Effect of Osmotic Shock on Some Intracellular Solutes in Two Filamentous Fungi

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Hypoosmotic and hyperosmotic shock experiments confirmed the importance for osmoregulation in *Penicillium chrysogenum* and *Chrysosporium fastidium* of glycerol and the osmoticum used in the medium. Growth ceased following both types of shock treatment for a time, depending on the magnitude of the shock and the species. Regrowth following hypoosmotic shock took place some distance behind burst tips. Following hyperosmotic shock, growth was initiated by branching at the apex, but if the magnitude of the shock was greater than 10 MPa, it did not occur in either species although osmotic adjustment was observed. Shock experiments did not implicate the higher polyols in osmotic adjustment. Hypoosmotic shock to *C. fastidium* resulted in a rapid loss of all internal solutes, while decrease in glycerol in *P. chrysogenum* following this treatment was more gradual. Transfer of *C. fastidium*, a species which does not grow on media of high salt concentration, to isoosmotic KCl did not result in loss of turgor although growth was inhibited. Glucose was lost from the hyphae but K^+ and Cl^- were taken up.

INTRODUCTION

Glycerol and the osmoticum used in the medium were found to be the principal solutes accumulating in two Fungi Imperfecti tolerant of low steady state water potentials (Luard, 1982). However, this accumulation may have occurred as an indirect result of stress and it is necessary to demonstrate the appearance or loss of solutes following osmotic shock in order to implicate them in osmotic adjustment. Such experiments are easily accomplished with the experimental technique employed which uses colonies grown on cellophane overlying solid media.

Penicillium chrysogenum Thom was subjected to both hyperosmotic and hypoosmotic shock. In an attempt to explain the obligate xerophily of *Chrysosporium fastidium* Pitt and its intolerance of salt, this species was subjected to hypoosmotic shock and to isoosmotic KCl, conditions under which it will not normally grow.

METHODS

Fungi and media. *Penicillium chrysogenum* and *Chrysosporium fastidium* were maintained and grown for experiments as described by Luard (1982). The growth medium was Czapek-Dox agar to which 5 g Difco yeast extract l^{-1} had been added (CYA). Water potential was controlled osmotically by the addition of either glucose or KCl to the medium (Luard & Griffin, 1981). The osmotic potential of unsupplemented CYA was -0.56 MPa. For convenience the theoretical potential of the added solute has been referred to throughout and designated ψ_s . The true potential of the media would be close to 0.56 MPa less than ψ_s .

Plastic Petri dishes (9 cm diam.) containing 30 ml medium were overlaid with cellophane and inoculated centrally with a 4 mm diameter plug cut, in the case of *P. chrysogenum* from a spore suspension in 2% (w/v) water agar, and in the case of *C. fastidium*, from a colony whose growth rate was linear. Colonies were grown to a radius of about 20 mm under steady state conditions at 25 °C. An osmotic shock was applied by lifting the cellophane with the colony from the surface of the agar on which it had grown and laying it carefully on the agar of a fresh plate of the desired potential; the procedure took a few seconds only.

Analysis of hyphae. At intervals following each transfer colonies were sampled and analysed for water, cation, chloride, sugar and polyol content, and hyphal osmotic potential was measured (Luard & Griffin, 1981; Luard, 1982).

Growth. In a separate series of experiments the two species were grown in 47 mm Petrislides with lids (Millipore) containing about 3 ml agar overlaid with cellophane. Osmotic transfers were made between Petri slides as described above and observations were made directly on unmounted hyphae by phase contrast microscopy. Hyphal growth was measured with an eyepiece micrometer.

RESULTS

Growth

When *P. chrysogenum* was transferred from 0 to -10 MPa glucose, growth did not resume; but a shock from -2.5 MPa KCl to -10 MPa KCl resulted in regrowth after 7 h which was initiated by branching at apices. In both cases there was an initial massive shrinkage of the colony such that the leading hyphae recoiled and curled. Hypoosmotic transfer from both -10 MPa glucose and -2.5 MPa KCl to 0 MPa resulted in bursting of tips. In both treatments regrowth started within 2 h but this took place from 250 μ m behind the original colony margin.

Similarly, regrowth did not occur on transfer of *C. fastidium* from -10 to -20 MPa glucose but it did after transfer from -5 to -12.5 MPa, although only after a delay of more than 12 h. Regrowth following hypoosmotic shock of -20 to -10 MPa was also slow and took about 8 h; it was initiated about 350 μ m behind the margin. *Chrysosporium fastidium*, when transferred to isoosmotic KCl, ceased growing but the effect was not lethal since regrowth was possible following transfer back to isoosmotic glucose. When regrowth did occur in either species, it was at the steady state rate expected for the new potential.

Hyphal osmotic potential and water content

The change in hyphal osmotic potential of both species following hypoosmotic shock from -10 MPa glucose to 0 MPa was extremely rapid and virtually complete in a matter of minutes (Fig. 1). This rapid time course is indicative of passive water uptake rather than metabolic solute adjustment. The change in fresh weight to dry weight ratios supported this assumption (Table 1). Following hyperosmotic shock to *P. chrysogenum* from 0 to -10 MPa, there was a rather slower adjustment and the final new value was not reached until about 7 h. The effect of hypoosmotic shock on *C. fastidium* appeared to be the same as that on *P. chrysogenum* in that a positive turgor of several MPa was maintained at the high water potential for at least 24 h. Similarly, transfer to isoosmotic KCl had no effect on the osmotic potential and turgor was clearly maintained under these conditions (Fig. 1).

Ions

The $K^+ : Na^+$ ratios of colonies transferred to or from glucose osmotica are presented in Table 1. Following hypoosmotic shock to *P. chrysogenum* there was a slight decrease in the ratio for the first 4 h but it had recovered to the expected steady state value after 8 h. The $K^+ : Na^+$ ratio after hyperosmotic shock remained relatively stable; the slight increase observed after 8 h was principally due to a decrease in sodium. Following hypoosmotic shock of the same magnitude to *C. fastidium*, there was an immediate reduction in the $K^+ : Na^+$ ratio to a value close to that of the medium. When *C. fastidium* from -10 MPa glucose was transferred to isoosmotic KCl there was a rapid uptake of both ions in equal amounts (Fig. 2). The final K^+ content was 31% higher and Cl^- was 81% higher than when *P. chrysogenum* was grown on -10 MPa KCl (Luard, 1982).

Sugars and polyols

Glycerol accumulated slowly in *P. chrysogenum* following hyperosmotic shock to -10 MPa glucose (Fig. 3). After 8 h, 95% of the glycerol present in the steady state experiment (Luard, 1982) had formed. However, a far higher glycerol content was recorded initially in the hypoosmotic shock experiment (Fig. 4) where the colonies were also grown on -10 MPa glucose. If this value is taken as the steady state, then the content 8 h after hyperosmotic shock

Fig. 1. Effect of osmotic shock on the hyphal osmotic potentials of *Penicillium chrysogenum* (a) and *Chrysosporium fastidium* (b). Hypoosmotic shock (∇), hyperosmotic shock (Δ) and isoosmotic shock ($\blacksquare$). KCl osmoticum (closed symbols), glucose osmoticum (open symbols). Broken lines indicate true external potential of shock treatment.

Table 1. Fresh weight to dry weight and potassium to sodium ratios of *Penicillium chrysogenum* and *Chrysosporium fastidium* following osmotic shock

See text for details of potential difference of shock.

Time (h)	<i>P. chrysogenum</i>				<i>C. fastidium</i>		
	Hypoosmotic		Hyperosmotic		Hypoosmotic		Isoosmotic
	Fw:dw*	K ⁺ :Na ⁺	Fw:dw*	K ⁺ :Na ⁺	Fw:dw*	K ⁺ :Na ⁺	Fw:dw*
0	2.30	4.9	3.43	4.6	2.98	8.3	2.98
0.5	3.22	3.4	2.45	4.4	4.15	1.2	3.31
1	3.24	4.0	2.39	4.3	4.24	1.5	3.19
2	3.32	3.8	2.29	4.6	—	—	—
4	3.30	4.0	2.22	4.6	4.43	1.2	3.23
8	3.23	4.7	2.14	5.4	4.15	1.1	3.30
24	—	—	—	—	3.55	1.5	3.23

* Fresh weight to dry weight ratio.

was only 53% of the final amount. Glucose also increased (Fig. 3), but the level reached did not appear to be quite as high as the steady state value (Luard, 1982). The mannitol content appeared to remain fairly constant throughout. The erythritol level was somewhat higher than previously measured steady state levels and also remained fairly constant, while arabitol increased from a trace to 53 $\mu\text{mol (g dry wt)}^{-1}$ after 8 h (data not shown).

Fig. 2. Effect of isoosmotic transfer of *Chrysosporium fastidium* to -10 MPa KCl. Postassium (●), sodium (▲) and chloride (■).

Fig. 3. Effect of hyperosmotic shock on the principal soluble sugar and polyol content of *Penicillium chrysogenum* (a) and of isoosmotic transfer to KCl on *Chrysosporium fastidium* (b). Glycerol (○, ●), glucose (□, ■) and mannitol (△, ▲).

There was clearly a difference in the effect of hypoosmotic shock on the principal soluble carbohydrates between *P. chrysogenum* and *C. fastidium*. Glycerol was lost only gradually from *P. chrysogenum* but extremely rapidly from *C. fastidium*; 59% remained in the former but only 13% in the latter after 4 h (Fig. 4). This suggests that the shock triggered a change in permeability to glycerol in *C. fastidium* resulting in leakage, whereas in *P. chrysogenum* the slower time course suggests metabolic conversion to a compound not osmotically active. However, glucose was lost fairly rapidly from both species and in fact the level of glucose remained higher in *C.*

Fig. 4. Effect of hypoosmotic shock on the principal soluble sugar and polyol content in *Penicillium chrysogenum* (a) and *Chrysosporium fastidium* (b). Glycerol (○), glucose (□) and mannitol (△).

fastidium than in *P. chrysogenum*, and may have accounted for the positive turgor observed (Fig. 1). Mannitol, arabinol and fructose were all lost from *C. fastidium*, presumably associated with the change in permeability, whereas there was little change in other solutes in *P. chrysogenum* (data not shown).

Isoosmotic KCl did not appear to alter the permeability of *C. fastidium* to glycerol, at least there was only a slight loss after 24 h (Fig. 3). Glucose was lost, 16% remaining after 24 h (Fig. 3), in exchange for KCl (Fig. 2). There was little change in the level of mannitol. Erythritol appeared after 4 h [$63 \mu\text{mol (g dry wt)}^{-1}$] but was not detected after 8 h.

DISCUSSION

Osmotic shock experiments have been performed by a number of workers when investigating the mechanism of osmoregulation in micro-organisms. This has generally led to a well defined sequence of events. Following hyperosmotic shock, water is rapidly lost with a subsequent decrease in volume in wall-less cells, and some degree of plasmolysis in walled cells. This is followed by a gradual return, usually to the initial volume or turgor, dependent on water influx as the concentration of osmotically active substances increases internally. In single cells this process can conveniently be followed by change in turbidity of cell suspensions or by electronic measurement of cell volume. In filamentous fungi the process must be inferred by measurements

of osmotic potential accompanied by rates of change of internal osmotica. The present results indicate that the fungal colony responds in a manner similar to the single celled micro-organism, with a relatively rapid adjustment of internal osmotic potential, and confirm the importance of glycerol and the major solute in the medium in osmotic adjustment in *P. chrysogenum* and *C. fastidium* (Figs 3 and 4).

Recovery of volume of the unicellular alga *Dunaliella parva*, which took 15 to 180 min, was independent of ATP hence excluding dependence on glycerol synthesis, and it was concluded that small influxes of ions would probably account for recovery (Gimmler *et al.*, 1977). In another alga, *Platymonas subcordiformis*, there was a transient increase of ions to bridge the gap until sufficient mannitol was synthesized to balance the external potential (Kirst, 1977). The time course of adjustment to hyperosmotic shock in *P. chrysogenum* was of the order of hours rather than minutes (Fig. 1a) and there does not appear to have been a detectable influx of ions (Table 1) so that equilibrium by accumulation of glycerol (Fig. 3a) was not reestablished for several hours. The organism must therefore be able to survive conditions of low turgor for such a period, presumably by the cessation of growth. In fact it was apparent that growth did not resume following hyperosmotic shock of 10 MPa to either species, even though osmotic adjustment occurred. However, it did resume if the magnitude of the shock was less than 10 MPa. The time taken for resumption of growth following a shock of the same magnitude was greater for the slower growing *C. fastidium* than for *P. chrysogenum*, so that species characteristics as well as potential difference affect recovery.

Mannitol is accumulated by the alga *P. subcordiformis* (Kirst, 1977) and is lost from the hyphomycete *Dendryphiella salina* (Jennings & Austin, 1973) during osmoregulation. In the present experiments, the mannitol content on a dry weight basis remained fairly constant following shock treatment (Figs 3 and 4). Neither the hypoosmotic nor the hyperosmotic shock treatments suggest that mannitol was implicated as an osmoregulatory compound, although it appeared to be inversely correlated with steady state potential (Luard, 1982).

The hypoosmotic shock experiments have revealed a fundamental difference between the two species which may account for the obligate xerophily of *C. fastidium*. It seems probable that such a treatment induced irreversible changes in membrane permeability in *C. fastidium* resulting in loss of cation selectivity and leakage of internal solutes. It is of course arguable whether death results from the loss of membrane integrity or is a cause of it. Tips of both species burst when subjected to this treatment and an alternative explanation might be that healing is slower, or less effective, in *C. fastidium* so that more protoplasm is lost from the hyphae than can be tolerated. An increase in permeability and subsequent leakage of solutes at low water potentials was also noted in conidia of *Neurospora crassa*, but the damage was not lethal (Charlang & Horowitz, 1974). They did not observe such changes in the more xerotolerant species *P. chrysogenum* and *Aspergillus nidulans*. Isoosmotic KCl did not appear to alter permeability to glycerol in *C. fastidium* although ion selectivity was altered so that K^+ and Cl^- were taken up in large and equal amounts.

Differences in permeability to internal osmotica may account for the different range of water potentials tolerated by these two filamentous fungi. The yeast *Saccharomyces rouxii* is considerably more tolerant of low water potential than *Saccharomyces cerevisiae*, although both species are able to produce glycerol as an internal osmoregulator (Brown, 1978). When grown at the same potential, both species contained the same amount of glycerol. However, *S. cerevisiae* was less able to retain glycerol, so that a far higher extracellular concentration of glycerol was present in the culture medium of this species than that of *S. rouxii*. Glycerol accumulated by *D. parva* and *D. tertiolecta* for osmotic adjustment did not leak following hypoosmotic shock (Ben-Amotz, 1975; Kessly & Brown, 1981). Although external concentrations of glycerol were not measured in the present experiments, the time course involved suggested that conversion to non-osmotic compounds rather than leakage took place following hypoosmotic shock. The mechanism by which glycerol, which permeates readily through membranes, is retained against large concentration gradients remains unknown. The nature of the signal controlling membrane permeability is also unknown but Coster *et al.* (1977) have proposed that changes in turgor can directly alter membrane properties including permeability to ions.

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REFERENCES

- BEN-AMOTZ, A. (1975). Adaptation of the unicellular alga *Dunaliella parva* to a saline environment. *Journal of Phycology* **11**, 50–54.
- BROWN, A. D. (1978). Compatible solutes and extreme water stress in eukaryotic micro-organisms. *Advances in Microbial Physiology* **17**, 181–242.
- CHARLANG, G. W. & HOROWITZ, N. H. (1974). Membrane permeability and the loss of germination factor from *Neurospora crassa* at low water activities. *Journal of Bacteriology* **117**, 261–264.
- COSTER, H. G. L., STEUDLE, E. & ZIMMERMANN, U. (1977). Turgor pressure sensing in plant cell membranes. *Plant Physiology* **58**, 636–643.
- GIMMLER, H., SCHIRLING, R. & TOBLER, U. (1977). Cation permeability of the plasmalemma of the halo-tolerant alga *Dunaliella parva*. I. Cation induced osmotic volume changes. *Zeitschrift für Pflanzenphysiologie* **83**, 145–158.
- JENNINGS, D. H. & AUSTIN, S. (1973). The stimulatory effect of the non-metabolized sugar 3-O-methyl glucose on the conversion of mannitol and arabitol to polysaccharide and other insoluble compounds in the fungus *Dendryphiella salina*. *Journal of General Microbiology* **75**, 287–294.
- KESSLY, D. S. & BROWN, A. D. (1981). Salt relations of *Dunaliella*. Transitional changes in glycerol content and oxygen exchange reactions on water stress. *Archives of Microbiology* **129**, 154–159.
- KIRST, G. O. (1977). Ion composition of unicellular marine and fresh-water algae, with special reference to *Platymonas subcordiformis* cultivated in media with different osmotic strengths. *Oecologia* **28**, 177–189.
- LUARD, E. J. (1982). Accumulation of intracellular solutes by two filamentous fungi in response to growth at low steady state osmotic potential. *Journal of General Microbiology* **128**, 2563–2574.
- LUARD, E. J. & GRIFFIN, D. M. (1981). Effect of water potential on fungal growth and turgor. *Transactions of the British Mycological Society* **76**, 33–40.