On the Possible Biological Significance of Some Physical Changes Encountered in the Cooling and the Rewarming of Aqueous Solutions

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Abstract

After a review of the evidence for structural changes occurring in the course of the cooling or the rewarming of aqueous solutions, between 0 and −150°C, when the temperature crosses ranges of molecular instability, the possible factors of injury to biological material and the conditions under which injury might occur are examined and discussed. The ranges considered—selected because they did not receive much attention from the cryobiologists as factors of injury—are: (1) the range of the *glass transition*, in the case of vitrifiable material (even if vitrification is only partial); (2) the range of *maximal nucleation rate*; (3) the range of *maximal rate of crystal growth*; the combined action of these two rates may affect considerably the degree of injury; furthermore, the partly amorphous material may become unstable and crystallize, or recrystallize, in those two ranges, during rewarming; (4) the range of *recrystallization*. Four types and a few subtypes of recrystallization are described and illustrated; and their possibly injurious action is examined.

The paper refers to a research program now in progress, which may be described briefly as consisting in: (a) establishing a sort of "phase diagram" (as drawn in Fig. 16) that will give the temperature ranges enumerated (in addition to the melting point), for various concentrations of various solutes; (b) establishing similar diagrams for some selected biological materials; (c) determining the injurious action of the changes occurring at these temperatures.

Introduction

The bibliographical lists of cryobiological investigations contain quite a large number of papers dealing with the injurious action of the formation of ice in tissues and in cell suspensions and a few papers on the injurious action of melting. It seems that these two phenomena have held all the attention of cryobiologists and that other physical changes—some of them quite important, like recrystallization—have been overlooked. The purpose of the present paper is to call to the cryobiologists' attention a few such changes which take place either during the cooling or during the rewarming of solutions, and to discuss the extent to which these changes may be of significance in causing injury to biological material.

In a recent analysis, Luyet (1966) examined "indications for the existence, in the range of temperatures extending from the absolute zero to the melting point, of regions of structural instability and increased molecular mobility" in which phase transitions, like melting, may take place, or other changes, like the passage of the rate of crystal growth to a maximum at some point on gradually decreasing temperatures. (Changes of the latter type represent departures from the regular decreasing rates that one would expect to obtain, during cooling, in processes controlled principally by temperature. Such

departures correspond to discontinuities in the course of molecular stability.) The "cases of instability" defined in the paper just cited are precisely the items of which the biological significance is to be discussed here. Their physical aspects will be examined in an introductory part of the paper.

In order to have general conditions and not to limit the study to particular cases, I shall consider the changes encountered both during the cooling and the rewarming of solutions, when these two processes take place at various rates, and when the solidified material is either crystalline or amorphous, or contains fractions in each of the two states.

Some of the data to be reported represent the latest developments in my attempts, during the last thirty years, at establishing the physical basis of the biological effects of freezing.

I. Physical Aspects of the Changes under Study

These introductory considerations contain in a condensed form the information given in Luyet's paper (1966) to which the reader is referred for more details. They also contain some complementary information obtained in experimental tests just completed.

I shall first present a list of the changes to be considered, and then examine the physical characteristics of those changes which are of particular interest for this study.

(A) *List of changes to be surveyed*

Three particular changes, indicative of temperature-dependent variations in molecular stability, are encountered when the temperature of solutions decreases from the freezing point to the absolute zero: (1) nucleation (passage of its rate to a maximum at a particular temperature), (2) crystal growth (passage of its rate to a maximum at another temperature), and (3) in the case a part of the material has remained amorphous, the glass transition. Similarly, three main changes are encountered when the temperature of a solidified solution is raised: (1) the glass transition, (2) the resumption of a crystallization which may have been interrupted during a previous cooling, and (3) the recrystallization of material which has undergone a previous crystallization. (Melting is not included in this program which is limited to changes generally overlooked by the cryobiologists.)

Some of the changes named in this list occur in various ways or on different substrates; the resulting variations should be classified as subtypes. Thus, not only water in a solution may undergo nucleation and crystallization, but also the solutes and the eutectic mixtures of solvent and solutes.

Recrystallization appears in various forms which have been classified into four categories: (a) irruptive, (b) slow-pace, (c) premelting and (d) reorientative. The first category itself has been subdivided into: (a′) induced or spontaneous recrystallization and (a″) recrystallization of rapidly or of slowly grown units.

(B) *Characteristic features of the changes to be surveyed. Introductory note on the classification of crystallization units*

Some of the changes to be examined affect a particular type of the crystallization units present in a system; a glance at the types most commonly encountered should then precede the examination of the changes.

One may have: (1) fairly well-formed crystals which consist of six axial columns developing around a center of crystallization and branching in a more or less regular manner (Fig. 1-1); these are formed when the rate of crystallization is very low; (2) at higher rates, the units lose their regularity; they assume various forms, such as that reproduced in Fig. 1-2, which are classified in the category "irregular dendrites"; (3) at still higher freezing rates, the number of ice spears developing from the center of crystallization is enormous, and there seems to be no branching; the formation is of the type "spherulite" (Fig. 1-3); when the rate of cooling reaches values of the order of several hundred degrees per second, the radii of the spherulites become invisible, and the units which are transparent in ordinary light (Fig. 1-4) are designated as "evanescent

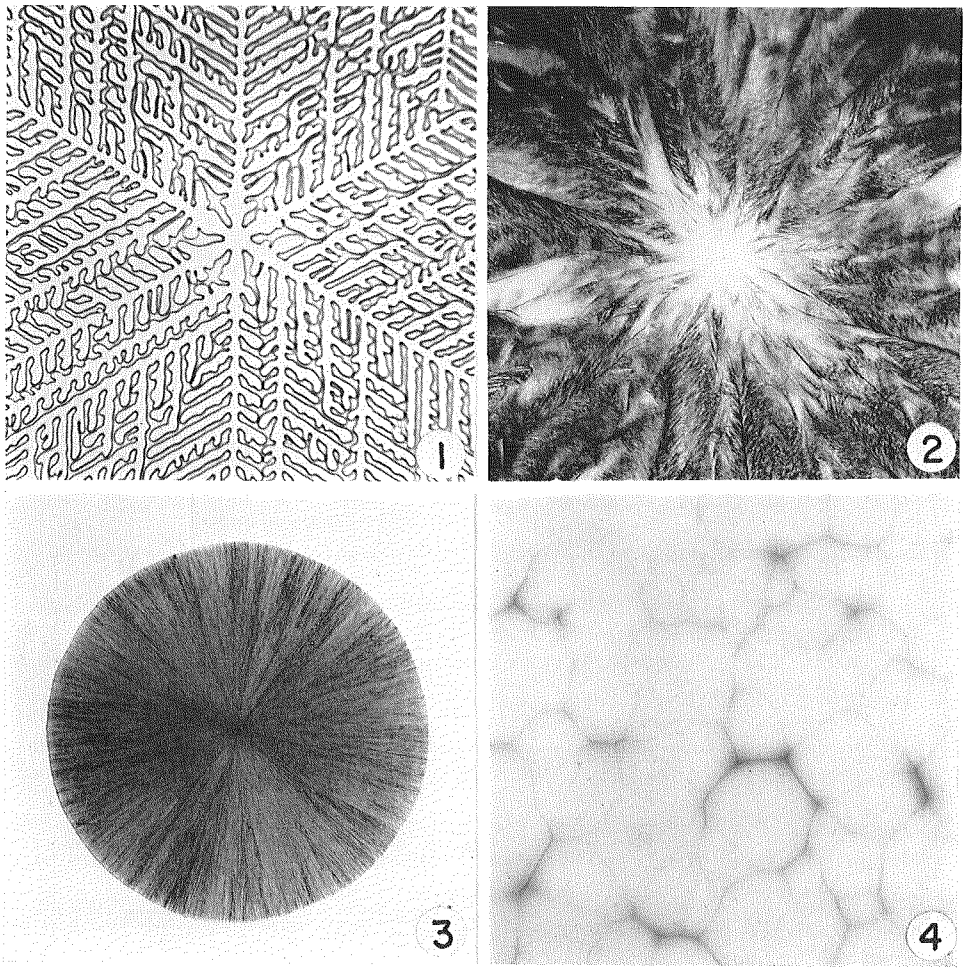

Fig. 1. The main types of ice formations developed in aqueous solutions. *Type, solute, conc.* and *temp.*: (1) hexagonal form, 35% albumin, formation started at −2°C and developed at −3°C; (2) irregular dendrite, 10% sucrose, −40°C; (3) spherulite, 6 M glycerol, −60°C; (4) evanescent spherulites, 40% gelatin, −60°C. *Thickness of preparations*: (1)-(3) approximately 20 μ; (4) 50 μ. ×95. (From Rapatz and Luyet's files)

spherulites". Viewing them in polarized light leaves no doubt about their crystalline nature.

I shall now examine the five changes named in the list above (under A) in the order of their historical development and recognition.

1 & 2. NUCLEATION AND CRYSTAL GROWTH

At the beginning of this century Tammann (1903) reported, on the basis of numerous experimental determinations, that the curves representing the rate of nucleation and the rate of crystal growth in terms of temperature have maxima which are often quite far apart on the temperature scale and sometimes far below the melting point. For example, he found that piperine, which melts at 129°C, has a maximal density of nuclei at 40°C (Fig. 2–A), and reaches a high growth rate at 100°C. To illustrate the trend in growth

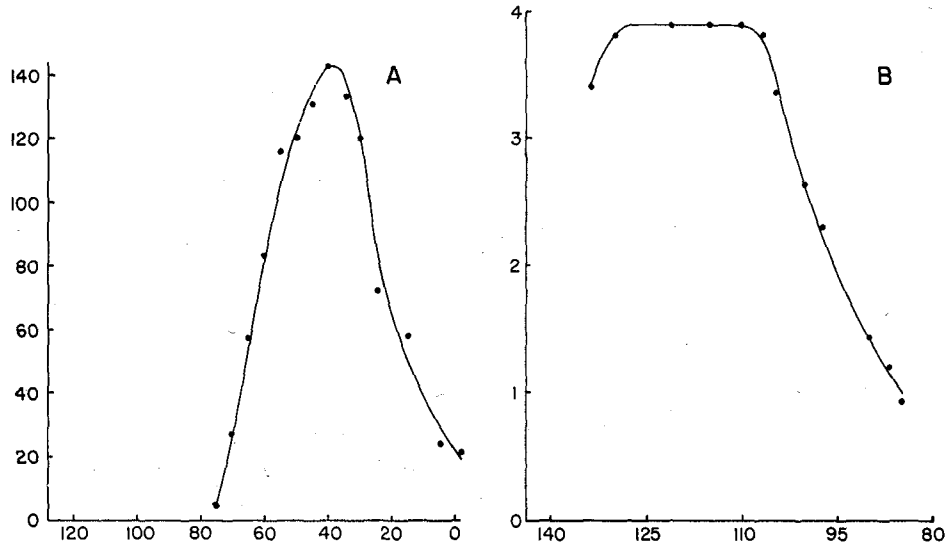

Fig. 2. A: Number of crystallization units (grains) formed in a given volume of piperine exposed for 10 minutes to the temperature given in abscissa and for 4 minutes to 100°C. The melting point is at the origin of the abscissa (drawn from Tammann's data, 1925)

B: Linear crystallization velocity in mm per minute in triphenylguanidine at the temperatures indicated in abscissa. The melting point is at the origin of the abscissa (drawn from Lautz's data, 1913)

rate at decreasing temperatures below the melting point, I reproduce in Fig. 2–B, a curve obtained by Lautz (1913) and cited by Tammann in his book "The States of Aggregation" (1925).

A number of papers have been published on the same subject during the last sixty years or so. Recent reviews (such as Van Hook's, 1961) contain tables of factual data on rates of nucleation and of crystal growth, and on factors controlling those rates, as well as discussions of the theoretical background. Tammann's views have been criticized but the fundamental facts established by him on nucleation and growth rate curves with maxima at different temperatures were confirmed. The occurrence of these changes indicate stages of molecular instability which fall directly in the program of this paper.

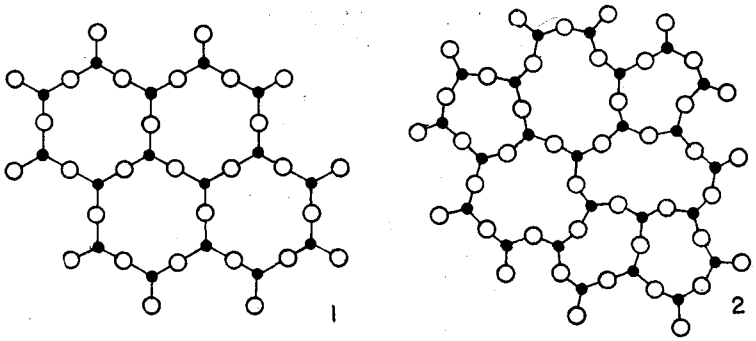

Fig. 3. Diagrams representing, in a plane, the difference in structure between crystalline and vitreous silica (diagrams 1 and 2, respectively) (from Zachariasen, 1932)

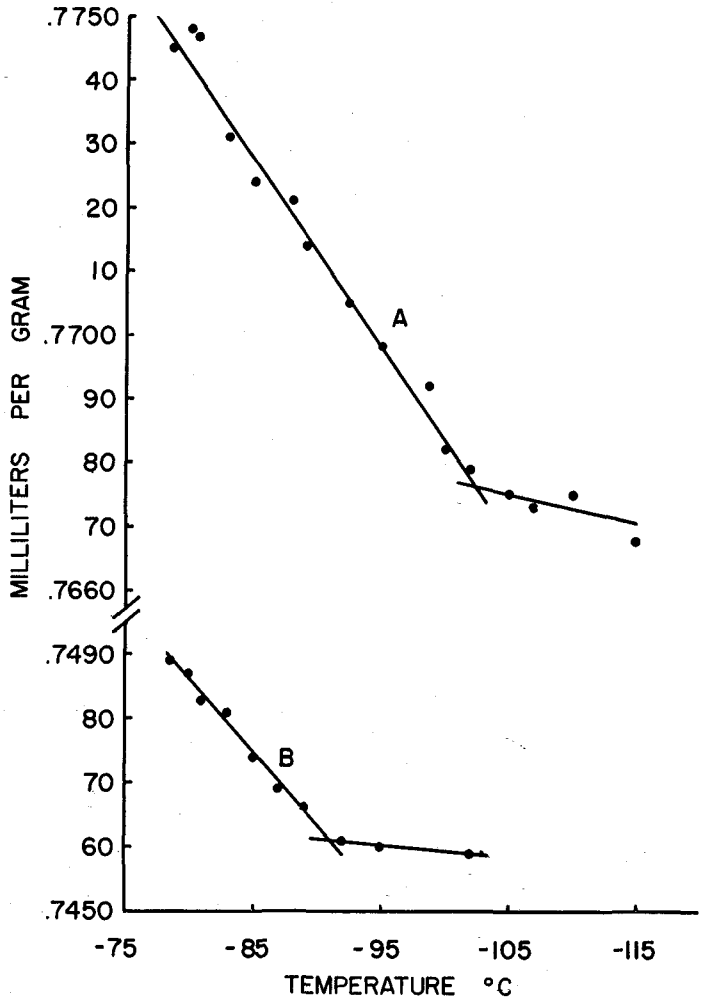

Fig. 4. Specific volume of an 80% glycerol solution (Curve A), and of a 97.3% solution (Curve B), cooled to the temperature indicated in abscissa (from Kroener and Luyet, 1966; with permission of Biodynamica)

3. THE GLASS TRANSITION

The molecules of a liquid occupy random average positions with reference to one another. In their state of continuous agitation, they exhibit three sorts of motion: translational, rotational and vibrational. Since temperature is, by definition, the rate of motion of the molecules, a lowering of temperature means a lowering of activity. When the molecules, which, during cooling, move gradually closer to each other, are practically in contact, their translational motion becomes impossible; they form a sort of rigid framework where they are still permitted vibrational motion but no side-wise displacement. Their situation is comparable to that encountered in the crystalline state, in which translational motion has also ceased and the molecules form a rigid framework, the crystal lattice; but the "liquid" so solidified differs from the crystal in that its constituent particles are in a state of disorder, while in the crystal they are orderly arranged. Diagrams 1 and 2 of Fig. 3 represent, in a plane, a diagram of the two structures, as pictured in a similar case by Zachariasen (1932).

In the glass transition, that is, in the passage from the state still permitting translational motion to the state where such motion is inhibited, there should occur an abrupt change in expansion coefficient. Figure 4 shows the change actually measured by Kroener and Luyet (1966) in a dilatometric study of a glycerol solution undergoing the glass transition.

The physical behavior of the two states, above and below the glass transition, is so marked that the students of the vitreous state insist on using two different terms for

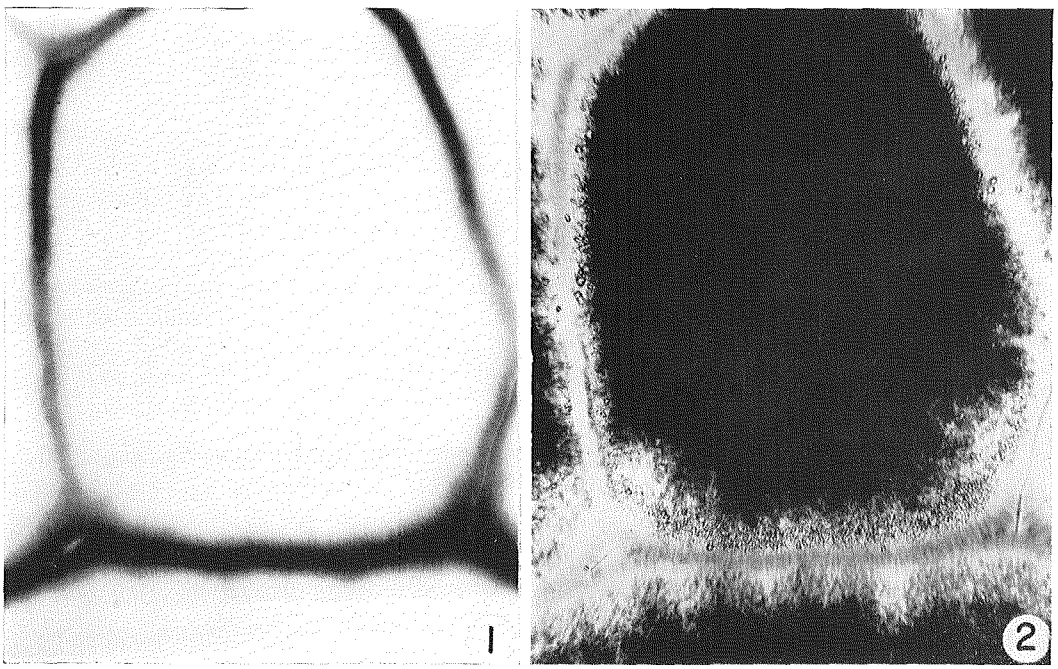

Fig. 5. Recrystallization of ice in a 35% bovine albumin solution. Preparation frozen at −30°C (1), and recrystallized at −7°C (2). *Thickness of preparation*: 20 μ. ×95. (From Rapatz and Luyet's files)

them: they call "supercooled" the material above the glass-transition temperature, and "vitreous" the material below that temperature.

4. RECRYSTALLIZATION

(a) *Irruptive recrystallization.* When a thin layer of solution has been cooled rapidly (at a rate of a hundred degrees per second or more), its water crystallizes into transparent spherulites (Figs. 1-4 and 5-1). When the preparation is rewarmed to a given temperature, which is characteristic of the solute—for example, −10°C for solutions of 30 to 45% gelatin, −31°C for 1-2 M solutions of sucrose—the specimen becomes suddenly opaque (Fig. 5-2). The opaque material contains ice particles which measure several micra.

(a′) *Spontaneous, irruptive recrystallization.* The spherulites of the transparent preparation shown in Figs. 5-1 and 6-1, have opaque borders. Apparently the latent heat of crystallization developed during freezing has raised locally the temperature to the recrystallization point. By using freezing baths at higher temperatures, one obtains larger opaque borders (Figs. 6-2 and -3). By using a freezing bath only 15 degrees below the recrystallization temperature one has a completely opaque preparation (Fig. 6-4). It seems that the phenomenon is entirely comparable to that reported under (a), except that the turning opaque of the specimen was there "induced" by rewarming, while here it occurs "spontaneously" upon freezing.

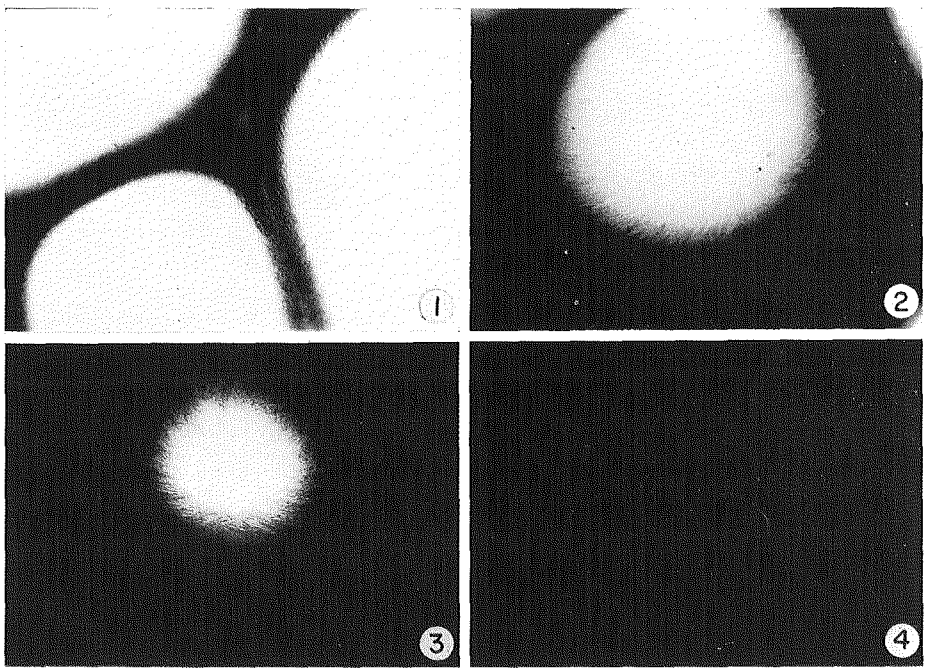

Fig. 6. Spontaneous irruptive recrystallization in a preparation 100 μ thick of a 30% gelatin gel. The recrystallized (opaque) areas are gradually larger when the temperature of the freezing bath is higher. *Freezing temp*: 1, −50°C; 2, −40°C; 3, −30°C; 4, −25°C. *Thickness*: 100 μ. ×70. (1, 2 and 3 from Luyet and Rapatz, 1958; with permission of Biodynamica)

(a″) *Irruptive recrystallization after slow freezing.* Figure 7-1 shows a rosette formed in slow freezing, which turned opaque (Fig. 7-2) when the temperature was raised to the same recrystallization temperature as the spherulites formed upon rapid freezing.

(b) *Slow-pace recrystallization.* The slow transformation from a fine-grain crystalline structure to a patchwork of large ice plates (Fig. 8) when the temperature of a layer

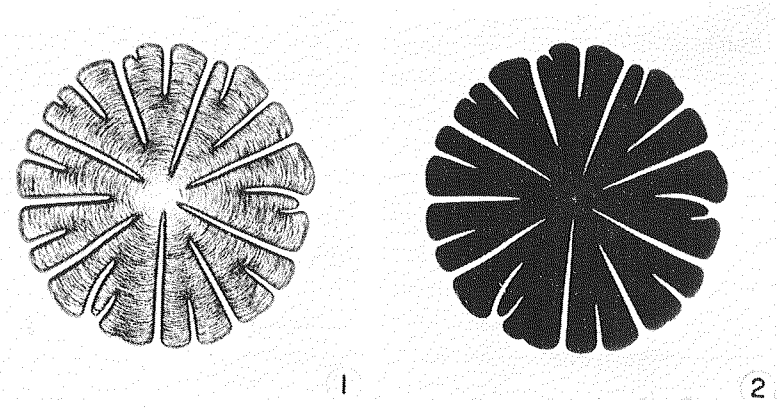

Fig. 7. Irruptive recrystallization in a slowly grown irregular dendrite (rosette). 1: rosette formed in 50% gelatin gel frozen at −30°C; 2: same rosette after the temperature has been raised to −8°C. ×95. (From Rapatz and Luyet's files)

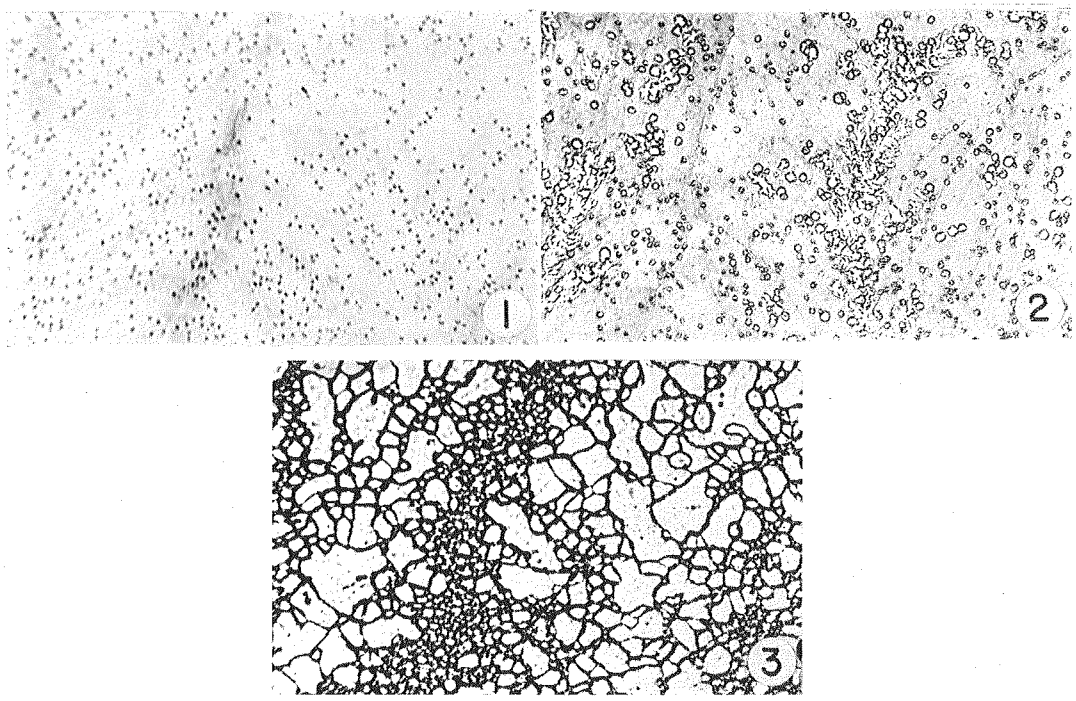

Fig. 8. "Slow-pace" recrystallization in a layer of intracellular fluid of frog's red cells (mostly hemoglobin). 1: preparation frozen at −40°C; 2 and 3: same field after the temperature has been raised to −6°C (2) and to −3°C (3) and held at each temperature for 5 minutes. ×100. (From Rapatz and Luyet, 1960; with permission of Biodynamica)

of plasma frozen at −20°C was raised gradually to −3°C will serve to illustrate this form of recrystallization.

(c) *Premelting recrystallization.* When a preparation is held near the melting point, one may observe a rapid growth of large ice particles at the expense of the small ones, which are seen to melt away immediately before the melting of the large ones. This phenomenon, designated as premelting recrystallization, is shown in Fig. 9–1 to 9–4 which are excerpted from time-lapse motion pictures (Luyet, Gehenio and Sager, 1966).

A recent work by Amrhein and Luyet (1966) suggests that an incipient melting may take place at the recrystallization temperature.

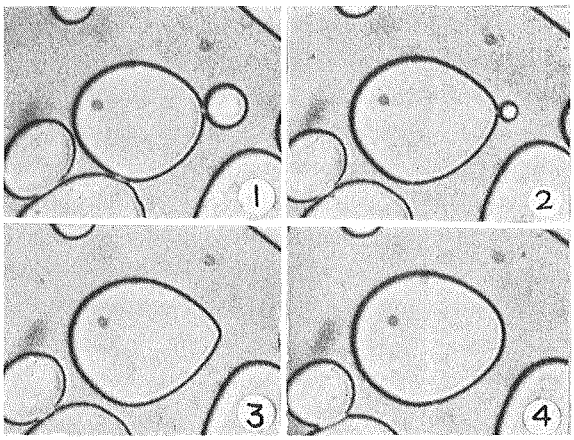

Fig. 9. "Pre-melting" recrystallization in a layer of 10% albumin solution. Sequence of photographs excerpted from a motion picture film showing the decrease in size and disappearance of small ice particles, and the simultaneous growth of larger ones which are seen to bulge out in the proximity of the disappearing particles. *Temperature*: close to the melting point. ×440. (From a paper in press by Luyet, Gehenio and Sager; with permission of Biodynamica)

(d) *Reorientative recrystallization.* If the reports according to which, in some aqueous materials, under certain conditions, water freezes first into cubic ice and then, upon rising temperatures, becomes transformed into hexagonal ice are confirmed, the process would be a recrystallization involving a molecular reorientation. (The formation of cubic ice at one freezing temperature and of hexagonal ice at another, in the case of gelatin gels, has been questioned by Luyet *et al.*, 1963.)

5. RESUMPTION, UPON REWARMING, OF A CRYSTALLIZATION INTERRUPTED DURING COOLING

In all the cases just discussed (in Section 4) some crystallization had taken place during cooling and the processes reported upon rewarming can be considered as forms of recrystallization. In some instances in which a part of the material was amorphous and a part crystalline, and some of the amorphous part crystallized upon being rewarmed (as shown by the onset of opacity at the recrystallization temperature), we used the term recrystallization in its broad sense of resumption, upon warming, of a crystallization interrupted during a previous rapid cooling (see comments by Gehenio and Luyet, 1959,

p. 84, on that designation).

But the problem appeared in a new light recently when we discovered by differential thermal analysis (Luyet, Rasmussen and Kroener, 1966) that, in the case of glycerol solutions, the resumption, upon warming, of the crystallization interrupted during cooling occurs at a lower temperature than the recrystallization marked by the onset of opacity. This observation led to a distinction of two "cases of instability" at different temperatures: in one of them the stability would be reestablished by the resumption of an interrupted crystallization, evidently at the expense of the amorphous material, and it would involve a release of heat; in the other the stability would be reestablished by a recrystallization, that is, a growth of the larger ice particles at the expense of the small ones (formation of the recrystallization cloud) and this would not involve an appreciable release of latent heat. In some cases, the two processes might occur in the same range of temperatures.

Note. The foregoing considerations concern transformations (1) from one crystalline structure to another, or from one type of crystalline formation to another, or from one size of crystalline particles to another, (2) from amorphous to crystalline material when some crystallites or nuclei are present, ready to grow at the expense of the amorphous phase. The transformation of entirely amorphous, non-nucleated material into crystalline ice, which would be a true devitrification, is not mentioned. Whether entirely amorphous material not nucleated during cooling nucleates when it passes, upon being rewarmed, through the temperature range of maximal nucleation remains an open question. (For more details see Luyet, 1966.)

II. The Possible Injurious Effects of the Physical Changes Reported

In the following analysis of the possible injurious effects of the physical changes reported in the introductory part of this paper, I will examine these changes in the order of the position that they usually occupy on the scale of ascending temperatures, namely: (1) the glass transition, (2 and 3) nucleation and crystal growth, (3′) resumption of interrupted crystallization, treated as an appendix to No. 3, (4) recrystallization.

Since the changes to be examined are selected from the list of those which received little attention from investigators, the data from the literature are meager or nonexistent; this analysis will, therefore, consist mainly in pointing out the possible factors of injury in the changes in question, and in attempting to correlate their biological effects with their physical characteristics. The injurious effects of the two major phase transitions: freezing, as such, and melting, are not included in the program, although the possible injurious effects of the two component processes of freezing: nucleation and crystal growth, from the point of view of their variations in rate in different ranges of temperature, are an integral part of this study.

1. THE GLASS TRANSITION

(a) *General considerations: the main factors of injury.* Two features in the glass transition phenomenon appear as liable to cause injury: (1) The stresses resulting from the fact that, upon cooling, the material cannot continue to contract at its normal rate, because of a rigidified framework, may cause some disruption of biological structures. (2) The resumption of translational molecular motion, upon rewarming, may contribute

to render the system less stable; the increasing fluidity could favor some slow, still uncharted changes.

(b) *Stresses and cracks and their relationship to the glass transition.* In a study of the formation and disappearance of cracks during the cooling and the rewarming of glycerol solutions, we observed that cracking occurs mainly when, upon cooling, the temperature is below the glass transition point, and that the cracks disappear, upon warming, at temperatures above those of the glass transition (see Fig. 10). This behavior seems to be explainable by the changes in physical properties known to take place at the glass transition: the passage into a more rigid state upon cooling and the return to the more fluid state upon rewarming.

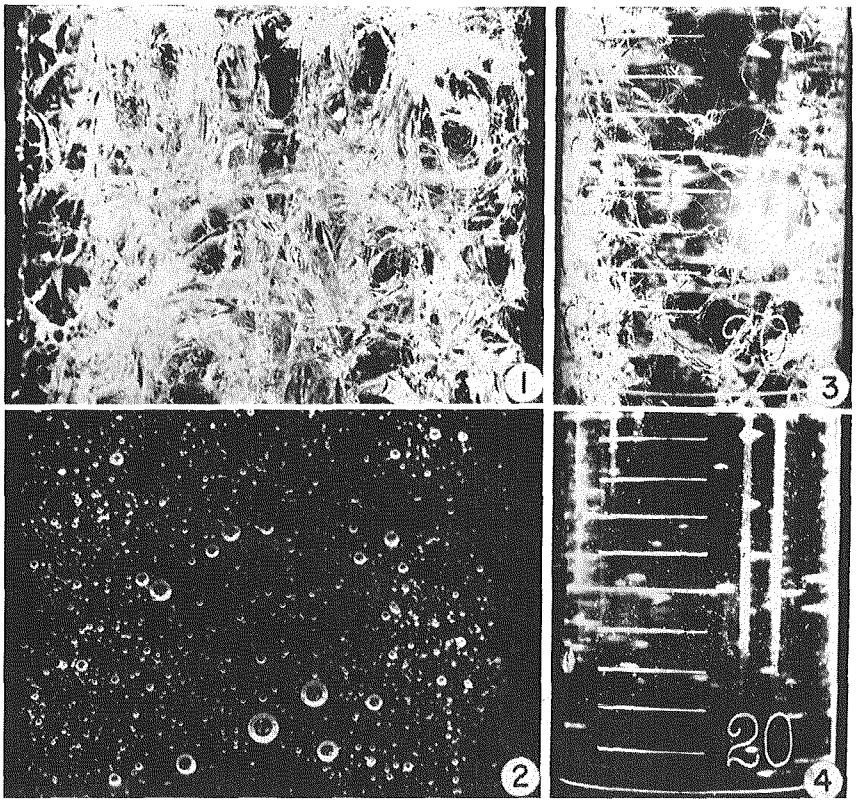

Fig. 10. Formation of cracks in a vitrifiable solution of glycerol (97.3%), cooled below the temperature of the glass transition, in a flat container (1), and in a cylindrical container (3); and disappearance of the cracks in the same samples when their temperature is raised above that of the glass transition (2 and 4). ×2.5. (From Kroener and Luyet, 1966; with permission of Biodynamica)

(c) *Possible injurious action of vitrification.* One may note that the concept according to which protoplasm vitrified by ultra-rapid cooling should not be injured, since the structure that it had in the liquid state is preserved, overlooks the possibly damaging changes in structure involved in the glass transition. It also ignores the possibility of damage caused by the occurrence of the events just described at temperatures below and above

the glass transition.

(d) *Molecular instability and the glass transition.* The idea has spread among cryobiologists that biological material cannot be safely preserved in the frozen state at temperatures higher than −130°C. The basis for that idea is not clear from the literature; in particular, it is not clear whether the assumed instability is attributed to the glass transition or to devitrification. According to Pryde and Jones (1952), whose work was authoritative at the time the notion developed, vitrified water would undergo the glass transition at temperatures estimated to be between −125 and −150°C and it would become crystalline at −129°C. According to the more recent data of McMillan and Los (1965) the two events take place at −135 and −124°C, respectively. But, no matter how the idea originated, and no matter whether the instability is related to the glass transition or to crystallization, the fact is that the temperatures of occurrence of the two phenomena are much higher in aqueous solutions than in pure water. This fact, in the case of the glass transition, is illustrated in Fig. 11.

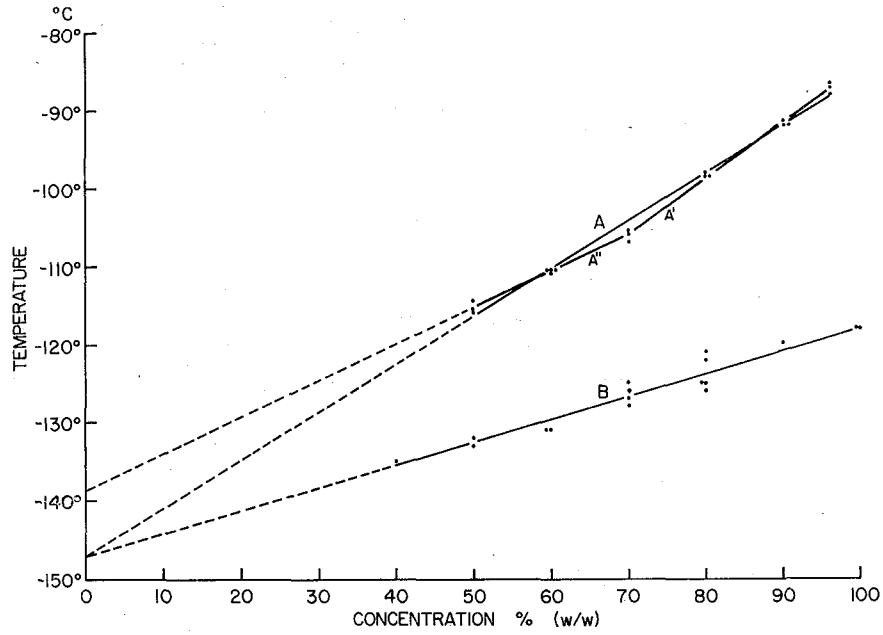

Fig. 11. Temperatures of the glass transition in solutions of glycerol (Curve A) and of ethylene glycol (Curve B), of the concentrations indicated in abscissa. (See text for comments on lines A′ and A″.) (From Luyet and Kroener, 1966; with the permission of Biodynamica)

(e) *Possible damage during freeze-drying of partly vitrified biological material.* When biological material is frozen rapidly enough to be partially in the amorphous state, the crystalline portion will sublime, while the amorphous portion will merely dry (pseudo freeze-drying) and, in doing so, it will shrink. The injurious effects of such pseudo freeze-drying and of its consequent shrinking remain to be investigated, and the question arises of whether the behavior of the specimen is different when it is pseudo freeze-dried while it is in the supercooled state, above the glass transition, or in the vitreous state,

below the glass transition.

2 & 3. NUCLEATION AND CRYSTAL GROWTH

(a) *General considerations.* To have an idea of the effects of the rate of nucleation on the structure of a frozen aqueous system one has only to compare preparations nucleated at temperatures where the rate of growth is high with preparations nucleated at lower temperatures; the former have only one or a few large crystallization units, the latter contain multitudes of tiny units. The observation of this striking difference has been the starting point of a research program, now in progress in our laboratory, in which (1) we are making systematic quantitative determinations of the rates of nucleation and of crystal growth in various solutions at various temperatures, particularly solutions of cryoprotective substances and of blood constituents and (2) we are studying the effects of the interrelated action of the two processes on biological material, particularly blood. To illustrate the type of information thus acquired, I reproduce in Fig. 12, the values obtained with a solution of 50% polyvinylpyrrolidone. The study of the dependence of

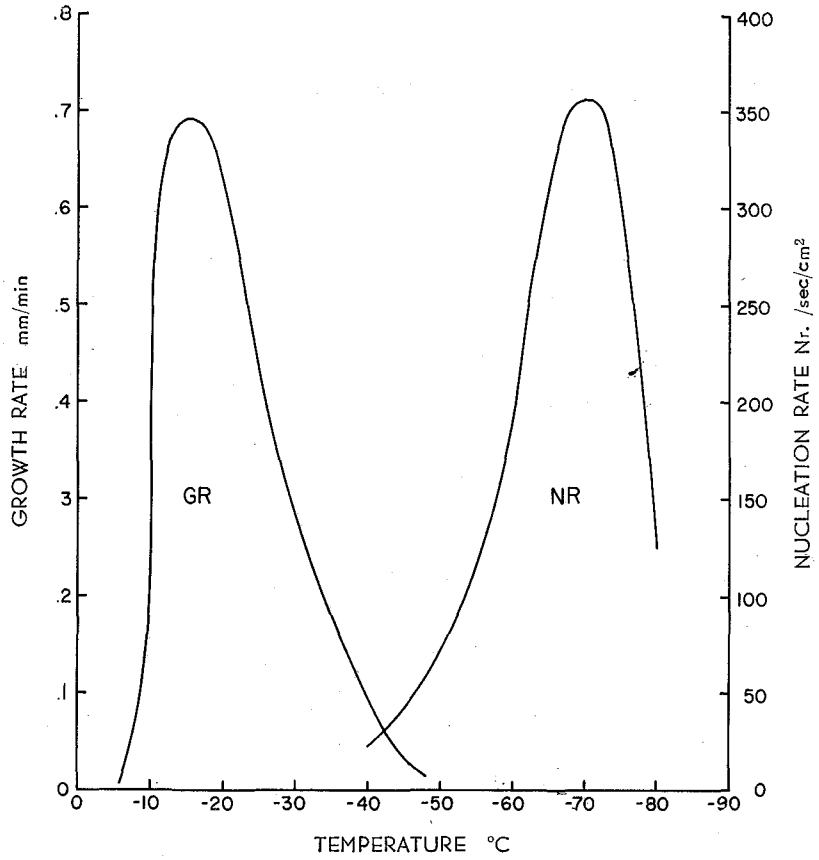

Fig. 12. Curves representing the rate of crystal growth GR (on left side) and the rate of nucleation NR (on right side), in terms of the freezing temperature, in a 50% solution of polyvinylpyrrolidone. Ordinate at left: radial growth rate; ordinate at right: nucleation rate (curves constructed from still unpublished data)

hemolysis on the interrelated action of nucleation and crystal growth is to follow.

(b) *Particular cases in which injury may depend on the rates of nucleation and crystal growth*

(1) Nucleation and composition of protoplasm. The rate of nucleation, under a given set of conditions, may be different for intracellular and extracellular fluids, or for cytoplasmic and nuclear contents. The latter possibility is suggested by the electron micrograph reproduced in Fig. 13, which represents a freeze-dried frog erythrocyte. The

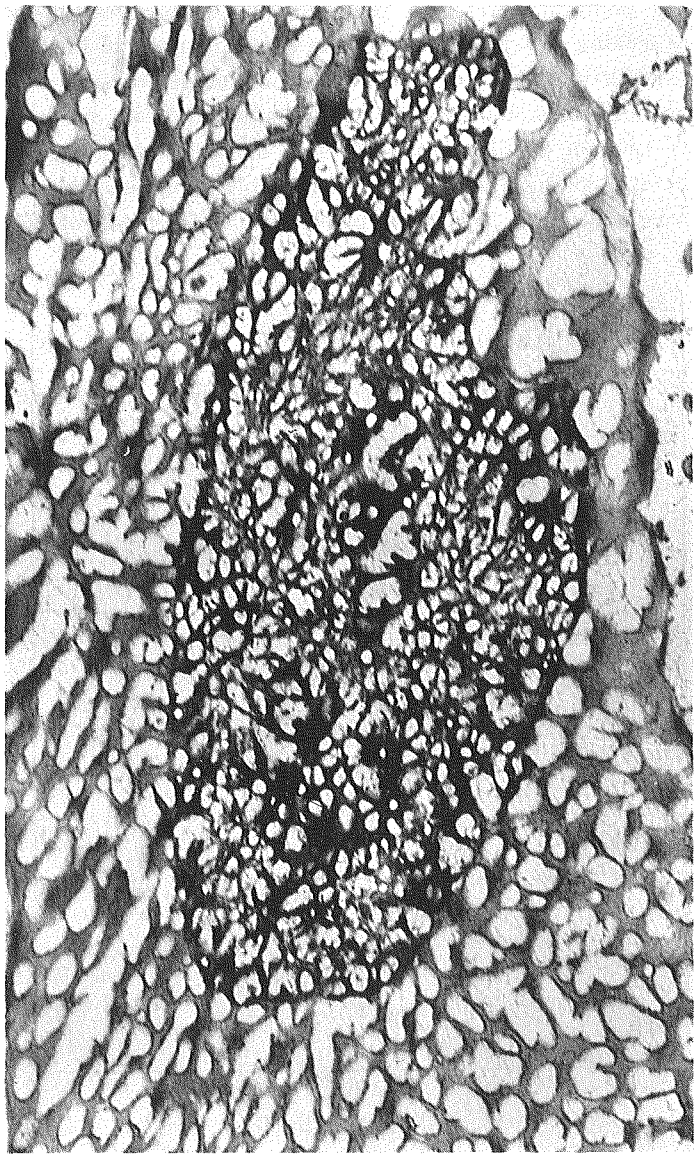

Fig. 13. Electron micrograph of a section through an erythrocyte in rapidly frozen frog blood. *Freezing temp.*: −30°C; *Freeze-drying temp.*: −60°C; *Thickness of prep.*: about 10 μ; *Mode of mounting*: between sheets of aluminum foil. ×19,000. (From Rapatz and Luyet's files)

"ice cavities", that is, the cavities left over after sublimation of the ice (the blank spaces) are of different sizes in the nucleus and the cytoplasm. The possible biological implications of this feature are obvious; it may control the formation of intracellular or intranuclear ice.

(2) Nucleation and solute concentration. It was observed that generally the nucleation rate is gradually higher, up to a certain value, at increasing solute concentrations. This observation raises the question of whether extracellular freezing, by increasing the concentration in and around the cells, may facilitate circumcellular or intracellular nucleation.

(3) Nucleation and cryoprotective action. One of the effects of cryoprotective substances might be to displace the temperature ranges in which the nucleation rate and the crystal growth rate have their maxima, or to modify these rates. We are now testing that possibility in solutions of various cryoprotective agents, as was already said, and in biological fluids containing various concentrations of such agents.

3′. RESUMPTION, UPON REWARMING, OF AN INTERRUPTED CRYSTALLIZATION

The observation that in a rapidly cooled glycerol solution a heat-liberating crystallization takes place, upon rewarming, at a temperature quite far below that at which the preparation turns opaque (recrystallization temperature) suggests that the temperature of resumption of the interrupted crystallization may be the same as that of maximal crystal growth rate. Three questions confront the cryobiologists in relation to this problem: (1) Do the two phenomena (resumption, upon rewarming, of an interrupted crystallization, and recrystallization) coincide or not in biological materials? (2) If they do not coincide, does the resumption of crystallization take place at the temperature of maximal crystal growth? (3) What are, in fact, the injurious effects of the molecular instability possibly involved in these phenomena?

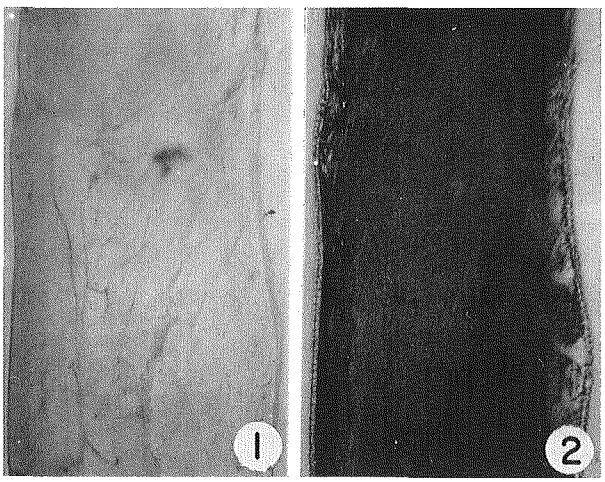

Fig. 14. Recrystallization in muscle fiber. 1: muscle fiber cooled rapidly by immersion in an isopentane bath at −150°C; 2: the same fiber after having been rewarmed to −10°C. ×450. (From Rapatz and Luyet, 1959; with permission of Biodynamica)

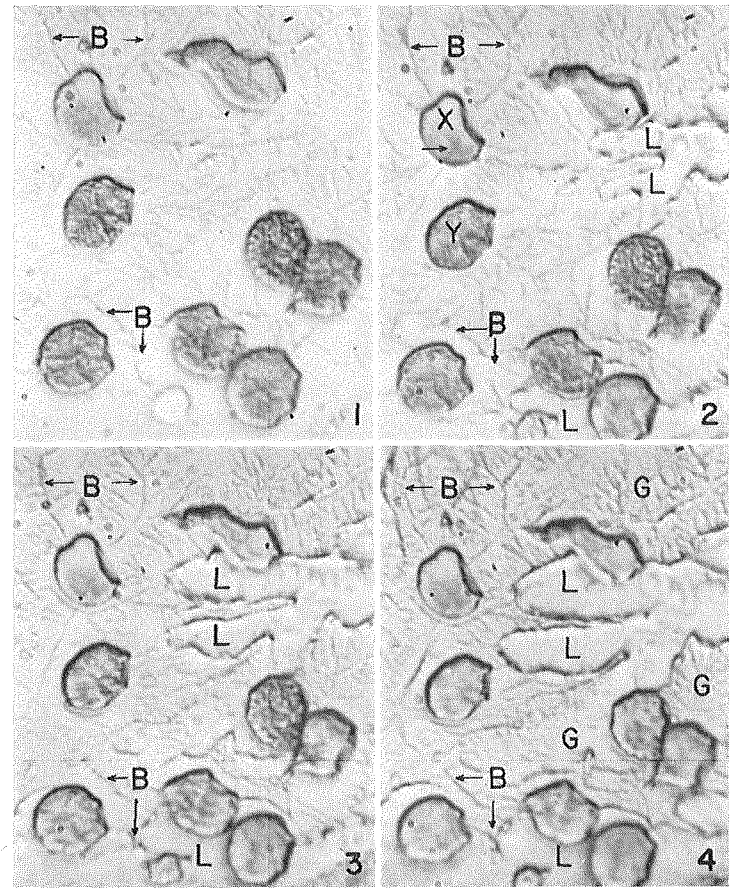

Fig. 15 A. Changes observed when a film of blood, mounted between plastic cover slips and frozen at −20°C (1 of Fig. 15 A), is gradually warmed to about −16, −14 and −12°C (2, 3 and 4 of Fig. 15 A, respectively), and to −10, −9, −7 and −6°C (1 to 4 of Fig. 15 B, respectively). B: borderlines between arborescent units in frozen plasma; C: channels of concentrated unfrozen plasma; G: granular, incompletely recrystallized areas; L: lobes of recrystallized ice; P: pools of melted fluid; X: thin layer of ice apparently extending from the plasma over the cells. ×1,190. (From Luyet and Pribor, 1965; with permission of Biodynamica)

4. RECRYSTALLIZATION

(a) *Irruptive recrystallization*

(1) General considerations. One may expect that the striking changes which take place during irruptive recrystallization and are marked by the development of the "recrystallization clouds" (the specimens becoming intensely opaque) exert a devastating action on the microstructure of biological material and be highly injurious. The ice particles pass from submicroscopic to microscopic dimensions, that is, they grow in a range of dimensions which are of the same order as several microsomic constituents of living matter; one would expect considerable interaction between the microsomes and the growing ice particles.

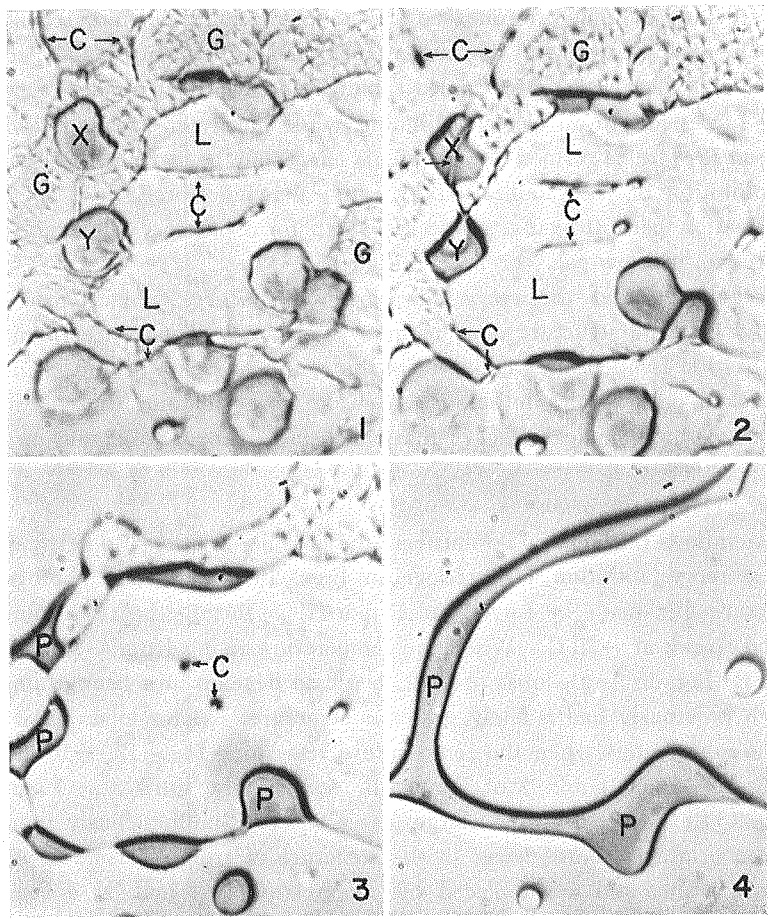

Fig. 15 B. ×1,250. See Fig. 15 A for explanation

The question arises of how frequently irruptive recrystallization, which is observed typically after very rapid freezing, has a chance to occur outside of special laboratory experiments. One should note, in this regard, that it occurs also in slowly frozen material, though it may not be as readily detectable there. Its occurrence in biological systems, frozen in nature or in the laboratory, may thus not be negligible. Furthermore, one should not overlook the observation that not only the type of crystallization units which turn opaque recrystallize at the temperature of irruptive recrystallization, but also the type which undergo the so-called slow-pace recrystallization. Thus, the passage through that temperature may represent a dangerous step in the rewarming of all living matter.

(2) Observations on blood and muscle and on their components. Rapatz and Luyet (1959, 1960) have demonstrated the phenomenon of irruptive recrystallization in the rewarming of rapidly frozen blood plasma and muscle juice They also made similar observations on entire muscle fibers as shown in Fig. 14.

In the case of blood, where a high cooling velocity exerts a beneficial action, we have a research program on the way to determine the possible effect of recrystallization, during rewarming, on hemolysis, both in the absence and in the presence of cryoprotective

agents. (For details on that program see Luyet, Rapatz and Gehenio, 1963.)

In experiments by Luyet and Thoennes (1938) and by Thoennes (1940) it was found that small bundles of muscle fibers cooled rapidly and rewarmed rapidly responded some 20 times in succession to an electric stimulus, while hardly any response was obtained when the bundles were rewarmed slowly. This effect may be attributable to a prevention or partial inhibition of recrystallization by rapid thawing but, if it is so, we have no factual information permitting us to specify which form of recrystallization is involved, whether irruptive, or slow-pace, or premelting.

(3) Freeze-drying and the recrystallization temperature. MacKenzie and Luyet (1965) reported that the structure of aqueous systems freeze-dried at temperatures above the recrystallization temperatures collapses in the course of freeze-drying. The phenomenon was checked on several solutions (sucrose, dextrose, albumin), but no study has as yet been made of the biological effect of that collapsing. (For more details on the process see MacKenzie, 1965.)

(b) *Slow-pace recrystallization*

(1) Observations on blood. To illustrate the possible relationship between hemolysis and slow-pace recrystallization, I reproduce in Figs. 15 A and 15 B photographs from a motion picture film made by Luyet and Pribor (1965) during the rewarming of a thin layer of blood frozen at −20°C. When the temperature rising from −20°C (Fig. 15 A-1) reaches −16°C (Fig. 15 A-2), lobes of ice L begin to develop in a background of frozen plasma which previously had a finely granular structure. These lobes grow larger and become more conspicuous when the temperature rises to −14, −12, −10 and −9°C (3 and 4 of Fig. 15 A, and 1 and 2 of Fig. 15 B, respectively) until at −7 and −6°C (3 and 4 of Fig. 15 B) they have formed large plates of ice. Simultaneously the borderlines B between the original arborescences in the structure of frozen plasma (B in Fig. 15 A) become more distinct and are replaced by channels (C in 1 and 2 of Fig. 15 B) and finally by pools of fluid P (3 and 4 of Fig. 15 B). The red cells, which had originally a granular appearance (Fig. 15 A-1) become smooth (Fig. 15 A-4) possibly as a result of intracellular recrystallization, and at −9°C (Fig. 15 B-2) lose their more or less circular contours and hemolyze, leaving behind pools of hemoglobin solution (P in 3 and 4 of Fig. 15 B). These observations indicate some relationship between recrystallization and hemolysis, but they leave open the question of the respective effects of recrystallization, of incipient melting, and of solute concentration in causing hemolysis.

(2) Observations on plant cells. Luyet and Gibbs (1937) reported that epidermal cells of plants frozen at high subzero temperatures have a finely granular structure immediately after being frozen, but acquire a coarser structure when standing for a short time at those temperatures. The phenomenon is illustrated in photographs and diagrams in the original paper.

(c) *Premelting recrystallization.* The rapid changes which characterize premelting recrystallization might well be among the principal offenders in the effects reported to occur upon thawing; but we have no data to support that notion.

Additional information on the physiological conditions of slowly thawed muscle fibers, and on the mechanism of injury in them, possibly involving premelting recrystallization, may be found in two recent papers by the author and his collaborators (Luyet and Pribor,

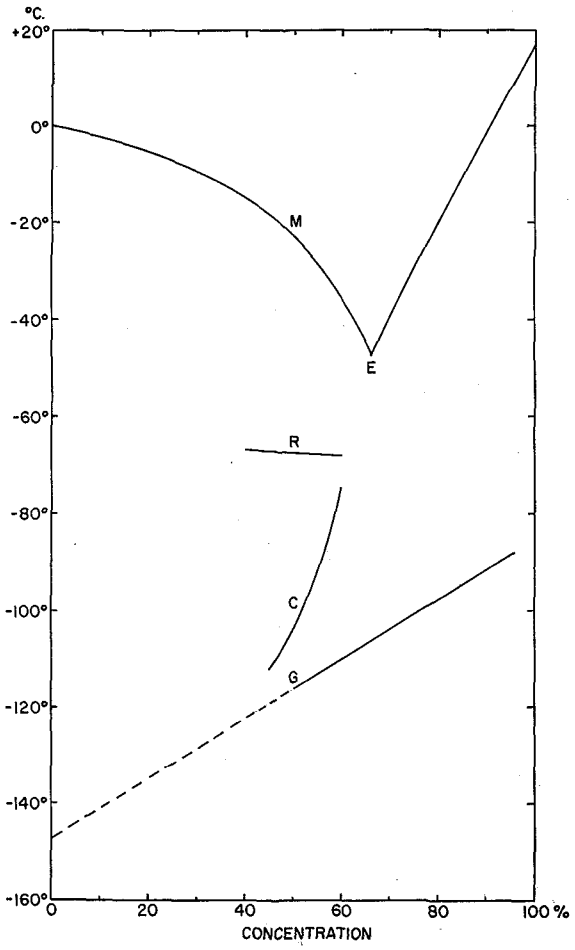

Fig. 16. "Phase diagram" representing the temperatures at which the glass transition G, a crystallization C, a recrystallization R and melting M occur during the rewarming of rapidly frozen solutions of glycerol of various concentrations. (See text for sources of curves M, R and G; curve C is constructed from still unpublished data)

1965; Luyet and Gupta, 1965).

(d) *Reorientative recrystallization.* One should also consider the possible damage caused by the transition from cubic to hexagonal ice, if such is confirmed in biological material.

4′. INCIPIENT MELTING

If the idea according to which the temperature of irruptive recrystallization is the temperature at which melting begins when a specimen is rewarmed is confirmed, the three forms of recrystallization, irruptive, slow-pace and premelting would correspond to three stages of the melting process. The mechanism of the damage appears then under a new light which may help in the clarification of some of its aspects.

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