Centrifugal Freezing. I. A System for Rapid Freezing of Aqueous Cell Suspensions [1]

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SUMMARY

A centrifugal method for ultrarapid freezing of cells and subcellular particles has been developed, in which small droplets are centrifuged at high speed through liquid nitrogen. When material such as water or blood was injected from a syringe into a rotor, increased rotor speeds gave progressively smaller frozen droplets. Droplet size of materials introduced from a sprayer was uniform, smaller than the smallest droplets obtained from a syringe injection, and independent of rotor speed. Cell survival was found to be inversely related to increasing droplet size and directly related to rotor speed and warming rate.—Nat Cancer Inst Monogr 21: 415–430, 1966.

THE TWO chief causes of injury in frozen and thawed cells appear to be the concentration of solutes produced during freezing and the physical consequences of ice crystal formation within the cell (*1*). Intracellular ice formation can be avoided by cooling cells sufficiently slowly so that they dehydrate (*2*), but they then may succumb to concentration of intracellular and extracellular solutes. Conversely, damage from solutes can be eliminated by increasing the cooling velocity to shorten the exposure time, an expedient that may result in the formation of intracellular ice and cell death.

Some years ago, Luyet and Gehenio (*3*), proposed that if cells were cooled rapidly enough, it might be possible to vitrify the intracellular water to avoid lethal injury. It now appears unlikely that truly amorphous ice can be produced by cooling water in bulk; however, high cooling velocities can produce very small ice crystals and may, in fact, produce ice that is partly crystalline, partly amorphous—especially in cells and tissues. Electron microscopy of rapidly cooled cells shows that crystal size decreases with increasing velocity, and that the highest velocities result in specimens in which ice crystals or spaces left by ice crystals are not resolvable (*4*, *5*).

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The survival of ultrarapidly cooled cells is much higher than that of cells cooled at intermediate velocities. Meryman and Kafig (*6*), Rinfret and Doebbler (*7*), and Luyet *et al.* (*8*) observed this effect with red blood cells, Doebbler and Rinfret (*9*) with a variety of microorganisms, and Moore and Mühlethaler (*5*) with yeast.

Small and imperfect ice crystals produced during rapid cooling will remain so only if they are warmed and thawed rapidly. If they are warmed slowly they grow by recrystallization (*10, 11*). Such slow warming is usually lethal to cells (*12*).

A specimen must have a high surface-to-volume ratio in order to be cooled or warmed rapidly. One attractive way of achieving this high ratio in suspensions of cells is to subdivide the suspension into droplets and spray the droplets into a coolant. Meryman and Kafig (*6*) introduced a technique, subsequently used by Rinfret and Doebbler (*7*), of spraying from a syringe into flowing liquid nitrogen.

One difficulty with the use of boiling liquid nitrogen as a coolant is that the warm, immersed specimen immediately becomes surrounded by a layer of gaseous nitrogen, and heat transfer through a vapor is much slower than through a liquid. However, the density of the vapor is much less than that of the liquid, and this difference suggested a method by which the vapor layer around the specimen could be reduced or prevented. In this method the cell suspension is directed into liquid nitrogen under centrifugal acceleration.

An aqueous fluid stream directed against liquid nitrogen spinning at a reasonably high speed in a hollow-bowl rotor will break up into very small droplets as it is accelerated by the moving liquid nitrogen surface. Centrifugal force will then move the droplets centrifugally at a speed dependent upon the rotor velocity and the density and viscosity of the liquid nitrogen. At high speeds, no insulating gas layer should exist around the aqueous droplets. Instead, the liquid nitrogen may form gas bubbles behind the sedimenting droplets or the gas may be stripped from the droplets as it is formed because of its low density and the velocity of the droplets. In addition, the boiling point of the liquid nitrogen would tend to be depressed near the rotor edge as a result of the pressure gradient.

The reverse problem, rapid thawing, may be approached by allowing the frozen droplets to fall against a warm aqueous solution spinning in a hollow-bowl rotor similar to the one used for freezing. In this case the frozen particles move rapidly through the warm fluid during acceleration to the tangential velocity of the liquid surface. With the proper choice of densities, the frozen droplet will float back to the surface since the density of ice (0.917 g/cc) is lower than that of the surrounding water, even at 37° C (0.9934 g/cc). Cold fluid at the surface of the frozen particle will be denser than either the particle or the suspending medium, and, in a centrifugal field, will tend to move rapidly away.

The question to be asked is simply: Will this technique permit cells and particulate material to be frozen and thawed rapidly enough to observe experimentally the theoretical advantages of ultrarapid cooling?

MATERIALS AND METHODS

Rotor Design

Three rotor designs were used. The first was designated "rotor F-I-f" [4] and is similar to that shown in figure 1. The brass outer shell of the rotor fits the drive spindle of a refrigerated centrifuge.[5] The shell supports a closely fitting inner cup machined from methacrylate plastic and drives it by friction. The cup must be made from a solid piece of plastic, since glued models invariably crack at low temperatures.

To allow rapid transfer of frozen droplets to the thawing bath, the original cup was modified to accept a plastic transfer cap. This second rotor type, F-II-f, is shown in figure 1. The opening at the top of the cup is a truncated cone, and the conical surface extends to the cup. A film of silicone stopcock grease was applied to the threads and sealing surface before attachment of the rotor. Rotor F-II-f has been especially useful for the transfer of suspensions into rotor F-III-t (fig. 2), which was built to evaluate the effect of centrifugal warming on the frozen suspensions. This rotor was fabricated by hollowing out a Duralumin angle-head rotor.[6] In use, cell suspensions frozen in rotor F-II-f were expelled into phosphate plating diluent held in rotor F-III-t, which is spun at 1800 rpm.

Freezing Procedure

The rotor was mounted in the centrifuge and chilled by repeatedly pouring in liquid nitrogen. Before the sample was introduced, the rotor was taken to operating speed and again filled with liquid nitrogen to the top lip of the insert.

Two methods of introducing samples into a rotor were used. Initially aqueous suspensions were pulse-injected into the rotor through a 23-gauge cannula fitted to a hypodermic syringe inclined to the axis of rotation. A typical injection is shown in figure 3. Observation of the procedure, positioning of the syringe, and protection of the experimenter were facilitated by a transparent plastic cover fitted to the centrifuge. Syringe injection did not yield particles of uniform size. A sprayer, shown in figure 4, was fabricated to produce small droplets in aerosol form. Formation of the aerosol had no apparent effect on the viability of yeast. Filtered air was used to form the spray, and the flow of air was controlled by a needle valve placed at the filter.

The yeast, *Saccharomyces cerevisiae*, used in the survival studies was cultured as described by Mazur (*13*) and used while in stationary growth phase. The cells were washed free of culture medium by centrifugation and diluted in triply distilled water to a volume containing approximately 4×10^8 cells per ml. In each experiment, approximately 4 ml of sus-

[4] The upper case letter indicates the rotor series, the numeral the modification, and the lower case letter the use, *i.e.*, freezing or thawing.

[5] Model PR-2, International Equipment Company, Boston 35, Mass.

[6] Number 855, International Equipment Company.

pension was introduced into a rotor. The exact amount was variable because some material was lost during transfer into the rotor or into warming solutions.

Thawing Procedure

Frozen droplets were recovered from rotor F-I-f by pouring the liquid nitrogen and suspended particles from the insert or by spooning them out with a chilled spatula after the coolant had evaporated. Frozen droplets were recovered from rotor F-II-f by first removing the insert from the brass shell and then attaching the chilled transfer cap. When nearly all of the liquid nitrogen had evaporated from the rotor, it was inverted and the contents expelled by gas pressure arising from vaporization of the remaining coolant.

To obtain relatively rapid thawing of frozen droplets, three warming procedures were used. Early in this study, droplets in liquid nitrogen were transferred from a rotor to a small aluminum dish in the bottom of a 1500 ml beaker. When nearly all coolant had evaporated, 1 liter of 0.067 M potassium phosphate solution was poured rapidly into the beaker over the droplets. In subsequent experiments, the droplets were transferred in liquid nitrogen to an insulated cup. When approximately 10 ml of suspension in liquid nitrogen remained, the droplets in approximately 1 ml aliquots were transferred to a beaker containing 1 liter of diluent at 35° C stirred by a propeller agitator. Reproducibility of results was maximal when the agitator was positioned near the wall of the beaker and driven at 2000 rpm.

In a centrifugal warming procedure, the cell suspension injected from a syringe and frozen at 5000 rpm in rotor F-II-f was expelled into 1 liter of 0.067 M diluent at 35° C in rotor F-III-f at 1800 rpm. The diluent containing the thawed cells was transferred immediately to beakers for subsequent viability assay.

To obtain slow thawing of droplets, the frozen suspension was left in the plastic rotor at room temperature until melted.

Evaluation Techniques

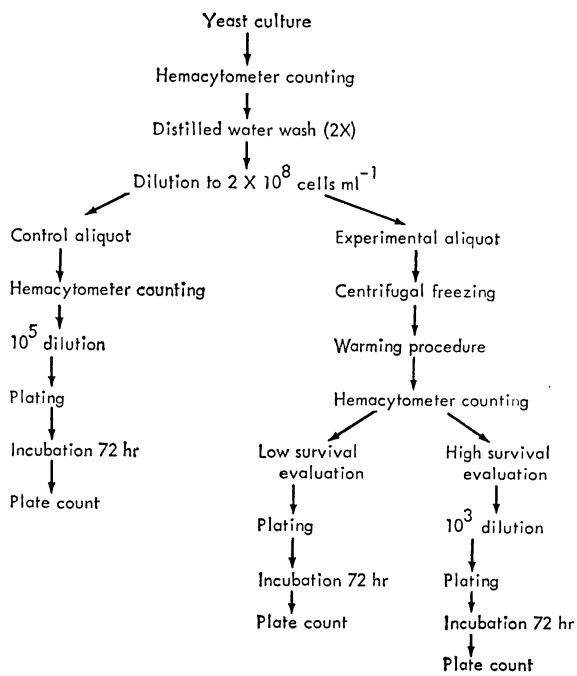
a. Droplet formation and freezing.—Experiments were performed with three materials to evaluate the performance of the centrifugal freezing techniques—distilled water, whole blood, and yeast suspensions. In an initial series of tests designed to study the mechanics of droplet formation at various speeds, distilled water was pulse-injected into a rotor, the frozen droplets recovered by pouring them in coolant into a chilled petri dish, and the dish observed under a low-power microscope. At the moment the last vestiges of coolant had evaporated, the droplets were photographed. In another series of experiments, rat blood, treated

with a chelating anticoagulant, was injected from a syringe into the rotor to evaluate the droplet freezing process with a cell suspension. The same photographic technique was used.

b. Assay of cell viability.—Cell viability was assayed by plating techniques described by Mazur (*13*), which are shown in text-figure 1. Triplicate samples were plated either directly from the thawing medium, or, when high survival rates were anticipated, from 1000-fold dilutions. Because the total number of cells recovered after freezing and thawing was somewhat variable, the determination of percentage of survival required some modifications. The total number of cells in aliquots of the thawed suspension and in unfrozen controls was determined by hemacytometer counts, and the total number of viable cells by agar plating. Percentage of survival, S, was calculated as:

$$S = \frac{F_v C_t}{F_t C_v} \times 100 \tag{1}$$

where F_v and F_t represent the number of viable frozen and total frozen cells per ml and C_v and C_t the number of viable and total control cells per ml, respectively. Appropriate multipliers were introduced for dilution factors. To offset the possibly deleterious effects of aerosol formation, control samples were sprayed into a liter of diluent, when that mode of sample injection was used.



TEXT-FIGURE 1.—Diagrammatic representation of experimental procedure with yeast suspensions.

RESULTS AND DISCUSSION

Droplet Formation

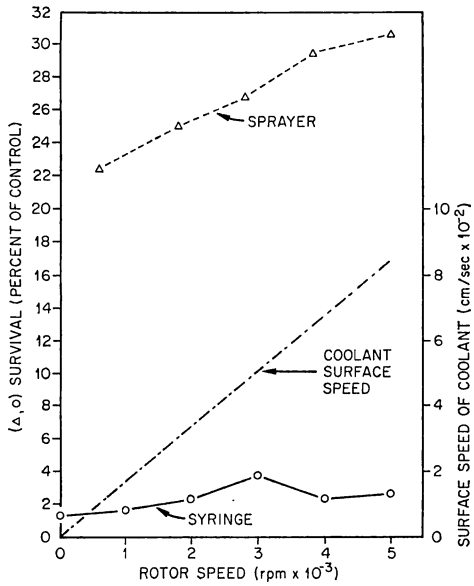
Photographs of droplets obtained when water was injected into liquid nitrogen in rotor F-I-f at rest and at speeds of 1000, 3000, and 5000 rpm are shown in figure 5. When the rotor was stationary, particles varying from 0.1 to 10 mm in diameter were formed, and, although smaller droplets were spherical, larger droplets were irregularly shaped. When the rotor was driven at increasingly greater speeds, successively smaller and, generally, spherical particles were produced. Figure 6, a photograph of whole blood injected into rotor F-I-f at 5000 rpm, illustrates that the injection technique yielded a spectrum of sizes of frozen particles. Many of the larger droplets cracked during photography. Such fracturing was peculiar to cell suspensions and did not occur when distilled water was frozen and warmed.

The following sequence of events during centrifugal freezing was inferred from photographic studies. On contact with the surface of the liquid nitrogen in a spinning rotor, a large droplet breaks into many smaller droplets, which remain fluid until surface tension produces a spherical shape. The particles are engulfed in the coolant and then accelerate and move to the rotor wall. Owing to the initial inertia of a particle and the velocity it attains in the centrifugal field, it is unlikely that a normal insulating gas envelope forms, but, rather, that either the particle leaves a trail of superheated liquid nitrogen in its wake, which vaporizes after the particle has passed, or that movement of the particle in the coolant and the lesser density of vaporized fluid results in reduction or removal of the gas. Heat transfer is probably rapid, since the absence of clumping and the relative sphericity of the frozen droplets indicate that they are sufficiently solidified to prevent distortion when they contact the rotor wall after passage through 1.2 cm of liquid nitrogen.

Cell Survival

Preliminary evaluation of centrifugal freezing with respect to cell survival has involved varying three parameters—droplet size, rotor speed, and rewarming rate of frozen suspensions. The effect of variation in the first two interrelated parameters may be inferred from the data in text-figure 2, and the gross effect of rewarming may be determined from table 1.

Particle size effects.—From the spraying mode of sample injection frozen droplets small and uniform in size were obtained. In contrast, syringe injection gave a spectrum of particle sizes at each rotor speed. The consistently higher survival values obtained from the spraying mode probably reflect the greater cooling and rewarming rate of the smaller droplets resulting from steeper thermal gradients. In addition, physical stress, such as shearing, is probably less with small droplets than with large droplets, which are fragmented at the surface of the coolant.



TEXT-FIGURE 2.—Survival of centrifugally frozen cells injected or sprayed into rotor F-I-f at various speeds.

TABLE 1.—Effects of warming and sample introduction mode on the survival of centrifugally frozen yeast

Experimental series	Rotor	Sample introduction mode	Relative warming rate	Percent survival*
Warming rate effects	F-I-f	Injected from syringe	Slow	0. 002
		"	Rapid, diluent pouring	10. 5
	F-II-f F-III-t	"	Rapid, centrifugal	6. 8
Sample introduction effects	F-I-f	"	Rapid, agitation	5. 09†
	"	Sprayed	"	16. 32†

*Corrected for viability of controls.
†Average of 8 experiments.

Rotor speed effects.—In general, as shown in text-figure 2, the survival of the cells increased with increasing rotor speed. With samples injected from a syringe, the survival rate rose to a maximum at 3000 rpm and declined. Although differences were small, results were consistent within experiments. With samples sprayed into the coolant, the survival rate rose throughout the rotor speed range.

Warming rate effects.—Mazur (*2*) showed that at high cooling rates yeast cells freeze internally since water cannot diffuse from them rapidly enough to prevent freezing. Survival of such cells depends upon the rewarming rate. So, too, does the survival of centrifugally frozen yeast cells. As shown in table 1, rapid warming yielded 5,000 times as many viable cells as slow warming did.

With optimum delivery procedures and rotor speed, the centrifugal freezing–rapid warming technique yielded survivals of 33.8 percent

(text-fig. 2). For comparison, Doebbler and Rinfret (*9*) obtained survivals of 42 percent by spraying suspensions of yeast on a moving surface of liquid nitrogen and Moor and Mühlethaler (*5*) reported survivals of 30 percent when a 0.2 mm^3 droplet on a copper disk was immersed in liquid propane at −190° C. Neither group, however, examined the effects of slow warming.

Although Rinfret (*14*) has calculated that the edge and center of a 1 mm droplet of water introduced into liquid nitrogen will cool at 60° and 300° C per second, respectively, from 0° to −60° C, no one has yet devised a method for actually measuring the cooling rates.

The survivals with the centrifugal freezing technique were not superior to those reported in other published procedures for rapid freezing. This could mean that, contrary to our expectations, centrifugal freezing did not produce a higher cooling velocity. Or it could mean that not more than 40 to 50 percent of the yeast cells will survive no matter how rapidly they are cooled. Two pieces of evidence argue against this last statement. One is that Moor and Mühlethaler (*5*) reported that 100 percent of yeast survived cooling in liquid helium II at an estimated (but unmeasured) cooling velocity of 10^4 °C per second. The other is that, in the present experiments, there appears to be a slow progressive rise in survival with increasing rotor speed.

The matter of cooling velocity is one of the unanswered questions with centrifugal freezing. The present technique yielded respectable survivals with yeast, and suggests that centrifugal freezing may be a valuable tool, especially when large quantities of rapidly frozen material are required. The data also indicated some avenues for improving percentages of survival. These include improvements in the techniques for forming and delivering small, uniformly sized droplets; methods for increasing the velocity of the liquid nitrogen surface; more attention to the matter of achieving still higher and more reproducible warming rates (*e.g.*, centrifugal warming); determining the applicability of centrifugal freezing to cells other than those of yeast, to subcellular particles and viruses, and to other techniques such as the preparation of materials for electron microscopy.

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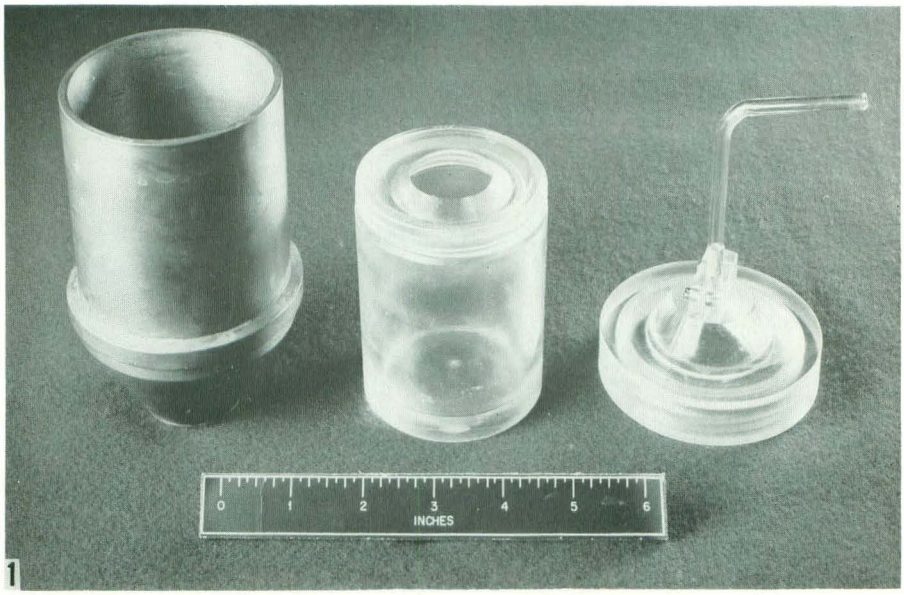

FIGURE 1.—Components of Rotor F-II-f. Rotating brass holder shown *at left*, plastic cup used to contain liquid nitrogen shown *in center*, and transfer cap used to transfer the frozen particles is shown *at right*.

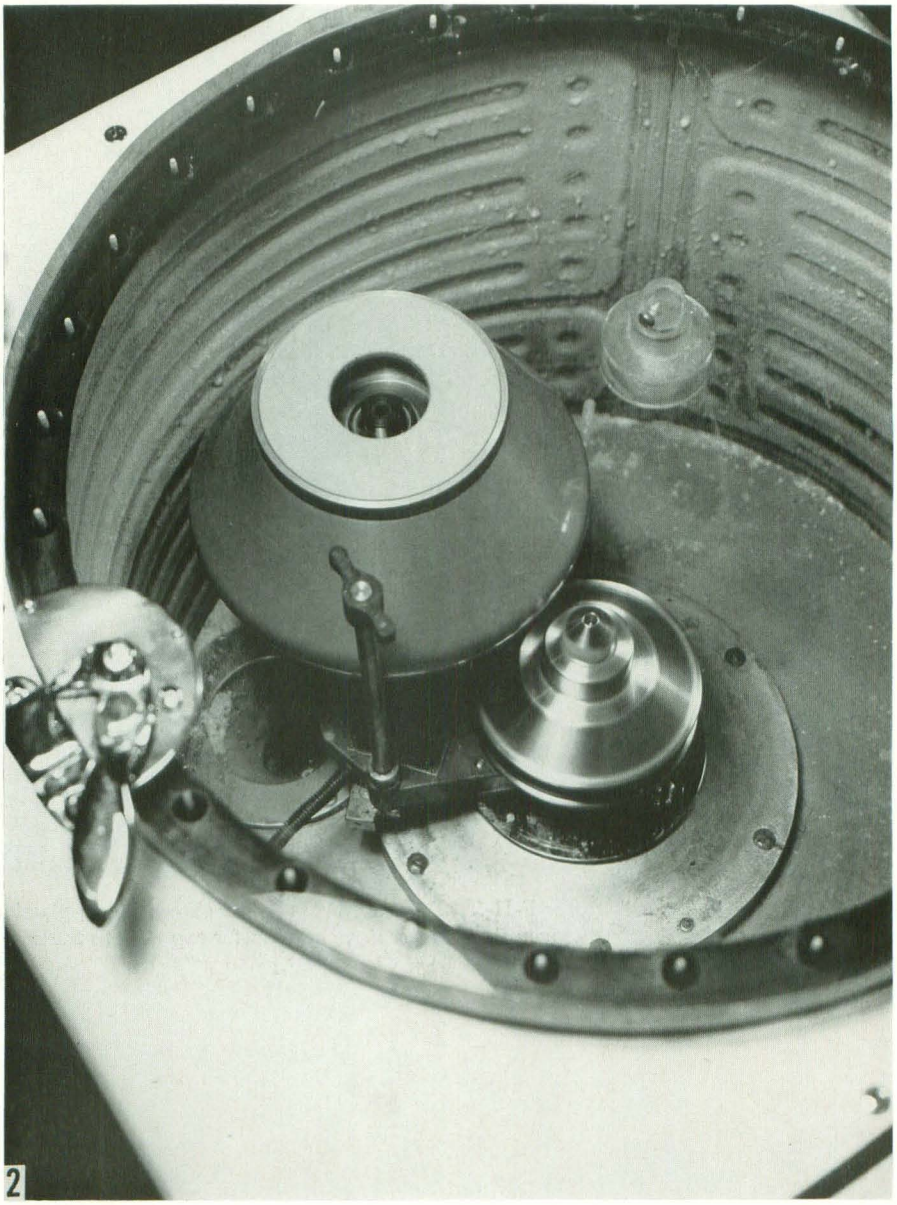

FIGURE 2.—Rotor F-III-t in the PR-2 centrifuge. Rotor is hollow and is used for rapidly thawing cells.

FIGURE 3.—Shows technique for centrifugal freezing of cell suspensions by injection of sample into liquid nitrogen in a spinning rotor.

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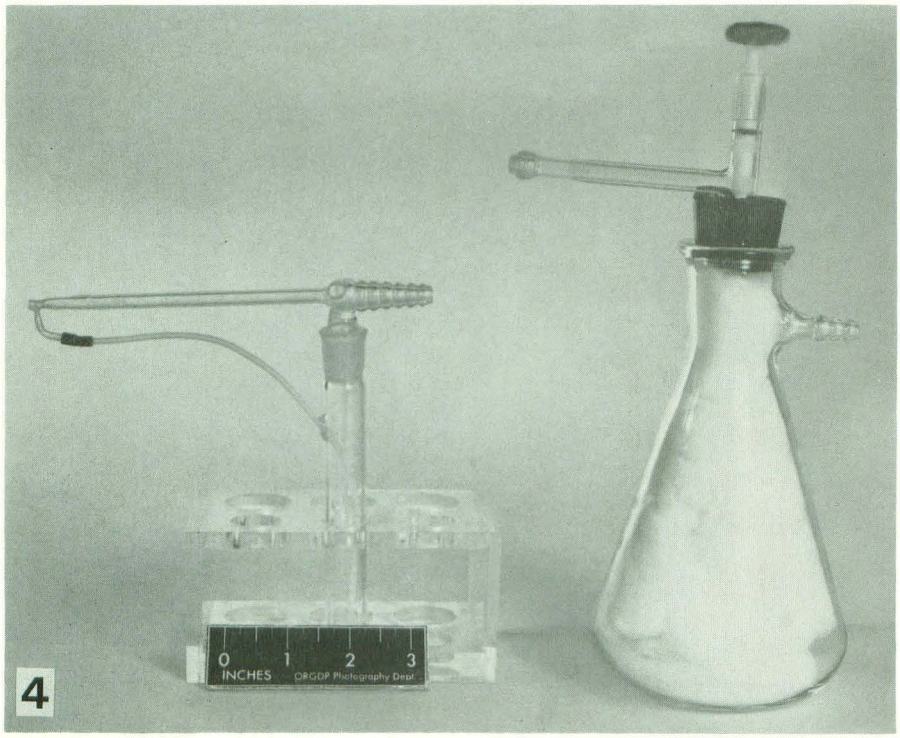

FIGURE 4.—Sprayer used to obtain fine droplets of cell suspension in aerosol form. Flask *on right* contains cotton filter and has needle valve on the outlet side. Sprayer *on left* includes small reservoir for cells connected by a plastic tube to the air jet. Direction of spray would be to left.

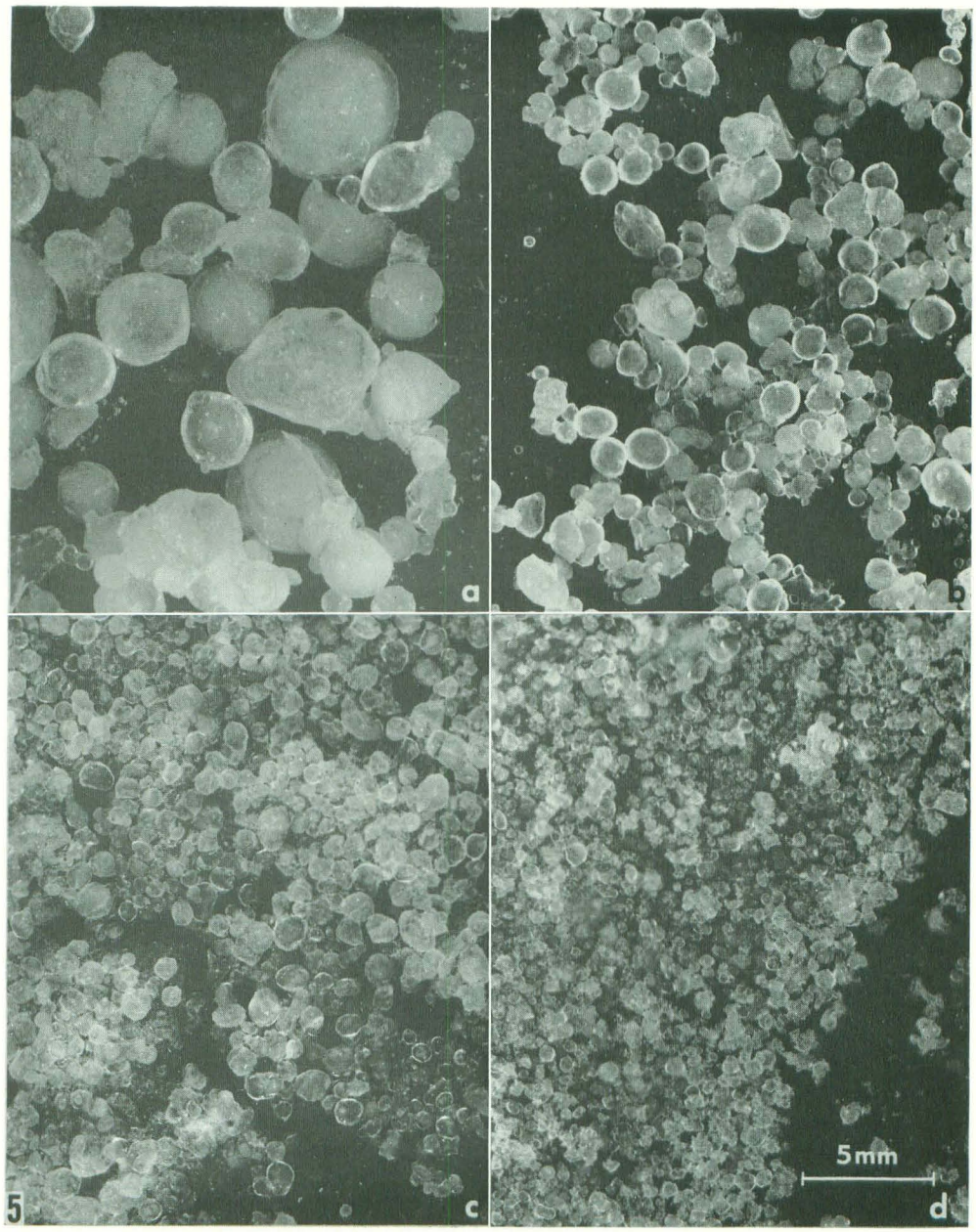

FIGURE 5.—Water injected into liquid nitrogen at rest (*a*) and in rotor F-I-f at 1000 rpm (*b*), 3000 rpm (*c*), and 5000 rpm (*d*).

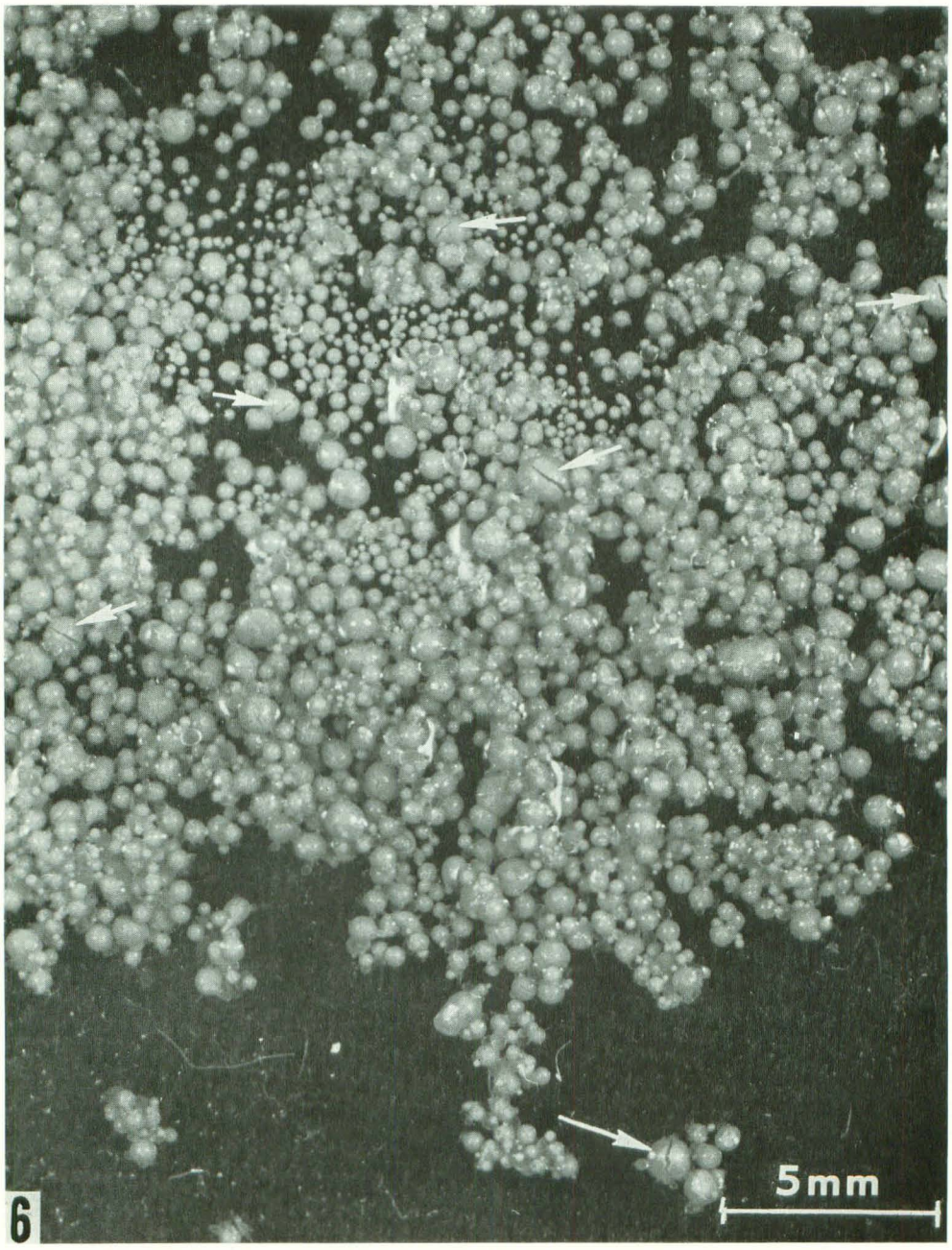

FIGURE 6.—Whole, versenated rat blood injected into liquid nitrogen in rotor F-I-f at 5000 rpm. *Arrows* denote cracking that appears in larger particles during observation.