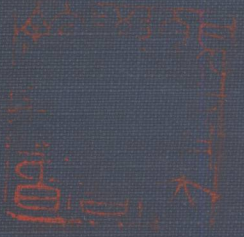
Biological Applications of Freezing and Drying

Edited by

R. J. C. HARRIS



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Foreword

A method for drying frozen biological material by sublimation of the ice *in vacuo* has been known for more than fifty years, but the advantages of the process were not fully appreciated until just before the outbreak of the second World War when apparatus for laboratory and small-scale commercial use was first becoming available. Enormous quantities of blood plasma and penicillin were required during this war. Freeze-drying was found to be an excellent method of preservation, and the procedure emerged in 1946 as a fully tested technique. Since that time, too, there has been rapid progress in work on the effects of low temperatures on cells and tissues with important practical applications for the long-term storage, for example, of spermatozoa for artificial insemination, of red blood cells for transfusion, and even of tissue "spare parts" for surgical purposes. At the present time, freeze-drying is used for the preservation not only of blood products and antibiotics but also of human milk, bacteria, tissue-culture media, and viruses, and for the preparation and preservation of tissues for morphological and histochemical studies, and of specimens for electron microscopy.

The authors of many of these chapters need no introduction, for they are the pioneers in their particular fields; the techniques which they have devised are being used and will be used increasingly in laboratories throughout the world, and apparatus similar to that which they first described is now being manufactured in many lands.

The aim of this volume is to provide for the first time a comprehensive and authoritative treatise on such aspects of freezing and of freeze-drying. It is intended to serve as a work of reference for all those already using such techniques and as a guide to those who may wish to employ them.

I should like to express my thanks to the publishers for their cooperation in, indeed for their toleration of, the editorial whims, and especially to Miss Christine Kilburn for her invaluable help in the preparation of typescripts.

The pleasure which I have found in the editing of this book has been marred by the tragic death of Joseph Singer, the author of Chapter 5, in an air accident in the summer of 1953.

R. J. C. Harris

March, 1954

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CHAPTER 1

Effects of Low Temperatures on Living Cells and Tissues

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I. INTRODUCTION

Accurate thermometers were invented by Fahrenheit in 1714 and by Réaumur in 1731, and were rapidly taken into use by biologists. Réaumur himself was one of the first to study the action of cold on insects (1736). Forty years later Spallanzani recorded observations on the effects of low temperatures on animalcules, insects, fish, amphibia, reptiles, birds, mammals, and man, as well as the results of cooling spermatozoa and eggs of several species (Spallanzani, 1787). Spallanzani, who referred to the Réaumur and to the Fahrenheit thermometers, succeeded in producing a temperature of 24 degrees below zero by adding spirits of niter to a mixture of ice and rock salt. He found that butterfly and silkworm eggs survived exposure to 17 degrees below zero under outdoor conditions

and to 24 degrees below zero in the laboratory, and that spermatozoa survived 15 degrees below zero although the animals from which the gametes were derived were killed at about 7 degrees below zero.* He showed that, when the medium was supercooled, animalcules survived lower temperatures than when ice formation had occurred. Spallanzani was aware that temperatures 30 degrees below zero were common in Quebec and St. Petersburg, and as low as 70 degrees below zero in Siberia where various plants and animals, including man, survived either by withstanding freezing of the tissues or by mechanisms which maintained body temperature despite intense environmental cold.

In the late eighteenth and early nineteenth centuries much further information was gathered about the effects of chilling on plants and animals. It became evident that there was remarkable species variation in sensitivity, some organisms having a capacity for withstanding extreme cold whereas others were killed or damaged at temperatures little below or even above the freezing point of water (Luyet and Gehenio, 1940).

During the latter half of the nineteenth century the stage was set for spectacular advances in the field of low-temperature biology by fundamental discoveries in new sciences as well as by developments in physics and chemistry. Thus, Pasteur's work on fermentation was the starting point of bacteriology and microbiology, and Claude Bernard's research laid the foundations of modern physiology. In the same period, Liebig and Fischer applied organic chemistry to the study of the products and components of animal and plant tissues, and Buchner demonstrated enzyme activity in cell-free extracts of yeasts. Biophysics stemmed from the work of Graham, who studied the properties of colloidal solutions and the phenomenon of diffusion. Raoult investigated the effect of organic substances on freezing points of solutions, and Pfeffer and van't Hoff developed the modern theories of osmosis. Notable progress was also made in pure physics. For instance, oxygen was liquefied in 1877 by Cailletet and by Pictet, liquid air was produced in quantity by Linde in 1895, and three years later, in 1898, Dewar succeeded in liquefying hydrogen. Great strides had meanwhile been made in thermometry, so that very low temperatures could be measured and recorded.

Biologists were quick to take advantage of the new knowledge and facilities. Brown and Escombe (1897), working in Dewar's laboratory, found that the germinative power of seeds was not affected by slow cooling to and storage for 11 hours at −182° to −192°C. in liquid air, and Thiselton-Dyer (1899) obtained similar results with seeds exposed to liquid hydrogen. Soon after, Macfadyen and Rowland (1900a, b) showed

* It is not clear to which scale Spallanzani is referring at this point, but severe cold is clearly indicated.

that bacteria retained unimpaired vitality and enzymes unaltered activity after exposure to or storage in liquid air and liquid hydrogen. Other methods of cooling and higher storage temperatures, on the other hand, proved effective in killing or checking the growth of microorganisms (Hilliard and Davis, 1918; Haines, 1934).

During the same era new feats of engineering had produced steamships with screw propellers and steel hulls capable of carrying refrigerating plants, so that in 1879 the first consignment of frozen meat was brought to England from Australia (Williams, 1933). Popular interest in the biological effects of low temperature was aroused on the one hand by the need for more food for the growing industrial populations of Europe, and on the other by the exploits of Nansen and other scientific explorers in the Arctic who drew attention to the paucity of medical knowledge about frostbite and other pathological effects of cold.

The stimulus of the varied discoveries and inventions and the potentialities of their practical application resulted in investigation from many angles of the action of low temperatures on living cells. Between 1890 and 1940 a vast literature grew up. Viruses, bacteria, yeasts, microorganisms of many kinds, plants, flowers, fruits, invertebrate animals, amphibia, reptiles, birds, and mammals of many species were exposed to low temperatures and studied during and after cooling and rewarming. Parts of whole animals and limbs, organs, tissues, and cells isolated from many species were similarly examined. The chemical components of cells and tissues, including proteins, lipids, carbohydrates, water, and solutions of electrolytes and non-electrolytes have all been cooled to different extents and under different conditions. The properties of colloidal solutions and gels and the activity of vitamins, hormones, and enzymes subjected to similar treatment have been investigated. The reports of results have been scattered throughout journals of every branch of academic and applied science, including medicine, agriculture, engineering, and food science. Much of the work up to 1940 has been summarized in classic monographs by Bělehrádek (1935) and by Luyet and Gehenio (1940).

Since 1940 there has been a further spate of work, so that the field is now too wide to be reviewed in a single article. The action of frost on plants and plant products was reviewed by Kidd (1929), Modlibowska and Field (1942), Rogers (1952), and at the 8th International Congress of Refrigeration (1951), and is outside the scope of this article. Extensive reviews on freezing meat and other foods have been published by Bate-Smith (1944), Callow (1952), and Reay (1951). The effect of low temperatures on tumor tissues was recently summarized by Passey and Dmochowski (1950), and physiological and medical aspects of cold have been dealt with exhaustively by Edholm (1953). No attempt will there-

fore be made to review these topics again or to deal in a comprehensive manner with every aspect of the action of cold on living things. Attention will, instead, be directed to certain fundamental aspects of the subject, particularly in the light of work done between 1940 and 1952 on isolated cells and tissues of animal origin. It has been possible to make reference to only a few of the many relevant papers published in 1953.

Even with this restriction, terminological difficulties arise. For instance, there is no agreement on the meaning of the expression "low temperature." Thus, to clinicians, 15°C. is a very low temperature. To inhabitants of the Tropics, ice and snow at 0°C. represent severe cold. To refrigeration engineers −20°C. is low, and −40°C. a very low temperature. Some physicists, on the other hand, regard temperatures as low only when close to the absolute zero, −273°C. (de Klerk, 1952). In this article the following classification will be adopted:

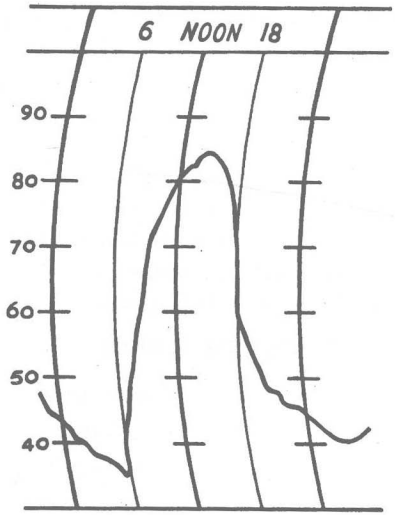
Temperature Range	Description
+20° to 0°C.	Subnormal
0° to −70°C.	Low
−71° to −273°C.	Very low

Differences of outlook have similarly led to confusion over rates of cooling. Meteorologists consider a fall in the air temperature of 50°F. (28°C.) in 8 hours as rapid (Hawke, 1944; Manley, 1952) (Fig. 1a). In the meat industry a change in temperature from +5° to −5°C. in 30 minutes is regarded as quick (Bate-Smith, 1944; Callow, 1952; Moran, 1935). By contrast, biophysicists of Luyet's school would call the rate of fall rapid only if the temperature changed from 0° to −190°C. in a matter of seconds (Fig. 1b.)

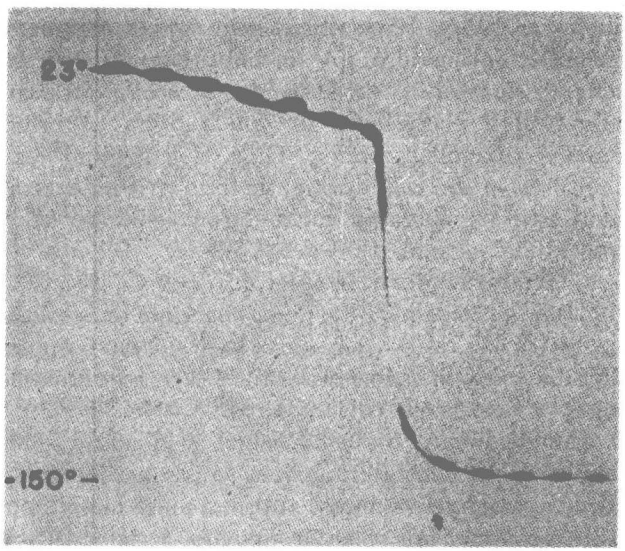
We shall arbitrarily adopt the following classification:

Temperature Range	Time	Description	Reference
0° to −190°C.	2 sec. or less	Ultrarapid	Luyet
0° to −79°C.	2 sec. to 5 min.	Rapid	Smith and Polge
0° to −79°C.	10 min. or more	Slow	Smith and Polge; Polge and Lovelock
20°C., any range	1 hr. or more	Very slow	

Another problem is raised by the loss of meaning of the term "living" (Pirie, 1937). Animals used to be regarded as dead when the circulation and respiration had ceased. Andjus (1951) has shown, however, that this is not necessarily the case. In dealing with isolated cells, irritability, motion, metabolism, growth, and reproduction were at one time regarded as criteria of life. During the last fifty years it has become increasingly clear that these criteria are no longer infallible. In the present state of knowledge it is impossible to say whether, at very low tempera-



(a)



(b)

FIG. 1. Contrast in cooling rates. (a) Extreme temperature gradients in a Hertfordshire frost hollow. (Fig. 55 from Manley, 1952.) (b) Ultrarapid cooling in *iso*pentane at −150°C. (Abscissa: time; each dash corresponds to $\frac{1}{2000}$ second.) (Fig. 9 from Luyet, 1951.)

tures, animation is completely suspended or very greatly slowed. Cells and tissues will, therefore, be described as living when they retain the capacity to exhibit metabolic activity and to perform specific functions under suitable conditions at a normal environmental temperature.

Finally, it may be pointed out that the study of biological effects of low temperatures has again entered a phase of very active growth, since the discovery of the protective properties of glycerol (Polge *et al.*, 1949), and that, although facts in the sense of reproducible observations will remain as such, interpretations and conclusions must be regarded as tentative.

II. THERMAL SHOCK

1. *At Subnormal Temperatures*

It has long been known that a sudden fall in temperature to a subnormal level above 0°C. has a harmful or lethal effect on a variety of living cells to which a gradual temperature change over the same range or a steady subnormal temperature is innocuous. The phenomenon which has come to be known as temperature shock, but which will be referred to here as thermal shock, is particularly common in higher plants (Kidd, 1929; Modlibowska, 1951). It occurs also in simple organisms such as algae and was noted, for instance, by Kylin (1917) with *Nitella clavata* in which complete cessation of plasma streaming occurs after sudden but not after gradual cooling between +20° and +3°C. Certain bacteria are extremely sensitive to sudden cooling. Thus, Hegarty and Weeks (1940) found that cultures of *B. coli* during their logarithmic growth phase showed great sensitivity to rapid cooling from +37° to 0°C. Sherman and Cameron (1934) found that 95% of *B. coli* in very young cultures were killed by sudden transfer from +45° to 0°C., whereas gradual cooling, taking 30 minutes to make the same temperature change, caused no injury. It has even been suggested that bacteriophage is susceptible to thermal shock between +60° and +2°C. (Smith and Krueger, 1952). Among protozoa slow cooling to 0°C. often permits adaptive changes. For example, *Stentor coeruleus*, which is killed by sudden cooling to 0°C., undergoes encystment during slow cooling and resumes an active form when its temperature is raised to normal after many hours at 0°C. (Greely, 1901). A gradual fall in environmental temperature is particularly important in insects for development of cold hardiness, an adaptation which depends partly on decreased water content (Payne, 1926; Salt, 1936, 1950; George, 1953). With higher animals a gradual fall in environmental temperature induces hibernation in some species and in others promotes increased growth of hair and laying down of fat to improve insulation.

Among isolated mammalian cells and tissues the deleterious effects of sudden temperature change between +40° and 0°C. are best known with spermatozoa. The term "temperature shock" originating from Milovanov in 1934 (Anderson, 1945) was first and is still widely used to describe the irreversible decrease in motility of bull and ram semen which occurs on sudden cooling, particularly between +15° and 0°C. Thermal shock can be avoided by gradual cooling over the same range (Chang and Walton, 1940). Sensitivity to thermal shock is decreased by the use of egg yolk diluent, so that the rate of cooling can be increased (Phillips

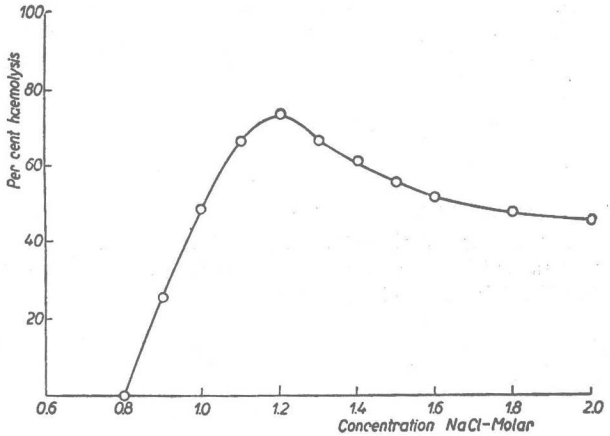


FIG. 2. The hemolysis which occurs when red blood cells suspended in sodium chloride solutions of various strengths are suddenly cooled from 30° to 5°C. (Fig. 7 from Lovelock, 1953a.)

and Lardy, 1940), but even so the change should not exceed 5°C. in 20 minutes (Salisbury *et al.*, 1941).

Until quite recently it was not realized that the majority of mammalian cells are, to some extent, subject to thermal shock and that their susceptibility to it can be increased or decreased by altering the composition of their medium. Red blood cells, for example, are not normally affected by sudden chilling; in the presence of sodium chloride solutions stronger than 0.8 *M*, however, they hemolyze readily when cooled suddenly from +30° to +5°C. (Lovelock, 1953a) (Figs. 2 and 3).

The mechanism of thermal shock to bull spermatozoa at subnormal temperatures has not so far been explained. The nature of the palliative action of egg yolk is also obscure, although lecithin is thought to be the active agent (Tosic and Walton, 1947). Luyet and Gehenio (1940) thought that abrupt cooling might cause gelation of protoplasmic sols, followed immediately by contraction of the gel and spontaneous expulsion of fluid,

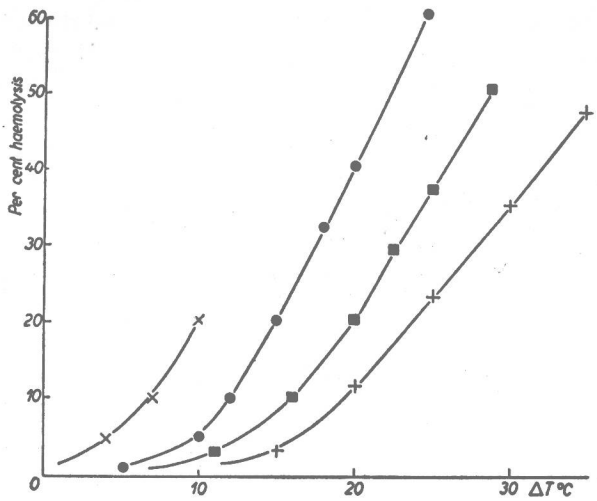


FIG. 3. The hemolysis which occurs when red blood cells suspended in 1.0 *M* NaCl are suddenly cooled through various temperature intervals (ΔT), and from different initial temperatures. Initial temperature 0°, x — x; +20°, ● — ●; +30°, ■ — ■; +45°, + — +. (Fig. 8 from Lovelock, 1953a.)

and that such syneresis would cause death. This hypothesis has not so far been proved.

2. *At Low Temperatures*

In spite of various earlier observations, it was only recently recognized that thermal shock, or something superficially similar, could occur in ranges far below 0°C. Rahm, for instance, had shown in 1922 that certain rotifers, nematodes, and tardigrades in the wet state survived exposure to low temperatures only if cooled slowly. Some years later Breedis and his colleagues found that mouse leukemia cells survived and retained their ability to transmit the disease after freezing to −70°C. only if cooled slowly (Breedis *et al.*, 1937; Breedis, 1942). Slow cooling also permitted better survival of various pathogenic protozoa than was obtained with rapid freezing (Weinman and McAllister, 1947). Parkes (1945) demonstrated that human spermatozoa survived exposure to and storage at −79° or −196°C. when frozen in bulk, but not when frozen at the maximum rate in microscopic films or capillary tubes by the technique of Hoagland and Pincus (1942). Nevertheless, rapid or ultrarapid freezing of living cells was, until a few years ago, still advocated and widely practiced to reduce or avoid damage by ice crystals (*v. infra*, and Sections IV and V of this chapter).

The occurrence of thermal shock between 0° and −79°C. and its