RECENT RESEARCH IN FREEZING & DRYING

Edited by

A. S. PARKES
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FOREWORD

By A. S. Parkes

The First International Symposium on Freeze-Drying, organized by the Institute of Biology in 1951, was a most stimulating and informative meeting, and the proceedings published by the Institute under the title *Freezing and Drying* formed a valuable work of reference. Advances in this field, however, were so rapid that in 1958 the Institute thought it desirable to organize a second symposium on similar lines. Some hesitation was felt at thus trying to repeat a successful experiment, but, in the event, the second symposium was even more successful, valuable and well-attended than the first one. Many distinguished workers took part and again it was a great pleasure to welcome participants from overseas, including such old friends as Professor Luyet, Commander Hyatt, Dr. Meryman, Dr. Rey, Dr. Spaander, Professor Kreyberg, and many others. As in 1951, Professor Flosdorf played a prominent part in the 1958 symposium, and his death shortly afterwards was a tragic blow to all those engaged in the study of freeze-drying. Three overseas contributors to the programme were unfortunately unable to attend—Professor Kalabukhov, Dr. Nei, and Dr. Obayashi—but their papers were delivered on their behalf or read in title, and are included in this volume.

Much of the symposium dealt with the effect of freezing and it was noticeable that the use of glycerol or other protective substances, in its infancy at the time of the earlier meeting, now received major attention. In the field of freeze-drying the symposium was remarkable for the fact that one speaker after another recorded striking advances, especially in the preservation of potential function or viability of biological material. In general, the proceedings left no doubt that the study of the biological application of freeze-drying is one of the active growing-points of science. In closing the meeting, the Chairman expressed great satisfaction at this and other evidence of the upsurge of biology in a world currently thought to be dominated by physics and chemistry, and concluded with the time-honoured saying: 'The impossible we do immediately; miracles may take a little longer'. The appropriateness of this remark is well shown by Dr. Meryman's recent report of the survival of viability and fertilizing capacity in freeze-dried and reconstituted bull spermatozoa.

The success of the second symposium in 1958, like that of the first in 1951, depended in the first place on the excellence of the organizational work by Dr. R. J. C. Harris, Chairman of the Symposium Committee, assisted by Dr. S. T. Cowan and Mr. L. G. Beckett. The Institute, also, has to thank all those who gave papers and took part in the discussions, and in so doing set up another milestone in the history of freeze-drying.

4 *November* 1959.

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PART I

Biophysics of Freezing

ON THE MECHANISM OF GROWTH OF ICE CRYSTALS IN AQUEOUS SOLUTIONS AND ON THE EFFECT OF RAPID COOLING IN HINDERING CRYSTALLIZATION

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As, in the study of the effects of freezing on biological systems, the need for a better knowledge of the basic physical principles of ice formation became more and more pressing, my research associates and I have concentrated our efforts, during the last few years, on the problem of the mechanism of growth of the ice phase in physical systems. I intend to present here some of our main findings and to discuss them from the particular point of view of their relationship to the hindering of ice growth by rapid cooling.

I shall, first, say a few words on the techniques employed. Then I will describe the types of ice formations that we obtained in the presence of different solutes, at various concentrations of these solutes and at various freezing temperatures. Next I will examine the mechanism of construction of the ice phase from the water molecules as the building blocks, and the retarding and disturbing action of the molecules of the solutes on those of water in that construction. In a last section of the paper I will discuss the effect of rapid cooling in hindering crystallization, with special reference to vitrification, the vitreous state and devitrification. This will call for some remarks on semantics.

Techniques

A drop of an aqueous solution of a crystalloid, mainly sucrose and glycerol, or of a colloid, mainly gelatin and albumin, was placed between two cover glasses and the preparation thus obtained was dropped abruptly into a bath precooled to temperatures from 0° to −150°. The preparation, held in a specially constructed dropping mechanism (Figure 1) fell to a prefocused position in front of the objective of a microscope placed horizontally (Figures 1 and 2), where it could be observed, photographed or cinematographed. In the latter case, the dropping mechanism automatically triggered a motion picture camera at the desired time. (For further details on the apparatus and the procedure see Luyet and Rapatz, 1957; and Rapatz and Luyet, 1957.)

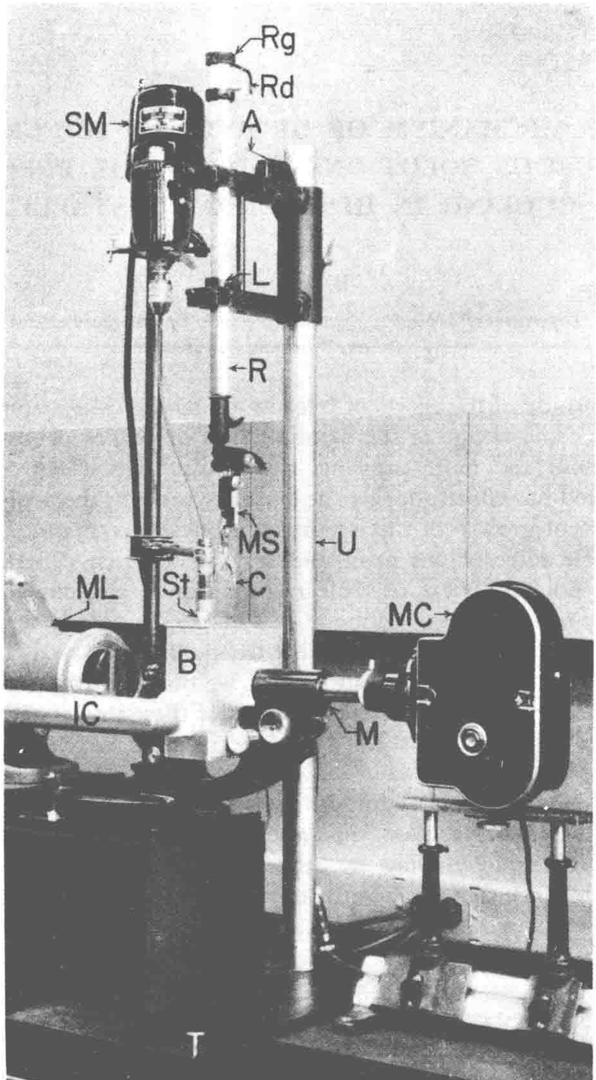

FIG. 1. Photograph of apparatus for photomicrography and cinemicrography during rapid freezing. B, container of cooling bath; M, microscope; MC, motion picture camera; R, dropping rod; U and T, upright and base of dropping mechanism; C, clamp holding the preparation; MS, mechanical stage; L, lock releasing the dropping rod; Rg, ring carrying a rodlet Rd which closes a circuit by contact with the arm A of a microswitch and thus triggers the motor of the camera; St, stirrer, SM, motor for stirrer; ML, microscope lamp; IC, insulated conduit for refrigerated air. (From Rapatz and Luyet, 1957.)

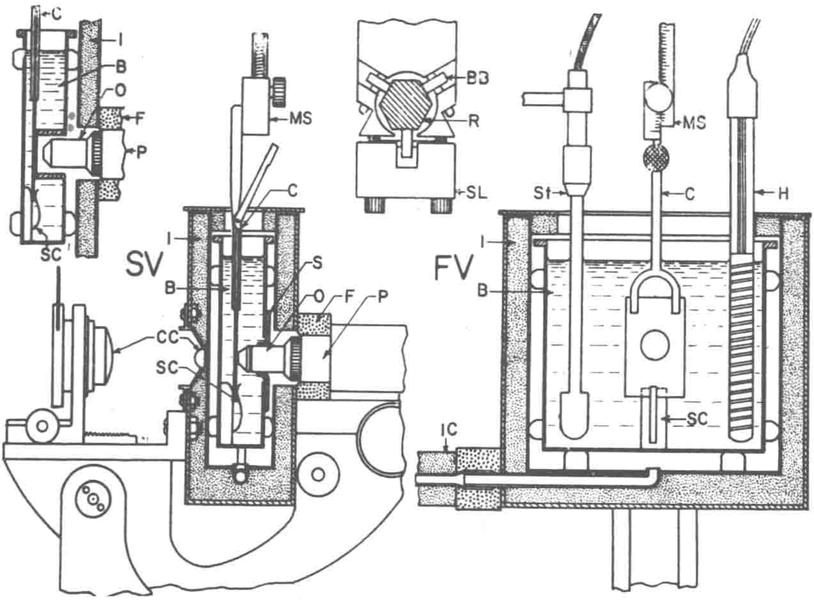

FIG. 2. Diagrams showing the details of some of the parts of the apparatus represented in Fig. 1. FV: front view; SV: side view. (The same parts are represented by the same letters in the two views.) B, cooling bath; I, 'styrofoam' insulation of bath container; St, stirrer; H, heater; O, objective of microscope; S, rubber sleeve connecting objective and bath container; P, plastic tubing connection; C, clamp holding the preparation; MS, mechanical stage; SC, stage clamp; CC, two components of the condenser system; F, foam rubber muff; IC, insulated conduit for refrigerated air. The insert at the upper left corner shows the arrangement used with low power objectives. (The letters refer to the same parts as in the side view SV of the apparatus with the immersion objective.) The insert in the centre shows the details of the ball bearing slide for the dropping rod: R, dropping rod; BB, ball bearings; SL, spring loading unit. (From Rapatz and Luyet, 1957.)

The cooling or freezing curves were traced by a high speed recording galvanometer, the 'Visicorder', built by the Heiland Company of Denver. The sensing element of that instrument was a 1-mil, or a 3-mil, copper-constantan thermocouple, of which one junction was inserted in the preparation.

TABLE 1

STRUCTURE OF SOLIDIFIED MATERIAL AND TYPES OF CRYSTALLINE FORMATIONS OBSERVED IN THE FREEZING OF AQUEOUS SOLUTIONS

Structure:	*Arrangement:*	*Forms:*
Crystalline	Hexagonal	1. Prisms
		2. Stars
		3. Rosettes
		4. Skeletons
		5. Transition Forms
	Radial	6. Spherulites (Plain)
		7. Spherulites (Evanescent)
		8. Lobed Formations (Irreg. Rosettes)
	Diffuse	9. Recrystallization Clouds
Amorphous.		

I. Types of Ice Formations

The types of ice formations obtained may be classified, on the basis of their morphology and their structure, into nine categories, as shown in Table 1.

I shall now examine briefly those various structures.

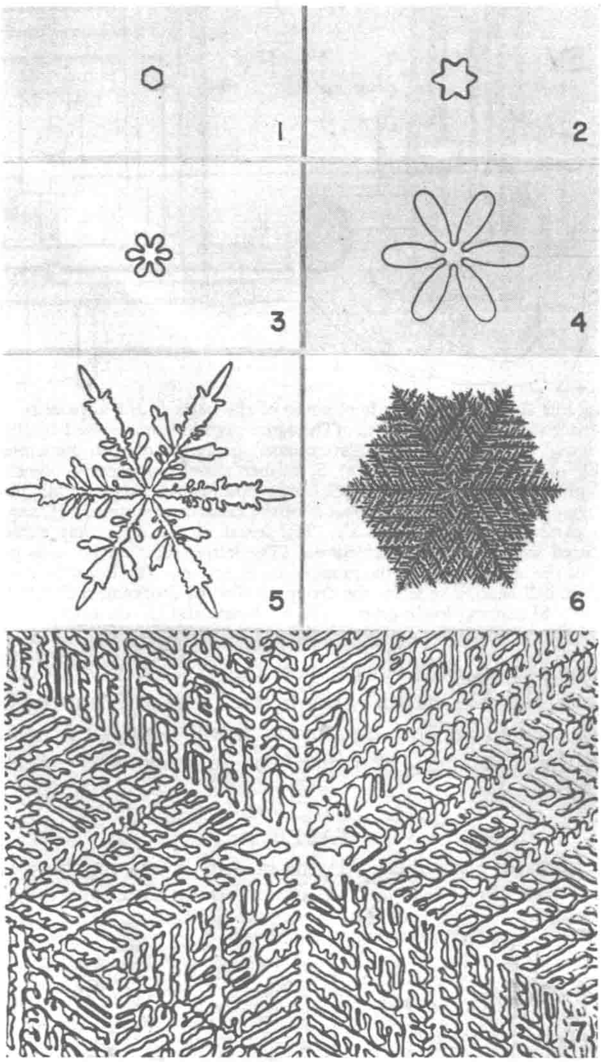

FIG. 3. Forms of hexagonal symmetry obtained in the freezing of 35% bovine albumin solutions at bath temperatures of $-1\cdot5°$ to $-3°$. (*1*) Hexagonal prism; (*2*) Star; (*3*) and (*4*) Rosettes; (*5*) and (*6*) Skeletons, in formation and fully formed; (*7*) Skeleton grown at a lower rate than that shown in (*6*). (As the original negatives showed very little contrast, because of the nearly equal transparency of the ice crystals and the solution, the contrast was intensified by two successive photographings on 'contrast' emulsions.) Magnif.: 95×.

*Forms of Hexagonal Symmetry**

Biologists, studying the freezing of tissues, often wonder why they generally find spheroidal masses of ice, instead of the hexagonal crystals described as characteristic of that substance and well known in snow flakes. The main reason, at least in the case of solutions, seems to be a too rapid freezing. We succeeded, by controlling the freezing temperature and the solute concentration, in regulating the rate of growth of the ice phase in physical systems and in obtaining, at will, hexahedral forms such as those shown in Figure 3.

The first hexahedral forms that we saw to appear, generally at high temperatures and high concentrations, were solid prisms of tabular habit (Figure 3.*1*).† When these had grown to a certain size, we observed a faster development along three of the axes of the prisms than in other directions. This mode of growth brought about the formation of six-sided stars (Figure 3.*2*) and six-sided rosettes (Figures 3.*3* and 3.*4*). Then secondary branches sprouted out of the six main stems (Figures 3.*5* and 3.*6*), tertiary branches from the secondary ones, etc., the final result being a dendritic skeleton (Figures 3.*6* and 3.*7*).

*Forms of Transition from Hexagonal Symmetry to Radial Arrangement**

At lower temperatures (higher cooling velocities) the patterns of hexagonal symmetry become gradually more irregular (Figures 4.*1* and 4.*2*) and finally they give way to formations in which the component elements are arranged radially around a centre (Figure 4.*3*).

Spherulitic Discs

The structure then becomes of the type described by crystallographers as 'spherulites'; in our flat preparations, we have 'spherulitic discs' (Figures 5.*1*, 5.*2* and 5.*3*). They present a particularly spectacular sight in polarized light, between crossed nicols (Figure 5.*6*).

When the cooling velocity becomes high and the water content low, the fibres of the spherulitic discs become very thin and one sees the crystalline phase extend through the preparation in the form of a flimsy veil. Then the veil itself fades away and the field appears uniformly transparent under ordinary light (white areas in Figures 5.*4* and 5.*5*). I designate this type of ice formation as evanescent spherulitic discs, and distinguish them from the plain spherulitic discs.

* Whereas hexagonal structures are the result of an arrangement of molecules, spheroidal structures appear as the result of an arrangement of larger units (crystallites). The former possess a true hexagonal 'symmetry', in the sense attributed to those terms by crystallographers. The latter have a relatively coarse arrangement of an indefinite number of component elements radiating from centres of crystallization; I describe it summarily here as radial 'arrangement'.

† Most of the photographs of ice formations reproduced in this report are taken from two papers, now in press (*Biodynamica*, Vol. 8), one by B. Luyet and G. Rapatz, the other by G. Rapatz and B. Luyet. The reader is referred to these papers for full details.

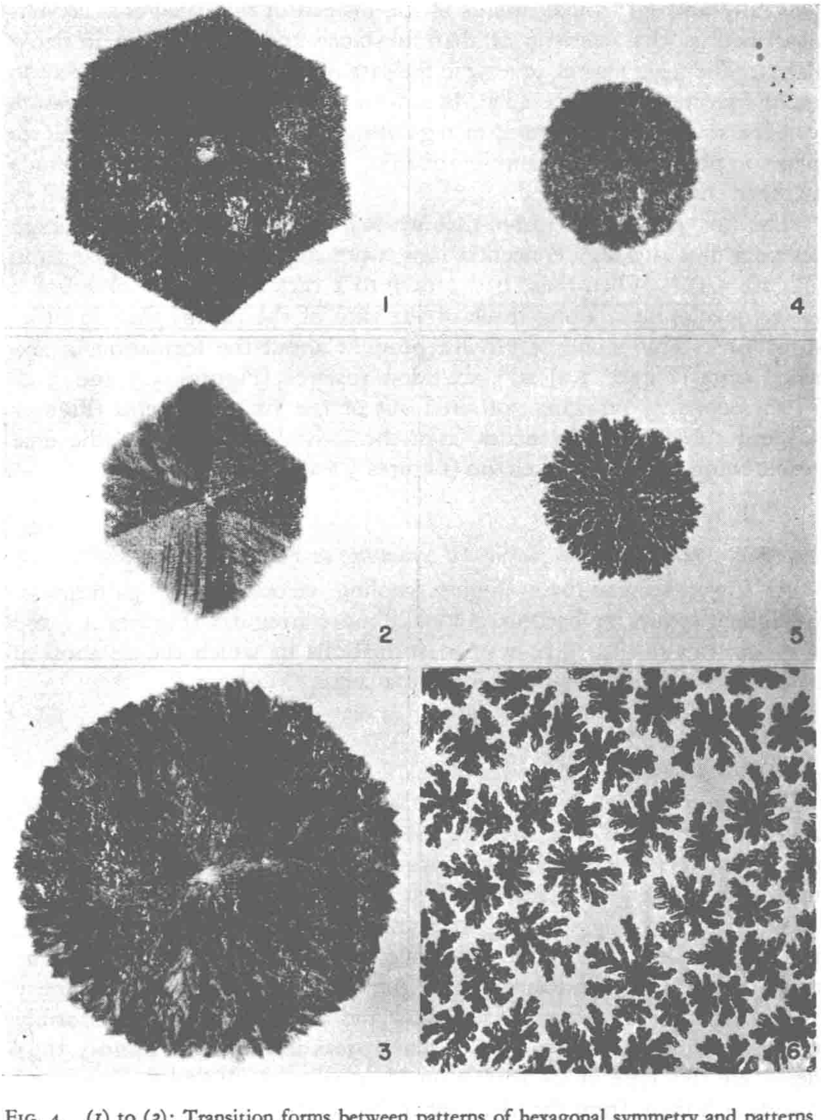

FIG. 4. (*1*) to (*3*): Transition forms between patterns of hexagonal symmetry and patterns with radial arrangement, obtained in solutions of sucrose. Conc.: 50%; Bath temp.: −20°, −20° and −25°, respectively. (*4*) to (*6*): Transition forms in the development of 'irregular rosettes', obtained in solutions of polyvinyl pyrrolidone. Conc.: 50%; Bath temp.: −30°, −45° and −55°, respectively. Magnif.: 80×.

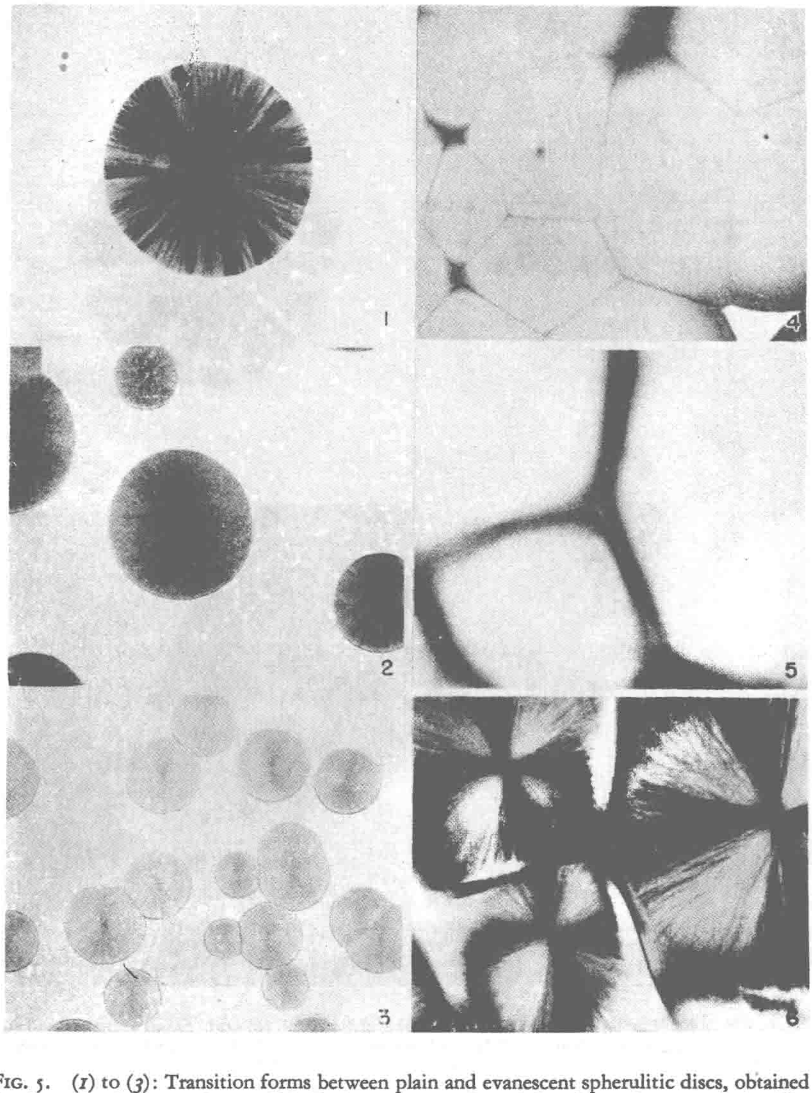

FIG. 5. (*1*) to (*3*): Transition forms between plain and evanescent spherulitic discs, obtained in glycerol solutions. Conc.: 6M; Bath temp.: −55°, −65° and −70°, respectively. The photographs were taken before the discs had had time to cover the field. (*4*) to (*6*): Evanescent spherulitic discs obtained in 50% sucrose (*4*) and in 30% gelatin (*5* and *6*). Bath temp.: −45°, −40° and −40°, respectively. The photographs were taken after the discs had completely invaded the field. The areas on which adjacent discs came in contact became dark, as a result of recrystallization. (*6*) shows the same formations as (*5*) in polarized light, between crossed nicols. Magnif.: 65×.

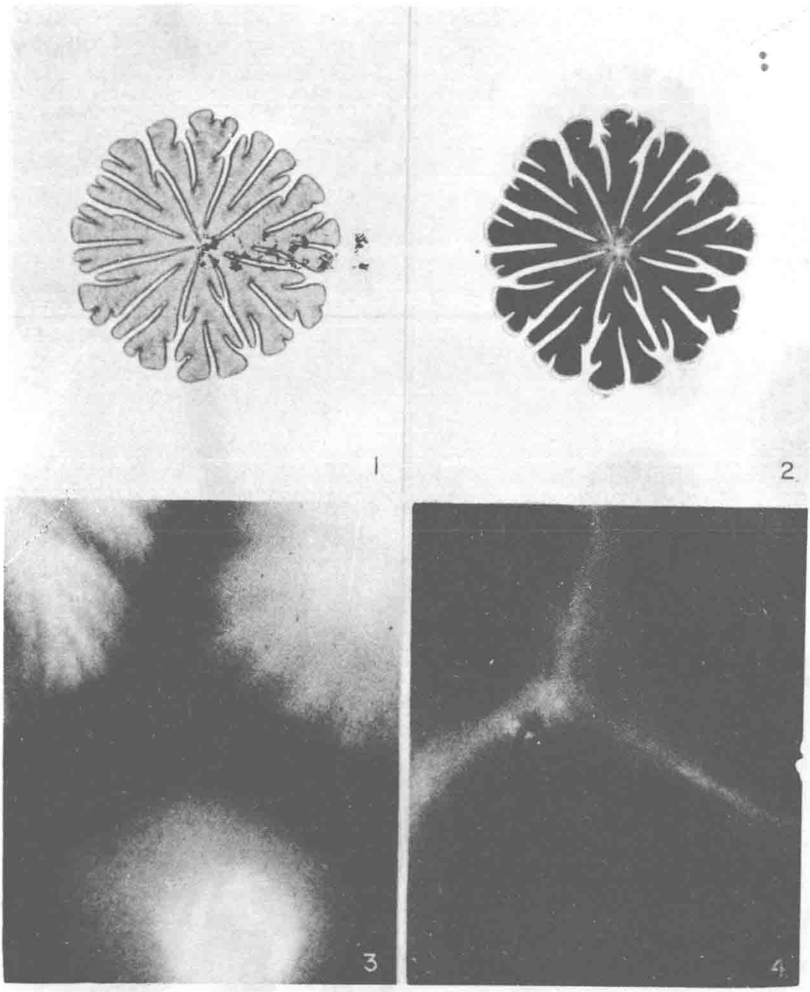

FIG. 6. Rec ystallization in a rosette and a spherulitic disc. (*1*) and (*2*): Rosette formed in a 50% gela in gel, in a bath at −30° (*1*) and rewarmed to −10° (*2*). (*3*) and (*4*): Evanescent spherulitic discs formed in a 30% gelatin gel, in a bath at −30° (*3*) and rewarmed to −10° (*4*). Magnif.: 80×.

Irregular Rosettes

Instead of the plain spherulitic discs, with radiating fibres, solutions of gelatin and polyvinyl pyrrolidone give rise to formations which consist of broad lobes growing from crystallization centres. In our flat preparations, these formations had the shape shown in Figure 6.*1* and Figures 4.*5* and 4.*6*. I shall designate them as 'irregular rosettes'.

Recrystallization Clouds

When the spherulitic discs and the irregular rosettes, formed at relatively low temperatures, are rewarmed, they assume a milky appearance and become opaque, as a result of the formation, in them or on them, of a dense cloud of minute crystals (Figure 6). (The temperature at which they become opaque is about $-10°$ for gelatin, $-30°$ for sucrose and $-65°$ for glycerol solutions.) This phenomenon is generally described as 'devitrification'. We have shown (Luyet and Rapatz, 1957a) that it is a recrystallization, as it occurs in material which had already crystallized. (For further details about it, see below.)

The formation of such clouds which, as was said, generally takes place upon rewarming, may be observed in the course of the original cooling, at places where evanescent spherulitic discs meet their neighbours (Figures 5.*4*, 5.*5* and 6.*3*).

Amorphous Phase

Under the proper conditions of solute concentration, temperature and cooling velocity, the material solidifies without showing any change in its appearance and in its optical properties; this material seems to be truly amorphous (Figure 6.*1*, the space around the rosette).

II. On the Mechanism of Growth of the Ice Phase

The study of crystalline growth presupposes some knowledge of the structure of the materials used, the nature of the forces involved and the architecture of the edifice to be built. For information on those matters we depend on the work of the physicists; and I shall, in the considerations to follow, adopt, without further discussion, the generally accepted views of modern physicists on the structure of (1) the water molecule, (2) liquid water, (3) ice crystals and (4) vitreous material. (For some bibliographical references see Luyet, 1957.)

Construction of an Ice Crystal

The molecule of water, H–O–H, is pictured as a V-shaped body (Figure 7), in which the hydrogen nuclei occupy the ends of the two legs of the V, and the oxygen nucleus is at the bifurcation point. That body is endowed with forces which are centred in two positive charges carried by the hydrogen atoms (charges which give rise to the hydrogen bonds) and in two corresponding negative charges on the oxygen side (signs + and –, respectively, in Figure 7).* The reciprocal attraction of these charges causes the molecules to become firmly attached to each other; in the liquid state, the connections were loose and labile.

The mode of arrangement of the molecules of water may be analysed

* The reciprocal action of the molecules of water in orienting and displacing themselves, before adhering to each other, was demonstrated with spherical wood models, in which magnets were inserted, in such a way that charges of one sign were represented by two north poles and charges of the opposite sign by two south poles.