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CRYOPREPARATION OF TISSUE FOR ELECTRON MICROSCOPY

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Abstract

An apparatus able to remove amorphous phase tissue water without recrystallization or rehydration has been produced. Application of this technique to biological samples achieves both the preservation of ultrastructure and the retention of cellular macromolecules and solute without redistribution or modification.

Small pieces of fresh tissue were cryofixed by the method of bounce free metal-mirror freezing on polished copper bars at liquid nitrogen temperature. Tissue samples were then placed under liquid nitrogen in a copper sample holder equipped with a thermocouple and feedback controlled heating circuit. Under liquid nitrogen the sample block was placed in a stainless steel sample chamber which was then evacuated to a hydrocarbon-free ultrahigh vacuum ($1x10^{-8}$mbar). Equilibrium temperature prior to the onset of the drying cycle was -192°C. Tissue was dried by increasing the temperature of the specimen block 1.33°C each hour while monitoring the rate of water removal with a partial pressure analyzer. Results indicate that drying is complete below the devitrification temperature of amorphous phase tissue water. After drying, tissue was fixed with osmium tetroxide vapour, vacuum embedded in low viscosity epoxy resin, sectioned, stained and viewed with the electron microscope. Tissue processed in this manner exhibits excellent morphological preservation without the need for prefixation or cryoprotective agents. In addition, by avoiding prefixation and solvent contact during resin embedding, this method provides the basis for combining ultrastructural preservation with optimum material for immunocytochemical staining and elecron microprobe analysis.

Key Words: Freeze-drying, Rapid freezing, Electron Microscopy, Ultrastructure, Plastic embedded.

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Introduction

Since the advent of electron microscopy, freeze drying as a preparative method for biological materials has had theoretical appeal (Coulter and Terracio, 1977). However, despite early attempts (Richards et al., 1942) poor ultrastructural preservation compared to fixation in aqueous solutions has limited its widespread use.

Cryofixed, freeze dried biological material has two important intrinsic advantages. Firstly, the almost instantaneous arrest of physiological processes which occurs with ultrarapid freezing (Van Harreveld et al., 1973). Secondly, the stabilization and retention of soluble cell constituents through the elimination of prefixation (Van Harreveld and Malhotra, 1966) and aqueous or solvent phase extraction. These advantages have led to its use for specialized applications, such as autoradiography of soluble tracers (Stirling and Kinter, 1967), laser microprobe mass analysis (Kaufmann, 1982) and electron microprobe analysis (Ingram and Ingram, 1984).

Despite improvements in the freeze drying techniques (Coulter and Terracio, 1977), the major disadvantage of cryofixation and freeze-drying of biological materials has remained the presence of cytoplasmic reticulation due to ice crystal formation followed by capilliary and/or thermal collapse (Dubochet and McDowall, 1984.) This is largely a consequence of the need to heat the tissue so as to increase the saturation vapour pressure of water in the solid phase and allow sublimation to proceed in a reasonable time. The addition of heat to vitrified tissue water causes it to undergo glass transition (-120°C to -130°C) (Rasmussen and MacKenzie, 1971) allowing water molecule migration and ice crystal formation to occur. This process of devitrification is likely to occur if the sublimation of vitreous phase tissue water is carried out above -120°C (Rasmussen and MacKenzie, 1971). This paper will present in detail, a technique for drying rapidly frozen biological material in a controlled high vacuum environment. The efficacy of this method is demonstrated by the ultrastructural preservation achieved.

The Freezing of Biological Tissues

The freezing process is a limiting factor in the preservation of fine structure by freeze-drying. During freezing, loss of cellular ultrastructure occurs when water molecules leave

the cytoplasmic solution and aggregate into well ordered crystals. The size of the individual ice crystals determines the amount of visible damage to cell ultrastructure. In addition to the physical effect, ice crystal growth causes chemical changes that may lead to thermal collapse and further ultrastructural damage. As water freezes out, the remaining cytoplasm becomes concentrated into aggregates forming what is visualized as reticulations. It has been suggested that the concomitant changes in solute concentration and pH in the cytoplasm may lead to the abnormal cross linking of macromolecules, plus the denaturation and precipitation of proteins. The integrity of the biological structures is guaranteed only if the cryofixation brings about solidification of water in a microcrystalline state in the absence of any cryoprotectants (Dubochet and MacDowall, 1984).

Water molecules pass from the relatively amorphous liquid state to the relatively amorphous solid state (ice crystal size below the limit of E.M. detection) if heat is removed faster than it can be produced by crystallization. As a consequence, nucleation is inhibited and at least part of the water may reach the vitreous state.

A major practical limitation to quick-freeze physical fixation arises from the high cooling rate required in the initial step. Maximal rates appear to be obtained by applying tissue to a highly polished pure copper bar in liquid helium temperature (4°K) (Heuser et al, 1979). Nevertheless, only the first 15 to 20 micrometers of tissue from the surface contacting the copper are well frozen because of the poor heat transfer properties of biological tissue. Furthermore since helium is a limited natural resource there are limitations on its use, particularly outside of the United States. Fortunately copper bars cooled in readily available liquid nitrogen (77°K) give similar minimal ice crystal formation in the superficial 5 to 15 micrometers of the tissue. We have been using the "Gentlemen Jim" (Phillips and Boyne, 1984) device to quick-freeze biological material.

Drying of Frozen Tissue

Once an appropriate freezing method has been chosen and implemented, if electron microscopy is intended, dehydration must be carried out under conditions which minimize recrystallization of the solid water. This can be accomplished by either freeze substitution (Van Harreveld and Crowell, 1964) or by freeze-drying (Stumpf and Roth, 1967). Although freeze substitution has resulted in excellent morphological preservation, it is contradictory to one of the principal reasons for freezing tissue in the first place, that is, the elimination of solvent contact and thus retention of soluble components.

Theoretically, freeze-drying, or the sublimation of water in vacuo is the ideal method for removing water without disturbing other cell constituents. In practice, as described above, heating the tissue sample to allow sublimation to occur in a reasonable time results in glass transition (Dubochet et.al., 1982, Rasmussen and MacKenzie, 1971), devitrification (Dubochet and McDowall, 1984) and recrystallization.

Most of the freeze-drying methods that have appeared in the literature involve placing the tissue in a vacuum chamber at constant temperature until drying is complete. To avoid impractical drying times, these techniques employ relatively high specimen temperatures. Gersh (1956) used the temperature of -30°C, Williams (1952) used -50°C and a number of investigators used temperatures in the range of -68° to -80°C (MacKenzie and Luyet, 1962, Sjostrand and Baker, 1958, Stirling and Kinter, 1967, Stumpf and Roth, 1967). In all cases the tissue is subjected to these temperatures before vacuum sublimation begins and hence the chances for devitrification and recrystallization are very high. Although the majority of ice crystal damage is probably a product of the initial freezing, it is likely that further ice crystal growth and ultrastructural damage occur during drying.

An additional problem of current freeze drying techniques is the potential loss or redistribution of molecular species during resin infiltration. Burry and Lasher,(1978) found in studies on frozen-dried neuronal cell cultures, that previously incorporated tritiated-gamma-aminobutyric acid was extracted from the cells during infiltration at atmospheric pressure, but was retained when the specimens were vacuum infiltrated. Frederick and Klepper (1976) have shown under similar conditions of vacuum infiltration that negligible amounts of tritiated steroids were extracted. Ingram and Ingram (1984) have shown that intracellular electrolyte distributions are not adversely affected by vacuum infiltration and embedding following freeze-drying. These three studies indicate the susceptibility of frozen-dried specimens to solvent extraction and the need for careful handling of the tissue if extraction is to be minimized or eliminated.

In this new freeze-drier design, the tissue is placed in a copper specimen block equipped with a heating circuit and a thermocouple to allow careful control of the environmental temperature surounding the tissue. The specimen block is inserted into a stainless steel freeze-drier bathed in liquid nitrogen and the drier is connected to an ultra-high vacuum pump. Over a period of four days the copper block is gradually warmed to room temperature. Heat from the specimen block is transferred to the adjacent tissue surface, creating a temperature gradient within the tissue. Water is sublimed from the surface of the tissue, resulting in an insulating dry shell of tissue. Partial pressure analysis during the drying schedule indicates that drying is complete at a specimen block temperature of -90°C with a vacuum of 2×10^{-8} mbar (Linner et al, 1985) . The efficacy of this method is supported by the high quality of the electron micrographs produced. This freeze-drier design also permits frozen tissue to be dried, vapor-fixed and infiltrated with resin without breaking the vacuum. Maintenance of vacuum throughout processing avoids exposing the specimen to moisture from the ambient air, aqueous osmium or hygroscopic resin all of which may contribute to the random movement of diffusible substances within the specimen.

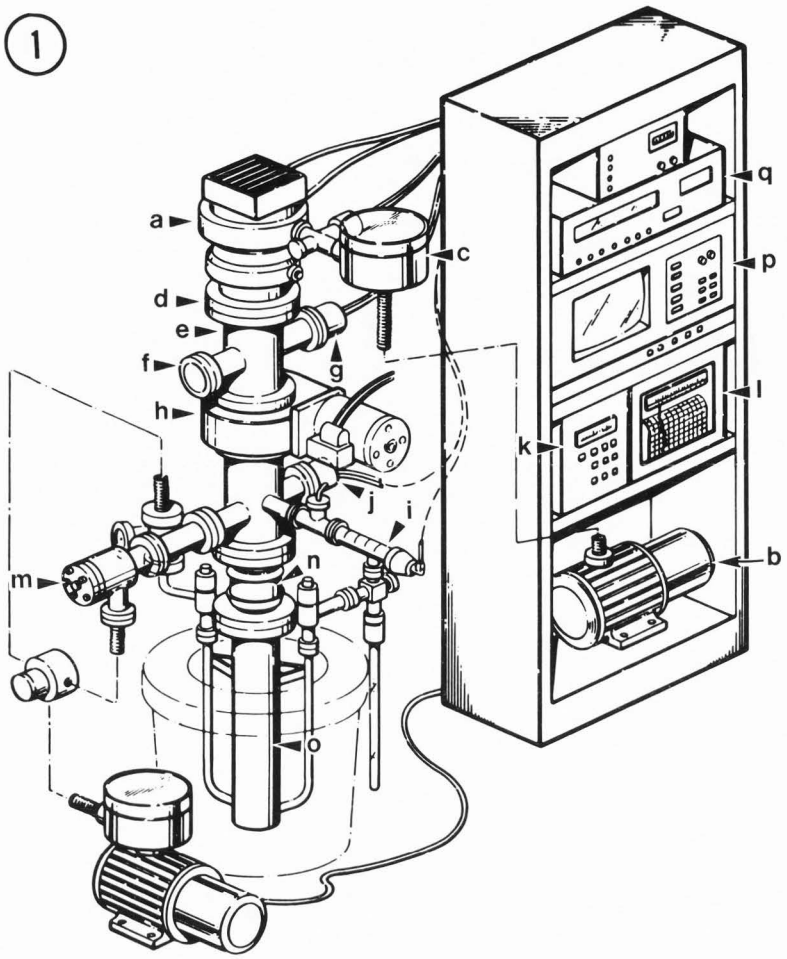


Figure 1. A schematic illustration of the freeze-drying apparatus and control panel.
(a) turbo-molecular pump, (b) mechanical vane pump, (c) absorption trap, (d) connecting Conflat flange, (e) four-way cross, (f) flange for residual gas analyzer sensing head, (g) ultra-high vacuum ion gauge, (h) electropneumatic gate valve, (i) roughing vacuum gauge, (j) electrical feedthrough for thermocouple and heater wire, (k) Barber-Colman 570 temperature controller, (l) chart recorder for specimen temperature, (m) valve for roughing vacuum pump, (n) ceramic insulation nipple, (o) specimen chamber, (p) controller for the residual gas analyzer, (q) digital vacuum gauge.

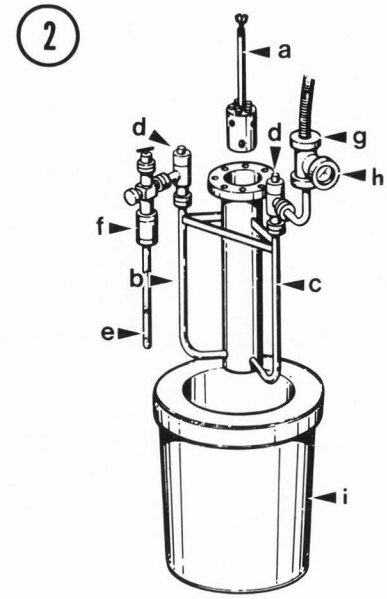


Figure 2.
A schematic illustration of the specimen chamber.
(a) specimen holder, (b) tubing feedthrough for osmication, (c) tubing feedthrough for resin embedding, (d) metal sealed ultrahigh vacuum valve, for osmication (e) glass tubing for crystalline osmium, (f) glass-to-metal adapter, (g) resin reservoir, (h) glass window, (i) liquid nitrogen Dewar.

Materials and Methods

The freeze-drying apparatus is constructed of stainless steel components (Leybold-Heraeus) and all of the seals are metal-to-metal (Conflat*) flanges. It was designed to achieve an ultrahigh vacuum (1 x 10^{-8} mbar) and withstand baking to 250°C. A schematic diagram of the freeze-drying system is illustrated in Figure 1. A turbo-molecular pump (a)(Leybold-Heraeus) (TMP 360) is used to obtain an ultra-high vacuum. The turbo-molecular pump is backed by a vane pump (b) (Trivac D16A Leybold-Heraeus) through a hydrocarbon trap (c) filled with aluminum oxide. The throat of the turbomolecular pump is continuously heated (250°C) by a bakeout jacket (d) and is connected to a weldment consisting of two Conflat flanges (f and g) and a reducing flange that is bolted to an electropneumatic gate valve (h). Flange (f) is used to connect the sensing head of an Inficon QI 200 residual gas analyzer (p) which is removed for clarity. Flange (g) provides connection for an ultra-high vacuum ion gauge (IE 211) that has a digital readout and controller (Combivac IT230D). The gate valve is attached to another weldment which consists of: one Conflat flange that provides a connection for a low vacuum gauge (i), another flange for the thermocouple and heater feedthroughs (j) which connect to a Barber-Colman 570 temperature controller (k) and a Barber-Colman chart recorder (l) (Model E4D2), a third Conflat flange providing connection to a vacuum roughing valve (m). This weldment is attached to a ceramic insulating nipple (n) with Conflat flanges on both ends, one of which is used for attachment of the sample chamber schematically illustrated in Figure 2.

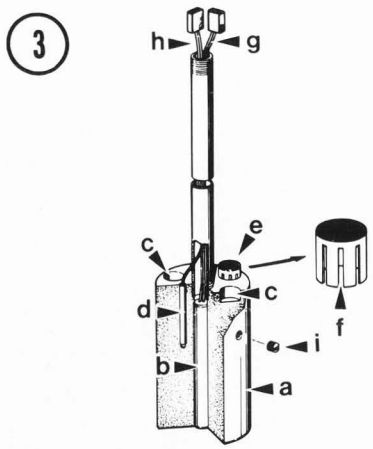


Figure 3. A schematic illustration of the specimen holder. (a) solid copper block, (b) Chromolox heater, (c) specimen reservoir, (d) thermocouple, (e) specimen reservoir cap, (f) specimen reservoir cap in detail (g) wire leads for thermocouple, (h) wire leads for Chromolox heater, (i) teflon spacers.

The specimen chamber contains the copper specimen holder (a) illustrated in Figure 3. The specimen chamber is a stainless steel cylinder closed at the bottom and with a Conflat flange welded to the top. Two tubes, 13mm in diameter, are welded to the base of the chamber 90 degrees apart. Each tube is connected by a Conflat flange by a gold-sealed ultrahigh vacuum valve (d). Tube b is for the introduction of osmium vapor. Crystalline osmium is contained in a glass tube (e) which is connected to the valve by a glass-to-metal adapter (f). Tube c provides a means for introduction of embedding resin from reservoir (g) into the specimen chamber while the vacuum is maintained. During the specimen drying, the specimen chamber is immersed in liquid nitrogen contained within the Dewar (i). Not shown in the illustration is a liquid nitrogen level controller (John's Cryogenics, Inc., Pittsburg PA. Model SN2-6) which maintains a predetermined level of LN_2 in the Dewar (i). The specimen holder schematically illustrated in Figure 3 consists of a solid 5cm copper (99.9% pure) cylinder (a). The center of the block has been bored out to allow a Chromolox cartridge heater (b) (Type CIR 1030,240 volt,200 watt) to be press-fit into place. Six flat bottomed cylinders (c) have been milled into the upper surface of the block 12 mm deep and 12 mm wide. A type T thermocouple (d) has been peened into a small hole between two of the cylinders. Each cylinder is covered with a stainless steel mesh cap (e) which fits snugly into the cylinder. The walls of each cap have slots (f) which provide for a tight fit and flexibility under liquid nitrogen. The thermocouple and the heater wires pass through a teflon tube 1 cm O.D. and connect to their respective feedthroughs from above by way of plugs g and h respectively. The teflon rod provides a strong insulating handle for maneuvering the heavy copper block. Teflon spacers (i) center the copper bar in the chamber and prevent direct contact of the bar with the walls of the chamber except at the base.

Tissue Preparation

All tissues (liver, brain, and kidney) were excised from anesthetized male Sprague Dawley rats and immediately frozen with a commercially available "Gentlemen Jim" bounce free freezing device (Phillips and Boyne, 1984). Tissue blocks were cut into 1 mm cubes before freezing. Frozen blocks were stored in Nunc (Cryomed, Inc., Mt Clemens, MI) tubes in a liquid nitrogen storage container until they were used. Tissue handling techniques are of extreme importance. At no time during transfer of specimens should the temperature of the tissues be allowed to exceed -140°C.

As the freeze-drying apparatus is highly automated, individual attention is only necessary to initiate the process. Drying schedules are programmed into the Barber-Colman 570 temperature controller and a program initiated at the desired time. Tissue specimens are transferred from the stored Nunc tubes to the copper block specimen holder under liquid nitrogen. The specimen holder is then transferred to the specimen vacuum chamber which has been partially filled with liquid nitrogen. The specimen chamber is then bolted onto the rest of the apparatus and rough pumped with a Leybold-Heraeus vane pump, model D16. The presence of an aluminum oxide trap is important to prevent backstreaming of vane pump vapor. Applying a vacuum to liquid nitrogen results in the formation of nitrogen slush at -210°C which is difficult to remove with a rough vacuum. Therefore, at this point the temperature controller program is initiated. The initial segment of the drying program (Figure 4) warms the specimen holder from -200°C (A) (the lowest our thermocouple gauge will read) to -150° C (B) in ten hours to heat the nitrogen slush and convert it to the vapor phase, which is removed from the specimen chamber by the roughing pump. At this point the roughing valve is closed and the vane pump is turned off. The ultrahigh vacuum valve (main valve) is then opened to the specimen chamber and the tissue exposed to ultrahigh vacuum. The next segment of the drying schedule is programmed for a rise of 80 degrees from -150°C (B) to-70°C (C) in 70 hours, or an increase of 1.33 degrees C per hour. At the end of this segment the tissue is considered to be dry and the temperature can be rapidly increased from -70°C to 25°C(D) in ten hours. The temperature is maintained at 25°C while the liquid nitrogen in the outside Dewar flask evaporates, a process that usually takes approximately 50 hours. As the liquid nitrogen level in the Dewar flask drops, water vapor condensed on the inside walls of the specimen chamber is pumped. When the partial pressure of water vapor, as monitored by the residual gas analyzer, drops to an acceptable level and the system has equilibrated to room temperature (25°C) (e), the ultrahigh vacuum valve is closed.

Osmication

Tissue is vapor phase osmicated by exposing the tissue to OsO_4 crystals (Ted Pella, Inc. Tustin Calif.) in a vacuum of approximately 1 x 10-3 mbars for 1 to 3 hrs (E). Osmium is recovered from the system by immersing the test

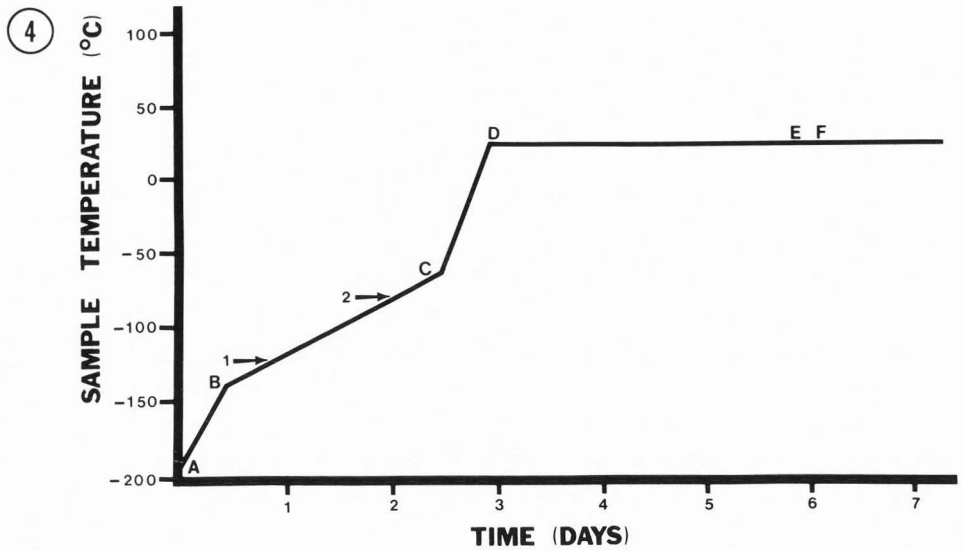


Figure 4. Freeze-drying schedule, time vs. temperature plot. Point A is the equilibrium temperature of the system (-192 C). B is the starting point of the gradual warming period (-150 C). Points 1 and 2 indicate the beginning and end of the molecular distillation process. At point C (-70 C), tissue is considered to be dry, and the temperature is raised rapidly to D (25 C). E indicates the beginning of osmication and F the infiltration of the tissue with resin.

tube which previously contained the osmium in liquid nitrogen for 30 minutes. This causes recrystallization of the osmium onto the walls of the test tube in a safe manner.

Embedding

Tissue is routinely embedded in Spurr's resin (Spurr,1969). Degassed pure resin is introduced through an ultra-high vacuum valve to infiltrate the tissue (F). At this point the system is opened to atmosphere and the specimen holder is removed. The tissue is placed in embedding molds and the plastic is polymerized 8 hours in a 70°C

Discussion and Results

These experiments were undertaken to remove vitreous water from tissue samples previously frozen in the vitreous phase by bounce-free impact freezing on a liquid nitrogen cooled, polished copper bar. (Phillips and Boyne, 1984). The goal of our drying technique is to remove this water at a temperature below the devitrification point to avoid ice crystal formation. Our initial experiments indicated that it takes about 25 hours to remove the water from tissue samples in the temperature range of -123° C to -90°C (evaporation rate equals 2.5 x 10-4 nanomoles per second). Thus, an ultra-high vacuum apparatus capable of maintaining a vacuum better than 1 x 10-8 mbars and a temperature of -140°C for a period of about 7 days was fabricated.

The essential features which are addressed and accomplished by this approach include: 1. low equilibrium temperature of -192°C at the beginning of the drying process; 2. use of ultrahigh hydrocarbon free vacuum; 3. computer controlled incremental heating of the specimen to achieve complete removal of amorphous phase tissue water prior to devitrification; 4. equipment design to incorporate a residual gas analyzer to allow measurement of vacuum chamber partial pressures and therefore analysis of vacuum chamber contents during the drying schedule; 5. the ability to vapor fix and infiltrate with resin prior to breaking of the vacuum.

The efficacy of the method is demonstrated by the ultrastructural preservation together with the potential for artifact free localization of molecular entities.

Ultrastructure

The ultrastructure of the tissue (Figures 5, 6, 7, 8, 9, 10) indicates that we have maintained unit membranes and electron dense material within the endomembranous compartments of the cell which we think are normally lost due to solvent content in previous fixation and embedding attempts. The ultrastructure of the nucleus seems to be most susceptible to freeze damage or reticulation.

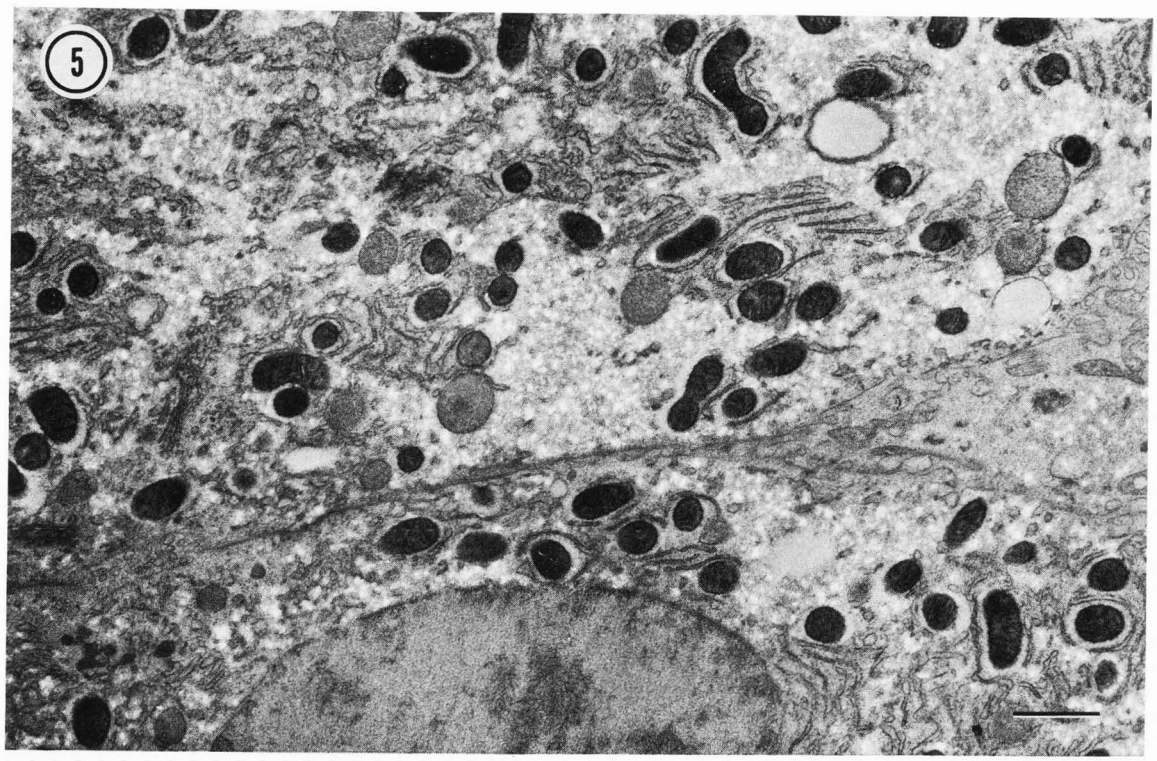
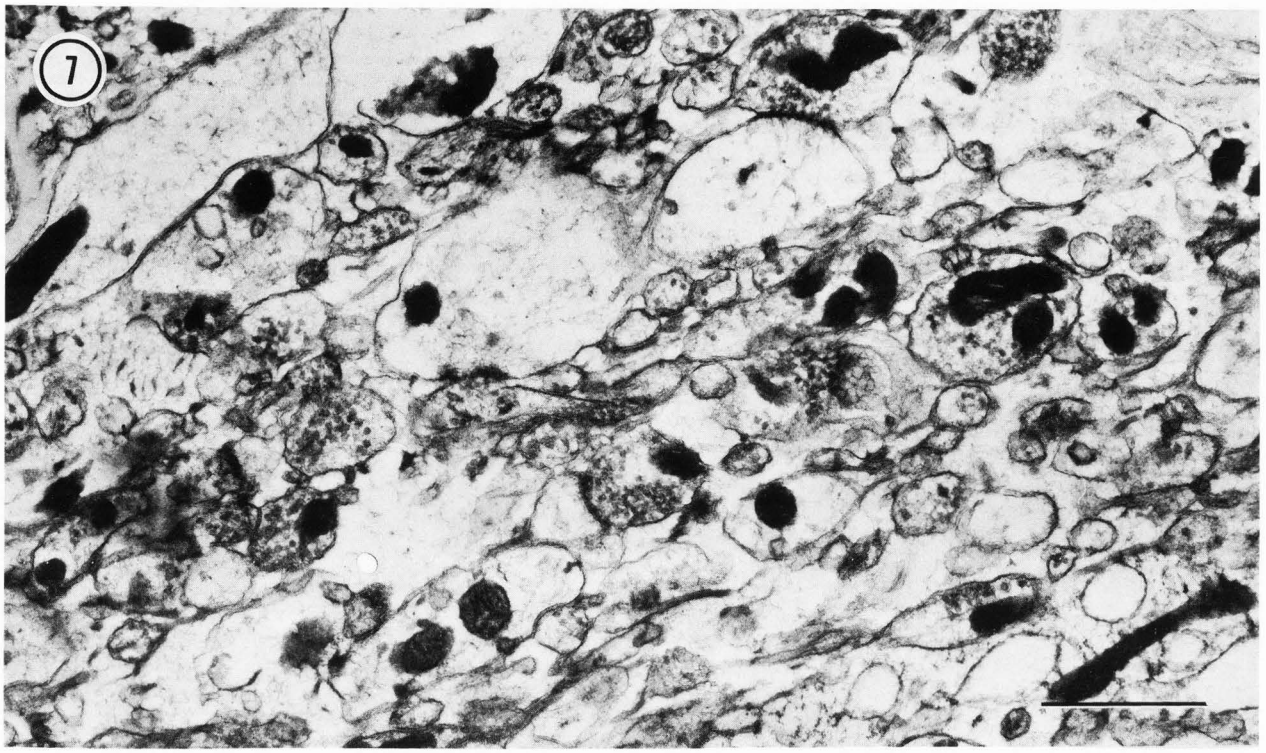


Figure 5. Normal rat liver. Quick-frozen and freeze-dried, osmium vapor fixed, resin embedded, and not post-stained. Bar = 1.0 μm.

Figure 6. Normal rat liver. Quick-frozen and freeze-dried, osmium vapor fixed, resin embedded, and post-stained with uranyl acetate and lead citrate. Bar = 0.1 μm.



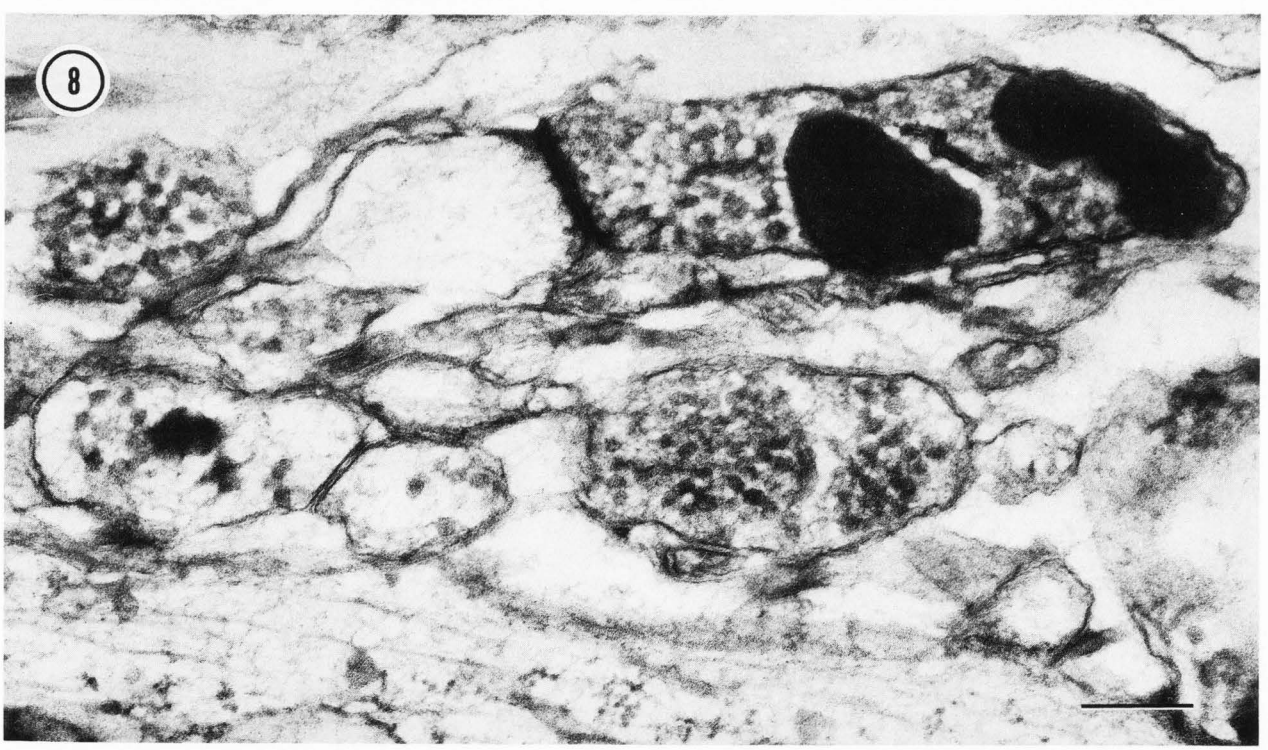
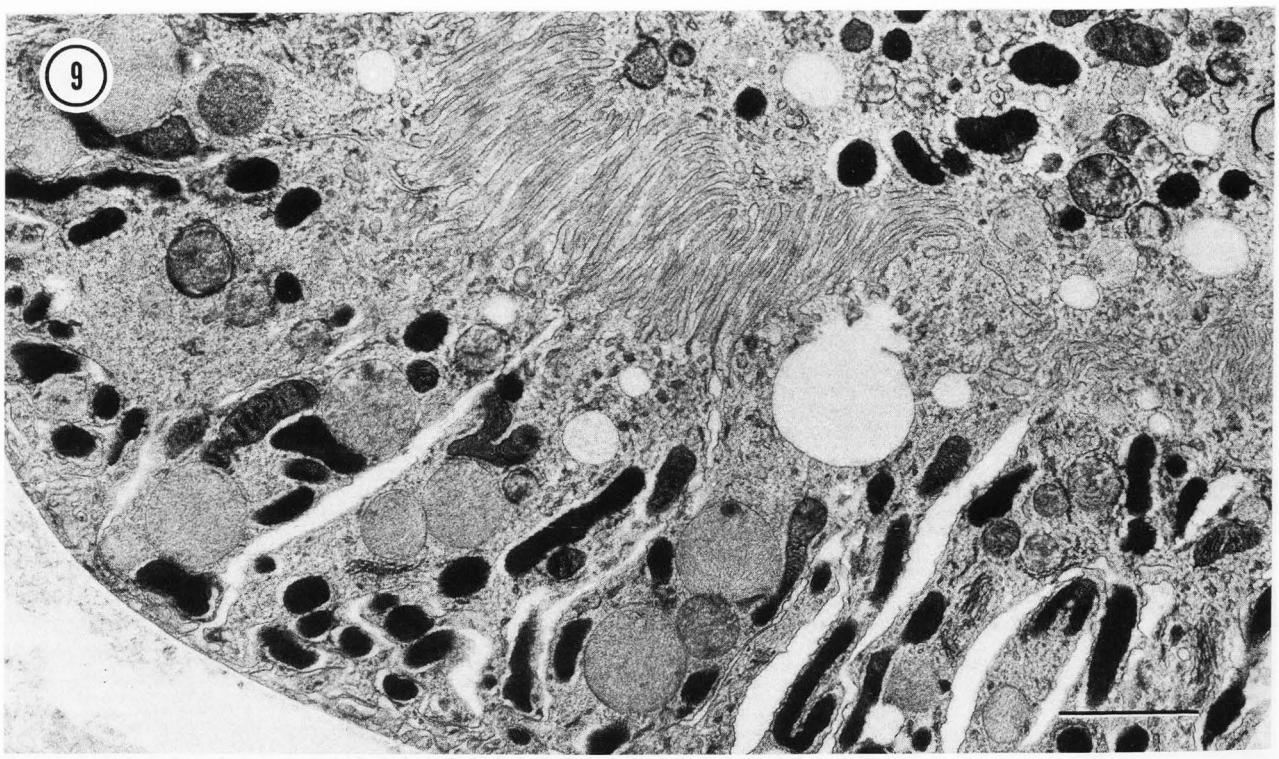


Figure 7. Normal rat cerebral cortex. Quick-frozen, freeze-dried, osmium vapor fixed, resin embedded, not post-stained with uranyl acetate and lead citrate. Bar = 1.0 μm.

Figure 8. Normal Rat cerebral cortex. Quick-frozen, freeze-dried, osmium vapor fixed, resin embedded, and post-stained with uranyl acetate and lead citrate. Bar = 0.5 μm.



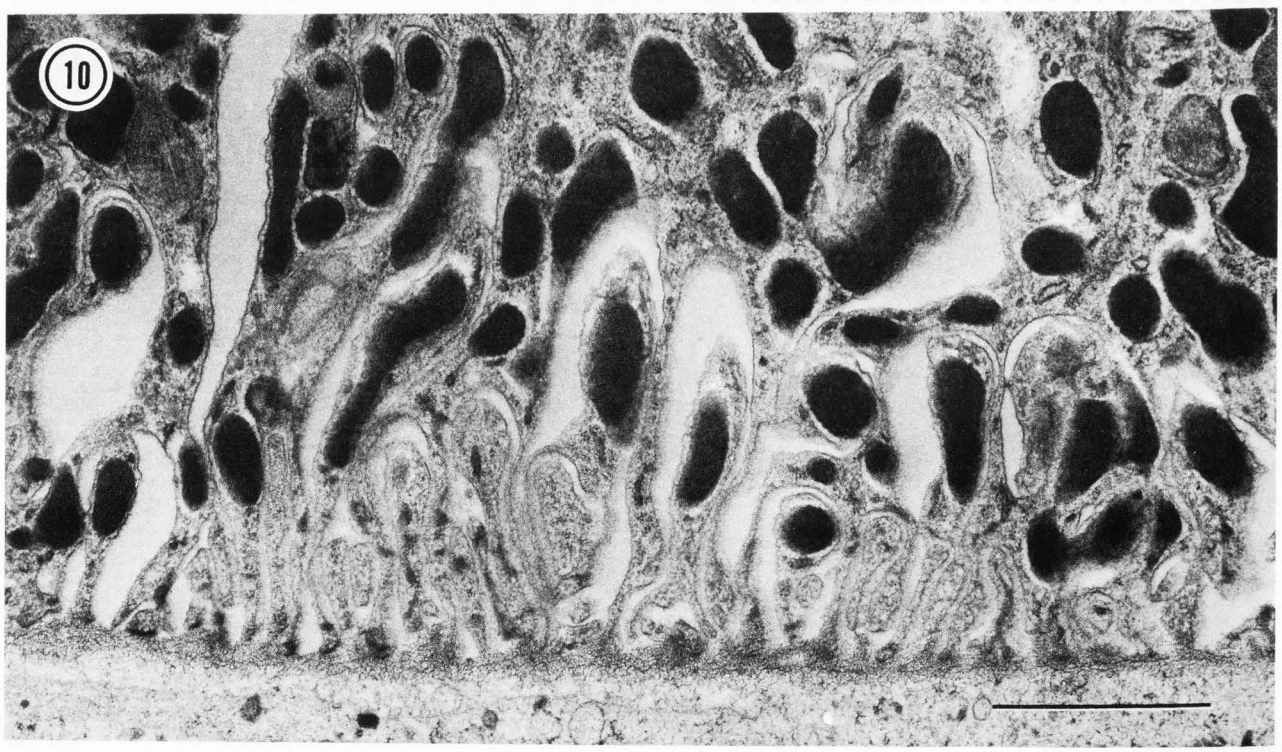


Figure 9. Normal rat kidney, proximal tubule. Quick-frozen and freeze-dried, osmium vapor fixed, resin embedded and not post-stained. Bar = 1.0 μm.

Figure 10. Normal rat kidney proximal tubule. Quick-frozen and freeze dried, uranyl acetate and lead citrate stained. Demonstrates well preserved basolateral membrane systems. Frozen edge is to the bottom of the micrograph. Bar = 1.0 μm.

This may be due to the fact that the nuclear components contain a large amount of free water and are therefore less readily frozen in the vitreous phase (Figures 5, 6). Nuclear preservation seems to be the best if the nucleus is found within 5 or 10 microns of the surface that hit the polished copper bar at liquid nitrogen temperature (Figure 6). The rough endoplasmic reticulum is well preserved with numerous ribosomes still attached to the membranes (Figure 6). The mitochondria appear to be extremely osmiophilic with dark crystae and they are somewhat susceptible to shrinkage during the drying process, at least more susceptible than other components of the cell's cytoplasm (Figure 5, 7, 8, 9, 10). The Golgi apparatus appears to be well preserved and contains electron dense material (Figure 5). Peroxisomes are well preserved, filled with electron dense material, and in some cases show the presence of nucleoids (Figure 5). Canaliculi in liver tissue are well preserved with no mechanical deformation. The cerebral cortex of rat brain has numerous easily identified synapses between axons and dendrites (Figure 9, 10). The axon terminals are filled with electron dense presynaptic vesicles and very large dense mitochondira (Figure 10). The post synaptic terminal contains no synaptic vesicles but contains a very fine trabecular network. The synaptic cleft in many instances is filled with very dense electron opaque material which may be lost with other preparative techniques.

Conclusions

Optimal preparation of biological material must maintain both ultrastructure and molecular integrity, that is, native molecular structure and spatial cellular location. Current methods of chemical fixation and freeze substitution although preserving ultrastructure, due to the nature of the fixative or of solvent phase extraction cause alteration, redistribution and loss of molecular entities (Harvey,1982). Freeze drying techniques to date, although able to retain cellular constituents, do so at the expense of ultrastructural preservation (Ingram and Ingram, 1984). The apparatus described in this paper, by the use of low equilibration temperature, ultrahigh hydrocarbon free vacuum and incremental sample temperature increase, achieves excellent morphological preservation. The theoretical potential of this material as optimum for molecular probing is currently being investigated with immunoelectron microscopy and microprobe analysis.

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