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Non-Equilibrium Freezing Behaviour of Aqueous Systems [and Discussion]

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Non-equilibrium freezing behaviour of aqueous systems

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[Plates 1 and 2]

The tendencies to non-equilibrium freezing behaviour commonly noted in representative aqueous systems derive from bulk and surface properties according to the circumstances. Supercooling and supersaturation are limited by heterogeneous nucleation in the presence of solid impurities. Homogeneous nucleation has been observed in aqueous systems freed from interfering solids. Once initiated, crystal growth is often slowed and, very frequently, terminated with increasing viscosity. Nor does ice first formed always succeed in assuming its most stable crystalline form. Many of the more significant measurements on a given system can be combined and displayed in the form of a 'supplemented phase diagram', the latter permitting the simultaneous representation of thermodynamic and non-equilibrium properties. The diagram incorporates equilibrium melting points, heterogeneous nucleation temperatures, homogeneous nucleation temperatures, glass transition and devitrification temperatures, recrystallization temperatures, and, where appropriate, solute solubilities and eutectic temperatures. Taken together, the findings on model systems aid the identification of the kinetic and thermodynamic factors responsible for the freezing – thawing survival of living cells.

Introduction

Many factors help to determine whether or not an aqueous system and, in particular, a biological system, tends to the thermodynamic state of lowest free energy during cooling. Metastable thermodynamic states frequently persist, at least at higher sub-freezing temperatures; complete conversion, once begun, to other, stabler states is often subject to kinetic hindrance. Kinetic and thermodynamic factors may, moreover, interact and prove very difficult to distinguish. The complexities are multiplied wherever systems are subject to very rapid cooling to lower sub-zero temperatures. They are further complicated when living cells and other similarly compartmented systems are employed. We will attempt in this presentation to draw attention to the principal non-equilibrium phenomena most frequently encountered in the attempted freezing of representative aqueous systems. It will be convenient and, we hope, useful to examine these phenomena in the approximate order in which they are encountered in practice, and to interrelate them by reference to various biophysical and physicochemical concepts. Beginning with supercooling we will consider rapid freezing from supercooled states, proceeding after that to reconstruct kinetic and thermodynamic phase behaviour, following which it will be helpful to discuss some biological implications.

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Supercooled water and heavy water

The equilibrium freezing of aqueous systems requires, among other things, that an ice crystal nucleus be made available at the equilibrium freezing point of the system, and that appreciable temperature differences not exist. Supercooling arises where one or other, or each of these conditions is not met. Being, therefore, an integral part of non-equilibrium freezing processes, supercooling excites considerable interest. One wishes to know both the properties of the supercooled states and the limits to which supercooling proves possible.

Earlier efforts to study supercooled states were often hindered by premature freezing, this being a factor very difficult to prevent in bulk specimen studies (Hallett, for example, measured the viscosity of carefully purified, supercooled water in the range 0 to −24 °C but found that the water froze, generally between −20 and −25 °C, this despite the knowledge that water could be supercooled in the absence of impurities to about −40 °C). Only when it was found that water could be subdivided into very small droplets and maintained in stable emulsion in circumstances in which the properties of the water were not affected by the emulsification, was it possible readily to extend the investigations of the properties of supercooled aqueous systems to lower temperatures (Angell, Shuppert & Tucker 1973; Hindman & Svirmickas 1973; Hindman, Svirmickas & Wood 1973; Rasmussen & MacKenzie 1973). The subdivision of the aqueous phase allowed, apparently, the creation of numbers of droplets orders of magnitude greater than the numbers of impurity particles present in the original sample. All but an insignificant fraction of the droplets obtained in the emulsification process behaved, therefore, as though the water were totally pure. Similar emulsification procedures have been applied to negate the effects of solid impurities in other systems, metallic and non-metallic (Turnbull 1952; Cormia, Price & Turnbull 1962; Miyazawa & Pound 1974; Rasmussen & Loper 1975, 1976; D. H. Rasmussen & A. P. MacKenzie, unpublished experiments).

The above-mentioned emulsification procedures made possible the study of the systems in question to their respective *homogeneous nucleation temperatures*, the temperatures at which the solvent molecules present, water for instance, undergo spontaneous crystallization in the absence of any impurity (the crystallization of a component of the system *on* the latter is called *heterogeneous nucleation*). Where conditions were appropriate, such 'clean' systems were found to pass, with sufficient cooling, to stable amorphous states in which case both heterogeneous and homogeneous nucleation were circumvented.

Several studies of the properties of water and heavy water in the temperature range between the equilibrium freezing point and the respective homogeneous nucleation temperature are now available (Angell *et al.* 1973; Rasmussen & MacKenzie 1973; Hindman & Svirmickas 1973). Rasmussen & MacKenzie (1973) measured specific heats and volumes of water and heavy water emulsified in 5% by mass solutions of sorbitan tristearate (SPAN 65) in *n*-heptane. They used standard dilatometric and classical, drift-type calorimetric methods. Angell *et al.* (1973) applied differential scanning calorimetric methods to obtain essentially the same specific heat capacity with somewhat higher accuracy from generally similar systems. The dilatometric data are reproduced in figures 1 and 2, in each of which one notes the decreased dilatometer readings with decreasing temperature (curves A, A). The dilatometer readings reflect the overall contraction of the water and the carrier fluid in each case. One notes a sudden increase in volume at about −36.5 °C in figure 1, and −31 °C in figure 2, corresponding to the freezing of the water in the separate droplets by homogeneous nucleation. Further cooling and rewarming caused the

systems to follow the respective curves B, B (figures 1 and 2, again) during which the water and the heavy water remained completely frozen, up to their respective equilibrium melting points. Generally it proved possible, once the ice was melted, and the system returned to curve A, to achieve a cooling to the same homogeneous nucleation temperature before the observation of the sudden volume change indicating the freezing of the water present. The respective emulsions were therefore stable to the repeated freezing and thawing of the water present.

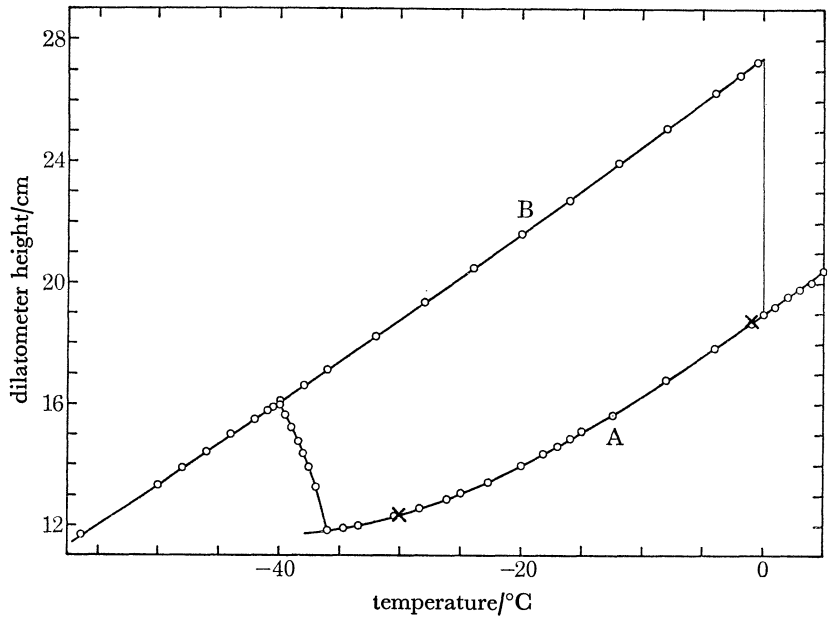

FIGURE 1. Temperature dependence of the volume of an aqueous emulsion. Curve A, supercooling and eventual freezing of emulsified water. Curve B, further cooling and rewarming of the frozen emulsion. Xs indicate readings obtained when the melted emulsion was cooled a second time to −30 °C, and rewarmed, without further cooling, to −1 °C. (By permission of the American Institute of Physics.)

Converting the primary data to specific volume, one obtains the values reproduced in figure 3, in which we have also inserted the known values of the specific volumes of ice and heavy ice. It is immediately evident that the measurements suggest far greater changes in specific volume with supercooling than might have been predicted on the basis of observations at higher sub-freezing temperatures. It would appear, however, that the extrapolation of the plots describing the specific volumes of the supercooled states suggests the intersection of the same and the plots describing the specific volumes of the respective crystalline states at temperatures 50–60 °C below the respective equilibrium freezing points. It is obvious, in any case, that the supercooling attained before homogeneous nucleation is accompanied in each instance by a very remarkable expansion.

Correspondingly unexpected results were obtained during the determination of the specific heats of supercooled water and heavy water (Rasmussen, MacKenzie, Angell & Tucker 1973). While deviations were small in the first 10 °C below the respective equilibrium freezing points ever larger increments in specific heats were observed with deeper supercooling. It appeared, in fact, to a rough approximation, that the specific heat capacities of the supercooled liquids rose asymptotically to the respective homogeneous nucleation temperatures, this despite the near-certainty that the experiments were carried out in the absence of any premature freezing. By the

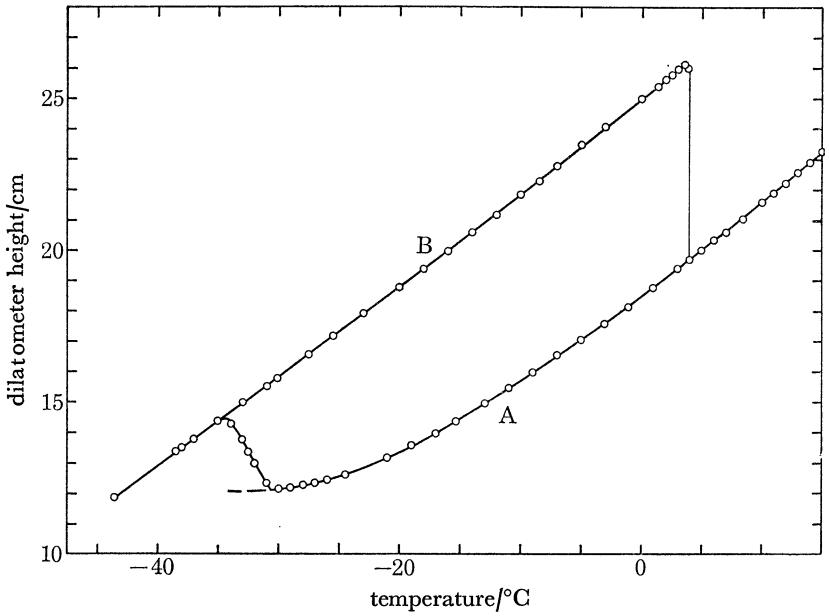

FIGURE 2. Temperature dependence of the volume of an emulsion containing heavy water. Curve A, supercooling and eventual freezing of emulsified heavy water. Curve B, further cooling and rewarming of the frozen system. (By permission of the American Institute of Physics.)

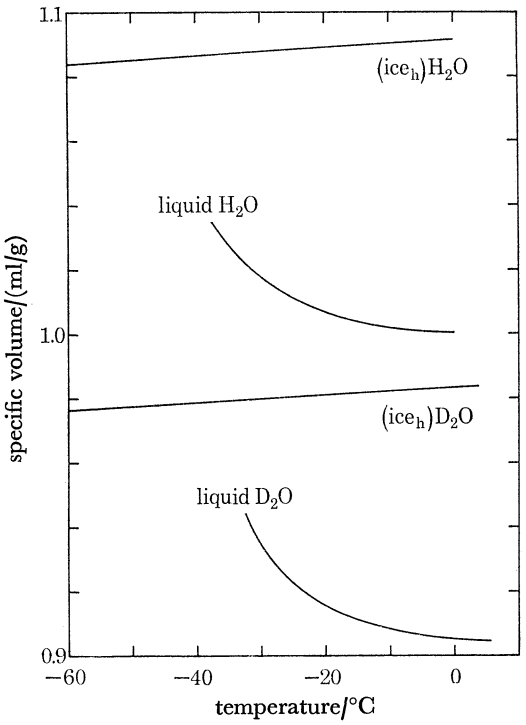

FIGURE 3. Temperature dependence of the specific volumes of water, ice, heavy water, and heavy ice.

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time the water and the heavy water were supercooled 35 °C below their equilibrium freezing points, the specific heat capacities had risen by roughly the specific heat capacity of ice at the melting point.

Various detailed explanations for the observed changes in specific volume and specific heat capacity have been advanced (Schufle & Venugopalan 1967; Zheleznyi 1969; Angell *et al.* 1973; Rasmussen & MacKenzie 1973). According to preference, it is quite likely that increased supercooling promotes a greater, generalized, non-icelike structuring in the presence of which an increasing fraction of the water molecules tend to form discrete, geometric clusters. It is assumed that it is the presence of clusters having hexagonal symmetry that is responsible for the observed homogeneous nucleation (Rasmussen & MacKenzie 1973). It has been suggested, on the other hand, that the anomalous behaviour of the supercooled water and heavy water is best explained in terms of a more and more orderly bond lattice, the hydrogen bonds between the water molecules strengthening as ordering increases with supercooling (Angell *et al.* 1973). Suffice it to say, for the present, that the properties of supercooled water and heavy water are not properly explained as yet by any known one- or two-state model. Nor does a single interpretation explain both specific volume and specific heat anomalies.

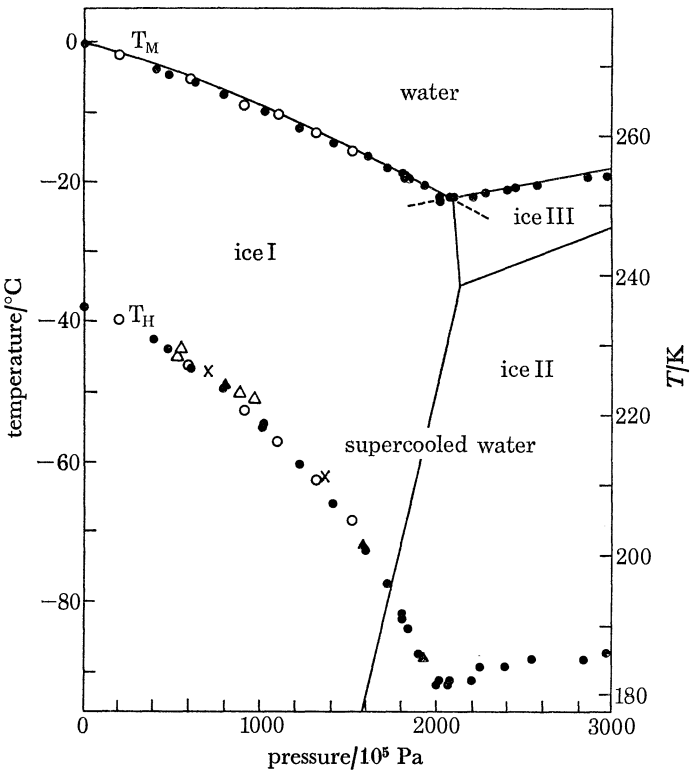

FIGURE 4. Pressure dependence of the temperatures of homogeneous nucleation and equilibrium melting of ice formed in aqueous emulsions (with the kind permission of the authors (Kanno *et al.* 1975) and *Science*).

carrier fluid	apparatus 1	apparatus 2
heptane	△	○
pentane	×	
MCH	▲	
MCH + MCP		●

The studies on the supercooling of water have, so to speak, been carried into another, most interesting dimension by Kanno, Speedy & Angell (1975) who reported the supercooling of water under pressures as high as 3 kbar (300 MPa). Their findings, reproduced in figure 4, demonstrate a minimum homogeneous nucleation temperature in pure water at 2.0 kbar (200 MPa) and −92 °C, at which temperature supercooling appears to have attained a maximum. It will be seen that the homogeneous nucleation temperature plot exhibits the same form as the equilibrium melting point plot, suggesting in the process that the local molecular order in the supercooled liquid 'changes systematically with pressure in the direction of the ice III local structure' (Kanno *et al.* 1975). With a means at hand for preventing freezing except by homogeneous nucleation, the area between the T_M and the T_H curves in figure 4 can be considered available for further studies on supercooled water.

Hardly more than a beginning has been made on the study of the thermodynamic and other properties of supercooled water and heavy water and much further information is badly needed. Heat capacities and densities might well be redetermined as functions of external pressure; nuclear magnetic resonance studies offer promise (Hindman & Svirmickas 1973); infrared measurements could be made. More than other primary properties, however, the vapour pressure of supercooled water needs to be determined to the lowest possible temperatures. A knowledge of the vapour pressure of supercooled water could, it appears, become a most important factor in the development of a general theory of the hydration of water soluble substances at sub-zero (°C) temperatures, and in the further development and understanding of the thermodynamics of the freezing process.

The supercooling of aqueous model systems – lower temperature limits

Having thus described some of the properties of supercooled water we come to the question of the precise extent to which water can be supercooled before it freezes by homogeneous nucleation. It will become apparent that the question is central to the subject of this presentation. It will also be seen that homogeneous nucleation is essentially kinetic in nature, and that the findings contrast with the thermodynamic measurements on the metastable states resulting from 'stable' supercooling. The homogeneous nucleation of water has, in fact, been found to be a function of droplet size and cooling rate, smaller droplets and higher cooling rates yielding lower homogeneous nucleation temperatures (Wood & Walton 1970; D. H. Rasmussen, private communication). The detailed interpretation of the latter findings is still a matter of debate. We will describe some of the findings on pure water, extending the description to the determination of the temperature of homogeneous nucleation of ice in numerous aqueous solutions, and in cells suspended in aqueous solutions.

To measure homogenous nucleation temperatures we employed the method of differential thermal analysis, which we conducted during slow cooling. Specimen and inert reference materials were placed in identical glass capillary tubes which we inserted in adjacent holes in a metal block, the latter being surrounded by a copper coil, the whole assembly being well insulated from external perturbations. 5–10 μl samples were employed. Cooling rates were adjusted according to the rate at which nitrogen gas at −196 °C was drawn through the copper coil. Sample emulsions were generally inserted at room temperature, or at 2 °C, into a block adjusted to the same temperature. Thermal junctions were inserted in sample and reference, one

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in the former, two in the latter. The set-up allowed the recording of the difference between the sample and the reference temperatures (δT) and the temperature of the reference (T). In the absence of singular thermal events in the specimen, δT remained small or, in the ideal case, non-existent.

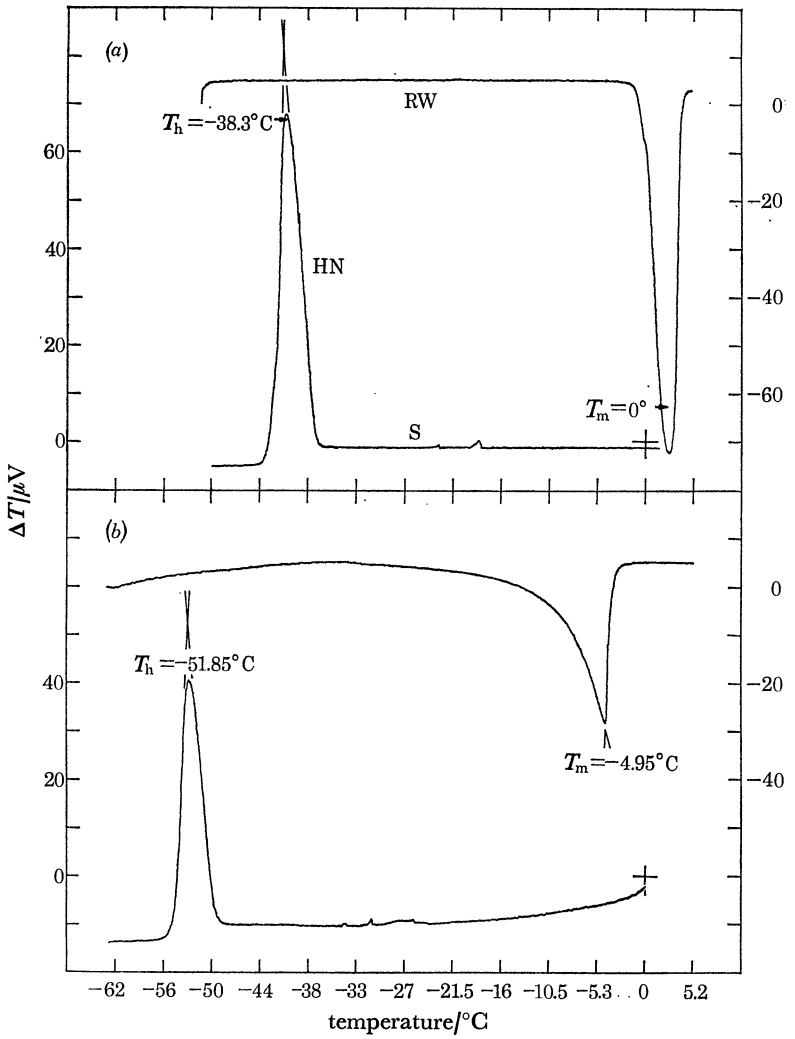

FIGURE 5. Thermograms obtained by the slow cooling and subsequent rewarming of aqueous emulsions. (*a*) distilled deionized water. Lower curve: supercooling (S) and homogeneous nucleation of ice (HN). Upper curve: rewarming (RW) and melting of ice. (*b*) corresponding study on a 40% (by mass) aqueous sucrose solution.

Figure 5 (*a*) reproduces the supercooling of an aqueous emulsion of water in *n*-heptane during which a cooling rate of about 2 °C/min was employed. The results are displayed in the form of an *X*–*Y* plot obtained in the course of the supercooling. A stable *dc* amplifier was used to raise the level of the δT signals to those required by the *X*–*Y* recorder. A steady horizontal trace (S) denotes the supercooling of the water to about −38 °C, at which temperature a sudden rise in the trace indicates the release of the latent heat of fusion of the water present. The peak (HN) sums the separate contributions of the *ca.* 10^9 droplets in the sample. Further cooling below HN appears not to induce any other thermal events.

Thermal analysis can be continued when the same sample is subjected to a controlled rewarming (a heating coil was incorporated into the metal block for the purpose). The upper curve (RW, figure 5*a*, again) denotes the rewarming of the frozen emulsion. (The δT offset was adjusted the better to separate the recording of the various thermal events.) It will be seen that the curve RW demonstrates only an equilibrium melting of the ice at 0 °C, in accordance with expectation (the lowering of the melting point with droplet diameter was too small to detect). It proved possible, as mentioned previously, to cycle such a sample a second time, with identical results. Individual droplets proved, moreover, in other experiments, to be separately stable to freezing and thawing. Homogeneous nucleation temperatures varied from −36.5 to −39 °C, according to the droplet diameter and the cooling rate. Slightly smaller supercoolings were obtained with heavy water (from +3.8 to *ca.* −31 °C). Similar findings have since been reported by Lafargue *et al.* (1974). The results have been the subject of a detailed kinetic analysis (Rasmussen & MacKenzie 1973; D. H. Rasmussen, private communication).

We soon found we could make emulsions containing a great variety of aqueous solutions and subject them to the same sort of differential thermal analysis by slow cooling (and subsequent slow warming). Hexane and heptane served to furnish the same inert continuous phases employed to suspend pure water. Stearate-containing surfactants soluble only in the non-aqueous phase allowed, as before, the stabilization of the emulsion. It was only necessary to avoid those aqueous solutes likely to partition between the water and the hexane or the heptane. Numerous salts, sugars, polyhydric alcohols, and water soluble polymers, natural and synthetic, were studied in aqueous solution in different concentrations (Rasmussen & MacKenzie 1972).

Figure 5(*b*) reproduces the differential thermogram obtained when an emulsion made from equal volumes of hexane containing 5% sorbitan tristearate and 40% aqueous sucrose (by mass) was cooled at 2 °C/min to −60 °C. As in the distilled water emulsion, the homogeneous nucleation of ice was indicated by a single, smooth, nearly symmetrical peak, the peak temperature (T_h) being in this case −51.9 °C. Slow warming yielded the upper plot (Figure 5*b*, again) from which an expected equilibrium melting point (T_m) of −5 °C was readily obtained. One notes the gradual melting of the sucrose solution, beginning at about −30 °C. One distinguishes the broad left hand shoulder to the melting well in the upper curve in figure 5*b* denoting the gradual dissolution of ice and the dilution of the sucrose phase from the narrow well in the upper curve in figure 5*a* attributable to the sudden melting of ice in the absence of solutes at 0 °C.

The measured homogeneous nucleation temperatures and the measured (and, in some cases, excellently documented) equilibrium melting points can each be plotted against the concentration of the solute in the aqueous phase. The results are seen in figure 6. The upper set of curves reproduces some well known, and in the case of the polymer solutes, some less well known melting behaviour. The lower set illustrates the dependence of the homogeneous nucleation temperature on solute concentration which, plotted on a solute (mass) content basis, separates the behaviour of the various systems rather nicely. Inorganic salts, depressing T_m very sharply, can be seen to depress T_h still faster. Organic solutes with similar or slightly higher molecular masses depress T_m less rapidly. Being more soluble, however, or, in some cases, totally miscible with water, they allow a comparable or a greater ultimate depression of the T_h. Quantitative comparisons aside, figure 6 is, in any event, remarkable for the very low temperatures to which many, more concentrated aqueous solutions can be cooled before stable supercooling is interrupted by the homogeneous nucleation of ice.

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The information contained in figure 6 can be replotted without explicit reference to the concentrations of the different solutions to considerable advantage. Figure 7 plots the reduction in the homogeneous nucleation temperature (ΔT_h) against the corresponding depression of the melting point (ΔT_m). Measured values of T_h were, that is, subtracted from $(T_h)_{H_2O}$, and the differences plotted against the value: $(T_m)_{H_2O} - (T_m)$. Interesting results are obtained. One notices, first, that, regardless of the shapes of the individual melting point curves in figure 6,

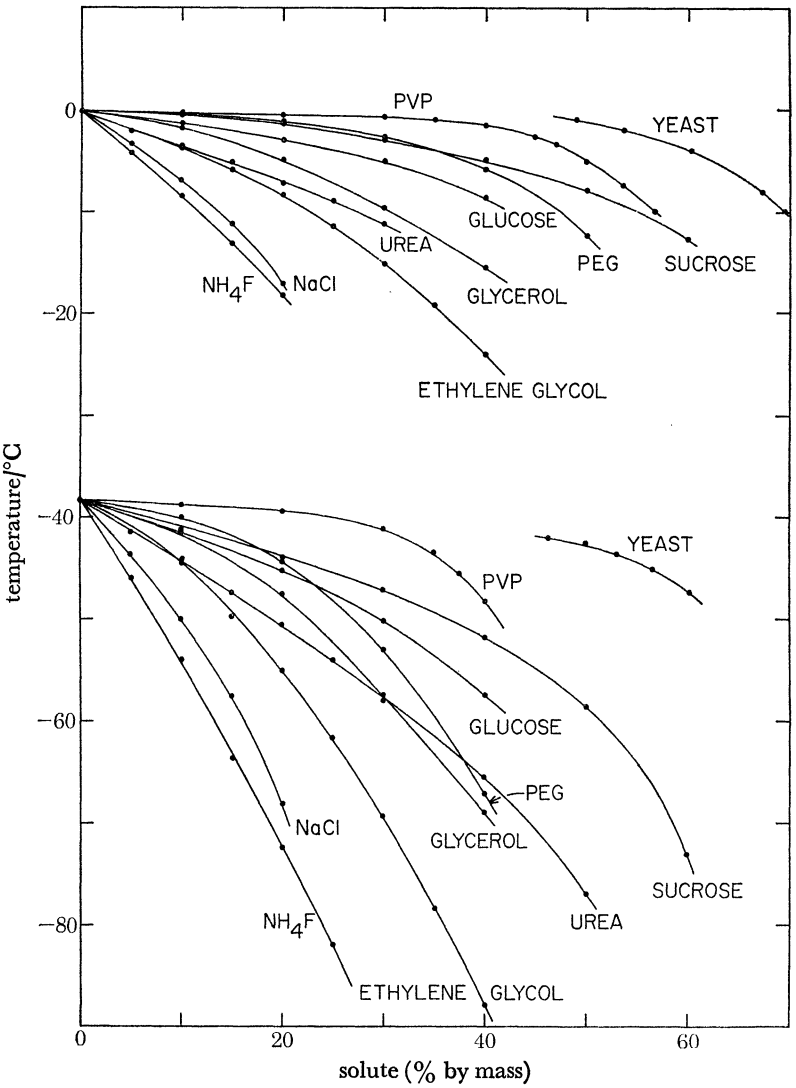

FIGURE 6. Dependence of the equilibrium freezing point and the temperature of the homogeneous nucleation of ice on the nature and the concentration of the solute.

the plots contained in figure 7 are almost all grouped into one band with a gradient of 2.0 ± 0.2. Only the polymer solutions yield plots with higher gradients. More remarkable yet, the plots turn out to be linear within experimental error, a finding we have thus far been unable to interpret. It has, in fact, been a surprise to find that T_h, a value determined in many respects by kinetic considerations, should be related in such a simple and direct way to a melting point

depression, a property entirely thermodynamic in its nature. Clearly the explanation must lie in the changing structure of the water with increasing solute concentration and decreasing temperature. The question demands further careful consideration.

The finding that ΔT_h was roughly five times greater than ΔT_m in the case of aqueous solutions of polyethyleneglycols of various molecular masses (the same result was obtained with mol. masses of 1000, 4000, 6000 and 9000) was employed to special advantage to determine the temperatures at which the water in yeast cells froze by homogeneous nucleation. *Saccharomyces cerevisiae* were suspended in aqueous polyethyleneglycol solutions of various concentrations.

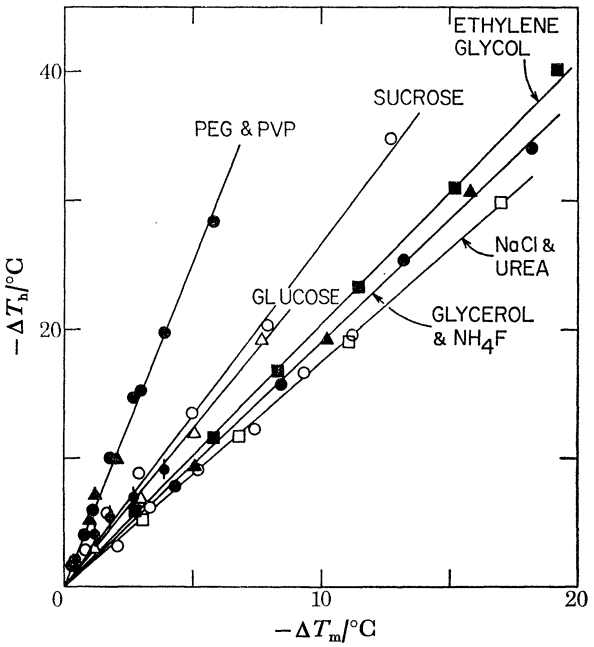

FIGURE 7. Relation between the depression of the homogeneous nucleation temperature (ΔT_h) and the depression of the equilibrium melting point (ΔT_m). Data from figure 6.

Where the polyglycol solution was hypertonic to the yeast, the cells lost water, equilibrating with their surroundings in a short time. When equilibrated suspensions were emulsified in non-toxic, surfactant-containing mineral and plant oils, emulsions suitable for thermal analysis were obtained. Slow cooling furnished well-defined thermograms from which the homogeneous nucleation of ice in the cell contents could be determined; slow warming confirmed the equilibrium melting points (Rasmussen, Macaulay & MacKenzie 1975). The ΔT_m values of the suspending medium and the yeast cells being the same in each case, the greater ΔT_h of the suspending aqueous polyethylene glycol allowed the freezing of the system 'from the inside out'. Had sucrose, for example, been used to dehydrate the yeast the outer medium might well have frozen first, in which case the independent freezing of the cell contents would not have been observed. The T_m and the T_h plots obtained from the studies on *S. cerevisiae* have been included in figures 6 and 7. One observes, in this connection, that the homogeneous nucleation behaviour of the water in the yeast cells is much like that of the water in a typical aqueous solution. It is especially significant that the cells were found to survive the supercooling, and that a homogeneous nucleation of ice was in fact observable (Rasmussen *et al.* 1975).

The supplemented phase diagram

The information from the previous section can be combined with numerous other data, equilibrium and non-equilibrium, to furnish a 'supplemented phase diagram' describing many of the first and second order phase transitions observed during the cooling and/or the subsequent rewarming of an aqueous solution (Luyet 1960; Luyet & Rasmussen 1968; Rasmussen & Luyet 1969; MacKenzie 1972). We will show how various measurements obtained by what has recently become routine differential thermal analysis contribute to such a diagram. Taking the water – sucrose system for the sake of illustration, we will distinguish the equilibrium from the non-equilibrium changes represented in the diagram. The nature of the time dependence of many of the phenomena will, perhaps, become apparent from brief descriptions of earlier studies on recrystallization by rewarming, and on the warming rate dependence of the glass transition. The dependence of the quantity of ice formed during the freezing of an aqueous gel on the freezing treatment will be seen to particular advantage in studies on a hydrated, cross-linked dextran system.

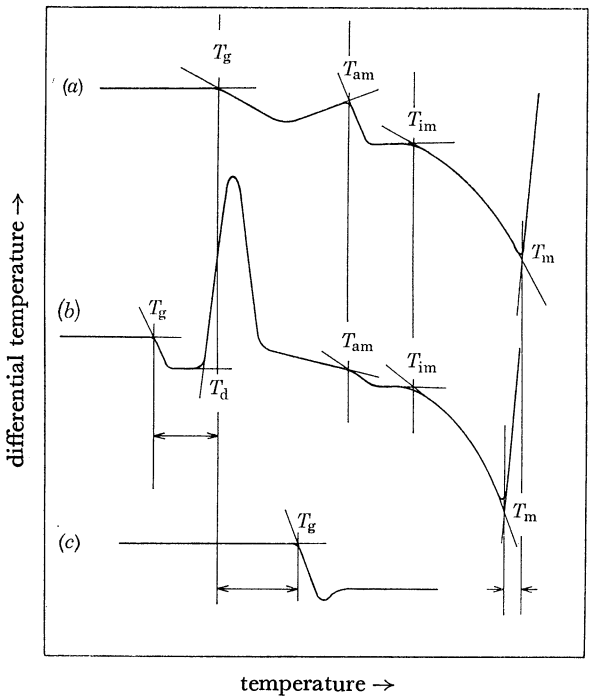

Figure 8. Schematic presentation of representative thermograms obtained by slow warming of rapidly cooled aqueous solutions. (*a*) warming of a 20% aqueous solution; (*b*) warming of a 30–60% aqueous solution; (*c*) warming of a 70–80% aqueous solution. T_g, glass transition temperature; T_d, devitrification temperature; T_{am} and T_{im}, ante- and incipient melting temperatures, respectively; T_m, equilibrium melting point. See text for further information.

When 5–10 µl volumes of more or less dilute (0–20%, by mass) sucrose solutions are cooled in capillary tubes as rapidly as possible and subjected to differential thermal analysis by 'slow' rewarming at several degrees Celsius per minute, thermograms of the type seen in figure 8*a* are obtained. One identifies a glass transition, an 'antemelting', an 'incipient' melting, and an equilibrium melting at T_g, T_{am}, T_{im}, and T_m, respectively. Essentially similar results are obtained after less rapid cooling. Experience shows in each case that all the freezable water is

converted into ice during cooling. When the same quantities of moderately concentrated aqueous sucrose (30–60 %, by mass) are similarly cooled, slow warming yields a thermogram incorporating an additional feature (figure 8*b*) revealing the formation of ice on warming. Careful analysis of the data shows the aqueous sucrose to have cooled, e.g. to −196 °C without freezing. Only after the system warmed above a characteristic glass transition temperature was a freezing (or a 'devitrification') observed. The further warming of the frozen system showed the

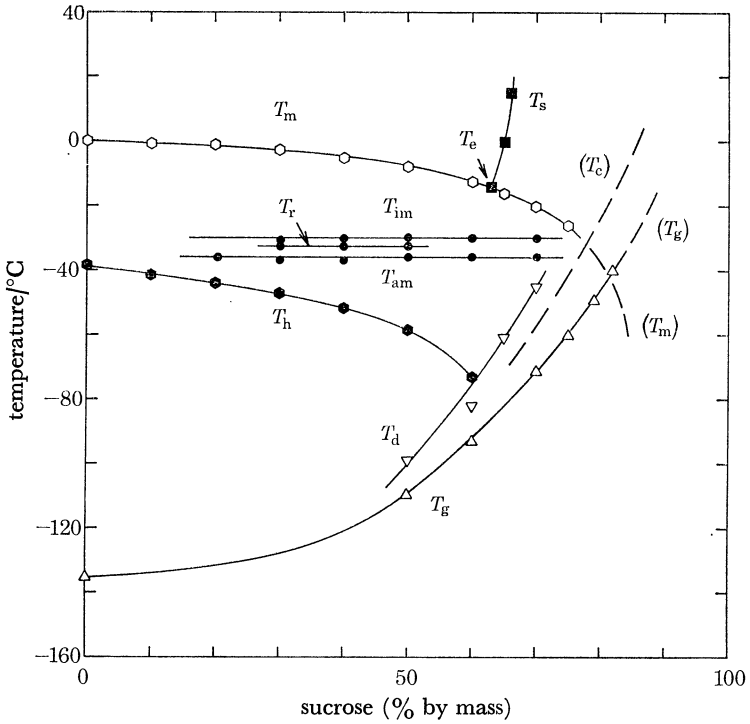

FIGURE 9. Supplemented phase diagram describing the low temperature behaviour of the system: water–sucrose. T_m denotes the measured melting point curve, (T_m) its extrapolation. Crystalline sucrose dissolves according to the curve T_s, the latter being terminated by the point T_e, the eutectic temperature. T_g describes glass transition behaviour, (T_g) the probable values at higher temperatures. (T_c) indicates probable 'collapse temperatures' determining freeze-drying behaviour. T_d, T_{am}, T_r, and T_{im} denote, respectively, devitrification, antemelting, recrystallization, and incipient melting on rewarming. T_h describes freezing by homogeneous nucleation (from figure 6). (By permission of Academic Press.)

same transitions seen in figure 8*a*. When the same moderately concentrated solutions were cooled less rapidly the characteristic devitrification was not observed during rewarming. Still more concentrated (70–80 %, by mass) aqueous sucrose yielded thermograms devoid of any evidence of devitrification, antemelting, incipient melting, or equilibrium melting, regardless of the initial cooling treatment. Devitrification is, it appears, a feature associated only with moderately concentrated solutions and very rapid cooling. Less readily defined thermograms were obtained from 20–30 % and 60–70 % by mass aqueous sucrose, the concentrations being ones in which the behaviour was in each case of an intermediate nature.

Figure 9 represents the supplemented phase diagram. Equilibrium melting points and homogeneous nucleation temperatures have been taken from figure 6. Glass transition temperatures, antemelting and incipient melting points have been taken from the various other thermograms, as have the equilibrium melting points, the last of these confirming the findings in

figure 6. Recrystallization temperatures by visual inspection of frozen specimens have been inserted between T_{am} and T_{im}. Handbook solubilities for crystalline sucrose at different temperatures have also been inserted, though the crystallization of the sucrose was never observed in the course of the many experiments conducted in the completion of the work. In the absence of the crystallization of the solute the formation of ice during slow cooling follows the melting point curve, T_m, without reference to the solute solubility curve, T_s. (Ice formation is *not* completed at T_e, the true eutectic point, where the crystallization of the solute has not occurred. References to 'eutectic freezing' and to the formation of 'frozen eutectics' are, accordingly, out of place, and often very misleading except where a true eutectic freezing has been demonstrated.) The slow freezing of aqueous sucrose proceeds, as we said, to the right and down the T_m curve, in the direction of the presumed intersection with the glass temperature curve, T_g.

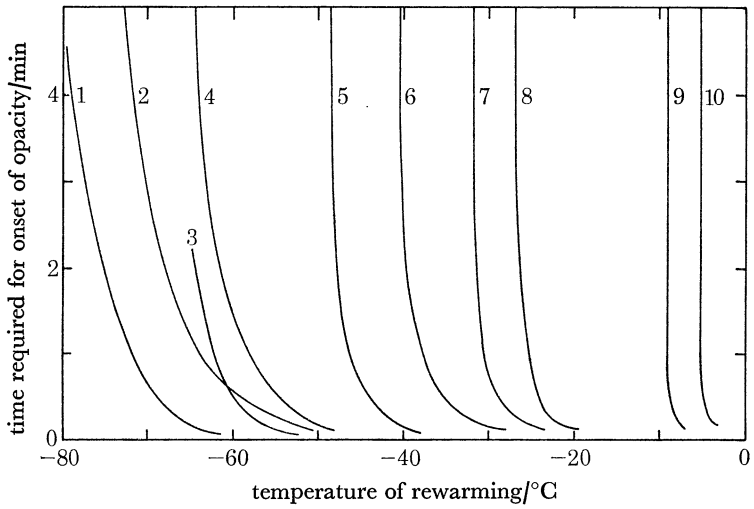

FIGURE 10. Curves describing the time required by rapidly frozen solutions to demonstrate the recrystallization of ice by rapid rewarming. 1, formaldehyde; 2, ethylene glycol; 3, polyethylene glycol (av. mol. mass 4000); 4, glycerol; 5, fructose; 6, glucose; 7, sucrose; 8, raffinose; 9, dextran; 10, starch.

The region of the intersection of the T_m, T_{im}, T_r, T_{am}, T_d, and T_g plots is a sort of focal zone in the supplemented phase diagram in that it appears that there is a sucrose concentration above which ice cannot be frozen out during slow cooling, even by deliberate seeding, or by undercooling and subsequent slow warming. It is hard to confirm the precise intersection of the T_m curve in that signals by differential thermal analysis become much smaller with increasing concentration in this interval. It will also be seen that the homogeneous nucleation temperature curve (determined during cooling experiments) intersects the devitrification curve (determined during warming experiments) at a lower sucrose concentration and a lower temperature than the point at which the curve T_m appears to meet the T_g plot. It would seem, therefore, that there is a rather well defined range of solute concentrations (and temperatures) in which freezing during cooling is only possible where ice nuclei already exist. The zone is marked by rapidly changing glass transition temperatures and is clearly very interesting.

The recrystallization temperatures inserted in figure 9 were derived from measurements of the type reproduced in figure 10, in which the times taken by rapidly frozen specimens to show visible recrystallization, or grain-growth of ice are plotted against the temperatures to which the

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individual specimens were warmed (Luyet 1939, 1960; MacKenzie 1966). It is apparent that, for a given system, the time required for visible recrystallization decreases very sharply with increasing temperature. It will also be seen that the time required appears to *increase* asymptotically with *decreasing* temperature, and that there seems in each case to be a temperature below which recrystallization is not observed at all in practice. It is this last threshold temperature which has been inserted in figure 9. One notes that the threshold temperature is generally lower, and that the asymptote is less definite, the time scales being equal, the lower the molecular mass of the solute. Certain solutes having higher molecular masses, polyethylene glycols, for example, have lower recrystallization temperatures; other solutes having a common molecular mass, fructose and glucose, for example, are distinguished by their different threshold temperatures. We may note, lastly, that the same recrystallization temperatures are obtained from 30, 40, and 50 % aqueous solutions of the solutes in question where the freezing treatments effect the same dehydration of the solute phase. An entirely different recrystallization behaviour is obtained wherever the solute crystallizes to yield a true eutectic.

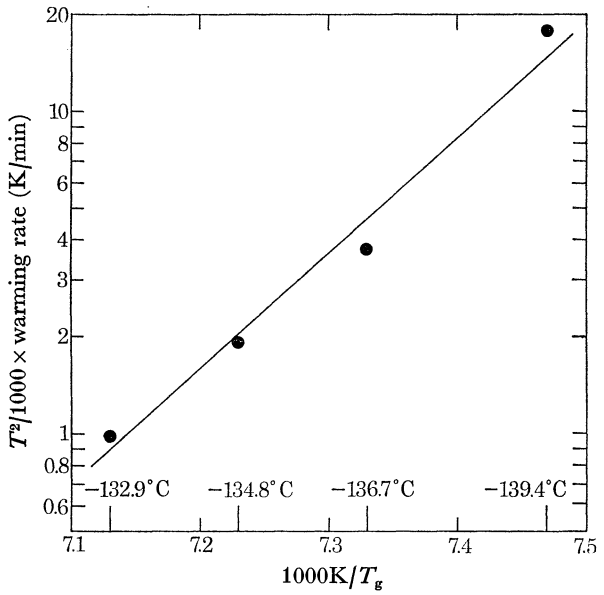

FIGURE 11. Effect of the heating rate on the glass transition temperature in amorphous water (Rasmussen & MacKenzie 1971).

Pursuing the same notion of a time-dependent phenomenon, we may usefully re-examine the question of the glass transition, a topic itself the subject of a recent conference (Goldstein & Simha, in press). Figure 11 offers a derived plot based on a series of measurements yielding extrapolated glass transition temperatures for pure water (Rasmussen & MacKenzie 1971). Glass transition temperatures in aqueous methanol, ethylene glycol, and glycerol solutions of differing composition were obtained at different warming rates by differential thermal analysis. Warming rates were varied in each instance from 1 to 20 °C/min to yield extrapolated glass transition temperatures for pure water from −139 to −133 °C. Figure 11 reproduces a derived plot furnishing a linear relation. The findings emphasize the kinetic nature of the glass transition.

Stated in somewhat simpler terms, lower warming rates allow longer intervals during which measureable changes are more likely to occur in glassy states at lower temperatures. Lower

cooling rates will, by the same argument, allow the observation of the viscous state to lower temperatures. It follows that the T_g curve in figure 9 is only one of a family of glass transition temperature curves characterizing the water – sucrose system, according to the warming rate chosen for the measurements. It will also be seen that the T_d curve must be determined by the warming rate, being itself one of a family of T_d curves. We may recall that we have already noted the time dependence of the 'recrystallization temperature', T_r, and the cooling rate dependence of the homogeneous nucleation temperature, T_h. The warming rate dependence of the ante-melting temperature remains in doubt and a pictorial significance has yet to be attached to this last transition.

The possibility that rapid freezing will cause a specimen not to follow the equilibrium freezing point curve is illustrated rather nicely by studies on various rapidly frozen gels. Figure 12 reproduces thermograms obtained when a Sephadex G-25 cross-linked dextran gel was em-

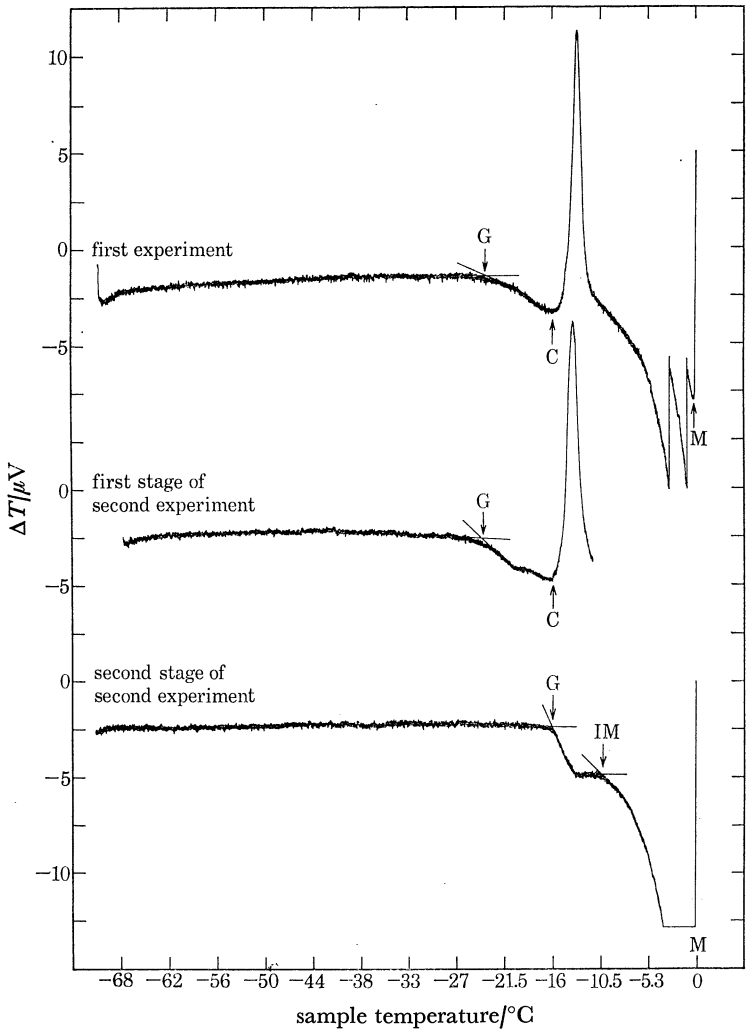

FIGURE 12. Thermograms obtained by differential thermal analysis of Sephadex G-25 cross-linked dextran gel beads. First experiment: rapid freezing of fully hydrated gel beads followed by slow warming through the melting point (freezing not shown). Second experiment: rapid freezing of fully hydrated gel beads followed by slow warming to about −11 °C (freezing not shown), after which the specimen was cooled a second time (below −100 °C) and rewarmed through the melting point. G, glass transition; C, crystallization of ice; IM, incipient melting; M, equilibrium melting of ice.

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ployed. The gel comprised a dextran network covalently cross-linked with epichlorhydrin; the final product contained only glucose and glycerol residues. Obtained in bead form and swelled with water to yield a system containing *ca.* 30 % gel solids by mass, the drained beads were frozen in capillary tubes in liquid nitrogen and subjected to differential thermal analysis by slow rewarming, with the following differences.

The rapidly frozen specimens warmed without any sign of a thermal event until about −24 °C, at which temperature a glass transition corresponding to a softening of the dextran phase was observed (figure 12, first experiment). A subsequent rise in the thermogram about −16 °C and a sharp peak around −13 °C revealed the formation of a quantity of ice. The entry of the $T/\Delta T$ trace into a deep well at a still higher temperature demonstrated the melting of a quantity of ice much larger than that forming at −16 °C during warming. Melting was completed very close to 0 °C much as we might have expected. A second experiment was conducted with a fresh specimen to duplicate the first to a temperature between −12 and −11 °C at which the warming was interrupted by moderately rapid cooling to −100 °C, or thereabouts. It will be seen from figure 12 (first stage of second experiment) that the second specimen duplicated the behaviour of the first. When the second gel specimen was subjected to a second rewarming the $T/\Delta T$ trace revealed a glass transition shifted to a markedly higher temperature (to about −16 °C) after which the ice in the gel very simply melted. It would appear that the single thermal cycling sufficed to freeze all the available water remaining unfrozen after the rapid freezing in liquid nitrogen. The further dehydration of the dextran phase during the first stage of the second experiment is also shown by the rise in the glass transition temperature (from −24 to −16 °C; figure 12, again; compare the first and second stages of the second experiment). We may note that slowly frozen cross-linked dextran specimens furnished thermograms essentially indistinguishable from those obtained by very rapid freezing and subsequent thermal cycling.

Many factors most likely contribute to the apparent stability of the rapidly frozen dextran systems to such high temperatures. The covalently cross-linked structure of the dextran gel, the tendency for neighbouring dextran chains to associate by hydrogen bonding, and the possibility that microporous regions maintain small quantities of water in effective isolation must all be kept in mind. It is doubtless significant that lower glass and threshold temperatures for further ice formation were observed when quantities of glycerol were added to the aqueous gel before freezing. The subject awaits a quantitative treatment. We should also note the very markedly lower glass temperatures obtained wherever freezing is completely circumvented. These are exemplified in figure 9 by the vertical descent of a specimen to the T_g curve. Slow freezing causes ice to separate and the resultant solute phase to traverse the equilibrium melting point curve to the intersection with the glass transition temperature curve. It will be seen that rapid freezing yielding less than maximum possible quantities of ice creates situations stabilizing solute phases possessing intermediate glass transition temperatures. There is a possibility that gel

Description of plate 1

Figure 13. Light micrographs of spherulitic ice obtained by the very rapid freezing of aqueous solutions. (*a*) 30 % (by mass) aqueous sucrose. (*b*) 30 % (by mass) aqueous gelatin. (Specimen thicknesses: 10–20 μm. Crossed polarizing filters. Magn. × 115.)

Figure 15. Electron micrographs illustrating patterns obtained by the rapid freezing of a 20 % (by mass) aqueous KCl solution at −150 °C. (Method of MacKenzie & Luyet (1962*a*). Magn.: *a*: × 12000; *b*: × 60000.)

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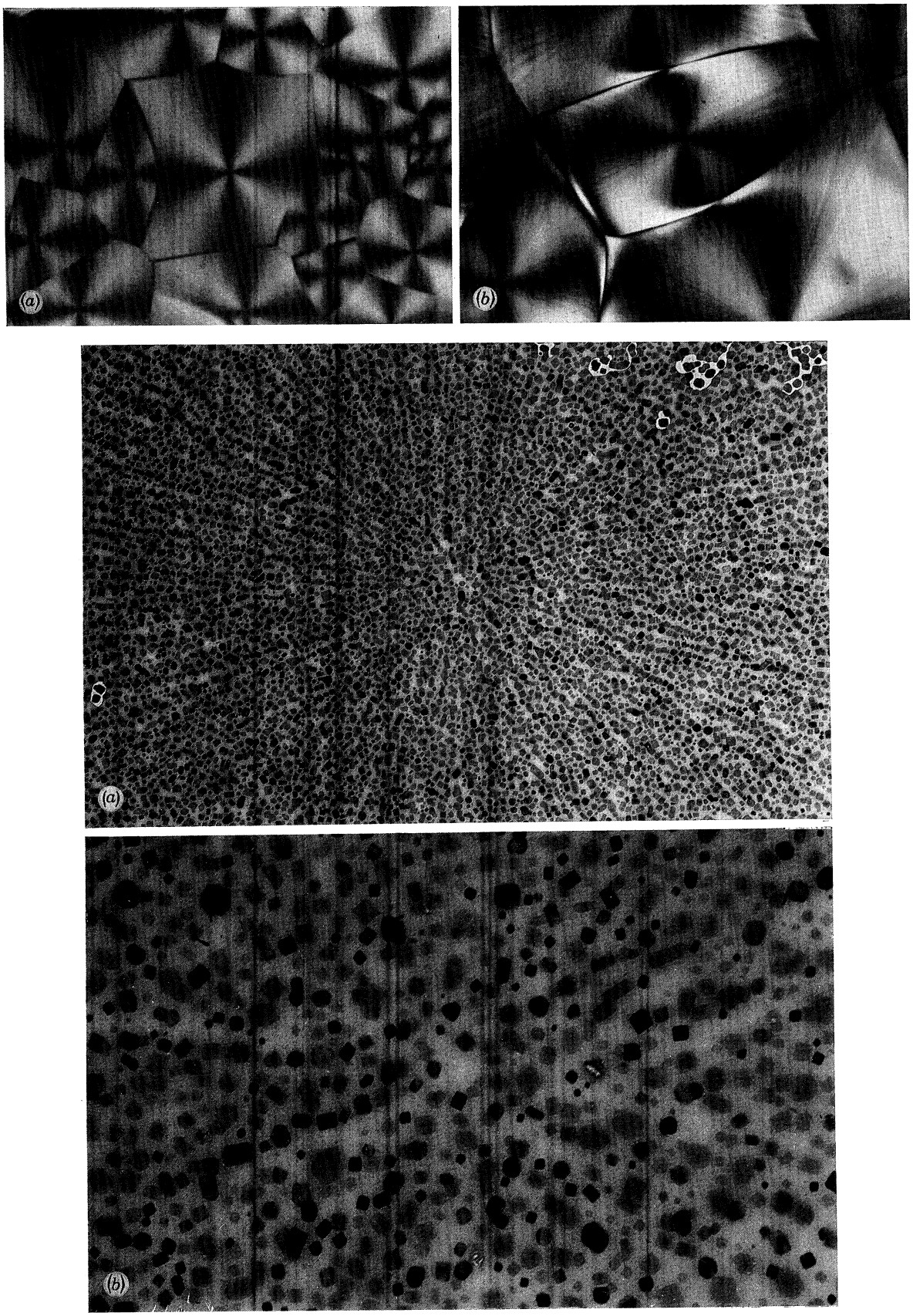

FIGURES 13 AND 15. For description see opposite.

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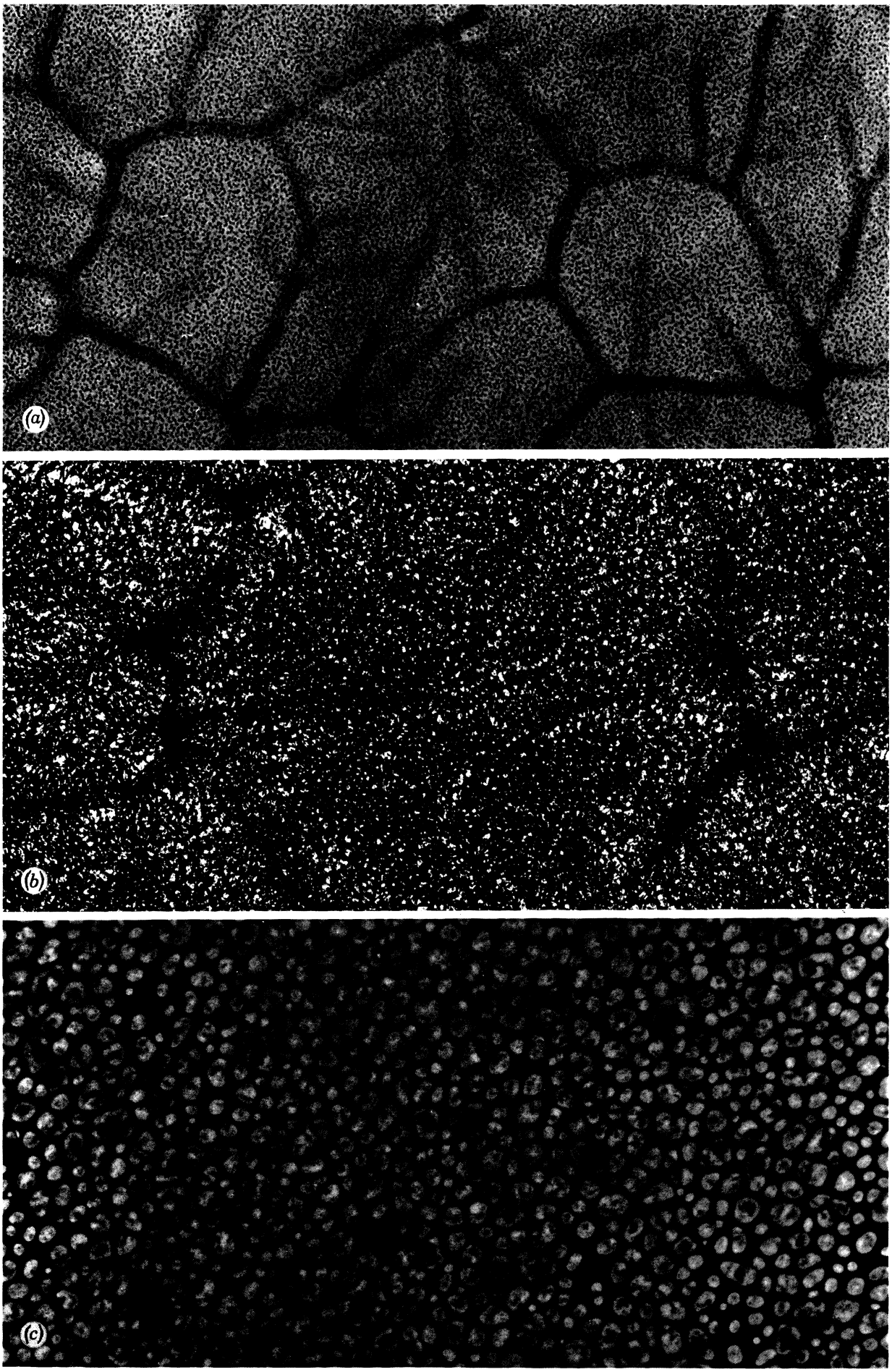

FIGURE 14. For description see opposite.

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structures tend to broaden the range of cooling rates in which this intermediate behaviour is likely to be found. This being the case we can expect to see signs of the same phenomenon during and after the rapid freezing of living cells. It would appear in any case that further studies are needed the better to characterize the supplemented phase diagrams of the more complex aqueous systems.

Non-equilibrium microcrystallinity

Just as faster cooling *tends* to stabilize metastable states retaining greater quantities of unfrozen water, so faster freezing, once begun, *tends* to promote a finer subdivision of the ice. Higher cooling rates tend, within limits, to increase both rate of nucleation and rate of crystal growth. While space will not allow a very detailed analysis it will, perhaps, be helpful if we describe some representative observations in the light and electron microscopes. We will outline the nature of the states resulting from the rapid freezing of thin layers of aqueous solutions discussing, in particular, spherulitic ice and its subsequent recrystallization. We will then attempt to relate the findings to the very rapid freezing of small three-dimensional specimens. The crystallization of the solute will also be described.

The light micrographs seen in figure 13, plate 1, demonstrate the presence of spherulitic ice in representative aqueous solutions subjected to very rapid cooling in layers 5–10 μm in thickness (Luyet & Rapatz 1958; Luyet 1960; Rapatz & Luyet 1966). Freezing is seen in each case to originate from a limited number of points and to proceed to completion by space-filling growth to afford a system of common boundaries resembling a net. Crystal growth proceeds around each centre with a radial symmetry more nearly perfect the higher the cooling rate. Little can be seen by transmitted light without a pair of crossed polarizing filters. The Maltese crosses seen in polarized light attest to the spherulitic nature of the crystallization. The same results are often obtained in the crystallization of low molecular mass organic compounds from the melt, and in the solidification of organic polymers (Keith & Padden 1963, 1969; Sharples 1966; Knight 1967; Amrhein 1975). While the precise size, shape, and orientation of the component crystallites comprising the spherulites are still not known it is nonetheless certain that, where the frozen systems are transparent in ordinary illumination, the crystallites are smaller in one dimension than the wavelength of blue light. It is also very likely that the birefringence is mostly due to the form of the crystallites, and to their distribution, though the anisotropy of the $(\text{ice})_h$ they seem to contain may make its own contribution. Higher cooling rates generally induce the formation of larger numbers of necessarily smaller spherulites, nucleation rates, heterogeneous and homogeneous, each rising faster than growth rates with more effective heat transfer from the specimen. Situations arise, on the other hand, where nucleation rates continue to rise and growth rates finally fall with increasing viscosities. Spherulites have, for example, been observed to form and grow in concentrated aqueous glycerol at very low temperatures at radial rates of the order of millimetres per hour (Rapatz & Luyet 1966). It will be seen that spherulitic ice resulting from just such a slow freezing can still demonstrate typical spherulitic microcrystallinity.

Description of plate 2

Figure 14. Electron micrographs illustrating patterns obtained by the freezing and subsequent rewarming of bovine blood plasma. (*a*) rapid cooling to −150 °C; (*b*) rapid cooling to −80 °C; (*c*) rapid cooling to −80° C followed by rewarming to −20 °C for 10 min. (Method of MacKenzie & Luyet (1962*a*). Magn.: *a* and *b*: ×10800; *c*: ×6700.)

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Transmission electron microscopic studies have allowed still better pictorial descriptions of rapid and ultra-rapid freezing. Different preparative approaches have been taken, according to the specimen. Thin film methods have been particularly useful in that they permitted the preparation and observation of uniform layers in the electron microscope. Trapped between collodion films several tens of nanometres thick, aqueous solutions 1–200 nm thick were frozen and freeze-dried prior to observation in the transmission instrument at room temperature (MacKenzie & Luyet 1962*a*, *b*, 1963). Preparations were examined intact without the need for sectioning, staining, or replication, and demonstrated contrast sufficient to permit photography. Figure 14, plate 2, reproduces three bovine blood plasma preparations photographed after freeze-drying. Figures 14(*a*) and (*b*) represent thin layers of plasma subjected only to rapid cooling to −150 and to −80 °C, respectively. Figure 14(*c*) reveals the effect of a deliberate rewarming to −20 °C after a rapid freezing to −80 °C.

Figure 14(*a*) suggests the presence of spherulitic ice prior to freeze-drying and the probable formation, during freezing, of crystallites too small to resolve, even in the electron microscope, with the techniques available. The finely divided background most likely results from the crystallization of the salts present in the plasma, protein solutions dialysed before preparation and observation being free from such grains. The plasma preparation illustrated in Figure 14(*b*) resulted from a somewhat less rapid freezing and offered a different appearance. Spherulites are distinguished by their border network and by a rather well resolved microcrystalline ice. Observations on a great many such preparations suggested a threshold freezing rate above which well resolved, particulate, intra-spherulitic ice was not obtained. Though still comparatively rapid, lower cooling rates were correlated with greater numbers of larger intra-spherulitic particles.

The deliberate recrystallization of a preparation that would, before rewarming, have looked much like figure 14(*b*) is pictured in figure 14(*c*). We note the absence of any trace of an original freezing pattern and the presence, instead, of a uniformly well resolved globular ice devoid of any particular arrangement. It would appear that recrystallization progresses by the dissolution of the smaller original crystals and the continued growth of the larger ones with the eventual annihilation of the original freezing pattern. The process would seem to be limited only by the time made available and the dimensions of the preparation. Returning to the clearly different appearance of the spherulites seen in figures 14(*a*) and (*b*), we might now conclude that the well resolved crystallites are the forerunners of the larger crystals we see in figure 14(*c*). It is, it appears, entirely possible and very likely that recrystallization of ice begins during rapid freezing and that it is interrupted only when the freezing specimen cools below a rather well defined temperature (MacKenzie & Luyet 1963, 1967). Whether or not there can exist cooling rates above which recrystallization is prevented remains to be determined.

The question of the development of a microcrystalline ice phase during rapid freezing is further complicated when the solute crystallizes simultaneously, or just afterwards. Figure 15, plate 2, reproduces electron micrographs of a freeze-dried specimen made from a 20% aqueous KCl solution frozen at −150 °C. Freeze-drying was completed at −70 °C, some 60 °C below the eutectic temperature, the temperature below which the system tended to assume a totally crystalline state. It appears from the micrographs that the ice formed first (we have shown only a part of one of the many spherulites observed). It would further appear from the random orientation of the KCl that the latter crystallized in the channels created during the growth of the spherulitic ice. It is hard to tell how the KCl was nucleated. Each crystal could have arisen

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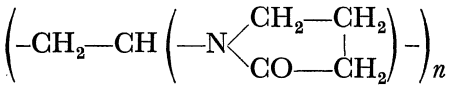
from a separate homogeneous nucleation. The solute phase could, alternatively, have arisen from a much smaller number of nuclei, and the patterns seen in figures 15 (*a*) and (*b*) could have resulted from a mutually accommodating recrystallization of ice and KCl, even at −70 °C, before freeze-drying. We may note that solutes, like solvents, will tend to follow their own homogeneous nucleation temperature curves where their crystallization has been observed (Rasmussen & Loper 1975; Rasmussen & MacKenzie, unpublished experiments).

Much of the information gained from the study of essentially two-dimensional freezing can, it appears, be applied to questions relating to the very rapid freezing of small three-dimensional specimens. Heat transfer and crystal growth proceed, for the most part, in mutually perpendicular directions in thin films, creating something of a special case. Rapid heat transfer often results, on the other hand, in a considerable supercooling in small three-dimensional specimens after which nucleation can occur at points some distance inside the outer surface. Where this is observed, crystal growth proceeds in every direction, albeit somewhat asymmetrically, into the supercooled surroundings. These are the conditions required for spherulitic growth. Spherulitic ice has been observed in small three-dimensional aqueous model systems and in cells suspended in aqueous solution (see, for example: Moor 1964; Nei, Kojima & Hanafusa 1964; Rapatz & Luyet 1965; Shimada, Asada & Asahina 1971; Buchheim 1972; Nei & Asada 1972; Plattner, Fischer, Schmitt & Bachman 1972; Benedetti & Favard 1973). A significant supercooling is a prime requirement. Where ice forms immediately at an outer surface, heat transfer from the interior of the specimen is greatly reduced. Crystal growth proceeds by the transfer of heat in an essentially opposite direction. The latter rather different situation calls for new model system studies.

Components incapable of crystallization

Figure 16 reproduces a supplemented phase diagram describing the phase behaviour of the water soluble polymer polyvinylpyrrolidone



in aqueous solution (MacKenzie & Rasmussen 1972). Polyvinylpyrrolidone (PVP) having an average molecular mass of 30000 was dialysed and concentrated by freeze-drying before it was mixed with water in various proportions to make the measurements. Figure 16 therefore represents the behaviour of a water–polymer system free from undesired low molecular mass components. It will be seen that the phase diagram resembles that of the water–sucrose system (see figure 9), though the molecular masses of the two solutes differ by a factor of about 100. The equilibrium melting point curve descends less steeply at first but turns nonetheless with rising solute concentration, becoming essentially vertical about 0.65 mass fraction PVP. We note a homogeneous nucleation temperature curve, a glass transition temperature curve, and a recrystallization plot. All efforts to crystallize PVP having failed, we do not, however, have a solute solubility curve. Nor can we expect to obtain a true solubility curve from such a hetero-disperse material.

The failure of the PVP and other water soluble materials to crystallize, regardless of the circumstances, raises important questions relating to the behaviour of biological material. To the extent that the water–PVP system characterizes the behaviour of other water–water soluble polymer systems, it would, however, appear that figure 16 describes, partly at least, the

likely response of many biological materials to rapid freezing (and, of course, to less extreme freezing treatments). Too little is known as yet of the non-equilibrium freezing of biological systems to test the notion. We may, on the other hand, note the indication that the water–PVP system is less than truly representative (MacKenzie 1975). Either way, the determination of the non-equilibrium phase behaviour of a number of other aqueous macromolecular systems would seem to be a priority.

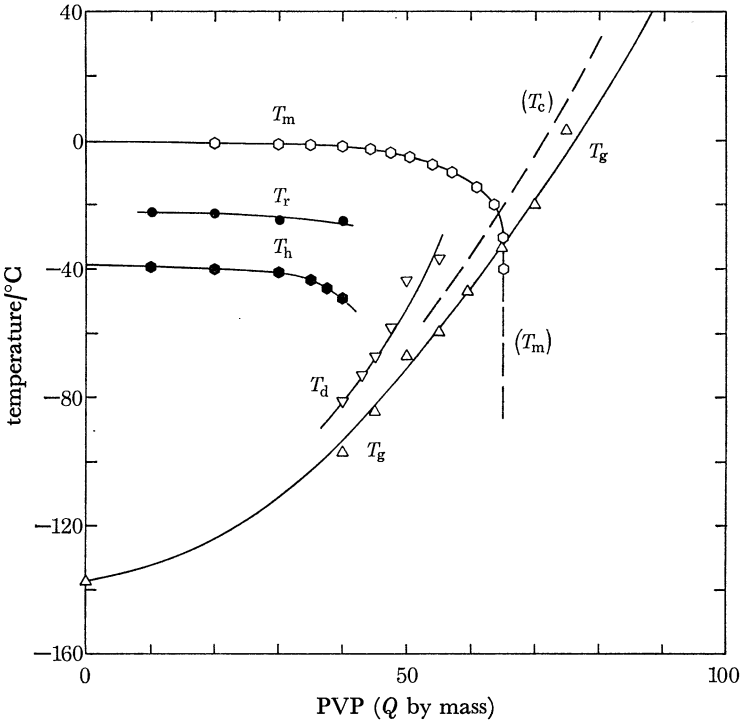


FIGURE 16. Supplemented phase diagram describing the low temperature behaviour of the system: water-polyvinylpyrrolidone (PVP). T_m denotes the measured melting point curve, (T_m) its extrapolation. T_g describes glass transition behaviour. (T_c) suggests probable 'collapse temperatures' determining freeze-drying behaviour. T_d and T_r denote devitrification and recrystallization, respectively, on rewarming. T_h describes the homogeneous nucleation of ice on slow cooling (data from figure 6). (By permission of Academic Press.)

CONCLUDING REMARKS

Much of the information obtained from the studies on model systems can be applied to the qualitative and, in many cases, the semi-quantitative interpretation of the freezing behaviour of cells and tissues. Knowing the composition and the concentration of many cell fluids, we can, for example, hope to predict homogeneous nucleation temperatures, glass transition and devitrification temperatures, ante- and incipient melting temperatures, and equilibrium melting points. But the course of events during rapid cooling and, where it is examined, slow rewarming, appears to depend in many cases on the compartmentation of the cell or the tissue, and the consequence of a particular freezing treatment proves to be less predictable than we might have supposed. The case of the cell is further complicated by the presence of a variety of essentially insoluble structures each of which affects, and is affected by, the freezing of the system.

The finding that the contents of many winter-hardy plant cells supercool, even in the presence of extracellular ice, to the homogeneous nucleation temperature of the contents (George, Burke,

Pellett & Johnson 1974) further complicates the situation. Clearly the barriers interposed by the cell wall and plasma membrane sufficed to prevent the intrusion of external ice. Nor could there have been any effective heterogeneous nuclei present in the cell (George *et al.* 1974; Rasmussen *et al.* 1975). It would appear that in these, possibly also in other cases, the nature of the non-equilibrium freezing was determined largely by the need for the independent nucleation of ice in the extra- and intracellular spaces. Further studies are urgently needed.

The non-equilibrium freezing of a wide variety of living cells less well protected by the process of evolution appears, on the other hand, to follow a course involving the growth of ice through the plasma membrane (Mazur 1965; Mazur *et al.* 1970), though the aforementioned penetration of the ice has not been demonstrated in the microscope. In a continuing series of definitive studies, Mazur and co-workers have shown that higher cooling rates leave less time for water to migrate from unfrozen cell interiors to surrounding ice. With less time left to dehydrate, and faster cooling, these same cells are more likely to seed by growth of ice through a previously undamaged membrane. Whether or not the same cells freeze by homogeneous nucleation rather than by trans-membrane seeding above a still higher cooling rate has not been determined. We may, in this connection, note the unusual observation of the seeding of the extracellular suspending medium following the internal freezing of supercooled yeast by homogeneous nucleation (Rasmussen *et al.* 1975). Only the lower homogeneous nucleation temperature of the hypertonic polyglycol-containing suspending medium allowed the observed sequence of events.

Further studies of the non-equilibrium freezing of cells and tissues await the direct determination of the phase behaviour of the systems in question. Differential thermal analysis and differential scanning calorimetry appear to be well suited to such measurements, to which we would add electron microscopic studies by freezing and freeze-fracture replication, freezing and freeze-drying, and, wherever possible, direct observation in the frozen state. One hopes also to see the greater use of low temperature X-ray diffraction analysis and nuclear magnetic resonance in the investigation of the non-equilibrium freezing of aqueous systems. These last methods have proved effective in studies on various models. We look forward to their early application to corresponding studies on living cells.

The author recognizes the joint nature of much of the work reported. He greatly appreciates the very extensive contributions by his several former colleagues, most notably those made by Dr D. H. Rasmussen.

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Discussion

W. Derbyshire (*Department of Physics, University of Nottingham*). Could you give an indication of the dependence of nucleation temperature upon sphere diameter in the emulsion studies? Is there a trivial explanation for the asymmetric shape of the warming curve in the emulsions of sucrose?

A. P. MacKenzie. (*a*) Assuming no surface effects, we may safely state that the probability of homogeneous nucleation is a constant per unit volume. The probability of a nucleation occurring somewhere in a given drop will therefore vary with the third power of the drop diameter. Homogenous nucleation of ice in water obeying a relationship of the form: log (nucleation rate per unit volume) = a constant $\times 1/T^3\Delta T^2$, where T is the absolute temperature and ΔT is the supercooling, the dependence of the nucleation temperature on the drop diameter can be determined. (*b*) The asymmetric shape of the well in the warming curve in figure 5*b* is explained by the increasing dissolution of ice in the concentrated amorphous sucrose with increasing temperature above T_{im}.

D. S. Reid (*Unilever Research, Sharnbrook, Bedfordshire*). When a frozen system consists, at low temperatures, of a mixture of ice and a concentrated aqueous phase from which no more ice can be formed under normal experimental conditions, further cooling does not change the composition of this concentrated phase. In view of the fact that on further cooling water mobility continues to decrease, and the G_{w} of ice, relative to hypothetical supercooled water also decreases, how well can sorption isotherms be applied to this concentrated phase, which at least coexists, at different temperatures, with a range of different activities of water (as ice) depending on the temperature?

A. P. MacKenzie. The subject is an intriguing one and demands an answer as long as a water mobility can be demonstrated in the concentrated amorphous phase. The question is perhaps best addressed in terms of a changing water structure with decreasing sub-zero temperature (MacKenzie 1975) by which it can be shown that unfrozen water present in roughly constant mass fraction might maintain a G_{w} approximately equal to that of ice, though the temperature continue to fall.

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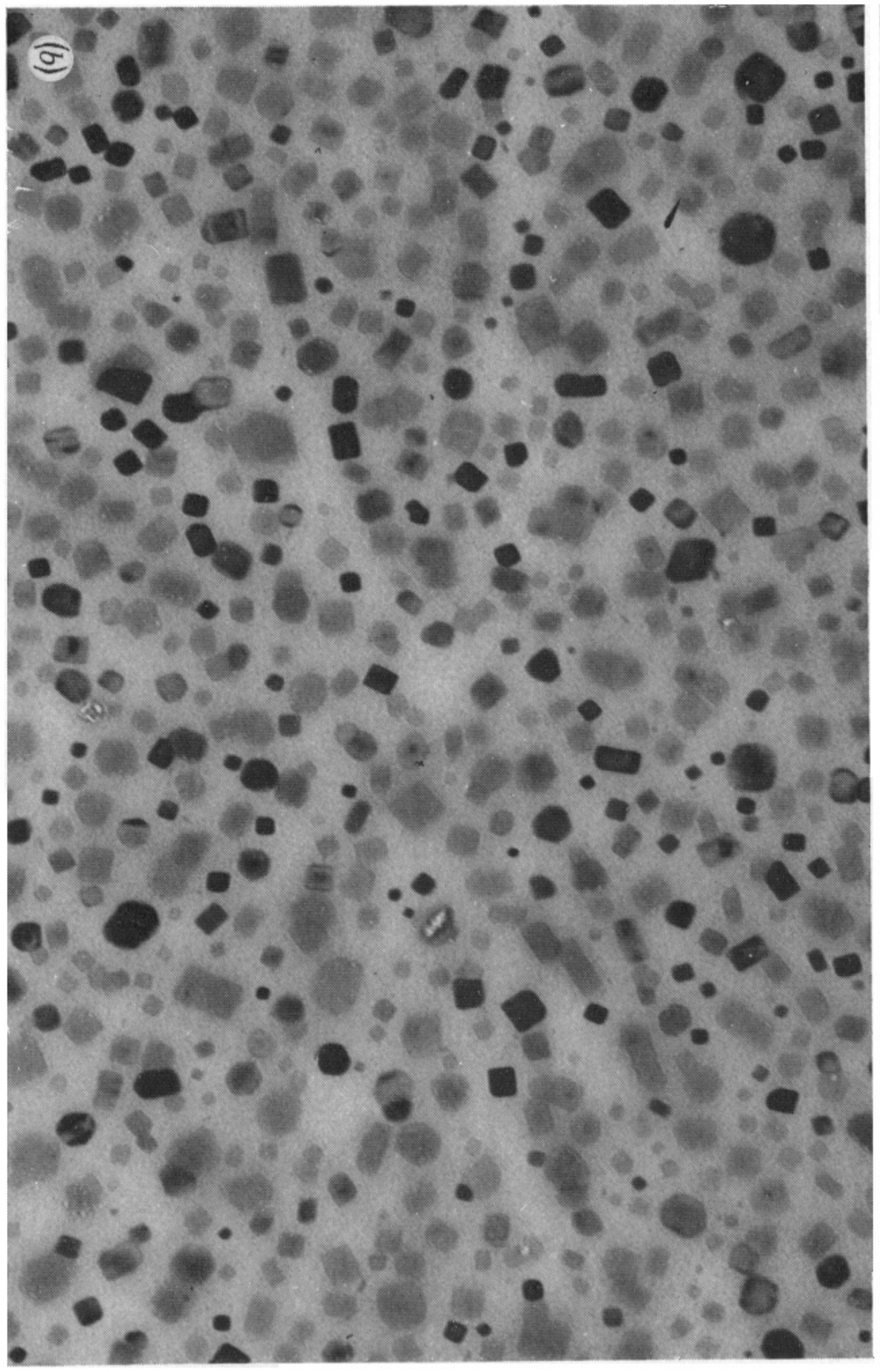

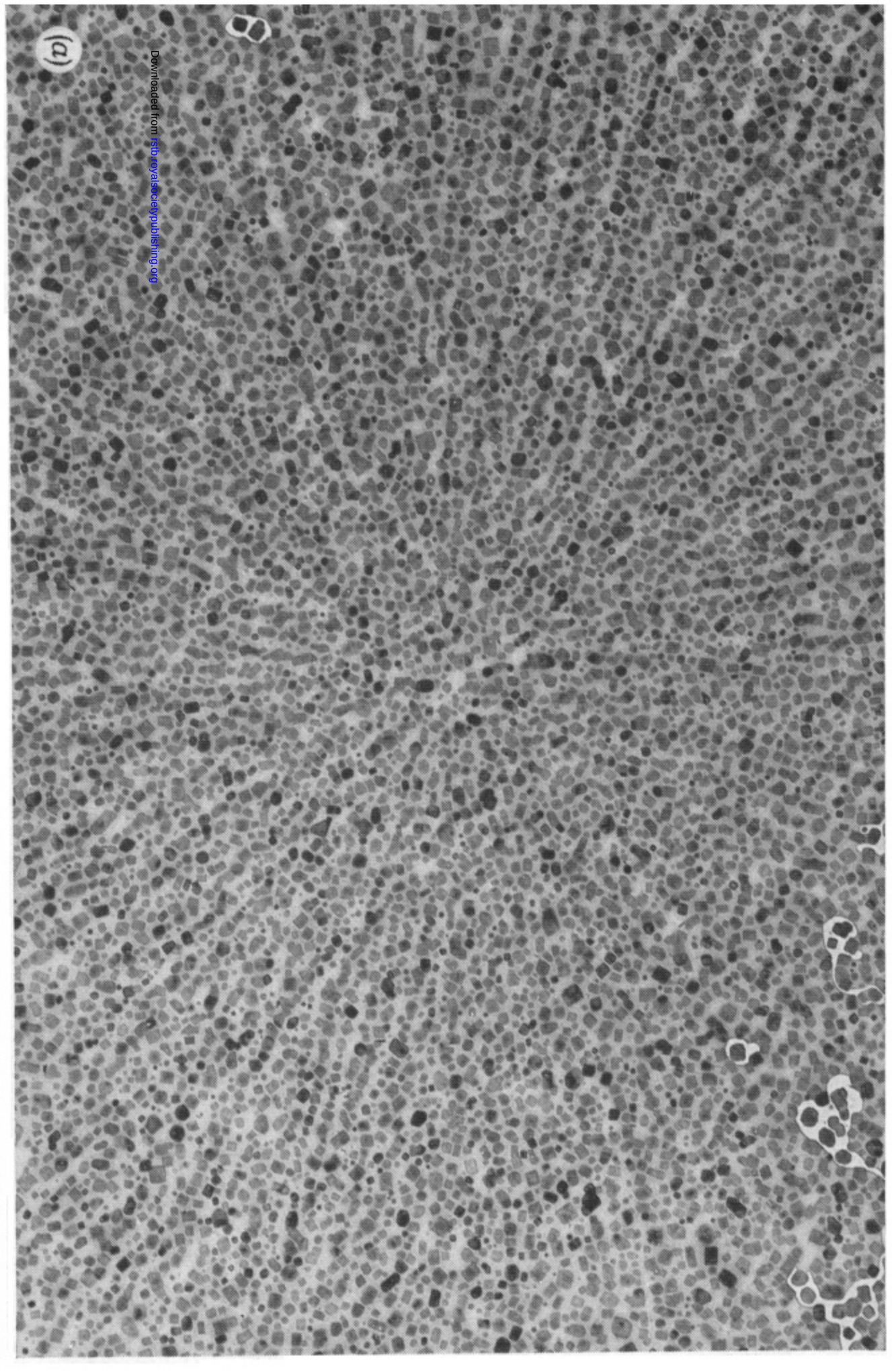

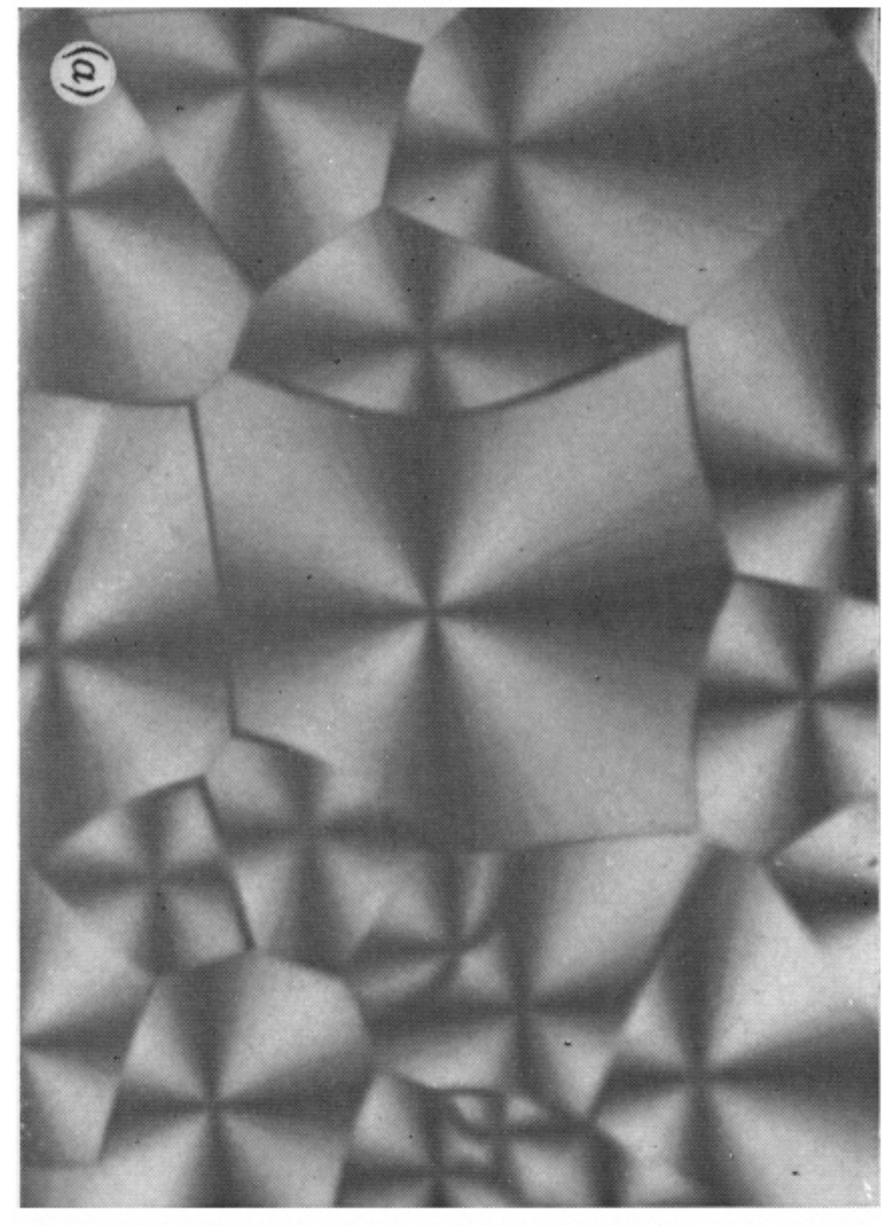

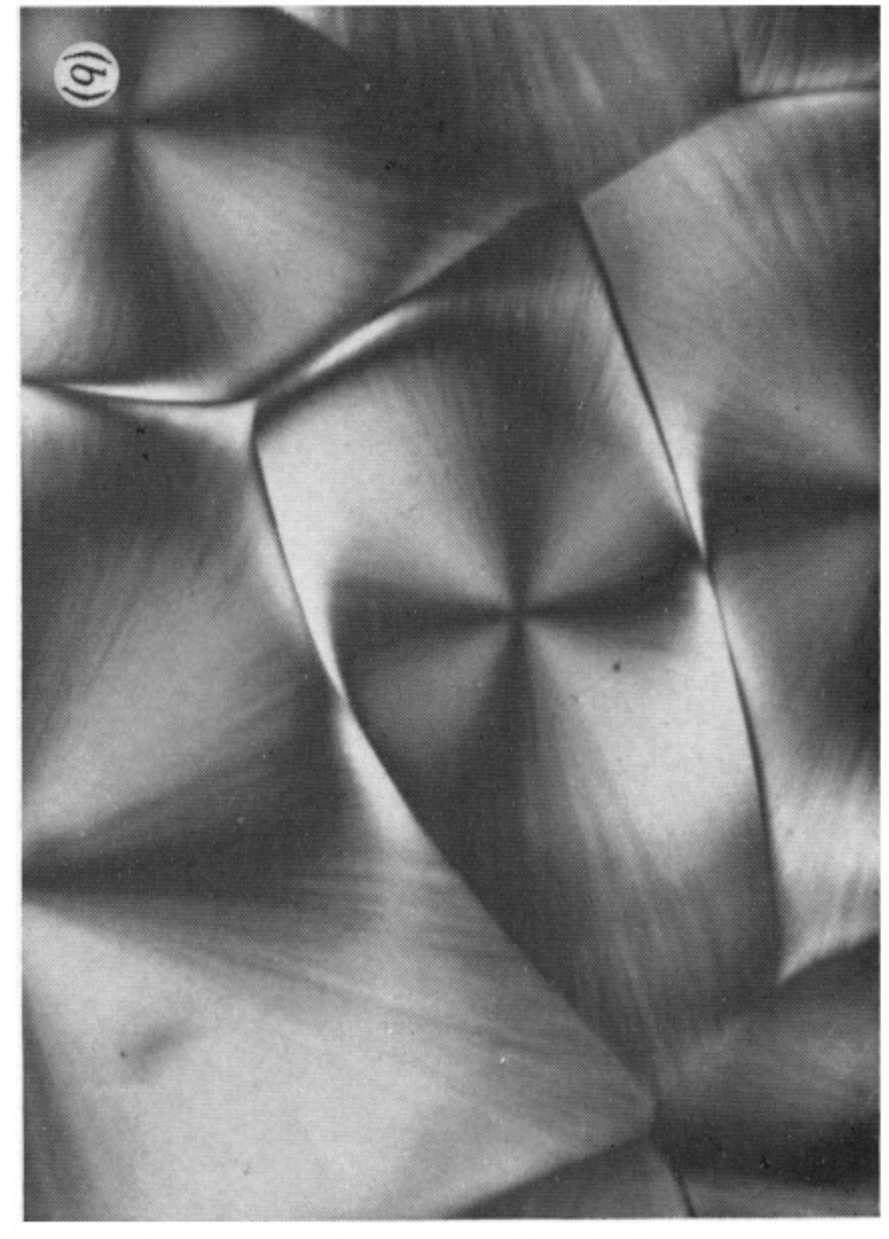

FIGURES 13 AND 15. For description see opposite.

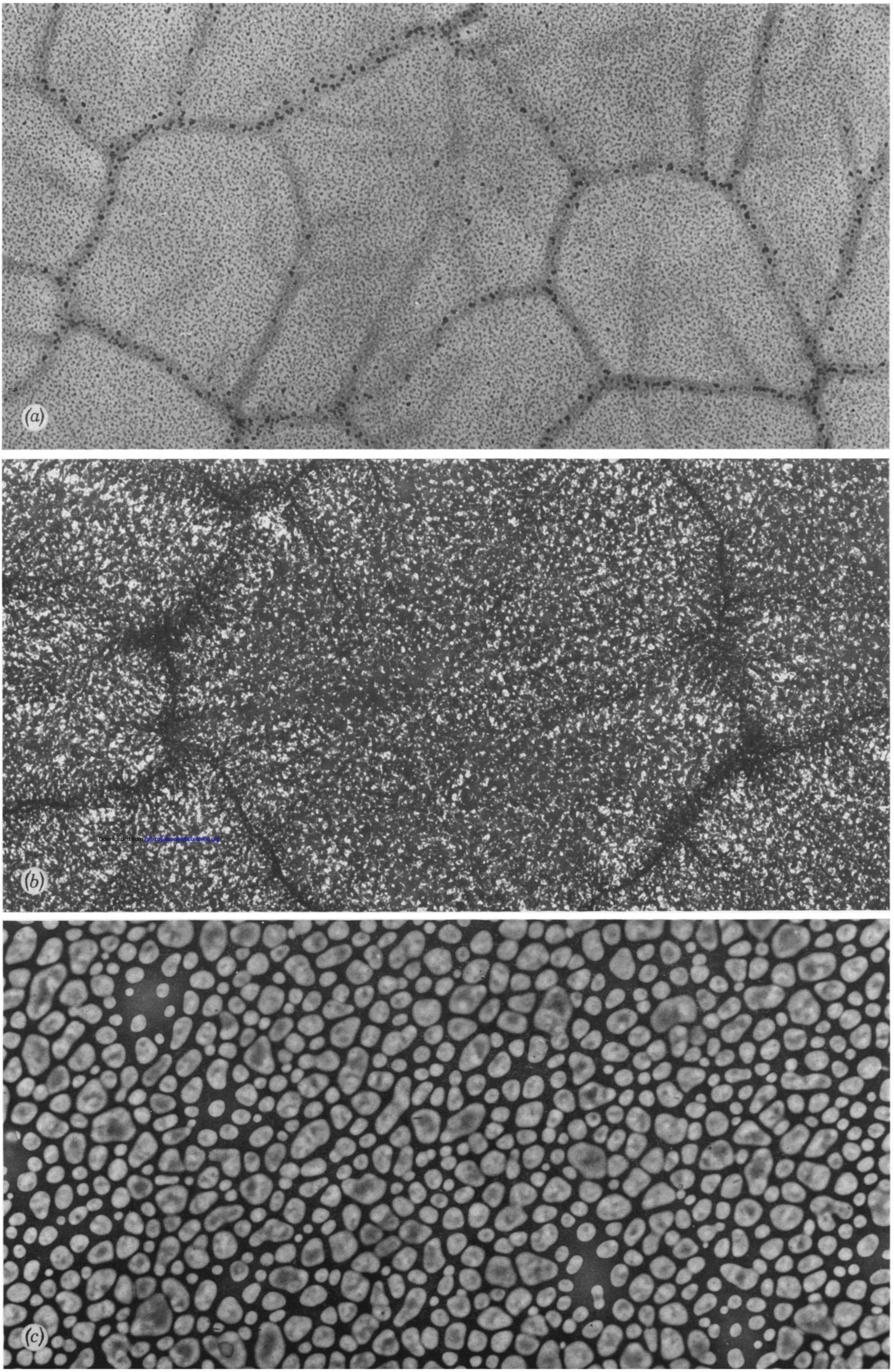

FIGURE 14. For description see opposite.