

Controlled Environment Vitrification System: An Improved Sample Preparation Technique

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ABSTRACT The controlled environment vitrification system (CEVS) permits cryofixation of hydrated biological and colloidal dispersions and aggregates from a temperature- and saturation-controlled environment. Otherwise, specimens prepared in an uncontrolled laboratory atmosphere are subject to evaporation and heat transfer, which may introduce artifacts caused by concentration, pH, ionic strength, and temperature changes. Moreover, it is difficult to fix and examine the microstructure of systems at temperatures other than ambient (e.g., biological systems at in vivo conditions and colloidal systems above room temperature). A system has been developed that ensures that a liquid or partially liquid specimen is maintained in its original state while it is being prepared before vitrification and, once prepared, is vitrified with little alteration of its microstructure. A controlled environment is provided within a chamber where temperature and chemical activity of volatile components can be controlled while the specimen is being prepared. The specimen grid is mounted on a plunger, and a synchronous shutter is opened almost simultaneously with the release of the plunger, so that the specimen is propelled abruptly through the shutter opening into a cryogenic bath. We describe the system and its use and illustrate the value of the technique with TEM micrographs of surfactant microstructures in which specimen preparation artifacts were avoided. We also discuss applications to other instruments like SEM, to other techniques like freeze-fracture, and to novel "on the grid" experiments that make it possible to freeze successive instants of dynamic processes such as membrane fusion, chemical reactions, and phase transitions.

INTRODUCTION

Fluid, labile systems like colloidal dispersions, microstructured liquids, and living cells are a formidable challenge to the electron microscopist. Volatile constituents evaporate into the vacuum that surrounds the microscope stage, altering the composition of the specimen and often inducing phase change with attendant artifacts. Low viscosity specimens can deform and flow under the subtle forces that develop during specimen preparation and imaging. Chemical reactions induced by the electron beam volatilize parts of a specimen or partially polymerize and cross-link other parts, causing stresses

that lead to grossly altered microstructure. Moreover, contrast between different parts of the microstructure of aqueous and organic systems is often low. Ever since the pioneering of transmission electron microscopy (TEM) by Knoll and Ruska (1931), fixation techniques have been used to reduce vapor pressure and to immobilize fluid, labile specimens; to reduce susceptibility to radiation damage by the electron beam; and, not in-

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frequently, in conjunction with staining, to heighten contrast.

Any fixation technique may produce artifacts, thereby altering the morphology (size, shape, connectivity, and topography), texture, composition, or state of parts of the specimen. The artifact potential of negative staining and other chemical fixation techniques is thoroughly documented (Talmon, 1983), but they nevertheless do find applications, particularly to specimens containing nonlabile types of microstructures. Thermal fixation by rapid cooling to the solid state has emerged as the technique of choice, especially for the more labile types of specimens (Talmon et al., 1979; Talmon, 1987). The speed of local quenching is controlled by the temperature of the cryogen employed, the nature of the cryogen's contact with the specimen, the penetration rate of the cold thermal wave into the specimen, and the thickness or diameter of the specimen (Bald 1984, 1985). The ultimate in thermal fixation, or cryofixation, is *vitrification*: cooling so fast that even the most fluid parts of the specimen are converted into glassy states, so viscous as to be solid, devoid of detectable crystallization, and so low in vapor pressure as not to evaporate or sublimate appreciably. Because vitrification is necessarily very rapid, it can avoid the artifacts of flow and thermally driven rearrangement. Not only does vitrification prevent phase separation and the artifacts brought on by crystal growth, it also avoids the imaging problems associated with a crystalline matrix, e.g., bend contours, thickness fringes, moiré fringes, and contrast reversal. However, water, dilute aqueous solutions, and other systems with low viscosity parts are particularly difficult to vitrify.

Adrian et al. (1984) first demonstrated a practical method of vitrifying thin specimens of water and of aqueous dispersions for transmission electron microscopy. They achieved the needed cooling rate by plunging thin films of the fluid specimen into liquid ethane at its melting point. The specimen films were formed across the holes of a bare electron microscope grid or, preferably, in the holes of a "holey film" (used for astigmatism correction and to support thin sections as described by Sjöstrand [1956, 1957]) supported on a grid. In either case the liquid film was initially held in the hole by surface tension in conjunction with partial wetting of the grid or holey film. Two

factors heightened the cooling rate. First was the choice of ethane as cryogen, for its thermal contact with the specimen is superior to that of lower boiling point liquids like nitrogen;¹ second was the avoidance of even the thinnest of protective films over the surfaces of the specimen films, for it turns out that vitrification proceeds faster than any detectable deformation of the specimen by the shear it suffers as it is plunged into the ethane.

Details of cryofixation methods are given by Mayer (1985), Bald (1985), and Newbury et al. (1986). Various plunge-cooling schemes and precautions to ensure high cooling rates are discussed by Elder et al. (1982) and Costello et al. (1982). Robards and Severs (1981) compared cooling rates achieved in the propane jet device with those in plunge-cooling; they concluded that plunge-cooling gave higher and more reproducible cooling rates. The thin-film vitrification method pioneered by Dubochet and his colleagues (Adrian et al., 1984) has now been applied by several researchers to a large variety of specimens, including membrane proteins by Lepault (1983); phages and catalase by Lepault et al. (1983); ribosomes, bladder membranes, and gap junctions by Milligan et al. (1984); phospholipid vesicles and virus particles by Dubochet et al. (1985); helical particles (TMV and T4 polyheads) by Lepault (1985); natural and synthetic liposomes and vesicles by Talmon (1986); and surfactant micelles and microstructures by Bellare et al. (1986b).

THE NEED FOR ENVIRONMENTAL CONTROL IN SAMPLE PREPARATION

What microstructure is vitrified depends on how a sample is handled between its formation, or withdrawal from the larger or bulk system of interest, and its quenching by plunge-cooling or jet-freezing. This handling is *sample preparation* and warrants thorough consideration and careful control, as illustrated by the following example of a procedure, now routinely used, for producing vitrified specimens from droplets of microstructured, multicomponent liquids.

An aqueous droplet of ~5 μl , held in a pipette, is placed on a carbon-coated, cellulose acetobutyrate film full of holes around

¹The normal boiling point (T_b) and melting point (T_m) of ethane are 184.4K and 89.7K ($T_b - T_m = 94.7\text{K}$), respectively; those of nitrogen are 77.3K and 63.1K ($T_b - T_m = 14.2\text{K}$).

5 μm in diameter (Fukami and Adachi, 1965) and supported on a grid. The drop is spread over the film by moving the pipette tip over all parts of the grid during placement. Most of the liquid is removed by careful blotting from the face of the grid with absorbent filter paper until all that remains is a film thin enough to show interference colors; at this stage the holes in at least some regions of the film contain biconcave films of the liquid that are thin enough, at least in their central areas, for subsequent imaging. The sample is then plunged into liquid ethane, transferred to liquid nitrogen, mounted on a cold-stage, and transferred to a transmission electron microscope for examination. Such specimens are called bare- or holey-film specimens because the sample, suspended within the holes of the grid or its support film, is not confined between protective films over their surfaces.

The problem is that the microstructure vitrified by plunge-cooling may not be the microstructure of the liquid in the original droplet. Spreading and blotting may concentrate particulates and colloidal microstructures nonuniformly in the liquid that fills the holes. Temperature variations in the surrounding air are transmitted to the liquid. Hygroscopic liquid absorbs humidity from the surrounding air; evaporation of more volatile components concentrates the less volatile ones, including protons (raising pH) and other ionic species (altering electrolyte balance and ionic strength), in the remaining material. Evaporation also pre-cools the sample, as may premature exposure to cold vapors of the liquid ethane or of the liquid nitrogen (used to cool the ethane). Temperature and concentration gradients that affect surface tension may drive microconvective flows that partially segregate components in the liquid that remains after blotting; differential evaporation may further segregate components in the liquid films within the holes. Temperature and concentration changes in turn change osmotic pressure and alter microstructure, only mildly if they induce no phase change, but seriously if they do.

The susceptibility of a sample to phase-change artifacts escalates the closer it or any part of it is to saturation, i.e., the nearer the state is to a phase boundary in the thermodynamic phase space of temperature and composition at ambient pressure. If, because

of unwanted phenomena, the phase boundary is crossed, the sample becomes supersaturated, and, unless the rate of phase change is too slow, previously absent structures such as crystals of salt or liposomes of liquid crystal appear. With colloidal dispersions, a stability boundary may be crossed so that particles agglomerate or an emulsion inverts. Without adequate knowledge of the thermodynamic space around the state of the sample it is impossible to forecast what dangers lurk. Consequently a method that uses evaporation to form thin films of material for cryofixation (e.g., that of Jaffe and Glaeser [1984]) can be unreliable.

When samples have to be prepared at a temperature different from ambient, e.g., biological systems at *in vivo* conditions and surfactant systems at elevated temperatures, the dangers of microstructural change during handling are still greater. Special precautions are required in dealing with samples that are toxic or infectious, as discussed recently by Jeng et al. (1988).

Thus, there has been a need for appreciation of the realities of sample preparation and for means of avoiding the various artifacts that can arise. We describe in this article a system for isolating and controlling the environment—temperature and partial pressures of volatile components—of fluid, labile samples during their preparation for thermal fixation by plunge-cooling. Earlier versions of the system were reported by Bellare (1986) and Bellare et al. (1986a). More recently, Murray and Ward (1987) described a saturated-environment fast-freezing system. The system described here permits control of both saturation and temperature and therefore allows a wider class of samples to be prepared. Our description is followed by some examples of its use in cold-stage transmission electron microscopy and a discussion of its potential for further applications including conventional freeze-fracture-replication methods as well as freeze-fracture techniques with cold-stage scanning electron microscopy. In the final section of the article, we comment on a novel class of applications that the CEVS facilitates: physical and chemical modification of samples.

THE CONTROLLED ENVIRONMENT VITRIFICATION SYSTEM (CEVS)

There are four subsystems, shown in Figure 1: first, the environmental chamber

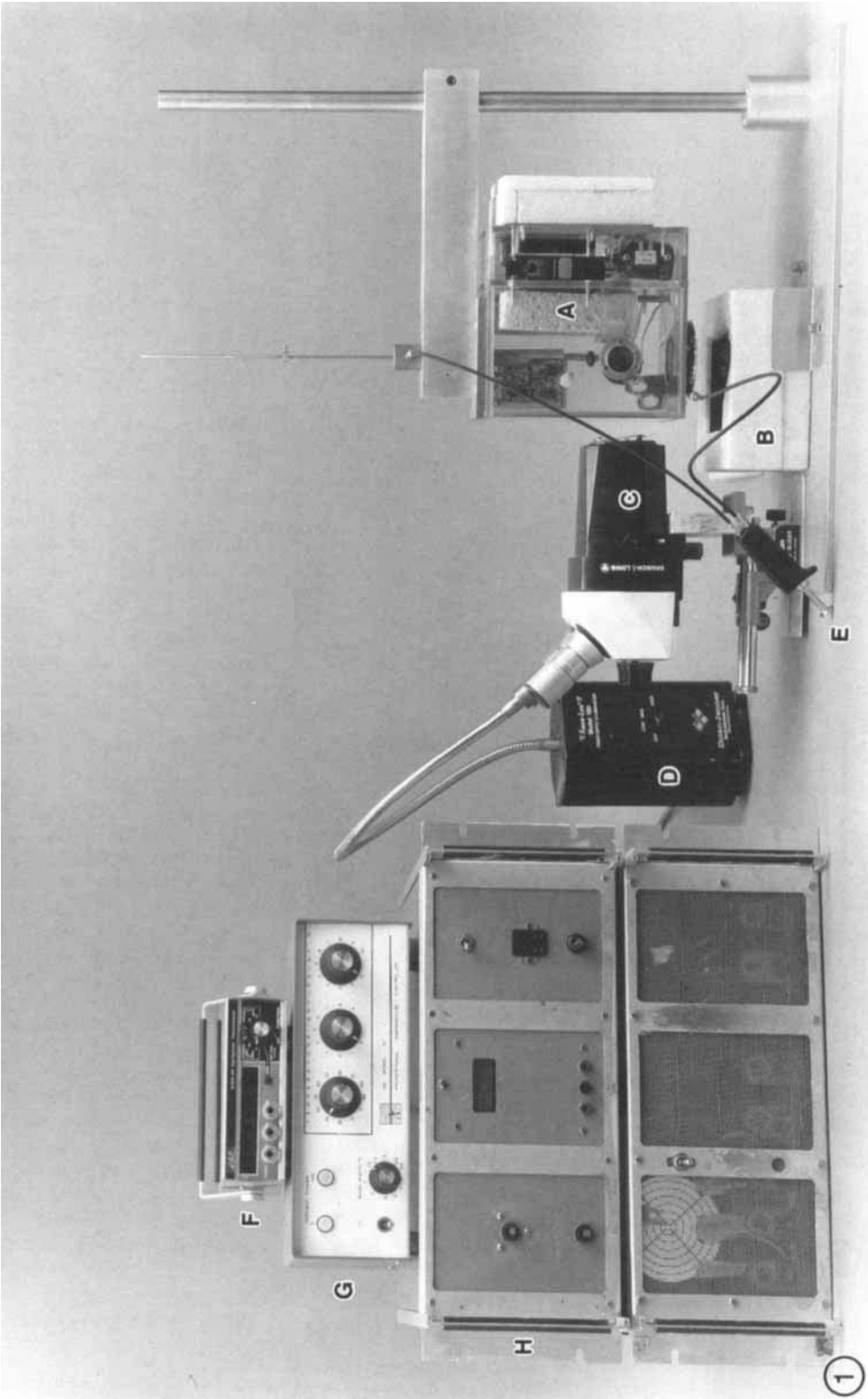


Fig. 1. The controlled environment vitrification system (CEVS): environmental chamber (A), cryogen reservoir (B), stereomicroscope (C), illuminator (D), double-cable release (E), instrument cluster with digital thermometer (F), temperature controller (G), and humidity meter (H).

within which samples are prepared or modified and from which they are propelled into a cryogen; second, a cluster of instruments for measuring and controlling temperature and humidity in the chamber; third, the cryogen reservoir into which the samples are plunged; and fourth, the assembly by which they are transferred from the reservoir to a storage dewar without suffering temperature rise or frost deposition.

The environmental chamber (Figs. 2-4) encloses a 3-mm-diameter² stainless steel rod that slides vertically in a custom-built phenolic bearing and at its lower end bears the sample. This assembly is the heart of the system. The sample support usually is a TEM grid, bare or carrying a holey film, held by a tweezer (e.g., type 3c, Dumont, Zürich, Switzerland) with a sliding O-ring for clamping it or held by self-locking tweezers. (Other sample supports such as standard scanning electron microscopic [SEM] stubs or freeze-fracture plates can be used if desired; their use is discussed below.) The tweezers have a magnetic strip attached in such a way as to mate with another magnetic strip at the bottom of a recessed block in the lower end of the rod. The recess is precisely machined to receive the tweezers so that the latter, and with it the grid, can be mounted repeatedly in exactly the same position. This allows viewing of the sample with a long working distance stereomicroscope (e.g., Stereozoom 7, Bausch & Lomb, Rochester, NY) mounted outside the chamber; the sample and support can be illuminated at near-normal incidence by directing a fiber-optic light source down one ocular, while the other is used for monocular viewing. This viewing system is useful when working with bar-grid specimens; with holey-film specimens the holes are generally too small to resolve with a light microscope.

The shaft bears a collar that compresses a spring mounted within the phenolic bearing. The spring thereby exerts downward force on the shaft. The shaft can be locked in a cocked position with a slotted block that is forced, with a second spring, into a groove in the shaft. The purpose of this feature is explained below.

The chamber itself is made of polycarbonate 0.6 cm thick and is 10 cm wide, 10 cm

deep, and 20 cm high. Its walls are assembled into two parts, each in a C form: The top, bottom, and front walls form one C; the two side and back walls form the other C. The two assemblies slide together in grooves machined in the faces. Thus the chamber can be easily opened to gain access to its interior and then sealed shut again. It is suspended by a frame so that the bottom wall is 12 cm above a base plate.

The bottom wall has a spring clip to hold a stoppered 1.2-cm-diameter specimen vial so that the sample can equilibrate with the temperature of the gas in the chamber before sample preparation is started. On the back wall are mounted small reservoirs for two or more liquids (water and organics); extending upward from each reservoir is a porous polyurethane sponge so supported as to saturate the gas within the chamber. The top wall has a port with a nozzle (Fig. 3Q) through which inert gas can be fed, and, if desired, a partial vacuum can be drawn to operate safely with a toxic or infectious sample. Stainless steel fins protruding inward from the back wall of the chamber make contact through that wall with an insulated reservoir mounted on the outside of the wall (Fig. 4). That reservoir can be charged with refrigerant (e.g., ice/water, dry ice/acetone, or liquid nitrogen), or, alternatively a refrigerant can be circulated through it to remove heat from the gas within the chamber. Heat is added as needed with a compact source, a 600-W halogen-quartz lamp (ANSI code DYS, Sylvania, Danvers, MA), across which air is continuously circulated by a low-profile, brushless fan (6-W and 2,000 rpm, Model 99XW, Etri, Monroe, NC). Stainless steel baffles cover the chamber corners to streamline the air flow. The tweezer, when mounted within the chamber, is situated in the air-stream such that air does not directly blow onto the face of the grid held at its tip. The air flows along the length of the tweezer, preventing any holes from being blown in the specimen film.

A pair of thermistors is mounted in the flowing gas 1 cm below the sample support; one is connected to a digital thermometer (Model 8502-25, Cole Parmer, Chicago, IL), the other to a proportional controller (Model 72, YSI, Yellow Springs, OH) that regulates the current flow to the lamp. A desired temperature set-point can be selected by dials on the controller front panel. Perfor-

²Dimensions in metric units are nominal. The apparatus was built in a workshop that used stock based on the English measurement system.

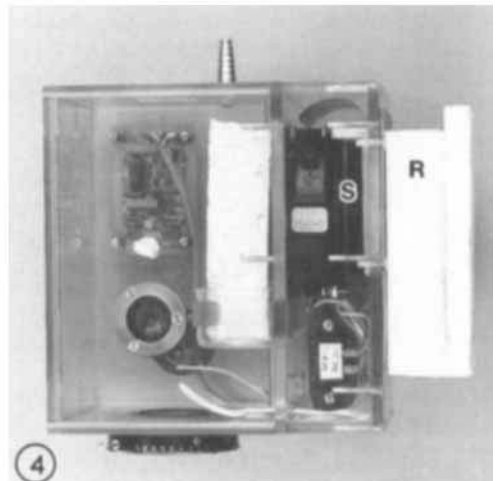
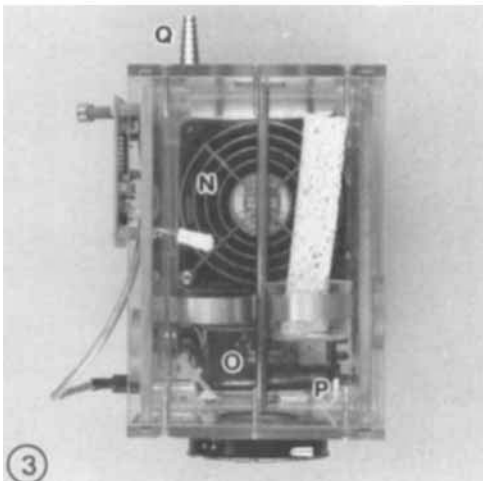
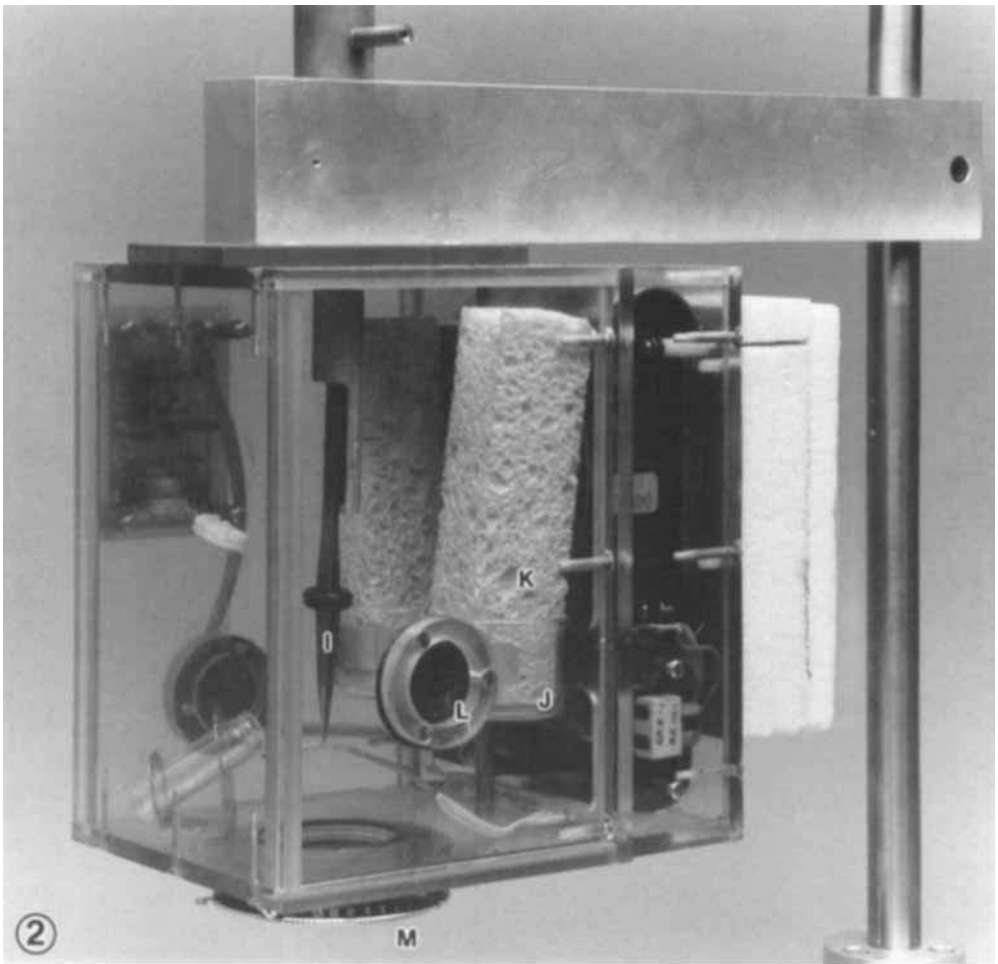


Fig. 2. The environmental chamber (with insulation, used for operation from 80 to 90°C, removed for clarity) with tweezers (I) on the plunger shaft, saturating reservoirs (J) with sponges (K), port with split rubber septum (L), and shutter (M).

Fig. 3. Front view of the environmental chamber

(with one reservoir removed for clarity) showing the fan (N), heater lamp (O), thermistors (P), humidity sensor, and gas inlet nozzle (Q).

Fig. 4. Side view of the environmental chamber showing the cooling reservoir (R) and cooling fins (S).

mance tests established that the chamber gas temperature can be maintained constant to within 0.1°C at any value between -5°C and 90°C . One may use, of course, different sensors and controllers to achieve comparable levels of temperature control.

Additional thermistors can be placed in the saturating wicks and connected to the digital thermometer to provide wet-bulb readings from which partial pressures and thus chemical potentials of volatile components can be evaluated (provided the requisite psychrometric data are available). A capacitance sensor (Phillips Sensor, Mark Worley, Round Rock, TX), mounted on the left side wall, is more conveniently used to measure water vapor concentration (relative humidity) when the chamber is filled with an air/water-vapor mixture, between 5°C and 70°C . The capacitance sensor is connected to a digital voltmeter.

There is a port in the left wall and another in the right wall, each covered with a split rubber septum through which forceps, pipette, and filter medium can be introduced as needed. The forceps are used to remove the sample-vial stopper. The pipette is used to withdraw a droplet of sample from the vial and place it on the grid, all within the chamber. The filter medium used for blotting is wrapped around a stainless steel strip $0.5 \times 10 \times 100$ mm. There is also a port in the bottom wall of the chamber. This opening is closed with a precision, iris-type, self-cocking, camera lens shutter with a 0.22-cm to 3.0-cm variable aperture (Prontor Press 1-12, Prontor-Werk, Wildbad, FRG). The lens shutter is set to "T" (for "time") so that the shutter remains open after firing, i.e., depressing the shutter release. The open shutter can be closed by partially depressing the release.

The instrument cluster (see Fig. 1) contains the digital thermometer, heater controller, power switch for the fan, and the humidity meter. All are connected to their counterparts in the chamber by a multiconductor shielded cable.

The cryogen reservoir (Fig. 5) is a 9-cm-wide, 9-cm-long, 4.5-cm-deep, and 0.8-mm-thick brass, five-sided open chamber, externally insulated on all five sides with 2.5 cm thick styrofoam and provided with a styrofoam lid, 14 cm square and 2.5 cm thick, to cover the sixth, open side. A 2-cm-diameter, 3.2-cm-deep brass cup, in which ethane is liquefied by the cooling action of liquid

nitrogen placed in the surrounding reservoir, is fixed inside the reservoir with a screw. A horizontal brass platform, 3 cm wide, 6 cm long, and 0.6 cm high, is attached on the cup. It has a 1.5-cm-diameter and 0.45-cm-deep blind hole with a 0.3-cm-diameter dowel pin fixed at the circumference of the hole and protruding into it. This hole is the receptacle for the specimen transfer box, which has a keyway that mates with the pin. The function of the keyway and pin is described below. The box can thus be held submerged in the cryogen. The reservoir, and therefore the cup, can be reproducibly positioned 2 cm directly below the shutter in the environmental chamber by sliding the reservoir on the base-plate against two L-shaped locator plates. These are screwed at right angles to each other in the base plate and serve as stops.

The main item of the transfer assembly is the four-slot specimen transfer box (Fig. 5U). This is made from a standard grid storage box and patterned after a similar item designed by Gatan (Model 626-0314, Gatan, Pleaston, CA) for their coldstage/work station. After each sample has been plunge-cooled, the grid that supports it can be placed in one of the slots for later transfer to a 5-liter dewar of liquid nitrogen and storage there. Before it is transferred, the box is closed by a cover (Fig. 5V) that has a screw that mates with a threaded hole in the box. The keyway-pin combination (described in the previous paragraph) prevents the box from rotating while the cover is screwed into it. The cover has a carrying handle to facilitate transfer to the storage dewar, and subsequent transfer to a cryo-transfer system for loading the grid in a cold-stage. The specimen transfer box fits the Gatan Model 626 cryotransfer Work Station.

When a specimen is ready for plunge-cooling, a photographic double-cable release (Model AR-7, Nikon, Garden City, NY) is activated by abruptly depressing its shaft and then unactivated by releasing it. One leg of the cable fires the shutter, and the other leg pushes a pin that moves the slotted block that holds the shaft cocked, allowing it to accelerate downward. The shutter is arranged, by adjusting the length of the pin, to open about 50 msec before the tweezers-held grid at the end of the downward-propelled shaft arrives at the shutter on its way to the cup in the cryogen reservoir.

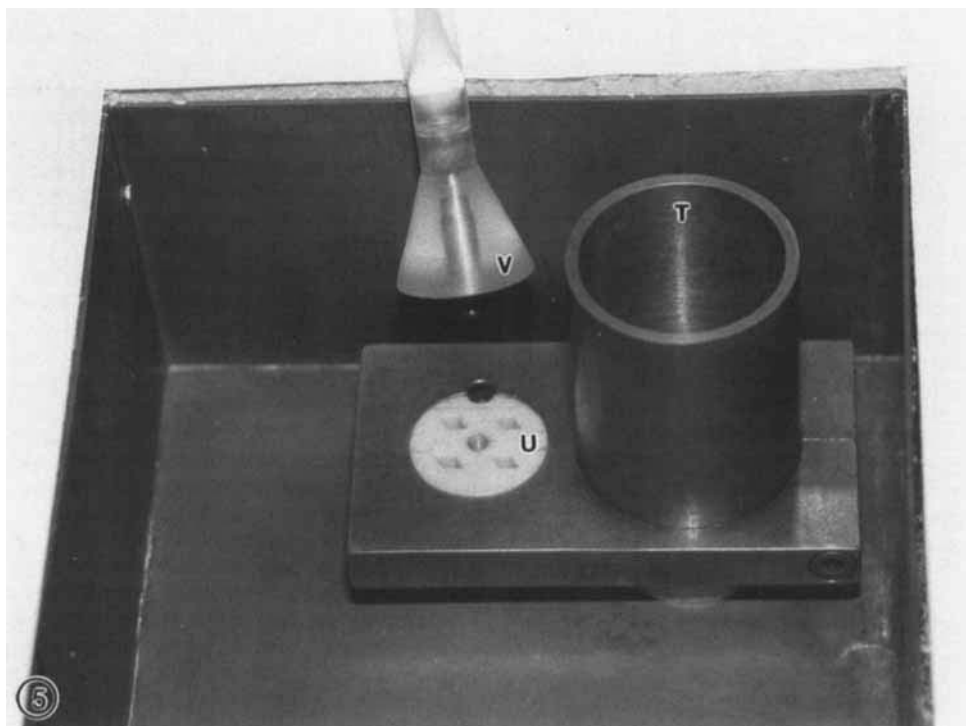


Fig. 5. The cryogen reservoir with vitrifying medium cup (T), specimen transfer box (U), and specimen transfer cover (V).

Thus, the chamber is kept gas-tight and insulated until the sample is plunged. The sample is protected from intervening air and cold vapor boiling off the cryogen except during the last 2 msec or so before it enters the cold liquid.

The speed of the sample at entry is about 2 m/sec. The distance the sample travels into the cryogen, i.e., the plunge depth, can be adjusted from 0 to 30 mm by positioning a resilient foam rubber-faced mechanical travel-stop on the shaft. The shutter release, now unactivated, allows the slotted block to retract; the block imposes a drag on the shaft that decelerates the sample as it nears the end of its travel in the cryogen. This cushioned and damped stop reduces any possible deleterious effect (e.g., movement of the frozen specimen and holey film relative to the grid, cracking of frozen specimen films in the holes, and rupturing of the specimen film centers) of rapid deceleration and resulting stress waves reflecting back and forth along the bar and tweezers.

Costello et al. (1982) and Elder et al. (1982), in their discussions of how to optimize plunge conditions for rapid cooling, recommend a plunge velocity of ~ 2 m/sec and a plunge depth of ~ 30 mm, parameters that fall within the operating range of the new CEVS. Construction plans for the apparatus are available.³

SAMPLE PREPARATION WITH THE CEVS

Described here is the step-by-step procedure for thermally fixing TEM specimens on a bare grid or holey film supported on a grid. The materials required in addition to the system described in the preceding section are 1) a 1.2-cm-diameter stoppered vial with at least 5 μl of sample; 2) a 0–10- μl repeating dispenser with pipette tips; 3) about 30 ml of each of the volatile components of the

³Construction plans (including engineering drawings, circuit diagrams, and list of parts and suppliers) can be purchased. Requests may be directed to Antony Strauss, Patents and Licensing, University of Minnesota, 1919 University Avenue, St. Paul, MN 55104.

sample (used to saturate the environmental chamber gas); 4) a 400-mesh bare grid or a carbon-coated holey polymer film supported on a 200-mesh grid; 5) a 90-mm circle of filter paper (type 1, Whatman, Hillsboro, OR) or glass filter medium (type 30, Schleicher & Schuell, Keene, NH); 6) long surgical forceps (20 cm long); 7) a pair of tweezers (e.g., type 3c Dumont); 8) bottled ethane, 99% pure (a size 3 cylinder, Matheson, Joliet, IL, with a single-stage pressure regulator and valve is convenient); 9) a 30-cm length of 4-mm-diameter Tygon tubing (to pipe ethane gas from the regulator) with a 16-gauge hypodermic syringe needle at its end (to direct the gas into the cup in the cryogen reservoir); and 10) liquid nitrogen.

Optional materials are 1) salt solutions or streams of dry and saturated air (if controlled humidity below saturation is desired in the environmental chamber—discussed in step 2 below); 2) bottled nitrogen or other inert gas (if the sample reacts with air or with water vapor that is normally present in air, so that the chamber must be filled with an inert atmosphere); 3) a vacuum aspirator (if the sample is toxic or poisonous); and 4) water-ice-brine or dry ice-acetone mixture (if the environmental chamber has to be operated at or below ambient temperatures).

Cryogens other than ethane can be used, such as freons and propane. Ethane is preferred because the large difference between its melting point (184.4K), and its boiling point (89.7K), together with its low viscosity (0.009 poise vs. 0.087 poise for propane), inhibits film boiling and increases heat-transfer coefficients, thus leading to faster cooling of the sample during plunging. Ethane is also advantageous because of the crust it forms on the sample when, wet with liquid ethane, the latter is transferred to liquid nitrogen and stored there. The crust readily sublines away when the sample, finally placed on the cold-stage of the microscope, experiences vacuum in the microscope goniometer prepump chamber.

Ethane presents a fire hazard, and thus when it is used the CEVS must be placed in a fume hood. Because skin contact with liquid ethane (unlike liquid nitrogen), even for short periods, can result in painful freeze-burns, rubber or cotton gloves and a face shield should be worn by the operator.

The steps to make a bare- or holey-film specimen are as follows.

1. The environmental chamber is opened by sliding backward the two sides and back, which form one C-assembly.

2. The environmental chamber is now saturated by filling the reservoir with a suitable saturator (Fig. 6). The best saturator is more of the sample itself. The goal is to have an environment inside the chamber such that the chemical potentials of each of the sample components in the vapor phase are equal to the respective chemical potentials in the liquid phase of the sample that will be subsequently exposed to this vapor. Because chemical potentials are difficult to measure and control, this requirement is not easily met. However, it can be realized by allowing vapor-liquid equilibration between the chamber atmosphere and the sample itself, or phases in equilibrium with it, rather than pure components of the sample. Unfortunately, in reaching equilibrium the sample will change composition; components will evaporate from or condense onto the sample. This problem can be overcome by sequentially placing a batch of the sample in the chamber, allowing vapor-liquid equilibration, and then replacing that altered sample with a fresh batch. This is repeated until none of the sample components evaporate into the vapor phase, or any of the vapor components condense onto the sample: the sample must be in mass equilibrium with its surroundings. Controlled humidity less than 100% relative humidity (RH) is often required to prevent evaporation from or condensation onto aqueous samples. In such cases the chamber atmosphere humidity can be controlled by placing salt solutions in the reservoirs instead of pure water. By suitable choice of salt and operating temperature, accurate and reproducible humidities can be obtained. Ganzer and Rebenfeld (1987) have recently reviewed the application of this technique. A chemistry handbook (e.g., Perry et al., 1984; Weast and Astle, 1981) will provide tables that can be consulted for this selection; Young (1967) provides an extensive compilation of such data. Alternatively, the humidity meter output can be used to control a valve that mixes streams of saturated air and dry air (or other gas) that can be fed through the chamber nozzle. (Intentional departures from equilibrium may be desired for some samples: This can be achieved by humidifying the chamber to an RH less than or more than the vapor pressure of the sample.)

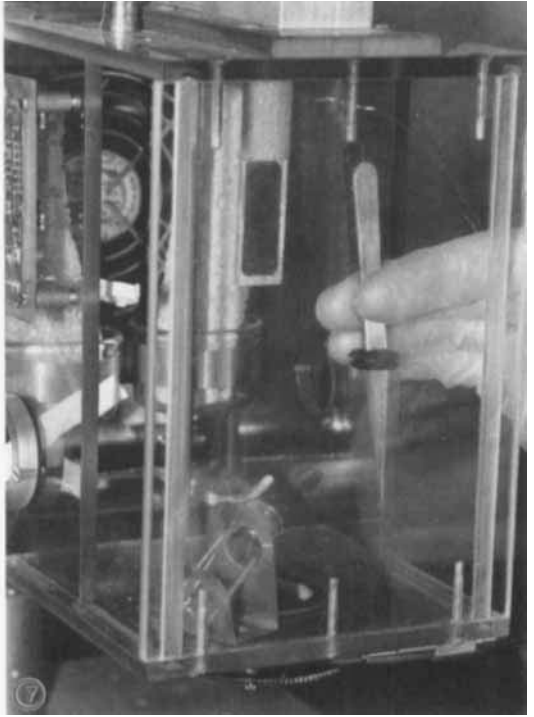
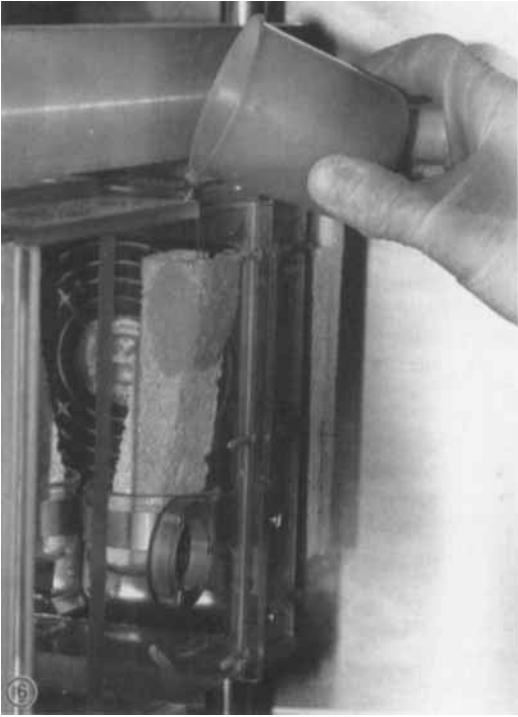


Fig. 6. Begin sample preparation by filling the reservoir with the saturating liquid(s).

Fig. 7. Clamp a grid on the CEVS tweezer and mount the latter on the magnetic clamp on the shaft.

Set the desired temperature on the temperature controller and allow the environmental chamber to equilibrate.

3. A bare or holey-film-covered grid is mounted in the CEVS tweezers by sliding the O-ring to clamp the tweezers closed. If a film-coated grid is used, the film side should face the operator. The tweezers are mounted in turn onto the shaft with the magnetic clamp (Fig. 7).

4. The shaft is manually raised through its bearing until it locks after compressing the spring.

5. The stoppered vial of sample is placed inside the chamber in the spring clamp.

6. The environmental chamber is closed by sliding forward the C-assembly formed by the two sides and the back. The shutter is closed by partially depressing the cable release or by depressing the release on the shutter.

7. If an inert atmosphere is desired inside the chamber, a stream of the desired gas is fed into the chamber nozzle until at least 10 liters (equal to 5 chamber-volumes) have purged the air in the chamber. The fan

should be turned on during this procedure to avoid channeling flows that would otherwise prevent significant purging. If a hazardous sample is to be used, the pressure in the chamber can be lowered by connecting a vacuum aspirator to the chamber nozzle. This prevents outward diffusion of the chamber gas through joints in the chamber. The discharge of the aspirator can be disposed of with suitable precautions (Jeng et al., 1987). The environmental chamber, together with its enclosed parts and wiring, can be dry-sterilized after sample preparation, if desired.

8. The fan, digital thermometer, and temperature controller are turned on, and the temperature-controller set-point is set to the desired temperature.

9. If the sample has to be at or below ambient temperature, the cooling reservoir on the back face of the chamber (Fig. 6) is filled with a refrigerant whose temperature should be close to the final desired chamber

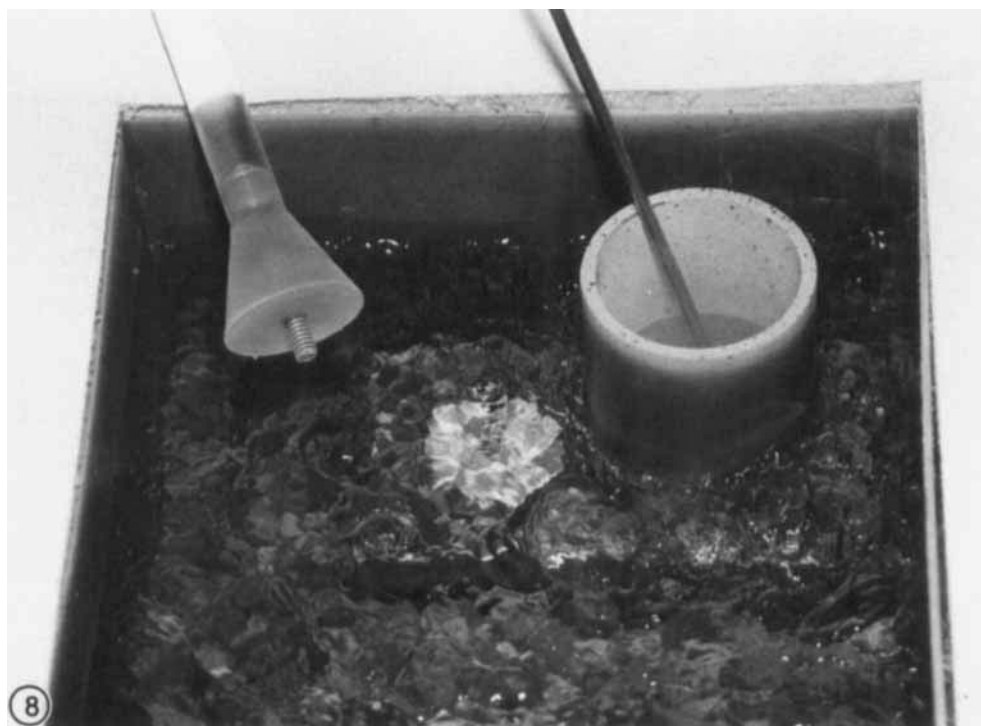


Fig. 8. Fill the cryogen reservoir with liquid nitrogen, and condense ethane gas in the cup.

temperature. Water-ice-brine or dry ice-acetone mixtures are satisfactory. Liquid nitrogen can be used for this as it is at hand; however, it is not recommended because the resulting large temperature gradients can reduce temperature stability and because it boils away rapidly and requires frequent refilling. The refrigerant can be circulated from a large auxiliary reservoir if extended operation without refilling is desired. Because of resistive heating of fan windings, the fan in the environmental chamber heats the gas by about 3°C above ambient temperature at steady state. Therefore, the cooling reservoir must be used if this temperature rise is undesirable. Moreover, the opposing effects of the appropriately positioned cooling reservoir and the heater result in more precise temperature control near ambient temperature than is provided by the heater alone.

10. The temperature and humidity are monitored at the instrumentation cluster as they approach the set-point. It takes about

20 min for the chamber atmosphere to reach 90°C , but only 5 min to reach 30°C .

11. While the temperature and humidity reach the desired set-point as measured by the digital thermometer and the humidity meter, the filter medium is wrapped around a stainless steel strip and inserted into the left port septum.

12. When the temperature and humidity have reached their desired values, liquid nitrogen is poured into the cryogen reservoir. After the vigorous film boiling has subsided, ethane gas is directed into the cup inside the cryogen reservoir, with the syringe needle connected to the Tygon tube leading from the ethane gas regulator (Fig. 8). The ethane is allowed to flow and condense as heat is removed through the cup walls to the boiling liquid nitrogen, until the cup is filled to the brim with liquid ethane. The cryogen reservoir is covered with the styrofoam lid to prevent frost deposition and water absorption until the sample is ready for plunging.

13. The long forceps is inserted into the right port septum, the cover of the vial is removed and placed on the inside bottom face of the chamber, and the forceps is withdrawn from the port.

14. The repeating dispenser volume is set to 5 μl if a bare or coated grid is used as the sample support. Because other specimen supports may require different volumes of sample, e.g., the freeze-fracture plates require 2 μl , a variable-volume pipette is useful. A pipette tip is placed on the dispenser, and this assembly is inserted into the right port septum. The tip temperature is allowed to equilibrate with the chamber by waiting for about 1 min. Alternatively, the pipette tip can be stored in the environmental chamber on the same spring-clip in which the sample is held and mounted on the pipette by inserting the latter into the tip through the port.

15. Five microliters of sample is withdrawn into the pipette (Fig. 9) and placed on the front face (i.e., the face toward the operator; also the film-coated face if such a grid is used) of the grid (or on the holey film) (Fig. 10). Perfectly wetting samples spontaneously cover the grid; partially wetting samples can be made to cover the grid (or film) completely by moving the tip around the grid face while depositing the sample. Support plates for nonwetting samples must be replaced or suitably altered (e.g., by placing them in a plasma discharge) so that the sample at least partially wets it. The pipette is withdrawn from the port septum.

16. The sample drop on the grid is converted to a thin film, suitable for TEM, by removing excess liquid: This is most conveniently done by blotting away part of the drop with the filter medium previously placed in the left port by pressing the filter surface against the back face of the grid for about 1 sec (Fig. 11). This forces loose ends and loops of fibers of the filter medium through and into the holes in the grid (and its holey film, if present) and draws the sample through the grid (and holey film) holes as the liquid is sucked into the filter medium by the capillary forces developed by the liquid as it advances into the pores of the medium. The filter medium is pulled away from the grid before all the liquid is blotted and is then pressed against the front face of the grid to blot any excess liquid present there (Fig. 12). The back and front filter-grid contacts are repeated with fresh,

unwetted areas of the filter medium until thin specimen films, ~ 250 nm thick (as estimated from the interference colors seen in bare-grid specimens with reflected light), are left spanning the grid (or film) holes. The thickness of the resulting liquid film depends on the interfacial tension between the sample and the filter medium as well as the grid or its film, the contact time, the sample viscosity, and the absorbency of the filter medium. Because these factors are not always well characterized or reproducible, a few trials may be required before a suitable thickness is obtained. The specimen should be thin enough that chromatic aberration caused by multiply-scattered electrons does not degrade the image; ~ 250 nm is adequate for aqueous specimens. When working with a bare grid, the operator can use the stereomicroscope to view the specimen during blotting. It is then possible to determine when the film thickness is acceptable by observing interference colors. The sample at the center of the grid hole becomes black when the film thickness there is less than one fourth of the wavelength of light. The biconcave liquid specimen films are then ~ 100 nm thick at the center and thicken to the thickness of the grid bars (~ 50 μm) at the edge of the grid hole, including a central zone < 250 nm thick, suitable for TEM. Figure 13 shows successive stages in the blotting of a water film on a bare grid as photographed through the light microscope. With a holey-film-coated grid the hole size, < 5 μm , is too small to resolve the liquid film spanning the holes with a long working distance microscope that has a numerical aperture (NA) of ~ 0.1 . However, better (and usually more expensive) microscopes (e.g., Model QM1, Questar, New Hope, PA) with larger NAs can be employed if desired. Alternatively, shorter working distance microscopes (which have larger NAs) can be used if the attendant loss of working space between the grid and the microscope objective can be tolerated. In this case the microscope objective, if hermetically sealed, can be mounted inside the environmental chamber.

17. The specimen is now ready to be quenched. But it can be kept as long as necessary in the chamber, where chemical potentials are properly controlled, without evaporation or unintended temperature changes, to allow relaxation from shear imposed during blotting. If dynamic rheolo-

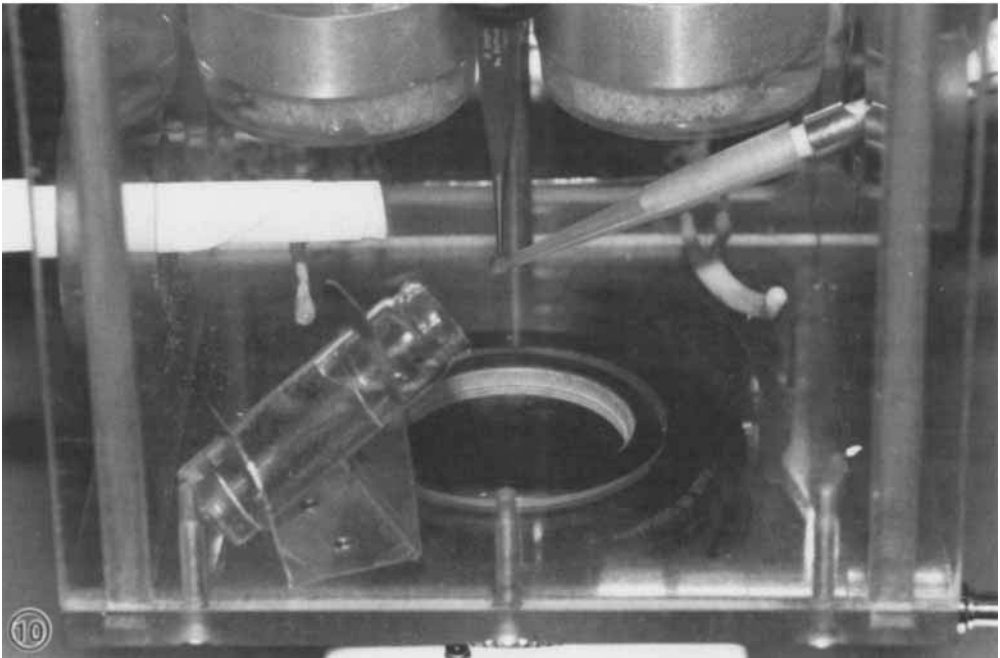
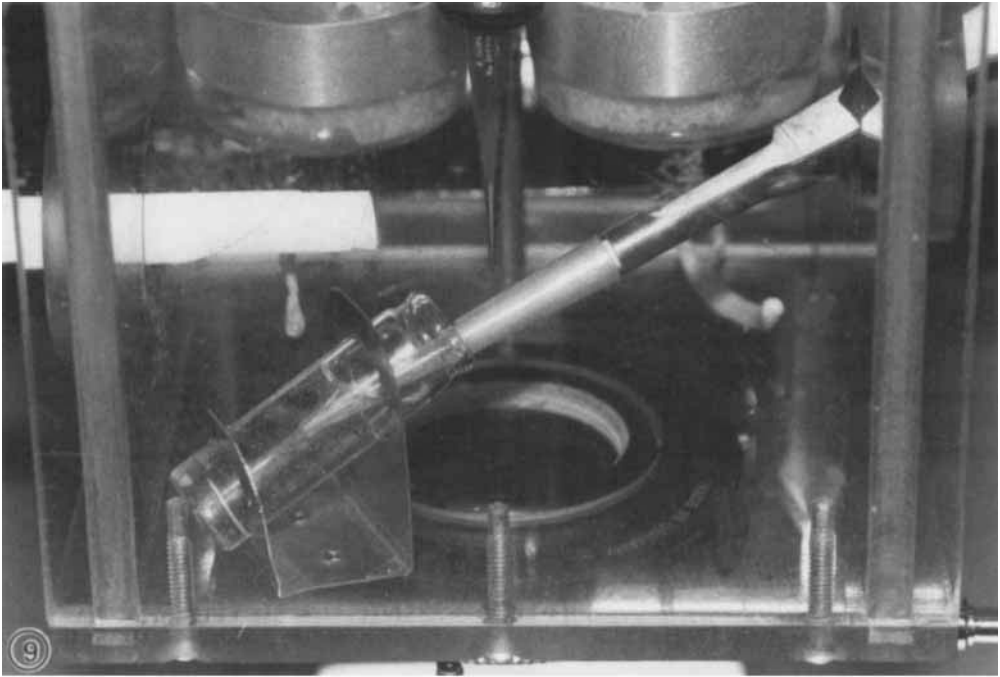


Fig. 9. Withdraw $5 \mu\text{l}$ of sample, from a vial previously placed in the environmental chamber, into a pipette inserted in the right port of the chamber.

Fig. 10. Dispense the sample onto the grid surface facing the operator (the front face).

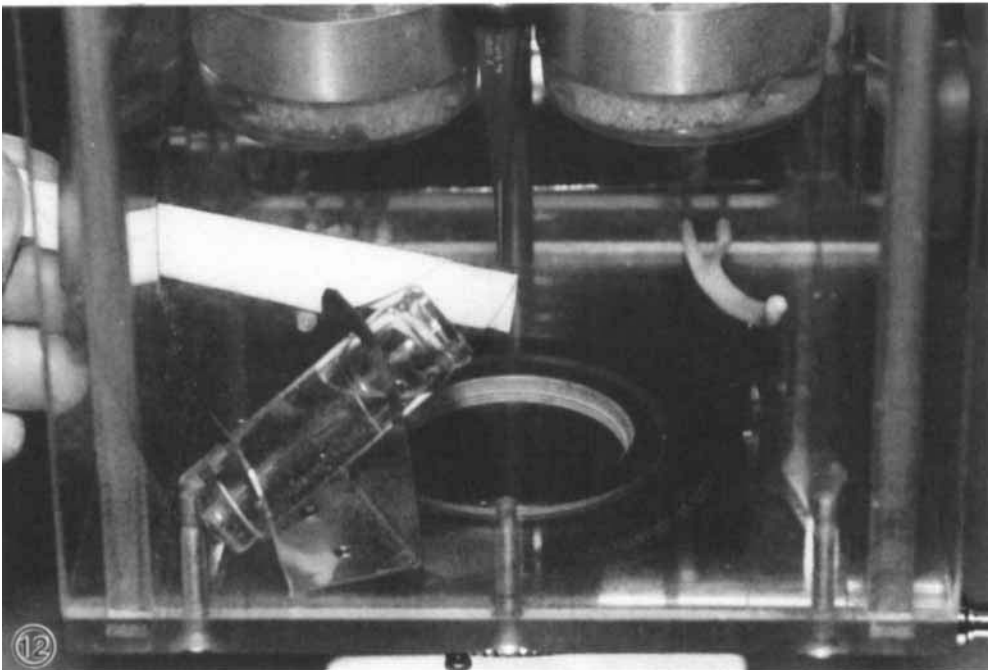
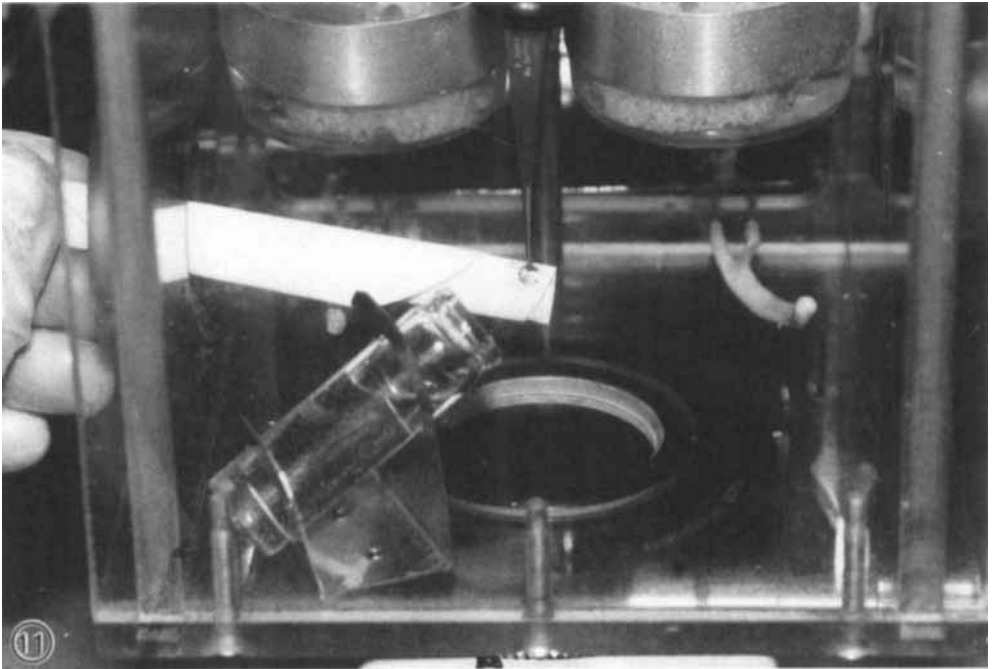


Fig. 11. Blot the sample drop by pressing a filter medium to the back face of the grid for about 1 sec.

Fig. 12. Form a thin film of sample by touching the filter medium to the front face of the grid.

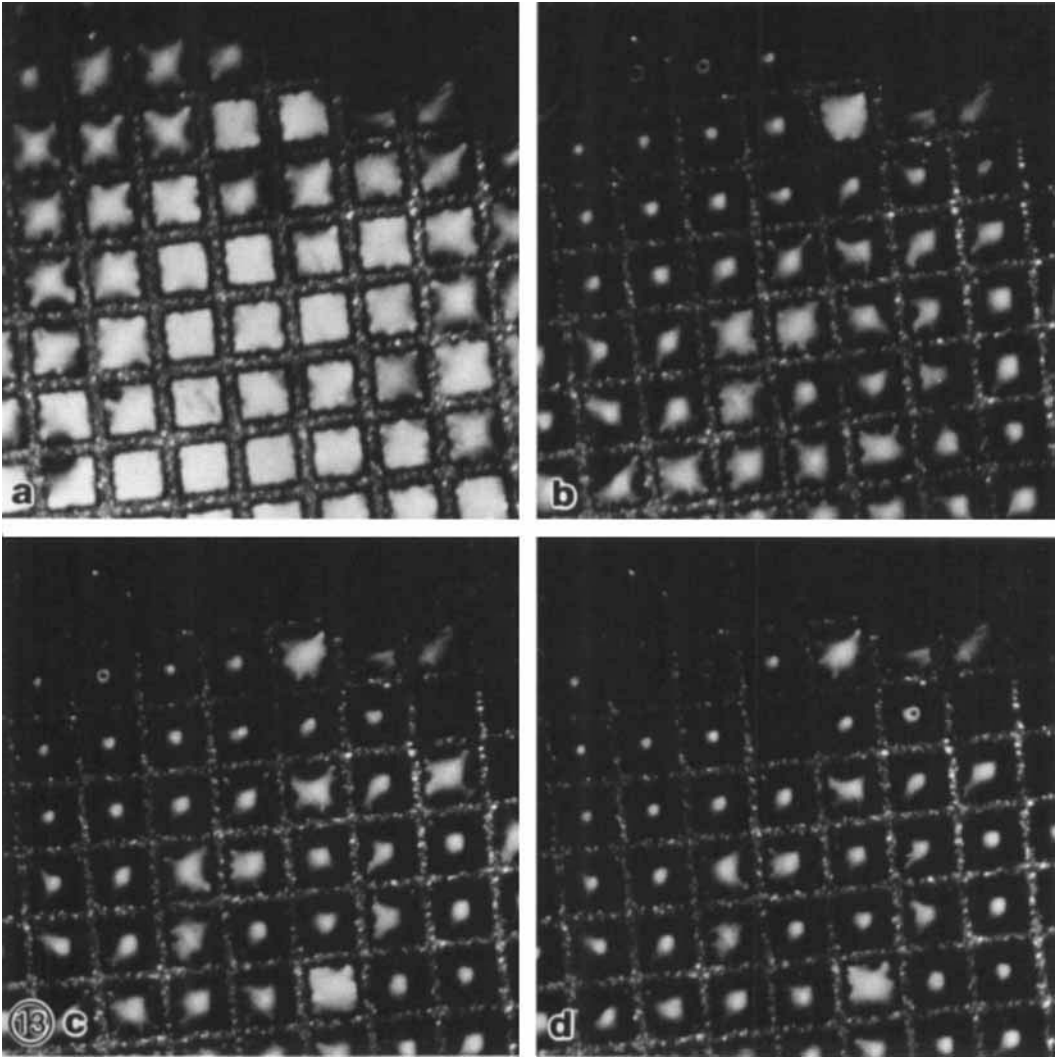


Fig. 13. Observe the sample during blotting through the light microscope. Shown here are successive stages (a-d) in the blotting of a water drop on a bare grid as

photographed through the light microscope. The thin films that span the holes are evident.

gical data of the sample are available, the relaxation time is known; in the absence of such data, successively longer times have to be employed with a series of specimens until no change in microstructure is seen in the images. On-the-grid processes may be conducted at this stage, e.g., the sample can be heated to drive a chemical reaction or cooled to induce an intentional phase change. A chemical reagent can be placed on the grid to initiate a reaction, e.g., gelling of a sol

(Bellare et al., 1987 a,b) or membrane fusion (Talmon et al., 1988), followed by further blotting to re-thin the specimen.

18. When the specimen is ready to be cryofixed, the cryogen reservoir is placed on the base plate under the environmental chamber, against the locator plates, and the styrofoam cover is removed. The ethane, cooled by the liquid nitrogen, may be frozen and solidified now at the cup wall, leaving a cylindrical pool, about 1.5 cm in diameter, of

liquid at the center. If the ethane is completely solid, then its central part is melted by directing a stream of ethane gas through the needle into its core. The presence of the frozen ethane is essential because it ensures that the liquid is at its lowest equilibrium temperature, and it therefore provides the highest cooling rate.

19. The cable release is depressed. This opens the shutter and rapidly plunges the specimen into the liquid ethane (Fig. 14).

20. The second pair of tweezers is cooled by immersing its tip for 15 sec in the liquid nitrogen surrounding the ethane cup. Then it is brought above the liquid ethane and used to force the tweezers holding the grid open so that the latter falls to the bottom of the cup (Fig. 15).

21. The shaft is raised and locked, and the cryogen reservoir is removed from beneath the environmental chamber and placed nearby in the hood. The CEVS tweezers is detached from the shaft, and the shutter is closed to permit the environmental chamber to return to the temperature and humidity set on the controller.

22. The specimen grid is removed from the ethane with the tweezers (Fig. 16) and rapidly (<1 sec) moved near the surface of the ethane cup, through the cold nitrogen vapor that is stably stratified above the liquid nitrogen-filled part of the reservoir, and immersed in liquid nitrogen surrounding the ethane cup. It is then placed in a slot of the specimen transfer box. After all four slots are filled with vitrified specimens, the transfer box is covered by screwing on its cover.

23. The specimen transfer assembly is removed from the cryogen reservoir and is quickly (within 1 sec) transferred into a dewar of liquid nitrogen. The latter is placed next to the cryogen reservoir. It is used subsequently to transport the specimen to the electron microscope or for further processing (e.g., freeze-substitution or -fracture and replication) if desired. Liquid nitrogen that is carried in the slots of the specimen transfer box, now covered, protects the specimen from the detrimental effects of frost deposition or heating. The specimen can be transferred into a cold-stage and into the microscope, without the above-mentioned detrimental effects, following well-established techniques (e.g., Perlov et al., 1983).

Additional samples can be prepared by

placing a new grid in the CEVS tweezers, readying a new piece of filter medium, and allowing the chamber temperature and saturation to return to their desired values. The time it takes to ready the CEVS for a new sample—the cycle time—depends on the operating temperature and is typically 5 to 15 min in our experiments.

SAMPLE RESULTS

Several systems were selected to illustrate, first, the pitfalls of conventional vitrification techniques and, second, how they can be overcome with the CEVS. There are surfactant dispersions that make ideal test specimens to evaluate preparation procedures, because their phase behavior, i.e., number and compositions of coexisting phases at equilibrium, have been, in many circumstances, well characterized by physicochemical techniques such as light microscopy; light-, X-ray-, and neutron-scattering; rheology; and conductivity.

Surfactants are molecules that have distinct ends of which one or more are hydrophilic and one or more are hydrophobic. When dissolved or dispersed in water or hydrocarbon, they form a variety of structures, e.g., vesicles (closed shells made of surfactant molecules arranged in a bilayer), liposomes (multilayered vesicles), micelles (aggregates with surfactant hydrophobic moieties directed toward a point and their hydrophilic moieties directed away from the point toward the water), inverted micelles, equilibrium microemulsions, and truly molecular solutions, depending on type of surfactant, temperature, concentration, and presence of additional components such as alcohols, oils, and salts. Vesicles and liposomes are associated with a thermodynamic phase that is distinct from the solution in which they reside. Images of vesicles and liposomes from light microscopy and, in particular, video- and computer-enhanced differential interference light microscopy (Miller et al., 1987a) can be used to check images from electron microscopy because particles with diameters of $\sim 1 \mu\text{m}$ can be observed by both methods. If the surfactant system, when represented in a phase diagram, is near a phase boundary, then small variations in compositions or temperature can cause a phase transition and with it a drastic change in microstructure. Thus, if the results of electron microscopy do not agree with comparable data from other

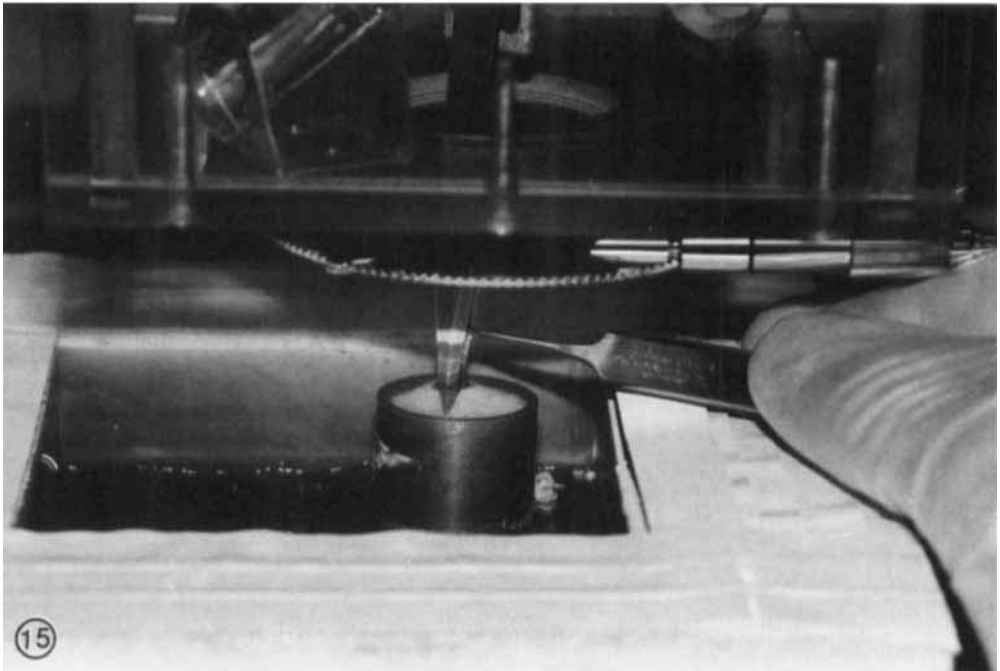
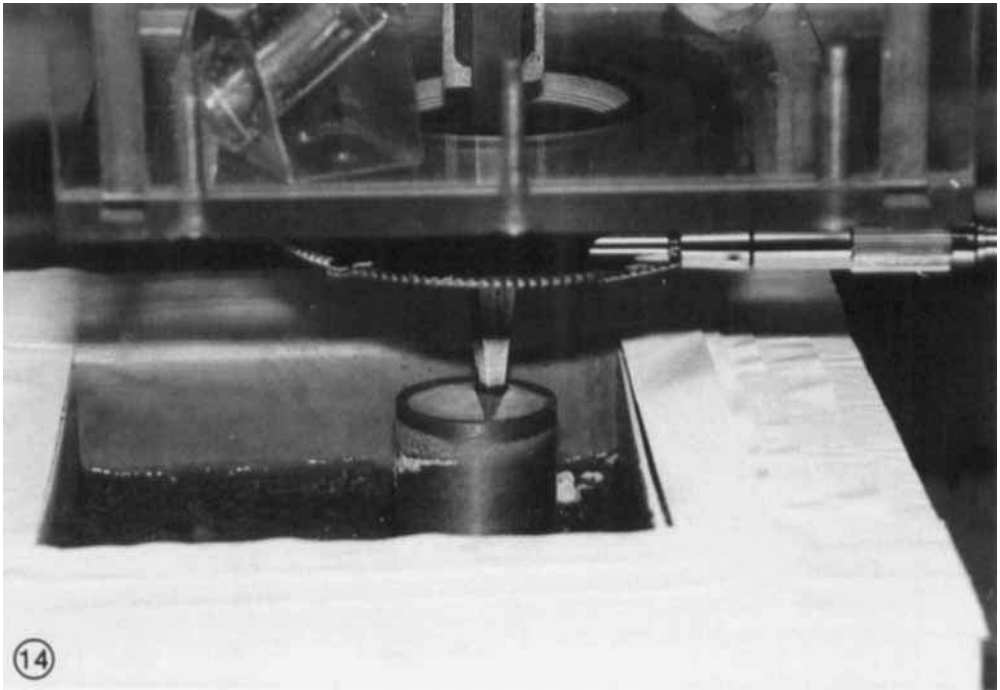


Fig. 14. Depress the cable release. This plunges the sample into the ethane cup by triggering the spring-loaded shaft; the shutter opens just before the specimen falls through.

Fig. 15. Force open the tweezers holding the grid with a second, precooled tweezer and let the specimen fall to the bottom of the ethane cup.

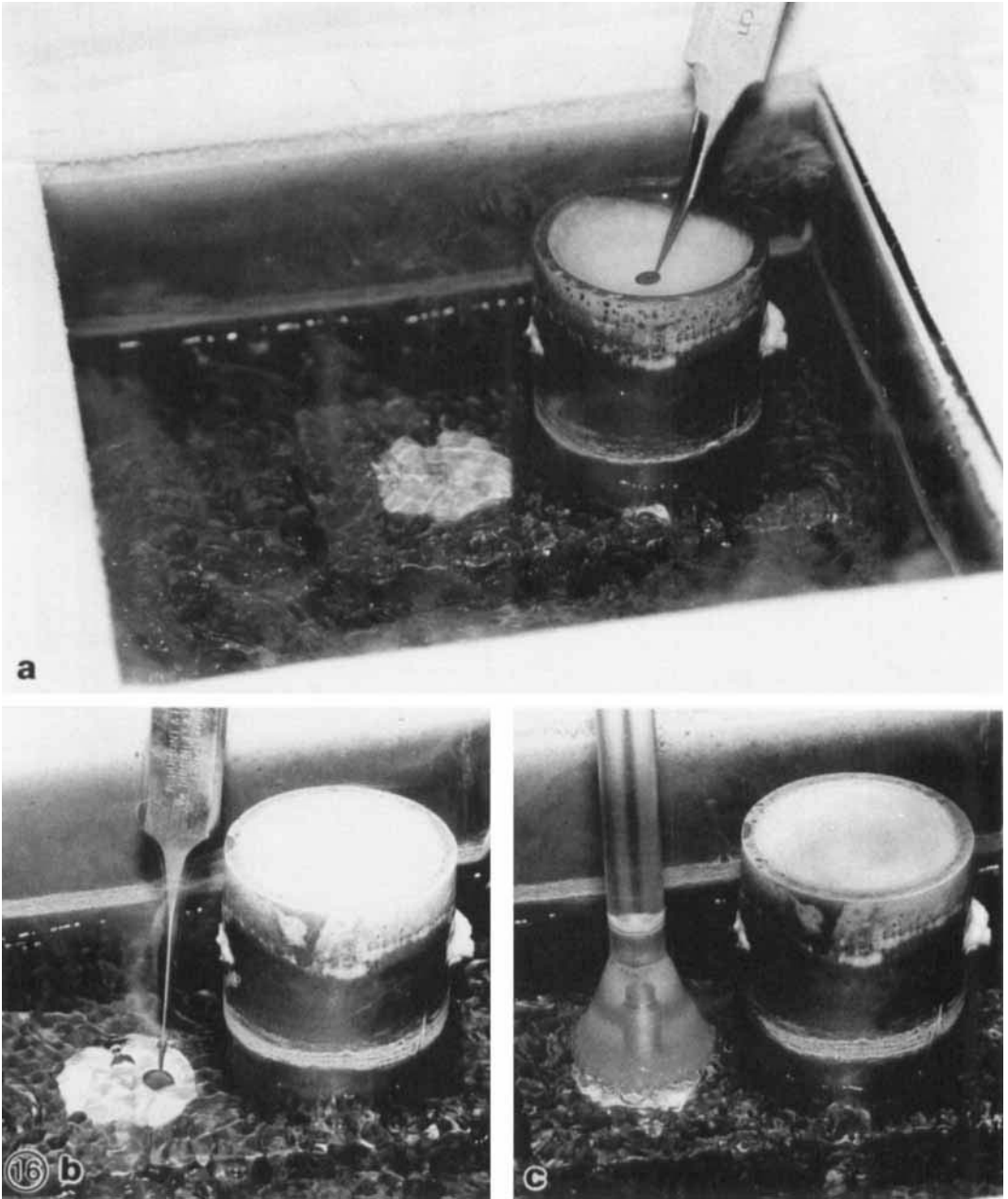


Fig. 16. a: Remove the vitrified specimen from the ethane. b: Transfer it through the cold nitrogen vapors boiling in the cryogen reservoir into a slot of the specimen transfer box. c. Screw the specimen transfer cover

onto the box and transfer the box into a liquid nitrogen-filled holding dewar. Sample preparation is now complete. Mount the specimen onto a cold-stage and examine by TEM.

techniques, the applicability of the sample preparation technique to the system is suspect. We have used three surfactant systems to evaluate the performance and establish the advantages of the CEVS.

Materials

Sodium 4-(1'-heptylnonyl)benzene sulfonate (SHBS) was synthesized at the University of Texas (Austin) and purified as described by Franses (1979). Octyldodecyl-dimethylammonium bromide (ODDDAB) was generously provided by Dr. R. Zana of CNRS (Centre de Research sur les Macromolecules, Strasbourg). Dioctadecyldimethylammonium bromide (DODDAB) was provided by Dr. D.F. Evans (University of Minnesota, Minneapolis). The surfactants were dissolved or dispersed in doubly distilled water further purified by dialysis. Dispersions were made by pouring water into weighed quantities of surfactants and repeatedly inverting the container by hand at about 0.5 Hz for about 10 min.

Methods

Vitrified-hydrated-unstained (VHU) specimens were made on holey-carbon-film-coated grids in the manner described above. Unless otherwise stated, the specimens were prepared with the environmental chamber at 25°C with 98% RH.

Specimens were examined in a JEOL 100CX analytical microscope, operating at 100 kV in the conventional TEM mode. The VHU specimens were mounted into a modified JEOL EM-SCH cooling holder by means of the cold-stage transfer module described by Perlov et al. (1983). The specimen holder temperature in all experiments was -168°C. Images were recorded on Kodak SO-163 film exposed for maximum rated speed and developed for 12 min in full-strength Kodak D-19 developer.

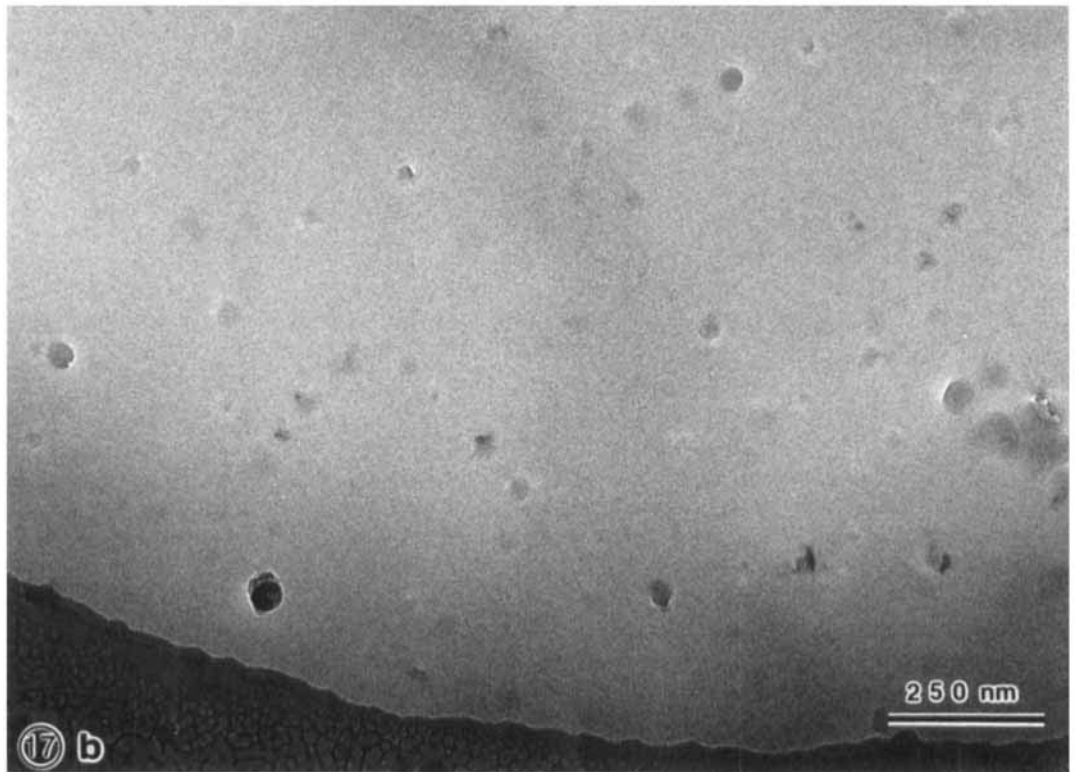
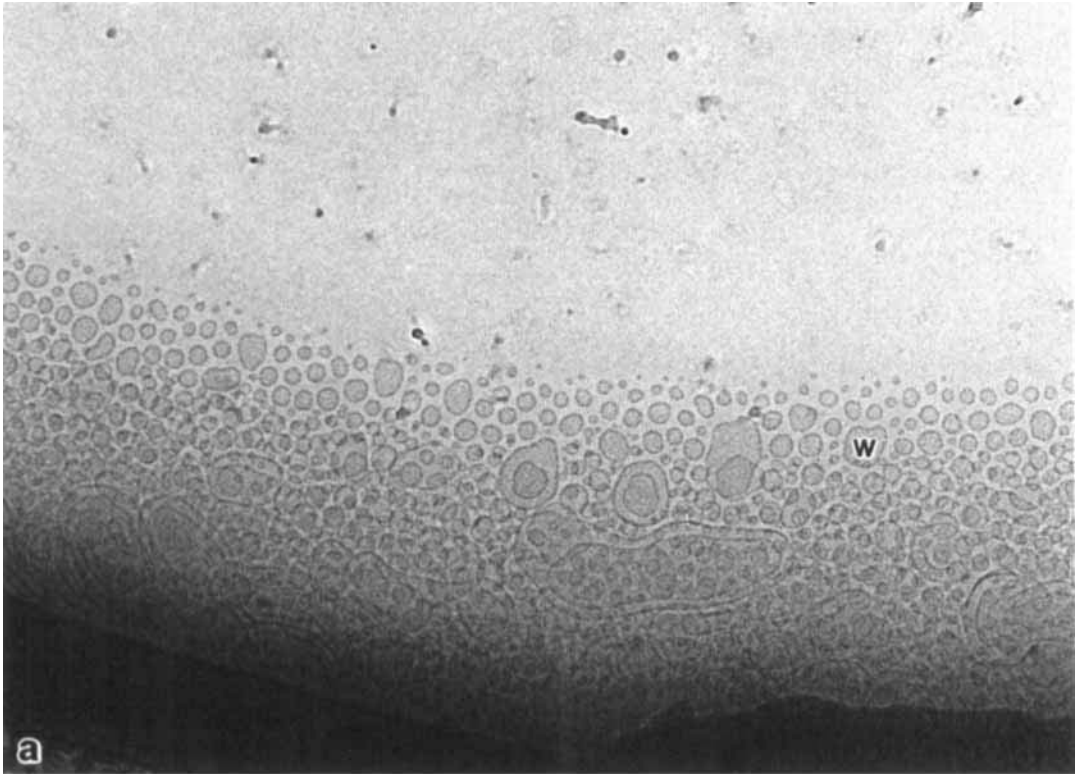
Results

A 2.0% solution of ODDDAB at 20°C was prepared for TEM; the resulting images are shown in Figure 17. No vesicles are expected to be seen, for at this concentration the surfactant solution or dispersion is optically clear; indeed, it is known that the surfactant forms what are apparently equilibrium aggregates smaller than 5 nm. However, at higher concentrations dispersions are turbid because of the formation of

large aggregates. Specimens prepared without environmental control (with the environmental chamber at the ambient humidity of 40% RH and without water in the saturating reservoirs) (Fig. 17a) have become sufficiently concentrated by evaporation that they have thus crossed a phase boundary (~2.5%, 20°C). The solution has become supersaturated with respect to a lamellar liquid-crystalline phase that precipitates as vesicles, which are, of course, absent from the original specimen. The sample preparation procedure concentrates the vesicles at the edge of the vitrified films, near the thicker regions at the grid-coated holey film. Furthermore, they segregate by size along the thickness gradient from the center of the vitrified specimen-film to its edge. However, specimens prepared in the water-saturated environment of the CEVS show (Fig. 17b) no evaporation-induced artifacts, demonstrating the importance of saturation and evaporation control that the CEVS achieves. Smaller aggregates, possibly micelles, may be present in the system, but cannot be resolved without further image processing.

DODDAB has a phase transition from crystal to lamellar liquid crystal at ~30°C. Figure 18a shows a 0.5% aqueous dispersion, sonicated for 30 min at 50°C to reduce the crystal size, prepared from 20°C, and examined by cryo-TEM. Large surfactant sheets and smaller lens-shaped structures are seen: these are not characteristic of liquid-crystalline phases. Samples prepared from 50°C using the CEVS show (Fig. 18b) vesicles and multilamellar liposomes, but no large sheets or lens-shaped structures. Preparing the specimen from 50°C would have been a difficult task without the temperature control provided by the CEVS.

Previous, limited evidence (Franses, 1979) indicates that 0.1% aqueous dispersions of SHBS at 90°C were molecular solutions. The CEVS allowed specimens to be prepared at 90°C from a water-saturated environment without evaporation. As Figure 19 shows, vesicles and liposomes can be seen in such samples; these microstructures have also been observed by hot-stage light microscopy (Javadi, G., personal communication). Thus, with the CEVS we were able to determine the high-temperature microstructure of this dispersion—relevant to enhanced oil recovery research—which was previously unknown.



Other applications of the CEVS

Miller et al. (1987 a,b) recently applied the CEVS technique in conjunction with video-enhanced light microscopy to study the temperature-induced transition from vesicles to micelles in alkylammonium acetate and bromide dispersions. We have also applied the CEVS to study microstructural changes in the transformation of a sol to a gel—a process important in manufacturing novel ceramic precursors (Bellare et al., 1987a,b). The value of the CEVS to microstructural studies of colloids is evident from these results.

DISCUSSION

Sample preparation may be the most important step in imaging the microstructure of fluid and labile systems by electron microscopy of vitrified specimens, because it determines what microstructure is fixed. There are thermodynamic and fluid-dynamic factors to control: heat transfer, mass transfer, spreading, and blotting. These may introduce artifacts like temperature-change- and concentration-change-induced phase transformation, segregation, and crystallization into the specimen. The controlled environment vitrification system solves the thermodynamic problems. By providing a closed atmosphere in which the temperature and saturation of volatile components are controlled, CEVS prevents evaporation, temperature variation, and phase-change and makes it possible to prepare samples at temperatures and saturations differing from ambient. The synchronous shutter prevents specimen precooling by cryogen vapors. By preventing temperature and concentration gradients that affect surface tension, it reduces convective flows that may segregate components in the specimen. However, the segregation effects, exemplified in Figure 17a, resulting from spreading and blotting still exist. Whereas the problems of coating and thinning the sample to make bare- or holey-film specimens without segregation remain to be

solved, the CEVS permits control of the thermodynamic variables—temperature, composition, and chemical potentials.

Preparing samples for cryo-ultramicrotomy

In addition to bare- and holey-film specimens for direct TEM visualization, the CEVS can be used to fast-freeze sample blocks or drops for cryo-ultramicrotomy; the resulting sections can be directly imaged by cryo-TEM. With such sections it is possible to avoid the effects of flow and rearrangement that occur when a liquid is blotted to prepare bare-film specimens; but the artifacts and difficulties associated with sectioning—compression, chatter, and skipping—must be dealt with.

Preparing samples for freeze-fracture replication and cryo-SEM

The CEVS has been used to quench specimens for freeze-fracture replication, as shown by Burns and Talmon (1988). A specimen sandwich is made by placing a drop of sample on a small gold cup and squeezing the drop into a film by forcing a second cup toward the first until a ~0.05-mm-thick layer of liquid is formed. This assembly is mounted on the CEVS tweezer and quenched. The sandwich can also be assembled inside the environmental chamber by holding the gold cups or copper plates in specially designed tweezers (Bellare et al., 1988). The sandwich is quenched, and the plates are separated to fracture the specimen; the resulting fracture surfaces are coated with a conductive material such as carbon after mounting on a cold-finger in a thermal or electron-beam evaporator. The sample can then be directly examined by cryo-SEM. Alternatively, the complementary fracture surfaces can be shadowed with a metal and reinforced with carbon; the resulting replicas are warmed to room temperature, cleaned of the original sample, and examined by conventional TEM without requiring a cold-stage. With the CEVS freeze-fracture replication technique, Bodet

Fig. 17. Vitrified-hydrated-unstained specimens of a 2.0% aqueous solution of octyldodecyldimethylammonium bromide (ODDDAB). No vesicles are expected at this concentration. However, a: samples prepared without environmental saturation show vesicles (W) formed because the solution has become concentrated enough (by evaporation) to precipitate a new lamellar phase. b:

Samples prepared with saturation using the CEVS have not precipitated a new phase and therefore do not show the vesicles. Vesicles in a are concentrated in the thicker regions of the vitrified water film near the holey film. They also segregate by size along the thickness gradient from the center of the vitrified film to its edge. Bar = 250 nm.

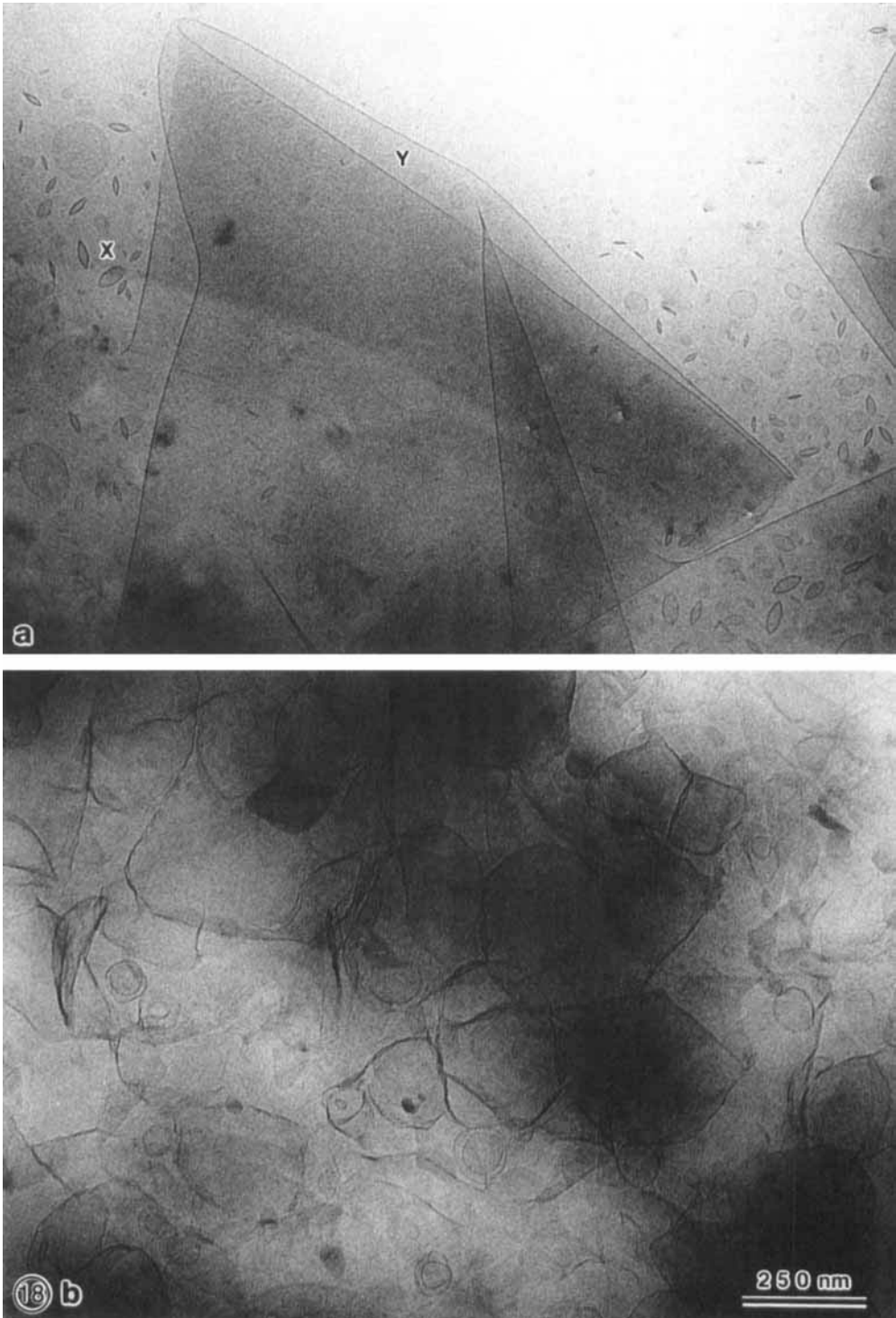


Fig. 18. Vitrified-hydrated-unstained specimens of 0.5% aqueous dispersion of dioctadecyldimethylammonium bromide (DODDAB). a: Sample prepared with

CEVS from 20°C. b: Sample prepared from 50°C. Note absence in b of lens-shaped structures (X) and large sheets (Y) seen in a. Bar = 250 nm.

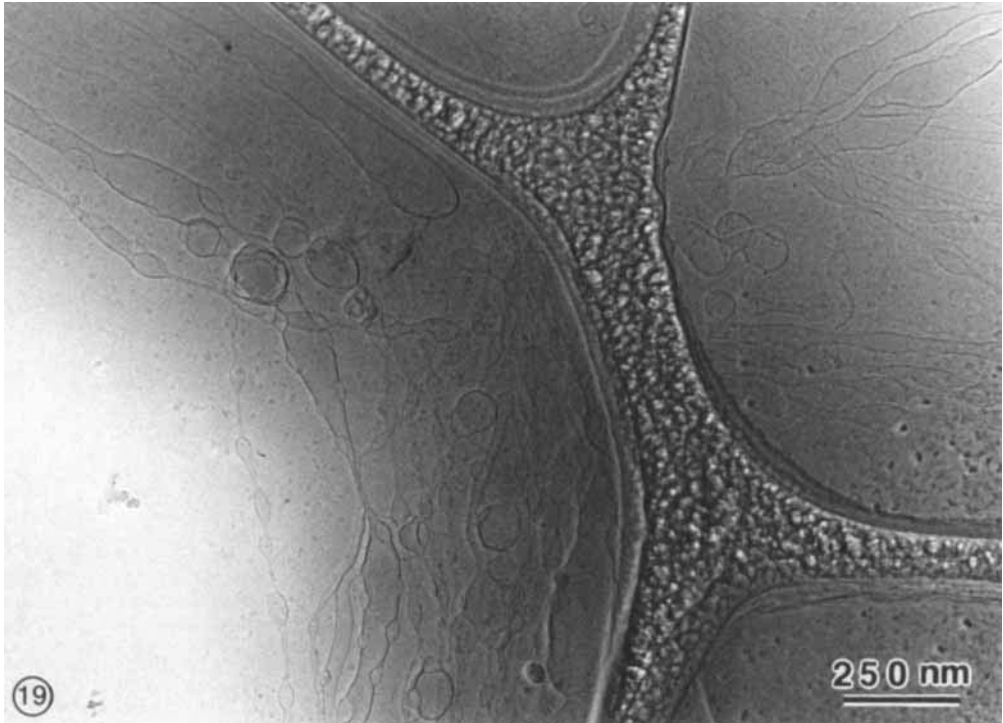


Fig. 19. Vitrified-hydrated-unstained specimen of 0.1% aqueous dispersion of sodium 4-(1-heptylnonyl)benzene sulfonate prepared from 90°C. From limited, nonmicroscopic evidence (Franses, 1979), it was

previously believed that the system, at 90°C, was a true molecular solution. However, these micrographs show that the system is an aqueous dispersion of liposomes and vesicles.

et al. (1988) recently mapped for the first time the transition of a microemulsion from globular to bicontinuous state.

“On-the-grid” experiments for dynamic processes

The controlled environment chamber can be used as a minilaboratory to create or to modify specimens. A chemical reaction can be conducted on the grid by applying, in succession or simultaneously, two or more drops of reactants or catalysts to the grid, blotting the resulting mixture, and then

waiting for the product to be formed in the thin-film. The specimen can be heated or cooled to alter it physically or chemically. Processes can be initiated, propagated, and terminated by changes in temperature or saturation, on-the-grid mixing, irradiation (e.g., ultraviolet light), or a suitable permutation and combination of these changes. Such procedures are particularly useful for gel specimens, which, because of the high viscosity of gels, cannot be easily thinned. The gelling process can be conducted in a thin film of its precursor sol, which has low

viscosity, resulting in a thin film of gel. Furthermore, by controlling the interval between sample preparation and cryo-plunging, the CEVS permits microstructural imaging of successive, nonequilibrium stages of dynamic processes in progress on the grid, without evaporation or temperature variations. Such experiments have already been applied to the successful study of sol-gel transformations (Bellare et al., 1987b) and vesicle fusion (Talmon et al., 1988); they provide a new approach to investigate processes such as membrane fusion, phase transitions, and chemical reactions.

Instrument development

Further additions to the CEVS under consideration are a programmable temperature controller, a solenoid-driven electrical triggering device to initiate the plunging action, and a high-resolution, long working distance light microscope. These will permit the CEVS to be interfaced with a microprocessor so that 1) timing can be controlled more precisely and reproducibly and 2) the trigger can be controlled by an external event such as the output of a spectroscope that monitors a reaction on the grid. This will allow desired instants of dynamic events to be more accurately captured. The light microscope will permit the holey-film support to be resolved and therefore will allow determination of sample thickness. It may also permit on-the-grid micromanipulation, e.g., deforming of vesicles, before vitrification. Improvements to the cooling reservoir that would prevent complete solidification of the ethane and maintain the ethane at its freezing point are also planned (Vinson, 1987).

SUMMARY

The success of the controlled environment vitrification system in reducing artifacts by preserving conditions very similar to the native state of the sample and the ability of the CEVS to perform "on-the-grid" experiments with controlled timing open new possibilities for research in membranes, viruses, liquid crystals, micelles, microemulsions, polymers, and ceramics.

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