CHANGES IN THE SHAPE OF MITOCHONDRIA FOLLOWING OSMOTIC STRESS TO THE UNICELLULAR GREEN ALGA CHLAMYDOMONAS REINHARDII

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SUMMARY
The effects of various stresses on mitochondrial activity and structure within the unicellular green alga Chlamydomonas reinhardii were investigated using the fluorescent probe rhodamine 123. Within control cells, treatment with rhodamine 123 stained an intense fluorescent network, which was considered to be mitochondrial from the similarity in structure to models of mitochondria reconstructed from serial-section electron microscopy, and because this pattern of staining was abolished following the addition of metabolic inhibitors.

Following osmotic shrinkage and rehydration, fragmentation of the mitochondrial network was observed within potentially viable cells. This was reversible within 1 h of resuspension in isotonic medium. Exposure of cells to hypertonic solutions of rapidly permeating compounds did not induce similar structural alterations. These changes in the mitochondria were confirmed by thin-section electron microscopy. In the presence of higher osmolalities of non-permeating compounds, which induce a greater loss of viability, rhodamine 123 stained cells uniformly. Following the osmotic stresses induced by slow rates of freezing and subsequent thawing no fragmentation in mitochondrial staining was observed. These findings demonstrate that shrinkage and rehydration may induce alterations to the structure and function of organelles and may be factors in determining cellular viability following osmotic stress.

INTRODUCTION
The response of cells to short-term osmotic stress and subsequent resuspension in isotonic medium is often considered solely in terms of mechanical stress within the plane of the plasmalemma (Steponkus, Wolfe & Dowgert, 1981; Coster, Steudle & Zimmermann, 1977). During osmotic dehydration of eukaryotic cells, however, the organelles will also be exposed to hypertonic solutions and will in turn undergo shrinkage followed by rehydration on return of the cell to isotonic conditions. In addition, elements of the cytoskeleton important in determining the relative position of organelles may be destabilized by changes in cell shape and volume. Shrinkage and rehydration may therefore induce alterations in the structure and function of the organelles and this may be a factor in determining cellular viability following osmotic stress.

Whilst several reports on the effects of hypertonic stress on isolated organelles, including mitochondria (Araki, 1977) and chloroplasts (Heber et al. 1981), have been

Key words: Chlamydomonas reinhardii, mitochondria, rhodamine 123, osmotic stress.
published, they do not necessarily reflect the situation in vivo. In this study we examine the morphology of the mitochondrial network within the unicellular green alga *Chlamydomonas reinhardii*, using the fluorescent probe rhodamine 123, which has been shown to be a useful indicator of mitochondrial function in mammalian tissue culture cells (Johnson, Walsh & Chen, 1980; Johnson, Walsh, Bockus & Chen, 1981). This cationic compound exhibits a potential-dependent interaction with the mitochondrial membrane of living cells (Johnson et al. 1981), enabling any rapid changes in mitochondrial morphology and activity to be followed by microscopy. The ultrastructure of the mitochondria was also examined by thin-section electron microscopy. *C. reinhardii* was selected for these studies as it has been previously demonstrated to be extremely sensitive to osmotic dehydration, both at a constant temperature and during freezing and thawing (Clarke, Coulson & Morris, 1982). In addition, the mitochondrial network within *C. reinhardii* has been investigated by serial sections, allowing three-dimensional modelling (Arnold & Blank, 1980; Blank & Arnold, 1980; Blank, Hauptmann & Arnold, 1980) and the specific effects of metabolic inhibitors on mitochondria (Blank & Arnold, 1981) to be determined.

**MATERIALS AND METHODS**

**Cells and cell culture**

Strains of *Chlamydomonas reinhardii* were obtained from the Culture Collection of Algae and Protozoa (CCAP), Cambridge. Strain CCAP 11/32c is the 'wild-type' from which CCAP 11/32 CW15 was isolated and characterized as a cell wall-free mutant by Hyams & Davis (1972).

Cells were cultured in CCAP medium 6 (Asher & Spalding, 1982) for 5 days at 20 °C in the light, at which time they were in the early stationary phase of growth. For some studies it was necessary to maintain cells in the dark to reduce the intensity of autofluorescence from the chloroplasts. The osmolality of the growth medium as determined by a freezing point depression osmometer (Fiske, model G66) was 45 mosM.

**Exposure of cells to osmotic stress**

The response of cells to hypertonic solutions was determined by adding 0.5 ml of the cell suspension to an equal volume of additive to give the final required concentration. After 5 min incubation at 20 °C the cell suspensions were diluted and viability was assayed by colony formation in agar. Additives were prepared in growth medium and, following the addition of an equal volume of cells, the sample was centrifuged and the osmolality of the supernatant determined. In each experiment there were five replicates for each treatment.

**Freezing and thawing**

Cell suspensions (1 ml) were placed in 12 mm × 35 mm sterile polypropylene tubes (Nunc) and cooled to −5 °C in a low-temperature bath (Fryka, model KB 300). Where appropriate, ice formation was induced in cooled samples by the introduction of ice crystals via a Pasteur pipette. The samples were cooled further, after freezing at a rate of 0.25 deg. C/min, to the required temperature. Ampoules were warmed or thawed (until no ice was visible within them) by agitation in a water bath at 30 °C.

**Technique of staining**

Cells from either 5 ml of a dark-grown or 1 ml of a light-grown culture were concentrated by centrifugation and resuspended in 1 ml of rhodamine 123 (Sigma) (10 μg ml−1 in distilled water)
Osmotic stress and mitochondria

for 5 min at 20°C. The cells were then washed twice in growth medium. To examine the effects of inhibitors of mitochondrial function on rhodamine staining, cells were incubated in growth medium containing either sodium azide, 2,4-dinitrophenol or valinomycin (Sigma).

Fluorescence microscopy

A Leitz Dialux 22 microscope equipped with epifluorescence was used with a blue excitation filter (390–490 nm) and a 515 nm suppression filter (Ploempak A). Observations were made with a 40/0.70 NPL fluorar objective combined with a 2× magnification changer and photographs were taken on a Wild 35 mm camera using Kodak Tri-X Pan uprated to 6400 ASA and developed with Kodak HC110 developer (dilution B) for 10 min. Exposure times for fluorescence were 1–2 s and for phase-contrast 1/60 s.

Electron microscopy

Control cells and cells rehydrated from 0.3 M-glycerol were fixed in glutaraldehyde (4%) in 0.025 M-sodium cacodylate buffer (pH 7.1) for 75 min at room temperature. The fixed cells were washed four times in 0.025 M-sodium cacodylate buffer, post-fixed for 60 min in 2% osmium tetroxide in 0.1 M-sodium cacodylate buffer (pH 7.1), dehydrated in a graded ethanol series, and embedded in Spurr's low viscosity resin (Spurr, 1969). Ultrathin sections were stained with 2% aqueous uranyl acetate and Reynolds' (1963) lead citrate and examined with a Jeol 100 CX electron microscope.

RESULTS

Cellular viability following hypertonic stress

The viability of light-grown C. reinhardtii CW15+ upon resuspension from hypertonic solutions of non-penetrating or slowly permeating additives is dependent on the osmolality (Fig. 1) and relatively independent of the nature of the solution.

![Graph](image)

**Fig. 1.** Recovery (%) of C. reinhardtii CW15+ following exposure to hypertonic solutions of KC1 (○) or glycerol (●) for 5 min at 20°C.
Table 1. Concentration (mosm) of additives inducing a 50% reduction in viability in C. reinhardii CW15

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration (mosm)</th>
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<tbody>
<tr>
<td>Sorbitol</td>
<td>460</td>
</tr>
<tr>
<td>Glucose</td>
<td>480</td>
</tr>
<tr>
<td>Glycine betaine</td>
<td>520</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>605</td>
</tr>
<tr>
<td>Glycerol</td>
<td>630</td>
</tr>
<tr>
<td>Ethyleneglycol</td>
<td>1125</td>
</tr>
<tr>
<td>Dimethylsulphoxide</td>
<td>1300</td>
</tr>
<tr>
<td>Methanol</td>
<td>&gt;2500</td>
</tr>
</tbody>
</table>

Cells were exposed to various concentrations of additives for 5 min at 20°C and then diluted rapidly into isotonic medium. Viabilities were determined by colony formation in agar and the concentration of additive inducing a 50% reduction in viability was estimated graphically.

(Table 1). In contrast, the more rapidly permeating compounds (dimethylsulphoxide, ethyleneglycol and methanol) become cytotoxic only at higher osmolalities. The responses of C. reinhardii 11/32c and of dark-grown C. reinhardii CW15+ were not significantly different from that of light-grown C. reinhardii CW15+.

Rhodamine staining

Following rhodamine 123 treatment of control, dark-grown cells of C. reinhardii CW15+, an intense fluorescent network was evident (Fig. 2A). In light-grown C. reinhardii CW15+ and C. reinhardii 11/32c similar structures were apparent but somewhat masked by the autofluorescence of the chloroplasts. In addition, in C. reinhardii 11/32c, rhodamine 123 also stained the cell wall.

These networks show a similar pattern to the mitochondrial models reconstructed from serial sections of C. reinhardii (Arnold & Blank, 1980). The addition of sodium azide (10^{-4}M), 2,4-dinitrophenol (10^{-5}M) or valinomycin (5 μg ml^{-1}) to cells for 15 min before the addition of rhodamine 123 completely inhibited this pattern of staining. With cells initially treated with rhodamine 123, the subsequent addition of inhibitors of mitochondrial function abolished the specific staining and induced a general fluorescence within the cell. From these data it was assumed that in C. reinhardii, as in other cell-types (Johnson et al, 1980, 1981), rhodamine 123 is a useful probe for examining mitochondrial morphology and activity.

The effects of hypertonic stress on rhodamine staining

Cells were exposed to solutions of glycerol or KCl of less than 300 mosm; under these conditions viability upon resuspension was greater than 80% (Fig. 1). In cells preloaded with rhodamine 123 and then placed in hypertonic solutions mitochondrial fluorescence was apparent, even though extensive cellular dehydration had occurred (Fig. 2D). If rhodamine 123 was added to cells following hypertonic exposure and
Fig. 2. Phase-contrast (A,C,E) and fluorescence (B,D,F) photomicrographs of the same fields of C. reinhardtii CW15* stained with rhodamine 123. Cells were either in growth medium (A,B), suspended in 0·3 M-glycerol (C,D) or exposed to glycerol (0·3 M) and then resuspended in isotonic medium (E,F). × 1760.
subsequent resuspension into isotonic medium, the normal pattern of mitochondrial staining was greatly modified (Fig. 2f); the mitochondria apparently fragment into smaller structures, which are of greater diameter than mitochondria in control cells. This swelling and fragmentation of the mitochondria is reversible and within 1 h of return to isotonic conditions all cells contained networks of mitochondria similar to those in untreated cells. These alterations were induced by solutions of glycerol and KCl in both strains of *C. reinhardii* examined.

At higher concentrations of KCl and glycerol, mitochondria were not apparent either in the shrunken state or upon resuspension. In cells preloaded with rhodamine 123, exposure to hypertonic solutions greater than 500 mosM induced an overall diffusion of fluorescence within the cell similar to that observed following the addition of mitochondrial inhibitors. Upon resuspension in isotonic medium cells contained neither normal nor fragmented mitochondria, but a uniform staining by rhodamine 123 was observed. The relative proportions of these different staining patterns following resuspension from different solutions of KCl (Fig. 3) demonstrate that the loss of normal mitochondrial staining occurs at lower osmolalities than the reduction in cellular viability (Fig. 1).

Following exposure to methanol (2.5 M) and resuspension in isotonic medium the mitochondria were similar to those in untreated controls. Methanol rapidly penetrates algal cells (Morris, Clarke & Fuller, 1980) and does not induce large changes in cell volume or loss of viability (Table 1). The fragmentation or loss of mitochondrial morphology observed at lower osmolalities of KCl or glycerol is caused by shrinkage and rehydration rather than a decrease in water activity.

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**Fig. 3.** The fragmentation of the mitochondrial network within *C. reinhardii* CW15+ following exposure to hypertonic solutions of KCl for 5 min at 20°C. Immediately following resuspension in isotonic medium rhodamine 123 was added.
During freezing, at low rates of cooling, cells are exposed to hypertonic solutions as a result of the removal of liquid water as ice, and upon thawing cells are returned to isotonic conditions. Following freezing to, and thawing from, −1, −3, −5 and −10°C, no fragmentation of mitochondria was evident within *C. reinhardtii*, even though at −5 and −10°C extensive cell damage and loss of viability occurs (Clarke *et al.* 1982). Cells either contained branched mitochondrial networks or stained uniformly. Cells cooled slowly to, and then maintained at, −5°C in the undercooled state, i.e. in the absence of ice, for periods of up to 1 h were indistinguishable from untreated controls in their rhodamine staining.

**Electron microscopy**

The mitochondria of *C. reinhardtii* frequently occupy positions between chloroplast and plasmalemma (Fig. 4A). Following exposure to, and removal from, glycerol (300 mosM) the mitochondria swell extensively and the intramitochondrial organization is severely modified (Fig. 4B).

**DISCUSSION**

Alterations in the mitochondrial network of *C. reinhardtii* are induced by shrinkage and rehydration and these changes have been observed by two independent techniques: rhodamine 123 staining (Fig. 2) and electron microscopy (Fig. 4). Following the addition of non-toxic, rapidly permeating compounds such as methanol, no alteration in mitochondrial morphology is apparent. The combined stresses associated with shrinkage and rehydration are necessary to induce such changes in mitochondrial organization, normal mitochondria being apparent in shrunken cells. In contrast, cellular dehydration induced by slow rates of freezing does not induce equivalent changes in mitochondrial staining. The effects of slow rates of freezing and subsequent thawing of cells have often been modelled by isothermal osmotic shrinkage and rehydration (e.g. see Steponkus *et al.* 1981). However, the results presented here demonstrate that mitochondria within *C. reinhardtii* react differently to osmotic stress at 20°C compared to dehydration induced during freezing, and suggest that factors other than osmotic stress may account for injury to *C. reinhardtii* during freezing and thawing.

The alterations observed in mitochondrial organization occur within potentially viable cells (Fig. 1) and appear to be completely reversible. Similar changes in the mitochondrial network have been observed during cell division of *C. reinhardtii* by serial-section techniques (Arnold & Blank, 1980; Blank & Arnold, 1980; Blank *et al.* 1980). Exposure of *C. reinhardtii* to solutions of non-permeating compounds greater than 500 mosm, which induces a greater loss of viability, results in an overall staining of the cell by rhodamine 123, and from a comparison with studies using metabolic inhibitors this is interpreted as being a pathological reaction. Whilst it is apparent that alterations in mitochondrial morphology and activity occur before loss of viability, it is not possible in this study to determine to what extent these alterations contribute to the loss of viability. In contrast, in *Saccharomyces cerevisiae* alterations in the
Fig. 4. Thin-section electron micrographs of *C. reinhardii* 11/32c. A. Control cells; B, following exposure to glycerol (0.3M) for 5 min at 20°C and resuspension in isotonic medium. \( \times 40000 \).
rhodamine 123 staining of mitochondria are observed only following osmotic stresses that induce loss of viability (Coulson & Morris, unpublished data), and in this cell-type are clearly pathological reactions.

This study demonstrates that shrinkage and rehydration may modify the structure and activity of mitochondria within viable cells of *C. reinhardii*. Further studies on the effects of changes in cell volume and shape on other organelles and elements of the cytoskeleton may elucidate the factors responsible for the sensitivity to osmotically induced damage in this cell-type.

REFERENCES


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