Basic principles of cryopreservation

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Cryopreservation in Gene banking

For gene banking we may establish collections of various types of frozen cells or tissue. For instance semen, oocytes embryos ovary, somatic cells….

- Semen will be cryopreserved with so called slow cooling methods.
- For the other cells/tissues also vitrification methods may be used.

This presentation

Explain the basic principles of various cryopreservation methods

- Knowledge of the fundamental principles will allow to access ‘new’ cryopreservation methods and implement variations or improvements in existing methods.
- Understanding of the causes of cryoinjury helps to minimize these causes to occur in our methods and handling routines.

Freezing in nature

Frozen wood frog

- Freezes internally
- Survives -8 ℃

Alaskan beetle (larvae)

- Survive -60 easily.
- May survive -100℃

Tardigrades

They survive

- Desiccation (1% water)
- -270 ℃
- + 150 ℃
- Complete vacuum
- Extremely high pressure
- 10 days in outer space

Fundamental Cryobiology

- Freezing
- Slow freezing
- (ultra) rapid freezing
- Vitrification
- Freeze-drying
- Glass transition

Common points:

- Take water out of the equation
- Glass transition
- Prevent intracellular ice formation

While these may be different things, they all relate to the same physico-chemical relationships

This can be explained by first explaining what happens during slow freezing

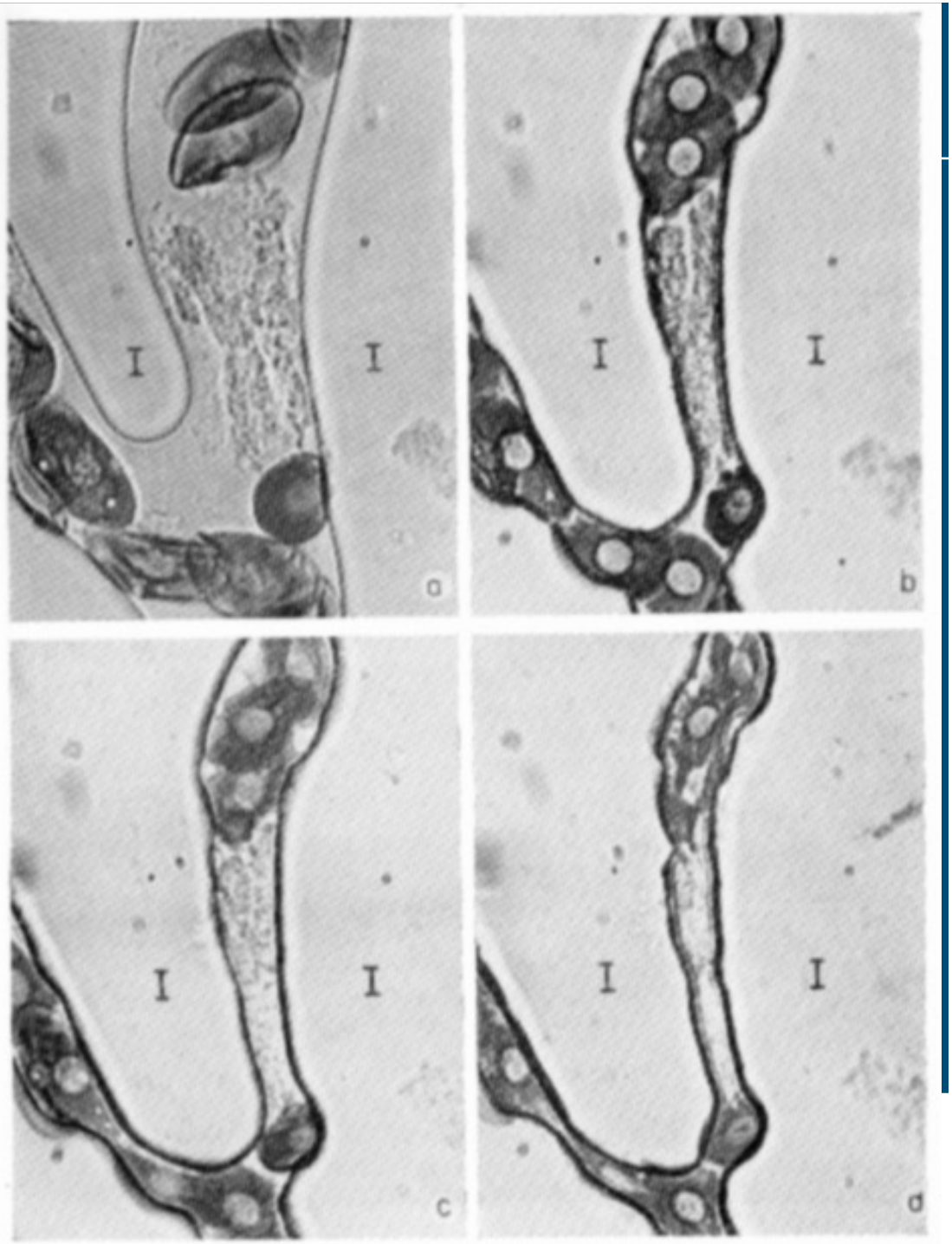
Slow Freezing

Erythrocytes at

-1,5 -5;

- 7 -10 ºC.

Rapatz & Luyet, 1960



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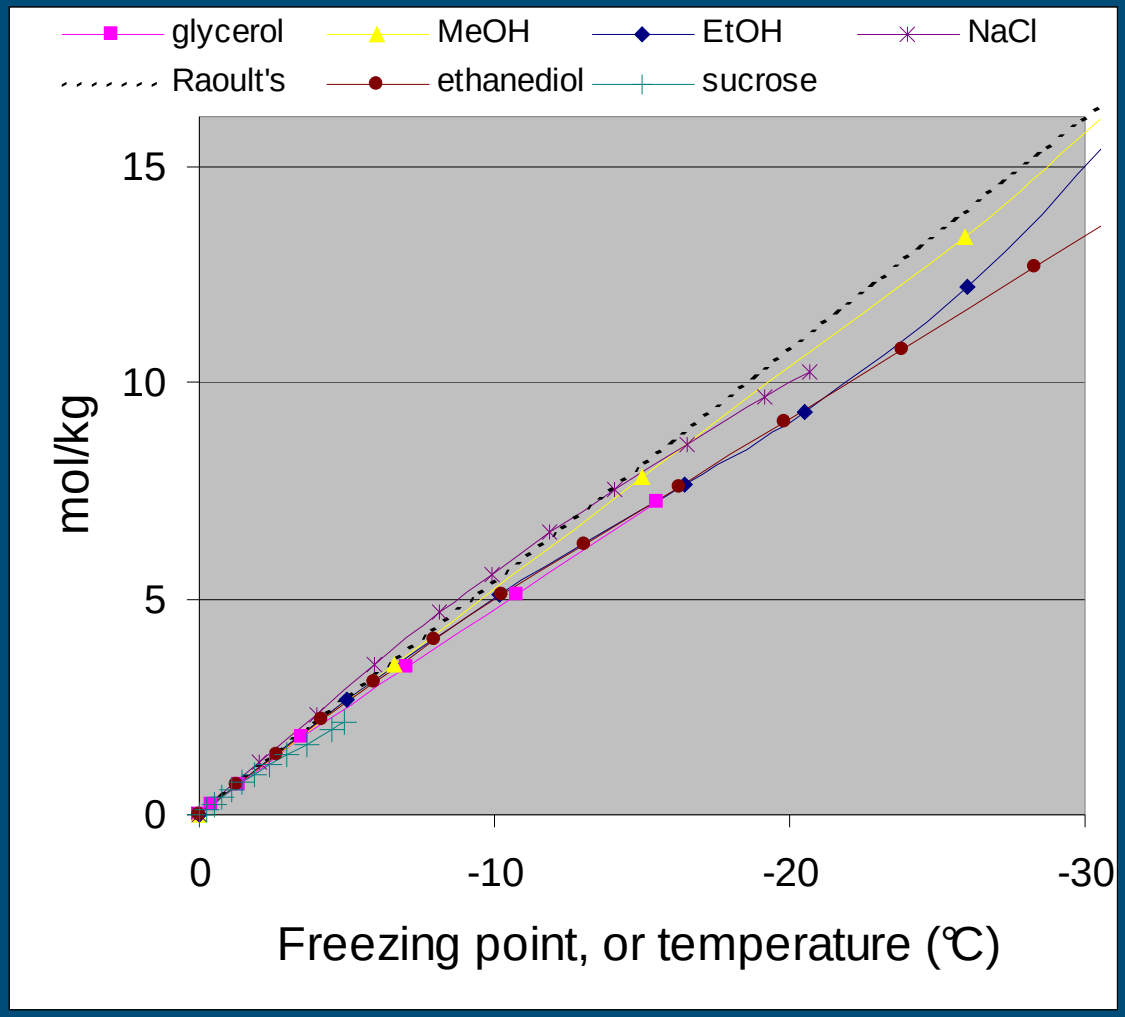


Woelders and Chaveiro 2004

Slow Freezing

Water freezes (extracellularly) as pure ice

An unfrozen fraction remains that contains all solutes

- The volume of unfrozen fraction ↓
- Water content ↓
- Solute (salt) concentration ↑
- Osmotic pressure ↑
- Viscosity ↑

While IIF is prevented!

At some point of temperature and concentration → Glass transition

Glass transition

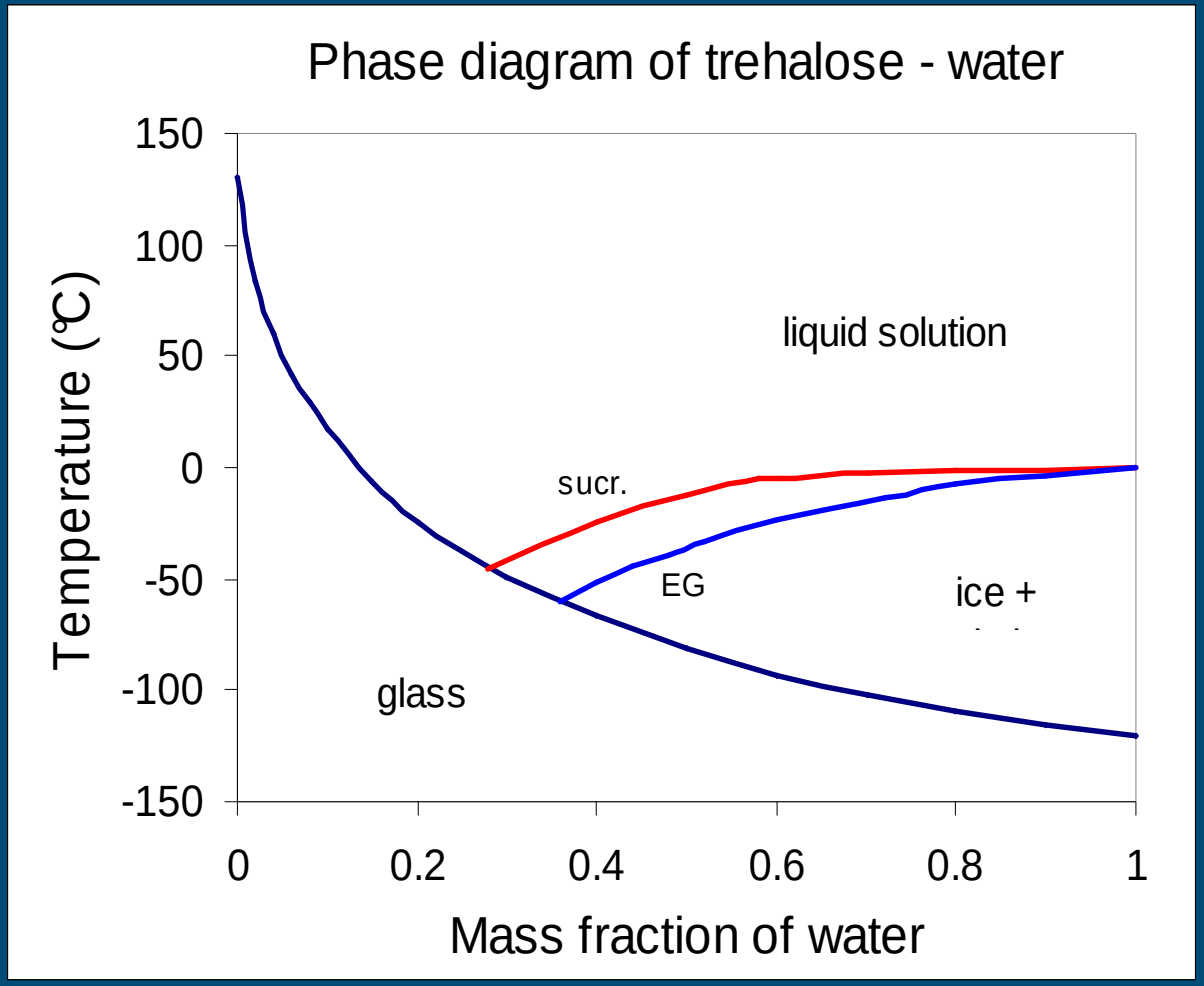
Glass transition means that a liquid becomes solid in an amorphous state. The lateral mobility of molecules becomes practically zero.

A glass is stable because (by definition) molecules have lost the ability of lateral movement. No significant biological or chemical changes will take place.

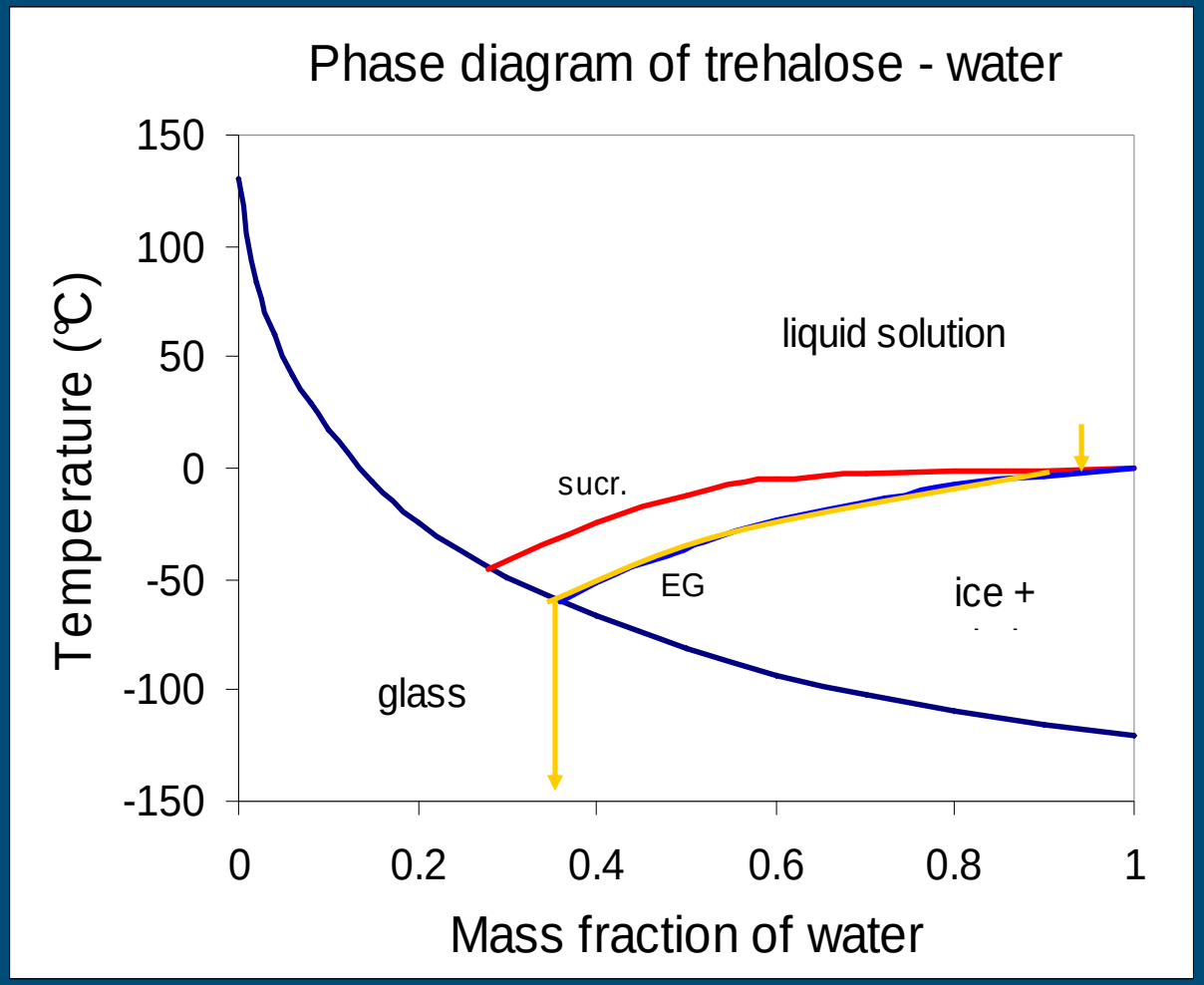
Phase diagram

Phase diagram of

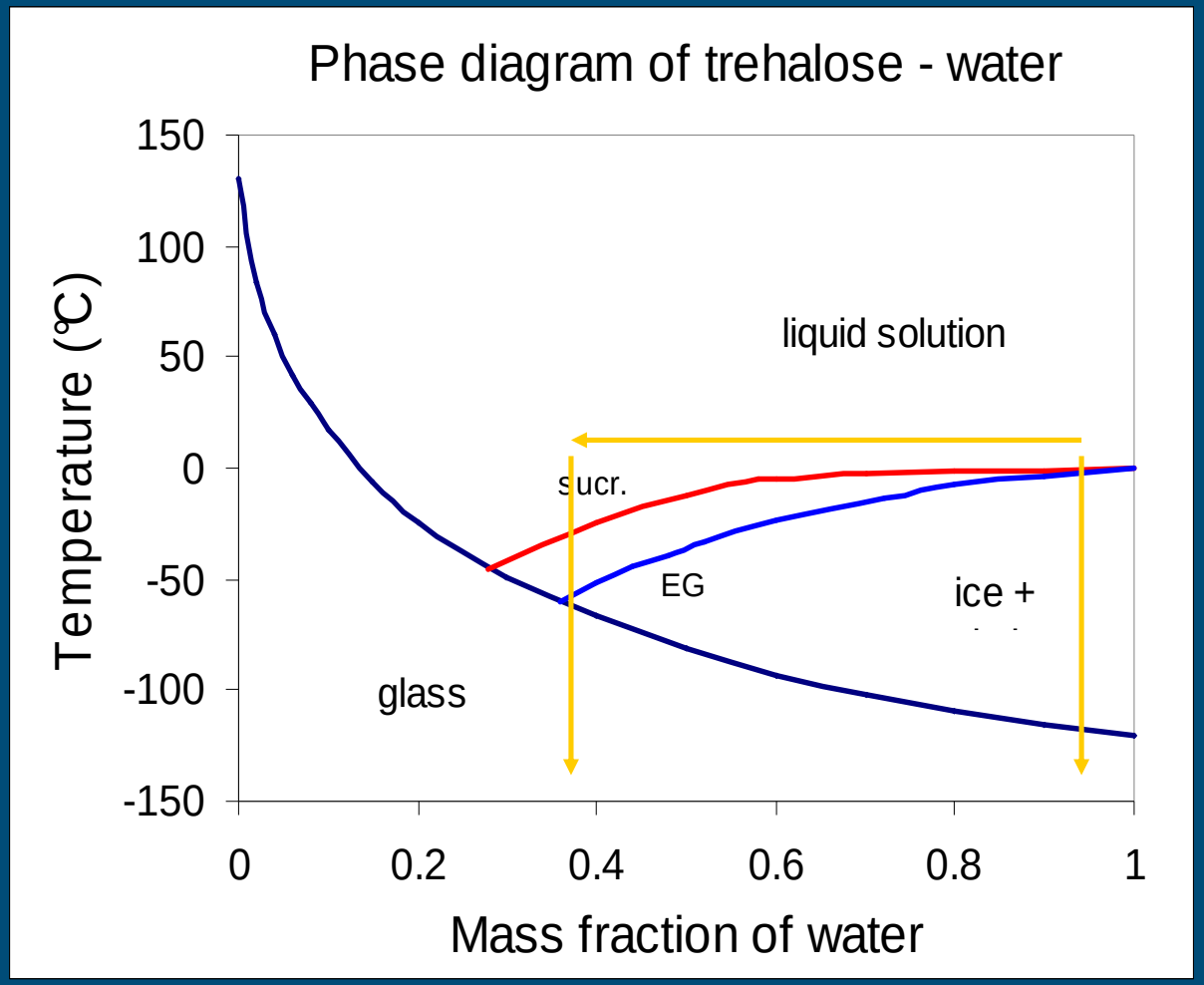
trehalose-water



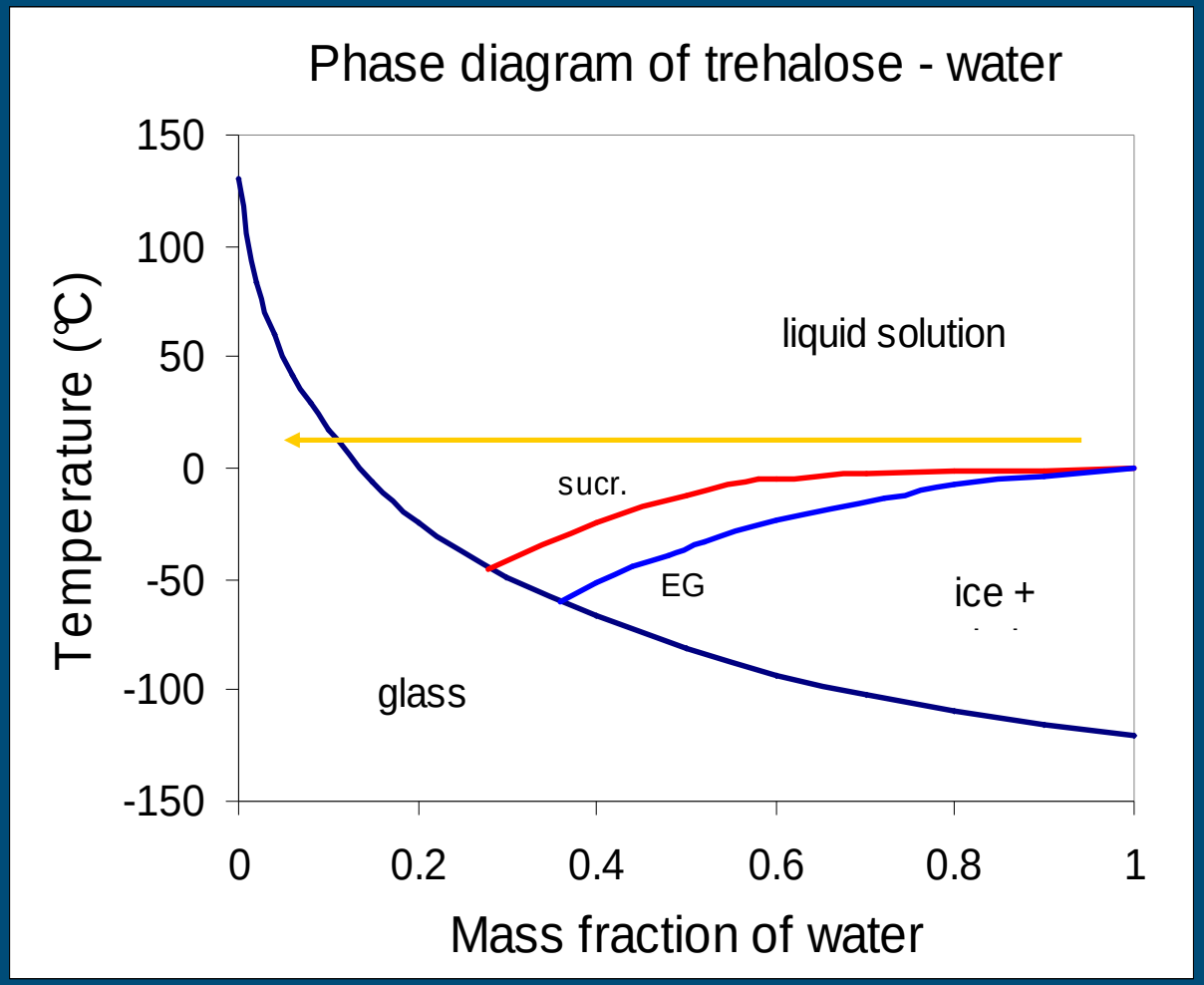
Slow-Freezing



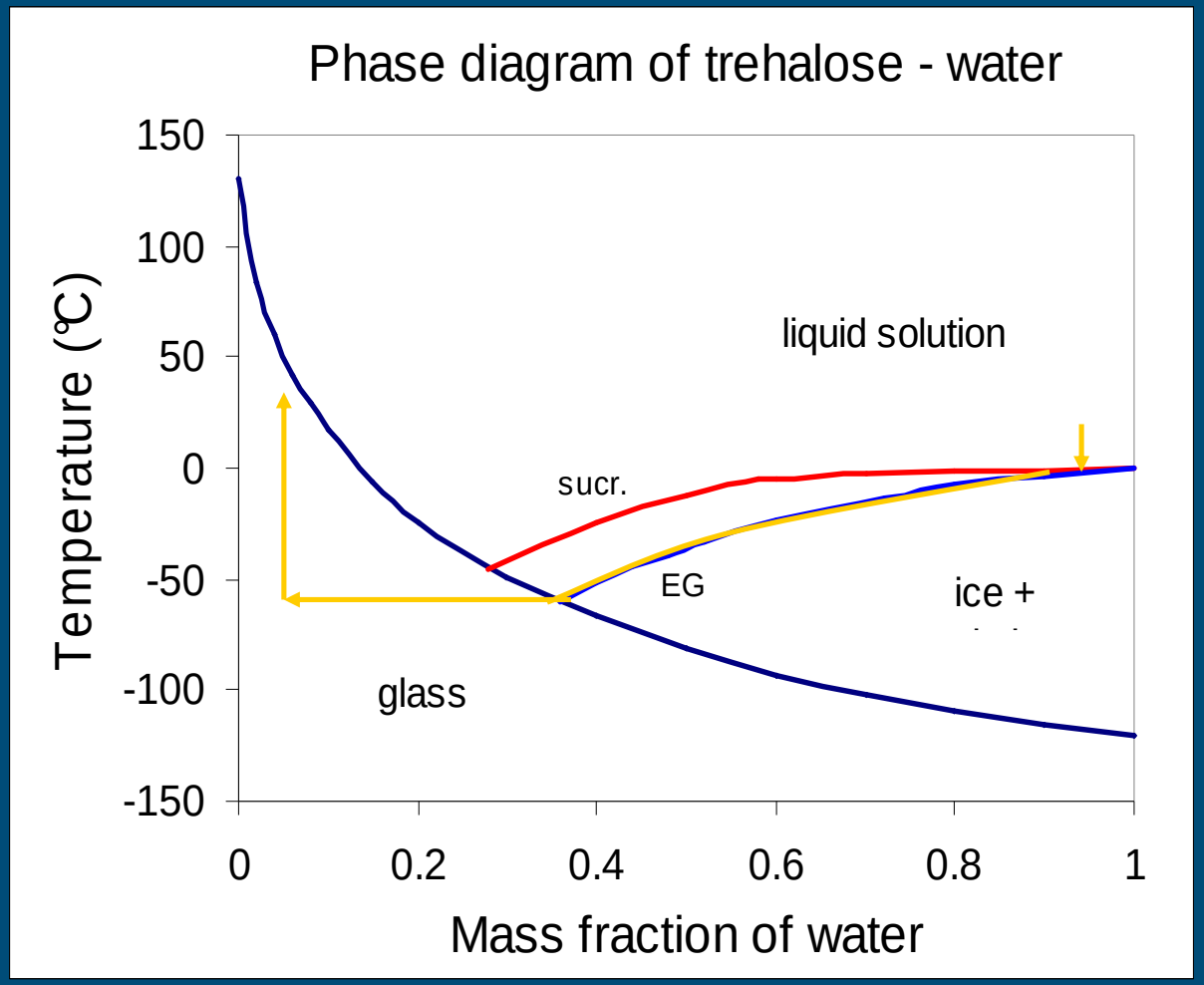
Vitrification



Drying (and/or increase solutes)



Freeze Drying



Stable preservation

- The common denominator in all these methods is that stable storage requires glass transition.
- For cold storage you must go to glass transition while preventing IIF
- The phase diagram explains how this is achieved in the various cryopreservation methods.

Cryoprotective Agents (CPAs)

So at some stage, the solute concentrations will be very high.

Negative effects:

- Cell shrink excessively
- Salt loading
- Destabilisation of proteins

By replacing part of the solutes by membrane permeating solutes this can be alleviated

Membrane permeating CPAs

- Replace part of the water. → Less ice can be formed.
  - The volume of the ‘unfrozen fraction’ remains larger.
  - Also the cells shrink less during freezing
  - The salt concentration inside and outside the cell is less

Membrane permeating CPAs

Examples of such compounds:

- propane triol (= glycerol)
- propane diol (= propylene glycol or PG)
- ethane diol (= ethylene glycol or EG)
- butane diol
- ethanol
- methanol
- dimethyl sulfoxide (= DMSO)

Positive effect of Glycerol was discovered ‘by accident in 1948 by Polge et al.

Not permeating CPAs

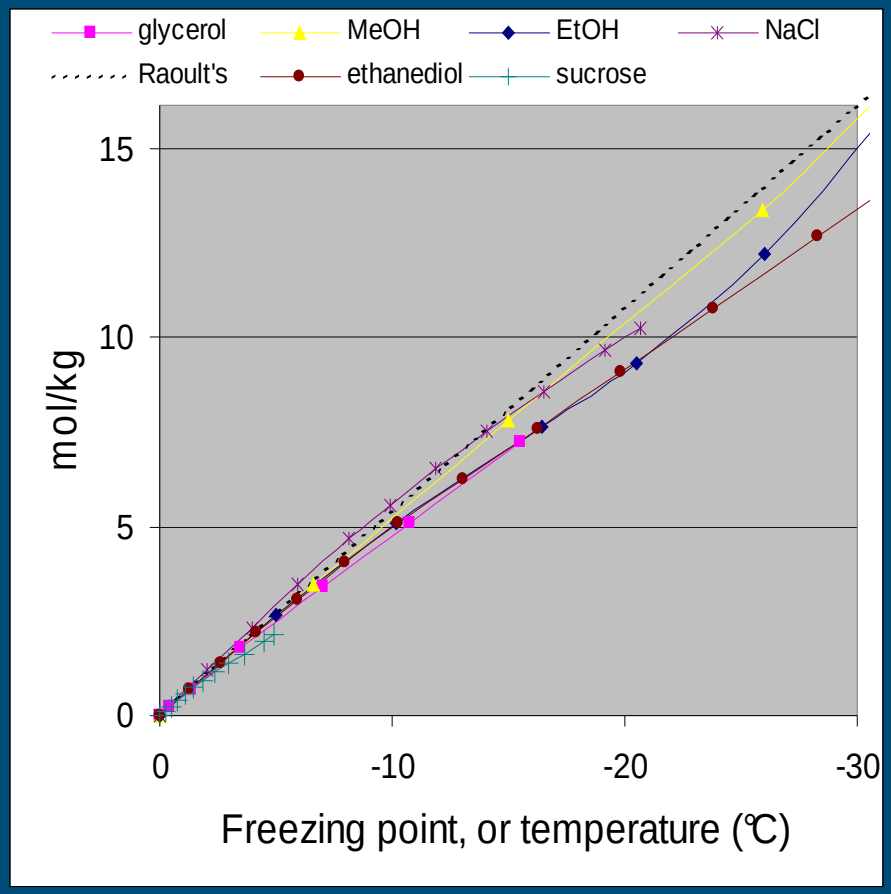
Not permeating cryoprotectants can also be used in addition

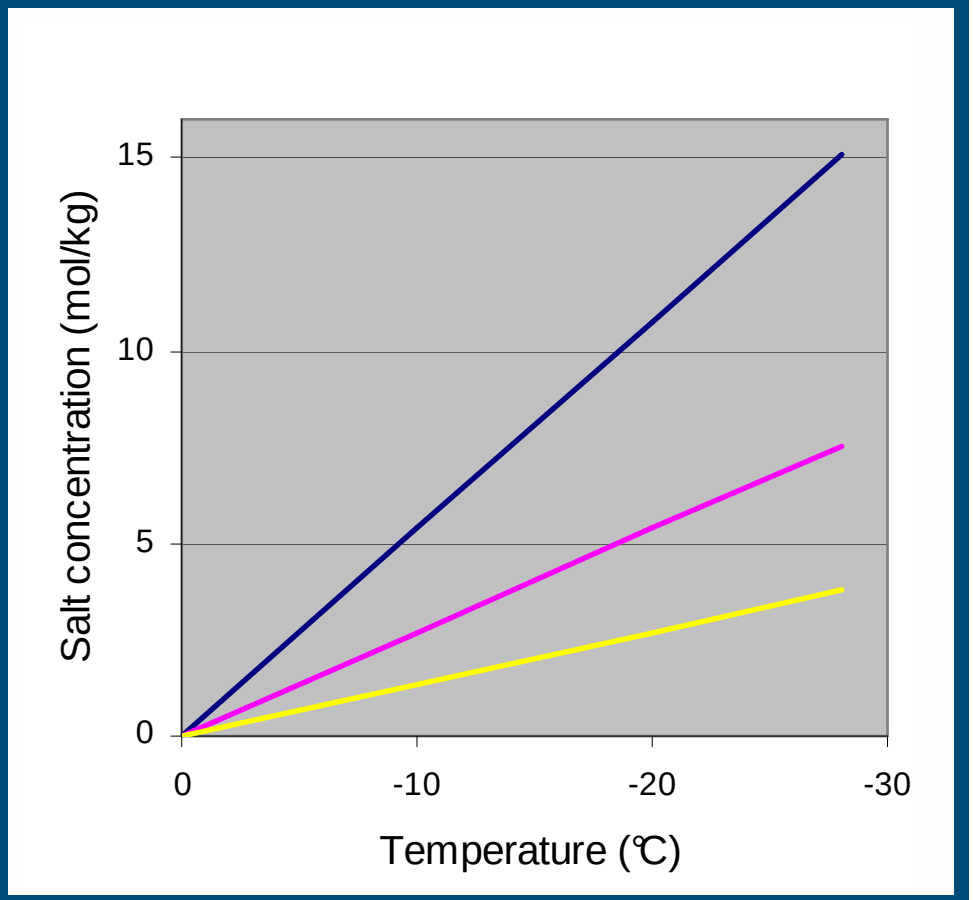
For instance macromolecules that increase the viscosity

- PVP
- PVA
- Ficoll
- Hexa-ethyl Starch (HES)

Or sugars (sucrose, trehalose)

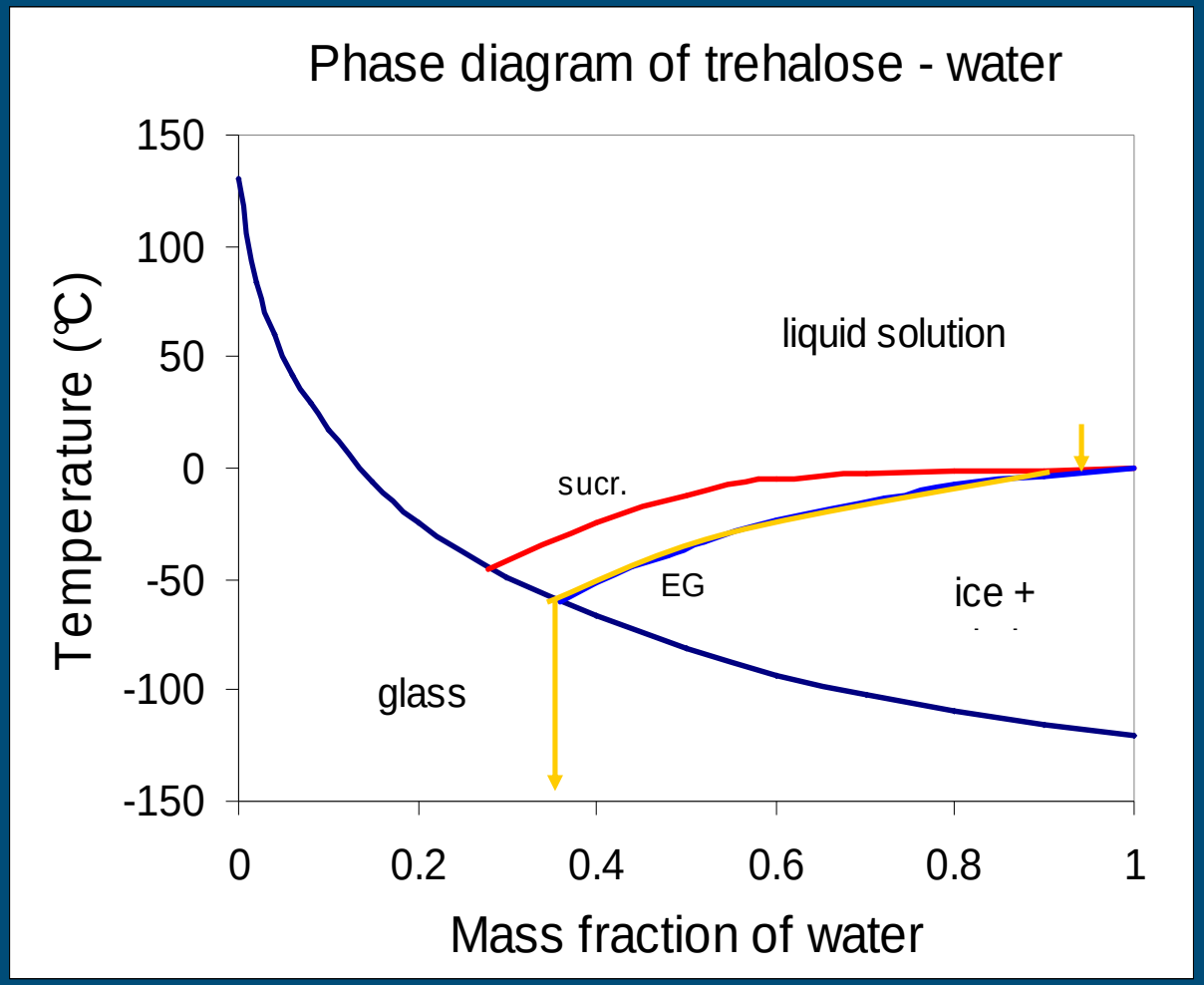
CPAs





Mazur & Rigopoulos, 1983

Again: Slow Freezing



Slow Freezing: The Cooling Rate

Why “Slow” freezing? and How slow?

The purpose of slow freezing is:

Increase solute concentration to glass transition while preventing IIF

IIF is prevented by cooling slow enough to allow the cells to dehydrate enough to prevent intracellular supercooling.

The question arises: How slow is “slow-freezing”

Cooling Rate

Two-Factor Hypothesis (Mazur et al, 1972)

<u>At too high rates</u>

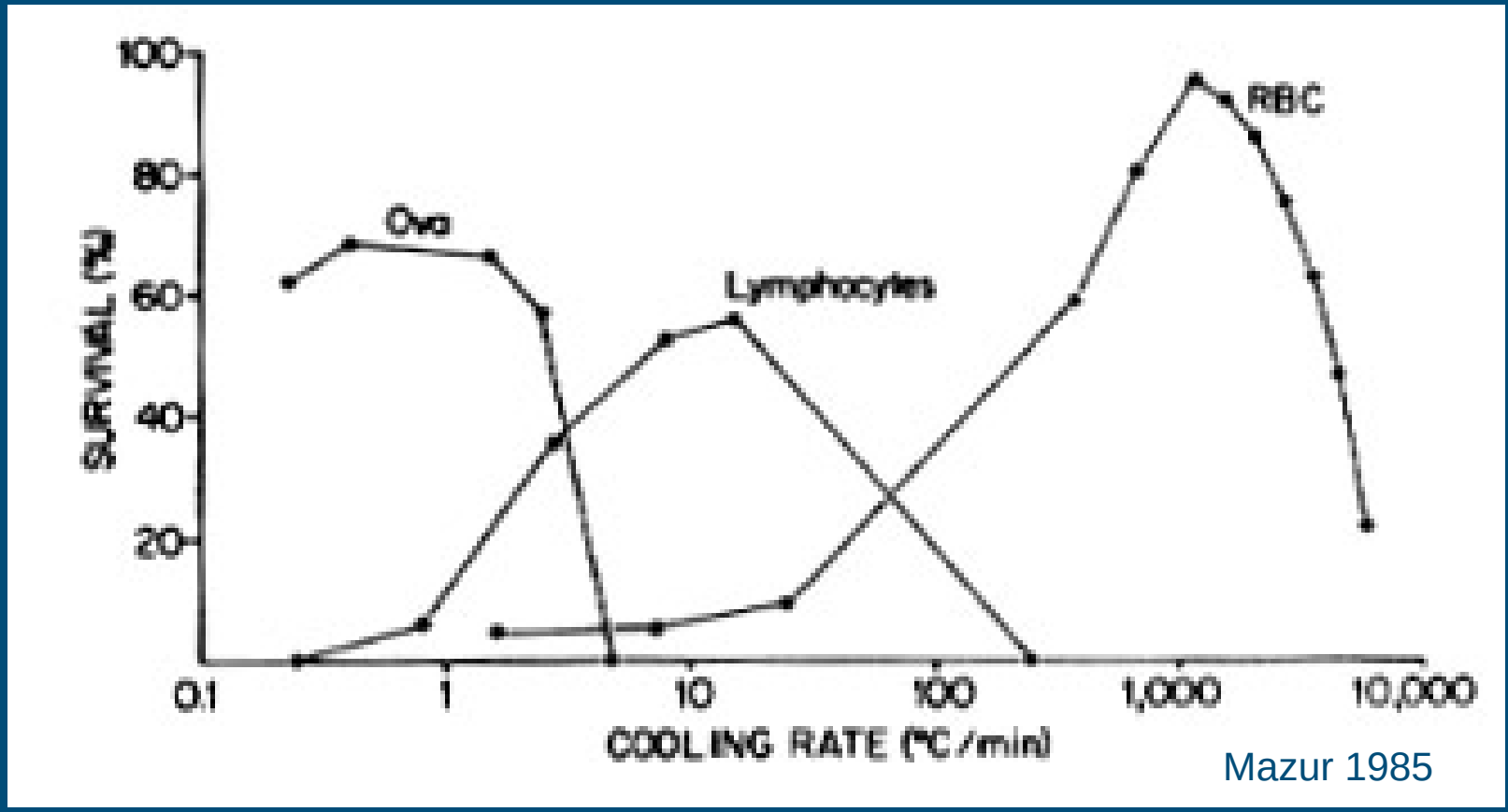
- IIF
- (or pore erosion at high water efflux)
- (or too rapid (ultra)structural changes)

<u>At too low rates</u>

- Cells shrink too much
- Cytoplasm too high salt
- Unfavourable conditions last longer

Cooling Rate

Optimal rate somewhere between ‘too slow’ and ‘too fast’



Mazur 1985

Theoretical Model (Woelders and Chaveiro 2004)

A mathematical model was developed that predicts the 'optimal' freezing programme, in which:

- The cooling rate is always as high as possible (to prevent so-called "slow cooling damage")
- While conditions that could lead to "fast cooling damage" are precluded.
  - No IIF
  - Membrane flux of water and CPA must not be too strong
  - Transmembrane osmotic pressure difference must remain within limits

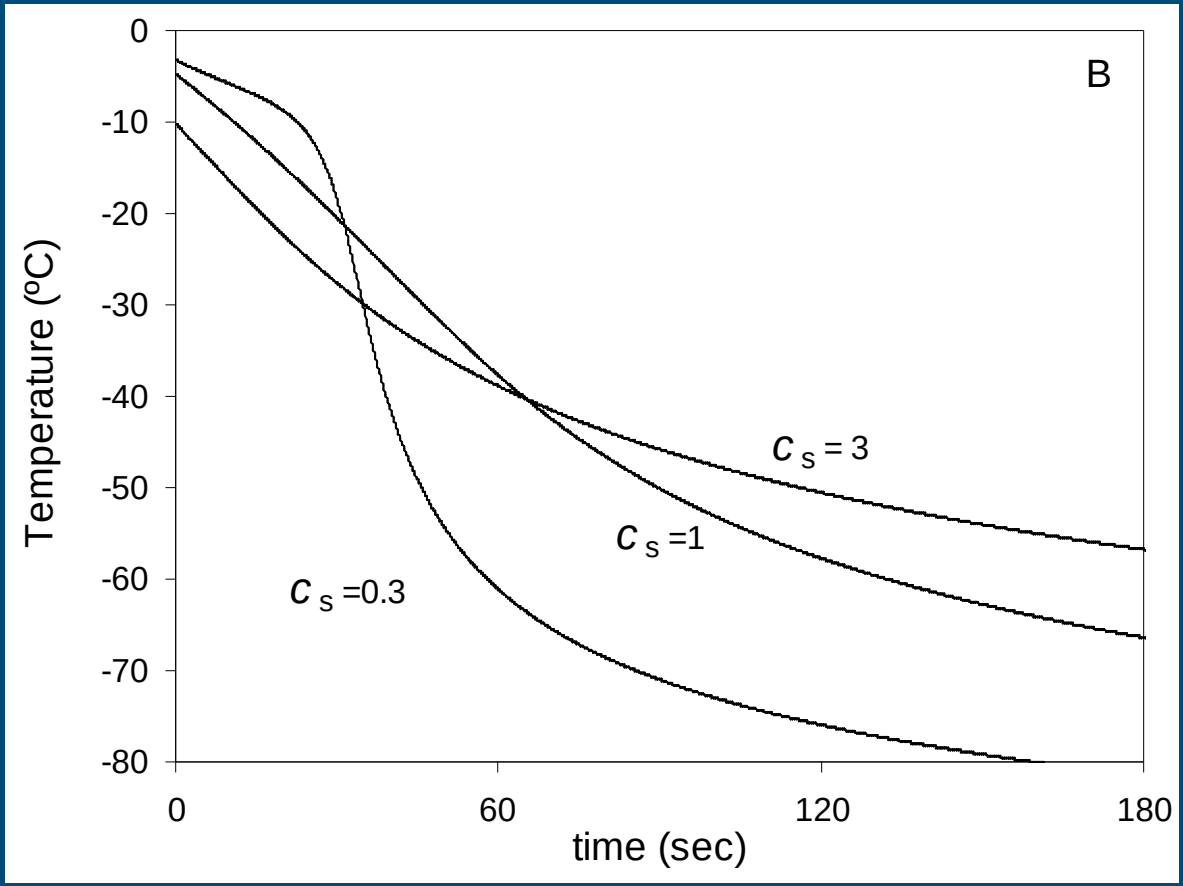
New Theoretical Model

Due to the chosen boundary conditions, the model predicts the ‘optimal’ freezing programme, in which:

- The cooling rate is always as high as possible (to prevent so-called “slow cooling damage”)
- While conditions that could lead to “fast cooling damage” are precluded.

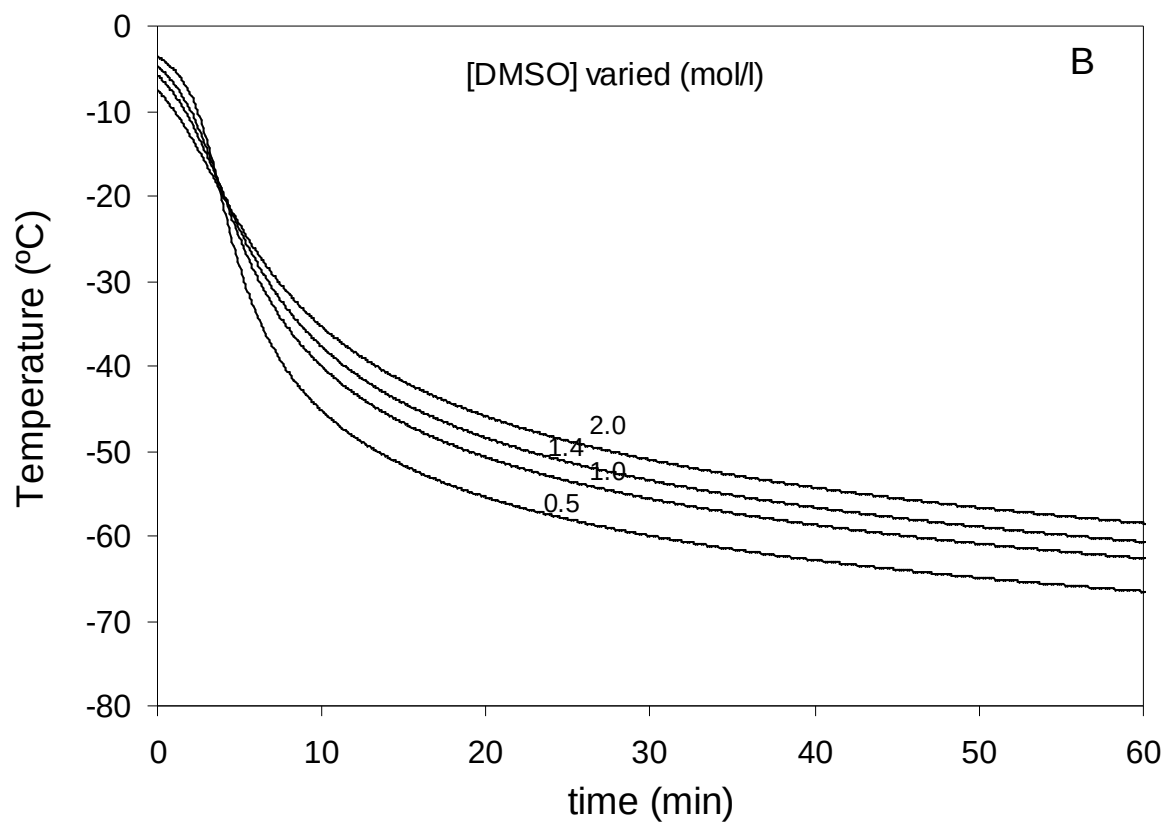
New Theoretical Model

Bovine spermatozoa (Woelders and Chaveiro 2004)



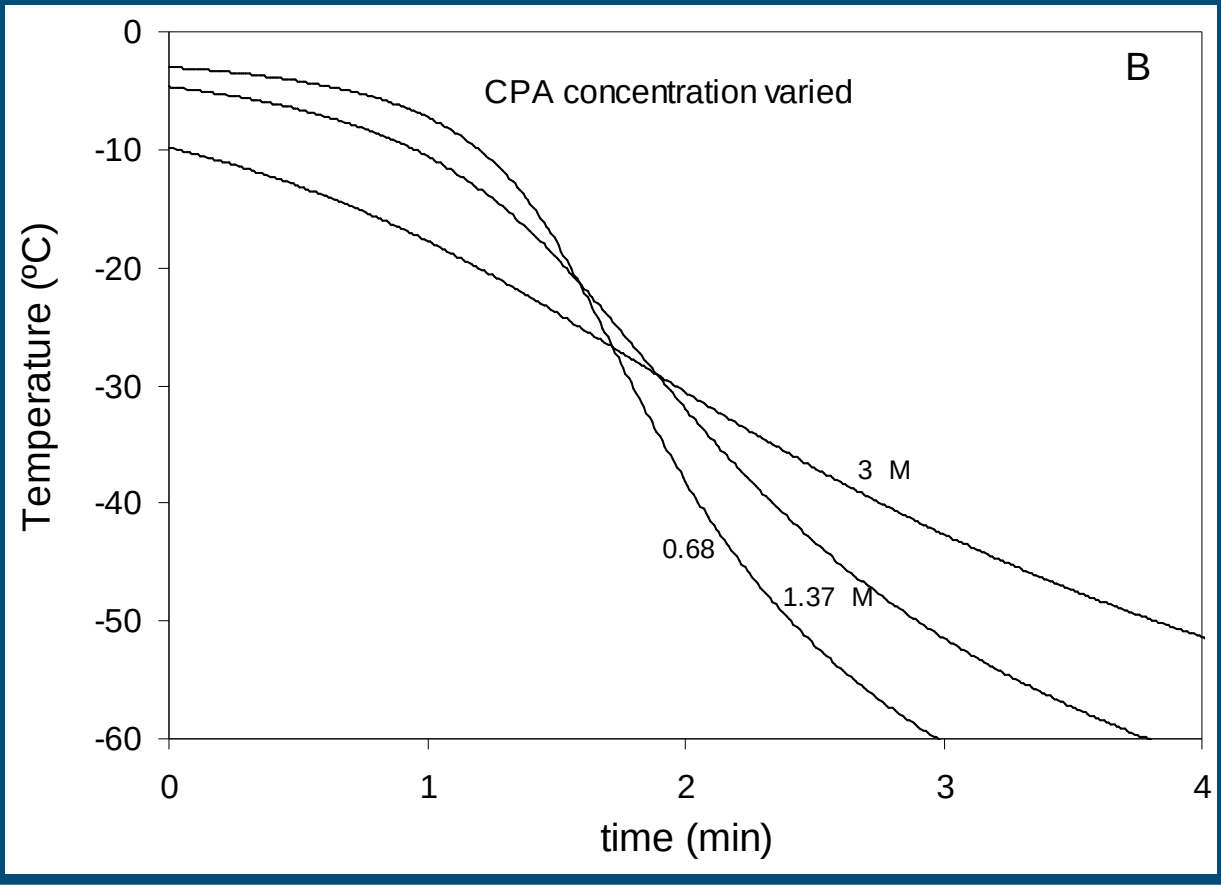
New Theoretical Model

Human blood stem cells (Tijssen et al 2008)



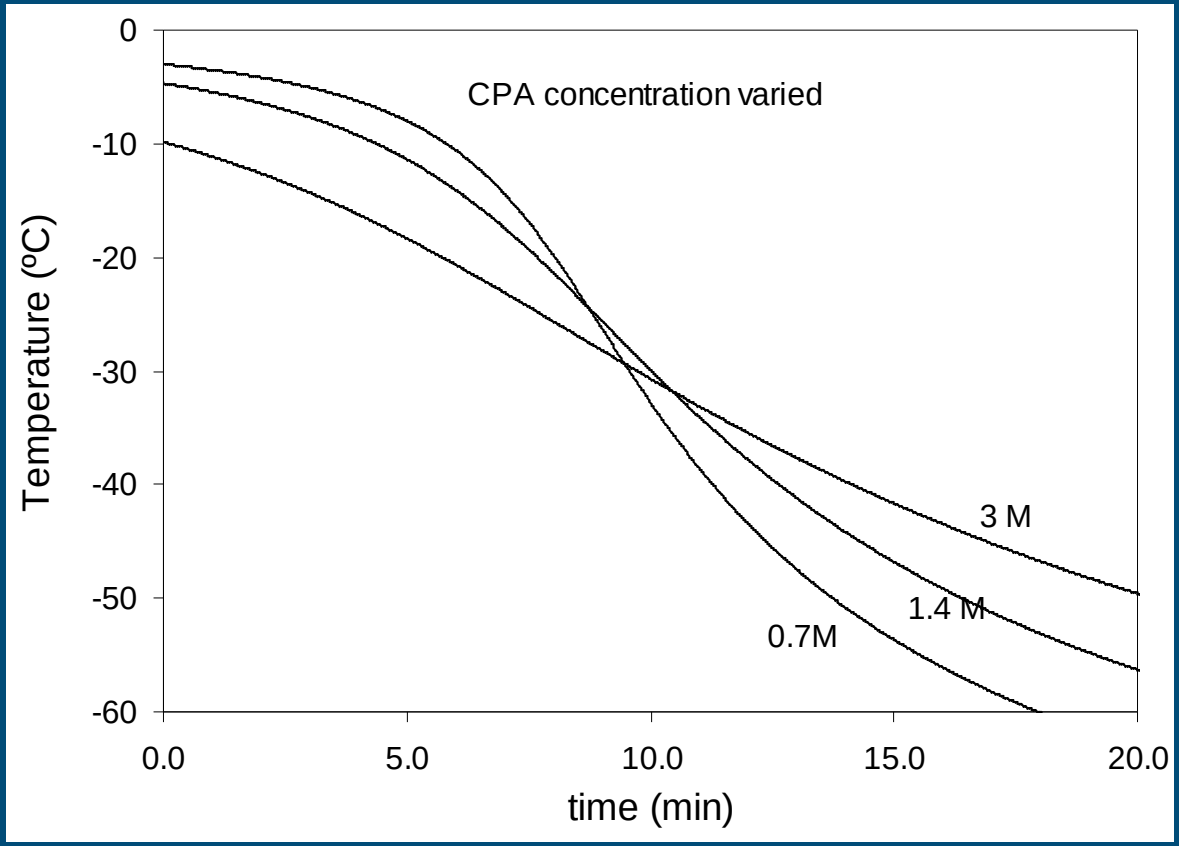
New Theoretical Model

Bovine morulae (Woelders et al 2007)



New Theoretical Model

Human oocytes (Woelders, unpublished)



Modelling Cell volume changes

- Cells shrink and swell when CPA is added
- Cells shrink during ice formation (slow freezing method)
- Cells swell and shrink when CPA is removed

- Simulations can show what happens in terms of cell volume but also in terms of intra and extracellular concentrations of solutes.

- Therefore, these simulations help make decisions on type of CPA and protocol.

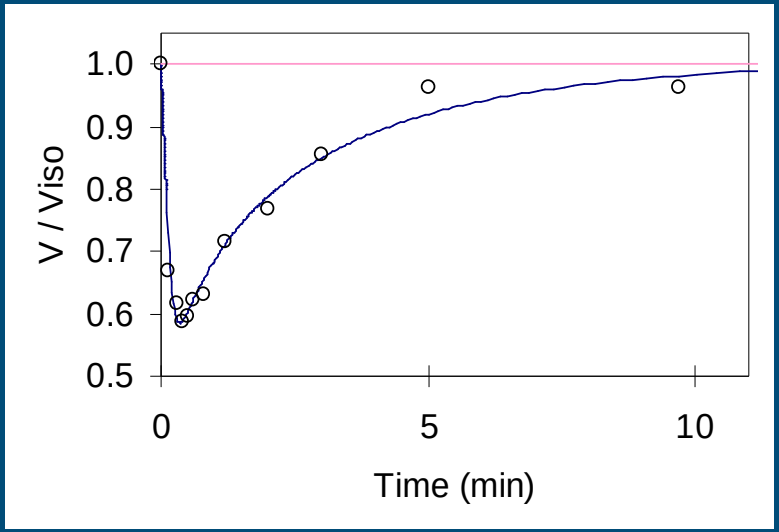
Volume excursion after adding CPA

- Addition of a permeating CPA
  - Water leaves the cells
  - Permeating solute enters the cells
  - This draws in water into the cells

  →Cells first shrink, then reswell

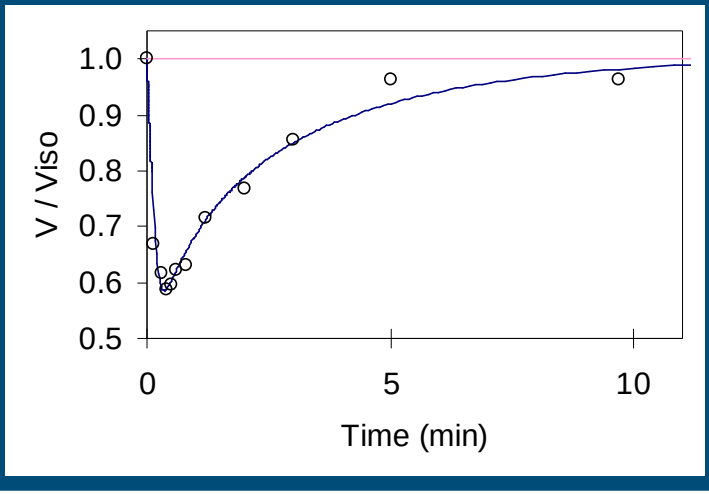
- Magnitude of volume excursion depends on
  - Ratio of Lp and Ps
  - Concentration of CPA

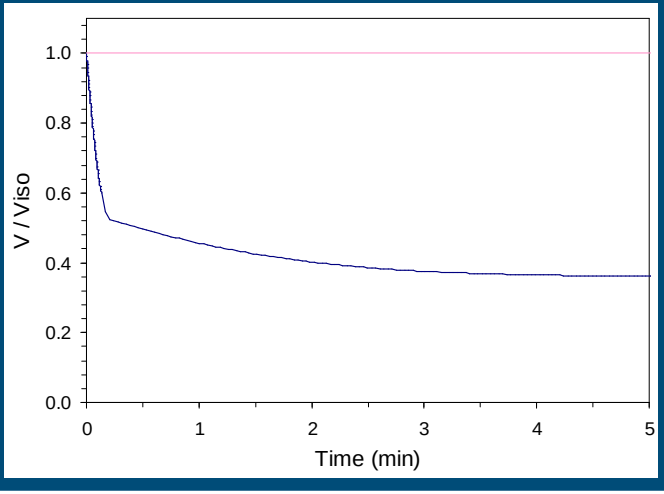
- May harm cells when exceeding osmotic tolerance limits



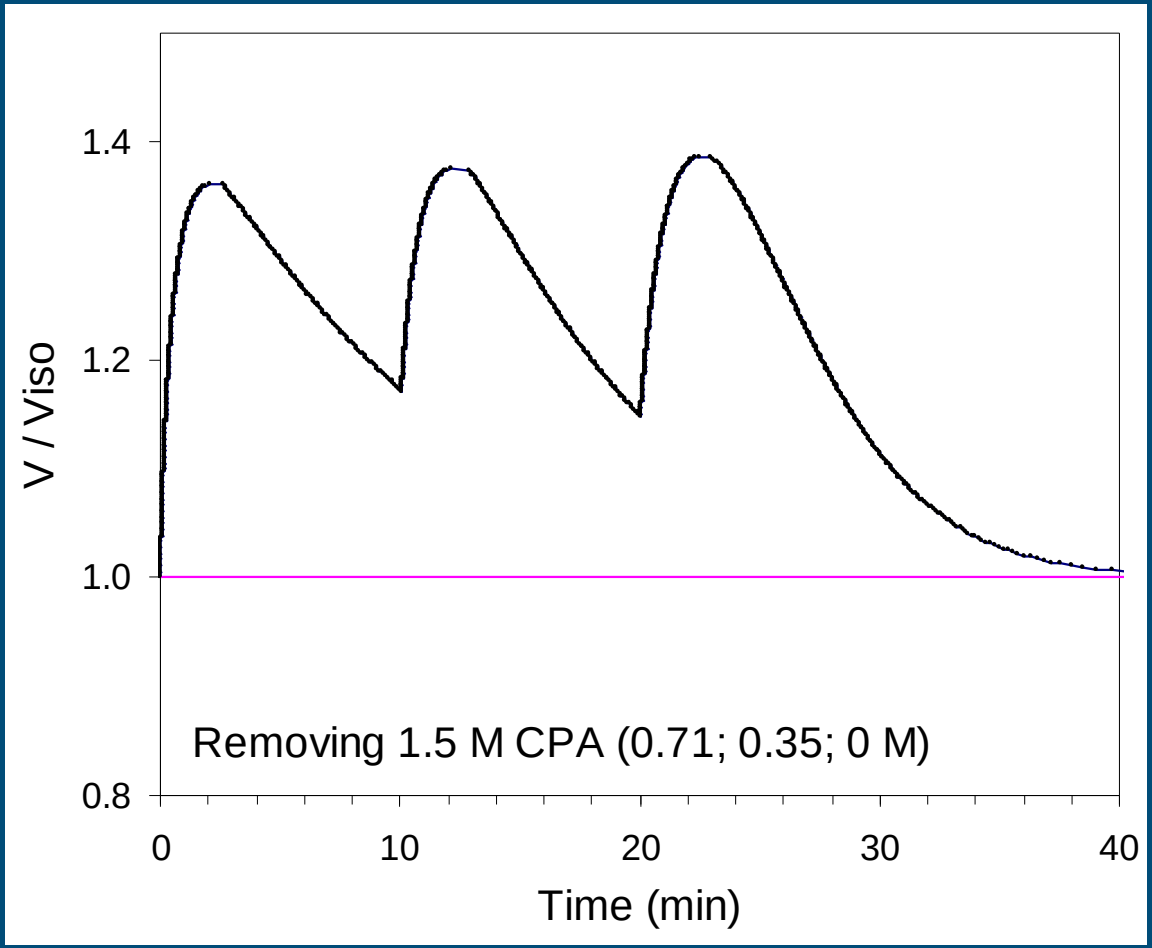
Volume changes in a vitrification method

- First step: Equilibration with a relatively low concentration of CPA's (e.g. 7.5% DMSO + 7.5% EG) during 20 minutes at room temperature.
  → shrink-swell cycle.
- Second step: Short exposure (<60 seconds) to a solution with 15% DMSO + 15% EG + 0.5 M sucrose (also at room temperature).
  → cells shrink rapidly.
- Plunge in liquid nitrogen





Volume excursions Removal of CPA



Human oocytes:

Lp = 0.55; Ps = 8.00

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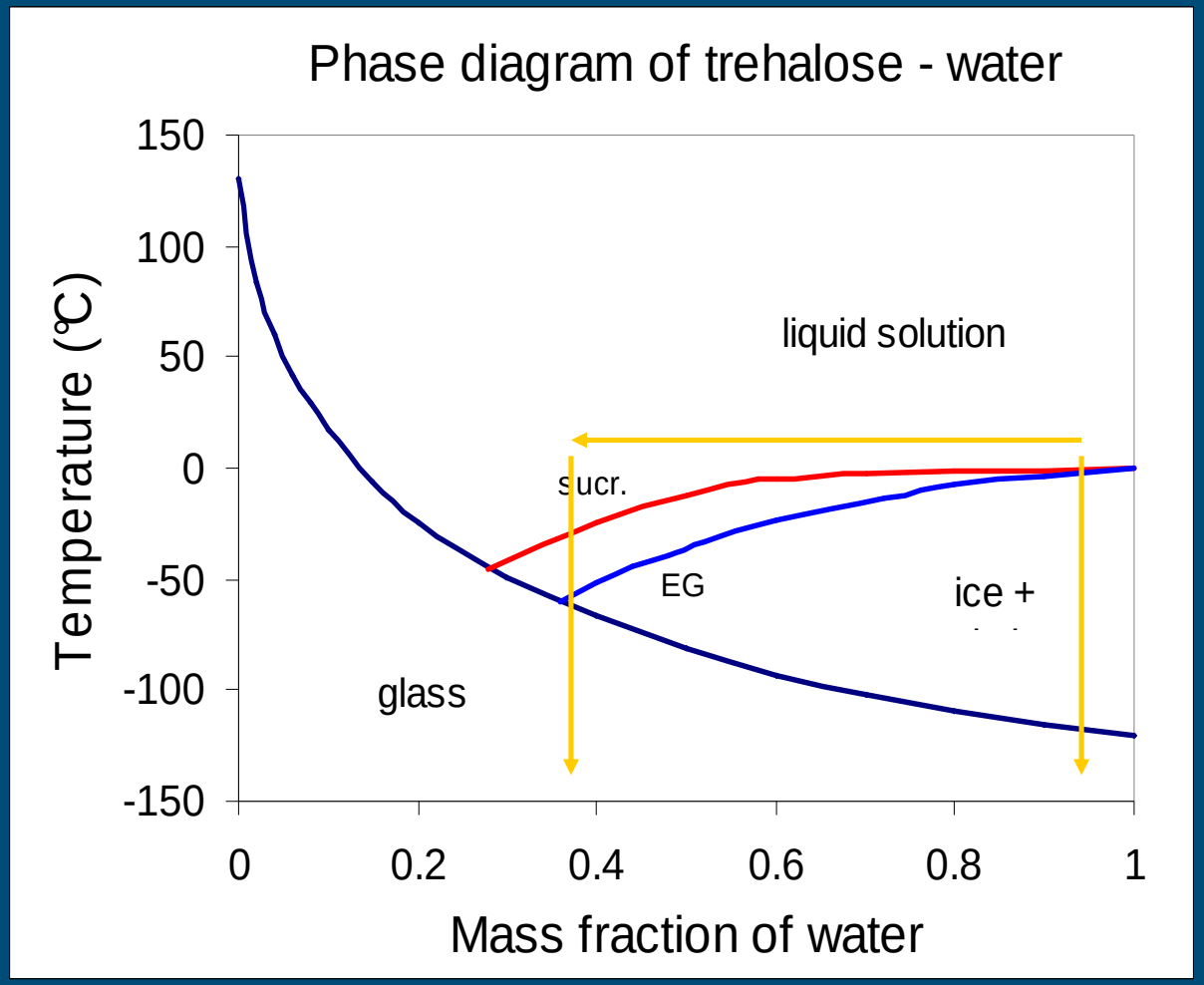
Cooling rate in Vitrification methods



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Cooling rate in Vitrification methods

- Very high cooling and thawing rate enable vitrification with lower CPA concentrations

- Ultrarapid cooling to outrun:
  - Spindle depolymerization
    (but see Stachecki et al 2004; Rienzi et al 2005)
  - Lipid lateral phase separation
  - Other hypothermia induced changes

Cooling rate in Vitrification methods

Very high cooling and thawing rate by techniques like

- Minimal volume (cryoloop, o.p.s., cryotop etc.
- N_2 at freezing point versus at boiling point (Vitmaster)

Applications, e.g.

- Semen vitrified without any CPA (Isachenko et al 2003)
- Oocytes vitrified with relatively low CPA concentration (e.g. Lucena et al. 2006; Antinori et al. 2007)

Thawing rate

Slow freezing

- (Too) rapid freezing may cause tiny sub-lethal intracellular ice crystals.
- During slow thawing, however, these small crystals could still grow in spite of the rising temperature because of *Recrystallisation*
- Recrystallisation means that larger crystals grow and fuse at the expense of the energetically less stable smallest ice crystals.
- Fast thawing may thus be needed when relatively high freezing rates have been used.

Vitrification

- In ultrarapid vitrification methods the CPA concentrations are too low for stable vitrification. During warming, ice formation is possible.
- Therefore, also ultrarapid warming is needed.

Other causes of cryoinjury

Cold shock and chilling injury

Before the actual freezing, cells may be damaged by cooling below body temperature:

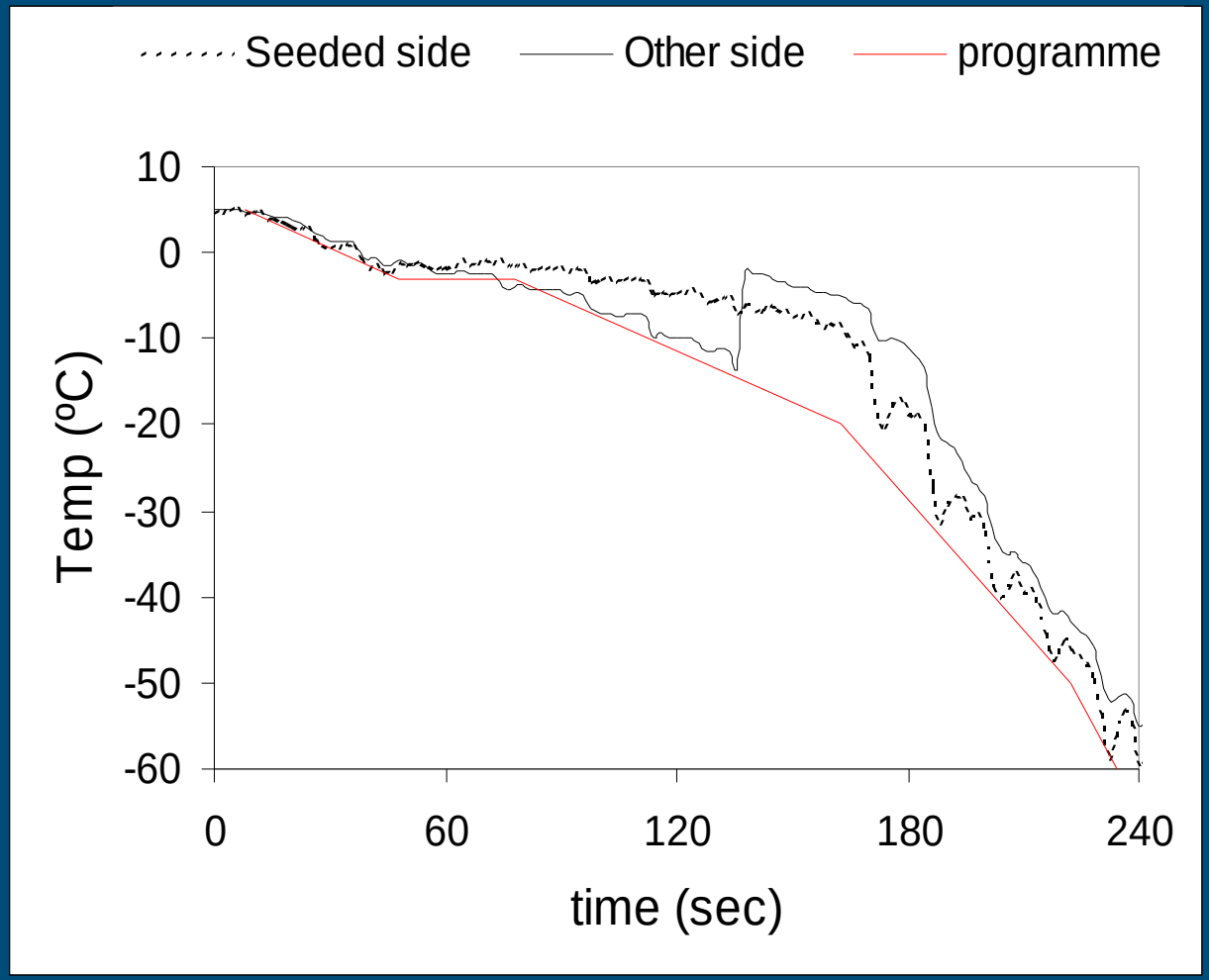
- Cold shock: damage by sudden cooling
- Chilling injury: damage by the low unphysiological temperatures *per se*

Boar semen for example is very sensitive to both cold shock and chilling.

Oocytes are very sensitive to chilling: At lower temperatures, the metaphase spindle disintegrates.

By choosing extremely rapid cooling rates these negative effects can be 'outrun'

Damage by supercooling



Damage by supercooling

- Boar semen
- Freezing on controlled-rate metal surface
- Split-sample comparison

Seeded at	% Live	% NAR
-5 ºC	65.3 ± 3.2	70.1 ± 2.6
-15 ºC	41.4 ± 3.9	23.3 ± 4.6

means ± S.E., n = 6

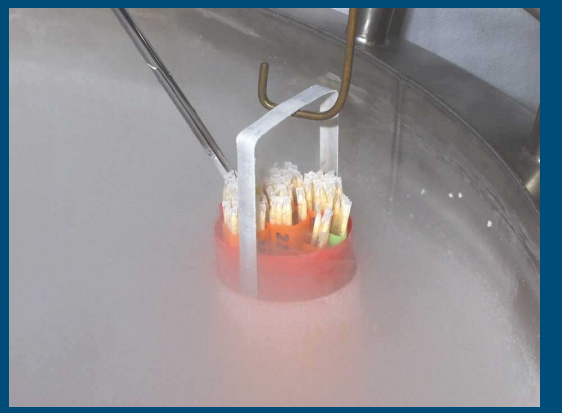
Recrystallisation and IIF during storage

- Recrystallisation means that larger crystals grow and fuse at the expense of the energetically less stable smallest ice crystals.
- Too rapidly frozen cells may contain sublethal ice crystals.
  - Melt during fast thawing → Cells are rescued.
  - Grow during slow thawing or during storage above the glass transition temperature → Cell death.
- Care must be taken during storage and during inspection or rearranging the content of a cryogenic storage tank.

Types of germplasm in AnGR gene banking

Types of genetic resource material

- Spermatozoa
- Spermatozoa + Oocytes
- Embryos
- Somatic cells



Feasible?

Practical?

Economical

Semen

advantages

- Existing ‘infrastructure’ for semen collection and for insemination in a number of species
- Survival of sperm after thawing is adequate in most cases

disadvantages

- Back-crossing is required, at least for 6 generations
- Mitochondrial genes are not conserved.
- In some species no ‘infrastructure’ is available

Semen collection

Small cattle breeds: Bulls not on AI station, but on farm.

On farm collection is agains EU regulation

Allowed in UK, Italy and NL. But strict in France and Finland

Other species:

Animals may be too ‘wild’ for semen collection

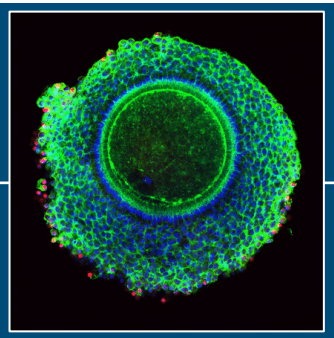
→ electroejaculation, or use epididymal semen

Epididymal ram semen

- Semi-quantitative collection from the caudae epididymidis of slaughtered rams.
- Post-thaw motility and Fertility of epididymal semen seemed to be better than that of ejaculated semen.

- On average **20 billion epididymal spermatozoa per ram**. = 108 doses of 0.2 billion sperm/dose.

Oocytes (plus semen)



- Full complement of chromosomal and mitochondrial genes
- Flexible in combining genotypes

- Many human babies born from vitrified and slow-frozen oocytes
- In farm animals, fewer successes reported
  Less incentive to develop methods in farm animal species?

- Live born young reported in cattle, horse, mouse and rat.

Embryos

- Full complement of chromosomal and mitochondrial genes
- Farm animal species reported life born young:
  Cattle, sheep, pig, horse, goat, rabbit
- ‘Easy’ in cattle and sheep
- In some species ‘infrastructure’ for ET is available.

Somatic cells → SCNT 'cloning'

- Collection of suitable somatic cells is possible and easy.
- Somatic cells, e.g. skin fibroblasts, can be readily cryopreserved.
- Using relatively simple cryopreservation techniques
- Full complement of chromosomal, but no mitochondrial genes, after transfer of nuclei (NT)

- Live offspring in: sheep, goats, cattle, pigs, horse, mule, mice, rats, rabbits, ferrets, cats, and dogs.

Somatic cells (2)

- For cattle and sheep only 0 - 5% of NT embryos result in live young
- Many pregnancies aborted, or result in malformed young.
- For pigs and horses, higher success rates are reported, with near normal rates of malformed young.
- Viable litters of cloned pigs are now obtained routinely

Somatic cells (3)

- In general though, techniques for cloning seem not safe and efficient now.
- However, future developments on the longer term are very likely.
- Therefore, cryopreservation of somatic cells could be a cheap and easy “insurance” of present day genetic diversity to be used only very much later

Possibilities

	Semen	semen plus oocytes	embryos	somatic cells
Samples needed to restore breed	10000	2 x 100	200	Depends on future efficiency of cloning
Backcrossing	Yes	No	No	No
Mitoch. genes	No	Yes	Yes	No
collection possible	Mostly	Yes, various species. Routine for bovine	Yes, various species. Routine for bovine	Always
Cost of collection	$$	$$$	$$$	$
Life young	Yes	human, cattle, horse, mouse, and rat.	Many species. Routine for Bovids, sheep, and human.	Yes (easily and cheaply)
How to use	Surgical or nonsurgical Insemination. Backcrossing ≥ 6 generations	ICSI → In vitro culture → surgical or nonsurgical ET	Surgical or nonsurgical ET	Culture somatic cells, isolate, culture, enucleate oocytes → Transfer somatic nuclei to oocytes → in vitro culture → surgical or nonsurgical ET
Cost of use	$	$$$	$$	$$$$
Possible?	Yes	Yes	Yes	Low efficiency and clear risks. Future development is likely!

Choice of cell type

Depends on goal(s) of gene bank

- Long time storage, no updates or regular use
- Medium-term storage, but with regular exchange with *in situ* populations. E.g. semen used to support breeding schemes.

Semen and embryos can be updated regularly and be readily used in the field.

In the Netherlands

- Using semen
- Starting with embryos (endangered cattle breeds)
- Only beginning to consider somatic cells

Thank you for your attention

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Questions?

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