Freezing and thawing serum and other biological materials: optimal procedures minimize damage and maximize shelf life

HYCLONE SERA

In general, serum should be frozen rapidly, stored at the lowest possible temperature, and thawed properly. Rapidly frozen serum has higher oxygen content than slow-frozen. As a consequence, the oxidation of some serum constituents will be increased in rapidly frozen serum. Here, we describe how to properly protect sera, media, and cells against auto- and photo-oxidation. It is recommended that serum and other medium components stored in freezers are held below the temperatures at which they freeze to a complete solid (1). Hence, we investigated conditions for preserving serum and other biological solutions.

What happens when biological solutions freeze?

Sera or other complex organic solutions can deteriorate when they are frozen. Water is the first serum component to freeze, and as ice forms, dissolved serum components are excluded from the ice crystals. Water expands and becomes less dense as it freezes, it floats and forces salts and other unfrozen serum components downward. These other components eventually become supersaturated and viscous, and might crystallize, which can damage the serum.

It is not feasible to store serum at a temperature at which all water is frozen. Water in different parts of a solution periodically goes through solid, liquid, and vapor phases. As a result, solutions that are not tightly capped (or solids such as meat that are not carefully wrapped) will dehydrate by sublimation. As water in localized areas of serum is repeatedly frozen and thawed, solutes continue to move toward the bottom of the container and, if not mixed, will further compromise serum quality.

Kaighn showed that when sucrose solutions were frozen at -20°C and -70°C, the solutions appeared to freeze within 30 min (2). However, when tubes of solutions frozen at the two temperatures were thawed 1 h and 18 h later, it was apparent that sucrose had moved toward the bottom of the tubes (Fig 1). One hour after the tubes were subjected to the subzero temperatures, the concentration of sucrose toward the bottom was greater in the solutions frozen at -70°C. After 17 additional hours at these temperatures, sucrose continued to move toward the bottom of the tubes held at -20°C but not in the tubes held at -70°C. After 18 h, there was more sucrose at the bottom of the tubes held at -20°C than in tubes held at -70°C. There was also a greater concentration of sucrose near the wall of the test tubes.

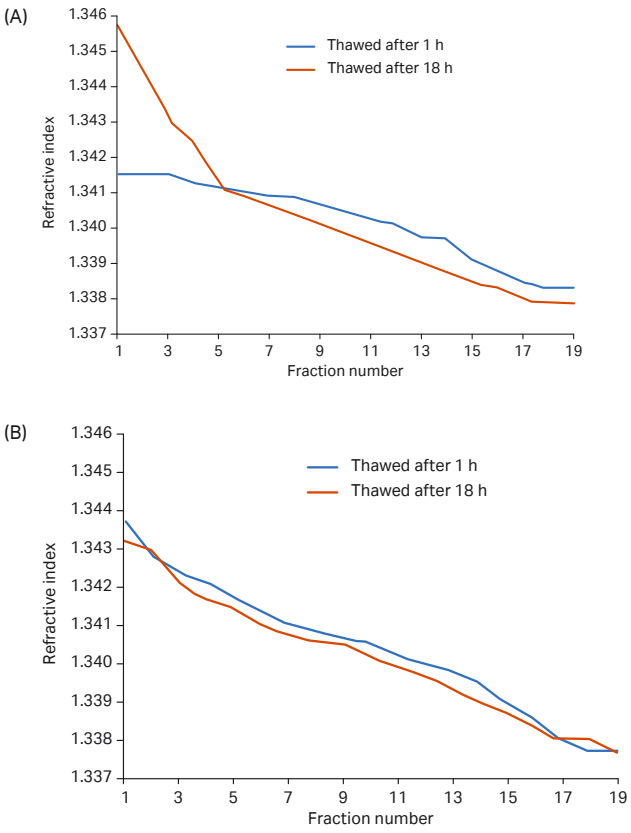

Fig 1. Gradient formation during freezing of sucrose solutions at (A) -20°C and (B) -70°C.

Adenosine deaminase and lactate dehydrogenase frozen at -20°C also tend to sediment at the bottom of tubes (2). Sedimentation of these enzymes frozen for 18 h at -20°C was greater in Tris buffer than in a sucrose solution.

Properties of water in biological materials

Nagashima and Suzuki classified water in frozen biological materials into three groups: (i) free water (ii) weakly bound water, and (iii) tightly-bound water (Fig 2) (3). Free water freezes first. The weakly-bound water is more difficult to freeze, while the tightly-bound water will not freeze until there is a much greater decrease in the temperature. This finding is primarily due to the increased solute concentration in bound water, which lowers both the freezing point and vapor pressure (evaporation potential) of the water, in addition to other colligative properties. When free water is frozen, the remaining unfrozen water in the solution is composed of weakly bound water, tightly bound water, and water that is supersaturated with salts and other solutes. This unfrozen water is responsible for the deterioration in serum quality. Even at a temperature of -35°C, which is 15 degrees lower than the temperature at which serum is usually stored, considerable amounts of unfrozen water exist to affect low-temperature chemical reactions. Each complex biological mixture has its own unique freezing curve, and thus, its own optimum storage temperature.

Van den Berg and Bruin report that tightly bound water in foods is difficult to remove by evaporation (4). Water is sorbed at active polar groups, usually by hydrophilic materials such as proteins and polysaccharides, and indirectly bound in maintaining spheres of hydration around solute ions or molecules. Weakly bound water is intermediate; it evaporates less readily than unbound water, but more readily than tightly bound water. Water that does not freeze enhances solution processes and rates of chemical reactions.

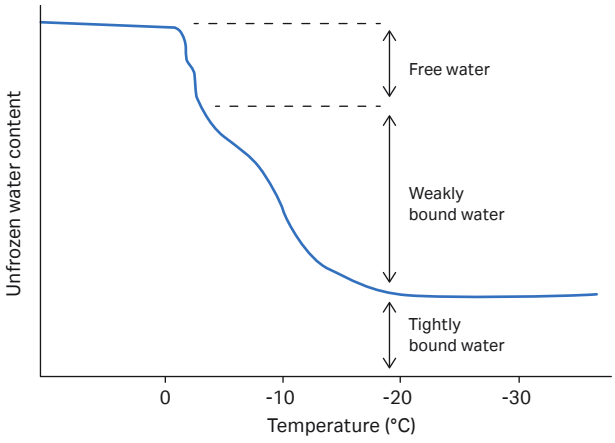

Fig 2. Tentative definition of weakly bound water and tightly bound water on freezing curve. Modified from Nagashima and Suzuki (3).

Influence of freezing on chemical reactions

Fennema discussed changes in protein solutions exposed to temperatures below ambient, and noted that many researchers neglect to pay attention to the effects on proteinaceous samples when stored at low temperature. Instead, it is simply assumed that storage at a lower temperature is better (5).

Freezing protein solutions can affect functions such as enzymatic activity and protein structure (5). These effects vary with cooling, freezing, and thawing rates; with storage time and temperature; and with the composition of the solution. The difference in the responses of proteins to freezing means that no single set of conditions is optimal for all proteins or serum pools. Some enzymes are more tolerant to freeze-thaw treatments than others. For example, fibrillar proteins are more susceptible to freezing damage than globular proteins (5). Molecular changes that occur in proteins held at low temperatures are dissociation of oligomers into subunits, rearrangement of subunits within oligomers, aggregation, and conformational changes.

The different reactions that occur in frozen solutions have different temperature optima (6). No single storage temperature would eliminate auto-oxidation, browning (a Schiff-type reaction forming a stable Amadori product), and enzymatic activity. In the hypothetical example shown in Figure 3, the most practical temperature would be at which the browning and enzyme activity curves intersect. It is usually not possible to store serum at the hypothetical optimal preservation temperature (where the auto-oxidation curve intersects with the other two curves).

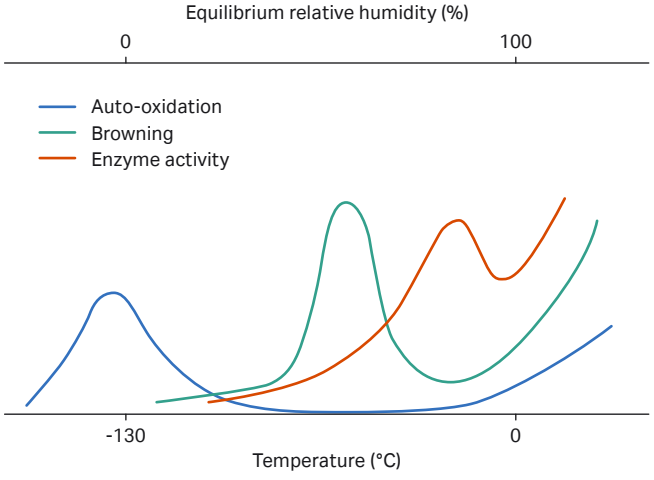

Fig 3. The influence of temperature and water on auto-oxidation, browning, and enzyme activity (partly theoretical). Modified from Poulsen and Lindelov (8).

Malondialdehyde is a decomposition product of lipid auto-oxidation in frozen materials (6). Malondialdehyde cross-links proteins by reacting with amino groups. This cross-linkage with trout myosin was greater at -20°C than at 0°C (7).

Poulsen and Lindelov described how freezing can accelerate chemical reactions (Table 1) (6). These reactions affect the deterioration of serum and other solutions that contain biological materials.

Table 1. Mechanisms by which freezing alters the rates of chemical and physical changes

1. Concentration of solutes during freezing causes:
   a. An increase in the rate of some chemical reactions.
   b. Protein precipitation and denaturation as the ionic strength increases.
   c. An increase in viscosity.
   d. A decrease in water activity.
   e. Changes in surface and interfacial tension.
   f. An increase in oxygen concentration and in oxidation.
   g. An increased salt concentration that produces a decreased stability of fat.
2. pH shift
   a. A lowering of pH can accelerate lipid oxidation and protein denaturation.
3. Enzyme reactions
   a. The rate of some reactions accelerates as freezing occurs.
4. Formation of antioxidants
   a. The maximum rate of formation of antioxidants produced by the interaction of amino acids and aldehydes is -24°C.
5. Ice surfaces
   a. Might act catalytically in a similar manner to other inert surfaces.
6. Proton mobility
   a. Can be greater in ice than in water.
7. Dielectric constant
   a. Is more favorable in ice than in water, so promotes nucleophile associations.

Reactions that result from concentration of solutes during freezing

Among the chemical reactions that can increase in frozen liquids are enzymatic activity, nonenzymatic browning (NEB) and auto-oxidation (Fig 4).

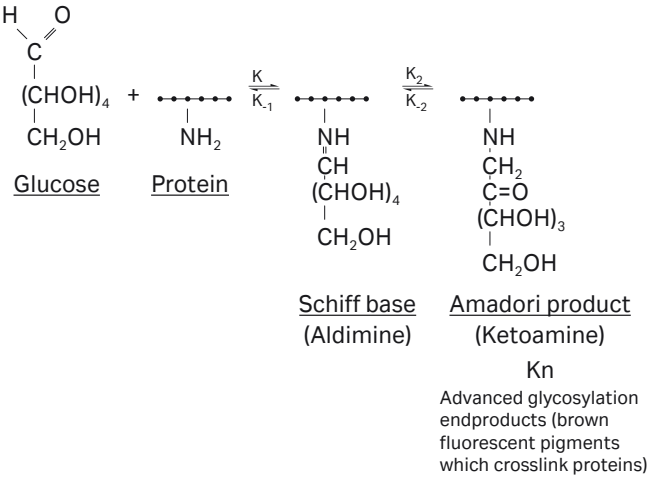

Fig 4. Nonenzymatic browning reaction of glucose and proteins. From Lee and Cerami (9).

Enzymatic activity

As expected in classical enzyme systems, enzymatic activity decreases as the temperature above freezing decreases. However, enzymatic activity increases as the temperature drops below 0°C, as reactive molecules are brought into closer proximity when solute concentration increases.

Browning

Much of the information about browning, which changes colors and alters taste and odor, has been obtained by food scientists. Non-enzymatic browning occurs as the ring structures of sugars in solution open (pentoses, hexoses, and disaccharides in descending order of reaction rate). Maillard described a series of reactions, in which acyclic reactive carbonyl sugar compounds react with amines to form a Schiff base, which subsequently forms a more stable compound known as an Amadori product (8). The reactive moieties can form crosslinks with adjacent molecules. Acyclic sugars can attach to and inactivate the functions of proteins and nucleic acids (9).

Bucala *et al*. found that infectious bacteriophage DNA was inactivated more rapidly by glucose-6-phosphate than by glucose (10). They hypothesized that lysine would compete with DNA in the formation of Amadori products, but found that inactivation rates were slower for a short period but that DNA was eventually inactivated more rapidly in the presence of lysine.

As diabetics age, blood glucose levels have been observed to increase, probably because glucose-derived Amadori products dehydrate to form advanced glycosylated end products (AGE's), which cross-link with molecules and accumulate (11). These browning reactions have been associated with changes in the heart and lung muscles; increasing rigidity in blood vessels; tightening of ligaments and tendons; and development of cataracts, atherosclerosis, and cancer.

Auto-oxidation

Oxygen levels in rapidly frozen biological fluids can be as much as 25-fold higher than when solutions are frozen slowly (O. Fennema, personal communication). This oxygen can be available for oxidative processes at subfreezing temperatures. Molecules can react without heat activation by "tunneling" into the new state, perhaps when the barrier width is sufficiently small (12). Tunneling can occur even at extremely low temperatures. The difference between tunneling and heat activation reactions is illustrated in Figure 5.

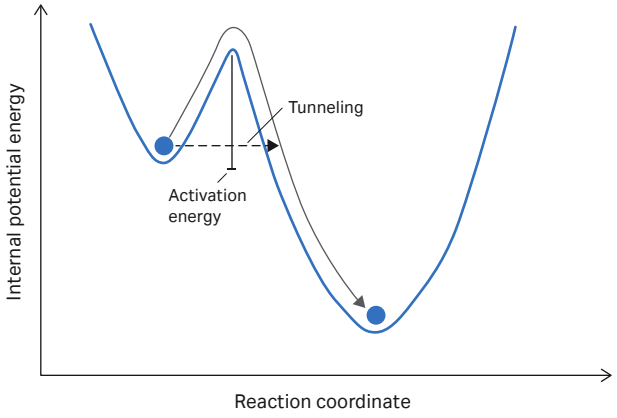

Fig 5. Tunneling circumvents the requirements for activation energy. Modified from Goldanskii (12).

pH

Changes in pH can be a major cause of instability in frozen samples, including irreversible conformational changes in protein that can be inactivated by freezing (13–15).

Dimethyl sulfoxide and glycerol, two cryoprotectants, prevent ice crystal formation that occurs during freezing and can protect NADPH from degradation (13). In investigations, some buffers that do not undergo pH shifts during freezing have been listed.

Pikal found a shift in pH when a citric acid-disodium phosphate buffer system was frozen. The shift occurred when the basic buffer component, disodium phosphate, became concentrated and crystallized (15) (Fig 6). The crystallization of sodium chloride that accompanies concentration of a solute is illustrated in Figure 7.

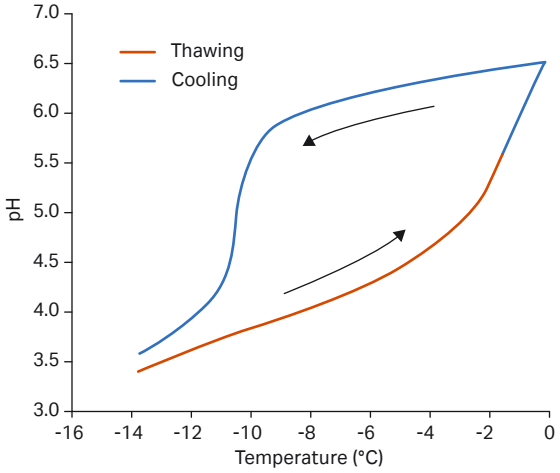

Fig 6. The effect of freezing on the pH of a citric acid-disodium phosphate buffer system. Modified from Pikal (15).

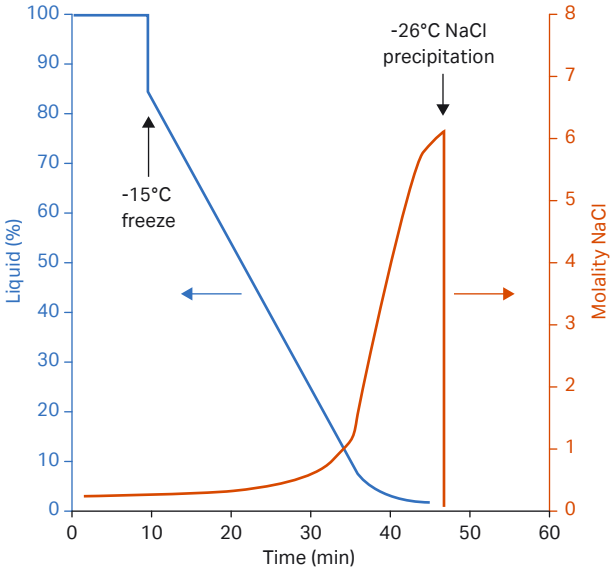

Fig 7. Concentration of solute during freezing, calculated for freezing 5 mL of 0.9% NaCl in 20 mL glass tubing vials. Modified from Pikal (15)

Optimal rates for freezing and thawing serum

Slow freezing exposes serum to increasing solute concentrations for longer time periods. Rapid freezing minimizes this "salting out" caused by increasing solute concentration, but increases the concentration of oxygen that could enhance oxidation (O. Fennema, personal communication). Carlebjork *et al*. increased the recovery of factor VIII in frozen plasma from 0.7 to 1.0 IU/mL by decreasing the freezing time from 10 h to 40 min (16). For this reason, Cytiva uses a quick-freeze process after serum is filtered.

Frozen serum should be thawed rapidly. If thawed slowly, serum near the container walls and in the bottom where salts are concentrated will thaw first, thereby increasing the exposure of serum components to higher than desired salt concentration. It is essential to employ the following procedures when thawing serum. Resuspend the viscous solutes by periodically agitating serum during thawing. Agitation also prevents serum proteins and other labile solutes from exposure to undesirably high temperature when, for example, thawed in a 37°C water bath.

Shelf life of frozen serum

Even though numerous changes can occur when serum is frozen and thawed, we have found that serum handled properly has a shelf life of five years, and is satisfactory for culturing cells that do not have exceptionally demanding nutritional requirements.

Some factors that influence shelf life are:

- Concentration and stability of essential serum components
    - Each cell type has unique requirements for cell growth and function. Any serum that contains concentrations of serum components that barely supports optimal growth (or if the components lose activity as a result of exposure to the environment) will have a short shelf life for that cell type. On the other hand, the same serum can have a long shelf life for other cells. Freezing and thawing, and auto- and photo-oxidation are the most destructive factors.
- Freezing and thawing of serum
    - The degradation of serum components by freezing and thawing is outlined in Table 1. However, cell culture performance of serum is not significantly diminished by this degradation.

Auto- and photo-oxidation

Oxidation results in the formation of free radicals, which are molecules with an unpaired electron. Almost every serum component can be adversely affected by free radicals. There are many pro-oxidants in serum that cause auto-oxidation but, hemoglobin and xanthine oxidation reactions are two of the most important. Xanthine oxidase is not inactivated by the customary heat inactivation procedure but users can minimize the effect of auto-oxidation by selecting serum that contains a low concentration of hemoglobin.

Photo-oxidation occurs when serum, media, or cells are exposed to laboratory lights or to light from windows (17).

What is Cytiva doing to improve the shelf life of HyClone serum?

HyClone™ serum is frozen in a rapid-freezing chamber and stored below -10°C in a validated and temperature-mapped freezer. Information on the effects of freezing and thawing serum is evaluated, disseminated, and applied. Our serum products are protected against auto- and photo-oxidation. HyClone serum contains low levels of the pro-oxidant hemoglobin. Serum protected against free-radicals will have higher levels of antioxidants (free-radical scavengers).

How can Cytiva's customers get maximum benefits from their HyClone sera?

Serum should immediately be stored at -10°C or below. Once thawed, serum can be stored at 2°C to 8°C for up to six weeks with quality maintained. If the serum needs to be stored longer than six weeks after opening, it is recommended that the serum is aliquoted into convenient volumes and refrozen. Handle bottles that have been stored in the freezer carefully. Avoid large temperature shifts and protect the serum from exposure to light. Refer to safety data sheet for any safety recommendations. Storage requirements are listed on the product label.

Upon use, remove serum from storage at -10°C or below and place in a refrigerator overnight at 2°C to 8°C. Transfer the serum to a 37°C water bath, agitate periodically to mix the solutes concentrated at the bottom of the container. Do not hold the serum at 37°C any longer than necessary after thawing. Thawing serum in a bath above 40°C without mixing can denature the concentrated proteins at the bottom of the container and precipitates might form in the bottle. Thawing serum at higher temperatures is not recommended.

Alternatively, bottles can be placed directly from storage at -10°C or below into a 37°C water bath. Bottles should be agitated to enhance mixing and thawing. Turbidity and flocculent material might be present after thawing or after prolonged storage.

Experience indicates that regardless of the method used to thaw serum, it is critical that it is mixed during the thawing process to prevent the formation of gradients and subsequent precipitation. Because of differences in thawing rates of different components, serum will form a gradient if it is not mixed as it thaws. If serum is allowed to remain in such a gradient state, precipitation is likely to occur.

Be sure to mix thawed serum before it is heat inactivated or added to a medium. Serum that is not mixed before heat inactivation might gel due to the high concentration of protein at the bottom of the container.

If not all of the serum is used, aliquot and refreeze serum the first time the serum is thawed. Serum that will be used within several days can be stored at 2°C to 8°C.

Shake and supercool serum so it refreezes rapidly (this might not be possible with large serum volumes). Do not store serum in a self-defrosting freezer, which repeatedly exposes serum to slow cycles of freezing and thawing. Protect the serum from exposure to light. Do not leave lights on in coolers where sera, media, and other biological materials are stored.

Frozen serum can precipitate if not handled properly

Cytiva employs procedures that minimize the formation of precipitates in HyClone serum during filtration and freezing. However, the serum components that are prone to precipitate during freezing remain in the serum and can precipitate later upon use. While this can induce initial concern, there are no adverse effects caused by precipitates on the performance of the serum in cell culture.

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