each fraction was measured using a Clifton nanoliter osmometer (55). Active fractions were pooled, lyophilized, and rechromatographed on Sephadex G75 as above. Fractions containing peak amounts of antifreeze activity were pooled and stored lyophilized in a freezer (-20C) until use. SDS-PAGE and high performance liquid chromatography (HPLC) analysis indicate that greater than 95% of the Sephadex G75 purified materials are antifreeze polypeptides (61,62,63). Reverse phase HPLC reveal 12 independently active components, eight of which have been sequenced (62).

Figure 21 shows long-term treatment of AFP blocks reloading of the CA internal store. Cells were pretreated

with AFP-containing solution for 3 min before stimulation. Cells were stimulated three times with carbachol, once in control condition and two more times in the presence of AFP. AFP eliminated the plateau and also prevented refilling of the internal store, because the second stimulation of the cells in the presence of AFP was blocked. This effect is similar to that of La and low pH, which block both the plateau and also reloading of the internal store. This experiment is the average of 10 cells from one gastric gland, and is typical of 3 different experiments (2 animals).

Figure 22 shows short-term treatment with AFP eliminated the carbachol-induced plateau, but allows some refilling of the intern al store. Cells were stimulated four times with carbachol. The first stimulation shows the control response, and the second shows the effect of AFP to block the plateau, while leaving the spike intact. When the AFP was washed out and the cells were stimulated again, the internal store had partially refilled, because the third and fourth carbachol-induced spikes were approximately 60% the size of the second. The plateaus of these third and fourth stimulations were also reduced. This experiment is the average of 10 cells from one gastric gland, and is typical of 3 different experiments (2 animals).

While not wanting to be bound by theory, the protective effect of the AFGP is probably associated with the particular chemical structure of the molecule. It is possible that the protection afforded to the cell membranes during exposure to cryogenic temperatures is a consequence of bonds formed between the hydrophilic parts of the membrane proteins and the AFGP's. There is evidence that the protection afforded by the AFGP's is concentration-dependent in a nonlinear fashion, which suggests that for complete protection all the bonds must be established and no survival is possible with partial interaction between the cell membrane and the AFGP.

The following examples are presented to further

explain, describe and define the present invention. They are not to be construed to be limiting in any manner.

GENERAL EXPERIMENTAL

The phosphate buffer solution (PBS) standard solution and may be supplemented as indicated herein, e.g. the higher molecular weight AFGPs 1 to 5 seems to be strongly related to the modification of the ice crystal structure, the biological function of the low molecular weight AFGPs remains unclear. They are less efficient in depressing the freezing point than the larger glycopeptides yet they seem to be present in the serum at much higher concentrations.

AFGP used from Fractions 1-8 (Table 1) are in essentially the same ratio to each other as is found in the Anarctic fish. Fraction 6 is present in a trace amount in the fish, and its presence or absence in the following experiments (in the concentration of Fraction 1-8) is assumed to have a negligible effect on the experiment.

A preferred concentration of AFP or AFGP in aqueous solution in this invention ranges between about 1 and 50 mg/ml., especially Fraction 1-8, Table 1. For some applications, a range of 20-40 mg/ml is preferred.

As can be observed, ice crystal formation has caused major disruption of the blood vessels and the surrounding cell tissue and cell membrane.

As can be seen the cell membrane structure shows minimum disruption. The cell tissue appear to have remained discrete, the cell membrane appear essentially intact and the blood vessels are not significantly enlarged.

EXAMPLE 1

FREEZING OF LIVER TISSUE

(a) Adult female Sprague-Dawley rats, ages 45 to 50 days were anesthetized with ether throughout the surgical procedure. The abdomen was exposed via a midline incision to expose the liver. The portal vein was exposed and cannulated. Immediately, one thousand units of heparin were injected into the vein. This procedure was followed by the injection of a 5-ml solution of physiological saline

containing 200 mg of AFGPs from an Antarctic fish (Dissostichus Nawsoni) (see Table 1), in a physiological composition of Fractions 1-5 and 7,8 (25/75). Optionally, glycerol/saline is perfused through the liver. The AFPs used are those shown in Table 1 above. The AFPs for Dissostichus mawsoni and for the AFGPs of Table 1 have essentially the same molecular weights and ratios to one another. A combination of antifreeze glycopeptide No. 1-5 and No. 6-8 are used in a ratio of 1/3, w/w. The portal vein was immediately clamped to prevent back flow. Within a period of 2 minutes, several rectangular samples of the liver, 8 by 4 by 3 mm in size, were sectioned with single radial razor cuts approximately 3 mm from the periphery of the lobe and were placed lengthwise on two No. 1 coverslips. A total of four animal experiments were performed.

The first coverslip was immediately plunged into nitrogen slush maintained under vacuum at -213°C. No boiling was visible. The cooling rate during freezing was estimated at about 4000° C/min. At the same time, the other coverslip was transferred to the directional solidification stage described earlier. The samples were frozen from an initial temperature of 25° C to a final temperature of -35°C, with a cooling rate of 4°C/min. The time of freezing was approximately 15 min. After freezing, the frozen samples were immediately immersed in the liquid nitrogen slush and transferred to an AMRAY 1000 low temperature scanning electron microscope (LTSEM). The samples were fractured in the cryochamber of the microscope, exposing an area approximately 2 mm from the outer surface of the lobe, gold-coated and transferred in a frozen hydrated state to the refrigerated stage of the LTSEM.

Photographs obtained from the LTSEM are two-dimensional images of an irregularly fractured three-dimensional surface. The photographs are taken at magnifications varying from 200 to 5000 times.

Figures 2A, 2B and 2C show results from liver tissue perfused with AFGPs and frozen in nitrogen slush. These

photographs demonstrate that the AFGPs modify the freezing pattern in mammalian tissue. Figures 2A and 2B illustrate the frozen tissue photographed at a magnification of 1000 times. Figures 2A and 2B were obtained after slight radiant etching of the frozen tissue, show the outline of ice crystals. Shown are individual cells and, in several of the cells, the nucleus is also visible. The ice crystals in the cells are different from those in the blood vessels. The ice crystals inside the cells are similar to typical ice crystals formed from plunging tissue in liquid nitrogen slush. These ice crystals are round in shape with dimensions in the micron range and are uniformly distributed throughout the cells. However, the ice crystals in the blood vessels perfused with AFPs are markedly different. The ice crystal structure is spicular with dimensions in the submicron range. It is also very similar to that observed during freezing of aqueous solutions of AFPs. See Figure 1C. Figures 2A, 2B, and 2C show spicular ice crystals in all the longitudinally and transversely fractured blood vessels and that the spicular ice crystals are oriented in the same direction independent of the relative orientation of the blood vessel.

These results demonstrate that water present in the tissue in the presence of AFGPs have ice crystals which do not propagate in the direction of the blood vessels but, rather, grow with a stable c-axis orientation, presumably in the direction of the temperature gradient. This is consistent with earlier reported research, which show that during freezing in a solution of AFGPs, the solutes entrapped between the spicules, stabilize and force the ice crystal to grow only in the direction of the c-axis. Therefore, the crystal growth is different from the freezing of solutions without AFGPs, where the ice crystal can grow along the different orientations of the ice crystal hexagonal prism facets, allowing a change of direction whenever the ice crystal encounters obstacles, such as the cell boundary. In the presence of the AFPs, the growth in the direction of the

c-axis is extremely stable and the orientation of the ice crystal cannot change when the ice crystal encounters a cell boundary. All the spicular ice crystals terminate at the blood vessel boundaries. The ice crystals in the blood vessels also do not cause the nucleation of the water in adjacent cells.

A higher magnification of a micrograph of the spicular ice crystals in a larger blood vessel is illustrated in Figure 2C. The significant difference between the spicular submicron size of the ice crystals in the blood vessel and the rounded micron size ice crystals in the adjacent cells in evident. The small dimensions of the spicular ice crystals suggest another potential application of the AFPs. Currently, extremely high cooling rates (e.g. 40,000 to 100,000°C/min, several orders of magnitude higher than the cooling rates in the present invention, are used for preparation of tissue samples with very small ice crystals for microscopy. Freezing tissues perfused with AFPs of the present invention is used to produce small ice crystals in tissue with much lower cooling rates which can be easier to achieve experimentally.

Figures 3A, 3B, 3C, 3D and 3E demonstrate the effect of the AFPs on the freezing pattern of mammalian tissue frozen with low cooling rates. The structure of liver tissue frozen with a cooling rate of 4°C/min in the presence of AFPs is illustrated in Figures 3B, 3C, and 3D. These Figures are compared with Figure 3A which shows the structure of liver tissue frozen with a cooling rate of 4°C/min without AFPs and with Figure 3E which shows the structure of liver tissue frozen without AFPs with a cooling rate of approximately 4000°C/min.

(b) For comparison purposes Figure 3A shows the typical structure of liver tissue frozen at low cooling rates without AFPs. The large continuous ice crystals along the sinusoids an the completely dehydrated hepatocytes surrounding the blood vessels are evident. Because of the dehydration of the hepatocytes in liver tissue frozen with

low cooling rates, the tissue is not able to fracture along the cell membrane boundaries and, therefore, shows fractures through large ice crystals.

(c) The morphology of liver tissue frozen with low cooling rates in the presence of AFPs is markedly different. Figures 3B and 3C show cross-sections through a large blood vessel and the adjacent tissue, at a magnification of 1000x and 2000x, respectively. The submicron size spicular ice structures, typically found in the freezing solutions with AFPs, (Figure 1C) are evident in the blood vessel. All the ice crystals in the blood vessel have the same orientation and they terminate at the blood vessel boundary. The structure of the spicular ice crystals in Figures 3B and 3C are markedly different from the smooth single ice crystal structures observed in the blood vessels of tissue frozen with the same cooling rate but without AFPs, Figure 3A. Figures 2A, 2B, 2C, and 3A, 3B, 3C, 3D and 3E obtained for cooling rates of 4000°C/min and 4°C/min, respectively, demonstrate that the AFPs generate a similar, submicron size stable spicular ice crystal structure when freezing mammalian tissue over a large range of cooling rates.

(d) The fracture in Figures 3B, 3C, 3D and 3E is along the cell membrane, where the cells are removed in a staggered form, leaving behind stair-like arranged hepatocytes. The micrographs show contours of box-like shaped hepatocytes, which do not appear dehydrated and actually retain their normal shape. Comparing Figures 3B, 3C and 3D with Figures 3A and 3E, it is found that the figures possess much more resemblance to Figure 3E, showing identifiable box-like hepatocytes with typical dimensions, fractured at the cell membrane. Figure 3A which shows dehydrated hepatocytes and ice crystals in the expanded sinusoids, is significantly different. The surprising result is that while Figures 3B, 3C and 3D are photographs taken from samples perfused with AFPs and frozen with a cooling rate of 4°C/min, which is similar to the cooling rate used for Figure 3A, Figure 3E is a photograph of liver tissue frozen, without AFPs, in liquid

nitrogen slush with a cooling rate of approximately 4000°-C/min. As expected, freezing at these high cooling rates retains the normal structure of the hepatocytes, and shows the bile duct with bile along the cell membrane of Figure 3E. For freezing with low cooling rates (4°C/min) without AFPs, it is impossible to fracture the dehydrated hepatocytes along the cell membrane, and these micrographs always show ice crystals. The observation is that the structure of liver tissue frozen with 4°C/min in the presence of AFPs resembles that of tissue frozen with a cooling rate of 4000°C/min. This result illustrates the significant effect of the AFPs on the freezing pattern in mammalian tissue.

EXAMPLE 2

CRYOPRESERVATION OF WHOLE ORGAN

(a) Cryopreservation of a whole liver from a rat as described in Example 1 is adapted for a whole organ. The rat liver is surgically removed, and held in an aqueous solution at 24°C. The portal vein vessel is cannulated. The well known Langendorf perfusion system (with a first bottle containing Krebs solution) is used. See procedure, for example, D.E. Pegg et al. (1986), Cryobiology, Vol. 23, pp. 150-160.

A second bottle of solution contains a saline solution and appropriate quantities of glycerol, dimethyl sulfoxide, ethylene glycol, polyvinyl chloride glucose or mixtures of these substances which are known as protectants for cells together with antifreeze glycopeptides at a concentrations of 40 mg/ml.

A computer controlled mixing switch provides intermediate amounts of solution 1 (Krebs solution), solution 2 (glycerol/saline/AFGP solution) bursts of time lengths controlled by the computer, for example 0.01 sec. to 0.1 sec. to 1 sec. time lengths. The two solutions mix in a specially provided mixing chamber.

These two bottles of physiological solution are connected to a mixing valve having known adjustable flow rates (e.g. about 5 ml/sec) and a computer to accurately

vary the flow rate and mixing of each bottle's contents immediately prior to perfusion. The perfusion using the solutions of bottles 1 and 2 is well known in the art as described by G. N. Alink et al. (1976), Cryobiology, Vol. 13, pp. 295-304; (1977) Cryobiology, Vol. 14, pp. 409-417 and 399-408; and (1978) Cryobiology, Vol. 15, pp. 44-58, and K.E.F. Hobbs et al. (1969), Cryobiology, Vol. 6, pp. 239-245. The Krebs solution is perfused through the liver held at 24°C at a rate of 4 ml/min.

The glycerol/saline/AFGP concentration in the perfusate is slowly increased at a rate of 0.001-mole/0.1 sec. until a concentration of about 3 mol glycerol and 40m/ml AFGP is perfused. The tissue is then perfused with the 3 mol glycerol/saline for an additional 20 min. The perfused liver in solution (AFGPs 0.001 M in the organ) is next placed in a cooling stage (US Patent - 4,531,373) and the temperature of the whole perfused liver is then cooled at a rate of 1°C per minute until -150°C is achieved. The liver is then cooled using a liquid nitrogen slush to -196°C and held at this temperature for 72 hrs. The frozen liver is then carefully thawed at a rate of between about 0.1 to 10°C per min. (preferably about 1°C per min.) using known techniques with warm fluids. Alternatively, carefully controlled microwave heating is used to thaw the perfused liver. When the thawed liver reaches about 0°C, a nutrient solution of Krebs is perfused through the large cannulated blood vessel. When warmed to 37°C, the thawed liver recovers not only tissue function but also organ function. The viability of the organ is measured by the production of bile following the freezing and careful thawing.

(b) When the rat liver in subpart (a) above is replaced with a rat kidney and the procedure is repeated, a thawed kidney having viable tissue function and recovered organ function is obtained.

(c) When the rat liver of subpart (a) is replaced with a rat heart, some additional procedures particular to heart tissue for perfusion, including immediate removal of blood

from the heart chambers, are observed. After freezing of the antifreeze polypeptide perfused heart, careful thawing and perfusion with appropriate biological fluids, the reactivated heart having viable tissue function and viable organ function is obtained. The viability of the heart is measured by observing restored contractions of the heart muscle.

EXAMPLE 3

CRYOPRESERVATION OF A WHOLE RAT HEART

(a) The cryopreservation of a whole rat is adapted from Examples 1 and 2. An adult rat is anesthetized and the heart is removed under the conditions described in Examples of U.S. Patent 5,075,210 having 20 mg/ml AFGP.

The rat heart is then cooled to -150°C and held at this temperature for 7 days. The frozen heart is then carefully thawed at a rate of 1°C/min to 0°C in the presence of oxygen/nitrogen (20/80, v/v). A nutrient blood substitute solution such as Krebs, Euro Collins, UW solution containing is perfused through the heart via veins, the frozen heart is warmed slowly to its normal biological temperature (about 37°C). Tissue function and viable rat heart function is recovered completely.

EXAMPLE 4

CRYOPROTECTION OF IMMATURE PIG OOCYTES AND PIG EMBRYOS

Immature pig oocytes were isolated from selected follicles of cyclic sows 20 minutes after slaughter at 20° C, according to the procedure by Mattioli, et al. (20). The two-cell stage pig embryos were collected from prepubertal gilts (average weight 90 kg.). Estrus induction was carried out by administration of 1250 I.U., Pregnant Mare Serum Gonadopin (PMSG), (SIGMA, St. Louis, Missouri), followed 56 hours later by administration of 750 I.U., Human Chorionic Gonadropin (HCG), SIGMA, St. Louis, Missouri). Two artificial inseminations were performed after 34 hours and 46 hours from the HCG injection. The two-cell embryos were collected from the animal by mid-ventral laparoscopy under general anesthesia 60 hours after the HCG injection.

In preparation for low temperature exposure, the embryos and the oocytes were first introduced in one ml of PBS containing 0.1 M sucrose and 20% FCS at 22° C. This was followed by a three-minute gradual mixing with one ml PBS containing 5% glycerol, 0.1 M sucrose and 35% propylene glycol, according to a procedure developed by Arav (21). The embryos and oocytes were transferred to slides containing 0.1 μl droplets of either AVS or AVS with AFGP or AFP, one embryo or oocyte per droplet. (The experimental conditions and parameters are summarized in Table 4.) Prior to cooling, the pig oocytes and pig embryos were incubated on the slide for 6 minutes at 22° C.

Droplets containing oocytes and embryos were exposed in separate experiments, to the cooling/warming protocol described earlier. The cooling/warming process was monitored using a recording video camera attached to a Leitz Diaplan microscope with magnification of 120x and 340x. Fig. 4A illustrates the typical appearance of pig oocytes inside transparent droplets, during cooling with a rate of 1700° C/min to -130° C. In all experiments, the droplets remained transparent at magnification of 340x indicating the absence of visible ice crystals. During warming at 1,700° C/min, the transparent droplets retained an appearance identical to that in Figs. 4A and 6A. The microscopical evidence shows that the morphology of the embryos and oocytes did not change during cooling and warming.

After warming, in preparation for viability assays in cell culture, the pig oocytes and pig embryos were introduced for three minutes in 1 ml PBS, with 20% FCS and 1 M sucrose at room temperature (22° C), followed by transfer to and equilibration in PBS containing 20% FCS for 10 minutes at 22° C.

Prior to cell culture, all embryos and oocytes were washed three times in cell culture media. The pig oocytes were cultured in TCM-199 medium which was modified in that it contained 5 μg/ml of sheep luteinizing hormone (NIH S20), pig follicle stimulating hormone (LER 441-2) and 20 ng/ml of

pig prolactin (LER 2073). The pig embryos were cultured in Brinster culture medium without glucose. After equilibration in cell culture medium, the oocytes and the embryos were incubated at 37° C under 5% CO_2 in air, the pig oocytes for 44 hours, and the pig embryos for 24 hours.

The pig oocytes were fixed after 44 hours incubation in acetic alcohol (1:3 v/v) and stained with lacmoid stain. The viability of immature pig oocytes was assessed using phase contrast microscopy (20), by their ability to develop from the germinal vesicular, (g.v.) stage to the first metaphase (MI) or second metaphase (MII) stage in vitro, and to present a normal morphology (cytoplasmatic compactness, integrated oolemma, visible nuclear stage). The viability of the two-cell stage pig embryos was assessed by their ability to develop to the four-cell stage in culture, while maintaining integrated morphology (cell membrane and cytoplasm). The in vitro culture was stopped at the four-cell stage because many times early stage pig embryos encounter the four-cell block when cultured in vitro (22), and therefore, further incubation would not be useful to an experimental goal to assess the viability of the embryos after exposure to cryogenic temperatures.

EXAMPLE 5

CRYOPROTECTION OF MOUSE EMBRYOS

The procedure of Example 4 was followed except for the following changes.

Mouse embryos at the two-cell stage, were obtained from four-week old $C_{57}Bl/GJ$ mice which were paired singly with CBA/CaJ males. The females were induced to superovulate by intraperitoneal injection of 5-7.5 I.U. PMSG (SIGMA, St. Louis, MO) followed 48 hours later by 5-7.5 I.U. HCG (SIGMA, St. Louis, MO). Forty hours after insemination, the oviducts were excised from the mice and the two-cell embryos flushed out and stored in phosphate buffeted saline, (PBS) medium.

In preparation for low temperature exposure, the mouse embryos were introduced in 1 ml of PBS and FCS as described

in Example 3. (The experimental conditions are also summarized in Table 7.) Prior to cooling, the mouse embryos were incubated on a slide for 12 min. at 4° C.

Fig. 6A illustrates the typical appearance of mouse embryos inside transparent droplets during cooling with a rate of 1,700° C/min to -130° C. In all experiments the droplets remained transparent at a magnification of 340x indicating the absence of visible ice crystals. During warming with 1700° C/min. the transparent droplets retained an appearance identical to that in Figs. 4A and 6A. The microscopical evidence shows that the morphology of the embryos and oocytes did not change during cooling and warming.

After warming, in preparation for viability assays in cell culture, as described in Example 3, the mouse embryos were exposed for three minutes at 4° C to 1 ml of PBS, with 20% FCS and 1 M sucrose, followed by transfer to and equilibration in PBS containing 20% FCS for 12 minutes at room temperature (22° C) (21). Prior to cell culture, all embryos were washed three times in cell culture media. The mouse embryos were cultured to T_6 Whittingham medium (21).

After equilibration in cell culture medium, the mouse embryos were incubated at 37° C under 5% CO_2 in air for 72 hours.

The viability of the mouse embryos after exposure to cryogenic temperatures was assessed by their ability to develop in vitro to the blastocyst stage while showing normal expanded morphology. Table 1 lists the experimental results which were discussed earlier.

EXAMPLE 6

CELL MEMBRANE ELECTRICAL POTENTIAL

During the experiments in which pig oocytes were vitrified in the presence of AFGP's, it was discovered that only 24% of the oocytes and 26% of the pig embryos survived the rapid cooling. However, it was surprisingly observed that close to 100% of the cell membranes remained intact. On the other hand, without the AFGP's (100%) of the cell membranes

were destroyed. The effect of the AFGP's at temperatures higher than 0° C for pig oocytes was examined because it is reported that they could not survive at temperatures lower than +10° C.

Immature pig oocytes were obtained from selected follicles of cylcic sows 20 minutes after slaughter, at 20°C, according to the procedure by Mattioli et al. The oocytes were then introduced in vials containing different concentrations of AFGP (Fractions 1-8) in a saline supplemented with 0.4 w/v microm/ml BSA (Bovine Serum Albumin), 0.34 mM pyruvate, 5.5 mM glucose and 70 micromol/ml kanamycin). The AFGP's used in this work were obtained from Antartic fish belonging to the family Notoheniidae (Dissostichus Nawsoni). A physiological composition of AFGP's was used in most of the experiments having one part by weight of AFGP's 1 to 5, and three parts by weight of AFGP's fractions 7 and 8. (available form A.L. DeVries supra.) Experiments were also performed with AFGP's fractions 1-5 and AFGP's 7 and 8, separately. The different experimental parameters are listed in Table 1. To determine the protective effect of the AFGP the oocytes were exposed for 4hr and 24hr to a constant temperature of 4°C, in a constant temperature chamber. After removing the oocytes from the 4°C environment the integrity of the oolema was determined by measuring the resting membrane potential of the oocytes at room temperature, 22°C, according to a procedure by Mattioli et al. Intracellular voltage measurements were made using single microelectrodes made from borosilicate glass tubes (Hilgenberg, FDR). The electrodes were pulled on a horizontal puller and filled with 2M KC1. The resistance of the electrodes was 10-20 Megaohms (Mo). To record the membrane potential the tip of the microelectrode was maneuvered to the surface of the cell using a micromanipulator controlled through 400x magnification with a Leitz Fluovert microscope equipped with Nomarski optics. When the tip just dimpled the surface of the cell, the final penetration was achieved by briefly causing an electrical oscilla-

tion induced by turning the capacity compensation of the amplifier. The electrical potential values, which remained constant for at least 1-2 sec, were recorded. The resting membrane potential is a very sensitive criteria for membrane integrity. In addition, experiments were performed to determine the viability of certain oocytes following exposure to the hypothermic conditions. Several of the oocytes exposed for 4 hr to 4°C, with 40mg/ml AFGP 1-8 in the basic PBS solution and without, were incubated in TCM-199 medium, which was modified in that it contained 5 microg/ml sheep luteinizing hormone (NIH S20), pig follicle stimulating hormone (LER 441-2) and 20 nanog/ml of pig prolactin (LER 2073) at 37°C under 5% carbondioxide for 44 hr. After incubation the oocytes were fixed in acetic acid/ethyl alcohol (1:2 v/v) and stained by lacmoid stain. The viability of the immature pig oocytes was assessed using phase contrast microscopy by their ability to develop from the initial germinal vesicular (g.v.) stage to the first or second metaphase, MI or MII in vitro and to present a normal morphology (cytopolasmatic compactness, integrated oolema, visible nuclear stage). The microscopic observation also allows a qualitative evaluation of the structural integrity of the membrane.

For each experiment the results for both criteria are given, as shown in Table 4. The mean of all means was -31 mv, and the mean standard distribution, 4.5 mv. These values are within the normal range of membrane potential for pig oocytes. It should be emphasized that measuring potential is a very sensitive and recognized measure of membrane integrity. It is apparent from Table 4 that the combined AFGP's fractions 1-8 protect the olema gainst damage induced by exposing the oocytes to hypothermic conditions. Since there are no ice crystals present at 4°C the protection must occur through an interaction between the Antarctic fish glycoproteins and the oolema. Therefore, this part of the experiment demonstrates that the AFGP's directly protect membranes, which is a property of the

AFGP's that has never been reported. The level of protection is not a linear function of the AFGP concentration; it reaches saturation at about 1 mg/ml in the perfusion and drops to low values at 0.1 mg/ml. This is a typical property of protein-protein interactions, which may indicate that the glycoproteins may offer protection by binding to the available sites on the oolema and can provide their protection only if all the sites are occupied. These sites could be the membrane proteins. Table 4 also shows that the whole physiological combination of AFGP's 1 to 8 is needed for protection and that AFGP's 1 to 5 and AFGP's 7, 8 separately do not protect the membrane. This result is extremely surprising because studies on the effects of AFGP's on depressing the phase transition temperature and ice crystal formation show that AFGP's fractions 1-5 depress the phase transition temperature almost as effectively as the whole combination of AFGP's fractions 1 to 8. On the other hand, it is apparent that AFGP's fractions 1 to 5 separately do not protect the cell membrane, and neither do AFGP's fractions 7,8 separately. A possible explanation for this phenomena is that all the different proteins with different lengths are needed to bind to all possible sites on the membrane and to block all the possible leaks sites and ions channels.

The microscope evaluations of the oocytes exposed to 4°C for 4 hr and incubated for 44 hr to verify the results obtained through measurements of membrane resting potential. In the absence of AFGP's only 2 of 20 oocytes retained an integrated oolemma (10%), and none of the oocytes matured in vitro, (0%). Figure 7A illustrates the appearance of an oocyte preserved at 4°C in PBS without AFGP. The oolema is apparently not integrated and the cytoplasm is degenerated. In contrast, in the presence of 40 mg/ml AFGP, 11 of 18 oocytes retained an integrated oolemma, 61% (Figure 7B). This result further demonstrates that the AFGP's protect the oolema of cells exposed to damaging hypothermic conditions and is consistent with the measurements of the resting

electrical potential. Close to 25% of the oocytes survived and matured to the MII stage as illustrated by (Figure 7C).

EXAMPLE 7

CRYOPRESERVATION EFFECT OF THE AFGP'S ON RAT LIVER

The procedure is essentially identical to that described in Example 1.

Experiments were performed with adult female Sprague-Dawley rats, ages 45 to 50 days. The rats were anesthetized with ether throughout the surgical procedure. The abdomen was exposed via a midline incision to expose the liver. The bile duct was exposed and cannulated. Bile was collected and used as a criteria for viability. The portal vein was exposed and cannulated. Immediately one thousand units of heparin were injected into the vein. The liver was released and flushed with a basic Krebs solution through the vein. In some of the models this was followed by an injection through the vein of 3 ml solution of Krebbs containing 20 mg/ml AFGP Fractions 1-8 (in a physiological composition found in the fish) (from the Antarctic fish belonging to the family Nototheniid, Dissostichus Mawsoni). The liver was then introduced into a refrigerator at 4° C for 6 hours. After that time, the liver was removed, perfused with a Krebbs solution at body temperature and kept on a plate maintained at body temperature 38°C. The production of bile was measured as a criteria for viability. This test is a well accepted criteria considered to provide the best overall indication of viability. The results show that with AFGP's, the bile rate of formation was about 85% of the initial level after 6 hours at 4° C. In the absence of the AFGP, bile rate dropped to about 20% of the normal level. Three animal experiments were performed for both the control and the solution with AFGP.

EXAMPLE 7A

EFFECT OF AFGP ON LONG-TERM VIABILITY OF INTACT RAT LIVER TISSUE

Surgical Procedure

Livers from Spague-Dawley rats (35) of ages 50-55 days

are surgically removed. The peritoneal cavity is entered under nembutal anesthesia. The bile duct is cannulated with a PE-30 polyethylene catheter, and the bile is collected for 10 min while the surgery proceeds. After partial mobilization of the liver from adjacent tissue, a 16-gauge TEFLON® intravenous catheter is introduced into the portal vein, and 3.0 mL of perfusion buffer containing 1000 units of heparin is rapidly infused using a 3 mL syringe. The inferior vena cava is transected distally, and the portal vein catheter infused with Krebs solution pre-equilibrated to a 95:5 mixture of O_2 and CO_2 at 0°C from the remainder of the surgical procedure. The inferior vena cava is ligated above the renal veins and freed from adjacent retroperitoneal tissues, and a PE-205 polyethylene catheter is secured in the superior vena cava through an incision in the right atrium. Samples of the effluent are collected at this time. The entire liver is then carefully cut free from the surrounding tissue and washed with warm saline.

Storage and Isolated Organ Perfusion

For the test livers, the perfusion line of TEFLON® is removed and a 3 mL solution of Krebs solution containing 20 mg/mL of AFGP fraction 1-8 from Antarctic notothenoiidae fish (D. Mawsoni) is injected into the catheter. The whole liver is then immediately placed into a container containing cold Krebs solution and is returned to the constant temperature apparatus. The apparatus and liver are kept at a constant 4°C. The liver is stored for periods of 6, 12 and 24 hr.

After the storage process, the liver is removed and inject with a 20 mL of Krebs solution at ambient temperature to remove the AFGP solution. The liver is immediately inserted in the single pass Largends rf type perfusion circuit described by containing Krebs solution 37°C pre-equilibrated with a mixture of 95% oxygen and 5% carbon dioxide. The flow rate is then increased from 5 to 25 mL/min with careful attention to the position of the liver and catheter. The liver is perfused for 50 min. The

effluent from the liver is collected continuously for the intervals 0-5, 5-10 and 10-25 min. In addition, bile is collected in 15 min intervals.

Control

For a stored liver control study, the liver was injected with a 3 mL of Krebs solution. The procedure then followed the test study conditions described above without AFGP addition. For the warm control liver, the liver was immediately inserted in the single pass perfusion circuit, and the necessary effluent and bile samples was taken.

The collected bile from each liver was measured and tabulated. The collected effluent was tested for activity of lactic dehydrogenase (LDH). Enzyme assays for LDH is performed using standard colorimetric techniques (Sigma Diagnostics KIT 500. A UV-visible spectrophotometer is utilized.

Experiments were performed with 35 rats. Each experimental point represents between 3 and 5 animal experiments.

Bile flow commenced within 3-5 mins. after the liver was connected to the single pass perfusion system. The bile flow was well maintained during the 50 min perfusion. Since rat livers do not produce bile salt, the production of bile from any excised liver could not be sustained for much longer than about 50 minutes. The production of bile reaches a plateau during the second collection and maintains this level for the duration of the perfusion. Figure 10 is a plot of the bile production from the second collection versus the storage period 6, 12 and 24 hrs. Figure 10 shows the gradual decrease of bile production as the storage period increases. The solid line column represents the bile production from liver stored with AFGP in Krebs solution. The dashed line column represents livers stored with Krebs solution only. There is a significant increase in bile production from livers stored with the AFGP at all the storage times. Although the bile flow decreased significantly after 24 hr. of storage, the bile production with AFGP showed an improvement over liver stored with Krebs

solution only.

In the 24 hr storage experiments with AFGP, the typical LDH activities during the perfusion process is shown by the solid line column in Figure 10. The dotted line column represent the results from the control liver perfusion, and the liver stored in Krebs solution are represented by the dashed line column. For the AFGP-perfused stored liver, the release of the enzymes is at a maximum during the first 5 min of the perfusion, and then decreases at later times to near control levels. The Krebs solution stored liver also reaches a maximum during the first 5 min. of perfusion. However, the decrease in activity level at later times remains significantly higher than control levels.

In utilizing the enzyme colorimetric tests, these results from LDH activity show the membrane protective capability of the AFGP. Since leakage of higher levels of LDH activity is reported to be associated with membrane damage, this test provides an indication of the integrity of the hepato cellular membrane. The results of the LDH tests show a significant decrease in activity for livers stored in AFGP, which indicates lessor damage to the cellular membrane. From these results, AFGP clearly provides cellular membrane protection during the storage process. As indicated by the increase in bile production, the AFGP protection of membrane leads to better preservation capability compared to Krebs solution alone.

EXAMPLE 7B

PRESERVATION OF RABBIT HEART

A preliminary experiment and a control experiment were performed in parallel using adult rabbit heart.

Two white laboratory rabbits (2-3 kg each) were anesthetized. Each heart was surgically removed. The control heart was perfused with Krebs solution for 30 sec. at 5°C and the aorta chamber injected with 5 ml of standard Krebs solution (5°C). The aorta was injected with a standard Krebs solution (at 5°C) containing 20 mg/ml of AFGP's-Fractions 1-5 and 7-8 (Table 1) (25/75, w,w) purified

by standard electrophoreses. Each heart was immediately placed in a small test tube containing Krebs solution at 5°C and placed into an ice/water bath at about 0°. The other heart was perfused with Krebs solution for 30 sec. at 5°C.

The control heart (without AFGP) was held at 0°C for 4 hr, then connected to a Langendorf perfusion system, and perfused with Krebs solution at 37°C (optionally containing some glucose) for one hr. During this time, the aorta beat weakly (or fluttered). At the end of one hour at 37° (physiological temperature), the aortic pressure was about 27 mm of water, aortic flow was negligible, and the flow rate through the heart was about 2 cc/min. This heart was nonvigorous, beating at about 30 beats per minute. Visually the portions of the heart tissue appeared to be dead or dying.

The experimental heart (with AFGP) was also held at 0°C for 4 hr, then connected to a Langendorf perfusion apparatus and perfused with Krebs solution at 37°C (optionally containing glucose). At the end of one hour at 37°C, the aortic pressure was over 100 mm water, aortic flow was 12 cc/min, and a cardiovascular flow rate of about 47 cc per min. was measured. This heart was vigorous, beating about 160 beats per minute. Visually, the heart looked robust, these measurements were close to the values for a normal heart.

EXAMPLE 8

Mouse embryos at the two cell stage were introduced in a T6 Whittingham medium with 40 mg/ml antifreeze glycopeptides and without an atmosphere of 5% CO_2 and a temperature of 37°C for 72h. Accidentally the concentration of CO_2 increased to about 8% and the temperature fluctuated while being most of the time at temperatures higher than 40°C. After incubation at these non optimal environmental conditions close to 80% of the mouse embryos developed to the blastocyst stage in the presence of the antifreeze protein while less than 50% developed to the blastocyst stage without the antifreeze protein. This result demonstrates

another useful property of the antifreeze proteins at hyperthermic temperatures and in a chemical environment that is not compatible with the cells.

EXAMPLE 9

MATERIALS AND METHODS FOR BOVINE OOCYTES

Immature bovine oocytes, in multilayered dense cumulus and with no alternation of the cytoplasm were obtained from selected follicles (2-6 mm) of cow and heifer ovaries brought to the laboratory in warm saline (0.9%). The follicles were aspirated with a 15 gauge needle within 80 minutes of slaughter. The oocytes were kept in standard buffer solution, PBS, (Dulbecco's phosphase buffered saline supplemented with 0.4 v/v BSA (bovine serum albumin), 0.34 mM pyruvate, 5.5 mM glucose and 15 mM Kanamycin). Prior to the hypothermic experiments the oocytes were introduced into Eppendorf vials containing 0.5 ml of the following solutions: (a) PBS; (b) PBS with 20 mg/ml AFP of Type I (Winter flounder), (c) PBS with 20 mg/ml AFP of Type II (Sea raven), (d) PBS with 20 mg/ml AFP of Type III (Ocean pout). (See Table 1A). (A concentration of 20 mg/ml was chosen because it is within the physiological range for all the "antifreeze" proteins). All three antifreeze proteins were purified were purified from fish blood plasma using Sephadex G875 as described in Kao et al. (55). Additional control experiments were performed with oocytes in a PBS solution with 0.1 M sucrose and in a PBS solution with 20% v/v FCS (fetal calf serum). The oocytes were incubated in the various solutions at 4°C for 24 hr.

When the oocytes were removed from the 4°C environment the integrity of their oolemma was determined using three different tests; morphological examination, fluorescein diacetate (FDA) staining, and trypan blue (TB) exclusion. The morphological examinations were performed using a Leitz microscope with phase contrast. The FDA staining test employs the fact that fluorescein diacetate is converted to a fluorescent compound only when it contacts hydrolases in the live cell. Oocyte viability and oolemma intactness

results in intense fluorescence, while nonfluorescence indicates nonviability (44,64,65). In our experiments the oocytes were exposed to PBS solution containing 5μg/ml FDA as described in Didion et al. (44). After 3 min exposure to the FDA the oocytes were placed on a slide under a coverslip. The oocytes were screened in a fluorescent microscope (Leitz), to which a photoreceptor was connected. The photoreceptor can evaluate quantitatively the intracellular fluorescence. Prior to the experiments with oocytes exposed to hypothermic conditions, similar, control experiments were performed with fresh oocytes, and used to establish a quantitative criteria for evaluating the integrity of the oolemma. An oocyte was considered to have an intact oolemma after hypothermic exposure if the quantitative reading of the photoreceptor was within two standard deviations of the mean in fresh oocytes. Trypan blue exclusion (TB) is commonly used to determine the membrane integrity (44). Exclusion of trypan blue is indicative of an intact oolemma. In the TB exclusion test an aqueous solution containing 0.1% TB was added to the oocytes in the PBS solution for 3 min. The oocytes were then examined with a phase contrast microscope (Leitz), to determine if the trypan blue was excluded.

Additional tests were performed to determine the ability of the oocytes to undergo in vitro maturation and in vitro fertilization. Following hypothermic exposure, the oocytes with their cumulus cells were incubated in a standard maturation medium, TCM-199 with 10% v/v FCS supplemented with 500 mg/ml hormones, LH (NiH-LH-B9) and FSH (bFsH-LER-1596-1) and granulosa cells. The granulosa cells were used at concentration of approximately 5 x 10^6 cells/ml and were obtained by dissection of small, fresh follicles, which were washed and recollected in the maturation medium. The oocytes were cultured in the incubation medium at 39°C in an atmosphere of 5% CO_2 in air, for 24 hr. After incubation, the oocytes were fixed in an acetic acid: alcohol mixture (1:3 v/v) and stained after 24 hrs with

lacmoid stain to determine the percentage which had undergone in vitro maturation. Microscopic evidence of the second metaphase stage, MII, and/or extrusion of the first polar body was used to determine oocyte meiotic maturation. Incubated oocytes which were not used to study in vitro maturation were fertilized, in vitro. Immediately prior to insemination the oocytes were transferred for 5 min to a PBS solution containing 0.1% hyaluronidase (Sigma) to remove part of the cumulus cells. Then the oocytes were placed in an insemination medium, (Brackett and Oliphant) B-O, (67), supplemented with 1.9 mg/ml caffeine. Semen frozen in straws was used for in vitro fertilization. After thawing the straws in 35°C water the semen was washed by centrifugation in B-O medium. Capacitation was achieved by incubating the sperm for 15 min in B-O medium supplemented with heparin (100 mg/ml) as described by Fukui et al., (66). Capacitated bull sperm at a final concentration of 10^6 cells/ml were then coincubated with in vitro matured oocytes. After 14-16 hr of culture the oocytes were fixed and stained using the technique described earlier for studies on maturation. Oocyte morphology (cytoplasm, oolemma) and fertilization were evaluated.

EXAMPLE 10

MATERIALS AND METHODS FOR ION CURRENT FOR PIG GRANULOSA CELLS

Pig granulosa cells were harvested from healthy antral follicles, 3 to 6 mm in diameter, obtained from pig ovaries collected immediately after slaughter. The cells were washed twice in a standard buffer solution, PBS (Dulbecco's phosphate buffered saline supplemented with 0.4 v/v BSA (bovine serum albumin), 0.34 Mm pyruvate, 5.5 Mm glucose and 14 Mm Kanamycin) and resuspended in a recording solution containing (Mm), 130 NaCl, 3 KCl, 10 $CaCl_2$, and 10 HEPES Ph 7.2 Recording was carried out using the patch clamp technique in the whole cell configuration. The electrodes were made of borosilicate glass using a horizontal puller, had an outside diameter of 1-2 μl and a resistance of 5 to

20 Ω when dipped into the bath solution. The whole cell electrode solution contained (mM0 140 KC1, 1 EGTA and 10 HEPES. A List EPC7 amplifier was used to measure the cell current and an ITC16 interface connected to an Atari Mega 4 computer and the program Patch Program Instrutech to store and analyze the data. During typical experiments, performed at room temperature, 22°C, the granulosa cells were introduced in 100 μl drops under the microscope and currents were elicited using 200 ms depolarizing pulses from -80 Mv to -30mV and 1000 ms depolarizing pulses from -40 Mv to 60 Mv for the calcium and potassium currents, respectively. After the normal currents were established and recorded, the AFP in a bolus of 5 μl, were injected in the droplet using a micromanipulator. The concentration of the AFP in the bolus was set to achieve concentrations of 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml in the 100 μl droplet. The time of the injection of the bolus was coordinated with the current reading to generate complete current records at the instant of the injection and every 20 seconds thereafter, Separate experiments were performed for the K^+ and Ca^{+2} currents. At least three experiments were performed for each of the AFP concentrations listed above. Negative control experiments were also performed in 100 μl droplets with 0.1 M bovine serus albumin and 0.1 M soybean trypsin inhibitor.

While only a few embodiments of the invention have been shown and described herein, it will become apparent to those skilled in the art that various modifications and changes can be made in the preservation composition and method to preserve viable plant or animal cells, or viable tissue or viable organs or plants viable animals which cells, tissue, organs or plant or animal are viable upon thawing without departing from the spirit and scope of the present invention. All suc h modifications and changes coming within the scope of the appended claims are intended to be carried out thereby.

WE CLAIM:

1. A composition useful in the protection and preservation of viable cells and cell membranes of an animal independently subjected to :

(i) hypothermal temperature conditions from the physiological temperature to about 0°C,

(ii) vitrification temperature conditions from about 0°C to about -190°C,

(iii)freezing temperatures from between about -0.5°C and 4K,

(iv) hyperthermal temperature conditions from the physiological temperature up to about 10°C above the physiological temperature, or

(v) nonphysiological chemical conditions, or

(vi) or combinations thereof which composition comprises:

one or more thermal hysteresis proteins; and

a biologically compatible aqueous preservation solution.

2. A method of preserving cells, cell membranes and animal tissue for human or animal food consumption, which method comprises:

contacting the animal tissue with an effective concentration of the composition of Claim 1.

3. The composition of Claim 1 wherein the cell and cell membranes are from organized tissue selected from the group consisting of skin, brain, cornea, liver, lung, heart, kidney, bladder, stomach, spleen, pancreas, muscle, testes, uterus, and ovary;

the hypothermal conditions (i) are from the physiological temperature of the animal to about 0°C; the one or more thermal hysteresis protein has a molecular weight of between about 2,000 and 100,000 daltons; and

the biologically compatible aqueous preservation solution is perfused through the vascular network of the organized tissue or otherwise contacted with the cell membrane.

4. The composition of Claim 3 wherein the animal cell or cell membrane to be preserved are selected from fish, mammal or bird;

the thermal hysterisis protein has a molecular weight of between about 2,000 and 50,000 daltons; and

the biologically compatible aqueous solution is selected from sterile normal plasma, normal saline containing 0.9% by weight sodium chloride, normal saline containing 0.95% by weight sodium chloride, Ringers solution, mammalian Ringer UK and Canada solution, lactated Ringer's solution, acetated Ringer's solution, Locke's solution, Tyrode's solution, Krebs solution, Krebs-Henseleit solution, Krebs Ringer phosphate solution, Krebs serum substitute solution, Krebs Improved Ringer II solution (calcium ion free), or Krebs Improved Ringer III (low bicarbonate, low phosphorus), Schumarach liver solution, Krebs kidney perfusion solution, Bahlman kidney perfusion solution, University of Wisconsin preservation solution, Collins solution, EuroCollins solution, Ross-Marshall solutions purified plasma from the vertabrate from which the organ or tissue is obtained, Fulgaff perfusion solution, Wikman-Coffelt solution or combinations thereof; and

the thermal hysteresis protein is present in the aqueous preservation solution in between about 0.01 and 50 mg/ml.

5. The composition of Claim 1 wherein the cell and cell membrane are selected from generally unorganized small cell moieties selected from the group consisting of ova, sperm and embryos;

the thermal hysteresis protein is selected from protein having a molecular weight of between about 2,000 and 100,000 daltons,

and the biologically compatible aqueous solution is selected from a vitrification solution wherein no ice crystals exist in the solution at temperatures below about 0°C to the unaided eye or an apparent vitrification solution wherein no ice crystals are visible in the solution at

temperatures below -56°C at a magnification of 300 to 400 power.

6. The composition of Claim 5, wherein the vitrification solution further comprises glycerol, ethylene glycol, propylene glycol, dimethyl sulfoxide, polyvinylpyrrolidone, glucose, propanediol, carboxymethyl cellulose, or mixtures thereof.

7. The composition of Claim 1 wherein the thermal hysteresis protein is selected from peptides substantially identical to antifreeze glycopeptide obtained from the Antarctic nototheniidae fish as fractions 1 - 5, 6, 7 and 8.

8. The composition of Claim 7 wherein, of the antifreeze glycopeptide present, about 25% by weight comprises fractions 1 - 5 and about 75% by weight of fractions 7 and 8.

9. The composition of Claim 1 wherein the thermal hysteresis protein are selected from antifreeze protein or antifreeze glycoprotein useful to control and to inhibit ion transport across cell membranes.

10. The composition of Claim 9 wherein the antifreeze glycoprotein is selected from Antarctic Nototheniidae fish and the antifreeze protein is selected from Group (I) winter flounder, Group (II) sea raven or smelt or Group (III) ocean pout or eel pout, and the ions inhibited independently comprise Ca^{+2}, K^{+}, N^{+} and combinations thereof.

11. The composition of Claim 1 wherein the antifreeze glycoprotein is selected from Antarctic Nototheniidae fish and the antifreeze protein is selected from Group (I) winter flounder, Group (II) sea raven or smelt or Group (III) ocean pout or eel pout, and the ions inhibited comprise Ca^{+2}, K^{+}, Na^{+} and combinations thereof.

12. A method of protecting or preserving viable plant or animal cell membranes which method comprises contacting the viable plant or animal cell membrane with an acceptable concentration of the composition of Claim 1.

13. A method of protecting or preserving viable animal cell or membranes which method comprises contacting the

viable animal cell membrane with an acceptable concentration of the composition of Claim 1 under freezing temperatures of from between about -0.5°C and 4K.

14. The method of Claim 1 wherein the concentration of the biologically compatible substance is present in an organ of an animal in between about 0.1 and 50 mg/ml of solution within the lumen space of the blood vessels or interstetial space between cells.

15. The composition of Claim 1 wherein the thermal hysteresis protein comprises a polypeptide, glycopolypeptide, or a polypeptide or glycopeptide covalently bonded to a carrier, or mixtures thereof.

16. The composition of Claim 1 wherein the thermal hysteresis protein is a polypeptide or glycopolypeptide is obtained from a natural animal source or is substantially identical in amino acid sequence to a polypeptide or glycopolypeptide obtained from a natural animal source.

17. The composition of Claim 16 wherein:

the polypeptide comprises multiple regions of alanine-alanine-threonine- or -alanine-alanine-alanine-, or

the glycopolypeptide which comprises multiple regions of alanine-alanine-threonine, wherein covalently attached to substantially all of the threonine residues is the disaccharide, ß-D-galactopyranosyl-(1→3)-2-acetamide-2-deoxy-α-D-galactopyranose.

18. The composition of Claim 17 wherein the molecular weight of the polypeptide or glycopeptide is between about 2,000 and 50,000 daltons.

19. The composition of Claim 1 which further independently includes compounds independently selected from glycerol, dimethyl sulfoxide, ethylene glycol, poly-vinyl-pyrrolidone, glucose, propanediol, carboxymethyl cellulose or mixtures of these compounds which are known to protect individual cells from damage by freezing.

20. The composition of Claim 1 wherein the thermal hysteresis protein is obtained from a fish, amphibian, bird, invertabrate, or reptile.

21. The composition of Claim 20 wherein the antifreeze peptide is similar in amino acid sequence to, obtained from or derived from the serum or fluids of an animal selected from amphibians, reptiles, insects, worms, Arctic fish, or Antarctic fish.

22. The composition of Claim 15 wherein the biologically compatible carrier thermal hysteresis protein is selected from an antibody, gelatin, biocompatible polymer, peptide, sugar or carbohydrate.

23. The composition of Claim 1 which is useful in the survival, functionality, stability and structural integrity of biological materials, selected from proteins, lipids, enzymes, carbohydrates cell membranes, microorganisms, animal cells, tissues, or organs subjected to nonphysiological temperatures, either higher or lower than the normal physiological temperatures or nonphysiological chemical environments by interacting with the proteins, lipids, carbohydrates or the cell membranes.

24. The composition of Claim 1 which is useful in improving survival, functionality, stability and structural integrity of proteins, enzymes, lipids, carbohydrates, cell membranes, animal cells, microorganisms, tissues, or organs independently subjected to:

temperatures lower than 0°C in the presence of ice crystals by modification of the structure of the ice crystals in the immediate vicinity of the proteins, enzymes, lipid or cell membranes;

temperatures lower than 0°C by reducing the number and the size of the ice crystals or by completely eliminating the ice crystals in the immediate vicinity of the proteins, enzymes, lipids or the cell membranes, or

temperatures lower than 0°C in the presence of ice crystals by modifying the mode in which solutes are rejected by the ice formation and thereby changing the chemical composition of the solutions surrounding the proteins, enzymes, lipids or cell membranes.

25. The composition of Claim 1 which is useful in

binding other macromolecules or conjugates to proteins, enzymes, lipids, carbohydrates or cell membranes.

26. The composition of Claim 1 wherein the thermal hysteresis protein are antifreeze protein having an amino acid sequence similar to, obtained from, or derived from the body fluids of animals selected from insects, amphibians, reptiles, worms, fish, from Arctic, Antarctic, North Temperate or South Temperate zone, or from genetic recombinant DNA processes or synthetic peptide processes.

27. The composition of Claim 26 wherein the thermal hystereis protein is obtained or derived from the body fluids of Antarctic or Arctic fish.

28. The composition of Claim 27 wherein the thermal hysteresis protein is an antifreeze glycopeptide substantially identical to or obtained from or derived from the Antarctic fish is selected from the family Nototheniidae, including the species D. Mawsoni and P. Borchgnevinki, or antifreeze protein independently selected from Arctic winter flounder (Type I), sea raven or smelt (Type II) or the Antarctic eel pout (Type III) Rhigophile dearborni.

29. The composition of Claim 1 wherein the thermal hysteresis protein is selected from a polypeptide, a glycopeptide, a polypeptide covalently bonded to biologically acceptable carrier, a glycopolypeptide covalently bonded to a carrier or mixtures thereof.

30. The composition of Claim 1 used to protect and stabilize membranes are also used in preservation of food; in cosmetics to restore, preserve or repair skin tissue; or in diseases associated with the instability of cell membranes.

31. The composition of Claim 1 used to control or to inhibit ion channels in treating diseases associated with the intracellular-extracellular ion transport across the cell membrane.

32. The composition of Claim 1 used to attach and interact with cell membrane, in attaching various macromolecules or conjugates to the antifreeze proteins and facili-

tating their attachment to the cell membrane.

33. A method for enhancing the preservation, survival, functionality, stability and structural integrity of biological materials, at nonphysiological temperatures or in nonphysiological chemical compositions, including animal proteins, enzymes, lipids, carbohydrates, cell membranes, cells, microorganisms, tissues, or organs, which method comprises:

(a) contacting with the thermal hysteresis protein composition of Claim 1 in sufficient concentration to interact with the animal proteins, enzymes, lipids, cell membranes, cells, microorganisms, tissues or organs,

(b) exposing the combination of step (a) to the nonphysiological condition, and

(c) removing the thermal hysteresis protein and returning the proteins, enzymes, lipids, carbohydrates, cell membranes, animal or plant cells microorganisms, tissues, organs, to a physiological temperature and environment composition.

34. The method of Claim 33 wherein the nonphysiological condition include hypothermal temperatures between about physiological and 0°C for preservation of animal proteins, enzymes, lipids, cell membranes, cells, microorganisms, tissues, or organs.

35. A method for the preservation of animal proteins, enzymes, lipids, cell membranes, cells, microorganisms, tissues, or organs at temperatures below 0°C to 4K, which method comprises:

(a) bringing in contact with the thermal hysteresis protein of Claim 1 optionally with addition the other cryoprotective compounds;

(b) cooling to lower temperatures by cooling means and either vitrifying or freezing the system according to the various concentrations and cooling rates using higher concentrations of additional compounds, such as propylene glycol or glycerol and higher cooling rates

which lead to vitrification and to lower freezing temperature.

(c) maintaining the animal proteins, lipids, cell membranes, animal cells, microorganisms, tissues, or organs at these temperatures for time periods between about 24 hr and 10 years,

(d) warming by warming means to physiological temperature conditions, and

(e) removing the antifreeze glycoproteins and the other compounds and replacing them by physiological compatible solutions.

36. The composition of Claim 1 wherein the viable cells and cell membranes of the animal are subjected only to (iii) freezing temperatures of between about -0.5°C and 4K.

37. The composition of Claim 36 wherein the temperature is between about-0.5°C and -180°C.

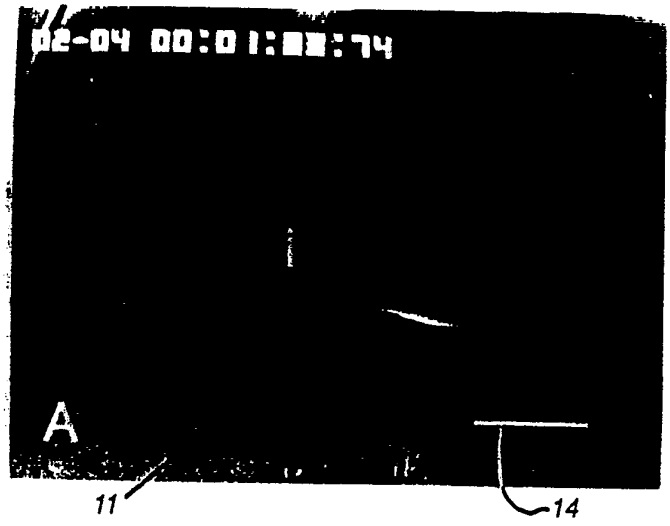


FIG._1A

FIG._1B

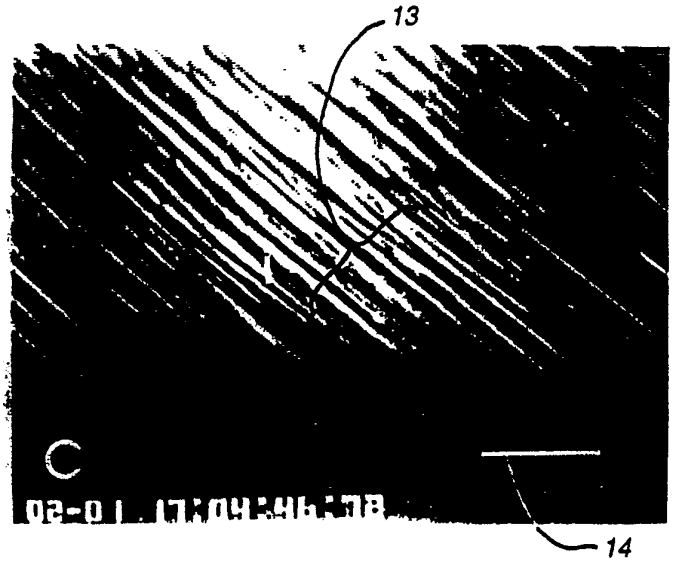


FIG._1C

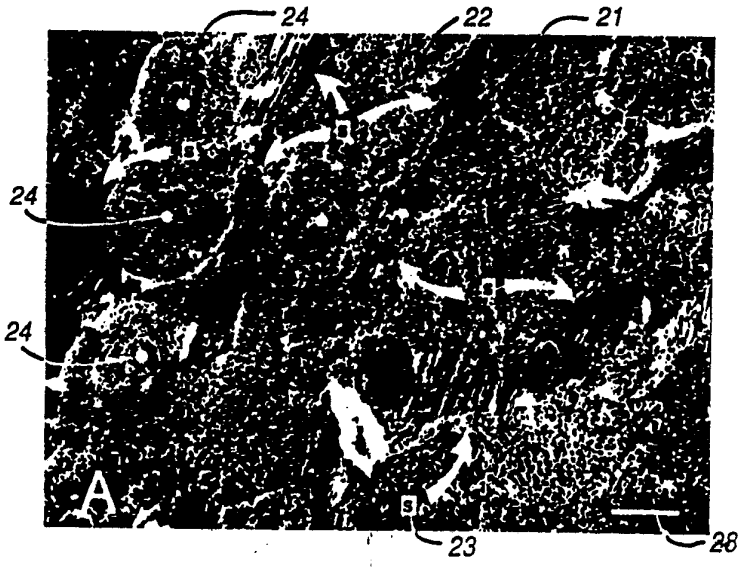


FIG._2A

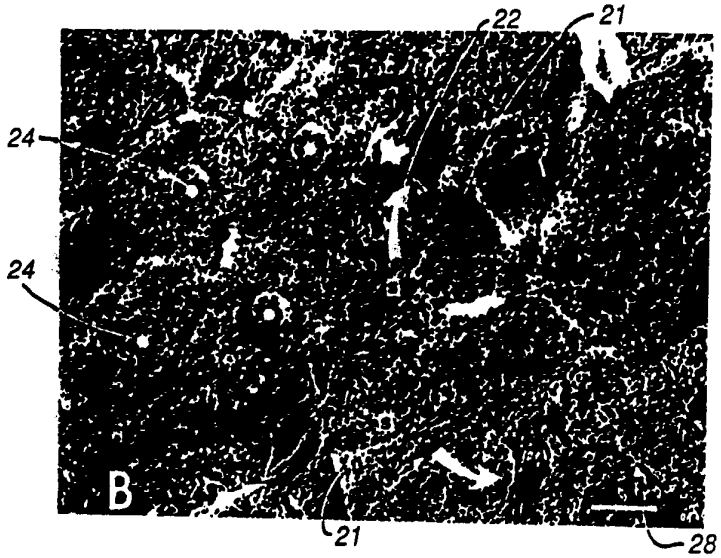


FIG._2B

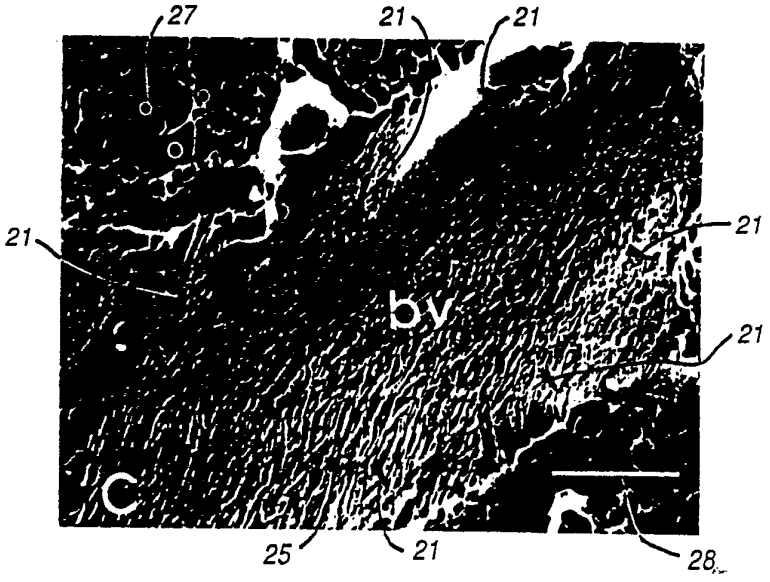


FIG._2C

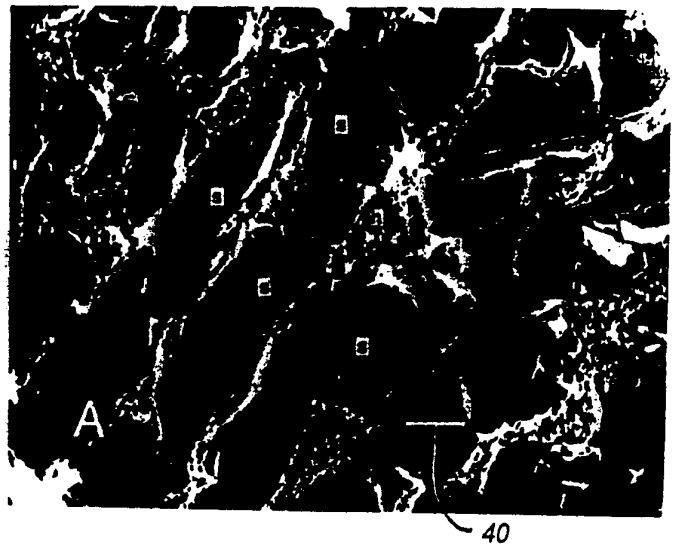


FIG._3A

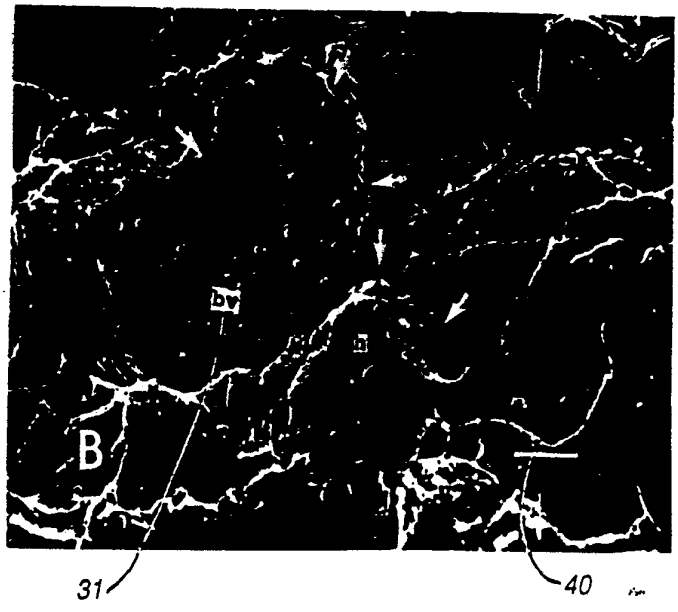


FIG._3B

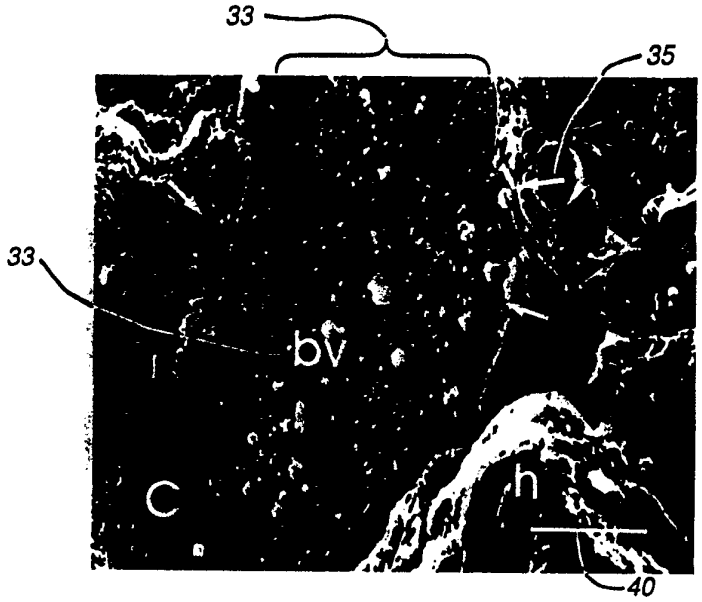


FIG._3C

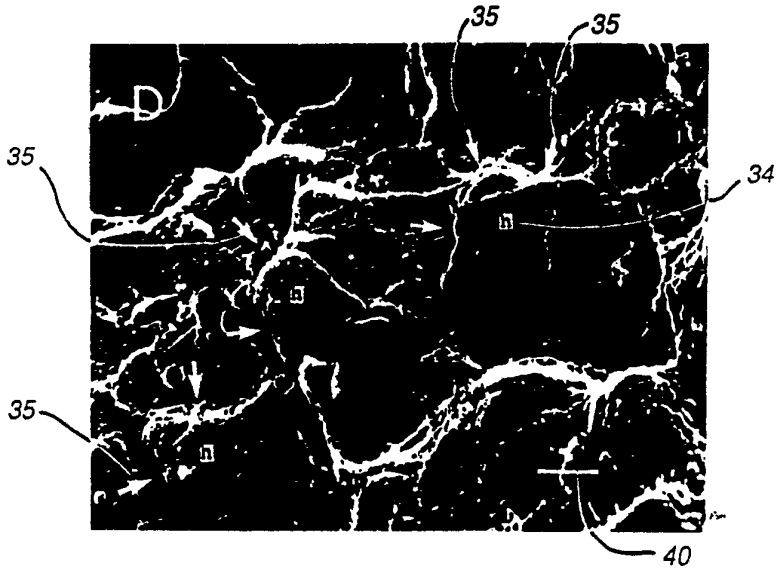


FIG._3D

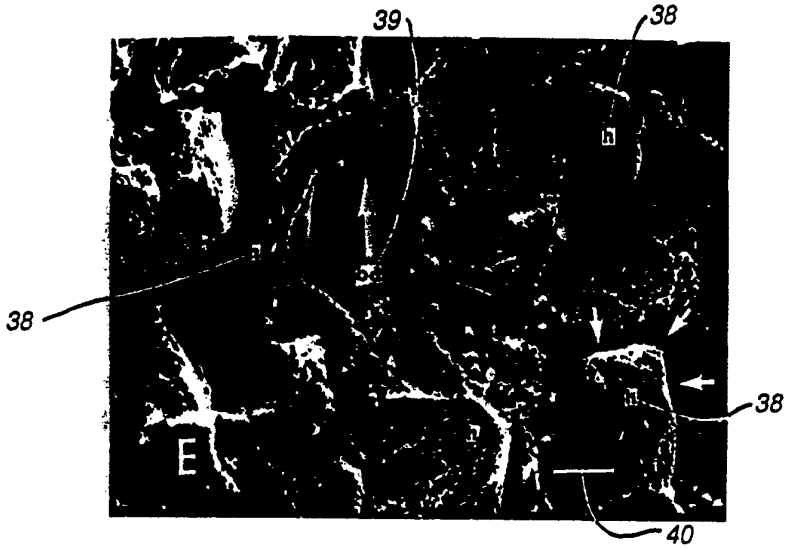


FIG._3E

FIG._4A

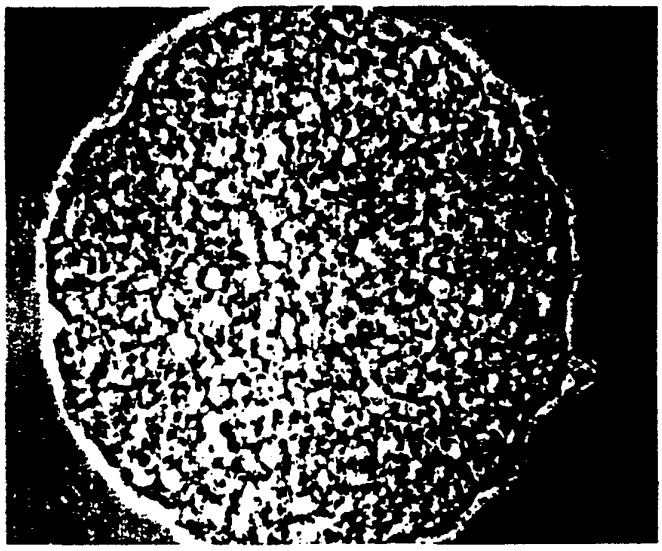


FIG._4B

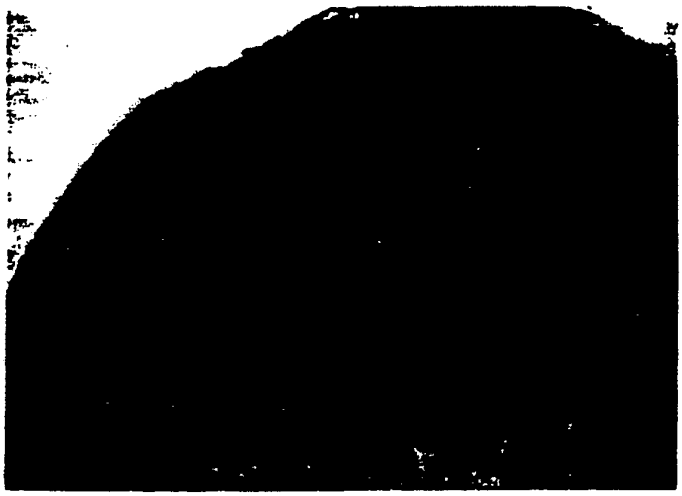


FIG._4C

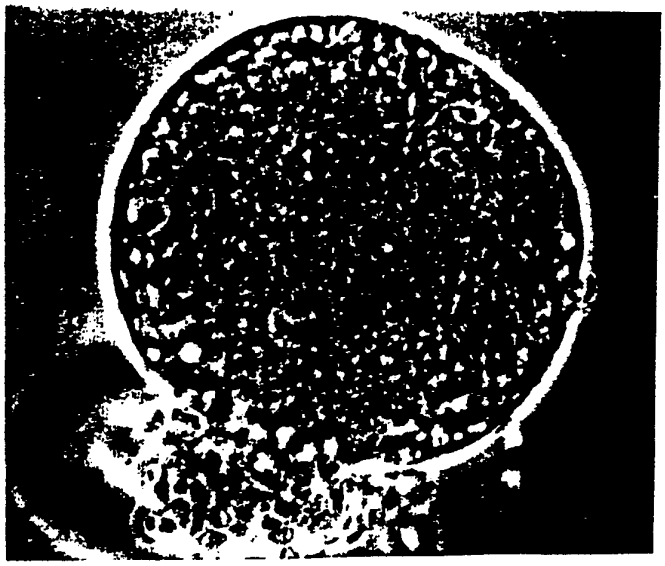


FIG._4D

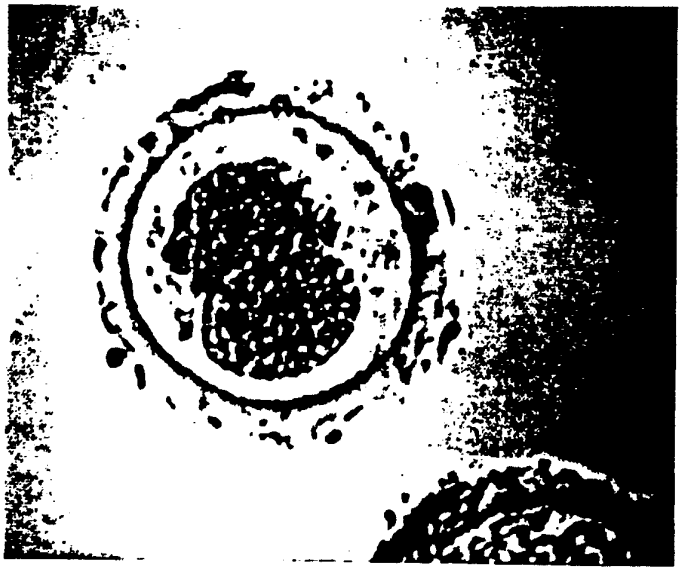


FIG._5A

FIG._5B

FIG._6A

FIG._6B

FIG._6C

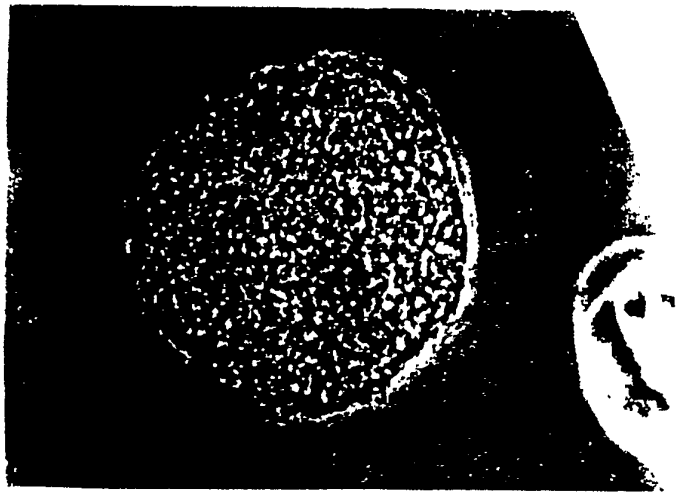


FIG._7A

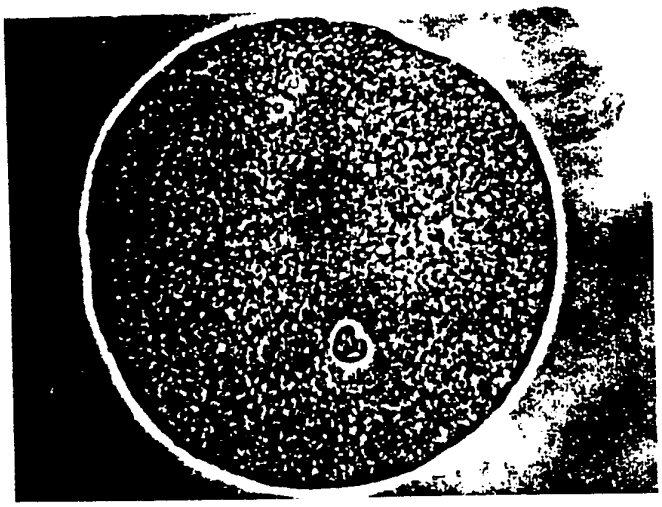


FIG._7B

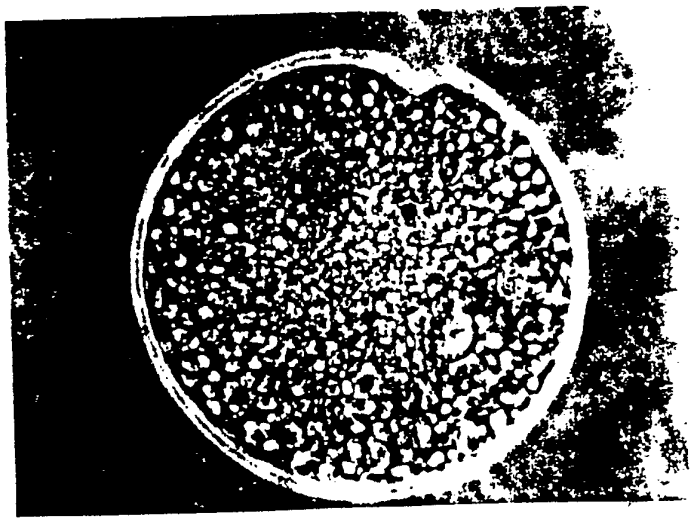


FIG._7C

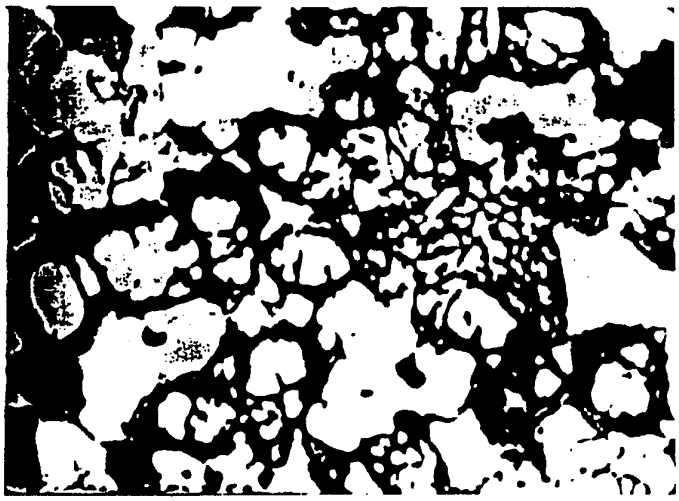


FIG._8

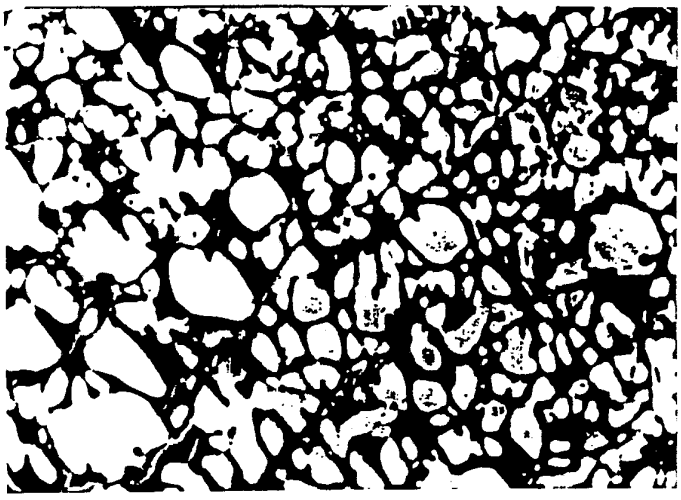


FIG._9

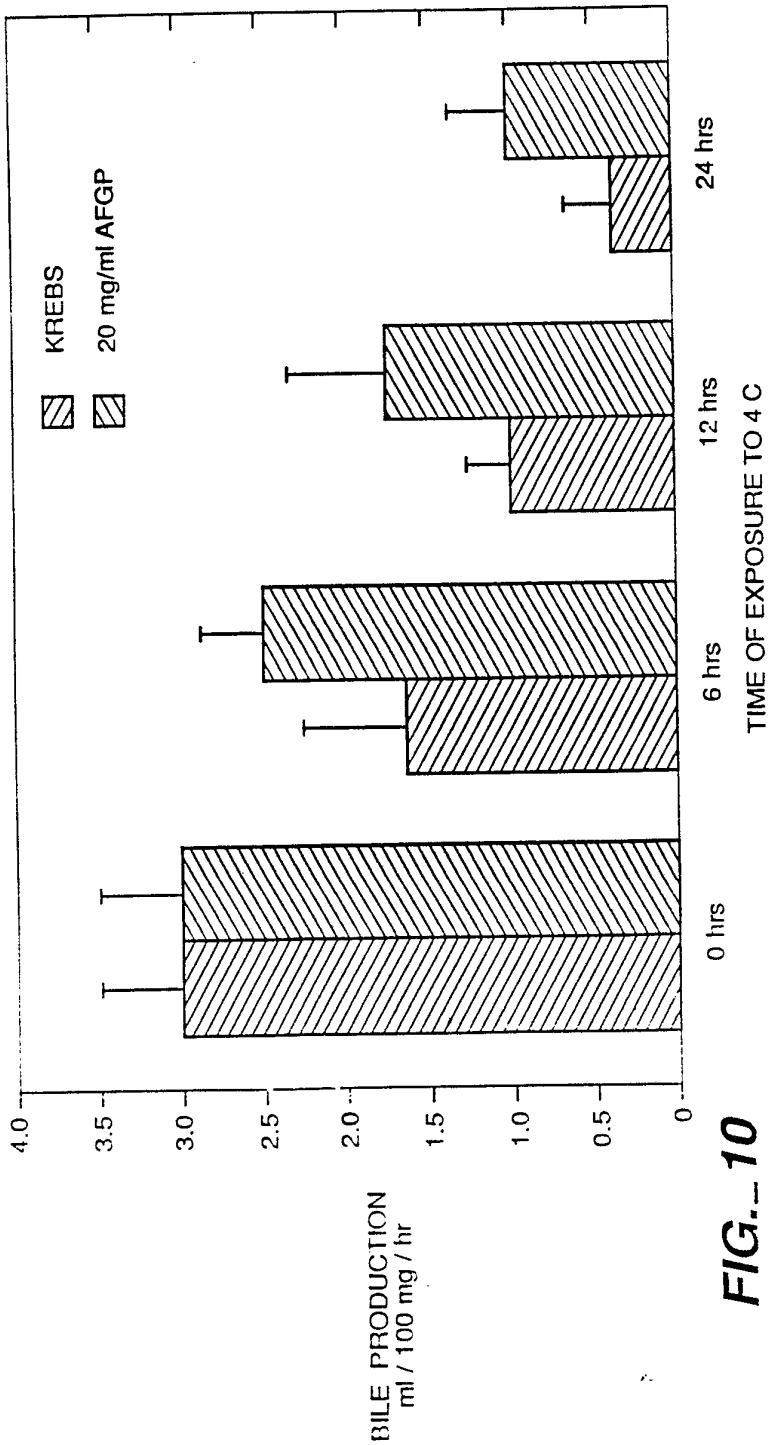

KREBS
20 mg/ml AFGP
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
BILE PRODUCTION
ml / 100 mg / hr
0 hrs
6 hrs
12 hrs
24 hrs
TIME OF EXPOSURE TO 4 C

FIG._10

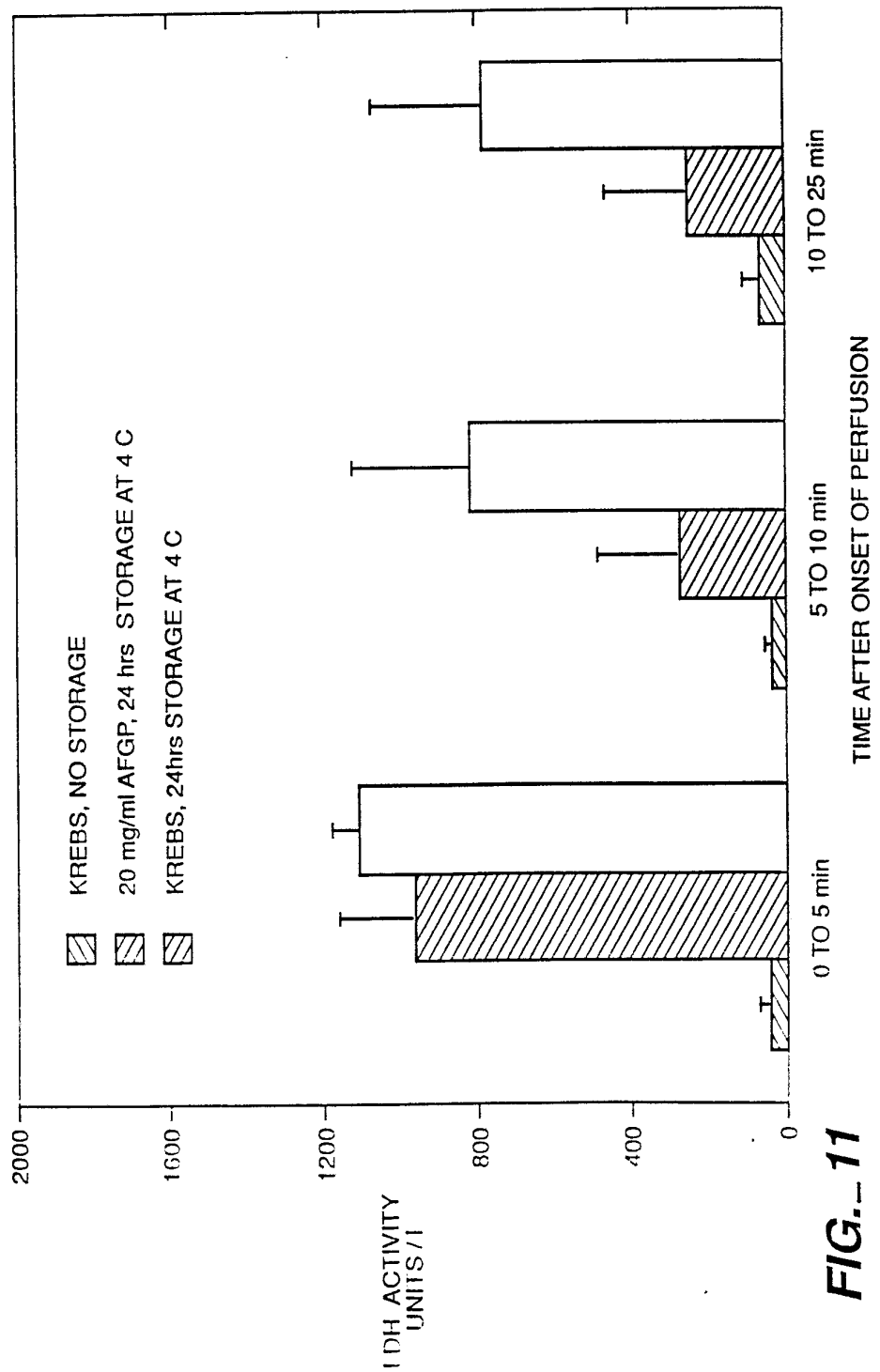

KREBS, NO STORAGE
20 mg/ml AFGP, 24 hrs STORAGE AT 4 C
KREBS, 24hrs STORAGE AT 4 C
LDH ACTIVITY UNITS/l
2000
1600
1200
800
400
0
0 TO 5 min
5 TO 10 min
10 TO 25 min
TIME AFTER ONSET OF PERFUSION

FIG._11

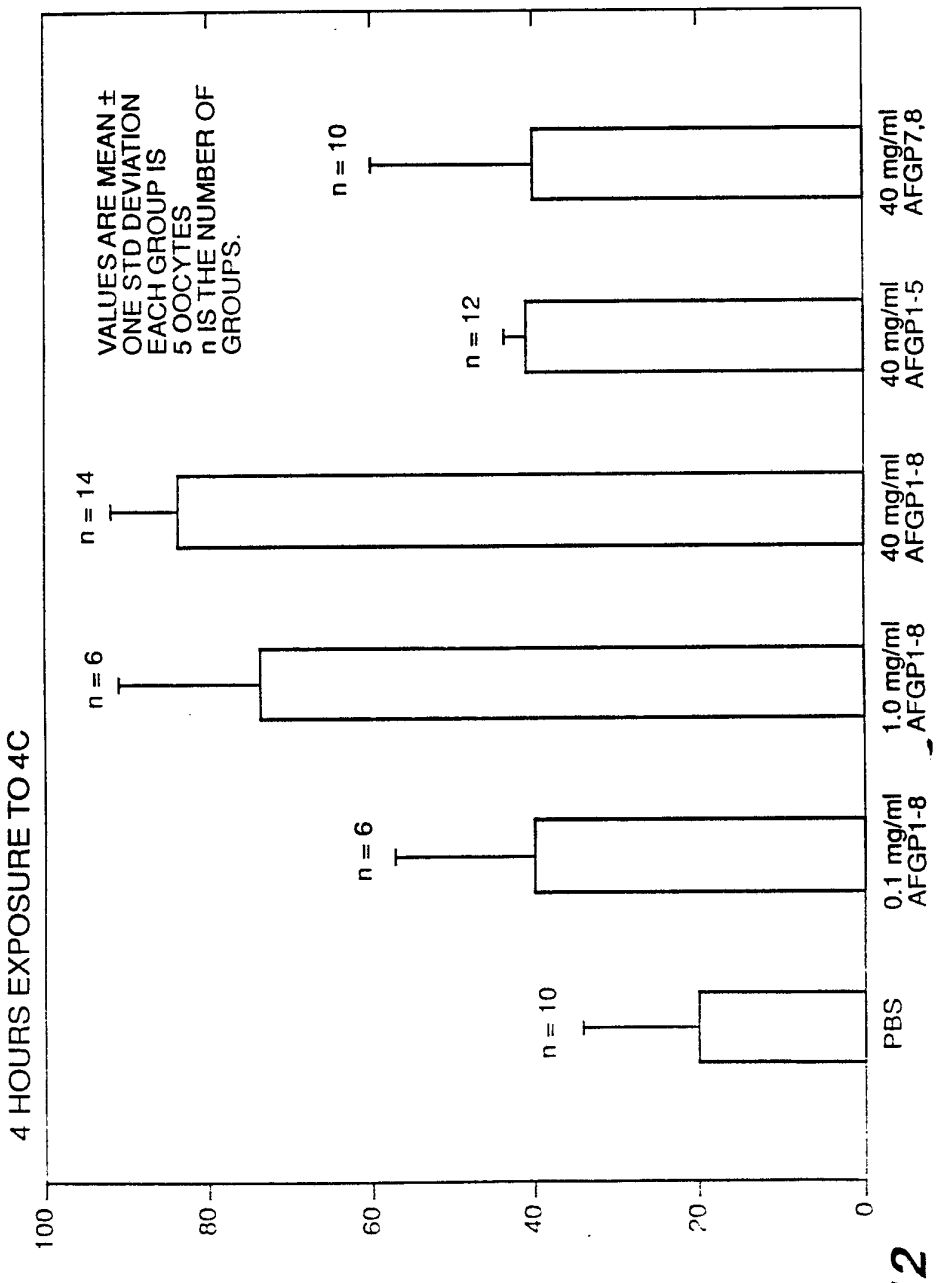

4 HOURS EXPOSURE TO 4C
VALUES ARE MEAN ± ONE STD DEVIATION EACH GROUP IS 5 OOCYTES n IS THE NUMBER OF GROUPS.
n = 10
n = 6
n = 6
n = 14
n = 12
n = 10
PBS
0.1 mg/ml AFGP1-8
1.0 mg/ml AFGP1-8
40 mg/ml AFGP1-8
40 mg/ml AFGP1-5
40 mg/ml AFGP7,8
0
20
40
60
80
100
PERCENTAGE OOCYTES WITH NORMAL MEMBRANE POTENTIAL AFTER HYPOTHERMIC EXPOSURE

FIG._12

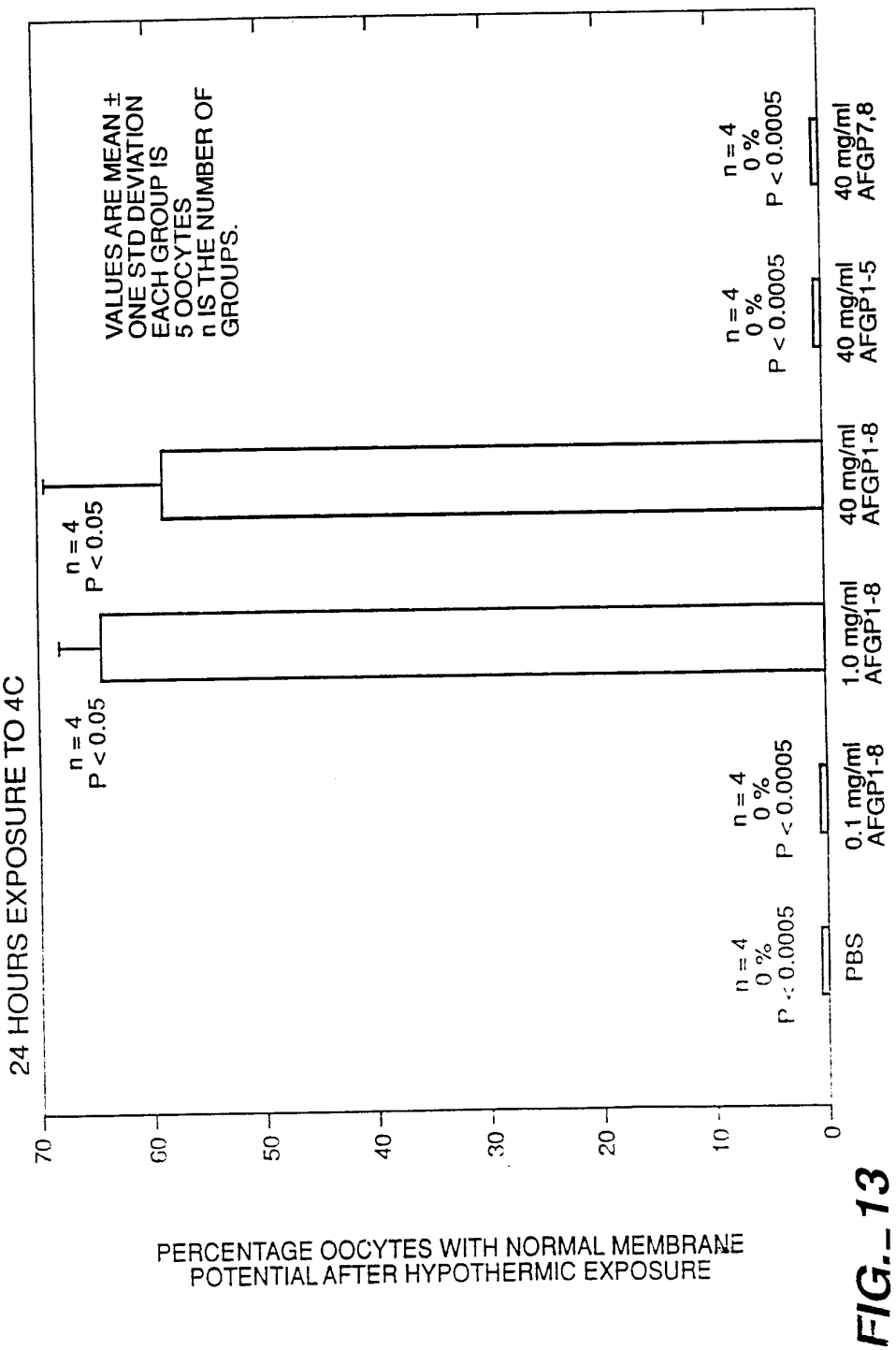

24 HOURS EXPOSURE TO 4C
VALUES ARE MEAN ± ONE STD DEVIATION EACH GROUP IS 5 OOCYTES n IS THE NUMBER OF GROUPS.
n = 4 P < 0.05
n = 4 P < 0.05
n = 4 0 % P < 0.0005
n = 4 0 % P < 0.0005
n = 4 0 % P < 0.0005
n = 4 0 % P < 0.0005
70
60
50
40
30
20
10
0
PBS
0.1 mg/ml AFGP1-8
1.0 mg/ml AFGP1-8
40 mg/ml AFGP1-8
40 mg/ml AFGP1-5
40 mg/ml AFGP7,8
PERCENTAGE OOCYTES WITH NORMAL MEMBRANE POTENTIAL AFTER HYPOTHERMIC EXPOSURE

FIG._13

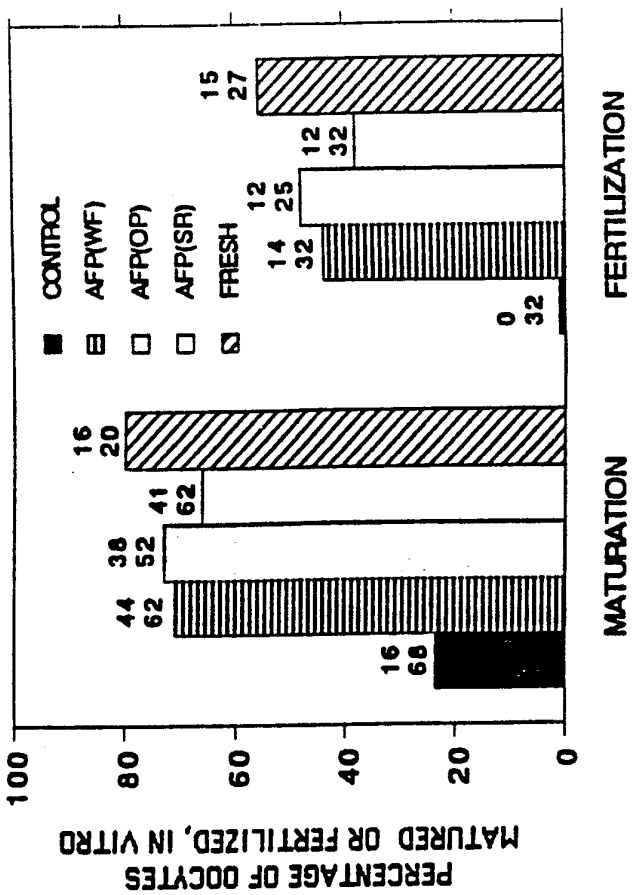


FIGURE 15

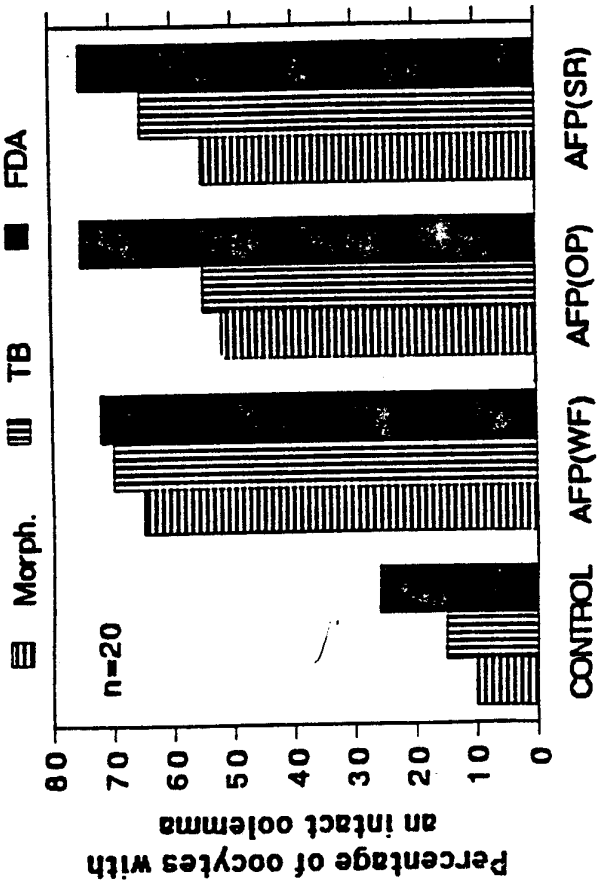


FIGURE 14

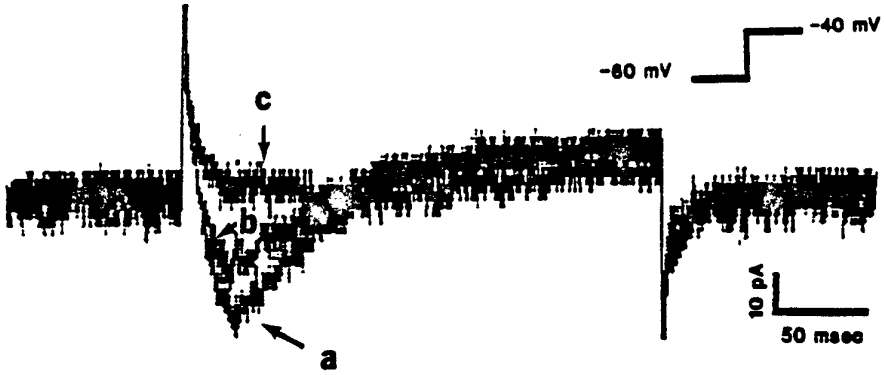


FIGURE 16

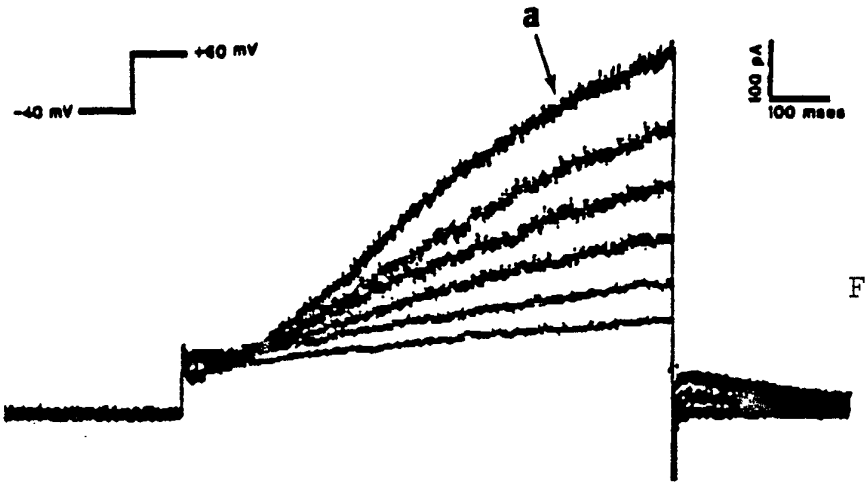


FIGURE 17

FIGURE 18

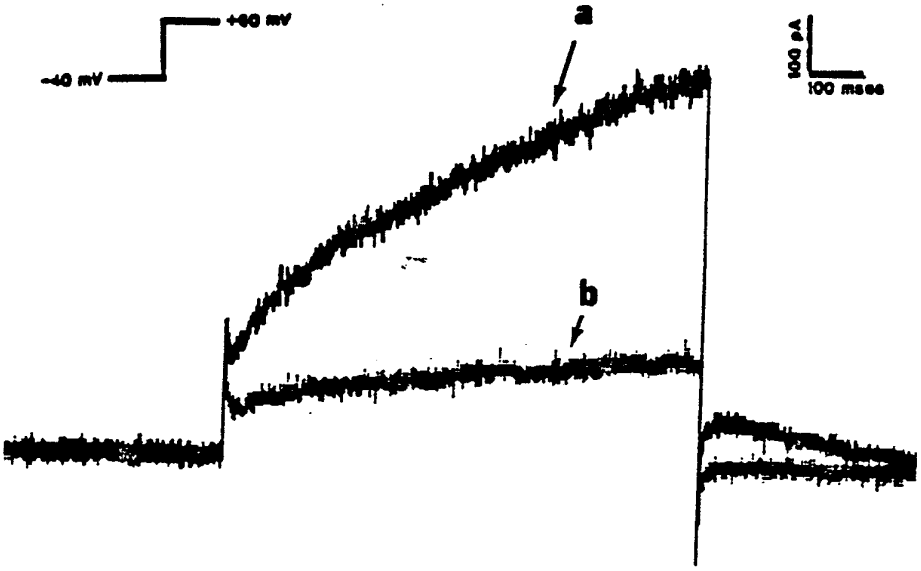


FIGURE 19

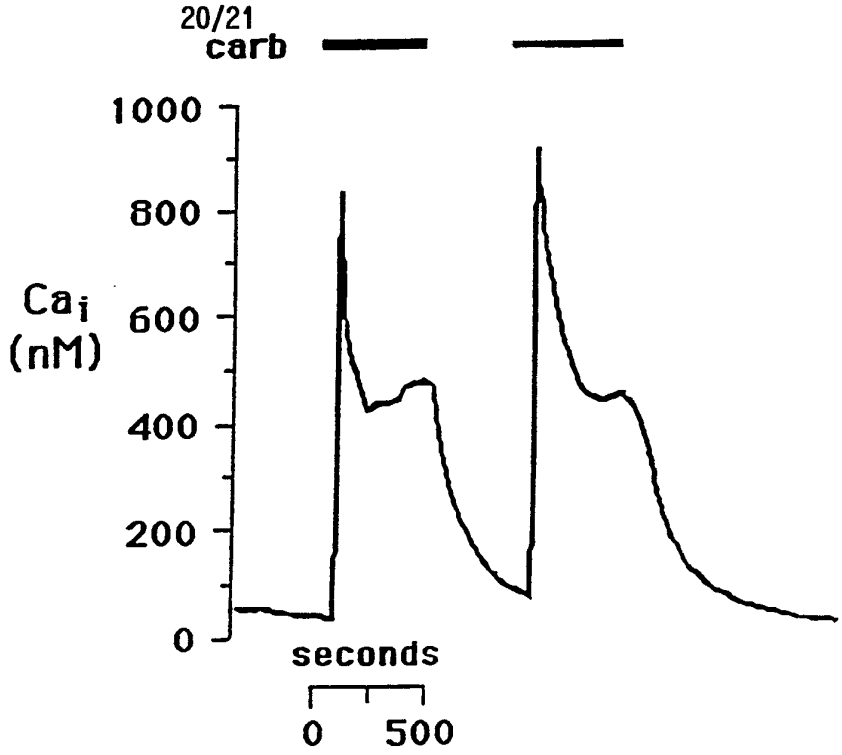


FIGURE 20

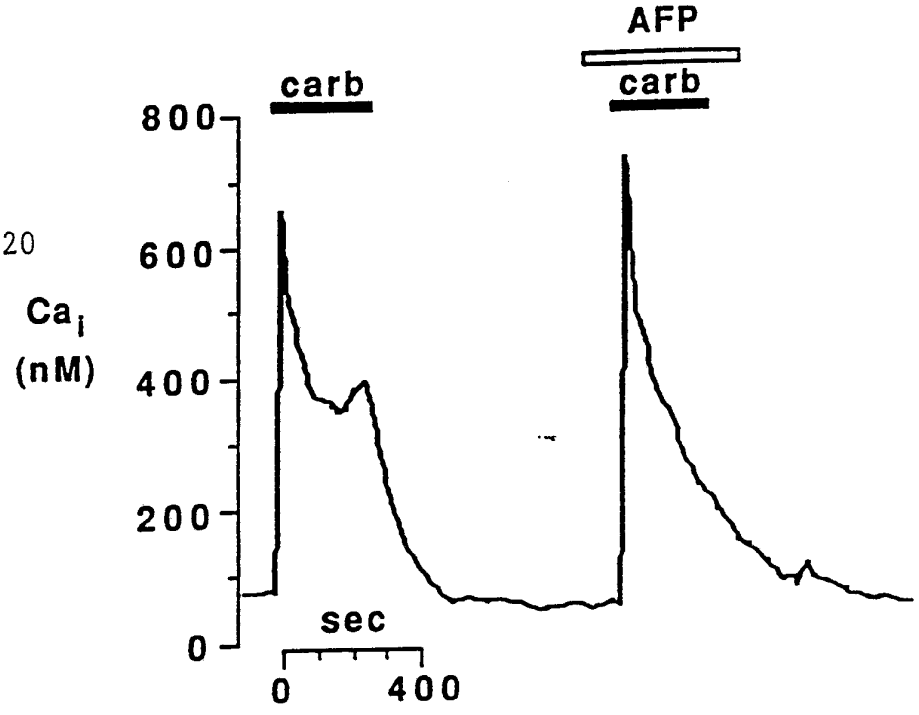


FIGURE 21

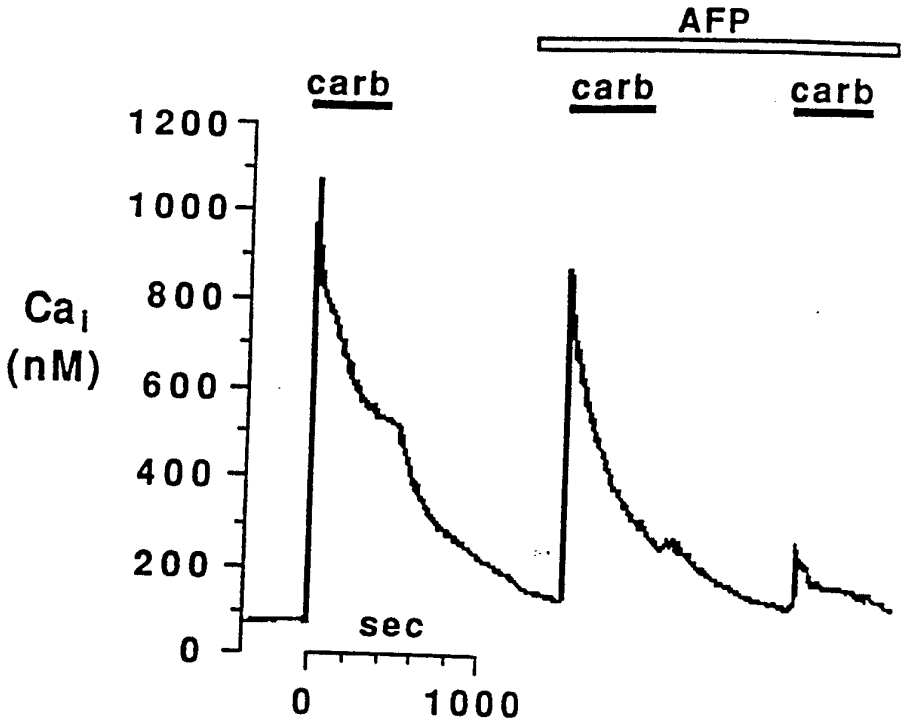
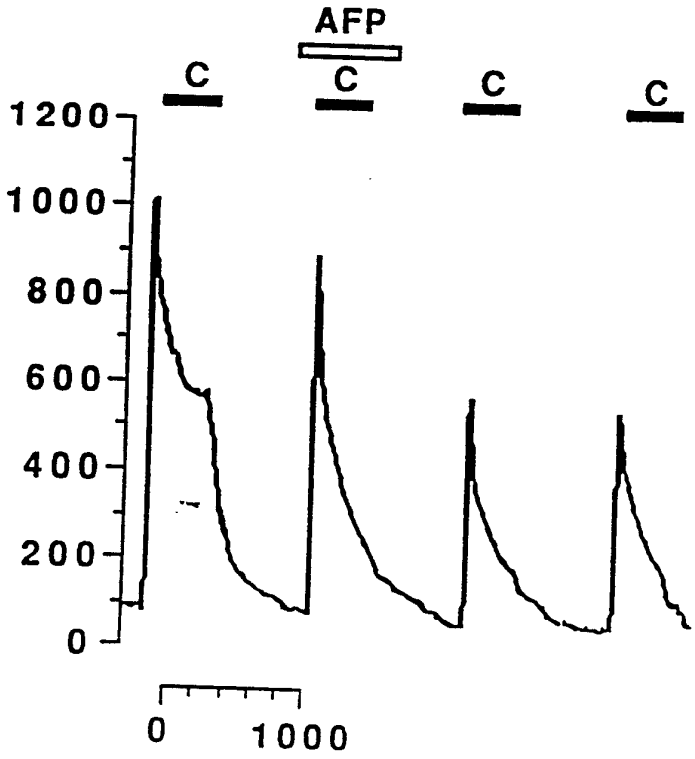


FIGURE 22



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00452

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 37/00
US CL : 514/12

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	514/12

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

PERSONAL FILES ON SUBJECT

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	ANN.REV.PHYSIOL., Volume 45, issued 1983, Duman et al., "The Role of Hemolymph Proteins in the Cold Tolerance of Insects", pages 261-270, see entire document.	1-37/ 1-37
A	ANN. REV. PHYSIOL., Volume 45, issued 1983, DeVries, "Antifreeze Peptides and Glycopeptides in Cold-Water Fishes", pages 245-260, see entire document.	1-37
Y	A. Karow et al., "Organ Preservation for Transplantation", published 1974 by Little, Brown and Company (Boston), pages 86-107, see entire document.	1-37
A	COMMENTS AGRIC. & FOOD CHEM., Volume 1, No. 3, issued 1988, R. E. Feeney, "Inhibition and Promotion of Freezing: Fish Antifreeze Proteins and Ice-Nucleating Proteins", pages 147-181, see entire document.	1-37
X/Y	CRYOBIOLOGY, Volume 25, issued 1988, Knight et al., "Solute Effects on Ice Recrystallization: An Assessment of Technique", pages 55-60, see entire document.	1-37/1-37

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
16 APRIL 1992	07 MAY 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Jon P. Weber, Ph.D.

Form PCT/ISA/210 (second sheet)(May 1986) ß

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	CAN. J. ZOOL., Volume 61, issued 1983, Hew et al., "Presence of Cystine-Containing Antifreeze Proteins in the Spruce Budworm, Choristoneura fumiferana", pages 2324-2328, see entire document.	1-37
A	BIOPOLYMERS, Volume 24, issued 1985, Brown et al., "Direct Evidence for Antifreeze Glycoprotein Adsorption Onto an Ice Surface", pages 1265-1270, see entire document.	1-37
A	D.E. PEGG ET AL., "THE BIOPHYSICS OF ORGAN CRYOPRESERVATION", published 1987 by Plenum Publishing Corporation, see pages 117-140, see entire document.	1-37
Y,P	US, A, 5,001,047 (LIBERMAN) 19 MARCH 1991, see entire document, especially Background.	1-37
Y	US, A, 4,980,277 (JUNNILA) 25 DECEMBER 1990, see entire document.	1-37
Y	CRYOBIOLOGY, Volume 27, issued February 1990, MacFarlane et al., "Recent Insights on the Role of Cryoprotective Agents in Vitrification", pages 345-358, see entire document.	1-37
Y	CRYOBIOLOGY, Volume 26, issued 1989, De Antoni et al., "Trehalose, a Cryoprotectant for Lactobacillus bulgaricus", pages 149-153, see entire document.	1-37
X/Y	US, A, 4,059,967 (ROWE ET AL.) 29 NOVEMBER 1977, see entire document.	1-37/1-37
Y	US, A, 4,688,387 (CONAWAY) 25 AUGUST 1987, see entire document.	1-37
Y	US, A, 4,186,253 (YOKOYAMA ET AL.) 29 JANUARY 1980, see entire document.	1-37
Y	CRYOBIOLOGY, Volume 21, issued 1984, Collins et al., "Studies in Cryoprotection", pages 1-5, see entire document.	1-37
Y	US, A, 4,155,331 (LAWRENCE ET AL.) 22 MAY 1979, see entire document.	1-37
Y	J. BIOCHEM., Volume 151, issued 1985, Hew et al., "Structures of Shorthorn Sculpin Antifreeze Polypeptides", pages 167-172, see entire document.	1-37
A	CAN. J. ZOOL., Volume 64, issued 1986, Kao et al., "The Relationship Between Molecular Weight and Antifreeze Polypeptide Activity in Marine Fish", pages 578-582, see entire document.	1-37
A	J. BIOL. CHEM., Volume 259, No. 23, issued 10 December 1984, Gourlie et al., "Winter Flounder Antifreeze Proteins: A Multigene Family", pages 14960-14965, see entire document.	1-37
A	US, A, 4,931,361 (BALDESCHWIELER ET AL.) 05 JUNE 1990, see entire document.	1-37

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
A	CAN. J. ZOOL., Volume 64, issued 1986, Fletcher et al., "Antifreeze Peptides Confer Freezing Resistance to Fish", pages 1897-1901, see entire document.	1-37
Y	NATURE, Volume 333, issued 19 MAY 1988, Yang et al., "Crystal Structure of an Antifreeze Polypeptide and its Mechanistic Implications", pages 232-237, see entire document.	1-37
A	NATURE, Volume 333, issued 19 May 1988, Pain, "Helices of Antifreeze", pages 207-208, see entire document.	1-37
X	BIOCHIM. BIOPHYS. ACTA, Volume 495, issued 1977, DeVries et al., "Structure of a Peptide Antifreeze and Mechanism of Adsorption to Ice", pages 388-392, see entire document.	1-37
A	CRYOBIOLOGY, Volume 25, issued 1988, Morris et al., "Freezing Injury in Saccharomyces cerevisiae: The Effect of Growth Conditions", pages 471-482, see entire document.	1-37
A	NATURE, Volume 325, issued 08 January 1987, Franks et al., "Antifreeze Activity of Anarctic Fish Glycoprotein and a Synthetic Polymer", pages 146-147, see entire document.	1-37
A	J. BIOL. CHEM., Volume 260, No. 21, issued 25 October 1985, Li et al., "Structure of an Antifreeze Polypeptide and its Precursor from the Ocean Pout, Macrozoarces americanus", pages 12904-12909, see entire document.	1-37
A	ANN. REV. PLANT PHYSIOL., Volume 35, issued 1984, Steponkus, "Role of the Plasma Membrane in Freezing Injury and Cold Acclimation", pages 543-584, see entire document.	1-37
A	NATURE, Volume 328, No. 6127, issued 16 July 1987, Harrison et al., "Ice Growth in Supercooled Solutions of Antifreeze Glycoprotein", pages 241-243, see entire document.	1-37
Y	PROC. R. SOC. LOND.:B, Volume 234, issued 1988, Rubinsky et al., A Mathematical Model for the Freezing Process in Biological Tissue", pages 343-358, see entire document.	1-37
Y	PROC. NAT. ACAD. SCI.(USA), Volume 79, issued January 1982, Davies et al., "DNA Sequence Coding fo an Anitfreeze Protein Precursor from Winter Flounder", pages 335-339, see entire document.	1-37
Y	J. BIOL. CHEM., Volume 259, No. 14, issued 25 July 1984, Davies et al., "Antifreeze Protein Genes of the Winter Flounder", pages 9241-9247, see entire document.	1-37

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	ANN. REV. PLANT PHYSIOL., Volume 27, issued 1976, Burke et al., "Freezing and Injury in Plants", pages 507-528, see entire document.	1-37
A	PROC. NAT. ACAD. SCI.(USA), Volume 78, No. 3, issued May 1981, Lin et al., "Molecular Cloning and Characterization of Winter Flounder Antifreeze cDNA", pages 2825-2829, see entire document.	1-37
A	EUR. J. BIOCHEM., Volume 143, issued 1984, Pickett et al., "Sequence of an Antifreeze Protein Precursor", pages 35-38, see entire document.	1-37

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.