THE PROBLEM OF STRUCTURAL INSTA-BILITY AND MOLECULAR MOBILITY IN AQUEOUS SOLUTIONS "SOLIDIFIED" AT LOW TEMPERATURES

(Present Status and Future Prospects)

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GENERAL INTRODUCTION

It is often assumed, rather summarily, that, when a solution has been frozen, its structure remains stable unless the temperature is raised to the melting point. There are, however, 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. The purpose of the present paper is: (1) to examine the factual evidence for such happenings, (2) to take stock of the state of our knowledge of the phenomena encountered, and (3) to consider the research to undertake and the methods to use in a further study of these phenomena. The paper will, accordingly, be divided into three parts.

In general, it is assumed that the solutions studied have been refrigerated in such a way that a part of their crystallizable water remained amorphous. I designate that condition by saying that the material has been "solidified" at low temperatures.

As I outlined in a previous publication (Luyet, 1960), the cases of instability in a given solution can be represented in diagrams of which the temperatures of instability are

plotted in terms of the concentration of the solute. In that publication I plotted, for glycerol solutions, three transitions: (1) melting, (2) "recrystallization" and (3) the glass transformation (only rudimentary information was available on the latter two).

In addition to diagrams of this type which represent the temperature and concentration ranges of instability for "solidified" material, one may consider diagrams representing ranges of instability encountered during cooling, when the liquid material undergoes nucleation and crystallization. It is possible that the points in those ranges at which, during cooling, the rates of nucleation and of crystal growth reach their maximum be also points of instability for the "solidified" material during rewarming. If it is so, two additional cases of instability should be indicated in the diagram for "solidified" material.

The instability of aqueous solutions "solidified" at low temperatures has been a subject of predilection for the searchers of this laboratory and of its predecessor at St. Louis University. The published papers deal with: (a) the devitrification or recrystallization temperatures (Luyet, 1939, 1941; Luyet and Gomez, 1947); (b) the nature of that transition (Luyet and Rapatz, 1957); (c) various phase transitions occurring in solidified solutions (Luyet, 1960, 1962 and 1965).

The method of gradually rewarming a rapidly cooled preparation of an aqueous solution lends itself well to the observation of changes in properties which reflect changes in molecular mobility in partly crystalline and partly amorphous material. Information obtained by that method and with that material will be used extensively—though not exclusively—in this work.

Aqueous solutions have been studied by our group of investigators as models of the more complex biological systems, in which instability may play an important role as a factor of injury. Although the present paper deals only with solutions, the research proposed in Part III is to be

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extended to biological materials, as will occasionally be mentioned.

The two main variables involved in most of the relationships to be studied in this paper are temperature and the concentration of the solutions. A third variable, often of great importance, is the cooling rate. Unless indicated otherwise, the statements about temperature and cooling rate refer, respectively, to the temperature of the refrigerating bath into which thin layers of the solutions are immersed, and to the rate of cooling of the specimen which one obtains in that immersion.

PART I

THE BASIC FACTS

As stated, the facts to be examined belong to five categories; they will be presented in five corresponding sections. A sixth section will mention other facts which may indicate molecular instability and are included because of their possible significance in that connection, although they will not be discussed further.

I. MELTING

Melting of either the solvent, the solute, or the eutectic mixture constitutes one of the most important phase transitions for both the physicist interested in molecular mobility and the cryobiologist interested in the resulting damage to living cells. But since the subject is adequately treated by the physicists, I shall not elaborate further on it in this paper.

II. RECRYSTALLIZATION

The phenomena tentatively classified under the heading "recrystallization" are very complex. In the following description of the facts, I shall distinguish four types of recrystallization and subdistinguish two varieties in the first type, as indicated in Table 1. Then I will examine the principal characteristics of each type.

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TABLE 1
TYPES OF RECRYSTALLIZATION
(A) Irruptive
    (a) Induced or Spontaneous
    (b) After Rapid or After Slow Freezing
(B) Slow-Pace
(C) Premelting
(D) Reorientative

(A) *Irruptive Recrystallization.* One of the most conspicuous changes is that observed when thin layers of solutions of concentrations extending from about 25 to 50% are rewarmed, after having been cooled rapidly by immersion in baths at temperatures below $-50°C$. The material, transparent after the rapid cooling, becomes rather suddenly opaque when its rising temperature reaches a value characteristic of the solute present. This phenomenon, which I called "irruptive recrystallization" (Luyet, 1960), is illustrated in Fig. 1, and the temperature at which it occurs in a certain number of solutions is given in Table 2. The "recrystallization temperature", which shows little
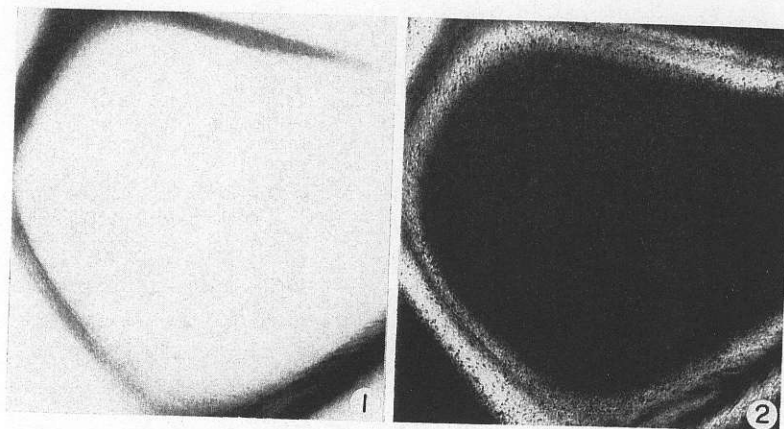

Fig. 1. Irruptive recrystallization of an evanescent spherulite.— Phot. 1: evanescent spherulite formed in a 30% gelatin gel frozen at −30°; Phot. 2: same field as in Phot. 1 after the preparation has been warmed to −10°.—MAGN.: 60×. (From Rapatz and Luyet, 1959.)

dependence on concentration, seems to depend mostly on the molecular weight of the solute.

(a) "Induced" and "Spontaneous" Recrystallizations. In the experiment just described recrystallization (the turning opaque of the preparation) is "induced" by a rewarming. There are cases in which the preparation turns opaque in the course of cooling. Thus, as illustrated in Fig. 2, Phot. 4, a gelatin gel may become completely opaque when cooled by immersion in a bath at –25°. The phenomenon has been designated as "spontaneous" recrystallization.

(b) Recrystallization of Rapidly and of Slowly Frozen Specimens. We dealt so far with cases in which the material, transparent after having been *rapidly* frozen, turns opaque. Material frozen *slowly*
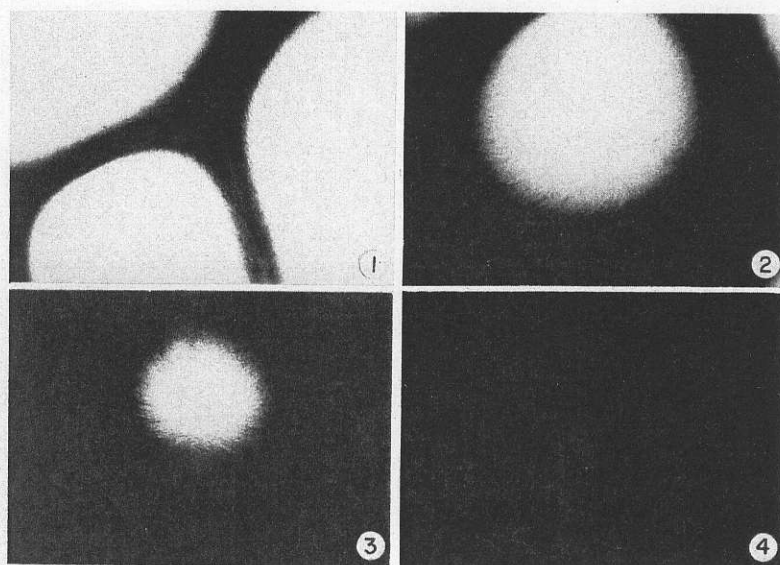

Fig. 2. Spontaneous irruptive recrystallization in a preparation 100 micra thick of a 30% gelatin gel. The recrystallized (opaque) areas are gradually larger when the temperature of the freezing bath is higher.—FREEZING TEMP.: Phot. 1: –50°; Phot. 2: –40°; Phot. 3: –30°; Phot. 4: –25°.—MAGN.: 56×. (Phots. 1, 2 and 3 from Luyet and Rapatz, 1958.)

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TABLE 2

TEMPERATURES OF "IRRUPTIVE RECRYSTALLIZATION" OF VARIOUS AQUEOUS SOLUTIONS

The temperatures given in this table are those at which thin layers of solutions of concentrations between 25 and 50% become intensely opaque in one minute.

Solute:	Temp. °C:	Solute:	Temp. °C:	Solute:	Temp. °C:
Hemoglobin	−3.5	Gelatin*	−11.5	Dextrose	−38
Soluble		Polivinyl-		Glycerol	−58
Starch	−5.0	pyrrolidone	−14.5	Ethylene Glycol	−63
Albumin	−5.3	Raffinose	−25.4	Acetamide	−65
Dextrin	−9.9	Sucrose	−30.5	Formaldehyde	−72

* A thin layer of gelatin solution turns intensely opaque in one minute at −11.5°, and in a few seconds at −10°. This last figure will be the one generally used in the present paper.

may also turn opaque; and this happens at the same temperature as with rapidly frozen material. Fig. 1 illustrates, as was said, the recrystallization of a rapidly frozen specimen (which has crystallized into transparent spherulites) and Fig. 3 the recrystallization of a slowly frozen one
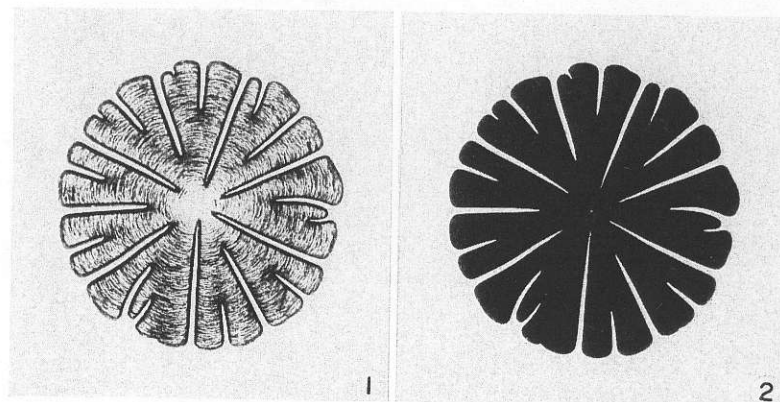

Fig. 3. Irruptive recrystallization in a slowly grown irregular dendrite (rosette).—Phot. 1: rosette formed in 50% gelatin gel frozen at −30°; Phot. 2: same rosette after the temperature has been raised to −8°.—MAGN.: 70×.

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₴	−65
₁yde	−72

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(which has crystallized into an irregular dendrite, in this case, a rosette). (For the literature on the subject, see Luyet and Rapatz, 1958; Gehenio and Luyet, 1959; Rapatz and Luyet, 1959; Persidsky and Luyet, 1959.)

The turning opaque of a preparation described in the last two paragraphs involves the combined action of several factors. To facilitate the analysis of its mechanism (analysis which is to follow in Part II), I shall describe here the cases in which we observed the phenomenon in gelatin gels. The complex series of events is illustrated in Fig. 4, where: (1) columns 2 and 3 represent the patterns of crystallization obtained when thin layers of gelatin of the concentrations given in column 1 freeze relatively slowly in a bath at −30° (column 2) or more rapidly in a bath at −100° (column 3); (2) columns 4 and 5 show what happens when the specimens frozen at −100° are rewarmed, respectively, to −30° (column 4) and to −10° (column 5); (3) column 6 illustrates the change resulting from a rewarming to −10° of the preparations frozen at −30° (column 2).—Freezing at −30° causes the formation of "irregular dendrites", such as, at lower gelatin concentrations, the rosette shown in column 2, horizontal row 3; when the concentration increases, the number of lobes decreases and one finally has balls, without lobes (column 3, row 8). Freezing at −100° causes the formation of "evanescent spherulites" of the type shown in Fig. 1; a dozen of them are seen in Fig. 4, column 3, row 1, at a concentration of 35%, hundreds of them in row 2 at 38%, and still more at higher concentrations; at 50% and above, nothing develops (column 3, rows 6–10). (From Persidsky and Luyet, 1959.)

In summary, opaque preparations are encountered in six particular cases, namely: (1) when a rapidly cooled solution of concentration varying from 20 to 50% is rewarmed to −10° (Fig. 4, column 5, horizontal rows 1 to 3 and 5 to 6, for concentrations beginning at 35%); (2) when a slowly cooled solution of 20 to 65% concentration is rewarmed to −10° (Fig. 4, column 6, for concentrations beginning at
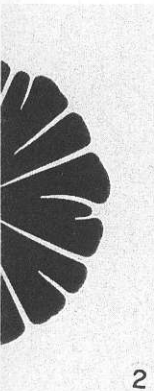
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1 Concen- tration	2 Frozen at −30°	3 Frozen at −100°	4 Frozen at −100° Rewarmed to −30°	5 Frozen at −100° Rewarmed to −10°	6 Frozen at −30° Rewarmed to −10°
1	35				
2	38			N O	
3	40			C H A N G E	
4	42				
5	45				
6	50				
7	53				
8	55				
9	60				
10	65				
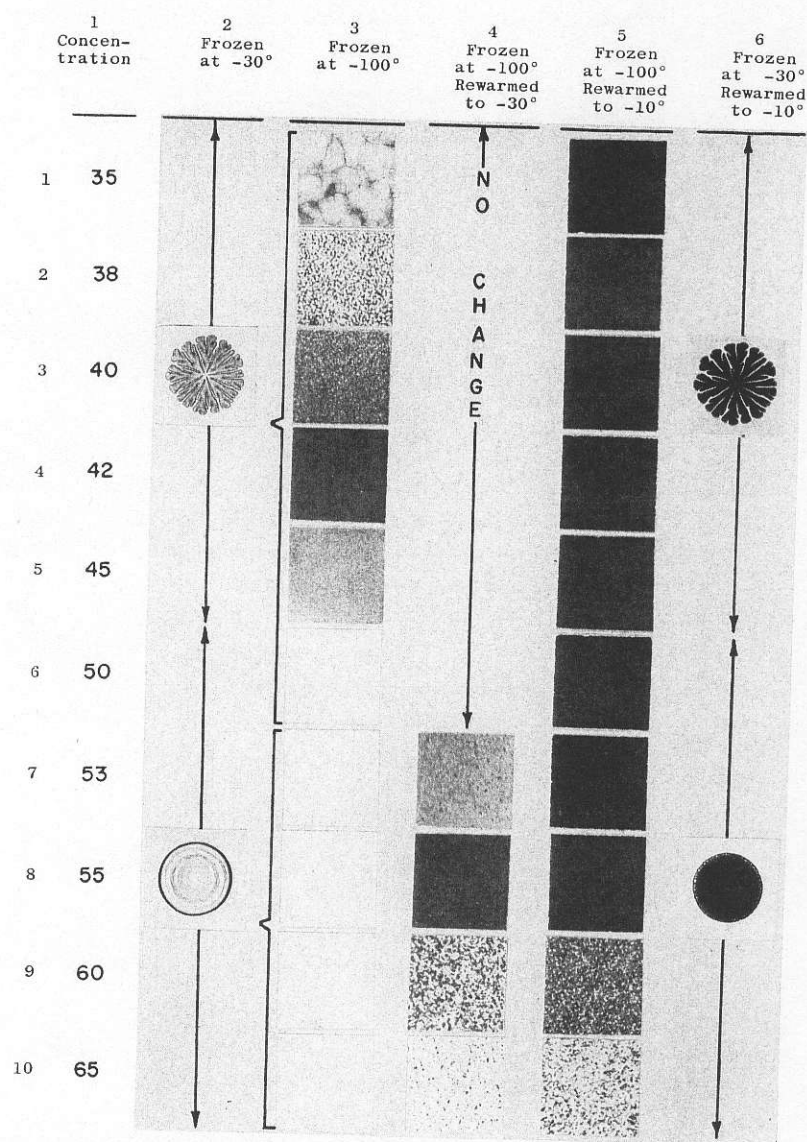
Fig. 4. Photomicrographs of patterns of crystallization and recrystallization observed in gelatin gels of various concentrations (as indicated in column 1), after (I) rapid freezing at −100° (column 3) and rewarming to −30° (column 4), or to −10° (column 5); or (II) after slower freezing at −30° (column 2) and rewarming to −10° (column 6).—MAGN.: 20×, except for the balls in columns 2 and 5 which are magnified 40×. (From Persidsky and Luyet, 1959.)
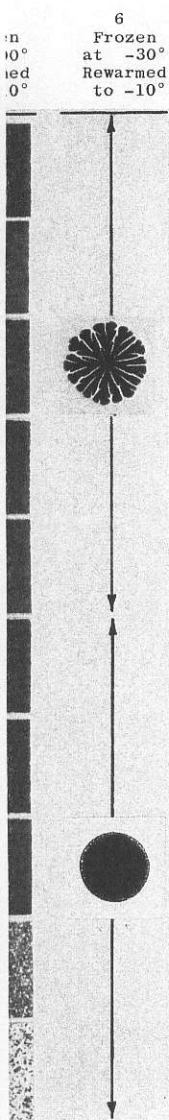
35% ; see also Fig. 3) ; (3) when a 42% solution is rapidly cooled by immersion in a bath at –100° (column 3, row 4) ; (4) when a 30% solution is rather slowly frozen at –25° (Fig. 2, Phot. 4) ; (5) when a rapidly cooled solution of 55% concentration is rewarmed to –30° (column 4, row 8) ; and (6) when a rapidly cooled 53% solution is rewarmed first to –30° and then to –10° (column 5, row 7).

(B) *"Slow-Pace" Recrystallization.* Another form of recrystallization which, in our laboratory, was observed repeatedly in biological fluids (Rapatz and Luyet, 1960; Luyet and Pribor, 1965; Gupta and Luyet, 1965) consists in the passage from a fine-grain structure at a given low temperature (–20° or thereabouts in the case of blood plasma) to a gradually coarser crystalline conglomerate at gradually rising temperatures and longer exposure until, finally, near the melting point, there are only large ice blocks. I shall refer to this process, which is illustrated in Fig. 5, in the case of a 10% albumin solution, as "slow-pace" recrystallization. (See legend of Fig. 5 for details.)

(C) *"Premelting" Recrystallization.* Luyet and Gehenio (1965) described a form of recrystallization, which occurred immediately before or during melting and which they designate as "premelting" recrystallization. The phenomenon, as photographed by them, is illustrated in Fig. 6. The photograph shows a thin layer of ice melting on a glass support. The melting front, which progresses upward from the lower edge of the support, has reached the level R. Between the melted portion M and the still frozen portion F, at R, one observes the formation and growth of polyhedral crystals which float in the last-melted portion before they themselves melt out. The phenomenon is particularly spectacular in polarized light, between crossed Nicols.

(D) *Recrystallization Involving a Molecular Reorientation.* Dowell, Moline and Rinfret (1962) reported a transition from cubic to hexagonal ice during the rewarming of a rapidly cooled 50% gelatin gel. The facts, as observed in a similar work in our laboratories (cf. Luyet, 1965, Fig. 7)
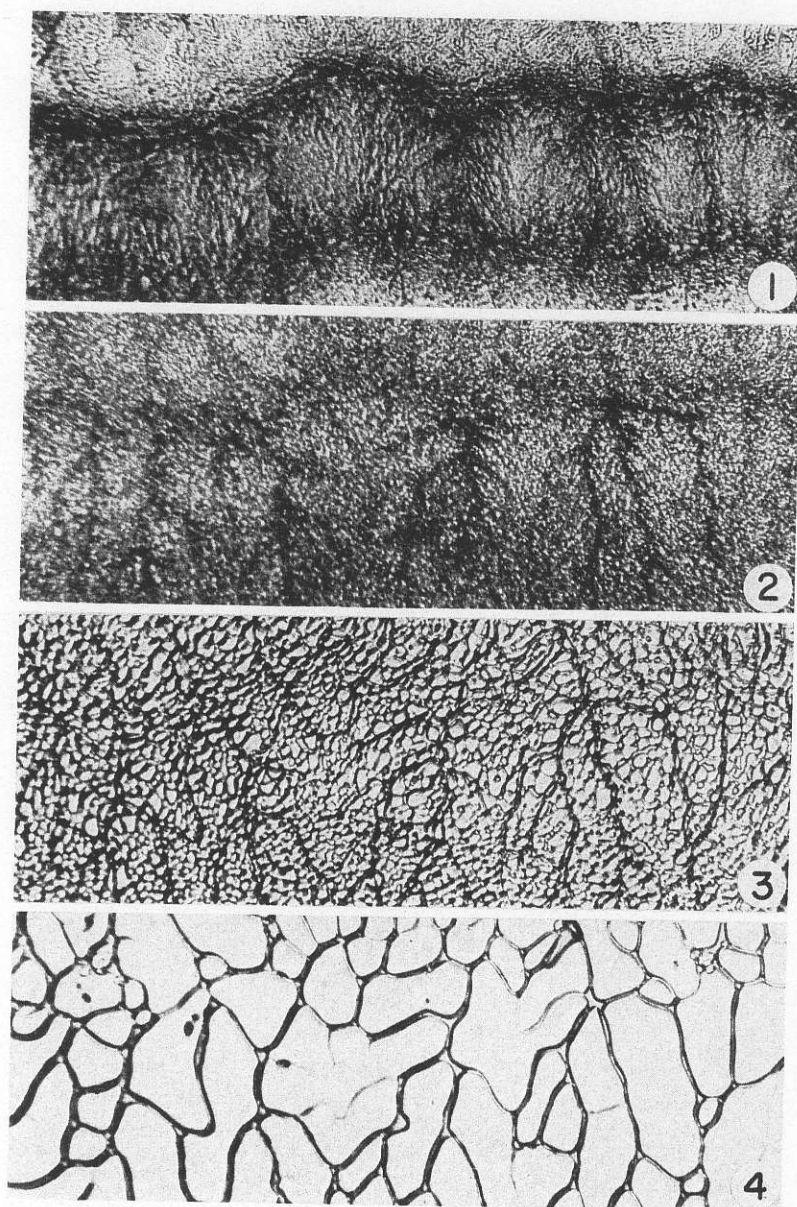
Fig. 5. "Slow-Pace" recrystallization of ice in a solution of 10% albumin.—Phot. 1: specimen frozen at −40° and warmed to −7°; Phot. 2: same field as in Phot. 1 after the temperature was raised to −3°; Phot. 2: same field after the temperature was raised to −2°; Phot. 4: same field after the temperature was raised to −1°.—MAGN.: 95×.
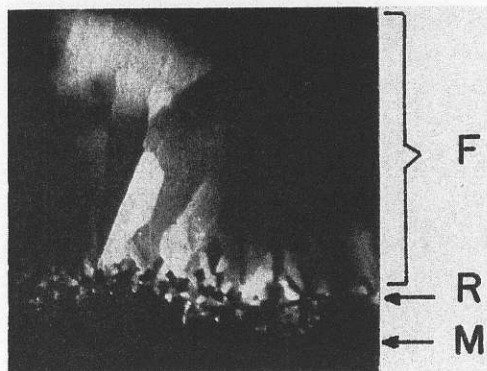
Fig. 6. "Premelting" recrystallization of ice (at R), when a film of water frozen on a glass coverslip (at F) has melted on one side of the coverslip (at M), and the melting front, which is moving upward, is at the level R.—MAGN.: 6×.

are as follows: the X-ray diffraction peaks common to the cubic and hexagonal systems are among the first to appear in the course of rewarming; they are fairly well delineated at −30°; gradually other peaks characteristic of the hexagonal system arise, but the complete set of sharp peaks develops only when the temperature approaches −10°. The order of appearance of the peaks was interpreted as indicating a transition from cubic to hexagonal ice, which would imply a state of structural instability resulting in a molecular reorientation.

III. THE GLASS TRANSITION

A change much less conspicuous than most of those mentioned in the previous section is the so-called glass transition which is manifested by discontinuous variations in specific heat, in coefficient of expansion, in refractive index, in electric resistivity, etc., when a solution of the proper concentration of certain solutes is cooled rapidly enough through a certain range of temperatures to be at least partially vitrified, or is rewarmed through that range. The facts about those changes, which have been the object of numerous observations, from the pioneering work of Gibson, Parks and Latimer (1920), Simon (1922), Gibson and

Giauque (1923) to the recent determinations by modern
techniques (McMillan and Los, 1965), are now well estab-
lished. (Cf. review by J. D. Mackenzie, 1960.)

IV. AND V. NUCLEATION AND CRYSTAL GROWTH

As stated in the General Introduction of this paper, our
interest in nucleation and crystal growth is focused on the
question of the temperatures of maximal rates of these two
processes.

Tammann pointed out long ago that the curve of the rate
of nucleation and the curve of the rate of crystal growth
have their maxima in different ranges of temperature (for
references see Tammann, 1925). As factual evidence for the
existence of two maxima at different temperatures, I shall
mention, in addition to the investigations by Tammann on
several organic liquids, the work of those who have estab-
lished the method of obtaining the crystallization of gly-
cerol. As reported by Miner and Dalton (1953, p. 276), the
method consists in exposing glycerol to temperatures be-
tween –50° and –55° for several hours to permit nucleation
and then to bring the nucleated material to the crystal-
growth temperature, near 0°, where it should remain for
several additional hours. (The melting point of glycerol is
17°.)

VI. MISCELLANEOUS OTHER CHANGES

The extensive investigations of Hüttig and his collabora-
tors on sintering of powders (cf. Williams, 1962), in which
they found changes of different types occurring in six dif-
ferent ranges of temperature, call for a comparison of these
changes with those occurring or possible in aqueous
solutions.

The question of the possibility that water may crystallize
into mesomorphic states has hardly been raised. The
tendency may be to exclude that possibility for the reason
that substances known to assume the nematic or smectic

state differ radically by their nature from water. But, could not this become another case in which experimentation will denounce theoretical previsions?

PART II

STATE OF OUR KNOWLEDGE OF THE FACTS REPORTED; UNANSWERED QUESTIONS

One of the categories of facts reported, the category "recrystallization" raises more questions than the others and has been studied more extensively; it will, therefore, be treated first and at greater length.

I. RECRYSTALLIZATION
AND POSSIBLY RELATED PHENOMENA

(A) *Irruptive Recrystallization.* (1) TYPICAL CASES. The turning opaque, upon rewarming, of rapidly cooled solutions has been diversely interpreted by the investigators. As the transparent material was thought to be vitreous, its turning opaque was classified as a passage from the vitreous to the crystalline state and was called *devitrification*. But Luyet and Rapatz (1957) pointed out that most of the specimens examined after rapid cooling (in general those of solute concentrations extending from 25 to 50%) had a microcrystalline (spherulitic) structure and that the parts of a specimen which turned opaque were only those located in the territories occupied by spherulites. The opacity then seemed due to a growth to visible size of the crystallites which made up the spherulites and to result from a *recrystallization*. In fact, photomicrographs show that the cloud in opaque preparations consists of particles of ice one to several micra in diameter. But these observations leave unanswered the question of whether the socalled recrystallization particles originate from preexisting crystallites or from amorphous material. In the former alternative, the process is a true recrystallization, in the latter it is a crystallization. In most cases of rapid cooling one would expect that it be both.

(a) Spontaneous Recrystallization. Whereas, in the cases just examined, the specimen turns opaque *when it is rewarmed,* in other cases, the same effect seems to be produced spontaneously *in the course of cooling.* Thus, as illustrated in Fig. 1, Phot. 1, and in Fig. 2, Phot 1, when a thin preparation is cooled rapidly and forms spherulites, the regions along the borderlines between two or several spherulites become opaque, apparently because the latent heat released during crystallization is sufficient to warm to the recrystallization temperature a small territory along the borderlines. To test this interpretation we froze gelatin solutions of 30% concentration to temperatures gradually closer to that of recrystallization (−10°) and observed that the opaque area becomes gradually larger until the entire preparation is opaque. The four photographs of Fig. 2 show the differences at bath temperatures of −50°, −40°, −30° and −25°.

In spontaneous, like in induced recrystallization, the growth of crystalline particles large enough to cause opacity may take place at the expense of amorphous material (a crystallization), or of preexisting crystals (a recrystallization), or of both.

(b) Recrystallization of Slowly Frozen Specimens. As for the turning opaque upon rewarming to −10° (column 6) of the rosettes or balls obtained by slow cooling to −30° (Fig. 4, column 2), the similarity in mode of occurrence with the turning opaque of spherulites obtained by rapid cooling (column 5) indicates a change of the same nature in the two cases; but, while in the case of rapid cooling we know that an appreciable amount of water remains nonfrozen and is available for crystallization, in the case of slow cooling we would not expect much. How much and where is that nonfrozen water located with reference to the crystalline portion remains to be investigated.

(2) COMPLEX CASES. Of the six cases in which a preparation turns opaque (as listed in the summary paragraph at the end of subsection A, in section II of Part I, and illus-

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trated in Fig. 4), case 1 is the prototype of irruptive recrystallization, case 2 apparently belongs to the same category, and cases 3 and 4 have the characters of the subtype "spontaneous" irruptive recrystallization. But case 3 calls for some comments: when a thin layer of a gelatin gel of 42% concentration is immersed in a bath at –100° (Fig. 4, column 3, row 4), a very large number of tiny spherulites is formed, of which the spontaneously recrystallized dark borders fill the field.—As for cases 5 and 6, of which the classification requires closer attention, they will now be examined (subsections a and b, respectively).

(a) Resumption of an Interrupted Crystalization of Slowly Growing Units. What happens in case 5, in which a rapidly cooled solution of gelatin of 55% concentration turns opaque upon being rewarmed to –30° (Fig. 4, column 4, row 8), seems closely related, from the point of view of the mechanisms involved, to what happens in a 50% solution which remains transparent after rapid freezing at –100° (Fig. 4, column 3, row 6). The two cases will now be examined (under a' and a'', respectively) and compared.

(a') The gradually decreasing opacity shown in column 3 of Fig. 4, when the concentration increases from 42 to 50%, and the complete transparency at 50% are apparently explainable by the gradually increasing number of nuclei and gradually decreasing size of the *spherulites* at rising concentrations; at 42%, as was said, the number of spherulites formed is such that their spontaneously recrystallizing borders just fill the field; at 50%, the units are so numerous that they have already filled the field before they could grow to visible size.

(a'') In rapidly cooled solutions of concentrations varying from 53 to 65% (column 3, rows 7–10, including row 8 which represents case 5), something similar and something different happens: like with the 50% solution, nucleation would occur and crystallites of submicroscopic size would develop, so that the preparations would remain transparent

(column 3, rows 6–10) ; but, unlike with the 50% solution, slowly growing *balls* would be formed instead of the rapidly growing spherulites, and these slowly growing units, not able to reach visible size at –100°, would accelerate their growth when the temperature is raised to –30° (column 4, rows 7–10) ; the field would then darken in proportion to their number and size.

Thus, the partial or total opacity brought about by the rewarming of the 53 to 65% solutions to –30° (case 5 included) would not be due to a recrystallization proper, but to the *resumption of an interrupted crystallization*. (Gehenio and Luyet, 1959, who described the phenomenon as recrystallization, point out (p. 84) that they use the term in a broad sense, including "a resumption of a previously initiated crystallization.")

The interpretation just given seems in fitting with the fact that the transparent preparations of 50% concentration do not turn opaque when rewarmed to –30°, as the 55% preparations do. In the former case, most of the crystallizable water would already have crystallized into a great many very small *spherulites;* in the latter, the smaller number of slowly growing balls would find enough water available around them (in the nonnucleated portion) to grow when the temperature is raised to –30°, a temperature favorable to the growth of balls.

(b) Resumption of Crystallization, and Recrystallization of Slowly Growing Units. Case 6, in which a 53% solution, transparent after treatment at –100°, becomes partly opaque at –30° and completely opaque at –10° (Fig. 4, row 7) is explainable on the presumption that nucleation takes place during the cooling to –100°, that crystallization units of the type balls grow at –30° and recrystallize (become opaque) at –10°.

Solutions of 60 and 65% concentration (Fig. 4, rows 9 and 10) would undergo the same changes, but, in them, the number of crystallization units is smaller, and there are, between them, blank spaces consisting of nonnucleated

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(B and C) *Slow-Pace and Premelting Recrystallization.*
The fact that in slow-pace recrystallization one can observe
the passage from a fine-grain structure to a coarse one
offers strong support to the view that the molecules of wa-
ter migrate from the small to the larger particles. Dorsey
(1948) designated as "migratory" a recrystallization phe-
nomenon described as consisting in the growth of the large
crystals or of the large ice particles in an aggregate at the
expense of the small ones, as a result of the migration of
molecules from the small to the large particles. The process
may be of quite general occurrence and may be responsible
for most of the types of recrystallization described, in par-
ticular for the types included under the heading "irruptive
recrystallization". The same mechanism is probably re-
sponsible for premelting recrystallization, in which the
migration of molecules apparently takes place through the
liquid phase; in other words, at a given temperature, the
small particles melt while the larger ones grow.

(D) *Reorientative Recrystallization.* Luyet, Tanner and
Rapatz (1962) have pointed out that the X-ray diagrams
obtained with 50% gelatin gels are more adequately ex-
plained on the basis of a preferred orientation in the
growth of ice crystals than by a transition from cubic to
hexagonal ice. Since, in solutions of this sort, the tempera-
ture of the assumed transition to hexagonal ice is that of
irruptive recrystallization (about −10° for 50% gelatin),
one of the unanswered questions is whether the so conspicu-
ous change marked by the turning opaque of the prepara-
tion is due to a recrystallization of the migratory type (that
is, in which the molecules migrate from small to large
particles) or to a transition from cubic to hexagonal ice.

II. THE GLASS TRANSITION

The actually observed changes in physical properties
occurring at the glass transition support the idea that the
molecules are capable of translational motion above the

temperature of the transition, but not below, so that, below that temperature, they are practically in contact, forming a rigid framework comparable to that of crystals (the lattice). But, in contrast to the crystals, in which the molecules are orderly arranged, in glasses they occupy more or less random positions in the framework. The coefficient of expansion should change abruptly when the structure of the specimen passes from that of a rigid framework to that of a fluid with translational motion, or *vice-versa*. Our further investigations on the glass transition and its significance for the cryobiologist will be based on that concept.

III. AND IV. NUCLEATION AND CRYSTAL GROWTH

I refer the readers to special treatises and articles for information on the rather elaborate modern concepts on nucleation and crystallization. Since, in this paper, we are using the data on nucleation and crystal growth rates for their possible relation to instability of the "solidified" material, our immediate task is to establish the curves of nucleation and growth rates in terms of temperatures and solute concentration for a certain number of selected solutions and to determine the temperature ranges at which these rates reach their maximum, and the immediate unanswered question is whether or not those ranges represent danger zones in which aqueous systems "solidified" at low temperatures are liable to become unstable.

PART III
RESEARCH PROJECTS
GENERAL CONSIDERATIONS

I shall now present a research program planned (a) to answer some of the questions raised in Part II, in particular those concerning the nature of the processes reported, and (b) to identify the "cases of molecular instability" in aqueous systems "solidified" at low temperatures.

The problems to be studied and the modes of approach to be used in that study may be presented in summarized form

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as follows: The occurrence of a state of instability in "solidified" aqueous material is well established experimentally in three cases: (1) when the material undergoes the glass transition, (2) when it recrystallizes, (3) when it melts. There are indications for a fourth case: a crystallization interrupted during cooling may be resumed during rewarming; and there is a question about a fifth case: whether material cooled rapidly enough to have escaped nucleation during cooling, might nucleate during rewarming. It is possible that the last two presumed changes, nucleation and crystal growth during the rewarming of "solidified" material, take place at the temperatures at which the rate of nucleation and rate of crystal growth, as measured during cooling, are maximal. Because of that possibility, studies on nucleation and on crystal growth are included in the present research plan. This would bring to five the number of changes to be considered, three occurring in the course of rewarming and two in the course of cooling. But, since melting is not included in the program of the paper, we are left with four cases: two well authenticated, and two questionable.

Looked upon from another point of view, the program may be described as dealing with two kinds of "phase diagrams", one showing the temperatures at which instability is encountered during the rewarming of "solidified" materials of various solute concentrations, and the other showing the temperatures at which the nucleation and crystal growth rates, in the course of cooling, reach their maximum for various solute concentrations.

The outline of the program will be divided into four sections corresponding to the four changes mentioned. Within each section the projects will be grouped into series according to the subject matter treated. These series will be designated by the initials of the subject of a section (G, R, N and C for, respectively, Glass transition, Recrystallization, Nucleation and Crystallization), followed by an order number, 1, 2, . . . In each group, the first series, that is G_1, R_1, N_1 and C_1, will be concerned directly with the

gathering of the data for the construction of the two "phase diagrams" mentioned.

The projected research is to be conducted on aqueous solutions and on biological materials. Both will be studied at various temperatures and solute concentrations, the higher concentrations in biological materials being obtained by dehydration. But, as was already said, this outline will be limited to solutions.

The solutes to be selected will be substances of interest to the cryobiologist, either because of their presence in biological material: salts, carbohydrates, amino acids and proteins, or because of their protective action against freezing injury: glycerol, ethylene glycol, dimethyl sulfoxide and polyvinyl pyrrolidone. (Some substances, like glycerol, are also of interest from another point of view, they can readily be vitrified and, as such, they become material of choice for students of glasses and for cryophysicists.)

This research program is presented here in general terms; the titles given indicate the subject matter of the *series* of projects; the individual projects in a series will not be specified.

I. SERIES OF PROJECTS DEALING WITH THE GLASS TRANSITION

Three main questions arise in regard to the glass transition: (1) At what temperature does it occur in solutions of various concentrations of various solutes and in biological materials? (2) Which structural changes does it involve? and to what extent do these changes bring about instability? (3) Are those changes of a type that disrupt biological structures? The following three series of research projects, designated as G_1, G_2 and G_3, are proposed in partial answer to these questions:

(1) *Series G_1: Temperatures of Occurrence of the Glass Transition at Various Solute Concentrations.*

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on the two coordinates: temperature and solute concentration). These data will supplement the limited information now available on the glass transition in aqueous solutions. Furthermore, other variables which are known to affect or which may affect the glass transition, such as the rate of cooling, the proportion of material in the amorphous state, will be included in the research program.

(2) *Series G_2: Changes in Properties Caused by the Passage through the Glass Transition.*

Among the properties reported to change abruptly at the glass transition, the principal are: the specific heat, the specific volume, and the refractive index. The extent to which the system may be rendered unstable by the transition will be made apparent by the size and the degree of abruptness of the changes in properties.

(3) *Series G_3: Formation of Cracks during Cooling, and their Disappearance during Rewarming, in Relation to the Glass Transition.*

Since the damage possibly produced when biological material undergoes the glass transition is probably attributable to tensions and stresses which may result in cracks, a study is planned of the formation of cracks under the stresses and of their disappearance upon release of the stresses.

II. SERIES OF PROJECTS DEALING WITH RECRYSTALLIZATION

(4) *Series R_1: Temperatures of Irruptive Recrystallization in Terms of Solute Concentrations.* This series of projects is an extension of the determinations reported in Table 2 to more solutions and more biological materials. Plotting temperatures of recrystallization in terms of solute concentrations will provide the basic "phase diagrams" needed in our systematic study of the recrystallization phenomenon.

In previous work on the subject, we used four methods: (a) direct observation of the onset of opacity, (b) deter-

mination of the progress and completion of opacity by a photocell, (c) detection of the changes by thermometry, and by calorimetry, (d) tracing of the development of the process by X-ray diffraction.

(5) *Series R₂: Patterns of Recrystallization.*

To what extent solutions of various solutes, and biological materials of various sorts undergo the four types of recrystallization enumerated in Table 1 for gelatin solutions, and to what extent they reproduce the stages of irruptive recrystallization illustrated in Fig. 4 remains to be investigated. This series of projects constitutes a very elaborate program which will result in two series of graphs, one giving the four main types of recrystallization, the other, the various forms of irruptive recrystallization (as shown in Fig. 4) encountered at given temperatures and solute concentrations, for various kinds of materials, treated in various ways.

Concerning the range of temperatures in which slow-pace recrystallization occurs, I reported (Luyet, 1962) that, in four cases studied, that type of recrystallization became noticeable only above the temperature of irruptive recrystallization. If this becomes an established fact, the three changes described as irruptive, slow-pace and premelting recrystallization appear as three stages of the same phenomenon beginning at the temperature of irruptive recrystallization and continuing at a gradually accelerated pace until, at the melting point, its rate becomes asymptotically high.

Series R₂ should also include further investigations on the characteristic features of slow-pace and premelting recrystallizations, on which we have only rudimentary information. The events described by us as recrystallization phenomena occurring near the melting point seem to have several characters in common with those reported by others, under various denominations, such as, regelation, coalescence, sintering, ripening, formation of colloidal ice, formation of quasifluid interfacial film on ice crystals (see,

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(6) *Series R₃: Rates of Recrystallization and Resulting Number and Size of Ice Particles.*

Another field on which information of practical use for the cryobiologist is meager is that of the rate at which the particles of ice grow at a given temperature and under other given conditions, in either irruptive, or slow-pace, or premelting recrystallization. The need is felt for tables of quantitative data on the relationship between dimensions of particles and the factors which control recrystallization.

The dimensions of recrystallization particles actually observed extend from a few hundred angstroms to several millimeters. A research program on particles of submicro-scopic dimensions should, of course, include the use of methods permitting the examination of such particles, in particular electron microscopy.

(7) *Series R₁: Cases in which Recrystallization May Be Combined with the Resumption of an Interrupted Crystalli-zation, or May Involve Other Transitions.*

The passage from a transparent to an opaque preparation has been generally designated as a recrystallization, in the broad sense of the word, which includes the possibility of a resumption of an interrupted crystallization. In some cases, such as that illustrated in Fig. 4, column 5, horizontal row 1, the process seems to be primarily a recrystallization; in others, like in that shown in column 4, row 8 of the same figure, it seems to be only a crystallization; in still others, like that in column 5, row 7, it appears to be both. We need means of separating the two. One of the most direct means would be to check some physical properties which behave differently in the two processes. Three properties seem to qualify for such a test: (a) the release of heat which may be observed by thermometry and preferably by differential thermal analysis, (b) the change in dielectric constant, and (c) the change in volume. Electron microscopy may also

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answer several of the questions. Experimental tests will be devised accordingly.

To have more adequate information on the conditions leading to the occurrence of both a recrystallization and a resumption of an interrupted crystallization, one should determine the amounts of ice formed, and of material which had remained amorphous in the course of the previous cooling. Such determinations, which are planned as a continuation of the dilatometric studies conducted in our laboratories during the last several years, stand by themselves as a subseries in Series R_4.

(8) *Series R_5: Nature of the Recrystallization Phenomenon.*

When the recrystallization phenomenon is separated from the concomitant events, as proposed under R_4, and when the characteristics of the various forms of recrystallization are established, as planned under R_2, there will still remain the question of the basic nature of recrystallization, which is specifically the object of Series R_5.

III. SERIES OF PROJECTS DEALING WITH NUCLEATION

(9) *Series N_1: Temperatures of Maximal Nucleation Rate at Various Solute Concentrations.*

This series of projects involves two steps: (a) a determination of the rates of nucleation at various temperatures for various solute concentrations, and (b) a determination, from the curves thus obtained, of the temperatures of maximal nucleation rate.

The method proposed for that study is the one described by Luyet and collaborators (1964 and 1965), in which they determined, on motion pictures of thin preparations taken under the cryomicroscope, the number of crystallization units becoming visible in a given area, in a given length of time, at different temperatures and at different rates of cooling, in solutions and in biological fluids of various concentrations. Then, when the data thus obtained permitted

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Whereas the physicist interested in nucleation *per se* wants to know the rate of nucleation in a substance free of nucleating motes, the cryobiologist, whose primary concern is the effect of the nucleation rate on the number and size of the crystallization units actually obtained, may derive more useful information by studying the natural products, such as blood plasma, as they are, with their normally present "contaminating" motes.

The observations made on gelatin gels (Fig. 4) in which apparently two types of crystallization units are formed, spherulites and rosettes (including balls), suggest that the student of nucleation look into the matter of the possible formation, in some cases, of two or more types of nuclei.

(10) *Series N_2: Evidence for Instability at the Temperature of Maximal Nucleation Rate in Material "Solidified" by Cold.*

Material treated in various ways so as to contain various proportions of amorphous and of crystalline components, will be exposed for some lengths of time, in the course of rewarming, to the predetermined temperatures of maximal nucleation rates, and examined for the occurrence of changes.

A question of particular interest is that of whether or not material not nucleated during cooling will nucleate upon being rewarmed to the temperature of maximum nucleation. One would, then, have cases of "true devitrification".

IV. Series of Projects Dealing with Crystal Growth

(11) *Series C_1: Temperatures of Maximal Rate of Crystal Growth at Various Solute Concentrations.*

Like series N_1, series C_1 involves two steps: (a) determinations of the rates of crystal growth at various temperatures for given solute concentrations, and (b) determinations, from the curves thus obtained, of the temperatures

of maximal rate of crystal growth at various solute concentrations.

The method that we have used, so far, in such determinations consisted, again, in making motion pictures or still photographs, under the cryomicroscope, of crystallization units growing in thin preparations, between coverslips, and in measuring the length of the radii of the circular units.

The curves obtained in the second step of C_1 will not only tell about the maximal growth rate, but also about the null growth rate, an information which is needed in the study of the combined effects of different rates of nucleation and of crystal growth.

When various types of crystallization units, like irregular dendrites and spherulites, are formed, one needs to study their different growth rates and other different characteristics.

(12) *Series C_2: Evidence for Instability, at the Temperature of Maximal Rate of Crystal Growth, in Incompletely Frozen Material.*

Using material of a given concentration, in which we determined the temperatures of maximum crystal growth during cooling, and which we treated in such a way that a part of it is amorphous, we will examine it for the occurrence of changes when it is rewarmed to the temperature of maximal growth. Since the material is partly crystalline, the amorphous phase is in contact with the ice crystals and should readily crystallize when its molecules have acquired sufficient motility. The phenomenon, if it occurs as predicted, would apparently be a mere resumption of an interrupted crystallization.

(13) *Series C_3: Patterns of Ice Formation.*

This research will consist in extending to various solutions and to biological materials the studies made on gelatin solutions by Luyet and Rapatz (1958) and by Persidsky and Luyet (1960). The results should lead to the construction of graphs showing, for each solution or aqueous

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material, the crystallization patterns obtained at particular concentrations and temperatures (the coordinates of the graphs). Some of the effects of concentration and of temperature (and also of cooling rates) on the patterns of crystallization are illustrated in columns 2 and 3 of Fig. 4, in the case of gelatin solutions.

(14) *Series C_4: Amounts of Ice Formed.*

For more adequate information on the development of the ice phase in aqueous systems, one should obtain quantitative data on the fraction of the water in the sample which is transformed into ice, the fraction which is freezable but not frozen under a particular set of conditions, and the fraction not freezable. In our previous work, we used the methods of calorimetry and dilatometry for such determinations.

(15) *Series C_5: Combined Effects of the Rates of Nucleation and of Crystal Growth on the Number, Size and Type of Crystallization Units.*

After the curves of the rates of nucleation and of crystal growth in terms of temperature, for a given concentration of a particular solute, are established, it should be possible to calculate the approximate number and size of crystallization units of a given type which will grow under a particular set of conditions.

These data will permit the construction of a graph indicating, for a particular pattern, the size of the crystallization units grown at various temperatures and concentrations. Such a graph could be superimposed on the one constructed with the data of the preceding series (C_3), the size of the units determined in C_5 being marked on the territory of a particular pattern in the graph of C_3.

Column 3 of Fig. 4 illustrates the effect of gradually higher concentrations of gelatin in reducing the size and increasing the number of the crystallization units.

Since the number and size of crystalline particles depend greatly on the cooling rate, this series of projects calls for an accessory determination of the cooling rates obtained

when preparations of given configuration and dimensions are immersed in a bath at a given temperature.

The inhibition of nucleation and/or of growth could also be recorded in the graphs. The resulting partial development, or complete absence of crystallization units would account, at least in part, for the occurrence of some of the partially opaque, or entirely transparent samples represented in Fig. 4.

In all cases in which the existence of submicroscopic particles is suggested, a study with the electron microscope is planned.

CONSPECTUS ON THE RESEARCH PROGRAM OUTLINED

My plan in presenting here the 15 series of projects just enumerated was to integrate the work to be done with the work already done. Some of the projects listed were completed and the results were published several years ago; some have been completed for some time, though the results are not yet published; some, planned during the last few years, are still in progress; and some are still to be activated.

SUMMARY

1. Aqueous solutions "solidified" by cold (it is assumed that they are partly crystalline and partly amorphous) can be shown to undergo changes in molecular stability when, upon being rewarmed, they reach three temperatures: that of the glass transition, that of recrystallization, and the melting point. It is suggested that they might also become unstable at two other temperatures, namely, when they are rewarmed to the points at which the rate of nucleation and the rate of crystal growth would have been maximal in the previous cooling. Since one of these five "cases of instability", melting, is adequately covered in classical texts, it is not included in this survey.

2. Three aspects of the problem of instability are examined: (I) the facts on which the notion is based, (II) the

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Part I: The Basic Facts

3. Four types of recrystallization are described: (1) one in which the specimen turns rather suddenly opaque when rewarmed to a given temperature, the so called "recrystallization temperature"; (2) one in which some of the ice particles grow slowly larger at the expense of the small ones; (3) another in which a rapid growth of some ice particles takes place near the melting point; (4) and a fourth which involves a molecular reorientation, such as would be required for the passage from cubic to hexagonal ice. Four varieties of the first type are also described: recrystallization may be (a) induced or (b) spontaneous, and it may affect (c) rapidly grown or (d) slowly grown crystallization units.

4. The temperatures at which recrystallization of type (1) occurs depend primarily on the molecular weight of the solute and are only slightly affected by its concentration. The values obtained with solutes of various kinds are presented in a table. Recrystallization of type (2) occurs at temperatures above those reported for type (1).

5. The complex changes which take place in solutions of gelatin of various concentrations, frozen at −100° or at −30°, and rewarmed to −30° or −10°, are systematically arranged in a table of 28 photographs (Fig. 4); particular changes of questionable nature are then selected for further study.

Part II: Present State of Our Knowledge of the Facts Reported

6. Most of the cases reported in Part I seem explainable on the basis that a part of the material which has crystallized during cooling recrystallizes upon being rewarmed, and that a part which has remained amorphous merely crystallizes. Some of the cases may involve the separate,

others the simultaneous occurence, during rewarming, of a recrystallization and a resumption of an interrupted crystallization.

7. The changes designated as irruptive, slow-pace and premelting recrystallizations may all be accountable by a migration of molecules from the small to the larger particles (migratory recrystallization).

8. The fact that the changes observed during the rewarming of rapidly cooled gelatin gels, and interpreted as indicative of a transition from cubic to hexagonal ice, occur when the temperature approaches that of recrystallization, raised again the question of whether the X-ray diagrams recorded do not indicate a preferred orientation at the time the crystals are growing rather than a reorientation from a cubic to a hexagonal crystal.

9. The commonly accepted interpretation according to which, above the temperature of the glass transition, the material has the properties of a supercooled liquid in which the molecules are capable of translational motion, and below that temperature, it has the properties of a "solidified" glass, in which translational motion is suppressed, seems to account for the facts observed.

10. The superimposition, in various ways, of the curves of the rates of nucleation and of the curves of the rates of crystal growth, plotted in terms of temperature, apparently explains the behavior of different solutions in regard to the patterns of crystallization and to the number and size of crystallization units actually observed.

Part III: Research Projects

11. In an attempt at coordinating the research projects undertaken or to be undertaken on the structural instability and molecular mobility in aqueous solutions "solidified" at low temperatures, I outlined 15 series of projects, classified into 4 categories (3 on the glass transition, 5 on recrystallization, 2 on nucleation and 5 on crystallization) ; some of these projects have been conceived years ago, others more

recently; some of them have been completed, some are in progress, others have not yet been activated; the results of some of the projects have been published, the results of some are in press, the results of others are being analyzed.

12. The whole research program may be presented as aiming at the construction of "phase diagrams" which would give the temperature of occurrence of each of the events mentioned as indicative of molecular instability, in terms of the concentration of one or more solutes in aqueous solutions and in biological materials. The research projects listed are planned to furnish the data required for the construction of such diagrams, to permit the identification of the "cases of molecular instability", and to contribute to the elucidation of the nature of the processes involved.

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