FREEZE ETCHING OF CELLS WITHOUT CRYOPROTECTANTS

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ABSTRACT

The technique of spray-freeze etching was applied to unicellular organisms. The superior freezing rates obtainable with this method gave excellent cryofixation on *Chlorella*, *Euglena*, and spermatozoa without the use of antifreeze agents, and cell damage due to ice crystal formation was never observed. In many instances the resultant morphology differed significantly from that obtained from glycerol-treated, freeze-etched cells. Furthermore, viability studies of spray-frozen *Chlorella* compared favorably with cells frozen by other methods.

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INTRODUCTION

One of the most serious problems in freeze-etching is the production of artifacts during cryofixation since the freezing rates obtained by the present standard methods are too slow to keep all colloidal and solute material in a true "lifelike" distribution. Gross cell damage, due to the formation of large ice crystals, has, to a large extent, been bypassed by the application of cryoprotectants, such as glycerol or dimethylsulfoxide. However, rearrangement or segregation phenomena still occur when cryoprotectants are used, albeit on a smaller scale. This is best illustrated by the partitioned appearance of freeze-etched solutions of glycerol, sugar, colloids, etc. (Moor et al., 1961; Riehle, 1968; Schmitt et al., 1970). Furthermore, cryoprotectant media themselves may have a toxic effect on the cell (Moor, 1964; Fineran, 1970). In addition, considerable change in membrane properties, as revealed by enzyme assays (Misch and Misch, 1967; Graham and Pace, 1968; Pribor and Nara, 1969), and alterations of the fracturing patterns of both artificial (Buckingham and Staehelin, 1969) and biological membranes (Plattner et al., 1969; Fineran, 1970) have been reported.

Recently, two freezing procedures have been introduced which promise the possible elimination of cryoprotectants. These are the high pressure method (Riehle, 1968; Moor and Riehle, 1968; Moor and Hoechli, 1970) and the spray-freeze-etch method (Bachmann and Schmitt, 1971 *a*, *b*). Both of them have already proved to yield superior freezing rates as judged by the greatly decreased segregation of glycerol-water solutions. Spray-frozen solutions of ferritin, dextran, polyvinylpyrrolidone, etc. gave freeze-etched pictures practically devoid of particle agglomeration, contrary to standard freezing procedures.

The spray-freeze-etch method has the advantage that it requires no elaborate equipment and thus can be applied in any laboratory with a freeze-etching apparatus. The purpose of the present study was to investigate its applicability to unicellular biological specimens and to see whether or not it could become a useful alternative to freeze etching with cryoprotectants.

Three different cells were used: *Chlorella*, *Euglena*, and spermatozoa. *Chlorella* was chosen as a rather rugged organism, the electron microscope

morphology of which is well known from ultrathin sections after chemical fixation, and acceptable pictures of which have been obtained by freeze etching of glycerol-treated but unfixed specimens. For *Euglena*, on the other hand, glycerol has proved to be very toxic, and previous attempts at freeze etching have so far resulted in heavily distorted cells (Schwelitz et al., 1969; Holt and Stern, 1970). In addition, *Euglena* was also thought to represent a cell type more sensitive to mechanical damage by spraying. Bull spermatozoa were chosen since their high viability after slow freezing in the presence of cryoprotectants is well known, and the morphology of such slowly frozen specimens has already been investigated by freeze etching. It was expected to find characteristic morphological differences caused by the two extremely different types of cryofixation.

MATERIALS AND METHODS

Cell Material

Chlorella pyrenoidosa (strain 211-8b, algal collection of the Pflanzenphysiologisches Institut, Universität Göttingen) was cultivated autotrophically suspended in the medium of Kuhl (1962) at room temperature under continuous illumination (6000 lux; Philips fluorescent tubes: 3 × TL 40 w—$1/32$ and 2 × TL 40 w—$1/55$). The cultures were permanently aerated with oil-free compressed air.

Euglena gracilis (wild type strain 1224/5125 from the Botanisches Institut, Universität München; isolated by Dr. G. Richter, Universität Tübingen) was cultivated under mixotrophic conditions, suspended in the medium of Hutner et al. (1956), and supplemented as indicated by Kratz and Myers (1955). All algal cultures were handled under sterile conditions.

Spermatozoa were obtained from breeding bulls. No diluents were used for preparation by spray freezing.

Application of the Spray-Freeze-Etching Technique

The rationale of spray-freeze etching is to increase the cooling rate, as compared with the standard method, by spraying small droplets (approximately 10 μ diameter) of the specimen into liquid propane at −190°C. In a cryostat kept at −85°C, the propane is evaporated and the remaining minute frozen particles are suspended in butylbenzene, a liquid which melts at −89°C. Drops of this particle-butylbenzene suspension are transferred onto precooled Balzers specimen mounts (Balzers AG, Balzers, Liechtenstein) which are then thrown into liquid nitrogen. Thus, specimens are formed which can be handled for freeze etching in the usual way. It should be emphasized that this technique has nothing to do with freeze substitution, since none of the organic media used can penetrate into the frozen aqueous suspensions. A detailed description of the procedure is given elsewhere (Bachmann and Schmitt, 1971 *b*).

The cellular suspensions were concentrated, before spraying, by very gentle centrifugation. The cells should occupy about one-third of the suspension volume in order to yield enough cells in the replica. The freeze cleaving was done in a Balzers BA 360 M unit at −100°C. The etching time varied between 30 sec and 2 min. After the replication the samples were immersed in acetone in order to dissolve the butylbenzene and then transferred onto water with a pipet. The cells were then removed as usual by floating the replicas on 70% sulfuric acid for several hours. Since replicas of single cell material break rather easily, mounting on collodion films and reenforcement by carbon evaporation was often found advantageous.

Treatment of Controls

CHLORELLA: The glycerol treatment was performed according to the procedure used by Staehelin (1966): glycerol was added to the cultures to a final concentration of 20% (v/v), and samples were taken after 1 and 2 hr, respectively. Cryofixation of these specimens was performed by the "standard method," i.e., approximately 1 mm^3 of the suspension was placed on a commercial Balzers gold-specimen mount and dropped into liquid Freon-12 at −158°C. Glycerol-treated cells were also frozen at a slower than standard rate by immersion into liquid nitrogen. Untreated material (i.e., without glycerol) was frozen in Freon by the standard method. Finally, original cultures, without glycerol, were fixed in potassium permanganate and processed by conventional ultrathin-sectioning technique.

EUGLENA: Controls of original and glycerol-treated cultures were prepared by the standard freezing method.

SPERMATOZOA: The controls were treated according to the procedures commercially used for artificial insemination by freeze-stored semen. They were incubated for 4 hr at 4°C in a Tris–citric acid–buffered medium containing egg yolk and fructose and having a glycerol concentration of 6.5%. Cryofixation was done by slow freezing (approximately 20°C/min as practiced by Koehler (1966). Further details are given by Plattner (1971).

Viability Tests

Colony formation on agar was used as the viability criterion for *Chlorella* and *Euglena*.

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The agar plates used for *Chlorella* provided autotrophic conditions; they contained 1% agar, 0.02% (w/v) KNO_3, 0.002% $MgSO_4 \cdot 7H_2O$, and 0.002% K_2HPO_4, but no glucose. The cultures were kept under reduced illumination (approximately 3000 lux) with alternating light (14 hr) and dark (10 hr) periods. The agar dishes were evaluated with the light microscope 5 days after plating. Randomly chosen areas of the cultures were evaluated, using a superimposed lattice as a guideline. Glycerol-treated *Chlorella* cells were plated either directly from the glycerol-containing medium (experiments 5 and 7) or after being thoroughly rinsed with glycerol-free medium (experiment 6). This was done by repeated vacuum filtering with Millipore filters (Millipore Corporation, Bedford, Mass.) and resuspending in an excess of culture medium, a procedure which by itself did not affect the cells. To test the influence of freezing on the viability of *Chlorella*, the cells treated by the different methods were either frozen in Freon according to the standard procedure or spray frozen in propane. For thawing, the standard specimens were simply dropped into culture medium at room temperature. After the propane was pumped off, spray-frozen material was transferred into the culture medium with a spatula cooled by liquid nitrogen. From the culture medium the cells were then plated onto agar.

The plates used for *Euglena* were prepared with 1.5% agar, 1% tryptone, 0.05% yeast extract, and 0.02% glucose, thus providing mixotrophic conditions. Before use, the plates were allowed to dry until the water content was low enough to prevent a dispersion of the divided cells. The illumination cycle of the cultures was the same as with *Chlorella*.

ELECTRON MICROSCOPE MORPHOLOGY

Chlorella

When *Chlorella* cells were frozen by the standard method in Freon without antifreeze, severe ice damage was usually observed. After glycerol treatment these cells appeared generally similar to those obtained by other investigators with this

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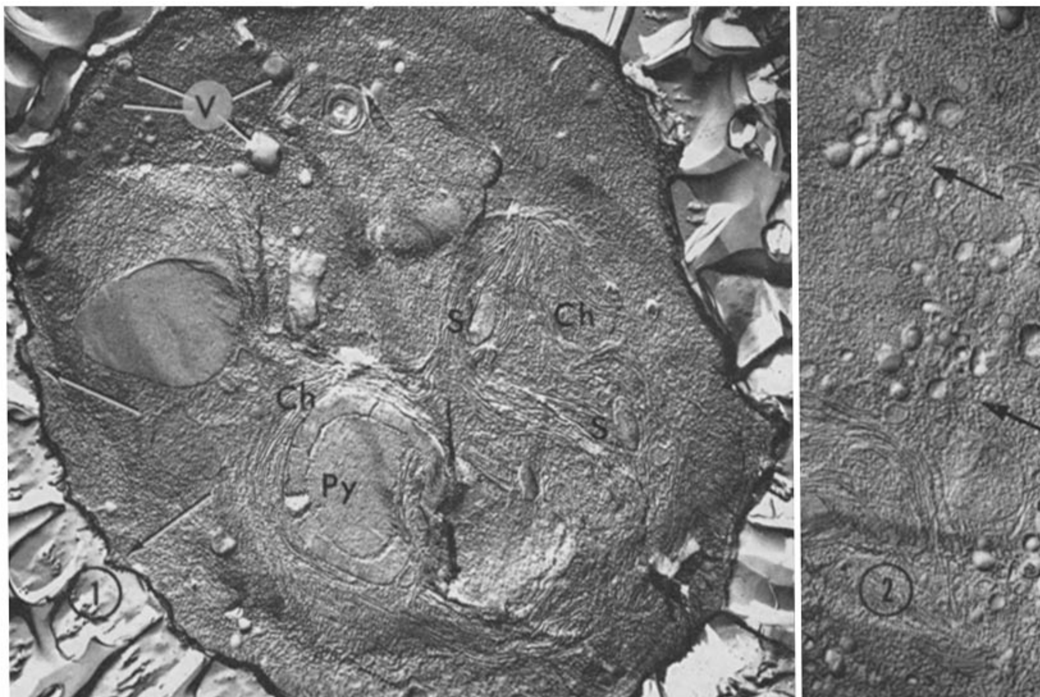
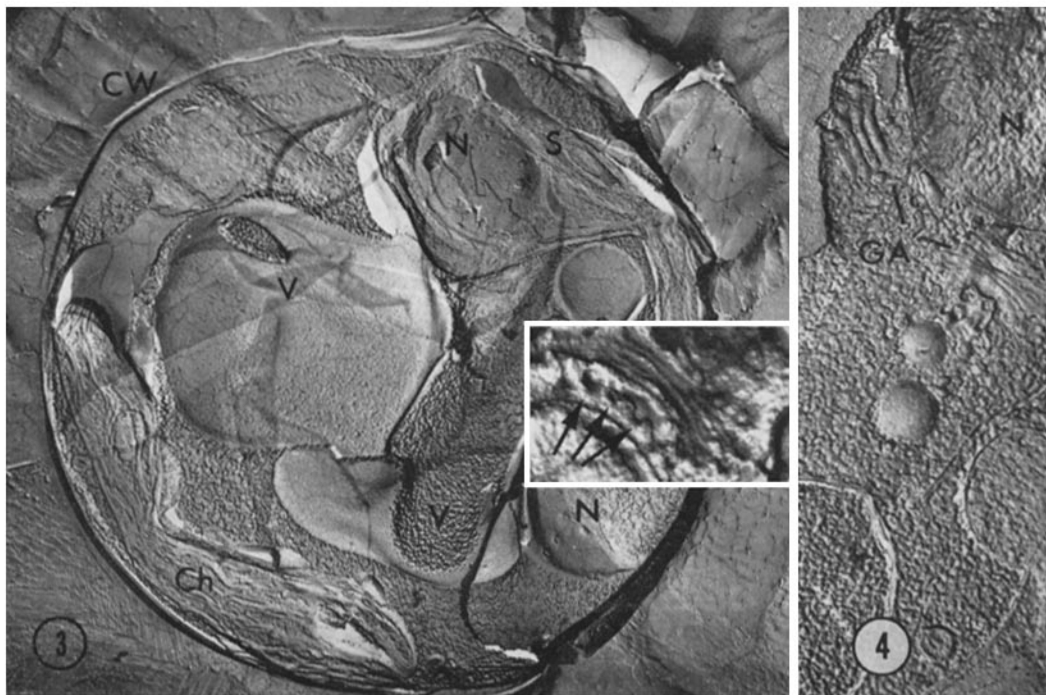


FIGURES 1 and 2 *Chlorella* frozen by the standard method in Freon after 2 hr treatment with glycerol. Fig. 1: Note the extracellular segregation of glycerol and water, whereas the cytoplasm displays a rather fine background relief. The freezing rate was not high enough to avoid some shrinkage of the cell (arrows). The chloroplast-lamellae (*Ch*) are somewhat irregularly arranged; they enclose starch grains (*S*) and a pyrenoid (*Py*). *V*, small vacuoles. × 12,800. Fig. 2: Groups of small vesicles (arrows), which are thought to arise from the glycerol-induced changes in the Golgi apparatus. × 17,600.

method (Staehelin, 1966; Guérin-Dumartrait, 1968). Compared with cells which were spray frozen without glycerol, the cytoplasm shows the same or a slightly smoother appearance (Figs. 1–4; note different magnifications of Figs. 2 and 4). The cytoplasm showed the smoothest appearance when glycerol-treated cells were intentionally frozen at a slower rate by immersion into liquid nitrogen. This observation indicates that an improved homogeneity of the cytoplasm cannot be used as a criterion for an increased freezing rate when cryoprotectants are used. In glycerinated standard frozen specimens, the number of cells that showed some distortion (Fig. 1) was much higher than in spray-frozen samples (Fig. 3). This type of cell distortion was much more pronounced when glycerinated specimens were frozen more slowly in liquid nitrogen. Glycerol-treated cells frequently contained more than one pyrenoid per cross-section. This is unusual, since we have rarely observed more than one pyrenoid per cross-section in untreated *Chlorella* of different developmental stages either in ultrathin sections (Fischer, 1972) or in spray-frozen material. The dictyosomes of glycerol-treated cells were rarely found as well-defined stacks in parallel arrangement. Instead, aggregates of vesicles, approximately 0.1–0.3 μ in diameter, were observed (Fig. 2). Numerous vesicles were located free in the cytoplasm, and occasionally they occurred also between the plasmalemma and the cell wall. On the other hand, the dictyosomes of spray-freeze-etched cells which were not treated with glycerol usually exhibited parallel lamellae with much fewer vesicles (Figs. 3 and 4). The same appearance is observed in ultrathin sections of permanganate-fixed *Chlorella*.

Euglena

The morphology of *Euglena* cells can hardly be studied by freeze etching (Moor and Hoechli,



FIGURES 3 and 4 *Chlorella* cells, spray frozen without antifreeze. The relief in the cytoplasm is about the same as in Fig. 1 or slightly rougher. Note that only large vacuoles (*V*) occur and that chloroplasts (*Ch*) maintain their well-defined outlines. *CW*, cell wall; *N*, nuclei (with pores); *S*, starch grains. × 12,800. The *insert* shows an intact dictyosome with small vesicles (arrows) that are in contact with the cross-sectioned lamellae. × 26,000. Fig. 4: Two dictyosomes of the Golgi apparatus (*GA*) exhibit the typical perinuclear (*N*) location and intact appearance. × 22,400.

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1970). Only the pellicle, storage granules, and chloroplasts have been investigated so far, partially on prefixed material (Schwelitz et al., 1969; Holt and Stern, 1970). Freezing by the standard method without antifreeze results in considerable ice damage. We were not able to establish a quality gradient between cells located at the periphery of the frozen droplet and those located in the center. The pellicle appears well preserved; the nucleus and chloroplasts can be clearly recog-

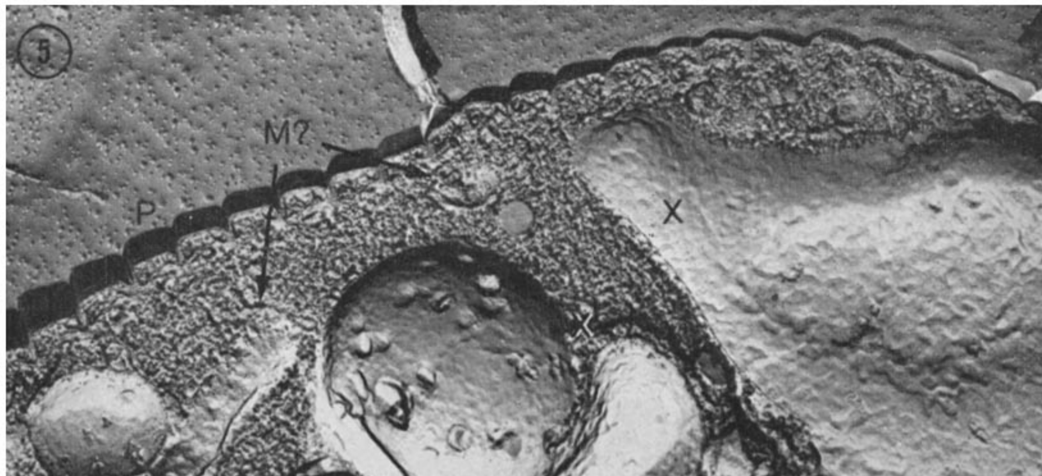

FIGURE 5 *Euglena* frozen in Freon by the standard method without antifreeze. The electron micrograph, although representing one of the best-preserved cells that we could find after standard freezing, still shows considerable ice damage and some distortion of the membranes which impairs the identification of smaller organelles, e.g., mitochondria (*M*). Compare with Fig. 7 which shows a similar portion of a spray-frozen cell. Pellicle, *P*; large membrane-bounded bodies, *X*. × 14,000.

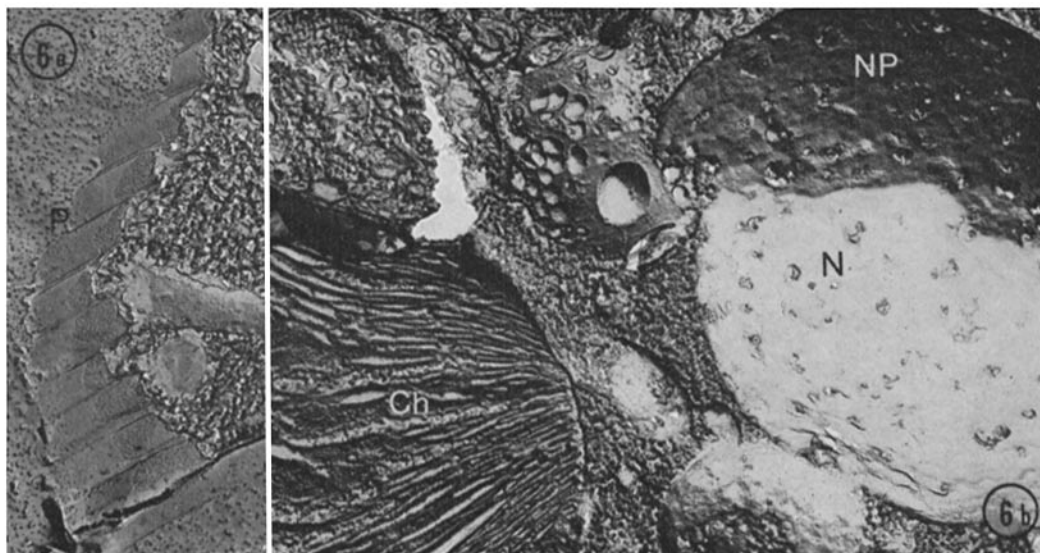

FIGURE 6 *Euglena* frozen as in Fig. 5. (*a*) The pellicle (*P*) is well preserved; the fixation of the cytoplasm is poorer than in Fig. 5, representing a more typical appearance after standard freezing. × 14,000. (*b*) Fairly well-preserved chloroplast (*Ch*) and nuclear envelope (*N*) with pores (*NP*); other structures are poorly preserved. × 14,000.

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nized, although they are more or less distorted; smaller organelles can hardly be identified (Figs. 5 and 6). Glycerol treatment causes instantaneous gross damage to the cells, as observed with the light microscope: deformation of the cells, loss of flagella, and total immobility. In addition, glycerol treatment in our hands did not improve the quality of freeze-etched specimens but in many cases made it even worse.

Good and reproducible results were obtained

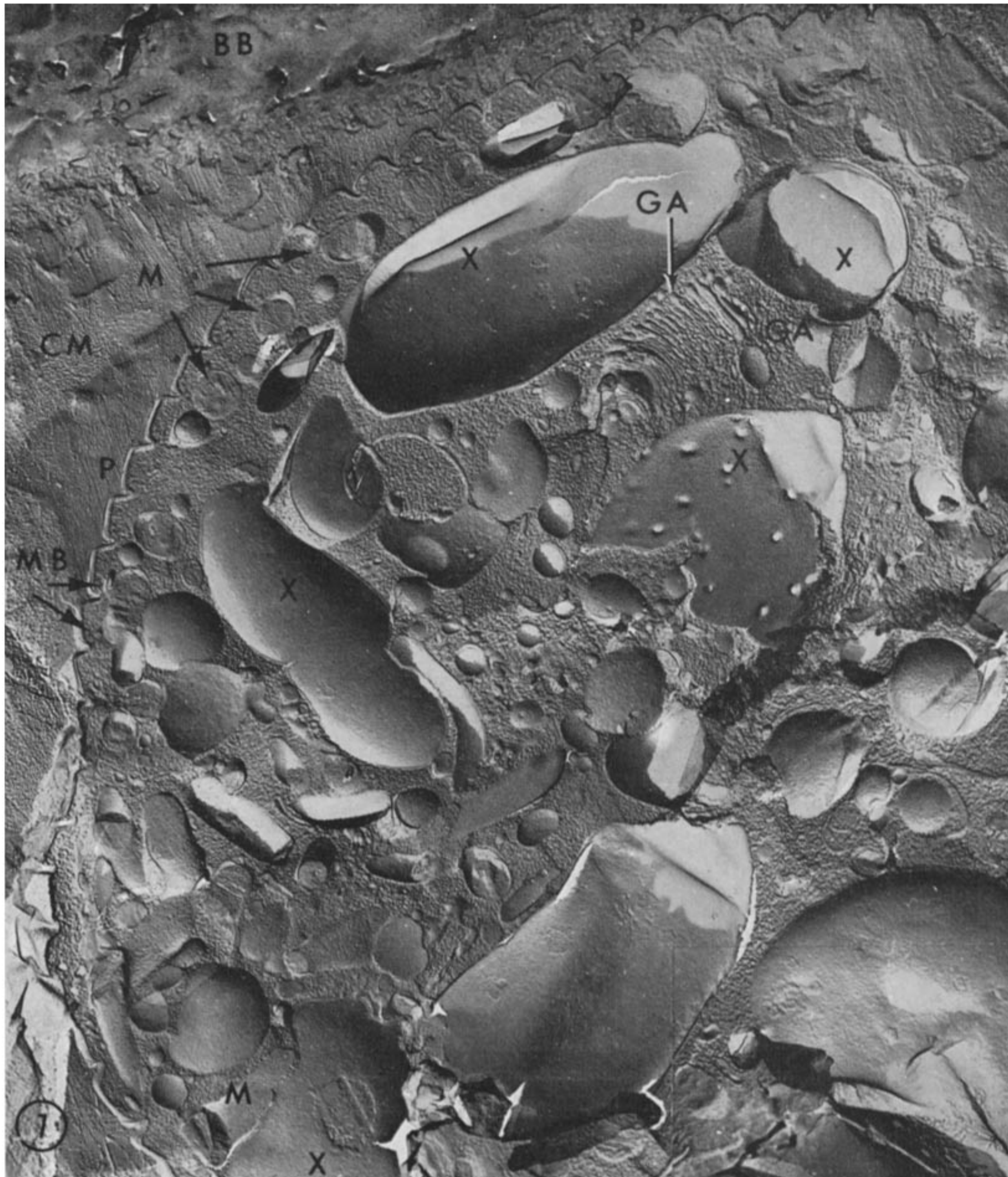

FIGURE 7 *Euglena* cell, spray frozen without antifreeze treatment. A thin layer of frozen culture medium (*CM*) surrounds the cell and can be distinguished from the butylbenzene (*BB*). A variety of structural details as known from ultrathin sections can be observed, e.g. the serrated pellicle (*P*), underlying "muciferous bodies" (*MB*), numerous mitochondria (*M*) with smooth outlines and cristae inside, an elaborate Golgi apparatus (*GA*), and large membrane-bounded bodies (*X*) which may correspond—at least partly—to chloroplasts. × 14,000.

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by spray freezing *Euglena* in their culture medium. Fig. 7 shows a typical example. No particular fine structural features were found which could be attributed to damage during spraying. Cells were intact, and organisms with their locomotory flagellum preserved have been observed. The Golgi apparatus was organized as flat membrane stacks (Fig. 7). Further structural details which could be observed (cf. Buetow, 1968) included: the canal and reservoir, nucleus (with pores), chloroplasts, vacuoles of different size, the serrated pellicle, and underlying muciferous bodies. The mitochondria showed regular outlines. We have not observed mitochondria with an irregular "euglenoid" shape as described after chemical fixation (Leedale et al., 1965; see also Buetow, 1968).

Spermatozoa

The appearance of "slowly frozen" pretreated material was very similar to that described by Koehler (1966) for corresponding specimens (Figs. 8–10). Compared with spray-frozen sperms (no pretreatment at all, or gentle centrifugation only) (Fig. 11), the slowly frozen, protected cells show considerable shrinkage. The posterior sperm head region has a "bumpy" surface relief which is not seen on spray-frozen material; along the middle piece, wrinkles with regular distances—corresponding to the diameter of the underlying mitochondria—occur (Fig. 10). "Granules" of diameters up to 200 A were, in most cases, found either on the shrinkage folds or on the areas between them. In spray-frozen sperm heads, the only surface structures observed were the parallel elevations on the posterior head region, just in front of the "neck" (for terminology, cf. Fawcett, 1970); sometimes these "parallel striations" were missing on slowly frozen cells (e.g., Fig. 8). More details are given by Plattner (1971).

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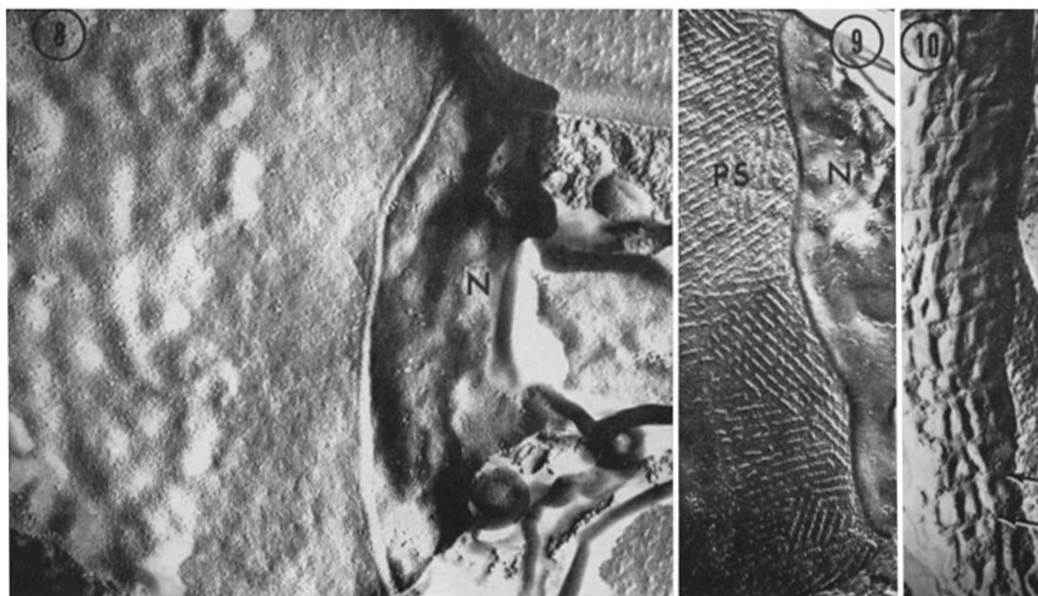

FIGURE 8–10 Slowly frozen bovine spermatozoa, incubated in protecting medium. Fig. 8: The posterior head region in front of the neck (*N*) displays a very coarse bumpy appearance. × 40,000. Fig. 9: The posterior head region, just in front of the neck (*N*), exhibits frequently linear structures in parallel arrangements (parallel striations, *PS*). × 47,200. Fig. 10: The middle piece, seen here from the outside, is characterized by elaborate surface structures after slow freezing. The distances of the wrinkles at a right angle to the tail axis correspond to about the size of the underlying mitochondria. Granules are abundantly arranged along these wrinkles (arrows). × 29,000.

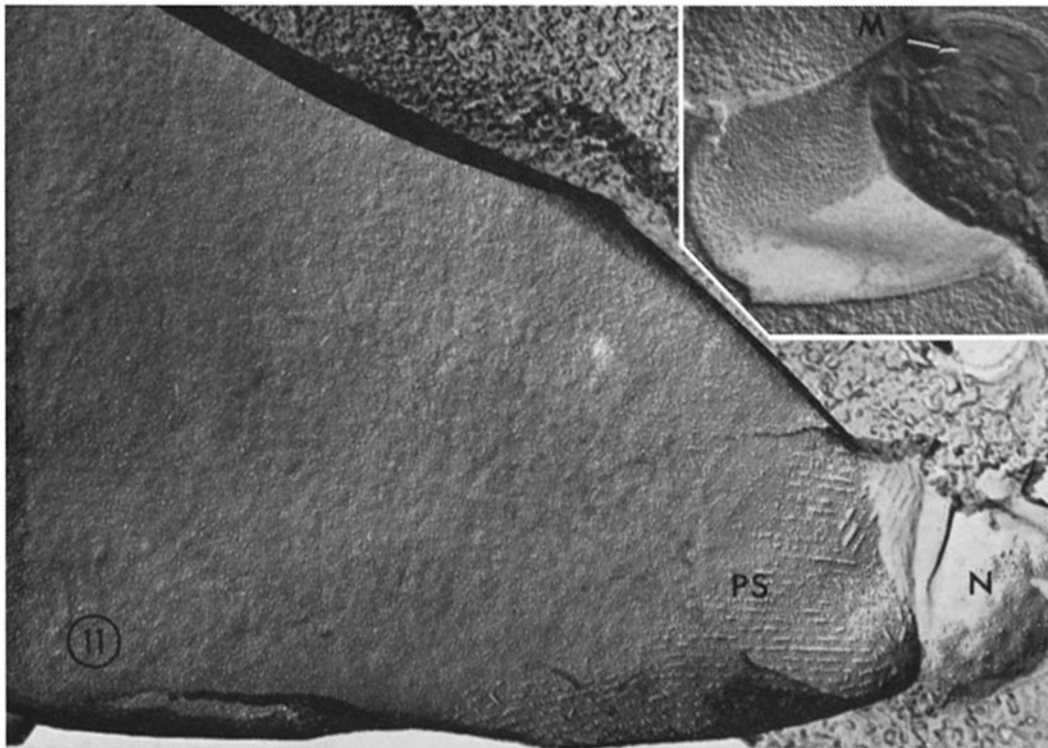

FIGURE 11 Spray-frozen bovine spermatozoa, no antifreeze pretreatment. The posterior head region has no bumpy appearance, but displays the parallel striations (*PS*) in front of the neck (*N*). × 30,000. The *insert* shows the middle piece region; note the elongate mitochondrion at *M*. After spray freezing, neither the complicated surface pattern, found after slow freezing, nor the regular arrangement of granules was detected. × 40,000.

EVALUATION OF CELL DAMAGE CAUSED BY THE VARIOUS PROCEDURES

Mechanical Damage due to Centrifugation and Spraying

Chlorella cells showed no change in the light microscope after centrifugation and spraying, even when intentionally sprayed with a pressure of 4 atm instead of the normal operating pressure between 0.5 and 1 atm. Their viability was also not impaired (Table I).

Euglena and Spermatozoa: Immediately after centrifugation and spraying under low pressure (0.5 atm), the majority of *Euglena* and sperm cells showed no visible damage in the light microscope but their motility was reduced. However, loss of flagella was frequently observed after spraying with too high a pressure ($\geq$ 1 atm).

Euglena cells were also sprayed by low pressure onto agar dishes as described in Materials and Methods. After 5 days of incubation, we found 65–90% divided cells exhibiting euglenoid (peristaltic) movement, 10–30% morphologically intact but immobile and undivided cells, and 3–5% burst cell fragments. These viability studies compare favorably with the high pressure tests of Moor and Hoechli (1970).

Impairment of Viability due to Glycerol Treatment and Freezing

Cryofixation can be applied for a number of reasons: one can be the need to keep unicellular organisms, spermatozoa, or tissues alive for

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TABLE I

Survival Rates of Chlorella Cells, after Various Treatments, Judged on the Basis of Colony Formation on Agar after 5 Days under Autotrophic Conditions

When glycerol was used, the concentration was 20% v/v. Chi squared tests were applied in comparing all the experimental groups. Yeates correction was used where necessary. Experiment 12 is significantly different from experiments 5 and 6 ($P < 1\%$).

Exp. No.	Treatment	% dividing cells	Number of cells counted
	Influence of mechanical treatment (no antifreeze, no freezing)		
1	None	98.5	339
2	Centrifuged only	96.3	235
3	Sprayed at 1 atm	97.3	260
4	Sprayed at 4 atm	97.9	230
	Influence of glycerol (no freezing)		
5	2 hr glycerol, cells not washed after glycerol	11.8	228
6	Cells rinsed with culturing medium after 2 hr glycerol treatment	11.2	251
7	4 hr glycerol, otherwise like experiment 5	<1	213
	Influence of various freezing procedures and pretreatments		
8	Frozen by the standard method in Freon, no anti-freeze pretreatment	<1	250
9	Frozen by standard method in Freon after 2 hr glycerol, cells not washed after glycerol	<1	242
10	Frozen in Freon after 2 hr glycerol, cells washed after glycerol	<1	151
11	Spray frozen in propane after 2 hr glycerol	<1	293
12	Spray frozen in propane, no antifreeze pretreatment	19.6	409

storage, and another one the need to preserve certain biochemical systems. Electron microscopists are interested in cryofixation mainly for the preservation of morphology and for the retention of soluble compounds. Cryobiology has given ample evidence that these different aims can often be achieved best by using different freezing methods. Cells which prove to be viable after freezing and thawing sometimes show serious morphological distortions, while cells better preserved morphologically show poor or no survival rates (Mazur, 1965). Nevertheless, an interesting aspect of freeze etching has always been the fact that this method permits the electron microscope study of cells which are potentially alive. Survival of unicellular organisms after freezing and thawing has frequently been used as an indicator of the extent to which their intravital ultrastructure is preserved, although this should be used only in connection with other experimental evidence (Mazur, 1970). Since viability tests are widely used in freeze etching, we also wanted to provide some comparison between standard freezing and spray freezing. In addition, we wanted to investigate to what extent the material was impaired by the various pretreatments before being frozen.

The possibility that glycerol itself might be toxic was tested by incubating the cells for 2 and 4 hr in 20% glycerol before agar-plating. Table I shows that the cells degenerate with increasing time in glycerol (experiments 5 and 7). This indicates that the damage is not or not only caused by osmotic shock. The damage seems to be irreversible, because even the careful removal of glycerol did not improve the rate of colony formation (experiment 5 versus 6). After glycerol treatment, large but nondividing cells were frequently observed. Structural alterations which have already been discussed (i.e., increased occurrence of pyrenoids and changes in the Golgi apparatus, as observed in both ultrathin sectioning and freeze-etch studies) might be associated with this reduced dividing activity.

The influence of freezing and thawing is also shown in Table I. Spray freezing without any pretreatment (experiment 12) yields the highest rate of dividing cells (~20%). All other experiments (8–11) showed extremely poor colony formation (below 1%).

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DISCUSSION

The morphology and viability of unicellular organisms frozen under different conditions confirm the findings obtained on model systems (Bachmann and Schmitt, 1971 *a* and *b*) and demonstrate that spray freezing indeed yields cooling rates substantially higher than those obtainable by the standard method. On the other hand, we found no morphological evidence that proper spraying introduced any new artifacts. Nor did it impair viability and motility to a serious extent. Other organisms or cell fractions might of course be more sensitive to mechanical damage. Spray-freeze etching of *Euglena*, frozen in their culture medium, resulted in pictures clearly superior to those obtainable by the standard methods.

Chlorella cells when frozen by the standard method without cryoprotectants usually show severe ice damage. This was never the case when the same cultures were spray frozen. Viability tests are in agreement with this finding. The rate of colony formation of nonprotected *Chlorella* cells is much higher after spray freezing than after standard freezing.

Glycerol treatment of *Chlorella* prevents ice damage when these cells are frozen by the standard method. However, we found indications that this procedure still results in some loss of intracellular water. The heavy cell distortion combined with the smooth appearance of the cytoplasm of glycerol-protected cells frozen slowly in liquid nitrogen indicates even more dehydration. Similarly, cell distortions were observed on protected spermatozoa when frozen at low cooling rates. This is in accordance with the finding that slowly frozen spermatozoa (Koehler, 1966) display the same surface patterns when freeze etched as when air dried and then stained or shadowed (Randall and Friedlaender, 1950; Hancock, 1953). In contrast, after spray freezing the cells show no noticeable shrinkage in the head region (Fig. 11 vs. Fig. 8) nor the wrinkles and the regular arrangement of granules in the middle piece (Fig. 11 insert vs. Fig. 10).

In *Chlorella* we found evidence that glycerol can cause morphological changes. Glycerol-treated *Chlorella* cells showed a disintegration of dictyosomes and an occurrence of numerous small vesicles in concentrated groups, contrary to observations on cells which were either chemically fixed or spray frozen without antifreeze. We also consider the enhanced occurrence of pyrenoids to be a pathological finding (Wanka, 1968). The toxic effect of glycerol became even more evident in our viability tests. Whereas almost the whole population of untreated *Chlorella* "germinated," colony formation after 2 hr of treatment with 20% glycerol dropped to 12%. Even washing of the pretreated cells with culturing medium did not change the results. This reduced viability after glycerol treatment differs drastically from that obtained by Staehelin (1966). However, we used more rigorous conditions by omitting glucose in the agar, thus including the functional integrity of the photosynthetic apparatus in the test. We are also aware of the possibility that other methods of glycerol incubation than the one used by Staehelin (1966) and ourselves might yield different results.

This study shows that by spray freezing the cooling rate is increased sufficiently to eliminate the need for cryoprotectants, at least for the three different kinds of cells which were investigated. The problem of an artifact-free cryofixation, even of dispersed material, is of course not completely overcome by spray freezing. Segregation phenomena are still visible in model systems (e.g., glycerol water solutions) although they occur on a much reduced scale (Bachmann and Schmitt, 1971 *a*, *b*). However, as this paper demonstrates, the use of antifreeze can introduce more serious artifacts before the specimen is even frozen. In addition, although it eliminates ice damage to the cell, it does not necessarily prevent other segregation phenomena (Schmitt et al., 1970). It also makes deep etching less reliable or impossible and the interpretation of the replicas more difficult. We therefore believe that spray-freeze etching offers a relatively simple and improved technique for studying unicellular organisms. We also believe that it can be applied to the investigation of organelle and membrane fractions. The high cooling rate which the method offers might also be used as an additional experimental parameter when studying the complex problems of cryofixation itself.

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