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Water Transport and Cell Survival in Cryobiological Procedures [and Discussion]

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Water transport and cell survival in cryobiological procedures

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[Plates 1 and 2]

Living cells may be cooled to 77 K (liquid nitrogen) either to destroy them selectively or to store them for long periods. Water transport across the cell membranes during freezing and thawing is a primary factor determining whether the cells survive. These water movements are controlled by phase changes both intracellular and extracellular and by other factors such as the nature of any cryoprotective agent present, and the rates of cooling and thawing. The relation between cooling procedure, water transport and cell survival is discussed. In particular, the crucial rôle of dilution shock is emphasized: this is the damage to cells induced during the dilution that occurs both as ice melts during rewarming and when any cryoprotective additives are removed after thawing.

Apart from the usefulness of understanding these processes for maximizing preservation or controlling selective destruction, the diverse responses of cells to different combinations of water transport and temperature changes appear likely to provide basic information on the properties of cell membranes.

Cell injury during freezing and thawing

The survival of living cells that have been stored at very low temperatures is determined in a large part by the transport of water across cell membranes. There are several ways that the transport of water can affect the extent of cellular injury. One way is by altering the amount of water in the cell and the volume of the cell before the stresses of cooling and rewarming. This has a fundamental effect on cell survival. In addition, the transport of water into and out of the cell can itself be damaging both during cooling and rewarming and during the post-thaw recovery period.

The main objective of applying cryobiological procedures to living cells is the practical one of storing cellular functions for long periods for biological, medical or surgical purposes. The storage environment is usually liquid nitrogen (−196 °C). In addition, when mixed cell populations, for example as in blood, are frozen together, it is possible to carry out some degree of selection by destroying one population in preference to another. Another application of cold is in surgical procedures, where often the only objective is to destroy the cells, as in the freezing of a tumour *in vivo*.

There are several comprehensive reviews on the basic background and use of cryobiological procedures (Smith 1961; Meryman 1966; Wolstenholme & O'Connor 1970; Leden & Cahan 1971; Weiner, Oldham & Schwarzenberg 1973). Potentially damaging events occur during the cooling and rewarming of cells: providing that the temperature is low enough (thought to be below the glass point) deterioration does not take place during storage. The biologist has few weapons that he can use in order to avoid or control the damage during cooling and rewarming although those that he has can be very effective. These techniques include the modification of

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the solution around the cell before cooling; and manipulation of the rates of temperature reduction and increase.

In general, damage to cells can be reduced by the incorporation of cryoprotective additives into the cellular suspension before freezing. The usual way to modify the cooling conditions is to alter a more or less continuous rate of cooling between about 0 and −60 °C over the range of 0.1 to 3000 °C/min. One major effect of altering the rate of cooling is to determine the amount of water within the cells when intracellular freezing first occurs.

Control of rates of cooling and use of cryoprotective compounds

The discovery of the cryoprotective properties of glycerol made by Polge, Smith & Parkes (1949) initiated an enormous advance in the preservation of different cell types. It became clear that survival was improved by a combination of the correct concentration of the cryoprotective agent and the best rate of cooling. However, if cooling is too rapid the recovery of functional spermatozoa (Polge 1957), erythrocytes (Chaplin & Mollison 1953) and many other cell types is worsened even in the presence of a cryoprotective agent. At first sight, the shorter exposure to the potentially hazardous conditions provided by the more rapid rates of cooling would be thought to be beneficial but this did not prove to be the case.

With a limited number of cell types the details of the interrelationship between the nature and concentration of cryoprotective agent and the cooling rate giving the best survival have been determined (Rapatz & Luyet 1965; Mazur & Schmidt 1968; Mazur, Farrant, Leibo & Chu 1969; Leibo *et al.* 1970). Several general points emerged.

(*a*) The cooling rate giving the best survival varied between different cell types in one species, and between species for the same cell type.

(*b*) Survival was lower at both slower and faster rates than the 'optimal' rate implying that there were at least two damaging mechanisms oppositely dependent on the rate of cooling.

(*c*) Most cryoprotective agents improved survival mainly by 'protecting' against the damage caused at slower cooling rates. The incorporation of a protective compound or increasing its concentration thus had the effect of slowing the cooling rate that gave the best survival.

Several fundamental findings became apparent. First, freezing within a cellular suspension began by heterogeneous nucleation extracellularly. At rapid cooling rates (defined as those faster than that giving optimal survival), intracellular supercooling became so great that intracellular ice formed and the cells reached the lower temperatures essentially unchanged in volume and with ice both within and without. In contrast, at rates of cooling slower than that giving highest survival, the extracellular freezing induced some of the water to leave the cell and by the lower storage temperatures the cells had lost a high proportion of their internal water and were grossly shrunken (figure 1).

In addition, it became clear that increasing the osmolality of the initial solution before freezing, by incorporating a cryoprotective additive such as glycerol, dimethyl sulphoxide (DMSO), or sucrose, reduced the amount of ice present at any temperature during freezing. This had the consequent effect of moderating the extent of the rise in concentration of the normal solutes necessary in a biological solution to maintain the viability of the cells (mainly electrolytes) (Lovelock 1953, 1954). The accepted neat analysis of the system can then be described as follows: during 'slow' cooling cells are damaged by some mechanism due to increased concentration of solutes, particularly electrolytes, and the extent of this concentration

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rise and consequent damage is moderated by cryoprotective additives; during 'rapid' cooling the cells do not have time to shrink and are damaged by some mechanism caused by the presence of intracellular ice within the cell (figure 1).

In the search for understanding the mechanisms of freezing injury during slow and rapid cooling the problem has been to isolate the different physical events that occur during freezing in order to find out their relative importance on the ultimate cell survival.

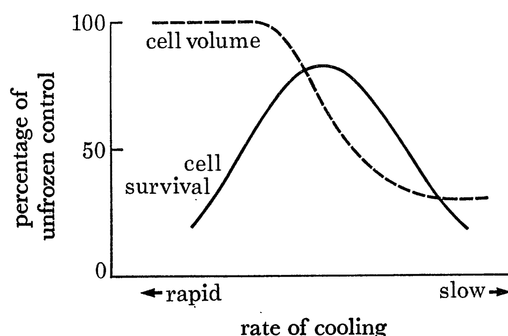

FIGURE 1. Diagrammatic representation of cell survival and cell volume (after cooling but before rewarming) at different cooling rates. Cells cooled too rapidly do not shrink during cooling, contain intracellular ice and are damaged. Cells cooled too slowly have time to shrink during cooling but are damaged by exposure to concentrated solutions. Intermediate cooling rates, that vary with cell type and cryoprotective additive optimize the survival.

It is clear that during the freezing and thawing of a cellular system a large number of interrelated physical events occur. These include:

the formation of extracellular ice
increased extracellular osmolality
increased extracellular ionic strength
the formation of intracellular ice
increased intracellular osmolality
increased intracellular ionic strength
osmotically driven water movements during freezing
osmotically driven water movements during thawing
increased concentration of cryoprotective agents outside the cells
increased concentration of cryoprotective agents inside the cells
solute transport across the cell membrane.

Although for most practical preservation procedures cooling rate techniques are used, it is almost impossible to isolate the separate effects of these different damaging stresses using techniques in which temperatures change continuously.

This paper discusses the hypothesis that the damage or survival of living cells during freezing and thawing is primarily linked to bulk water transport across cellular membranes. In the temperature range in which damage occurs (0 to −60 °C), the extent and direction of water movements are controlled by the occurrence and amount of extracellular and intracellular ice during a particular freezing and thawing schedule. It will therefore be best to consider the freezing of a cell in suspension under the general headings of extracellular ice and intracellular ice.

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Extracellular ice

Formation of extracellular ice

As has already been discussed freezing of a cellular suspension usually originates outside the cell by a process of heterogeneous nucleation. If cryoprotective additives are present there is some depression of the melting point but the major physical effect that is relevant to cell survival is that less ice is formed in the system at any temperature during freezing (Lovelock 1953). This is true both for low molecular mass protective compounds and also for polymers (Farrant 1969). The latter do not affect the melting point of the system significantly but do reduce the amount of ice during freezing since there is a steep rise in their osmotic coefficients as their concentration increases.

Damaging effects

It is unlikely that cells are injured by the contact of extracellular ice crystals disrupting their membranes since most recent work shows that extracellular ice is perfectly compatible with cell survival. There is a report by Nei that in the freezing of erythrocytes at relatively high subzero temperatures the liquid volume available is smaller than the volume of the cells. He suggests that the observed haemolysis could be due to direct pressure by the ice on the cells (Nei 1970).

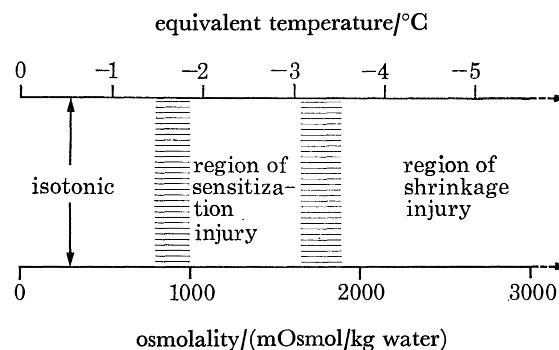

Figure 2. Regions of high osmolality that damage erythrocytes at a constant temperature (+4 °C). Above 1800 mOsmol/kg water membrane injury is linked to cell shrinkage whether the hypertonic solute is sodium chloride or sucrose. Between about 800 and 1800 mOsmol/kg water the cells were injured in sodium chloride but not sucrose but only if a subsequent stress was applied. The top abscissa gives the subzero temperatures at which the different osmolalities are reached.

If it is not the direct action of ice that damages the cell during slow freezing two other possibilities are, first a direct attack on the cell membrane by the increased concentration of extracellular solutes and secondly the osmotic effect on the cell of the high concentration of solutes. Meryman has suggested that during slow cooling there is a progressive withdrawal of cell water until no more water can leave the cell due to the internal bulk of structures and solutes such as haemoglobin (Meryman 1968, 1974). He has suggested that this sets up a hydrostatic pressure gradient across the cell membrane that is a damaging process. However, the minimal cell volume observed, used as a justification of this hypothesis, may be a consequence of cell injury rather than its cause. Once the cell membrane is damaged the volume of the cell will not continue to decrease during slow freezing since rapid transport of solutes would now be possible through the membrane. We have reported that red cells exposed to increasing concentrations exhibit membrane damage at an osmolality of about 1800 mOsmol/kg water providing that the

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solute does not penetrate the cell membrane readily (Farrant & Woolgar 1972*a*, *b*). Thus at osmolalities above this figure it appears that the cell membrane is damaged as a result of cellular shrinkage. This is true whether the solute is ionic or not. Figure 2 shows that this occurs at relatively high subzero (°C) temperatures.

Figure 2 also shows that at lower osmolalities (higher temperatures during freezing) there is a region that can be termed 'sensitization injury'. This phenomenon was first reported for red cells by Lovelock and appears to be present when the solute that is being concentrated by the freezing is ionic (Lovelock 1955). In the less severe conditions leading to sensitization injury the cell membrane is not damaged directly. Damage occurs only when an added stress is present. When a stress is applied the membrane is injured as judged by a large increase in its permeabilities to cations and normally non-permeant solutes such as sucrose (Daw, Farrant & Morris 1973). Typical stresses that induce injury include a simple reduction in temperature (the so-called thermal shock phenomenon) and a water flux across the cell membrane such as that induced when the cells are returned from the high ionic strength solution to isotonic conditions (Farrant & Morris 1973). It can be seen that the increase in ionic strength accompanied by a fall in temperature are phenomena that occur during freezing, and that a reduction of ionic strength back to isotonic conditions occurs during thawing. We have evidence that during the hypertonic exposure of the cell to the sensitization injury range there is no significant increase in intracellular solute concentration, so one would expect that the cell volume would return to normal without injuring the cell. This however is not so and it appears that the cell cannot withstand a water flux that should be innocuous. In the more severe conditions present above 1800 mOsmol/kg water (the region of shrinkage injury) extra solute molecules do enter the cells during hypertonic exposure and on return to isotonic conditions (as would occur during thawing) there is an excessive uptake of water leading to cell disruption. Thus, during conditions of slow freezing the uptake of water is a stress that can injure the cell both in the presence and absence of extra molecules of intracellular solute.

Cryoprotective substances

These compounds reduce the amount of ice and thus reduce the build up in ionic strength. In addition they alter the water balance of the cell in that they can shrink the cell before cooling (if they are non-permeant) or provide osmotic fluxes during and after thawing as they leave the cells (if they are permeant). There is also evidence that during freezing and thawing normally excluded solutes (like sucrose for erthyrocytes) can enter the cells (Daw *et al.* 1973).

It also seems to be possible that cryoprotective agents can have a direct action on cell membranes that alters the response of the membrane to a stress when it is applied.

Intracellular ice

Rate of cooling that prevents shrinkage

First we can consider cells cooled rapidly in one step to a very low temperature (e.g. −196 °C) in a solution without any added cryoprotective agent. Because of the high rate of cooling both the extracellular and intracellular compartments will supercool rapidly. Even if freezing begins outside a cell before it does inside there will be insufficient time for any significant amount of intracellular water to leave the cell osmotically before the intracellular water will freeze also. Essentially then the cell will reach the low storage temperatures before any mass transport across

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its cell membrane has taken place. At these low temperatures physical processes such as water movement across membranes and deposition of water onto ice virtually cease. The stored cell will contain intracellular ice and there will be extracellular ice also. The limiting rate of cooling that is fast enough to prevent any significant osmotic loss of water from the cell during cooling is primarily a function of the water permeability of the cell and its temperature dependence.

There have been several attempts to predict the effect of different rates of cooling on cell volume during freezing, from primary cell data such as cell area, water permeability and the temperature dependence of water permeability (Mazur 1963; Ling & Tien 1970; Mansoori 1974). The original approach was that of Mazur (1963). The difficulty of applying equations concerned with the rate of water loss from cells during linear cooling in the presence of extracellular ice is chiefly that of obtaining data for such variables as the temperature dependence of water permeability at subzero temperatures. Despite these limitations excellent agreement has been obtained between theoretical analysis of predicted water loss and directly observed cell volume changes using an elegantly designed cryomicroscope to observe the freezing process directly (Diller & Cravalho 1971).

If intracellular ice is damaging, several questions remain. Is it the presence or amount or size of intracellular ice crystals that is important? What is the mechanism of damage, is it by interactions between the ice itself and the cell components or is it an indirect effect on the cell *via* the intracellular liquid phase or by water transport during and after melting?

Formation of intracellular ice

The first question to consider is how does intracellular ice form. When biological systems of cells in suspension are frozen *in vitro* freezing always begins in the solution bathing the cells. This is presumably due to heterogeneous nucleation from particulate matter in the solution. The melting point of biological solutions is of the order of −0.56 °C; however, spontaneous freezing of a biological solution (in the absence of any added cryoprotective agent) usually occurs in the temperature range −1 to −10 °C.

Intracellular water can freeze either by nucleation within the cell or by the penetration of ice crystals through the cell membrane from the extracellular environment. Let us first consider the homogeneous nucleation of water within cells. In pure water this process begins at −39 °C. It has been shown that the presence of solutes depresses the temperature at which homogeneous nucleation begins rather than elevating it (Rasmussen & MacKenzie 1972). It thus seems probable that if a cell avoids internal freezing by heterogeneous nucleation during progressive cooling, that at about −40 °C or below the cell water will nucleate by the homogeneous process. This could explain the result we have shown with Chinese hamster cells frozen for 5 min to a series of subzero temperatures before thawing. The temperature of about −40 °C was the lowest limit of a reasonable recovery of functional cells.

Description of plate 1

Figure 3. Electron micrograph of freeze-etched rat ascites tumour cells primarily frozen at −27 °C. A cell with large intracellular ice crystals is shown beside a dehydrated cell with fine granulous crystals (from Shimada & Asahina 1975). (Magn. × 7500.)

Figure 4. Electron micrograph of freeze-etched rat ascites tumour cells subsequently warmed to −17 °C from −27 °C. Ice crystal grain growth was remarkable after a period at −17 °C suggesting that the original crystals had formed at −27 °C (from Shimada & Asahina 1975). (Magn. × 7500.)

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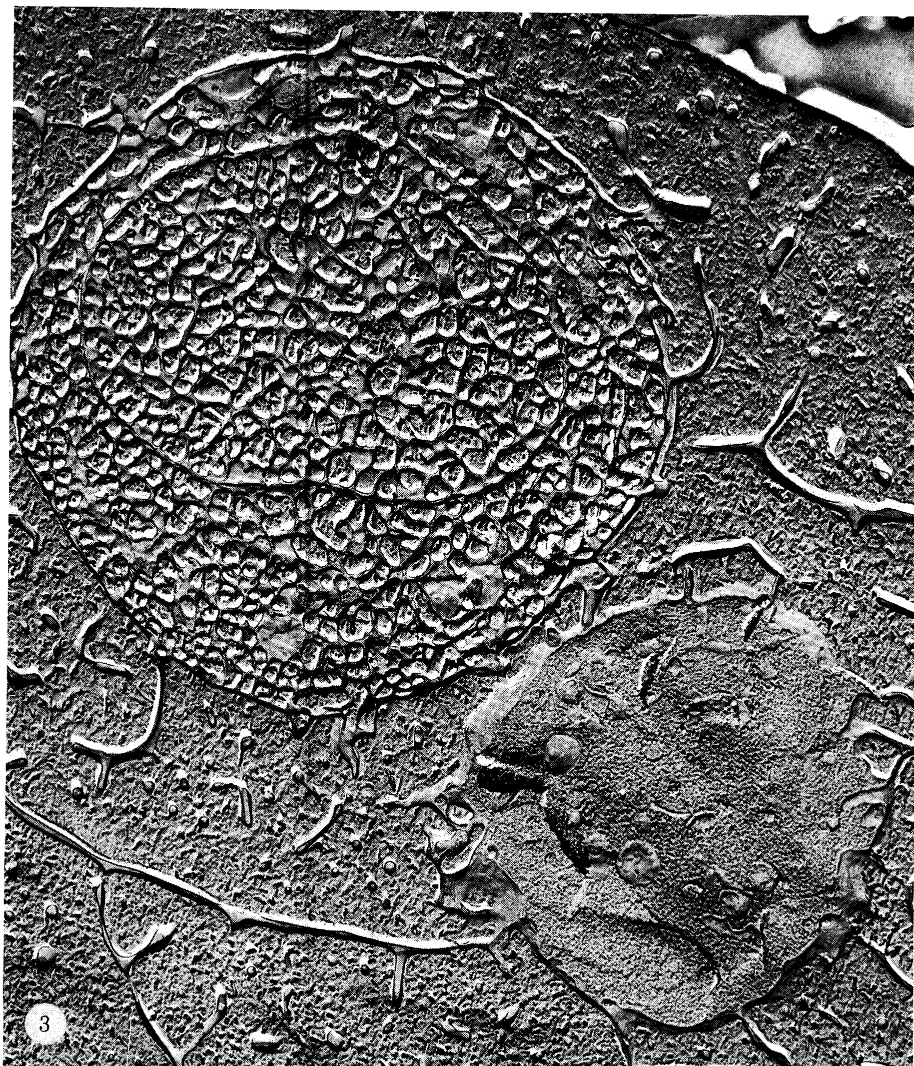

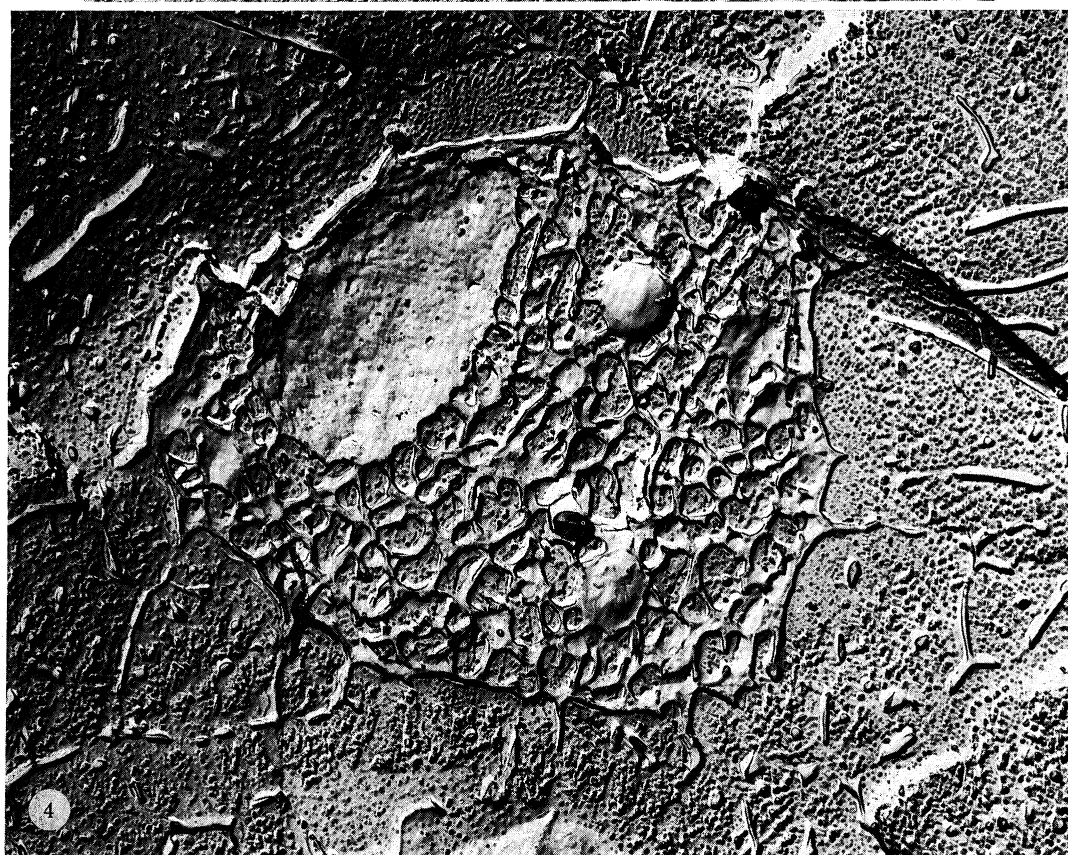

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FIGURES 3 AND 4. For description see opposite.

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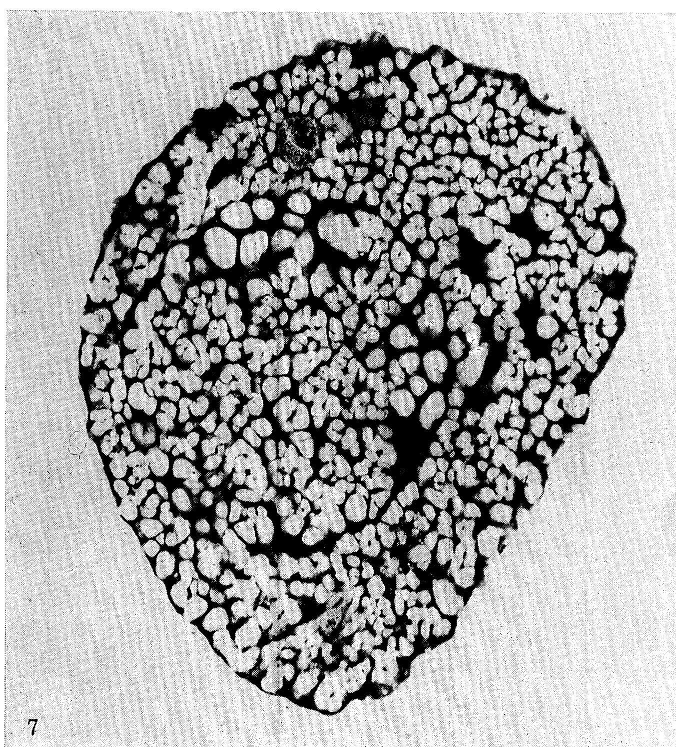

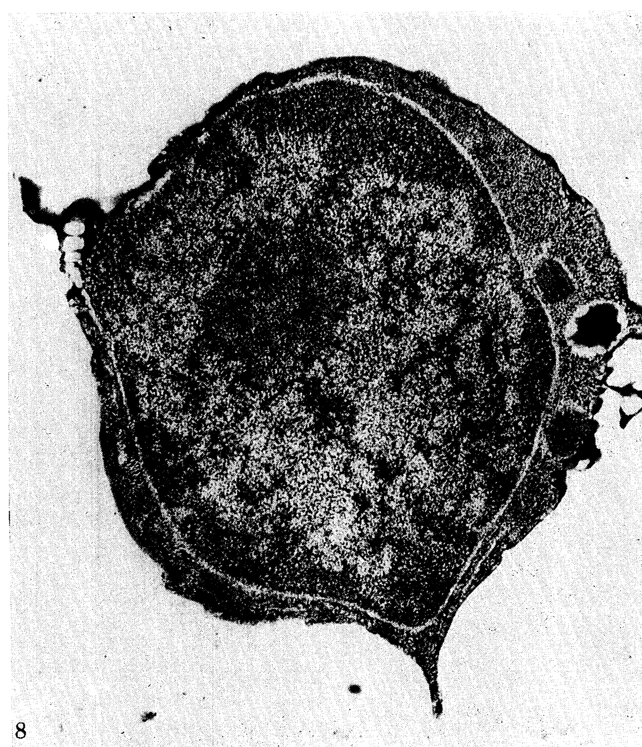

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FIGURES 6–8. For description see opposite.

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However, many cells do not escape intracellular freezing even at relatively high subzero temperatures despite the fact that cell membranes can act as barriers to the growth of ice. In 1932 Chambers & Hale showed that amoebae will only freeze internally when they are punctured with an ice-tipped pipette even in the presence of an extracellularly frozen solution. Mazur has suggested that in some cells the membrane becomes permeable to ice nuclei at about −10 °C. He suggests that this phenomenon is governed by the Kelvin equation that determines the minimum radius of spherical ice nuclei at different temperatures, together with the pore diameter in the particular cell membrane (Mazur 1966).

Although there is little evidence in the literature that the interior of intact cells contain any particulate material that can initiate heterogeneous nucleation, the recent results of Asahina and his group do suggest that this process may occur. They have shown that intracellular ice forms in very rapidly cooled isolated tumour cells even at temperatures as high as −27 °C (Shimada & Asahina 1975). Ultrastructural examination (see figures 3 and 4, plate 1) suggests from the size and nature of the intracellular ice that it has been formed by heterogeneous nucleation within the cell rather than by penetration of ice through the membrane. Asahina has suggested from his results, that the reason why intracellular ice is not usually seen at high subzero temperatures is because the rate of cooling is usually too slow. Clearly if cooling is slow enough to permit any water loss from the cell into the extracellularly frozen environment then intracellular nucleation will be inhibited to some extent.

As the rate of cooling of any aqueous solution is increased, the size of ice crystals formed gets smaller and smaller until a cooling rate is reached when ice crystals cannot be detected by microscopy or by X-ray diffraction techniques (Luyet, Tanner & Rapatz 1962). For some time it was thought that this implied a process of vitrification but later it was shown that ice crystals were present either in the form of evanescent spherulites difficult to distinguish in normal light, or even in the form of nuclei too small to detect but which could grow during rewarming (Luyet 1955). At cooling rates less than about 10000 °C/s (much faster than that feasible for the cooling of a biological system for practical purposes) ice nucleation is inevitable. True glass formation within a living cell may thus be virtually impossible.

Before considering the way that water transport may be involved in the damage to living cells containing intracellular ice during freezing and thawing, it will be useful to consider the protective effect on cell survival of cell shrinkage before intracellular freezing. Loss of cell water can either be induced by slow cooling or by a period at a subzero holding temperature (two-step freezing) providing that intracellular ice did not form during the rapid temperature fall to the holding temperature.

Description of plate 2

Figure 6. Electron micrograph of lymphocyte freeze-substituted at −80 °C after being frozen to −196 °C in dimethylsulphoxide (5% by volume) by a two-step technique involving 60 min in the frozen state at −26 °C. The cell appears grossly shrunken and this procedure allowed a high survival of cellular function on thawing (from Walter, Knight & Farrant 1975). (Magn. × 24000.)

Figure 7. Electron micrograph of lymphocyte freeze-substituted at −80 °C after being frozen directly to −196 °C. This procedure allowed little survival of function. The cell showed little shrinkage and contained large intracellular ice particles (from Walter *et al.* 1975). (Magn. × 14650.)

Figure 8. Electron micrograph of lymphocyte freeze-substituted at −80 °C after being frozen directly to −196 °C. This procedure allowed little survival of function. Again the cell showed little shrinkage but the intracellular ice cavities were finer and less obvious (from Walter *et al.* 1975). (Magn. × 15400.)

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The two-step freezing technique is effective for preservation of spermatozoa (Luyet & Keane 1955; Polge 1957), for erythrocytes (Rapatz & Luyet 1973) and for tissue culture cells and lymphocytes (Farrant, Knight, McGann & O'Brien 1974).

Two-step freezing

In this procedure cells in samples of small volume are cooled rapidly from ambient temperature by immersing them into a subzero constant temperature bath or refrigerator at some preset temperature (e.g. −25 °C). During the initial fall in temperature, the sample freezes. The sample is then maintained at the constant holding temperature for a period before cooling again rapidly to the storage temperature, usually −196 °C (liquid nitrogen) (figure 5).

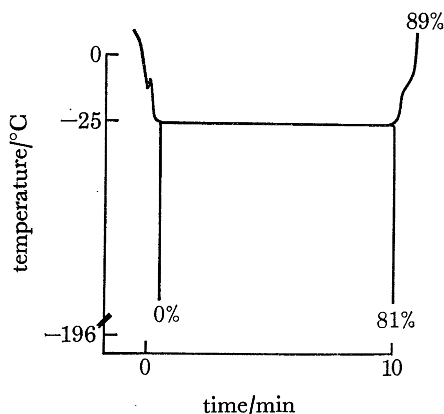

FIGURE 5. Survival (%) of Chinese hamster tissue culture cells in dimethylsulphoxide (5 %) after cooling by different procedures using a two-step technique. Time (10 min) at −25 °C in a frozen suspension protected the cells against injury on further cooling to −196 °C.

When the conditions are optimized an appreciable recovery of the function of many types of cells is observed that must be conferred by the period at the subzero holding temperature, for cells from samples cooled rapidly from ambient into liquid nitrogen without the period at the subzero holding temperature are dead (figure 5).

During two-step cooling two sets of conditions that lead to cell injury can be separated. At the holding temperature itself, the cells are acquiring 'protection' against damage that would otherwise occur during rapid cooling into liquid nitrogen. In addition the period at the subzero holding temperature can itself lead to cell injury. These two effects can be distinguished by freezing samples to the holding temperature and at the end of the designated period thawing one (giving the effect of the damage at the holding temperature) and plunging the other into liquid nitrogen before thawing. Any lack of protection against damage during the second stage of cooling to −196 °C and thawing is detected by the extra damage observed in the plunged sample in relation to the thawed sample. Figure 5 shows this procedure for tissue culture cells cooled in a medium containing DMSO (5 %) as protective agent.

Under appropriate conditions, the period at the holding temperature not only allows the cell to shrink before the second temperature reduction to the storage temperature, but also allows the functional recovery of the cell after thawing from liquid nitrogen. Freeze substitution of lymphocytes at −80 °C after exposure to liquid nitrogen (Walter, Knight & Farrant 1975) showed that when the procedure involved a period at a holding temperature the functional cells recovered appeared to be shrunken and contained no apparent intracellular ice (figure 6,

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plate 2). In contrast, cells plunged directly into liquid nitrogen contained much intracellular ice that was more or less obvious and were less shrunken (figures 7 and 8, plate 2). There is a clear correlation between the amount of intracellular ice and cell damage.

Is intracellular freezing invariably lethal?

For some time there has been a discussion in the literature on whether any cells could survive after they had been frozen internally (Sherman 1962; Bank 1974; Shimada & Asahina 1975). A lot of data suggested that the formation of intracellular ice during freezing correlated with the death of the cells but there were also isolated reports of cells surviving despite the presence of intracellular ice.

Asahina and his group have recently obtained strong evidence that some cells survive intracellular freezing providing that thawing was extremely rapid, especially over the temperature range of −30 to 0 °C (Asahina, Shimada & Hisada 1970; Shimada & Asahina 1975). If thawing over part of this temperature zone was done slowly, cells were killed and this destruction was accompanied by observations of the recrystallization of ice from the small crystals produced during rapid cooling to larger crystals (Shimida & Asahina 1972). That damage is caused by the larger crystals formed by recrystallization during thawing is an idea that has been put forward by several workers (Rapatz & Luyet 1963; Moor 1964; Nei & Asada 1972; Bank 1973). Apart from the presence of intracellular ice, and the size of each crystal the total amount of ice is another factor that may be relevant to cell survival.

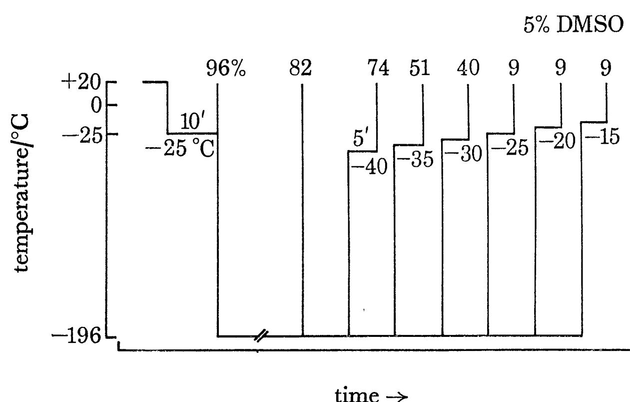

FIGURE 9. Survival (%) of Chinese hamster tissue culture cells frozen in dimethylsulphoxide (5 % by volume) by a two-step procedure involving 10 min at −25 °C. The cells were protected against rapid thawing but could not tolerate interrupted thawing, particularly at high subzero temperatures.

Damage during rewarming

There is a good deal of evidence that the damage to cells caused by intracellular ice occurs during rewarming and not during cooling. For example, Asahina has shown that some cells with intracellular ice can survive if thawed very rapidly (Shimada & Asahina 1975). It has also been shown that cells cooled by cooling rate techniques at rates faster than the optimal rate (conditions favouring intracellular ice) are more sensitive to a slowing of the rate of thawing than are slowly cooled cells (Mazur *et al.* 1969; Leibo *et al.* 1970).

In a similar way we have shown that following a two-step preservation procedure cells will survive a rapid thaw but will not survive if thawing is interrupted by a short period at a high

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subzero temperature. By this means the temperature region in which damage can occur during thawing can be isolated (figure 9). It is interesting that if the experiment shown in figure 9 is done again with the holding temperature during cooling being at −35 °C instead of −25 °C that the cells can tolerate slow thawing even at high subzero temperatures. This suggests that the greater cell shrinkage presumably possible at −35 °C protects against injury during interrupted or 'slow' thawing.

Injury by intracellular ice

It is thus clear that the presence of intracellular ice is not damaging *per se*. As yet there has been little to isolate the specific mechanism of damage. Mazur has suggested that the injury due to intracellular ice is caused by the rupture of the membranes of intracellular organelles by ice growth within a frozen organelle. It is the direct action of large ice crystals that is proposed as the damaging factor. A general consensus of views is that the large crystals formed during thawing can disrupt membrane systems within the cell.

Water transport as the damaging mechanism

In this paper I would like to suggest another possibility for the causes of cellular injury by intracellular ice. This hypothesis involves water transport as the damaging mechanism. It seems clear that once intracellular ice forms, osmotic movements of water into and out of cells virtually cease. The chemical potential of water in a system containing ice at equilibrium is determined solely by the temperature. Thus when freezing first occurs extracellularly it is possible for water to leave the cell. However, once freezing occurs within the cells by whatever mechanism this water transport will stop. Thus intracellular freezing virtually seals cells to water movement. Once freezing continues to lower temperatures than that at which the intracellular ice first appears more ice will form both inside and outside the cell and it is probable that the removal of liquid water by this process will be more rapid than any movement of water across cellular membranes, particularly at these low subzero temperatures although specific data are not available.

One implication of this idea is that the growth of intracellular ice during the freezing of a cellular system can only be within each cell at the expense of the water already in the cell at the moment that the intracellular ice first formed. Thus if a cell freezes internally in its unshrunken state there will be a large amount of intracellular ice formed at the lowest storage temperatures. In contrast if a cell has had time to shrink and lose its water osmotically before intracellular ice occurs then the final amount of intracellular ice at the lowest temperatures will be much less. In general it seems that the same argument can be put for processes during the thawing of a system of cells containing intracellular ice. The growth of larger ice crystals during the recrystallization process can only be at the expense of ice or perhaps water that is already within the cell.

The above analysis is a generalization assuming no temperature gradients across the cell membrane during both cooling and thawing and complete equivalence of the chemical potential of ice in both compartments. I believe however that the key to understanding the mechanism of injury by intracellular ice can be shown by the incompleteness of the above analysis and I would like to suggest two possible sources of inequality of the chemical potential of water across the cellular membranes during thawing.

The first source of inequality is due to the different sizes of the ice crystals in the two compartments inside and outside the cell. Usually extracellular ice crystals are larger than intracellular ice. However, as the rate of cooling is increased intracellular ice is formed in smaller and smaller

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units. These crystals are small enough to have a lower equilibrium melting point due to the change of interfacial energy between the ice and the liquid water. This process is described by the Kelvin equation. Thus at a temperature during freezing below the recrystallization temperature the chemical potential of water buffered by the presence of ice is different on each side of the cell membrane purely as a result of the small size of the intracellular ice crystal. This constitutes a driving force for water transport.

It is interesting to examine the effect on this system of different thawing conditions. We know that in most systems rapid thawing is less harmful than slow thawing. During rapid thawing there is less time for recrystallization of ice to occur and therefore less time for the cells to exist during rewarming with the driving force for water movements imposed by the small crystals.

The second possible course of inequality of the chemical potential of water across cell membranes during thawing is that due to the imposition of non-isothermal conditions during rewarming. During the rewarming of a system containing different amounts of ice and different sizes of ice crystals the absorption of heat by the ice will retard the temperature rise locally in the vicinity of the larger ice crystals and thus impose temperature gradients throughout the system. In addition the smaller ice crystals will melt first. During rapid rewarming of a cellular system therefore it seems logical to expect that the intracellular ice crystals will melt before the large extracellular crystals. Thus during rapid thawing the melting of intracellular ice before significant amounts of recrystallization has a chance to occur implies the imposition of a chemical potential gradient across cell membranes that will act as a driving force for the loss of water from the cell during the period that the extracellular ice remains. From the data already discussed this is a less damaging procedure than the events that occur during slower thawing. In those circumstances there is sufficient time for recrystallization of ice within the cell and this will reduce any transmembrane temperature gradients. The driving force for loss of cellular water by this mechanism will thus be less. Another factor to be considered is the latent heat evolution if recrystallization is rapid (Luyet 1955). Although it is not yet possible to distinguish between this hypothesis involving bulk water transport and the suggestion that intracellular ice damage is caused by direct disruption produced by the damaging crystals formed during recrystallization, one point in its favour is that during slow cooling damage can be caused by water movements rather than by the direct effect of ice.

The difference between 'slow' cooling and 'rapid' cooling is not that between conditions where intracellular ice can or cannot form. It seems to be inescapable that even the most grossly shrunken cell produced by slow cooling techniques or by a long period at a subzero holding temperature in the two-step process must freeze internally during the reduction of temperature to liquid nitrogen. The fact that there is little water to freeze inside a grossly shrunken cell does not mean that it will not freeze. This conclusion must be so if the homogeneous nucleation temperature is higher than the glass point. The shrunken cell that is 'protected' from freezing injury thus does not escape intracellular ice but very little is formed.

Conclusion

The bulk movement of water is an important factor in the survival of cells following freezing and thawing. In particular it can be a stress that damages cells directly as when erythrocyte membranes are 'sensitized' by high ionic strength solutions. In addition, at higher osmolalities in 'slow' freezing, cellular shrinkage is itself a cause of injury.

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The injury associated with intracellular ice and 'rapid' freezing appears to occur during rewarming and it is suggested that this damage is due to changes in transmembrane water transport during thawing rather than to a direct disruptive effect of intracellular ice.

I would like to thank Professor E. Asahina, Professor T. Nei, Dr Stella Knight and Dr C. A. Walter for helpful discussions. I am also grateful to Dr K. Shimada and Dr C. Walter for allowing me to use their electron-microscopical data.

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Discussion

P. Molyneux (*Pharmacy Department, Chelsea College, Manresa Road, London, SW3 6LX*). There seems to be an interesting parallel between your results and the work presented by Professor Gould on the formation of endospores by bacteria. It would appear that the most efficient preservation of cells in a cryobiological procedure is obtained when the cells are held at a moderately reduced temperature (−25 °C) during which they are transformed into a shrunken and largely dehydrated state, before being taken down to liquid nitrogen temperatures. Such a state is thus analogous to that of the bacterial spores. If this analogy is correct, it would seem to serve as a useful guide in the devisal of the optimum cryobiological régime.

J. Farrant I agree. We are using the extremely high osmolalities present at −25 °C in a frozen aqueous system to shrink the cells minimizing the amount of intracellular ice and thus preventing damage on thawing. Perhaps bacterial spores have a sufficiently low water content that they do not suffer injury during freezing. I am not sure about that. However, shrinkage of mammalian cells does have a limit, for as I have shown for temperatures above 0 °C, shrinkage can itself correlate with injury.

J. B. Hasted (*Department of Physics, Birkbeck College, Malet Street, WC1E 7HX*). Perhaps the removal of intracellular water might be assisted by electrophoresis or electroendosmosis during the first freeze?

J. Farrant. Perhaps, but I do not know.

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P. Meares (*Chemistry Department*, *University of Aberdeen*, *Meston Walk*, *Old Aberdeen*, *AB*9 2*UE*). When intracellular freezing takes place the latent heat of freezing must flow outwards through the cell membrane. This would produce a relatively large heat flux under a small temperature gradient. It seems not possible that a coupling of this heat flow with a water flow, as in thermo-osmosis, might lead to an appreciable contribution to the overall water movement which occurs, despite the near equality of the chemical potentials of water inside and outside the cells when ice is present inside and outside. I wonder whether any attention has been given to the possibility of coupling between heat and matter flows in cryobiological procedures.

J. Farrant. The suggestion that I have made that the melting of intracellular ice can lead to a water flow across the cell membrane affecting survival is relatively recent and I am not aware of any quantitative investigation, either theoretical or practical, into the possibilities of the coupling between heat and mass flows being relevant to cellular survival. However, I am heartened that you think the effect of such a coupling may be appreciable.

P. Echlin (*Botany School*, *Downing Street*, *Cambridge CB*2 3*EA*). It would appear that a paradoxical situation exists in the examination and analysis of biological material which has undergone freezing and/or thawing as part of the preparative procedures for light or electron microscopy. The evidence presented here at this meeting and elsewhere in the scientific literature suggests that if the morphology of the specimen is good, as judged by our somewhat subjective assessment as to what constitutes a good image, then the survival rate of such cells is poor. The reverse also appears to be true. Cells which have a high survival rate after freezing and thawing tend to have a rather poor morphology. The question now remains as to what criterion of excellence we should adopt to judge whether our specimen preparative procedures are preserving cells and tissues in as near a life-like state as possible. This question is probably of more academic (even philosophical!) than practical importance when considering tissues which have also been subjected to exposure to fixatives and polar organic fluids. The question assumes much greater significance in situations where tissues have only been subjected to rapid freezing, and which are subsequently examined and analysed in the frozen-hydrated state. Depending on the natural water content of the cells, a good morphological image is probably indicative of frozen-dried protoplasm whereas a poor morphological image is indicative of frozen-hydrated protoplasm and the latter situation more closely resembles the cell in its natural state. The argument comes full circle when one considers what has been known for a long time by light microscopists that one can never see as much in *living* cells as in cells which have been killed, fixed and stained.

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J. Farrant. The shrunken cells that are produced by a period at the holding temperature in the two-step process were observed by freeze-substitution techniques before thawing. The ultrastructure of these cells after thawing might show 'good' morphology correlating well with good survival.

The second and more important point that you raise is the relevance of the 'good' morphology seen with rapidly cooled cells that on subsequent thawing are shown to be dead. There is rather a cunning way to deal with this problem. In the paper, I have discussed the mechanism by which intracellular ice damages cells during thawing. The rapidly cooled cell that is

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examined by an ultrastructural technique at low temperatures is thus visualized in an as yet undamaged state. The damage to these cells only occurs during thawing, perhaps because our thawing techniques are too slow. The morphologist therefore should perhaps cease to worry about the discrepancy between 'good' morphology and 'bad' survival and instead direct his complaints at the cryobiologist for the inefficiency of his thawing techniques.

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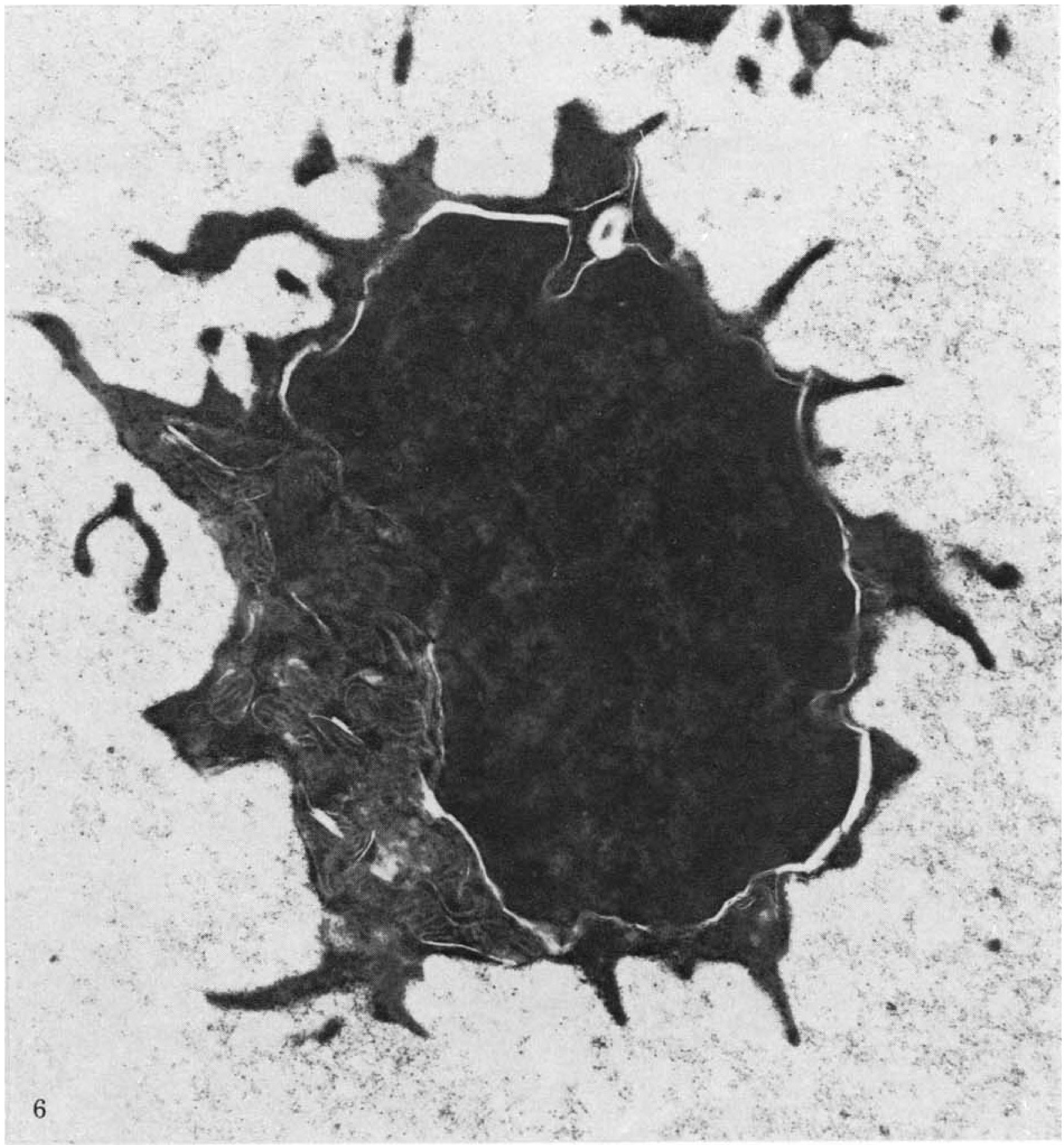
FIGURES 3 AND 4. For description see opposite.



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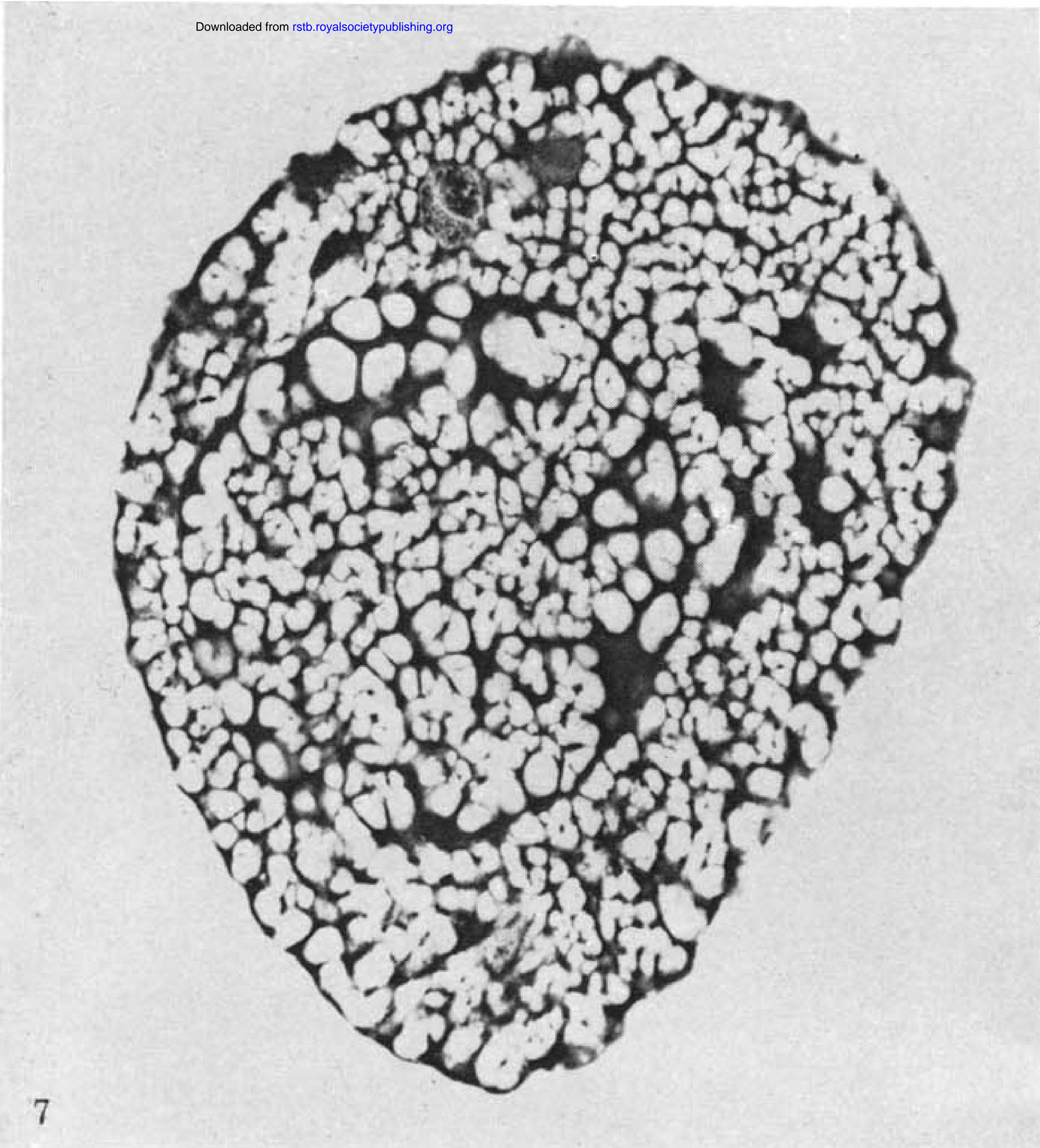

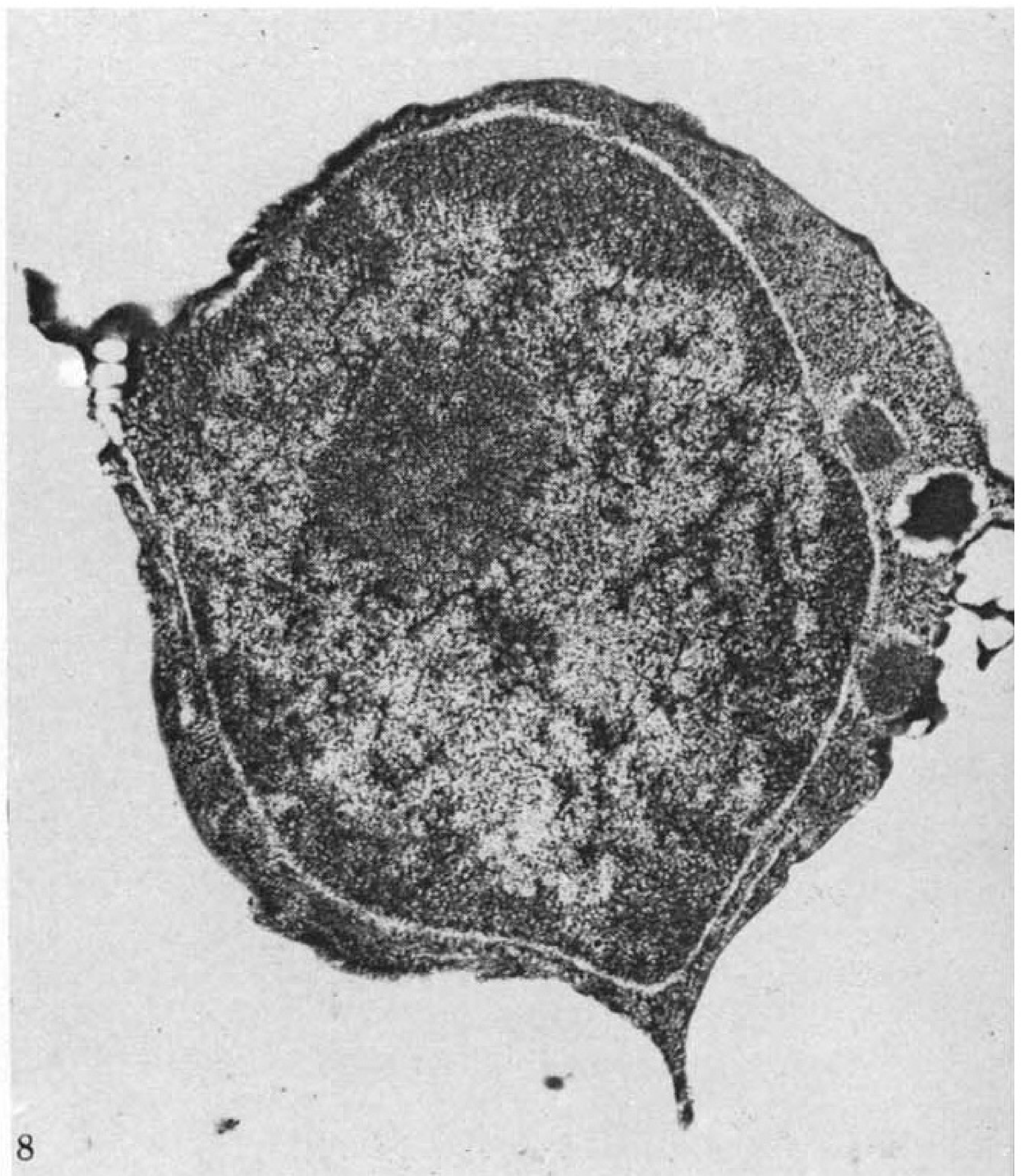

FIGURES 6–8. For description see opposite.

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