BEYOND THE CUTTING EDGE OF COLD

The way to freeze organs without ice may be clear as glass

By STEFI WEISBURD

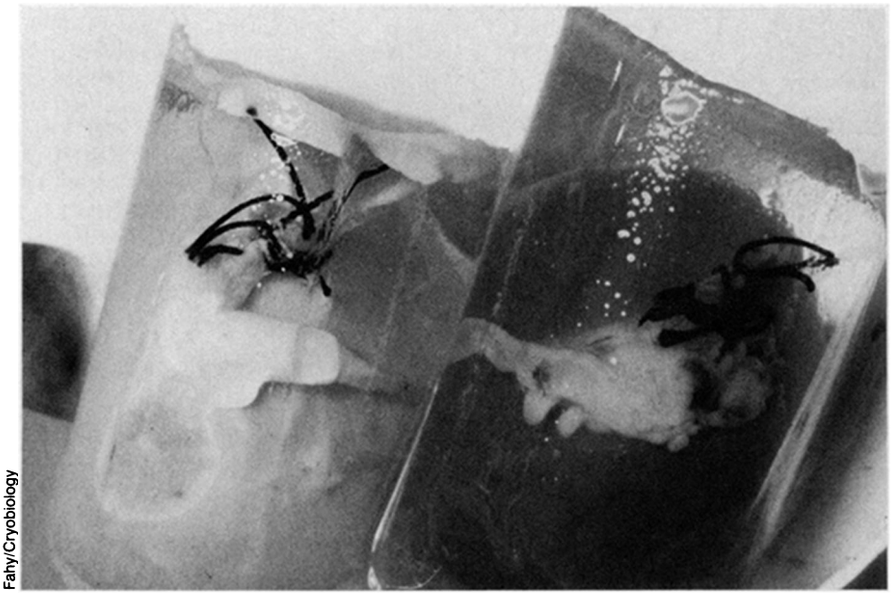
Some frogs freeze. They can be frozen hard as rock during the chill of winter and still leap back to life with the first spring thaw.

Nature has provided frogs, hundreds of insect species and other invertebrates with the ability to endure the ravages of freezing. We mammals, however, are another story. If we can't prevent ice crystals from forming in our cells, blood vessels and organs, the ice will do enormous damage. This fragility to frigidity hasn't hindered our survival, since we've invented down coats and other strategies to keep warm. But it has thrown cold water on the premier goal of cryobiology: to use the immobilizing effects of cold to indefinitely preserve biological materials, especially human organs that are donated for transplantation.

Cryobiologists have had some success in freezing, thawing and reviving some kinds of cells and tissues. But with conventional techniques, they're approaching a dead end in their attempts to cryopreserve anything nearly as complex as an organ. The problem has spurred a resurgence of interest in a technique called vitrification, which was first explored by biologists in the 1930s. The intent of vitrification is to cool a sample extremely rapidly or manipulate it in other ways so that water molecules are robbed of the opportunity of forming ice crystals. Instead, the material is transformed into a highly viscous liquid, an amorphous glass.

Scientists have yet to successfully vitrify biological samples that couldn't already be cryopreserved by conventional means. But recent studies have shown sufficient progress for the cryobiology community to be optimistic about the vitrification process.

"I look on the recent developments in vitrification as the most important in cryobiology since [scientists first showed in 1949] that living cells could be artificially cryopreserved with glycerol [an event that ultimately made the storage of all sorts of cells and tissues routine]," Harold T. Meryman of the American Red Cross Transplantation Laboratory in Rockville, Md., told the Society for Cryobiology at a recent meeting in Edmonton, Alberta. "I think we will look back at this particular period of cryobiology as being a real turning point."



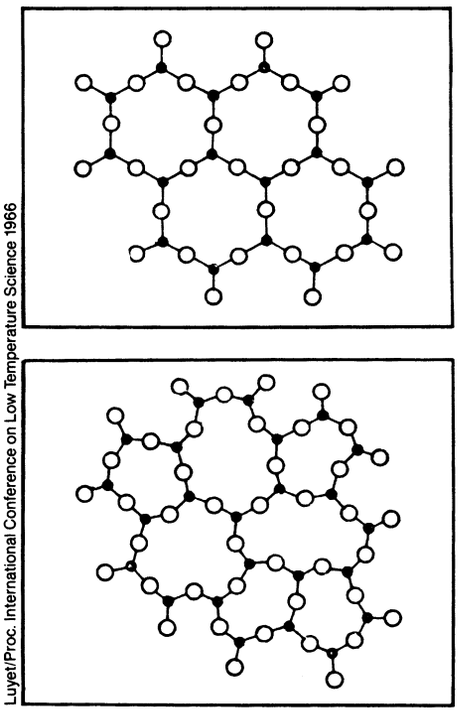
Fahy/Cryobiology

A tale of two kidneys: Both of these rabbit kidneys have been cooled to -140°C. The organ on the right was perfused and immersed in a solution that discourages the formation of ice. Researchers were indeed unable to find any evidence of ice when the vitrified kidney was subsequently split open with a hammer and chisel. The kidney on the left, however, was only immersed, not perfused, in a vitrification solution. It looks like a snowball because it has frozen and is riddled with ice.

Although there's still considerable debate over how freezing and ice crystals injure cells, many scientists believe that intracellular crystals crush cell parts or destroy the fine network of filaments that keeps the cell intact. To avoid this damage, cryobiologists usually cool their samples slowly so that water has enough time to come out of cells before it freezes. They also add cryoprotective agents such as glycerol, which are thought to act as antifreeze solutions and which may protect cell parts by stabilizing membranes and proteins.

Cells are also thought to be damaged during freezing and thawing by dehydration. According to a traditional view, if the cooling rate is too slow and too much water is drawn out of the cell during the formation of extracellular ice, it can leave behind high concentrations of salts that denature proteins and destroy cell structure. However, there is growing evidence that dehydration damage is caused by changes in cell volume: The exodus of water makes the cells shrink and lose so much membrane material that they leak or burst upon rehydration during thawing. The addition of cryoprotectants may

prevent some of this shrinkage, but, like salt, these can be toxic in high concentrations. By carefully balancing the cooling rate and cryoprotectant levels, scientists have learned in some cases how to navigate a safe passage between the dangers of dehydration and ice.



Luyet/Proc. International Conference on Low Temperature Science 1966

The structure of a material, in this case silica, is far more orderly when it is frozen (above) than when it is vitrified (below).

Using this basic method, cryobiologists can preserve human blood cells (SN: 10/7/78, p.250), corneas, skin, pancreatic islets (SN: 7/18/87, p.47) and other whole tissues and tissue culture cells. Thousands of healthy human infants have been conceived with frozen sperm and dozens of children have been born from once-frozen embryos — and the numbers are likely to grow as *in vitro* fertilization clinics become more popular. Cryopreserved animal sperm and embryos are used routinely by dairy farmers to improve cattle breeds, by zoos to bolster populations of endangered species and by scientists to maintain cell lines and research animal colonies that would otherwise suffer from "genetic drift," or random mutations that alter the genetic makeup over the generations (SN: 9/16/78, p.202).

But the problem with using the conventional approach on more complex tissues and organs is that they contain a multitude of cells and cell types, each of which may require a unique freeze-thaw regimen. And conventional cryopreservation methods, although they may avoid ice formation inside cells, still permit ice to form outside where it can damage blood vessels and threaten the architectural integrity of the organ. The big hope is to avoid ice damage by avoiding ice formation altogether. The big question is how.

One experimental approach to vitrification is being taken by Gregory M. Fahy at the American Red Cross Transplantation Laboratory. He has vitrified rabbit kidneys that have been perfused with highly concentrated mixtures of cryoprotectants and then cooled to about -150°C under 1,000 atmospheres of pressure (SN: 5/24/86, p.326). The pressure, Fahy believes, counteracts the expansion of water necessary to form ice and it reduces the amount of potentially toxic cryoprotectants needed.

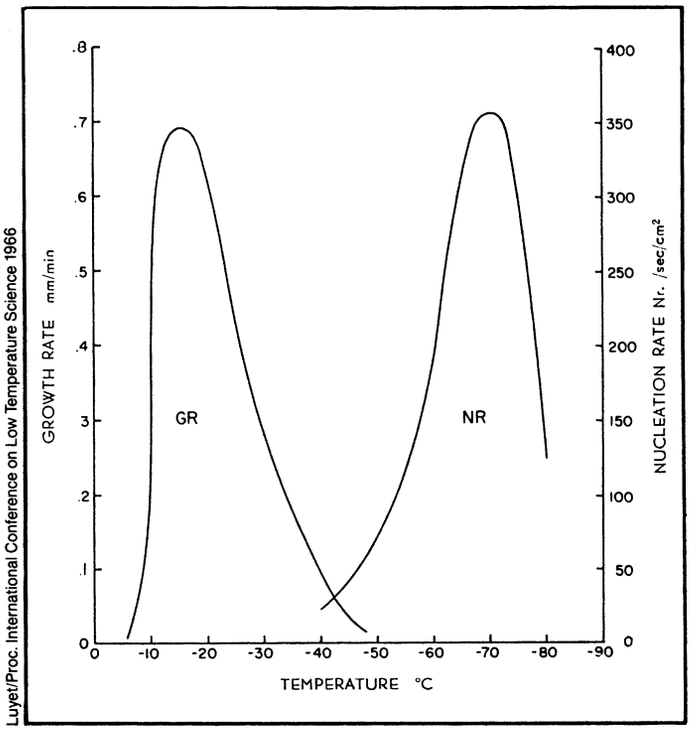
The cryoprotectants associate with water molecules, making it more difficult for the molecules to coalesce into an orderly ice crystal lattice. These agents lower the freezing point and raise the temperature at which the material turns into an amorphous glass. Fahy says that with a *mixture* of cryoprotective agents he can minimize the toxicity, maximize the material's glass-forming ability and balance the cryoprotectant levels inside and outside the cells. With the cryoprotectants and pressure, he also doesn't have to cool the samples at very rapid rates to avoid ice formation. This is important, he says, considering that it is difficult to quickly and thoroughly cool objects as large as organs.

Cryoprotective mixtures, he notes, are also used in nature: "There are some findings in comparative biology that suggest that the kidneys I'm treating with 7.5-molar cryoprotectant might have something in common with coelacanths, those ugly prehistoric deep-sea fish everyone thought were extinct until fishermen caught one in the Atlantic in the early 1900s," he says. "They use an apparently similar mixture of cryoprotectants."

Before testing whether the rabbit kidneys he has vitrified are viable, Fahy has first wanted to make sure that the organs can tolerate his cryoprotectant concoction. In his most recent work, he has shown that some of the perfused kidneys are indeed able to perform renal functions while the kidneys are temporarily connected to the animal's circulation. He suspects that with additional tinkering, he could get the other organs to survive as well.

One of the next steps, he says, is to transplant these kidneys back into animals to see if they can support life. Then there are other questions to be addressed, such as whether the kidney cells can withstand high pressures at a variety of temperatures and, if some ice does form, how much the organ could tolerate. Moreover, the physics of water is such that ice crystals actually form more readily during warming than during cooling, so to avoid ice crystal growth, Fahy must devise ways of reheating the organ very rapidly and uniformly. He and others are now experimenting with microwave or radiofrequency heating for this purpose. Finally, assuming he is successful with the rabbit kidneys, there is still another slew of problems surrounding the application of his work to human organs.

"We are many steps away from an organ bank right now," says Fahy. "But for the first time ever I think there's a lot of light at the end of that tunnel."



Luyet/Proc. International Conference on Low Temperature Science 1966

Researchers have to be more wary about ice forming while rewarming a vitrified sample than while cooling it down. The reason is explained by this graph, which shows that ice crystals, once seeded, grow most rapidly in a temperature zone that is warmer than the zone at which nucleation, or the configuration of water molecules into tiny ice-crystal seeds, occurs. Ice crystals can form relatively easily during warming because the warmed water encounters the nucleation zone before the region of rapid crystal growth. To get around this problem, scientists are investigating the use of microwaves to rewarm a sample rapidly and uniformly.

Because of the potential problems associated with the high pressures and high cryoprotectant levels used by Fahy, S. Randolph May and his colleagues at LifeCell Corp. in The Woodlands, Tex., have taken another route. They have developed equipment that can cool thin samples at rates of about 100,000°C per second, faster apparently than water molecules can form into ice crystals. In collaboration with John G. Baust and his colleagues at the State University of New York in Binghamton, and using a variety of diagnostic instruments, May's group has recently demonstrated that this technique will vitrify pieces of rat liver.

From the standpoint of organ preservation, however, the main drawback of the method is that it can vitrify only to a depth of less than 100 microns into the sample. May and Baust plan to experiment with a process called induction cooling, which may help them vitrify the entire sample when it is placed in and then out of a magnetic field.

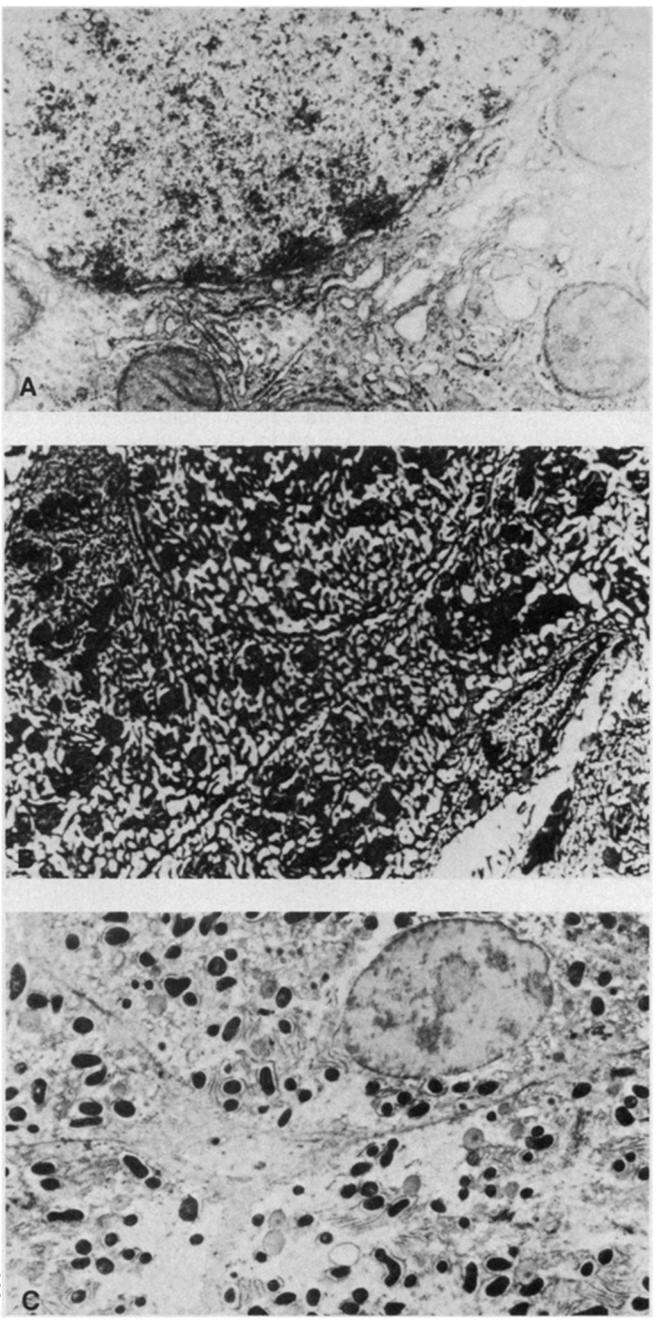
Even if they don't succeed, May says their vitrification process would get deep enough to vitrify most transplanted tissues, such as heart valves, skin and dura mater (a membrane around the brain). But where May and others say the technique will have its greatest punch is in electron microscopy.

According to May, the traditional methods of preparing tissue samples for inspection under the electron microscope either alter the tissue's biochemical makeup with fixation chemicals or destroy cell and tissue structure if the samples are frozen and ice crystals form. The complete LifeCell method, which is basically an improved form of freeze-drying, involves neither chemicals nor ice formation, and so, May thinks, will "revolutionize electron microscopy" by enabling microscopists for the first time to "see the cells the way they really are."

After vitrifying a sample, LifeCell researchers put it into a strong vacuum until most of the water has diffused out — a process called molecular distillation drying. Once the water has left, says May, ice crystals can't form and biochemical processes that degrade the sample are unable to proceed. The researchers can then warm the sample to room temperature and store it on a shelf until it is needed.

May believes the vitrification-drying method also holds promise for tissue and cell preservation. His group has vitrified and dried cultured cells, stored them for six weeks at room temperature and then rehydrated them. While some cells died, says May, the survival rate was better than that of cultured cells frozen by conventional cryopreservation.

The group has also applied the process to corneas, grafting revived rabbit corneas onto rabbits and both a human and baboon cornea onto baboon eyes. May says the corneas cleared, one indication that they were functional.



LifeCell

Conventional methods of preparing a sample for electron microscopy have their drawbacks. Chemical fixation can change the chemical composition of cells (A), and freeze-drying often causes formation of ice crystals that damage cellular and tissue structure (B). Researchers say that these kinds of damage are avoided with vitrification-drying (C).

Other researchers agree that May's group has probably made an extremely important contribution to electron microscopy, but some doubt that either this process or previous freeze-dry methods will ever produce living tissues that are healthy enough for transplants.

The biological systems that scientists have so far been able to vitrify with unquestioned success include human red blood cells, monocytes (a type of white blood cell), ova and pancreatic islets. At Rio Vista International Inc. in San Antonio, Tex., William F. Rall and some British colleagues have implanted vitrified mouse embryos into mice which delivered normal offspring. The group has also vitrified cow embryos and placed them into cows that are scheduled to give birth this month.

Rall's technique originally required that workers prepare embryos for vitrification in the uncomfortable environs of cold rooms. Now, however, the preparations can be performed at room temperature. Of embryos treated in this way, says Rall, 64 percent developed into normal offspring — a survival rate comparable to that of embryos preserved with conventional freezing methods.

Besides eliminating the opportunity for ice to cause damage, the main advantage of vitrifying embryos, he notes, is that it doesn't involve the slow freezing of conventional cryopreservation, which takes a long time and requires elaborate machines. "People would be able to cryopreserve embryos essentially without using any equipment," he says. "All

they would need would be the vitrification solutions and a canister of liquid nitrogen [to drop the samples into]."

Meryman says he is particularly encouraged by the success researchers have had in vitrifying embryos. The studies show that "there is not some mysterious injury that is going to take place at temperatures where everything stops," he says. "I think the vitrification of the embryo is probably the most convincing of all things one could do because the 'life forces' appear to survive that particular insult."

By de-animating biological materials and halting the chemical processes that cause their decay, vitrification offers a way of arresting biological time. Sometimes, however, scientists would be satisfied if biological time were merely slowed down a bit. Liver cells, for example, die within a matter of hours, and it is very difficult to run a series of comparative tests on one group of cells all in an afternoon. If these cells, which can't be stored or cultured with conventional methods, could be somehow preserved for weeks or even just days, research would be advanced considerably.

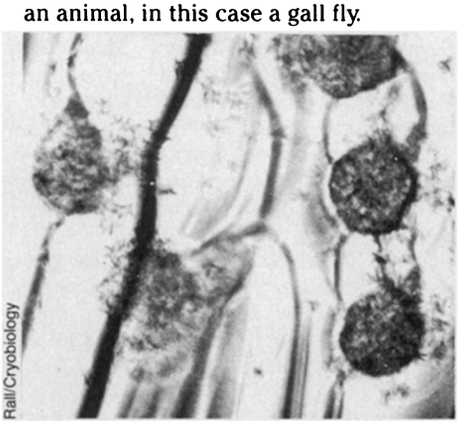
For such biopreservation problems, Felix Franks, Sheila Mathias and their colleagues at Pafra Ltd. in Cambridge, England, have elaborated on a technique called undercooling or supercooling. While water normally freezes at 0°C, scientists have found that water droplets – which because of their small volume are relatively free of the particles that seed ice crystals – can be cooled to -40°C and still remain liquid, without the creation of ice.

In the process developed by Franks's group, cells are undercooled, without the use of a cryoprotectant, down to -30°C in droplets that are suspended in an inert oil emulsion. The researchers have geared their technique toward a number of different biological materials that at present either cannot be frozen or freeze-dried at all, or that suffer from a low recovery rate with these methods. The group has been able to store red blood cells, platelets, plant cells and a bacterium used to make sauerkraut and pickles. According to Mathias, they have also undercooled and preserved some enzymes for periods of up to a year.

Undercooling probably is not suited for the preservation of solid organs or large amounts of biological materials, or for preservation for posterity, she says. "But because conventional cryopreservation doesn't work in every case, undercooling presents a relatively simple, low-cost alternative to low-temperature preservation" for quite a lot of biological systems.

Mathias notes that undercooling, like the use of cryoprotectant mixtures in Fahy's work, appears to have a precedent in nature. Some kinds of Antarctic fish, she says, are able to undercool because they contain chemicals that surround ice crystal seeds and discourage water molecules from joining the lattice (SN: 11/22/86, p.330). In all of cryobiology, says Meryman, "it's always humbling to discover that in most cases nature was there ahead of you." After researchers began to use glycerol as a cryoprotectant for red blood cells, for example, they discovered that insects had been doing this to protect their cells for millennia.

As for vitrification, nature seems to have been doing a little experimentation as well, at least inside cells. Fahy says the cells of some plants appear to vitrify below about -25°C. And at the recent Society for Cryobiology meeting, Baust, John M. Wasylyk and their co-workers presented what Wasylyk says is the first example of vitrification in an animal, in this case a gall fly.



Rall/Cryobiology

Deadly ice can form not only during cooling but also during thawing. None of these vitrified mouse embryos survived after they were warmed slowly enough for ice crystals to grow.

Baust's lab has been recording the monthly variations in sugar production in the blood of one gall fly species capable of surviving the low temperatures of winter. The researchers found that the concentrations of two sugars, fructose and trehalose, remained constant year round, but that sorbitol and glycerol were produced only when temperatures fell.

They then made up a solution of these four sugars and used nuclear magnetic resonance spectroscopy and a calorimeter to analyze its properties as it cooled. According to Wasylyk, at the proper sugar concentrations, most of the mixture remained in the liquid state and was transformed into a glass well below the freezing point. Moreover, the most glass was formed when all four sugars were used. Wasylyk suspects that the sugars are somehow engaging many of the water molecules and preventing their rearrangement into an ice lattice.

"It's been suggested before that the possible roles of the sugars are not only to lower the freezing point, but also to induce glass formation as opposed to ice," says Wasylyk. "But the proof was lacking." He believes that other insects will be found to vitrify because many are also known to produce sugar mixtures during cold temperatures.

Baust believes that researchers trying to vitrify mammalian cells, tissues and especially organs may harvest many clues from the study of such insects, which appear to survive winters by using mixtures of cryoprotective agents and vitrifying at fairly warm temperatures and at relatively slow cooling rates. "Maybe we need to look at little more at these natural approaches in designing cryopreservation procedures of whole organs, because these . . . insects have organs [too]," he says.

But Wasylyk cautions that extrapolating from such studies to human organs will be no simple feat, because human organs are much larger, far more complex and may have to be stored at much colder temperatures than what insects and frogs encounter in even the harshest of winters. Trying to vitrify organs "is something out of Jules Verne 1987," he says. "It may happen in the future, but I'm leery."

This caution is echoed somewhat by May, who notes that protective chemicals used by insects and frogs aren't the whole answer to the animals' survival. "Their bodies also adapted [during evolution] to whatever damage ice crystals cause," he says. "So [for example] if they had fragile membranes, they doubled their membrane size" and made other structural changes that hardened them against whatever ice does form.

With evolutionary adaptations, says Meryman, nature has indeed had the advantage of time. And nature has also had the ability to put certain compounds into cells, a feat that researchers have been hard pressed to duplicate. "But we have two big advantages," he says. "While nature has to devise systems that work in the face of unpredictable environments, we can control freezing and warming rates and the solutes we're going to use. It's obvious . . . that our successes in dealing with cells and tissue that are not naturally adapted to vitrification and freezing are largely the result of our ability to control the environment." The other advantage, he adds, is that researchers don't have to rely on evolution as the test for their ideas, but have developed far more efficient and less time-consuming methods in the laboratory.

The next item on the agenda, says Meryman, is to move to larger specimens and try to achieve vitrification in the absence of extracellular ice, a task that appears to have eluded even nature.

Will vitrification move cryobiology out of the ice age? "Maybe it's a fad, but I don't think so," says Baust. For the moment at least, vitrification "is *the* hot topic in a cold science." □