# CELL ON ICE

Research on the reaction of cells to freezing temperatures has opened the way to deep-freeze storage of plant seeds and of sperm, eggs and embryos of many animal species

BY JULIE ANN MILLER

*This is the first of two articles on cryobiology — the art and science of freezing living tissue.*

Biology is entering an ice age. Interest in low-temperature phenomena gradually spreads from laboratory to laboratory. And in the wake of the research, immediate benefits to medicine, agriculture and animal husbandry crystallize.

More than three thousand infants have already been born of once-frozen sperm. The Carnation Co. is beginning to commercially freeze, thaw and implant cattle embryos. Collections of frozen wild-animal sperm, plant seeds and microorganisms are kept in attempts to protect genetic diversity. And the freezing probe is becoming a tool in more and more surgical procedures.

The progress in practical cryobiology rests on an increased understanding of the characteristics of tissue as it freezes and thaws. Also essential is discovery of methods to protect tissue from damage. "The challenge to cells during freezing is not their ability to endure the very low temperature required for long storage; it is the lethality of an intermediate zone of temperature (−15°C to −50°C) that a cell must traverse twice — once during cooling and once during warming," says Peter Mazur of Oak Ridge National Laboratory. At liquid nitrogen temperature, −196°C, there can be little damage because biological and chemical processes have been halted.

Ice crystals and high salt concentrations are a two-edged sword threatening cells as they freeze and thaw. If cells are frozen rapidly, ice crystals within may destroy delicate membranes. If a cell is frozen slowly, it dehydrates and its interior becomes so concentrated in salt that cell proteins may be damaged. Therefore, neither very fast nor very slow cooling guarantees a safe course between destruction by ice or by salt.

Yet scientists have innocuously frozen and thawed various types of cells. Mazur, Stanley Leibo and colleagues have demonstrated that the optimal procedure differs with cell type. Human red blood cells, for example, survive best when cooled more than 500 times faster than the optimal cooling rate for mouse embryos. And mouse bone marrow cells survive best at an intermediate rate.

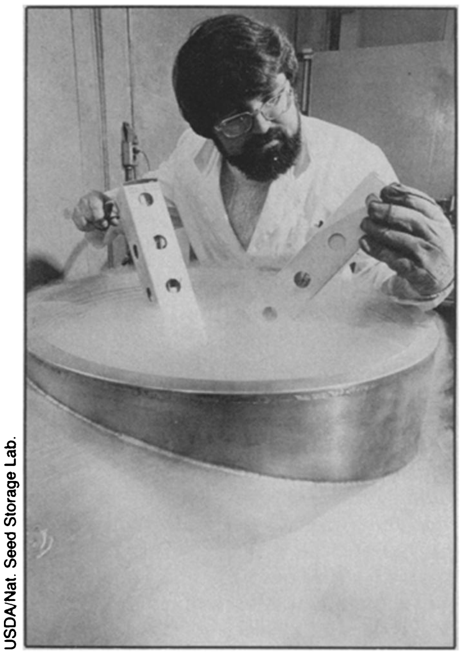
Mazur and colleagues make calculations based on physical chemistry to evaluate the parameters involved in cell freezing. From these calculations they can predict ice formation in different cell types by the permeability of the cells to water and by the cell surface-to-volume ratio. Such ice formation is usually, but not inevitably, lethal. Mazur is currently examining the more subtle "solution effects," which include the increasing salt concentration and the cell shrinkage. Those effects result from water leaving a slowly cooled cell as ice forms outside it.

Researchers have also found that the rate of warming affects cell survival after freezing. Cells cooled very rapidly usually survive best if they are rapidly thawed; cells cooled slowly often fare better if slowly warmed.

Much of the practical progress in cryobiology is due to the empirical discovery of protective chemicals. Thirty years ago Audrey Smith and Christopher Polge in England reported that glycerol protects sperm during freezing. (Those researchers had been unsuccessfully freezing turkey sperm in sugar solutions when they observed fantastic survival rates in a solution accidentally made with glycerol.) Thus began the practice of freezing animal and human sperm for artificial insemination. Today about half of the cattle born in the world are produced from previously frozen sperm.

The theoretical basis for the chemical protectants is still debated. The most common, glycerol and dimethyl sulfoxide (DMSO), are really not antifreezes, Mazur says. They protect against the injury caused by slow-freezing, but not against ice formation in rapidly cooled cells. Mazur believes the important role of these protective chemicals is to decrease the maximum concentration of salt outside (and perhaps inside) the freezing cell. Another possibility is that the protective chemicals limit damaging cell shrinkage. Among the more unusual protective agents used experimentally are a small protein from winter-hardy spinach and "antifreeze" glycoproteins from the blood of arctic fish.

Most of the success with freezing techniques has been with individual cells, rather than with more complex organs. Sperm and red blood cells, besides being the first cells successfully frozen, are economically and medically the most important.

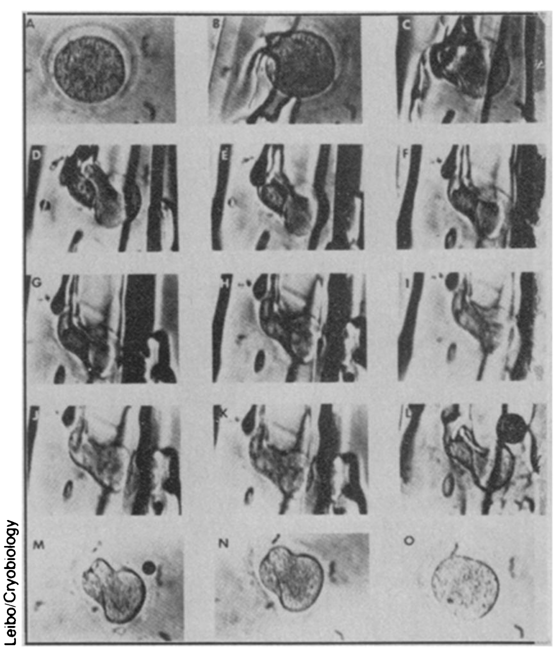


USDA/Nat. Seed Storage Lab.

*Plant seeds are retrieved from ultra-cold freezer for testing by Phillip Stanwood.*

A reliable procedure for freezing sperm allows storage for years as well as simple transport from donor to recipients anywhere in the world. Thus, in animal breeding a superior animal can fertilize progeny even after its death. And rare animals (for instance, a breed of dogs with a genetic disease useful in medical research) can be maintained by a much smaller colony than if it had to guarantee continuous availability of breeding males.

Success in sperm freezing varies among species. Cattle and human techniques are relatively simple, achieved more than twenty years ago. Ram sperm, however, proved much more difficult. A current listing of species in which frozen sperm, once

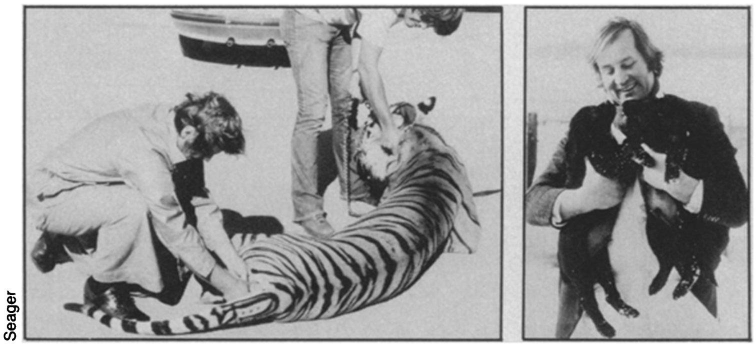



Leibo/Cryobiology

*Mouse ovum shrinks as it is slowly cooled at 1.2°C per minute from 0°C (A) to −135°(I). It re-expands as it warms (J-O). Black stripes are ice outside the cell.*

thawed, is at least half as effective as fresh sperm in fertilizing a female includes bull, boar, stallion, ram, goat, dog and probably man. This year Chinese scientists announced they can freeze water buffalo sperm. (That animal is the equivalent of a tractor in southern China's rice producing areas.) Chicken and turkey thawed sperm are viable, but not always effective for fertilization.

The cryobiologists have not limited sperm freezing to mammals. Sperm of several species of trout, salmon and cod are able to fertilize eggs after surviving freezing procedures. Even honey bee sperm has been successfully stored at liquid nitrogen temperature. Entomologist John R. Harbo of the U.S. Bee Breeding and Stock Center laboratory, who has produced progeny from the thawed bee sperm, says the practical importance of the procedure is that frozen sperm would reduce the cost of maintaining laboratory bee colonies.

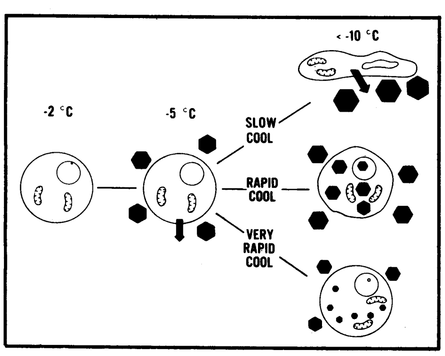


Seager

*Stephen Seager collects semen from a tiger and holds the first pups born from frozen sperm.*

One frozen sperm bank specializes in the exotic. Stephen W. T. Seager and colleagues at the Institute of Comparative Medicine, Texas A and M University/Baylor College of Medicine in Houston are freezing semen of captive wild animals, especially those on the endangered list. Seager anticipates that zoos around the world will order semen of rare breeds for inseminating animals that cannot be persuaded to reproduce naturally. In some cases, frozen sperm is a solution to a geographical problem. Sending a frozen vial across a continent is certainly a much simpler, safer matter than transporting, say, a male polar bear or a 3,000-pound rhinoceros. The fitness of zoo animals also might be improved by insemination with sperm collected from animals in the wild. Inbreeding of zoo animals could be avoided without reducing the wild populations.

In 1973 Seager and his associate, Carrol C. Platz, demonstrated that artificial insemination with frozen sperm actually can succeed in a normally wild animal. The proof was a number of Canadian timber wolf pups born at Olympic Game Farm in Sequim, Wash. Currently, Seager and colleagues are attempting to produce pregnancy with frozen sperm in a jaguar at a Houston zoo. The researchers have investigated semen collection and freezing techniques for 80 species of zoo animals including lion, tiger, leopard, cheetah, mountain lion, camel, rhinoceros, pygmy hippopotamus, gorilla and polar bear. Future studies, Seager says, will include reptiles and birds. Much of their work is done in collaboration with the National Zoo in Washington and zoos in St. Louis and Texas and is supported by Houston's Blaffer Foundation.




Mazur/ORNL

*Cooling rate affects cell's fate. Slow cooling allows water to leave, so no ice forms. Very rapid cooling permits only tiny ice crystals. Rapid cooling is most damaging.*

The wild animal techniques derive from Seager's earlier work on domestic dogs and cats (funded in part by the American Kennel Club and Ralston Purina Co.). He first reported pregnancy from frozen canine sperm in 1969. Since then 1,074 puppies, fathered through frozen semen, have been born in his laboratory. Those include five generations. Seager told the recent meeting of the International Society for Cryobiology in Tokyo that he observes no decline in effectiveness of dog semen frozen 10 years and no adverse effects of the procedure on pups or parents. Seager also leads a National Institutes of Health project banking sperm of animals with inherited disorders that are useful models of human disease. That collection includes frozen sperm from dogs with kidney problems, heart conditions, narcolepsy, lipid deficiency diseases, blindness and deafness.

Seager stresses that he is not trying to replace Cupid. "These are sometimes totally stopgap measures," he says. "Artificial means of breeding should be applied only where natural methods have failed or are impractical due to cost or risk of injury or death to the animals involved."

Natural insemination sometimes fails for people, as well as for zoo animals. Freezing semen is now a widespread practice for overcoming certain fertility problems. The first human pregnancies with sperm frozen to −196°C succeeded in 1964. Today J. K. Sherman of the University of Arkansas School of Medicine, the researcher who developed the method, says there have been more than 3,000 human births from frozen sperm. Normal births have occurred from human semen stored at least ten years. Although no one has been able to obtain information on all the human births, Sherman has surveyed about 1,500 children, and Japanese researchers have data on about 200. Both groups find no increase in abnormalities and abortions resulting from conceptions from frozen semen.

Current work on both human and animal sperm is aimed at increasing the number of sperm that survive freezing and developing new methods for evaluating the thawed sample. Especially for large animals and for humans, researchers need a method more practical than impregnating a female to judge whether the sperm are capable of fertilization. Many laboratories look at motility as a sign of healthy sperm, but the correlation with fertility is imperfect. Carp sperm, for example, are motile after freezing, but have nonetheless lost their capacity to fertilize.

Preserving the capacity to germinate, rather than the ability to fertilize, is the goal of another economically important area of deep-freeze research. The National Seed Storage Laboratory at Fort Collins, Colo., has just reported that seeds of 45 crops stored in liquid nitrogen for up to six months generally sprouted successfully. The laboratory now stores billions of seeds, representing almost 100,000 varieties, in vaults kept above −20°C, and researchers there must run germination tests on samples of each stock every five years. If no genetic changes are found in frozen seeds, freezing would allow long-term storage without the time-consuming testing. Seed storage is necessary to allow botanists to preserve seed stocks while they develop higher yielding and more disease- and insect-resistant crops.

The mammalian seed—fertilized ova or early embryos — has been successfully frozen and recovered for six species. Mouse embryos were the first to be frozen — by David Whittingham and Leibo and Mazur (and independently by S. Wilmut in England) in 1972. Since then, embryos of rabbit, cattle, sheep, goat and rat have yielded viable offspring after being frozen, thawed and transferred to the wombs of foster mothers. The successful specific procedures vary among the species. Generally, very slow cooling rates are used; for instance, mouse embryos survive cooling best at less than one degree per minute.

Biologists and animal breeders are excited about embryo freezing for somewhat opposing reasons, S. M. Willadsen of the Animal Research Station in Cambridge, England, has suggested. The breeders see it as a way to speed up evolution. It allows

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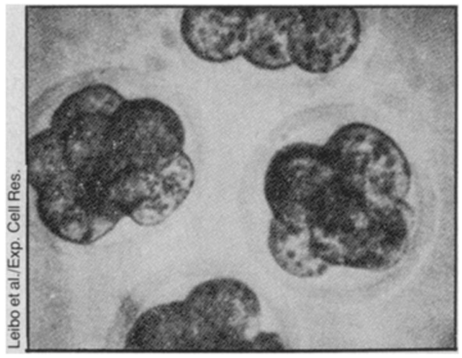
them to obtain 25 calves, instead of one, from a donor cow in a year. If embryos of a superior breed—with shapely cows giving 20,000 pounds of high-fat milk annually—are transferred into inferior animals, in a single generation the quality of the herd can be greatly improved. And distance is no limit. "The main thing is to be able to transport embryos to Asia, Africa and European countries," says Krish Karihaloo, research director of the Carnation Co. in Hughson, Calif.

Biologists, on the other hand, often want to slow down evolution and preserve diverse genetic strains. At a 1977 Ciba Foundation Symposium (*The Freezing of Mammalian Embryos,* Elsevier Publishing Co., 1977), Jan Klein of the University of Texas Health Science Center in Dallas described numerous instances in which geneticists would have saved years of research had there been an established bank of frozen embryos. The advantages are similar to those of frozen sperm but even stronger, because by using frozen embryos the genetic mother as well as the father can be bypassed. For instance, frozen embryos would travel better than colonies of mice; thus researchers could change laboratories and exchange specimens without traumatizing or killing valuable animals. Also, strains with interesting characteristics, once discovered, could be



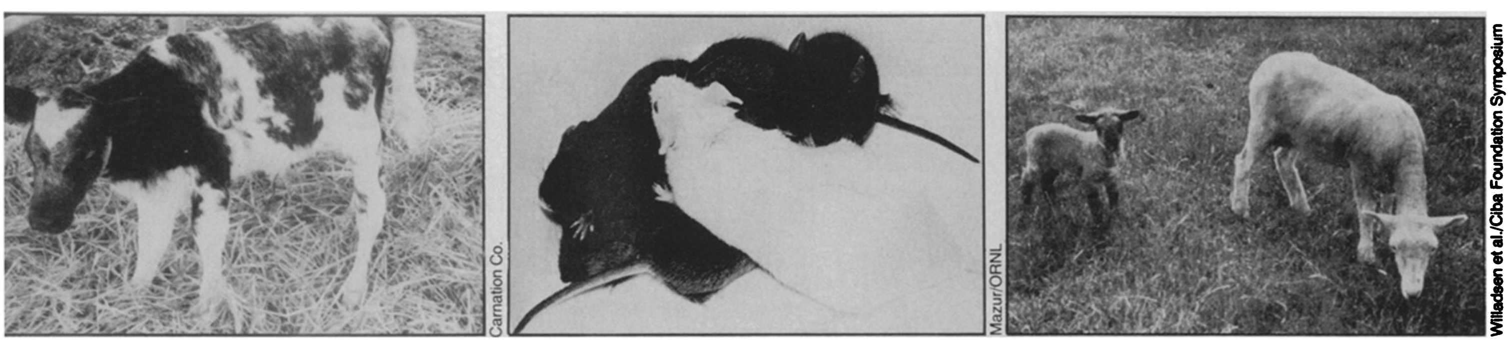

*Eight-cell stage of mouse embryo is the most successfully frozen.*

Klein says mouse genetics is now "a discipline dependent on a large and almost logarithmically increasing collection of variants." Every year some of the variants are inadvertently lost. "Such a loss is often comparable to a loss of an invaluable art object, and, monetarily at least, it amounts to tens of thousands of dollars," Klein says. The Jackson Laboratory and the Medical Research Council Laboratory Animal Center in Cambridge, England, are now beginning to use frozen embryos to preserve potentially valuable mutant mouse lines, especially those not used day to day.

So far the most extensive frozen embryo research has been on mice. Eggs are fertilized normally in a female and the resultant embryos flushed from the uterus.

ceed). A British company has done more than 200 thawed embryo implants, with a 50 percent success rate. Researchers are now working on techniques for freezing and implanting slightly older embryos, which can be identified as male or female. Then breeders could specify the sex of the product of the pregnancy they order.

In the wake of the "test-tube" baby (SN: 8/5/78, p. 84), one must wonder whether the next step will be a "freezer" child. Researchers on human reproduction certainly have the freezing of embryos in mind. Whittingham told the Ciba Foundation Symposium that he had tried freezing two human eggs fertilized in the laboratory, but had had no success. Robert G. Edwards and Patrick C. Steptoe, the scientific parents of human laboratory fertilization and implantation, outlined potential benefits of freezing such embryos. They explained that a procedure for removing eggs from infertile women involves treatment with ovulation-inducing hormones. If the embryo must be transferred into the patient immediately, the womb is not in its correct hormone condition. However, if the embryo could be frozen until a later menstrual cycle, it would experience the natural sequence of hormones. Freezing would also provide the time for embryos to be analyzed for inherited disorders. Finally, frozen embryos could be stored for successive transfers, Edwards says, "so




*Animals born of frozen embryos: first American heifer (1978), first mouse pups (black) with white foster mother (1972) and sheep of embryos removed at the same time, but frozen for 3 months or 15 months. Ewe (right) was foster mother to lamb at left (1977).*

preserved without the space and expense of maintaining breeding colonies. All that would be required for reviving a strain would be the appropriate frozen embryos and an animal to serve as foster mother.

Avoiding "genetic drift" is another possible advantage of embryo freezing. Donald W. Bailey of the Jackson Laboratory in Bar Harbor, Maine, explains that a highly inbred strain of mice, or of anything else, will not remain genetically identical from one generation to the next. Random mutations gradually change the strain's genes. Thus important characteristics, for example those crucial in cancer research, could disappear. Bailey and colleagues are in the midst of a five-year study using more than 12,000 mice to determine whether the number of mutants that arise in frozen embryos is really smaller than those accumulating in conventionally bred mice.

The first mice born from frozen embryos lived a normal lifespan and their progeny are normal now, six to eight generations later. Mouse embryos frozen at −196°C for four years have thawed and developed as usual. The greatest success with mice has been with 8-cell embryos that are grown in laboratory culture for one day after thawing before being transferred to foster mothers. Sheep embryos, in contrast, can be frozen at a fairly wide range of developmental stages.

Much of the work with farm animals has been done by British researchers. However this year the Carnation Co. announced the first American birth of a calf from a once-frozen embryo. Carnation has just begun freezing embryos on a commercial basis at its research facility. Karihaloo says 42 percent of the frozen embryos they transfer produce live births (65 percent of fresh embryo transfers suc-

that a whole family could be established by a single laparoscopy [surgical egg removal]."

The implications of cryobiology have a science fiction tone. Although the researchers do not envision adult bodies being frozen for future thawing, freezing of embryos is mind-boggling enough. Leibo speculates, "It is already possible to freeze mammalian embryos to within a few degrees of absolute zero. It is conceivable, therefore, that an embryo that would otherwise develop into an adult in the 20th century could be frozen and stored close to 0° Kelvin for a millenium, to begin its life in the 30th century. The freezing of biological systems, then, offers the potential for the human being to control time, rather than the reverse." □

*An upcoming article will describe research on frozen blood cells, frozen organs and cryosurgery.*