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THE ULTRASTRUCTURE OF CRYO-SECTIONS AND INTACT VITRIFIED CELLS - THE EFFECTS OF CRYOPROTECTANTS AND ACCELERATION VOLTAGE ON BEAM INDUCED BUBBLING

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Abstract

Chemically fixed pancreas was infiltrated with various cryoprotectants to obtain homogeneously vitrified samples upon cooling. The suitability of these samples for cryo-ultramicrotomy was tested. Contrast was hardly detectable initially in thin cryo-sections but increased upon irradiation, irrespective of the cryoprotectant (glycerol, propylene glycol, methanol) used. Contrast and beam damage were analyzed in vitrified thin films from collagen, phospholipid vesicles and various concentrations of glycerol. Glycerol increased the beam sensitivity of both collagen and phospholipid vesicles, but diminished the contrast between matrix and lipid vesicles or collagen fibers. The effects of glycerol as observed in thin films explain some of the effects of cryoprotectants in thin cryo-sections. To reduce beam damage in vitrified specimens two approaches are proposed. Firstly, when vitrified films are prepared, dilute suspensions should be used without cryoprotectant. In some cases, such as (thin) intact cells, the composition of the suspended material can only be marginally influenced. Then a second approach can be used involving the application of higher accelerating voltages (e.g. 300 kV). This has two advantages; the increase in mean free path-length of the electrons causes less beam damage on one hand and allows better resolution of thick specimens on the other hand. Micrographs from *E. coli* bacteria vitrified from suspension illustrate some of the potentials of "intermediate voltage" cryo-electron microscopy.

Key Words: cryo-sections, cryoprotectants, beam damage, vitrified thin films, contrast, intact cells, intermediate voltage microscopy, phase-contrast.

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Introduction

Cryo-electron microscopy, the low temperature observation of hydrated objects by transmission electron microscopy, as introduced by Taylor and Glaeser (1975, 1976) has become an important tool in ultrastructural research. The cryo-observation of hydrated objects became feasible when it was discovered that water could be vitrified (Brüggeler and Mayer, 1980; Dubochet and McDowall, 1981) and suspended macromolecules could be observed by cryo-electron microscopy (Dubochet *et al.*, 1982) in the vitrified and hydrated state. The observation of vitrified and hydrated objects has several advantages, one of these is the protection against beam induced damage to organic material. When the cryoprotection is estimated by the vanishing of diffraction spots from (hydrated) catalase crystals, protection by a factor ten (room temperature vs. -160 to -170 °C) came out as the average, determined by several independent groups (Chiu *et al.*, 1986). The vanishing of diffraction spots is a form of beam damage which is accessible to quantitative determinations. Beam induced bubbling (Glaeser and Taylor, 1978; Dubochet *et al.*, 1982) is a form of beam damage typical for hydrated specimens and difficult to quantitate (see also Talmon, 1987). In non-crystalline specimens this is often the first visible sign of beam damage. This form of beam damage was especially prominent in the cryo-observation of cryo-sections prepared from chemically fixed and cryoprotected (glycerol, methanol, propylene glycol) pancreas. The material properties of pancreas prepared in this way furthermore allow a comparison with resin embedded material in relation to the nature of the sectioning process. The beam induced bubbling in cryo-sections from fixed and cryoprotected pancreas is preceded by an increase in contrast in the cryo-sections as will be reported in this paper. These observations are in strong contrast with observations on vitrified thin films prepared from phospholipid suspensions. In dilute suspensions and a range of film thicknesses it was virtually impossible to induce bubbling by electron beam irradiation (Frederik *et al.*, 1989b). By the addition of virus particles

or collagen fibers to a phospholipid suspension differential effects were observed in vitrified thin films. Beam induced bubbling started in the virus particles or in the collagen fibers, leaving the rest of the film unaffected. In this paper, we will report on the increase in beam sensitivity of vitrified thin films when glycerol is added to a phospholipid suspension. By the addition of glycerol it was possible to induce bubbling in vitrified thin films of collagen fibers at approximately the same time as in the rest of the film. Furthermore, it will be shown that the addition of glycerol to a phospholipid suspension changes the contrast between bilayers and solute. These data indicate that beam induced bubbling in cryo-electron microscopy is enhanced by the presence of organic material (e.g. virus, collagen) and depends on the concentration (e.g. glycerol, dilute and concentrated lipid). It is therefore advised to use dilute suspensions without cryoprotectants when preparing vitrified thin films for cryo-electron microscopy. In applications where "intact" biological specimens such as blood platelets (Frederik *et al.*, 1991a) or bacteria (to be reported in this paper) are to be observed in vitrified thin films, the chemical composition of the specimens has to be taken for granted. Beam induced damage of such specimens is hard (if not impossible) to prevent if conventional acceleration voltages (up to 120 kV) are used to produce micrographs. At higher acceleration voltages (e.g. 300 kV) the proportion of inelastically scattered electrons has diminished. This has a two-fold effect; firstly, image blurring in thick specimens is reduced (Frederik *et al.*, 1991a) and secondly, beam induced bubbling is reduced. Data presented in this paper on bacteria vitrified in suspension will illustrate the ultrastructure as observed at 300 kV.

Materials and Methods

Vitrified cryo-sections from pancreas

In order to have homogeneously vitrified tissue blocks, pancreas samples (removed from anesthetized rats) were cut into small pieces, fixed in 2% formaldehyde, 0.2% glutaraldehyde (w/v in phosphate buffer pH 7.4) and infiltrated with a graded series of cryoprotectant. Several cryoprotectants have been used for this purpose and the final concentrations employed were 67% w/w for glycerol, 80% methanol, 80% methanol with 20% PVP (polyvinylpyrrolidone) and 80% propylene glycol (w/w). Thermodynamic properties of these solutions and data relevant to contrast formation are presented in Table 1. These solutions qualify as vitrification solutions since they vitrify at low cooling velocities and do not form crystals upon rewarming. This can be checked by vitrifying a drop of the vitrification solution in liquid nitrogen and observing that the glass-like appearance remains when the drop is rewarmed (a rather crude test for recrystallization, see Forsyth and MacFarlane, 1986).

We assume that pancreas samples infiltrated with these high concentrations of cryoprotectant are vitrified upon cooling. Furthermore, we may assume that the water-containing matrix can only undergo viscosity changes (notably the glass transition) upon cooling and rewarming. Segregation of ice crystals from the water-containing matrix can thus not interfere with cryo-sectioning or cryo-observation at any temperature.

Cryo-sections were obtained on dry glass knives or a cryo-diamond knife (Diatome) using an LKB Cryo-Nova ultramicrotome. It was found that each "embedding" had its own optimal sectioning temperature (see Table 1). At lower sectioning temperatures the material became brittle yielding only fragments, at higher temperatures sections stuck to the knife. The cryo-sections were collected with an eyelash probe on grids supported with a Formvar film. The cryo-sections were observed at -169 °C using a Gatan 626 cryotransfer system in conjunction with a Philips CM 12 microscope. To observe dose-related changes in cryo-sections we calibrated the exposure meter of the microscope with a Faraday-cage (see also Stols *et al.*, 1986). Consecutive micrographs were taken at a preset dose rate. Thickness determinations of cryo-sections were carried out by densitometry of the micrographs using the method of Bahr and Zeitler (1965) and Zeitler and Bahr (1962, 1965, also see Frederik *et al.*, 1989a).

Vitrified thin films

Two types of vitrified thin films were used in this study. The first type consisted of collagen, combined with phospholipid vesicles prepared in various concentrations of glycerol. These films were used for the estimation of the dose required for the first visual signs of beam induced bubbling (at 100 kV). The second type of vitrified thin films was prepared from a suspension of *E. coli* bacteria. From these films it was practically impossible to obtain micrographs without beam damage at 100-120 kV and these films were used for observations at 300 kV.

Phospholipid films. For the formation of thin films spanning a specimen grid, the synthetic lecithin dimyristoylphosphatidylcholine (DMPC, Sigma No. P-0888) was used. A suspension (16 mM) was prepared in glycerol/distilled water (0, 10, 20, 30 % w/v respectively) by vortexing. From this suspension unilamellar vesicles were prepared by extrusion through Nuclepore filters (pore size 0.1 μm) at 30 °C using the extruder described by Hope *et al.* (1985). After several passages through the extruder the suspensions were used for thin film formation and vitrification. The grids used for thin film formation (bare grids, 700 mesh, honeycomb pattern) were precoated with collagen fibers. An acid collagen solution (collagen reagent, Hormon Chemie, prod. nr. 10500) was diluted 1:1 with glycerol/water (0, 20, 40, 60% w/v respectively). A drop of this solution was placed on parafilm and a specimen grid

Table 1. Properties of cryoprotectants.

Compound	Formula	Concentration W/W %	Glass transition °C	Density g/cm^3	$(\rho\ x)_c$[5] g/cm^{2}x 10^{-6}	Sectioning temp. °C
Methanol	H_4CO	80	-161[1,2]	0.84	8.16	-120
Propylene glycol	$H_8C_3O_2$	80	-109[3]	1.04	8.7	-115
Glycerol	$H_8C_3O_3$	67	-110[4]	1.18	9.0	-105

1 Vuillard and Sanchez (1961);
2 Miller and Carpenter (1964) described a methanol monohydrate decomposition at -102.2°C;
3 Boutron and Kaufmann (1979);
4 Luyet and Rasmussen (1968);
5 $(\rho\ x)_c$ is the mass per surface area that statistically leads to one scattering event per incident electron. Data have been calculated according to Carlemalm *et al.* (1985a).

was floated on top of this solution. Subsequently, the grid was washed with two drops of glycerol having the same glycerol concentration as the glycerol in the phospholipid suspension used for thin film formation. Thin films were formed by dipping and withdrawing the collagen coated grid in the phospholipid suspension. After withdrawing from the phospholipid/glycerol suspension the grid was blotted against filter paper; in the spaces between the grid bars and the collagen fibers, the thin films formed "soap films" (also see Frederik *et al.*, 1989a,b). Thin films were vitrified into ethane cooled to its melting point by liquid nitrogen. A gravity-powered guillotine was used to guide the tweezers, holding the grid, into the ethane (Dubochet *et al.*, 1982). Cryotransfer and cryo-observations were performed as described for the cryo-sections (see above).

Thin films of suspended bacteria. *E. coli* bacteria grown in suspension were concentrated by centrifugation to obtain a density of approximately 10^9 bacteria per ml. From this suspension thin films were formed using essentially the same procedure as described for lipid suspension. Only blotting was done more gently, excess liquid was sucked away with the pointed tip of a piece of filter paper placed at the periphery of the grid, and an additional piece of filter paper was placed between the tweezers holding the grid. Thin film formation thus took several seconds and was followed by vitrification in melting ethane. Cryotransfer and cryo-observations (at 300 kV, Philips CM 30 microscope) were performed as described for the cryosections.

Results and Discussion

Beam damage in cryo-sections

Cryo-sections, from chemically fixed and cryoprotected pancreas, hardly showed any contrast when "virgin" pictures are taken at first exposure (Figs. 1-3). This occurred despite the fact that the density of the cryoprotectant solution varied (0.84 for 80% methanol vs 1.14 for 67% glycerol) and also the electron scattering properties of the aqueous embedding (see Table 1). We expected on the basis of calculations of the scattering properties (see Carlemalm *et al.*, 1985a) that high concentrations of glycerol would obscure the contrast, e.g., between zymogen granules and the surrounding matrix; whereas in methanol-infiltrated pancreas this contrast would be enhanced compared to the contrast in cryo-sections from freshly frozen pancreas (no chemical fixation or cryoprotection). It can be questioned if cryo-sections, when observed in their native (not freeze-dried) state, are thin enough to reveal subcellular details. In thick sections blurring of image details is caused by the relative large proportion of the incident electrons that is elastically and/or multiply scattered upon interaction with the specimen. We therefore measured the thickness of the cryo-sections by densitometry. It turned out that most sections had a thickness between 160 and 280 nm (with all cryoprotectants used) with only an occasional exceptionally thin section in between. Micrographs of such a section are presented in Fig. 2. Even in such a thin section hardly any subcellular detail could be observed in a low dose picture. In consecutive pictures subcellular details seem to develop gradually as a result of beam exposure (see Figs. 1-3). This can also be demonstrated by continuous recording of the ultrastructure using a Gatan video-camera with image inten-

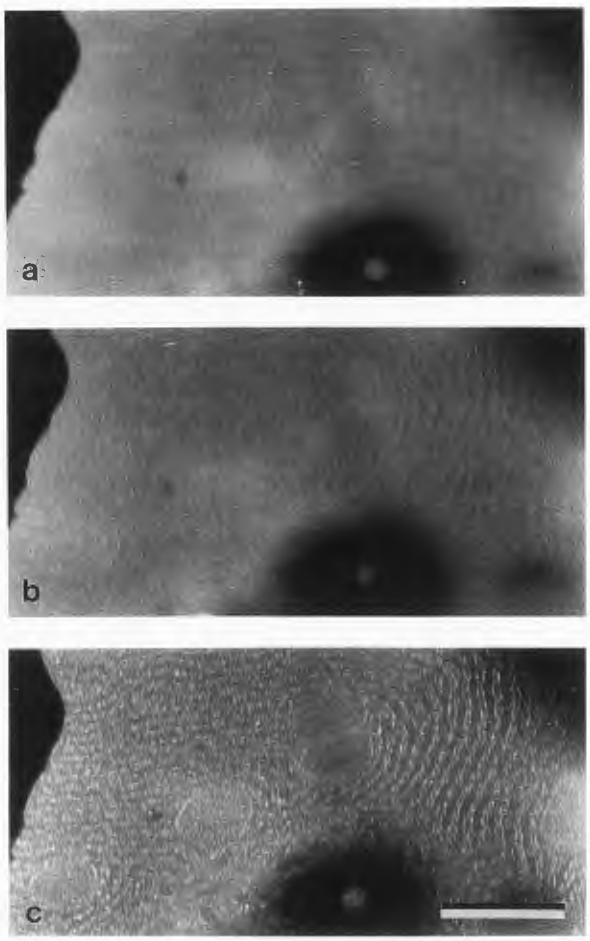

Figure 1. Cryo-section from rat pancreas (chemically fixed) infiltrated in 80% w/w propylene glycol. Successive micrographs demonstrate the effect of irradiation; (a) 400 e.nm^{-2}, (b) 1000 e.nm^{-2}, (c) 1600 e.nm^{-2}. Note the increase in contrast and visibility of subcellular detail in successive micrographs and the appearance of beam damage (voids) in (c). Bar represents 1 μm.

sifier (data not shown) using dose-rates which are unpractical for photography (integration over time periods of 12 sec or more). The areas in the cryo-section where contrast develops are also the areas where beam induced bubbling is initiated as observed after longer exposure times (e.g. Fig. 1). In fact the electron beam can be used to "stabilize" the ultrastructure (see Fig. 3) upon subsequent warming of the cryo-section (also see Hagler and Buja, 1986).

The observations reported above made us curious about the mechanism of contrast development during irradiation as well as the initial absence of contrast in cryo-sections. For further investigations in this context we used vitrified thin films of various chemical compositions.

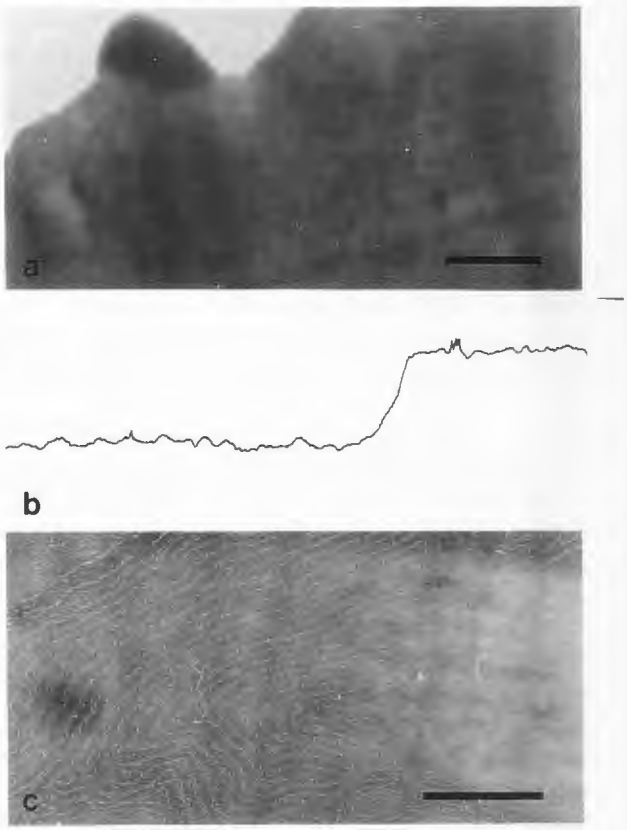

Figure 2. Cryo-section from rat pancreas (chemically fixed) infiltrated in 80% w/w methanol. (a) Micrograph (dose less than 500 e.nm^{-2}) from an exceptionally thin section. (b) Densitometer tracing from micrograph (a) used for thickness determination (120 nm). (c) Micrograph from an area adjoining the area shown on (a), showing the effect of beam exposure. Bar represents 1 μm.

The cryo-sectioning process

The aim of using chemically fixed and cryoprotected tissue was to obtain samples which were vitrified as a whole (and not only a thin surface layer) with only a partial extraction of the tissue water. Most of the extraction of lipids (e.g. phospholipids) and denaturation of proteins occurred in the final steps of a dehydration procedure. Essentially the same rationale was used to develop the "progressive lowering of temperature" technique of Carlemalm *et al.* (1982, 1985). It was also hoped that by infiltration with various vitrifying solutions, or mixtures of these solutions, the sectioning properties of the embedded tissue could be improved.

From our experiments with cryo-sectioning we conclude that the optimal sectioning temperatures are related to the devitrification temperature of the various compounds. This further supports our idea (Frederik *et al.*, 1984) that sectioning is essentially a "flow" process involving devitrification (and revitrification?) at the knife edge to separate the sec-

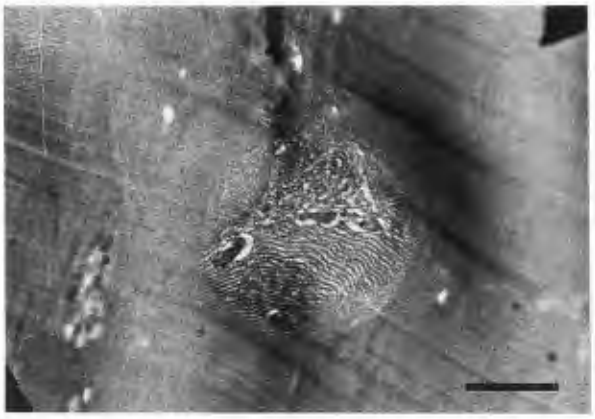

Figure 3. Cryo-section from rat pancreas (chemically fixed) infiltrated in 80% w.w. methanol. Structure was developed by focussing the beam in a small spot and taking a micrograph after defocussing the beam. This micrograph demonstrates that the low contrast observed at low dose (the periphery of the micrograph) is not caused by improper focussing. Bar represents 2 μm.

tion from the block and/or to lubricate the (sliding) contact between section and knife. Although this hypothesis seems attractive, there are some points which deserve further clarification. First of all, the devitrification temperatures reported in the literature are usually determined by differential scanning calorimetry (DSC) using slow heating rates. At other heating rates other devitrification (or glass transition) temperatures are found since this transition is a "kinetic-change". From the machining of polymers it is known (J.H. Dautzenberg, Technical University Eindhoven, personal communication) that the mechanical properties of these compounds strongly depend on the deformation speed; the sectioning speed divided by the thickness of the affected zone ($\leq$ section thickness). The extreme conditions found in micron-machining and ultramicrotomy cannot be approximated by standardized stress-strain experiments. Data from such standardized stress-strain experiments on a number of embedding resins, as well as a number of other mechanical properties of resins are found in a paper by Acetarin *et al.* (1987). These authors consider a low elastic strain, a small plastic flow and noticeable flexibility as important mechanical properties of an embedding resin. Such properties are supposed to favor cleavage sectioning (as opposite to machining) as a cutting mechanism in ultramicrotomy. Cleavage sectioning involves the formation of cracks and crazes (consisting of distorted, deformed and flowing macromolecules) in advance of the knife edge.

In the same paper determinations of the glass-transition

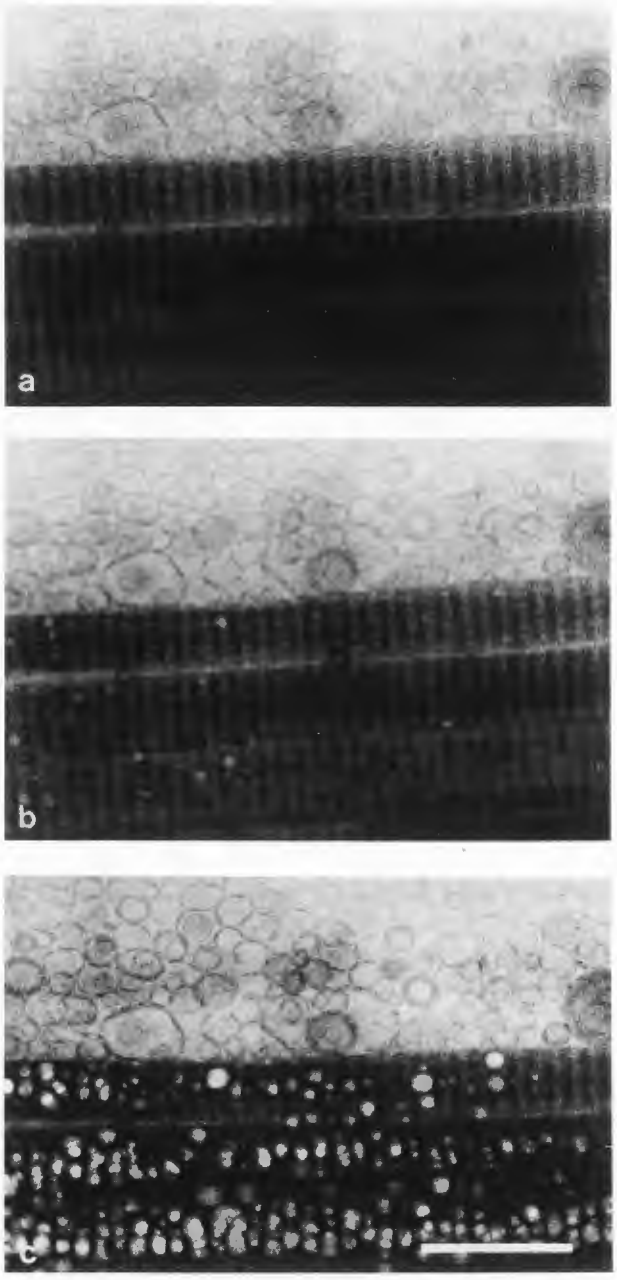

Figure 4. Vitrified thin film containing collagen and phospholipid vesicles (DMPC 16 mM), no glycerol added. Serial micrographs taken at cumulative doses; (a) 6000 e. nm^{-2}; (b) 12.000 e.nm^{-2}, (c) 18.000 e.nm^{-2}. Note the appearance of bubbles (voids) in collagen fibers in (b) and rows of voids in (c). No signs of bubble formation are observed in the phospholipid vesicles at these doses. Bar represents 0.5 μm.

temperatures of various polymers (including Epon) are presented. These temperatures as estimated by DSC, fall in a range between 47°C and 97°C. The fact that Epon (standard Luft mixture) can be sectioned on "dry" glass knives at temperatures below -70°C (Frederik and Klepper, 1976) is thus an exception to the preliminary conclusion that cryo-sectioning is optimal in the temperature range of the glass transition temperature. Furthermore, we used cryo-sectioning of Epon to check if the microtome and knife combinations were limiting factors in obtaining smooth and thin cryo-sections. Both with glass knives and a diamond knife dry cut sections (temperatures between -80°C and -110°C) from Epon could be obtained below 100 nm (range 40-100 nm, as estimated by densitometry).

Exactly at these temperatures the same knife-microtome combination routinely produced sections of a thickness between 160 and 280 nm (with a few exceptions, see above) from chemically fixed and cryoprotected pancreas. This observation reinforced our opinion that the mechanical properties of cryoprotected tissue are at present the limiting factor in obtaining thin cryo-sections.

In searching for a better mechanically tailored vitrifying solution to be used for "embedding" tissue samples for cryo-ultramicrotomy one should also consider the contrast relations and the beam sensitivity. These properties are important when sections are used for the study of biological material in their "native" (partly dehydrated) state. Vitrified thin films have been used for further investigations of contrast as well as beam induced damage.

Vitrified thin films

In recent papers we have described the factors involved in thin film formation and vitrification (Frederik *et al.*, 1989 a,b). Thin films prepared from phospholipid suspensions have been found to be suited for the study of thermotropic lipid phases (Frederik *et al.*, 1991a) and of lipid polymorphism (Frederik *et al.*, 1989c, 1991b) as well as for the study of the fusion between virus and lipid-membranes (Burger *et al.*, 1990). On basis of our experience with thin films prepared from phospholipid suspensions, we designed our experiments on beam induced damage in thin films of a mixed composition. As reported earlier (Frederik *et al.*, 1989b) the phospholipid used, dimyristoylphosphatidylcholine, was hardly sensitive to beam induced bubbling when vitrified in a dilute suspension. We observed (Fig. 4) that beam damage started in collagen fibers, when these fibers were added to a thin film of DMPC vesicles. Upon further exposure beam damage gradually developed, decorating the cross-striations of collagen with rows of voids (Figs. 4, 5). With glycerol we observed an earlier onset of beam damage (compare Figs. 4-6). When different dose rates (beam intensities) were considered the dose at the onset of radiation damage was the same (within the limited accuracy of the method) for each glycerol concentration.

At increasing glycerol concentrations the contrast between the lipid bilayers and its surrounding solute diminished (Fig. 5). At high glycerol concentrations (in excess of 30%) only faint outlines of collagen fibers could be observed initially and lipid vesicles only became apparent when the outlines were decorated by beam induced bubbling.

From these experiments it can be concluded that glycerol increases the sensitivity of "hydrated" collagen to beam induced bubbling. Within the collagen, voids are formed in rows parallel to the cross striations. Phospholipid vesicles which are hardly sensitive to beam induced bubbling when vitrified in distilled water become sensitive in vitrified glycerol solutions. The data show a considerable variation. We attribute this to variables which have not (yet) been taken into account when collecting the data. One of these variables is the diameter of the collagen fibers. For instance, we observed in crossing fibers that the first onset of radiation damage is observed in the overlapping areas.

In other micrographs non-overlapping thin and thick fibers were found, and in those series of micrographs the thicker fibers showed the first signs of damage. Consistent results were obtained when one (isolated) fiber was followed along its course and small non-overlapping areas were used for the determination of beam damage. In such an approach reproducible results (within 10%) were obtained using a fixed beam intensity as well as when using different beam intensities (onset of radiation damage at the same dose).

The preliminary conclusion that glycerol increases the beam sensitivity of collagen as well as of phospholipid vesicles has to be taken as qualitative statement which has to be refined by further experimental work. The unique feature of vitrified thin films in this respect is that we can now consider "solute concentration" as parameter in contrast formation as well as in the development of beam damage. With respect to contrast in vitrified thin films it was found that the addition of glycerol obscures structural details in collagen as well as in phospholipid vesicles. The experiments with thin films were not conducted with the high glycerol concentrations employed in "cryoprotection" of pancreas samples because the viscosity of phospholipid suspension became too high to allow reproducible thin film formation. Nevertheless the experiments with thin films clearly indicated that high concentrations of glycerol have an adverse effect on the beam sensitivity of cryo-sections from vitrified pancreas. The experiments furthermore indicated that decoration effects of bubble (void) formation may be responsible for the development of contrast during irradiation of cryo-sections.

The data presented strongly discourage the use of glycerol as an additive to organic material that is to be observed in the vitrified state. One may question if these observations on glycerol solutions in thin films can be extrapolated to

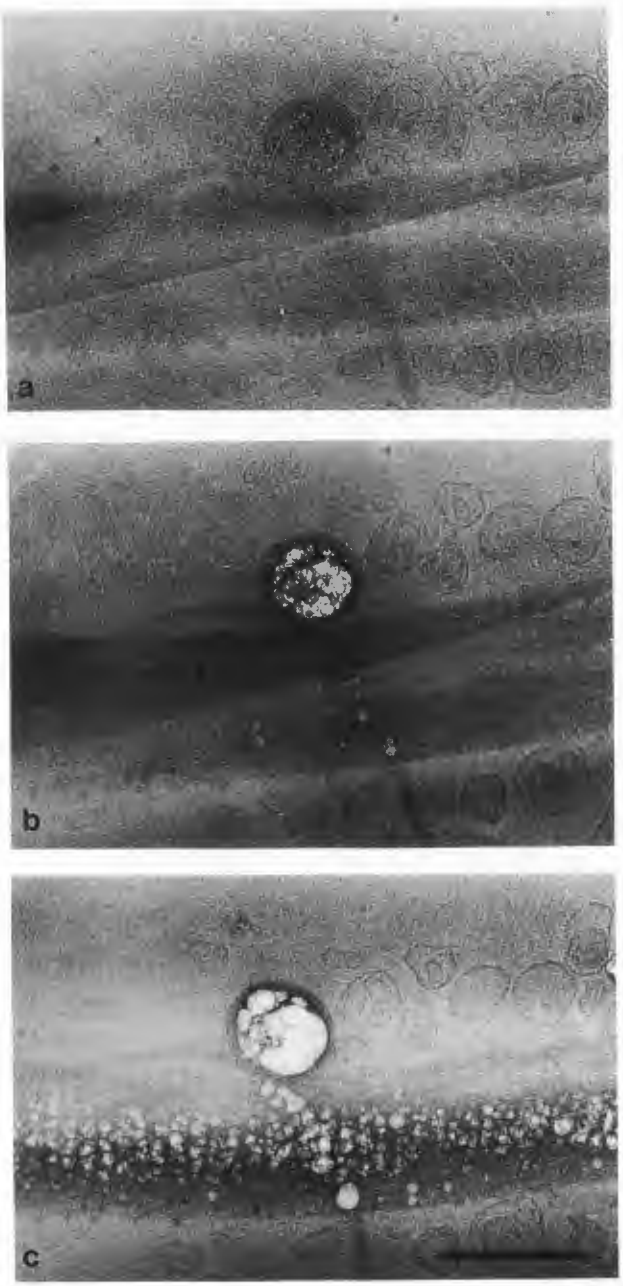

Figure 5. Vitrified thin film containing collagen and phospholipid vesicles (DMPC 16 mM) in 20% w/v glycerol. Serial micrographs taken at cumulative doses; (a) 2400 e. nm^{-2}, (b) 6000 e.nm^{-2}, (c) 12.000 e.nm^{-2}. Note the appearance of bubbles (voids) in collagen fibers and in a multilamellar vesicle in (b) and rows of voids in collagen (c). Bar represents 0.5 μm.

other cryoprotectants or to organic materials in general. Observations of other cryoprotectants, for instance sucrose,

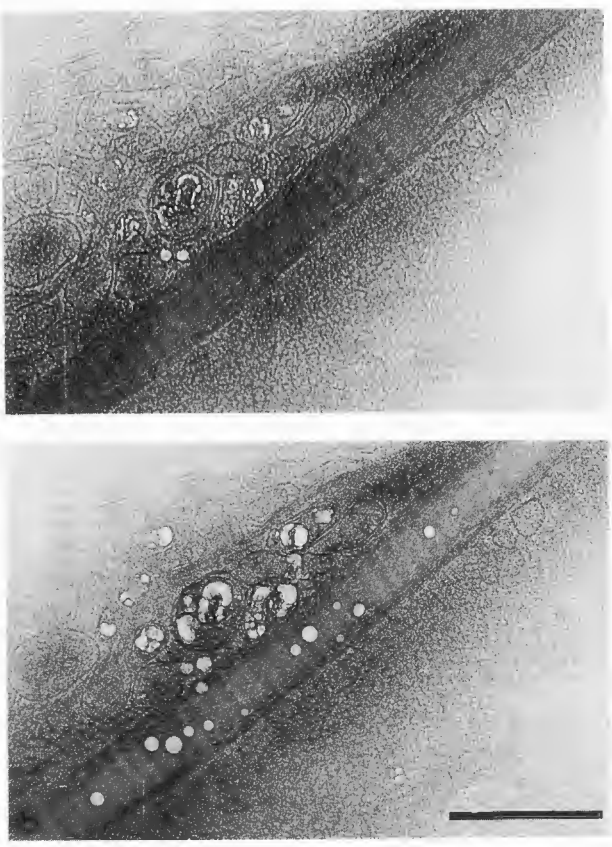

Figure 6. Vitrified thin film containing collagen and phospholipid vesicles (DMPC 16 mM) in 30% glycerol. Serial micrographs taken at cumulative doses; (a) 1540 e. nm^{-2}, (b) 5720 e.nm^{-2}. Note the appearance of bubbles (voids) in multilamellar vesicles in (a) concomitant with only minor signs of damage to collagen. Bar represents 0.5 μm.

indicate that this compound increases the beam sensitivity as well, although no systematic data are (yet) available.

We postulate that high (local) concentrations of organic material in a vitrified matrix are sensitive to beam induced bubbling. Note for instance that multilamellar vesicles (Figs. 5, 6) of phospholipid are more sensitive to beam damage than unilamellar vesicles. The chemical nature of the matrix (e.g., glycerol at increasing concentrations) can amplify this effect. Thus, if suspended material is to be studied in the frozen hydrated state we would advise the use of a dilute suspension without any additives, apart from the additional surface active compounds (e.g. phospholipid) needed to support thin film formation. This advice would almost rule out the observation of "whole-mounted" cells in their vitrified state. And in fact,beam damage at 100-120 kV often appeared in low-dose pictures taken from bacteria or blood-platelets vitrified in suspension. We therefore investigated these specimens at 300 kV. Results from blood

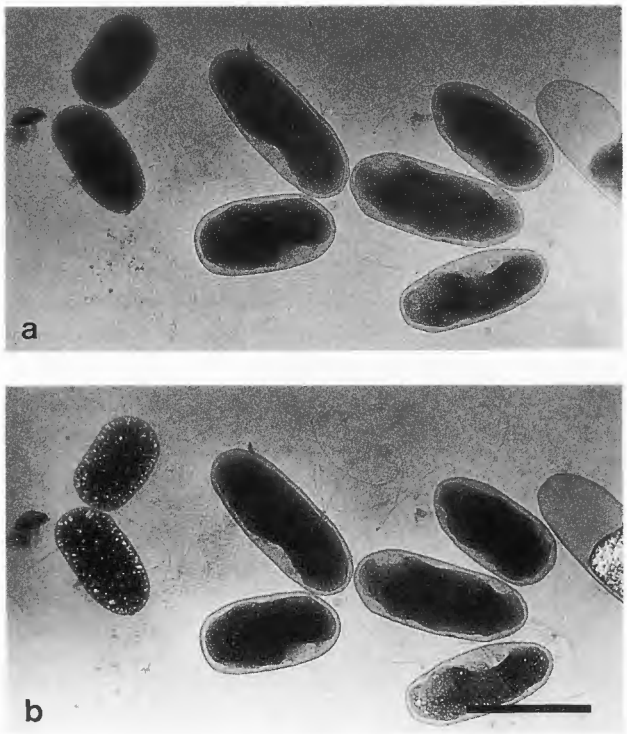

Figure 7. Escherichia coli bacteria grown in suspension and vitrified in a thin film. Low dose picture (a) taken at 300 kV and a second micrograph (b) taken within one minute. Fine details of the bacterial structure can be observed in (a) (e.g. bacterial wall, flagellae), beam damage becomes prominent in (b). Bar represents 0.5 μm.

platelets will be reported in a forthcoming paper (Frederik *et al.*, 1991a). In this paper we present micrographs from *E. coli* bacteria vitrified in suspension. The micrographs (Fig. 7) show that subcellular detail (bacterial wall, flagellae) can be observed and that in the next micrograph (taken within a minute after the first) the structures are destroyed. The advantage of using the higher acceleration voltages of "intermediate voltage" instruments for the observation of (small) biological organisms is thus obvious; it can distinguish a good micrograph from a micrograph with beam damage. An additional advantage of the better penetration power of the electron beam is also the better definition of subcellular details in "thick" specimens as was demonstrated with blood platelets. It should be noted that rather extreme defocus values (10-20 μm) have been used in combination with a small (10 μm) objective aperture to obtain the results presented in this paper. Under these conditions we do not use the full resolving power of the instrument. Instead, we use the instrument as an electron-optical phase-contrast microscope for the observation of cells in their native (no chemical fixation, dehydration or staining, whatsoever) state. In this approach it should be possibile to study dynamic events in (small) intact cells, using the time-resolution of vitrification (10^{-5} sec, see Bachmann and Mayer 1987).

Conclusions and Perspectives

The (cryo-)sectioning properties of tissue can be altered by partial dehydration and infiltration with cryoprotectants. Such a procedure (e.g. sucrose infusion of chemically fixed tissue) is more or less routine in immunocytochemical procedures. For the observation of cryo-sections in their "native" (partially dehydrated) state the addition of cryoprotectants is contraindicated. Cryoprotectants may increase the sensitivity of tissue components for electron beam irradiation, and cryoprotectants may alter contrast relations. Evidence for these effects of cryoprotectants was obtained with vitrified thin films with suspended material (collagen, phospholipid vesicles). Thus, if possible, the composition of a sample should be adjusted (e.g. dilute the suspension without cryoprotectants) to the requirements of cryo-electron microscopic observation. This is not always feasible, especially not when intact (thin) cells are to be studied. In these cases we recommend the use of higher acceleration voltages for cryo-electron microscopy. Because of the increase in mean free path length of the electrons, less damage is done to the specimen and subcellular details can be resolved even in rather thick (micron-range) specimens. Small cells grown in suspension and thin cells growing as a monolayer on a substrate can thus be vitrified entirely (in a time span in the order of 10^{-5} sec) and studied by cryo-electron microscopy without the need of sectioning. Spatial resolution and time resolution are both important aspects of vitrification. Both aspects are of equal importance when dynamic events in intact cells are to be studied.

References

Acetarin J-D, Carlemalm E, Kellenberger E, Villiger W (1987) Correlation of some mechanical properties of embedding resins with their behaviour in microtomy. J Electron Microsc Techn **6**, 63-79.

Bachmann L, Mayer E (1987) Physics of water and ice: implications for cryofixation. In: *Cryotechniques in Biological Electron Microscopy* (RA Steinbrecht, K Zierold, eds) Springer Verlag, Berlin, pp. 3-34.

Bahr GF, Zeitler E (1965) The determination of the dry mass in populations of isolated particles. Lab Invest **6**, 955-977.

Boutron P, Kaufmann A (1979) Stability of the amorphous state in the system water-1,2-propanediol. Cryobiology **16**, 557-568.

Brüggeler P, Mayer E (1980) Complete vitrification in pure liquid water and dilute solutions. Nature **288**, 569-571.

Burger KNJ, Knoll G, Frederik PM, Verkleij AJ (1990) Influenza virus mediated membrane fusion: the identification of fusion intermediates using modern cryotechniques. In: *Dynamics and Biogenesis of Membranes* (JAF op den Kamp, ed) Nato ASI series H40, Springer Verlag, Berlin, pp. 185-196.

Carlemalm E, Gravito RM, Villiger W (1982) Resin development for electron microscopy and an analysis of embedding at low temperature. J Microsc **126**, 123-143.

Carlemalm E, Colliex C, Kellenberger E (1985a) Contrast formation in electron microscopy of biological material. Adv Electron Electr Phys **63**, 269-334.

Carlemalm E, Villiger W, Acetarin J-D, Kellenberger E (1985b) Low temperature embedding with Lowicryl resins: to new formulations and some applications. J Microsc **140**, 55-63.

Chiu K, Downing KH, Dubochet J, Glaeser RM, Heide HG, Knapek E, Kopf DA, Lamvik MK, Lepault J, Robertson JD, Zeitler E, Zemlin F (1986) Cryoprotection in electron microscopy. J Microsc **141**, 385-391.

Dubochet J, McDowall AW (1981) Vitrification of pure water for electron microscopy. J Microsc **124**, RP 3-4.

Dubochet J, Lepault J, Freeman R, Berriman JA, Homo J-C (1982) Electron microscopy of frozen water and aqueous solutions. J Microsc **128**, 219-237.

Frederik PM, Klepper D (1976) The possibility of electron microscopic autoradiography of steroids after freeze drying of unfixed testis. J Microsc **106**, 209-219.

Frederik PM, Busing WM, Persson A (1984) Surface defects on thin cryosections. Scanning Electron Microsc 1984;I: 433-443.

Frederik PM, Stuart MCA, Bomans PHH, Busing WM (1989a) Phospholipid, nature's own slide and coverslip for cryo-electron microscopy. J Microsc **153**, 81-92.

Frederik PM, Stuart MCA, Schrijvers AHGJ, Bomans PHH (1989b) Thin film formation and the imaging of phospholipid by cryo-electron microscopy. Scanning Microsc Suppl **3**, 277-284.

Frederik PM, Stuart MCA, Verkleij AJ (1989c) Intermediary structures during membrane fusions as observed by cryo-electron microscopy. Biochim Biophys Acta **979**, 275-278.

Frederik PM, Stuart MCA, Bomans PHH, Busing WM, Burger KNJ, Verkleij AJ (1991a) Perspective and limitations of cryo-electron microscopy. From model systems to biological specimens. J Microsc **161**, 253-262.

Frederik PM, Burger KNJ, Stuart MCA, Verkleij AJ (1991b) Lipid polymorphisms as observed by cryo-electron microscopy. Biochim Biophys Acta **1062**, 133-141.

Forsyth M, MacFarlane DR (1986) Recrystallization revisited. Cryo-letters **7**, 367-378.

Glaeser RM, Taylor KA (1978) Radiation damage relative to transmission electron microscopy of biological specimens at low temperature; a review. J Microsc **112**, 127-138.

Hagler HK, Buja LM (1986) Effect of specimen preparation and section transfer techniques on the preservation of ultrastructure, lipids and elements in cryo-sections. J Microsc **141**, 311-317.

Hope MJ, Bally MB, Wiebb G, Cullis PR (1985) Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim Biophys Acta **812**, 55-65.

Luyet BJ, Rasmussen DH (1968) Study by differential thermal analysis of the temperature of instability of rapidly cooled solutions of glycerol, ethylene glycol, sucrose and glucose. Biodynamica **10**, 167-191.

Miller GA, Carpenter DK (1964) Phase diagram of the system methanol-water. J Chem Engin Data **9**, 371-373.

Stols ALH, Smits HTJ, Stadhouders AM (1986) Modification of a TEM goniometer specimen holder to enable beam current measurements in a (S)TEM for use in quantitative X-ray microanalysis. J Electron Microsc Techn **3**, 379-384.

Talmon Y (1987) Electron beam radiation damage to organic and biological cryospecimens. In: *Cryotechniques in Biological Electron Microscopy* (RA Steinbrecht, K Zierold, eds), Springer Verlag, Berlin, pp. 64-84.

Taylor KA, Glaeser RM (1975) Electron diffraction of frozen, hydrated protein crystals. Science **186**, 1036-1037.

Taylor KA, Glaeser RM (1976) Electron microscopy of frozen hydrated biological specimens. J Ultrastruct Res **55**, 448-456.

Vuillard G, Sanchez M (1961) Vitrification et cristallisation dans le système binaire eau-methanol (Vitrification and crystallization in the binary system water-methanol). Bull Soc Chim France **294**, 1877-1888.

Zeitler E, Bahr GF (1962) A photometric procedure for weight determination of submicroscopic particles. Quantitative electron microscopy. J Appl Phys **33**, 847-853.

Zeitler E, Bahr GF (1965) Contrast and mass thickness. Lab Invest **6**, 946-954.

Discussion with Reviewers

L. Edelmann: You stated that it can be questioned if cryosections, when observed in their native (not freeze-dried) state, are thin enough to reveal subcellular details. What is your interpretation of published micrographs of frozen hydrated cryo-sections which show subcellular details? Which experiments are necessary to convince you that a micrograph shows a fully hydrated cryo-section?

Authors: The data presented in this paper indicate that "concentrated" solutions of organic material are extremely sensitive to the electron beam. Furthermore the data show

that the electron beam has differential effects on various tissue components (e.g. lipid vesicles and collagen). Evidence is now accumulating (P.M. Frederik *et al.*, submitted) that at a given electron dose an aqueous matrix may show mass-loss whereas suspended material may show bubbling (or not yet bubbling). These data imply that the electron dose used to record the ultrastructure of a "fully hydrated" section should be kept as low as possible and carefully documented. The "development" of further contrast by electron irradiation should be followed as function of the dose and data used to extrapolate contrast to a zero electron dose. There are no data yet available (to our knowledge) which meet the criteria outlined above. We expect that other groups (Martin Müller, personal communication) may soon report such data and thus bring new impetus to cryo-ultramicrotomy of vitrified material.

J. Dubochet: Methanol (density < water) and glycerol (density > water) are used as cryoprotectant. Both reduce (or suppress) contrast. Will the authors please comment on this surprising observation?
Authors: The density of the vitrified matrix depends on the thermal history of the sample and in particular on the glass-transition temperature were the temperature-volume curve deviates from the liquid line to follow a course parallel to the temperature/volume curve of the crystalline phase (MacFarlane, 1987). The density of a vitrified solution can thus be modified by the degree of subcooling e.g. by 'fast' or 'slow' vitrification or by addition of cryo-protectant. We assume that the cryoprotectant solutions employed can lower the temperature of the glass-transition and thus lead to a higher density of the matrix with a suppressed contrast as a result.

J. Dubochet: Could you give quantitative values for the bubbling dose observed in non cryoprotected tissue and when cryoprotectant is added? The value for glycerol is of special interest.
Authors: Thin vitrified films prepared from glycerol (< 20% w/v) only will show mass-loss and no bubbling when observed at -170°C. Thin films prepared from higher glycerol concentrations may show bubbling depending on the cooling velocity (see answer to your first question), and the temperature of observation. (P.M. Frederik *et al.*, submitted). These data have implications for possible contrast 'development' by the electron beam when vitrified material is observed.

J. Dubochet: I wonder if the possibility of medium-high-voltage is not somewhat overemphasized. In a first approximation, the mean free path of the electron increases with $\sqrt{V}$. Going from 100-300 kV should therefore allow an increase of specimen thickness by a factor of 1.7. This is significant but not dramatic. Beam damage is reduced with increasing voltage; however, contrast is also reduced. In order to record the same signal to noise ratio, the dose must be increased with increasing voltage. Is there something to gain?
Authors: At 'intermediate' voltages intact vitrified specimen can be studied at the resolution of subcellular organelles. This conclusion holds for objects smaller (thinner) than one micron and thicker specimens can only be studied with a lower resolution. This still opens the possibility to study membrane dynamics in bacteria and platelets (Stuart *et al.*, in preparation) without the need of (cryo) sectioning.

J. Dubochet: Can the authors offer an explanation for the pairs of small inclusions ($\approx$ 500 Å diameter) in most of the bacteria (Fig 7.)
Authors: The dense spots are thought to be the result of the culturing conditions (N. Nanninga, personal communication).

E. Kellenberger: It is not clear what 80% of methanol with 20% PVP really means. Is there still some 15-20% of water?
Authors: In a 80% methanol solution 20% PVP is dissolved. PVP dissolves easily in a methanol solution and may bind some water which is nevertheless still part of the (viscous) solution.

E. Kellenberger: Table 1 needs some better discussions: E.g. discuss the curious fact that with a glass transition occurring at -161°C sectioning is possible at -120°C. (Melting point methanol -97.9°C).
Authors: The data reported on the glass-transition temperatures are usually obtained by differential scanning calorimetry using vitrified material that is analyzed during slow heating. For ultrastructural studies we mostly use high cooling velocities and the glass-transition may occur at a much higher (warmer) temperature (see also discussion with J. Dubochet). Therefore we think that we have sectioned vitrified material (containing methanol) although we cannot exclude the possibility that the "matrix" consists of methanol hydrate (also see footnote to Table 1).

E. Kellenberger: The bacteria show the well-known phenomena of plasmolysis. The two cells, upper left, are the only "normal" ones!
Authors: Plasmolysis is probably caused by resuspending of the bacteria (grown in nutrient rich medium) in physiological saline prior to film formation and vitrification. In recent experiments we could prevent plasmolysis by resuspending the bacteria in a low-salt medium (< 100 mosm).