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A NEW VERSATILE SYSTEM FOR FREEZE-SUBSTITUTION, FREEZE-DRYING AND LOW TEMPERATURE EMBEDDING OF BIOLOGICAL SPECIMENS

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Abstract

A universal system for freeze-substitution (FS), freeze-drying (FD) and low temperature embedding (LTE) has been developed, suited to perform standardized procedures of cryoprocessing biological and medical specimens as well as systematic studies of dehydration and embedding at various low and high temperatures. In a 35 l Dewar vessel with 110 mm neck diameter an aluminum tube is mounted to the bottom of the liquid nitrogen (LN_{2x}) reservoir and extends to the lower part of the cylindrical neck. At its top an aluminum plate serves as a contact surface for either the FS chamber or the FD chamber. FS and subsequent LTE are carried out in an environment of dry cold nitrogen gas provided by evaporating nitrogen from the dewar. Different capsules and moulds may be used for cryodehydration and LTE. FD of bulk specimens or cryosections takes place in an absolutely clean vacuum provided by a cryosorption pump integrated in the FD apparatus. Most of the H_2O molecules from the frozen specimen are trapped by large cold surfaces inside the drying chamber. Due to the low LN_2 consumption during FS or FD (3-4 l LN_2/day) both procedures may be carried out for 8-10 days without refilling the dewar. A few representative results show that well frozen biological material is stabilized by prolonged FS or FD at temperatures of about -80°C without use of chemical fixatives like OsO_4 in the substitution medium during FS or by OsO_4 vapor fixation after FD.

Key Words: Freeze-substitution, freeze-drying, cryosectioning, low temperature embedding, X- ray microanalysis

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Introduction

Cryopreparation of biological material for electron-, ion- and light microscopy or laser microprobe mass-analysis (LAMMA) and ion spectroscopy is now of increasing interest in biological and medical research. This is due to the fact that many artefacts introduced by conventional chemical fixation, dehydration and embedding procedures can be avoided by cryomethods (Kellenberger, 1991; Kellenberger *et al.*, 1985; Pearse, 1961; Robards and Sleytr, 1985; Sitte *et al.*, 1989; Steinbrecht and Zierold, 1987). After cryofixation (Moor, 1987; Sitte *et al*,. 1987a; Studer *et al.*, 1989) stable specimens can be produced by freeze-substitution (FS) or freeze-drying (FD) and subsequent resin embedding at low temperature (LTE). A considerable number of reviews and reports describe the physical base, the methodology and the results of these cryomethods (compare e.g., for FS: Condron and Marshall, 1990; Edelmann, 1991; Harvey, 1982; Humbel and Müller, 1985; Murray, 1992; Steinbrecht and Müller, 1987; for FD: Boyde and Echlin, 1973; Chiovetti *et al.*, 1985; Condron and Marshall, 1990; Coulter and Terracio, 1977; Dudek *et al.*, 1982; Edelmann, 1986, 1994; Elder *et al.*, 1986; Elder *et al.*, 1992; Kulenkampff, 1955; Mellor, 1978; Meryman, 1966; Murray, 1992; Neumann, 1952, 1958; Pfaller and Rovan, 1978; Steinbrecht and Müller, 1987; Stephenson, 1953; Stumpf and Roth, 1967; Terracio and Schwabe, 1981; Umrath, 1983; Wroblewski and Wroblewski, 1984. and for LTE: Carlemalm *et al.*, 1985; Carlemalm and Villiger, 1989; Chiovetti *et al.*, 1985; Edelmann, 1991; Humbel and Müller, 1985; Wroblewski and Wroblewski, 1984).

It is expected that by using optimized cryoprocedures one may obtain biological material captured in a state quite close to the living state. Progress in morphological and immuno-cytochemical research of recent years is mainly due to improved cryomethods. In addition, meaningful detection of cellular and subcellular ion distributions by analytical methods such as X-ray microanalysis, electron spectroscopic imaging (ESI),

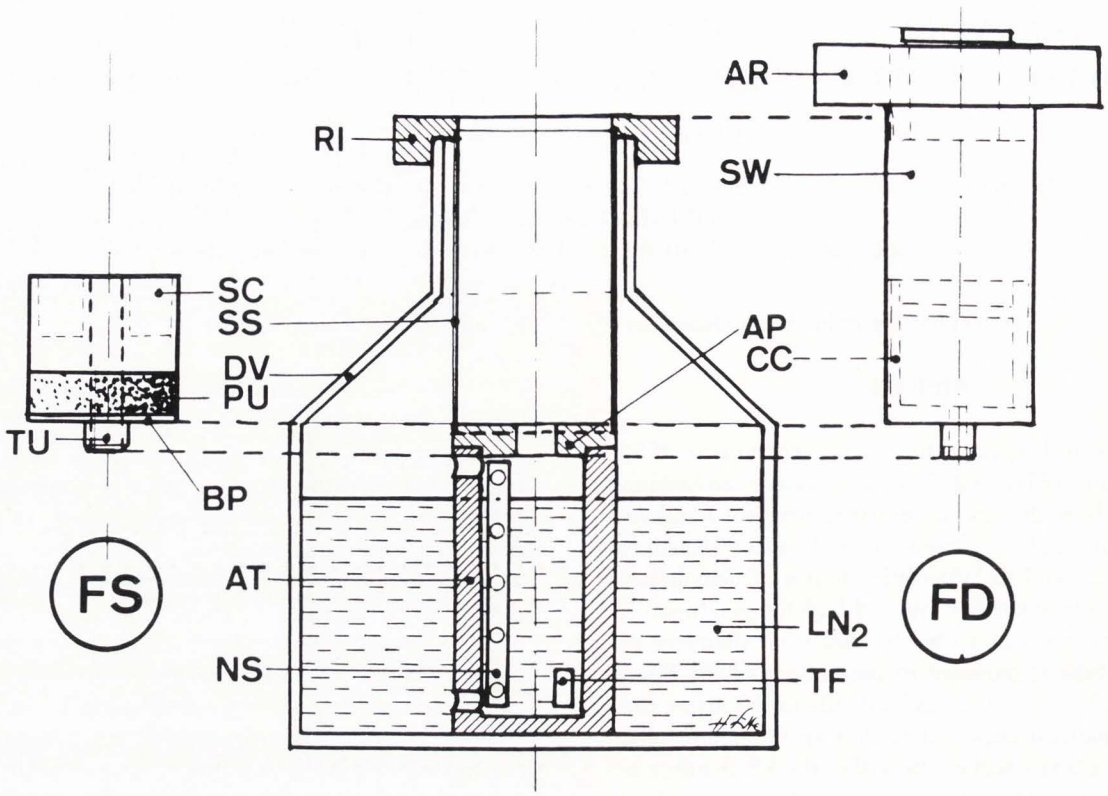


Fig. 1: Schematic cross section of the Dewar system. The system consists of the LN_2 filled Dewar vessel DV with aluminum tube AT covered by aluminum plate AP and the steel tube SS in the wall held by the ring RI on the upper border of the vessel neck. The space inside SS takes up either the chamber SC for FS or the chamber SW/AR for FD which are positioned on the plate AP with a fairly constant temperature of approx. -185°C. The base plates of FS and FD chambers are made of metals with a good heat conductivity and have contact surfaces complementary to plate AP. Compare also with Figs. 2, 4 and 6. For additional explanations see text.

electron energy loss spectroscopy (EELS), LAMMA and ion spectroscopy is greatly dependent on stable cryo-preparations (sections of embedded material or freeze-dried cryosections) with good structure preservation and minimized ion redistribution artefacts (Edelmann 1986, 1991, 1994). The possibility to carry out all preparation steps including polymerisation of Lowicryls or LR Gold by ultra-violet (UV) irradiation at subzero temperatures cannot be emphasized enough for progress in biological research. However, the toxic properties of the acrylic mixtures for LTE have probably prevented a widespread application of LTE methods so far.

The purpose of this paper is to present a universal system for FS, FD and LTE to carry out these cryo-procedures in a safe and reproducible manner. With the possibility of choosing any temperature between -140°C and +80°C, and any desired temperature change (heating or cooling rate up and down) during the different preparation steps following a preset automatic control of the temperature/time sequence systematic studies to optimize FS, FD and LTE can be performed. Due to its low consumption of liquid nitrogen (LN_2) during operation the new system is particularly well suited for long-term FS and FD procedures necessary to obtain stable preparations of high quality without using chemical fixatives.

Design of the Dewar System

In order to achieve long-term drying a 35 l Dewar vessel (DV) was chosen (Taylor-Wharton LD 35). The neck diameter 110 mm of this vessel is suited to take up rather large chambers for FS and FD. According to Fig. 1 an aluminum tube (AT) covered by the aluminum plate (AP) is mounted to the bottom of the vessel DV. The cross section of the tube AT (approx. 28 cm^2) guarantees a nearly constant temperature of approx. -185°C of this plate. The upper surface of AP serves as the contact

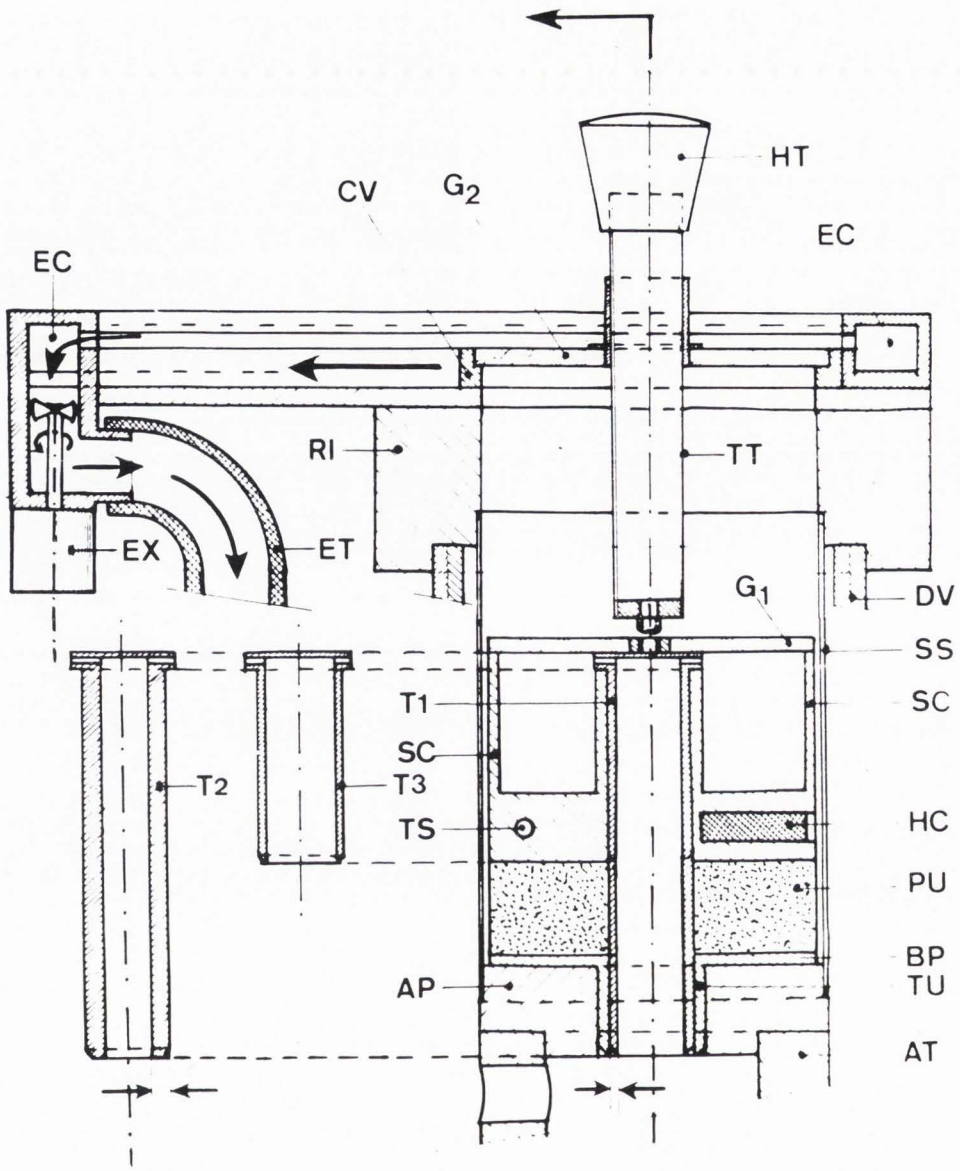


Fig. 2 : Schematic cross section of the substitution chamber SC with central tube for refill of LN_2 during operation. The chamber SC made of aluminum contains a heating cartridge HC and a Pt lOO temperature sensor TS for thermostatic control of the substitution temperature. On the upper ring RI of the dewar vessel DV (see Fig. 1) a table is connected, which is surrounded by an exhaustor channel EC (exhaustor EX, exhaustor tube connection ET). The substitution chamber SC (see Figs. 3 to 5) can be closed by glass plate G1. For additional description and explanation see text and Figs. 3-5.

plane for the chambers for FS or FD inserted in the neck of the vessel DV. A heater (TF) and a printed circuit board (NS) with several Pt 100 level sensors for the indication of the LN_2 filling level are mounted inside the tube AT. A thin stainless steel tube (SS) is mounted in the dewar neck, connecting the aluminum plate AP to the aluminum ring (RI), the temperature of which is thermostatically controlled. This is especially important during periods of increased evaporation of LN_2 by the heater TF (e.g., cryotransfer operations).

Chamber SC for Cryosubstitution

As shown in Fig. 1 and 2 the cylindrical substitution chamber (SC) can be placed inside the stainless steel tube SS on plate AP in the dewar system DV. The main components of the chamber are (see Fig. 2): an upper part with the concentric substitution container SC made

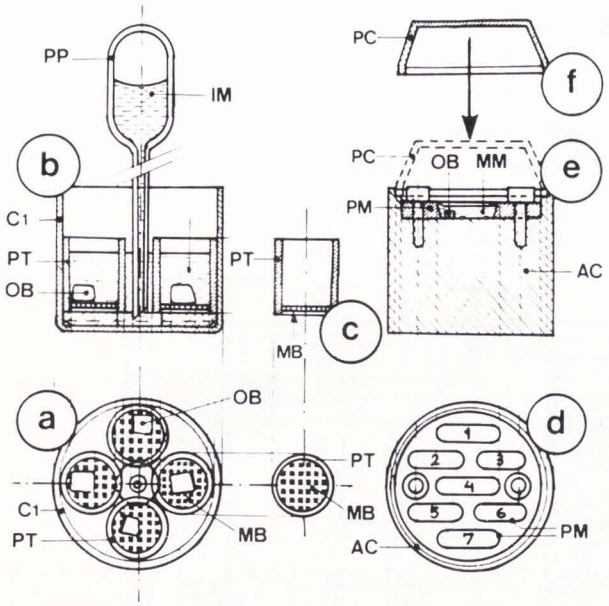


Fig. 3: Schematic cross sections (upper half) and birds eye views (lower half) of the containers for substitution (C1/PT), of incubation in monomeric resin MM and of flat embedding of objects OB in plastic moulds PM/AC. Compare with Fig. 4, for additional explanation see text.

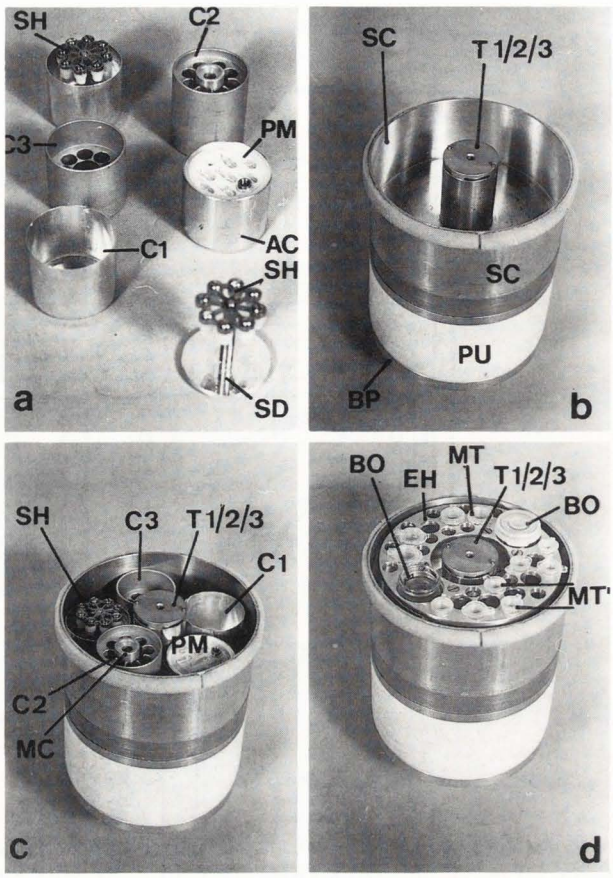


Fig. 4: Components for FS and LTE. (a) Parts for FS and LTE used in chamber CS. - (b) Substitution chamber with upper part made of aluminum (SC), polyurethane insulation PU and bottom plate BP. In the central tube of SC the cover of one of the tubes T1/T2/T3 is visible. - (c) Parts as set out in (a) placed in chamber SC. - (d) System EH for FS and LTE in Eppendorf microtubes MT/MT' placed in chamber FS. Glass bottles BO for precooling of media. - See also explanations in the text and Figs. 3 and 5.

of aluminum, a cylinder of polyurethane foam (PU) and an aluminum bottom plate (BP) with the tube (TU) which fits into the central hole of the plate AP (Fig. 1). The temperature of SC is controlled thermostatically by the heater (HC) and the Pt 100 sensor (TS) (Fig. 2). PU serves as thermal insulation between plate AP (-185°C) and substitution container SC, the temperature of which can be thermostatically controlled between -140°C (FS) and +80°C (heat polymerisation). For FS and LTE at temperatures between -100°C and -50°C, the metal tube T1 is inserted into the central hole of the container SC. T1 bridges the gap between the aluminum elements SC and TU by a metal cross section, which just allows the extraction of heat from SC necessary to reach -100°C. Thus the consumption of LN_2 is minimized (< 3 l/d). Lower temperatures down to approx. -140°C can be obtained with a tube T2 (larger cross section), causing a higher LN_2 consumption. For temperatures above -50°C (e.g., LTE in Lowicryl K4M at -30°C) the shorter tube T3 without metal contact between SC and TU is used. All tubes T1, T2 and T3 direct the escaping nitrogen gas (GN_2) into the container SC which takes up the substitution vessels and moulds (see Fig. 4).

To exclude ice formation, the container SC can be closed by the glass plate G1. This plate can be mounted by a screw connection to a thin-walled tube TT with handle HT. The thermal contact between TT and G1 can be interrupted completely during incubation. To open the chamber SC the glass plate G1 is connected to tube TT, then lifted into the highest position and shifted horizontally together with the whole cover (CV) including glass plate G2 (see Fig. 2, arrows). When the chamber is opened, the heater TF in the dewar vessel DV (see Fig. 1) is automatically activated. Thus GN_2 is evaporated escaping through the central hole in TU/PU/SC flushing the chamber SC. In this way the chamber remains always filled with dry inert GN_2 and stays frost-free. The substitution chamber SC may be opened very fast, since only the dry GN_2 inside SS/SC is convected. When the container SC is opened, GN_2 together with toxic vapors

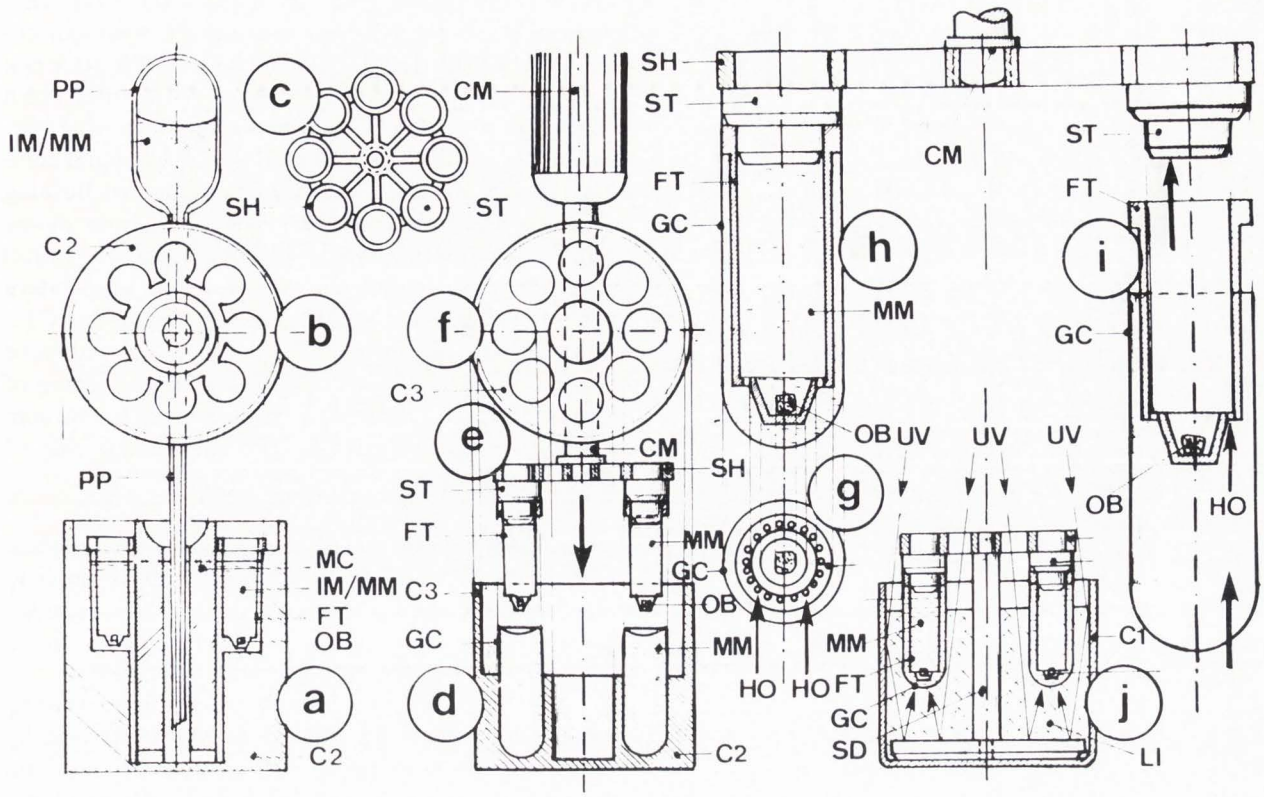


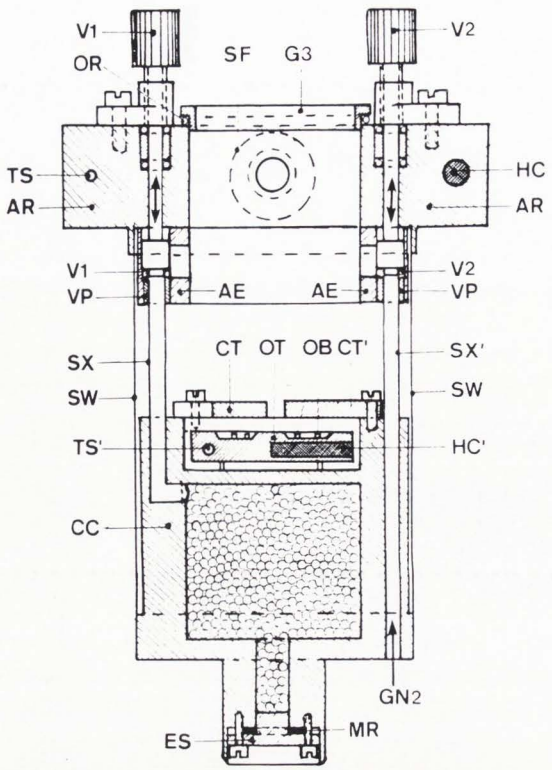
Fig. 5: FS and LTE in flow through capsules FT according to L. Edelmann. - (a) Incubation of objects OB in container C2 (see Fig. 4), filled with media IM/MM by plastic disposable pipette PP. Schematic cross section. - (b) Container C2 in birds eye view. - (c) "Spider holder" SH for synchronous picking up and transfer of 8 FT-capsules. - (d) Schematic cross section of container C3 for 8 gelatine "shell" capsules GC, filled with fresh monomeric mixture MM. - (e) Introduction of "spider holder" SH with 8 FT capsules loaded with fresh monomeric mixture MM and impregnated objects OB (see "d"). - (f) Birds eye view of container C3. - (g) Bottom of FT-capsule with holes HO for entrance/exit of media IM/MM. - (h) Enlarged view of capsule system FT/GC loaded with objects OM and fresh monomeric mixture MM. - (i) Components of capsule system before assembling. - (j) UV-polymerisation of monomeric mixture MM in double capsules FT/GC in container C1. Fenestrated "spider holder" SH on stand SD allows UV-entrance. Reflected UV cures MM firstly at the lower regions with the objects OB. Liquid LI as "sink" for polymerisation heat. - Compare with text and Figs. 2 and 4.

of the acrylic mixture for LTE escape through the open top. This vapor is sucked off through the exhaust channel EC by the exhaustor EX. The flexible tube ET may be introduced into a fume hood or into the open air. Chamber SC is closed by reverse operation: the cover CV/G2 is shifted over the chamber SC/SS, the glass plate G1 lowered and separated again from TT/HT. Finally TT/HT is lifted to the highest position.

The system is suited for different procedures of FS and LTE described in the following section. Both FS and LTE can be carried out either in flat embedding moulds or in capsules. Incubation and embedding of different specimens following an identical protocol can be simply realized either in plastic containers and plastic moulds or in double capsules designed by one of us (L. Edelmann). They offer the most efficient handling. For different processing of different specimens a holder for Eppendorf Microtubes was designed. All systems allow the processing without risk of skin contact with toxic acrylic mixtures or the inhaling of their vapors.

FS and Flat Embedding at Low Temperature

The incubation in the different media IM is performed in simple metal containers C1 (volume approximately 25 ml) suited for insertion into the substitution container SC (see Figs. 3-5). Each container C1 takes up maximally 4 plastic tubes (PT) with objects (OB). It is



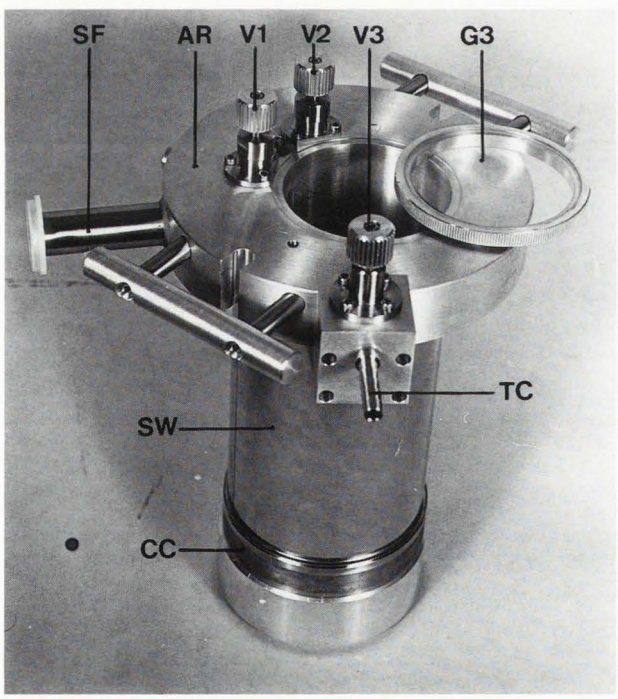


Fig. 6: FD-chamber (see Fig. 1 and text). - (a) Schematic cross section. Copper container CC with molecular sieve MS for cryosorption. Side wall SW (0.5 mm thick cylindric stainless steel) of FD-compartment connected both with CC and upper aluminum ring AR. Valves V1 and V2 for sealing of the cryosorption compartment CC against FD-compartment and for flushing of FD-compartment with dry cold GN_2 from dewar vessel. Top opening sealed with cover glass G3. Object table OT with objects OB and electrical temperature control HC'/TS' below additional cold trap wings CT. - (b) Opened FD-chamber: Note valve V3 with tube connection TC for "booster pumping" after loading of the chamber. SF: standard flange connection for vacuum sensor and other accessories. IR: intermediate ring of aluminum as support.

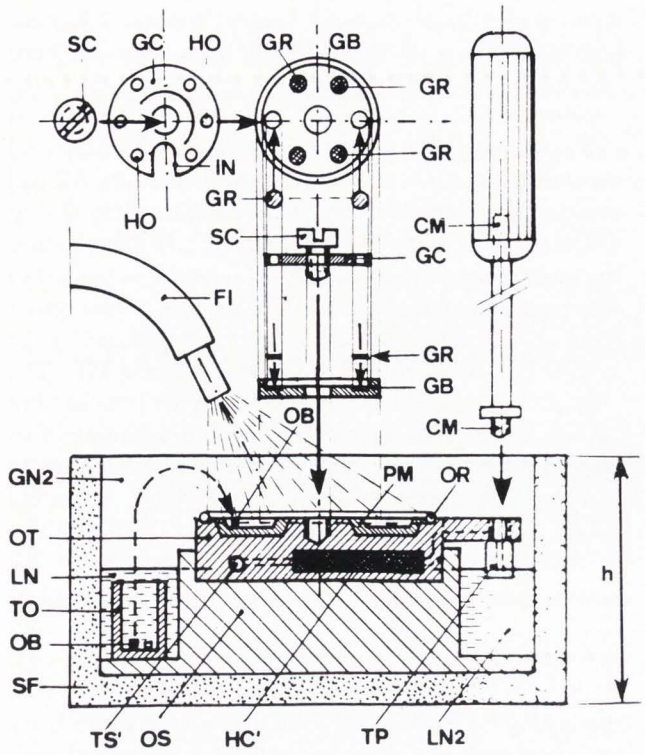
possible to fill and empty the plastic tubes PT with mesh bottom (MB) simultaneously within one container C1 by a plastic disposable pipette PP without risking to lose specimens (Fig. 3 a-c) For this operation the plastic pipette (PP) with the incubation medium IM is inserted between the tubes PT as shown in Fig. 3b. In order to reduce concentration gradients in the liquid, medium IM can be agitated either by lifting and lowering the single tubes PT or by taking up liquid with pipette PP.

After FS and complete infiltration with embedding medium the monomeric mixture (MM) is removed by PP. The impregnated objects OB are transferred within the inert GN_2 atmosphere of the chamber SC into plastic moulds (PM) mounted on the aluminum cylinder (AC) (Fig. 3d and e). After filling the moulds PM with fresh monomeric mixture MM the system AC/PM/OB/MM is covered and tightly sealed by the plastic cover PC (Fig. 3f). For polymerisation the open chamber is closed by a reflector system equipped with a UV light source (Philips TW6W). This UV reflector is similarly used for curing monomeric mixtures in the other systems described in the following chapters.

FS and LTE in Flow-Through Double Capsules According to Edelmann

The double capsule system is suited both for simultaneous low temperature and for standard processing of up to 8 different specimens following an identical protocol (see Fig. 5). For FS the frozen specimens are transferred into precooled flow-through plastic capsules FT in metal container C2. Up to 4 containers C2 can be inserted in the substitution chamber SC (see Fig. 4).

Each capsule FT has a series of holes (HO) (diameter 0.3 mm) near the bottom allowing the free transfer of the different incubation media IM/MM for FS and LTE. After insertion of all frozen specimens OB therefore all capsules FT are filled simultaneously with the incubation medium IM through the central hole of the metal cylinder MC with a plastic disposable pipette PP



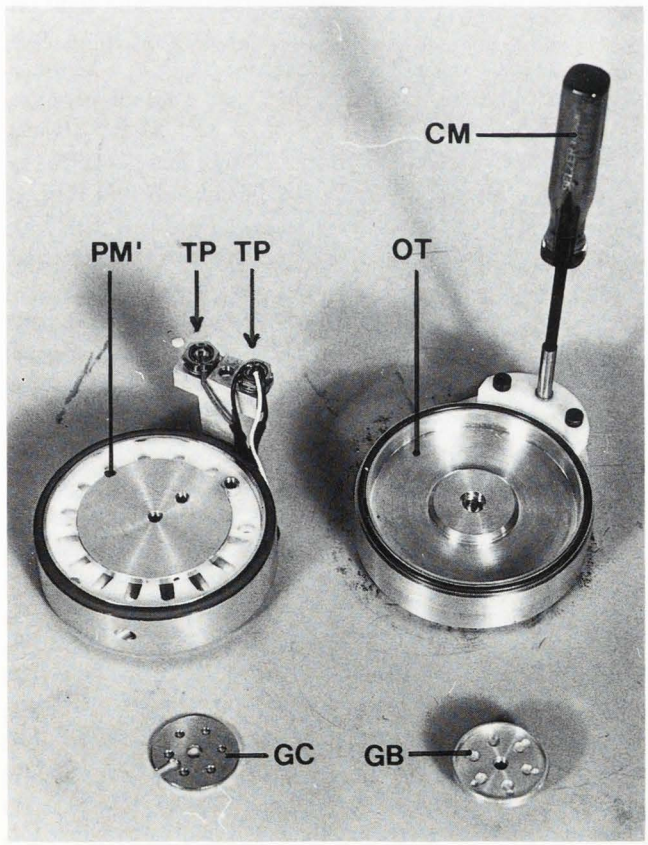


Fig. 7 : Loading of the object table OT for FD-procedure (see Fig. 6 and text). - (a) Schematic cross section of OT inside styrofoam container SF partially filled with LN_2. Transfer tube TO with objects OB. Transfer into plastic moulds PM' is made within dry cold GN_2 atmosphere. Transfer of OT into FD-chamber with cryo manipulator CM. Preparation under fiber optic FI illumination. Accessory GB/GC for FD of cryosections on grids GR. SC: screw, GC: coverplate, HO: holes, GB : base plate, for explanation see text. - (b) Prototype object tables OT with plastic moulds PM' and cryo manipulator CM. Grid holder GB with transfer cover GC below.

as shown in Fig. 5a. Efficient agitation of medium IM is possible by lifting and lowering the metal cylinder MC. The media IM/MM are exchanged by using plastic pipettes PP to remove and add media. After FS and complete infiltration with the monomeric mixture MM all 8 capsules are now mounted en bloc on to the stoppers ST of a fenestrated holder SH ("spider"). For this procedure SH is mounted to the cryomanipulator CM. The FT-capsules are simultaneously taken up by pressing down ST/SH/CM against the FT-capsules. Then the FT-capsules are transferred from container C2 into gelatine capsules GC located in container C3 already filled with MM (Fig. 5d and e). The excess of MM flows into the central hole of C3. After this operation all plastic capsules FT with the objects OB are sealed by the gelatine "shell capsules" GC. Both capsules FT and GC are now completely filled with monomeric mixture MM. The "spider holder" SH/ST with the filled double capsules FT/GC/MM/OB is now simply transferred into container C1 and deposited on the stand SD (Fig. 5j). The container C1 is partially filled with a suited liquid LI (e.g., ethanol) for thermal equilibration during the subsequent UV-light curing. Since the stoppers (ST) prevent direct UV irradiation of monomeric mixture MM from the top, the curing is achieved exclusively by indirect irradiation with UV reflected by the walls and the bottom of container C1/SD as recommended by the Lowicryl manufacturer.

FS and LTE in Eppendorf Microtubes

For incubation of different specimens following different protocols a holder (EH) for Eppendorf microtubes with 0.5 ml (MT) resp. 1.5 ml (MT') volume was designed (see Fig. 4d). This holder EH fits into the substitution chamber SC. It offers 20 openings for the smaller tubes MT (0.5 ml volume) or alternatively 10 openings for the larger tubes MT' (1.5 ml volume). The UV-light for curing enters through the openings for the

other tube size not used. Direct irradiation is prevented by shadowing rings covering either the tubes MT (two rings with different diameters) or the tubes MT' (one ring of a medium size). During the exothermic polymerisation reaction (see Ashford *et al.*, 1986) thermal equilibration is achieved by partial filling of the substitution container SC with a suited liquid (e.g., ethanol). This liquid serves as a heat sink for the polymerisation heat preventing undesired heat polymerisation. Openings for two bottles BO with a volume of 12 ml each within the holder EH allow storage and precooling of media for FS and LTE.

Freeze-drying by Cryosorption using Molecular Sieve and Cold Traps

Freeze-drying by cryosorption (e.g., Stumpf and Roth, 1967) offers the advantage of an absolutely clean vacuum free of carbohydrate traces in combination with simplicity of design and saving investment costs for highly sophisticated pumping systems. The efficiency of the cryosorption pumping can be enhanced considerably by large area cold traps cooled by LN_2. In this way the partial vapor pressure of H_20 can be lowered to negligible values. A very simple FD system based exclusively on a cryosorption pump and a cold trap has been described by Edelmann (1978, 1979). A similar system was designed by Elder *et al.* (1986). The convincing results obtained with the Edelmann system in our laboratory led to a redesign of this FD chamber. Our goal was to improve the handling without losing the advantages of the combination of cryosorption with an efficient cold trap. The desired improvements were the following : (a) Easier closing and opening of the chamber. - (b) Avoiding of a metal sealing ring between LN_2 cooled parts. - (c) Preventing of N_2-accumulation by molecular sieve during the opening (makes frequent exchanges or regeneration of sieve necessary). - (d) Controlled flushing of the chamber with dry cold N_2 gas. - This redesign and some representative results are described and discussed in the following sections.

New FD Chamber with Cryosorption Pump and Cold Trap

Basically the new FD chamber (see Figs. 1 and 6) consists of a copper container CC connected via a thin stainless steel tube SW to an aluminum ring AR at the top. The copper container CC forms a compartment for the molecular sieve MS. The base of CC is in direct contact with the aluminum plate AP in the Dewar vessel DV (Fig. 1). Therefore the copper surfaces are cooled down rather rapidly after insertion of the FD chamber to -185°C and held almost constant at this low temperature. The stainless steel tube SW (height approx. 180 mm, diameter approx. 100 mm, wall thickness 0.5 mm) minimizes the heat flux from the upper ring AR to the copper base CC. Therefore the ring AR remains at ambient temperature during FD-operation without special measures. Thus it is possible to connect the ring AR and the stainless steel wall SW vacuum-tight by an O-ring OR or by an epoxide glue. Because of LN_2 temperature the lower end of the stainless steel tube SW is welded to the copper container CC. For exchange of molecular sieve MS the copper container CC can be opened by the stopper ES sealed by a disposable metal ring MR. The copper container CC is connected with the FD chamber by valve V1. With respect to possible evaporation of large amounts of adsorbed gases after warming up to ambient temperature this valve is designed as safety valve: the spring loaded valve opens automatically if a pressure is built up within CC. A second valve V2 connects the FD chamber with the GN_2 compartment of the Dewar DV. It allows to flush the FD chamber with dry inert GN_2 evaporated by a heater TF (see Fig. 1). Both valves V1 and V2 are thermally connected with the upper ring AR of the chamber: the valve plates VP are surrounded by aluminum elements AE connected with the upper ring AR. They are mounted vacuum tight on steel tubes SX/SX' (diameter approximately 7 mm, wall thickness 0.5 mm). Since the upper ring AR is thermostatically heated by heater HC (Pt 100 temperature sensor TS), the O-rings of these valves are always warm: this guarantees a proper sealing function. This is especially important, when the FD chamber is flushed with cold GN_2 during cryotransfer operations (Loading with frozen objects OB or transfer of freeze-dried specimens).

The object table OT is thermostatically heated by heater HC' (temperature sensor TS'). The whole surface of the copper container CC acts as a cold trap with a temperature of approx. -185°C. Additional cold traps CT can be mounted on CC and swung out over the objects OB on plate OT if needed.

The upper aluminum ring AR shows additional openings : one opening is sealed by valve V3 (Fig. 6b) and serves for initial "booster pumping" (tube connection TC for pump). Both a water jet pump, a membrane or a turbo molecular pump are suited to eliminate most of the rare gas atoms together with N_2 and O_2 molecules without introduction of carbohydrate traces. Another opening simply consists of a standard flange SF for connection of other vacuum elements (e.g. vacuum sensor, additional connections for electrical elements like an additional temperature sensor). Finally the wires for thermostatical heating of the object table OT are intro-

duced through the ring AR. The FD chamber itself is closed during operation by a glass plate G3 sealed by an O-ring OR. Refilling of LN_2 into Dewar vessel DV is possible during FD operation trough a stainless steel tube (diameter approx. 10 mm x 0.25 mm). This tube is guided through the upper ring AR (O-ring sealing) and the copper container CC (sealing by welding).

Special measures are necessary for the transfer of the frozen objects or cryosections on grids into the FD-chamber and for the safe transfer of the freeze-dried specimens resp. cryosections out of the chamber for subsequent preparation or transmission (TEM) or scanning (SEM) electron microscopical investigation (see Fig. 7). The loading of the object table OT occurs outside the FD-chamber in a simple styrofoam box SF partially filled with LN_2 (aluminum support OS for OT). During the loading operation the table OT is always completely covered by cold dry GN_2. Aside the support OS a place is also available for transfer elements TO for frozen objects OB or cryosections on grids. They can be safely transferred under GN_2 or LN_2 from the corresponding sectioning chamber of the cryo-ultramicrotome or from the cryofixation system used. Since the height h of SF is below 100 mm, the preparation can be controlled under a standard stereo microscope. For "cold" illumination a glass fiber system FI is used. After the preparation (dividing and positioning of the specimens in the plastic moulds PM' on OT) the object table OT (temperature < -190°C) is mounted on the cryomanipulator CM and transferred into the FD-chamber. If necessary the object table OT can be covered by a cold or insulating plate during this cryotransfer operation. The electrical connection of the heating cartridge HC' and the thermal sensor TS' in OT is realized by two triax plugs TP. The counter parts to TP are mounted on the copper container CC (see Fig. 6a) over an intermediate plate. This plate reduces the heat transfer between OT and CC by TP to the desired value for a proper electronic temperature control during FD or during flushing with cold GN_2.

For drying of cryosections on grids GR a special grid holder was developed, which consists of a base plate GB and a coverplate GC (Figs. 7a,b). Both plates GB and GC can be mounted on the object table OT by screw SC. The cover GC has an incision IN for loading and holes HO for FD. During the cryotransfer to the FD-chamber the cover GC is turned (arrow) to a position, where all grids are covered by the metal plate GC. For our experiments 6 places (6 holes HO in GC and corresponding 6 support cavities for grids GR in GB) have been proven to be sufficient. If necessary a grid holder with a higher capacity may be designed.

Freeze-dried objects OB can be incubated with a monomeric epoxide mixture for subsequent heat polymerisation in the same way as described earlier (Edelmann, 1979). Alternatively there is the possibility of LTE inside the inert dry GN_2 atmosphere of the flushed FD chamber. For this operation a special sealing of the object table OT has been developed (e.g. O-ring OR combined with a suited counter part) This sealing avoids free evaporation of volatile components of the monomeric mixture inside the FD chamber and subsequent condensation on cold surfaces. The inert dry GN_2 excludes the risk of H_2O uptake by the freeze-dried objects OB which may cause redistribution of ions and other cellular components.

Methods and Results

Both the FS system and the FD chamber described above have been tested during two years (FS) resp. one year (FD) in our laboratory. Our main interest was focused on cryoprocedures without employing any chemical fixative, because it is expected, that biological specimens which have not been treated with chemical fixatives are ideal preparations for meaningful elemental analysis and for progress in immunocytochemistry. So far it was demonstrated that good structure preservation can be obtained after sufficient periods of drying. In addition, staining of sections after LTE posed no difficulties.

In the following a few representative results obtained with chemically unfixed striated muscle show that biological material can be stabilized by FS or FD and processed for transmission electron microscopy without the use of chemical fixatives like OsO_4 either in the substitution medium or after FD as OsO_4 vapor. Skeletal muscle was chosen as a test specimen (1) since it is very difficult to preserve without chemical fixatives its delicate ultrastructure by FS or FD and (2) since it reveals a well known internal standard (A band width 1.5 μm) to judge shrinkage artefacts during FS and FD. Isolated frog sartorius muscles from *Rana pipiens* Schreber are cryofixed by rapid contact with a LN_2 cooled copper plate as described by Edelmann (1989).

FS and LTE in K11M is carried out as follows: FS in pure acetone for 1 week at -80°C; infiltration in K11M (70% K11M + 30% acetone, 5h, -80°C; 100% K11M, 5 h, -60°C; 100 % K11M, 12 h, -60°C); transfer into fresh embedding medium in flat embedding moulds and polymerisation by UV irradiation at -60°C for 24 h. The polymerised specimens are warmed up to room temperature without further UV irradiation and removed from the moulds. They are immediately suited for sectioning. A Diatome diamond knife is used for ultrathin sectioning (Fig. 8).

FD and embedding in Spurr's medium is carried out as described by Edelmann (1986): FD of frozen specimens together with frozen drops of evacuated Spurr epoxy resin for 3 days at -80°C and 6 days at -60°C; venting of the FD chamber with dry cold GN_2; the specimen support is warmed to -15°C and maintained for 12 h at this temperature : the Spurr medium liquefies and infiltrates the specimen; the specimen support is warmed up to room temperature (10°C/h) and removed from the FD chamber; the tissue samples are transferred into fresh embedding medium and polymerised at 40°C for 1 week (Fig. 9).

Cryosections are cut with a Diatome diamond knife in a Reichert Ultracut-E/FC4 and freeze-dried in a Zeiss EM 10 CR as described in Figs. 10 a and b. Other cryosections are cut in a Reichert Ultracut-S/FCS, freeze-dried in the described FD chamber at -100°C for 33 h. After this drying period the FD chamber was filled with cold dry GN_2 as mentioned above. The dry sections are transferred to a Zeiss EM 902 (Fig. 10c).

Finally some specimens are freeze-dried, infiltrated under dry cold GN_2 in Lowicryl HM20 and polymerized in the described FD chamber in plastic moulds at -30°C. Care was taken to avoid any evaporation or condensation of monomeric components of the mixture on the cold surfaces within the chamber by a tight sealing of the embedding mould. UV polymerisation at -30 °C was carried out for 24 h. After this curing by UV irradiation the flat embedded specimens could be sectioned immediately with a Diatome diamond knife (Fig. 11).

Discussion

Both FS and FD depend on the removal of water molecules from frozen specimens. Both processes are temperature dependent. During freeze-drying the number of escaping molecules per time and surface unit increases with temperature according to the Knudsen equation (see e.g., Mellor, 1978; Neumann, 1952; Umrath, 1983): The absolute rate of sublimation of pure (e.g., hexagonal) ice G_s (in $g.cm^2.s^{-1}$) is

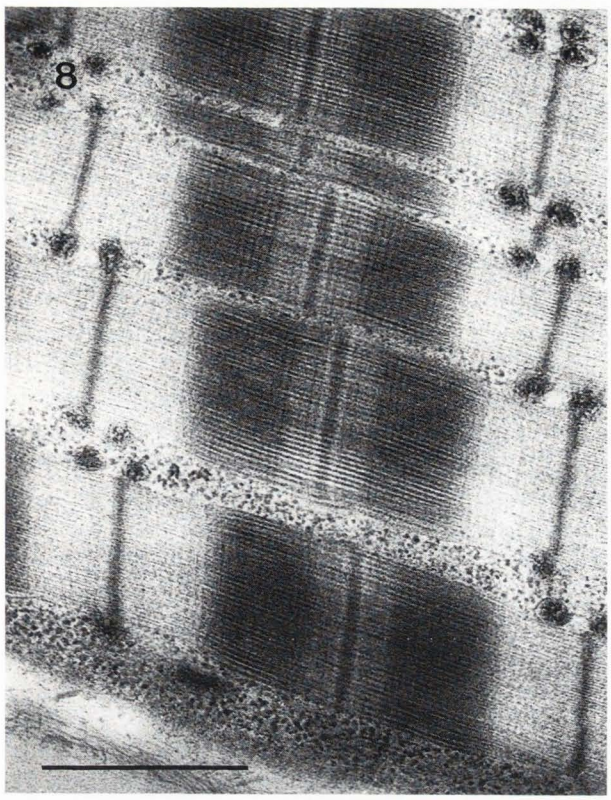
$$G_s = \alpha P_s(M/2 \pi RT)^{0,5} \quad 0 < \alpha < 1 \qquad (1)$$

where α = coefficient of evaporation, P_s = saturated vapor pressure of ice, M = molecular weight of water, R = gas constant, and T = absolute temperature. In deriving this equation it is assumed that the number of escaping water molecules depends on temperature of the ice surface, whereas the number of returning water molecules depends on the pressure and temperature of the adjacent vapor phase. That means, that in an "absolute vacuum" (vapor pressure zero) only the temperature of the bulk ice influences the sublimation rate. The lower the pressure the closer this condition is approached.

In principle the Knudsen equation in a slightly modified version should also be valid for FS processes. In this case the partial H_2O pressure of the substitution medium has to be considered. A correction coefficient should introduce the different hydrophilia (polarity) of different liquids resp. the different ability to attract H_2O at low temperatures. For example Humbel (1984) has measured, that methanol in comparison to acetone has a stronger tendency to attract and substitute water (see also Humbel *et al.*, 1983). On the other hand the water attraction by ether is lower than the attraction by acetone.

All empirical data and theoretical considerations lead to the conclusion, that the dehydration by FS and FD have a similar physical base namely the temperature dependent probability of detachment of water molecules from the ice and the removal of these liberated water molecules from the immediate neighborhood of the ice. Differences of results obtained with the different dehydration techniques must be attributed to the presence or absence of a liquid dehydrating medium with its physicochemical properties. E.g., certain cellular components may be stabilized near their original sites during FD at low temperature but may be dissolved and redistributed during FS by the given substitution medium.

In FD even the sublimation of pure ice from bulk specimens at temperatures below -120°C is too slow for practical work (see Fig. 12): the sublimation of a 1 μm thick layer of pure ice in one direction requires considerable more than 1 year. Even the sublimation of a 1 μm thick layer of pure ice under the same conditions requires at least one day. Work at lower temperatures << -120°C leads to even longer sublimation periods (e.g., -150°C: approximately 1000 years/μm or approximately 1 year/μm, respectively). These theoretical values given by Umrath (1983) are of course considerably below all real times for frozen biological bulk specimens or cryosections of such objects. Any material dissolved in the bulk lowers the H_2O vapor pressure. The freezing point depression of cellular and extracellular water demonstrates this reduction. Both cellular and extra cellular phases of biological specimens have a rather high content of molecular dispersed constituents and ions, which reduce the partial pressure of H_20. In addition to this phenomenon, the influence of the numerous diffusion barriers between cells and cell compartments must be considered: both lipid/protein bilayers and cell wall structures must be expected to be severe diffusion barriers for H_2O molecules. They influence (reduce) heavily the escape of H_2O from layers



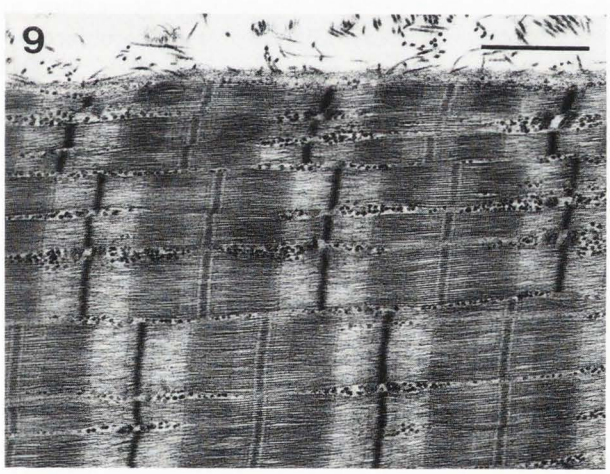


Fig. 8: Ultrathin section of frog sartorius muscle after FS in pure acetone and LTE in K11M (see text), stained with uranyl acetate and lead citrate. Bar = 1 μm.

Fig. 9 : Ultrathin section of frog sartorius muscle after FD and embedding in Spurr's resin (see text), stained with uranyl acetate and lead citrate. Bar = 1 μm.

below the first cell layer (object surface), which is mostly opened by sectioning during excision. Stephenson (1953) has calculated the delay of H_2O evaporation by

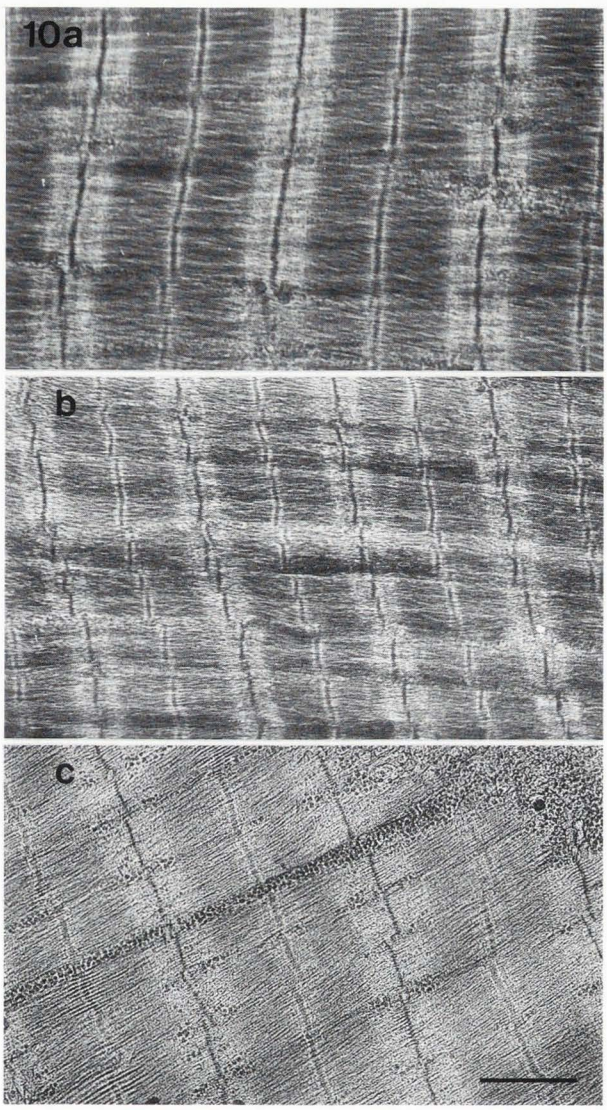


Fig. 10: Cryosections of frog sartorius muscle. - (a) FD for 1 h at -100°C in a Zeiss EM 10CR, 6 h at -80°C, temperature rise to room temperature (10°C/h, manual operation) and cooling down to about -170°C. Section thickness about 0.2 μm. - (b) Cryosection from the same muscle preparation as shown in (a) kept for 1 h at -100°C in the EM, removal of the Dewar vessel used for cooling of the grid holder, uncontrolled warming up of the grid in the EM overnight, cooling down of specimen to about -170°C. Note the shrinkage of this freeze-dried cryosection (same magnification as (a) !). - (c) Cryosection (section thickness about 0.1 μm) after FD for 33 h at -100°C. Bar for a, b and c = 1 μm.

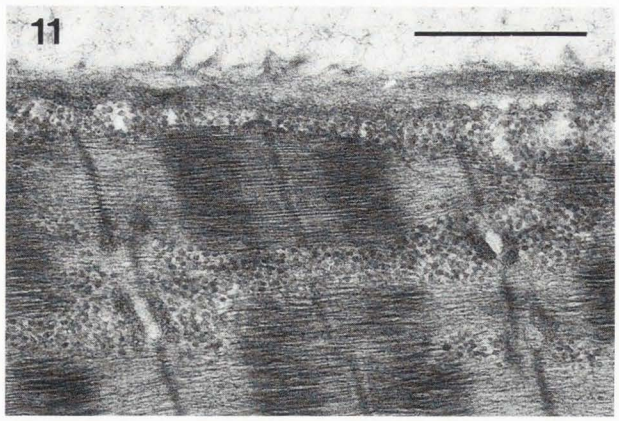


Fig. 11 : Freeze-dried frog sartorius muscle, embedded in Lowicryl HM20. FD : 2 days -80°C, 1 day -60°C, 2 days -50°C. Infiltration with HM20 at -30°C for 6 h. UV polymerisation at -30°C for 1 day. Section stained with uranyl acetate and lead citrate. Bar : 1 μm.

these layers and published a prolongation factor P of approximately 1000 times. Other prolongation values are collected by Umrath (1983). They vary between 20 and 15,000 times. Even in the best case (P = 20) the unidirectional drying of a 1 mm bulk layer at -120°C would need more than 20 years.

The temperature and the prolongation of drying times caused by dissolved material and different intermediate layers represent only two parameters. Additional parameters are given by the vacuum, the geometry of the drying system and the polymorphic modifications of H_2O at low temperatures. The influence of the total vapor pressure (measured vacuum) in the drying system is limited and not as severe as often expected. Vapor pressures above 10^{-2} mbar may influence to some extent the drying time or ice sublimation, respectively, by repelling escaping H_2O molecules to the frozen specimen: if they hit the specimen again, there is some probability, that they are captured again by the bulk. In this case the sublimation rate indeed may be lowered. Repelling is dependent on the mean free path of gas molecules or atoms in the vacuum, which decreases with increasing pressure according to Fig. 13. This mean free path between one and the next following contact with another particle measures between 10 and 100 mm at 10^{-3}mbar. The probability of a return by repelling to a small specimen with a diameter below 0.5 mm under these geometrical conditions evidently must be very small. If an LN_2 cooled surface is positioned at a distance similar to the mean free path over the specimen, the influence of repelling phenomena decreases nearly to zero. Much more important than the total pressure is the partial pressure of H_2O in the vacuum. This partial H_2O pressure slows down the evaporation of H_2O molecules from the specimen surface. A partial H_2O pressure equal to the pressure of the water molecules escaping from the specimen surface stops indeed the FD process. It results in a dynamic equilibrium of loss and uptake of H_2O molecules on the specimen surface. Under the preconditions discussed above (factor $P > 1000$) FD will stop even at rather small partial H_2O pressures within the FD chamber. It is therefore of great importance, to minimize the partial H_2O pressure around the specimen. This can be done easily, if cryosorption is combined with an efficient cold trap. In this way the partial pressure of H_2O can be kept below 10^{-13} mbar in a simple way, if LN_2 cooled surfaces of sufficient area are positioned close to the specimens on the object table inside the vacuum chamber (see Fig. 6a and 13). Without H_2O other measures these cold surfaces capture rapidly most of the H_2O molecules from the gaseous phase. On the other hand other molecules and rare gas atoms can not influence the sublimation rate considerably at total pressures below 10^{-3} mbar.

Under the conditions discussed above the geometry of the system used for FD is of great importance. This geometry has an influence both on pressure and temperature within the micro areas around the specimen. Theoretically the condition depicted in the scheme of Fig. 14 would be ideal: we assume that the object OB has a constant overall temperature of -80°C and is surrounded by an LN_2 cooled hollow sphere (HS). A pumping system PS eliminates most of the gas molecules. The pressure inside the sphere may therefore be below 10^{-3} mbar. If the distance between specimen and cold surface is < 10 mm, nearly all escaping H_2O molecules are trapped on the condensation surface. Optimal drying is realized, since practically no H_2O molecule returns to the object OB. In reality (see Fig. 14b) a heated support (e.g. object table OT, see Fig. 6a) is used. Even if the object OB is inserted in a hole HO, considerable thermal exchange is given with the nearby LN_2 cooled plate (CP) above. Within the spatial angle ω the object surfaces remain in a heat exchange with this cold trap by infrared radiation. The other specimen surfaces interact mainly with the heated object table OT. Surface temperatures at different surface areas of the bulk object OB will therefore differ considerably as a result of these different heat exchange rates by radiation.

Two other parameters have to be considered: the best vacuum is not able to eliminate the repelling of H_2O molecules by the solid state surfaces of OT inside the receptor hole HO. Finally one has to imagine according to Fig. 14c that after an initial drying of the outer border the center of a biological specimen still represents a bulk

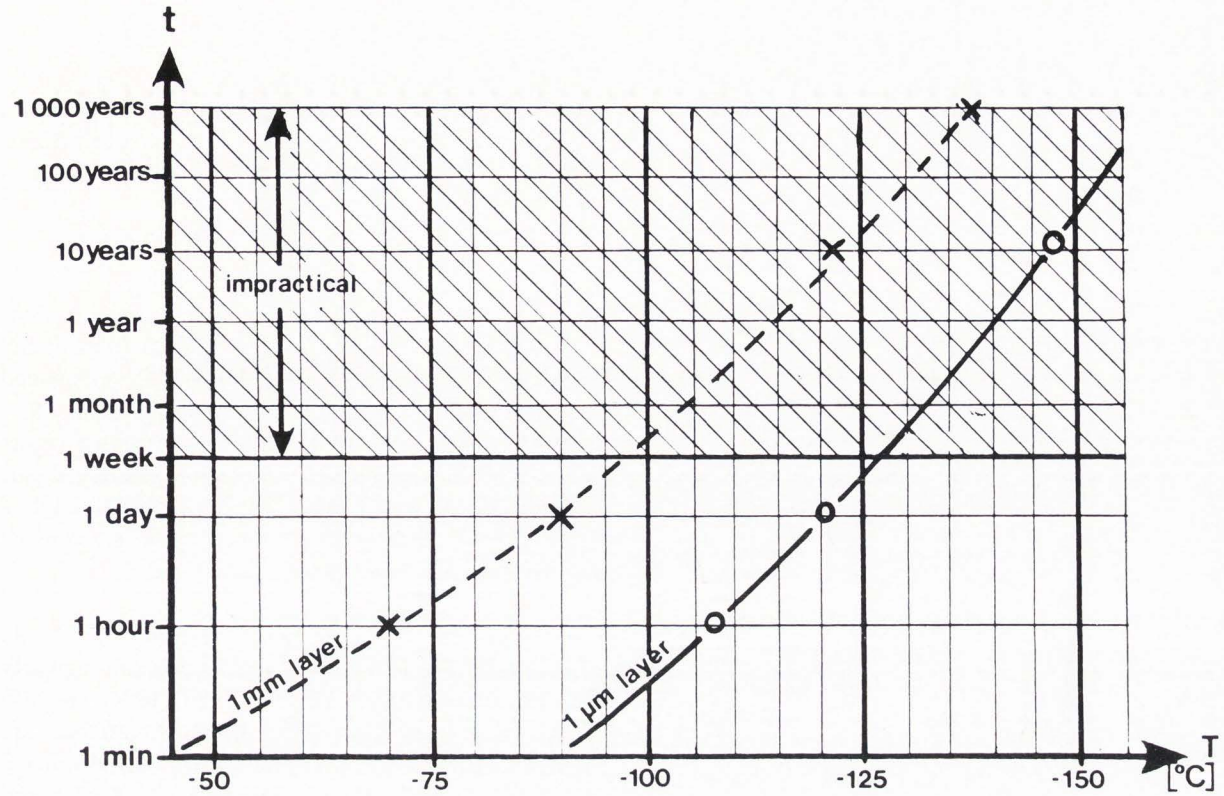


Fig. 12 : Required times t for sublimation of 1 mm (---x---x---) resp. 1 μm (---o---o---) thick layers of pure ice at different temperatures between -50 and -150°C. Note that even an 1 μm thick layer of pure ice requires more than a day for drying at temperatures below -120°C (redrawn from Umrath, 1983).

of frozen material (ice matrix IC) surrounded by a light weight porous shell LW similar to a porous styrofoam insulation. It may be, that the real temperature of the solid state ice core IC differs from the temperature of the surrounding solid state surfaces of the hole HO in the object table OT: such differences in temperatures could e.g., occur by the escape of H_2O molecules with higher kinetic energy levels. It will therefore be very difficult if not impossible to calculate even in a rough approximation the real temperatures and pressure values in the different micro areas of such systems.

The influence of the heat exchange by radiation according to Fig. 14b and c was mentioned by Elder *et al.* (1992), reporting about the influence of cooled plates like CP. One of us (L.E.) has observed droplets of pure water frozen on a metal surface (diameter approximately 1 mm) inserted in a system similar to that described in Fig. 14b. Such frozen droplets sublimate faster in the lower regions. This indicates a higher surface temperature at the bottom region due to heat exchange by radiation. It appears that the influence of repelling phenomena are of minor importance. These observations agree very well with the findings of Elder *et al.* (1992): the real temperature of the specimen may deviate considerably from the temperature in the solid state object table OT indicated by a temperature sensor (e.g., TS' in Fig. 6a).

In some discussions the question was posed, if FD from amorphous frozen biological specimen is possible and advantageous (Linner *et al.*, 1986; Livesey *et al.*, 1989). According to Bachmann and Mayer (1987) devitrification of amorphous to cubic ice occurs at different temperatures between -140°C and -110°C depending on the sample and the measuring technique (heating rate). Most measurements of the devitrification temperature are made at rather high rates of temperature increase around 10°C/min. These rates are not comparable with common FD procedures at nearly constant temperatures. Cryosections of amorphous frozen specimens devitrify on a TEM cryostage to a cubic ice matrix at approx. -135°C (Dubochet and McDowall, 1981). Linner *et al.* (1986) and Livesey *et al.* (1989) claim, that FD of vitreous ice following their protocol of "Molecular Distillation" should be possible. In this

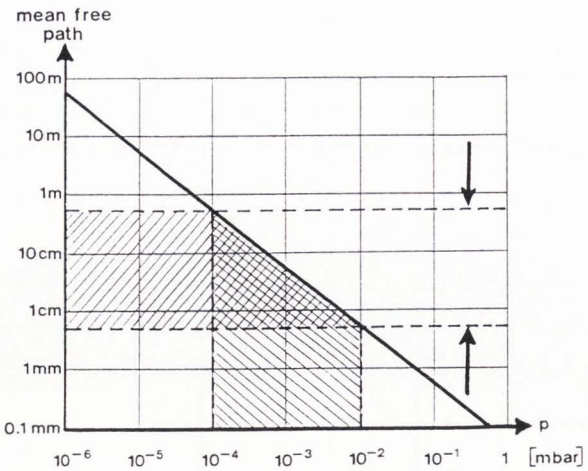


Fig. 13 : Mean free path of water molecules as a function of pressure. The logarithmic diagram shows that for a FD chamber in which the distance between frozen specimen and cold trap is between about 5 mm and 50 cm (arrows) a vacuum between 10^{-2} and 10^{-4} mbar is sufficient for FD because nearly all water molecules escaping from the specimen are trapped by the cold surface. FD at pressures below 10^{-4} mbar is not necessary (redrawn from Meryman, 1966).

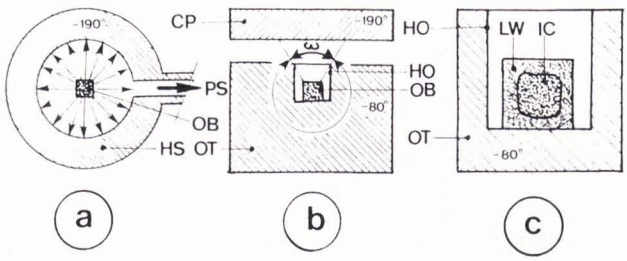


Fig. 14 : Schemata concerning theoretical considerations of FD (compare with text). - (a) Optimal (unrealistic) conditions of an object OB, heated to -80°C, surrounded by a cold trap HS. Vacuum by pumping system PS. - (b) Real conditions : OB in hole HO of heated object table OT. Cold trap plate CP above. - (c) Enlarged part of (b) showing a schematic cross section through a partially dried object with inner solid state ice core IC and outer already dried "shell" LW.

schedule the heating rate between -150 and -70°C is held constant at 1°C/h. That means, that the specimens following this protocol are heated for more than 30h from -135 to -100°C. Following the values given by Umrath (1983) FD is extremely slow within this temperature range. Furthermore the temperature range between -90 and -70°C (where FD accelerates considerably) is passed too fast (approximately 1 day) to yield almost dry specimens. After our own experience this period is considerably too short to freeze dry the specimens sufficiently and to avoid artificial shrinkage. This shrinkage probably occurs mainly during the last step of the drying protocol, which includes the rapid heating with a rate of 4.5°C/h. The most important question is, if any advantage of the "Molecular Distillation" protocol in comparison to our elongated schedule (-80°C for several days) is visible. Most of the pictures show "halo phenomena" (e.g. see Linner *et al.*, 1985, Figs. 5, 9 and 10; Linner *et al.*, 1986, Fig. 11; or Livesey *et al.*, 1989, Fig. 1 and discussion with the reviewer, p. 239 ff). Contrary to the statements of Livesey *et al.* we do not believe, that these "halo effects" represent "compression zones due to growing ice crystals in the environment of mitochondria" or "distortion and plastic flow phenomena during subsequent sectioning". "Halo effects" according to our own experience result regularly from insufficient FD (Edelmann 1986, 1994; see also Dudek *et al.*, 1982). "Halo effects" therefore are common artifacts of FD-procedure and document probably "thermal collapse phenomena" (Kistler and Kellenberger, 1977; Kellenberger, 1991; Kellenberger *et al.*, 1985) typical for shortened drying protocols not sufficient for proper stabilization of the ultrastructure. According to our own observations these artefacts can be completely and reproducibly avoided if longer FD periods in the temperature range around -80°C are used. Of course this elongated drying at a higher temperature excludes the preservation of the amorphous frozen state of the ice matrix in the frozen specimen. Nevertheless it leads to an evidently better structural preservation and excludes both "halo effects" and most of the artificial shrinkage.

Both theoretical considerations and practical experience lead to the conclusion, that only experiments may be really helpful to establish a proper drying protocol. Under these preconditions it is indeed almost impossible to transfer results from one specimen into preconditions for another specimen. Nevertheless the following rules are generally accepted and fully understandable: (1) The specimen should be well frozen and as small as possible. - (2) If bulk specimens are considered, thin strips with a thickness below 0.5 mm dry well, even if the total size is fairly large. - (3) Thin foils in thicknesses within the μm range or below (e.g. cryosections) also dry very well. - (4) Nevertheless in both cases (2) and (3) the drying times needed for optimal results are mostly underestimated. Drying artifacts mostly result from drying schedules too short for a nearly complete evapo-

ration of the frozen H_2O matrix. - (5) Incomplete drying in most cases results in an artificial shrinkage down to 30 % of the original volume. Artificial shrinkage probably is one feature of the "thermal collapse phenomenon" discussed by Kellenberger *et al.* (1991). The dimension of these shrinkage phenomena is often different for different structural components : inclusions with a higher tendency for thermal collapses probably lead to "halo phenomena" in their immediate environment. As mentioned above secretory granules and mitochondria are often surrounded by empty spaces ("halo phenomena"), if the drying protocol was too short.

Cellular structures with an "internal standard" offer a very simple way to study this artificial shrinkage. E.g., the periodic pattern in striated muscle can serve as a tool to study this artifacts. As shown in Fig. 9 it is possible to reduce artificial shrinkage by elongation of a total FD period to 7 or even 10 days. The protocols employed for Figs. 9 and 10 show, that elongated drying times result in very small and often negligible shrinkage of frozen bulk specimens or cryosections. After elongated drying periods the application of OsO_4-vapor is not necessary, and both infiltration in monomeric resin and staining with uranyl acetate and lead citrate pose no severe problems (see Fig. 9-11; for further documentation see Edelmann, 1994).

Vacuum resin impregnation after FD is difficult as far as acrylic resins for LTE are concerned. Monomeric mixtures of these resins contain components of rather high vapor pressure, which evaporate under vacuum and are trapped right away by the cold surfaces inside the FD chamber described above. Also at ambient pressure under GN_2 a considerable part of such acrylic monomers condensates on the cold surfaces. Care has therefore to be taken to separate this liquid phases completely from all cold surfaces. This is accomplished by closing the embedding molds completely during UV-polymerisation. Under these conditions good results with LTE under inert dry GN_2 are obtainable (see Fig. 11). In contrast to the earlier cryosorption system of Edelmann (1978, 1979) the new FD chamber can be used for several runs of long FD periods. This is due to the possibility to close the container CC by the valve V1, if the chamber is opened (see Fig. 6). An additional advantage is given by the use of a membrane pump (Vacuubrand, type MZ2/1.9) to evacuate the chamber to 10 mbar before the valve V1 is opened again. We routinely follow this schedule. If necessary, the molecular sieve MS is regenerated by heating (100°C overnight) on a laboratory hot plate with thermostatic temperature control. With respect to the bottom profile (see elements ES/MR in Fig. 6 a) the FD chamber is positioned on an aluminum intermediate ring IR (Fig. 6b) as support between the bottom of the chamber and the surface of the hot plate.

One of the main practical problems of FS and LTE in the described FS system was a proper and efficient handling of the frozen specimens at low temperatures. Our experimental base was a system for automatic FS developed approx. 10 years ago. This system was produced and distributed by Reichert-Jung Vienna since 1985 under the commercial name "CS-auto" (see Sitte, 1984; Sitte *et al.*, 1986, 1987b). Working on the same principle (substitution container in the neck of a dewar vessel), the system was not purpose designed but later on adapted for LTE. The described new FS apparatus is based on years of experience with the CS-auto system. The main differences between the new system and the former CS-auto are a much larger volume of the Dewar vessel (35 l instead of 8 l), a larger neck diameter (110 mm instead of 80 mm) and a much simpler set up (e.g., omission of any casing, replacing the motor driven stopper by a simple hand operated cover). The motor driven stirrer of the former CS-auto could be omitted, since it did not convect effectively the monomeric mixtures for LTE. The described simple manual convection of the media guarantees better results for media with different viscosities. Additionally the automatic temperature/time control was completely redesigned. (*Note*: According to our proposals the new microprocessor controlled electronic unit was developed by Leica AG, A-1171 Vienna (formerly Reichert-Jung Optische Werke AG). Both the FS and the FD system described in this paper will be manufactured and distributed as AFS (meaning "Automatic Freeze Substitution") and CFD (meaning "Cryosorption Freeze Dryer") by Leica AG, A-1171 Vienna. Contrary to the former CS-auto, the new AFS is equipped with an integrated exhaustor and a completely new set of tools. It is experience from the former model, that different studies need a completely different equipment. The FS/LTE processing of different single specimens, following different protocols is considerably simpler and less time consuming with Eppendorf microtubes according to Humbel and Müller (1985). On the other hand the processing of a couple of similar specimens (e.g. several blocks of the same tissue) following the same protocol is much simpler in grid containers with mesh bottom in combination with plastic moulds for UV-light flat embedding similar to the former CS-auto equipment. Finally, one of us (L.E.) developed a completely new system for the processing of different frozen specimens following an identical protocol.

The described flow through capsules FT (see Fig. 5) in combination with different containers and a universal manipulator ("spider holder" SH/ST) simplify the

processing considerably: 8 specimens can be processed simultaneously from substitution to polymerisation without touching the specimens. All different possibilities are safe, since any skin contact with allergens and inhaling of toxic vapors can be completely excluded. Nevertheless an efficient work is possible in the cold area without a special skill. The mechanical possibilities are supported by an efficient electronic system which executes complicated temperature/time schedules both upwards (FS -> LTE) and downwards (PLT -> LTE, according to Carlemalm *et al.*, 1985).

Experimental work has shown, that also FS without chemical fixatives can be improved, if elongated schedules at low temperatures are used (see Fig. 8 and Edelmann, 1991). It seems, that the commonly used short protocols are not suited to dehydrate the specimens at low temperatures to such an extent that they are stabilized without chemical fixation. Of course short FS with additives like OsO_4 (Van Harreveld and Crowell, 1964), uranyl acetate or aldehydes (Humbel, 1984; Humbel and Müller, 1985) may be carried out for purely morphologic or morphometric studies. But if the conformation of sensitive macromolecules for subsequent histochemistry (e.g., immunoreactions) has to be preserved, these additives should be avoided. The same applies to specimens prepared for elemental analysis. As demonstrated, it is possible to omit additives without loss of structural preservation by elongation of the protocol. The new system both for FS and FD offers this possibility, since the larger Dewar vessel allows long-term procedures of 7 to 10 days without refilling of LN_2.

Acknowledgements

Our colleague Klaus Neumann contributed considerably to the design of the laboratory prototypes for FS and FD. Many important details and breakthroughs result from his cooperation. The instrumental design was partially supported by Leica Aktiengesellschaft, A-1171 Vienna (formerly Reichert-Jung Optische Werke AG) and by the Forschungsförderungsfonds der Gewerblichen Wirtschaft Österreichs, A-1015 Vienna, Austria. Reinhard Lihl, Gerhard Kappl and Hubert Goll from the Research and Development Department of Leica Vienna contributed considerably to the design of the electronic control and the commercial instruments AFS and CFD. Christa Rosinus always perfectly assisted in the laboratory and was engaged together with Barbara Reiland in the preparation of the manuscript. We sincerely thank them all.

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Discussion with Reviewers

T. von Zglinicki: A couple of years ago I suggested differential freeze point depression and subsequent osmotic water flow between compartments during relatively slow freezing as one possible reason for the "halo effect" (Von Zglinicki (1988) Intracellular water distribution and aging as examined by X-ray microanalysis. Scanning Microsc. **2:** 1791 - 1804). This explanation was prompted by the observation that in one and the same section haloes were absent in the well-frozen area close to the specimen surface but could be found deeper within the specimen together with larger ice crystals. Which mechanism would you suggest for halo formation during fast freeze-drying? Could it also account for the case that the specimen was never warmed up to above say, -60°C?

K. Zierold: Shrinkage of structures by dehydration is supposed to be caused by removal of "bound water" which is necessary to maintain macromolecular structures. Theoretical data on freeze-drying, e.g. Fig.12 are based on sublimation of bulk water usually existing as hexagonal ice. Do you know from your experiments with striated muscle (e.g. by measurements of the length of the A-band after different preparation protocols) the critical temperature of the beginning collapse of macromolecular structures?

Authors: Halo formation during fast freeze-drying is a differential shrinkage phenomenon. E.g. mitochondria shrink to a higher degree than the surrounding cytoplasm. One conceivable mechanism of the generally observed severe shrinkage during fast FD has been described by Edelmann (1994, Discussion with Reviewers): "It appears that shrinkage values exceeding 5 % linear shrinkage are only obtained if a specimen is warmed up (even during continuous freeze-drying) above a critical temperature before it is sufficiently dehydrated. In this case the water molecules which are more or less immobilized at the low temperature become more and more mobile with increasing temperature (whether there is a definite melting point for the remaining water or whether this water is unfrozen remains to be determined). As a result the attractive forces between macromolecules or between intramolecular sites are no longer balanced by the immobilized intermediate water molecules, the macromolecules aggregate at many areas almost simultaneously and the whole system shrinks. Only if the water is removed slowly at rather low temperature certain few sites are dehydrated first which

may combine with sites of opposite polarity thereby stabilizing the system. Further slow dehydration causes step by step more cross links and the tremendous shrinkage can be avoided. This mechanism is similar to that proposed by Kellenberger *et al.* (1986, p. 11) for FS at low temperatures." In the case of differential shrinkage after FD one may now assume that different areas are dehydrated to different degrees after low temperature FD. During a subsequent (fast) temperature increase (with or without continuous FD) the compartments containing more water will then shrink more than those dehydrated already to a higher degree. It was this consideration which led to the conclusion that prolonged (e.g. 5d) FD at -50°C (after initial FD for 3d at -80°C and 6d at -60°C) should dehydrate further those compartments still rich in water (mitochondria) to such an extent that differential shrinkage and hence halo formation is prevented. The results obtained with freeze-dried mouse kidney (Edelmann, 1986) confirmed this expectation.

Whether halo formation during FD of well cryofixed specimens may occur at or below -60°C is unknown to us. Another unanswered question is whether differential shrinkage may occur during warming up of fully hydrated well cryofixed specimens to -60°C or higher temperatures (e.g. caused by fast growing hexagonal ice crystals outside mitochondria).

The critical temperature of the beginning collapse of macromolecular structures has not been determined but may be investigated systematically by freeze-drying cryosections within the described CFD. Noteworthy is the finding that the muscle shown in Fig. 11 has been shrunken considerably (A band width about 1.2 μm instead of 1.5 μm) although - according to Fig 12 - this muscle should have been dehydrated to a higher degree (FD: 2d -80°C, ld -60°C, 2d -50°C) than that shown in Fig. 9 (FD: 3d -80°C, 6d -60°C). One may conclude that severe shrinkage may start already below -50°C or may be caused by events taking place below -50°C. The fact however, that severe shrinkage can be prevented (see Figs. 9 and 10 a) by specific long FD at low temperature shows that there is no critical subzero temperature range in which collapse of macromolecular structures inevitably must occur.

K. Zierold: I agree with your skeptical estimation of the "molecular distillation" method reported in the literature. However, your theoretical arguments are not convincing: Freeze-drying times (e.g. those presented in diagram 1) are based on sublimation of water from crystalline ice. Vitrified water could have a very low sublimation energy allowing freeze-drying in reasonable times at a temperature below 150 K. Please comment!

Authors: At present there is no experimental evidence for the view that e.g. cryosections of biological material containing only vitrified water can be freeze-dried faster at low temperature than cryosections with hexagonal ice. On the contrary, there is the possibility (discussed by Edelmann, 1994) that a relatively strong protein-water interaction is preserved in vitrified biological material which could make it even more difficult to evaporate this vitrified water than to sublime hexagonal ice from frozen specimens. Systematic studies of the freeze-drying of cryosections containing different modifications of water or ice should be carried out to tackle the discussed problem.

K. Zierold: How do your results showing freeze-substituted and freeze-dried striated muscle compare with frozen-hydrated cryosections?
Authors: This is an important question because artefacts introduced by dehydration and embedding techniques can only be identified by comparison of the images obtained after FS or FD with the visualized frozen-hydrated control. The feasibility of comparative studies has been demonstrated by Edelmann (1994, Fig. 4). However, the preparation of "beautiful" frozen-hydrated cryosections of untreated muscle (not cryoprotected) still poses severe difficulties (Edelmann, 1994, Fig. 4 and Discussion with Reviewers).

G.M. Roomans: In order to support the points that the authors are making about shrinkage I propose to include quantitative data. Do you have quantitative values of the sarcomere length or A band width obtained with the different preparative methods?
Authors: At present it appears not to be very useful to give quantitative values of the sarcomeres or A bands from the micrographs taken so far. For instance, Figs. 10 a and b, obtained from the same cryofixed muscle, are not suited for a quantitative comparison because these micrographs have been taken after two single FD experiments under uncontrolled conditions (see legend of Fig. 10b). Fig. 10c is obtained from one FD experiment. Nevertheless these figures show - without giving numbers - that the generally observed linear shrinkage of freeze-dried cryosections of about 20 % can be produced and that longer FD at low temperature does reduce the shrinkage artefact considerably (to about 5%). Also, muscle pictures similar to that shown in Fig. 11 are only available from one FD and embedding experiment carried out according to the given procedure. Nevertheless this figure shows 1) the feasibility of FD and subsequent LTE and 2) a reduced A band width when compared with Fig. 9 which was obtained following a different procedure (longer FD at low tempera-

ture).

Quantitative studies should of course be made. In a meaningful quantitative study one should compare values obtained from frozen-hydrated cryosections (see Edelmann, 1994, Fig. 4) with values from the same cryosections after different FD procedures, and with values from the same muscle after different FS or FD and embedding procedures. Most important for quantitative studies are frozen-hydrated controls because neither the sarcomere length of different cryofixed muscles is constant nor - contrary to general belief - the A band width (see e.g., Periasmy A, Burns DH, Holdren DN, Pollack GH, Trombitas K, 1990. A-band shortening in single fibers of frog skeletal muscle. Biophys J **57**: 815-828). Although the A band is rather constant in a resting muscle it may shorten considerably in a damaged or activated (contracting) muscle fiber.

T. von Zglinicki: How was muscle contraction directly before freeze immobilization excluded?
Authors: Cryofixation of isolated frog sartorius muscles has been carried out as described by Edelmann (1989). Briefly, a muscle is mechanically fixed in a slightly stretched position on a frame constantly bathed in Ringer's solution. Care is taken to avoid any damage or stimulation of muscle fibers during preparation which is carried out under the control of a stereo light microscope equipped with fiber optic illumination. Then the frame with the attached muscle is transferred to a specimen support, the liquid adhering to the surface of the muscle is removed by means of a wet filter paper, and immediately afterwards the muscle is frozen by a falling Cu-block cooled to LN_2 temperature. For more details see Edelmann (1989). Despite these precautions it cannot be guaranteed that all fibers of a frozen sartorius muscle are captured in a perfect resting state.

T. von Zglinicki: Could you indicate in Figs. 8 to 11 the depth within the specimen.
Authors: The muscles shown in Figs. 8 to 11 have not been cut perpendicular to the contact plane of cryofixation. Furthermore, the depth of the surface coat of the different muscles (mainly connective tissue) has not been determined. Hence, it is impossible to give the precise depth within the specimens from which the figures have been taken. Earlier experiments have shown however that the muscle tissue is well preserved after cryofixation until a distance of about 7 μm from the contact plane of cryofixation (see Sitte *et al.*, 1987 a, Fig. 5 a). It is assumed that the shown figures have been taken from a well cryofixed area.