Studies on the action of liquid nitrogen on cultures

_in vitro_ of fibroblasts

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[Plate 21]

Fragments of living tissues, mostly from the chick embryo heart are frozen at various rapid rates of cooling, stored at liquid nitrogen temperature, then thawed and cultured _in vitro_. Without any previous glycerol impregnation no survival is to be found. If the tissues are soaked before freezing in glycerol-saline solutions the viability is preserved with a maximum for 25 to 30 % of glycerol. The results are discussed with regard to different theories related to cold injury.

INTRODUCTION

The study of the action of cold on living tissues and organisms is a very old one and many investigations have been done in this field since the first experiments of Réaumur (1736). However, in the past 10 years, accurate techniques of cooling and rewarming isolated cells, organized tissues, and whole organisms have been worked out and a great many important data have been published on the alterations which are to be found after the exposure to low temperatures.

Although it is true that scientific study of death due to cold has been made the object of numerous studies from the theoretical point of view, most of the work in this line has been carried out in hopes of finding favourable conditions which permit survival of tissues that are frozen and their preservation over long periods of time at low temperatures.

One of the essential points in this study, then, is the ability to estimate exactly the viability of tissues after the experimentation. To that end, various procedures may be used.

The histological examination and the histophysiological procedures, such as measuring of respiratory activity, show only very little as regards the degree of survival and do not allow many conclusions to be drawn as to the viability of the fragment of tissue after freezing.

On the other hand, experience with grafting, in which the tissues are frozen, defrosted and grafted in an animal, permits one to determine whether it is still viable. As far as the endocrine tissues are concerned, very striking results have been obtained by Dr A. S. Parkes, Dr Smith and their collaborators. But for some other materials (skin, bone, arteries) one still may hesitate to draw conclusions for fear of being wrong as to the apparent prolonged survival. The graft may be invaded by a cellular proliferation coming from the host and although viable in appearance, may be nothing but an inert support.

Another test is afforded by the culture _in vitro_ under sterile condition, of fragments of frozen tissues. If there is growth, one can be definitely assured that the tissue is still viable.

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Being interested in the problem of banking tissues consisting mainly of fibroblasts, our main intention in these experiments was to determine the conditions in which viability of fibroblasts can be preserved after deep freezing and storage in liquid nitrogen. We have seen in preliminary studies that the tissues were undergoing dramatic changes in their physico-chemical properties during the freezing and the thawing processes.

First, from a chemical point of view we must notice that, as the crystallization takes place, there is an important increase of the salt concentration inside the tissue. As water freezes out and ice is separated in a pure form, we must note that, in proportion to the crystal growth, the volume of the interstitial liquid phase diminished at the same time as its saline concentration increases (Lovelock 1954).

This increase of concentration may produce an irreversible damage. The strong ionic solutions dissolve the lipoproteins, develop sensitivity to thermal and mechanical shocks. The viscosity and the pH of the cellular medium change, and as a result there are marked disturbances in the colloidal systems of the living cell. On the other hand, the dynamic equilibriums inside the protoplasm and the nucleus are upset.

Likewise there may be an enzymatic disequilibrium; in the same reactions, with the enzymatic activity decreasing faster than the diffusion processes, reactions no longer can be equilibrated.

In the chains of reaction, various enzymes are not inhibited in the same manner. As a result, inside the protoplasm, there may be an abnormal accumulation of intermediate metabolites which normally have a transitory existence and which may either prove to be toxic or to orient metabolism in a different direction.

A mechanical disorganization of the relations between the enzymes and their substrates may take place. A change in the configuration of proteic molecules may produce a defect or failure of enzymic coaptation with the receptor portion of the molecule and then the reaction may be incomplete or totally inhibited.

Finally, in the course of defrosting, as a result of dissociation of certain proteic molecules, particularly of the lipoproteins, there may appear some active enzymes which disturb normal metabolism.

From these various observations it follows that, in the course of slow freezing and slow thawing, there occurs a general breakdown of biochemical equilibriums. There are two opposing theories on how to prevent these changes.

First, it is necessary to produce slow freezing in order to prolong the period of adaptation of the cell to the conditions in its new environment; likewise to expose it for a longer time to the injurious effects.

Secondly, it is preferable to produce a very rapid freezing in order to avoid this period of internal disequilibrium and to block all metabolism, while congealing cellular colloids. Such a brutal transition then may have very injurious effects—that is the thermal shock.

**Material and methods**

In order to verify these different theories we have made experiments, using different methods of freezing. We have worked on fibroblasts, mostly from the chick embryo heart, and we have checked viability by the tissue-culture method.
according to the technique of Albert Fischer. The explants, before freezing, are soaked for $\frac{1}{2}$ h in a balanced salt solution (Earle's solution) containing 1000000 units of penicillin and 1 g of streptomycin per litre for sterilizing purposes. This solution is either pure or contains increasing concentrations of glycerol from 5 to 40% and has been proved to be non-toxic for the fibroblasts.

**Freezing techniques**

*Medium velocity.* The explants (about 0.25 to 1 mm large) are mounted on a glass coverslip inserted inside a Pyrex tube which is immersed in liquid nitrogen. The freezing is completed in about 50 to 60 s.

![Diagram of freezing techniques](image)

**Figure 15**. For rapid freezing the tissues are dipped in liquid nitrogen. Medium velocity is obtained in the Pyrex test-tube where an air-gap delays the cooling. High velocity is realized by holding the tissues on a thin aluminium strip.

**Figure 16**. For ultra-rapid freezing liquid propane at liquid nitrogen temperature is used.

*High velocity.* The explants are put on aluminium strips and dipped directly in liquid nitrogen. The freezing is done in about 2 to 3 seconds.

**Ultra-rapid freezing**

To check the theory of Luyet & Gehenio (1940) we have tried to 'vitrify' the explants; rather, to cool them in such a way that the ice keeps a cryptocrystalline structure. The liquid nitrogen is not a good medium for quick freezing. Being a liquid at its boiling point, there is a calefaction effect which delays the freezing because there is poor thermal contact between the specimen and the cooling bath. For that reason we have, for several years, been using for ultra-rapid cooling liquid propane which may be kept in the liquid state at liquid nitrogen temperature in an atmosphere of hydrogen.
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Being 150° C below its boiling point, there is no calefaction effect and the thermal contact is excellent. Furthermore, the propane does not dissolve the ice, so that the specimen is protected from the deleterious effects of the propane itself by a thin pellicule of frozen balanced salt solution all around its surface. The freezing occurs in a fraction of a second and after 5 to 6 s the specimen holder is put in liquid nitrogen which surrounds the propane inside the same Dewar flask.

**Figure 17.** In the thermostatic atmosphere maintained at any fixed temperature in between 0 and 150° C the tissues can be frozen at two different rates; medium velocity in the Pyrex test-tube, high velocity on the coverslip placed at the surface of the metallic block.

_Freezing by steps_

This is done by putting the explant for ½ h at an intermediate temperature in a thermostatic atmosphere and then plunging it into liquid nitrogen. We have been using for that purpose a special device with a freezing chamber cooled by cold nitrogen gas, which can be kept at any fixed temperature of between 0 and −150° C with an accuracy of one-tenth of a degree.
Storage and thawing techniques

The tissues are kept in liquid nitrogen for some hours and then thawed. Preliminary experiments have shown that rapid thawing is necessary, and this is done by immersing the coverslips or the aluminium strips in the solutions used for soaking, maintained at room temperature. We have not obtained better results by using a solution at the incubator temperature.

After thawing the explants are put into culture on normal coverslips, or Maximow slides, in a medium consisting of fowl plasma, embryo extract and a nutrient solution, and then incubated.

Results

Without glycerol

Our first results show that, with this kind of tissue, glycerol is necessary for maintaining viability. This is in agreement with what has been described in previous experiments by different authors.

![Graph of growth vs glycerol percentage](http://rspb.royalsocietypublishing.org/)

**Figure 18.** Growth of the cultures of fibroblasts 72 h after thawing and which have been soaked in different glycerol-saline solutions before freezing. \(-196^\circ C\) (- -); \(-45 \text{ to } -196^\circ C\) (---); \(-30 \text{ to } -196^\circ C\) (----).

The discovery of the effect of glycerol led to some most remarkable work done in London at the National Institute for Medical Research by a group of investigators, A. U. Smith, J. E. Lovelock, C. Polge, under the direction of Professor A. S. Parkes (Parkes & Smith 1952; Polge, Smith & Parkes 1949).

Actually there is no growth in the cultures without glycerol except sometimes in two or three migrating cells. It may be of some interest to know why some peculiar cells do survive after the exposure to liquid nitrogen. It seems unlikely that they have not been crystallized and the reason for their survival might be found in a special resistance of those cells to cold injury; it would be then interesting to follow them and check the behaviour of this strain to extreme temperatures.

Why is the main part of the tissue killed by rapid cooling in liquid nitrogen? We cannot claim that these alterations occur during the freezing process. Actually, we think, according to our first observations with the freezing microscope, that they occur during the thawing process. This may be provoked by a recrystallization from the amorphous state as has been shown by Meryman & Kafig (1955). There may also be an osmotic shock, or, in a very imprecise way, a thermal shock.
Figure 19. Culture of fibroblasts ultra-rapidly frozen in 25% glycerol-saline and cultured 72 h. Note organogenic growth and thickness of the culture (living).

Figure 20. Culture of fibroblasts ultra-rapidly frozen in 5% glycerol-saline and cultured 72 h. Note no organogenic growth around the explant (living).

Figure 21. Phase-contrast micrograph of living culture of fibroblasts frozen in 10% glycerol saline (72 h).
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With glycerol

The protective effects of glycerol increase with its concentration, with a maximum of 25 to 30% where the growth is equivalent to the growth of the controls. For concentrations as low as 10% there is no organogenic growth around the explant; this appears at 15% and is normal for 20% (figures 19 to 21, plate 21).

Rates of cooling

As for the different rates of cooling, we have found that, in the presence of an adequate concentration of glycerol there is no significant difference between slow, rapid or ultra-rapid cooling. We may say that there is no evidence of a thermal shock. On the other hand, if the concentration of glycerol is not sufficient, there develops a great sensitivity to a prolonged stay at an intermediate temperature where we must think that there is still a liquid phase in the tissue. These alterations are then greater if the temperature is higher.

To sum up these different results we may say that glycerol is necessary to protect the cells from the deleterious effects of freezing and thawing. We suppose that glycerol has several effects.

Acting as a diluent agent in the course of freezing, this allows the avoidance of high saline concentrations.

It lowers the temperature for the formation of eutectics, prolonging the period of adaptation in the absence of toxic effects.

Finally, it lowers considerably the speed of crystallization and renders the size of crystals smaller. We have shown by experiments in capillary tubes that, for instance, for whole blood at −12°C the addition of 2% of glycerol brings the velocity of crystallization from 13 mm/s to 7 mm/s, and to 3 mm/s for 5% glycerol (Rey, unpublished).

Future trends

We are now following these experiments with a special phase-contrast microscope with freezing stage that we have built in our laboratory in the past 2 years.

The stage consists essentially of a cylinder of german silver: 26 mm in diameter, 10 mm high and 5 mm thick, closed above and below by plates of optical glass. Two channels pass through the metal wall of the cylinder and connect with inlet and outlet tubes. A refrigerated gas or fluid is circulated through these channels. The air enclosed in between the lower glass of the cell and the optical condenser is kept dry by a desiccant held in a water-tight groove in the metal wall. This prevents condensation and frosting below the specimen. The specimen is mounted on the upper glass plate and its temperature is recorded from a thermocouple. The objective of the microscope is surrounded by a cylindrical jacket which makes contact with the upper glass plate of the cell by the intermediation of a plastic ring. The air between the front lens of the objective and the specimen is dried by a desiccant kept in a water-tight groove in the wall of the jacket. Frosting above the specimen is thus avoided. The cell and the whole cooling circuit is carefully insulated.
It is possible with this apparatus which has been illustrated and described in detail elsewhere (Rey 1957) to reach $-150^\circ$ C on the specimen-holder either within a second or in an hour. For slow freezing we use a circulation of cold hydrogen. For quick freezing we use a circulation of liquid propane pushed under hydrogen pressure. This device is entirely automatic and has a thermostatic control so that the temperature may be kept constant at a fixed level during a long period of observation. An interconnecting system allows quick thawing by circulating warm isopentane (Rey 1957).

We have found that if a very quick freezing was performed there are no morphological changes inside the cells and no crystallization appears even without glycerol. It is better to say that it is impossible to see any crystal formation. If afterwards there is a slow thawing, the crystals develop from the amorphous structure at as low as $-60^\circ$ C and at $-20^\circ$ C the cell is nearly destroyed.

We are now developing more extensive studies on tissue-culture specimens with this apparatus and we hope to get more information on the phenomena occurring during freezing and thawing.

References (Rey)


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Discussion

The effect of rate of cooling on survival of frozen tissues*

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[Plate 22]

Cell damage by freezing has sometimes been attributed to the compression and shearing forces generated by the growth of ice crystals that result in rupture and laceration of cytoplasmic membranes. It cannot be denied that such purely mechanical factors may be important under certain special conditions, yet ample

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