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The relative efficiency of various fluids in the rapid freezing of protozoa

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KEY WORDS. Protozoa, freezing, flagella, cilia, convection, electron microscopy, freeze-substitution, heat transfer, fluids, ethane.

SUMMARY

The cooling efficiencies of various fluids at low temperature were compared by measuring the temperature decay in 3 μl water samples plunged into them. A simple model of cooling was used in order to discuss the results. Liquid ethane was found to produce a cooling rate of 660 K s^{-1}, about twice that of liquid propane, while ethanol was almost as effective as ethane between 273 to 223 K. The heat-transfer coefficient of liquid ethane was estimated to be between 1500 and 5000 W m^{-2} K^{-1}, depending on the physical state assumed for the water sample. Samples of flagellated organisms, after being frozen rapidly in the above way, were freeze-substituted by the method of Barlow & Sleigh (1979). Although this fixation did not give good definition of the microtubules of the flagellar axoneme, it exhibited reasonable tissue preservation in thin sections of the cell body. The fixation method resulted in preserved flagellar wave shapes, which were observed under the light microscope and in critical-point dried cells examined by scanning and conventional electron microscopy. It was concluded (a) that methods for preserving the wave shape of the flagellum and for preserving its internal structure may not be compatible, and (b) that although the present cooling method (with ethane) approaches the speed required to arrest a flagellar wave, further improvements in the speed of the method are desirable.

INTRODUCTION

In the study of motility in flagellated microorganisms it is of interest to preserve the wave shape of the flagellum by a rapid fixation technique. In this way one hopes to observe in the electron microscope the external and internal structure of the flagellum, in a state as close as possible to a transient one in the cycle of flagellar beating. In this particular field some of the first attempts were by Afzelius (1961) in fixing the metachronal wave of the cilia in ctenophore swimming plates, using 40% osmium tetroxide in carbon tetrachloride, and by Satir (1963) in fixing gill cilia, again with osmium tetroxide. Woolley (1974) preserved spermatozoa by rapid cooling and more recently Barlow & Sleigh (1979) have described a rapid-freezing method followed by a carefully developed system of freeze-substitution in preparation for the scanning electron microscopy of ciliates. The literature of rapid-freezing methods has been amply reviewed by Franks (1977) and Costello & Corless (1978). A method of fast chemical fixation has recently been reported by S. A. Baba, Zoological Institute, University of Tokyo (personal communication) who used very thin (100 μm) liquid films of suspended organisms, immersed rapidly in osmium-tetroxide vapour. Although chemical methods may be developed that are fast enough to 'stop' the flagellar wave in its progress, there is always the possibility that the

fixation process may itself produce an alteration in the beat pattern as a response to the chemical. For this reason a rapid physical method is perhaps to be preferred in the initial fixation, and we were interested to learn of the method of Barlow & Sleigh (1979) in which small (3 μl) drops of liquid are plunged into propane at liquid-nitrogen temperatures. These authors were only able to measure the cooling rate in 10 μl drops, within various coolants, but we were interested in the actual rate for the smaller drops. In addition we wondered whether the cooling rate could be improved since one wishes to achieve a fixation time which is as short as possible, not only for good preservation (Farrant *et al.*, 1977) but in relation to the flagellar beat period. Lastly, we wished to adapt the method for use with the conventional transmission electron microscope (CTEM) rather than the scanning electron microscope (SEM).

For the above reasons we have investigated the cooling rates of small (3 μl) drops of water in in various fluids. To assist in our choice of suitable fluids and in an attempt to understand the cooling process, we have used an unsophisticated model (described below) which, however, gives some insight into the relative importance of the sample properties and those of the coolant in determining cooling rates. We also report our progress in adapting the Barlow-Sleigh technique (freezing, freeze-substitution and critical-point drying) to transmission electron microscopy.

HEAT LOSS INTO A LOW-TEMPERATURE FLUID

In considering mechanisms of heat loss one can ignore the radiation and conduction of heat in the present context, as on calculating the heat exchange between a 10 μl spherical drop at room temperature and a given cooling fluid at liquid-nitrogen temperatures, one obtains initial cooling rates much smaller than those obtained experimentally. Radiation alone gives a rate about 2000 times smaller, and conduction alone a rate at least 10 times smaller, than those published by Barlow & Sleigh (1979). One thus assumes that convection (natural or forced) is the main method of heat loss.

For most of the time in which a specimen is being cooled (after the initial period, in which phase changes in the liquid are occurring) we have a solid sample (ice) in which heat transfer to the surface is by conduction while the rate of heat loss from the surface into the cooling fluid is primarily due to convection (Fig. 1a). In a small temperature range, the rate of heat loss from the surface is proportional to the temperature difference between the surface and the bulk fluid. This situation is analogous to the diffusion of molecules in a closed volume towards its surface, where they then evaporate (Fig. 1b). Similar equations apply in both cases (Chapman, 1974; Crank, 1956) and analytic solutions are available for a spherical sample and a thin sheet of infinite extent.

A point of interest is that these solutions contain a dimensionless factor, B (the Biot modulus) which characterizes the cooling situation and affects the way in which heat is lost (see also Bald

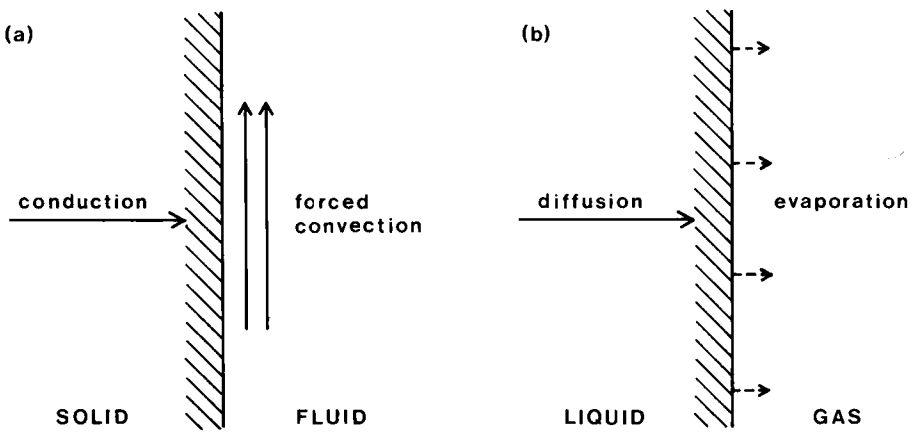


Fig. 1. (a) Model for heat loss from a solid sample. (Heat loss is proportional to temperature difference.) (b) Diffusion analogue of (a). (Mass loss is proportional to concentration difference.)

& Crowley, 1979). $B = lh/\lambda$ where l is the specimen dimension (radius or half-thickness) h is the convective heat-transfer coefficient (heat loss per second per unit area, per unit temperature difference) and λ the thermal conductivity of the *sample*. For example, if the sample were a thin metal foil of high conductivity, in a poor coolant, $B \ll 1$, while if the sample were thick and of low conductivity, in a highly efficient coolant, $B \gg 1$. In the latter case it is the properties of the sample which limit the speed of cooling, while in the former it is those of the coolant.

In the experiments to be described below, the Biot modulus was of the order of 0·1, showing that it is worth searching for better coolants in this particular context.

EXPERIMENTAL

(a) *Measurement of cooling rates*

Circular loops about 0·25 mm thick were sliced from plastic tubing with 1·78 mm internal diameter. The loops were cemented to the ends of micro-pipettes pulled from 2 mm diameter glass tubing. The loops were filled with sample drops of measured volume (3 or 10 μl) or with

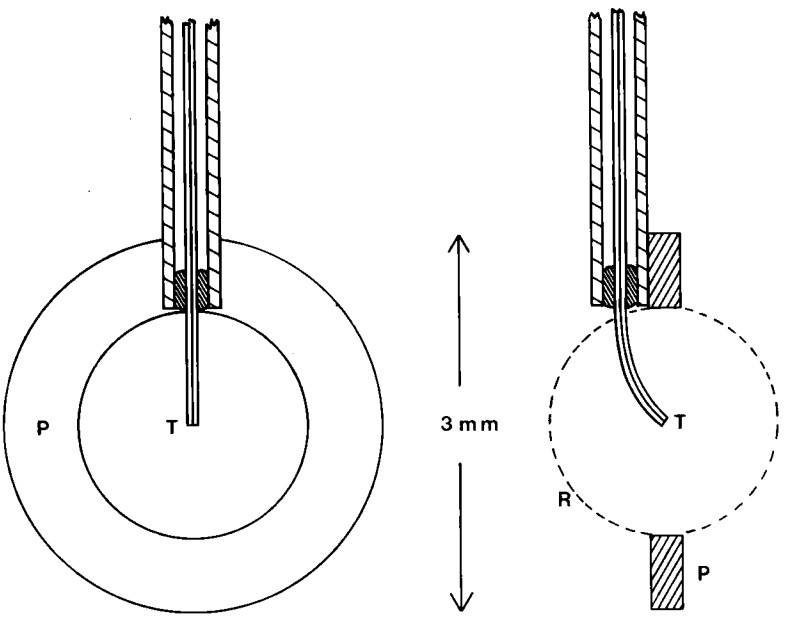


Fig. 2. Diagram of the sample holder and thermocouple used in measurements: P, plastic loop; T, thermocouple junction; R, radius of 3 μl spherical drop.

approximately plane liquid sheets of the thickness of the loop. For temperature measurement a Cu–Cu/Ni thermocouple was inserted down the glass micro-pipette so that its junction projected into the centre of the sample in the loop (Fig. 2). The junction diameter was 0·1 mm. The e.m.f. of the junction relative to that provided by a cold-junction reference unit (Comark Ltd) was amplified to be in the range $-2{\cdot}5$ to $+2{\cdot}5$ V. The amplifier output was sampled through the AR11 analogue/digital interface of a PDP11/10s computer. Recording rates of up to 1000 values per second were used. The amplified thermocouple e.m.f. was calibrated at three temperatures, 373, 273 and 77 K, and a parabolic curve used to interpolate between them. The sample was plunged into the coolant manually, at an estimated speed of $1 \pm 0{\cdot}2$ m s^{-1}.

In order to measure the data as the sample drop was plunged into the liquid coolant, a program was written which continually updated the temperature readings stored cyclically in a 'ring' buffer in the computer. After the temperature passed through 273 K the program updated the following three-quarters of the readings contained in the buffer and then stopped. The remaining quarter of the buffer then still contained temperatures recorded before the sample

entered the coolant. Thus although the recording system was triggered by entry of the sample into the coolant, the cooling curve was available from room temperature downwards. The response time of the whole recording system was tested by simulating a sudden temperature drop by short-circuiting the input to the thermocouple amplifer. Several cooling curves were recorded for each coolant and sample volume.

(b) *Preparation of coolants*

The following fluids were investigated: (i) propane, ethane, methane; (ii) chloroethane, Freon 12; (iii) ethanol, methanol, acetone, diethyl ether, propanol, 2-methyl pentane, ethanethiol; (iv) liquid nitrogen. Their melting points and boiling points are given in Table 1. The

Table 1. Melting points and boiling points of fluids at atmospheric pressure.

Fluid	m.p. (K)	b.p. (K)
Nitrogen	63·1	77·2
Propane	83·3	230·9
Ethane	89·7	184·4
Methane	90·5	109
Freon 12	115	243·2
2-Methyl pentane	119·3	333·3
Ethanethiol	128·6	308
Chloroethane	136·6	285·3
Propanol	146·5	370·4
Ethanol	155·7	351·5
Diethylether	156·8	307·5
Acetone	177·5	329·2
Methanol	179·1	338

gases in group (i) were condensed in a copper cooling coil immersed in liquid nitrogen, after first flushing air and water vapour from the coil with dry nitrogen at room temperature to prevent later blockages. The coil, with its axis vertical, surrounded a plastic centrifuge tube (4 cm diameter) suspended in the liquid-nitrogen Dewar, and the end of the coil delivered the liquefied gas into this tube. Gases of group (ii) emerged already as liquid from their cylinders and were delivered into the cooled centrifuge tube directly, in the same manner as group (iii) liquids. Liquid-nitrogen experiments were done directly in the Dewar, without use of the smaller container. Experiments were performed with the normally liquid coolants as near to their freezing points as was practicable; the actual coolant temperatures may be inferred from the base lines of the cooling curves in Figs. 3–7.

(c) *Safety*

Many of the coolants are highly flammable, with combustion levels in air as low as 2% or 20,000 ppm (propane). Liquefaction of gases was performed in the open air before bringing them, at liquid-nitrogen temperature, into the laboratory. All spark sources (stills, refrigerators, ovens, etc.) were disconnected during the experiments and suitable notices displayed at the laboratory entrance. A flammable-gas sensor (R.S. Components Ltd) was built into a circuit which gave an audible alarm at levels above one-tenth of the combustion level, and a meter reading below this. The sensor was tested with measured concentrations of ethanol vapour. Waste coolants were allowed to evaporate slowly in a suitable fume-cupboard.

(d) *Electron microscopy*

A 10 ml sample of a 5–6-day-old culture of *Crithidia oncopelti* was centrifuged into a pellet, washed in distilled water and resuspended in 1·0 ml of distilled water to give a cell-protein concentration of 1·35 mg/ml (Lowry *et al.*, 1951). To enable single cells to be processed for thin-section electron microscopy, the cells were suspended in a fibrin clot in the plastic loop used for freezing (see (a)). Thus after freeze-substitution the cells could be handled for dehydra-

tion and embedding in the same way as a piece of tissue. To produce the clot, 0·2 ml of the cell suspension was mixed with 0·2 ml of a fibrinogen solution (containing 0·3% (w/v) fibrinogen, 0·8% (w/v) sodium chloride and 0·16% (w/v) trisodium citrate) for 10 s, after which 0·2 ml of a thrombin solution at a concentration of 50 units/ml was added. The plastic loop was lowered by its glass 'handle' into the suspension and twisted in one direction only to cause the 'veil-like' strands of fibrin to be wrapped around the loop. The cells in the fibrin clot were immersed in the coolant as described earlier (a) and after about 5 s the loop was transferred quickly to 10 ml of the freeze-substitution liquid (methanol 50% (v/v), ethylene glycol 40% (v/v), acrolein 10% (v/v) and mercuric chloride 12·5% (w/v)). The freeze-substitution liquid was maintained at approximately 193 K with solid CO_2 in a Dewar. After 48 h the sample was brought to room temperature over a period of 5–6 h. (Faster times than this could not be used due to CO_2 bubbles being liberated in the sample.) Once the sample was at room temperature the medium around the sample was gradually exchanged with methanol. Specimens were embedded in Spurr's resin and silver to gold-coloured sections were obtained using an LKB1 ultramicrotome. Sections were collected on 400-mesh uncoated copper grids and were double-stained with 20% (w/v) uranyl acetate in methanol in the dark for 20 min and lead citrate (Reynolds, 1963) for 3 min.

Cells that were to be critical-point dried were freely suspended in distilled water in their loop, without fibrin. Freeze-substitution was done in the same manner as described above, except that the frozen sample was suspended in a small plastic bucket (Beem capsule) which had a Millipore filter as its base as detailed by Barlow & Sleigh (1979). The bucket stood in a reservoir of freeze-substitution liquid which could exchange with the sample through the Millipore filter. After the cells had been freeze-substituted and brought to room temperature the freeze-substitution liquid was gradually exchanged for methanol through the filter base of the plastic bucket. (At this stage specimens were examined in the optical microscope using dark-field optics.) The sample in its Beem capsule was critical-point dried using methanol as the exchange medium and CO_2 as the transitional fluid. For scanning electron microscopy the Millipore filter was cut from the Beem capsule and mounted on a stub for viewing. For transmission microscopy the dried specimens were collected on carbon-Formvar coated 200-mesh copper grids and viewed in a Philips 300 TEM.

RESULTS

(a) *Cooling rates*

The response time (time-constant) of the recording system was found to be approximately 2 ms. Representative experimental cooling curves for 3 μl drops are given in Figs. 3–7; they are so arranged that all curves pass through 273 K at the same nominal time. Figure 3 shows the three fastest coolants, ethane, ethanol and propane. Ethane produces a mean cooling rate of 660 K s^{-1}, about twice that of propane (358 K s^{-1}) over the same range of 273–173 K. Although ethanol closely matches ethane over the first half of this range (273–223 K) the cooling rate then decreases as the final temperature of the ethanol is approached. This temperature was on occasions below the quoted freezing point and the ethanol became very viscous under these conditions.

Figure 4 shows a homologous series of coolants which were investigated in the search for some pattern in the cooling properties, while Fig. 5 compares the effectiveness of the three smallest alcohols and acetone. Figure 6 is plotted on a different time scale from the previous figures as it shows the less efficient coolants, nitrogen, methane, 2-methylpentane, Freon 12 and two ethane derivatives; since ethanol appears in each of the other figures it is included here as a reference curve. Figure 7 shows the effect on the temperature at the centre of a 3 μl sample of *removing* it from a coolant into the atmosphere at about 293 K.

In performing the cooling experiments it was noted that only with liquid nitrogen and liquid methane did the immersion of the sample lead to bubbling in the liquid.

In an experiment with a 0·25 mm plane sheet of water in a plastic loop of the same thickness, cooled in ethane at 88 K, the cooling rate was 694 K s^{-1} at 191 K. Assuming that the cooling equations mentioned earlier for an infinite sheet can be applied to this specimen, two values for

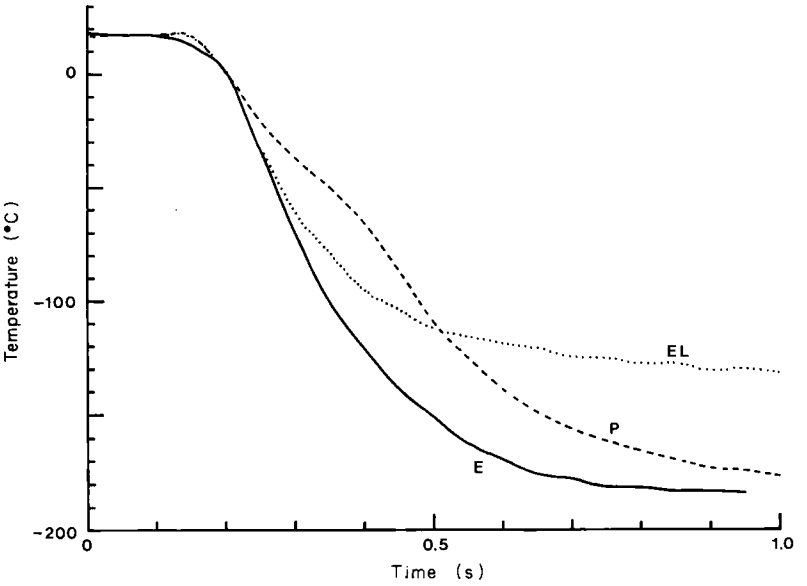


Fig. 3. Experimental cooling curves for a 3 μl water drop in ethane (E) propane (P) and ethanol (EL).

B were obtained which would fit such a cooling rate. The larger, 1·025, was based on the assumption that the thermal properties of the specimen were those of water. The smaller, 0·095, was obtained using the thermal properties for ice. From these values of B the heat-transfer coefficient h for liquid ethane was estimated as 4840 or 1670 W m^{-2} K^{-1} respectively, under the conditions of the experiment.

(b) *Microscopy*

In preparation for the electron microscopy, three freeze-substitution media were studied which varied only by the concentration and type of halogen salt they contained. A freeze-

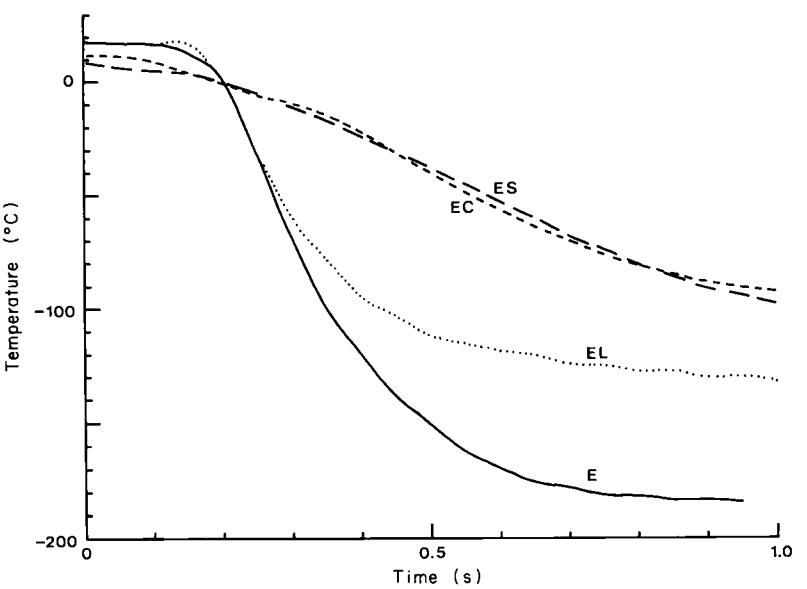


Fig. 4. Experimental cooling curves for a 3 μl water drop in various ethane derivatives: ethane (E), ethano (EL), chloroethane (EC) and ethanethiol (ES).

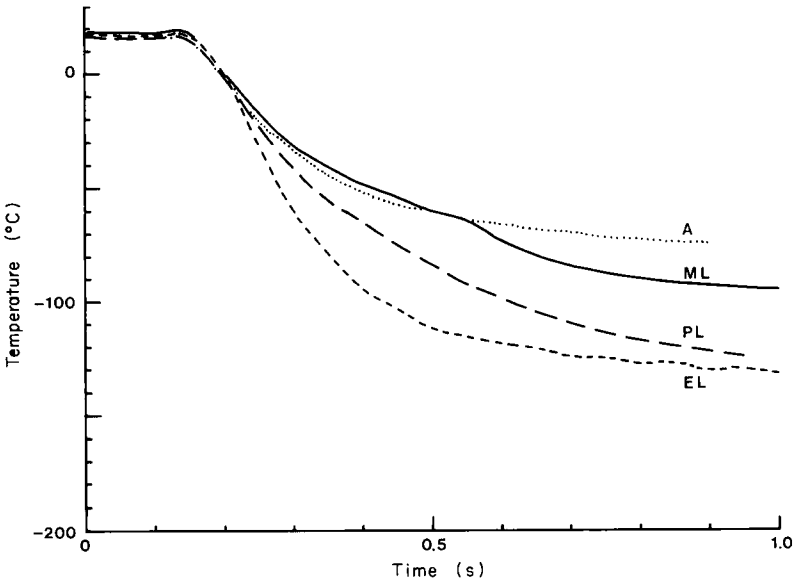


Fig. 5. Experimental cooling curves for a 3 μl water drop in various alcohols and acetone: methanol (ML), ethanol (EL), propanol (PL) and acetone (A).

substitution medium containing 8·0% (w/v) $HgCl_2$ and another containing 8·0% (w/v) $HgCl_2$+ 3·0% (w/v) $CaCl_2$ resulted in poor cellular preservation, and the latter medium also gave rise to excessive cytoplasmic extraction. The medium giving the best cytoplasmic preservation, as judged by comparison with glutaraldehyde-fixed cells, was one containing 12·5% (w/v) $HgCl_2$ which was used for all further preparations. Sections of freeze-substituted cells (Fig. 8) when compared with glutaraldehyde-fixed cells (Fig. 9) appeared similar; however, there were minor

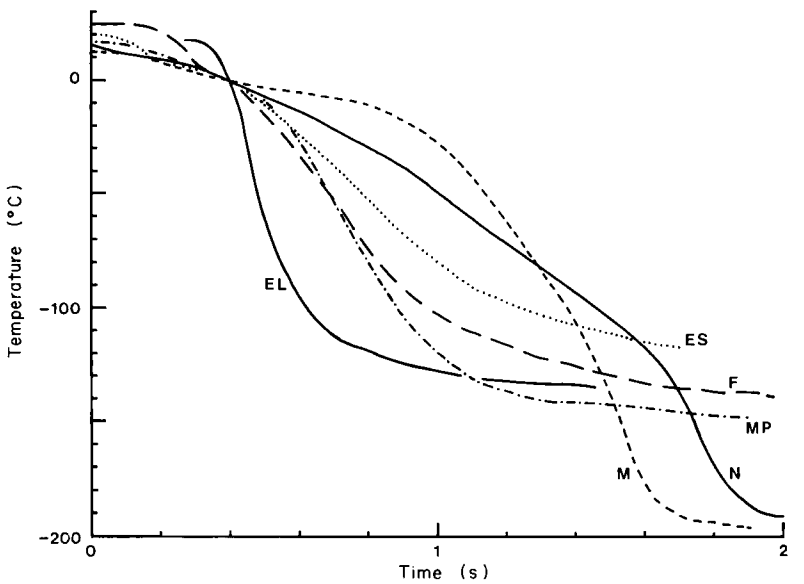


Fig. 6. Experimental cooling curves for a 3 μl water drop in various fluids: methane (M), nitrogen (N), 2-methylpentane (MP), Freon 12 (F) and ethanethiol (ES). (Note change of time scale compared with Figs. 3–5.) Chloroethane follows almost the same curve as ethanethiol—see Fig. 4. Ethanol (EL) is also shown, for reference.

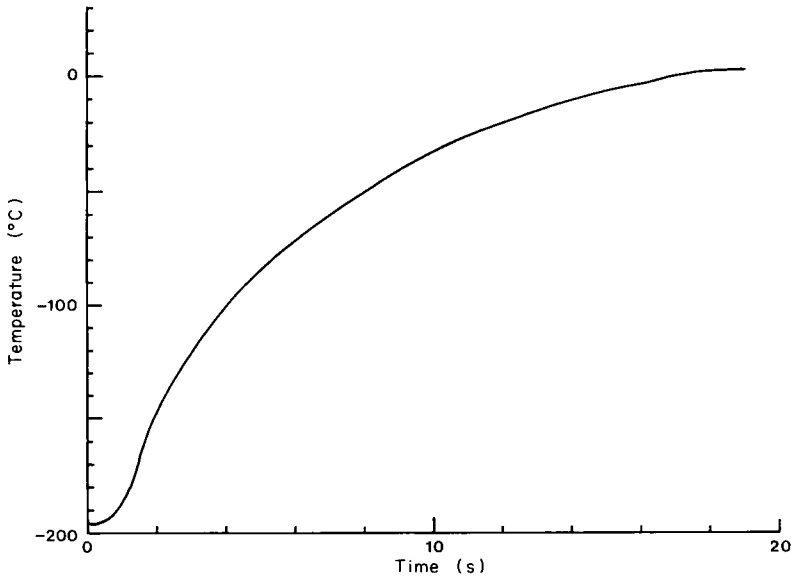


Fig. 7. Experimental warming curve from a 3 μl water drop removed from liquid nitrogen into the atmosphere at *c.* 293 K.

but distinct differences. The cytoplasm of the freeze-substituted cells was more granular than that of glutaraldehyde-fixed cells and had electron-translucent voids of similar size to the 0·5 μm lacunae reported by Barlow & Sleigh (1979). The lipid globules (which could be distinguished from bacilliform bodies by the smaller size and greater abundance of the former) were less electron dense, and the nuclear periphery was darker and more distinct, than corresponding features in conventionally processed cells.

A comparison of the flagella showed that the microtubules of the axoneme in freeze-substituted cells (Fig. 11) were not clearly outlined and that the flagellar matrix was more densely stained than in glutaraldehyde-fixed flagella (Fig. 10). The state of preservation of wave shapes on the flagella was judged by comparing the appearance of freeze-substituted organisms in the light microscope with that of normally swimming ones. The basic wave shape *in vivo* exhibits one-and-a-half to two wavelengths ($\lambda = 10$–14 μm) per flagellum, with an amplitude of 4–7 μm. Approximately 60–70% of freeze-substituted cells (as judged by visual inspection) had flagella that conformed to this basic pattern. The remaining flagella were either straight or exhibited a single curve of low amplitude along their entire length.

Figs. 8–9. Thin sections of an ethane-cooled freeze-substituted cell body and a glutaraldehyde-fixed cell body, respectively: b, bacilliform bodies; g, lipid globules; n, nuclear periphery. × 20,000. (Length scale 1 μm.)

Fig. 10. Transverse section of a flagellum after glutaraldehyde fixation, showing the characteristic '9 + 2' arrangement of microtubules. × 175,000. (Length scale 25 nm.)

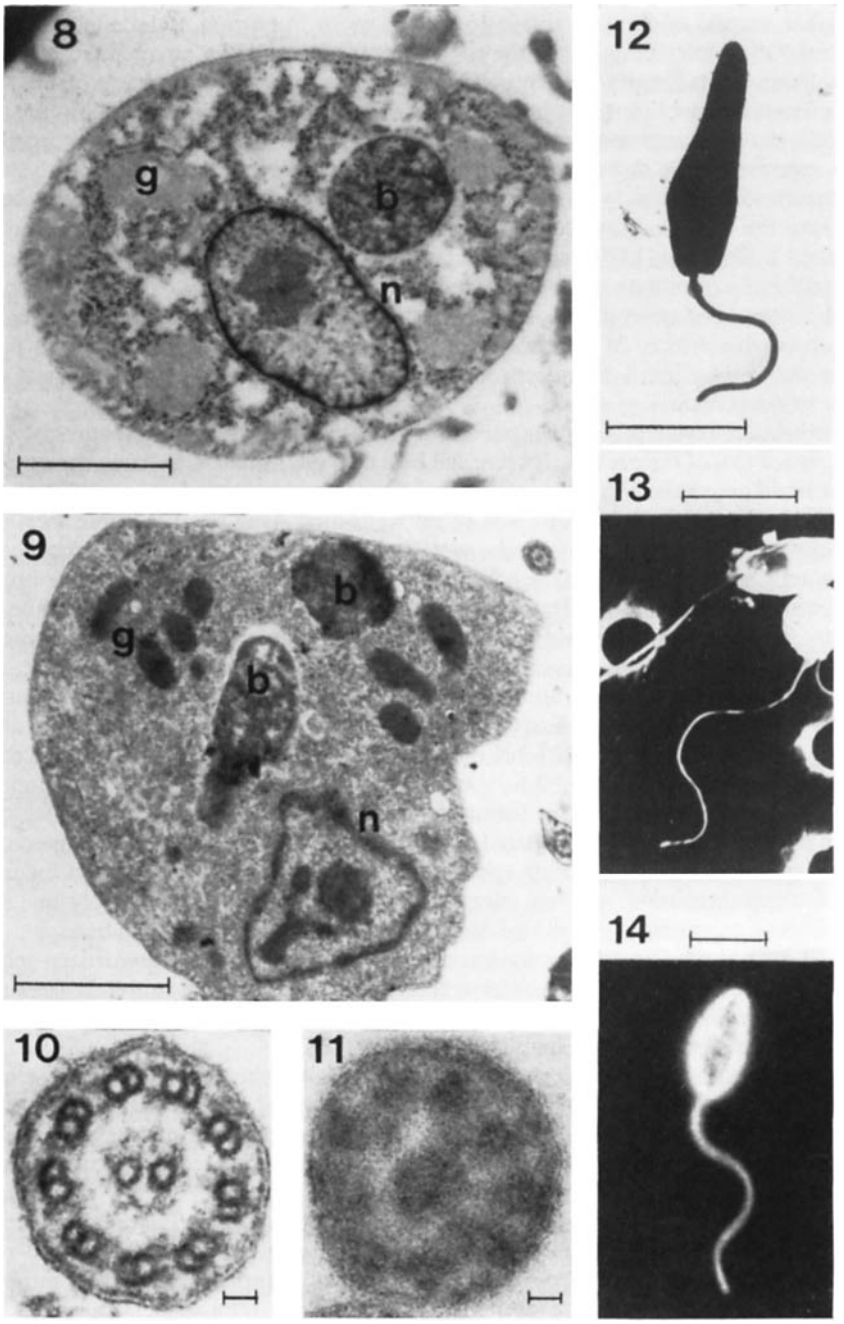
Fig. 11. Transverse section of a flagellum after ethane cooling and freeze substitution. Flagella prepared in this way have poorly defined microtubules. × 175,000. (Length scale 25 nm.)

Fig. 12. CTEM image of a propane-cooled freeze-substituted cell after critical-point drying. × 3600. (Length scale 5 μm.)

Fig. 13. SEM image of propane-cooled freeze-substituted cells after critical-point drying. × 3000. (Length scale 5 μm.)

Fig. 14. Dark-field photomicrograph of a live organism with an actively beating flagellum. × 2000. (Length scale 5 μm.)

When freeze-substituted cells were critical-point dried, some difficulty was experienced in recovering enough of the cells for transmission electron microscopy. Partly this was because the was because the cells could not be settled down on conventional EM grids during the substitution process, as the substitution medium was corrosive. (Attempts to use nylon grids were unsuccessful as they were unstable in the electron beam after exposure to the substitution



Figs. 8–14

medium.) Micrographs of critical-point dried cells taken in the TEM and SEM (Figs. 12 and 13) showed good surface preservation, with bends on the flagellum not unlike those of actively swimming cells viewed in the light microscope (Fig. 14).

DISCUSSION

The quantitative accuracy of the cooling-curve results depends on many factors. The response time of the recording system and the accuracy of the thermocouple calibration (± 1 K) are negligible sources of error in this case; much more important is the effect of the thermocouple on the sample volume. Since the thermocouple wire was in an air-filled glass tube over all but *c.* 1 mm of its length in the coolant, its ability to conduct heat *out* of the sample we assume was small. However, it may have acted as a heat sink, allowing heat to flow *into* the sample as it cooled—the measured cooling rates would then be under-estimates. The recorded temperatures are supposed to be those at the centre of the sample but it is doubtful if the wire could be positioned to better than $\pm 0{\cdot}1$ mm and the sample drop itself could, of course, be distorted on entry into the coolant. However, under the conditions of these experiments (Biot modulus $B=0{\cdot}1$) Bald & Crowley (1979) imply that temperature gradients within the sample are small, so that positional errors may not be important. The main uncontrolled factor in the measurements was the speed of entry of the sample into the coolant, which depended on manual dexterity and had observable effects on the cooling curves if the entry into the coolant was deliberately slow. The curves in Figs. 3–6 are representative ones and do not show the fastest or slowest rates for a given coolant.

The significant result of our comparison of coolants is that liquid ethane gives a cooling rate about twice that of the previously recorded best coolant, propane. Since in the type of experiments described above the behaviour of the coolant is a predominant factor ($B \ll 1$) any improvement in the heat-transfer coefficient h is to be welcomed. Our general impression is that the best coolants are those of lowest molecular weight, but exceptions to this rule occur for H-bonding compounds with high thermal conductivity (e.g. ethanol), gases of very low boiling point which vapourize around the specimen (methane, nitrogen) and liquids which, while otherwise very efficient, do not remain liquid at low enough temperatures to be useful (methanol). The conventional wisdom that coolants of higher boiling point should be more effective, since they prevent a 'vapour jacket' forming round the specimen, seems to be of doubtful application except in the range of very low boiling points. For example, 2-methylpentane, ethanethiol and chloroethane (b.p. 333·3 K, 308 K and 285·3 K respectively) are in fact much *less* effective than ethane (b.p. 184·4 K) or propane (b.p. 230·9 K). Only in the cases of nitrogen (b.p. 77·2 K) and methane (b.p. 109 K) did we observe vapour formation and a consequent low heat-transfer coefficient. One other point of interest is that ethanol is such a rapid coolant, especially if supercooled, that it might be quite suitable for freezing specimens down to 193 K in preparation for freeze-substitution if the substitution medium already contained ethanol. Alternatively one might use an ethanol-based mixture as a combined coolant and freeze-substitution medium.

The warming curve (Fig. 7) emphasizes the need for speed in handling frozen specimens of small dimensions. When transferring the specimen from the coolant to the freeze-substitution medium only a few seconds in air may be enough to raise the temperature to a value at which devitrification and crystal growth may occur (Franks, 1977).

It is also interesting that only in the slower cooling experiments (Fig. 6) did any effects of the release of latent heat appear in the cooling curves as obvious plateaus or 'shoulders', whereas in the paper by Barlow & Sleigh (1979) pronounced shoulders are apparent in their cooling curves for 10 μl samples, even in propane. However, on calculating a cooling curve (based on the model discussed previously) to fit the experimental results given above for the plane sheet of water cooled in ethane, we found marked differences between the two curves. The experimental curve resembled that for ethane in Fig. 4 but the theoretical curve, while it fitted reasonably well below 193 K, showed an increasingly steep slope as it approached 293 K, rather than the rounded appearance of the experimental curves in this region. We suggest that the slow initial cooling exhibited by the experimental curves is due to (a) the smaller thermal diffusivity of water,

compared with that of ice, which obtains at the higher temperatures and (b), more importantly, the release of latent heat as the ice/water interface advances into the specimen. Thus, the 'smoothness' of our experimental cooling curves is no indication of the absence of latent heat effects.

The estimate of a heat-transfer coefficient for ethane illustrates the difficulties of this type of measurement. Ideally one requires a test object of well-defined size and shape, of which the thermal conductivity, specific heat capacity and density are known at every temperature, and in which there is no change of phase. Instead, we have a specimen with a composition probably intermediate between that of a single ice crystal and that of an amorphous structure, surrounded by a plastic ring. However, the order of magnitude of h for ethane (between 1500 and 5000 W m^{-2} K^{-1}) appears reasonable in the light of calculations of h for ethanol during forced convection over a vertical flat plate. On inserting tabulated values of the properties of ethanol at 223 K in a standard equation for h (Thewlis, 1961) and putting the characteristic dimension of the 'plate' as 3 mm, one obtains a value of about 3500 W m^{-2} K^{-1} at a fluid velocity of 1 m s^{-1} relative to the 'plate'. The same procedure for *natural* convection, at a temperature difference of 50 K between plate and bulk fluid, gives about 450 W m^{-2} K^{-1}.

While the ultrastructural preservation of freeze-substituted cells may depend initially upon the rapidity of freezing it also depends on the quality of fixation by the freeze-substitution medium. Clearly, if the fixation is poor the preservation of cellular morphology will be poor regardless of how effective the freezing may have been.

The three freeze-substitution media used in this study were considered by Barlow & Sleigh (1979) to be among the best of over 200 fixation regimes investigated for preserving ciliated surfaces. However, the abilities of these three media to preserve ultrastructure may differ from that required for cell-surface preservation.

The inability of the best freeze-substitution medium to allow clear resolution of the microtubules may be explained in two ways: either (a) the composition of the medium is not optimal for microtubule preservation, or (b) the preparative method prevented or reduced the elution of diffusible solutes from the flagellar matrix and lumen of the microtubules, which would otherwise have occurred had the material been conventionally fixed at between 277 and 293 K. On staining, in case (b) the flagellar matrix would be denser than usual and consequently the contrast between the microtubules and the matrix would be reduced.

We have not established which of the two explanations above is the correct one, but it is likely that both contributed to the loss of resolution. However, Pfaller & Rovan (1978) have also reported a lack of definition of the microtubules of the flagella of bull spermatozoa prepared by freeze-spraying and they concluded that this effect was due to the preservation of diffusible solutes which were then stained in the flagellum. Similar effects have been reported by other authors, e.g. Werner *et al.* (1972).

The preservation of the external shapes of the flagella in the present work appears to be reasonably successful, in spite of the complications we envisaged. It was expected that some differences in the wave parameters might be observed between swimming cells and freeze-substituted ones, even with ideal preservation, because of the different hydrodynamic wall effects originating from the glass cover-slip and the plastic loop respectively. In addition, it was thought possible that the temperature drop experienced by the cells before freezing might induce a traumatic change in flagellar behaviour. Neither of the above expectations appears to have been realized. The inability of the technique to preserve wave shapes on every flagellum in the sample, even with the fastest coolant, is probably due to the thickness of the sample. Cells at the centre of the sample must experience a slower cooling rate than those at the surface in contact with the coolant and inevitably there will be a variation of freezing rates and a range of states of preservation.

Since the flagella of *C. oncopelti* beat at frequencies between 20 and 30 Hz it is clear that for bends to be well preserved the temperature must be reduced from 293 to near 273 K in less than 30 ms. Although the present technique is approaching this criterion, it is evident that much faster cooling rates are at least theoretically possible, even with the present sample volume, since it is not the sample so much as the external cooling system which limits the cooling rate ($B \ll 1$).

Although some improvements in the present convective cooling method are feasible—faster fluid flows and more efficient coolants—it seems that the extraction of heat by conduction into a solid may be more efficient (e.g. freeze-spraying). However, the response of flagellated organisms to the shear strains imposed in spraying and the mechanical shocks involved in sudden contact with a solid have yet to be investigated.

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