Journal of Microscopy, Vol. 121, *Pt* 3, *March* 1981, *pp.* 273–282.
Revised paper accepted 18 *July* 1980

The design and use of a simple device for rapid quench-freezing of biological samples

by DEAN A. HANDLEY, JACK T. ALEXANDER *and* SHU CHIEN, *Department of Physiology, College of Physicians and Surgeons, Columbia University*, 630 *West* 168*th Street, New York, N.Y.* 10032, *U.S.A.*

SUMMARY

The detailed design of a simple device for rapid quench-freezing of biological samples under reproducible conditions is presented. With spring-augmented descent, sample immersion velocity of 10 m s^{-1} into a cryogenic liquid is achieved. Biological samples, loaded in Balzers planchets, Denton holders, or a newly designed 'titanium envelope', are suitable for rapid-freezing with this device. Using 4 μm titanium foil, light weight (1 mg) streamlined holders can easily be made to enclose cell suspensions or tissue samples. The foil envelope is designed for efficient heat dissipation while protecting the sample from possible impact or flow distortions occurring from spring-augmented immersion. Human erythrocytes, quench-frozen in the titanium envelope, were prepared for electron microscopy by the freeze-substitution technique. Two opposing 25–30 μm surface zones were frozen in the apparent absence of ice. The extended depth of cryofixation is attributed to the advantages of thin foil in the titanium envelope design and the use of rapid-immersion technique.

INTRODUCTION

Preservation of cell structure by chemical fixation is a relatively slow process, incapable of arresting rapid membrane interactions (Heuser *et al.*, 1976; Van Harreveld *et al.*, 1974), and often inducing cellular anoxia (Bernard & Krigman, 1974) leading to redistribution of electrolytes and water (Van Harreveld *et al.*, 1965). Cell preservation by freeze fixation obviates many of the limitations of the chemical fixation and is capable of capturing transient cellular events so that their ultrastructural basis may be studied by electron microscopy. Several methods have been developed for freeze fixation of biological samples, e.g. spray-freezing (Bachmann & Schmitt, 1971; Ververgaert *et al.*, 1973), pressure-freezing (Moor & Riehle, 1968) or contact-freezing with metal at low temperatures (Dempsey, 1974; Dempsey & Bullivent, 1976; Heuser *et al.*, 1976). In each method, the cooling rate is sufficiently rapid to suppress ice crystal formation, thus averting the need for pretreatment with cryoprotectants and the artefacts that occur with their use (Farrant *et al.*, 1977; Franks, 1977; Niedermeyer *et al.*, 1977). Samples frozen by these methods are suitable for freeze-fracture as well as freeze-substitution studies.

In spite of the successful use of these methods, they are occasionally limited in application to certain samples or require specialized equipment not readily available to most investigators. In many cases the alternative method of quench-freezing in cooling baths is an easy and inexpensive technique suitable for general application (Umrath, 1975). However, the cooling rate achieved with quenching is insufficient to suppress ice crystal formation, thus restricting its use as a suitable alternative method for freeze fixation of biological samples. In order to improve the cooling rate attainable by this method, we have designed a device for effective quench-freezing of biological samples through spring-augmented immersion into a liquid

coolant. In this way, the sample immersion velocity (10 m s^{-1}) is controlled while the variability of hand plunging is eliminated. Easily constructed from inexpensive materials, the main feature of this device is its simplicity of design and operation. We have developed an envelope holder in which samples, enclosed between one folded sheet of 4 μm titanium foil, are suitable for rapid-immersion quenching. The design of the envelope foil holder prevents accidental evaporation from small specimens as it shields against possible impact and flow distortions occurring at high immersion velocity. Rapid quench-freezing of human erythrocytes contained in the envelope resulted in two opposing 25–30 μm surface zones that are frozen in the apparent absence of ice as evidenced by freeze-substitution ultrastructure. The degree of cryofixation obtainable by this method is discussed in terms of theoretical factors that affect the rate of heat dissipation from enveloped samples.

MATERIALS AND METHODS

1. *Design and materials for the freezing device*

The design of the rapid freezing device is shown in Fig. 1, with the materials for construction corresponding to the identification numbers. (1) Styrofoam container (round or square) with molded insert top. (2) Brass adaptor tube (in this case from an ICE International Centrifuge Model UV, tube capacity 65 ml). (3) 20 mm diam. copper pipe cap. (4) 7 mm plywood sheet. (5) Wooden dowels (0·1 × 25 cm). (6) Brass tubing (i. diam. 1 cm, length 15 cm). (7) Steel wire (0·3 × 20 cm). (8) Two calibrated extension springs (length 9 cm, 0·7 mm wire, 14 coils cm^{-1}, each spring with a k value of 3·5 kg m^{-1}). (9) Two retaining hooks.

2. *Construction and assembly of freezing device*

A plywood disc (part 4A, cut from the plywood sheet) was cemented to the styrofoam top and a centred 10 mm hole was cut through both. Four lengths of steel wire (part 7C), previously bent to the configuration shown in Fig. 1, were soldered to a 10 cm length of brass tubing (part 6B), with the four in a 90° position from each other to form support struts. These were cemented to the disc with the brass tubing axially aligned in a vertical position to serve as a guide during the descent of the shaft assembly.

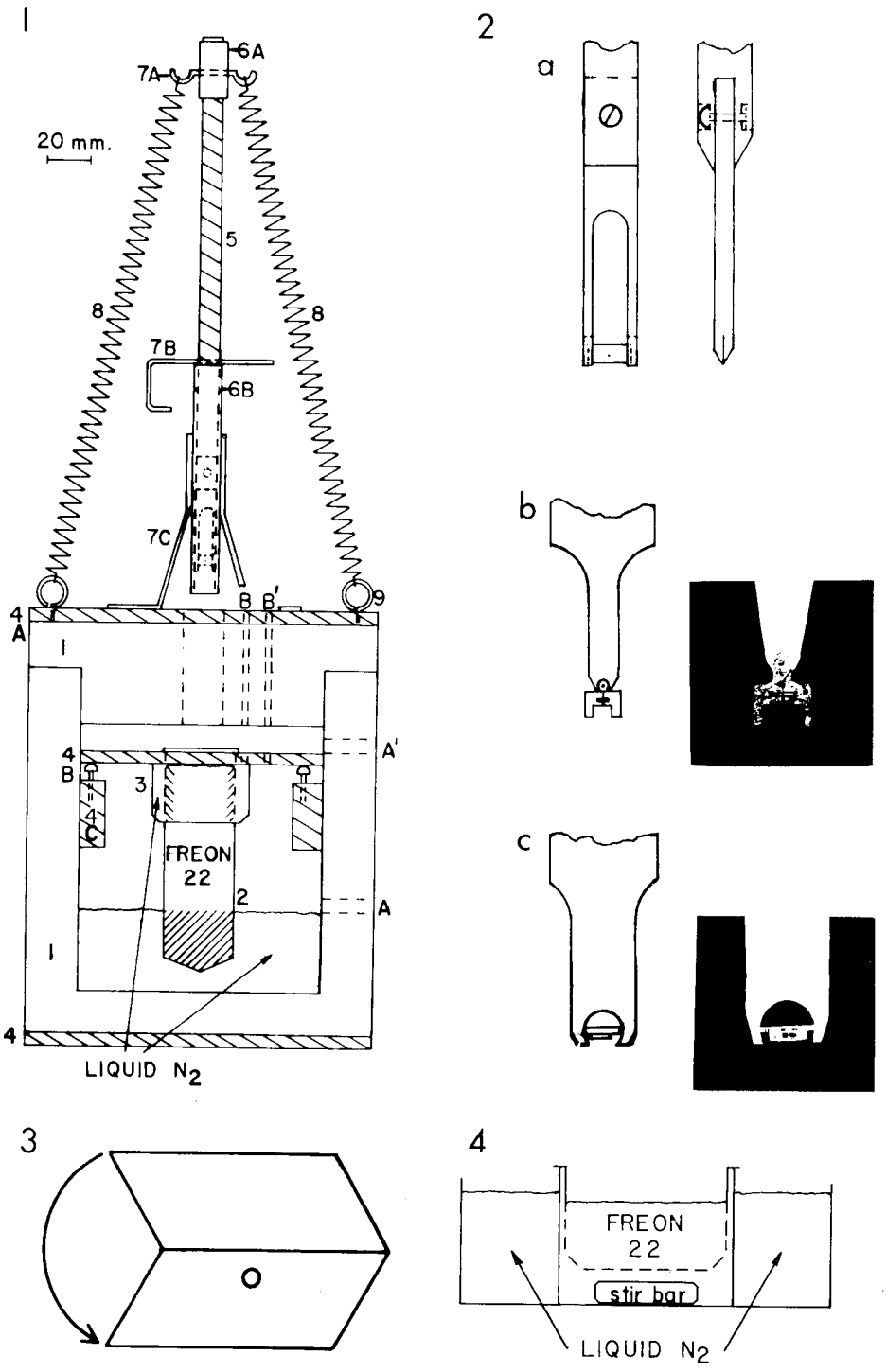
A second plywood disc (part 4B) serves as a shelf inside the styrofoam container to support the brass adaptor tube (part 2) in a suspended position. After cutting a 4 cm hole in the centre of the copper cap (part 3), the cap was soldered to the brass adaptor tube to form a leak-proof seal. This assembly is supported by three pieces of wood (part 4C) cemented to the inner wall of the styrofoam container, with leveling screws that allow for height adjustment. Two 6 mm holes (A and A′) allow gas vapours to escape, thereby avoiding pre-cooling of the specimen before entering the coolant. A second set of holes (B and B′) allow charging of the jacket (part 3) and the lower reservoir, respectively. Polyethylene tubing inserted through holes B and B′ served as filling tubes for recharging of the liquid N_2 during use.

Fig. 1. Scale design of the rapid-freezing device, with dimensions in millimetres. Styrofoam container (1) and cooling jacket (3) are charged with liquid N_2 and the adaptor tube (2) with propane or Freon. Samples to be frozen (titanium envelopes, Balzers planchets or Denton holders) are plunged into liquid coolant to a depth of 3 cm. The descent is abruptly stopped causing the sample to be released from the holder. See text for complete details of numbered materials and construction.

Fig. 2. Design of the Teflon tips whch accommodate titanium enveloped samples (a), Denton hinged-holders (b), and Balzers gold planchets (c). In (a), the tapered ends are slit to allow insertion of the foil envelope; this streamlined design causes minimal splash upon entry into the coolant. In (c), the planchet is facing the coolant to prevent sample loss during the spring-accelerated descent.

Fig. 3. Schematic configuration of the titanium envelope. Samples (fluid or tissue) are placed in the centre depression, and the upper half of the foil is folded over to ensure complete enclosure. Depressions of different types (i.e. troughs) may be used to accommodate various tissue samples.

Fig. 4. Tri-container assembly with liquid N_2 in the outer container and Freon in the inner containers. The centre container retains the frozen samples during opening of the titanium envelope. Continual magnetic stirring ensures that the Freon is at a uniform and maximally cooled temperature.


1
20 mm.
6A
7A
5
8
8
7B
6B
7C
9
B B'
4
A
1
4
B
A'
3
FREON
22
2
A
1
4
LIQUID N2
2
a
b
c
3
O
4
FREON
22
stir bar
LIQUID N2

Figs. 1–4

Each shaft was made with a 25 cm length of wooden dowel (part 5) with a 3×13 mm rectangular notch cut into the lower end. A 3 cm piece of the brass tubing (part 6A) was cemented to the upper end of the shaft. Two 3 mm transverse holes were drilled: into the upper hole through the brass tubing and the shaft, a 5 cm length of metal wire (part 7A) was inserted, bent to the configuration shown (Fig. 1), and glued in place; the other hole is 5·5 cm from the lower end to accommodate the retainer pin (part 7B).

The Teflon tips were easily trimmed from the Teflon sheet ($0{\cdot}3 \times 5 \times 10$ cm) to hold titanium enveloped specimens (Fig. 2A), Denton hinged-holders (Fig. 2B), or Balzers gold planchets (Fig. 2C). The Teflon tip was slit at the tapered ends to retain the enveloped sample. Among the different tips that were tested, this tip resulted in minimal splash upon entry into the coolant. The tip to accommodate the Denton holder was roughly trimmed from the Teflon sheet and a 1·0 mm hole drilled through the end of the tip. Fine trimming, to allow retention of the holder by the hinged portion, is seen in the enlarged photographic view (Fig. 2B). Tips for the Balzers planchet are similarly cut, drilled (2·8 mm), and trimmed to the configuration shown (Fig. 2C). Each tip was inserted into the notched end of the dowel (part 5 in Fig. 1) and securely held in place with a small nut and machine screw (Fig. 2A), which were recessed to allow for smooth travel through the guide tube (part 6B in Fig. 1).

3. *Titanium envelope design and method of sample loading*

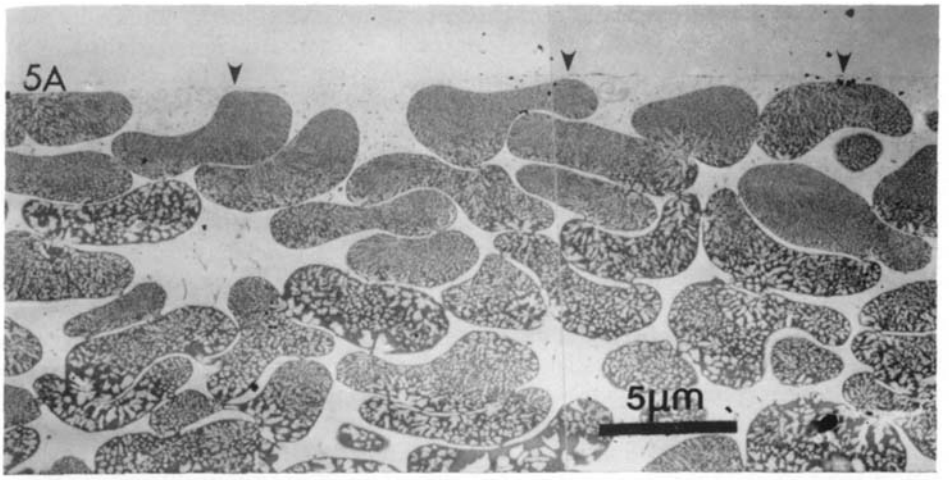
Titanium envelopes were made from titanium foil (99·999% pure, 4 μm thick, Ventron Corp., Mass.). Titanium was selected for the foil envelopes due to its high tensile strength and resistance to tarnish (Milek, 1948). These properties permit formation of durable envelopes of extremely thin foil to withstand high velocity immersion. The foil was cut into 6×8 mm pieces and rinsed in acetone. Before sample loading, the foil was bent at its midline (8 mm axis) to an obtuse angle and a small discoid depression (0·4 mm diam $\times$ 0·2 mm depth) was formed in the centre of one half of the foil to accommodate fluid or tissue samples (Fig. 3). Human blood was withdrawn in heparinized containers and washed three times in 0·15 mol Tris–saline–albumin buffer (305 mOsm), pH 7·4. The cell suspension was adjusted to an 80% haematocrit and frozen without further treatment. After a drop (5–10 μl) of the cell suspension was placed in the depression, the upper half of the strip was folded directly over the lower half to enclose the sample. Small punctures at the sides of the envelope seal to prevent accidental opening during immersion.

4. *Freezing procedure*

The brass adaptor tube (part 2 in Fig. 1) was filled with Freon 22, and the styrofoam container (part 1) filled with liquid N_2 level with the lower exhaust hole (A in Fig. 1). This causes the lower portion of the Freon coolant in the brass adaptor tube to solidify. The copper cap, when fillled with liquid N_2 (15 ml), acts as a cooling jacket to provide maximally cooled liquid Freon at the surface where specimen entry occurs. Liquid N_2 in this reservoir may be refilled (hole B in Fig. 1) as needed.

The enveloped sample was placed into the slits at the end of the Teflon tip (in a vertical position with the folded axis downward) and the shaft positioned into the guide tube with the sample 8 cm above the coolant surface. The retainer pin (part 7B in Fig. 1), inserted through the plunger shaft, holds it in place while the extension springs (part 8 in Fig. 1) are stretched into position. Removal of the pin sends the shaft plunging into the liquid coolant at 10 m s^{-1} (calculated from spring k value) so that the specimen penetrates to a controlled and extended depth of 3 cm in 3 ms. In this way, the convection currents passing over the foiled sample result in

Fig. 5. Red cell suspension frozen in titanium envelop by gravitational descent of 1·25 m s^{-1} into Freon. Cells nearest the foil surface (arrows) contain small ice cavities, indicating that the freezing rate was insufficient to suppress ice formation. The selected area, shown in Fig. 5B, shows the radiating pattern of ice formation occurring at points of cell-to-cell contact and the thread-like connections spanning cells severely distorted by ice formation (brackets).



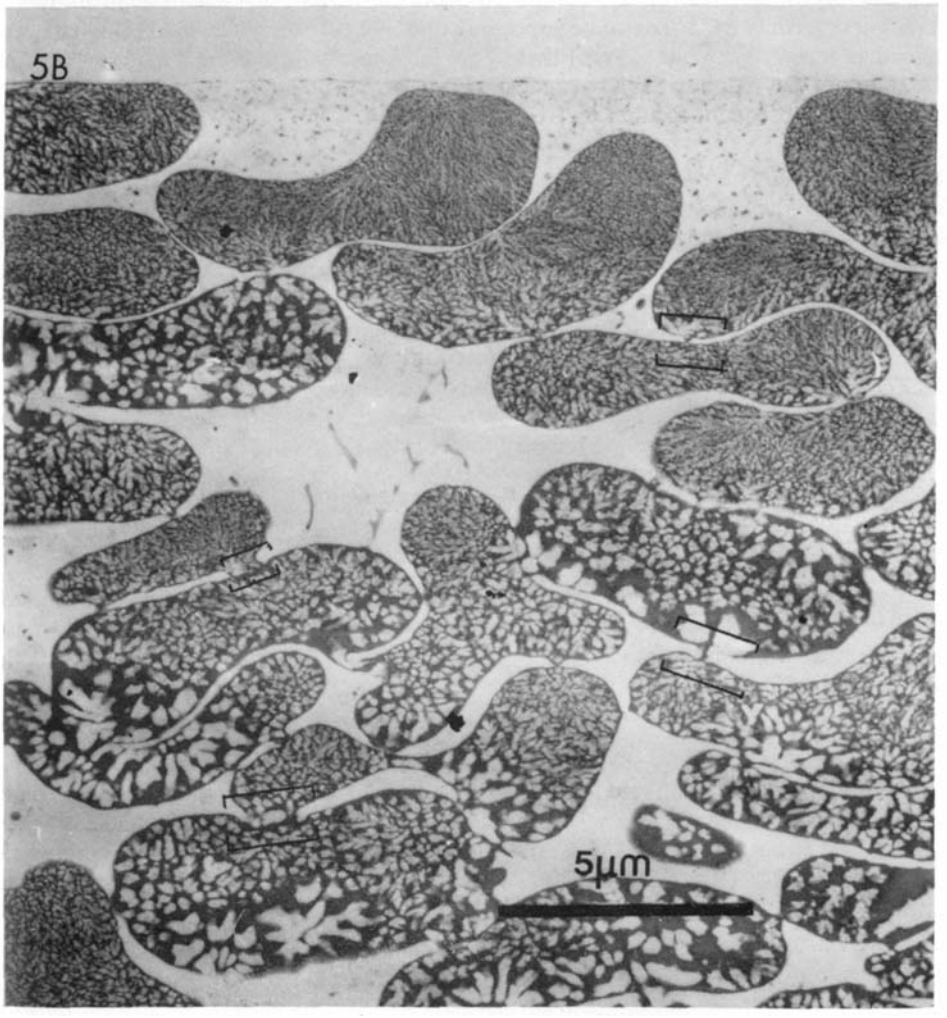


Fig. 5

effective heat dissipation through continual exposure to maximally cooled liquid. The descent is abruptly stopped when the upper brass sleeve (part 6A) meets the lower one (part 6B), so that the titanium envelope is released from the Teflon tip to settle on the frozen coolant. Similar immersion and release occurs when freezing samples contained in the Balzers or Denton holders. The springs are taut at the end of the descent, thus preventing recoil of the shaft. Only a small amount of spring fatigue (10%) has occurred with over 500 firings.

In addition to spring-augmented descent, titanium enveloped samples were similarly loaded in the plunger shaft and quench-frozen by gravitational descent from a height of 8 cm above the coolant. From this position, a theoretical maximal entry velocity of 1·25 m s^{-1} into the coolant can be reached (ideal conditions, Newtonian physics), thereby requiring 25 ms to travel 3 cm into the coolant.

After the desired number of samples were frozen, the brass adaptor tube (part 2) and lower plywood disc (part 4B) are removed, slightly warmed, and the contents (liquid and solid Freon) poured into the inner container of a tri-container assembly (Fig. 4). The liquid N_2 in the jacket spills against the disc and is not collected with the coolant. The design of the tri-container assembly allows for indirect cooling of the Freon by liquid N_2 in the outer well. Continual magnetic stirring of the Freon eliminates the temperature gradient that would occur in the static conditions and permits the Freon to be supercooled to 109 K (Somlyo *et al.*, 1977). The centre container has several openings (2 mm) that allow for intermixing of the Freon but are small enough to retain the titanium foiled samples. The samples are exposed by slowly opening the foil to allow for subsequent fixation by freeze-substitution.

6. *Freeze-substitution*

After opening, the titanium foil pieces (with the samples adhering to the foil) were collected in Beem capsules while under the Freon. The Beem capsules (filled with Freon) were rapidly transferred and submerged in substitution fluid at 173 K. This type of transfer is required to prevent possible warming of the frozen samples (Steinbrecht, 1980). Freeze-substitution was in 4% OsO_4 in acetone at 193 K for 18 h, with anhydrous conditions maintained with molecular sieve (4 A Linde pellets, Union Carbide). The sample temperature was increased (203 K, 253 K, 277 K) at 1 h intervals to room temperature (Van Harreveld *et al.*, 1965), and the sample stained with 1% uranyl acetate. The samples were dislodged from the foil with a forceful stream of acetone and the titanium envelope retained for additional freezing experiments. The red cell sample, although originally frozen as a suspension, was retrieved from the foil depression after freeze-substitution as a firm discoid-shaped pellet and embedded in Spurr's resin (Spurr, 1969).

7. *Electron microscopy*

Thin sections (60 nm) were cut perpendicular to discoid axis to allow for cross-sectional examination. Ice crystals were easily recognized as cavities within the red cell cytoplasm as no subcellular organelles were present to obscure identification. Sections were stained in lead citrate (Reynolds, 1963) and examined under a Zeiss 9S electron microscope at 60 kV.

RESULTS

Cross-sectional examination revealed that cells frozen in the titanium envelope by gravitation acceleration into the coolant (1·25 m s^{-1} entry velocity) contained large ice cavities, which severely distorts the cell structure (Fig. 5A). However, the ice cavities are smallest within those cells nearest the foil surface (Fig. 5B). Areas of cell-to-cell contact serve as mutual nucleation sites for intracellular ice formation. One frequently observed artefact was the thin bridge-like

Fig. 6. Red cell suspension frozen in titanium envelope by spring-augmented descent of 10 m s^{-1} into the Freon, resulting in a well-frozen 25–30 μm surface zone (A). The enlarged area shows ice crystal patterns that occur at points of cell-to-cell contact (brackets) (B). Insert shows absence of microscopically visible ice and a smooth profile of cell membrane (C).

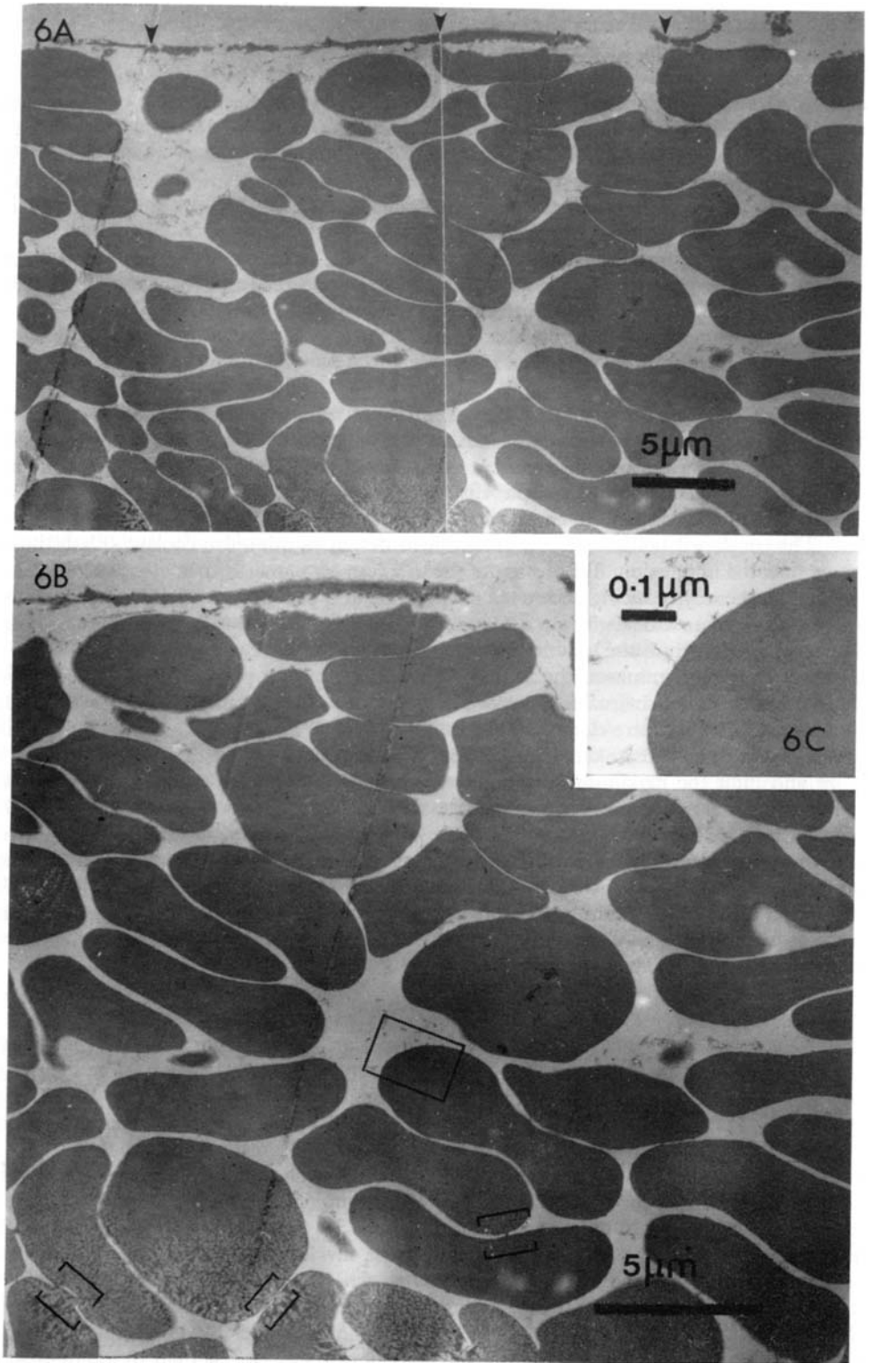


Fig. 6

connections occurring between adjacent cells, perhaps the result of expansion pressures exerted at the cell periphery during ice formation (Fig. 5B).

In comparison, immersion of the foiled sample by spring-augmented acceleration (10 m s^{-1}) resulted in a freezing rate sufficiently rapid to produce 25–30 μm surface layer of cells devoid of ice cavities (Fig. 6A). Extracellular nucleation occurring from cell-to-cell contact again is a primary factor for intracellular ice formation but only at a depth greater than 20 μm (Fig. 6B). In addition, as red cells are readily deformed by mechanical stress (Chien, 1979), the biconcave cell shape indicates that the spring-augmented acceleration did not adversely affect the sample.

The first visible ice cavities occur at 25–30 μm from the surface and increase to maximum dimensions at the sample centre, after which the ice cavity dimensions decrease to approach a minimum at the opposing sample surface. This pattern of ice distribution was observed in samples ranging from 80 to 150 μm in thickness.

DISCUSSION

This device for quench-freezing is easily constructed from inexpensive, readily obtainable materials. The use of Teflon as a sample holder is ideal as it does not interfere with the freezing process and is not embrittled during repeated exposure to the coolant. The Teflon tips, designed to allow release of the enveloped sample after entry into the cryogenic liquid, may be easily trimmed to accommodate titanium enveloped samples, Balzers gold planchets or Denton hinged-holders. The enveloped holders are of a streamlined design to minimize coolant splash during spring-augmented immersion. The design of the titanium envelope ensures complete enclosure of the sample, thereby reducing accidental evaporation and preventing possible impact distortions that would occur with high velocity immersion into the coolant. Two opposing 25–30 μm surface zones are frozen in the absence of microscopically visible ice.

Of the factors that influence the rate at which biological samples (unfixed without cryoprotection) can be quench-frozen, sample size, type of coolant, sample holder weight and composition, and immersion velocity are the most important (Costello & Corless, 1978; Gulik-Krzywicki & Costello, 1978; Skaer *et al.*, 1978). It is the latter two which are often difficult to optimize and limit the maximum freezing rate obtainable by quench-freezing. To overcome these two limitations, we have designed a light weight (1 mg) sample holder using 4 μm foil that can be immersed at a high velocity (10 m s^{-1}) into a liquid coolant. The advantage of using thin foil in the envelope design is related to the theoretical time required to cool the inner surface of the foil envelope (next to the sample) from 293 K to 193 K. The time interval required for this temperature decrease should be as short as possible to reduce potential cryoinjury resulting from ice crystal formation (Costello & Corless, 1978). Assuming instantaneous immersion of the foiled sample into maximally cooled Freon (113 K), the time (t) is given by:

$$t = 0{\cdot}36\, l^2 \rho\, c / T \qquad (1)$$

where 0·36 is a value previously measured for linear heat flow through a sheet of thickness l (Carslaw & Jaeger, 1962), l is the thickness of the titanium foil (0·0004 cm), ρ is the density of titanium (4510 kg m^{-3}), c is the specific heat (523 J kg^{-1} K), and T is the thermal conductivity (2·19 Wm^{-1} K). The theoretical time required for a 100 K decrease is 0·62 μs, yielding a cooling rate of $1{\cdot}6 \times 10^8$ K s^{-1}, which is several orders of magnitude greater than the 1×10^4 K s^{-1} needed for vitrification (Ververgaert *et al.*, 1973). Although such calculations are of limited use, it becomes apparent that the foil thickness is of critical importance for rapid heat conduction, as heat dissipation occurs in only one spatial direction, i.e. perpendicular to the sides of the titanium foil. Thus, for any particular metal used in the envelope design, the theoretical cooling rate can be improved by further reducing the foil thickness. Although silver and copper have better heat conductive properties than titanium, their limited tensile strength (130 MPa silver; 211 MPa for copper) compared to that of titanium (860 MPa; Milek, 1948) renders them unsuitable for thin foil application. We found that silver or copper foils less than 10 μm thick were difficult to manage, susceptible to tears by handling and distortions during high velocity immer-

sion into the coolant. Conversely, titanium is a rigid foil that will easily retain the envelope design even with a thickness of 4 μm. In addition, copper, and to a lesser extent silver, are susceptible to atmospheric oxidation, leading to the formation of surface oxides that decrease heat transference capacity. Although removable by polishing, thin silver or copper foils do not easily withstand such treatment. Titanium is extremely resistant to atmosphere oxidation, such that its heat conduction properties are not compromised by surface oxidation.

In spite of the advantages of 4 μm titanium foil, the theoretical cooling rate of equation (1) is based on the assumption of instantaneous sample immersion into maximally cooled Freon. Although not instantaneous, only 1·5 ms is required for the foiled sample to travel the first 15 mm into the coolant in spring-augmented descent. Apparently, effective heat dissipation from the titanium envelope fully occurs with an entry velocity into the coolant similar to the one we have used. During spring-augmented immersion, the design of the envelope permits optimal fluid flow over the foil surfaces, thus ensuring effective heat transfer through continual exposure to the coolant. In addition, the liquid nitrogen cooling jacket maintains maximally cooled liquid Freon at the surface where specimen entry occurs.

It appears that the centre of the sample, which had the largest ice crystals, is frozen the slowest. This may be a reflection of its distance from the inner foil surface, indicating that the cooling process occurs by a moving front model of freezing (Williams & Hodsen, 1978). Heat dissipation from the foil surface is sufficiently rapid to induce temperature gradients within the sample, and as a consequence the rate of cooling is slowest at the sample centre. With the use of freeze-substitution, the relative frequency and size of ice crystals can be determined easily, thus permitting effective comparison of different areas within one sample and the merits of gravitation versus spring-augmented acceleration. However, a description of cell thermal histories by freeze-substitution ultrastructure does not truly define surface boundary conditions of the sample nor does it identify actual cooling rates (Bald & Crowley, 1979). Likewise, it is difficult to distinguish primary artefacts induced by freezing from secondary artefacts caused during subsequent tissue preparation (Steinbrecht, 1980). Although accurate cell thermal histories are obtainable by thermal couple measurements of the sample during the freezing process, the small sample size used in the envelope design precludes this type of analysis. Albeit these limitations, the ice cavity profile within the cell sample is consonant with expected patterns from the cooling process that occurs by a moving front of freezing.

In conclusion, this device is designed for spring-augmented immersion of biological samples into a liquid coolant. The selection of 4 μm titanium foil is ideal for use as a light-weight and durable sample holder. The envelope holder results in effective heat dissipation, yielding two opposing 25–30 μm surface zones frozen in the absence of microscopically visible ice crystals. The foil envelope is equally applicable for freezing either tissue or liquid samples.

ACKNOWLEDGMENTS

This work is supported by assistance from NIH Grants HL 16851, HL 19454 and T 32-HL 07114, of which D.A.H. is a postdoctoral fellow. We thank Dr Kung-Ming Jan and Dr Martin Blank for advice during manuscript preparation. We profited from the technical suggestions of Mr Steven Vallespir and thank E. Goodrich and R. Taborn for manuscript preparation.

REFERENCES

Bachmann, L. & Schmitt, W.W. (1971) Improved cryofixation applicable to freeze etching. *Proc. nat. Acad. Sci. U.S.A.* **68**, 2149.

Bald, W.B. & Crowley, A.B. (1979) On defining the thermal history of cells during the freezing of biological materials. *J. Microsc.* **117**, 395.

Berndard, S.A. & Krigman, M.R. (1974 Ultrastructural analysis of the deeper layers of freeze-substituted guinea pig cortex. *Brain Res.* **76**, 325.

Carslaw, H.S. & Jaeger, J.C. (1962) *Conduction of Heat in Solids*. Oxford University Press, London.

Chien, S. (1979) Blood rheology. In: *Quantitative Cardiovascular Studies—Clinical and Research Applications of Engineering Principles* (Ed. by N. H. C. Hwang, D. R. Gross and D. J. Patel). University Park Press, Baltimore.

Costello, M.J. & Corless, J.M. (1978) The direct measurement of temperature changes within freeze-fracture specimens during rapid quenching in liquid coolants. *J. Microsc.* **112**, 17.

Dempsey, G.P. (1974) Ultrastructural preservation related to rapidity of freezing. *8th International Congress on Electron Microscopy*, Vol. II, p. 42.

Dempsey, G.P. & Bullivant, S. (1976) A copper block method for freezing non-cryoprotected tissue to produce ice-crystal-free regions for electron microscopy. I. Evaluation using freeze-substitution. *J. Microsc.* **106**, 251.

Farrant, J., Walter, C.A., Lee, H., Morris, G.J. & Clark, K.J. (1977) Structural and functional aspects of biological freezing techniques. *J. Microsc.* **111**, 17.

Franks, F. (1977) Biological freezing and cryofixation. *J. Microsc.* **111**, 3.

Gulik-Krzywicki, T. & Costello, M.J. (1978) The use of low temperature X-ray diffraction to evaluate freezing methods used in freeze-fracture electron microscopy. *J. Microsc.* **112**, 103.

Heuser, J.E., Reese, T.S. & Landis, D.M.D. (1976) Preservation of synaptic structure by rapid freezing. *Cold Spring Harbor Symp. quant. Biol.* **40**, 17.

Milek, J.T. (1948) Titanium. In: *Metals Handbook* (Ed. by T. Lyman). American Society for Metals, Cleveland, Ohio.

Moor, H. & Riehle, U. (1968) Snap-freezing under high pressure: a new fixation technique for freeze-etching. *Proc. 4th Eur. Conf. Electron Microsc.* **2**, 33.

Niedermeyer, W., Parish, G.R. & Moor, H. (1977) Reactions of yeast cells to glycerol treatment. Alterations to membrane structure and glycerol uptake. *Protoplasma*, **92**, 177.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208.

Skaer, H. le B., Franks, F. & Echlin, P. (1978) Non-penetrating polymeric cryofixatives for ultrastructural and analytical studies of biological tissues. *Cryobiology*, **15**, 589.

Somlyo, A.V., Shuman, H. & Somlyo, A.P. (1977) Elemental distribution in striated muscle and the effects of hypertonicity: electron probe analysis of cryosections. *J. Cell Biol.* **74**, 828.

Spurr, A.R. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**, 31.

Steinbrecht, R.A. (1980) Cryofixation without cryoprotectants. Freeze substitution and freeze etching of an insect olfactory receptor. *Tissue and Cell*, **12**, 73.

Umrath, W. (1975) Rapid freezing in open cooling baths. *Arzneim-Forsch.* **25**, 450.

Van Harreveld, A., Crowell, J. & Malhotra, S.K. (1965) A study of extracellular space in central nervous tissue by freeze-substitution. *J. Cell Biol.* **25**, 117.

Van Harreveld, A., Trubatch, J. & Steiner, J. (1974) Rapid freezing and electron microscopy for the arrest of physiological processes. *J. Microsc.* **100**, 189.

Ververgaert, P.H.J., Verkleij, A.J., Verhoeven, J.J. & Elbers, P.F. (1973) Spray-freezing of liposomes. *Biochim. biophys. Acta*, **311**, 651.

Williams, L. & Hodson, S. (1978) Quench cooling and ice crystal formation in biological tissues. *Cryobiology*, **15**, 323.