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LIFE AND DEATH AT LOW
TEMPERATURES

by

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*The authors dedicate this book to all
those straining in the pursuit of Truth*



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INTRODUCTION

The problems to be discussed in this monograph are primarily those of the preservation of life and of the mechanism of death at low temperature. On account of their immediate connection with the questions of the structure of living matter and of the nature of life, these problems are of fundamental philosophical importance. It is precisely this fundamental aspect which will be our main concern throughout this work. None of the practical applications of low temperature research will be considered except inasmuch as they directly involve some phase of our topic. Though the fields of *Cold Hardiness in Plants* and of *Refrigeration Industry* are quite closely related to our subject we shall not include them; each one of these fields constitutes a science in itself which will be better reviewed by a specialist.

Our knowledge on death by cold consists of observations concerning: 1. Which organisms are killed by or survive various low temperatures; 2. How the organisms affected are physically modified by the action of cold; 3. What are the causes and the mechanism of cold injury and cold death. The study of these three questions will constitute, respectively, Parts I, II and III of this book.

Bibliographical references which only incidentally concern the subject treated will be incorporated in the text, in italics; references dealing primarily with the topic under discussion will be given at the end of each part in which they were used; a *tentatively* complete bibliographical list including also references not utilized in our work, will be appended at the end of the monograph.

Saint Louis, September 15, 1940,

THE AUTHORS.

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PART I

THE LOWER LIMIT OF VITAL TEMPERATURES

INTRODUCTION

DEFINITIONS AND PRELIMINARY REMARKS

In a study of the mechanism of cellular or of protoplasmic death by low temperatures, one needs to know, first of all, at what low temperatures life is destroyed. The following pages present a review of the literature on this subject.

According to the effects that they produce on living matter, the temperatures are classified into *vital*, *lethal*, *survival* and *anabiotic*. If the same effects are obtained over a range of temperatures, there must be distinguished a maximum and a minimum in each range; thus there would be a maximal and a minimal lethal, a maximal and a minimal survival temperature, and so on. The multiple aspects originating from so many distinctions on one hand, and the overlapping of the ranges on the other, have resulted in some confusion. But more important misunderstanding has arisen from the widely different senses in which the various authors use the terms just mentioned, and from the impossibility of defining clearly a death temperature, in the present state of our knowledge of the cause of death by cold. This will be illustrated by the following analysis of the notion of lethal temperatures.

The death point has sometimes been considered as a definite temperature at which an organism passes from the living to the dead state in somewhat the same manner as, at the freezing point, a substance passes from the liquid to the solid state. So understood, the death temperature would be specific for each organism in the same sense as the physical constants are specific for each sub-

stance. A clear and adequate definition could then be established.

Much evidence, however, has been accumulated for an entirely different notion of death temperatures. In some plants, for example—and the same might be true of single cells—death seems to result from the unequal slowing down by cold of the various metabolic activities. If, for instance (in the case of a higher plant), less moisture is absorbed by the roots than is liberated by the leaves, desiccation and death will follow. An exposure to a temperature as high as 5° might become lethal if enough time is allowed for the material to dry; a temperature a few degrees lower will dehydrate the plant to death in a shorter time. It is evident, then, that the time factor must be introduced into the definition of the death temperatures. On the other hand, the degree of moisture of the atmosphere, the amount of light present, the water-content of the soil, etc., will also affect the duration of death and the temperature at which it occurs. The lethal temperatures, therefore, represent simply conditions unfavorable for life, in the same sense as the scarcity of light or of oxygen or of water. In cases of this kind, the definition of death temperatures is rather involved; instead of a lethal point one should use the notion of a lethal region; the time necessary for death should be mentioned, as should also the other conditions which hasten death or delay it. The temperature at which the plant dies, by desiccation, in an arbitrarily chosen very long time, can be called the maximal lethal temperature. The lethal zone extends from that maximum downward, but it has no minimum since there is no temperature, however low, which will kill the plant instantaneously by putting its metabolic processes out of balance.

If such a plant is cooled rapidly to the freezing point, the injury produced by the congelation of its juices may suffice to cause it to die. But death results here from an entirely different cause, and the plant has, besides the death zone mentioned in the preceding paragraph, a death

point which coincides with the freezing point. A precise definition can be given of that death point, as well as in the case in which death was supposed to result from a cooling to a specific minimum. The solidification of a given proportion of moisture takes place at a definite temperature. The time factor has little importance; it is simply related to the total mass of material to be killed in the same way as the time necessary to melt a certain quantity of metal depends on the amount present and bears no relation to the melting point.

But an organism which is killed when left to congeal at the freezing point might not be killed if brought rapidly to some hundred of degrees below zero, where congelation cannot take place on account of the high viscosity of the material (cf. Luyet, 1937). Thus, temperatures below the lethal point might be non-lethal.

One and the same organism may, therefore, possess a zone of lethal temperatures above zero, a sharp death point slightly below zero, and a zone of non-lethal temperatures some hundred degrees below zero. And these are only a few of the many possibilities that one can imagine.

It is clear, then, that the notion of lethal temperature depends on the idea that one has of the mechanism of death and, therefore, is susceptible of multiple interpretations. There being no possible general definition of the death point, of the survival point, etc., we shall, in this review, use these expressions in the sometimes unprecise sense given to them by the various authors, without attempting to define them more exactly.

The most important factors to be considered in a study of the temperatures at which animals or plants, organs, tissues, cells, or protoplasm, die, are the following: 1. The species or the type of material studied; 2. The external temperature to which the material has been subjected; 3. The time of action of this temperature; 4. The

temperature within the dying material; 5. The time of action of this latter temperature; 6. The rate of cooling and, if the material was thawed, the rate of warming; 7. The presence or absence of ice in the tissue, in the cells or in the protoplasm; 8. The state and the conditions of the material, before and during cooling, such as, its acclimatization to low temperatures, its water content, its thermic insulation, etc.

In most of the older works on death by low temperature, only the first two of these factors, that is, the type of organism and the external temperature, are recorded. This literature, therefore, does not bring much substantial information on the point at issue in the present work, and we shall mention only some of the most outstanding results obtained by the older investigators. Those papers of later date which contain enough of the data mentioned in the previous paragraph to be significant will be summarized and some of them will be discussed briefly.

Immersion in liquid gases has been the usual method of subjecting living matter to extreme cold. The liquefaction temperatures of most of the gases used are given in the following list and will not be indicated for each particular case in the text.

Absolute Zero	-273° C.
Temperature of liquefaction of Helium	-269° C.
Temperature of liquefaction of Hydrogen	-253° C.
Temperature of liquefaction of Nitrogen	-196° C.
Temperature of liquefaction of Air	-192° C.
Temperature of liquefaction of Oxygen	-183° C.
Temperature of sublimation of Carbon dioxide	-78.5° C.
Temperature of liquefaction of Sulphur dioxide	-73° C.

Some conditions of the experiments, which follow as natural consequences from other fully described condi-

tions, will often be omitted. For instance, when the infracellulars and the monocellular organisms are exposed to the temperature of liquid air, in water suspensions, or in nutrient media, for an hour or so, it is certain that the medium is hard frozen and hence this will not be stated explicitly.

The ordinary life stage and the usual culture conditions are to be assumed if nothing to the contrary is mentioned. For example, bacteria and yeasts are supposed to be in the vegetative form and in aqueous media (solid or liquid), if it is not specified that they have sporulated or that they have been dried.

The data to be reviewed will be arranged in sections according to the type of organisms studied, the generally accepted classification of plants and animals being followed. Within a section, except if otherwise indicated, the order is that of decreasing temperatures. There results an unavoidable separation of related genera or species. But, since the subject-matter is indicated in italics, the reader will be able, without much effort, to re-establish the natural correlations. Finally, then, each separate paragraph usually contains an investigation on a given subject, at a specified temperature and by a given author or group of authors.

The temperature shall always be given in degrees centigrade except if otherwise indicated.

The terms "freeze" and "congeal" shall be used exclusively when it is meant that the fluids of the material under experimentation solidify.

I. INFRACELLULARS

By our inclusion of the infracellulars with living matter we do not mean to imply anything as to their real nature.

1. *Vitamins and Hormones.* The only information that we found in the literature, on the action of low temperatures on vitamins and hormones, was the observation that

these substances do not lose their activity in cold storage. Thus, Wright (*J. Soc. Chem. Ind.*, 42, 509, 1923) reported that pork, kept for 9 years in the frozen condition, still contained some active *vitamin A*; Weill, Mouriquand and Michel (*C. r. Soc. Biol.* 79, 189, 1916) showed that *vitamin B*, in beef, was not affected by a storage of 8 months at a temperature of -8° to -10° ; etc. It is generally thought that non-protein substances should not be affected, as proteins are, by low temperature and by the congelation of the aqueous medium in which they are dissolved or suspended.

2. *Enzymes*. Spoilage of refrigerated food is, in some instances, attributed to the action of *proteolytic enzymes* elaborated by bacteria. This type of enzyme has been found by many authors to be active at sub-zero temperatures, in some cases, even at -9° . (Cf. Glage, *Ztschr. f. Fleisch. u. Milchhyg.* 11, 131, 1901, and Talyract, *Ann. Hyg. Pub. Ser.*, 45, 166, 1901.)

Kovchoff (1907) observed the conversion of protein nitrogen into non-protein nitrogen in various plant tissues, after they had been frozen for 24 hours. The plant *proteases*, he concluded, survive freezing.

According to Pennington and Hepburn (*J. Am. Chem. Soc.*, 34, 210, 1911), *lipase* was active in the abdominal fat of chickens after the latter had been kept hard frozen for more than 7 years at temperatures of -9.4° to -12.2° .

Hepburn (1912) demonstrated the presence of *oxidase*, *peroxidase*, *catalase* and *reductase* in the same material maintained at the temperature indicated for several months or even several years.

Gertz (1926), studying the oxidizing enzymes of the alga *Delesseria sanguinea* and of potato tubers, found that, after a stay of 24 hours in the frozen state at -15° , the tubers yielded unimpaired *oxidase*, as did also the algal sap, previously extruded, and kept frozen for 98 hours at the same temperature. But, after an exposure of 1 hour to -80° , the reaction of these enzymes was weaker.

Thunberg (1920), who observed that the animal *oxidases* are also impaired by exposure to -80° , suggests that they might be modified in their structure in such a way that they act only on some particular fraction of the substances on which they acted before.

According to d'Arsonal (1892), yeast, subjected for 24 hours to -42° or for 45 minutes to about -95° , furnished an active *sucrase*; but if the enzyme, in a glycerin solution, was maintained for 45 minutes at -95° , it lost its power of splitting sugar.

Pietet and Yung (1884) subjected yeast successively for 108 hours to -70° and for 20 hours to -130° ; they found that it lost its power to raise bread.

Yeast juice, exposed by Macfadyen (1900) for 20 hours to the temperature of liquid air, furnished an active *zymase*.

Buchner obtained *zymase* by grinding yeast in liquid air or by grinding a mixture of yeast with solid carbon dioxide ("Die Zymasegähmung." Munich and Berlin, 1903. pp. 67 and 226).

Rennet, in liquid preparations, exposed for 30 minutes in liquid air, coagulated milk as effectively as did the controls (Chanoz and Doyon, 1900).

Pozerski (1900) investigated the action of an exposure to the temperature of liquid air on the following enzymes in solution (in test tubes): *salivary enzymes* from human saliva, *sucrase* from yeast, *sucrase*, *amylase* and *inulase* from *Aspergillus niger*, *trypsin*, *pepsin* (a glycerin solution) and *rennet*. The activity of all these enzymes, after 45 minutes exposure, was found to be identical with that of the controls.

The coagulating enzyme of the blood was not destroyed when oxalated dog blood, contained in a glass tube, was exposed for 13 minutes to the temperature of liquid air; coagulation took place normally upon addition of calcium chloride after thawing (Chanoz and Doyon, 1900). Since the blood was oxalated, the fibrin-ferment itself was not formed and the enzyme acted upon was the *prothrombin*.

According to Hepburn and Bazzoni (1915), *oxidase* was still active after an exposure of 3½ hours to the temperature of liquid air, while an aqueous solution of *urease* retained 96.29% of its initial activity after having been kept in liquid air for 100 hours.

Bickel (1905) subjected *pepsin* (in gastric juice) to liquid air temperatures for 22 hours, after which treatment the proteolytic activity of the pepsin was not impaired. Nor did repeated freezing in liquid air and thawing (up to 6 times within an hour) produce any effect.

Solutions of *trypsin* contained in glass tubes and frozen in liquid air, then thawed rapidly in tap water, and refrozen in the same manner 12 times, showed 70% of the trypsin inactivated when the 5% stock solution was diluted 1-500, and 17% when it was diluted 1-10 (Rivers, 1927).

Galvialo (1937) subjected *catalase* from human blood and *diastase* from saliva (either undiluted or diluted with water) to liquid air temperatures; the catalase was not affected, but the activity of the diastase was reduced to one half.

The literature on the action of low temperature on enzymes, up to 1913, has been reviewed by Hepburn (1915).

3. *Enzymoids*. According to Tetsuda (1912), sera frozen in an ice chest, for times varying from a few hours to several weeks, and either kept continuously in the frozen state or frozen and thawed repeatedly, did not show any alteration of *complement*, of *agglutinin*, of *hemolysin*, of *precipitin*, or of the properties of *inducing* and of *attenuating anaphylaxis*. (Freezing was used as a method of concentrating these various substances in the sera.)

The toxin of *Clostridium botulinum* showed no decrease in toxicity after having been stored at -79° for 2 months or at -16° for 14 months (Tanner and Wallace, 1931).

Complement, frozen 12 consecutive times, in undiluted guinea pig serum, or in 1-10 dilutions, was not affected; but it was inactivated when frozen the same number of

times in serum diluted 1–100 with physiological salt solution (Rivers, 1927).

Complement, as well as *amboceptor*, in rabbit serum immersed in liquid air for times varying from 10 to 30 minutes, kept their unaltered activity, according to Lüdke (1905).

Tetsuda (1912) also reported that *complement* and *antibodies* were not affected by maintaining the sera for several days in liquid air.

A 24 hour-old broth culture of Eberth bacilli, maintained frozen in liquid air for 20 minutes, did not lose its *agglutinability*. Sheep serum, frozen in the same manner, was as efficient in agglutinating these bacilli after the freezing as before (Chanoz, Courmont and Doyon, 1900).

Maefadyen (1902a) used the juices extracted from the Eberth bacilli by trituration in liquid air, to produce immunizing sera. The latter possessed the normal *anti-toxic* and *antibacterial* properties.

Cobra *venom*, in a 1% solution, maintained in liquid air for 9 days, presented an unaltered toxicity (Lumière and Nicolas, *Province médicale*, Sept. 21, 1901).

According to Pictet (1893), *ptomaines* were affected by exposure to temperatures of -100° to -200° .

4. *Viruses*. A strain of *bacteriophage* active on *B. coli* and one active on staphylococci, frozen at -78° (with solid CO_2) and thawed 20 times consecutively, did not lose any of their activity (Sanderson, 1925).

D'Herelle ("The Bacteriophage and Its Behavior," p. 300, Baltimore, 1926), however, reported that while the phage (one for staphylococci and one for dysentery bacilli) was not affected in young filtrates, it was inactivated by 1 to 3 freezings in liquid air, when treated in filtrates more than 17 days old.

Rivers (1927), using 19- to 90-day-old filtrates of a phage lytic for *B. coli*, observed that, after 12 freezings in liquid air, the phage was completely inactivated when physiological salt solution was used as a diluent, and that it was partially inactivated when the diluents were Locke's

solution, distilled water, or broth, the degree of inactivation decreasing in the order given. He then experimented with dilutions of 1-10 and 1-1000 of the stock filtrate and found that an increased dilution with salt and Locke's solution increased the percentage of phage inactivated while, on the contrary, increased dilution with distilled water or broth did not.

According to Stockman and Minett (1926), the virus of *foot and mouth disease* was not destroyed by repeated freezing in ammonia brine.

Pictet and Yung (1884) reported that the *cow-pox vaccine* was inactivated after two consecutive exposures of respectively 108 hours to -70° and 20 hours to -130° .

According to Barrat (1903), an exposure of the *rabies* virus to the temperature of liquid air for 1 to 5 hours did not inactivate it.

Salvin-Moore and Barrat (1908) found that a stay of 30 minutes in liquid air did not affect the graftable *mouth cancer*.

Gaylord (1908) obtained identical results with the same material maintained in liquid air for 80 minutes.

Rivers (1927) observed that the *herpes* virus in a brain emulsion was not inactivated by 12 freezings in liquid air but was inactivated when frozen 24 times in an emulsion diluted 1-20 or more with Locke's solution.

Similar results were obtained by the same author with *vaccine* virus, which resisted 12 freezings in a testicular emulsion diluted 1-10, 1-100, and 1-1000, but the titer of the virus decreased after 24 freezings at dilutions of 1-10,000 and 1-100,000 and the virulence was completely destroyed after 34 freezings at a dilution of 1-100,000.

Rivers investigated also *virus III* and found it to be readily inactivated after 12 freezings at dilutions of 1-10 of his stock emulsion.

The results on the infracellulards can be summarized as follows: 1. The enzymes, toxins, bacteriophage and viruses investigated are not affected by a freezing of their

solutions or suspensions in liquid air nor by a stay of short duration at about -190° . 2. Some of them are inactivated by prolonged or repeated freezing at that temperature, especially in very dilute solutions.

II. MONOCELLULARS

SECTION I. PROTOPHYTA

1. *Bacteria and Bacterioids.* The numerous investigations on bacteria will be classified into two groups: *A.* Those in which the organisms were found unaffected by low temperature; *B.* Those describing lethal or injurious effects.

A. Many authors have reported that bacteria support temperatures of 0° or of a few degrees below 0° , as used in cold storage; we shall not attempt to name all these investigators.

McLean (1918) has described 4 species of bacteria which he isolated from ice, from snow and from frozen algae of the Antarctic where the mean annual temperature is about -20° .

Smart (1935) observed that some bacteria, found alive in fruit held for 3 years at -9.4° , then isolated and subjected in pure culture to a temperature of -8.9° for 1 year, produced growth at this temperature.

Brehme (1901) reported that the *cholera* vibriones, in bouillon cultures, survived a continuous freezing for 57 days at -1° to -16° ; nor were they killed when subjected 40 times alternately to -15° and to 15° . In similar conditions *typhus* bacilli survived freezing at -2° to -16° for $4\frac{1}{2}$ months and were not killed when frozen and thawed 40 times at respectively -15° and 15° .

Tanner and Wallace (1931) found that spores of *Clostridium botulinum* survived freezing at -16° for 14 months, and that commercially frozen fruits and vegetables were not sterile even after 2 years at -16° .

Citoviez (1928) put an emulsion of streptococci of *scarlet fever* into capillary tubes, where they were subjected to temperatures of -17° to -18° for at least two weeks; the

organisms were not killed. It is important to remark that in these experiments, the emulsion was not frozozn but subeooled.

Prucha and Bramman (1926) isolated *B. typhosus* from ice cream kept for 28 months at -20° .

Gladin (1898) froze *B. pestis* at -20° daily for 40 days. Such repeated freezing and thawing did not kill the organisms.

Kasansky (1899) found no loss of viability in *B. pestis* after a stay of $5\frac{1}{2}$ months at -31° .

According to Pictet and Yung (1884), bacteria, in sealed glass tubes, did not show any impairment of their activity after having been subjected for 108 hours to at least -70° and for 20 more hours to -130° .

In 1893, Pictet reported that he had exposed 30 to 35 kinds of bacteria to -200° and that they had apparently suffered no injurious effects.

Bouillon suspensions of the vegetative form of *B. anthracis* and of the bacillus of *chicken cholera* were immersed in liquid air for 15 hours by Belli (1902). No change of the morphological characters nor of the virulence of the organisms could be observed. In another experiment the author placed in a sterilized test tube a small piece of spleen from a rabbit which had just died of anthrax, and then immersed the test tube in liquid air for 15 hours. The treated specimen was found to be just as virulent as another untreated specimen from the same spleen. In a last experiment, small strips of filter paper were impregnated with bouillon cultures of *B. anthracis* and of the bacillus of chicken cholera, and these strips were then suspended in liquid air for 8 hours. No diminution of virulence could be observed.

Paul and Prall (1907) found that dried *Staphylococcus pyogenes aureus* can remain alive for weeks in a mixture of dry ice and ether, and for 125 days in liquid air.

Gonococci, held for 10 days at -20° and for 24 hours at -195° , in liquid nitrogen, were found to be viable by Lumière and Chevrotier (1914).

Macfadyen (1900) exposed vegetative and spore forms of 10 kinds of bacteria (*bacilli*, *spirilla*, *staphylococci* and a *photobacterium*) to liquid air for 20 hours. The cultures were either in broth or on solid media (gelatin, agar, potato). No effect whatsoever was observed; all the physiological properties: curdling of milk, fermentation of sugar, indol production, pigmentation, pathogenicity and photogenicity, appeared to be normal.

In another experiment, Macfadyen liquefied the air of his laboratory and cultured the bacteria present in it; he found several species of *bacilli*, *cocci* and *sarcinae*. The temperature during the liquefaction of the air reached about -210° .

Macfadyen and Rowland (1900a and 1902) left *B. coli*, *B. typhosus* and *Staphylococcus pyogenes aureus* in liquid air for 6 months. The bacteria, suspended in small loops of platinum wire or on cotton-wool swabs, were directly in contact with liquid air. No impairment of vitality was observed in any of the organisms.

Lipman (1937) exposed for 48 hours to the temperature of liquid air bacteria in the actively growing state. The organisms were cultivated on agar slants in test tubes. The latter were immersed in liquid air after having been sealed. The 9 species of bacteria experimented upon survived and 8 of them presented an abundant growth. Particular attention was given to the choice of the species in order to exclude the possibility of spore formation.

Macfadyen and Rowland (1900b) observed no sign of injury on various bacteria left for 10 hours, in sealed tubes, in liquid hydrogen.

De Jong (1922) summarizing the results of Beek (1919) and Ongkiehong (1922), says that *B. coli*, *B. faecalis alcaligenes*, *B. lactis aerogenes*, and the bacteria of *typhoid*, of *paratyphoid A* and *B*, and of *enteritis*, resisted equally well exposures to temperatures of -20° , -190° and -253° .

Kadisch (1931) found that *B. coli* (in 0.85% NaCl solution) could resist -252° (liquid hydrogen) for 3 hours,

and that *Staphylococcus pyogenes albus* and the *tubercle bacillus* could withstand the same temperature for 50 hours. In further experiments, the same investigator reported that these organisms were not killed after two consecutive exposures of respectively 4 hours at -268.8° and $1\frac{1}{2}$ hours at -271.8° . Cooling was relatively slow, it requiring about 24 hours for the entire experiment.

Zirpolo (1932) observed no injurious effect when luminescent bacteria (*Bacillus* and *Micrococcus pterantonii*), in the fresh vegetative state, were exposed for several hours to the temperature of liquid helium.

Lipman (1936a) immersed in liquid helium, at temperatures varying from 4.2° K to 1.35° K, spores of common bacilli (species not mentioned), smeared on fragments of cover glasses, and desiccated for 2 weeks. The preparations were kept 2 hours at the lowest temperature and 42 hours below 4.2° K. The spores presented as abundant a bacterial growth as would normal spores.

We shall not review here the literature accumulated during the last 3 years on the method of drying bacteria at low temperatures for preserving them. We shall mention only that, in these experiments, the physiological properties of the organisms were found unimpaired (Cf. the fundamental work of Flosdorf and Mudd, 1935).

B. Park, Williams and Krumwiede ("Pathogenic Microorganisms," 8th ed. p. 698, New York, 1924) kept 21 strains of water-inoculated *B. typhosus* at about -2° to -7° . Six cultures were sterile after 5 weeks and all were sterile after 22 weeks.

Hilliard, Torossian and Stone (1915) and Hilliard and Davis (1918) subjected water suspensions of *B. coli* to freezing for 3 hours at 0° or at -15° and determined the percent of survivors by the usual method of bacterial counts. At 0° , 26.4% to 67.7%, and at -15° , 99.4% to 99.7% of the organisms were killed. After intermittent freezing for the same length of time (4 freezings), the number killed was slightly greater.

In another series of experiments, the *B. coli* were suspended in glucose solutions of different concentrations and then subjected to varying degrees of cold, from -0.5° to -6° . The concentrations of glucose were such that the solutions did not solidify at the temperatures used, so that the effect of cold alone, without crystallization, could be determined. The results were compared with those obtained with tap-water suspensions of bacteria subjected to the same temperatures. At -0.5° , an average of 93.6% of the organisms were killed in the frozen tap water, while in the unfrozen glucose solution 46.4% were destroyed. At -6° , an average of 99.2% perished in the frozen medium, and 49.5% in the unfrozen solution. When both the glucose and the tap water suspensions were frozen solidly at -10° for 3 hours, the percent killed in the former varied from 77.5% to 99%, while in the latter it ranged between 98.1% and 99.8%.

Tanner and Williamson (1928) exposed 4 species of common bacteria to temperatures of -13° to -15° for 2 to 16 weeks, and found that this prolonged freezing destroyed some species completely, while others were still alive after 160 weeks at -15° .

According to Onorato (1902), the *Hemophilus influenzae* is destroyed in blood broth after a stay of $2\frac{1}{2}$ hours at -15° or $1\frac{1}{2}$ hours at -20° .

Smith and Swingle (1905) exposed *B. typhosus*, in bouillon cultures, to -17.8° for 2 hours and found that, on an average, 99.5% were killed.

Keith (1913) observed that when *B. coli*, in solidly frozen tap water, were kept at -20° , only a fraction of one percent remained alive after 5 days, and that storage of a few weeks at that temperature resulted in a complete destruction of the bacteria. Moreover, when *B. coli* were in pure milk or in milk diluted to various degrees with water, a larger proportion of bacteria survived the freezing of the undiluted than of the diluted milk.

According to de Jong (1922), the bacteria of *dysentery* were killed after a relatively short exposure to -20° .

For certain strains, -190° was more lethal than -20° , while for some others it was less.

Kasansky (1899) found that *B. pestis*, cultured on agar and kept at -31° in the frozen condition for 4 months, but thawed 8 times during the interval, showed a weakening of its virulence.

Sanderson (1925) gives the following figures as representing the proportion of *B. coli* which were killed after, respectively, the first, the tenth, and the fifteenth freezing at -78° : 16%, 86%, 94%.

According to Klepzzoff (1895), bacteria are killed when exposed to the temperature of liquid air.

D'Arsonval (1898) reported that the chromogenic function of *B. pyocyaneus* was slightly impaired by exposure to liquid air temperature. The effect was more accentuated if the cultures had previously been dried.

Macfadyen and Rowland (1900a) mention a slight weakening in the activity of some species of bacteria, after the latter had been left for 7 days in liquid air.

According to White (1901), a culture of bacteria, exposed for 2 hours to liquid air temperature, presented a large number of killed organisms; only the more resistant ones survived.

Smith and Swingle (1905) subjected bouillon cultures of *B. typhosus* to liquid air temperatures for 2 hours, and found that, on an average, 99.3% were killed but the few organisms which survived produced normal colonies.

Rivers (1927) froze a broth suspension of *B. coli* 12 successive times in liquid air. Before freezing, there were 180,000,000 viable organisms per cc; after the 12 freezings and thawings, this number was reduced to 40,000. Greater dilution of the suspension decreased the percentage of survivors.

According to Park, Williams and Krumwiede (*loc. cit.*, p. 295) 30% of the *staphylococci* subjected to freezing in liquid air for 30 minutes, remained viable.

A brief review of the investigations on pathogenic bacteria has been published by Hampil in 1932.

2. *Yeasts.* The investigations on yeasts, like those on bacteria, will be arranged in two sections: A. Those reporting negative results; B. Those reporting injury or death.

A. Several authors have observed that some yeast species are physiologically active at sub-zero temperatures. Thus, Berry (1934) found a species which reproduced actively in beer wort at -2.2° .

Salimovskaja-Rodina (1936) described 19 species of yeast (?) isolated from colored snow (*Arch. Biol. Nauk*, 43, 229).

Smart (1935) found that several species of yeast, cultured on agar slants and left for 1 year at -8.9° , produced abundant growth when transferred to room temperature.

Molisch (1897) described a deformation and a shrinking of about 10%, but no loss of growth or of fermentation power when yeast cells, growing actively in a liquid medium, were frozen at -5° to -9° and observed under the microscope during the congelation of their culture medium.

Kadisch (1931) exposed several sorts of pathogenic yeasts, suspended in 0.85% sodium chloride solutions, to temperatures of -20° to -30° for 2 months, subjecting them to 11 successive freezings and thawings during this time. No harmful effects were observed.

Zopf (1890) made thin smears of vegetative cells and of spores of *Saccharomyces Hansenii* on mica sheets and subjected them for 4 hours 20 minutes to -83° ; death did not ensue. Furthermore, no harm was done when this frozen material was transferred directly into water at room temperature ("Die Pilze," p. 275, Breslau).

Melsens (1870), after having observed that yeast resists -91° , investigated the combined action of pressure and cold. He put a yeast suspension in a steel bomb calculated to resist a bursting pressure of 8,000 atmospheres and froze the suspension at a temperature low enough to burst the bomb; the cells remained active after this treatment.

Doemens (*Allg. Brau. u. Hopf. Ztg.*, 2, 2225, 1897) exposed beer yeast to the temperature of liquid air for 6 minutes; the yeast retained its vitality.

Käreher (1931) found that *Saccharomyces cerevisiae*, cultured on agar slants, survived a 1 to 8 day action of a temperature of -70° , as well as a 13 hour exposure to -183° to -192° in liquid air.

Macfadyen and Rowland (1902) kept yeasts which had been washed, pressed and wrapped in paper, immersed in liquid air for 6 months; neither the vitality nor the physiological properties of the organisms were impaired.

According to Kadisch (1931), pathogenic yeasts suspended in 0.85% sodium chloride were not killed after 24 hours at -180° , nor after 3 hours at -252° , nor after 2 hours at -268.8° , though the latter treatment caused a considerable delay in development. In a further test he cooled the yeasts by degrees from room temperature to -268.8° , where they stayed for 2 hours; thereupon he brought them down to the temperature of -272° , which was maintained for $1\frac{1}{2}$ hours. He then raised the temperature to -268.8° and kept it at this point for another 2 hours; finally, he brought the organisms by degrees back to room temperature. There was no loss of vitality, but merely a slowed development, and the author concludes that cold alone cannot produce the death of the micro-organisms.

B. Tanner and Williamson (1928) exposed ampullae containing 2 cc. of a suspension of yeast in physiological saline, to -15° for varying lengths of time up to about 3 years; most of the yeasts were markedly injured after that time.

Schumacher (1874) observed that in fresh yeast, exposed for 15 minutes to -113° , many of the cells were killed. The younger ones with smaller vacuoles and those still devoid of vacuoles remained alive and were capable of budding.

Pietet and Yung (1884) exposed *Saccharomyces cere-*

visae for 108 hours to -70° and for a further 20 hours to -130° , after which the organisms were no longer capable of raising bread, although no alteration, microscopically visible, could be detected.

Doemens (*Allg. Bran. u. Hopf. Ztg.*, 2, 2225, 1897) reported that, when he exposed a suspension of yeast to liquid air and thawed it too rapidly in water, the power of development was impaired.

3. *Monocellular Algae*. It may be mentioned here that several species of monocellular algae are found in the encysted form in snow and give it a green or red coloration. These organisms become active in the melting snow. It is reported that some of them cannot resist temperatures higher than 4° . We did not find any data on the lower limit of temperature that they can support. For the literature on this subject, we refer the reader to a series of papers by Kol, E., and Chodat, F., in *Bull. Soc. Bot., Genève*, 25, 1934.

According to W. West and G. West, *Closterium* (a desmid) was found motile in water which had been frozen for 14 days. *Microsterias* (another desmid) was also found alive under the same conditions. Since this last genus was not observed in the cultures in the spore form, the authors remark that it resisted freezing in the vegetative state (*Ann. of Bot.*, 12, 1898, p. 33).

Wislouch (1910) exposed *Stichococcus bacillaris*, in sterilized water, to various degrees of cold. A very small number of cells survived a 2 hour exposure to -75° , while about 50% resisted a 6-7 hour exposure to -21° .

According to Pictet (1893), various species of *diatoms* in water cultures, frozen at about -200° , were uninjured and, after the thawing of the medium, they were seen to extend pseudopodia-like protrusions.

Edlich (1936) subjected *Pleurococcus vulgaris*, *Apatococcus minor* and *Stichococcus bacillaris* on their natural bark substratum to temperatures ranging from -20° to -80° for varying lengths of time, after a previous ex-

posure to different degrees of atmospheric moisture (relative humidity 25% to 100%). *Pleurococcus* was found to withstand -80° for at least 24 to 26 hours, irrespective of the humidity to which it had previously been subjected. *Stichococcus* and *Apatococcus* proved themselves less resistant; they were killed at -80° in two hours and one hour respectively. However, with the relative humidity reduced to 50% they were equally as resistant as *Pleurococcus* to a temperature of -80° .

Warburg (1919) maintained in liquid air for one hour suspensions of *Chlorella* in Knopp's solution. The cells sustained no injury from the treatment. The chlorophyll granules were unaltered while they became enlarged and showed structural changes in *Euglena* treated in a similar manner (*Bioch. Ztschr.*, 100, 234).

Kärcher (1931) exposed *Stichococcus bacillaris*, cultured on agar slants in test tubes, to -70° for 1 to 8 days, or to -183° to -192° for 13 hours. The algae were not killed.

Becquerel (1932d and 1936) found that *Protococcus* and *Pleurococcus* grew normally after having been subjected in the dry state to the lowest available temperatures. The cells, obtained from cultures in synthetic liquid media (or in a previous experiment from a piece of bark) were dried on barium oxide for 3 months at $+35^{\circ}$; they were then sealed into glass tubes evacuated to 10^{-5} mm. of mercury, and subjected to -190° for 480 hours and to -269° to -271° for $7\frac{1}{2}$ hours. Some specimens had been kept dried in the vacuum for 25 years before being cooled. In one experiment a temperature of 1.84° K. was maintained for one hour. The material so treated yielded living *Pleurococcus vulgaris*, *Chlorella vulgaris*, *Stichococcus bacillaris*, *Hantzschia amphioxys*, *Pinnularia viridis*, *Chlorococcum humicolum*, and *Palmella miniata*. The same was also true for cultures not subjected to high vacua, but exposed directly to the action of the liquefied gases.

SECTION II. PROTOZOA

1. *Rhizopods*. According to Külme (1864), *Amoeba* and *Actinophrys* are killed when their culture medium freezes, but they suffer no injury when they are kept at 0° for several hours, as long as the medium is not frozen.

Molisch (1897) observed under the microscope *Amoeba* freezing in drops of water. He found that the organisms die as soon as the ice forms in their proximity or within them, that is, at a temperature slightly below zero.

Chambers and Hale (1932), who proceeded in a similar manner, state that the internal freezing of *Amoeba*, which they induced by inserting an ice-tipped pipette into the interior of the organism, occurs at -0.6° . Internal freezing always kills these animals, while, if ice is not present in them, temperatures as low as -5° produce no damage. These authors describe how the ice spreads through the protoplasm in the form of fine feathery crystals radiating in all directions from the point touched by the pipette.

According to Deschiens (1934), *dysenteric Amoebae*, in the vegetative form, were destroyed in a short time when their medium froze, at a temperature of -5° . They could be kept alive, though immotile, for 56 hours, at 0.0° , in the unfrozen medium.

Fol (1884) obtained growing *Amoeba* from dried earth which he put to freeze in Pictet's apparatus at a temperature of about -100° .

Becquerel (1936) revived several species of rhizopods from dried soil cooled to the lowest available temperatures. He kept in a vacuum, with barium oxide, at 35° , for 3 months, samples of soils containing various microorganisms. When the soils were thoroughly dried he sealed one portion of them in glass tubes evacuated to 10^{-5} mm. of mercury and placed another portion in glass tubes closed with cotton plugs. Thereupon he subjected all the tubes to liquid helium (-269° to -271°) for $7\frac{1}{2}$ hours and subsequently to liquid nitrogen (-190°) for 480 hours. From both groups of soils he obtained, after this treatment, living *Amoeba proteus*, *Amoeba limax*, *Amoeba dactylifera* and *Actinophrys sol*.

2. *Ciliates*. Greeley (1901) observed that when cultures of *Stentor coeruleus* were cooled slowly, ciliary movement continued for 1 to 3 hours at 0° , after which the cilia and gullet were absorbed, the ectosarc was thrown off, and the endosarc transformed into a spherical cyst-like cell. When the temperature was again raised, these resting cells underwent a reverse process, and resumed their normal activity. If the temperature was lowered rapidly and the culture medium was solidly frozen, the organisms were killed.

According to Efimoff (1924), *Paramecium* can withstand freezing (Ausfrieren) at -1° for 30 minutes, but is killed when frozen at the same temperature for 50 to 60 minutes. Various infusoria (*Paramecium caudatum*, *Colpidium colpoda*, *Spirostomum ambiguum*) die in less than 30 minutes when exposed to temperatures below -4° . Rapid and short subcooling (not below -9°) produces no injury, but, if it is prolonged, the paramaecia become spherical and swell up, increasing their volume 4-5 fold, while the other infusoria shrivel up and assume irregular contours.

To study the effect of low temperatures on *Paramecium* in a subcooled medium, Wolfson (1935) enclosed these organisms singly or in numbers from 2 to 116 in capillary tubes and observed them microscopically during cooling in a specially constructed, metal, glass-bottomed well, cooled by stout metal leads connected with a brass plate on which pieces of dry ice were placed as desired. Warming was effected by means of a heating coil wound about these same conducting leads. He found that both bodily and ciliary movements could be observed down to -14.2° . Then, all bodily movements ceased, but the cilia continued to beat for some time yet. While organisms subjected momentarily to -16° were found to recover completely, after a longer cooling at that temperature morphological degeneration set in, manifesting itself in a rounding up of the organism, an apparent increase in cell volume and a marked visibility of the nucleus, followed by

complete disintegration of the organism. In no case did a paramaecium ever survive the freezing of its medium.

Fol (1884) isolated living ciliates from dried soil frozen at about -100° .

Taylor and Strickland (1936) found that immersion in liquid air for $13\frac{1}{2}$ hours does not affect the viability of air-dried cysts of *Colpoda cucullus*. In another experiment these authors obtained some excystment (percentage unknown) from cysts first subjected to a vacuum of about 10^{-5} mm. of mercury for 2 or 3 days, thereupon exposed to liquid air temperatures for $12\frac{1}{2}$ days, and then warmed to room temperature within 2 minutes.

Beequerel (1936) found living *Paramaecium bursaria* in dried soil kept for $7\frac{1}{2}$ hours at a temperature of about 2° K.

3. *Flagellates*. Mainx (*Arch. f. Protistenk.*, 60, 387, 1928) did not find any evidence in favor of the belief that *Euglena* can withstand freezing. He observed that partially or completely frozen cultures of *Euglena gracilis*, *E. viridis* and *E. deses* gave rise to very few living forms, and to these only if cysts were present previously.

According to Jahn (1933), when *Euglena* cultures were kept in the frozen state, at -4° , for 1 hour, most of the organisms, but not all, were killed. A temperature of -0.2° maintained for the same time, without freezing of the medium, showed no harmful effect.

On the other hand, Günther (*Arch. f. Protistenk.*, 60, 556, 1928) reported that he observed a specimen of *Euglena terricola* which had been frozen while in the process of mitosis and which completed its division, on the microscope, in a normal manner when the ice melted, after having been congealed for 8 days at -12° .

Klebs (*Ztschr. f. wiss. Zool.*, 55, 265, 1893) observed that *Euglena*, in the free swimming state, was not killed by repeated freezing.

Trypanosoma gambiense, according to Gaylord (1908) can resist being immersed in liquid air for 20 minutes, but is killed after 40 minutes.

Trypanosoma lewisi was found alive after immersion of the culture in liquid air for 75 minutes, but it was killed after 24 hours (according to Doflein's "Lehrbuch der Protozoenkunde," 3rd ed., p. 230, Jena, 1911).

De Jong (1922) reported the results obtained in his laboratory by Zandbergen (1922) on *Trypanosoma* as follows: *Trypanosoma lewisi* was killed at -20° in 8 minutes, at -30° in one hour and at -190° in 2 hours; *Trypanosoma equiperdum* lost its motility and pathogenicity by a stay of $3\frac{1}{4}$ hours at -20° ; at -39° , -65° and -145° it lost its motility but not its pathogenicity; at -191° it was still motile and pathogenic after 21 days. *Trypanosoma venezuelense* and *brucei* behaved in the same manner at *T. equiperdum*.

Becquerel (1936) found living *Euglena viridis* in dried soil subjected to liquid helium in a high vacuum.

A general survey of the monocellulars reveals that: 1. Most of the bacteria, bacterioids, yeasts, algae of the type *Chlorella* or *Stichococcus* and *flagellates* of the type *Trypanosoma*, in the vegetative state and in their aqueous culture medium, are not destroyed by the lowest available temperatures acting for some hours; 2. When the bacteria and bacterioids are affected by the temperatures of the liquid gases, the influence of the factors: species, time of exposure, concentration of the medium, and repeated congelation has been emphasized but is not clear; 3. The rhizopods, ciliates, and flagellates of the type *Euglena*, in the vegetative state, and in an aqueous medium, do not resist more than a few degrees below zero and are usually found dead after they had been congealed; 4. All the monocellulars resist temperatures near the absolute zero in the encysted and dry state.

III. GERM CELLS, SPORES AND SEEDS

1. *Spermatozoa*. Our knowledge of the resistance of spermatozoa to cold is gleaned mostly from scattered data obtained as secondary results in the study of other prob-

lems. Consequently some important details concerning the method of exposure to low temperatures are often missing.

H. Weber (1936) could maintain *bull* spermatozoa motile *in vitro* at 0° for 96 hours (Dissert., Leipzig, 1936; quoted from *Ber. f. ges. Physiol.*, 103, 294, 1938).

According to Spallanzani (1787), *frog* spermatozoa, frozen hard for half an hour, are able, after thawing, to initiate egg development, but if they are kept in the frozen state for several hours, this power is lost.

The spermatozoa of *Periplaneta orientalis* (a *cockroach*), and those of *man* are capable of surviving freezing for 10 to 11 hours, according to G. N. Pawlow (1927).

Schenk (1870) reported that the spermatozoa of the *frog* and of the *turtle*, frozen at temperatures of -4° to -7°, resumed motility after being warmed to 38° or 40°, but all attempts to fertilize eggs with these spermatozoa failed. Resumption of motility was also observed on the spermatozoa of *rabbits* and *dogs* after exposure to -6°.

Prevost (*C. r. Ac. Sc.*, 11, 907, 1840) froze excised *frog* testes at -8° to -10°, and then thawed them slowly in cold water. The water was found full of motile spermatozoa.

Quatrefages (1853) made several observations on the action of cold on fish sperm. His results can be summarized as follows: 1. *Carp's* spermatozoa mixed with ice water on a microscope slide stopped their motion immediately. *Barbel's* sperm, under the same conditions, maintained its motility for 50 seconds. 2. *Pike's* spermatozoa could be observed to be motile after having been directly in contact with ice for 50 hours. In a series of experiments in which pieces of ice were put in a dish full of sperm or in which the sperm was stored with ice in a container devised to allow the water from the melting ice to flow out, the organisms remained motile for 64 hours. 3. Pikes, dead for 24 hours and maintained in ice, furnished motile spermatozoa, while dead fish kept at 13° to 15° showed less viable sperm. 4. Pike's spermatozoa left to freeze overnight either on a plate or wrapped in moist

paper, or in a dish full of water, were active the next day, after thawing. 5. To kill Pike's sperm by cold, it took a 5-hour exposure of the ice-sperm mixture to a temperature of -10° to -12° . In the course of these experiments it was observed also that: *a.* completely mature sperm was less resistant than immature sperm; *b.* spermatozoa aggregated in clumps resisted longer than those dispersed in water; *c.* diluting the material was apparently more injurious than cooling it.

Mantegazza (*Rendic. r. Inst. Lomb.*, 3, 183, 1866), as quoted from Davenport (1897), reported that *human* spermatozoa were not killed by exposures to -17° .

2. *Eggs.* We shall classify the literature on eggs according to species.

Gemmules of 2 kinds of *sponges* are said by Waltner (*Arch. f. Naturgesch.*, 59, 257, 1893) to have resisted respectively 17 days and 59 days in ice.

Essex and Magath (*Am. J. Hyg.*, 14, 700, 1931) placed ova of the *tapeworm*, *Diphyllobothrium latum*, in water and subjected them to a temperature of -10° for 48 hours. This treatment killed them all.

Zawadowski (1926) reports that fertilized eggs of *Ascaris* can withstand -5° for 1 month, and show normal development after subsequent warming. After 14 days at -20° , 50 per cent of the eggs degenerated; after 2 months of exposure to -24° , there was still a possibility of development on warming, yet the embryos finally died. An exposure of $1\frac{1}{4}$ years to -10° produced a similar degree of injury. (From Belehradek, 1935.)

A wealth of data on the death temperatures of *insect* eggs is found scattered in the observations of the entomologists of the last two centuries. Bakhmetieff (1901), Bachmetjew (1901) and Uvarov (1931) reviewed the subject. We refer the reader to these authors' works for more complete information and we mention here only a few of the more striking results. Some of them indicate a high cold resistance, others a high cold sensitivity.

Spallanzani (1787) observed that the eggs of *Bombyx mori* can support an air temperature of -50° for 4 hours.

According to Pictet (1893), the eggs of *Bombyx* can be stored at -40° ; this temperature is not only non-lethal to the eggs but it is injurious to the germs which might infect the eggs.

Dönhoff (1872) exposed for 5 hours *Bombyx* eggs contained in small glass vials to the temperature of -15° to -21° of an ice-salt mixture. The eggs were then placed into little bottles tied shut with linen and the bottles put into a box which Dönhoff carried on his breast by day and took to bed with him by night. After a few weeks all the eggs hatched.

On the other hand, according to Pictet (1893), *ant* eggs are extremely sensitive to cold. They are all killed between 0° and -5° . The sensitivity increases with the stage of development and some more advanced eggs died when exposed to 5° for a few hours.

Bach and Pemberton (*J. Agr. Res.*, 5, 657, 1916) found that no eggs of the *Mediterranean fruit fly* survived cold storage at 4.5° to 7.2° for 7 weeks, or at 0.5° to 4.5° for 3 weeks or at 0.0° to 0.5° for two weeks. (From Uvarov, 1931.)

According to Hase (*Ztschr. f. Parasitenk.*, 2, 368, 1930), an exposure of *bedbug* eggs for 3 to 20 days to a temperature of 2° was harmful enough to prevent the hatching of a number of eggs, in which only an initial development was found to take place.

According to Rahm (1920 and 1923), the eggs of the *tardigrad*, *Echiniscus*, in moist moss, survived two successive freezings of 35 minutes each at -81° and those of another *tardigrad*, *Macrobiotus*, were not killed by a sudden immersion, in the moist condition, in liquid air or in liquid hydrogen.

Schenk (1870) exposed fertilized eggs of *Rana temporaria* to -3° for one hour during which time the jelly layer had frozen hard; he found that, after thawing, the eggs developed. Fertilized eggs exposed for one hour to -7° showed no further development after thawing. Mature unfertilized eggs of *Bufo cinereus*, taken from the body of

the mother, and possessing a jelly layer, were exposed to -4° for 1 hour and then artificially fertilized and placed in favorable conditions for development. In the first hours after thawing, no externally perceptible changes indicative of fertilization could be observed, but 14 hours after fertilization, the cleavage process commenced and thereafter proceeded at the normal rate.

Fischer-Sigwart (*Vierteljahrsch. d. Naturf. Ges. Zürich*, 62, 1897) records finding masses of *frog* eggs which had been frozen solid for 2 days, during which time the temperature sank to -8° . When the eggs were gradually thawed out, they underwent a normal process of development, though this was somewhat slower than usual.

Pietet (1893) says that he cooled *frog* eggs to -60° for several hours and that they developed into tadpoles.

Several investigations are found in the older literature on the death point of *bird* eggs, but many authors overlooked the fact that it takes a relatively long time for the interior of the larger eggs to reach the equilibrium of temperature with the surrounding atmosphere. There thus result some apparent contradictions which partially vanish when one considers the temperature of the bath or of the atmosphere in the various experiments, the time of exposure, the size of the eggs and the other factors which determine heat conduction. A short review of some of the literature will be found in Moran (1925). We shall describe here a few of the more typical investigations on chicken eggs.

Lipschütz and Illanes (1929) reported that of 33 *hen's* eggs, exposed for 3 to 7 hours to an air temperature of -4° to -6° , 20 developed embryos or yielded chicks, these latter retaining their normal appearance throughout an observation period of several months. In another series of experiments, the temperature within the eggs was determined by inserting a thermometer into a hole in the egg. Temperatures of -2.5° to -4.5° were thus recorded.

Rabaud (1899) put to freeze in an ice-salt mixture at -18° , 30 sets of 18 eggs each. He left them in the mixture

for half an hour, after which time most of the shells were cracked. Some of these frozen eggs were then incubated immediately at 38° , others were kept in a cool chamber and incubated after one day, and others, finally, were kept for 3 days before being incubated. All of them, together with 6 control eggs in each set, were opened after a 3-day incubation. About one third of the eggs in each of the different series of experiments presented embryos, although badly deformed, while the other two thirds contained blastoderms extended over the yolk, but showed no embryo formation. The author concludes that freezing did not prevent cell proliferation but tended to inhibit cell differentiation.

As a thoroughly worked out investigation on the death temperatures of the *chicken* egg and on the time required for death at various temperatures we shall describe Moran's work (1925). He placed 100 eggs in a constant temperature room at -2.9° and 100 in another room at -4.6° . Every few hours some eggs were taken out and tested, the power of incubation being taken as the index of vitality. In a previous determination of the velocity of cooling, made with a thermo-couple, it was found that 24 hours elapsed before the center of the egg reached -2.9° and 30 hours before it reached -4.6° . The general results were that some eggs were still capable of developing after having been for nearly 47 hours at -4.6° and 118 hours at -2.9° . But even above zero the power of incubation was soon destroyed. The limit of the time that the eggs could be stored at 0.7° was 10 days and at 10.4° , 34 days. In these experiments cooling and warming were graded and slow. Moran concludes that the germination capacity is probably destroyed immediately at -6° to -7° , while at the higher temperatures death requires increasingly longer times. It seems evident that all the eggs studied were subcooled; the question of whether or not the chicken egg withstands congelation, is left untouched in this work.

3. *Spores*. Strasburger (*Jena. Ztschr. f. Naturwiss.*, 12, 612, 1878) observed *swarm spores* of the *algae*, *Haema-*

tococcus and *Chilomonas* moving about in partially frozen drops of water, among the ice crystals.

According to the same author, fully frozen (“völlig eingefrorene”) *swarm spores* of *Haematococcus*, *Ulothrix*, *Bothrydium* and *Chilomonas* were found dead after thawing, in an experiment in which the temperature of the surroundings had not dropped below -1° .

Kjellmann (quoted from *Bot. Ztg.*, 33, 771, 1875) reported that some *marine algae* form and discharge *swarm spores* when the sea temperature lies between -1.5° and -1.8° .

According to Wettstein (*Sitzungsb. d. Wiener Akad.*, 91, 33, 1885), the *conidia* of *Rhodomycetes Kochii* (a *fungus* parasitic on man) are, for a large part, incapable of germination after having been exposed for 2 hours to -7° .

Smart (1935) found that some species of the *fungi*, *Dematiium*, *Monilia*, *Oidium* and *Penicillium* failed to grow after one year at -8.9° . Other species of *Penicillium*, however, did grow after the same treatment.

According to Noack (1912), spores of *fungi* belonging to the genera, *Mucor*, *Thermoascus*, *Thermoidium*, *Thermomyces*, and *Actinomyces*, suspended in hay decoction in test tubes, allowed to remain for several hours at room temperature, and then exposed to -20° for 13 hours, did not lose their germinating power.

Teodoresco (1906) subjected the *zoospores* of *Dunaliella* (a *Volvocinea*) to temperatures down to -32° , in concentrated salt solutions (in which they are known to thrive). Some of these organisms survived freezing at -28° to -29° for one hour. However, since many of them were killed by this treatment, Teodoresco thinks that only those *zoospores* survived that were able to find interstices between the crystals of the hard-frozen mass.

Irmscher (1912) exposed water suspensions of *moss spores* belonging to 4 genera to -20° to -21° , for 16 hours. All of them germinated normally. In a second experiment the spores of the same mosses were exposed, under the same conditions, for 18 hours to -32° to -40° , after which no germination occurred.

Chodat (1896) reported that spores of the mould, *Mucor*, spread on agar or suspended in liquid Raulin's medium and put for 2 hours in a freezing chamber at -70° to -110° , germinated, but with a considerable delay over the controls. The filaments originating from the treated spores were 1 cm. long when those from the controls were 2 to 3 cm.

Alexopoulos and Drummond (1934) exposed to the action of liquid air for 1 hour the spores of the fungi, *Melanconium*, *Coniothyrium*, *Eurotium*, and *Cytospora*, either suspended in sterile water or inoculated on corn meal agar slants (in both instances they were contained in test tubes) or dried on a glass rod. In all cases, germination and normal growth followed.

According to Kadisch (1931), the spores of some 10 species of pathogenic filamentous fungi (*Epidermophytes*) could resist -20° to -30° for at least 34 days. In liquid air, some species were killed in one hour, others in 24 hours, still others in 48 hours, while some were not at all affected. Further experiments of Kadisch showed that two species (called the *Ach. Gyps.* and *Kaufmann Wolf's fungus*) survived after respectively 3 and 50 hours at -252° and were not killed either by a stay of 2 hours at -268° followed by 4 hours at -268.8° and $1\frac{1}{2}$ hours at -272° .

Becquerel made a series of observations on the germination of various spores and of pollen after exposure to the temperature of liquid gases. His results are summarized in the following paragraphs.

Spores of 4 kinds of *phycomycetes* (*Mucor*, *Rhizopus*, *Sterigmatocystis* and *Aspergillus*), thoroughly dried and sealed in tubes where the vacuum was made to 10^{-4} cm. of mercury, were immersed in liquid hydrogen for 77 hours. They were then left 2 years in the vacuum. Germination was normal (Becquerel, 1910).

Spores of 6 genera of mosses (*Dicranella*, *Atrichum*, *Hypnum*, *Leucobryum*, *Funaria* and *Brachythecium*), not specially dried, were immersed for 10 days in liquid nitro-

gen, directly in contact with the fluid. Nothing abnormal was observed in their germination. In another experiment, spores of 2 of the above genera, previously desiccated and maintained in a vacuum of 10^{-6} mm. of mercury, were immersed in liquid helium at 4° K. for 9 hours and at 1.84° K. for 1 hour. No injurious effects resulted (Becquerel, 1932a).

The spores of the fern, *Polystichum filix mas*, previously desiccated, sealed in the highest obtainable vacuum and maintained for 6 hours in liquid helium between 5° and 3° K. and for 5 hours at 3° K., germinated like the controls (Becquerel, 1930).

Pollen grains of *Antirrhinum* and *Nicotiana*, thoroughly dried, in sealed tubes, in a vacuum of 10^{-5} mm. of mercury, were put in liquid helium for 7 hours, the lowest temperature reached being 1.3° K.; this did not weaken the germinating power of the grains (Becquerel, 1929).

4. *Seeds.* Seeds have been a subject of predilection for investigators of the effects of low temperatures.

Edwards and Colin (1834) subjected seeds of *barley*, *oats* and *wheat* for 15 minutes to -40° (by the evaporation of sulphur dioxide in the vacuum) and noticed no impairment in their germinating power.

Wartman (1860 and 1884) exposed 9 species of seeds, from 20 minutes to 2 hours, to temperatures from -78° to -110° (ether and carbon dioxide) and observed no injurious effect in them. The seeds were enclosed in sealed tubes.

De Candolle and Pietet (1879) subjected various seeds to temperatures between -40° and -50° for about 4 hours, and in another experiment, to -80° for more than one hour; in no case did they observe any action of the cold on germination. In a later experiment (1884), the same authors subjected seeds of 8 species, dried in the air and enclosed in sealed tubes, to a temperature lower than -100° (by evaporation of carbon dioxide) and they extended the time to 4 days. This prolonged action of low temperature had no effect on the seeds.

De Candolle (1895) investigated the action of repeated freezing on seeds not previously subjected to a special desiccation. The seeds, wrapped in tin foil and enclosed in a sealed metal box, were exposed 118 times to a temperature lower than -100° , the duration of each exposure varying from 8 to 20 hours. No harm was done to *wheat*, *oats*, and *fennel* by such treatment, but most of the *lobelia* seeds did not germinate.

Pietet (1893) mentions exposure of seeds to -200° without injury.

According to Brown and Escombe (1897), seeds of 12 kinds from 8 families, dried in the air, that is, still containing 10 to 12% of water, were enclosed in thin glass tubes and put in liquid air for 110 hours. Neither their power of germination nor their growth was impaired in any way.

In 1899 Moissan presented to the French Academy a note received from Dewar where the latter stated that he had succeeded in solidifying hydrogen and that seeds cooled to the temperature of liquid hydrogen did not lose their germinating power. (*C. r. Ac. Sc.*, 129, 434, 1899).

Thiselton-Dyer (1899) subjected to the temperature of liquid hydrogen various seeds chosen for their diversified nature (farinaceous or oily), for their various sizes and shapes (ellipsoidal, flattened, etc.). The seeds, simply air dried, enclosed in sealed tubes and cooled first in liquid air, were left half an hour in liquid hydrogen (more than 1 hour it is said elsewhere in the paper). No change was noticed and germination was normal. In a second series of experiments, the seeds were immersed for 6 hours in liquid hydrogen with which they were directly in contact. No attempt was made this time to graduate the cooling. All the seeds germinated in the normal manner.

Adams (1905) put in liquid air, for about 24 hours, 6 sorts of seeds, some of which were dry, while the others had been buried in the soil for 3 days so as to absorb water. All the dry seeds germinated while none of the moist ones did, except timothy which is known to imbibe water with

difficulty. The author states that seeds should contain more than 12% water to be killed by the temperature of liquid air.

Beequerel (1905) showed that the higher the degree of desiccation the more resistant are the seeds to the temperatures of the liquid gases. In a first series of experiments he soaked decorticated seeds in water for 12 hours and immersed them in liquid air; the seeds did not survive the treatment. On seeds dried in the vacuum at 40° for a month and immersed for 132 hours in liquid air he did not observe any injury. But, out of 16 kinds of seeds, simply dried in the air, 6 only survived the immersion in liquid air and these were the species which contained less water (6 to 12%). In later experiments (1909), in which Beequerel intended to bring the seeds into a state of complete inactivity, he desiccated them in the presence of barium oxide for 2 weeks at 35°, then put them in sealed tubes evacuated to 10^{-4} mm. of mercury, and immersed the tubes in liquid air where they stayed 3 weeks and in liquid hydrogen where they stayed 77 hours and, finally, he maintained them sealed for 1 year. When he opened the tubes, the seeds germinated in the usual manner. In a last experiment (1925), he used liquid helium. Seeds desiccated as described above, put in a vacuum of 10^{-6} mm. of mercury and kept immersed in liquid helium at 3.8° K. for 10½ hours germinated like the untreated controls.

Lipman and Lewis (1934) investigated the action of a prolonged exposure of seeds to liquid air temperatures. Nineteen sorts of seeds, previously dried over calcium chloride and enclosed in glass tubes, were left in liquid air for 30 days, in a first experiment, and for 60 days, in another. Neither the germinating power of the seeds, nor their growth, nor any of the characters of the plants grown from them, differed from the controls.

Lipman (1936a) immersed in liquid helium 9 kinds of seeds previously desiccated for 2 weeks over sulphuric acid in a partial vacuum and contained in glass tubes 1 cm. wide. The temperature was 4.2° K. for about 40 hours,

1.35° K. for 2 hours and some intermediate temperature for another 2 hours. He obtained 1.35° K. by application of vacuum to liquid helium. The treated seeds grew like the controls. The author gives photographs of the plants obtained with the exposed seeds and he announces that he intends to study the possible delayed effect of cold on the next generations.

To summarize: 1. The spermatozoa, even those of the homoiotherms, were found to resist sub-freezing temperatures; they might well belong to the type of organisms which, like the bacteria and some smaller flagellates, resist the lowest temperatures, but we did not find enough evidence in the literature to substantiate this view; 2. The eggs of higher animals (birds, amphibia) were, in general, found to be killed after having been exposed to a few degrees below zero; the eggs of lower animals (insects, parasite worms) often resisted -30 to -40° ; 3. The few motile forms of spores investigated were rather sensitive (as were also the larger flagellates); 4. The spores of fungi, ferns and mosses, and the seeds resisted, after drying, and some of them even in the moist condition, the lowest available temperatures.

IV. ISOLATED CELLS AND TISSUES

SECTION I. PLANT MATERIAL

1. *Hair Cells*. Klemm (*Jahrb. f. wiss. Bot.*, 28, 641, 1895) exposed to low temperatures *Triana* and *Momordica* hair cells in a test tube immersed in a freezing mixture. The temperature was read on a thermometer placed in the tube with the material. After 15 minutes exposure to -13° , the cells were dead, the protoplasmic streaming had permanently ceased. After a shorter exposure or at higher temperatures (-6°), the streaming was often resumed; exposures to temperatures above -2° did not stop the streaming.

Külme (1864) found that if isolated staminal hairs of *Tradescantia*, placed in a drop of water on a slide, were

frozen on an ice-salt mixture, the protoplasm was completely destroyed and, after thawing, it broke up into crumby coagulated clumps. If the hairs were put, without the addition of water, into a thin platinum crucible and the latter lowered into the freezing mixture, the protoplasm could be kept alive for 5 minutes at -14° ; after thawing, the protoplasmic streaming was resumed. At 0° *Tradescantia* hairs could be kept alive for at least one hour.

Molisch (1897) who observed, under the microscope in a cold chamber, the freezing of *Tradescantia* hair mounted in water, described the cessation of cytoplasmic movement when the temperature reached -2° and the sudden congelation of the cell content at about -6.5° (temperature of the air within the freezing chest in which the material had been for several hours). After thawing, the nucleus was disorganized and the plasma coagulated. When the hair cells were exposed directly to the air temperature of the chamber, without water as a mounting medium, they withstood 6 hours at -5° to -9° . When they were mounted in oil, they did not freeze even at -9° ; congelation and death occurred only at -15° . In air and in oil the cells could be subcooled to a much lower temperature than in water, and they stayed alive as long as they were in the subcooled state. The *epidermal hairs* of *Episcia*, *Ageratum mexicanum* and *Pelargonium* had a higher resistance than *Tradescantia* hairs.

2. *Epidermal Tissue*. Molisch (1897) reported that in sections of the epidermis of *Tradescantia*, mounted in water between slide and cover-slip, and observed under the microscope while exposed in an air chamber to a temperature of -5° , many cells were seen to freeze in sudden flashes. On thawing, after 6 hours exposure, they were dead, as judged by the disorganized, shrunken, and sometimes stainable nuclei, by the coagulated cytoplasm and by the fact that the cell membranes absorbed the anthocyanin present. Some cells did not freeze during that time and at that temperature, which would indicate that -5° is within the range of the lethal temperatures for the epidermal cells.

Luyet and Gibbs (1937) repeated the experiments of Molisch, using the epidermis of the *onion*. They followed this author's procedure in all details except that their material was vitally stained with neutral red before being cooled, and that their low temperature chamber was set to about -10° . With the higher cooling velocity furnished by this lower temperature, all the cells were observed to freeze in 5 to 6 minutes. Sudden flashes during freezing indicated that the individual cells were subcooled and that they congealed at once. After thawing, they were all dead, as shown by the release of the stain.

Zacharowa (1926), in a study of the effect of hydrogen ion concentration on cold resistance, put to freeze epidermal sections of *red cabbage* in test tubes immersed in freezing mixtures and containing a little quantity of either distilled water or a weak solution of sodium hydroxide (0.01 to 0.001 N) or sulphuric acid (0.0001 to 0.00001 N). The material, seeded with an ice particle, was left to freeze and cool for one hour. It was then thawed and its vitality was tested by plasmolysis. Half of the cells were found alive after exposure to -3.9° and one-fourth after exposure to -5.75° , in distilled water. The sections in the sodium hydroxide solution were slightly more resistant, and the sections in the sulphuric acid solution less resistant than those in distilled water.

Chambers and Hale (1932) followed under the microscope the gradual freezing of single epidermal cells in precisely known temperature conditions. Epidermal strips of the red *onion* were placed, together with the tip of a thermo-needle, in a hanging drop of liquid paraffin and the temperature of the whole system was slowly lowered, in a cooling room, while inoculation with an ice-tipped pipette prevented subcooling and subsequent too rapid freezing. When the temperature reached -7° to -10° , ice was formed between the cellulose wall and the protoplast but the latter was usually still capable of plasmolysis. At -10° , the cytoplasm and nucleus disintegrated, but the tonoplast still resisted. These phenomena

seem to indicate that the lowest limit of non-lethal temperature, under these conditions, is in the neighborhood of -10° .

Becquerel (1937) immersed in a liquid nitrogen bath *onion* epidermises mounted on thin glass slides and he left them at the temperature of that bath for 10 minutes, 24 hours, or 3 weeks. The cell contents, observed with the microscope while they were in the liquid gas, were congealed and, after thawing, coagulation could be observed. Death had always set in.

Luyet and Thoemes, in an attempt to test a theory according to which, in a very rapid cooling, the protoplasm would vitrify, that is, solidify without crystallizing, succeeded in maintaining alive *onion* epidermises after immersion in liquid air. The material, vitally stained with neutral red, was partially dehydrated by a rapid plasmolysis in a sodium chloride solution; it was then immersed at once in liquid air and brought back into the plasmolysing agent. After this treatment, the majority of the cells held the vital stain and could be plasmolysed to a further degree or deplasmolysed. (Paper in press; Abstract presented at the Indianapolis meeting of the Am. Soc. of Plant Physiol., Dec. 1937.)

Stomatal cells, which are known to be resistant to a number of injurious agents, such as heat, drought, decay, etc., have been found by several investigators to be also exceptionally resistant to cold. Molisch (1897) put leaves of different kinds (*Primula*, *Nicotiana*, *Campanula*, *Hyanthis*, *Episcia*, *Cyclamen*) in test tubes in which he had a thermometer and he immersed the tubes in cold mixtures. After two hours, when the temperature between the leaves was -7.5° , a plasmolysis test with 10% NaCl revealed that the stomatal cells were alive while all or almost all of the neighboring cells were killed. In *Piper*, treated in a similar manner, the stomatal cells were also killed at that temperature. In *Maranta*, they were killed by an exposure of one hour to -6° . In *Dahlia*, all the cells except those of the stomata were killed after exposure

to -3° , and in *Pelargonium*, after exposure to -6° . In *Nicotiana*, one hour at -12° did not kill the stomatal cells; they died only when brought to -15° to -17° . In leaves which die by exposure to temperatures of $+2^{\circ}$ to $+3^{\circ}$, the stomatal cells were also observed to be alive after the other epidermal cells had been killed.

3. *Tuberous Tissue*. The potato tubers, on account of the large masses of homogeneous tissue that they present, were often used in investigations on the freezing point of living material and on the effects of low temperatures. The death point has been investigated, in particular, by the following authors.

Göppert (1830) reported that when the temperature in the interior of a potato (measured by inserting a thermometer in it) had dropped by only one degree below zero and all the sap had been converted into ice, the potato was dead after thawing and turned brown.

According to Müller-Thurgau (1886), potatoes exposed to an air temperature of -6° for a time long enough to bring the temperature in their center to -1.5° , were dead throughout after thawing. A thermometer was inserted in the middle of the potato. Death was diagnosed by the blackening and the soft consistency of the tissue.

Apelt (1909) who was asked by his master, Mez, to test a theory on the existence of a death temperature, specific for each organism and lying below the freezing point, found that each race of potato has its own death point, although the latter can be raised or lowered by the conditions of the previous storage. He determined the temperature within the material with a thermocouple and diagnosed the vitality of the cells by the plasmolysis method, with potassium nitrate solutions containing a few drops of methylene blue. The low temperatures were produced by ice-salt mixtures. The death points registered extended from -1.71° to -3.63° . They were lower than the freezing points by quantities varying from 0.10° to 1.17° . The "hundredth of a degree," always scrupulously given, and the idea of a death point determinable to

the third decimal place have amused some later investigators.

Maximow (1914) repeated the experiments of Apelt, also using plasmolysis as a criterion of vitality. He obtained the following results: 1. Potato tissue frozen to -2.11° (internal temperature) had all its cells alive after thawing; 2. The number of dead cells was quite considerable after freezing to -2.26° ; 3. The cells were practically all dead at -2.66° . The death temperatures would then lie in the neighborhood of -2° .

In other experiments, Maximow (1914) showed that the velocity of freezing was a factor to be considered in the problem of death temperatures. A piece of potato, frozen to -1.82° in 13 minutes, had almost all its cells alive; another, frozen to the same temperature in 3 hours 52 minutes, had most of its cells dead. Measuring then the amount of ice formed before death occurred he came to the conclusion that a slower freezing, in the second experiment, had caused more water to freeze in the tissue, and that death depends, in the last analysis, on the quantity of ice formed. These results fit with the notion of the death point, according to which death is conditioned by a number of variable factors and does not take place within sharply defined temperature limits.

Luyet and Condon subjected to slow freezing in an air chamber potato tissue cut into cylindrical shells having a wall thickness of 2 mm. and slipped around the bulb of a thermometer; they stopped the cooling process in successive experiments, after congelation had proceeded for various lengths of time and they counted the number of living cells, using the plasmolysis test combined with a vital staining method. They found that as long as the freezing curve stays at an approximately constant horizontal level, in general above -2° , the cells are practically all alive even after a freezing of 12 to 15 minutes. Death begins when the temperature drops below -2° , and, if cooling is continued at the same slow rate, the death of all the cells requires about 10 more minutes; the temperature,

when all the cells are killed, is then in the neighborhood of -5° . The lethal temperatures extend, therefore, from -2° to -5° . (Unpublished data; paper presented before the Indianapolis meeting of the Bot. Soc. of America, Dec. 1937.)

4. *Tissue from Leaves, Stems, Roots, etc.* In this section, we shall deal with plant organs rather than with plant tissues. In some cases the investigators, in their studies of organs, have endeavored to find which tissues were involved, but most of the time we have no such information.

The agricultural and horticultural literature is rich in observations made in the open on death temperatures of leaves, buds, flowers or stems of various species of plants. Tables of observational and also of experimental data have been published. The authors of most of these tables, however, intended primarily to furnish the agriculturists with instructions easy to follow, and they do not give such factors as the time during which a plant organ can be exposed to a given degree of cold or the internal temperature of the organ. Instead of quoting these survey tables, we think it, therefore, more useful for our purpose to describe some of the more significant experiments.

The leaves of some plants are killed by the action of temperatures above zero. Molisch (1897) exposed the isolated *leaves* of *Episcia* to temperatures of 2.5° to 4.4° . After 24 hours, several leaves became brown and spotted and after 4 days, they were all brown. The plasmolysis test revealed that the cells were dead. On another experiment, the leaves were put in ice water between 0° and 1° . In three hours, they exhibited brown spots and in 24 hours they were entirely brown.

Müller-Thurgau (1880), using as a death criterion the change in color that the petals of some orchids undergo during protoplasmic disintegration, put to freeze in a cooling chamber *petals* of *Phajus grandifolius* wrapped around the bulb of a thermometer. The temperature sank first to a subcooling point, then rose suddenly to the freezing point, stayed there for a short time and finally dropped

again slowly. The author observed that the white petals became blue when the temperature, in the last phase of the curve, began to fall below the freezing point. The latter was found to be -0.8° .

The same author reported that an *onion bulb*, exposed to an external temperature of -4° for 7 hours and hard frozen, was found alive. A thermometer previously inserted in the middle of the onion showed that the tissue had first been subcooled to -3.1° and that it then froze for 4 hours between -0.7° and -0.95° . According to the author's idea that death takes place when most of the water content is frozen, the death temperature would lie immediately below the freezing temperature registered.

Walter and Weismann (1936), in studies on the difference in the position of the freezing point in living and in dead tissue, observed that in the *roots* of *Daucus carota* frozen 3 times in succession (lowest temperature reached: -1.28°) more than half of the cells were alive. The temperature was read on a thermometer inserted in the material, and the loss of vitality was ascertained by the stainability of the tissue with eosin. In another series of 6 successive freezings of the same object, the lowest temperature reached having been -1.50° , a number of cells were still alive. But repeated freezing, even at temperatures becoming gradually higher in the successive experiments, resulted in an increased number of dead cells.

According to Apelt (1909), the death point of *potato shoots* lies between -2.16° and -2.74° . (For the details of the experimentation see, above, the description of Apelt's work on potato.)

Maximow (1914) found that in the *root* of the *red beet*, where death was diagnosed by the release of the pigment, the freezing point lay at about -2° , the death point at about -3° , while ice continued to form, after death, at lower temperatures. (The experimental procedure has been described under the heading "Tuberous Tissue.")

According to Harvey (1919), *leaves, petioles* or *stems* with waxy epidermal coverings resist lower temperatures

than the less protected plant organs; they can be more easily maintained in the subcooled state, the ice-inoculation being prevented by the epidermal coverings. Subcooling to 5 degrees below the freezing point was found not uncommon in these less exposed plants.

The cold resistance of growing *rye seedlings* was investigated by Zacharowa (1926). She exposed them, when the rootlets were 1.5 to 2 cm. long, to various degrees of cold in a freezing chamber, and occasionally determined the internal temperature with a thermocouple. As a criterion of death, the change in coloration and the facility of the subsequent drying were used. The results were that: 1. An air temperature of -2.9° for several hours did not injure the roots; 2. A similar exposure to -2.9° to -3.9° resulted in the death of the cortex and the root hairs; 3. An exposure to -5.75 for 1 hour killed the entire root except 1 to 1.5 mm. at the tip; 4. The meristem of the tip died after an exposure of 1 hour to -7.8° , after which time the interior of the root had reached that temperature; 5. Without ice formation, exposures to -11.1° were harmless. Similar experiments on seedlings of wheat, pea, corn and buckwheat showed a decreasing resistance in the order given, the meristematic tip of the buckwheat rootlets being killed at -2.9° .

We have mentioned above Mez' theory (1905) according to which death occurs at a minimal temperature specific for each plant or plant organ. Mez contended also that congelation protects the tissue against death by releasing heat and delaying the further drop of temperature. His disciple, Voigtländer (1909), claims to have confirmed the theory in a variety of plant tissues. The specific minimal temperatures that he obtained are in general low, some of them being about 10 degrees below the freezing point. The extreme cooling rapidity that he used in his experiments (often 10 to 15 degrees per minute at 0°) makes his results the most doubtful, since it has subsequently been shown that the time the material takes to freeze or the time that it stays at a given sub-freezing temperature is to be considered in the damage caused.

The abundant literature on winter hardiness, which we do not treat in detail, is sometimes summarized in the statement that, in *juicy plant tissues*, death occurs when the temperature drops a few degrees below zero, while in *conifers* and several other *evergreens* the death temperatures are lower by some 10, 20 or more degrees. Some investigations, however, like the following one by Winkler seem to indicate that the problem is not so simple, there being important seasonal variations in the same species.

Winkler (1913) experimented on the cold resistance of *buds, leaves, cambium, cortex* and *wood* of branches, 5 to 6 years old, of numerous species of *trees*. The branches were cut into pieces 20 to 25 cm. long, split longitudinally, and placed upright in a brass cylinder, with their lower ends immersed in tap water in a glass dish set into the bottom of the cylinder. The brass container was lowered into an ice-salt bath and the temperatures recorded by a thermometer suspended in the air between the twigs. Subcooling was avoided by placing a few ice crystals in the water in the dish. After having been exposed for 12 hours, the material was thawed and its vitality investigated by plasmolysis. The results were as follows: 1. In experiments made in the Spring, the buds of 28 species of deciduous trees, exposed to -3° , were found uninjured, only those of *Quercus pedunculata* having been killed at that temperature; at -5° , all the buds were killed. Similarly, the leaves or needles of 13 out of 14 species of evergreens were killed at approximately -4° . The cambium, cortex, and wood of all the trees experimented upon survived exposure to -10° , but were all killed after 12 hours at -18° . 2. In the Summer, the cold resistance of deciduous trees drops considerably, the death point of the cambium, cortex and wood lying at about -9° , while the resistance of the same tissues in the evergreens undergoes little change. 3. In the Fall, the resistance is on the upgrade again, and it was found that in November, all the buds of both deciduous and evergreen trees withstood -17° , while those of 17 out of 29 deciduous trees, and 5

out of 14 evergreens were not killed at -19° . 4. The maximum resistance is reached in January, at which time the buds of all the evergreens (14 species) and of 27 out of 29 species of deciduous trees were not killed at -20° ; most of them survived -22° as well. In none of the species were the cambium, cortex and wood injured at this time by -20° or -22° . 5. Branches, whose death point had been determined as about -21° , were frozen repeatedly at -13° , and, after each freezing, they were kept for 24 hours under a bell jar at 18° . Most of the trees were killed by a 6-times repeated freezing at -13° ; the leaves and needles of the evergreens were, in general, found more resistant.

While, as we have said above, the cells of juicy leaves are generally thought to be killed when they are subjected to temperatures of a few degrees below zero, Iljin (1933) showed that sections of *red cabbage leaves* could be maintained alive after freezing at much lower temperatures if the withdrawal and the reabsorption of water, concomitant respectively with freezing and thawing, were gradual. According to him, death results usually from a tearing of the protoplast in a too rapid plasmolysis during freezing or mostly in a too rapid invasion of the cell by water when the ice melts. Freezing slowly and thawing in hypertonic solutions would prevent such injury and maintain the cells alive. So, he cooled the material by steps, letting it, for example, one day at about -5° , one day at -8° , one day at -11° , etc.; then he warmed it to about -5° and immersed it in cooled salt solutions which were approximately isotonic with the frozen cell content; he then let the tissue thaw slowly in that solution. Proceeding in this manner, he obtained living cells (capable of plasmolysis) after an exposure of $2\frac{1}{2}$ hours to -80° and after longer exposure to the higher temperatures of -15.8° , -11.3° , etc. If the observations of Iljin, which favor the old theory that death is due primarily to a too rapid thawing and not to congelation or to an injury at some given low temperature, are confirmed, the classical notion of death

temperature must be entirely modified. Which is the lowest temperature reached, how long the material has been at that temperature, and even how much ice has been formed, become factors of secondary importance if death results from a mechanical injury during thawing.

Becquerel (1932b) investigated the action of the temperature of liquid gases on the *tuberous roots* of some *Ranunculaceae*. In preliminary experiments he found that the roots can be dried in the air for 6 months or in a vacuum for 2 months without losing their ability to produce new sprouts when remoistened. The roots, dried in the air and in a vacuum, withstood an immersion of 18 days in liquid nitrogen; the roots, dried and subsequently soaked so as to re-imbibe respectively 45, 98 and 160 per cent of their previous water-content, died.

Lipman (1936b) immersed in liquid air for 50 hours sealed tubes containing *protonemata* of 8 genera of *mosses*, previously dried in a vacuum over sulphuric acid. No injury resulted from the treatment, growth was normal and a microscopic examination revealed healthy cells, and chloroplasts in a good condition. The author published photographs of the plants grown from the exposed protonemata.

According to Becquerel (1932c), *sprouting seeds of wheat, rye, lucern and Helianthus*, which can be dried in a vacuum and revived, do not suffer from exposure in the dry state to any low temperature. The material, consisting of seeds with radicles no more than 10 mm. in length, was first air-dried; then it was desiccated in a vacuum of 10^{-4} mm. of mercury in presence of barium oxide; finally, it was put for 18 days in liquid nitrogen. Most of the rootlets could be revived after this treatment. Some specimens were immersed in liquid helium for 9 hours and stayed one hour at 1.84° K. in a vacuum of 10^{-5} mm. of mercury, without suffering any injury.

SECTION II. ANIMAL MATERIAL

1. *Blood.* To study the action of low temperatures on *white blood corpuscles*, Schenk (1870) placed the blood of

tritons, frogs or turtles in a shallow glass and set this on a freezing mixture; the temperature was recorded by a thermometer immersed in the blood. The white cells cooled to 0° , -3° , -5° , or -7° for a short time, retained their vitality and resumed amoeboid movement when the temperature was again raised. If the blood was kept for several hours at low temperature, movement was only seldom resumed; thus, a specimen kept for 8 hours at -2° to -3° , did not resume its activity. The white blood cells of the warm-blooded animals, especially of the rabbit, retained their vital properties after exposure to -3° , only if the time of exposure was not more than 10 to 15 minutes. In Schenk's experiments the blood corpuscles were apparently never congealed.

Pouchet (1866) reported that the *red blood cells* were considerably altered by congelation. *Frog's* blood was allowed to drop into a dish cooled to -15° . After thawing, the nuclei were found free in the plasma. In other experiments, he froze entire animals such as *frogs, toads, an eel, a young cat*, etc., and observed that the blood corpuscles were crenated and reduced in size. From these observations he developed the theory that the damage done in the body of an animal by freezing was in direct ratio to the number of blood cells injured.

2. *Embryonic Tissue.* Simonin (1931) subjected fragments of embryonic tissues of *mouse, rat or ox*, to temperatures from 0° to -15° , in boxes immersed in a freezing mixture. He could obtain some growth in culturing these tissues after they had been at 0° for 20 days or at -5° for 5 days, provided no ice had been formed in them; -15° was always lethal in a short time. *Nerve cells* and *liver tissue* were most sensitive, while *heart, lung, and intestine* tissue showed a greater cold resistance.

Gaylord (1908) observed that growing *epithelium* from young *mice* embryos was killed after immersion in liquid air.

The embryos of the birds were always found more resistant to low temperatures than the adults. But almost

nothing is known on the time at which the resistance drops. Moran (1925) remarks in a general manner that partially incubated eggs were less resistant than fresh eggs to low-temperature storage. According to Edwards (*J. Physiol.*, 6, 351, 1902), *chick embryos* responded like cold-blooded animals to temperature changes during the first 20 days of incubation. This statement would indicate a very late change in resistance.

On the more fundamental problem of the mechanism of the decrease in cold resistance during incubation, as related to the mechanism of the passage from the poikilotherm to the homoiotherm state, still less is known.

3. *Ciliated Epithelium.* According to Schiff ("Lehrb. d. Physiol. d. Menschen," 1, 12, 1858), a ciliated *frog* epithelium, immersed for several hours in iodine serum at a few degrees above zero and brought into a warm room for observation, presented a complete absence of movement; but, with rising temperature, a slight motion began which was soon followed by an intense ciliary activity. This experiment could be carried out repeatedly on the same preparation.

Roth (*Arch. f. path. Anat. u. Physiol.*, 37, 188, 1866) made more accurate determinations of the temperature of cessation of ciliary motion in *Anodonta*. The experimental object, with a thermometer and enough water to cover the bulb of the thermometer, was placed into a reagent glass, and this was set into an ice-salt mixture. Recovery was found possible after congelation at -3° to -4° , when this temperature was not maintained for more than a few minutes. At -6° , death always set in. Temperatures above zero, when they acted long enough, produced a transient cessation of motion; this happened, for example, at 0.4° after 6 minutes. By freezing and thawing, the endosmotic relations of the ciliated cells were extraordinarily changed; the movement, temporarily resumed, was soon definitely lost, and the cells swelled up; very often the cuticle, with the cilia attached to it, separated from whole cell rows and sometimes it rolled up

into a tiny cylinder which, with the protruding cilia, looked like a fox-tail.

According to Pietet (1893) a ciliated epithelium from the *frog's* mouth, frozen hard by exposure to a temperature of -90° , resumed its beat after thawing, but exposures to temperatures lower than -90° killed the tissue.

4. *Muscular Tissue.* In describing the investigations on the muscles and on the heart we shall follow the chronological order.

Kühne (1864) reported that *frog* muscles, frozen at -7° to -10° for 3 hours, were irritable after thawing, and that the irritability was observed to last for 6 hours at 15° .

Jensen and Fischer (1909) demonstrated the fundamental fact, often confirmed by later investigators, that some water can be congealed in the muscle without injuring the latter, but that the solidification of more water induces death. The gastrocnemius of *Rana esculenta* was cooled to its lowest subcooling point (about -9°), then it was left to freeze till past the end of the horizontal portion of the freezing curve, or to various points in the subsequent drop of the curve. The cooling process was then interrupted and the reactivity of the muscle tested. The temperature was indicated by a thermocouple. Cooling to about -1° and solidification of the greater part of the free water produced no serious injury. With further cooling, the reactivity and conductivity of the muscle decreased rapidly and disappeared at about -3° . Since, in this temperature interval, the more firmly bound water began to freeze out, the death of the muscle, it is suggested, might be correlated with this process.

Brunow (1912) determined the death temperature of the isolated *frog's* muscle in the following manner. He drew the gastrocnemius of *Rana fusca* firmly into a narrow glass tube, centered this in a second wider tube, letting an air space between them, and immersed the whole into an ice-salt mixture, the temperature of which was kept constant to within 1° . The temperature of the muscle was determined by means of a thermo-needle in-

serted into the middle of it. Isolated muscles thus treated could be frozen and subjected to a temperature of -2.9° without losing their irritability completely. The death point would then lie at about -3° .

In another series of experiments by the same author, determinations were made on the *frog* muscles *in situ*, for which purpose the frog's leg was pulled through a small glass tube, as before, the frog being meanwhile fastened to prevent movement. The muscle, not cut off from the circulation by any ligature, was still irritable after having been frozen to -4.06° . Its death point is given as lying at about -4.1° to -4.2° .

Moran (1929) exposed to freezing temperatures excised *frog*'s muscles (sartorius and gastrocnemius) in constant temperature rooms and, after freezing and thawing, he tested the vitality of these muscles by observing their response to an electric stimulus, their osmotic properties and also their electric resistance. Cooling was very slow, a long time being allowed for the establishment of the temperature equilibrium. In a previous determination, the freezing point of the material was found to be -0.42° . Moran's results can be summarized in the following points: 1. The muscles brought to temperatures below -2° were killed; 2. The muscles frozen hard but exposed to temperatures above -2° for less than a day could be revived after thawing; 3. The muscles left for a long time in the frozen condition above the critical temperature of -2° were killed; the lethal time of exposure was about 7 days at -0.9° , 4 days at -1.5° and 20 hours at -2° . In his attempts at rapid freezing, by immersion in liquid air for example, Moran could never obtain revival. He thinks that at the critical point an irreversible change takes place in an inappreciable time.

Chambers and Hale (1932) reinvestigated the problem of the death temperature of isolated muscles, using some of the methods that Chambers had developed for micro-manipulation. Portions of muscle fibers, 3-5 mm. long, from the sartorius of the *frog* were placed in a hanging

drop of liquid paraffin. The coverslip with the hanging drop was mounted on a chamber on the stage of the microscope, and the temperature ascertained with a thermocouple inserted into the drop near the tissue. It was found that muscle fibers could be subcooled at -6.5° for 2 hours or at -15° for 3 hours without losing their power of contraction. Surface freezing, induced by touching the fiber with an ice-tipped pipette, occurred at -1.2° , and after a stay of about 15 hours at -1.2° to -1.3° , a slight contraction could still be produced. Internal freezing occurred at -1.6° , but fibers in which such freezing was complete were always found to be dead on thawing.

Heubel (1889) studied the effect of freezing on the *frog's heart in situ*, as follows. He directed a stream of ether from an atomizer upon the exposed, ligated and therefore relatively blood-free heart, thus freezing it hard through and through. The operation lasted 20 to 30 seconds. When the ligature was removed and the blood forced into the heart, it soon started to beat and, shortly after, it functioned normally again. Hearts subjected to the cooling action of the ether for more than 1 minute could never be revived, nor could hearts which were exposed for 10 or for 17 minutes (1 experiment in each case) to an air-temperature of -18° .

According to Cameron and Brownlee (1913), excised *frog's hearts*, placed in test tubes immersed in freezing solutions, recovered after having been frozen at temperatures of -2.5° to -3° for two hours, but not after treatment at lower temperatures. Recovery consisted in a reestablishment of the beat after it had stopped. These authors made about 25 determinations of death temperatures on excised hearts.

Britton (1924) mentions a *skate* exposed for 16 hours to -20° and solidly frozen, the *heart* of which resumed its beat after thawing, though the fish did not recover. The author states that the heart of the skate can be solidly frozen for several hours and recover.

5. *Nerve Tissue.* Bühler (1905) observed that, when the temperature of an isolated *frog's nerve* (Ischial of

Rana temporaria and *Rana viridis*) was lowered slowly, the stimulus conduction dropped imperceptibly down to a certain point (a phenomenon not due to cold), then it sank suddenly to a very small value, and finally it dropped slowly again to complete disappearance. The "critical temperature" at which the sudden drop occurred was found to be between -2° and -10° . With a thermocouple inserted in the nerve it was possible to observe a remarkable temperature rise coincident with the decrease in conduction. Evidently the tissue was then freezing. Upon warming, a slight conduction reappeared sometimes, either below or above the freezing point; the original conduction, however, was never reached again; and some nerves did not recover at all. When there was a return of conduction, it took place between -11° and 7° . With a too sudden cooling, convulsive twitches, due to stimulation by cold, disturbed the experiments.

In 1932, Bahrman repeated Bühler's experiments on the same material, but he determined the electrical conductivity of the nerve simultaneously with the stimulus conduction at decreasing temperatures. He found that there was a gradual increase in resistance and decrease in stimulus conduction as the temperature was slowly lowered down to about -6.4° to -7° , at which temperature there occurred abruptly a large rise in resistance and simultaneously an abrupt drop in stimulus conduction, followed by the absolute cessation of the latter. Thawing, as judged by the change in resistance, occurred between -1.5° and 0° . Upon warming, stimulus conduction was never resumed below -1.8° and often it was not resumed at all. As a whole, Bahrman's experiments would show that death or serious injury results from freezing after a subcooling of about 6° .

Summary: 1. Most of the tissues studied in this section: plant epidermises, juicy tissue from leaves, stems or roots, embryonic animal tissues, ciliated epithelium, muscular tissue and apparently also nerve tissue, are

killed by frost at a few degrees below zero. Usually some ice can be formed in them but the solidification of a larger proportion of their water becomes lethal. 2. Experiments on plasmolysed material seem to indicate that some tissues can acquire an exceptionally high resistance by partial dehydration. 3. Considerable seasonal changes in cold hardiness are reported which might be due primarily to a natural dehydration and rehydration. 4. The tissues which can be dried withstand any low temperature.

V. METAPHYTA

1. *Fungi*. We have already treated the action of cold on the spores of fungi; we shall deal here with the vegetative forms. But, in most of the investigations to be summarized in this section, the authors did not attempt to exclude entirely the presence of spores from their cultures and in many instances the resistance attributed to the mycelium should probably be ascribed to remaining spores. When, in a research, it was evident that the material experimented upon contained spores and that the resistance observed was due to the latter, we excluded such a research from this section and treated it in the section "Spores" even if the author did not present it under that heading.

Growth of fungi at and below zero has been reported by several investigators. But, inasmuch as this phase of the problem is not directly related to death temperatures, we shall give here only a few typical instances (the next 6 paragraphs), most of which are taken from a review of the subject by Berry and Magoon (1934), to which we refer the reader for further information.

Schmidt-Nielsen (1902) reported the growth of several species of *Actinomyces* at 0°.

According to Horowitz-Wlassowa and Grinberg (1933), some fungi of the genera *Mucor*, *Penicillium* and *Cladosporium* can grow and multiply at -3°.

Brooks and Hansford (*Food Inv. Bd.*, Spec. Rpt. No. 17, 1923) found that *Cladosporium* not only grew, but also developed fresh spores on meat at about -7.8°.

According to Smart (1935), who exposed some species of *Penicillium* cultured on agar slants, to -8.9° , a slight growth could be observed at that temperature.

Bidault (*C. r. Soc. Biol.*, 85, 1017, 1921) observed growth in *Penicillium*, *Cladosporium* and *Botrytis* between 0° and -6° , and in *Choetostylum* and *Hormodendron* at -10° .

Haines (1930) found that *Sporotrichum carnis* grew at -5° to -7° , the lower limit of growth of this fungus on supercooled Czapek's agar being near -10° .

Noack (1912) studied the cold resistance of thermophilic fungi belonging to the genera *Mucor*, *Thermoascus*, *Anixia*, *Thermoidium*, *Thermomyces* and *Actinomyces*. Spores suspended in hanging drops were germinated in a thermostat, and when the germinating filament had reached a length equal to $\frac{1}{2}$ to 10 times the diameter of the spores, the cultures were exposed for 4 days to 5° to 6° . After this treatment, granulations could be seen in the filaments, and no further growth took place when the cultures were put back into the thermostat. Vegetative colonies of these fungi, either in fluid or on solid nutrient media were also killed after an exposure of 2 to 6 days to the temperature mentioned. In general the cold resistance was found to be, to a large extent, independent of the previous culture conditions, and it could not be raised by an increased concentration of the medium.

Kühne (1864) observed that when the myxomycete *Aethalium septicum*, in the active, moving state, was cooled by immersion of its container in ice water, it became motionless; its contours presented many amoeba-like protrusions, which, during the gradual rewarming, constricted off as shiny spheres. The rest of the myxomycete (apparently the uninjured central portion) resumed its normal activity. However, when *Aethalium* and *Didymium* were exposed to the lower temperature of a freezing ice-salt mixture, they were killed.

Lindner (1915) subjected the submerged mycelia and air hyphae of *Aspergillus niger* and *Penicillium glaucum*, cultured on 3 per cent gelatin, to temperatures of -10° to

-13° for varying lengths of time. After three hours of freezing, 95 per cent of the cells were killed in the submerged mycelia of a 24-hour-old culture, and after 12 hours all the cells were killed. Death was determined by the inability to grow, which was observed to correspond with the inability to plasmolyse. In a 48-hour-old culture, 90 per cent of the cells were destroyed after 4½ hours and all were dead after 24 hours. So, the older cultures had a higher resistance. When the submerged mycelia of a 48-hour-old culture were kept subcooled at -13° for 8 hours, only a few cells were killed; after 24 hours of subcooling many of the cells were dead, but a few older cells still survived. The air hyphae showed no visible signs of injury after 4½ hours at -11°, while, after 7½ hours at -13°, disorganization occurred in the basal cells of the hyphae, and after 24 hours all the air hyphae were dead. According to Lindner, the duration of the exposure is an important factor in causing death.

Molisch (1897) observed that *Phycomyces nitens* growing on bread and exposed over night to a temperature which reached -9°, presented the next day filaments which were turgescient and growing. Hyphae of the same species, mounted without water between slide and coverslip and observed under the microscope during an exposure of 8 hours to -10° to -12°, showed no stiffening of the cellular fluids. When the temperature was lowered to -17°, ice crystals separated from the protoplasm, disaggregating the latter.

Rumbold (*Naturw. Ztschr. f. Forst. u. Landw.*, 6, 110, 1908) found that the mycelia of *Coniophora* and of *Meruleus*, in gelatine cultures, did not survive being hard frozen at -6° for 12 hours, while the gemmae of *Coniophora* were not killed when kept over night in the open at about -20°, even though their aqueous medium was hard frozen.

Bartetzko (1910) subjected germinating spores, with mycelia 70 to 200 micra long, of *Aspergillus*, *Penicillium*, *Botrytis* and *Phycomyces* to low temperatures in liquid

nutrient media, in test tubes. None of the moulds were injured by a 2-hour subcooling at -14° . *Aspergillus* survived subcooling for 4 days at -6° to -11° . Increased concentration of the medium resulted in increased cold resistance of the suspended fungi. In the frozen condition, in 1 per cent glucose solution, *Aspergillus* was killed after 2 hours at -12° , while in 50 per cent glucose, no injury was apparent after 2 hours at -26° . The other three fungi gave similar results, though their resistance was not quite so high. In general, the resistance of the hyphae increased with age.

Bartram (1916) exposed a number of fungi, cultivated on agar slants of varying composition, to the cold of winter for 4 months during which time a minimum temperature of -29° was reached. All the *Sclerotinia*, *Cephalothecium*, *Glomerella*, *Venturia*, and *Ascophyta* survived, while *Alternaria*, *Cylindrosporium*, *Plowrightia* and *Phytophthora* survived on some media but not on others. *Sphaeropsis* and *Fusarium* were killed.

According to Richter (1910), the mycelia of *Aspergillus niger*, cultivated in liquid nutrient media, survived a 24-hour exposure to -10° to -13° . In another series of experiments, he exposed the material to -12° for two days, then allowed it to grow for 3 days at $+30^{\circ}$, thereupon exposed it to about -80° for 12 hours, again kept it at $+30^{\circ}$ for 24 hours, and finally exposed it over night to -11° and let it thaw rapidly. No injurious effect of the treatment could be observed.

Chodat (1896) exposed for 4 hours to temperatures of -70° to -110° *Mucor* mycelium growing on agar or on liquid medium. After thawing, the filaments seemed to be dead, but some growth originated from them later. A part of the mycelium which was entirely formed within the liquid medium, showed, after thawing, a tendency to roll into a ball as dead mycelium does, but, after a few days, new growth originated from it.

Heldmaier (*Ztschr. f. Bot.*, 22, 170, 1929) subjected the mycelia of *Schizopyllum* and *Collybia*, cultured on agar

slants in test tubes, to -5° to -7° for 6 weeks, or to -78° for 7 hours, or to -185° for $\frac{1}{2}$ hour. The plants were not killed.

Kärcher (1931) cultured several genera of fungi on agar slants in narrow test tubes, and, when the colonies had spread over about $\frac{1}{4}$ of the surface of the agar, she exposed them to varying degrees of cold. *Coprinus* was killed when the temperature of the agar was lowered to -40° , even when it was thereupon immediately thawed. *Lepiota*, *Boletus*, and the submerged mycelia of *Phycomyces* were similarly killed by a momentary exposure to -60° . The mycelia of the following survived a 1- to 8-day action of -70° as well as a 13-hour exposure to liquid air: *Collybia*, *Schizophyllum*, *Hypholoma*, *Clitocybe*, *Placodes*, *Armillaria*, *Xylaria*, *Aspergillus* (spore-bearing, and young spore-free mycelia) and *Phycomyces* (mycelia with sporangia).

Lipman (1937), considering that the fungi used by Kärcher might have formed spores (the same objection could be raised against most of the above described investigations), undertook a series of experiments in which he made sure by direct observation that the fungi experimented upon were in the actively growing state, that no spores were present at the moment of the treatment. Twelve species of fungi belonging to the genera, *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Absidia*, *Mortierella*, *Rhizoctonia*, *Armillaria*, *Trichoderma*, *Pythium* and *Fusarium*, were cultivated for 24 or 48 hours on synthetic agar-media or on potato-agar. The cultures, on slants in sealed test tubes, were immersed in liquid air for 48 hours. The previous cooling and the subsequent warming were gradual. In 14 cultures out of 26, and in 8 species out of 12, Lipman observed some growth, either in the tubes in which the fungi were treated or after a transfer to fresh media. A *Rhizopus*, a *Rhizoctonia*, an *Aspergillus* and an *Armillaria* were killed. A higher percentage survived when exposed to low temperatures at the age of 48 hours than when exposed at the age of 24 hours and the author

suggests that some change took place in the protoplasm by aging.

2. *Algae*. According to W. West and G. S. West, *Spirogyra cataeniformis* was in excellent vitality after having been imbedded in ice for 2 weeks while in the process of conjugation (*Ann. of Bot.*, 12, 33, 1898).

Cohn (1871) studied the vitality of *Nitella syncarpa* cooled on a freezing stage on the microscope. Branches of the alga were placed under a few mm. of water in a shallow glass which was exposed to -20° . A thermometer indicated the temperature of the water in the glass. Active protoplasmic streaming could be observed at 0° and slow movement could still be seen at -2° . After exposure to lower temperatures, the cells were frozen and killed, although, in 2 cases, cells taken from the ice at -3° were alive. Repeating these experiments with *Nitella* branches not surrounded with water, Cohn observed the protoplasmic streaming until the air temperature near the alga registered -2° . Ice formed in the cells between -3° and -4° and the protoplasts shrunk. After thawing, the cells were dead.

Molisch (1897) froze filaments of *Spirogyra* between slide and coverslip at -3° to -6° and found, after thawing, disorganized chloroplasts and swollen nuclei characteristic of dead cells. Death was observed also in *Cladophora* frozen at -8° in the same conditions, and in *Derbesia* and *Codium* frozen at -11° .

Klemm (1895), using the method that we described above under the heading "Hair Cells," observed that *Chara* sprouts and *Spirogyra* filaments were killed after an exposure of 15 minutes to -13° , while protoplasmic streaming could be resumed after exposures to higher temperatures (-6°) or for shorter times and was only slowed after a cooling to -2° .

Kylin (1917) experimented on the effects of freezing on sea algae. The material, immersed in sea water, was cooled successively to the temperatures of -2.9° , -4.0° , -5.7° , -7.8° , -10.7° , -16.8° , and -18° to -20° and the

time necessary to kill the algae at any of these temperatures noted. As a sign of death, he used the changing color exhibited by the brown and the red algae and the loss of the plasmolysing power for the green algae. He found that these plants were never killed by low temperature in the subcooled condition, the pigment not being released when ice was not formed. The death temperatures and the corresponding lethal times of exposure were as follows: *Trilliella*, -2.8° , 3 hours; *Delesseria* and *Laurencia*, -4° , 10 hours; *Laminaria* (1 year old) -5.7° , 6 hours; *Ceramium*, -5.7° , 10 hours; *Laminaria* (several years old) -16.8° , 3 hours; *Chondrus*, -16.8° , 10 hours; *Cladophora*, -18° to -20° , 3 hours; *Pylaiella*, -10° to -20° , 10 hours; other species such as *Nemalion*, *Porphyra*, *Fucus*, *Enteromorpha* were alive after 10 hours at -18° to -20° . Kylin obtained thus a surprisingly large difference in the death temperatures of the various kinds of algae.

According to Kärcher (1931), *Pediastrum* and *Hormidium* cultivated on agar slants in glass tubes, and immersed for 5 hours in a freezing mixture at -70° , were not killed.

Becquerel (1936) investigated the vitality of dried algae subjected to the temperatures of liquid gases. He took samples of soils containing various algae and gradually dried them in a vacuum with barium oxide, at 35° , for 3 months. He then placed a portion of the soils in glass tubes closed with cotton plugs and sealed another portion in tubes evacuated to 10^{-5} mm. of mercury. Thereupon he subjected all of them to liquid helium (-269° to -271°) for $7\frac{1}{2}$ hours, and to liquid nitrogen for 480 hours. Both groups of soils yielded living *Oscillatoria*, *Glaeotila*, *Hormidium*, *Siphonema* and *Pediastrum*.

According to the same author (1932b), the filamentous alga *Tribonema elegans*, thoroughly dried on a piece of bark, kept in a high vacuum for 22 years and exposed for several hours to temperatures from 4° to 1.84° K., grew after that treatment.

3. *Lichens*. The lichens are known to be plants which, in the natural conditions, are among the most resistant to low temperatures. They flourish in the northern climates and on the high mountains in the proximity of the glaciers.

Jumelle (1890 and 1892) attributed the high cold resistance of the lichens to their ability to lose water at the onset of the cold season. In confirmation of his views, he gives the fact that the ratio between the fresh weight and the dry weight of *Physcia* and *Parmelia*, collected during the winter, was found to be as low as 1.10 to 1.14; also that lichens of the same kind, soaked in water, absorbed an amount of moisture equivalent to $3\frac{1}{2}$ times their weight. But determinations by the same author of the quantity of oxygen and of carbon utilized by lichens previously soaked in water and then frozen resulted in the conclusion that respiration was still active after 8 hours at -8° , and photosynthesis after several hours at -30° and -40° . The author attributes this activity to the presence of free water, not yet frozen at these temperatures. If a high water content does not increase the injurious effect of freezing, as the second part of Jumelle's work seems to show, one can hardly see how a natural dehydration could explain the high cold resistance, as it is assumed in the first part.

According to Becquerel (1932d), lichens of the genera *Parmelia*, *Xanthoria* and *Cladonia*, desiccated in the air and exposed for 18 days in liquid nitrogen, became green again when brought back to favorable conditions, their gonidia resumed growth.

4. *Mosses*. Irmscher (1912) studied the cold resistance of about 34 species of mosses belonging to 27 genera. The gametophytes, freshly gathered, were put into tubes 1.5 cm. in diameter, and the tubes immersed for 18 hours in an ice-salt freezing mixture at -5° , -10° , -15° , -20° , and -30° . Two parallel series of experiments were carried on, one in which the material was in air, the other in which it was in water. The leaf cells were then tested for

their vitality by plasmolysis. It was found that: 1. A stay of 18 hours at temperatures down to -10° caused no essential injury; 2. At -15° , a considerable number of cells in most of the species, and almost all in some species, were destroyed; 3. At -20° , all the leaf cells of most of the species were killed; 4. At -30° , they were all killed. The apical cells and the cells of the dormant buds were more resistant than the rest of the plant. The setae and the protonemata did not differ essentially from the other organs in their cold resistance. Gametophytes, previously dried for 3 days in air at 20° , then made fully turgescient again by immersion in water, and thereafter frozen, showed a higher frost resistance than specimens not previously so treated. Repeated freezing at -10° to -15° was found to increase the number of dead cells so as to become equivalent to a single freezing at -15° to -20° . Between the plants frozen in water and those frozen in air there was no significant difference.

Bequerel (1932d) obtained some traces of growth in the moss *Hypnum*, desiccated in the air and immersed for 18 days in liquid nitrogen. He thinks that cells of the stem must be particularly resistant.

5. *Higher Plants.* The isolated organs or tissues of higher plants, (in contrast to those of higher animals), are capable of independent life. Consequently, almost all the studies on death temperatures in plants are concerned with the death of organs or tissues (while in animals it is the organismal death which is considered primarily). Since we have treated the tissues and organs previously, little remains to be added here.

As stated above, most of the plant tissues are killed at a temperature slightly below the freezing point of their sap. But, since given tissues may have various freezing points, depending on their water content, their structure, their age, etc., they die at different temperatures; and since their death influences the death of the whole plant, the lethal temperature of the latter will vary to a great extent and hardly any precise statement concerning it can be made.

Of particular interest are the plants reported to die at temperatures above zero. Molisch (1897), among others, investigated their behavior. We refer the reader to this author's charts for details concerning the comparative cold sensitivity of these plants. Most of them are from tropical countries and adaptation is thought to play a rôle in inducing sensitivity. As to the mechanism of death by chilling, the commonly accepted view is that of a disturbed balance between the various metabolic functions, mostly transpiration and absorption of water by the roots, the temperature coefficient of these functions being different.

Another group of higher plants of special interest for their cold resistance is that of some conifers. The trees of the Siberian forests (*Larix sibirica* in particular) are often mentioned in the literature as typical in that respect. Not only do they withstand exposures of several months to -30° or -40° , but they thrive in climates in which such low temperatures are not uncommon. This resistance can evidently not be attributed to any insulating layer, there is no known substance which would possess the low heat conductivity required to protect the cambium of these plants against the external temperature for that length of time. Such cold hardiness is generally ascribed to the nature of the colloidal cell contents and to the ability of the protoplasm to undergo seasonal changes in its composition and concentration. But the fundamental mechanism of this phenomenon is still little known.

Summary: 1. Most of the fungi and algae are killed when exposed to temperatures of -10° to -15° . 2. It seems definitely established, however, that some fungi, in the actively growing stage, can withstand the temperatures of the liquid gases, although they show less resistance than the monocellulars to these temperatures. 3. The algae which, like the *Horridium*, are more closely related to the monocellular forms, also resist very low temperatures. 4. The large majority of the higher plants

are killed when frozen at a few degrees below zero; some, however, resist -30° or -40° ; some others are killed by cold at temperatures above zero.

VI. METAZOA

In the sections of this chapter in which the investigations are more closely coordinated toward the solution of some fundamental problems, we shall follow the chronological order so as to show in a clearer manner the historical development of these problems.

1. *Coelenterates*. Payne (1930) investigated the action of repeated freezings on the coelenterates, *Mnemiopsis leidyi* and *Pennaria tiarella*. She states that 3 successive freezings and thawings "break the colloid structure" of *Mnemiopsis*, its constituent parts "going into solution," and that the animal "disappears," the disappearance being sudden and definite. For *Pennaria*, freezing 5 to 7 times, or maintaining the temperature at -10° , produced the same effect. As described, this process, we think, is unique in the literature.

2. *Helminthes*. Oliver (*Lancet*, p. 357, 1910-I) reported that *larvae* of the *hook-worm* recovered after having been frozen solid in water and thawed slowly.

Kjava (*Finska Laek. Handl.*, 55, 707, 1913) showed that *plerocercoids* of *Diphyllobothrium latum* were killed when kept for 48 hours at -9° .

According to Magath and Essex (*J. Prev. Med.*, 5, 239, 1931), all the *larvae* of *Diphyllobothrium latum* in 10 heavily infected fish (*Stizostidion vitreum*) were killed when the fish were exposed to -15° for 24 hours. Ten larvae that had been kept in fish at -10° for 88, 40, and 16 hours respectively were fed to each of 3 dogs; no worms developed.

Schmidt, Ponomarer and Savellier (1915) studied the resistance of *encysted Trichina* to cold. They found that a temperature of 0° for 11 days does not affect this parasite, that it can also support -6° for 10 days, that -9° is sometimes but not always fatal, and that -15° to -16° is always lethal.

The Helminthes, *Moniezia*, *Ostertagia*, *Nematodirus* and *Trichostrongylus*, were reported by Griffiths (1937) to resist the cold of a Canadian winter in which the temperature reached -26° , and to be capable, the next spring, of infecting the grazing sheep.

Augustine (1932) subjected to freezing *trichinous* meat that he fed to experimental animals on which he studied the subsequent infection. The temperature within the meat was recorded with thermocouples. He found considerable infection after exposure of the meat to -21° , less after exposure to -27.6° to -30.9° , and none after the action of a temperature of -33.7° .

Rahm (1920, 1921, and 1923) observed that the *nematodes*, *Plectus*, *Tylenchus* and *Dorylaimus*, in the dry state, could be cooled without injury to the lowest available temperatures (boiling helium), and that in the moist state, they resisted a slow cooling to the temperature of liquid hydrogen. The author did not mention the revival of any nematode after a rapid cooling to the latter temperature. The dried worms, heated to 136° or to 151° and then cooled abruptly to -190° or to -81° , died; they supported a heating to 50° followed by a sudden immersion in liquid air.

3. *Rotifers*. The last-mentioned author (*loc. cit.*) exposed to -80° for 17 hours specimens of moss with their natural fauna which consisted, among other organisms, of rotifers belonging to the genera *Callidina* and *Adineta*. The material, air-dried for periods extending from 18 days to 14 months, was immersed directly in a bath of ether and solid carbon dioxide. All the organisms survived and resumed motion when remoistened. In other experiments, the temperature of liquid air for $\frac{1}{2}$, $5\frac{1}{2}$, 25, 26 or 125 hours, that of liquid hydrogen for 26 hours, or that of liquid helium (-269 to -271.88°) for $7\frac{3}{4}$ hours was found harmless to the dried animals. A previous desiccation of 3 months and a stay of a week in a vacuum of $1/10$ mm. of mercury did not change their cold resistance.

While the preceding observations of Rahm are in good fitting with those made by other investigators on dried

material, the results that he reports on moist rotifers are probably not paralleled in the literature. When the dried animals had resumed their motion after immersion in water, they were frozen either slowly, by steps, to -81° , to -190° and to -253° , or rapidly by sudden immersion in liquid air and in liquid hydrogen. After having stayed 2 days and 1 day respectively in the two last-mentioned liquids, they could be revived, although the rapid refrigeration seemed to have lengthened the duration of the recovery and to have been somewhat more harmful to some individuals than was the gradual cooling. The author remarks that possibly some organisms, counted as survivors, may have been hatched after the exposure to low temperature. This would, of course, modify the problem entirely.

In another series of experiments, Rahm succeeded in maintaining alive some of the above-mentioned rotifers after they had been thoroughly dried in air for a month, then immersed in liquid air for 5 hours and finally heated in an oven to 140° to 151° for 15 minutes. Heating the dry organisms first and cooling them afterwards gave the same results.

Becquerel (1936) found the rotifers, *Adineta gracilis*, *Rotifer vulgaris*, and *Callidina angusticollis*, alive in samples of soil previously dried in a vacuum for 3 months and exposed to liquid helium for $7\frac{1}{2}$ hours and to liquid nitrogen for 480 hours, either in cotton-plugged glass tubes or in highly evacuated sealed tubes.

4. *Annelids*. According to Doenhoff (1872), *leeches*, exposed for 1 hour to a temperature of -1.5° , survived though they had been frozen so stiff that they could be bent only with difficulty. These animals, when cut with scissors, exhibited a cross section whitish with ice. Similarly, leeches exposed for 3 hours to -1.5° revived, but could no longer crawl and died after a few days. Leeches, subjected for a few minutes to -6.25° , died.

According to Schmidt and Stehepkina (1917), *earthworms* could be revived after an exposure of 8 hours to

0° without congelation, or an exposure of 2 to 3 hours to -1.2° in the congealed state; they were killed after having been frozen at -2° for 2 hours. The authors, who are interested in the study of the anabiotic state, consider the temperature range from 0° to -2° as the favorable one for that purpose. They reported also the somewhat surprising fact that dried worms, that is, worms in which 30 to 40 per cent of the normal water-content was removed, were killed when the temperature was lowered below -1.2°, though drying alone did not affect their vitality. (The normal water-content was 82.8 per cent of the body weight.)

5. *Mollusks*. Roedel (1886), who exposed to low temperatures land or water *snails*, either in the air or in water, by immersing into freezing mixtures glass tubes containing the animals, reported that *Planorbis* and *Limnaeus* were killed in times varying from 15 minutes to 5 hours at air temperatures from -8° to -4°, the younger and smaller animals offering less resistance, and that *Helix pomatia* was killed in 10 hours at -10°. None of the mollusks investigated ever survived a complete freezing. In the unfrozen state, in ice-water at 0°, *Limnaeus* could be kept alive for days. It would follow from these data that congelation is the main lethal factor.

According to Yung (1888), (cf. also Pictet, 1893) the *snail*, *Helix pomatia*, survived after having been for 20 hours at an air temperature of -130° in Pictet's refrigerating "well" (*puits frigorifique*). This surprising result is always described in the literature among the several achievements on reviviscence reported by Pictet.

Fischer (1930) found that *Helix pomatia*, kept for 8 hours at an air temperature of -5°, survived, while at temperatures of -6°, -7° and -10° they always died in less than 5 hours, in spite of all precautions for slow thawing. According to him, the air temperatures favorable for hibernation are between 0° and -5°.

Kapterev (1936) asserts that he "often succeeded in reviving mollusks (*Planorbis*) that had been frozen in the

ice." Judging by the thickness of the ice from which the animals were taken and from the climate of the country (Far Eastern Russia) he thinks that these organisms must have been subjected to -20° .

Weigmann (1936) attempted to determine the body temperature of *snails*, and of *Helix pomatia* in particular, during death by cold. For that purpose he used a thermocouple inserted through the shell. The surrounding air temperature was lowered slowly to a minimum where it stayed for several hours. He found that when the snails, with or without operculum, were frozen and when their body temperature dropped to -3° to -4° , they were always killed. Whether they were also killed at higher temperatures (between -2° and -3°) when ice was formed in them, we cannot ascertain from Weigmann's paper. He states, p. 310, that none of the species examined withstood freezing ("ein Einfrieren übersteht also keine der untersuchten Arten") and, p. 306, that some animals survived freezing at -2° ("ein Einfrieren bis auf -2° überleben"). Besides, a freezing curve is given (fig. 1) for an individual which is reported to have survived (table 1). As to the difference between operculated and non-operculated animals, Weigmann observed that the former could resist for 4 to 5 hours air temperatures down to -6° , while the latter were killed in $4\frac{1}{2}$ hours at -4° . The water snails *Limnaea* and *Bythinia* are given as having a higher lethal temperature than the land snails, namely, -1.5° .

6. *Arthropods*. The action of low temperature on arthropods has been the object of a very large number of observations and of investigations. Several reviews have been published, of which we shall mention two: that of Bachmetjew (1901 and 1907), and that of Uvarov (1901). The reader will find in the former a detailed history and compilation of the older works, and in the latter a short compendium of the more important modern contributions.

Although the larvae and the adult arthropoda present a widely different cold resistance, we shall treat them together.

As far back as 1742, Réaumur, in his famous "Mémoires pour servir à l'histoire naturelle des insectes," described experiments which confirmed the popular opinion that insect larvae can be frozen hard without being killed. He froze caterpillars at -8.8° and revived them. It is of interest to notice that the method which was used by most of the investigators of low temperature effects and which consists in putting the organisms into a glass tube and immersing the latter into a mixture of ice and salt, was the one used by this pioneer two centuries ago. Réaumur was probably also the first to remark that some juices, in the caterpillars, do not freeze at the freezing point of most of the body fluids, he thought that they must congeal at a lower temperature which would be the death point. This idea has been developed and studied experimentally during the last 30 years.

From the middle of the eighteenth to the end of the nineteenth century, the revival of hard frozen insects and mostly of larvae has been asserted by a legion of naturalists. From a review of the literature of that period one gains the impression that it was the most generally accepted view. However, there were divergent statements. To explain the differences reported, one generally had recourse to specificity. In the review lists we find silkworms, chrysalids, caterpillars, grasshoppers, mosquitoes, bedbugs, scolopendrae, etc., among the animals reported to have survived hard freezing, while ants, aphides, butterflies, house flies, and particularly bees are perhaps more often quoted among the sensitive insects. Bees have been reported by many to die at a temperature of a few degrees above zero (cf. Bakhmetieff, 1901). But what rôle is played in cold resistance by specificity or by the stage of development, the age, the season, adaptation, and by other similar factors, has by no means been elucidated in older works. The improved methods and the more systematic studies of the last 50 years or so have brought some light on the action of a few of the above-mentioned factors.

Doenhoff (1872) placed *bees*, *spiders* and *meat flies* on the frozen ground, covered them with a wire cage, and exposed them thus for 5 hours to a temperature of -1.5° , as indicated by a thermometer laid on the ground. The bees were killed by this treatment, but the spiders and flies soon recovered. It took an 8-hour exposure to -2° to -3° under similar conditions to kill the spiders, while the flies survived even a 12-hour exposure to -3.75° to -6.25° . The flies were then put into a small glass, $1\frac{1}{2}$ inches long and $\frac{1}{2}$ inch wide and so immersed into an ice-salt mixture at -3.5° to -6.5° for 4 hours. They survived this treatment also. Finally, they were killed by a 3-hour exposure to -6° to -10° .

Plateau (1872) studied the cold resistance of *aquatic arthropoda*. They were placed in glass tubes containing a few cc. of water and a thermometer, and the whole was immersed in a cooling mixture. The animals were little by little surrounded by ice, and finally caught in it. Plateau measured the time during which they were caught in the ice and recorded their survival after thawing. He gives the following figures as the longest periods supported in ice: 10 to 30 minutes for 3 *coleoptera*, 2 to 10 minutes for 2 *hemiptera*, 20 to 30 minutes for the *larva* of a *neuropteron*, 2 minutes for the *larva* of an *orthopteron*, 10 minutes for the *crustacean*, *Asellus*, $1\frac{1}{2}$ minutes for *Daphnia* and 1 minute for *Cyclops*. In experiments conducted in sugar solutions which froze at -2° , *Asellus* always died in less than a minute after it was caught in the ice, but it did not die at -2° when no ice was present. For Plateau it is evident, that the aquatic insects die at the temperatures at which ice forms in the fluid medium around them.

Roedel (1886) made experimental determinations of the death temperature of some arthropoda with an apparatus consisting of 2 concentric tubes, of which the external one contained a freezing mixture and the internal one the experimental animals, each tube being provided with a thermometer. For slow cooling, he used the principle of

the lowering of temperature produced by the evaporation of water at the free surface of a porous tube immersed in ice water. He observed: 1. That *ants*, exposed to an air temperature of 0° , soon lose their ability to cling to the wall of a glass container, and in 15 minutes they are rigid, but they are killed only by a stay of 3 hours at -15° ; 2. That *bees* become stiff in a short time in the neighborhood of zero, and that they die after $3\frac{1}{2}$ hours at -1.5° ; 3. That several *coleoptera*, as also the adult *Bombyx*, are killed in less than one hour at -4° to -6° ; 4. That the *mosquito*, *Culex pipiens*, is killed in one hour at -4° ; 5. That house flies are killed in 5 minutes at -12° , in 20 minutes at -8° and in 40 minutes at -5° ; 6. That the *spider*, *Tegevaria*, dies after an exposure of 45 minutes to -9° , or of one hour to -6° ; 7. That the *water spider*, *Argyroneta*, suffers no injury at -5° as long as it does not congeal, but dies in 3 hours at -4° when it is frozen throughout; 8. That the *water mite*, *Hydrachna*, dies after half an hour's exposure to -4° when its body is congealed; 9. That, when the *larvae* of several species of *coleoptera* were congealed all through at temperatures lower than -6° , they never survived. (Actual internal congelation was ascertained by sectioning the animals); 10. That some *dipteran larvae* could sometimes be revived when they were taken out of a frozen medium but never when they were themselves frozen; 11. That the *caterpillars* of *Smerinthus* did not survive a lowering of temperature to -10° . To summarize: Roedel, whose work covers most of the groups of arthropoda, never obtained the survival of a completely frozen animal, not even in the larval stage; he found that they all die in a range of temperature extending to about 10° below zero and during times of the order of an hour.

According to Bachmetjew (1901), death occurs when an insect which has been subcooled to a certain temperature below zero and has congealed, has its temperature lowered a second time to the subcooling point. The lowest subcooling point that he observed was -15.7° and this

would be, consequently, the lowest death point. One does not see for what reason death would correspond to a second cooling to the subcooling point; and, besides, the conclusions of Bachmetjew have against them that his experimental results disagree with the conclusions in 27 per cent of the cases (cf. Payne, 1926).

Revival after hard freezing was reported by Duval and Portier (1922) for the *caterpillars* of *Cossus cossus*. These authors froze the caterpillars in test tubes immersed in cooling mixtures. The temperature was measured with a thermometer placed in the tubes in between the animals. They found that death resulted when the temperature dropped below -21° , while, at higher temperatures, -15 to -17° and -20° , the caterpillars could be frozen so hard as to be breakable, and be revived on warming, even if the thawing was rapid (in water at 40°). The portions broken off in the frozen state showed activity after warming. Caterpillars congealed at -15° to -17° , then immersed in liquid air or exposed to the temperature of boiling chloroform (-63°) for 50 minutes, or cooled only to -22° , could not be revived. The authors think that the rigidity of the frozen caterpillars above -21° is due to the congelation of the *body fluids*, while the *cellular fluids* would still be unfrozen. The latter would congeal at about -21° , thus inducing death. As a matter of fact, a sudden rise in temperature, indicative of a congelation, was sometimes observed in the neighborhood of -20° . The resistance of the caterpillars was found to be less in the Spring than in the Winter.

A work which perhaps explains some conflicting results of the older authors is that of Payne (1926a, 1926b and 1927). This author determined with thermocouples the freezing and the subcooling points of various insects and found these points to be always low in Winter and to correspond to a low water-content. The freezing point varied in one case from -2.4° in midsummer to -8° in midwinter, and the subcooling point from about -6° to -14° . The changes in freezing temperatures, and there-

fore in death temperatures, could be induced artificially by exposure of the animals to cold; changes in cold resistance were caused also by exposure to moisture. While aquatic insects consistently showed the same survival temperatures, insects which could be experimentally dehydrated presented a considerable lowering of the death point. The *Japanese beetle*, for example, which can lose half of its body weight in the form of water, exhibited a lowering of about 28 degrees in its survival temperature, when so dried. Several species of *oak borers* are self-dehydrating during the winter and show a natural periodicity in cold hardiness. Artificial softening, the reverse of artificial hardening, could be accomplished by exposure to high humidity or high temperature. As to the freezing points, the author distinguishes two of them, the first one being the freezing point of the blood and the second probably that of some tissues, such as nerve tissue, or of some tissue components. The insects were able to recover from exposure to the first freezing temperature but not from exposure to the second. The latter, therefore, might be considered the death temperature. The time factor was found to be important.

Another significant work along the same line is that of Sacharov (1930). He determined, by the dilatometer method, the quantity of ice formed in the body of insect *larvae* at various temperatures during hibernation and during the active state, and correlated these data with cold resistance. He found that, for example, in the active caterpillar of the *brown tail moth*, 44.85 per cent of the water-content was frozen at -7.8° , while in the hibernating stage only 5.06 per cent was frozen at -11.1° , and 15.22 per cent at -17.35° . The total water-content in the 2 stages was, respectively, 82.94 per cent and 71.83 per cent of the body weight. On the other hand, the active caterpillars died after exposure to -7.8° while the hibernating ones withstood -17.35° . Sacharov also thinks that the cold resistance bears some important relation to the fat-content, which was about twice larger in the hiber-

nating caterpillars. Adult *bees* were found to die when their temperature was lowered to -0.7° .

Robinson (1926) insisted on the importance of the time factor in death by cold. For exposures of *Sitophilus oryzae* to temperatures from 1.1° to -17.70° , he obtained lethal times decreasing from 98 to $1\frac{1}{2}$ hours. As to the cause of cold hardiness, Robinson seeks it in the amount of bound water, and the hardening process would consist in increasing water-binding.

According to Kalabuchov (1934), *bees, bumble-bees, wasps, and larvae of beetles* can revive after having been subcooled for 48 hours at temperatures from -2.9° to -17.1° (body temperatures). Death usually ensues a few minutes after the beginning of ice formation subsequent to subcooling. Partial freezing, without preliminary subcooling, is not fatal, nor is partial freezing following a slight subcooling of short duration. Complete freezing is always lethal.

Results, similar to those reported by Duval and Portier and by Payne, were obtained by Lozina-Lozinskij (1935) on the *caterpillars* of a *Pyrausta*. These animals, frozen at -21° , and breakable like glass, were revived after a stay of 8 days in the congealed state. Considerable changes in the position of the freezing point and in the cold resistance took place during the passage from one metamorphosic stage to the other.

Kapterev (1936) says that he "often" revived *cyclops* taken from layers of ice 3 to 12 cm. thick. But, what is much more remarkable, is the experiment in which he claims that he revived a *crustacean, Chydorus sphericus*, isolated from a piece of soil taken in the permanently frozen ground, at a depth of 3.5 meters, in the valley of the Great Never River (a tributary of the Amur). He thinks that the "age of the layers whence these samples were taken should be reckoned not in hundreds but in thousands of years."

Some investigators have reported injurious effects or death as a result of long exposures of insects to temperatures above zero. For references, see Uvarov (1931).

6. *Tardigrades*. On account of their particular ability to recover from the dry state, and to resist the action of the lowest temperatures when dried, the tardigrades deserve to be mentioned in a separate section.

The tardigrades, *Macrobiotus*, *Echiniscus* and *Milnesium*, were subjected by Rahm (1920, 1921 and 1923) to the drying and cooling treatments that he used for rotifers (see above). In the dry state, all these tardigrades survived a cooling down to about 1° K; when moist, they resisted a slow cooling to the temperature of liquid hydrogen, and some *Macrobiotus* likewise supported a rapid cooling to that temperature. Moreover, the dried tardigrades could withstand a sudden cooling in liquid air followed by abrupt heating to 140° to 151° .

Becquerel (1936) isolated the tardigrade, *Macrobiotus Hoffmanni*, from dried soils subjected for 71 hours to temperatures of -269° to -271° . (For the details of the experiment, see above, under the heading Rotifers.)

7. *Amphibia and Reptiles*. That frogs and toads can be revived after having been frozen hard is a popular statement frequently encountered. Many observations and experiments are reported in the older biological literature which simply confirm this popular idea. Frogs or toads, found rigid on the frozen ground, or maintained in water or in a cold atmosphere until they become inert and rigid, are described as resuming activity when warmed up. Being skeptical of these reports we tried the experiment ourselves. Frogs, wrapped in several layers of cheesecloth, were put for 8 minutes into cavities dug in solid carbon dioxide. The animals became hard and rigid like dead bodies, but, on being rewarmed, they regained movement and sensitivity. As we shall see by the following review, two important steps were taken toward the understanding of the apparently high cold resistance of lower vertebrates when the investigators realized that a certain body rigidity did not involve a complete freezing, and when they began to determine the internal temperature of the animals during cooling. The time necessary

for the temperature equilibrium to be established between the body of an animal of the size of a frog and its environment, was found to be much longer than previously thought. The body temperature can still be above the freezing point when the animal has been for a long time at an air temperature of several degrees below zero. Besides, during congelation, the temperature stays for a surprisingly long time in the neighborhood of zero.

Hunter, about a century ago (1837), measured the internal temperature of *frogs* during freezing experiments and gave -0.6° as the lowest point to which they could be subjected without fatal results.

Müller (1872) investigated the effects of freezing on the *frog* as follows. He placed brown grass frogs in a bottle almost filled with water and exposed them so to the cold. The frogs were kept beneath the surface of the water with a stick, till solidification took place. Thereupon the bottle was kept for a further 6 hours at an air temperature of -6° to -8.7° . The frogs recovered, both after rapid and after slow thawing. If, however, the water in the neck of the bottle froze before that in the lower portion, the bottle burst and the frog was killed, even though it had been for only a short time in the rigid state. The author thinks that in the last case, the animal has been injured internally by the action of the pressure developed by the ice.

Koch (1890) proceeded in about the same manner as Müller. He placed *Rana rosea* or *Rana vicedis* singly into 400 cc. beakers filled with water and exposed them thus to air temperatures of -4° , -10° and -15° . When the water was frozen, a hole was drilled through the ice, and a thermometer inserted to measure the ice temperature. The frogs were killed by a 6-hour stay in ice at -6° . In another series of experiments, frogs were placed, without the addition of water, into beakers and exposed to an air temperature of -4° . They at first became very excited and active, but, after one hour, even the most resistant ones fell prostrate and quickly froze hard: such frogs could never be revived.

Knanthe (1891) reported that frogs, laid on ice or imbedded in snow, mud, or moist moss, became rigid after 12 hours at -1° to -5° . Animals, treated in this manner and kept afterwards for several days at temperatures between -0.2° and $+0.5^{\circ}$, recovered completely on thawing, even though, in the frozen state, their heart had ceased to beat and circulation had, to all appearances, stopped. In another group of experiments, frogs (*Ranidae* and *Hylidae*) and toads (*Bombinatoridae* and *Bufoinidae*) were frozen so stiff that their extremities could no longer be extended, but not so hard that they could be broken, and were left in this condition for several hours at -0.5° . When they were thawed, only 10 to 15% of the frogs, and about 50% of the toads survived. None of the animals could withstand a further congelation nor a repeated freezing. Frogs and toads, frozen completely in water, were always killed. (What is meant by "completely" is not clear.)

According to Pictet (1893), frogs could be revived after having been frozen hard and brought to a temperature of -28° . To kill them, it required an exposure to -30° to -35° . The lack of information concerning the body temperature of the frogs and the time the animals stayed at that temperature deprives Pictet's so often-quoted observations of most of their value.

Kodis (1898) who investigated, among other subjects, the effects of a considerable subcooling on vitality, reported that whole frogs, with a thermocouple inserted into the thigh, suffered no injury when subcooled to -10° . Isolated frog muscles could be subcooled to -18° .

Maurel and Lagriffe (1900) called attention to the rigidity gradually acquired by frogs subjected to temperatures above zero. They observed that the animals, cooled slowly, were not capable of resuming their position when set on their back, when their buccal temperature was 8° ; that they showed a complete absence of reflexes at 4° ; and that they became rigid when they were caught in ice, the buccal temperature (not actually mea-

sured) being near zero. They could be revived after this treatment, but not if the temperature was 2 or 3 degrees lower.

According to Harris (1910), pithed frogs, whose visceral temperature was maintained at 0.0° for 1 hour, survived; one frog died after having sustained for 100 minutes a visceral temperature of -2.1° . As to the external temperatures supported, Harris summarizes his results by saying that a frog weighing 10 to 12 grams could probably withstand 1 hour but not 2, in water at -10° . He states also that frogs which die after freezing experiments, contain some subcutaneous or perivisceral ice, those which survive do not.

The most extensive investigation on the resistance of frogs to low temperatures is probably that of Cameron and Brownlee (1913). They inserted the bulb of a thermometer and, in some experiments, a thermocouple into the stomach of the animals, and exposed them to a constant low temperature in the air. They found that frogs, the stomach of which showed -0.5° for 8 hours, or -0.5° to -1.0° for 1 hour, recovered, while those cooled to -1.5° to -1.8° for 2 hours or to -2° to -2.4° for $1\frac{1}{2}$ hours, died. The authors conclude from these experiments that -1.8° is the lowest non-lethal internal temperature which can be maintained for a short time. The authors then determined the freezing temperature of the body of the frog as a whole and found it to be -0.44° . The freezing temperature being -0.44° and the lethal temperature -1.8° , it evidently follows that ice can be formed in the frog without killing it. Concerning the external air temperatures supported, the authors found that occasionally -25° could be withstood for 1 hour; which shows how slowly heat is withdrawn from the animal tissues by the surrounding air.

According to Kalabuchov (1934), the toad, *Bufo bufo*, could not be subcooled below -0.9° to -1.0° . When frozen without subcooling, it survived an exposure to sub-zero temperatures for 20 to 145 minutes during which time its

body temperature reached a minimum of -0.5° to -0.15° . Three out of four toads were killed after a 165–302 minutes' exposure, the minimum body temperature having been -0.65° to -5.75° .

The same author found that, in 18 out of 22 experiments, the tortoise, *Testudo norisfieldi*, could be subcooled to -2.6° to -5.3° and revived. If, however, ice-formation set in after subcooling, the organisms were killed in a few minutes, even though their body temperature had not dropped below -0.3° to -1.4° . When the tortoises were frozen without previous subcooling, they survived relatively long exposures, during which the minimal body temperature reached was -0.5° to -0.7° . No completely frozen animal could ever be revived.

Jecklin (1935) who investigated the cold resistance of *Salamandra maculosa* reported that, when the temperature was lowered slowly, the animals could, without injury, be subcooled to -1.7° or -2.2° ; they could likewise withstand for a short time a cooling to -5.2° , even when they had solidified; a temperature of -3.5° , with complete rigidity, was supported for 70 minutes. The freezing point of the blood was found to vary between -0.5° and -1.7° .

8. *Fishes.* The numerous reports, some of them rather startling, about the revival of hard frozen fishes, would make one think that the popular term "fish stories" might well have originated there. Indeed when the possibility of reviving hard frozen fish is mentioned to fishermen, it sometimes "reminds" them that they actually saw it themselves. Turner (1886) saw fishes revive in quite extraordinary circumstances. He reports that the Alaskan black fish, *Dallia pectoralis*, kept frozen in grass baskets for weeks, not only is fully alive on thawing, but that "the pieces thrown to ravenous dogs are eagerly swallowed; the animal heat of the dog's stomach thaws the fish out, whereupon the movements soon cause the dog to vomit it up alive." (Quoted from Borodin, 1934.)

As we shall see, the results of the investigators diverged widely on this very point of revival after hard freezing, until good determinations of the body temperature of fishes experimented upon were made.

Pouchet (1866) exposed to various low temperatures *gold fishes*, *small stickle-backs*, and *eels* contained in tubes full of water, that he immersed in freezing mixtures. He found that the eels were killed in 1 hour at -14° , the gold fishes in 2 hours at -19° , but that *stickle-backs*, the body of which was only half congealed after 2 hours at -19° , survived. He emphasizes that he never observed the revival of a completely frozen fish, and that when the animals were entirely caught in the ice they were dead. He could observe some revived fishes carrying pieces of ice attached to their body but the latter had been only partially caught in the ice.

According to Knauthe (1891), fishes of the genera *Cyprinus*, *Carpio*, *Carassius*, *Rhodeus* and *Misgurnus*, laid on ice and covered with snow or ice water, can be maintained alive if the surrounding temperature never drops below -4° , and if the animals do not stay in the rigid condition for more than one hour.

Pietet (1893) says he could revive *gold fishes* and *tenches* which had been left for 24 hours in water at 0° and were then cooled slowly to -8° to -15° , at which temperature they were brittle. He obtained no revival after a cooling to -20° . Pietet's experiments, which confirmed the popular opinion of the reviviscence of "hard frozen" fishes, have been generally considered as demonstrative by the biologists until more recent determinations have brought into evidence the fact that the body temperature in fishes is often above the bath temperature for a time much longer than it was generally thought.

The action of cold alone, without congelation, on fishes was investigated by Regnard (1895). *Carps*, accustomed to water containing 2.5% magnesium sulphate, were cooled slowly in this medium. Cooling was achieved by immersing the container in a refrigeration bath. Reg-

nard observed that when the temperature of the magnesium water came to 0° , the fishes became little by little immotile; when it reached -2° and -3° , the complete cessation of motion gave the animals a dead appearance. However, they were not rigid but "perfectly supple." On rewarming, they resumed motion and soon behaved normally.

Britton (1924) made a somewhat extensive study on the resistance of the *skate*, *sculpin*, *sea raven*, *flounder*, *eelpout*, *cod*, *toncod* and *pollack* to sub-zero temperatures. The fishes were immersed in refrigerated tanks containing sea water. The effects of cold, as exemplified in the case of the sea raven, were in general, as follows. After an exposure of 30 minutes to -1.9° , the rectal temperature was around -1° ; the body then became rigid (not by congelation but by some cold rigor; the freezing point of sea water being -1.6°), the respiratory movements, the jaw and fin reflexes and finally the heart-beat ceased. When rewarmed, most of these fishes were dead. The lethal body temperature was, then, that produced by an immersion of the fish from 20 to 60 minutes in a bath at -1.9° . At higher sub-zero temperatures, the fishes were also usually killed, although after a longer time. Slow and gradual cooling gave the same final result as rapid cooling by abrupt immersion. The elasmobranchs were more resistant.

Kalabuehov (1934) reported that the *carp* could not be subcooled to temperatures below -5° . When frozen without subcooling, the fishes survived minimum body temperatures of -0.2° to -0.5° ; they were killed when the latter dropped to -2° to -4.9° . Completely frozen fishes could never be revived.

Borodin (1934) studied the effects of low temperature on some 10 different kinds of fishes including, among others, the *mummichog*, *eel*, *gold fish*, young *carp*, *perch*, and *mud-minnow*. The low temperatures were produced by a Westinghouse household refrigerator within the freezing chambers of which the temperature could be kept constant

to within $\frac{1}{4}^{\circ}$. The fishes were put either directly into the aluminum ice trays lined with cellophane to prevent adhesion during freezing, or into a paraffined card-board box. The body temperature of the experimental animal was ascertained by means of a needle thermocouple inserted into the muscles of the back of the fish behind the dorsal fin. The various species were found to differ in frost resistance; *bullhead*, 6 to 7 inches long, recovered after $\frac{3}{4}$ hour at a chamber temperature of -18° , while *killifish*, 4 to 6 inches long, survived 1 hour at -14° to -15° , these being the hardiest of the species used. In general, the larger fishes of a species supported freezing more easily than the smaller ones. Moreover, a rapid freezing at a somewhat lower temperature proved less fatal than a prolonged freezing at a higher temperature; thus, fishes frozen for $\frac{1}{4}$ to $\frac{1}{2}$ hour at -14° to -15° recovered in most cases, while those frozen for $\frac{3}{4}$ to 1 hour at -10° to -12° generally did not revive. None of the fishes could withstand a temperature below -18° for more than 25 to 30 minutes. Death occurred when the body temperature of the fishes dropped below -0.6° to -1° which required 25 to 45 or more minutes depending on the temperature of the chamber and the size of the fish. The author concludes that the death point probably lies near the point at which the body fluids freeze. In a few rare cases, fishes with muscles, blood vessels and intestine frozen hard, but with the heart not yet frozen throughout, recovered when thawed. It is thought that only when the heart is hard frozen and kept so for some time, does the fish die. The author distinguishes between fishes that are "soft frozen," that is, stiff and apparently dead, but with the body fluids still liquid as is evidenced by the bleeding when they are dissected, and fishes that are "hard frozen," that is, "frozen through as hard as stone." The former recover rapidly and evince no ill effects of the frost, while the latter, in most instances, do not regain their vitality, or, if they should perchance survive, exhibit marked injuries.

Weigmann (1936) studied death temperatures in *sticklebacks* cooled slowly in water. He inserted a thermocouple into the body of a few specimens and showed that when the temperature of the water was lowered, that of the animal followed closely (how closely depends, of course, on the cooling velocity and the size of the animal). As to the death temperature, he observed that all the fishes died when their body became surrounded with ice but before being actually caught in ice. The maximal resistance registered was 20 minutes at bath temperatures of -2.5° to -3.2° , which would give for the lethal body temperature, -2° to -3° . The author remarks that a velocity of cooling varying from 0.02° to 0.6° per minute had no influence on death or survival.

Schmidt, Platonov and Person (1936) subjected to cold *carps* maintained in air, determining their internal temperature with thermocouples. They found that these fishes could be revived if their body temperature, measured at a depth of 1.5 to 2.5 cm, from the surface of the skin, did not fall below -0.72° to -0.92° . In the outer surface layers of the body, -3.33° was recorded. In another series of experiments, the same authors revived *bleaks* and *sticklebacks* subcooled to -3.06° (body temperature). None of the fishes experimented upon ever survived a congelation of its body.

Luyet (1938), in an attempt to test a theory according to which a rapid freezing would cause the formation of smaller crystals less injurious to the cells, studied the effects of a sudden immersion of *gold fishes* in liquid air. He could never obtain any sign of recovery if the immersion had lasted more than 15 seconds (for fishes 40 mm. in length, not counting the caudal fin). However, after that time, the fishes were not frozen throughout, it requiring about 35 seconds for them to become breakable. Immersions of 1 to 15 seconds caused gradually increasing injuries, corresponding to the gradually increasing thickness of body wall congealed. The author, who reports that a juggler had as a regular item in his program the

revival of hard frozen gold fishes, remarks that for a successful demonstration, the animals to be broken before the audience should be left for 35 seconds in liquid air and those to be revived should be withdrawn in less than 10 seconds.

9. *Homoiotherms*. It is well known that the warm-blooded animals cannot support any considerable lowering of their body temperature. Of the abundant literature on this subject, we shall select some reports establishing the general fact of the high cold sensitivity of homoiotherms, a few others describing experiments made in some unusual conditions, and still others which are of interest because of the exceptionally low temperature reached.

Walther (1862), working with *rabbits*, found that when the animals, immobilized but non-narcotized, were cooled to a body temperature of 18° or 20° , they were unable to recover spontaneously. However, the complete recovery of such animals could be effected either by warming them in air at about 40° , or by artificial respiration.

Coleman and McKendrick (1885) placed a *rabbit* whose rectal temperature was 37.3° , pulse 160 per minute, and respiratory rate 45 per minute, into a cold chamber at an air temperature of -69.4° , for 2 hours. The animal was then removed for a minute or two; it seemed unaffected, though the rectal temperature had dropped to 34.5° . Thereupon it was returned to the cold chamber, now at a temperature of -73° , for another hour, after which it was again taken out. The animal seemed to be comatose, reflex action had ceased, there were jerking movements of the limbs, the rectal temperature had reached 6.1° , the pulse rate was 40 per minute, and respiration was hardly perceptible. Placed in a warm atmosphere, the animal recovered completely.

Colin (1891) reported that *rabbits* could stay alive in winter after 5 or 6 days at an atmospheric temperature of -10° to -15° , in cages hanging on trees.

Pictet (1893) subjected a *dog* weighing $8\frac{1}{2}$ kilos to an air temperature of -90° to -100° in one of his refrigera-

tion "wells." The metal walls of the latter were covered with wood or cloth so as to avoid contact between the animal and the cold metal. The inguinal temperature increased by about $\frac{1}{2}$ degree during the first 13 minutes, it came back to normal during the following 12 minutes, and decreased by $\frac{1}{2}$ degree during the next 75 minutes. Then it dropped rapidly, while at the same time respiration and heart beat became slower and slower. When the temperature reached 22° the animal was withdrawn, inanimate, and it did not recover.

The same author reported that a *cat* which fell into a liquid refrigerating mixture at -30° to -35° died "almost suddenly." The more rapid cooling of the body when in contact with a liquid than when in contact with air, is considered the cause of the rapid death.

According to Winternitz (1894), a *rabbit*, whose body temperature drops to 34° to 31° , begins to shiver, it tends to fall asleep at 31° to 29° , and finally, when the temperature reaches 22° to 19° , breathing ceases and the animal dies.

Simpson (1902) placed a *monkey* (*Macacus rhaesus*), fully etherized, into a double-walled chamber made of thin sheet-iron, and cooled with lumps of ice inserted into the space between the walls. The animal could be observed through a sliding glass door. The rectal temperature, pulse rate and respiration rate were taken every $\frac{1}{2}$ or $\frac{1}{4}$ hour. After $3\frac{1}{4}$ hours, the body temperature had fallen to 14° , the heart beat had ceased and the respiration rate was reduced to 2 per minute. The ice-water was then removed and quickly replaced by hot water till the chamber temperature stood at 41.6° . When, after 5 hours, the rectal temperature had risen to 37.7° , the animal was removed from the chamber and placed in a room at 25° , where it recovered completely in a further $2\frac{1}{2}$ hours. Another monkey whose rectal temperature had been lowered to 12.5° , under the same conditions, succumbed.

According to Simpson and Herring (1905), *cats*, whose normal temperature is about 38° , can be cooled till their

rectal temperature reaches 16° and still recover, provided they are then warmed artificially.

Tait (1922) reported that, while the excised, perfused heart of an ordinary mammal stops beating at about 17° , that of a *hibernating hedgehog* or *woodchuck* will cease to beat only when it freezes.

The same author, in collaboration with Britton (1923), found that, in the *woodchuck*, respiration ceases without signs of asphyxia at 12° to 3° ; but, when warmed, the animal may recommence breathing, and spontaneously resume its former body temperature; with artificial respiration, it always recovers.

Britton (1923) repeated the earlier work of Simpson and Herring, using a cooling apparatus similar to that of these investigators, and working almost exclusively with deeply anaesthetized *cats*. He called attention to the fact that the "deep rectal" temperature (the thermometer bulb placed 8 cm. deep in the rectum) may be 0.5° to 2° higher than the "anal" temperature (thermometer bulb 4 cm. deep). Cats, whose deep rectal temperature had been lowered to 19° , could still spontaneously recover their previous body temperature, even though very slowly. Below 18° , recovery could be effected only by artificial warming. The lowest deep rectal temperature at which a cat gave evidence of being alive was 16° , while a *guinea-pig* could be cooled to 14° .

According to Kalabuchov (1934), the *bat Nyctalus noctula* can withstand a 5–15 minutes' subcooling to -2.9 to -5.9° . Of 10 bats of the same species, frozen after subcooling, 7 were killed, though the minimal body temperature reached only -0.5 to -1.5° . If, however, freezing set in without a previous subcooling, the animals could survive a 12–60 minutes' exposure, and a minimal body temperature of -0.8 to -1.9° , but were killed after a 67–145 minutes' exposure and minimal body temperature of -1.2 to -5.5° . *Myotis daubentonii* is not so resistant; 4 out of 5 bats of this species were killed when freezing without subcooling occurred during a 17–90 minutes' exposure.

the minimal body temperature reached having been -0.9 to -1.5° .

The same author reported that the *mouse*, *Mus musculus*, was killed when frozen till a body temperature of -0.9° was registered, or when subcooled to -3.7° to -7.1° . (One would naturally expect it.)

Murigiù (1937), repeating some old experiments of Horvat (1881), in which the *ground-squirrel*, *Citellus suslica*, could be revived after its body temperature was lowered to -0.2° , exposed 19 *Citellus pygmaeus* for 2 to 4 hours in cooling chambers to temperatures of -11° to -19° and found that 47% of the animals could be revived after their body temperature, measured by a thermocouple, had sunk to -0.5° and -1° . When the body temperature went below -1° , the animals died. Repeating a cooling experiment on the same individual seemed to result in a greater ability to resist a new lowering of temperature; one specimen survived -0.5° 3 times, another survived -1° twice. The smaller animals were more resistant than the larger ones. The author compares the special state of torpor which results from the lowering of the temperature, to the anabiotic state; he describes it as an "imaginary anabiosis."

Summary: 1. The invertebrates were found, in general, to be killed when frozen at temperatures of a few degrees or of some 10 degrees below zero. 2. The rotifers, the nematodes and the tardigrades, however, resisted, in the moist condition, extremely low temperatures. 3. The invertebrates which support desiccation could be cooled to the lowest available temperatures without injury. 4. Some insect larvae were alive, although hard frozen, at some 20 degrees or more below zero, but they died at lower temperatures, probably under a more complete congelation of their cellular fluids. 5. The cold-blooded vertebrates died when their internal temperature dropped a few degrees below zero. They could support the formation of some ice in their body. 6. Sub-cooling is not in-

jurious to the poikilotherms. 7. The warm-blooded animals died at above-zero temperatures except if, as in the case of hibernators, they had developed some adaptative properties.

GENERAL COMPENDIUM

By their resistance to low temperatures, the plants and animals considered in this review can be classified into 3 groups: 1. Those which can approach the absolute zero without being killed; 2. Those which die near the freezing temperatures; 3. Those which die above the freezing temperatures.

The first group comprises: a) The forms which support desiccation, such as, the seeds, the spores (bacterial and others), the protozoan cysts, the rotifers, the tardigrades, the nematodes. b) A number of microorganisms of the types, bacteria and yeast, some flagellates, some of the lower fungi, and the infra-cellulars. All these resist extreme temperatures without previous desiccation.

The third group, namely that of organisms which are killed at above-zero temperatures, includes only the homoiotherms and some of the higher plants.

The large majority of plant and animal phyla belong, then, to the second group, that is, to the kind of organisms which are killed at near-zero temperatures. There are, it seems, two classes to be distinguished in this group: 1. The organisms which die when their temperature is lowered slightly below the freezing point. Usually these organisms can support the formation of some ice in them but they are killed when a larger proportion of their water-content solidifies. This has been observed in several plant tissues, in some animal tissues and in entire organisms, such as, the mollusks, the amphibia, the fishes, etc. 2. The organisms which resist some 10, 20 or 30 degrees below freezing. Often a relatively large quantity of ice can be formed in them, they become hard and breakable, and they die, apparently when a last portion of their cellular fluids solidifies. This is probably the case of

insect larvae at certain periods of the year and, to a certain extent, of the cold-hardened woody plants.

Between the first and the second class, that is, between the organisms which are killed near the freezing point and those which resist some 30° below zero, there seems to be a continuous gradation.

In short, one can perhaps summarize this entire review as follows: With the exception of a few organisms which are affected by above-zero temperatures, all the plants, animals, tissues or cells either die in the neighborhood of their freezing point or are not killed at all by any low temperature.

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PART II

THE PHYSICAL STATES OF PROTOPLASM AT LOW TEMPERATURES

A substance may exist in four states, as a gas, as a liquid, as a crystal and as a glass. The relation between these states and temperature is illustrated in the diagram (Fig. 1), in which the temperatures are plotted on a horizontal axis, with the absolute zero at the origin. "A body is a gas at high temperatures, in the zone A; it is a liquid at lower temperatures, in the zone B; it becomes crystalline at still lower temperatures, in the zone C; and it hardens, but does not crystallize, that is, it takes the vitreous state, if it is brought (without being previously crystalline) to the low temperatures represented by the zone D." (Luyet, 1937, p. 1).

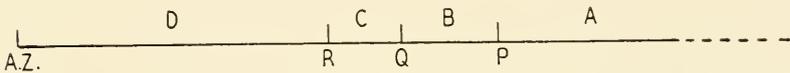


FIG. 1. Diagram representing the relationship between temperature and the four states of matter. A.Z.: Absolute zero.

Some physicists (cf. Tammann, 1925, p. 2) for whom the arrangement of the molecules in the aggregate is the essential feature in the definition of the states of matter, name only two of them: the crystalline state which possesses as a specific character an ordered arrangement of the atoms, and the amorphous state (gas, liquid and vitreous) in which the molecules are distributed at random in space. Most students of nature name three states: gas, liquid and solid, and make of the vitreous state a subdivision of one of the three. Some, considering the fact that a glass possesses the hardness and cohesion of the solid bodies, classify it as a form of the solid state; others, considering the deformability presented by some glasses during long periods of

time, prefer to call them liquids of high viscosity; still others, considering the method of obtaining glasses and their amorphous nature, insist on calling them super-cooled liquids. Defining a state by its relation to temperature (as illustrated in Fig. 1), by the consistency of the material and by the arrangement of the constituent particles, we shall distinguish four states of matter: vitreous, crystalline, liquid and gas.

This classification of the states requires a revision of the classification of the transitions from one state to another. The following list gives the various possibilities. (We coined a few names, when such were lacking; they are indicated by quotation marks.)

CHANGES OF STATE

1. From the vitreous to the crystalline state:	Devitrification
1'. From the crystalline to the vitreous state:	(Impossible)
2. From the vitreous to the liquid state:	“Vitromelting”
2'. From the liquid to the vitreous state:	Vitrification
3. From the vitreous to the gas state:	“Vitrosublimation”
3'. From the gas to the vitreous state:	“Gasovitrification”
4. From the crystalline to the liquid state:	Melting
4'. From the liquid to the crystalline state:	Crystallization
5. From the crystalline to the gas state:	Sublimation
5'. From the gas to the crystalline state:	“Gasocrystallization”
6. From the liquid to the gas state:	Vaporization
6'. From the gas to the liquid state:	Liquefaction

The liquid state can be maintained also at temperatures below the freezing point; one then obtains, besides the four states just described, a supernumerary one (called state in a broader sense), the supercooled state.

Of these five states there are only three which protoplasm can take at low temperatures, the crystalline, the vitreous and the supercooled; the liquid state obtains at ordinary temperatures and the gas state is unknown in protoplasmic substances. As to the changes of state, the most important for the biologist are: crystallization (that is, freezing), melting, vitrification, devitrification and “vitromelting.” Consequently we shall divide this work into the following chapters: I. Freezing, the Frozen State and Melting; II. Supercooling and the Supercooled State; III. Vitrification,

the Vitreous State, Devitrification and "Vitromelting." A preliminary chapter will be devoted to the study of the principles of heat conduction.

Within each section, after having discussed the fundamental laws, we shall consider the various biological materials which undergo the changes of state, in the order of the increasing complexity of these materials: pure water, solutions, suspensions, emulsions, colloids, dead tissues, living protoplasm, living cells and living tissues.

PRELIMINARY CHAPTER

FUNDAMENTAL PRINCIPLES OF HEAT CONDUCTION

In the study of the states of biological material at low temperatures the investigators, most of the time, "apply" principles "established" by the physicists, but often they overlook the numerous assumptions on which the physicists built up these principles. There results a considerable amount of confusion, particularly in the discussions concerning cooling, freezing and subcooling curves, freezing and entectic points, and even in more common problems such as the interpretation of the readings of a thermometer. Most of our knowledge on these questions is based on the fundamental principles of heat transmission. We shall here discuss briefly these principles.

In the last analysis, heat is understood to be the movement of the constituent particles of matter. When, in a body, a disturbance at one place causes the particles to move faster than in the surroundings we are accustomed to say that this point is warmer. The faster motion will be transmitted by collisions to the neighbouring particles and this is what we call heat transmission.

1. *The "Problem of the Wall," in the Steady State.* One of the best-known methods of analysis of heat transmission is that used in the so-called "Problem of the Wall" (Fourier). Let W (Fig. 2) be a wall of homogeneous mate-

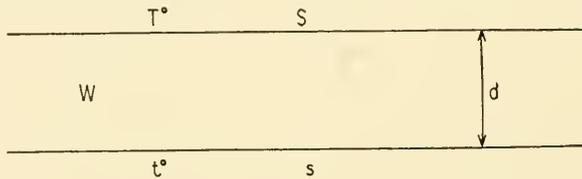


FIG. 2

rial, of thickness d , limited on two sides by two parallel surfaces S and s and unlimited in the other directions. Let T and t be the temperatures, respectively, at S and s , T being higher than t and both temperatures being maintained constant. Heat will flow through the wall from S to

s, gradually modifying the temperature of each intermediate plane within the wall until a state of equilibrium is established. Then heat will continue to flow but the temperature at each intermediate plane will stay unchanged. Before the establishment of this equilibrium the system is in a *variable state*; after the equilibrium is established the system is in a *steady state*. In the variable state, the amount of heat which enters through a given area on S is different from the amount which comes out through an equal area on s; in the steady state these amounts are the same. From theoretical considerations it has been concluded that, in the steady state, the quantity of heat Q which traverses the wall should be proportional to the difference of temperature T-t, to the area A through which heat flows, to the time z during which it flows and inversely proportional to the thickness d of the wall:

$$Q = K \frac{A(T-t)z}{d} \quad (1)$$

Experimental studies have confirmed this relation.

The constant of proportionality K in the formula (1) is the coefficient of heat conductivity. It is defined as the number of calories which, during one second, traverse an area of 1 cm² of a wall 1 cm. thick, when the temperatures of the two sides of the wall differ by one degree.

After the steady state is reached, each intermediate plane p is at a temperature θ which is proportional to the distance x from the plane p to the cooler surface (t being taken as the temperature origin, that is, t = 0):

$$\theta = a x \quad (2)$$

The constant of proportionality a is the ratio of the temperature difference T-t to the thickness of the wall d.

The formula (2) which is a necessary consequence of the principles admitted in formula (1) has also been verified experimentally.

The main assumptions in these relations are: 1. That the temperature on each side of the wall is constant; 2.

That the steady state is reached; 3. That the material of the wall is homogeneous; 4. That the wall is not limited in any direction other than the direction of flow of heat. In any application of the formulas these assumptions need to be considered.

It should be noticed that the specific heat of the material does not play any role in the flow of heat, in the steady state. This is of importance in several problems, as when, for example, the passage of heat through water is to be compared with the passage of heat through ice, two substances which possess a very different specific heat.

In any practical case, the wall is in contact with two media, one on the warmer side, the other on the cooler side. Heat flows from the warmer medium to the cooler one through the wall. But the constant temperature of these media (even if they are perfectly stirred) is not the temperature at the surfaces of the wall. In other words, when two bodies are in contact, their surfaces of contact are not at the same temperature or, what is equivalent, the interface between two substances in contact has a heat conductivity (sometimes called "contact-conductivity") which is different from that of either of the two substances. For having overlooked this point in his experiments on the heat conductivity coefficient in metals, Péclet, a pioneer physicist of the last century, was led to the evidently erroneous conclusion that all the metals have the same conductivity. Applications of the notion of contact-conductivity in biological investigations will be mentioned later.

Usually it is not with a single wall in contact with two media that one has to deal but with several walls in contact with one another. Furthermore, these walls are rarely limited by plane surfaces, they have any shape. Of the numerous problems which result from the combination of walls of various forms, the simpler ones, in particular that of the "multiple cylindrical wall" and that of the "multiple spherical wall," are studied in the treatises on mathematical physics.

As an application of the problem of the multiple

cylindrical wall we shall mention the analysis of the freezing curves obtained with arrangements of the type represented in Figure 3. A cylindrical shell of living tissue AC,

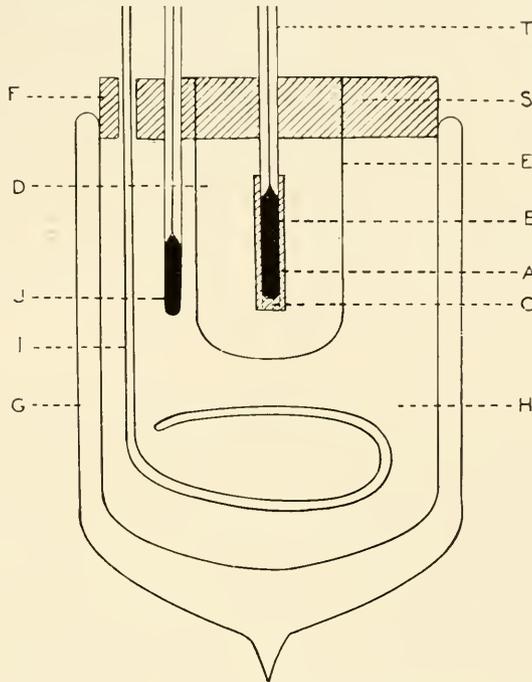


FIG. 3. Arrangement of apparatus illustrating the application of the "Problem of the multiple cylindrical wall."

slipped around the bulb B of a thermometer, is placed in the medium D contained in a tube E, which is itself immersed in a cooling bath H. During freezing, heat is then transmitted from the mercury of the thermometer and from the freezing object to the external bath, through the many cylindrical walls which are the glass of the thermometer, the tissue, the medium and the tube E. In such cases important errors have resulted from a too candid use of thermometers as temperature-meters, as their name suggests them to be, instead of as heat conductors which, in the last analysis, they are.

2. *The Problem of the Wall, in the Variable State.* The essential laws governing the relation between the tempera-

ture at a given plane in the wall, in terms of time and in terms of the distance from that plane to the warmer side, are deduced from a famous equation established by Fourier. Of the various conclusions he arrived at we shall mention here only the following one which is of some importance in the practical applications to biothermometry. The time y necessary for the establishment of the steady state is inversely proportional to the coefficient of conductivity c of the material and directly proportional to its specific heat s , to its specific gravity D and to the square of the thickness d of the wall

$$y \propto \frac{sDd^2}{c} \quad (3)$$

So the factor, specific heat, which had no influence on the amount of heat traversing the wall in the steady state, plays a role in determining the time necessary for the establishment of the steady state and, in general, in various problems of the variable state.

3. *The Problem of the Cooling Body.* A particular case of the problem of the wall is that in which a wall at a temperature ϑ is brought into contact, on its two sides, with a medium at a temperature t different from ϑ . The wall will then warm up or cool down at a certain rate; if it is in contact with the medium not only on two sides but all around, the problem becomes that of a body immersed in a warming or a cooling bath. This problem led to the establishment of the "Law of Cooling."

Newton, in 1701, on the basis of theoretical considerations, came to the conclusion that, when a body is exposed to a constant temperature bath, it should lose, during a unit of time, a quantity of heat Q/z (z being the time) proportional simultaneously to the difference between its temperature ϑ and that of the cooling bath t , to the area A of the body, and to a certain coefficient C which indicates the velocity with which heat is removed from the system:

$$\frac{Q}{z} \propto AC (\vartheta - t) \quad (a)$$

(One recognizes the same relation as in the problem of the wall.)

Cooling will result from the withdrawal of heat at a rate determined by the fact that every unit of weight of the body requires the removal of a number of calories represented by the specific heat in order to be cooled by one unit of temperature (say, from θ to θ_1). Calling m the mass of the body and s its specific heat, one has

$$Q = (\theta - \theta_1) ms \quad (b)$$

Bringing the value of Q from (b) into (a) one obtains

$$\frac{\theta - \theta_1}{z} \propto \frac{AC}{ms} (\theta - t) \quad (4)$$

that is, the *cooling velocity* is proportional to the area of the body, to the coefficient C , to the difference between the temperature of the body and that of the bath, and inversely proportional to the mass and the specific heat of the body.

A consequence of the assumption that the cooling velocity is proportional to the difference between the temperature of the object and that of the bath is that, when the time is increasing arithmetically by one unit, the temperature decreases to a set fraction of its own value, that is geometrically; in other words, the cooling curve is an exponential. Calling θ_z the temperature at the time z and θ_0 the original temperature of the object, one has

$$\theta_z = \theta_0 e^{-\frac{AC}{ms}z} \quad (5)$$

where e is the base of natural logarithms.

The formulas (4) and (5) express two aspects of Newton's "Law of Cooling."

The assumptions on which the law is based are fundamentally the same as those previously given for the problem of the wall. An additional condition is that the body be entirely surrounded by the bath.

If no heat escapes by radiation or convection and if all contact-conductivity can be overlooked, C is the coefficient of heat conductivity of the object.

The law of cooling was soon subjected to experimental tests. The classical verification is that of Richmann (the law itself is often called Richmann's law). Martine, in 1740, observed that for a good agreement between the theoretical law and the experimental data the temperature of the object should not differ from that of the bath by more than about 50° . Dalton showed that the cooling velocity is in reality more rapid than that indicated by Newton's law. Dulong and Petit, in 1817, undertook to establish more accurately the influence of each factor on the rate of cooling. They proposed corrections to render the formula applicable over a larger range of temperatures. Other corrections were introduced later by other investigators. But the fundamental law established by Newton, although correct only in a first approximation, is still usually employed in most of the practical applications. (Cf. Chapuis and Berget: "Leçons de Physique Générale." Paris, 1911, Vol. I, p. 622.)

Perhaps, when erroneous conclusions are drawn from the application of the law of cooling, it is not so much on account of its lack of accuracy but because some of the conditions for its application are not fulfilled; for example, the object to be cooled may not be entirely surrounded by the bath, as when use is made of a too bulky thermometer-stem or of too conductive thermocouple-leads which establish thermal connections with the outside.

All the problems so far treated have been analysed as particular cases of the original assumption of an ideal wall limited by two surfaces at constant temperatures. In many instances one or both of these temperatures vary. There results a large number of possible problems to be analysed by further application of the method of simplifying assumptions.

SUMMARY

The essential points brought out in the preceding pages can be summarized as follows: 1. Most of the investigations on thermometry and calorimetry to be described

or discussed later in this work are but applications of the fundamental principles of heat transmission. 2. These principles are briefly discussed under the form of: a) The "Problem of the Wall," in the steady state; b) The "Problem of the Wall," in the variable state; c) The problem of the cooling body. 3. The fundamental laws are sufficiently well established theoretically and experimentally in some simplified conditions, but in the more complicated conditions which obtain in nature and in the laboratory and in which they are supposed to apply, they are not always proven to be applicable. 4. The acceptance of "established" laws without previous analysis of the principles on which these laws are based led to misinterpretations and omissions. A notion almost always forgotten is, for example, that of "contact conductivity."

CHAPTER I

FREEZING. THE FROZEN STATE AND MELTING

I. INITIATION OF CRYSTALLIZATION

A. THE FORMATION OF CRYSTALLINE NUCLEI

Physics has acquired a wealth of information during the last 30 years on the structure of crystals, but very little has been learned yet on the mechanism of initiation of crystallization.

The condition *sine qua non* for the molecules of a liquid to be hooked up in a crystalline pattern is the reduction of their mean distance to a certain minimum, that is, the lowering of their temperature to the freezing point.

When the liquid to be solidified is at a convenient temperature and when a crystal of the substance is present, crystallization proceeds at once, but if a crystal is not there to initiate the process, that is, to give a direction to the molecules, the latter have first to orient themselves and build up the original nuclei. Crystallization nuclei are understood to originate from the collision of molecules coming in contact in certain definite conditions of orientation. After a first successful collision, a pair of molecules would be constituted, but this would not yet be the crystal unit wanted; other successive collisions under definite conditions would be required to complete the original crystallite.

Though our knowledge of the mechanism of formation of the first crystallization nuclei is to a large extent theoretical, their coming into existence as individual centers is a well observed fact; the centers can be photographed and counted. Figure 4 represents the formation of such centers in a solution of gelatine spread between two glass slides and immersed in a bath at -25° . Tammann (1925, p. 228) has shown that during a gradual decrease of temperature (below the freezing point), the number of crystallization centers formed per unit of time increases to a maximum and then decreases, following a curve of the

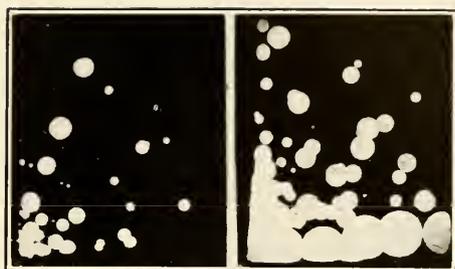


FIG. 4. Formation of crystallization nuclei in a film of gelatin solution enclosed between two thin glass plates and immersed in a bath at -25° . The lower left-hand corner was the first part of each of the two preparations to be immersed in the bath. (Original, Luyet and Geheuo.)

type represented in Figure 5. Unfortunately, little is known of the course of such curves for water, aqueous solutions and aqueous colloids.

Some physical chemists consider the congelation of water not as the crystallization of a liquid but as the precipitation of a solute from a saturated solution. What we ordinarily call water would be a solution of ice (a trihydrol

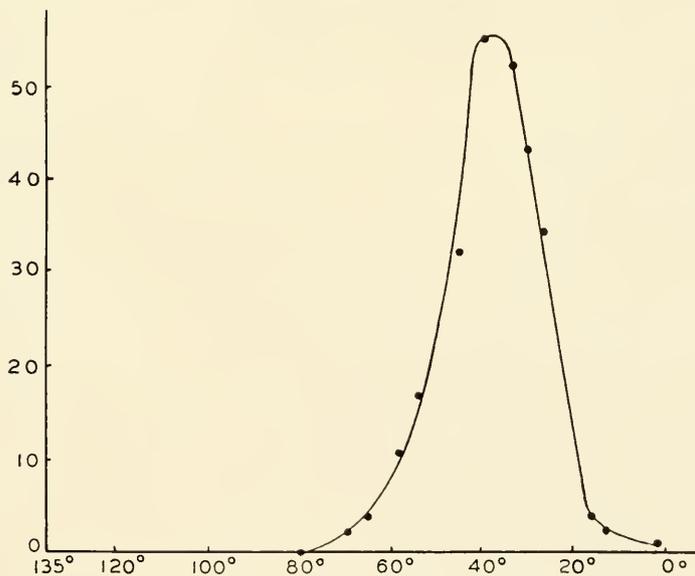


FIG. 5. Number of crystallization nuclei formed in piperine at various sub-cooling temperatures. (Curve drawn from Tammann's data, 1925.) Abscissae: temperatures (the melting point of piperine, 135° , being the origin); ordinates: number of nuclei.

of formula $(\text{H}_2\text{O})_3$ in liquid water (a dihydrol of formula $(\text{H}_2\text{O})_2$); at 0° the solution would be saturated and ice would precipitate. The mechanism of initiation of crystallization assumed above is consistent with this theory of water being a solution as well as with the usually accepted view that water is a definite compound.

A new light has been thrown on the subject by the investigations of Barnes ("Ice Engineering," Montreal, 1928, p. 7), who attempted to observe under the microscope the formation of ice in water. He states that: "The particles of ice as soon as they can be seen are devoid of crystal form and appear as a true colloid in small disc like particles. These flocculate and grow, passing through a crystal colloidal form to a true ice crystal." (*Cf.* Fig. 6). More

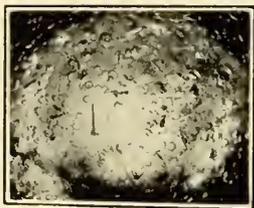


FIG. 6. Disc-like particles of colloidal ice. (From Barnes, 1928.)

experimental investigations in that direction might prove highly significant for the knowledge of the mechanism of initiation of crystallization.

Bernal and Fowler (1933) have suggested that liquid water might possess a quasi-crystalline structure. Five molecules would hook up together to form a tetrahedral figure (centered tetrahedron); these tetrahedra would aggregate, according to a definite pattern, in a mass of water. It is evident that, if the structure of liquid water is such, our notions of the passage from the liquid to the crystalline state, and in particular of initiation of crystallization, should be radically modified.

B. THE FREEZING POINTS

Most of the freezing point determinations of biological material have been undertaken for the information that

they furnish on the osmotic pressure of organic fluids and on the binding of the water molecule by protoplasmic substances. We shall summarize here some of the more representative works, but we refer the readers to reviews on the two subjects mentioned, for more complete data.

1. *Culture Media*. The freezing point of fresh water was found to be, in general, 0.03 or 0.04 of a degree lower than that of distilled water.

Backman and Runnström (1912) report that the water from which they collected frog's eggs froze at -0.060° .

Bialaszewicz (1912) found a freezing point of -0.01° to -0.02° for the tap water that he used for studying the development of amphibian eggs.

The freezing point of sea water, determined by various authors, mostly at marine biological stations, was found to vary from -1.09° at Kiel (Dakin, *Biochem. Jour.* 3, 258, 1908) to -2.29° at Naples (Bottazzi, *Arch. Ital. de Biol.*, 28, 61, 1897). At the American biological stations of Woods Hole (Mass.), Pacific Grove (Cal.) and Beaufort (N. C.), Garrey (1905) obtained respectively -1.81° , -1.92° and -2.04° . In a later work of the last mentioned author (1915), the reader will find tables of the freezing points of sea water at various dilutions and of solutions of NaCl and $MgCl_2$ at concentrations osmotically comparable to sea water.

Jensen and Fischer (1910) give -0.42° as the freezing point of the "physiological solution," that is, of a 0.7% solution of sodium chloride.

Of the substances used in culture media, sucrose was found to lower the freezing point more than was expected by the law of the freezing point depression. A gram molecular solution freezes at -2.775° , according to Garrey (1915). Loeb ("Artificial Parthenogenesis and Fertilization," Chicago, 1913, p. 130) pointed out the biological significance of this fact, which is usually attributed to a hydration of the molecules of sucrose. For more data and discussion see Morse, Frazer, Hoffman and Kenyon (*Am. Chem. Jour.*, 36, 39, 1906).

2. *Plant Juices.* A large list of freezing points of extracted plant juices is given by Dixon and Atkins (1910), in a table reproduced in *Tabulae Biologicae* (1, 431-434, 1925). The freezing points vary from -0.357° to -2.455° .

Harris, Gortner, Hofman and Valentine (1921) mention the exceptional case of a plant, *Atriplex nuttallii*, which was collected near the Great Salt Lake and which had a freezing point of -14.4° . The same authors obtained exceptionally high freezing points in cactuses.

According to Harris, Gortner and Lawrence (1917), the freezing point is higher in juices extracted from leaves near the ground than in leaves taken higher up. The higher osmotic pressure at higher levels is thought to play a role in the ascent of sap.

3. *Blood and Body Fluids.* Collip (1920a) measured the freezing point of the blood of animals belonging to various orders. He found values varying in mammals from -0.48° to -0.70° , in birds from -0.55° to -0.69° , in reptiles from -0.46° to -0.70° , in amphibia from -0.44° to -0.76° , in fresh water fishes from -0.45° to -0.69° .

The blood cells, in Collip's experiments, froze, on an average, at a temperature 0.043 degree *higher* than the serum.

According to various authors (quoted by McClendon and Medes, "Physical Chemistry in Biology and Medicine," Philadelphia, 1925, p. 300), the fluids of the mammalian body have, in general, a freezing point very near that of the whole blood. Such is the case for milk, gastric juice, pancreatic juice, bile, intestinal juice, cerebrospinal and spermatie fluid. Saliva, and sweat were often found to have a higher freezing point, reaching about -0.1° . Urine had a much lower value, sometimes as low as -2.6° .

Individual Differences. Some authors have thought that the freezing points of body fluids or of tissues might be specific characteristics of some animal (or plant) group. Atkins (1909a) attempted to study the individual differences in the freezing point of the blood of birds within the same species. He found—quoting only the

results that he considers reliable—0.1 of a degree difference, between the extremes, in the freezing points of the blood of 10 ducks and about 0.3 of a degree, between the extremes, in 7 turkeys.

R E L A T I O N B E T W E E N T H E E X T E R N A L A N D I N T E R N A L M E D I U M. The problem of the adjustment of the fluids of the body to the external medium has given rise to a series of observations on the comparative freezing points of sea or fresh water and of the blood of marine, fresh water or land animals. A first review of the subject has been made by Höber ("Physikalische Chemie der Zelle und der Gewebe," Leipzig, 1902, Chapters 2 and 12).

Since the publication of this review, Garrey (1915) reported that the freezing point of the blood of several marine animals, including large-sized fishes such as the shark, was very near that of sea water. The freezing point of fresh water animals, for example, fishes of the Mississippi River, varied from -0.48° to -0.52° in 6 species, and was, therefore, considerably higher than the freezing point of the blood of marine fishes and definitely lower than that of fresh water itself.

The blood of the marine turtles, unlike that of other sea-animals, had a freezing point of -0.69° , that is, markedly higher than that of the water in which they were living, which was -2.04° . To study the possibility of an adjustment of the internal to the external medium, in these animals, Garrey measured the freezing point of the blood of two marine species left for 2 months in a tank containing fresh water. He found the same value as for the animals taken directly from sea water. However, some adjustment in other animal groups, such as fishes, has been shown by Garrey himself in some of his previous investigations (1905) and also by other workers, in particular by Scott (1913).

The last mentioned author published, in 1916, a review in which the results of numerous investigators are tabulated. There are listed 111 species from the most important phyla of the animal kingdom.

Collip (1920a) made observations leading to the same conclusions as those of Garrey concerning the difference between fresh water and marine animals.

Duval (*C.R. Soc. Biol.*, 89, 22, 1923) showed that the freezing point of the serum of sea fishes was due, to a large extent, to factors other than the mineral salt content. The freezing point of the serum was -1.89° (that of sea water -1.84°) and that of a water solution of the ashes from the dried serum, diluted to occupy the same volume as the serum itself, was -1.06° . If there is an adjustment of the internal to the external medium, therefore, the modifications of the blood should involve more than a change in the salt concentration.

4. *Eggs.* Abundant data on the freezing points of insect eggs will be found in Bachmetjew (1901 and 1907).

Bialaszewicz (1912) and Backman and Rummström (1912) made several determinations on amphibian eggs (see below).

The chicken egg, according to Atkins (1909a), freezes at -0.454° , there being no difference between the freezing point of the yolk and that of the white.

Howard (*J. Gen. Physiol.*, 16, 107, 1932), like Atkins, reported that the yolk and the white had nearly the same freezing point.

Straub (*Rec. Trav. Chim. Pays-Bas*, 48, 49, 1929), on the contrary, found that the yolk froze more than 0.1 degree lower than the white.

Hale (1935), in a series of very accurate determinations, observed that the yolk never froze above -0.57° and the white never above -0.42° .

Hale made several other concomitant observations which deserve mention: a) The various layers of white had the same freezing point; b) The intact yolk froze at a slightly lower temperature than broken yolk; c) When the vitelline membrane was punctured, ice started at the punctured point; d) A yolk surrounded by a thin layer of white did not freeze when the latter froze.

According to the same investigator, the discrepancies

between the results of the authors mentioned above might be due to the fact that some worked with intact and some with stirred yolk.

Specific Differences. Bialaszewicz (1912) found -0.444° , -0.446° and -0.455° for the freezing points of the ovarian eggs of, respectively, *Rana fusca*, *Rana esculenta* and *Bombinator igneus*.

Atkins (1909a) gives -0.454° , -0.452° and -0.420° as the freezing points of the eggs of the chicken, the duck and the goose.

Individual Differences. The last mentioned author reported a difference of 0.05 degree between the extremes of 12 hen's eggs and 0.09 degree between the extremes of 7 duck eggs.

Moran (1925) observed individual differences of the same order for both the white of egg and the yolk.

Cycle in the Freezing Points of Developing Eggs. Atkins (1909a), investigating the difference between the osmotic pressure of the blood and that of the eggs, in birds, as related to the exchanges between the egg and the mother's body, obtained a freezing point 0.13 degree lower for the blood than for the eggs. He attributes this fact to a higher concentration of inorganic salts in the plasma of the blood, as a determination of the chlorine content showed.

Atkins (1909b) furthermore observed that the freezing point of the eggs drops, during incubation, by about 0.15 degree. The mixed content of the egg (after the separation of the embryo, at the end of the incubation period) froze at -0.611° , that is, at nearly the same point as the blood. The author suggests that the animals in the phylogenetic series might exhibit the same differences in the osmotic pressure of their internal fluids as do the embryonic forms in the ontogenetic series. According to this view, the birds, for example, should have body fluids of lower freezing point than the reptiles from which they descend.

Bialaszewicz (1912) gives the following figures for the

freezing points of the yolk of the chicken egg during its formation and development :

Ovarian egg, $\frac{3}{4}$ to 1 cm. in diameter :	- 0.632°
Ovarian egg 3 cm. in diameter :	- 0.613°
Eggs half way down the oviduct :	- 0.585°
Freshly laid eggs :	- 0.564°
Embryo at the 8th day of inc. :	- 0.496°
Embryo at the 18th day of inc. :	- 0.601°
Blood of the adult :	- 0.635°

So, when it is detached from the ovary, the egg has about the same freezing point as the blood. During the growth period, the freezing point increases to a maximum. During embryonic development, it gradually drops again to its original value.

The amniotic fluid froze at -0.582° and this point did not change significantly up to the 18th day of incubation. The freezing point of the allantoic fluid, on the other hand, rose from -0.513° to -0.431° from the 8th to the 18th day. The egg white froze at -0.458° in fresh eggs and at -0.444° after 8 days of incubation.

The same author reported a change in the freezing point of the frog's egg from -0.444° for the ovarian egg, to -0.294° on the 3rd day after fertilization and to -0.382° 12 days later. The blood of the adult frog froze at -0.479° .

According to Backman and Runnström (1912), the cycle of changes of the freezing point in the frog's egg and embryo (*Rana temporaria*) is as follows:

Ovarian egg :	- 0.48°
Fertilized, unsegmented egg :	- 0.045°
Stage of crescentic blastopore :	- 0.215°
Stage of spherical blastopore :	- 0.215°
Embryo, 5 days old :	- 0.230°
Larvae, 20-25 days old :	- 0.405°
Serum of the adult :	- 0.465°

These data confirm the general results obtained by previous investigators and, furthermore, they point out a remarkable rise of the freezing point at the time of fertilization.

5. *Protoplasm.* Chambers and Hale (1932) induced freezing in *amoeba proteus* by seeding the supercooled protoplasm with an ice-tipped micromanipulator needle at a temperature of -0.8° and below.

Gehenio and Luyet (unpublished work) determined the freezing point of the living plasmodium of the myxomycete *Physarum polycephalum*. Some 5 cc of protoplasm collected from agar cultures were used in each determination. The values found in 7 specimens average -0.17° .

6. *Tissues.* The determination of the freezing points of living tissues by any of the methods used involves an element of uncertainty. Ice-seeding, to prevent subcooling, or at least to prevent a too considerable degree of subcooling, is an essential procedure for any accurate freezing point determination. But ice-seeding through cell walls is not always efficient; only some cells might freeze after seeding or spontaneously, others not; the number of cells frozen might not suffice to bring the temperature of the subcooled object up to the freezing point. On that basis one should, perhaps, question most of the results obtained for the freezing points of living tissues.

Collip (1920b) determined the freezing point of the pulp obtained by grinding various tissues of the dog. He found values varying from -0.74° to -0.87° for the heart, the spleen and the lungs, -0.91° for the liver, and -0.76° for the brain.

Jensen and Fischer (1910) obtained, for the frog's muscle, in the living state, freezing points varying from -0.46° and -0.53° .

Cameron and Brownlee (1913) give -0.44° as the freezing point of an entire frog exposed to the freezing temperature with a thermometer in its stomach.

Living and Dead Tissues. Müller-Thurgau (1886) noticed that living tissues have a lower freezing point than dead ones, that is, than tissues killed by a first freezing. He found -0.98° and -0.55° , respectively, for living and dead potato tuber, and likewise, -0.8° and -0.4° for living and dead *Phaseolus* leaves. For the determina-

tions on potato, a mercury thermometer was inserted into a cavity bored in the tuber; for those on leaves, the bulb of the thermometer was wrapped in these organs. The cause of the differences observed is attributed to the fact that the capillary spaces between the intact membranes of the cells in the living tissues hold water more firmly and render freezing more difficult than in dead material.

Maximov (1914) observed similar differences in red beet; he obtained -2.15° and -1.25° for living and dead material, respectively, and -1.21° for the extracted beet juice. According to this author, the resistance presented by the living cellular membranes to the outward passage of water during freezing is responsible for the lower freezing point of the living material. In dead tissue, the sap forms a uniform fluid mass which freezes like a solution, there being no membrane resistance to overcome; in living tissue, the sap has to be extruded from the cells before it freezes; it is extruded in small quantities at a time and the heat developed by the freezing of such small quantities is not enough to raise the temperature of the whole tissue to its real freezing point.

Walter and Weismann (1936) found, in pieces of potato frozen several times in succession, a rise of the freezing point at each of the first few congelations (for example, -0.89° , -0.76° , -0.72° , -0.69° , in a series of 4 successive experiments). Thereupon the freezing points oscillated in an irregular manner. These authors determined by the browning of the tissue and the loss of turgor the increasing number of dead cells in successive freezings and they claim that it is the proportion of living cells which determines the level of the freezing point. Dead matter freezes first and the heat produced during its congelation, being absorbed by the bulk of surviving material, cannot raise the temperature of the whole to the same level as when all the cells are dead. Besides, in completely dead material, the freezing point may be high because the sap is diluted by the water which was bound in the living state and which is set free at death.

Jaccard and Frey-Wyssling (1934) reported a sharp rise in the freezing point of the tissue of the root of *Daucus carota* after the first congelation, but this rise was followed by an irregular drop in subsequent freezings. In pieces killed by heat, the freezing point remained constant. They attribute the rise in the freezing point at the second freezing to the fact that the water extracted from the cells during the first freezing dilutes the extruded sap around the thermocouple, which they used in their determinations. Walter and Weismann object to this interpretation, claiming that the water extruded should be reabsorbed if the tissue is still alive as Jaccard and Frey-Wyssling assume.

According to Luyet and Gehenio (1937), the freezing point of living potato tissue is, on an average, by as much as 1.5 degree lower than that of dead material. In a tentative explanation of this difference, they distinguish three kinds of water in protoplasm: 1. water which acts simply as a solvent in the cell sap, 2. water which behaves as a protoplasmic constituent, 3. water which, in dead as well as in living matter, is bound in such a way that it cannot freeze; and they attribute the higher freezing point in dead tissue to the fact that, at death, protoplasmic water is transformed into solvent, readily-freezable water.

This assumption, however, does not seem to be tenable in view of new findings of Gehenio and Luyet (unpublished) that protoplasm which has no cellular structure, like that of the myxomycetes (*Physarum polycephalum*), does not present the difference observed between living and dead tissues. So, finally, the lower freezing point of living tissues seems to be due not to a binding of water in a *protoplasmic* structure but to a hindering of the water activity by a cellular structure, probably the cellular membranes.

Jensen and Fischer (1910) observed that the freezing point was lower in dead than in living muscle. They attribute this fact to some binding of water at death.

Influence of Cooling Velocity on Freezing Points. The use of a high cooling velocity has been found by many investigators to result in a lowering of

the freezing point, particularly in living tissues. This subject will be discussed later in the section on Freezing Curves.

The Double Freezing Point of Living Tissues. Maximov (1914) described a very particular course in the freezing curve of the living tissue of the petiole of *Tussilago farfara*. After subcooling, the temperature rose rapidly, then it sank a little and either stayed at that level or rose again slightly (*cf.* Curves 1 and 2, Fig. 7). There were apparently two freezing points marked by two maxima on the curve. This author observed furthermore that only the living tissues presented the double freezing point, and that soaking the material in water favored the phenomenon, while drying the canal through which the thermocouple was inserted into the tissue prevented it. On the basis of these observations he attributed the first freezing point to the congelation of the extruded cell sap which surrounded the thermocouple.

Zacharowa (1926), also using a thermocouple and following Maximov's procedure, observed the same phenomenon in roots of rye seedlings (Curve 3, Fig. 7). She admits Maximov's interpretation.

Walter and Weismann (1936) confirmed the observations and interpretations of the previous investigators on potato tuber. They used a mercury thermometer (Curve 4, Fig. 7).

According to Mez (1905), one can observe, in the freezing curves of plant tissues, two plateaus which correspond respectively to the freezing and to the eutectic point of salt solutions.

Voigtländer (1909), following Mez' views, presented a vast amount of data which, when plotted, showed two periods of retardation in the drop of temperature (*cf.* Curve 5, Fig. 7). He took for granted that these periods corresponded to the freezing and the eutectic points (F and E in the figure).

Luyet and Gehenio (1937) made a special study of the factors involved in the doubling of the freezing point

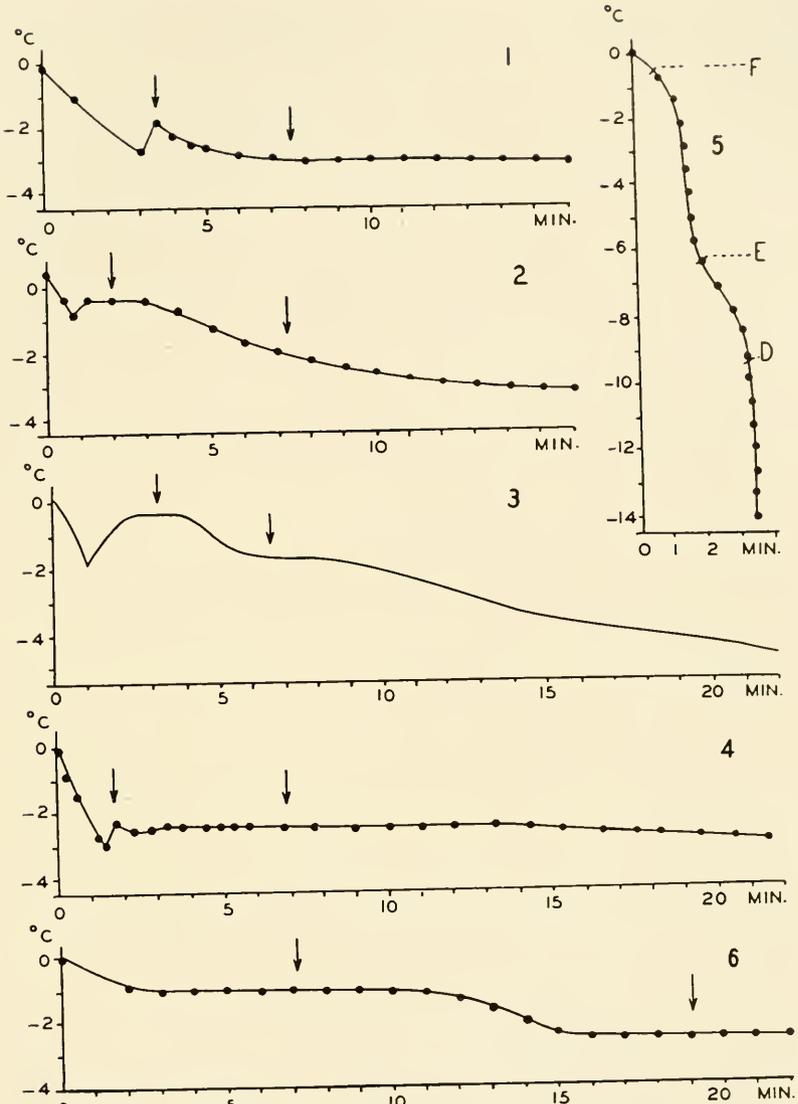


FIG. 7. The double freezing point as observed by various authors. For the sake of comparison, all the curves have been plotted to the same scale. (From Luyet and Gehenio, 1937.)

(Curve 6, Fig. 7). They used potato tissue in the form of hollow cylinders slipped about the bulb of a mercury thermometer. In order to control the cooling velocity and the

thermal gradient through the material, they separated the latter from the cooling bath by media of various thickness and of various heat conductivity, such as mercury or paraffin oil. Their findings can be summarized as follows: 1. The observations of previous investigators, according to which dead tissues do not present a double freezing point, were verified in two series of experiments: one on living tissues in which 33 out of 68 specimens showed the double freezing point, and one on dead tissues (killed by previous freezing) in which none of the 44 specimens experimented upon presented the double freezing point. 2. The observations to the effect that soaking the tissues induces double freezing, while drying prevents it, were also confirmed; the per cent of cases presenting a double freezing increased from 49 to 90 by a soaking of the tissue in water for 30 minutes or more and decreased to 0 by a drying in the air for 3 hours or more. 3. The first freezing point in normal living tissues varied from -1.2° to -2.0° , the second from -1.45° to -2.74° ; that of dead tissue was higher than both and varied from -0.5° to -0.75° . 4. The first freezing point was raised by soaking; in some soaked tissues it reached -0.05° . The second was not influenced by the imbibed water. 5. When congelation was stopped at the passage from the first freezing point to the second, the tissue was uninjured and the first freezing point could be obtained repeatedly in subsequent experiments. 6. The position of the first freezing point was not affected by a change in the cooling velocity, while that of the second moved from -1.45° to -2.3° for velocities varying from 2.5 to 5.1 degrees per minute.

In the light of these findings the authors discuss the following four theories on the doubling of the freezing point in living tissues:

1. The "Wound-Sap" theory, proposed by Maximov, according to which, as said above, the two points would represent, respectively, the freezing of the sap extruded from the cells through the wound caused by the insertion of the thermo-couple and the freezing of the water of the

tissue itself. An objection to this theory is that the quantity of water which congeals at the first freezing point is often considerably greater than that which can possibly be extruded in the wounding.

2. The "Thermal Gradient" theory, suggested to Luyet and Gehenio by the fact that the wave of congelation which moves from the external surface of the object inward and which liberates heat gives rise to a wave of heat which travels toward the thermometer. This stops or retards the cooling curve, thus producing an apparent freezing point. But cases were observed in which the temperature stayed constant at the first freezing point for nearly 10 minutes which was evidently more than the time necessary to dissipate a wave of heat, in the conditions of the experiments.

3. The theory of the "Eutectic Point" (Mez and Voigtländer), which considers the two points as, respectively, the freezing and the eutectic point of the tissue. The extremely high cooling velocities used by these authors in their determinations (*cf.* Curve 5, Fig. 7) render their results the most uncertain. (For a more detailed discussion see below, under Freezing Curves.)

4. The theory of the "Double Freezing Point," according to which the 2 freezings are not only apparent and attributable to the procedure or to the apparatus but real, one being the congelation of *intercellular*, the other of *intracellular* water (the latter would freeze after extraction from the cells by osmosis or within the larger vacuoles). Luyet and Gehenio, after proposing the theory, mention its agreement with most of the facts observed: the occurrence of the double freezing in living tissues only, the innocuous effect of a congelation below the first (but above the second) freezing point, the absence of a first freezing in dried tissues, its more frequent occurrence in soaked tissues, the rise of the first freezing point when more water is imbibed, etc. They point out, however, that the existence of two different freezing points, one for the intercellular and the other for the intracellular fluids would

involve a permanent osmotic disequilibrium between the cell content and the intercellular spaces.

II. PROGRESS OF CRYSTALLIZATION

A. THE GROWTH OF CRYSTALS

When a crystallization center is formed, it grows with a speed which depends primarily on the temperature. Under the same conditions, the different faces of a given crystal grow with different velocities, each set of faces having a velocity coefficient. To simplify the study of the crystallization velocity, the physicists consider separately the linear growth of each face of the crystal, that is, the growth in a direction perpendicular to that face. The same general laws of growth, with various coefficients, apply to all faces.

It is generally thought that the velocity of crystallization increases when the temperature decreases (below the freezing point), in other words, that the colder a body is, the faster it freezes. Several experimenters who studied this relation have obtained data which confirm the commonly accepted view and which follow a curve of the type represented in CDEF, Figure 8. But Tammann, amongst others, pointed out that an increase in velocity for a decrease in temperature would be contrary to the general laws governing physical and chemical transformations (*cf.* Tammann, *op. cit.*, 1925, p. 251). He then showed that the direct experimental determination of the temperature of crystallization always involved an error. The temperature measured by the experimenter is that of the liquid in which crystallization occurs and not that of the growing surface of the crystal. When the thermometer or thermocouple is placed as near as practically possible to the crystal, it is still relatively too far from the active surface to give any adequate measure of the temperature of this surface itself. The heat continuously produced by crystallization on the growing surface maintains the local temperature there higher than that of the surrounding medium where the thermometer is placed. Even the finest

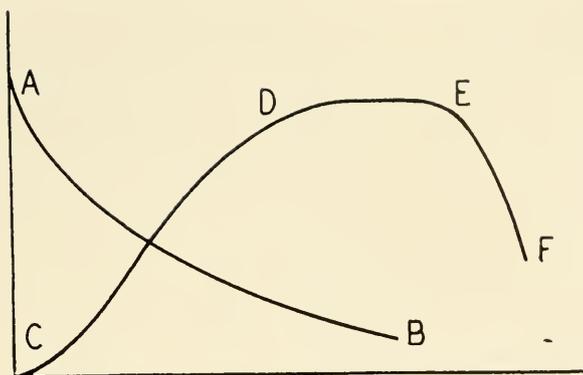


FIG. 8. Relation between crystallization velocity and temperature. (After Tammann, 1925.) The crystallization velocities are plotted in ordinates, increasing from the origin; the temperatures are plotted in abscissae, decreasing from the origin, the latter being the freezing point. The curve CDEF represents experimental data (it is a composite curve drawn from several of Tammann's observations). The curve AB represents the data that one should obtain if one could avoid the experimental errors inherent in the procedure.

available thermoneedles have too high a heat capacity to register this local temperature with any accuracy.

Another source of error pointed out by Tammann is that the heat liberated during crystallization at the growing surface of the crystals causes interruptions in the crystallization process, the latter being resumed only after the dissipation of the heat liberated in the preceding congealation; consequently, the duration of crystallization experimentally determined consists of a succession of crystallization periods and interruption periods and the resulting figure for velocity is too low.

Taking these facts into consideration, Tammann concluded that the relation between the velocity of crystallization and the temperature should be of the type represented by the curve AB, Figure 8. Crystallization would then proceed more slowly at lower temperatures. Indirect temperature determinations furnished an experimental verification of this relation.

It is of interest to notice that the physicists who, in general, are better trained than other experimenters in the use

of thermometric devices, were induced into error by a misinterpretation of the readings of thermometers. Their attempt to determine directly the temperature at the surface of a growing crystal by the finest thermocouple placed the nearest possible to the surface, is comparable to an attempt at measuring the temperature of a burning match with a giant thermometer (having a bulb several inches in diameter) separated from the match by a distance of a centimeter or so. In the last analysis, the error is due to the fact that too many experimenters *believe* in thermometers; they take for granted that a thermometer is an apparatus which gives the temperature at a point in space, while a thermometer is a complicated system of bodies in contact, through which heat flows, usually in an uncontrolled manner, and from the behavior of which we try to guess (or to use a euphemism, to calculate) what the temperature is at a point in the neighborhood of the system. Since such errors probably escape the attention of the investigators more often than is usually thought, we deemed it justifiable to discuss the fundamentals of thermometry, that is, heat conduction, in a preliminary chapter of the present work.

Strange as it may seem, then, the fact is that the lower the temperature is, the slower is the freezing (crystallization) and when the temperature reaches a certain minimum, freezing becomes impossible because of the too intense cold.

Since the maximum of the curve of the number of crystallization centers (Fig. 5) is at a lower temperature than the maximum of the curve for the velocity of crystallization (Fig. 8, CDEF), it is clear that at temperatures at which the number of centers is high and the velocity low, a relatively large number of small crystals will be formed, while at temperatures at which the number of centers is low and the velocity high, there will be a small number of large crystals. The latter condition is realized at higher temperatures near the freezing point, the former at lower temperatures.

The growth velocity coefficients for the different faces of a crystal do not vary at the same rate when the temperature is changed, as we indicated above; hence, some faces become prominent at certain temperatures and the crystal develops more in the direction of these faces. The temperature of crystallization determines, therefore, the shape of the crystals. In general when the velocity of crystallization is very low for one face it is low for all the faces and the crystal has the shape of a spherulite.

Walton and Judd (1914) studied the rate of growth of ice in a long glass tube previously filled with distilled water and subcooled to various temperatures. They initiated crystallization by ice-seeding at one end of the tube and measured the velocity of the congelation-wave as it moved toward the other end. They found a rate of 65 mm. per second at -8° .

Hartmann (1914), in experiments of the same kind, obtained 46.6 mm. per second at -7° .

Tammann and Büchner (1935a), who succeeded in keeping water subcooled to -13.4° , found 41.3 mm. per second at -7.2° and 96.8 mm. at -13.4° . They also measured the crystallization velocity when ice was formed from heavy water and obtained almost the same values as with ordinary water, at the same degrees of subcooling (that is, for temperatures calculated from $+3.8^{\circ}$ as the freezing point of heavy water and from 0° as the freezing point of ordinary water).

The same authors (1935b) determined the lowering of the crystallization velocity of water when substances such as sodium chloride, sulphuric acid, glycerine, alcohol and sugar, were dissolved in it. They found that, at 5 degrees below the freezing point, 0.85 mole of sodium chloride in 1000 gr. of water slows the rate of congelation to about 6 mm. per second; while 1.1 and 2.9 moles of sugar, in the same conditions, slow it to about 0.3 and 0.02 mm. respectively. Sugar, it was remarked, has a particularly strong retarding effect. It might be of interest to notice that in very dilute concentrations (about 0.01 mole per liter) the

crystallization velocity was slightly increased by the substances dissolved.

Callow (1925) studied the action of various concentrations of gelatin on the growth of ice crystals in gels of that substance. He found that 1% gelatin reduces the rate of crystallization of water to about half its value, 1½% renders the velocity 45 times lower, and 3% renders it 350 times lower.

The velocity of crystallization in biological material is entirely unknown.

B. THE PHASE SEPARATION

1. *Solutions and Suspensions.* It is well known that, in the freezing of a solution, water separates from the solute and freezes alone, while the solution becomes more concentrated. Some biologists have attempted to observe this separation under the microscope. Molisch (1897) says that, by mounting drops of 10% solutions of sodium chloride, potassium nitrate, magnesium sulfate, cobalt chloride, etc., on microscope slides and exposing them to low temperatures, he could see the water freeze out in several crystallization centers and form ice masses which wedged between them the concentrated salt solution; the latter, in its turn, crystallized in a different form of crystal. This separation of water was seen in the same manner in solutions of dyes.

According to Goeppert (1830), when plants which contain a milky sap, for example, *Rhus*, *Euphorbia*, *Papaver*, *Ficus*, are frozen, the sap solidifies into transparent ice. However, the yellow sap of *Chelidonium* gave yellow ice.

Molisch (1897) reported that, when he put to freeze at a temperature of -6° , on the stage of the microscope, the milky sap of the fig tree, *Ficus elastica* (a substance which consists of an aqueous solution and of droplets of rubber), he could see the water freeze out into ice crystals which separated rows of particles of rubber. On thawing, the two phases mixed again. Similar experiments were made with suspensions of carmine, indigo and gum and the same

results were obtained. With carmine the network of particles stayed after thawing, which fact the author attributes to the adhesion caused by the pressure exerted by the ice. The Brownian movement of the carmine particles had ceased.

2. *Colloids.* Bruni (1909) described colloidal isinglass and colloidal silicic acid in the frozen state as consisting of colloidal particles interspersed with ice crystals. In silicic acid the two phases, one of which was pure water, remained separated after thawing.

Moran (1925) noticed that, after a congelation, followed by melting of the ice, the liquid portion of the white of egg had increased and the colloidal portion had decreased. He compares this phenomenon with the exudation of water from gelatin, from jellies and from the muscle, after thawing.

According to Molisch (1897), a layer of a gelatin gel frozen on a slide, under the microscope, allows one to observe the ice separated from the gel. There remains a gelatinous network with ice particles in the meshes (Fig. 9). After thawing, the meshwork can be preserved for

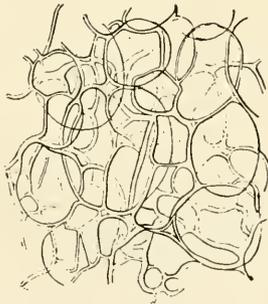


FIG. 9. Network left after the thawing of a frozen gelatin solution. (From Molisch, 1897.)

several days if the water content of the gel is low; it can also be fixed as a tissue, by formol, and kept indefinitely. With higher water contents, a reimbibition takes place and the meshwork disappears.

Bobertag, Feist and Fischer (1908) observed that, on thawing solutions of gelatin, carrageen, agar agar, isin-

glass, soap, etc., the water which had separated from the gelatinous mass during freezing was almost free from the dissolved substance. After complete thawing, one could still see the inhomogeneous mass to consist of clumps of the jelly and of a dilute solution.

The freezing of gelatin gels has been the object of extended studies by Moran (1926) and Hardy (1926). They observed three types of freezing: the compact surface freezing, the intermittent freezing and the disseminated freezing.

Compact Surface Freezing. Moran exposed to sub-zero temperatures discs of gelatin gels of various concentrations, measuring 3 mm. in thickness and 15 mm. in diameter. With water contents higher than 34% and temperatures from -3° to -19° , ice formed around the discs, while the inside gelatin core became more concentrated. The authors removed the ice shells and determined the amount of water so frozen and the maximal gelatin concentrations reached by the core. They found that these concentrations varied from 54.3% to 65.2% when the temperatures varied from -3° to -19° . If a preparation frozen at -3° was put consecutively at lower temperatures (even in liquid air) and brought back to -3° , the concentration was 54.3%, that is, the same as in gelatin frozen at -3° .

With very high water contents and low freezing temperatures, for example 88% and -11° , there were, in addition to the surface freezing, some centers of crystallization formed inside of the gelatin core where one could find places containing a "sponge of gel" (Fig. 10, A).

Briefly, when freezing was slow, water separated from the gel and came to the surface to solidify in a compact mass of ice; when freezing was rapid or when there was great abundance of water, crystallization took place inside the gel.

Intermittent Freezing. Moran, studying the congelation at -11° of gels containing 62% water, observed granulations, 2 to 3 mm. in diameter, of congealed material,

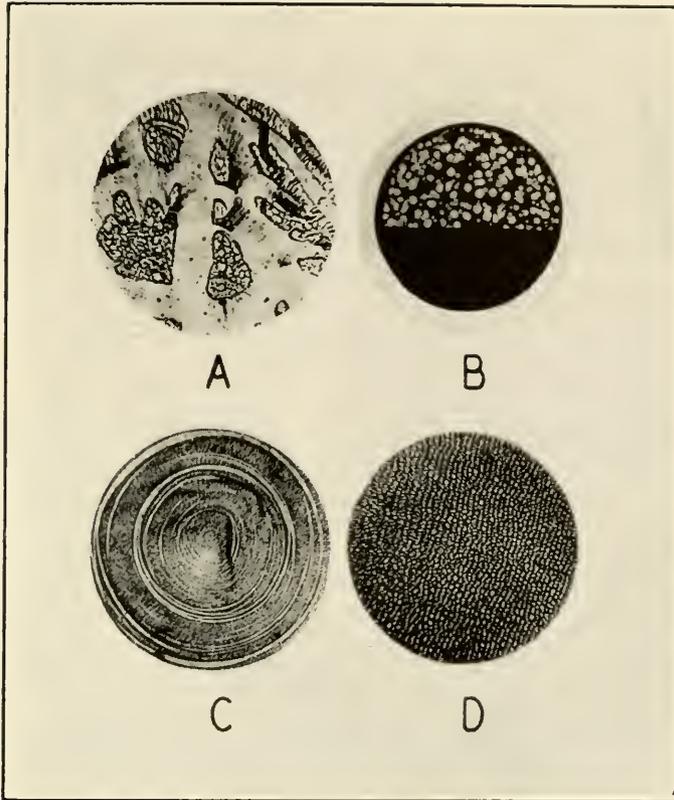


FIG. 10. Photomicrographs of sections through frozen gelatin gels. (From Moran, 1926.)

irregularly distributed in a non-frozen gelatin matrix (Fig. 10, B). Sections made through these granulations at -11° , with instruments maintained at that temperature, or at higher temperatures, after fixation with formol, revealed a structure consisting of alternating concentric layers (Fig. 10, C). Moran considers these layers as made respectively of ice, and of dehydrated gelatin.

Hardy (1926) studied Moran's spheres in greater detail. Layers of gelatin gels 0.5 mm. thick, containing the proper amount of water, were mounted between slide and cover-slip; they were, then, frozen at a convenient temperature and observed in a low temperature room. The concentric shells were seen to consist of two sorts of rings, one of

which formed "membranes" about 0.5 micron in thickness, while the other which seemed enclosed within these membranes, consisted of much broader layers. Outside of the rings, solidification continued in the form of rays (Fig. 11, A), the rings bulging into the base of the rays. The rays themselves were divided transversely into compartments by the curved membranes. At the tip of a ray, or at the



FIG. 11. Formation of "rays" at the periphery of crystalline discs in frozen gelatin gels. (From Hardy, 1926.) An entire ray is shown in A, and the terminal portion of a ray, at higher magnification, in B.

outer edge of a circle, one could see a series of fine etched lines (Fig. 11, B).

Moran explains the concentric structure by assuming that the heat developed in the formation of ice in a center of crystallization keeps the local temperature higher at that point for a time and prevents a further freezing. The gelatin in the immediate neighborhood becomes more concentrated on account of the withdrawal of the water transformed into ice and it cannot freeze. When the local temperature is lowered again, a new layer of ice will be formed. The concentric shells will result from such an alternation of warmings and coolings.

Hardy claims that, while the "membrane" phase consists of dehydrated gel, the layers between the membranes are not made of ice, as Moran thought, but of a solid solution of ice and gelatin. He bases his conclusions on the optical properties of the various phases of the system (in polarized light) and on the behavior of the material on thawing.

D i s s e m i n a t e d F r e e z i n g. With weaker concentrations or lower temperatures, for example, with 88% water at -19° or in liquid air, a third type of freezing occurred. Moran described it as follows: "The interior of

the gel was now occupied by a large number of clear spherical spaces each about 3 micra in diameter and arranged in rows." (Cf. Fig. 10, D).

Hardy called this kind of freezing "disseminated freezing." The spherical masses that he observed had a diameter as large as 20 micra. He showed, furthermore, that the spheres consisted of pure ice. To explain their formation, he suggested that they had started as crystallization centers, but that, owing to the rapid cooling, the viscosity of the material was soon high enough to prevent their further growth.

As it was said above, with high water contents and very slow freezing, some centers of crystallization are formed within the gelatin gel and there result irregularly distributed crystalline spots within the remaining non-crystalline gelatin. It seems that this type of freezing differs from the one just described under the name of disseminated freezing only by the shape of the crystals, which were prismatic in the first instance and spherical in the second. In both cases, the crystals are of pure ice, by opposition to what happens in the case of intermittent crystallization. So, we shall distinguish two types of disseminated freezing according to the shape of the crystals, and we shall call "Granular disseminated" the type in which the crystals are spherical and "Irregular disseminated" the other.

The researches of Moran and Hardy can then be summarized in the following points: 1. The irregular disseminated freezing was observed mostly in gels of high water content (88% water) but also with more concentrated gels if freezing was moderately rapid; 2. The granular disseminated freezing occurred with very rapid cooling (in liquid air and at -19°); 3. Surface freezing took place over a large range of water contents but it required a slow cooling; 4. Intermittent freezing extended also over a large range of water content and it occurred at intermediate cooling velocities (with an external temperature from -6° to -13.5°).

Moran pointed out another important fact, namely, that

a gelatin gel of which the water content is 34.5 % or less never freezes at any temperature.

The following table gives, in a comprehensive form, the conclusions of Moran and Hardy:

<i>Water Content</i>	<i>Cooling Velocity</i>	<i>Type of Freezing</i>	<i>Nature of Crystallizing Phase</i>
High, Medium	Low	Surface, Compact	Ice
High, Medium	High	Internal, Disseminated, Irregular	Ice
High, Medium	Very High	Internal, Disseminated, Granular	Ice
Medium	Medium, High	Internal, Intermittent	Ice-gelatin
Low	Any	No freezing	

3. *Coagulated Material.* Prillieux (1869b) described an exudation of ice from the white and from the yolk of a boiled egg when the latter was put to freeze under a cover, after the shell was removed. There was a layer of ice 1 mm. thick outside the white, and another of about the same thickness between the white and the yolk. The white was itself divided into several concentric layers of unfrozen, soft albumin, separated by shells of ice, some of which were about 1 mm. thick. The ice shells consisted of small ice columns with their axis perpendicular to the surface of the shells. There were tiny air bubbles along the axis of the columns.

4. *Porous material.* If the water which freezes out of a liquid is hindered in its withdrawal not only by the forces which hold it in solution or in suspension but also by capillary or osmotic forces, we should expect some difference in the form of the resulting crystals, their state of aggregation and their velocity of formation.

Rigaud (*London-Edinb. Phil. Mag.*, 2, 190, 1853) described a particular type of exudation of ice from the mortar of a stone wall. A plate of ice covered the wall. It consisted of juxtaposed ice columns which grew parallel to each other and perpendicular to the surface from which they arose. The columns were of all sizes up to several centimeters in length.

Leslie (*“Encyclopedia Britannica,”* vol, 3, art. Cold, p. 258, Supplement) observed a protrusion of ice filaments, like bundles of spun-glass, from a porous earthenware pan into which he put water and which he maintained in the presence of sulphuric acid in reduced air pressure. The water was conveyed through the pores and crystallized outside in ice shoots, perpendicular to the surface of the container.

Le Conte (1852) obtained a similar effect by soaking “the smaller portions of soft and spongy roots” of the cypress in potassium nitrate and letting them dry in the air. Crystalline fibers of the salt emanated at right angles to the surface. The author compares these fibers to the filaments of zinc sulphate which form at the surface of the earthenware cups used in batteries.

5. *Tissues. Surface Freezing.* The astronomer Herschel (the son of the famous William Herschel), in 1833, observed, on some decaying thistles and on the stumps of living heliotropes, ice ribbands with a fibrous structure, a silky surface and a frilled wavy shape (Fig. 12). The ribbands were formed longitudinally on the stem and per-

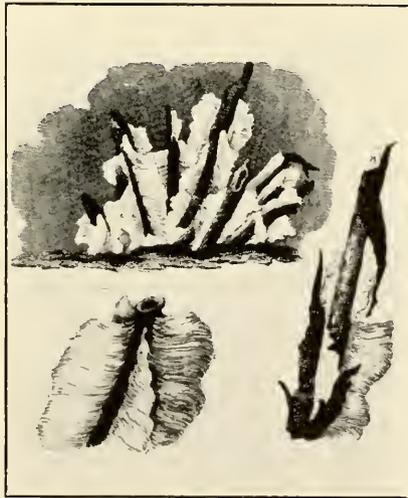


FIG. 12. Formation of ice ribbons on plant stumps. (From Herschel, 1833.)

pendicular to it. They originated on the wood, below the cortex, and bored their way through cracks in the latter. The ice fibers were perpendicular to the stem. The attachment of the riband to the stem was very light and did not correspond to any crack in the stem.

Dunal (1848) described on the square stems of some labiates, four ribbons of ice coming out from the sap wood, through the torn cortex.

Similar ice blades on plant stems were observed, two years later, in Georgia, by Le Conte who depicts the same details as the previous observers.

Sachs (1860) attempted to induce ice formations of the kind described, by exposing sections of beets to the frost, under a cover to avoid an excessive evaporation. He obtained plates of ice, of velvety appearance, made of ice columns perpendicular to the cut surface of the tissue. The author observed these phenomena on several types of vegetables. He noticed, furthermore, that the process could be more easily induced in succulent plants and when freezing was not too rapid.

Several attempts have been made to explain this exudation of ice in plant stems. Herschel notes that the water comes from within the plant and that it must finally come from the ground. The plant would work as a "chimney," moisture being exuded from the earth by "every open spiracle."

According to de Mohl ("Vermischte Schriften," Tübingen, 1845), the exudation of fluid from the tissues is due to the contraction of the latter under the action of cold.

Le Conte holds that water will begin to freeze at the external end of each capillary pore, where the contact with the air maintains a lower temperature. When the water freezes into ice columns, a lateral pressure would be exerted between the latter, due to the expansion which accompanies freezing. The ice plate constituted by the columns would then separate from the substratum in a direction perpendicular to its surface. When the ice columns have moved away from the broader open end of

the capillaries, water from within would fill the latter again and a new layer of ice would be formed as before.

Caspary (1854) suggests that, at freezing temperatures, for one reason or another, an exceptionally abundant amount of sap might ascend the plant through the vessels. That sap would traverse the walls of the vessels to freeze outside.

Sachs called the attention of the biologists to the fact that a body imbibed with a liquid is always surrounded by a film of that liquid. To show this, he covered with varnish a piece of a pig's bladder membrane and dipped it into water; the varnish, which before imbibition was adhering to the membrane, became loose, on account, says the author, of the formation of a film of water between the membrane and the varnish. Applying this principle to the present problem he assumed that the film of water present on each wet surface freezes first and that upon formation of a new film, it behaves as a removable layer.

Prillieux (1869a) objected to Le Conte that the size of the ice columns did not correspond to that of the capillaries. He objected to Sachs that a surface film of capillary size should resist congelation instead of initiating it. He furthermore remarked that the expansion of water between 4° and 0° is not sufficient to explain the exudation observed. Finally he proposed the following explanation. Water is held in the living cells or in the boiled white of egg by the forces of imbibition; the molecules of water which are farther away from the imbibing molecules and are not so strongly attracted by the latter, leave them and freeze. The crystals formed in that manner grow by attraction of new molecules of water. So, as we understand the author's interpretation, the columns of ice originate in larger intermolecular spaces and in pores.

It seems that, during the first half of this century, the attention of the biologists has been attracted by other questions and that the problem of the mechanism of these particular ice formations has been left unsolved.

6. *Tissues. Intercellular Freezing.* In 1817, du Petit-

Thouars described ice formations in the pith and in the cortical parenchyma of the stem of some plants (vine, elderberry, etc.) The ice crystals were occasionally so abundant that it was possible to obtain a dish-full of them. In some instances there was a complete cylinder of ice below the cortical layer. On melting, the ice collected in the tissues gave almost clear water.

Later de Mohl (*op. cit.*) discovered that, when the leaves fall after a freezing weather, a layer of ice can be seen at the base of the petiole, separating the leaf from the branch. The mechanism of the formation of this ice layer is probably the same as that of surface freezing of stems discussed above.

Caspary (*Bot. Zeitung*, 1854, p. 665) who had observed surface freezing on various plants, in the Schoeneberg garden, near Berlin, described also the formation of masses of ice in the interior of fresh stems, in particular, below the epidermis. He attempted an anatomical study of the tissues from which the ice originated. But he says that he could not ascertain whether the separation of the cortex from the wood and of the wood from the medulla was accompanied by a tearing of the cells or if the latter were only pushed apart.

Several observers, however, after Goeppert (1830), have pointed out that the cells, in frozen tissues, are not torn by ice crystals. Such an observation, together with that of the presence of ice in the intercellular spaces, contributed to establish the notion that water does not freeze in the cells but that it is withdrawn from them during the freezing process.

Sachs (1860, *cf.* also his "Textbook of Botany," Book III, Ch. III, Sect. 7), by cross-sectioning leaf stalks of a frozen artichoke, observed that the epidermis was separated from the parenchyma by a layer of ice, and that the parenchyma itself had split into several portions separated from each other by ice (Fig. 13). From pieces of leaf stalk weighing 396 grams he picked out as much as 99 grams of ice. This ice was almost pure water; after evaporation it left only 0.1% of solid substance.



FIG. 13. Section through the leaf-stalk of a frozen artichoke. (From Sachs, 1860.) The hatched spaces represent the ice masses, the black spaces, the cavities of the ruptured tissue.

Prillieux (1869a) insisted on the fact that, in frozen tissues, ice was found between the cells and not in them. This was observed in petioles, in buds, in stems, etc. Some stems presented, in a cross section, four masses of ice radially distributed, some three, some five, depending on their structural symmetry. A layer of ice always isolated the epidermis; the pith often seemed filled with ice crystals; the parenchyma was usually split into several portions separated by ice masses. Since the cell walls were not broken, Prillieux remarked that they could not have been traversed by ice crystals and that, therefore, it was in the liquid form, before freezing, that water had left the cells.

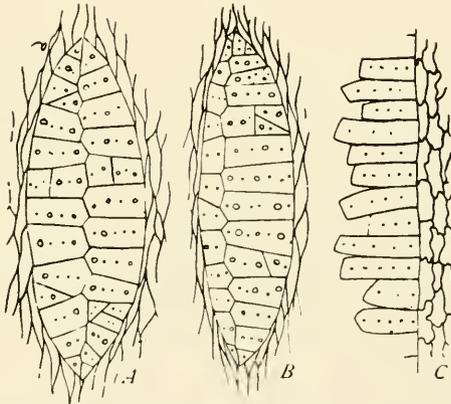


FIG. 14. Intercellular lens-shaped masses of ice in plant tissues. (From Wiegand, *Plant World*, 9, 26, 1906.) Each mass consists of two layers of ice pillars. In the middle of the tissue, (A) the pillars have the same length in the two layers; they are of unequal length nearer the external surface of the tissue (B); only one layer remains at the surface itself (C).

Müller-Thurgau (1886) studied the deposition of ice in the intercellulars by the following procedure. He sectioned with a cooled knife pieces of frozen tissues, such as beets, potatoes, dahlia tubers, etc., took out the little lens-shaped pieces of ice located between the cells and examined them on a cooled slide. These ice masses consisted of two layers of ice pillars (*cf.* Fig. 14). Each pillar was a more or less regular six-sided prism. Within the individual pillar, bead-like air-bubbles extended along the axis. The ice masses were of enormous size as compared with that of the cells. The cross section of the individual crystals was itself larger than that of the cells. The length of the columns was about the same in the two adjacent layers except if the ice mass had been formed near the external surface of the tissue, then the layer on the external side was thinner.

The surface freezing in compact ice crusts, as described before, is considered by this author as the limit in the series of forms that the ice masses take when they originate at points gradually nearer the outside surface. The formation of ice between the base of the petioles and the stem, described by de Mohl in his study of the fall of the leaves after a frost, is also considered a particular case of the same phenomenon.

As to the question of intercellular or intracellular freezing, Müller-Thurgau noted that a direct examination of sections of frozen tissues under the microscope shows the crystals between the cells, not in them.

Repeating an experiment previously made by Sachs, he determined the residue left after evaporation of 21.08 grams of ice collected inside of a cow-beet, and found 0.04 g. It was, therefore, practically pure water which filtered out through the cell walls during freezing.

Müller-Thurgau, described also ice masses of columnar structure in animal tissues, for example, around the alimentary canal of an earthworm left to freeze in the ground, at -6° .

7. *Cells. Surface Freezing.* Molisch (1897), exposing

yeast suspensions to a freezing temperature of -9° , states that a withdrawal of water was evidenced by a shrinking of the cells (by about 10% of the original volume) and by the fact that the vacuoles became indistinct. The cells themselves, he thinks, never froze.

The same author described also an exudation of water from various filamentous algae: *Spirogyra*, *Cladophora*, *Derbesia*, etc., observed under the microscope during the congelation of their medium. In a *Spirogyra* the diameter decreased by 62%. Then he froze these algae in olive oil and noticed that the extruded water formed a cylinder of ice around the plants. In some forms the ice exuded presented a characteristic filamentous pattern, with the filaments sometimes twisted like in a screw, a pattern that he also observed in moss leaves and in fern prothalia.

In staminal hairs of *Tradescantia*, Matruchot and Molliard (1902) observed, during freezing, a loss of turgor, a decrease in cell volume comparable to that which occurs in plasmolysis, and a decrease or disappearance of the large vacuoles. They consider all these features as resulting directly from the withdrawal of water.

8. *Cellular Constituents.* While the preceding investigations refer to the exudation of water from freezing cells, Matruchot and Molliard studied the evidence of withdrawal of water from the cell constituents, in particular from the nucleus. Plant tissues of various types were left to freeze for several hours (15 hours mentioned in one case) at temperatures from -4° to -7° ; then they were thawed, fixed and stained. The authors used cells with a somewhat abundant vacuolar content taken from the lacunar parenchyma of the leaves (*Narcissus*), from the cortex of the hypocotyl axis (*Phaseolus*), from the stem (*Lupinus*), from the parenchyma of the roots (*Hyacinthus*), from the floral peduncles (*Clivia*, *Tulipa*), and also cells of more compact cytoplasm, such as those found in the tissues of the nucelle (*Leucoium*) and of the ovary (*Tulipa*). The essentials of their results are represented in Figure 15. The *nucleus*, homogeneously granular in un-

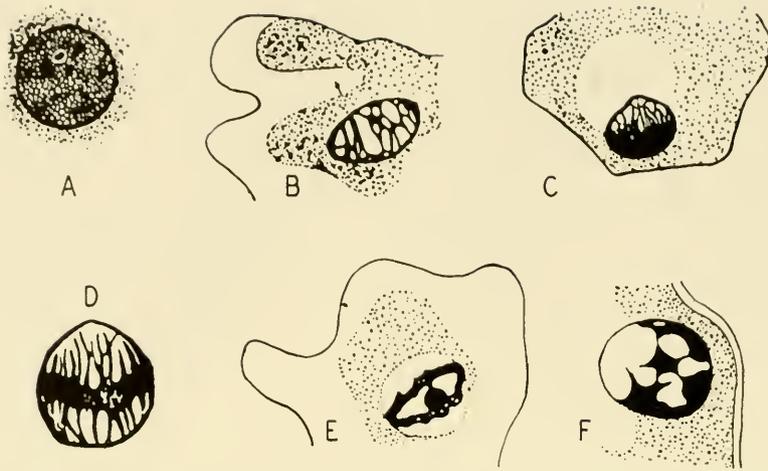


FIG. 15. Phase separation in the nucleus of frozen plant cells. (After Matruchot and Molliard, 1902.) The chromatin is represented in solid black masses.

frozen controls (Fig. 15, A), shows vacuolization in frozen cells. The chromatin forms a network with the meshes elongated in the direction of the vacuoles (Fig. 15, B). If the nucleus is between two vacuoles, the pattern is then bipolar (Fig. 15, B), if there is only one vacuole in the neighborhood of the nucleus, the pattern is monopolar (Fig. 15, C). The meshes fuse into larger masses at the equator of the nucleus in bipolar systems and at the pole opposed to the vacuole in monopolar ones. These masses of chromatin become more and more compact, the filaments projecting from them thin out gradually (Fig. 15, D) and finally there results (in bipolar systems), a crown of chromatin at the equator of the nucleus, separating two nuclear vacuoles which bulge out on each side (Fig. 15, E). In the cells with more condensed contents (from nucelles and ovaries), the chromatin meshes are thicker, the vacuoles within the meshes occupy a smaller volume, and the entire pattern is of a different type (Fig. 13, F). As to the cytoplasm, which is described as granular in the normal condition, it becomes spumous or spongy by vacuolization, after freezing. In general, in the cells studied by Matruchot and Molliard there was, on freezing, a separa-

tion of water from the chromatin or from the cytoplasm and an accumulation of that water in the vacuoles.

Luyet and Gibbs (1937) made a detailed description of the progress of freezing in the epidermal cells of onion. After the congelation of the moisture present at the surface of the mounted epidermis, some subcooled cells froze suddenly, becoming opaque. Two separated phases could then be seen in the frozen cells: ice and concentrated vacuolar sap. The opacity decreased during the few seconds subsequent to the sudden freezing, a phenomenon that the authors attributed to a rapid growth of crystals. There followed a slow transformation and growth of ice masses (Fig. 26) which lasted for hours.

Bugaevsky (1939), evidently unaware of the last-mentioned work, described the same process in epidermal and subepidermal cells of the "underground part" of wheat plants. The ice crystals are said to grow within the protoplasm itself; no mention is made of vacuoles.

Buck (unpublished work; *cf.* abstract in *Anat. Rec.*, 72, *Suppl.*, 125, 1938) obtained the curious picture represented in Figure 16 by freezing salivary gland chromosomes of *Chironomus tentans*. The nuclei, dissected out of the gland and contained in a drop of water, were let fall into a tube of petroleum ether cooled to about -70° . The content of the tube was then evaporated in a high vacuum at a temperature lower than -30° . One of the nuclei so obtained, photographed *in toto*, is shown in the Figure. One can distinguish several lobes of the coiled chromosomes. The parallel lines seen across the nucleus seem to bear no relation to the orientation of the chromosomes. The author suggests that they represent regions of compressed nuclear material, while the clearer stripes would represent regions of reduced chromosomal content previously occupied by ice (personal communication).

C. THE FREEZING CURVES

Many experimental investigations on death or injury by low temperature involve the study of freezing curves, that

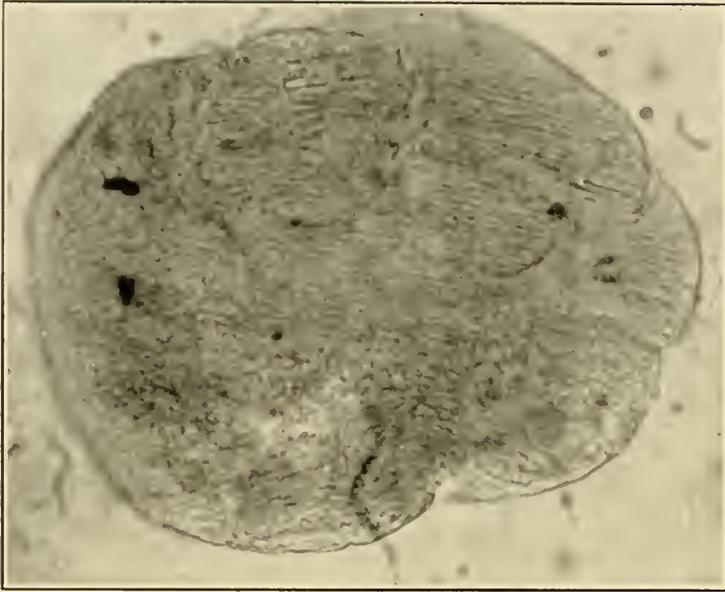


FIG. 16. Frozen nucleus from salivary gland of *Chironomus tentans*. (Original, Buck.)

is, of curves expressing the course of the temperature of a freezing object in terms of time. We shall analyse here briefly the essential characters of freezing curves and, to begin with, those of the freezing curve of water.

1. *Freezing Curve of Water.* If a mass of distilled water, perfectly stirred, at a temperature T (above zero), is exposed to a cooling bath at a constant temperature t (below zero), the temperature of the water will drop, in terms of time, according to a curve of the type represented in Figure 17. This composite curve consists of three limbs: AB, BC, and CD, which represent the course of the temperature before, during, and after freezing, respectively.

The limbs AB and CD are *cooling curves*. Under ideal conditions, as has been stated in the third section of the Preliminary Chapter (*cf.* in particular the formula 5), such curves are exponential.

AB and CD are not, however, identical. The curve AB

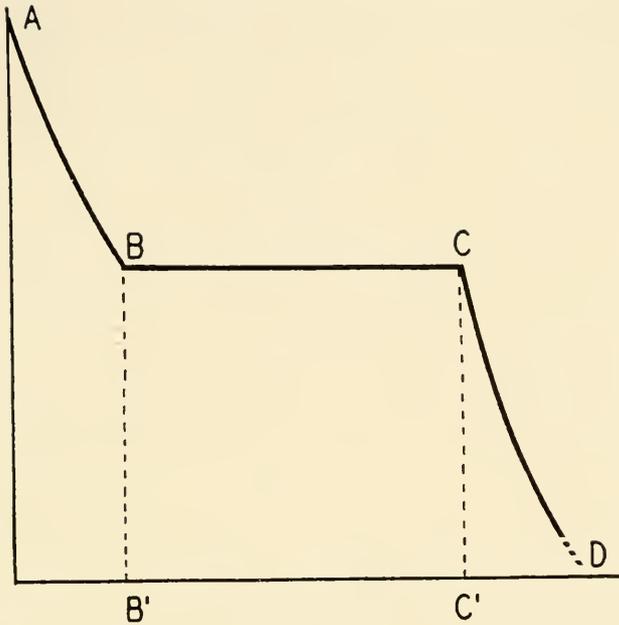


FIG. 17. Freezing curve of a liquid under ideal conditions. Abscissae: time; ordinates: temperature.

represents the cooling of water, and the curve CD the cooling of ice, that is, of a solid which cannot be stirred, and which possesses a heat conductivity about 4 times higher and a heat capacity about twice lower than that of water. The impossibility of stirring results in a non-uniform distribution of temperature; in a solid body which is being cooled the temperature decreases gradually from the center to the periphery. The high heat conductivity and the low specific heat of ice cause a more rapid cooling than in the case of water; consequently, the slope of the curve CD is steeper than that of AB.

To construct exponential curves one needs only two experimental points. Let us assume, for example, that a mass of water at $+10^{\circ}$ is exposed to a bath at -5° , and that the cooling rate is such that the temperature drops during the first minute from 10° to 8° , that is, if -5° is taken as the origin, from 15° to 13° (from $10 + 5^{\circ}$ to $8 + 5^{\circ}$). The two experimental values, 13 and 15, give the ratio of the geo-

metric progression representing the course of temperature: $13:15 = 0.866$. With this ratio one calculates the temperature after n minutes, which is 15×0.866^n . In general, there is a good agreement between the experimental and the calculated cooling curves.

As to the portion BC (Figure 17), it is a straight line in the ideal conditions assumed. When the decreasing temperature reaches 0° , water begins to freeze and, by liberating heat, it prevents a further drop of the temperature. Theoretically, if the entire mass of water were at 0° and if the heat liberated could be eliminated instantly, freezing would be completed at once. But a portion only of water is at 0° and freezes, the remnant is at a slightly higher temperature and stays liquid. The heat liberated by the part which freezes contributes to maintain the temperature of the non-frozen portion above 0° and delays the complete freezing of the mass. The curve then stays horizontal, there being an exact balance between the quantity of heat which is withdrawn by the bath and which would bring the temperature down, and the quantity of heat which is produced by freezing and which would bring the temperature up.

The length of the horizontal portion of the curve depends on two main factors: the velocity of withdrawal of heat, and the mass of liquid to be frozen. Concerning the rate of withdrawal of heat from the freezing mass one should remember that it does not remain constant during the process of freezing, even if the temperature of the cooling bath stays constant. While the material is being solidified, there is a gradual increase in heat conductivity and consequently the rate of withdrawal of heat increases. It is evidently necessary to take into account this change when one calculates the length of BC. The change in specific heat can be neglected in as much as the system consisting of the substances separating the freezing mass from the cooling bath can be compared to a wall limited by two surfaces at constant temperatures (see, above, the treatment of the "Problem of the wall"). Another factor

which considerably disturbs the process of withdrawal of heat from a freezing mass and which renders partially inapplicable the laws established under ideal conditions, is the gradually increasing inefficacy of stirring during the progress of crystallization.

The area $BC'C'B'$ is sometimes used as a measure of the quantity of ice produced during the time interval $B'C'$. If the heat Q produced by freezing is entirely transmitted to the bath, one can write that it is equal to the heat Q' dissipated:

$$Q = Q' \quad (A)$$

But the number Q of calories produced by crystallization is equal to 80 times the number I of grams of ice formed:

$$Q = 80 I \quad (B)$$

On the other hand, the heat dissipated Q' (see, above, the "Problem of the wall") is proportional to the difference y of temperature between the freezing water and the cooling bath, to the thickness d of the material separating them, to the heat conductivity c of that material and to the time t

$$Q' = kytd \quad (C)$$

where k is a constant of proportionality. From the equations (A), (B) and (C) one deduces

$$I = \frac{k}{80} yt cd \quad (D)$$

that is, the amount of ice I is proportional to the area yt of the rectangle $BB'C'C$ (when the heat conductivity c and the thickness d are constant).

The numerical value of the product $kytd$, that is, the number of calories lost by water in one unit of time, in the conditions of the experiment, can be determined by measuring, on the cooling curve, at the point B , the number of degrees by which the temperature drops in one unit of time and multiplying that value by the number of grams of water present (each gram requiring 1 calorie to lower the temperature by one degree).

As we said, the formula D is established on the assumption that the factors c and d are constant. But c , the heat

conductivity of freezing water, does not stay constant; it increases as more and more ice is formed. The formula D, therefore, should be considered correct only in a first approximation. The other factors that we mentioned above as affecting the length of the curve should also be taken into account in the study of the area limited by the curve.

Theoretically, at the points of junction of the three curves AB, BC and CD, there should be sharp angles; practically, the angles are rounded on account, mostly, of the lag in the conduction of heat. As we shall see later, with biological material the passage from one section of the curve into another is sometimes so gradual that it cannot be assigned to any definite point.

We said nothing in this discussion on the heat capacity of the thermometer, of the stirrer and of the container. The presence of these objects might sometimes have an important disturbing influence on the shape of the curve and it is not enough simply to correct the results by introducing their water-equivalent in the calculations.

2. *Freezing Curves of Solutions.* When freezing biological material we are always concerned with solutions or suspensions and not with pure liquids. The freezing curve of an aqueous solution in ideal conditions is represented in Figure 18. Such a curve consists of 4 limbs AB, BC, CD, DE.

The parts AB and DE are simple cooling curves of the exponential type studied in the preceding section.

The part BC requires a detailed analysis. When the temperature has dropped to a certain point below zero, water starts to freeze out of the solution. This point (B in the figure) is called, though improperly, the freezing point of the solution. The portion of water which freezes at B liberates heat and the drop in temperature is retarded. However, the curve cannot stay horizontal for any length of time; as soon as some of the water is solidified, the solution becomes more concentrated and its freezing point is lowered. When the temperature is down to this lower freezing point, more water freezes, more heat is liberated,

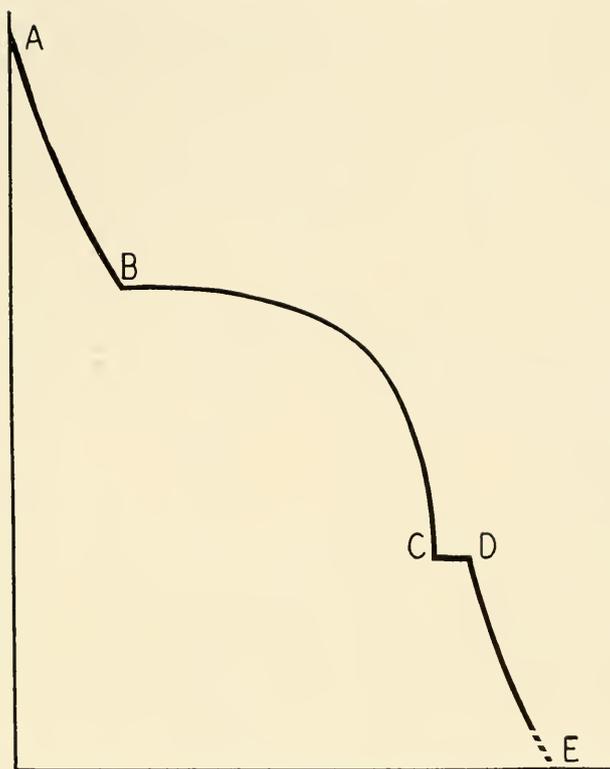


FIG. 18. Freezing curve of a solution under ideal conditions. Abscissae: time; ordinates: temperature.

a new delay results in the rate of cooling and the solution becomes again more concentrated. The curve slowly follows a downward path BC.

Such a curve is of the hyperbolic type, as is shown by the following considerations. A 0.01 weight-molar (molal) solution begins to freeze at -0.0186° . To lower the freezing temperature by a further 0.0186 degree, that is, to bring the freezing point to -0.0372° , the concentration should be doubled; for another lowering of the freezing point by 0.0186 degree the concentration should be tripled, and so on. To double, treble, etc. a weight-molar concentration, the weight of water in the solution should be reduced respectively to $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{4}$, etc., its original value. To reduce the weight of water to $\frac{1}{2}$, one should crystallize $\frac{1}{2}$ of it; to

reduce it to $\frac{1}{3}$ one has to crystallize the other $\frac{2}{3}$, etc. In these successive operations the quantities of water separated by crystallization from the original quantity present would be $1 - \frac{1}{2}$, then $1 - \frac{1}{3}$, then $1 - \frac{1}{4}$, etc. Assuming that the heat produced by the crystallization of these quantities of solvent is dissipated proportionally to the time one has the following values for the freezing temperatures in terms of time (the original concentration of the solution is supposed to be 0.01 weight-molar, its freezing point, therefore -0.0186° , and the unit of time is the time that it would take to freeze all the water of the solution):

<i>Freezing Point</i>	<i>Time</i>	<i>Freezing Point</i>	<i>Time</i>
$-0.0186^\circ \times 1$	$1-1 = 0$	$-0.0186^\circ \times 4$	$1-\frac{1}{4} = 0.75$
$-0.0186^\circ \times 2$	$1-\frac{1}{2} = 0.5$	$-0.0186^\circ \times 5$	$1-\frac{1}{5} = 0.80$
$-0.0186^\circ \times 3$	$1-\frac{1}{3} = 0.666$

The freezing point F decreases as an arithmetic progression; the time t increases as a harmonic sequence; it is known that the relation between two such quantities is hyperbolic. The formula of this relation is

$$F = \frac{k}{1-t} \quad (E)$$

where k is the freezing point depression of a weight-molar solution.

In the establishment of this formula many factors have been left behind, which should be taken into consideration in most of the quantitative studies on freezing curves: 1. The law of the freezing point depression holds only in dilute solutions of non-electrolytes; 2. The heat produced is not withdrawn proportionally to the time, its withdrawal depends on the difference between the temperature of the bath and that of the material, a difference which is continuously decreasing; 3. By the gradual formation of ice, the specific heat and the heat conductivity of the system are changing and this also affects the rate of heat withdrawal; 4. There is some heat of solution involved in the phase separation. The hyperbolic relation, therefore, should be considered only as a first approximation law.

One can see, by the preceding analysis, that, if the solution is dilute, the curve will stay practically horizontal for a long time since a relatively large quantity of water has to freeze before the freezing point is lowered noticeably. With very concentrated solutions, on the contrary, the horizontal portion of the curve might be so small that a sharp determination of the freezing point would be difficult.

At the temperature of congelation of the saturated solution, that is, at the eutectic point (C in the Figure), the two phases, solvent and solute, crystallize. The curve becomes parallel to the time axis (portion CD). For the analysis of this portion which is a freezing curve, we refer to what has been said in the preceding section on the freezing curve of water. A slight complication results, however, from the fact that there are 3 phases present, the liquid solution, the solid solvent and the solid solute, the latter two crystallizing separately, though at the same time.

It should be mentioned also that, for dilute solutions, the quantity of eutectic mixture is so little as compared to the quantity of ice present that the eutectic plateau almost vanishes while the freezing plateau is large. On the contrary, for concentrated solutions, in which, as we said above, the freezing plateau disappears, the eutectic plateau becomes considerably broader.

3. *Freezing Curves of Colloids.* Little is known on the forces which have to be overcome to freeze water out of colloids. The following investigation is one of the few that we found in the literature, on this subject.

Fischer and Bobertag (1909) described the curious fact that, when gelatin was dissolved in water by a previous heating, the freezing curve so obtained had a longer horizontal plateau than the freezing curve of a suspension of the same quantity of gelatin (about 9%) dispersed in flakes in the water (Fig. 19). A further investigation into the cause of this phenomenon might yield valuable information on the mechanism responsible for the anomalies of similar nature so often met with in the freezing of tissues.

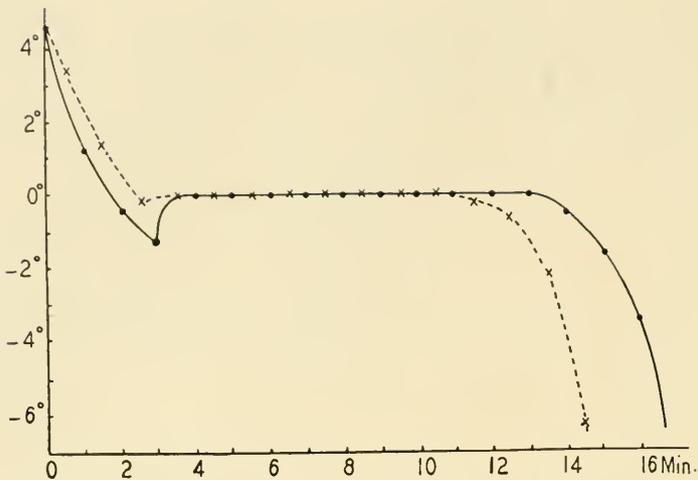


FIG. 19. Comparative freezing curves of a solution (xxx) and of a suspension (...) of gelatin. (Curves drawn according to the data of Fischer and Bobertag, 1909.)

4. *Freezing Curves of Tissues.* For a description and a discussion of the freezing curves of plant tissues, we refer the readers to Müller-Thurgau (1881 and 1886), Voigtländer (1909), Maximov (1914), Walter and Weismann (1935), and Luyet and Gehenio (1937). Some freezing curves of animal tissues were studied by Jensen and Fischer (1910) and some of entire animals by Cameron and Brownlee (1913) and by Weigmann (1936). These curves were established for the information that they furnish on such problems as the position of the freezing point, the quantity of water withdrawn at a given temperature, the mechanism of death by freezing, the relations between the death point, the freezing point and the quantity of ice present, the range of subcooling, and the existence of a eutectic point. We treated above the problems concerning the freezing points; those related to subcooling will be reviewed in the next chapter; the other problems mentioned will be studied here.

Living and Dead Tissues. The following differences were reported in the freezing curves of living and of dead tissues, studied under otherwise comparable con-

ditions: 1. The horizontal plateau was found higher in dead than in living tissues (for bibliographical references, see above under "Freezing Points"); 2. In dead tissues, the plateau was maintained horizontal for a longer time and then the curve dropped more rapidly than in living tissues (Fig. 20); 3. The freezing curves of living material often

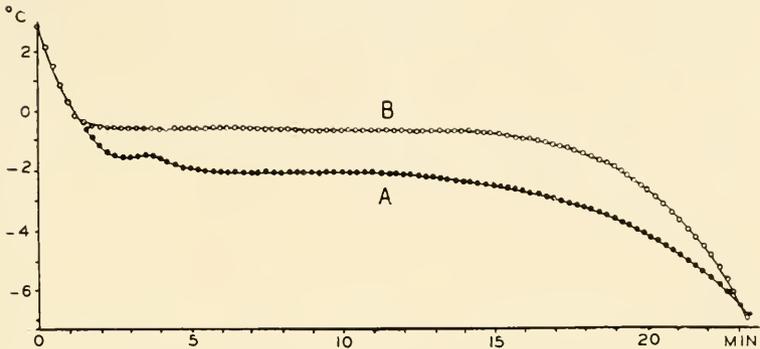


FIG. 20. Comparative freezing curves of living (A) and dead (B) plant tissue. (From Luyet and Gehenio, 1937.)

showed jerks or irregularities while those of the dead were smoother; 4. In dead material, the position of the horizontal plateau, that is, of the freezing point, was little affected by the cooling velocities used in the experiments, except at the very high velocities of 5 degrees per minute; in living material, the horizontal part of the curve was lowered by cooling velocities of some 3 degrees per minute.

Several of the authors mentioned above have attempted to explain these differences. In general, they attribute most of the results to the resistance offered by the living cell membranes and by the living protoplasm to the withdrawal of water. The dead tissues, in which the membranes have become permeable, and in which the protoplasmic structure has been altered, would behave as aqueous solutions; their water would be free. The *living tissues* in which it is thought, *water is held more firmly*, would release the latter only on the application of more force. The jerks are attributed by Luyet and Gehenio to the sudden release of various amounts of water or to the

occasional freezing of several vacuoles at a time, ice-seeding through the membranes of the living cells being often hindered.

The results of Jensen and Fischer (1910), on muscle, are quite different from the results obtained by the authors that we just mentioned, on plant material. The curves of dead muscles dropped more slowly after the horizontal plateau than those of living muscle (Fig. 21). The authors attribute this to a *firmer binding of water in dead material*.

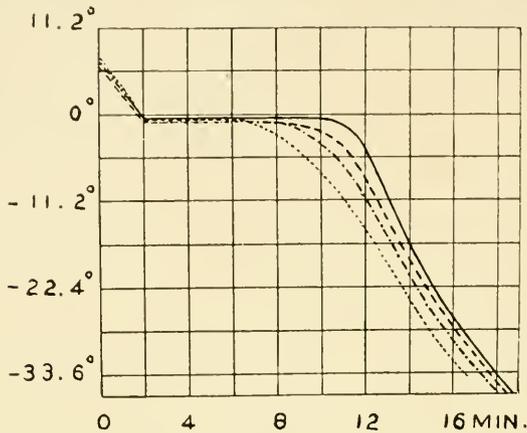


FIG. 21. Comparative freezing curves of living and dead muscle (From Jensen and Fischer, 1910: (—), fresh tissue; (---), tissue killed by freezing; (- · - · -), tissue killed by heating at 100°; (· · · ·), tissue killed by heating at 115°).

The freezing curve obtained by Cameron and Brownlee (1913) on an entire frog exposed to -10° with a thermometer in its stomach, seems to agree with the results generally reported rather than with those of Jensen and Fischer. The drop of the curve after the horizontal plateau is slower and does not present the relatively sharp turn exhibited by the hyperbolic curve of a saline solution frozen at the same time (Fig. 22).

Eutectic Point. Mez (1905) assumed that plant tissues should present a eutectic point just as true solutions do. He claimed, furthermore, that this point was always above -6° .

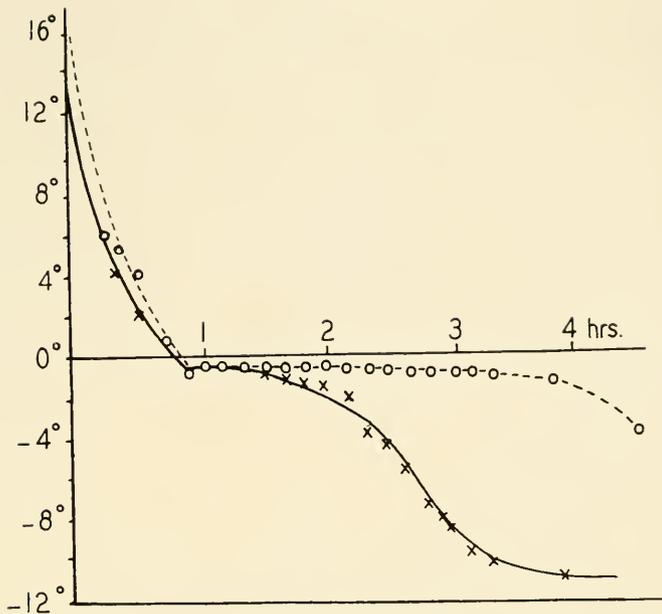


FIG. 22. Comparative freezing curves of an entire frog (x x x) and of a saline solution (o o o). (After Cameron and Brownlee, 1913.)

On Mez' suggestion, Voigtländer (1909) established a large number of freezing curves of various plant tissues. He observed that in most of them the cooling velocity showed a decrease at two points along the curves (Fig. 7, Curve 5, Points F and E), and he considered these retardations in the cooling rates as representing, respectively, the freezing and the eutectic points.

According to Fischer (1911), it is quite daring to speak of the eutectic point of the cell constituents which are mixtures of colloidal substances, some of which perhaps never crystallize. Jensen and Fischer (1910), who made a comparative study of the freezing curves of muscles and of saline could observe a faint trace of eutectic in the latter but their curves show no evidence of any eutectic in the muscle.

Maximov (1914) criticized Mez and Voigtländer's results on the ground that, in such a dilute solution as the cell sap, the retardation in the drop of the curve caused by a

eutectic freezing, should hardly be noticed and could not be as large as was observed. He expressed his surprise at Mez' contention that the eutectic was always above -6° , when it is known that solutions of several substances found in the cells have much lower eutectic points. He emphasized the fact that, in the numerous curves that he established with tissues or with extracted juices, he never observed any indication of a eutectic freezing. He also pointed out that, with the high cooling velocities used by Voigtländer, any retardation in the slant of the curve would be so attenuated that even the freezing points would be obscured. He finally suggested that the two retardations observed by that author were due, respectively, to a freezing of the cell sap extruded around the thermocouple by the insertion of the latter, and to the freezing of the tissue. This interpretation was confirmed by the fact, mentioned by Voigtländer himself, that extracted juice gave but one retardation.

Luyet and Gehenio (1937) showed that a living tissue which gives a curve with two long horizontal plateaus (Fig. 23, A), at a low cooling rate of 2.5 degrees per minute,

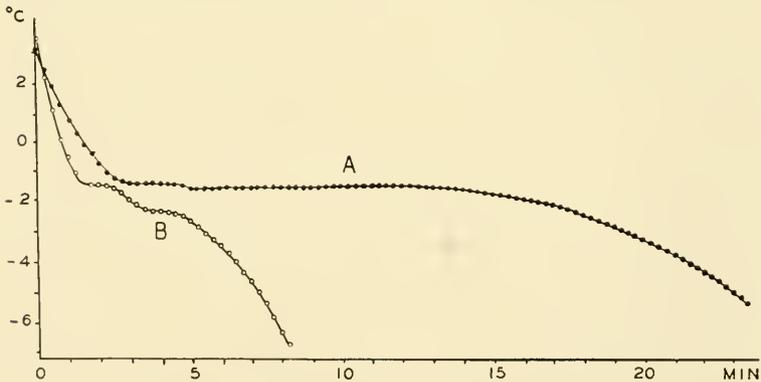


FIG. 23. Effect of cooling velocity on freezing curves. (From Luyet and Gehenio, 1937.) Cooling velocity at 0° : in curve A, 2.5° per minute; in curve B, 5.2° per minute.

gives a curve in which the plateaus almost vanish at a cooling velocity of about 5 degrees per minute (Fig. 23, B), and would be replaced by two slight retardations at the

cooling velocity of 10 to 14 degrees per minute used by Voigtländer. This confirms Maximov's view that Voigtländer's curves present a double freezing point but no eutectic.

Moran (1935), who defines the eutectic point as "the temperature at which all free water is frozen," determined this temperature in a concentrated filtrate of muscle juice. For that purpose he measured the electric resistance of the material when the temperature was lowered, there being quite a definite change in resistance at the eutectic. He obtained the value -37.5° , which he considers as the "eutectic temperature of muscle."

One must confess that there is little experimental evidence for the existence of a eutectic freezing in tissues, despite the fact that a considerable portion of plant juices and body fluids are true solutions, from which one would expect a eutectic freezing. The problem of the eutectic, therefore, remains almost entirely to be investigated.

Quantity of Ice Formed. In the study of death by low temperatures, biologists have attempted to find, from an analysis of the freezing curves, the proportion of water frozen at a given temperature, in order to determine if death by freezing is conditioned essentially by the quantity of water withdrawn from protoplasm. In doing so, they have generally observed that the descending portion of the freezing curve which follows the horizontal plateau is maintained for a long time at a level higher than one should expect if all the water were frozen.

Müller-Thurgau (1886), by recording the time necessary for the freezing curve to fall from the freezing point to -1° , from -1° to -2° , etc., concluded that, in a tissue, the amount of ice formed during the first part of the freezing period is considerably greater than that formed later; but there was still some crystallization when the curve had dropped several degrees below the freezing point. This was confirmed by the observation that the ice masses formed in the intercellulars of a tissue exposed for several hours to -10° were larger than those formed after an exposure to -4° .

Jensen and Fischer (1910) calculated, from the areas limited by the freezing curves, the comparative quantities of ice formed at various temperatures, in living, in previously frozen and in previously heated muscles, as also in physiological saline. The essentials of their results were discussed above.

Maximov (1914), using the method of the freezing-curve-area, noticed, among other interesting facts, that the quantity of ice formed in a tissue of given water content does not depend only on the lowest temperature reached but also on the cooling velocity, that is, on the temperature of the cooling bath (investigations on potato and beet).

Luyet and Condon (1938) found that the quantity of ice formed in small pieces of potato tissue weighing 2.25 gr. was 34.5, 45.5, 55.7 and 65.9% of the weight of the object, after, respectively, 15, 20, 25 and 31 minutes of freezing. The temperature had dropped during these periods of time to -1.9° , -2.8° , -4.6° and -8° , respectively. There was still evidence that some ice was formed between the last two temperatures.

In most of the investigations described in this last section, no attempt has been made to bring the temperature to very low values and to study the behavior of the curves at these temperatures. The last portions of the freezing curves have hardly been investigated.

III. COMPLETION OF CRYSTALLIZATION

Most of the authors who have studied the completion of freezing and its relation to temperature and time have done it by the calorimetric or the dilatometric method. We refer the readers who desire more complete information on this subject to reviews on "Bound Water" and we summarize here only a few papers more intimately related to our topic.

1. *Suspensions and Colloids.* Foote and Saxton (1916) determined with a dilatometer the formation of ice, at various low temperatures, in mixtures of sand and water, of lampblack and water, in moist calcium hydroxide and in

hydrogels of alumina, of silica and of ferric oxide. While freezing was completed at -4° in the sand mixture, it required a temperature of -28° to be terminated in the lampblack mixture. In most of the other substances studied, it was observed that at -20° some water was still crystallizing out.

Moran (1926), who had previously observed that, when gelatin gels in form of discs are frozen slowly, ice forms at the surface, determined the quantity of water left in the core of the discs, that is, the quantity of non-frozen water, after exposure to various low temperatures. The curve of Figure 24, constructed from his data, represents the amount of water found in the cores of discs exposed to temperatures from -3° to -19° . At this last temperature,

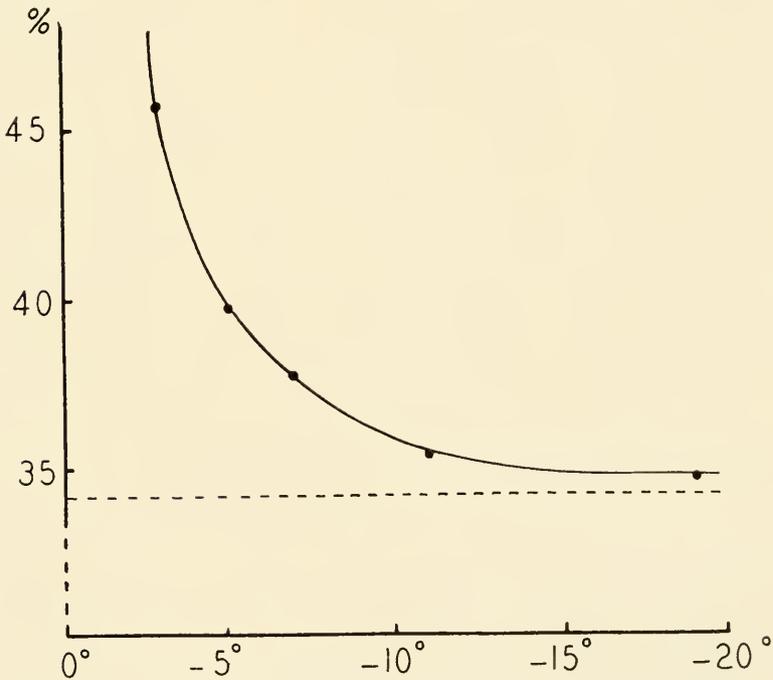


FIG. 24. Proportion of water which remains unfrozen in gelatin gels exposed to various temperatures. (Calculated from Moran's data, 1926.) Abscissae: temperatures; ordinates: unfrozen water in percent of the total weight. The horizontal dotted line represents the quantity of water which does not freeze at any temperature.

34.8% of the weight of the core was water. But this was almost entirely non-freezable water, since gels containing 34.5% water did not freeze at any temperature, even after immersion in liquid air. Consequently, almost all the freezable water was frozen at -19° .

Lloyd and Moran (*Proc. Roy. Soc.* 147, 392, 1934) extended these determinations to a temperature of -45° . They found no significant difference in the quantity of ice formed at -20° and at -45° .

Moran (1935), using the method of surface freezing observed that in solutions of agar, myogen and albumin (in collodion bags) there was still some freezing at -20° ; however, at this temperature the curves representing the proportion of water frozen approached the asymptotic position. In egg white, cessation of freezing would take place at -31° as determined by the change in electric resistance of the freezing material.

2. *Tissues.* Müller-Thurgau (1886) measured, by the calorimetric method, the quantity of water not yet congealed at about -15° in frozen apples. The apples were brought, after congelation, into a water-calorimeter at 16° and the resulting drop in the temperature of the water was recorded. The amount of ice was found to vary from 53.13 to 66.0 gr. in 100 gr. of apple, when the freezing temperatures varied from -4.5° to -15.2° . The water content of the apples was about 83%.

Jensen and Fischer (1910) noticed that some ice was still being formed in frog's muscle at more than 20 degrees below the freezing point. Their criterium is the slope of the freezing curves.

Rubner (*Abh. Preuss. Akad. Wiss.*, 1922), using the calorimetric method, found that only 75% of the water content was frozen in beef muscle at -20° .

According to Plank (*Z. Ges. Kalteind.*, 32, 141, 1925), the amount of ice present in ox muscle at -20° , determined calorimetrically, was 91% of the total water content.

Moran (1930), by the dilatometric method, found that 94% of the water was frozen at -10° and 98.2% at -20° , in muscles from beef, mutton and pork.

The same author (1935) studied the completion of congelation in amphibian muscles. He embedded the latter in discs of gelatin gels, exposed them to various low temperatures, and measured the amount of ice formed at the surface of the discs. He found that most of the freezable water froze between 0° and -5° , that about 10% of the total water froze between -5° and -20° and that less than 1% froze between -20° and -37.5° , at which temperature freezing ceased.

The duration of the freezing process at a given temperature is shown by an experiment of Moran (1926) on a 43.7% gelatin gel put to freeze at -11° , the volume of which was determined with a dilatometer. The increase in volume, rapid during the first few days, soon became very slow, but it ceased only after 26 days (Fig. 25).

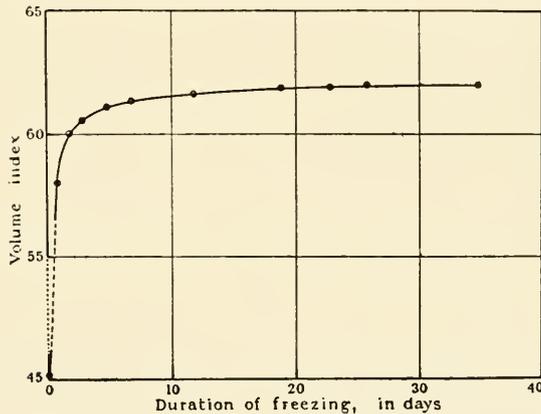


FIG. 25. Increase in the quantity of ice formed in a gelatin gel exposed for 26 days to -11° . (From Moran, 1926.) The ordinates represent dilatometer readings.

In all these investigations it was assumed, as a principle, and this was subsequently observed as a fact, that congelation is more complete when the temperature is lower. But, it is also a principle and an established fact, as we said above, that the velocity of formation of nuclei and of growth of crystals is gradually reduced to zero when one lowers the temperature. These two principles are in evident contradiction and one of them has to be sacrificed.

As to the factual observations they can probably be reconciled if the temperatures at which crystallization was found to be complete, according to the first principle, are precisely the temperatures at which the crystals cease to grow, according to the second. The first principle should then be abandoned since the facts allegedly explained by it are really consequences of the other principle.

IV. THE FROZEN STATE

PROPERTIES OF ICE AND OF FROZEN SYSTEMS

A historical and critical review of the investigations on the physical constants of ice, and a large bibliography, will be found in H. T. Barnes' Book "Ice Engineering" (1928). The subject having thus been reviewed, we shall merely mention the essential established data, indicate the use that the biologists have made of them and suggest some other applications. Most of our information is obtained from Barnes' work.

1. *Mechanical Properties of Ice.* The plasticity of ice is well exemplified in the movement of the glaciers; ice really flows. The velocity of flow depends on the temperature and on the pressure exerted and it is relatively high in large masses of ice, such as the glaciers (for figures, see H. Hess: "Die Gletscher" 1904).

Andrews (1885) studied ice deformability at different temperatures by measuring the degree of penetration of a steel rod applied to blocks of ice. He found that the resistance to penetration varied little when the temperature was raised from -40° to -9° but decreased rapidly from -9° to 0° . This considerable plasticity at a few degrees below the melting point is probably the cause of the transformation of the ice pattern observed by Luyet and Gibbs (1937) in frozen epidermal cells of plants (Fig. 26).

The flow of ice does not seem to result, as it is usually thought, from a partial melting followed by recrystallization, but it consists probably in a sliding of the crystalline planes one over the other (McConnell, 1891). In some other crystalline materials, gliding lines can occasionally

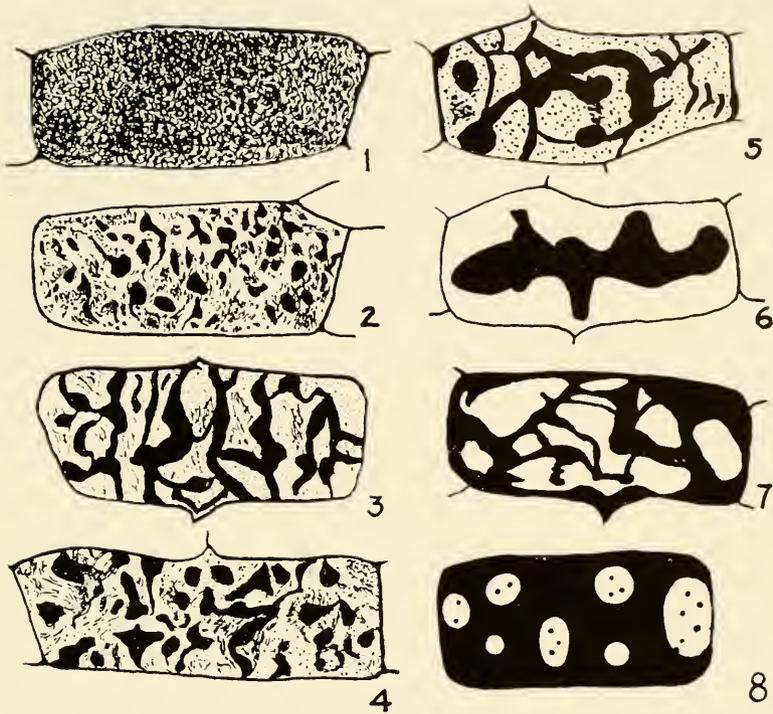


FIG. 26. Drawings 1 to 7: Transformations in epidermal plant cells while in the frozen state. (From Luyet and Gibbs, 1937.) Drawing 8: thawing cell. In all the drawings, the fluid sap is represented in black and the ice in white.

be observed and for some metals, the degree of ductility and malleability can be related to the number of gliding planes (*cf.* Tammann, 1925, p. 192, *sqq.*). Even the classical experiments known as “regelation” in which a piece of ice gives way under the pressure of a wire applied on it, should perhaps be explained, at least partly, by the gliding motion of the crystalline planes.

According to McConnell, single ice crystals are “perfectly brittle.” However, since, in general, the fluidity of a crystal depends on the temperature, some crystals being so fluid near the freezing point that their faces bulge out or that their edges bend in a curve line under the action of gravity (*cf.* Tammann, 1925, p. 291), it seems evident that the individual ice crystals should also show some

fluidity at temperatures near the melting point. A study of this property, namely, of the degree of fluidity of the ice needles in formation, near 0° , would help in solving the problem of the mechanical injury which can be produced in protoplasm by ice crystals.

Some physicists discuss the viscosity of ice and, in general, of crystalline substances. They assume that in a crystalline body there are layers of molecules in the liquid state which give to the system the consistency of a "viscous solid." Hess (1902) observed that the presence of sand and dust increases the viscosity of ice. This observation might have some importance for the biologists since, in biological material, a number of foreign substances admixed with ice can modify its viscosity.

Among the other mechanical properties of ice, let us mention the tensile strength and the compressibility which, according to Hess (1904), amount respectively to 7–8 kg. and 25 kg. per sq. cm., and the crushing strength which was found to vary from 23 kg. to 70 kg. per sq. cm. (*cf.* Barnes, 1928).

The strength of a layer of ice 18 inches thick would be such that it could support a railroad train.

2. *Specific Gravity.* Bunsen, in 1870, obtained 0.91676 as the specific gravity of ice at 0° . Barnes considers this value as a "good average of all the latest and best measurements." The specific gravity of water at 0° was found by Chappuis (1897) to be 0.9998674. Due to the high precision of density determination, the method of utilizing the difference in the specific gravity of ice and of water in the study of such problems as that of the quantity of ice formed in biological material at a given temperature, might prove valuable.

3. *Thermal Constants.* Maass and Barnes (1927) give 0.4873 as the specific heat of ice at 0° . Callendar (1912) found 1.00934 for water at 0° .

The biologist working with low temperatures occasionally needs to know the specific heat of ice from 0° C. to the absolute zero. We represented in Figure 27 the values

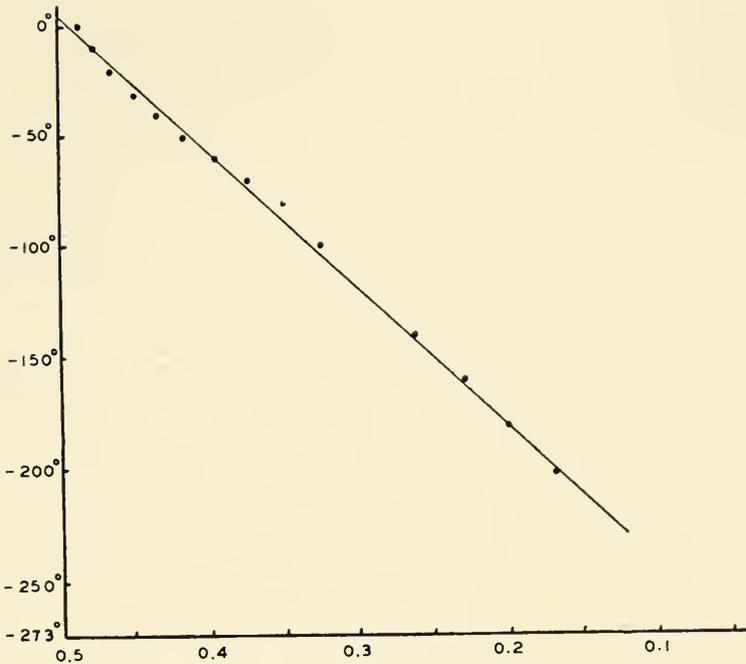


FIG. 27. Specific heat of ice in terms of temperature. (Curve drawn from the data of Maass and Barnes, 1927, and of Nernst, 1910.)

obtained, from 0° to -80° , by Maass and Barnes (1927) and, from -100° to -200° , by Nernst (1910). As an average between -188° and -252.5° , Dieterici (1903) gives the value 0.146° . Since the curve is practically a straight line at the temperatures plotted, one can, in calculations involving the amount of heat withdrawn for cooling some material between two points within this range of temperatures, take the average specific heat between the two points.

The heat conductivity of ice at 0° is 0.00573, according to Neumann (1862); that of water at 0° is 0.00120, according to H. F. Weber (1880).

The difference in conductivity along the axis of the crystals and in a perpendicular direction was found by Trouton (1898) to be practically negligible, the ratio of the two being 22:21.

We did not find any data on the heat conductivity of ice at very low temperatures. Lees (1908) has investigated

the heat conductivity of several metals at -160° . His results indicate that in some of them the conductivity is lower at low than at high temperatures while in others it is higher.

According to Dickinson, Harper and Osborn (1914), the heat of fusion of ice is 79.68 calories.

It is to be noticed that the heat of fusion decreases by 0.59 calories for every degree of lowering of the freezing point.

Barnes and Vipond (1909) found that the heat of evaporation of ice varies from about 600 calories in rapid evaporation to about 700 in slow evaporation. It is not the sum of the heat of fusion of ice (79 cal.) and of the heat of vaporisation of water (598 cal.), but it tends, in rapid evaporation, to become identical with the latter. This renders probable the assumption that ice evaporates without passing through the liquid phase, the molecules escaping from the ice in the state of a polymeric vapor.

According to Washburn (1925) and others, the vapor pressure of ice varies from 0° to -90° as follows:

<i>T.</i>	<i>V.P.</i>	<i>T.</i>	<i>V.P.</i>	<i>T.</i>	<i>V.P.</i>
0°	4.579	-30°	0.286	-70°	0.002
-5°	3.013	-40°	0.097	-80°	0.0004
-10°	1.950	-50°	0.030	-90°	0.00007
-20°	0.776	-60°	0.008

The coefficient of linear expansion of ice, as determined by Petterson (1893) is 0.000053 between -2° and -10° . The coefficient of cubical expansion is 0.0001620 according to Nichols (1899).

4. *Electrical Properties.* It is known that ice is a good insulator; its specific resistance at 0° is given by Johnson (1912) as 0.367×10^6 . That of distilled water at the same temperature is 0.5×10^6 .

We mentioned above that the difference in the electric resistance of ice and water has been used by Moran (1935) for the determination of the entectic point of the muscle.

The specific inductive capacity of ice at -5° was found by Thwing (1894) to be 2.85 (wave-length of measuring

current: 1200 cm.); that of water at 18° is 81.07 (wave-length practically infinite) as determined by Turner (1900). Ice at the temperature of liquid air has a specific inductive capacity of 1.76 to 1.88 (wave-length: 75 cm.) according to Behn-Kiebitz (1904). (For quotations see the Smithsonian Tables.)

5. *Optical Properties.* Ice is birefringent; the refractive indices, as given by Reusch (1864) are 1.30598 for the ordinary ray and 1.30734 for the extraordinary ray (red light).

V. MELTING AND MELTED MATERIAL

A. THE PROCESS OF MELTING

Although there is an abundant biological literature on the possible injurious and lethal effects of thawing, the process of fusion itself has been little investigated, far less than the process of freezing.

Müller-Thurgau (1881) attempted to study the course of thawing in a few tissues. He established, though with rather crude methods, some thawing curves. These, when compared with the corresponding freezing curves, presented some characteristic differences which later investigators discussed in more detail.

Fischer (1911), after discussing some of the results which he obtained with Bobertag (1909) in freezing and thawing various carbon compounds, analyzed the aforementioned results of Müller-Thurgau. He concluded that the amount of heat liberated in freezing is not the same as that absorbed in thawing and he attributes this difference to some endo- and exothermic processes concomitant with freezing and thawing.

Maximow (1914) compared freezing and melting curves of potato tissues and noticed, in particular, that the freezing point was more than half a degree lower than the melting point. As to the difference observed by Fischer between the heat liberated in freezing and the heat absorbed in thawing, he showed that it is a question of procedure; the difference is reversed if one changes the cooling velocity in the freezing curve.

One should naturally expect that the course of melting be different from that of freezing, since, in frozen tissue, the two constituent phases are separated, one of them, water, being in the intercellulars, the other, partly dehydrated protoplasm, remaining within the cell; a frozen tissue to be melted is, therefore, a quite different system from an intact tissue to be frozen.

More investigation on melting curves of tissues, in particular of tissues which survive a partial freezing and still possess semipermeable membranes, would throw some light on several questions related to osmosis during freezing and thawing and, in general, on the forces binding water in living protoplasm. A comparison between melting curves of living and of dead tissues might also prove illuminating.

B. ALTERATIONS OBSERVED IN THAWED MATERIAL

The fundamental alteration caused in aqueous solutions and suspensions, in aqueous colloids and in protoplasm by freezing is the separation of ice. If, when ice is thawed, the system can be restored to the condition in which it was before freezing, in other words, if the phase separation is reversible, the material might be unaltered. In this sense, the true solutions are, at least on first approximation, unaltered by freezing. Whether or not in suspensions, in colloids and in protoplasm the phase separation, or any other alteration, is reversible upon thawing, is the subject of the following inquiry.

1. *Suspensions and Colloids.* Vogel (1820) observed that starch paste loses its adhesive properties after being frozen. This is one of the oldest reports concerning a permanent change induced by freezing in a colloid.

According to Goepfert (1830), when the milky plants *Euphorbia* and *Ficus* are frozen, the milk is irreversibly transformed into a watery fluid.

Ljubavin (*J. Russ. Phys.-Chem. Soc.*, 21, 397, 1889) was the first, according to Lottermoser (1907), to show that a hydrosol can be converted into a gel by freezing. He also pointed out the influence of electrolytes on that conversion.

Bredig (*Ztsch. f. angew. Chem.*, 954, 1898) described a precipitation of platinum and of other metals from colloidal solutions, after melting.

Lottermoser (1907 and 1908) confirmed these results and, in particular, the fact that the lower the electrolyte content of a sol is, the easier it acquires the property of precipitating after thawing (although there are exceptions). Under ordinary conditions, hydrosols with high electrolyte content, such as silicic acid, undergo no change by being frozen and thawed; but when their electrolyte content has been reduced by dialysis they can be precipitated. The changes in consistency were concomitant with changes in electric conductivity. The author considers precipitation a consequence of the withdrawal of water during freezing. The fine structure of the gel, he suggests, is destroyed by the increase in volume of solidifying water. The particles of the colloid set free in that breaking down of the structure are wedged between the expanding ice crystals where they are transformed into leaflets which, after thawing, precipitate to the bottom of the vessel.

Bobertag, Feist and Fischer (1908) studied the action of congelation at -10° , -70° and -180° on a large number of substances belonging to various groups: true solutions of dyes, such as eosin, safranin, etc.; partly colloidal solutions, fuchsin, methyl violet, etc., colloidal solutions of congo red, benzopurpurin, etc.; colloidal solutions of tannin, gums, starch, agar-agar, gelatin, albumin and hemoglobin; colloidal solutions of metal and metal compounds. Some dyes were partly decolorized after thawing, but usually the previous color was resumed later, although in some cases it took 2 days for the restitution of the original condition. Tannin solutions precipitated above the freezing point; on thawing, the precipitate fell to the bottom of the container and, on rewarming, it dissolved again; this is the ordinary behaviour of a solute of which the solubility decreases at lower temperatures. Gum, starch and hemoglobin solutions were turbid immediately after thawing, but cleared up entirely afterwards.

Agar-agar and gelatin solutions showed, for quite a long time after thawing, a double phase structure consisting of clumps of jelly and of a dilute liquid. Albumin solutions stayed permanently turbid after treatment at -70° and -180° but not after being frozen at -10° . Platinum and gold were precipitated from their colloidal solutions by a freezing at -70° . Solutions of arsenic trisulfide and of antimony trisulfide also precipitated. A dilute solution of iron hydroxide (*liquor ferri dialysati*) did not precipitate, but presented an increased Tyndall effect. A solution of sodium silicate remained entirely unaltered. In silver preparations, such as protargol, kollargol and lysargin, the silver agglomerated into clumps, irregularly distributed in the ice; but, after thawing, the preparations were just the same as before freezing (a protective effect of albumin is thought to be responsible for the reversible action in these preparations).

The authors think that, in all the above solutions, solute particles are brought together during congelation and that, after thawing, they may or may not be dispersed again, depending on the nature of the substance. Besides, some colloids would be irreversibly dehydrated by freezing.

According to Bruni (1909), colloidal isinglass, frozen at -20° or at -80° , formed, after thawing, a gel which could not be distinguished from the original one. But colloidal silicic acid, treated in the same manner, consisted, after thawing, of two phases, one of which was pure water and the other, a precipitate of amorphous leaflets of hydrated silicic acid.

In 1911, Fischer reviewed the investigations of most of the previous workers, including those which he had made himself with various collaborators, and he concluded that colloids show an extreme variation in resistance to cold. In general, the changes which they undergo on freezing are reversible but, for some colloids, at some definite temperatures, irreversible changes take place. The temperatures at which these irreversible processes occur are some-

times determined by the age and previous history of the material.

Bottazi and Bergami (1924) studied coagulation by freezing in ox and dog sera and in *Octopus* blood. The material, in glass containers with parallel faces 15 mm. apart, was left for a given time in a freezing mixture, then allowed to thaw and its turbidity was judged by the degree to which it obscured a series of dark lines drawn against the back wall of the container. They found that a coagulation of ox serum at -20° gave, after thawing, a liquid as clear as before, except sometimes for a trace of opalescence. So, temperatures which solidify all the water of the proteins do not denature and coagulate them irreversibly. At higher, non-freezing temperatures, for example, at -0.9° , the sera would coagulate partly if they were dialyzed for 10 days and so deprived of the major part of their electrolytes, but the coagulation process was entirely reversible on rewarming. This partial coagulation was easier to obtain if the sera were diluted with water. The addition of NaCl to coagulable sera prevented coagulation. The addition of hydrochloric acid caused the sera to become turbid when the isoelectric point was approached; coagulation was, however, always reversible. The serum, it is concluded, is a rather stable colloidal system, which becomes labile when it loses its electrolytes and when it is acidified near the isoelectric point. The hemocyanin of the blood of *Octopus macropus* was found still more stable. It did not coagulate at -3° or -4° even after a month of dialysis.

According to Callow (1925), repeated freezing irreversibly destroys the structure of gelatin gels.

Moran (1925) observed that frozen egg yolk loses its fluidity on thawing and takes on a stiff pasty consistency. This change, however, occurs only if the following conditions are fulfilled: 1. The yolk must have been cooled below -6° (called by Moran the "critical temperature of freezing"); a stay of several months above -6° did not change the yolk consistency; 2. Congelation must actually

have taken place; eggs subcooled and maintained at -11° for 7 days were fluid on thawing; 3. The material must stay a certain length of time in the frozen condition; the degree of stiffness increases with that length of time up to about 20 hours and then it stays constant; 4. Thawing must not be too rapid; yolk immersed in liquid air (24 hrs.) and then thawed at room temperature was pasty, while it was fluid when thawed in mercury at $+30^{\circ}$.

Moran explains these facts by assuming that the protein of the yolk, vitellin associated with lecithin, which is known to be soluble in 10% NaCl, gets dissolved in the salt solution of the yolk when this solution is concentrated by freezing. The "critical temperature of freezing," -6° , would be the freezing point of that solution. On thawing, the protein would irreversibly precipitate out. In an attempt to verify this theory, he froze a dilute solution of NaCl containing some lecitho-vitellin and observed, on thawing, a persistent cloud.

During cooling and freezing, the yolk underwent some unexpected changes in volume, which were measured with a dilatometer. These changes can be summarized in the 4 following points: 1. The volume of the yolk exposed for 3 days at -11° and thawed decreased by 0.0084 of its original value (measured at 0°); 2. The coefficient of expansion of the thawed yolk was higher than that of the unfrozen yolk; 3. The contraction of the normal unfrozen yolk, which was linear with the temperature down to 0° , showed a higher rate in the subcooled condition from 0° to -7° ; 4. The volume of the frozen yolk, measured at -7° , increased by about 0.00015 of its original value when the material stayed for 3 days at -11° , but it decreased below the original value when the material stayed 9 days at -11° .

Moran ascribes the decrease in volume resulting from a long stay in the frozen state at -11° (under 4, above) to the dissolving of the lecitho-vitellin of the yolk in the salt solution; dissolving proteins has been observed often to result in a decrease in volume. The long time involved would be attributable to the breaking down of the struc-

ture (breaking down which results in the liberation of the proteins) rather than to the duration of the solving process. The increase in volume after a relatively short time in the frozen state (under 4, above) is attributed tentatively to the destruction of the capillary structure and to a reduction of capillary pressure. The decrease in volume on thawing (under 1 above) is more puzzling since a precipitation of the dissolved protein would increase the volume. Moran suggests that a process comparable to the swelling of proteins, which is known to result in a final shrinkage of the entire mass in which they are held, takes place in the precipitated flakes of the yolk. The decrease in volume during subcooling (under 3 above) is ascribed to an increase in the quantity of bound water, the degree of hydration of stable hydrates tending, in general, to increase at lower temperatures. The suggestion is made, finally, that this decrease in volume, observed in the subcooled state, might be the beginning of the same decrease which was observed after thawing, and which could eventually become an irreversible change if subcooling were maintained long enough.

Moran reported also that the irreversible changes just mentioned do not take place when the yolk is frozen in presence of a 10% sucrose solution (at -11°). He compares this phenomenon to what happens in plants which are rendered winter-hardy by an increase in their sugar content. (Such protective action, according to Shrivastava (*J. Phys. Chem.* 29, 166, 1925), would be due to an adsorption of sugar on colloidal particles.)

The permanent modifications produced in yolk by freezing were studied microscopically. Normal untreated yolk, fixed in Müller's fluid, consists of polygonal masses (the yolk particles). Frozen yolk, fixed in the same manner, presents a horny appearance. The author explains this by a rupture of the yolk particles on freezing, a rupture which can be obtained also by stirring.

Nord (1927-1938) and his collaborators investigated the effect of freezing, for various lengths of time, at tem-

peratures extending from 0° to -180° , on the physical constants of colloidal solutions of a large number of substances, such as, zymase, egg albumin, gelatine, gum arabic, saponin, metacholesterin, myosin, sodium oleate, casein, polyacrylic acid and its esters, polyvinyl alcohol, etc. In general, dilute solutions (0.001 to 1.0%) of these substances exhibited, after having been frozen and thawed, higher diffusion coefficient, interferometer value, viscosity, cataphoretic migration velocity, specific conductivity and also an increased absorption of such gases as ethylene and acetylene. More concentrated solutions (2%) showed, after freezing and thawing, lower values of these constants than did the unfrozen controls. The authors conclude that, in dilute solutions, freezing effects a disaggregation of the individual particles, whereas, at higher concentrations, it produces an aggregation of these primary particles.

In a study of the surface tension of frozen and thawed egg albumin and gelatin solutions, Nord (1934) found that this property increases with the duration of freezing but not with its repetition nor with the exposure to very low temperatures.

Heller (1934) studied coagulation by freezing in sols of FeCl_3 , $\text{Fe}(\text{NO}_3)_3$ and FeOOH , in terms of hydrogen-ion concentration, of duration of dialysis and of the length of the time of exposure to low temperatures. He found: 1. That coagulation never occurred when the pH was lower than 6.1; 2. That a dialysis of 69 days induced coagulation in a case in which a dialysis of 3 days was ineffective; 3. That the length of time the material was maintained in the congealed state, often increased the degree of coagulation. Comparing these results with those obtained in coagulation by stirring and noticing similar conditions in the two cases, he concluded that coagulation by freezing must result from a fusion of the colloidal particles under the compression exerted by ice. In freezing, as in stirring, coagulation would be possible only when the "double layer" at the surface of the micelles is weakened; such a

condition is obtained by dialysis. He attributed the influence of the length of time in the frozen state to a resulting slow increase in the size of the particles; in larger particles peptization after thawing would become impossible.

2. *Tissues.* The alterations caused by freezing in tissues consist in changes in the general appearance, color and consistency of the material, in a release of enzymes and other substances, in some chemical transformations, in a modification of the protoplasmic structure and in some cytological changes. A few examples of these various alterations will be described shortly, in the order given.

Some externally observable injurious effects of freezing in plants, and particularly in leaves, are well known by the agriculturists. Following Goeppert (1830), who is one of the earliest observers, we shall mention a blackening of the surface or of the entire organ, a more fleshy consistency, a somewhat transparent, shiny and glassy appearance, a loss of turgor, an exudation of water and a rapid desiccation. Flowers become, in general, dark brown.

Goeppert remarked a change of color of another type in some white orchids which, after freezing, become pale blue and finally dark blue. This property has been used as a criterion of death by several investigators in their studies of injury by cold.

Other changes in color have been described by Kylin (1917) in algae.

Molisch (1897) noted that some plants which contain coumarin release that odoriferous substance on being frozen.

The release of enzymes by freezing and the consequent increased enzymatic activity, in some tissues, is a commonly observed fact.

Formation of sugar under the action of cold has been reported by many investigators. The literature on winter hardening in plants contains abundant data on this subject.

Luyet and Grell (1936), in centrifuging onion root tips

which were previously frozen at about -80° and thawed, found that the cellular constituents could be stratified without difficulty while in tissues killed by heat no displacement was possible. Accordingly, they think that freezing does not induce protoplasmic coagulation as readily as do other lethal agents, such as, heat, toxic substances, high pressure, radiations, mechanical injury, etc. This is confirmed by the absence, in frozen tissue, of the characteristic opacity observed in tissue killed by the other methods.

However, Luyet and Gibbs (1937) published photomicrographs showing the protoplasm coagulated in frozen and thawed plant epidermal cells. The pattern of this coagulum was different than that obtained in death by heat. The authors remarked a difference in the consistency of the two types of coagula, which might explain why the components of one type could be separated by centrifugation while the components of the other could not. The coagulated material was found in a thin layer adhering to the wall of the cell in the same position as in the living state.

Bugaevsky (1939) also observed coagulated flakes in the cells of wheat after freezing and thawing.

The tearing of cell membranes by ice has been assumed by the earliest biologists. Goeppert (1830) contended that he could never observe any broken membrane. After him several authors have contributed to establish the fact that there is never a perforation of the membranes by ice crystals (for quotations see above, under the title "Phase Separation" in tissues). But, if the membranes are not broken, it is certain that they are damaged and that they lose their semipermeability, as is evidenced by the fact that the cellular fluids cannot be maintained any more within the cells and that the penetration of dyes becomes considerably easier. Prillieux (1869a) however, contended that there is more evidence for an injury to protoplasm than to its membranes.

Matruhot and Molliard (1902) reported the particular phenomenon of the vanishing of the chromosomes in a

terminal cell of the staminal hair of *Tradescantia* put to freeze at -12° . Some 18 hours after thawing, the chromosomes had reappeared and cellular division had progressed. The cell was apparently not killed.

Lenoir (1931) describes, in the cells of the ovary and of the nucelle of *Fritillaria imperialis*, after freezing, swollen chromosomes or spiremes and a disorganized linin network.

According to Detmer (1886), chloroplasts are disaggregated after freezing in the same way as when plants are killed by heat or by acids.

Several experiments on tissues described above under "Phase Separation" contain information on the subject discussed here.

SUMMARY

1. The formation of crystalline nuclei in a liquid is generally assumed to be initiated by collisions of molecules in some definite conditions of orientation.
2. Crystallization of water is sometimes considered as a precipitation of trihydrol dissolved in dihydrol.
3. There is some evidence that the formation of ice is preceded by a colloidal state.
4. It has been suggested that liquid water possesses a quasi-crystalline structure and that little molecular rearrangement is required for obtaining the crystalline state.
5. The freezing point of culture media varies from -0.01° to about -2.5° .
6. That of plant juices was found to lie between -0.3° and -2.5° for most species.
7. That of the blood of aquatic animals is, in general, about the same as that of their medium.
8. The individual differences in the freezing point of blood can be as much as 0.3 degree (in birds).
9. The freezing point of eggs follows a cycle, being nearly the same as that of the mother's blood during the period of their formation, then decreasing considerably and rising again during embryonic development.
10. Living protoplasm (myxomycetes) was found to freeze at the relatively high temperature of -0.17° .
11. Animal and plant tissues congeal at temperatures extending from about -0.4° to -3° .
12. The freezing point of living tis-

ness is, in general, lower than that of dead material. 13. Living tissues sometimes present two freezing points, one of which is probably that of the intercellular, the other, that of the exuded intracellular fluids.

14. The growth of crystals is less rapid when the temperature is lower. 15. The non-parallel variations in the velocity of formation of nuclei and in the velocity of growth of crystals, in terms of temperature, result in a dependence of number, size and form of crystals on temperature. 16. The velocity of growth of ice crystals is about 65 mm. per second at -8° ; the addition of solutes reduces this velocity (3% gelatine reduces it 350 times).

17. The separation of water during the congelation of solutions, suspensions and colloids can often be observed under the microscope. 18. In a gel cooled slowly, water comes out to the surface where it freezes in a compact mass, while, on a more rapid cooling, internal congelation takes place. 19. Internal congelation of gels sometimes results in the formation of spherulites within which one can observe two separated phases, ice and dehydrated gel, in alternating concentric layers. 20. In coagulated material, in porous structures and in tissues, water exudes to the surface during freezing and forms tiny ice columns which fuse laterally into ribbons or plates. 21. Cavities within frozen tissues are found filled with ice which has separated from the surrounding cells; intercellular spaces, in compact tissues, play the role of cavities and lens-shaped masses of ice accumulate in them. 22. In the case of single cells exposed to slow freezing, water exudes and congeals at the surface. 23. The withdrawal of water during freezing has been observed also in cellular constituents, in particular in nuclei, possibly in chromosomes.

24. The freezing curve of an ideal liquid consists of a linear and of two exponential portions. 25. The freezing curve of a solution consists of a hyperbolic, a linear and two exponential portions. 26. The plateaus of the freezing curves of most living tissues occupy a lower position, are shorter, less uniform and more influenced by cooling

velocities than those obtained with dead material. 27. There is little evidence of a eutectic freezing in tissues. 28. The quantity of ice formed per unit of time decreases gradually, in the course of freezing.

29. In colloids and in tissues some ice formation has still been observed below -20° . 30. It took 26 days, at -11° , for the completion of crystallization in a gelatin gel.

31. The following properties of ice are discussed: plasticity, fluidity of single crystals, viscosity, tensile strength, compressibility, supporting force; specific gravity; specific heat, heat conductivity, heat of fusion, heat of evaporation, vapor pressure, coefficient of expansion; electrical resistance, specific inductive capacity; birefringence, refractive indices.

32. The melting curves of tissues differ from the freezing curves in particular by the position of the melting point and by the "curve-area" representing the quantity of heat involved in each of the two processes.

33. After freezing and thawing, some colloids stay unaltered while others are irreversibly precipitated. 34. The causes of precipitation assumed by various investigators are: a compression between the ice masses, a change in electric charge during freezing or thawing, a change in chemical structure and the reaching of the saturation temperature. 35. Alterations of tissues by freezing include: changes in external appearance, release of enzymes, internal chemical changes, disaggregation of cellular constituents, and protoplasmic precipitation. 36. The latter seems to be of different nature than coagulation by other lethal agents.

CHAPTER II

SUPERCOOLING AND THE SUPERCOOLED STATE

A liquid is said to be supercooled (or subcooled, or undercooled) when it is still in the liquid state at a temperature lower than the freezing point.

The fundamental character of the subcooled state is to be unstable. The circumstances which control the passage of a subcooled liquid into the crystalline state are very incompletely known. The following are often mentioned in textbooks of physics: stirring, shaking, jarring, touching with a solid body, keeping in presence of impurities, maintaining in contact with the air, mostly with dust-containing air, etc. On the other hand, crystallization is said to be prevented when the liquid is heated before being cooled, when it is reduced to small droplets, when it is enclosed in capillary tubes, etc. It is difficult to say to what extent some of these various ways of inducing or preventing crystallization, which are sometimes effective and at other times not, have really been observed, and how frequently they entered the physical literature through the back door of a too conservative teaching of insufficiently controlled observations. A consideration of their mechanism of action might help one to correlate the data concerning their efficacy.

It is generally thought that the first crystallization nuclei originate from the collisions of the molecules and that any factor liable to bring the molecules in closer proximity should facilitate congelation while any factor rendering more difficult the collision of the molecules should delay congelation. Initiation of crystallization should, then, depend on the number of molecules present, on the time allowed for the molecules to collide and on the speed of motion, that is, on temperature. The presence of all-formed crystals, which need only to grow, is expected to induce congelation. A mechanical disturbance might, by a sudden change in the orientation of the molecules, increase the chances of successful collisions. On the

other hand, the destruction, by previous heating, of nuclei in formation, should render crystallization more difficult. The hindrance of molecular motion by capillary forces, in thin tubes or in small droplets, should delay the formation of centers. Finally, if the formation of nuclei is a random process subject to failure according to the laws of probability, one should expect some irregularity in the success of experiments designed to induce crystallization, under apparently identical conditions. We shall discuss below, after an introductory analysis of a typical subcooling curve, the most important of the factors just mentioned, which either cause or prevent crystallization in a subcooled liquid.

Subcooling is generally considered an exception to the rule that a substance crystallizes at a definite temperature. It seems more logical, however, to consider subcooling as the general condition, and crystallization at a fixed temperature as a particular case, since the fixed freezing temperature is only one, the highest, of the several temperatures at which a fluid can start crystallizing, and since congelation will not take place at the freezing point except in the particular case where a crystallization center, that is, an infinitesimal crystal, is present. Accordingly, it would be preferable, from this point of view, to define the freezing point not as the temperature of solidification but as the highest temperature of solidification.

The problems which concern the biologist in the study of subcooling are: 1. Whether, to what extent and under what conditions the fluids present in living animals and plants, in tissues, in cells and in protoplasm, are brought into, or barred from the subcooled state. 2. How life is affected by this state and, in particular, whether injury and death result from it or from its cessation? Only the first of these two problems belongs to the program of the present chapter, the other will be investigated elsewhere.

I. THE SUBCOOLING CURVE

When a liquid is subcooled, the curve representing the drop of temperature in terms of time, AB, (Fig. 28) con-

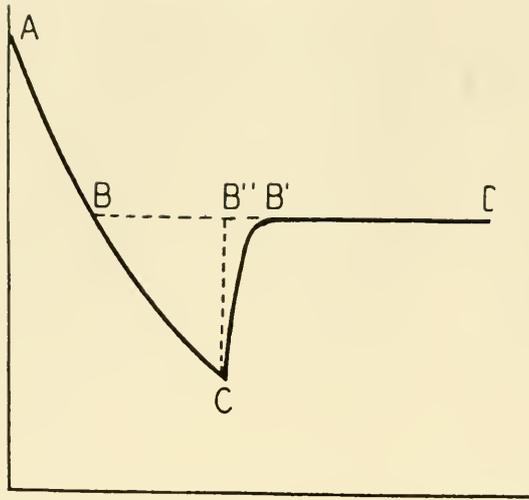


FIG. 28. Subcooling curve of a liquid under ideal conditions. Abscissae: time; ordinates: temperature.

times its course, past the line of the freezing point BB' , down to the *subcooling point* C . It then rises abruptly to the freezing point B' . The subcooling curve consists of 2 limbs AC and CB' . The temperature interval CB'' is the *degree of subcooling*.

AC is an ordinary exponential cooling curve.

Concerning the curve CB' we shall discuss briefly two questions: that of the maximum reached by the curve and that of the calculation of the freezing-curve-areas (see above, under "Freezing Curves") in cases in which there is some sub-cooling.

The problem of the maximum reached by the rising temperature can be treated as follows. Three factors influence the temperature, one of which tends to raise the maximum while the other two tend to lower it. These three factors are: 1. The amount of heat Q_1 liberated by freezing, during the time $B''B'$; 2. The amount of heat Q_2 withdrawn by the cooling bath, during the same time. 3. The amount of heat Q_3 used for bringing up the temperature of the material from the subcooling point. Q_1 is either equal to the sum $Q_2 + Q_3$, or it is smaller or larger

than this sum. In the first instance, the temperature will just reach the freezing point; in the second, the temperature will rise to a maximum somewhat below the freezing point; if the third were possible, the temperature would rise above the freezing point; but this cannot happen since the heat produced by the advancing head of a crystal in formation will first raise the temperature of the fluid immediately in contact with it, and when this local temperature, precisely in the area where the crystal is to grow, reaches the melting point, all crystal growth will stop. So, after subcooling, the temperature never rises above the freezing point, it reaches the freezing point in two of the three cases given and remains below the freezing point in the third.

When the freezing point cannot be reached, it is almost always because Q_2 is too high, in other words because cooling is too rapid. The following example gives an idea of the order of magnitude of the cooling velocity required to prevent the ascent of temperature to the freezing point. Let us suppose that, out of a total mass of 10 grams of water supercooled at -4° , 1 gram freezes out, and that one half of the heat liberated, that is, 40 calories, are withdrawn by the cooling bath during freezing (during the time B''B'), there is still enough heat left (40 calories) to bring all the water from -4° to 0° . To prevent the rise of temperature to 0° , the cooling velocity should then be such that more than 40 calories be withdrawn during the time B''B' (a fraction of a minute). This is a very high cooling velocity.

Some investigators, having simply admitted (perhaps from a too docile belief in classical statements) that, at crystallization after subcooling, the temperature reaches the freezing point, have made valueless determinations of the latter. With such cooling velocities as 10 degrees per minute, the temperature might not have the time to rise from the subcooling point to a maximum and it will simply be retarded in its drop (cf. Fig. 7, Curve 5); or if it reaches a maximum, there is no evidence that this is the freezing point.

In living tissues one has to consider the fact that water is withdrawn from the cells before it freezes, that crystal growth is then retarded by this slow osmotic process and that, therefore, less heat of crystallization is liberated during the time $B''B'$.

In the application of the method of the freezing-curve-areas to cases in which there is some subcooling, one should remember that this method is based on the assumption that the heat withdrawn by the bath is equal to the heat produced by freezing. When there is a subcooling, the heat produced is equal to the sum of the heat withdrawn and of the heat necessary to bring the temperature from the subcooling to the freezing point. The heat withdrawn is proportional to the area limited by the curve $CB'D$, (Fig. 28) the time axis and two abscissas, one at the beginning and the other at the end of the freezing period considered. To obtain the heat produced one should add to the number of calories found in the determination of this area the number of calories necessary to warm the material up to the freezing point.

II. FACTORS AFFECTING CRYSTALLIZATION IN A SUBCOOLED LIQUID

1. *Temperature.* By bringing the temperature far enough below the freezing point it is almost always possible to induce crystallization. The lowest temperature required for that purpose, that is, the maximal degree of subcooling, varies greatly with the nature of the substance studied. Some substances are classically known to present a very high degree of subcooling. Phosphorus and sulfur, which are often used in demonstration experiments, can be maintained liquid, respectively, at about 35 and 100 degrees below the freezing point; iron and nickel have also been observed supercooled by 100 degrees. According to Tammann (1925, p. 231), a supercooling of 20 degrees is quite common in organic compounds. Water is often still liquid at -3° or -4° , more rarely at -6° or -7° , and exceptionally, for short times, at -10° .

It is generally thought that biological material and, in particular, living matter can be subcooled to a larger extent than water and aqueous solution, and several biologists attribute to that property the high resistance offered by some plants to death by congelation. But systematic investigations on that subject are lacking.

While lowering the temperature induces crystallization in a subcooled liquid, warming the liquid is supposed to prevent freezing in a subsequent undercooling. The crystallization centers, it is assumed, are not quite completely destroyed when the temperature is raised above the freezing point, some molecular orientation would be maintained; but, if the material is warmed to a temperature far above the freezing point, the nuclei would be entirely destroyed and a high degree of subcooling or a longer duration of the subcooled state should be possible at a given temperature.

De Coppet (1907), in experiments on salol heated at temperatures up to 40 degrees above the freezing point (which is 42.6°), showed that the higher the temperature of the previous warming the longer the material stayed in the subcooled state at a given temperature. However, there were a number of exceptions.

Othmer (*Z. anorg. u. allg. Chem.*, 91, 235, 1915) studied, in a series of experiments on piperonal the number of crystallization nuclei formed at 2 degrees below the freezing point when the material had previously been heated to 3 and to 33 degrees above the freezing point. He found 228 and 33 nuclei respectively.

Concerning the time that the material is left at a temperature above the freezing point for destroying the crystallization centers, some physicists have expressed the opinion that it plays no significant role and that the temperature reached is the only factor to be considered. De Coppet (1907) conducted a few experiments on this question and he pointed out, in particular, the results of one experiment in which, after having maintained salol for one hour at 68 to 78 degrees above the freezing point, he could keep it in the subcooled condition for 6 years.

Luyet and Hodapp (1938b) investigated the destruction of crystallization centers by temperatures above the freezing point in potato tissue. They determined the proportion of subcoolings and of spontaneous freezings in material previously heated for two minutes at various temperatures. After exposure to 95° , 50° or 20° - 24° , the percentage of subcoolings was respectively 100, 95 and 85; after a previous freezing followed by exposure to 20° - 22° , 9° - 10° or 1.8° - 3° the percentage of subcoolings dropped to 75, 35 and 0 respectively (about 20 experiments at each temperature).

2. *Inoculation.* Crystallization of a supercooled liquid can be initiated by bringing the liquid in contact with a crystal of the same substance. This is called *seeding* or *inoculating*. The procedure is used as a routine laboratory method.

The fact that congelation starts when a crystal is present shows that the type of modification that a subcooled liquid undergoes to produce the first crystallite is an organization and orientation of molecules. When the first crystallite is already formed, no such modification is required, the molecules are simply attracted to the faces of the crystal on which they deposit.

Inoculation can be performed not only with crystals of the same substance as the liquid but also with isomorphous crystals, that is, with crystals in which the directions of growth are the same. This confirms the view that the mechanism of construction of a crystal, and evidently also of a crystallization center, consists essentially in controlling the directions taken by the molecules when they enter the crystalline structure.

Besides isomorphous crystals, some substances are said to be specific in starting the crystallization of some particular melts; for instance, traces of potassium hydroxide or of nitric acid are used for inducing crystallization of phosphorus. Assuming that this is an observed fact, one might attempt to explain it by the theory that certain substances contribute toward orienting the molecules of other

substances on account of some similarity of structure, even if that similarity is far from that presented by isomorphous crystals.

The biologists have tried to inoculate tissues or other biological materials by touching them with ice crystals. In general, the method is described as successful although, here and there, some authors express a doubt as to its efficacy.

Several plant physiologists used, for inoculating tissues or organs such as leaves, a method which consisted in putting a drop of water at the surface of the material, with the idea that water would freeze first and seed the adjacent tissue. Others who wanted to prevent crystallization, took precautions against any moisture on the surface.

Müller-Thurgau (1881) claims that potatoes cut into pieces subcool less than intact potatoes, because of the easier formation of centers on the cut surfaces.

According to Mez (1905), when a tissue was in contact, at one point, with the wall of a cold glass container, instead of being isolated from it by air, subcooling did not occur because of seeding by the ice formed at the cooler point. This author went so far as to say that he could guarantee a strong subcooling if a plant tissue was cooled in oil (he used castor oil) while there was no or little subcooling if the tissue was cooled in water.

Voigtländer (1909), to obtain subcooling in plant tissues, removed with absolute alcohol all traces of moisture on the glassware to come in contact with the tissues and on the thermo-needle to be inserted in them.

Harvey (1919), for preventing seeding by the extruded sap on the cut surfaces of leaves and petioles, dried them and covered them with vaseline. He also reported that leaves, stems and petioles covered with wax, bloom, or a thick mat of epidermal hair can be subcooled to lower temperatures than organs not so protected, or than plants in which the coverings were removed or broken. Such a protection is attributed to a prevention of seeding. A drop of water laid on the heavily covered samples did not seed the tissue below, as it did in cases of nude epidermis.

Wright and Taylor (1921) remarked that water or sap from bruises on the surface of a potato prevented that material from reaching the degree of undercooling that it would attain without the presence of such moisture.

On the other hand, Luyet and Hodapp (1938b) repeatedly obtained subcooling, and often to a considerable degree, when cylindrical pieces of potato tissue were exposed to low temperatures in glass vials previously filled with water. Moisture, in this case, did not facilitate freezing. One must notice, however, that this moisture might have lost its ordinary properties due to the fact that it was held, in the form of a capillary layer, between the material and the vial.

Concerning the efficacy of seeding by surface moisture, it seems that if such moisture can be brought to a lower temperature than the tissue itself (as, for example, by contact with a cooler object), or if it has a higher freezing point than cell sap, inoculation becomes understandable; but one cannot see why a film of moisture of capillary size, mixed with sap at the surface of a cut tissue, would not subcool as much as the tissue itself.

Luyet and Gibbs (1937) remarked that seeding through cellular membranes must be impossible in living tissues like onion epidermis, in which one can observe the subcooled cells freeze one by one, there being sometimes half-hour intervals between the congelation of neighbouring cells, as was recorded previously by Molisch (1897).

3. *The Time Factor.* Some authors claim that, if a liquid is maintained long enough in the subcooled state, the molecules will unfailingly have a chance of colliding in the conditions of orientation necessary for crystallization. This view is based on thermodynamic considerations, but what is somewhat puzzling is that the time required for the formation of nuclei at low and at high subcooling temperatures does not seem to vary in proportion to the change in thermodynamic activity of the molecules. While at low temperatures one can maintain the subcooled state for but a few seconds, at slightly higher temperatures one can sometimes maintain it for years.

The geologists contend that some rock inclusions are in the subcooled state and that they have been in that condition for millions of years. If this could be established as a fact it would serve the purpose of the longest imaginable experiment to check the theory according to which crystallization always occurs if enough time is allowed for it; and it would speak against this theory.

Among observations carried on over definite periods of time, let us mention that of de Coppet (1907) who maintained 16 solutions of sodium sulfate in the supersaturated state, far below the precipitation point, for 33 years.

Moran (1925) kept an egg yolk subcooled for a week at -11° (that is, more than 10 degrees below the freezing point), while, in almost all the other cases observed, subcooling of but a few degrees lasted only several hours.

These few facts, and many others, seem to indicate that the fundamental theories proposed for explaining the action of the time factor in initiating crystallization are none too reliable. More investigations on the relation between time and temperature and on the influence of the nature of the substance on that relation could pave the way to a deeper understanding of the problem.

4. *Mechanical Disturbance.* The types of disturbance usually mentioned as inducing crystallization are: shocks on the container of the subcooled liquid, vibrations of the building, the room or the table where the experiments are made, and shaking or stirring the liquid. The mechanism of action of the disturbance consists evidently in giving to the system which is in an instable equilibrium, the last impulse necessary to destroy that equilibrium. It is thought that this is done by some local compression on the path of the vibratory waves created by the disturbance.

Despretz, already in 1837, pointed out the frequent inefficacy of repeated shaking. Several investigators after him reported frequent negative results in attempts to induce crystallization by shocks or by stirring.

De Coppet (1907) could not obtain the congelation of salol by vigorous and repeated stirring, at a temperature

of 30 to 35 degrees below the freezing point. With supersaturated solutions of sodium sulfate, maintained at about 8 degrees below their saturation temperature, he succeeded in 10 out of 101 cases, in causing crystallization by a continued stirring of several minutes. In another series of experiments, in which the temperature was about 9 degrees below the saturation point, he obtained one crystallization out of 37 trials. When small sealed tubes containing the supersaturated solutions were dropped on a wooden table from a height of 10 cm., no congelation occurred.

Bachmetjew (1907) was not able to cause the freezing of a subcooled butterfly by hammering on the table on which the insect was lying, or by tapping the wire of the thermoneedle inserted in its body.

On the other hand, according to Wright and Harvey (1921), undercooling of potatoes maintained at -4° could be terminated at any time by a shock.

Wright and Taylor (1921) studied the effect of jarring resulting from rough handling or incidental to hauling, in causing the undercooled potatoes to freeze. They found that dropping them on a hard floor or even tapping them with a pencil was generally effective.

Luyet and Hodapp (1938a) determined the proportion of efficient mechanical shocks on the congelation of potato tissue. A cylindrical shell of tissue was fastened around the bulb of a thermometer, and a slight tap was given to the upper end of the thermometer with a wooden lever, when the tissue was subcooled to temperatures from -1° to -5° . In 63 experiments out of 122, crystallization occurred on tapping. The percentage of successful taps increased at lower temperatures, but to express numerically the efficacy of tapping, one must eliminate from this percentage that of possible coincident spontaneous crystallizations, which also increases at lower temperatures. Unfortunately, the latter percentage is unknown. Summarizing their results, the authors conclude that no doubt is left as to the efficacy of tapping in some experiments, and as to its inefficacy in others. They remark that such

a behavior is consistent with the theory that crystallization occurs as an event which follows the law of probability.

As to the degree of force necessary to induce crystallization, several observers have noted that it varies with the degree of subcooling. Fahrenheit, who is considered the discoverer of the subcooled state, remarked, already in 1724, that strongly subcooled water could be made to crystallize by a shock, while slightly subcooled water could not.

According to Mousson (1858), water can be maintained subcooled to -12° or -15° in a vacuum but then the slightest disturbance causes solidification. The same author could obtain a high degree of subcooling in tiny water droplets laid on a cold plate, but touching them with the point of a pin caused them to freeze at once.

De Coppet (1907) observed that a strong stirring could not cause congelation of salol subcooled 30 to 35° below the freezing point, while 10 degrees lower, crystallization took place readily, on the slightest disturbance. The same author said that when he gave to sealed tubes which contained supersaturated solutions of sodium sulfate, the slight inclination necessary to bring an air bubble present in them from one end to the other, he sometimes observed solidification.

A study of the minimal disturbance (friction) which is effective in causing the precipitation of supersaturated solutions, has been made by Young (1911).

Sensitivity to mechanical disturbance, as well as sensitivity to several other factors, depends on the nature of the material. Wright and Taylor (1921) observed that, while subcooled potatoes can be induced to freeze by a slight tap, berries subcooled to the same degree did not congeal under vigorous tapping.

Mez (1905) claims to have observed a rather peculiar behavior in the cell sap of *Impatiens*, pressed out, filtered, boiled, subcooled and subjected to shaking. A slight disturbance started crystallization at temperatures immedi-

ately below the freezing point and at the extreme lower limit of subcooling, while a vigorous shaking was ineffective in the intermediate zone of temperatures. He contends also that spontaneous crystallization occurred most of the time between -0.72° and -2.9° and between -5.01° and -8.17° and less frequently in the intermediate region. To establish the existence of such zones of stability, more statistical data would be necessary.

We shall mention, to finish this section, a curious but too concisely described observation of Wartman (1860). Some water that he had left during an entire night, at about -4° in a glass container 31 cm. high and 16 cm. wide (diameter ?), had stayed liquid, but when he took the container to empty it, in the morning, three walls of ice suddenly formed, making 60° angles (?) with each other, adhering at one side to the wall of the container and oblique to the axis of the latter.

5. *Capillarity.* Some of the oldest investigations on subcooling were made by Gay Lussac (1836) who observed that water can be subcooled to -12° when it is enclosed in small tubes.

Later Despretz (1837) reported that the congelation of a liquid is "retarded" by ten or twelve degrees if the liquid is enclosed in a thermometric tube or even in a tube having a diameter of one centimeter.

Mousson (1858) exposed to temperatures from -5° to -7° , 8 tubes which varied in diameter from 0.187 to 2.503 mm. and which were filled with water and sealed with sealing wax. After a night at the temperatures indicated, water was frozen in all the tubes which had a diameter larger than 0.9 mm. while it was liquid in the smaller ones. In other experiments he sprayed droplets of water less than 0.5 mm. in diameter on a dry surface and observed that the smaller the drops the longer they stayed subcooled. The fact that, in clouds, water remains liquid at rather low temperatures is attributed to the very small size of the droplets.

Dufour (1861) studied the subcooling properties of small

drops. He made suspensions of water in mixtures of chloroform or petroleum and sweet almond oil (mixtures which have the same specific gravity as water), and he observed that the smallest drops stayed liquid at -20° .

Bigelow and Ryckenboer (1917) attempted to determine the degree of subcooling in capillary tubes in terms of the diameter of the latter. They encountered difficulties with water which presents such a narrow range of subcooling temperatures and they decided to use other substances, in particular, sulphur. In a previous determination the same degree of subcooling was obtained in tubes 4 millimeters in diameter and in larger ones. It was then decided to use tubes of about four millimeters as standards of comparison and tubes of smaller size. Definite differences in the degree of subcooling were sometimes observed in tubes of different diameter; for example, a tube of 4.1 mm. gave an average subcooling point of 59.5° while a tube of 0.164 mm. gave 53.5° . But in other cases, the results were so inconsistent, and sometimes two tubes of the same size furnished such different average data that the authors questioned the significance of the experiment. The inconsistency of the results, especially in systematic investigations like those of Bigelow and Ryckenboer, renders doubtful the commonly accepted assumption that the degree of subcooling increases gradually with the decreasing size of the capillary spaces. There is some evidence that capillary forces exert an action in rendering congelation more difficult, but the data so far obtained are not sufficient to say anything on that relation.

The biologists have generally admitted without discussion that capillarity increases the degree of subcooling and they attributed to capillary forces in the intercellulars or within the cells the fact that plant and animal tissues subcool to a greater degree than water.

According to Müller-Thurgau (1886) living plant tissues undercool more than the extruded cell sap. The hindrance of molecular motion in living protoplasm is considered responsible for the maintenance of the subcooled condition.

Bachmetjew (1901) claimed to have observed a relation between the size of insect pupae and the degree of subcooling, the latter being less in larger pupae, and he explains his findings by the capillary properties of the tissues which he assumes to be related to the size of the pupae.

Mez (1905) stated that considerable subcooling is possible only in plants with very fine intercellulars, and that there is no or only a slight subcooling with larger intercellular spaces.

Voigtländer (1909) undertook to check Mez' statement. For that purpose he measured, on *camera lucida* drawings, the areas representing the intercellulars and compared them with the degrees of subcooling observed in 16 different plant species. For areas varying from 2.4 mm.² (*Strelitzia augusta*) to 25 μ^2 (*Ricinus communis*) he obtained an increase in the range of subcooling from 0 to 11 degrees. The increase was, in general, regular, although there were some inconsistencies. In the case of the presence of tracheae, these observations could not be confirmed. The same author studied the relation between cell size and degree of subcooling in tissues of 11 sorts of plants, using fifty or more specimens of each. His conclusion is that there is no relation between these two quantities.

Altogether considered, it seems that neither the physicists nor the biologists have obtained convincing evidence of the effect generally attributed to capillarity in maintaining the subcooled state.

6. *Impurities.* Among impurities assumed to be capable of starting the solidification of a subcooled liquid, that most often mentioned is air. We shall also describe a few experiments in which droplets of oil and colloidal particles were considered the inoculating agents.

According to one of the early investigators of the changes of state, Dufour (1861), one should, for obtaining a good subcooling of water, free it from air and maintain it in an atmosphere at reduced pressure.

Mez (1905) reports that, while he could not obtain subcooling invariably nor to any considerable degree with

pressed-out plant sap, even when, after repeated boiling and filtering, the liquid was clear and transparent, he did obtain subcooling at will, when he had previously frozen and thawed that clear liquid several times and let the air bubbles enclosed in the ice escape during thawing. To prevent the dissolving of air bubbles anew, he maintained a layer of oil at the surface of the material. The difficulty of subcooling tissues which contain tracheae is attributed by him to the abundance of air in them. He explains in the same manner the fact that the intercellulars freeze before the cell contents. Concerning the mechanism of action of the air bubbles, Mez suggests that their separation under the action of cold consumes heat and that the resulting local cooling favors the formation of crystal nuclei.

Some authors think that the action of air in inducing crystallization is indirect and that it should be attributed to dust particles or infinitesimal crystals contained in the air. But against this contention one has the observation of Moran (1925) that chicken eggs show an increase in the tendency to subcool when their shell is coated with vaseline.

Cases of crystallization of a subcooled liquid in a sealed glass container, at the breaking of the latter, are not unfrequently mentioned in the literature. Some authors attribute crystallization to a disturbance at breaking, others, to the sudden contact with the air.

Mez (1905) could not readily obtain subcooling in cell-sap which had been heated while it was covered by a layer of oil. He thinks that some emulsification took place and that the oil droplets in the sap play the same role as air bubbles in preventing subcooling.

The efficacy of colloidal micelles in inducing crystallization is suggested by Füchtbauer (1904) who expresses the opinion that some dust particles which prevent subcooling are of colloidal nature.

Mez (1905) considers the difficulty in subcooling turbid cell-sap as due partly to the action of mucous and gummy substances. He thinks that colloidal particles might give the start to the formation of nuclei.

Voigtländer (1909) attempted to investigate this suggestion. He used Malvaceae which are known to be rich in nuclei. Out of 71 specimens, 52 did not subcool and the others furnished but a very slight subcooling.

It is of interest to notice that, according to the last mentioned authors, colloids favor crystallization, while most of the physicists and biologists hold that anything which delays molecular motion should prevent the formation of nuclei.

Despretz (1837), assuming the possibility of inoculation by the presence of a foreign body, remarked that the delay in the congelation of water (subcooling) takes place as well in a copper or in a lead container as in glass.

The notion of impurity being conventional, the entire question of inoculation by an impurity resolves itself, in the last analysis, to that of inoculation by a body other than the subcooled liquid itself.

7. *Other Factors.* Among other factors which might have an influence in inducing crystallization we shall mention the cooling velocity and the concentration of the supercooled or supersaturated solutions.

Some data on the first of these two factors will be found in the work of Füchtbauer (1904) who concludes that the cooling velocity does not influence subcooling.

According to Bakhmetieff (1901) the relation between the degree of subcooling and the cooling velocity is a rather complicated one. At some cooling rates there would be a maximal subcooling, and at higher and lower cooling rates, subcooling would be less. The author observed many exceptions to that "law," and he attributes them to individual differences or to various degrees of development of the organisms investigated (insects). It seems that Bakhmetieff's notion of the method of establishing a law of nature was different from that commonly accepted.

Voigtländer (1909), from numerous experiments on plant tissues, concluded that there is no relation between velocity of cooling and degree of subcooling.

Jones, Miller and Bailey (1919), working with potato,

reported that when the temperature was lowered slowly to -5° the subcooling point of the material approached that temperature, while if cooling to -5° was rapid, subcooling ceased near -3° .

Wright and Taylor (1921) confirmed the observations of the last mentioned authors by showing that with a cooling temperature of -9° a subcooling point of -6.5° was obtained (on potato), while with a cooling temperature of -12.9° subcooling ceased at -5° .

As to the factor, solute concentration, a work of Jaffé (1903) would indicate that more concentrated solutions would be more sensitive to crystallization by shock.

Voigtländer (1909) attempted to investigate the influence of the osmotic concentration on the degree of subcooling, in plants. He worked with 20 different species and made at least fifty determinations on each. No relation was found; the subcooling point varied at random from -4.19° to -11.07° , and the maximum subcooling was about the same in plants isotonic with 1% and in plants isotonic with 4.6% KNO_3 . Unfortunately, in the same work, strange results were obtained for the freezing points, there being no relation between the latter and osmotic pressure; for example, materials isotonic with 2.6% and 4.4% KNO_3 were found to have the same freezing point. However, since at subcooling there is a reversal of the direction of the freezing curve, which can be more accurately determined than the simple slowing of the curve at freezing (*Cf.* Fig. 7, curve 5), Voigtländer's subcooling maxima might be sufficiently well observed to be taken in consideration.

That a higher concentration favors subcooling would result from an observation of Moran (1925) who exposed chicken eggs to evaporation until they lost about 1% of their weight and noticed, then, that subcooling took place more readily.

Weigman (1936) says that he obtained deep subcoolings with snails (*helix*) possessing an operculum, while uncovered snails presented only slight subcoolings. Since it is

known that, in the autumn, the snails lose a considerable amount of water before entering into the dormant state, the increased tendency to subcool should, perhaps, be attributed to the concentration of body fluids.

SUMMARY

1. Some fundamental principles on the nature of subcooling and the mechanism of crystallization in a subcooled liquid are outlined.

2. In an analysis of the subcooling curve, two problems are discussed: a. The maximal temperature reached by the ascending curve; b. The quantity of heat liberated as calculated by the method of the freezing-curve-area.

3. The factors inducing or preventing crystallization, as studied by the physicists and the biologists, are reviewed; the following topics are considered: a. Temperature, specific response to temperature, destruction of centers by previous warming, duration of previous warming; b. Inoculation, inoculation by isomorphous crystals and other bodies, inoculation of tissues through cell membranes mostly by surface moisture; c. Time factor, maintenance of the subcooled state for long periods; d. Mechanical disturbance, its frequent inefficacy, its undeniable efficacy in other cases, the magnitude of the disturbance required, the specific sensitivity to mechanical disturbance; e. Capillarity, the inconsistency of the experimental data; f. Impurities, air, oil droplets, colloidal particles.

CHAPTER III

THE VITREOUS STATE, VITRIFICATION, DEVITRIFICATION AND VITROMELTING

The subject of this chapter is new in biology. The first experiments on "Vitrification of Protoplasm" were reported by Luyet in 1937. Since the data are still scarce, instead of following our usual procedure which consisted in selecting the essentials in the literature, we shall attempt to give, in compact form, a complete account of the experiments made up to the present (May 1939) and shall supplement it with some observations and remarks suggested by our own experience with the subject.

The first part of this chapter will be devoted to a study of the principles, facts and methods relating to the vitreous state in *physical systems*, and the second will treat of the application of these principles and methods to *biological material*.

I. PHYSICAL SYSTEMS

1. *The Vitreous State.* The vitreous state has been known in silicates for hundreds of years, but it was generally assumed that the possibility of becoming vitreous was an exceptional property of these bodies. At the end of the last century (1898), Tammann pointed out that a large number of substances can be obtained as glasses and suggested that this property might be universal. Out of a series of 153 carbon compounds investigated, 59, that is, 38 per cent, could be vitrified.

The production of the vitreous state is conditioned by temperature in the manner illustrated in the diagram, Fig. 29. If the temperatures are plotted on a horizontal line, from the absolute zero up, the states, gas, liquid, crystalline and vitreous, are represented by the zones G, L, C, V, and the changes of state by the zone D and the points M and B. Upon a lowering of temperature, a body in the gas state becomes liquid at the liquefaction point B, it thereafter crystallizes at the freezing point M and stays

crystalline down to the absolute zero. But if, by abrupt cooling, one can bring a liquid through the zone C before it has the time to crystallize, it takes the vitreous state and stays vitreous at lower temperatures. If a body in the vitreous state at a low temperature is warmed up slowly, it devitrifies, that is, it becomes crystalline when it reaches the devitrification temperatures D, it becomes liquid at the melting point M and it is transformed into a gas at the boiling point B.

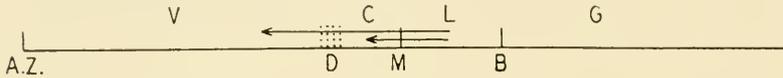


FIG. 29. Diagram representing, on a temperature scale of which the origin is the absolute zero, A. Z., the four states of matter: gas G, liquid L, crystalline C and vitreous V, and the three changes of state: boiling B, melting M and devitrification D. The upper arrow indicates the change of temperature for passing from the liquid to the vitreous state, and the lower arrow the change of temperature involved in subcooling.

A glass is amorphous, that is, its molecules are distributed at random, while, in a crystal, there is an ordered arrangement of the atoms. A glass is isotropic, its physical properties being the same in all directions, while most of the crystals are anisotropic, their physical properties having higher coefficients in some directions than in others. In polarized light, between crossed nicols, a glass is opaque, while most crystals are brightly illuminated. A glass differs from a liquid in that it is hard and breakable, while a liquid is fluid and deformable.

In the passage from one state to another, an intermediate state can, as we said above, be skipped over. A crystal can become a gas without taking the intermediate liquid state, a process which is known as sublimation. Similarly, when the crystalline state is skipped over by rapid cooling, the body passes from the liquid to the vitreous condition, it is said to vitrify. If the crystalline state is avoided by rapid warming, the vitreous body becomes liquid, a process which we call vitromelting or vitrofusion.

The two transition processes, melting and boiling, are

not entirely comparable to devitrification. Melting and boiling are reversible while devitrification is not. A gas becomes a liquid on cooling and a liquid becomes a gas on warming; a liquid crystallizes when one lowers its temperature and a crystal melts when its temperature is raised; while a glass crystallizes on warming but a crystal does not vitrify on cooling.

Furthermore, melting and boiling take place at temperatures which are practically points, while devitrification occurs over a larger range of temperatures. To illustrate this difference, we indicated in the diagram (Fig. 29) the boiling and melting points by the lines of separation B and M, and the range of devitrification temperatures by the zone D.

According to these principles, the behavior of silicates is not exceptional. Their vitreous zone (V in Fig. 29) extends from the absolute zero to about 1000° C. Since the atmospheric temperature, which prevails about us, is within this zone, we are familiar with the vitreous state of silicates. A silicate glass heated to the devitrification temperatures (below the melting point) becomes opaque by crystallization. At a higher temperature, the crystals melt.

From observations made on some silicates which devitrify very slowly, becoming opaque in the course of several years, it has been concluded that the vitreous state is unstable and that if enough time, *i.e.*, centuries or thousands of years, were allowed, devitrification would always take place. The fact that natural flint has an opalescent appearance, due probably to the presence of minute crystals, is sometimes considered an indication of the instability of the vitreous state, it being assumed that crystallization is still being completed. But there is no evidence that the opacity of flint has developed during the millions of years which have followed its vitrification and that the crystals now observed were not formed at the same time as flint itself.

It has been mentioned above that glasses are regarded

by many as supercooled liquids. This definition is useful, in a sense, since it reminds one of the fact that glasses resemble liquids in that they present a random arrangement of their molecules. But such a conception ignores the fact that, while a supercooled liquid is in so unstable a state of equilibrium that the contact with a crystal invariably produces its crystallization, a glass can be put in contact with a crystal without losing its stability. A supercooled liquid has been brought from the zone L (Fig. 29) into the zone C of crystallization temperatures, where it is highly unstable; a glass has been brought, from the zone L, below the zone of crystallization temperatures, into the zone V where it is practically stable. The change in temperature which leads to the formation of a glass is represented diagrammatically by the upper arrow in Fig. 29, that which leads to the formation of a supercooled liquid is represented by the lower arrow.

From what has been said it follows that a body can crystallize (*i.e.*, freeze, to use the common term) only within a limited range of temperatures (C and D, Fig. 29). In the zone of the vitreous state (V, Fig. 29) freezing is impossible, the temperature being too low. Since the crystallization range can be reached from above and from below, there are two ways of freezing a body: one is to cool it down from the liquid state, the other to warm it up from the vitreous state. We are so accustomed to the idea that freezing is more intense at lower temperatures, that the statement which precedes appears paradoxical.

As we shall see later, for aqueous colloids, the zone of crystallization temperatures extends over some tens of degrees below zero; consequently these colloids cannot be frozen (crystallized) if the temperature within them is below this range.

The reason why plants or animals freeze in nature, when the atmospheric temperature drops to -30° or -40° , is that the drop is too slow. The objects which are being cooled remain for a few minutes at the dangerous (freezing) temperatures. To avoid freezing, the temperature

should drop at a rate of some hundred degrees per second, within the objects themselves.

2. *Vitrification.* As a consequence of what has been said on the position occupied by the vitreous state at the lower end of the temperature scale and the impossibility of passing from the crystalline to the vitreous state, the only method of vitrifying a substance is to take it in the liquid or gas state and cool it rapidly so as to skip over the zone of crystallization temperatures in less time than is necessary for the material to freeze.

The ease with which an intermediate state can be skipped over depends on the range of temperatures at which that state obtains. The liquid state of CO_2 , for example, is easily avoided when solid CO_2 is warmed up, on account of the fact that the melting and the boiling point almost coincide.

The range of freezing temperatures, although large in some substances under ordinary conditions, can become narrower when such processes as subcooling occur. For example, in a gel of which the freezing zone extends from -2° to -12° , a subcooling to -7° will reduce this zone to one half its normal size, that is, to a range of from -7° to -12° .

Another factor of considerable import in the vitrification of a substance is its crystallization velocity. It is evident that when the crystals grow faster one must traverse the crystallization zone more rapidly if one wants to avoid crystallization.

The essential point in the vitrification technique being to overcome the velocity of formation of crystals, the main problem is to secure a high cooling velocity. Practically the only method used to cool a liquid or a solid body (for a gas, it is different) is to bring that body in contact with another at a lower temperature. The cooling velocity will be higher when the temperature of the cooling medium is lower and when the contact is better. Liquid air (-190°) is appropriate for the vitrification of aqueous colloids, aqueous solutions and protoplasm. Liquid hydrogen or

liquid helium would be still better, their boiling points being respectively about 60 and 80 degrees lower. A liquid bath cooled to about -75° with solid CO_2 also allows vitrification of most aqueous colloids, although with a lower efficiency.

It is important that the cooling bath be liquid; a liquid insures a better contact with the substance to be vitrified than either a solid or a gas. Attempts at vitrifying thin layers of gelatine gels by application of smooth plates of solid CO_2 on both sides gave poorer results than immersion in a liquid bath at the same temperature.

The fact that liquid air, when evaporating, forms a protective mantle around the object to be cooled has led the histologists who use rapid freezing as a method of fixation to look for another cooling liquid. They have adopted isopentane cooled in liquid air. Isopentane has a very low freezing point (-159°), it can be subcooled to -200° , and it has a relatively high boiling point (28°); because of this last property it does not boil when it comes in contact with the object to be cooled. Nevertheless, experimentation has convinced us that isopentane is not so satisfactory for the dissipation of heat as one might expect. Small quantities of a gelatine gel were placed in glass tubes about 1 millimeter in inside diameter, closed at one end. The tubes were immersed in a bath at -10° where their contents froze. Some of them were then immersed in water at 20° , the rest in isopentane at the same temperature, both warming media being well stirred. It was found that the time necessary for melting the gelatine was considerably longer in isopentane than in water. This is doubtless due, to a large extent, to the difference in heat conductivity of the two liquids (inclusive of the difference in contact conductivity).

The velocity of cooling also depends on the mass to be frozen and on its surface. One will obtain rapid elimination of heat by reducing the material to sheets with the smallest possible thickness and the largest possible area. Calculation shows that when a glass strip, 0.1 mm. thick,

is transferred from air at 20° to a liquid bath at -200° , the temperature drops about 200 degrees during the first second. It is generally admitted that, with liquid air as a cooling bath, the drop in temperature is somewhat slower because of the formation, around the object, of the protective air mantle mentioned above; however, the cooling velocity obtainable with liquid air amply suffices to vitrify objects whose thickness is of the order of 0.1 millimeter.

When the mass of material to be cooled is too large, only the most external layer vitrifies. The inner parts, which lose their heat too slowly, freeze.

Whenever crystallization begins at some point on an object, the conditions for vitrification of the rest are impaired. The portion which crystallizes liberates heat; this spreads to the surrounding parts and maintains their temperature at the freezing point. Hence, the whole mass might freeze.

In our research on the vitrification of colloids we have found, moreover, that their water content determines the possibility or impossibility of vitrification. In general, with 50% gelatin solutions, we have been able to vitrify layers 0.3 mm. in thickness (by the method of immersion in liquid air), while, with solutions containing 90% water, we could vitrify only smears a few micra thick.

Attempts at vitrifying pure water have been made by a few investigators. Burton and Oliver (1935) obtained, from steam, some solid water in which X-ray analysis did not reveal any crystalline structure.

Previously, Hawkes (1929) had mentioned an experiment in which a drop of solid amorphous water was obtained, by chance, during rapid cooling.

The difficulty experienced in vitrifying pure water has been attributed, in general, to the high velocity of crystallization of that substance. Walton and Judd (1914) who measured this velocity, found 65 mm. per second (an exceptionally high velocity).

According to Callow (1925), the addition of 3% gelatine to water reduces its velocity of crystallization to $1/350$ of its value.

This property has rendered possible our experiments on the vitrification of colloids (Luyet, 1937). We proceeded in the following manner: A drop of a 50% gelatin solution was put, when still hot, on a thin glass support and spread out so as to form a layer about 0.2 mm. thick. This preparation was then immersed in liquid air. When we took it out, the gelatin was vitrified, as shown by its transparency when viewed against ordinary light and its opacity between crossed nicols. After about 10 seconds of exposure to room temperature, the gelatin became opaque under ordinary illumination and reestablished light between crossed nicols (devitrification, *cf.* Fig. 30). This fundamental experiment has been the starting point of all the investigations which we made on the vitrification of protoplasm.

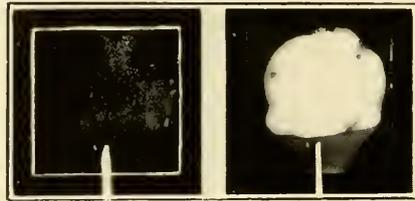


FIG. 30. Vitrified and devitrified gelatin gels on glass supports, photographed against a dark background. The figure on the left shows the vitrified gelatin almost transparent; the figure on the right shows the same preparation after it has been warmed up and has become opaque by devitrification (crystallization). (Original, Luyet.)

To sum up, the method of vitrification consists essentially in immersing a thin film of the liquid to be treated in a bath at a very low temperature.

Vitrification is a process of quite a different nature than that used in the refrigeration industry as "rapid freezing." When some hundred pounds of food are frozen in 10 or 15 minutes, small crystals are formed which cause less damage within the tissues than larger crystals, as those found in slower freezing, would. In vitrification one aims at avoiding crystallization altogether and, to achieve this, the cooling velocity should be about a thousand times higher than in the industrial process.

3. *Devitrification*: When one raises the temperature of a vitreous substance, it crystallizes. In the glass industry, this process is called devitrification or recrystallization.

Among the various methods of ascertaining the passage from the vitreous to the crystalline state, the simplest is that of observing the decrease in transparency. When a large number of small crystals are formed in a body in the vitreous state, light is scattered in all directions, the transparency is attenuated and, in some cases, the body becomes completely opaque.

This method, however, is not always infallible. In devitrifying concentrated solutions of magnesium chloride, we observed that the transparency remained altogether unaltered, although the material reestablished light between crossed nicols. The method of analysis by polarized light is, therefore, necessary in certain cases.

The best method of diagnosing the commencement of devitrification would be the analysis by X-rays. This might allow one to perceive crystallites of very small dimensions in most of the substances which we consider glasses.

The possible existence, in a body in the vitreous state, of these crystallites or of what we previously called nuclei of crystallization is suggested by the fact that, during devitrification, a glass passes from the condition of transparency to that of opacity by a continuous and uniform darkening. One cannot distinguish separate centers of crystallization in this case as one can when one induces crystallization from the liquid state. If the opacity of a devitrified body is due to the presence of crystals, the gradual darkening ought to be explained by an increase in the dimensions of the crystals already formed or by the formation of new crystals. So, when a preparation begins to lose its transparency, it does so either because nuclei of crystallization are forming, or because the already pre-formed nuclei, at first too small to cause the obscuration of the vitreous substance when it is observed with ordinary light, are growing in size.

When the temperature is sufficiently high, devitrification proceeds with an easily measurable velocity. For example, a 1M solution of sucrose, vitrified in a thin layer, devitrifies within 10 seconds when exposed to a temperature of -26° ; it devitrifies in a minute at about -30° , while it does not devitrify at all, even within an hour, at -35° . We call -26° , -30° , etc., "temperatures of devitrification," but it is evident that one must indicate the time required for devitrification at each temperature if one wishes to give to the notion of devitrification temperatures a precise meaning.

We have undertaken to establish the devitrification temperatures, or better, the time-temperature curves, for aqueous solutions of various organic (Luyet, 1939) and inorganic substances. The method employed was as follows: A small drop of the solution to be vitrified was placed between two glass strips, each about 0.1 mm. thick, kept apart by two bits of glass 0.1 mm. in thickness. This preparation was first immersed in liquid air; thereafter it was placed in an isopentane bath maintained at a constant temperature, and the time necessary for complete crystallization was determined. The opacity of a frozen preparation served as a term of comparison to show when the devitrification was complete.

The curves obtained seem to indicate that, contrary to the general belief, devitrification does not take place at all at very low temperatures. If one extrapolates the curve of Figure 31, for example, it becomes parallel to the time axis at a temperature a little below that at which devitrification occurs within a minute. The hypothesis of a very slow rate of devitrification, requiring hundreds of years for producing an observable result, seems, therefore, to be out of the question, at least for certain substances like those studied here.

Within a rather large range of concentrations, for example, from 0.9M to 2.2M for a sucrose solution, the devitrification temperatures change but little. Thus, 1M and 2M sucrose solutions have devitrification temperatures

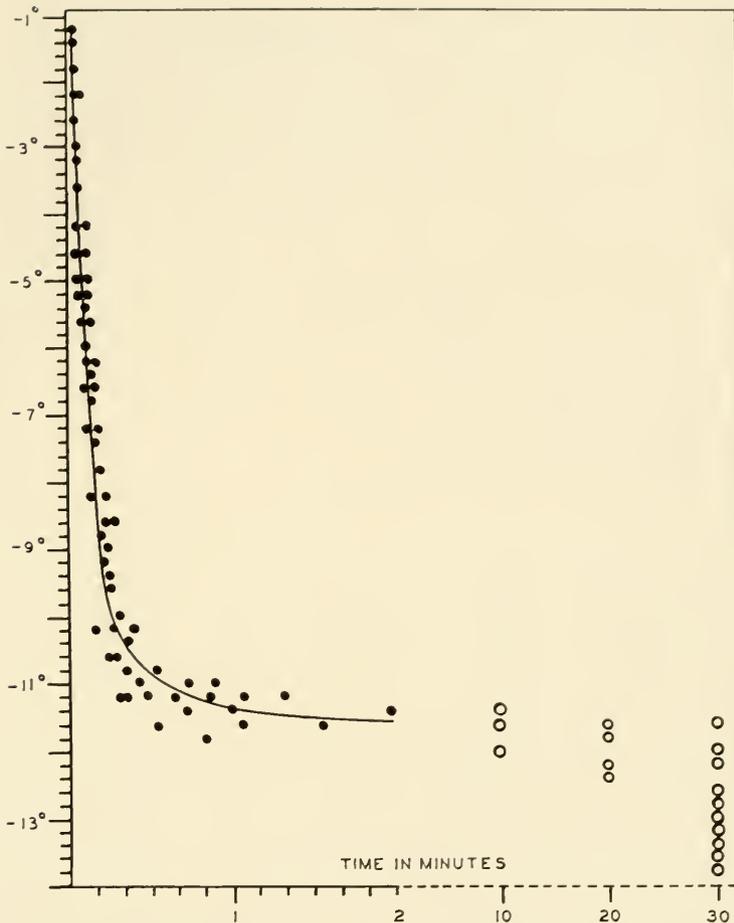


FIG. 31. Time-temperature curves of devitrification of a 20% gelatin solution. The dots indicate a complete devitrification; the circles signify that devitrification was not completed at the time and temperatures recorded.

which differ by only 0.4 degree (-31.4° and -31.8° respectively; devitrification in 5 minutes). For determining these temperatures, two drops, one of each solution, were mounted on the same preparation and the increase in opacity of the two was observed simultaneously.

This lowering of the devitrification temperatures with increase in concentration reminds one of the freezing point lowering of aqueous solutions. Nevertheless, we cannot say at the present time to what extent these two processes are referable to the same cause.

At concentrations higher than those of the above mentioned range, a devitrification of a different type obtains, which we shall describe farther on.

Since devitrification is a change of state comparable, in many respects to other changes of state like melting and boiling, it is probable that, like them, it takes place at a temperature which is a function of the chemical structure of the substance under investigation. One should expect a rise in the devitrification temperatures as one passes, within the same series, from compounds of simple molecular structure to those with a more complex one, as is the case with the boiling point, which rises when one passes, for example, from methane to pentane. We could establish the existence of such a relation. In a series of sugars we obtained (duration of devitrification, 5 minutes)¹:

Glucose	2M	$C_6H_{12}O_6$	-40.6°
Sucrose	2M	$C_{12}H_{22}O_{11}$	-31.8°
Raffinose	1M	$C_{18}H_{32}O_{16} \cdot 5H_2O$	-27.2°
Dextrin	2M x	$(C_6H_{10}O_5)_x$	-9.4°

The other high-molecular-weight substances which we have studied, such as, gelatine, albumin, gums, dextrin, have high devitrification temperatures (about -10°). Substances whose chemical composition is more or less similar to that of the sugars, but which have much lower molecular weights, such as glycerine, ethylene glycol and formaldehyde, have been found, in preliminary experiments, to have devitrification temperatures in the neighborhood of -60° to -70° .

However, the relation which we just noted cannot be simply compared to that between the molecular complexity of a substance and its melting or boiling point. In the one case, it is question of solutions, in the other of definite compounds.

It is to be expected that certain molecular groupings in the solute have a specific effect on the devitrification tem-

¹ Luyet, B. J., paper in press in *J. Phys. Chem.*

peratures of the solution. In preliminary investigations on this problem¹ we have observed that dextrose and levulose, which have the same empirical formula but differ in the structural arrangement of their atoms, have the same devitrification temperatures, while sucrose and lactose, which are likewise isomers, exhibit a slight difference therein.

There also exists a relation between devitrification temperatures and water of hydration or, more generally, between devitrification temperatures and the water-binding capacity of the solute (Luyet, 1939). With sucrose solutions we found that, when the concentration was higher than that corresponding to ten molecules of water per one of sucrose, devitrification did not take place at all, no matter what the temperature was. With lower concentrations, corresponding to one molecule of sugar for every 11, 12 or 13 of water, devitrification occurred at temperatures of about -50° , and the frozen mass exhibited a particular type of crystallization which we designate by "crystallization in tufts" there being tufts of crystalline needles in the vitreous mass. With lesser concentrations, a different type of devitrification set in: the preparation assumed an amber color which gradually darkened to complete opacity. The devitrification temperatures were about 15 degrees higher in this case. It is this type of devitrification which we have studied particularly and which has furnished the numerical data given above. One can, it seems, interpret the results observed in these three ranges of concentration by supposing that the first 10 molecules of water which come in contact with a sugar molecule attach themselves so firmly to it that they cannot be torn loose by the forces of crystallization. The next three molecules would be bound by a different type of bond, and, when more than 13 molecules of water are present per molecule of sugar, this excess water would be held only by the forces of solution and would be free to solidify. (In making this hypothesis, we are supposing

¹ In collaboration with Dr. C. and Miss M. Jordan.

that only water crystallizes during the successive devitrifications; but this point is to be investigated.)

The existence of three ranges of concentration with the properties just described has been observed in a great number of solutions. Thus, solutions of gum arabic¹ do not devitrify if their concentration exceeds 68%. They devitrify in the form of tufts between 68% and 64%, and in the amber-color form below 64%.

Devitrifications at more than two ranges of temperature were noticed with solutions of substances such as urea.²

With solutions of sodium chloride, two devitrifications of the amber-color type occur at different temperatures, one of them in the neighborhood of -28° . When the devitrified material is warmed up, a partial melting takes place at about -21° , the well-known freezing temperature of the eutectic mixture.

We have also applied the method of devitrification to the study of the mode of binding of water in substances which set as gels. A preliminary work³ has shown that a sugar solution to which pectin has been added and which has been allowed to set, devitrifies at temperatures slightly below those at which an identical but alkaline solution, which does not set, devitrifies.

From what we have said on the higher devitrification temperatures of solutions of substances such as gelatine, dextrin, the gums, etc., it follows that these solutions can freeze only from about zero to 10 or 12 degrees below zero.

4. *Vitrofusion.* The direct passage from the vitreous to the liquid state can be effected by a rapid warming. The conditions for assuring this passage and avoiding the intermediate crystalline state are fundamentally the same as those required for vitrification: 1. The greatest possible temperature difference between the warming bath and the object; 2. The smallest possible mass and the greatest pos-

¹ Research made in collaboration with Mr. J. Fulton.

² Studied in collaboration with Mr. H. Noe.

³ In collaboration with Mr. W. Schmiesing.

sible surface area of the latter; 3. A high heat conductivity of the warming bath and, in particular, a good contact conductivity (as it has been said in the previous section, a liquid bath is far superior in this respect to all others).

II. LIVING MATTER

1. *General Procedure.* The principle that the solidification of a liquid into a glass requires less molecular rearrangement than the transformation of that liquid into a crystal suggests that vitrification might not injure protoplasm in conditions in which crystallization kills it.

Our first attempts at vitrifying living matter have consisted in applying to it the methods of vitrification and vitrofusioin and then testing for its vitality. Only a few experiments, and these on but a few types of material, have been carried out to ascertain whether or not the treated protoplasm had actually been brought into the vitreous state. In the following account, therefore, the vitality of living matter treated by the vitrification methods is taken as indicative of a probability that the material was actually vitrified. Vitality after treatment does not, indeed, constitute a proof of vitrification, but, if masses of protoplasm, of small size and not too high water content, are still alive after rapid cooling and rapid warming, while they are killed in larger quantity, or when they have a higher water content, or when they are cooled or warmed slowly, vitality does afford at least indirect evidence that vitrification has been achieved.

In as far as living beings are comparable to the gelatin solutions which we studied previously, we can expect to succeed in vitrifying organisms whose thickness is about one third of a mm., if their water content is about 50% and smaller organisms of greater water content, provided the latter does not exceed 90%.

2. *Methods.* For rapid cooling, we used the method of immersion in liquid air. For rapid warming, we ordinarily immersed the material in water at a temperature of about 20°. Occasionally we employed water heated to

50 or 60°, or even boiling water, but in the last case, the object remained in the bath for less than one fifth of a second, whereupon it was immediately plunged into cold water. Immersion in mercury heated to 40° gave good results in the case of moss. Isopentane recommends itself particularly in experiments on protozoa because of its immiscibility with water. One can place on a thin cover-glass a small drop of water containing the protozoa, immerse this preparation in liquid air, then in isopentane, and repeat the operation several times; the drop is still intact with the organisms within it. But in spite of this notable advantage, it seems, on the basis of experiments reported above, that isopentane is too slow as a warming medium.

In order to reduce the heat capacity of the preparation, one must use only very thin supports. Microscope cover-glasses are often too thick. Frequently we substituted for them sheets of mica; since these cleave easily, one can get sheets which are only some ten micra in thickness. Metallic foil presents the inconvenience of not being transparent and so not allowing of microscopic observation.

Instead of thin supports, we sometimes used, with advantage, a ring about two millimeters in diameter, made of as thin a metal wire as possible, and fastened to a light, rigid rod. One simply dips this loop into the culture, and thus obtains, in the thin film within the ring, quite a considerable number of organisms. With the diameter of loop indicated, it is only seldom that the film breaks either when immersed in liquid air or when immersed in the warming medium. We often found it advantageous to reduce the thickness of the wire still further by flattening it with a hammer.

With protozoa we also employed another method which consisted in placing the culture in an atomizer and spraying it thence into liquid air. But the freezing of the water which encloses the organisms liberates heat and retards cooling.

We likewise tried to emulsify the culture with oil. When

this emulsion was placed on a thin support, each organism could be seen to be enclosed in a cell consisting of a droplet of nutritive medium surrounded by oil. The objection mentioned for the previous method applies to this one also.

Perhaps one might have success by spraying the cultures in thin sheets into liquid air by means of a syringe with a flattened and adjustable jet.

As for the methods of drying, we used, besides that which consists in allowing the preparation to evaporate in the air or in desiccators containing various concentrations of sulfuric acid, also the plasmolytic method of immersing the preparation into a solution of salt or of sugar.

3. *Experiments and Results.* We undertook a first series of experiments with euglenae. The organisms were first concentrated by centrifugation. A small drop of the concentrated culture was thereupon placed on a glass slide and left to evaporate in the air till only a swarming mass of animals remained. The preparation was then dipped into liquid air and after this into water at 20° or at 40°. No euglena ever came back alive from the ordeal. Thinking that the organisms contained too much water, we tried to carry the evaporation still farther or to reduce the water content by adding to the droplet concentrated solutions of sugar. But concentrations which killed the euglenae within one minute, did not suffice to produce the desired result. The use of strips of mica instead of glass supports, to lessen the heat capacity, was likewise ineffectual. On the whole, all our attempts to revive euglenae in the vegetative state were unsuccessful. The organisms were ordinarily not deformed, but they showed no sign of life. Whether they had actually been vitrified or frozen is uncertain.

We next repeated with paramecia all the experiments made with euglenae. The results were completely negative. But, while the euglenae did not suffer any deformation by the treatment, the paramecia always did, and often they were found completely broken in pieces when taken out of the liquid air.

Experiments with ciliates smaller than paramecia, that is, with colpoda, in the vegetative state, likewise gave negative results.

The same is true also of experiments with amoebae.

On the whole, our investigations on the three principal groups of protozoa, the rhizopods, the ciliates and the flagellates, did not allow us to revive a single organism. It seems probable that these animals could not really be vitrified on account of their too high water content.

However, revival was obtained in some experiments with myxamoebae (Gehenio and Luyet, unpublished). Out of thirty attempts made to vitrify these organisms by immersion in liquid air on the wire loop, five gave living myxamoebae whose contractile vacuoles resumed their function and maintained it for several hours. Though the percentage of animals revived is small, we consider the fact as highly significant.

Next we tried the spermatozoa of the frog (Luyet and Hodapp, 1938c). A smear on a thin cover-glass gave negative results. A second attempt on a sheet of mica was no more successful. A third series of experiments in which the spermatozoa were previously immersed in a 20% sucrose solution for being dehydrated before immersion in liquid air, yielded some motile organisms, less than 1% of the number treated. By increasing the concentration of the sucrose solution to 40% or 50%, we could increase the percentage of motile or non-disorganized forms to 20% or more. To sum up, by employing with the spermatozoa of the frog the method of mica sheets, of dehydration in concentrated sugar solutions and of rapid warming in water at 20°, we obtained some living forms in each preparation.

Studies on the duration of immersion in liquid air which the spermatozoa can support have shown that the number of survivors and their activity are the same after five days as after three seconds. This finding is in good agreement with the assumption that the material is vitrified and stays unaltered at low temperatures.

Experiments like those just described were also carried out with the spermatozoa of the rat, but not a single one could be revived.

Goetz and Scott-Goetz (1938) described experiments in which yeast previously treated by the vitrification methods was killed when exposed, in monocellular layers, in a wire loop, to temperatures of some few degrees below zero, while it was intact when exposed, under the same conditions, to 150° below zero. This seems to indicate that the very low temperatures, at which the vitreous state can be maintained, do not kill, while the higher temperatures which cause devitrification are lethal.

The epidermis of the onion, a classical subject in plant cytology, seemed to be particularly appropriate for our researches, especially because of the ease with which one can obtain very thin monocellular layers (Luyet and Thoennes, 1938b). A piece of epidermis, held on a small metal fork, was immersed into liquid air and then into water at 20° . The vitality of the cells was tested by plasmolysis. A first series of observations furnished only dead cells. Thinking that the quantity of water present in the large vacuoles of the epidermal cells rendered vitrification impossible, we tried to reduce this quantity of water by plasmolysis in salt solutions. No cell could be revived. But we had not taken account of the fact, reported by many investigators, that a direct immersion in water after plasmolysis in a concentrated salt solution is often fatal. The invasion of the strongly plasmolysed cells by water produces a too violent expansion which causes the bursting of the protoplast. We therefore tried to warm the cells rapidly by immersing them not directly into water, but into a saline solution at 20° . This time we found a considerable number of cells capable of being deplasmolysed or plasmolysed to a further extent.

A study, with polarized light, of the plasmolysed onion epidermis, in liquid air, showed that the cellular protoplasm, concentrated by plasmolysis, was isotropic, while the space which surrounded it and which contained only

salt solution, was anisotropic (Luyet and Thoennes, 1938a).

Plant leaves can be vitrified, at least partially, when their water content is not too high. We have shown this in the following manner. A leaf which, when examined against a source of light (an electric lamp) exhibits a certain degree of translucidity, is dipped into liquid air. It becomes hard and breakable but its degree of transparency has hardly changed when one takes the leaf out and again examines it against the lamp. After a few seconds of exposure to room temperature one sees the leaf become considerably opaque and, a few seconds later, it again acquires its original translucidity. It seems evident that all the water of the leaf was not frozen at the temperature of liquid air, that it froze only when the temperature rose to the zone of devitrification, and that it melted at a still higher temperature. The leaves treated in the manner described showed little or no vitality. Though this material allows of an easy observation of vitrification and devitrification, we think that it could not be subjected to vitrofusion (the transition from the vitreous to the liquid state without passing through the crystalline state), even when boiling water was used as the warming bath.

Moss gave results in perfect agreement with our anticipations (Luyet and Gehenio, 1938). Specimens of the genus *Mnium* were placed in containers in which solutions of sulfuric acid of known concentration maintained an atmosphere having a given degree of humidity. After a stay of less than 24 hours in one of these containers, a state of equilibrium was established. A portion of the moss was then taken out for a determination of the water content, while the remainder was immersed in liquid air and in water at 20°. The vitality of the cells was tested by plasmolysis. The results can be summarized as follows:

<i>Water Content</i>	<i>Method</i>	<i>Results</i>
More than 65%	Slow devitrification	Dead
More than 65%	Rapid warming	Survival
65% to 30%	Slow devitrification	Partly dead
65% to 30%	Rapid warming	Survival
Less than 30%	Slow devitrification	Survival
Less than 30%	Rapid warming	Survival

One sees that when the moss is sufficiently dried, it always survives treatment by low temperatures, whatever be the rapidity of cooling or of warming. When the water content is high, the plants die if one lets them freeze, but survive the vitrification if one prevents freezing by a rapid warming.

Finally we tried to vitrify muscle fibers (Luyet and Thoennes, 1938c). Bundles of 6 to 10 fibers, taken from a chloroformed frog and mounted on a metal frame, were immersed in liquid air and then into Ringer's solution at 20°. The fibers so treated were thereupon placed on the microscope and subjected to an electric shock. Some fibers, often most of the fibers present, contracted. However, they ceased to respond sooner than normal fibers to repeated electric shocks, and, for the same reaction, they required an induction current of higher potential. But whether the period of immersion in liquid air lasted several hours or only a few seconds seemed to make no difference.

In a series of researches which we have just begun, we intend to study the extent of the zone of crystallization in various organic fluids such as muscle plasma, moss juice, yeast extract, the protoplasmic fluid of the protozoa, etc. Perhaps these researches will reveal the reason why certain living beings are very resistant to low temperatures while others are not. The extent of the temperature interval within which they freeze might be the determining factor in their sensitivity. Our first results on muscle fibers seem to indicate that the temperatures at which the muscle dies during devitrification are the same as the temperatures of devitrification of muscle plasma, determined by the method previously described for aqueous solutions.

Although there are several unexplained exceptions, the results obtained in the vitrification of protoplasm, and in particular those furnished by moss, epidermal cells and muscle fibers, seem to confirm the view that a good vitrification is not injurious, there being no molecular disturbance, while an incomplete vitrification or devitrification and, *a fortiori*, crystallization, are injurious to the extent that they disrupt the living structure.

SUMMARY

1. At different temperatures, matter can exist in four physical states: as a gas, as a liquid, as a crystal and as a glass. The last-named state occupies the lowest zone in the scale of temperatures.
2. Crystallization is possible only within a limited range of temperatures. Below this range, matter is too inert to crystallize (in other words, too cold to freeze).
3. The vitreous state is obtained by cooling a liquid very rapidly. The rapidity has for its object to make the liquid traverse the zone of crystallization temperatures before it has the time to become crystalline.
4. By using, as a method of rapid cooling, the immersion in liquid air of material previously reduced to thin layers, we vitrified solutions of various organic and inorganic substances, such as gelatine, albumin, amino acids, agar, gums, dextrin, sugars, glycerine, formaldehyde, sodium chloride, sodium hydroxide, etc.
5. The more dilute a solution is, the more difficult it is to vitrify it. Pure water could not be vitrified by our methods.
6. A vitreous substance devitrifies, that is, crystallizes, when one raises its temperature. One can therefore cause the crystallization of a body by warming it up from the vitreous state.
7. The temperatures of devitrification of solutions rise when one passes from a solute of simple molecular composition to a more complex one, in the same series.
8. At high concentrations of a given solute (for example, one molecule of sucrose for 9 or 10 of water), devitrification does not take place. At intermediate concentrations (one molecule of sucrose for 10 to 14 of water), devitri-

fication occurs in a definite range of temperatures and the devitrifying mass acquires a tufty structure. When one passes to lower concentrations, the ranges of devitrification temperatures move suddenly higher and the devitrifying material appears as a transparent mass of amber color which gradually darkens to complete opacity. The behavior of these three groups of concentrations is attributed to three modes of binding of water. 9. Within the range of concentrations of the last group mentioned, the devitrification temperatures decrease slightly with increasing concentration, a phenomenon perhaps comparable to the depression of the freezing point of solutions. 10. By a rapid warming (immersion in warm water), one can avoid devitrification (crystallization) and pass directly from the vitreous to the liquid state; we call this change of state "vitrofusion."

11. Assuming as a working hypothesis that it is usually the formation of ice which kills protoplasm at low temperatures, we studied the vitality of organisms subjected to the vitrification and vitrofusion procedure. 12. The protozoa: euglena, paramecium and amoeba did not survive the treatment, but we have no guarantee that they have not been frozen. Some myxamoebae survived. 13. Some frog spermatozoa resumed their motility after solidification in liquid air; rat spermatozoa never did. 14. The cells of the epidermis of the onion were capable of plasmolysis after vitrification and vitrofusion. 15. Plant leaves could be vitrified, but we are not sure that their vitrofusion has succeeded. In general, they appeared severely injured after being brought back to room temperature. 16. Moss leaves have given results in perfect accord with the anticipations relative to the innocuousness of vitrification and the fatal action of freezing. The cells were always alive after rapid cooling and rapid warming and always dead after slow treatment. 17. Frog muscle fibers responded to electric stimuli after the vitrification and vitrofusion procedure.

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PART III

THE MECHANISM OF INJURY AND DEATH BY LOW TEMPERATURE

Some confusion in the study of death arises from the failure to distinguish between *organismal*, *systemic*, *cellular* and *protoplasmic* death. It is evident that a theory which holds that the death of a frog results from the destruction of the red cells in the frozen blood refers to *organismal* death; a theory, according to which the leaves of a tree die after a layer of ice has been formed at their point of attachment and severed their connection with the stem, considers *systemic* death; a theory stating that the death of a tissue results from a tearing of the cellular membranes by ice crystals is concerned with *cellular* death; finally, a theory which considers the fundamental physico-chemical processes involved in the destruction of living matter, such as the precipitation of colloids by congelation, regards *protoplasmic* death. In this work we are not concerned with organismal nor with systemic death by cold, though occasionally reference will be made to these; we are concerned primarily with protoplasmic death. But the investigators, in general, do not distinguish between cellular and protoplasmic death, their theories and their experiments concerning the two phenomena are so intimately related that we shall treat these two subjects together.

There is some evidence that the mechanism of injury and death by cold is different when death is accompanied by ice formation and when it is not. These two cases will, therefore, be discussed in separate chapters.

CHAPTER I

ACTION OF COLD WITHOUT ICE FORMATION

Observations and theories on the action of low temperature without ice formation can be classified into two groups: 1. Those concerned with organisms which die or are injured in the proximity of their freezing point; 2. Those which refer to the action of extreme cold, at temperatures at which the material solidifies without forming ice crystals, that is, vitrifies. The study of the first group will be subdivided into two sections: one, concerning the effects of chilling above the freezing point; the other, the action of cold in the subcooled state.

I. ACTION OF COLD IN THE NEIGHBORHOOD OF THE FREEZING POINT OF PROTOPLASM

A. ACTION OF COLD ABOVE THE FREEZING POINT

This subject has been competently reviewed by Belchradek (1935) in the chapter "Chilling, Chilling-Coma and Death by Chilling" of his monograph "Temperature and Living Matter". The reader will find in that chapter, presented in a condensed tabular form, the data of numerous investigators on the temperatures of cessation of protoplasmic streaming, ciliary movement, cellular multiplication, growth, metabolic activity and irritability. There follows a compendium and discussion of the observations and theories concerning injury and death by cold at temperatures above the freezing point. Numerous bibliographical references are given. Since the subject has been already reviewed, we shall present here only a brief outline of the essential points of interest. A few questions which have not been discussed by Belchradek will be considered in detail. For these only will the bibliographical references be given.

Practically all the authors cited by this reviewer have found that chilling becomes injurious or lethal only

after having exerted its action on protoplasm for a relatively long time. We encountered in the literature a few reports of rapid injury by cold. The mechanism of injury is apparently different in these two cases; a slow injury suggests a disturbance in the interplay of physiological functions while a rapid injury is more likely to be due to some sudden structural changes such as precipitation, solidification, etc. The two cases will be treated here separately.

1. *Slow Injurious Action.* A. OBSERVATIONS. Homiotherms enter into a state of coma when their body is cooled to some temperature above zero and they are killed on further cooling.

A state of rigor, induced by chilling, has been described in homiotherms and poikilotherms, in particular in fishes.

The numerous observations made on insects, either in the adult or in the larval stages, are contradictory. Both a high resistance and a high sensitivity to cold have been reported (cf. Bachmetjew, 1901 and 1907, and Uvarov, 1931).

Prolonged exposure of some plants to near-zero temperatures is sometimes fatal.

In general, organisms adapted to high temperatures are more easily affected by cooling. Among these one should mention, besides the tropical plants, some poikilotherms, in particular reptiles.

While the plants and animals just mentioned are higher forms, less differentiated or undifferentiated protoplasm has also been reported to be injured or killed by cold without freezing. Eggs are, according to some authors, very sensitive: ant eggs (Pictet, 1893), eggs of the Mediterranean fruit fly (Bach and Pemberton, *J. Agr. Res.*, 5, 657, 1916), bedbug eggs (Hase, *Ztschr. f. Parasitenk.*, 2, 368, 1930), chicken eggs (Moran, 1925). However, there are also reports claiming a high degree of resistance to cold in eggs.

B. Discussion. "Cold anesthesia", "cold rigor" and death by chilling, in homoiotherms and, in several cases, in poikilotherms, have been attributed to a specific injury to the nervous system, but nothing is known of the physico-chemical mechanism of this injury. Cameron and Brownlee (1913) consider the fact that an excised frog's heart is more resistant to cold than the entire frog, as indicating that cold must exert a special action on the nervous system; the latter is intact in the entire frog and absent in the excised heart.

Death at temperatures above the freezing point is in plants probably due to a disequilibrium resulting from a change in the rate of the various physiological functions, in particular, of water absorption from the ground and of transpiration.

In organisms adapted to high temperatures it is interesting to note that injury and death are due to the same factor which has produced adaptation, namely a change in the temperature. While high temperature, acting gradually for a long time, modified the organism so as to make it fit the new conditions, low temperature, later acting abruptly for a short time, exerted an injurious or lethal action.

While these interpretations deal almost exclusively with organismal death, the following structural changes have been invoked to explain the mechanism of protoplasmic death:

1. Chemical changes (very few specific suggestions have been made);
2. Changes in the velocities of interrelated chemical reactions;
3. Impairment of the functions of elimination and consequent accumulation of toxic products;
4. Impairment of the functions of osmosis and permeability;
5. Impairment of the functions controlling protoplasmic water relations and resulting dehydration;

6. Changes in the viscosity of protoplasm;
7. Changes in the adsorptive properties of some protoplasmic constituents;
8. Solidification of protoplasmic fats;
9. Precipitation of some of the components of the living structure;
10. Coagulation processes.

Belehradek discusses the interrelationship between several of these mechanisms. Many of them might be involved at the same time in causing death.

The observations concerning *dehydration* as a result of the action of cold without ice formation call for a few notes and remarks.

According to Molisch (1897), when *Tradescantia* hair cells are exposed to cold for a long time the protoplast separates in some places from the cell wall, indicating that water has been extruded.

Greeley (1901) described the same phenomenon in *Spirogyra* filaments cooled from 20° to $+1^{\circ}$ and kept at that temperature for 3 hours. When the filaments were cooled while immersed in olive oil he could see water droplets exuding from the cells into the oil.

Klemm (1895) looked upon such a protoplasmic contraction as an initiation of a disorganization which gradually will result in death.

Chambers and Hale (1932), however, noted that maintaining onion epidermis at -10° in the unfrozen condition for several hours does not induce plasmolysis. The apparent contradiction between this last observation and those previously recorded might be due to the fact that plasmolysis by cold is exhibited only by some types of protoplasm and only under some particular conditions.

Concerning the cause of dehydration, we wish to point out that the rounding up of protoplasm in *Tradescantia* hair, such as occurs under the action of cold, can also be produced by various forms of more or less violent stim-

ulation. A comparison of this phenomenon with the contraction of muscular tissue and of other forms of contractile protoplasm by cold might not be out of place.

At first it appears somewhat surprising that the withdrawal of water involved in this phenomenon be at all injurious and if it is injurious one would hardly expect it to lead to death.

2. *Rapid Injurious Action.* (a) In the ciliate *Stentor coeruleus*, Greeley (1901 and 1902) observed that while a slow cooling caused encystment or sporulation, a sudden cooling was lethal. (The absence of ice formation within the organisms themselves is evidently implied in the author's account.) In the last case, that is, after sudden cooling, the animals were deformed, their pigments were released and complete disorganization followed. In the former case, when the rate of cooling was about 10 degrees per hour, the following sequence of phenomena which is partly the same as that which often precedes death was observed: 1. A cessation of body movements and of ciliary motion; 2. The formation of vacuoles which gradually increased in size; 3. A change in the form of the cell which gradually became spherical; 4. The separation of the nuclear material; 5. A transformation of the protoplasm into a granular mass; 6. The throwing off of the ectosare; 7. The transformation of the cell into a cyst. The animal resumed its normal activity upon an elevation of temperature.

The fact that the final result of the action of low temperature was death or encystment depending on the velocity of cooling postulates that some similar mechanism must underlie these two processes.

The reduction of the water content, Greeley suggests, might be responsible for both, cystogenic and lethal action, as indicated by further experiments in which an immersion of *Stentor* in a strongly hypertonic M/10 solution of sucrose, produced the same effects as a

sudden lowering of the temperature, and an immersion in M/100 sucrose the same effects as a slow cooling.

In cooling experiments of the same type with *Monas*, Greeley (1902) described a typical sporulation caused by low temperature. At $+4^{\circ}$ resting cells were formed which, when exposed for 5 to 7 days to $+1^{\circ}$, divided into several spherical spores (from 2 to 25 in a cell). So the physiological processes involved in reproduction by spores seem to bear some similarity with those which lead to injury and death.

(b) Gehenio and Luyet (1939) found that the plasmodium of the myxomycete *Physarum polycephalum* was killed by sudden or moderately rapid exposure to cold at temperatures above the freezing point. They described the following phenomena as stages in the process of death, when the temperature of the organisms was lowered gradually from room temperature at the rate of one degree per minute: 1. At $+5^{\circ}$ to 0° , movement ceased; 2. At $+3^{\circ}$ and below, hyaline vesicles formed; 3. At 0° or below, the protoplasm underwent disorganization; 4. At the same temperature the pigment granules broke down. A sudden cooling to a given low temperature was considerably more injurious than a slow cooling to the same temperature.

The authors propose the following interpretation of their observations. Under the action of the lowering of temperature the protoplasmic sol would set gradually to a gel. They relate this hypothetical gelation to the increase in viscosity which was noted by Heilbrunn (1924) in protoplasm cooled to a temperature slightly above zero and to the cessation of protoplasmic streaming. But the gelation "would be a reversible process preceding death and not constituting death. . . .After complete setting, the gel would undergo a syneretic breakdown, squeezing out the dispersion medium enmeshed within it, and this would be the death process itself. At the periphery of the plasmodium the locally expressed fluid would appear

in the form of vesicles or blisters. Within the interior of the plasmodium, syneresis would manifest itself in the phase separation or disorganization."

This view, the authors state, fits in with the fact that the changes induced by cold are, in general, the same as those produced by other lethal agents; the approximation of the molecules which causes gelation would be a lowering of temperature in the present case, while it would be an electrostatic discharge or a dehydration or an adsorption, etc., in the case of other killing agents. The influence of the time factor (death produced in 5 seconds at -1°), the greater effect of abrupt cooling and the percentage of recovery after exposure to cold at a given temperature and for a given time, are also, the authors point out, consonant with the theory.

B. ACTION OF COLD IN THE SUBCOOLED STATE

1. *Observations.* The literature on the action of cold in the subcooled state is summarized in Table 1.

2. *Discussion. A. Mechanism of Injury and Death.* It readily appears that an injurious action of cold in the subcooled state, though definitely observed, is rather unfrequent. Subcooling probably affects some types of protoplasm more than others but it is, at present, impossible to say which types.

As in the case of injury above the freezing point, the time factor plays an important role. This and other similarities make it quite certain that, in the last analysis, when no ice is formed, the mechanism of action of cold on protoplasm is the same above and below the freezing point. What has been said in the preceding section would then adequately apply here.

On first consideration one might think that, in the cases in which cold injures protoplasm, subcooling must cause more damage than chilling, for the simple reason that the subcooling temperature is lower. But if injury is due to a disequilibrium of functions, the latter, in general, are

slower at lower temperatures (as is the case for osmosis, for example) and less damage should be produced during a given time in the subcooled state than during the same time at temperatures above the freezing point. If, on the other hand, injury results from a process akin to precipitation in a saturated solution, the lower the temperature the greater would be the chances of its happening. A comparative study of the degree of damage done in the same time in the subcooled state and above freezing might allow one to decide between these two possible mechanisms of injury.

TABLE 1
ACTION OF COLD IN THE SUBCOOLED STATE
(a) Injury by subcooling

Organisms	Temp. and Time of Exposure	Results	Investigators
<i>B. coli</i>	-0.5 or -6.0° (In glucose solution)	50% killed	Hilliard, Torossian & Stone, 1915; Hilliard & Davis, 1918
<i>Paramecium</i>	Below -4° 30 min. -16° Long time (In capillary tubes)	Marked swelling & death Swelling, visibility of nucleus & death	Efimoff, 1924 Wolfson, 1935
<i>Colpidium colpoda</i> and <i>Spirostomum ambiguum</i>	Below -4° 30 min.	Shrivelling & death	Efimoff, 1924
White blood cells of poikilotherms	-2° to -3° 8 hrs.	Death	Schenk, 1870
White blood cells of homiotherms	-3° >15 min.	Death	Schenk, 1870
Mycelia and hyphae of <i>Aspergillus</i> and <i>Penicillium</i>	-13° 24 hrs.	Death	Lindner, 1915
Germinating tubes of <i>Aspergillus niger</i>	-4.2° to -13° 9 days (In 50% glucose)	75% killed	Bartetzko, 1910
Cells of red cabbage leaves	-3.9° >1 day	Death	Iljin, 1934

(b) *Innocuousness of subcooling*

Organisms	Temp. and Time of Exposure	Criterion of Vitality	Investigators
Streptococci of scarlet fever	-17° to -18° 2 wks.		Citovicz, 1928
<i>Amoeba</i>	-5°		Chambers & Hale, 1932
<i>Paramaccium</i>	-9° Short time		Efimoff, 1924
	-14.2° to -16° Short time		Wolfson, 1935
<i>Colpidium</i> and <i>Spirostomum</i>	-9° Short time		Efimoff, 1924
<i>Euglena</i>	-0.2° 1 hr.		Jahn, 1933
Leucocytes of poikilotherms	-7° Short time		Schenk, 1870
Leucocytes of the rabbit	-3° 15 min.		Schenk, 1870
Chicken eggs	-4.6° 47 hrs.		Moran, 1925
	-2.9° 118 hrs.		Moran, 1925
Marine algae	-1.8°	Some physiological activity	Kjellmann (Quoted from <i>Bot. Ztg.</i> , 33, 771, 1875)
<i>Nitella</i>	-2°	Protoplasmic streaming	Kylin, 1917
Germinating spores of <i>Aspergillus</i> , <i>Penicillium</i> , <i>Botrytis</i> , and <i>Phycomyces</i>	-14° 2 hrs.		Bartetzko, 1910
<i>Aspergillus</i>	-6° to -11° 4 days		Bartetzko, 1910
Various fungi	-3°	Growth	Horowitz-Wlassowa & Grinberg, 1933
	-7.8°	Growth	Brooks & Hansford, (<i>Food Inv. Bd.</i> , Spec. Rpt. No. 17, 1923)
	-8.9°	Growth	Smart, 1935
	-6° and -10°	Growth	Bidault, 1921
	-10°	Growth	Haines, 1930

Organisms	Temp. and Time of Exposure	Criterion of Vitality	Investigators
Hyphae of <i>Phycomyces</i>	-10° to -12°	No stiffening of protopl. fluids	Molisch, 1897
Submerged fungal mycelia	-13° 8 hrs.		Lindner, 1915
Aerial hyphae	-11° 4½ hrs.		Lindner, 1915
Hair cells of <i>Trianea</i> and <i>Momordica</i>	-13° < 15 min.		Klemm, 1895
Hair cells of <i>Tradescantia</i> , <i>Episcia</i> and <i>Pelargonium</i>	-5° to -9° 6 hrs.		Molisch, 1897
Plant tissues (various)	-13°	Ability to plasmolyse	Voiglaender, 1909
Rye seedlings	-11.1°		Zacharowa, 1926
Embryonic mammalian tissue	-5° 5 days	Growth	Simonin, 1931
Frog gastrocnemius	-4° 2 days	No loss of irritability	Moran, 1929
Frog muscles	-15° 3 hrs.	No loss of contractility	Chambers & Hale, 1932
	-18°		Kodis, 1898
Bees, bumble-bees, wasps, and larvae of beetles	-2.9° to -17.1° 48 hrs.		Kalabuchov, 1934
Frogs	-10°		Kodis, 1898
Salamanders	-2.2°		Jecklin, 1935
Toads	-1°		Kalabuchov, 1934
Tortoises	-5.3°		Kalabuchov, 1934
Bleaks and Sticklebacks	-3.06°		Schmidt, Platanov, & Person, 1936
Carps	-3° -5°		Regnard, 1895 Kalabuchov, 1934
<i>Citellus suslica</i> and <i>Citellus pygmaeus</i> (ground squirrels)	-0.5° and -1°		Murigin, 1937
<i>Nyctalus noctula</i> (bat)	-5.9°		Kalabuchov, 1934

B. Role Played by Subcooling in Nature. Concerning the role played by subcooling in the preservation or in the injury of plants and animals in nature, a review of the literature reveals that the most diverse views have been held. These views depend on the theory accepted by each individual author for the mechanism of death by cold. They are, too often, rather theoretical and lacking in experimental evidence.

Since the generality of investigators have found that protoplasm is not injured in the subcooled state, they naturally concluded that subcooled animals or plants are "protected" against the damage of frost. The resistance of plants in winter to temperatures of several degrees below zero is often attributed to a tendency to subcool. The fact that trees in northern forests and poikilothermic animals in cold climates are not killed by long and severe winters is also thought by many to be due to a particular ability to undergo deep subcooling (Kalabuchov, personal communication).

Weigman (1936) pointed out that hibernating snails have a freezing point as high as -0.39° ; their survival to low temperatures therefore cannot be attributed to a lowering of the freezing point at the onset of the state of dormant life, it must then be due to the ability to subcool.

Harvey (1919) suggested another indirect protective role of subcooling, namely, that plants left for some length of time in the subcooled state might undergo adaptative changes and become hardened against frost.

The authors who held the theory of the specific minimum (see below) according to which death occurs at a certain given temperature, no matter whether ice is formed or not, took sides against the view of "protective" subcooling. For them, only that which prevents an organism from reaching the specific minimum is protective. Subcooling itself does not, of course, prevent the drop of temperature, while freezing, by liberating heat, does.

So they conclude, paradoxical as it may seem, that subcooling is dangerous while freezing is protective. A few of the arguments of the main proponent of this theory, Mez (1905), will suffice to illustrate it and to show how it has been applied to the most varied phenomena of nature.

According to this author, the tissues of the stem of *Impatiens* were killed sooner when freezing was preceded by subcooling than when it was not. But in these experiments the quantities of ice formed in the two cases were not compared, a factor which has been shown to be of fundamental importance in causing death.

Mez then applies his theory to the supposedly observed fact that trees and shrubs are more injured in "stagnant" air than in air agitated by winds. Shaking would, in the latter case, prevent subcooling which is assumed to be the damaging agent.

The frost resistance acquired by "hardened" plants is attributed by this author to the prevention of subcooling by oils formed during hardening.

The fact mentioned by Sachs and Molisch that a sprout of *Tradescantia*, exposed to -5° , half in water and half in air, had the latter part killed and the former unharmed, is explained by Mez on the assumption that the part frozen in air was previously subcooled to a greater extent.

II. ACTION OF EXTREME COLD

While injury and death can result from cold without ice formation at near-zero temperatures, it seems that, in general, extremely low temperatures are harmless if ice is not formed. So, instead of discussing the mechanism of injury by cold in this section, we shall consider the cause of the innocuousness of very low temperatures.

1. *Observations.* The organisms which resist the temperatures of liquefaction of "permanent" gases can be divided into two groups: 1. Those which support drying

and which, in the dry state, are not affected by the low temperatures in question; 2. Those which, either with their full water content or after having been only slightly dehydrated, are immune against extreme cold. This second class, in its turn, includes two subdivisions: 1. There are organisms which survive liquid air temperatures only when special precautions are taken, such as extremely rapid cooling and rewarming; 2. There are others which, without any of these precautions, can be immersed in liquid air, liquid hydrogen or liquid helium without injury. The experimental data concerning these three groups are presented in tables 2, 3 and 4.

2. *Discussion. A. Cause of the Resistance to Extreme Cold.* The fact that all the organisms mentioned in the first group (Table 2) resist very low temperatures when they are dried suggests that it is the absence of freezable water which immunizes them. The type of precautions which save the organisms of the third group (Table 4) from death, that is, rapid cooling and rewarming through the temperatures at which freezing of water would be possible (from 0° to some tens of degrees below 0°), supports the assumption that, if ice formation is prevented, danger is avoided. This assumption has naturally been applied to the organisms of the second group (Table 3), in other words, it has been thought that when bacteria, yeasts, fungi, algae, protozoa and germ cells in the vegetative state or nematodes, rotifers and tardigrades, with their full water content, resist immersion in liquid air, it is because their water does not freeze.

The cause of the assumed absence of ice in the organisms of the second group has been sought in the capillary forces within the intermicellar spaces. Some types of protoplasm (the bacteria, in particular) would be of a denser structure and the capillary spaces in them would be so small that "water will not be changed into ice at any temperature" (Lipman, 1939). Concerning this view

we wish to point out that it is not established experimentally that the degree of subcooling of water becomes increasingly greater when the size of the capillary spaces reaches the order of a micron or less. The generally assumed relation between degree of subcooling and capillary size already ceases to be consistent when the capillary sizes are of the order of 0.1 mm. (cf. our review, "The Physical States of Protoplasm at Low Tem-

TABLE 2
ORGANISMS WHICH, WHEN DRY, RESIST EXTREMELY LOW TEMPERATURES

Organisms	Temp.	Time of Exposure to Cold & Other Conditions	Investigators
<i>1. Bacteria and Fungi</i>			
Bacteria:			
<i>Staph. pyogenes aureus</i>	L. air	125 days	Paul & Prall, 1907
Common bacilli (Spores)	L. helium 4.2° to 1.35°K	6 hrs.; previously desiccated for 2 weeks	Lipman, 1936a
Myxomycetes:			
<i>Physarum polycephalum</i>	L. air	12 hrs.	Gehenio & Luyet (unpublished)
<i>2. Algae</i>			
<i>Pleurococcus vulgaris</i>	-190° and -269° to -271°	480 hrs. at -190° and 7½ hrs. at -269° to -271°; previously desiccated over barium oxide for 3 mos. and then sealed into tubes evacuated to 10 ⁻⁵ mm. of mercury	Becquerel, 1932d, 1936
<i>Chlorella vulgaris</i>			
<i>Stichococcus bacillaris</i>			
<i>Hantzschia amphioxys</i>			
<i>Pinnularia viridis</i>			
<i>Chlorococcum humicolum</i>			
<i>Palmella miniata</i>			
<i>Oscillatoria</i>			
<i>Glaeotila</i>			
<i>Hormidium</i>			
<i>Siphonema</i>			
<i>Pediastrum</i>			
<i>Tribonema elegans</i>	4° to 1.84°K	Several hours; previously dried and kept in high vacuum for 22 years	Becquerel, 1932b

Organisms	Temp.	Time of Exposure to Cold & Other Conditions	Investigators
<i>3. Protozoa</i>			
<i>Colpoda cucullus</i> (Cysts)	L. air	13½ hrs.; previously air-dried	Taylor & Strickland, 1936
	L. air	12½ days; previously subjected to a vacuum of about 10 ⁻⁵ mm. of mercury for 2 to 3 days	Taylor & Strickland, 1936
<i>Amoeba proteus</i> <i>Amoeba limax</i> <i>Amoeba dactylifera</i> <i>Actinophrys sol</i> <i>Paramecium bursaria</i> <i>Euglena viridis</i>	-190° and -269° to -271°	480 hrs. at -190° and 7½ hrs. at -269° to -271°; in soils previously dried in a vacuum over barium oxide for 3 months, then sealed in glass tubes evacuated to 10 ⁻⁵ mm. of mercury	Becquerel, 1936
<i>4. Spores and Seeds</i>			
Spores of the fungi, <i>Mucor</i> , <i>Rhizopus</i> , <i>Sterigmatocystis</i> and <i>Aspergillus</i>	L. hydr.	77 hrs.; previously dried and sealed in tubes evacuated to 10 ⁻⁴ cm. of mercury. Left for 2 years in vacuum after exposure to low temperature	Becquerel, 1910
Spores of the mosses, <i>Dicranella</i> , <i>Atrichum</i> , <i>Hypnum</i> , <i>Leucobryum</i> , <i>Funaria</i> and <i>Brachythecium</i>	L. nitr.	10 days; air-dried	Becquerel, 1932a
Spores of 2 genera of mosses	L. helium 4° to 1.84°K	9 hrs. at 4° and 1 hr. at 1.84°K; previously desiccated and sealed in tubes evacuated to 10 ⁻⁶ mm. of mercury	Becquerel, 1932a
Spores of the fern, <i>Polystichum filix mas</i>	L. helium 5° to 3°K	6 hrs. at 5° to 3° and 5 hrs. at 3°K; previously desiccated and sealed in the highest obtainable vacuum	Becquerel, 1930
Pollen grains of <i>Antirrhinum</i> and <i>Nicotiana</i>	L. helium Minimum 1.3°K	7 hrs.; previously thoroughly dried and sealed in tubes evacuated to 10 ⁻⁷ mm. of mercury	Becquerel, 1929

Organisms	Temp.	Time of Exposure to Cold & Other Conditions	Investigators
Seeds (various)	-180°	1 hr.	Dewar & McKendrick, 1892
	-200°		Pictet, 1893
	L. air	110 hrs.; previously air-dried (with still 10 to 12% water)	Brown & Escombe, 1897
	L. hydr.		Dewar, 1899
	L. hydr.	6 hrs.; previously air-dried	Thiselton-Dyer, 1899
	L. air	24 hrs.	Adams, 1905
	L. air	132 hrs.; previously vacuum-dried	Becquerel, 1909
	L. air and L. hydr.	3 weeks in liquid air and 77 hrs. in liquid H ₂ ; previously desiccated over barium oxide for 2 wks. and sealed in tubes evacuated to 10 ⁻⁶ mm. of mercury; kept sealed for 1 yr. after treatment	Becquerel, 1905
	L. helium 3.8°K	10½ hrs.; previously desiccated over barium oxide for 2 wks. and sealed in tubes evacuated to 10 ⁻⁶ mm. of mercury	Becquerel, 1925
	L. air	60 days; previously dried over CaCl ₂	Lipman & Lewis, 1934
L. helium 4.2° to 1.35°K	40 hrs. at 4.2°, 2 hrs. at 1.35° and 2 hrs. at 1.35° to 4.2°K; previously desiccated for 2 wks. over H ₂ SO ₄ in a partial vacuum	Lipman, 1936a	
<i>5. Metaphyta</i>			
Lichens:			
<i>Parmelia, Xanthoria and Cladonia</i>	L. nitr.	18 days; previously air-dried	Becquerel, 1932d
Moss:			
<i>Hypnum</i>	L. nitr.	18 days; previously air-dried	Becquerel, 1932d
Protonemata of 8 genera of mosses	L. air	50 hrs.; previously vacuum-dried over H ₂ SO ₄	Lipman, 1936b
<i>Mnium</i>	L. air	30 seconds	Luyet & Gehenio, 1938

Organisms	Temp.	Time of Exposure to Cold & Other Conditions	Investigators
Pteridophytes: <i>Selaginella</i>	L. air	2 hrs.	Luyet & Hartung, 1939 (unpublished)
Spermatophytes: Tuberous roots of <i>Ranunculaceae</i>	L. nitr.	18 days; previously air- and vacuum-dried	Becquerel, 1932b
Sprouting seeds of wheat, rye, lucern and <i>Helianthus</i>	L. nitr.	18 days; previously air- and vacuum-dried	Becquerel, 1932c
6. Metazoa			
Nematodes: <i>Plectus</i> , <i>Tylenchus</i> and <i>Dorylaimus</i>	L. helium	Air-dried	Rahm, 1920, 1921, 1923
Rotifers: <i>Callidina</i> and <i>Adineta</i>	L. air	125 hrs.; previously air-dried for 18 days to 14 months	Rahm, 1920, 1921, 1923
	L. hydr.	26 hrs.; previously air-dried	Rahm, 1920, 1921, 1923
	L. helium -269° to -271.88°	7¾ hrs.; previously air-dried for 18 days to 14 months	Rahm, 1920, 1921, 1923
<i>Adineta gracilis</i> , <i>Rotifer vulgaris</i> and <i>Callidina angusticollis</i>	L. nitr. and L. helium	480 hrs. to liquid N ₂ and 7½ hrs. to liquid He; previously vacuum-dried for 3 mes.	Becquerel, 1936
Tardigrades: <i>Macrobiotus</i> , <i>Echiniscus</i> and <i>Milnesium</i>	L. air	125 hrs.; air-dried for 18 days to 14 months	Rahm, 1920, 1921, 1923
	L. hydr.	26 hrs.; air-dried for 18 days to 14 months	Rahm, 1920, 1921, 1923
	L. helium -269° to -271.88°	7¾ hrs.; air-dried for 18 days to 14 months	Rahm, 1920, 1921, 1923
<i>Macrobiotus Hufelandi</i>	-269° to -271°	7½ hrs.; vacuum-dried for 3 months	Becquerel, 1936
Arthropods: <i>Artemia salina</i> (eggs)	L. air	Air-dried	Gilchrist, 1939 (unpublished)

TABLE 3

ORGANISMS WHICH, IN THE WET CONDITION, RESIST
EXTREMELY LOW TEMPERATURES

Organisms	Temp.	Time of Exposure to Cold & Other Conditions	Investigators
<i>1. Bacteria, Yeasts and Other Fungi</i>			
Bacteria:			
Bacteria (various)	-200°		Pictet, 1893
	-192°	1½ hrs.	White, 1899
	L. air	20 hrs.; in broth or on solid media; veg. and spore forms	Macfadyen, 1900
	L. nitr.	10 hrs.	Macfadyen & Rowland, 1900b
	L. hydr.		Beijerinck & Jacobsen, 1908
	-180°	8 days	Moussu, 1912
	L. air	48 hrs.; veg. forms on agar slants	Lipman, 1937
<i>B. typhosus</i>	L. air	6 months	Macfadyen & Rowland, 1900a
	-253°		DeJong, 1922
<i>B. coli</i>	L. air	6 months	Macfadyen & Rowland, 1900a, 1902
	-253°		DeJong, 1922
	L. hydr.	3 hrs.; in 0.85% NaCl solution	Kadisch, 1931
<i>Staph. pyogenes aureus</i>	L. air	6 months	Macfadyen & Rowland, 1900a, 1902
	L. hydr.	50 hrs.; suspended in 0.85% NaCl solution	Kadisch, 1931
	-252°		
<i>B. anthracis</i>	L. air	15 hrs.; bouillon suspensions of veg. form	Belli, 1902
<i>Spirochaeta pallida</i>	L. nitr.	14 days	Jahnel, 1937
Bacillus of Chicken Cholera	L. air	15 hrs.; bouillon suspensions of veg. form	Belli, 1902
Sodoku spirilla	L. nitr.	14 days	Jahnel, 1937
<i>Gonococcus</i>	L. nitr.	24 hours	Lumiere & Chevrotier, 1914
	-195°		
<i>B. faecalis alcaligenes</i>	-253°		DeJong, 1922
<i>B. lactis aerogenes</i>	-253°		DeJong, 1922

Organisms	Temp.	Time of Exposure to Cold & Other Conditions	Investigators
Bacteria of Parathyphoid A and B and of Enteritis	-253°		DeJong, 1922
Tubercle bacillus	L. air	6 weeks	Swithinbank, 1901
	L. hydr.	50 hrs.; suspended in .85% NaCl solution	Kadisch, 1931
	L. air	20 alternate freezings and thawings	Weinzirl & Weiser, 1934
Avian Tubercle bacillus	L. nitr.	200 alternate freezings and thawings	Kyes & Potter, 1939
Luminescent Bacteria	L. helium	Several hrs.; fresh veg. state	Zirpolo, 1932
Yeasts:			
Beer yeast	L. air	6 min.	Doemens, 1897
Yeasts (various)	L. air	6 months; washed and pressed	Macfadyen & Rowland, 1902
Pathogenic yeasts	-252°	3 hrs.; suspended in 0.85% NaCl solution	Kadisch, 1931
	-268.8°	2 hrs.; suspended in 0.85% NaCl solution	Kadisch, 1931
	-272°	1½ hrs.; suspended in 0.85% NaCl solution	Kadisch, 1931
<i>Saccharomyces cerevisiae</i>	L. air -183° to -192°	13 hrs.; cultured on agar slants	Kaercher, 1931
Fungi:			
Mycelia of <i>Schizophyllum</i> and <i>Collybia</i>	-185°	½ hr.; on agar slants	Heldmaier, 1929
	L. air	13 hrs.; on agar slants	Kaercher, 1931
Mycelia of <i>Aspergillus</i> and <i>Armillaria</i>	L. air	13 hrs.; on agar slants	Kaercher, 1931
	L. air	48 hrs.; on agar media	Lipman, 1937
Mycelia of <i>Hypholoma</i> , <i>Clitocybe</i> , <i>Placodes</i> , <i>Xylaria</i> and <i>Phycomyces</i>	L. air	13 hrs.; on agar slants	Kaercher, 1931
	L. air	48 hrs.; on agar media	Lipman, 1937

Organisms	Temp.	Time of Exposure to Cold & Other Conditions	Investigators
<i>2. Monocellular Algae</i>			
Diatoms	-200°	Frozen in water cultures	Pictet, 1893
<i>Chlorella</i>	L. air	1 hr.; suspended in Knopp's solution	Warburg, 1919
<i>Stichococcus bacillaris</i>	L. air -183° to -192°	13 hrs.; cultured on agar slants	Kaercher, 1931
<i>3. Protozoa</i>			
Trypanosomes (various)	L. air	5 to 25 min.	Laveran & Mesnil, 1904
<i>Trypanosoma gambiense</i>	L. air	20 min.	Gaylord, 1908
<i>Trypanosoma Lewisi</i>	L. air	75 min.	Quoted from Doflein, 1911
	L. air	1 hr.	Laveran & Mesnil, 1904
<i>Trypanosoma brucei</i>	} L. air	21 days	DeJong, 1922
<i>Trypanosoma venezuelense</i>			
<i>Trypanosoma equiperdum</i>			
Dourine Trypanosome	L. nitr.	20 hrs.	Jahnel, 1937
<i>4. Germ Cells and Spores</i>			
Eggs of <i>Macrobiotus</i> (Tardigrad)	L. air and L. hydr.		Rahm, 1920, 1923
Spores of various fungi	-252°	50 hrs.; suspended in 0.85% NaCl solution	Kadisch, 1931
	-268° to -272°	7½ hrs.	Kadisch, 1931
Spores of <i>Melanconium</i> , <i>Coniothyrium</i> , <i>Eurotium</i> and <i>Cystospora</i>	L. air	1 hr.; suspended in water	Alexopoulos & Drummond, 1934
<i>5. Metazoa</i>			
Rotifers (various)	L. hydr. -253°	2 days in L. air and 1 day in L. hydrogen	Rahm, 1920, 1921, 1923

TABLE 4
ORGANISMS WHICH, IN THE WET CONDITION, SURVIVE IMMERSION IN LIQUID
AIR, PROVIDED THEY ARE COOLED AND REWARMED SUDDENLY
(VITRIFICATION PROCEDURE)

Organisms	Conditions of Exposure	Investigators
Myxamoebae	Suspended in films of water in a thin wire loop	Gehenio and Luyet, 1939 (unpublished)
Moss Leaves (with full water content)		Luyet and Gehenio, 1938
Onion Epidermis	Previously plasmolysed in NaCl solution	Luyet and Thoennes, 1938a
Frog Spermatozoa	Previously plasmolysed in sucrose solution	Luyet and Hodapp, 1938
Frog Muscle Fibers		Luyet and Thoennes, 1938b

peratures"). However, the absence of experimental evidence is not an argument against the *possibility* of the theory.

Another reason which might be suggested for the resistance to extreme cold of the organisms of the second group is that their small size provides them with such a large surface area, in comparison with their volume, that they can lose water by exosmosis in the short time during which their culture medium freezes. The cells, being then dehydrated as a result of the congelation of the water around them, would naturally become resistant. An objection to this interpretation is that osmosis is known to be a comparatively slow process which would hardly account for the dehydration of even a small speck of protoplasm during the few seconds necessary for freezing a small drop of culture medium in liquid air.

Luyet and Gehenio (1939, p. 123) proposed a third explanation for the prevention of congelation in microorganisms which survive the lowest temperatures in the vegetative state. According to Luyet (1939) water solutions of substances which have a high molecular weight

have a narrow freezing range. For example, a concentrated solution of dextrin freezes only between about -1° and -9° . Similarly the substance of some types of protoplasm might have a very narrow freezing range. If, then, the degree of subcooling is such that the freezing range can easily be traversed, the formation of ice is avoided.

As to the organisms of the first group, those which can be dried, they resist not only cold but almost any injurious agent when they are in the dry condition. Such a general resistance is again attributed to the absence of water. But evidently the ability to support without injury the removal of water is due to some intrinsic property characteristic of some given types of protoplasm.

A priori it seems that the resistance to cold could be attributed directly to this intrinsic property rather than to the actual absence of water and consequent absence of ice. But experiments have shown that most of the desiccable organisms are killed when frozen without being previously dried. To mention one instance, Adams (1905) found that seeds which contain more than 12% water may be killed by freezing, while if they are dried to a further extent they remain uninjured. These experiments clearly speak in favor of the theory of the actual absence of freezable water as the cause of the resistance to extreme cold in desiccable organisms.

In the instance given, 12% of the weight of the seeds would then be unfreezable water. This proportion, it might be remarked, is low when compared to the 34.5% water content which has been found by Moran (1926) to stay unfrozen at any low temperature in gelatin gels. The quantity of water which, in several colloids, cannot be unbound by crystallization forces is considerably higher than is usually thought.

Coming now to the explanation of the survival of protoplasm treated by the rapid cooling and rewarming

method (third group above) we have evidence from all angles that if cooling and rewarming are sufficiently rapid to prevent the formation of ice and to really vitrify protoplasm, life is preserved, while if a good vitrification cannot be achieved, the damage is in proportion to the degree of crystallization (for experimental data cf. our review: "The Physical States of Protoplasm at Low Temperatures"). Death then seems to result from the disruption of the units which constitute living matter when the molecules of water are torn away from these units by the forces of crystallization, and the cause of the innocuousness of low temperature seems to be the absence of freezable water.

In this discussion of the three groups of cases in which extreme cold is innocuous, we assumed, on the basis of circumstantial evidence, that no ice was formed in the protoplasm. We know, however, only one direct observation of the actual absence of ice in protoplasm at very low temperatures: Luyet and Thoennes (1938a) reported that the plasmolyzed protoplasts of the cells of onion epidermis do not lose their isotropic properties when immersed in liquid air.

B. Resistance to Extreme Cold and the Structure of Living Matter. The experiments on vitrification show that living matter can be hardened into a solid without being killed. The passage from the liquid to the solid state is not lethal. On the other hand, the passage from the liquid to the crystalline state at near-zero temperatures and the passage from the solid amorphous to the crystalline state in devitrification experiments is lethal. Now, the change involved in the transformation of a liquid into an amorphous solid is simply an increase in cohesion connected with a closer approximation of the molecules, while crystallization involves a rearrangement of the molecules. The structure of living matter is, therefore, such that life is compatible with the increase in compactness and density

which occurs in vitrification but that it is destroyed by the molecular rearrangement which takes place in crystallization.

Furthermore, a considerable decrease in molecular motion has no injurious effect on living matter (cf. Lipman and Lewis, 1934). In the present state of our knowledge the influence of temperature on life can be represented diagrammatically as in the accompanying figure.

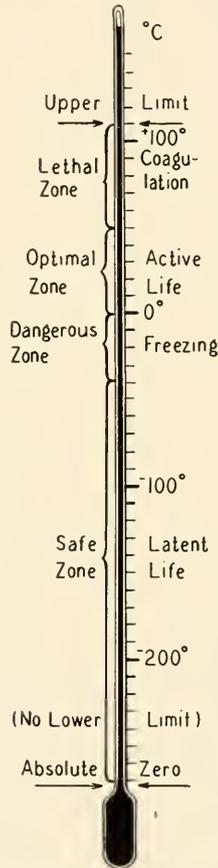


FIG. 32. Diagram illustrating the influence of temperature on vital processes.

While apparently one can always kill protoplasm by raising its temperature, that is, by increasing its molecular

motion, one cannot kill it, it seems, by lowering its temperature even to near the absolute zero, that is, by nearly stopping its molecular motion. In the last analysis, however, the mechanism of action of high and that of low temperature might not be so different as it appears. The theories of injury by high temperature (cf. Belehradek, 1935) attribute death to some indirect effect of heat, such as protoplasmic coagulation, the destruction of some enzyme, the vaporization of lipoids, etc., in the same way as death by cold is attributed to some indirect effect of temperature such as the freezing of water. It seems then that it is not temperature itself, high or low, in other words, it is not the rapid or the slow molecular motion, which disturbs the structural architecture of living matter, but the indirect changes caused by certain particular rates of molecular motion.

Water appears to play a role of fundamental importance not only, as is well known, in the *functions* of living matter at the temperatures of active life but also in the *structure* of the living units, as is shown by the destruction of protoplasm when the molecules of water are torn away, in the process of devitrification, for example.

Adams (1905) points out that at extremely low temperatures not only water should be solidified in protoplasm but also carbon dioxide, and that oxygen and nitrogen should liquefy or solidify too. The separation of these substances from protoplasm and the mechanical injury possible as a result of the reduced pressure caused by these changes of state, might be expected to cause damage. However, the fact seems to be that the separation of these substances is harmless, while the separation of water is highly injurious. The importance of the crystallization of water in death by low temperature is emphasized in the diagram above where one of the main lethal zones is at the freezing point of water.

The observation that living matter can stay for a long time near the absolute zero without showing any measur-

able amount of activity but, without losing its ability to become active again when brought back to higher temperatures, has led some thinkers (de Candolle, 1895; Chodat, 1896; Brown and Escombe, 1897; etc.) to consider the static aspect of life, which is usually overlooked in the classical definitions. An organism which resists extreme cold behaves like a watch which, though well wound, is stopped by some braking mechanism. This watch is in perfect condition as to its constructional features and it will start of its own accord as soon as the brake is removed. In a similar manner, the activities of living matter can be stopped entirely without destruction of the mechanism which conditions them. This state of affairs is consistent with the hypothesis that the force which controls the vital activities requires a special *structure* of matter, and that, when that structure is destroyed, the organism is dead, while, when the structure is maintained, the protoplasm is alive, though it might not be active. To use the comparison of de Candolle, an organism in the state of latent life is like an explosive which does not show any evidence of its tremendous potential energy as long as it is not fired.

SUMMARY

1. An injurious action of cold, above the freezing point of protoplasm, has been reported in all homoiotherms, in some poikilotherms, in some higher plants and in some undifferentiated living forms. 2. This action, in which the time factor is rather important, has been attributed to a disturbance of physiological functions, to chemical changes, to the accumulation of toxic products, to an alteration of permeability, to dehydration, to changes in viscosity and in adsorptive properties and to processes of solidification, precipitation and coagulation. 3. A rapid lethal action of cold, above zero, has been reported in a few cases; it has been attributed to syneresis preceded by gelation.

4. The majority of investigators have found subcooling innocuous. 5. When subcooling is injurious, the importance of the time factor suggests that the mechanism of action is the same as that of cold above zero. 6. In nature the subcooled state represents probably a condition of safety against injury, though there are some arguments in favor of the opposite view.

7. The organisms which resist extreme cold belong to three groups: a) some resist only in the dry state, b) some with their full water content, c) of the latter, some survive only if special precautions of rapid cooling and re-warming are taken. 8. In all these, the main cause of the resistance seems to be the impossibility of freezing. The formation of ice might be prevented by the bound state of the water in the partially dried organisms, by capillary forces, by a possible rapid exosmosis of water in the small living forms or by a narrow range of freezing temperatures.

9. Studies on the resistance of living matter to extreme cold indicate that: a) molecular rearrangements such as take place in crystallization are lethal, while solidification into the amorphous state is not; b) cold alone, that is, a decrease in molecular motion, is innocuous; c) water plays an important role not only in the functional activity of living matter but also in the structure of the living units; d) life is probably conditioned by some special structure which, at low temperatures, allows for a state of latent life and at higher temperatures furnishes the basic mechanism for vital activities; the destruction of this structure would induce death.

CHAPTER II

ACTION OF COLD ACCOMPANIED BY ICE FORMATION

The theories on the mechanism of death by freezing attribute the lethal injury to the following various causes which will serve as a basis for our classification:

1. A mere withdrawal of energy;
2. The attainment of a minimal temperature;
3. Mechanical injury;
4. Too rapid thawing;
5. Dehydration;
6. Various physiological, physical and chemical changes.

Often, when the experimental data are too few or too inconsistent to justify a pertinent discussion, we shall limit ourselves to a mere presentation of the theories and of the facts recorded in the literature.

I. THEORY ATTRIBUTING DEATH TO A MERE WITHDRAWAL OF ENERGY

In the last analysis, all the theories to be reviewed hereafter attribute injury and death to the withdrawal of energy, that is, to cooling, but the present theory considers the withdrawal of energy as the immediate and final lethal mechanism, while the others assume that cooling causes some intermediate action, such as the formation of ice, which is then considered the immediate lethal factor.

The idea that death might result from a decrease in the energy content of an organism is perhaps the first to come to one's mind when one considers death by cold in the warm-blooded animals. These creatures constantly produce energy so as to maintain their body at a temperature higher than that of their milieu. They compete with the milieu and, if they fail in this competition, death

follows. It is then natural to think that death results from the inability to produce enough energy to compensate for that which is withdrawn.

This theory has been applied to cold-blooded animals which, it has been claimed for a long time, succumb to cold when they lose more heat than they can supply. As a typical representative of the many authors who held this view, during the last century, we shall mention Plateau (1872). He claims to have observed that aquatic arthropoda immersed in freezing water die when they are caught in the ice in such a way that they cannot make any more movement and consequently cannot produce any more heat.

While the production of energy by cold-blooded animals seems to have been universally recognized by the earliest physiologists, the production of heat by plants was still a subject of controversy a century ago. Goepfert, in 1830, in his book entitled: "Ueber die Wärme-Entwicklung in den Pflanzen, deren Gefrieren und die Schutzmittel gegen dasselbe", states (p. 228) that the plants "do not possess the ability to produce their own heat" ("eigene Wärme"). His conclusion is that, since plants cannot resist cold by producing heat themselves nor by receiving heat from the ground, "their vital force ("Lebenskraft") is the first and most important and perhaps the only source from which the resistance to the harmful influence of cold arises" (p. 225). Each species would possess a certain vital force which requires a given withdrawal of energy for its destruction.

It is interesting to mention, in connection with this concept of specific energy content of living matter, an idea which was quite generally accepted during the last century, namely, that freezing could not take place in *living* plants and animals, but that life had to be destroyed before congelation could occur. The destruction of life, it was thought, was caused by cold alone, that is, by the withdrawal of energy.

The theory of the impossibility for an organism to freeze as long as it is alive was still accepted some fifty years ago since Müller-Thurgau (1886) deemed it necessary to point out that his own experiments, in which plants were found alive after freezing and thawing, spoke against such a view.

There is some resemblance between this apparently antiquated theory and some modern forms of the "bound water" hypothesis, according to which living matter would bind a certain quantity of water in such a way that the crystallization forces would have to tear away this water and, in doing so, kill the protoplasm, before freezing can occur.

II. THEORY ATTRIBUTING DEATH TO THE ATTAINMENT OF A MINIMAL TEMPERATURE

The theory that death is due to the removal of a given quantity of energy leads naturally to the idea that there is a definite death temperature for each species of animal or plant. In this form, the theory has been known as that of the "specific minimum". It was developed mostly by Mez (1905) and his pupils Apelt (1907), Rein (1908) and Voigtländer (1909).

Mez (1905), considering that some plants are killed by cold at temperatures above zero while others resist hard freezing, and that some seeds present a high cold resistance (*Avena*, *Triticum*) while others are rather sensitive to cold (*Lobelia*), developed the theory that for each plant species there is a temperature minimum below which the plant cannot live. This minimum, or death point would be characteristic of each species, under a given set of conditions, such as, water content, age, stage of development, etc., but it would change when any one of these conditions changes. Drying, for example, would lower the specific minimum. The development of a seed into embryonic tissues would raise the minimum.

Mez applies his theory to the explanation of various observations or assumptions on the behavior of plants at low temperatures. Adaptation to climate would consist in a change of the specific minimum. The resistance of plants which stay alive in ice would be partly attributable to the fact that the heat developed during ice formation protects them and retards the drop of the temperature to the specific minimum. Subcooling might then be rather harmful, as has been said in the preceding chapter. The injurious effect of rapid thawing is explained on the assumption that if the heat necessary to melt the ice be withdrawn too suddenly from the regions surrounding the thawing spot, the temperature of the internal portions of the material may be lowered to the specific minimum. To confirm this interpretation Mez says that, 5 mm. below the surface of a frozen apple thawed by the warmth of the hand, he could measure a lowering of temperature of 1.8 degree.

The idea that each animal or plant species is characterized by a given death temperature appeals to those biologists who are in quest of sharply defined, typical physical processes. It suggests that death might be correlated with some physical change, which would take place at a definite temperature, as is the case for melting. Apelt (1907) seems to have followed this line of thought. He evidently assumed that the death temperature of a plant species could be measured with the same precision as a melting point when he claimed to have established the range of death temperatures in some types of potato within 0.007 degree. His procedure consisted in exposing potato to a given temperature and diagnosing death by the change in color of the tissue and by the failure of the cells previously stained with methylene blue to plasmolyse.

While Apelt's precision has not been taken seriously by all biologists, several points in his argumentation and experiments deserve mention. He established, for ex-

ample, a clear distinction between the theory of death by withdrawal of energy and that of death at a temperature minimum and he rejected the former as contradicted by the following facts. Cells of potato tubers were not killed in one hour at a temperature 0.3 to 0.4 degree higher than that which was instantly lethal, neither were they killed in two hours at a temperature one degree higher than the death point. Death, then, is not attributable to a withdrawal of energy, since the same amount of energy could be withdrawn in a longer time at a higher temperature as in a shorter time at a lower temperature. Retaining the theory of the specific minimum, the author thinks that a protoplasmic disorganization when the temperature reaches that minimum constitutes the mechanism of death.

III. THEORY ATTRIBUTING DEATH TO MECHANICAL INJURY

Death has been considered as resulting in various ways from a mechanical injury inflicted during freezing or thawing. *A.* Some authors have thought that the expansion of ice during its formation bursts the cells, as it bursts a bottle filled with water; *B.* Others have assumed that the ice crystals in the cells or in the intercellulars damage the protoplasm or the cell parts by piercing or tearing them; *C.* Some investigators have invoked the pressure exerted by the expanding ice as the cause of death, its action being not to burst the cells, but to compress the protoplasm; *D.* A few biologists have attributed death to a mechanical injury inflicted, not by the ice itself, but by the jarring of the protoplast during plasmolysis and deplasmolysis, as a result of which the cytoplasmic mass would be torn off from the cell walls where it adhered.

A. Most of the reviewers have attributed to Duhamel and Buffon the idea that death results from a mechanical injury caused by the expanding ice in living tissues. We

could not find any statement to that effect in the authors mentioned. They say that big cracks can be produced in trees as a consequence of the formation of ice in their interior but their theory of death is entirely different and will be explained later.

According to Senebier (1800) ice might cause death by its expansion.

But soon several investigators showed that the cells are not broken by freezing. Goeppert (1830) says that the large cells of *Calla ethiopica* do not show, when their sap is congealed, any expansion of the cell wall observable under the microscope. He furthermore states that in the limp tissue, after thawing, the cellular structure remains intact and that the cell walls are never torn.

Morren (1853) confirmed the observation that there is no evidence of ruptured walls.

Nägeli (1860) calculated what should be the expansion of a cell during the formation of ice in it and showed that this expansion is never great enough to break the cells, the membranes of which are always sufficiently expandible. Besides, he observed that often the cells in contact with the intercellular masses of ice are actually not damaged.

Schacht (1854) noted that the juice exuded from potatoes after freezing does not contain starch and he concluded from that observation that the cells could not be lacerated.

Prillieux (1869), after describing the formation of ice in the intercellulars, states that ice may tear the tissues but never the cells and that there is no necessity of assuming that the membranes are torn to explain that the juices come out of the cells.

To summarize: The opinion that ice bursts the cells as it would a bottle has been advanced by some early biologists. Between 1830 and 1870 several authors demonstrated that it is not based on experimental and observational evidence. From the beginning of the pres-

ent century this idea has generally been abandoned by the physiologists, though occasionally some authors wonder if the reaction against it has been exaggerated. The extrusion of water from the cells during freezing and the absence of ice in the cells after the congelation of a tissue have generally been observed after *slow* freezing as it usually occurs in nature. With rapid freezing or when the water content of the cells is very high, intracellular congelation takes place (Molisch, 1897; and, more recently, Stuckey and Curtis, 1938). The mechanical injury in intracellular freezing may be quite different from that of the generally observed extracellular formation of ice.

Stuckey and Curtis (*loc. cit.*), who reported to have observed ice formation within the cytoplasm itself in the cells of the prothallia of *Polypodium aureum*, claim that death always resulted from such intracellular freezing. They consider death as due, according to all evidence, to a mechanical injury by ice.

B. While the authors cited above have established that ice does not destroy the cells by bursting them or by tearing their membranes, more recent investigators have supposed that tiny ice crystals tear the *protoplasm* itself. Maximov (1914) for example, attributes death partly to the destruction of the fine structure of the protoplasm as a result of mechanical injury.

Stiles (1930) thinks that the formation of a new phase, namely ice, constitutes a mechanical disturbance resulting in a breaking down of the colloidal system. The types of protoplasm in which the separation of the materials on freezing is followed after thawing by their restitution to the former state, would not be killed, the other types would. He assumes that, if the crystals be smaller, the mechanical injury would be less, and several types of protoplasm might resist freezing. As a means of inducing the formation of smaller crystals he proposes the use of lower freezing temperatures, according to the finding of Tammann (1898) on the relation between the temper-

ature, the number of crystallization centers formed and their velocity of growth. But the part of this suggestion concerning the possibility of avoiding injury by causing the formation of smaller crystals could not be confirmed experimentally, as the following observations show.

Iijin (1934), after comparing experiments on rapid and slow cooling of the cells of red cabbage leaves, concludes that sudden freezing is more injurious than slow freezing.

The abrupt immersion of sticklebacks in liquid air by Weigman (1936) killed the animals readily.

Identical experiments by Luyet (1938) on gold fish gave the same results.

Luyet and Thoennes (1938a) found that monocellular layers of plant epidermis presented only dead cells after rapid freezing in liquid air.

As is evident, these experiments which do not confirm the idea of a lesser injurious effect of smaller crystals, do not invalidate the theory of a possible mechanical injury on protoplasmic structure by ice.

Some have thought that a mechanical contact with ice might result in a coagulation of protoplasm, since it is well known that touching or piercing with a needle (Chambers) or pricking a cell may result in its coagulation. Lepeschkin (1936) apparently follows this trend of thought, when he speaks of "mechanical coagulation" by freezing.

C. The killing of protoplasm under the action of the pressure exerted by the expanding ice is often referred to in the biological literature. Among the authors who discuss this theory more extensively let us mention Plateau (1872) who attempts to show that there is no pressure within the ice in formation. It is a known physical principle, according to him, that the cavities in a solid body expand like the body itself. Therefore the cell contents cannot be crushed by the freezing of the tissue around them. He claims to have shown the absence of pressure

experimentally with an apparatus consisting of a glass tube on the end of which was a rubber bulb filled with a liquid and immersed vertically, the open end up, in a flask containing water. When the latter froze in the flask, the level of the fluid in the tube stayed the same, indicating that there was no pressure exerted on the rubber bulb. Plateau, it seems, did not notice that the results of his experiment disagreed with the principle that he invoked, he should have observed a lowering of the level of the fluid in the manometric tube, if the cavity around the bulb were expanding.

Hjtin (1936), discussing the case of plant cells frozen in water, conceives the mechanism of pressure by ice as follows: The water in which the material is immersed freezes first and forms a wall around the protoplast of each cell. When, later, the sap of the vacuole freezes, the protoplast is wedged between two masses of ice. On these assumptions he calculates, for different forms of cells, how much the volume of the protoplasm should give so as not to be crushed by the ice formed in the vacuole. His figures indicate that there is definitely a possibility of some crushing action. If the cells are frozen in air, it is assumed that the pressure might be exerted against the frozen cell walls. The fact that the congelation of the vacuolar sap is always followed by death is in agreement with the concept of an injurious pressure effect of the frozen sap.

As a whole, both the problem of the existence of a pressure in a frozen tissue and the problem of the efficacy of pressure in causing death call for more experimental evidence.

A consideration of the enormous pressure required to kill protoplasm makes one doubt the possibility of the existence of such pressures in cells. Most of the tissues of metazoa resist a hydrostatic pressure of several hundred atmospheres. Protozoa and bacteria are killed only at pressures of the order of 1,000 atmospheres

(cf. Cattell's review, 1936). Yeast takes more than four thousand atmospheres (Luyet, 1937). Under the action of pressures of this magnitude, ice should melt several degrees below zero. Besides, for obtaining such pressures it would be necessary to prevent the expansion of ice by holding it in some material more resistant than the walls of an animal or plant tissue.

The experiments of Melsens (1870) who found that, in a culture of yeast exposed in a steel bomb calculated to burst at 8000 atmospheres, there were living cells after the temperature was lowered until the bomb burst, are also significant in the discussion of this problem.

How enormous hydrostatic pressures have no action on protoplasm while pricking with a glass needle may, in some instances, start coagulation, is entirely unknown. The answer to this question might throw some light on the type of mechanical injury caused by ice.

D. Iljin (1934) presented a new theory of death by mechanical injury, applicable to typical plant cells which consist of a protoplast adhering to cell walls and filled with cell sap. He distinguishes two general cases: that in which death occurs during freezing and that in which it occurs during thawing, and he subdistinguishes two cases of death during freezing, that in which ice is formed only in the intercellular spaces and that in which there is ice also in the vacuoles. When death is caused by thawing, the too rapid invasion of the protoplasm by water would damage the living structure which is not capable of expanding rapidly enough and is torn by being pulled about. When death occurs during freezing but without congelation of the vacuolar content, the withdrawal of water from the vacuole would cause the latter to shrink and the protoplasmic layer, still attached on one side to the cell walls, would be stretched between these cell walls and the vacuole whose contraction it has to follow; this stretching would be injurious. In the case of formation of ice, both around the cell and in the vac-

uole, the protoplast would be killed by being squeezed between two masses of ice, when the vacuole expands on freezing. Iljin's experiments in which he succeeded in keeping alive, by cautious slow freezing and thawing, cells which otherwise would have been destroyed give much weight to his theory.

IV. THEORY OF DEATH BY TOO RAPID THAWING

For the investigators who maintain that cold, when not accompanied by ice formation, is not generally lethal and that freezing is usually a necessary condition for death, the question arose as to whether death occurs during freezing itself or during thawing. Almost all who favor the latter assumption think that it is the rapidity of the thawing which renders it dangerous. So the theory of "death by thawing" and that of "death by too rapid thawing" will be treated together.

The origin of the theory of "death by too rapid thawing" seems to be the old popular idea that, when a person has frozen limbs, he should be warmed gradually. This notion, frequent in the medical literature, is found here and there also in the biological literature. For example, Duhamel and Buffon (1737) say that when animals are frozen one puts them in snow, in water or in dung to warm them slowly. These authors also give as a well-known fact that frozen fruit decays if thawed too rapidly. They claim, furthermore, that they could save plants (orange trees and geraniums) which were coated with ice, by covering them so as to prevent a too rapid thawing by the sun, or by exposing them to a slight rain which also would cause a slow thawing. After refuting the idea that the injurious action of the sun on frozen trees might be due to a condensation of the rays by the lenses constituted by the droplets of melting water, as some have maintained, Duhamel and Buffon present the following tentative hypothesis for explaining the mechanism of action of rapid thawing: the vessels distended by

the increase in volume of the frozen sap cannot, in fast thawing, resume their normal size smoothly enough ("avec assez de douceur"), then they break, the sap evaporates and the plant dries up.

A similar idea is held by Pichel (1816, quoted by Müller-Thurgau, 1886). Injury in twigs would result from a tearing of vessels or of essential structures in the too rapid thawing of the unequally expanded outer and inner layers of the twigs. The free course of the sap would thus be disturbed.

Goeppert (1830) called into doubt the view of many of his predecessors that slow thawing saves frozen plants from death. He thawed slowly, at about 0° , in snow, frozen bulbs of onions, tulips, etc., and observed that they were killed.

The experiments of Sachs (1860; see also Sachs' *Handbuch der exp. Physiol. der Pflanzen*, Leipzig, 1865 and later editions) again revived the older theory that slow thawing can forestall the death of frozen plants. He froze pieces of beet and of pumpkin and leaves of beet, cabbage, bean, etc., at -4° to -6° R (-5° to -7.5° C) and thawed them in water at 0° , in air at 2° to 3° R or in water at 6° to 10° R. In the first case, with slow thawing at 0° , the plants were alive, in the last two cases they were killed. Sachs' interpretation is that when thawing is slow the molecules of water pulled loose from the protoplasm during crystallization can again take up their former position and reestablish the conditions existing before freezing, while if thawing is rapid some of the water may flow away and not be reabsorbed and thus the previous conditions of concentration and imbibition cannot be restored and death may result. As to the fact that a lower water content decreases the sensitivity to cold, Sachs explains it by assuming that when there is less water to freeze it can more readily be reabsorbed after thawing.

Goeppert (1871), more than forty years after his first observations, undertook a new series of experiments which led him again to the conclusion that it is during freezing and not during thawing that the plants are killed. He put to freeze orchids which contain indican and turn blue at death (*Calanthe*); he found that the blue color appeared in the frozen state before thawing.

Prillieux (1872) repeated these experiments and came to the conclusion that the indican plants became blue only after thawing. It seems that the different conclusions reached by these two authors are due partly to a disagreement on what is called blue. Before thawing the plants are of a steel blue color (Stahlblau) and after thawing they are of a deep dark blue.

Kunisch (1880), instead of utilizing the change of color of indican plants at death, tried to revive them and to put them to grow after freezing and slow thawing but he registered only negative results.

Müller-Thurgau (1880) also experimented with indican plants. Using the petals of *Phajus*, he could observe by a slow lowering of the temperature that the change in color took place during freezing, not however at the freezing point but when, after some ice formation, the temperature dropped to a lower level. Any attempt to keep alive by slow thawing petals which had turned blue on freezing failed. The same author (1886) summarizing the results of other experiments in which he subjected "several hundreds" of frozen plants to rapid or slow thawing at various temperatures (for example, potatoes thawed in sand at 45° and at 0°) concludes that there is no evidence that slow thawing ever saved these plants from death. However, a few years later (1894), he found that frozen pears and apples show considerable injury after thawing in water at 0° or in luke-warm water, whereas they show only slight or no injury if thawed more slowly in air at 0° or at 20°. (One might mention here that Müller-Thurgau pointed out the error made by previous in-

investigators who thought that thawing in water should be slower than thawing in air, at the same temperature).

Molisch (1897), who reviewed the problem of death by rapid thawing, himself made a large number of experiments on this point. He used mostly leaves of various higher plants, in particular, of *Ageratum* in which death can be diagnosed by the odor of coumarin released from the cells. He also performed some experiments with algae of the class *Florideae*, which change their color at death. Rapid thawing was carried out in water at 30°; for slow thawing the material remained 5 hours at -1°, 5 hours at 0° and then it was brought to 2°. The leaves were cut longitudinally in two, one half being thawed rapidly, the other slowly. The author concludes that the rapidity of thawing had no effect on survival except in one case, namely with the plant *Agave*.

According to Sorauer (Handbuch der Pflanzenkrankheiten, 1909) frozen leaves of *Cineraria* presented dead spots only at places where they had been thawed too rapidly between the warm fingers.

Hedlund (1912, quoted by Akerman, 1919), also found that plants frozen at moderately low temperatures can be saved by slow thawing.

Winkler (1913) on the contrary, came to the conclusion that survival is not controlled by the rapidity of the thawing.

Chandler (1913), experimenting on fruit and vegetables, found in exceptional cases some evidence of a more injurious action of rapid thawing; in general, however, the rapidity of thawing made no difference on the results.

Akermann reviewed this subject (1913) and himself made a systematic series of observations on the effect of rapid and slow thawing on various plants (red cabbage leaves, *Viburnum*, *Aucuba*, potato, *Tradescantia*). He concludes that slow thawing might play a role in saving from death some species of frozen plants but

only if they had not been exposed to too low a temperature.

Ijima (1934) revived the old theory of injury and death by too rapid thawing and established it on a new basis. He observed that when, during freezing, water is extruded by osmosis from the vacuoles of plant cells, the protoplast contracts and the opposite sides of the cytoplasmic sac approach each other and come in contact in the center of the cells, while they are still separated by cell sap in the outer portions. On thawing, water suddenly invades the contracted protoplast and tears it, if it cannot pull apart the adhering sides. The author says that he observed the same phenomenon, namely that the protoplast tears open, in too rapid deplasmolysis. In the case of death by desiccation he claims to have evidence that injury results from the too rapid invasion of the protoplasm by water on remoistening. In his experiments on low temperature effects he succeeded in saving from death cells of red cabbage frozen at the temperature of solid carbon dioxide. His method consisted in slowly restoring the water to the cells by letting cooled hypertonic solutions of sugar, glycerine or inorganic salts of gradually decreasing concentrations fall dropwise on the frozen sections of the tissue.

Of the few authors who found slow thawing more injurious than rapid thawing we shall mention here a recent work of Turner and Brayton (1939) on the spirochetes of relapsing fever. When rewarming and thawing took place in 30 seconds, (in water at 37°) or in 25 to 35 minutes (at room temperature), there were living spirochetes. As a test of vitality the authors used motility and pathogenicity for mice.

The numerous data recorded in this section do not allow one to draw any general conclusion. It is probable that the effects of slow thawing are different in different organisms, as a comparison of the results on

spirochetes, for example and those on the tissues of higher plants, seems to indicate. Besides there are evidently other factors in the problem which have not yet been analysed.

V. THEORY OF DEATH BY DEHYDRATION

After it had been observed that water comes out of the cells during freezing and passes into the intercellular spaces, the theory was proposed that death results from the fact that congelation deprives the protoplasm of its moisture. Death by freezing would then be identical, in the last analysis, with death by drought. Several eminent plant physiologists toward the end of the last and the beginning of the present century took sides on this question. We shall here summarize, in chronological order, the most important works which have contributed to the development of this theory.

Sachs (1860) is usually not considered an advocate of the dehydration theory of death by freezing, though some of his statements place him among the pioneers who pointed out the important alteration which results when water molecules are disengaged from a living structure. He says that the molecules of water belong to the structural organization of protoplasm and of the cell walls, and are in a certain state of equilibrium with the other constituent molecules. By freezing and thawing, the water molecules are pulled away from the structure and a new state of equilibrium is established in which the other molecules have a stronger attraction for each other than for water. This is precisely the mechanism which several later investigators who hold the water withdrawal theory assumed to explain injury and death by freezing.

Müller-Thurgau (1886), who is ordinarily held as the founder of this theory, proposed it in a rather hesitating phraseology. He says that death is usually considered as resulting from a destruction of the ordered arrange-

ment of the constituent parts of protoplasm, including water, and that such destruction has been attributed to one of the three following factors: temperature alone, the withdrawal of water during freezing, some processes which take place during thawing (three theories which, according to him, can be traced back to the previous century.) After discussing the pros and cons for the first and the third possibilities he gives his preference to the second, saying that, since water withdrawal is the most essential change which occurs during freezing, it could very well be the cause of death by cold.

He then goes a step further and gives a body to the dehydration theory by suggesting a mechanism by which death might result from the withdrawal of water. He considers that the solid constituents of protoplasm, *i. e.*, the micelles, are separated by the water phase, being farther apart in protoplasm which has a higher water content; the withdrawal of water will result in displacing these micelles from the positions that they occupied in the ordered arrangement of living protoplasm. How a molecular structure can be altered by freezing is exemplified by starch paste which, after congelation, loses its property of holding water of imbibition.

Müller-Thurgau also holds that: 1. The sudden withdrawal of water during the rapid freezing which follows subcooling is particularly dangerous; 2. Often the disturbance produced by dehydration would be reversible and the original structure could be restored if the water withdrawn by freezing would not evaporate after thawing before it can be returned to the cells. Consequently, he claims that a means of keeping alive frozen material is to prevent evaporation during and after thawing.

Several observed facts are interpreted by Müller-Thurgau as being in good fitting with his dehydration theory. The greater sensitivity to cold in plants with higher moisture content, as exhibited, for example, by soaked seeds, and the relatively higher resistance of less

hydrated material as in the undeveloped buds, are explained on the assumption that when the micelles are closer together, as in drier tissue, less damage is done since there is less water to be withdrawn.

The fact that many of the plants which resist cold are also those which resist drought, in particular, moss and lichens, is adduced as evidence for the theory.

Sachs' experiments in which plants thawed in water recovered while plants thawed in the air died, are interpreted as favoring the dehydration theory, since recovery would be possible when enough water is furnished to rehydrate the cells, while death would occur when too much water evaporates, as in the case of thawing in the air.

To the objection that some plants would be killed by a relatively slight dehydration in freezing while they resist considerable drying under other circumstances, he answers that, in freezing, the withdrawal of water is particularly sudden and acts by its suddenness.

Molisch (1897) contributed important experimental data to the theory of Müller-Thurgau which he accepted almost entirely. He first pointed out that the theory of a disturbance of the functional harmony cannot explain the cases in which death takes place immediately upon congelation. He remarked that the theory of a lethal action of temperature alone without ice formation is contradicted by the numerous observations of the innocuousness of subcooling. Finally, he showed that the theory of death by too rapid thawing does not apply in a large number of cases that he studied. Though each of these three theories, he concludes, might explain death in some particular instances, death is, in general, coincident with the formation of ice and with its attendant withdrawal of water.

The following points, several of which had already been indicated by Müller-Thurgau, are emphasized by Molisch: 1. A relatively large proportion of ice is formed

at the beginning of freezing at near-zero temperatures; 2. A further withdrawal of water at lower temperatures causes death. 3. Cold resistance is increased by dehydration (wilted tobacco leaves were found to resist freezing more than fresh ones); 4. The high concentrations which result from dehydration exert a toxic effect; 5. The differences in resistance to both freezing and drying in various plants are specific characters. Molisch furthermore attributes the resistance of bacteria, spores, seeds, moss, etc., to the fact that these organisms can be exposed to low temperatures without releasing their water.

According to *Matruchot and Molliard (1902)*, who had observed that, in frozen plant cells, water had been separated from the cytoplasm and from the nucleus, the dehydration theory explains most of the facts known on the action of low temperature. They discuss, in particular, the following ones: 1. Cytoplasmic streaming decreases and finally stops, when a cell is cooled; this would be due to the more solid consistency acquired by protoplasm on the withdrawal of water. 2. Numerous plants and animals revive after freezing or after drying when water is supplied to them; in both cases, the organism would come back to life if water could be re-imbibed by the protoplasm and the conditions which existed previously reestablished, while the organisms would die if the separation of water went so far as to constitute an irreversible process. 3. The injurious effect of too rapid thawing would be due to the sudden invasion of the dehydrated tissues by water, a too rapid imbibition rendering impossible the reestablishment of the previous state. 4. The various degrees of resistance offered by different species to both desiccation and freezing would be explainable by different specific water-holding capacities. 5. The high resistance to cold of plants with a thick or heavily cutinized epidermis could be attributed to the ability of these plants to retain water.

The first of these processes, namely the cessation of protoplasmic streaming, does not seem to be satisfactorily explained by dehydration. Protoplasmic streaming has been observed to become slower and to stop under the action of low temperature without ice formation, that is, without dehydration to any noticeable extent.

In an attempt to analyse the mechanism itself of death by dehydration, Matruchof and Molliard, following some views previously held by Dastre, distinguish 3 sorts of water in living matter: 1. *External* water, that is, the water of the cell sap, which does not enter into the make-up of living protoplasm; 2. *Interposed* water, the molecules of which move freely in the capillary spaces between the micelles, that is, within the meshes which constitute protoplasm; 3. *Constituent* water which is either a part of the protoplasmic molecules or is attached to them by adhesion forces. The withdrawal of external water would be harmless and would leave a still liquid protoplasm. The withdrawal of interposed water would not be usually lethal and would result in the production of that more solid sort of protoplasm found in seeds or spores. As to the constituent water, its separation from protoplasm would induce death.

Pfeffer (1904) formulated several objections to the water withdrawal theory. The essential points in his objections are the following: 1. There is a contradiction between the fact that desiccation of a plant increases its resistance to cold and the theory that desiccation by cold causes death. 2. More water can be removed without injury by transpiration and by plasmolysis than by freezing. 3. Death by cold is in several cases independent of the water content: some plants survive cold when they are in the turgescient state while others, like seeds, survive in the dry state. 4. To account for the death of some plants at temperatures far below the freezing point one would have to assume that some moisture does not freeze until these low temperatures are reached, an

assumption which is not in agreement with what is known on the rate of formation of ice in terms of temperature.

Some of these fundamental objections were resumed by *Mez (1905)*. This author wonders why dehydration should be harmful when caused by freezing and harmless otherwise. Concerning the formation of ice at temperatures far below the freezing point, he quotes his experiments with *Impatiens* stems in which he claims to have shown by the shape of the freezing curves that congelation is completed at -6° . Cooling at a lower temperature, therefore, would not induce any further desiccation and one does not see how it would be injurious, except if one admits the theory of the "specific minimum".

According to *Mez*, several experiments in which death was attributed by *Molisch* to freezing and interpreted as identical with death by desiccation (water algae, staminal hair of *Tradescantia*, potato) represent really cases of death by desiccation and not of death by freezing.

Apelt (1907), supporting the argument of *Mez*, says that in his experiments on potato tubers he always found that the death point was definitely below the freezing point. This fact is interpreted as signifying that death is not due to a desiccation during freezing.

According to the same author, the dehydration theory has against it that, in one experiment on potato, repeated freezing (more than 4 or 5 times) at a temperature slightly above the death temperature resulted in death. Since it is assumed that the amount of water congealed in each freezing at the same temperature is the same, one does not see how the repetition of the experiment could become injurious.

Gorke (1907) brought forth a new argument in favor of the dehydration theory by showing that proteins can be precipitated by freezing when the salt concentration

increases as a result of the removal of water (the details of this theory will be given below).

Voigtländer (1909), resuming the views of Mez, claims that the cell sap usually presents a eutectic point at around -6° . He, therefore, denies that there can be any further desiccation at the temperature of -30° at which some plants die.

The same author argues that if death is due to some water withdrawal, or to the salting out of some protein, or to the eutectic freezing of some complex mixture, some thermal effect should appear. None, however, has ever been observed.

One of the most natural explanations of the fact observed so many times that a tissue survives the formation of ice in the neighborhood of its freezing point and is killed on further freezing at a lower temperature is that, as long as there is free water to freeze, no damage is done, and that injury begins when more strongly bound water freezes. *Jensen and Fischer (1910)*, found, in a comparison of the freezing curve of frog's muscle with that of an isotonic salt solution, that only about 3.9% of the water of the muscle might be more firmly bound. Besides, the dead muscles also seemed to contain bound water. Death, therefore, could hardly be explained by the unbinding of that water.

According to *Irmischer (1912)*, the similarity pointed out by other investigators in the resistance of plants to injury by drying and by freezing can also be observed in moss. In general, the species which are more resistant to cold are also more resistant to drought. (There are, however, several exceptions.)

Another similarity between the mechanism of action of desiccation and that of freezing, pointed out by the same author, is that cold hardiness can be induced by exposure to drought as well as by exposure to cold. Moss which had been dried and which regained its full turgor by immersion in water was found more resistant to cold

than normal moss. The natural conclusion from this observation is that the protoplasmic changes induced by desiccation must be the same as those induced by cold.

Irmischer, furthermore, repeated with moss the experiments of Molisch on the difference between material frozen in water and that frozen in air. Moss partially immersed in water was exposed for 3 days to -5° to -6° . The submerged portion, all surrounded by ice, remained alive, while the tips which projected out were killed. The more complete dehydration in air is assumed to be responsible for the injurious effect.

Cameron and Brownlee (1913), discussing experiments in which frogs were killed after having been frozen by an exposure of several hours at or below the freezing point, attribute death to the excessive concentration of the cell contents caused by dehydration. (They note that such concentrated cell contents should not freeze.)

In order to clarify the problem of the correlation between ice formation and death in plants, *Maximov (1914)* undertook to study: 1. The death temperature as related to the freezing point; 2. The influence on death of the time the material is left at a given freezing temperature; 3. The relation between the quantity of ice formed at a given time and temperature and the occurrence of death. Using potato tissue in which the vitality of the cells was judged by the plasmolysis test, he found that the tissues were completely killed in a short time at -2.66° while almost all the cells were alive at -1.96° and -2.11° . Furthermore, a piece of potato left to freeze for 10 minutes between 0° and -1.82° was alive, while one left for about 3 hours at the same temperatures was practically all dead.

These and other experiments confirmed his views that death progresses step by step with ice formation and, consequently, with water withdrawal. So this author accepts, in general, the dehydration theory of Müller-Thurgau and Molisch, but modifies it by suggesting that

the damage by freezing results not only from the water withdrawal itself but also from a precipitation of the colloids of the protoplasm. Such a precipitation would be caused by both an increased concentration of the colloids subsequent to freezing and by a mechanical compression between the ice masses. The common action of these two factors would be to bring the colloidal particles of protoplasm in too close a proximity so that coagulation would result.

Having admitted a mechanical action of ice on the precipitation of colloids, Maximov considers as answered the objection of Pfeffer and others to the dehydration theory, namely, that some plants withstand desiccation while they are killed by freezing. The factor pressure, present in freezing, is absent in desiccation.

The fact pointed out by Maximov himself (1912) that immersion in a concentrated solution protects a tissue against the action of cold except if the temperature of the solution is below the cryohydrate point, is brought to support the theory; above the cryohydrate point the effect of pressure by ice is lessened by the fact that a part of the solution is liquid and gives way under pressure.

Maximov departs in some minor points from Müller-Thurgau's views; for example, he denies that the rapid water withdrawal after subcooling is the cause of death since he observed that the death temperature was about the same when freezing was preceded by a considerable subcooling and when there was no subcooling.

He criticizes Fischer's theory that death by cold consists in the loss by the protoplasmic colloids of the capacity for adsorption, saying that only a small portion of water is adsorbed in protoplasm, the rest is "free" water, held in the cell by osmotic forces, not by adsorption. The release of water after death is explainable, according to Maximov, who here quotes Nägeli (1861),

by an increase of permeability and it does not involve a loss of the adsorption capacity.

Twenty-five years later, Maximov (1938) held about the same fundamental views. The withdrawal of water during freezing would cause an alteration in the protoplasmic colloids; in particular, it would increase permeability. He could, in leaves, determine an increase in cell permeability which paralleled the degree of wilting and, consequently, he considers freezing and wilting as similar in their injurious action.

Kylin (1917) reported that various algae, for which the temperature of death by freezing in sea water was previously recorded, were killed at temperatures above freezing when the increasing concentration of the water in which they were immersed was high enough to have the same freezing point as the water in which the algae were killed by freezing. Accordingly death of the organisms is attributed to the concentration resulting from freezing and not to the congelation itself.

Moran (1929), who worked mostly with isolated frog's muscles, remarks that neither cold alone kills, as is shown by the well known innocuousness of subcooling, nor does the formation of a relatively large quantity of ice in a tissue, since muscles left for 48 hours in the frozen state at -1.5° (freezing point -0.42°) were irritable after thawing. Having rejected these two interpretations, he points out that it is the removal by freezing of more than a certain critical quantity of water which seems to be fatal, as is shown by the fact that a muscle loses its power to react to an electric stimulus when frozen at a temperature lower than -1.9° .

Comparing these results with those obtained in his study of death by desiccation, namely, that it was impossible to revive a muscle in which 78% or more of the water content had been removed, he pointed out that the temperature of death by freezing was precisely the freezing point of a solution of sodium chloride which was

concentrated by evaporation from its point of isotonicity with the normal muscle until it had lost 78% of its water. Death by freezing would then take place when more than this proportion of water freezes out from the already concentrated protoplasm. (A determination of the freezing point of a solution containing the salts of the muscle at the concentration reached after drying to the critical degree gave -1.6° .)

The similarity of action of freezing and drying was further evidenced by the similarity in the changes of the electric resistance observed in tissues which were killed by either of the two injurious agents.

Moran, however, remarks that the action of freezing and that of drying are not altogether identical on all sorts of materials. Drying is not known ever to produce an irreversible change in gelatin gels, while freezing does.

As to the mechanism by which a removal of water becomes injurious, he makes the following suggestions: 1. Dehydration may result in a destruction of "an interface, a catalyst, or a structure holding catalysts"; 2. It may upset the ionic balance and cause a change in the viscosity and permeability of protoplasm; 3. It might produce some internal strains of the type observed in gels which become anisotropic by being frozen and thawed.

Comparing death to the irreversible change brought about at -6° in frozen egg yolk and probably due to the action of concentrated electrolytes on the protein-lipid complex, Moran sees "closely similar relations" in the two cases; they would differ, however, in the fact that the irreversible change in yolk requires some time so that one can avoid it by rapid cooling, while "no sensible time appears to be needed for permanent destruction" of the living system and it is impossible, by rapid freezing, to avoid death.

Some of the authors who hold the dehydration theory of death by cold assume that if the water withdrawn can

be reabsorbed after thawing, death can be prevented. Concerning this question, a remark of Stiles (quoted by Jones and Gortner, 1932) is of interest. Hydrated gelatin loses 4 to 6 times more water on thawing when it has been frozen slowly than when it has been frozen rapidly. Jones and Gortner (1932) mention, as a natural explanation of this observation, the fact described by Moran (1926) and by Hardy (1926) that, in rapid freezing, a large number of small crystals are formed within the gelatin mass and a reabsorption of that water after thawing is easy and rapid, while, in slow freezing, the presence of larger quantities of ice at the same place and mostly at the external surface impairs or slows reimbibition after thawing.

A determination by Luyet and Condon (1938) of the time at which the cells of potato tubers are killed when gradually increasing proportions of water are withdrawn by freezing, led these authors to the conclusion that injury begins after the withdrawal of about 35% of the water content, at a temperature of 0.2 to 0.3 degree below the freezing point (between B and C in the graph)

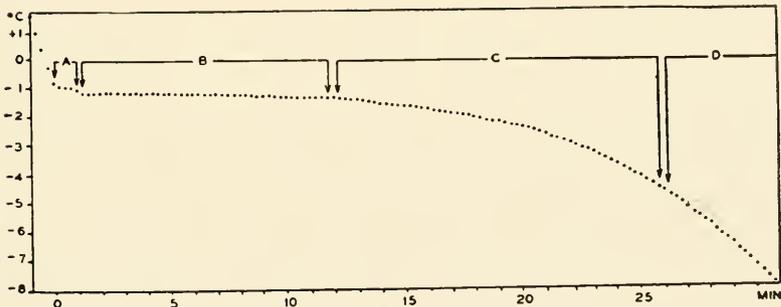


FIG. 33. Freezing curve of a piece of potato. Abscissae: time in minutes from the beginning of freezing; Ordinates: temperature. During the periods of time indicated by A and B no cell was killed; death occurred during the period C; freezing continued when all the cells were killed, during the period D. (From Luyet and Condon, 1938.)

and that all the cells are killed when about 70% of the water has been removed at 3.5 degrees below the freezing

point (between C and D in the figure). After death, more water freezes out when the temperature is lowered (region D in the figure). If death, in these experiments, took place while the material was frozen (and not on thawing), it did not correspond to the removal of the most tightly bound water, since some was still unfrozen after death.

This observation led *Luyet (1939)* to distinguish between *vital* and *bound* (unfreezable) water. There would be 5 kinds of water in protoplasm: 1. *Excess* water which can be removed without affecting the activity of protoplasm; for example, without decreasing the respiratory rate. 2. *Metabolic* water which influences the rate of living processes but can be withdrawn without inducing death. 3. *Vital* water, the separation of which is lethal. 4. *Remnant freezable* water which, in some organisms, stays unfrozen during death by congelation but can be frozen after death. 5. *Unfreezable* water which never freezes at any temperature. The author accounts for the specific differences in cold resistance by assuming that, in some types of protoplasm, the vital water is the most tightly bound, unfreezable water, while, in others, it is the freer, freezable water.

Becquerel (1939) claims that cells killed by freezing do not show any evidence of having been dehydrated by plasmolysis. He exposed epidermal strips of onion or of the petals of red hyacinth to temperatures extending from -150° to -25° and examined individual cells under the microscope before, during and after freezing and thawing. He observed that neither the cytoplasm, nor the vacuole nor the nucleus undergoes any change which might be interpreted as resulting from plasmolysis. He concludes that "almost all the physiologists so far have made the mistake" of considering as plasmolysed a coagulated cytoplasm slightly separated from the cell wall. It seems that the condition reported by Becquerel is due to the fact that he used a rapid method of freezing which did not allow plasmolysis, which is usually a slow process, to take place.

Levitt (1939), in a study of cold hardiness in cabbage, brings a quite new argument against the theory that resistance to injury by cold is simply resistance to dehydration. He determined by calorimetric methods the proportion of frozen and unfrozen water in hardened and in unhardened plants at their critical freezing temperatures, that is, at temperatures at which 50-75 per cent of the plants are killed (-5.6° for hardened, -2.1° for unhardened). The "unhardened tissue retained 3.5 times as much water in the liquid state per gram dry matter as did hardened (6.30 and 1.75 gm. respectively)". Hardiness, that is, resistance to injury by cold, "is therefore not determined simply by resistance to dehydration."

It is quite evident that as yet one cannot draw any *general* conclusion from the data here presented.

VI. THEORY OF DEATH BY VARIOUS PHYSIOLOGICAL, PHYSICAL OR CHEMICAL ALTERATIONS

Various physiological changes which accompany freezing have been assumed to cause injury and death. Though, in the last analysis, the mechanism of their lethal action might be physical or chemical, they will be treated here as physiological changes, as they were presented by their authors. The physical alterations, other than crystallization of water, which have been observed to result from freezing and thawing in aqueous colloids are: precipitation, agglomeration or dispersion of particles, coagulation, gelation, changes in transparency (turbidity, opalescence) and changes in consistency (cf. our previous review of this subject). Any of these physical alterations, as well as a number of chemical changes, might constitute the mechanism of injury and death by freezing. In the literature one finds such a general suggestion repeated again and again. But specific suggestions as to which protoplasmic constituents might be altered, and in what manner, are few and still fewer the experimental attempts to test these possibilities. We shall mention here the more specific suggestions and those which are backed by some experimental evidence.

1. *Physiological Changes.* A weakening of vitality by the action of cold on protoplasm has been assumed by Irmscher (1912) to explain how a prolonged or repeated exposure causes a gradually increasing damage. He had observed that some moss species which resisted one freezing at -20° were killed by 4 freezings at -15° and that moss which was not entirely killed by one freezing at -15° , this temperature being maintained for 18 hours, died entirely after an exposure of 4 days to -10° . (Similar experiments were reported by numerous other authors, in particular by Goeppert, 1830, on *Lamium* and by Apelt, 1907, on potato).

Schaffnit and Lüdtké (1932), who studied the influence of low temperature on the metabolism of plants subjected to various diets, came also to the conclusion that before death there is evidence of a gradual weakening under the action of cold. Death would then be due, finally, to disturbed metabolism.

To assume that low temperature weakens protoplasm and renders it more vulnerable to cold is in direct opposition with the observed fact that low temperature hardens against cold.

According to Krebs (1931), the muscles would lose their ability to synthesize glycogen and phosphagen after being frozen. Death would then occur when the energy demands exceed the supply.

Cold injury might be related to physiological changes which take place at some particular temperature. Moran (1925) cites, as an example of a change of the kind which might be invoked here, the observation of Vernon (*Jl. of Physiol.*, 17, 277, 1894) and of Castle (*Jl. Gen. Physiol.*, 7, 189, 1924) that respiration in the frog and in other animals and plants undergoes a critical change at around $+15^{\circ}$. It is possible that, in a similar manner, some unknown activity, perhaps some enzymatic process, is critically altered at the temperature at which

the water phase is separated by freezing from the protoplasmic substance.

Some authors, after Klemm (1895), have observed that freezing causes a granulation of protoplasm. The mechanism of this alteration, which is probably physiological in nature, is unknown.

Freezing might be more injurious to some vital activities than to others and destroy one particular function before it destroys them all at death. This assumption would explain the observation of Pictet (1893) that a wound made by freezing takes a much longer time to heal than a wound inflicted by other injurious agents. The subepidermal tissues would have lost their power of *regenerating* the dead portion.

2. *Physical and Chemical Changes.* Most of the physical changes assumed to take place at freezing temperatures are phase separations. The most important phase separation is evidently that of water; but other phases might separate either directly when a saturation temperature is reached or indirectly as a result of dehydration.

Gorke (1907) assumed that the proteins dissolved in the cell sap of plants might precipitate at death, such a precipitation being due to the gradually increasing concentration of the sap subsequent to the withdrawal of water by freezing. He attempted an experimental study of his theory by determining the amount of precipitable protein in the sap extracted from previously frozen and from unfrozen plants. The proteins were salted out and the nitrogen content of the precipitate was determined by the Kjeldahl method. The sap from frozen material contained only $\frac{2}{3}$ the quantity of nitrogen found in the sap from living plants. Unfortunately Gorke's procedure is subject to criticism (as Voigtländer, 1909, pointed out) in the fact that he extracted the sap by a slight pressure in frozen tissue, in which the fluid came out readily, while he crushed the living tissue; this difference in treatment might well account for the difference

in protein content observed. But this criticism does not apply to a second group of experiments in which Gorke measured the amount of protein which precipitated in the freezing of previously extruded sap. He found a noticeable quantity of nitrogen in the precipitate of the frozen specimens. From these and other experiments in which it was found that the precipitation of proteins in plants of varying cold resistance (for example, mustard leaves and spruce needles) requires either a different low temperature (-3° and -40° respectively) or a different salt concentration (2.7 N and over 5.4 N NaCl respectively), Gorke concludes that the specificity observed in the behavior of plants exposed to cold depends on the ease of precipitation of the proteins.

Nord (1934) remarks that if death is characterized by the transition from a clear, homogeneous protoplasm to a coarse, microscopically heterogeneous material, it is natural to think that such a transformation corresponds to the freezing point of some aqueous solutions and to the coagulation point of some proteins. Since his investigations on the action of freezing on various suspensions, colloidal solutions, and enzymes have resulted in the conclusion that the size of the colloid particles is modified by crystallization of water, he correlates death with changes of this kind.

Mirsky (1937a and b), who observed that extracted myosin coagulates by freezing or drying and that myosin within the muscle coagulates on being rehydrated after having been dried, postulates that death by freezing, in the muscle, results from a coagulation or aggregation of the myosin molecules caused by some change during freezing or during the reimbibition of water.

Harvey (1918) found a higher hydrogen ion concentration in the sap of frozen than of unfrozen plants. Whether this ionic dissociation is an immediate effect of freezing or whether it results from various intermediate transformations, it is impossible to say.

A liberation of ions on freezing has also been reported by Dexter, Tottingham and Graber (1930) who observed a higher electric conductivity and a higher electrolyte content in frozen than in unfrozen plant sap.

Fischer (1911) suggests that one of the modifications caused by freezing in colloids might be a loss of their power to absorb water. He attributes to this change the difference observed by some biologists in the quantity of heat liberated during the freezing of a tissue and the quantity of heat reabsorbed on thawing.

Becquerel (1937, 1938, 1939), considering the resemblance between a tissue killed by freezing and no longer capable of holding its water and a gel which loses its water when its structure breaks down (syneresis), proposes the theory that death is a structural disorganization identical with that occurring in such gels. He observed in cells of the epidermis of the onion, immersed in liquid air and thawed, a decrease of the volume of the nuclei which could be interpreted as comparable with the contraction of gels which lose their water by syneresis. Becquerel then pictures death as follows: During freezing (he speaks in particular of freezing in liquid air), syneretic phenomena take place which consist in a separation of water from the rest of the colloidal system and in an irreversible chemical change of the colloidal particles; on thawing, the particles so modified aggregate into larger masses and form a coagulum. The fluid extruded after thawing consists of the syneretic water and of the cell sap which easily traverse the now disrupted protoplasm.

Concerning the water-holding properties of living tissues, it is interesting to compare them with those of aqueous gels. Though these two systems might be quite similar in many respects, they differ, among other features, in their behaviour on freezing. As Jones and Gortner (1932) have pointed out, when one freezes (rapid freezing) a gelatin gel several times in succession, the

same amount of water crystallizes each time, at the same initial freezing point. This shows that no irreversible alteration takes place from one freezing to the other. In the case of living tissues, on the contrary, the freezing point is raised by a first lethal freezing and the quantity of water which crystallizes at a given temperature, in a second congelation, is different from the quantity solidified in the first.

Several authors have assumed that, at death, proteins are denatured in a salt solution which became concentrated by freezing. Concerning this theory, as applied to muscle proteins, Moran (1929) remarks that all attempts to produce synthetically a solution of the same composition as the fluid of the muscle have failed and he attributes this failure to the fact that the calcium is not simply in solution in the living muscle but that it is associated with the proteins. This remark suggests the hypothesis that the destruction of the complex, protein-calcium, might constitute death by cold.

SUMMARY

1. Death in frozen animals or plants has been attributed by some pioneer authors to a withdrawal of energy and the resulting impossibility on the part of the cooled organism to supply the energy necessary for vital activities. 2. According to this theory freezing is impossible as long as the organism is alive; life has to be destroyed to allow the formation of ice.

3. Other investigators have attributed death of frozen plants to the attainment of a given minimal temperature, characteristic of each species. 4. Accordingly, when this "specific minimal temperature" is below the freezing point, the formation of ice protects against injury and death, while subcooling is dangerous.

5. Death has been attributed to a mechanical injury consisting in a bursting of the cells by the expansion of the ice in formation; but the rupturing of cells after slow

freezing in nature could never be observed. 6. A mechanical injury resulting in the destruction of the fine structure of protoplasm by ice crystals has been invoked as the cause of death. 7. Some investigators have thought of a damage by pressure between growing ice masses. 8. Others have proposed that the protoplasm might be injured by the jarring involved in freezing and thawing.

9. A large number of investigators have studied experimentally the theory that the so-called death by freezing is caused by a too rapid thawing. No general conclusion can be derived from their results which seem largely to disagree.

10. Most plant physiologists have attributed death by freezing to a dehydration of protoplasm. Some consider dehydration as a step toward a precipitation or a coagulation; others think of a destruction of the structure of living molecules, perhaps of catalysts, by the withdrawal of their constituent water; for others dehydration might cause such essential changes as an increase in permeability, an increase in viscosity, an upsetting of the ionic balance, etc.; finally, some authors attribute death to the toxic concentration resulting from dehydration. 11. Arguments in favor of the dehydration theory of freezing are: the resemblance, repeatedly recognized, between the action of freezing and that of drying and the resemblance in the conditions of sensitivity to these two factors. 12. The main objections against the theory are: that in some plants more water can be removed without injury by other means than by freezing; that dehydration by freezing is assumed to be lethal, while dehydration by air-drying increases the resistance to cold.

13. Various physiological changes which accompany freezing have been assumed to cause injury: a weakening of vitality by cold, an impairment of the synthesizing ability, an impairment of functions such as regeneration, etc. 14. Among the physical or chemical changes which might be responsible for death by freezing the most im-

portant mentioned in the literature are: a precipitation, a denaturation, or a coagulation of proteins, an ionic disso- ciation, a loss of adsorptive or water-binding properties, a synergetic release of water, the destruction of some com- plex of the type protein-calcium.

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GENERAL BIBLIOGRAPHY OF THE LITERATURE ON THE PRESERVATION AND THE DESTRUCTION OF LIFE AT LOW TEMPERATURES*

(In Chronological Order)

1736

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1776

SPALLANZANI, L., Opuscoli di fisica animale e vegetabile. Modena; (Experiments on the action of low temperatures in Opusc. 1, Chapters V and VI).

1778

HUNTER, J., Of the Heat of Animals and Vegetables. *Philos. Trans.*, 68, 7-49.

*This bibliography is not a list of the references utilized in the present monograph—such a list has been printed, in sections, at the end of each Part—it is intended to be a compilation, as complete as possible, of the researches published up to 1940 in the field of "The Preservation and the Destruction of Life at Low Temperatures." The expression "complete bibliography" has become, at present, practically meaningless for a large number of topics; we present, therefore, this bibliographical list as a nucleus of the essential references, which is to be completed in future editions. The elaboration of monographic bibliographies, and the organization of some means of providing a periodical republication and completion of such works is probably to become an important task for the next generation of scientists (perhaps as important as the organization of research laboratories has been during the first half of this century). To emphasize this particular character of the present bibliography, and also to show the historical development of the research on the subject, we arranged the references in the chronological order.

1817

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1838

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Among subjects which have been deliberately omitted, though they concern rather directly our topic, we shall mention: *cold hardiness in plants, preservation of food by refrigeration* (and, as such, *cold injury to edible plants, fruit, etc.*), *physiological effect of cold on germination, growth, morphogenesis, habitat, etc.* (and, as such, *snow vegetation, arctic vegetation, etc.*), *properties of physical systems at low temperatures* (the properties of colloids, however, are included). These subjects either have been investigated enough to constitute separate units, or the accepted division of the sciences has assigned them to specialists in other branches, or they have already been or are reviewed by competent authors whose work it would be useless to duplicate.

When the title of a paper does not indicate what relation its content bears to low temperature, we added a note in parentheses to that effect.

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