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E. Kellenberger
University of Basel

E. Carlemalm
University of Basel

W. Villiger
University of Basel

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SEM Inc., AMF O'Hare (Chicago), IL 60666-0507, U.S.A.

PHYSICS OF THE PREPARATION AND OBSERVATION OF SPECIMENS THAT INVOLVE CRYOPROCEDURES

E. Kellenberger*, E. Carlemalm and W. Villiger

Department of Microbiology,
Biozentrum
University of Basel
Klingelbergstrasse 70
CH-4056 Basel, SWITZERLAND
Phone No: (061) 25 38 80

Abstract

With this introductory chapter we attempt a synthesis of old and new knowledge of the physical principles that govern cryomethods. Interface phenomena determine the increase or decrease of the number of particles observed in frozen-hydrated suspensions because they occupy the air-liquid interface according to their specific balance of hydrophobic versus hydrophilic properties. Biological macromolecules are surrounded by organised water, the hydration shell, that prevents them from sticking to each other. Partial or complete removal of these hydration shells by freeze-drying or freeze-substitution leads to collapses or aggregations. The solvent-induced aggregation is usually decreased by prior cross-linking with adequate chemical fixatives. A new finding is that aggregations are also decreased with lower temperatures. This allows us, for example, to preserve DNA-containing plasma from coarse aggregation even in cases where it has not been previously crosslinked.

When rapidly freezing a physiologically homogeneous population of bacterial cells without added cryoprotectants we find a 10-20 µm thick layer of cells without ice crystal formation. In deeper layers an increasing proportion of cells exhibits crystallization damage, although some cells are still well preserved.

Treatment of cells with aqueous solutions of OsO_4 and/or uranyl acetate leads to 10-20 % (w/w) heavy metal deposit. Direct staining of sections of resin embedded material results in 10 times more deposit. The location of these deposited metals is at first unknown and is best visualized through a comparison with totally unstained material. Sufficient contrast is achieved with the dark field, or the ratio-contrast mode of imaging. The latter has the advantage of giving less weight to thickness variations than does the former. When observing fully unstained thin sections by ratio contrast the influence of the surface reliefs is thereby virtually eliminated. With CTEM the dark field mode requires too great a dose to use it successfully on frozen-hydrated material. With STEM this is possible for both dark field and ratio contrast.

Key Words: Interface-denaturation, interface-phenomena, surface tension, ice crystal formation, aggregation in solvents, cryosubstitution, low temperature embedding, frozen hydrated specimen, ratio contrast, section staining artifacts.
*For reprints and other information, please contact E. Kellenberger at the above address.

Introduction

The freezing of samples containing water is involved in nearly all the cryo methods that we compare here. The influence of the freezing speed, the properties of the different forms of ice (vitreous, cubic = Ic, hexagonal = Ih) and their critical temperatures of transition (vitreous to cubic at 150°K) have been discussed by many authors (Hobbs, 1974; Franks, 1982; Mayer and Brüggeller, 1982; Plattner and Bachmann, 1982; Dubochet et al., 1983; Heide and Zeitler, 1984). The protocols of the different applications show numerous discrepancies and disagreements about the need for and the nature of cryoprotective additives, the temperatures of transitions, and the rates of cooling. The existence of real vitreous ice, for example, was contested until recently and is still questioned by many. The excellent results obtained recently (Adrian et al., 1984) begin to show that it is not very useful to prolong these theoretical disputes. What is important, after all, is the quality of the micrographs!

It seems that every type of specimen has particular requirements because of the temporal delays that affect both freezing (undercooling) and the transitions; their specific requirements are not yet predictable. The more one learns about obtaining good results through well controlled, reproducible experiments, the more other physical events emerge as influential: interface phenomena and drying, which occur before freezing particle suspensions, have again to be considered (as will be discussed later). When embedding by freeze substitution, the biological material is in an organic liquid at temperatures below 233°K and at 293°K with conventional methods. Biological macromolecules are in general not soluble in organic solvents and aggregations might be induced because dehydrated proteins and nucleic acids are very "sticky".

With both freeze fracturing and sectioning, the necessary mechanical events lead to plastic flow, and to additional distortions due to bending in the case of sectioning. With ice these distortions are still more disturbing than with resins.

There is a general tendency to avoid heavy metal staining if possible. Several observations suggest the existence of misleading staining "artefacts", e.g. the "double track appearance" of membranes. We discuss the means used for achieving sufficient contrast with unstained material by applying different imaging modes. Both dark field and ratio contrast provide very good contrast. When

using them in STEM, the dose can be kept as low or lower than with CTEM bright field.

We must also consider the ultimate limitation of beam-induced destruction and the possibility of reducing it by observing specimens at low temperatures (4-20°K, He or 80-100°K, N_2). The problems involved are discussed in the proceedings of two symposia (Zeitler, 1982, 1984). In the present contribution we only discuss them for cases where the beam damage interferes at the level of a visually interpretable micrograph (see "Imaging of unstained material and the problems of beam induced damages" below). We do not emphasize those problems which manifest themselves only through image processing and/or 2D-crystalline objects, despite the obvious importance of these modern techniques.

In the practical work with frozen-hydrated specimens, as we discuss below, the so-called "bubbling artefact" is the dominant limiting factor. (Dubochet et al., 1982). The results with CTEM indicated dose dependency of bubbling. In the STEM the dose-rate of the electron beam is very high. A priori it was thus not excluded that the dose-rate becomes equally or even more important a determinant than the dose. We show later that also in STEM bubbling turns out to be dose-dependent. The usefulness of the above-mentioned imaging modes, other than CTEM phase contrast can be exploited and will therefore briefly be discussed in the "Imaging of unstained material and the problems of beam induced damages" section.

Cryomethods in the preparation of particles from suspensions

The different methods in use are summarized in Table 1. There one also finds the very old method based on freeze drying (theory detailed by MacKenzie, 1977) which gave interesting results on viruses (Williams and Fraser, 1953) and glycogen (Arber et al., 1957). It was rapidly understood that frozen dried material had a strong tendency to show collapse phenomena (Kistler and Kellenberger, 1977). This is explained by thermal movements and the natural stickiness of dehydrated biological material (Anderson, 1954). Structures are brought into contact with each other through the thermal movements and then remain attached. Subsequently we observe collapses and aggregations (Kellenberger and Kistler, 1979). The section titled "Cryofixation, chemical fixation and solvent induced aggregations", explains why dehydrated macromolecules are sticky. A further phenomenon has been observed with air-dried macromolecules (Engel and Meyer, 1980) and could be described as a macromolecular explosion. We think that it is related, perhaps, to the wrapping phenomenon (Kellenberger et al., 1982). The supporting film wraps itself around about half the molecule, as far as elasticity allows. Upon complete drying the film tends to stretch itself leading to a disruption of the molecule as soon as the adhesions to the film are stronger than the cohesion forces within the molecule.

The success of high resolution microscopy of frozen dried particles is limited and only rarely can information which is as good as that obtained by negative staining be gotten (Engel et al., 1982). Freeze-drying provides invaluable help in mass determinations through the count of elastically scattered electrons in STEM (reviewed in Engel, 1982).

Collapses and related phenomena as described above should not occur when the biological material is sustained or supported by the surrounding ice. Glaeser's group (Taylor and Glaeser, 1976; Glaeser et al., 1979) was the first to observe frozen hydrated preparations with a conventional bright field electron microscope equipped with a liquid nitrogen cryostage. They prepared a thin layer of an aqueous suspension on a carbon film which was then quickly frozen by immersion into liquid nitrogen. They also investigated the presence and nature of ice by recording electron diffraction patterns.

A very interesting variation of this procedure was introduced by Dubochet and his group (Lepault et al., 1983; Adrian et al., 1984). These authors produced a self-supporting, very thin film of the suspension which they then froze by immersion into liquid ethane cooled by liquid nitrogen. Some of their results are shown in figs. 1 and 3. This method has potential advantages because an interface between the aqueous solution and the supporting film ("water-solid") is lacking and, at first sight, it appears as if only freely suspended particles would be observed. Personal discussions with J. Dubochet made it clear that the situation is not so simple. If one calculates the concentration needed for virus-size particles to form a good micrograph, one would need concentrations of about 10^{13} particles/ml. In practice one frequently gets beautiful preparations with 100 times less. According to Dubochet, this is true even when, during preparation of the film, drying is excluded. Dubochet also observed that under experimental conditions one needs more or less concentrated solution than theoretically calculated, depending on the nature of the particle. This curious behavior is again explained by the basic principles of interface phenomena as detailed in fig. 2. The interface between air and the suspension is enriched, or depleted of particles according to the distribution and relative amounts of hydrophobic and hydrophilic areas on the surface of the particle. During preparation of the liquid film, the air-water interface film would be retained on the specimen. Most of the remaining material would be sucked away.

This explanation could and should be verified experimentally. It is indeed well known to researchers working with particle suspensions in test tubes that interfaces with both glass and air are frequently very heavily "coated" with particles, particularly when these suspensions consist of proteins. It is also well known that proteins in suspension and reaching the air-water surface, become denatured (whatever that means). These interfaces are continuously renewed by shaking a suspension with air; the proportion of denatured particles (molecules) increases by "denaturation through foaming". The danger, herein, is that particles collected on such water-air interfaces and then observed in the frozen-hydrated state might not only be altered functionally, but possibly morphologically as well. It is well known, for example, that viruses easily lose their nucleic acid content through foaming. Similar phenomena might be ex-

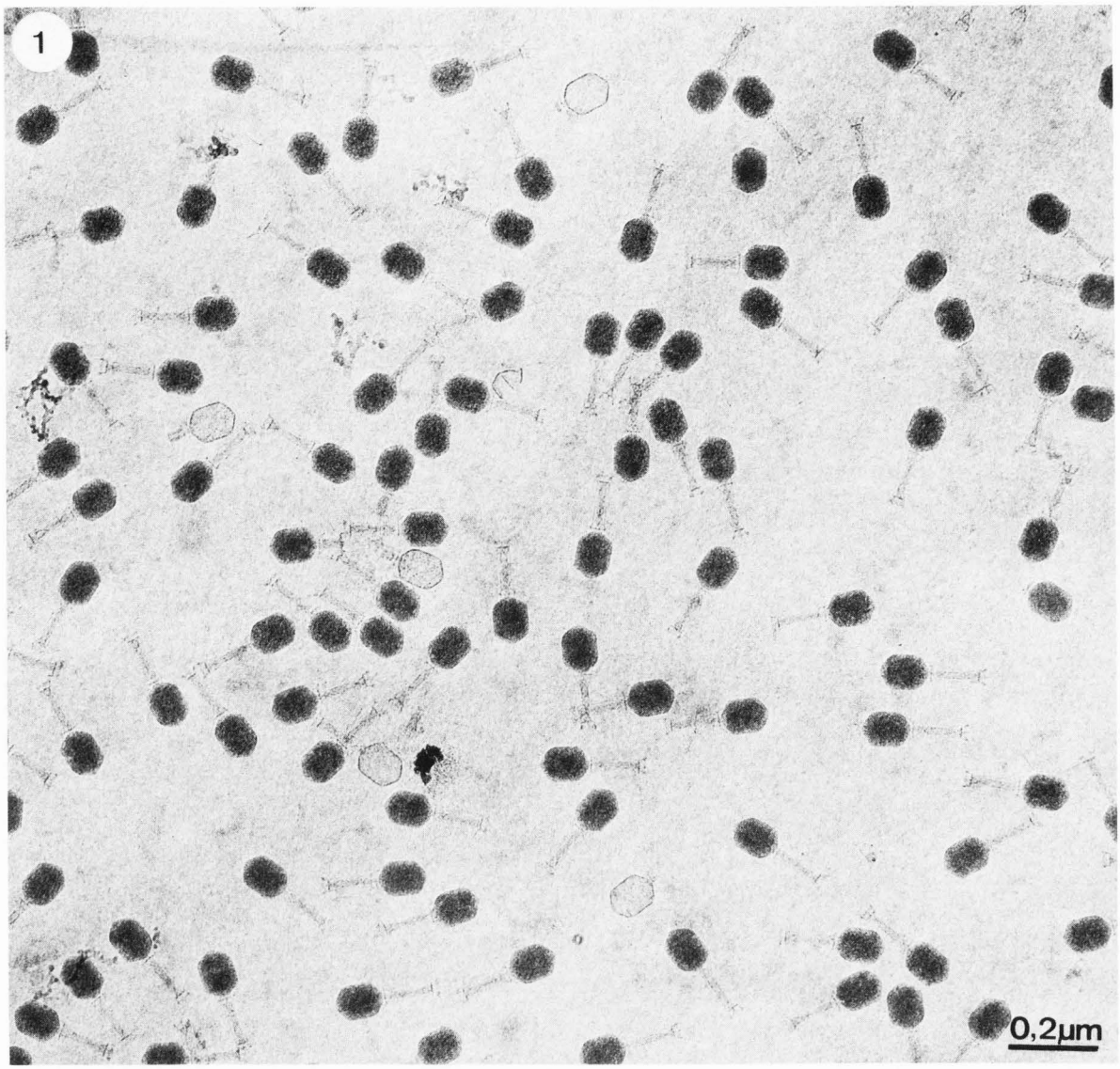


Figure 1. Frozen-hydrated bacterial virus T4, prepared by M. Adrian and J. Dubochet (EMBL, Heidelberg) without using a supporting film. A fine-mesh grid (or perforated film) is dipped into the virus suspension, withdrawn, and the excess liquid removed. It is then rapidly immersed into liquid ethane cooled by liquid nitrogen. The micrograph was taken by the same authors in a Philips 400 electron microscope with underfocus.

pected to occur during the preparation of the thin film of an aqueous suspension prior to freezing. It is also quite clear that during this period a slight drying occurs, with the concomitant increase in salt-concentration of the suspension medium.

With the possibility of deleterious effects due to the air-water interface, the difference between the older methods with and Dubochet's protocol using frozen-hydrated layers without supporting films may be less than imagined. Indeed, from time immemorial we have all used this interface to enrich particles by what we then called adsorption onto the supporting film. It was long suspected that in this water-solid interface, morphological alterations might occur, as exemplified in the complete flattening of tubular structures (Kellenberger and Kistler, 1979). Such an event would strongly depend on the respective surface charges of the structure and the supporting film. In cases of little or no adherence this flattening on the water-solid interface would not occur. This might be the cause of the relatively good preservation of the shape of viruses when prepared by agar-filtration (Kellenberger and Bitterli, 1976) or by spraying (Backus and Williams, 1950). It is clear from these examples that the well known air-water surface tensions will cause flattening as soon as the suspension medium is removed (e.g. by drying). Recently Brisson and Unwin (1985) made the observation that tubular structures prepared on a supporting film and observed frozen-hydrated, were flattened. This

Table 1

Preparation of suspended particles through cryoprocedures

Starting Material	Observation	Drawbacks	Advantages and/or uses
Quick-frozen layered on supporting film	Frozen-hydrated in cryo stage	Interface effects	Preservation (potential[1])
Quick-frozen as self-supporting layer	Frozen-hydrated in cryo stage	Interface effects	Preservation (potential[1])
Quick- frozen on supporting film and freeze-dried	Dry with or without shadowing	Collapses[2] and explosions[3]	Adequate for mass determinations and chemical analysis

[1] means that the advantage is in part a potentially better preservation. Numerical analysis of fine structure details have as yet not shown clearcut improvement over negative stain. The general shape, e.g., of virus, is however much better preserved. For discussion, see text.

[2] Kellenberger and Kistler (1979) / Kistler and Kellenberger (1977)

[3] Engel et al. (1982)

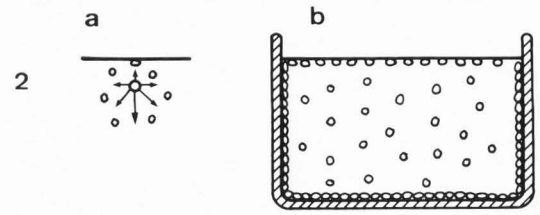


Figure 2. Interfaces of particle suspensions with air and solid (glass vessel or supporting film). In Fig. 2a the attractive forces acting on a solvent molecule near the surface are illustrated. The resultant force tries to pull the molecule back to the interior. This is impossible because the existing interface requires molecules to be there. Energy has to be supplied to occupy the surface positions; this is the surface energy or the surface tension. Because these interface energies depend on the substances involved, the interfaces are differentially occupied by particles from a suspension. This is illustrated by Fig. 2b, which shows a situation that might easily be encountered with viruses: the interface between liquid and air is occupied more densely than the suspension itself. The interface between solid and liquid (represented, for example, by a glass recipient) is likely to be tightly packed by the viruses giving a close packing). According to the nature of the suspended particles the situation may vary. It may be that the liquid-air interface is nearly unoccupied if it needs less energy to transport water to the surface than for particles.

observation might be a result of the above-mentioned flattening prior to freezing, but it could also be due to an extremely thin water film. Jean Lepault (EMBL, personal communication) has investigated the flattening of tubular structures when prepared frozen hydrated without support (fig. 3). Completely flattened tubular structures show a different type of diffraction pattern from the same lattice whilst the sample is still cylindrical. In the latter case, but not in the former, we have to consider Bessel functions. J. Lepault used polyheads, a well known tubular variant of the shell of the prohead of bacteriophage T4. Polyheads are cylindrical when observed intracellularly in sections, but most of them are completely flattened when prepared with negative stain on a supporting film (Yanagida et al., 1970). In frozen hydrated specimens (fig. 3) nearly all polyheads showed diffractograms of the type shown by cylindrical structures (fig.4). This excludes flattening at the interface with air, but does not exclude irregular cylinder deformation due to thermal agitation.

In conclusion, we should state that in frozen-hydrated specimens the interface forces also have to be considered. In some cases, (interface water-supporting film) their effects might start to be comparable to those due to the surface tension acting during air-drying. In most cases of water-air interfaces, however, we have justifiable hopes that they are much weaker. This is already indicated by the extraordinarily good preservation of the shape of viruses (Adrian et al., 1984) and also by information obtained by image reconstructions (Lepault and Pitt, 1984; Lepault and Leonard, 1985;

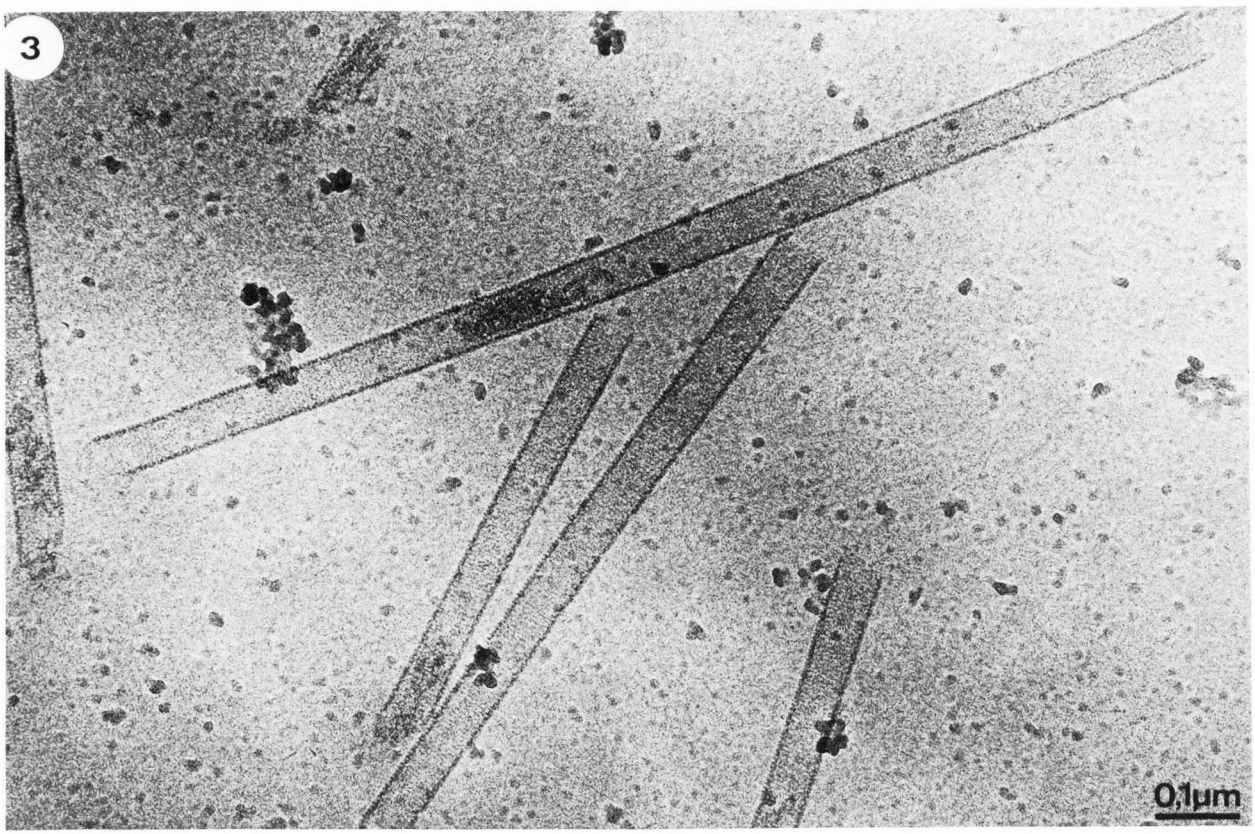


Figure 3. Tubular variant of the prohead shell of bacteriophage T4 ("polyhead") prepared by Dr. Jean Lepault, frozen-hydrated without supporting film. The micrograph is taken by the same author in a Philips 400, bright field at an underfocus of about 5 µm. Note the nice contrast. Preparations of this sort were used for diffraction as shown in figs. 4a, b(Courtesy Dr. Jean Lepault, EMBL Heidelberg).

Unwin and Ennis, 1984, see also "High resolution imaging on frozen-hydrated specimen from particle suspensions" section).

Preparations from bulk material

The freezing of bulk material

Our own studies (figs. 5,6, and 7) with frozen bacterial slurs without using cryoprotective agents have shown (fig. 6) a zone of 10-30 µm exhibiting no visible effects of ice crystal formation ("vitreous ice"). The depth of this zone depends strongly on the specimen and the freezing procedure used. The final result was assessed from the degree of preservation versus distortion of the bacterial cells after cryosubstitution . We are not yet sure whether the temperature, either 4°K or 90°K, of the coolant is the determinant. We have, however, the conviction that with copper block cooling at 4°K the vitrification penetrates to about twice as deep as with pentane immersion cooling. It is obvious that such small zones of correct preservation are problematic for the sectioning of tissues and large cells; not to speak of cryofracturing.

In Fig. 6 we also see some of the typical artefacts associated with freezing. A slight drying is very common during the preparation of the material for freezing. Bacteria are excellent indicators, because the slightest increase in the concentration of suspension medium results in a plasmolysis, i.e. a shrinkage of the protoplast so that it becomes detached from the outer layers of

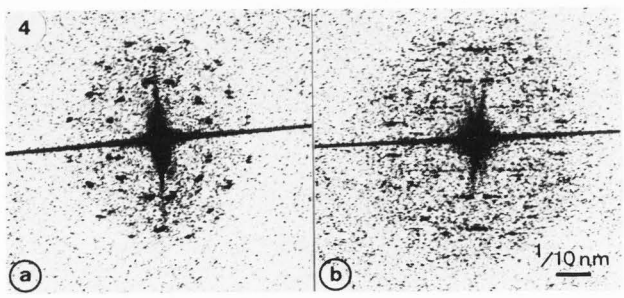


Figure 4. Diffraction from tubular prohead variants as shown in fig. 3. These studies were made by Dr. Jean Lepault (EMBL Heidelberg).
a) A relatively flattened particle exhibits the expected diffraction pattern which shows no equatorial spreading of the spots according to Bessel functions.
b) Non-flattened particles, as the major part of a frozen hydrated suspension is revealed to be, shows the equatorial spreading of the diffraction spots according to Bessel functions, as is expected for non-flattened tubes.

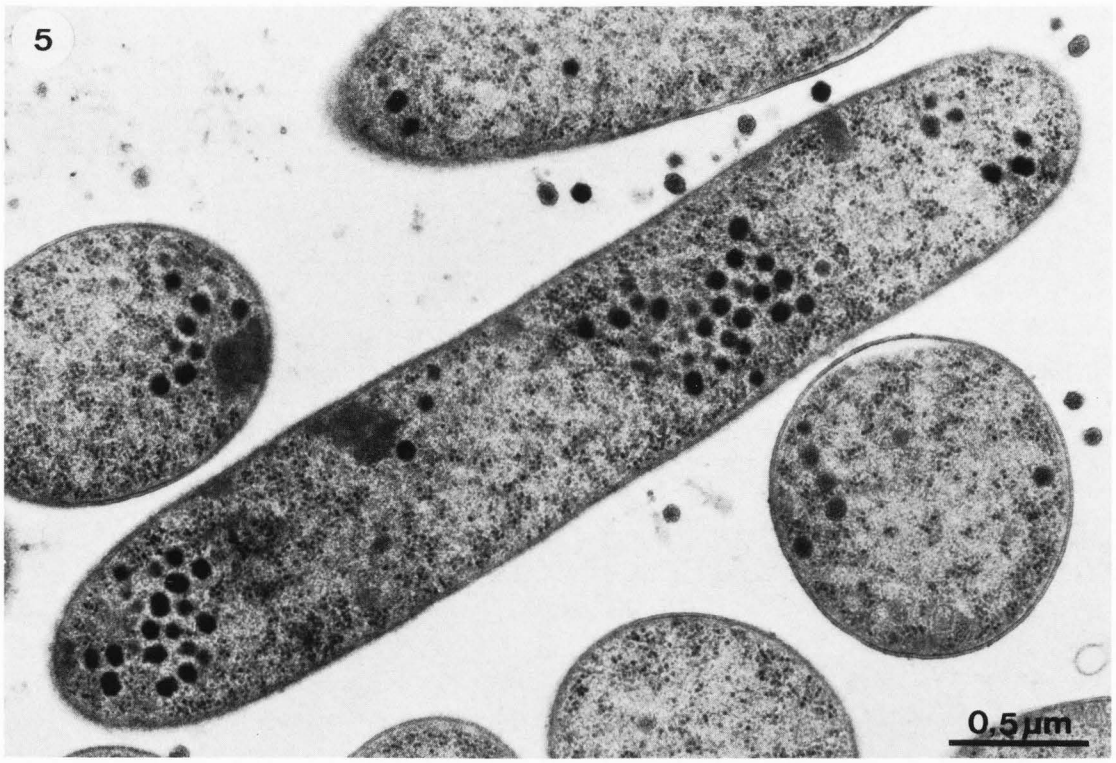


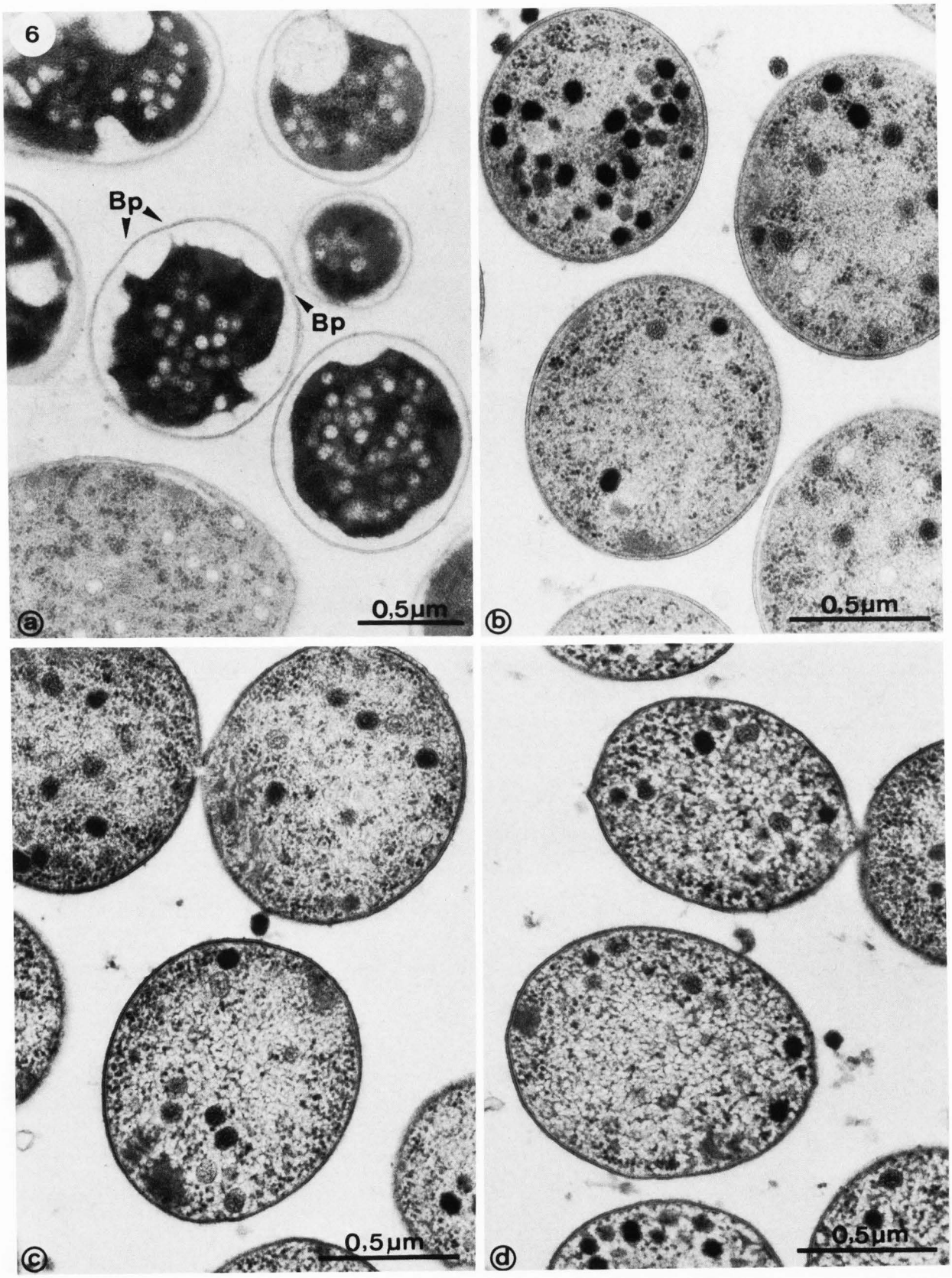
Figure 5. Shows phage T4 infected cells about 60 minutes after infection. Prepared by Cryosubstitution of a "rapid-frozen" sample (on helium cooled copper block). It was cryosubstituted at 188°K into acetone containing 1% OsO_4 over a period of 62 h. Later infiltrated into Epon, polymerized at 65°C and then sections were stained with uranyl acetate and lead citrate.

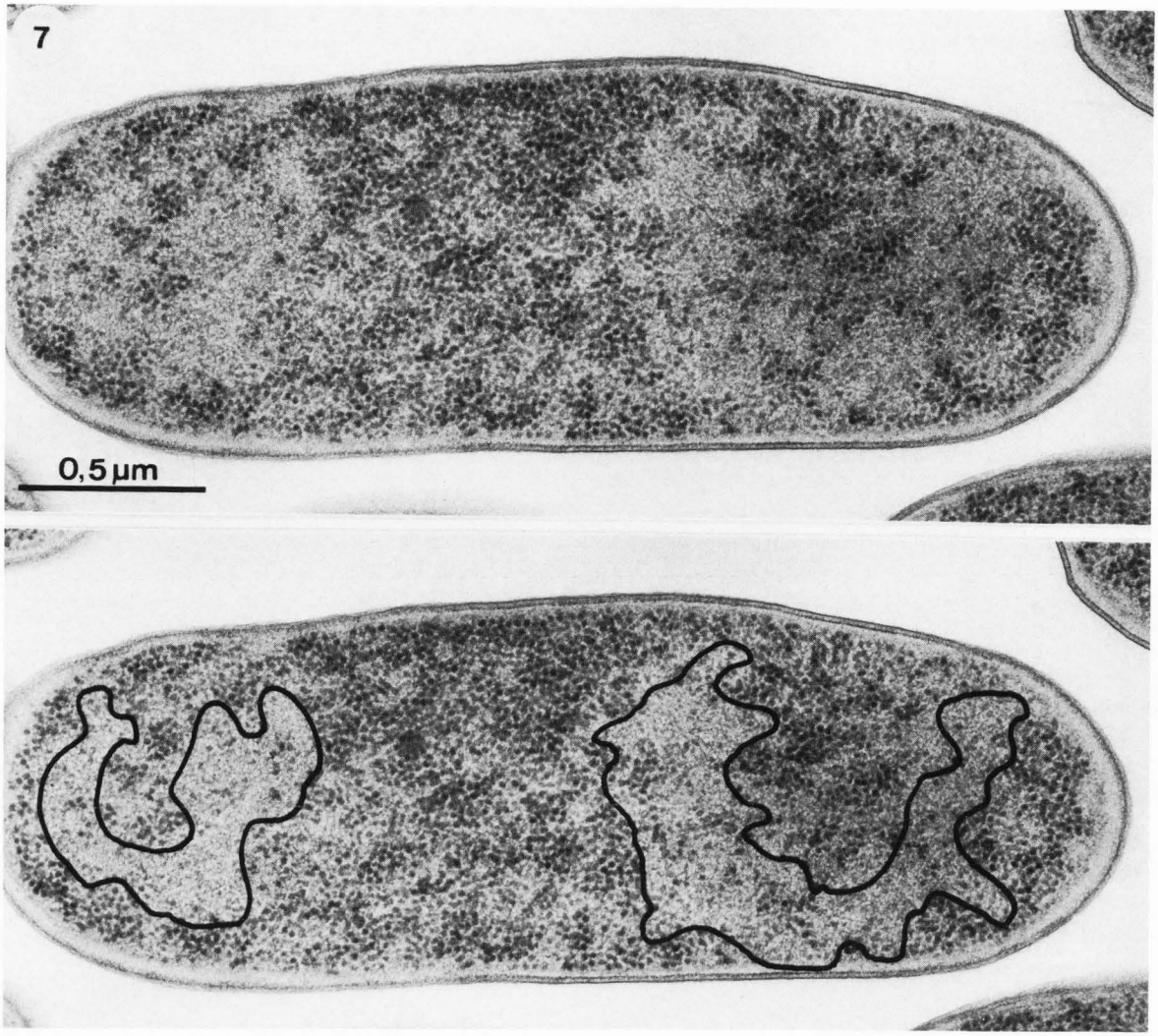
the cell envelope (Fig. 6a).

In experiments in which we did not use cryoprotective additives we followed the preservation of the bacteria from the coolest place to more distant parts and found a layer varying from 10-30 µm in which the cells were uniformly well preserved. Deeper inside, a non-homogeneous population appears; well preserved cells (fig. 6b) are found mixed with cells exhibiting typical intracellular ice formation. Progressing further inside, the size of ice crystals increases, and the number of well preserved cells decreases to nil (Fig. 6c). It is interesting to note that the heterogeneity of the population of well preserved cells intermixed with cells showing ice-crystal damage is absolutely reproducible, while the depth of the layer showing only well preserved cells is variable from experiment to experiment.

In addition to the two above-mentioned methods of freezing, namely "slamming" of the probe onto a helium cooled block (Heuser et al., 1979; Escaig, 1982) and immersion cooling (Costello and Corless, 1978), a third method has been very successful. This is the so-called "cryojets" method where two jets of liquid nitrogen cooled propane are directed from two sides onto a specimen (Müller et al., 1980). Finally, we should also mention the method of rapid freezing under the very high pressure of 2.1 kbar (Riehle and Höchli, 1973; Müller and Moor, 1984). Under these conditions vitreous ice should be preferentially formed. Due to the expense of the

Figure 6. The same T4-infected cells, as in fig. 5, shown in cross sections, harvested at different distances from the helium cooled copper block used for freezing, (figs. 6 b,c,d) or after slight drying between harvesting the cells and freezing (fig. 6a). 6a) shows cells having suffered different degrees of plasmolysis due to drying and the subsequent increase in the concentration of the medium. "Bayer bridges" (Bp, arrow heads) can be seen when the plasmolysis is very strong; they are not detectable at all with slight plasmolysis (Hobot et al., 1985). The cell at the lower left has probably become permeable so that the plasmolysis was reversed. However, it could also be that the pumping of K has already compensated the increased outside osmotic pressure.
6b) Some 10-20 µm from the copper block all cells are well preserved and show no signs of ice crystal formation, either outside or inside the cells.
6c) About 30-50 µm from the copper block the population shows a heterogeneous response ranging from no visible damage to more or less visible signs of ice crystal formation.
6d) Still further away from the "heat sink" we have predominantly damaged cells, though to a variable degree. Very few well preserved cells are still found, suggesting very variable delays in the onset of crystal formation.


6
Bp
Bp
0,5µm
a
0,5µm
b
0,5µm
c
0,5µm
d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0,5 µm

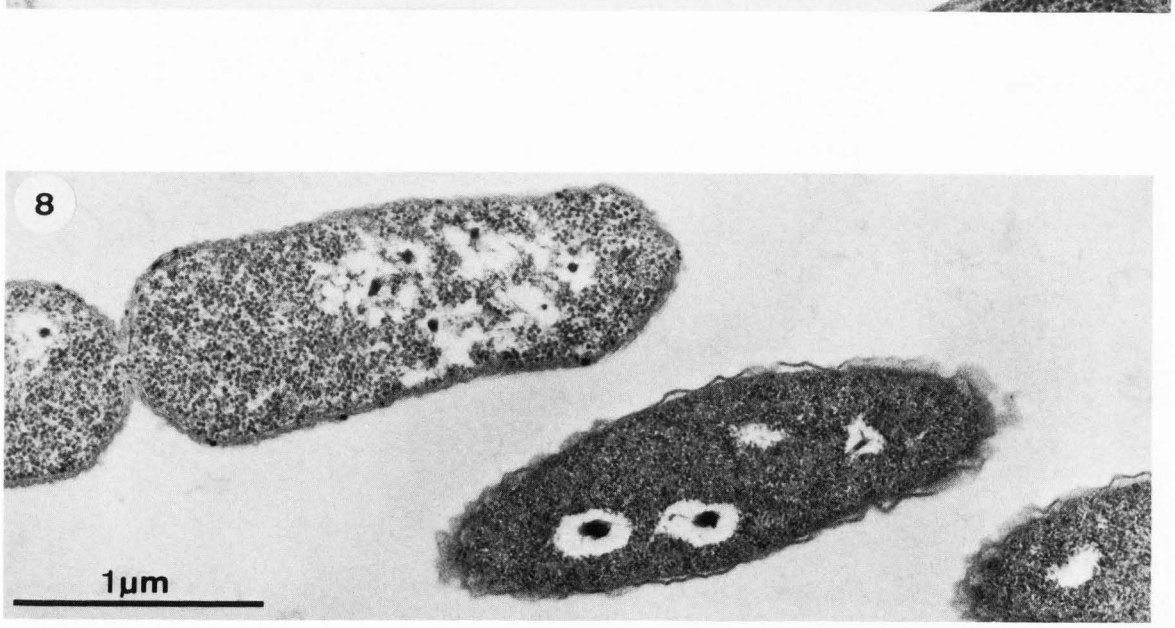

8
1µm

Figure 7. Cryosubstituted E. coli cells showing a new aspect of the bacterial nucleoid (Hobot et al., 1985). After rapid-freezing on a copper block cooled by liquid helium, substitution was done in an 0.1% OsO_4 acetone mixture at 188°K followed by embedding in Epon. The ribosome-free space is filled with rather grainy stuctures interdispersed with fibrous elements. The shape of this space has a cleft appearance. It has been drawn in for clarity in the (identical) micrograph at the bottom. This new aspect of the bacterial nucleoid is always seen, irrespective of the salt concentration of the growth medium. A change was previously observed when using osmium fixation and standard embedding techniques. The supposedly improved preservation of the bacterial DNA-plasm should now allow us to carry out immunolabelling studies of the protein partners of DNA by embedding into Lowicryl K4M at 238°K following the initial substitution step.

←

Figure 8. A mixture of E. coli B cells fixed with glutaraldehyde, 1%,120 min, room temperature (left) or OsO_4,1%,120 min, room temperature (right). After mixture the cells were "classically" dehydrated and embedded in Epon. The sections were stained with uranyl acetate and lead citrate.

Note the aggregated DNA of the nucleoids which appears as black particles in an empty "vacuole". According to the size of this "vacuole", the amount of DNA varies and gives aggregates of different sizes. According to the level of slicing the vacuoles appear as empty on many sections.

Note also the differential preservation of the ribosomes. They can really be compared here due to the constant section-thickness.

For specialists in bacterial nucleoids, it is interesting to see the different shapes after the two fixations.

→

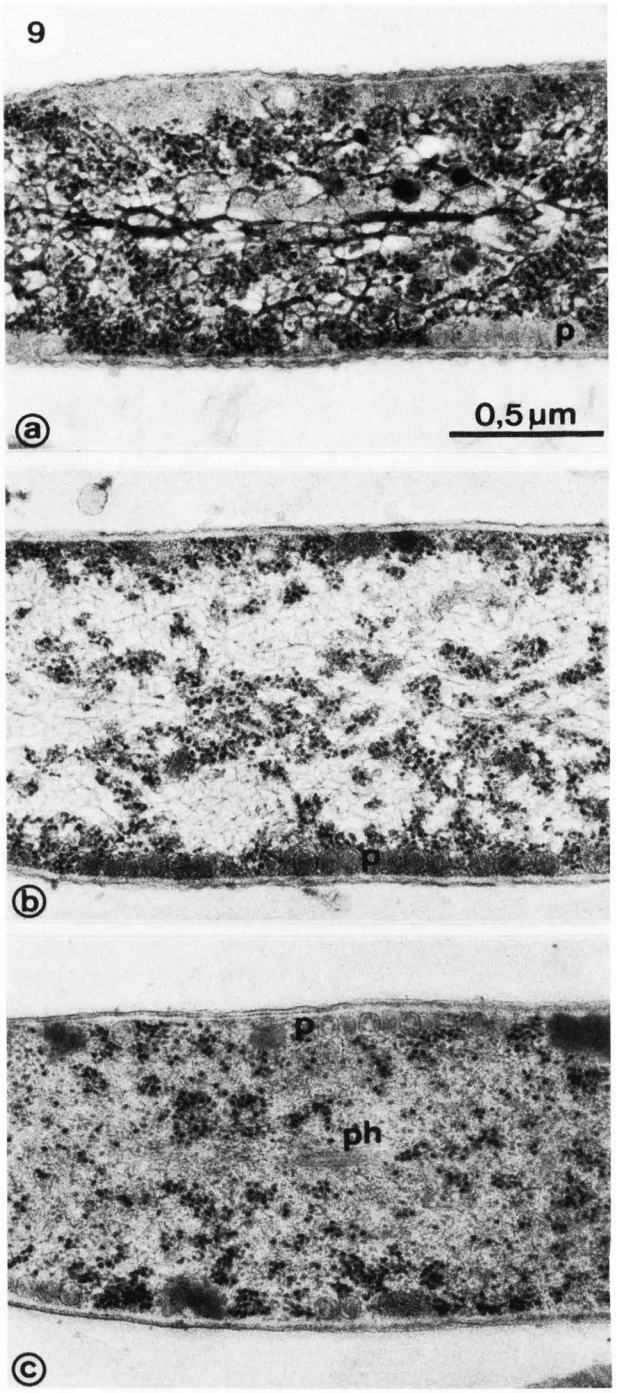
Figure 9. - E. coli cells after infection with a mutant (21⁻) of bacteriophage T4 that is arrested at the prohead (p) stage. Polyheads (ph) are also seen in some cells. The replicating phage DNA ("vegetative pool") is represented as coarse strands in (9a), already finer strands in (9b) and so fine as is nearly undetectable in (9c). Same magnification for 9a,b and c.

The individual preparation of the three samples were different:

9a) Fixation in 2% glutaraldehyde for 60 min. afterwards "classically" treated at room temperature by dehydration in ethanol and embedding with Epon. The sections were stained with uranyl acetate and lead citrate.

9b) In this case, after the same fixation, the sample was dehydrated by the procedure of "progressive lowering of the temperature" (PLT) and then embedded in Lowicryl K4M. The sections are stained as in 9a.

9c) In this case the sample was not fixed but rapidly frozen by the slam procedure at liquid He temperature, substituted into acetone containing 2.5% OsO_4 at 190° K during 64 h and then Epon embedded, stained as in (9a) and (9b) above.



necessary equipment, the experimental evidence is not yet sufficient to definitively establish the superiority of high pressure methods over others. Rapid freezing will be discussed in several places in these proceedings.

The further processing of the frozen blocks

In Table 2 we have summarized the methods in use. Either we fracture the frozen blocks and prepare shadowed replicas of the fracture surface as discussed later in this volume or we process the

Table 2

Preparation of bulk material through cryomethods

Starting Material	Treatment	Observation	Drawbacks	Advantages and/or Practicability
A quick-frozen	freeze-fracture, etching, shadowing and replication	of shadowed replica	immunolabel complicated; plastic flow, limits of shadowing	observation of surfaces and middle planes of membranes
B quick-frozen	direct sections	frozen-hydrated in cryostage[1)]	plastic flow and enormous distortions more than 100 nm thick; no labelling possible	excellent as check for cryo-substituted material (see C and D) no organic liquid ever involved
C quick-frozen	cryosubstitution[2)] and embedding into high temperature resin (Epon)	resin embedded sections[1)]	plastic flow and distortions possible temperature denaturation	easy to get very thin sections for relatively high resolutions
D quick-frozen	cryosubstitution[2)] and embedding into low temperature resins	resin embedded sections[1)]	plastic flow and distortions	easy to get very thin sections for relatively high resolutions. Ease of labelling on-section
E quick-frozen	directly sectioned and melted; possibly reembedded in thin layers (labelling in between)	reembedded	same as B above, plus possible collapse and rearrangement during melting and reaembedding	should provide theoretically the highest efficiency of labelling
F quick-frozen	freeze-dried and resin embedded	resin embedded sections	possible rearrangement and collapse due to freeze-drying and re-immersion into organic liquid	particularly suited for chemical mapping[3)]
G fixed (aldehydes)	dehydrated with progressive lowering of temperature (PLT) and embedded in low temperature resins	resin embedded sections[1)]	same as D but with chemical fixation	relatively fast and particularly suited for colloidal gold labelling

1) These methods, when completed totally unstained with ice or adequate resins, are potentially suited for improvement of resolution and contrast when imaging by ratio-contrast. For discussion see text.

2) Cryosubstitution might be done with a solvent containing chemical fixatives. The main advantages of cryosubstitution are lost if the material is fixed before freezing. In these cases method G is faster and provides comparatively good results (Exception: DNA plasms, see text). Immunolabelling is not possible after Os-fixation. With long aldehyde fixation the labelling efficiency decreases.

3) According to the resins used, labelling is obviously also feasible.

block into thin slices. For sectioning we distinguish direct cryosectioning and cryosubstitution. Other methods, like the progressive lowering of temperature are useful in many cases. Each of the procedures has its own advantages and disadvantages as are listed in table 2. Those procedures which allow for both good preservation and specific heavy metal labelling, e.g., immunolabelling with protein-A-gold, are much appreciated today. Unfortunately, immunolabelling is not possible on frozen-hydrated cryosections while it is easily feasible with high efficiency on Lowicryl K4M resin embedded section. The tissue or cells are embedded either by cryosubstitution or by the protocol of "progressive lowering of temperature" (PLT). As far as we know, cryosubstitution gives preservation comparable to direct cryosectioning. With both methods the periplasmic gel of gram-positive bacteria has been shown, the non-existence of mesosomes demonstrated, and a new characterization of the nucleoids achieved (Dubochet et al., 1983; Hobot et al., 1984; Hobot et al., 1985).

Recently described higher order structure in the insect flight muscle obtained by frozen hydrated cryosections (McDowall et al., 1984) justifies hopes that the predictions of better preservation through freezing become further substantiated.

Cryofixation, chemical fixation and solvent induced aggregation

It has long been known, but not widely recognized, that the DNA-plasm filling the bacterial nucleoid is not fixed by common fixatives and, therefore, it aggregates during dehydration (Fig. 8). With prior cryofixation, however, this DNA-plasm is now a mixture of fine fibrillae and grains (Fig. 7). Previously a fine fibrillar aspect was obtained by fixatives that are able to gel

concentrated DNA solutions, such as uranyl acetate, indium chloride, potassium permanganate and OsO_4 under RK conditions (Kellenberger et al., 1981). In this context it is interesting to note that glutaraldehyde treated cells (with unfixed nucleoids) showed aggregates which were finer as the temperature of dehydration was lowered. With PLT, the aggregates are already finer (Fig. 9b) than at room temperature (Fig. 9a), but not as fine as after cryosubstitution (Fig. 9c). An important result is that aldehyde or OsO_4 treatment prior to freezing and substitution does not lead to aggregates, establishing definitively, that aggregation occurs during dehydration. They are not induced directly by the fixative, but are due to a lack of adequate cross-linking as is indicated by macroscopic gelation. We conclude that dehydration at low temperatures, when done by cryosubstitution, does not lead to coarse aggregates. In other words, the aggregation in solvents is finer when the temperature is lower.

These observations allow very optimistic predictions for cryofixations without subsequent aldehyde or osmium fixation in the organic liquid. Since aggregation in DNA-plasms is virtually absent at low temperatures, one has to hope that the same might be true for proteins and nucleoproteins.

In living systems, as well as in their aqueous solution, nucleic acids and proteins are hydrated. Water molecules are strongly bound to the molecules and form what is called a "hydration shell". These prevent aggregation, "sticking", when two molecules approach each other. When the water is partially replaced by a water-miscible organic solvent, both species of macromolecules precipitate out of solution when the organic solvent reaches a 40-60% concentration. It is believed that the hydration shells of the macromolecules are removed by the solvents and that the now "naked" molecules have the tendency to stick to each other by virtue of weak interactions (van der Waal forces) so that aggregations occur. Why should the size of aggregates decrease at low temperature? Only one explanation is logical. The removal of hydration shells is in general slowed down by lower temperatures. Differences of hydration, according to the hydrophobicity of different surface areas, lead to greater differences in the speed of these removals. The areas which were "naked" first, would be "sticky" and allow a loose gel to be built up before the whole surface of each molecule becomes dehydrated and thus sticky. Once such a gel is formed, the established cross-links would no longer allow for stronger, more compact aggregation, even if the overall "stickiness" is increased.

Changes of folding of proteins and possible staining artefacts

In an aqueous environment the folding of a polypeptide chain into a protein occurs in such a way that a maximum of hydrophilic amino acid residues are on the surface of the protein while the hydrophobic ones are located inside. If a polypeptide chain becomes folded in an organic solvent, i.e. in an essentially hydrophobic environment, the situation would be reversed.

What is likely to happen when a protein is transferred from an aqueous medium into an organic liquid as we currently do when embedding? In principle, the polypeptide chains should change their folding, reorienting the residues. The protein would change conformation. At room temperature this change will be extremely slow because the folded chains are held together by a compact three-dimensional array of weak interactions. The thermal energy fluctuations certainly allow the opening of such interactions to occur singly and transiently but almost never would several occur together within the same area. Such simultaneous openings, however, are a necessary condition for rearrangement. Such events occur much more frequently at higher temperatures. From SDS-mediated changes we have learned that a few proteins already change conformation at room temperature, but nearly all molecules are changed at temperatures approaching 373°K.

What predictions can be made with respect to embedding? First, we can safely state that conformational changes will be slowed down when temperatures are lowered and secondly, that high temperatures, such as those involved in the curing of Epon (333-343°K), might change the conformation of many proteins by turning the hydrophobic parts outward.

It has been shown, some time ago, that hydrophobic areas of sectioned proteins are not stained by uranyl acetate (Garavito et al., 1982). The presence of matter, however, was demonstrated by ratio-contrast.

We therefore propose the following hypothesis: the apparent absence of matter is not necessarily due to extraction of proteins, as broadly believed, but more often is this lack of visibility due to a conformational change which concomitantly leads to hydrophobicity and thus to a lack of stainability. This hypothesis can easily be tested by ratio contrast which reveals matter without staining.

Plastic flow and other distortions associated with freeze fracture and sectioning

Freeze fracturing and sectioning are both essentially cleavage. A cleavage results from "pulling" matter apart. In material sciences the phenomena that are at the basis of cleavage are well known and described as follows: when a sample of matter is pulled apart with a force F and the resulting elongation measured, we obtain the results shown in Fig. 10. In a first stage the elongation is proportional to the force applied and is fully reversible; we have the elastic phase. The second stage, plastic flow, is characterized by an elongation which is no longer proportional to the force and no longer reversible. The probe is locally strongly deformed. This plastic flow continues until rupture occurs suddenly. After this, the elastic part of the elongation and deformation reverses, but not that due to the flow. The ranges of each of the two stages and the limits of rupture are specific for the matter considered. Something brittle, for instance, has only a very small range of plastic flow. Exactly the same phenomena occur during cleavage. Blocks composed of biological material embedded in ice or resins are non-homogeneous and, therefore, the ranges of responses are different for each component. It is thus obvious that after rupture, an irregular relief appears which only partly reflects the structure of the embedded material. The deformations of the cleavage surface of each component become more pronounced as the plastic flow character of the

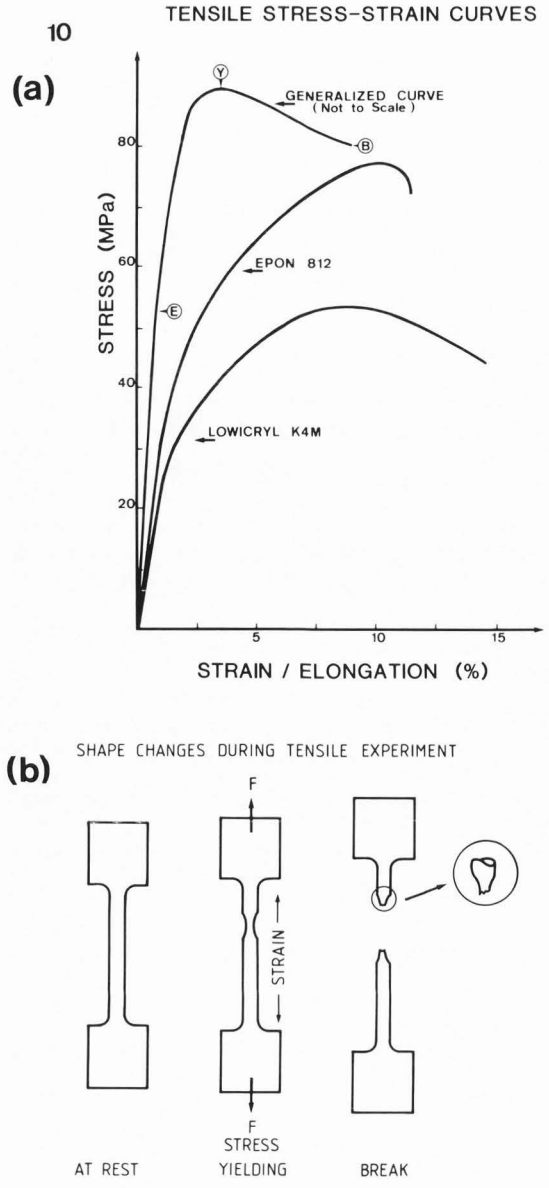


Figure 10. Elasticity and plastic flow in material sciences. Samples as depicted in (10b) are submitted to tension (F). The resulting elongation is then plotted as a diagram (10a). With the uppermost curve we show the generalized behaviour of spring steel. The elastic part is very long (up to E). After the elastic limit (E) additional plastic deformation occurs. In this phase the deformation is only partly reversible. At the yield point (Y), the material starts to flow and then breaks (B). After the point Y, the sample shows the typical, irreversible constriction (10b) resulting from flow. Afterwards, rupture occurs without producing any neat surface. Such broken surfaces are thus heavily distorted (see fig. 10b circled enlargement and fig. 11).

The experimental curves for resins of fig. 10a are from work of Dr. J.-D. Acetarin of our laboratory, to whom we are grateful for the complete figure.

matter under consideration increases.

With cryofracturing one is fortunate. It happens to be that biological membranes like to cleave in their middle. In contrast however ribosomes, and the like, are barely recognizable, and the results are grainy, rough surfaces.

In cryosections the situation is very interesting. Verkleij's group (Lennissen et al., 1984) has found that the surface of the block shows a relief, as expected for freeze fracturing. The surface of the section, however, has been smoothed. Has another deformation occurred which has led to a transient melting?

We should now consider, in more detail, the sectioning process itself. For the basics we have to refer the reader to Sitte (1983). In "normal" microtomy the resin-section after cleavage is bent and slides over the knife to float on the water of the trough. The water seems to lubricate the sliding, and only a little compression is observed. With ice, one has to cut dry, and consequently much compression and distortion occur. Recognizing the causes should allow a systematic approach to technical improvements (trying to find lubricants, reducing the knife angle, etc.). For resin-sections it had been proposed to coat the knife surfaces e.g. by tungsten (Roberts, 1975). We found indeed with this procedure some improvement as was also found by incorporating some camphor into the resin (but not by virtue of camphor also being a plastifier!). Analogous tricks should be tried for cryosections. The success of such efforts seems to us a sine qua non for the introduction of cryosectioning as a routine procedure.

Resin sectioning is certainly considered as an established routine today. We think, nevertheless, that improvements are still possible, mainly by attempting to reduce the radius of curvature of the bending of the sections. It is presently not possible to affirm that this bending occurs solely on the grounds of elasticity and therefore involves only reversible deformations. It is not excluded that the situation is worse, and that flows of matter do occur. If this is true and cannot be improved, there is little hope of ever being able to extract high resolution information from thin sections in a reproducible manner (see below).

Chemical mapping and specific labelling on thin sections

Cytochemistry on thin sections is presently one of the centers of interest because it allows us to bridge the gap between the enormous amount of information gained in biochemistry, and the morphological knowledge accumulated by electron microscopy. Three approaches are presently explored: (i) specific cytochemical reactions like the Feulgen-analogue osmium-ammine stain for DNA (Gautier, 1976), (ii) "chemical mapping" e.g. of calcium, magnesium, phosphorus (Harauz and Ottensmeyer, 1984) and of other biologically essential elements, and (iii) specific labelling, for example with antibodies, lectins and toxins (Roth, 1982, 1983).

Chemical mapping is currently done on the low resolution level by analysing the X-rays generated by a scanning electron probe. The energy loss of the transmitted electrons is correlated with the generation of X-rays. With STEM, the transmitted electrons might be dispersed according to energy by an adequate spectrometer. In CTEM equipped with a filter lens (Castaing and Henry, 1962; Ottensmeyer

and Andrew, 1980), one can use the electrons of a specific energy loss for imaging and, in principle, map the distribution of an element within a specimen. In practice this is somewhat more complicated because the specific energy losses are "buried" in a large background of electrons with non-specific losses ("Bremsstrahlung"). The sensitivity of the method is therefore substantially decreased. Because the concentration of the elements of interest are in general extremely low within biological structures, we should not overemphasize the potential of the method. An additional complication is due to the fact that sections have a relief (see below) and show thickness variations that easily produce differences in the amount of unspecifically scattered electrons which are comparable with, or even larger than, the expected peak of element-specific electrons. This problem can be avoided by ratio-contrast, where the influence of the thickness variations can be eliminated by dividing the inelastic signal by the elastic signal (as explained in "Ratio-contrast allows for a relief-independent, purely matter and concentration dependent imaging" section).

With respect to specimen preparation, there are two requirements for chemical mapping : (a) the resin used for embedding should not contain large amounts of the elements under consideration and (b) during preparation no movement or disappearance of these elements should occur. Freezing and chemical mapping on frozen-hydrated sections would be the ideal solution to these problems (Zierold, 1984; Somlyo and Shuman, 1982) if the electron doses needed for higher magnifications were not so high as to produce "bubbling" and eventually melting of the sample. Unfortunately this makes it necessary to return to resin sectioning. The procedure with which the least movement of elements is to be expected is that of freeze-drying the sample, followed by its direct immersion into the monomeric resin (see this volume). Obviously surface tension effects occur when the interface of the organic liquid enters the labile, freeze-dried structure. By capillary effects, loose bundles of fibers, for example, might become compacted and aggregated. Other examples of deleterious effects might be easily imagined. The practical experience gained in many laboratories showed, fortunately, that for lower magnifications one does not have to be too afraid of these possible artefacts. If required, they could even be substantially reduced by applying a (reversed) critical point procedure.

Protein-A-gold labelling of thin sections with specific antibodies, lectins and toxins (Roth, 1982, 1983) has been found to be quite efficient with Lowicryl K4M. Current embedding media, like Epon, are less adequate unless their surface is first chemically etched (Baskin et al., 1979). Indications also exist that low temperature procedures significantly increase the efficiency of this on-section labelling (Armbruster et al., 1983). Presently, resins of the same type but allowing work with still lower temperatures, have been designed (Carlemalm et al., these proceedings).

As a procedure that leads to adequate embedding and sections, one in general chooses mild glutaraldehyde fixation and the progressive lowering of temperature (PLT) for dehydration (see Carlemalm et al., this volume): the temperature of each step of dehydration is kept at a sufficiently low level so that the block entering this step will not have ice crystals formed within it. It is obvious that nothing speaks against using cryosubstitution instead of PLT, if it is not that the latter is so much faster and easier. In favor of cryosubstitution is the potential of being able to leave out all chemical fixations (see "Cryofixation, chemical fixation and solvent induced aggregations" above) and thus to gain possibly another increase of sensitivity in immunolabelling.

The reasons why sections with K4M are so much more suited for protein-A-gold labelling are supposedly two-fold. First, the chemical nature of K4M fortunately allows the unspecific background to be kept extremely low. Secondly, the antigenic sites are available and still active. When the cleavage surface follows the interfaces between the resin and the biological structures, then antigenic sites become exposed. If the resin adheres very strongly to the biological material, then the cleavage might go through the resin instead of the interface. This behaviour is likely to be linked with known properties of these resins; Epon is a representative of the epoxy family to which the best glues or cements known belong. Indeed, Epoxy will form covalent bonds with biological matter. This is not the case with the methacrylates and the other acrylates contained in the Lowicryls. These fundamental differences are indeed reflected in the surface relief of the sections (fig. 11) which are smoother in Epon than K4M.

Immunolabelling should obviously be much more efficient on melted cryosections than with sections of resin embedded material. Assuming that a 5 nm layer is accessible to antibodies on a K4M section, the cryosection being 100 nm thick, then a factor of 20 should be gained. Unfortunately this gain has not yet been demonstrated with a given set of antigens and antibodies.

The technique of antibody labelling of melted cryosections was pioneered by Tokuyasu (1973). By using procedures of re-embedding after immunolabelling the collapses due to surface tension on drying were progressively overcome. Re-embedding with L.R.-White-resin has recently given a beautiful result (Keller et al., 1984). One has to be aware, however, that the efforts and skills needed with cryosections are far higher than those involved in labelling of resin-embedded sections. As already mentioned, a major effort has to be made to improve cryomicrotomy.

Imaging of unstained material and the problems of beam induced damages

High resolution imaging of frozen hydrated specimens from particle suspensions

An interesting discovery was made whilst observing frozen hydrated, fully unstained material. In contrast to carbon films, the ice film did not show the characteristic "holographic" noise when observed in high coherence imaging (so called phase contrast). Contrast can therefore be increased photographically because it is no longer the signal to noise ratio that is limiting the detection of details (i.e. the "image resolution"). This fact allows one to obtain very good micrographs from

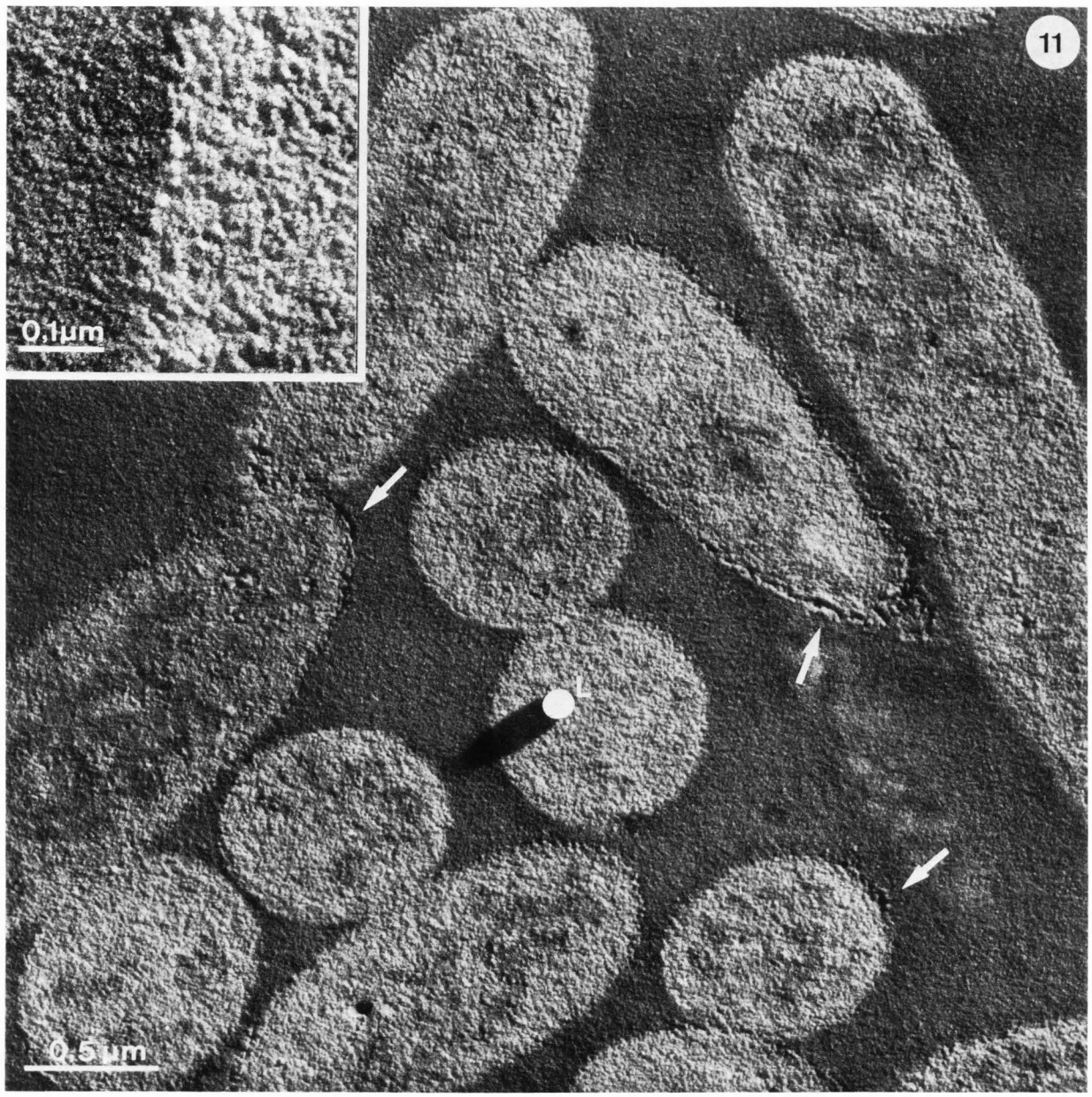


Figure 11. Cleavage surfaces of thin sections. E. coli embedded in Lowicryl HM20 has been sectioned with utmost care to about 40 nm thickness. The slices were then shadowed with tantalum/tungsten at an angle of 15°. The relief is clearly visible, particularly also in the inset of higher magnification. It shows the roughness inside and outside of the cell. Particularly strong reliefs are found at membranes as is indicated by arrows. Polystyrene latex sphere (L) shows the shadowing direction.

fully unstained, frozen-hydrated specimens. Unfortunately it is necessary to underfocus considerably. The contrast-transfer function (Hanszen, 1966; Thon, 1966) allows analysis of the size of details that are shown on a micrograph as a function of defocussing. Unfortunately this function tells nothing about the precise location of this detail in respect to the object itself. An additional Fresnel transformation would have to be introduced to accomplish this. It is clear, therefore, that the obtainable image resolution is again limited by the required underfocussing.

At present, all reports on averaged crystalline structures obtained from frozen-hydrated material do not show much higher order diffraction than those obtained from negatively stained specimens (Lepault and Leonard, 1985; Unwin and Ennis, 1984). It is, however, well understood that obtaining high-order spots is not always a proof for a faithfully preserved specimen. High-order

spots can also be produced by "sharpening" artefacts due to drying. Any abrupt change of density in a regular, well-ordered specimen will produce high orders. They do not demonstrate the presence of real details of this size. When each unit cell is changing its conformation into another specific one (e.g. by denaturation), the observed structure might be full of details, but still not represent the functional state we are interested in. In short, it may well be that a frozen-hydrated specimen is more faithful than a conventionally prepared one, even if the latter shows higher orders of diffraction.

We must also not forget that the resolution is necessarily lower with underfocussing, as explained above. Other modes of imaging should therefore be investigated, as is further explained.

Dark field imaging with CTEM needs more than 50 times as high a dose than bright field. This is a consequence of the photographic recording and efficiency of collection. With STEM, dark field imaging is possible with the same minimal doses as with bright field and CTEM. Dark field imaging gives extremely good contrast and unstained, freeze-dried small particles, like myosin, are depicted easily with high resolution and at very low doses (Walzthöni et al., 1984).

These possibilities of non-conventional imaging should be carefully investigated. This is difficult with the very low number of correctly equipped STEMs (presently only two in the world!). They should have a cold stage, if possible with tilt, a good spectrometer and should be able not only to take dark field pictures, as such, but also to record normalized ones, based e.g. on the simultaneous signals of scattered and unscattered electrons. In addition, imaging based on the ratio between elastically and inelastically scattered, low energy loss electrons, has potential advantages as discussed in the next section.

A major problem associated with the scanning mode is the question of whether the beam induced "bubbling artefact", that occurs on frozen-hydrated specimens (Dubochet et al., 1982) is entirely dependent on the dose or if the dose rate (= intensity) has some influence. Recent unpublished experiments with W. Tichelaar at EMBL (Heidelberg) show that the bubbling artefact occurs with STEM as soon as a critical dose is reached, as has been seen with CTEM. The "bubbles" are as large as with the latter, and they increase in size by regular steps after each successive passage of the scan. If these observations are confirmed for various materials then the excellent contrast achievable with dark field and ratio-modes might also be extremely useful for frozen-hydrated material.

We have shown that the ratio contrast responds much more strongly to added heavy metals than does dark field and brightfield (Carlemalm et al., 1982, 1985; and Carlemalm et al., this volume). A reversed ("negative") contrast effect was produced by incorporating some tin (Sn) into a resin. Similarly, with a frozen-hydrated specimen of particles an adequate amount of heavy metal containing solutes added to the suspension medium should, according to predictions, produce a strong contrast. If present in concentrations much lower than needed for high coherence imaging in bright field, their addition might not significantly increase the bubbling artefact.

Ratio-contrast allows for a relief-independent, purely matter- and concentration-dependent imaging

We learned in previous sections that cleavage involved in sections leads, at least with resin embedded material, to a surface relief on both sides which we estimated to be of the order of 5nm with the Lowicryls, and about 2-3 times less with Epon (Carlemalm et al. 1985, Armbruster et al., 1984). Because of plastic deformations caused by cleavage, the relief reflects the structures of the embedded material very poorly. Conventional imaging, dark field or bright field, depends essentially on differences of density ρ or of specimen thickness x. Calculations have shown that the relief on unstained sections is primarily responsible for the image contrast obtained (Carlemalm et al. 1985). The density differences between resins and biological material are too small to produce contrast. This situation changes as soon as the sections are stained, e.g. with uranyl acetate, which penetrates several microns into embeddings. The large amount of stain deposited changes the density drastically (Carlemalm et al. to be submitted).

We have shown that the contrast produced by the ratio of elastically scattered electrons over inelastically scattered, low energy loss electrons (LEL), is relatively independent of thickness variations. The contrast of organic material mainly reflects its hydrogen content (Carlemalm et al. 1985). Thin sections imaged by ratio contrast, therefore emphasize the information due to the material inside the section, having negligible dependence on the topographic relief. It is not astonishing, therefore, that ratio-imaged sections give much more precise information than dark field images (Fig.12; see also Carlemalm & Kellenberger, 1982). Ratio-imaging is thus ideal for unstained sections embedded either in resins or ice. It might also be useful for frozen-hydrated suspensions, although here we cannot predict its virtues as compared to simple, normalized dark field imaging.

Another application of interest would be to embed biological material in heavy metal containing resins (Carlemalm et al., 1982). Tin containing resins have been developed which allow one to follow previously outlined low temperature procedures (Carlemalm et al., this volume). The particular interest of this resin is illustrated by the possibility of determining intracellular concentrations of matter through use of ratio-contrast (Reichelt et al., 1985).

It might be mentioned here, that ratio contrast also allows for high contrast imaging with thick layers of"sustain" containing only minute amounts of heavy metal (as e.g. auroglucose and uranyl acetate diluted with sucrose). The relatively thick, about as thick as the specimen, layer allows excellent preservation of the shape of a virus (Fig. 13).

How far is it possible to reduce radiation damage by low temperature electron microscopy?

A few years ago experiments were reported where the observation of a specimen at 4-20°K would reduce its radiation sensitivity by approximately 100 times. This claim was weakened (Lepault et al., 1982) because the estimates were obtained by inade-

quate experiments. In the meantime other experiments with more suitable specimens led to an estimate of a "protection factor" of about 10 fold (for references see Zeitler, 1982, 1984). These estimates were always obtained from diffractions by observing the loss of high-order spots. It is well known that such a fading might be the consequence of disordering the crystalline structure as a whole and/or of variable conformational changes or variable damages to the matter in single unit cells. A measure of cryoprotection through a crystallographic approach is certainly a prerequisite for high resolution work on crystalline specimens. It might be of little relevance, however, for direct imaging. Ad hoc estimations are needed here to learn about beam induced deformations. Indeed, experiments suggest that thin sections become reduced to about half of their original thickness due to deterioration from interaction with the beam (Bennett, 1974; Crowther, 1984), although it is by no means established that a corresponding mass loss occurs.

Experiments are urgently needed here in order to find out which limitations are dominant in thin sections: beam induced distortions, deformations due to sectioning or staining artefacts.

There are not, at this time, sufficient studies comparing results obtained with cryostages at liquid nitrogen temperature (90°K) and cryolenses at 4-20°K due to the lack of available instruments. The stage in the supraconductive cryolens of Dietrich et al. (1977) does not yet allow screening of present biological specimens. The supraconducting cryolens of Jones et al. (1985) in a STEM constructed at EMBL in Heidelberg, where a conventional STEM with LN_2 cryostage also exists, should allow comparisons of the relative damages by beam-specimen interactions at these two temperatures (90°K, 4-20°K). It is hoped that these tests will be performed in the near future to see if all these new developments involving cryotechniques really improve the image resolution to approach the instrumental resolution. Even if this turns out not to be presently possible, we should already be pleased by the improvements in preservation due to cryotechniques in the field of sectioning, where these techniques have greatly improved the possibilities of specific labelling and chemical mapping.

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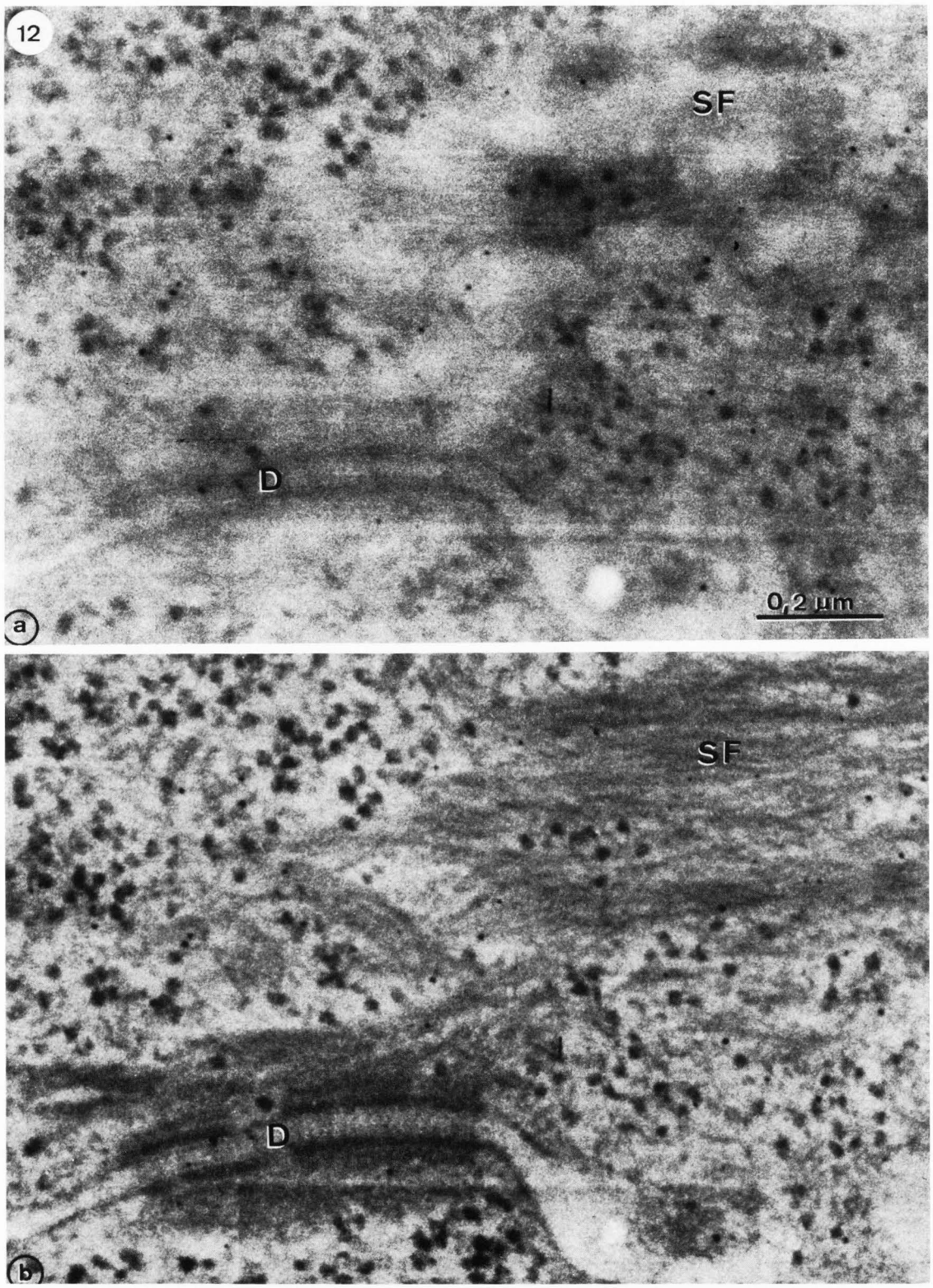
Figure 12. STEM micrographs of completely unstained *) thin sections of rat skin (D = desmosome, SF = stress fibers) at N_2-temperature. The rat was perfused with a mixture of 3% formaldehyde / 0.1% glutaraldehyde for 6 min and pieces of rat skin were postfixed in the same solution for 70 min by immersion. After dehydration in ethanol and embedding in Lowicryl K4M by the PLT technique, thin sections were made and mounted on 400 mesh copper grids. The unstained*) sections were then transferred to a VG HB5 STEM equipped with a cryostage.

Both micrographs are taken at the same correct focus; (12a) in the dark field mode and (12b) in the ratio mode (Carlemalm et al., 1985). While the ribosomes are about equal in both cases, although less sharp in (12a), the stress fibers (SF) are only visible in (12b). We explain this lack of sharpness by the very strong influence of the relief in (12a), which essentially determines the contrast, while in the ratio mode, the influence of the relief is very small and the material inside the section contributes mainly towards contrast. Since the surface is plastically deformed (see figs. 10 and 11) the dark field picture is "unsharp", while the other appears much clearer.

Note that we have to distinguish two levels of staining. The first occurs as a side product during fixation with OsO_4 or uranylacetate in aqueous medium and is between 10-20 weight percent (Carlemalm et al., to be submitted). A second deposit of stain is achieved by the on-section stain with uranyl- and lead salts. As judged from the resulting contrast, it is about 10 times more than the first deposit (Carlemalm et al., 1985 and to be submitted. OsO_4-fixed material is unfortunately called "unstained" by many people (Arsenault and Ottensmeyer, 1983). This has given rise to serious misunderstandings in the interpretations of ratio-contrast, where we use material that was not stained at all. We obviously cannot exclude heavy metals being present incidentally, in vivo or through buffers.

These pictures were taken in collaboration with Dr. W. Tichelaar, EMBL Heidelberg.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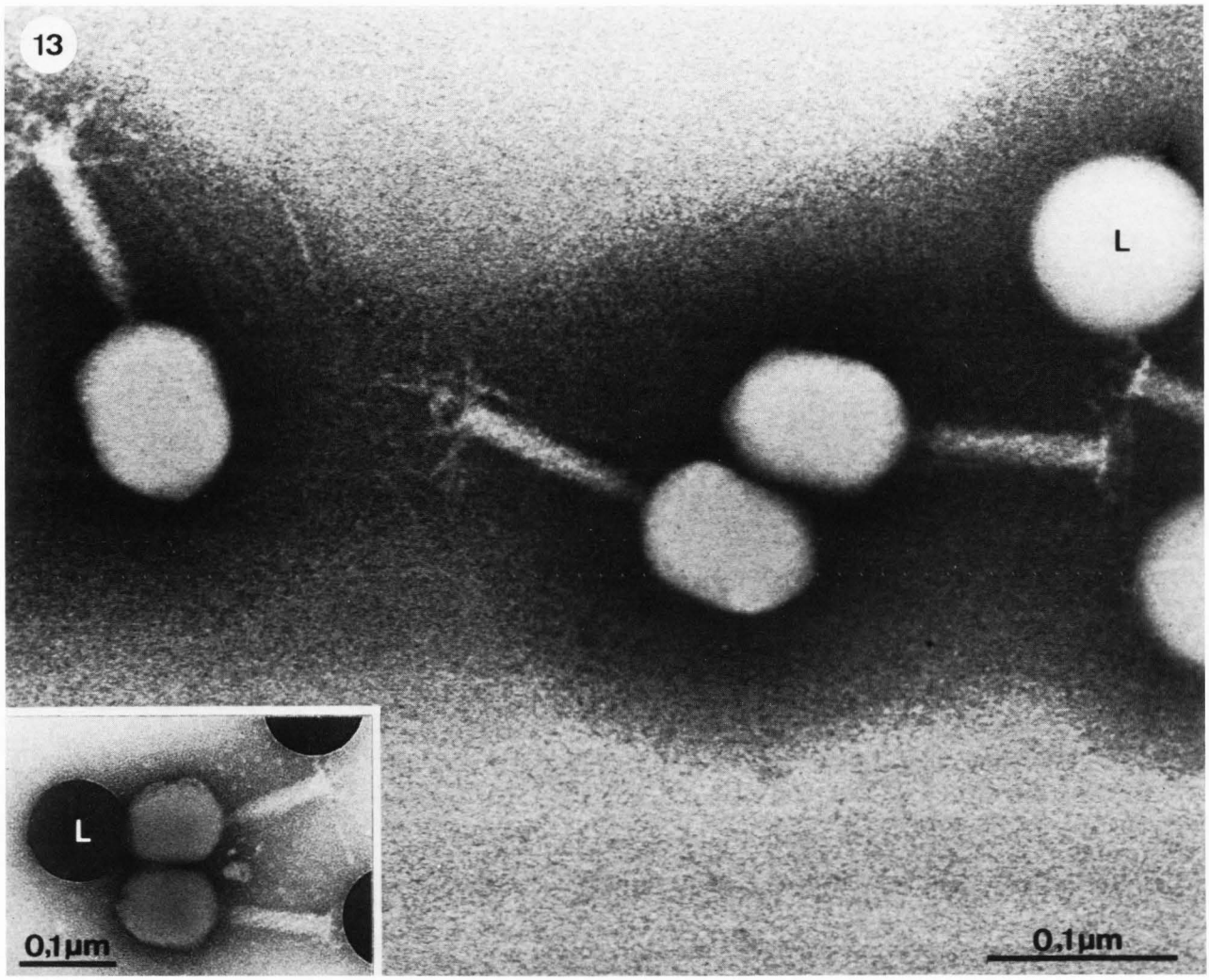

Figure 13. Bacteriophage T4 prepared in uranyl acetate diluted with sucrose. The same types of specimens were observed by ratio contrast and conventional bright field microscopy (inset). The visual difference in contrast is even stronger than the photographic presentations. By virtue of its viscosity a high content of sucrose in a negative stain leads to a thicker and more regular layer of stain. Such a thick surrounding layer protects the structure, at least partly, from collapses. The procedure is therefore frequently designated as "sustain" to distinguish it from pure (negative) stain. This procedure was used for measuring the (correct!) dimensions of viruses. With CTEM the resolution is poorer than with thin layers of concentrated stain without sucrose. The ratio contrast is much more sensitive to the presence of heavy atoms and therefore the negative contrast is much higher than with conventional imaging. Accordingly, the resolution of fine details is also increased.

Note also the differential contrast of the polystyrene latex spheres (L) in the two imaging modes: the ratio contrast depends for organic matter without heavy metals, on the hydrogen content. The scattering decreases with increasing hydrogen content. In conventional imaging the contrast is density (g/ml) and thickness dependent and thus explains the different response in the insert.

Micrograph taken by M. Wurtz and M. Häner of our laboratory.

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