The Role of Glycerol in Osmotolerance of the Yeast *Debaryomyces hansenii*

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Transfer of growing cells of the salt-tolerant yeast *Debaryomyces hansenii* to media of higher salinity resulted in an increased production and intracellular accumulation of glycerol, which was proportional to the magnitude of the shift in salinity. Stress solutes other than NaCl, when added in iso-osmolar concentrations, promoted the accumulation of similar amounts of glycerol. Cells grown at high salinity rapidly lost glycerol when returned to media of lower salinity and the loss was greater when the cells were transferred to more dilute media. A mutant strain of *D. hansenii* showed poor glycerol production and was inhibited by NaCl at concentrations about half the maximum tolerated by the wild-type. Growth of this mutant occurred at otherwise inhibitory NaCl concentrations if the medium was supplemented with a low concentration of glycerol. The added glycerol was intracellularly accumulated to levels that increased with salinity and were only slightly lower than the corresponding wild-type levels. Glycerol additions above the growth promoting level had little effect on growth rate but caused substantial shortening of the lag phase. Osmoprotectants other than glycerol did not permit growth to occur. The mutant was isolated as a glycerol non-utilizer but displayed growth in glycerol media at increased NaCl concentrations.

**INTRODUCTION**

Cellular growth is generally inhibited by extremes of environmental salinity. However, in a few groups of prokaryotic and eukaryotic micro-organisms there are species that have acquired the ability to tolerate wide ranges of salt stress (Brown, 1976; Gould & Measures, 1977). The yeast *Debaryomyces hansenii*, which is widely distributed in natural habitats, can be found in saline environments from sea-water (Norkrans, 1966) to concentrated brines (Onishi, 1963). As the extracellular water potential may fluctuate greatly the cells must be able to adjust their internal milieu to maintain metabolic functions and turgor pressure. Potassium ions are accumulated intracellularly while sodium ions are fairly well excluded, despite massive extracellular concentrations (Norkrans & Kylin, 1969; Hobot & Jennings, 1981). The intracellular Na*/K* content of growing cells is not sufficient to maintain osmotic equilibrium vis à vis the environment at high salinity and several studies indicate that the accumulation of an organic solute, glycerol, is correlated with growth at increased salt stress (Gustafsson & Norkrans, 1976; Gustafsson, 1979; Adler & Gustafsson, 1980; Adler et al., 1985; Nobre & daCosta, 1985). It has been proposed that glycerol acts as a compatible solute (Brown, 1978; Yancey et al., 1982) to increase the internal osmotic pressure without interfering with macromolecular structure and function at high or variable concentration.

A previous study described the pathways of glycerol metabolism in *D. hansenii* and a concentrative transport of glycerol was demonstrated (Adler et al., 1985). In the present work the behaviour of the glycerol pool in growing cells subjected to changes in environmental water potential is reported. It is shown that a mutant with a reduced salt-tolerance and a decreased ability to produce glycerol regains the ability to multiply at inhibitory concentrations of NaCl if the medium is supplemented with small amounts of glycerol. A preliminary account of part of this work has been given elsewhere (Adler, 1986).
**Organisms, media and growth conditions.** Debaryomyces hansenii (Zopf) van Rij strain 26 (Norkrans, 1966) and a mutant, strain 26-6, isolated as described below, were routinely maintained on nutrient agar WA (Wickerham, 1951). Cells were cultured in a defined liquid medium (Adler & Gustafsson, 1980) with supplements as stated in the text. Starter cultures containing 1% (w/v) glucose were prepared by inoculating 50 ml medium in a 300 ml Erlenmeyer flask with a loopful of yeast. These cultures were incubated for 48 h and used to inoculate 2-8 l Fernbach flasks containing 500 ml 0-5% (w/v) glucose medium to give a cell density of 2 x 10^6 cells ml^-1, unless otherwise stated. All liquid cultures were incubated at 27 °C on an orbital shaker (110 cycles min^-1). Solid media contained the same components as the liquid medium with 1% (w/v) carbon source, 1-5% (w/v) Bacto-Agar, and NaCl and other additions as stated.

Growth was monitored by measuring the optical density at 610 nm in a Beckman B spectrophotometer. Dry weight was routinely determined with cells washed twice in distilled water and dried at 95 °C for 48 h or from OD_{610} readings using standard curves for appropriate strain, growth phase and NaCl concentration.

**Transfer experiments.** Exponential-phase cultures were shifted to higher salinity/lower water potential by adding an equal volume of medium containing sufficient stress solute to give the desired salinity/water potential and a final cell density of 0.1-0.3 mg dry wt cells ml^-1. Immediately after the addition, the culture volume was adjusted to its original value (500 ml). A shift from high to lower salinities was done by growing a culture containing 12% (w/v) NaCl to a cell density of 0.8 mg ml^-1. Cells were recovered by centrifugation (5000 g, 10 min) and resuspended to half the original cell density, in fresh medium without glucose and with the desired lower concentration of NaCl. Glycerol was omitted since extracellular release of intracellular polyols has been reported for yeasts on exposure to glucose (Brown, 1974).

**Mutagenesis.** An exponentially growing culture was mutagenized by treatment with N-methyl-N'-nitro-N-nitrosoguanidine and mutants that were capable of growth on glucose but unable to grow on glycerol were isolated as described by Adler et al. (1985). The glycerol non-utilizer used in this study grows on other gluconeogenic carbon sources, such as acetate, pyruvate and ethanol.

**Extraction of glycerol.** For determination of intracellular glycerol the cultures were sampled by the following methods. (1) Two 1-5 ml samples were withdrawn and one of them rapidly centrifuged in an Eppendorf centrifuge. The uncentrifuged sample (a) and the clear supernatant (b) were heated in boiling water for 10 min in glass tubes equipped with pear-drop condensers. The heat-treated samples were centrifuged and the supernatants frozen until analysis. After determination of the glycerol content the difference between total (a) and extracellular (b) glycerol was taken as the intracellular glycerol content. (2) For experiments where different stress solutes were used or when glycerol was supplemented to the extracellular medium, 10 ml culture samples were filtered through a 50 mm HAWP Millipore filter (0.45 μm porosity) and washed twice with 5 ml growth medium without glucose, containing the appropriate concentration of stress solute. The filter was transferred to 5 ml distilled water in a glass tube equipped with a condenser and cells were heat treated as above. After centrifugation the clear supernatant was frozen until analysis. The two methods used gave values for the intracellular glycerol content that agreed within ±10%.

**Glycerol analysis.** Glycerol was analysed enzymically with a commercial glycerol assay kit (Boehringer Mannheim). The absorbance readings were recorded with a Shimadzu UV-Vis 240 spectrophotometer.

**RESULTS**

**Effects of changes in salinity/water potential on glycerol production and the intracellular glycerol pool**

The effect of a sudden shift in growth medium salinity on the glycerol production and the intracellular glycerol accumulation of a growing culture of *D. hansenii* is seen in Fig. 1. Exposure to higher salinities led to an increased rate of glycerol production and intracellular glycerol accumulation, and the levels reached increased in direct proportion to the magnitude of the shift in NaCl concentration (0.25-8% NaCl). The time taken for these changes in glycerol content (2-4 h) was about a generation time. The NaCl-induced glycerol was produced from the glucose of the medium rather than from cellular carbon reserves because there was insignificant glycerol production by cells transferred to 8% NaCl medium containing no glucose (data not shown). For cells subjected to the most severe salt-shocks there was an adjustment period before the maximum rate of glycerol production/accumulation was reached; this was most clearly seen for the cells transferred to 12% NaCl. Increases in salinity up to 2% NaCl were accommodated by the cells without visible disturbance of growth pattern, whereas cells subjected to an up-shift of a higher magnitude experienced a lag phase of growth (Fig. 1b, inset), which increased markedly at salinities above 4%.
Osmotolerance of D. hansenii

Fig. 1. Glycerol production (a) and intracellular accumulation (b) by D. hansenii wild-type after a shift (at \( t = 0 \)) to medium of increased salinity. The concentrations of NaCl after up-shift were: 12\%\( \downarrow \); 8\%\( \downarrow \); 4\%\( \downarrow \); 2\%\( \downarrow \); 1\%\( \downarrow \); 0.5\%\( \downarrow \); 0.25\%\( \downarrow \); and 0\%\( \downarrow \). The inset in (b) shows the length of the lag phase of growth after transfer to media of increased salinity.

Fig. 2. Effect of a sudden decrease (at \( t = 0 \)) of the medium NaCl concentration on the intracellular content of glycerol for D. hansenii wild-type grown at 12\% NaCl. The concentrations of NaCl after down-shift were: 10\%\( \downarrow \); 8\%\( \downarrow \); 6\%\( \downarrow \); 4\%\( \downarrow \); 2\%\( \downarrow \); and 0\%\( \downarrow \). The dashed line (\( \Box \)) shows the total amount of glycerol in the cultures after down-shift and the vertical bars indicate variation (±SD) in total amount between cultures of different final salinity.

To study the osmotic sensitivity of the intracellular glycerol pool, cells growing in 12\% NaCl were transferred to lower salinities and their intracellular glycerol contents were followed (Fig. 2). Such down-shift caused a rapid adjustment to a lower glycerol content, which changed little after the initial drop. For cells transferred from 12\% down to 4\% NaCl the glycerol content was comparable to that found in cells at these salinities after up-shock (cf. Fig. 1b). When shifted to 2\% and 0\% NaCl, the cells retained more glycerol (1.5 and 4 times, respectively) than expected.
An exponentially growing culture containing 12% NaCl was diluted in media containing 12, 6 or 0% NaCl. Samples of 0.1 ml were plated in quadruplicate on media containing the same concentration of NaCl as the diluting medium. The mean number of colonies ± SD was determined after 4–7 d.

<table>
<thead>
<tr>
<th>Final concn of NaCl (% w/v)</th>
<th>Colonies formed per plate</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>209 ± 8</td>
</tr>
<tr>
<td>6</td>
<td>202 ± 27</td>
</tr>
<tr>
<td>0</td>
<td>210 ± 30</td>
</tr>
</tbody>
</table>

Table 2. Glycerol accumulation by D. hansenii wild-type after exposure to various stress solutes added in equiosmolar concentrations

The solute concentrations used to adjust the water potential were as given in the Tables of Harris (1981). The concentrations of NaCl used for these adjustments were 0.6, 1.3 and 3.9%. The glycerol values shown represent plateau levels achieved 2–4 h after addition of the stress solute and the values are averages ± SD of three measurements made at time intervals. The control value obtained with no stress solute added was 0.09 µmol (mg dry wt)⁻¹.

<table>
<thead>
<tr>
<th>Stress solute</th>
<th>-5 bar</th>
<th>-10 bar</th>
<th>-30 bar</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.25 ± 0.01</td>
<td>0.53 ± 0.05</td>
<td>1.37 ± 0.11</td>
</tr>
<tr>
<td>KCl</td>
<td>0.24 ± 0.03</td>
<td>0.47 ± 0.11</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.25 ± 0.02</td>
<td>0.32 ± 0.05</td>
<td>1.21 ± 0.08</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.27 ± 0.02</td>
<td>0.43 ± 0.14</td>
<td>1.34 ± 0.31</td>
</tr>
</tbody>
</table>

from such comparisons. The adjustments of the glycerol pool after down-shock were effected by release of glycerol to the surrounding medium rather than by catabolism, as the total glycerol content of the cultures remained essentially unchanged during the period of adjustment. Since released glycerol may have originated from cells that were ruptured by the shock, the viability of cells subjected to down-shock was surveyed (Table 1). Cells were able to withstand the down-shock stresses imposed, with no loss in colony forming ability, suggesting that the glycerol contents measured represented equilibrium levels reached after osmotic adjustment.

To examine whether the accumulation of glycerol was affected by the chemical nature of the stress solute, media were adjusted with different stress solutes in iso-osmolar concentrations to yield water potentials of -5, -10 and -30 bar (1 bar = 10⁵ Pa) (Table 2). Approximately the same extent of change in the cell glycerol content was observed if KCl, Na₂SO₄ or sucrose were used in place of NaCl.

Effects of glycerol on the salt tolerance of the mutant strain 26-6

On examination of the growth characteristics of glycerol non-utilizing mutants of D. hansenii, it was observed that one of the mutants isolated had a clearly reduced salt tolerance. Colony formation did not occur on solid media containing more than 10% NaCl, while the wild-type retained colony forming ability on media containing 18% NaCl. When exposed to salt stress the mutant displayed a slower rate of glycerol accumulation than the wild-type strain and the glycerol levels attained were substantially lower than those of the wild-type (Fig. 3a, b). The reduced capacity for glycerol accumulation was due to a decreased ability to produce glycerol rather than a decreased capacity for intracellular retention of glycerol. The mutant retained 50–75% of the glycerol produced (data for total production not shown), as observed for the wild-type (cf. Fig. 1). Therefore an experiment was done to determine whether growth was stimulated by exogenously supplied glycerol at an NaCl concentration (14%) that is normally inhibitory to the mutant strain (Table 3). Glycerol additions ≥ 0.5 mM completely restored the colony forming
**Fig. 3.** Glycerol accumulation by *D. hansenii* wild-type (*a*) and mutant strain 26-6 (*b, c*) after a shift (at $t = 0$) to medium of increased salinity containing no (*a, b*) or 1 mM (*c*) glycerol. The concentrations of NaCl after up-shift were: 14%, ▽; 8%, ▲; 4%, Δ; and 0%, ▼.

**Table 3.** Colony formation by *D. hansenii* mutant strain 26-6, on 1% glucose media containing 14% NaCl supplemented with various osmolites

Cells grown in basal medium were diluted in saline (0.9% NaCl), samples of 0.1 ml were plated in triplicate for each incubation and the average ± SD of colonies formed per plate was determined after 5–14 d incubation. Plates showing no colonies were incubated further for 40 d. The number of colonies formed on media with no NaCl addition was 215 ± 20.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Conc. (mM)</th>
<th>Colonies formed per plate</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.1</td>
<td>165 ± 10</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.5</td>
<td>215 ± 15</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1</td>
<td>224 ± 14</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5</td>
<td>212 ± 26</td>
</tr>
<tr>
<td>Mannitol</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Arabinol</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Proline</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Trimethylaminoxide</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Betaine</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

ability of the mutant cells at this salinity, whereas a concentration of 0-1 mM gave about a 20% loss in colony formation. No colonies appeared on media lacking glycerol or on media where glycerol was substituted by other well-known osmoprotectants (cf. Yancey *et al.*, 1982) at 1 mM concentration. Since glycerol additions permitted growth to occur at otherwise inhibitory NaCl concentrations, it was of interest to determine if the exogenously added glycerol was accumulated by the cells. Accordingly, 1 mM-glycerol was provided to exponentially growing cultures of the mutant strain as the cultures were shifted to higher salinity. The glycerol accumulation proceeded rapidly in cultures receiving exogenous glycerol to levels that were only slightly lower than those reached at the same salinities by wild-type cells (Fig. 3c). Complementary determination of total and extracellular glycerol in parallel experiments confirmed that the intracellular glycerol levels were achieved by uptake of extracellular glycerol rather than by intensified glycerol production after glycerol supplementation (data not shown).
To examine the effects of glycerol on the growth characteristics of the mutant strain at high salinity, inocula were transferred to liquid media containing 16% NaCl and various concentrations of glycerol. As expected from the plate-count studies mentioned above, the mutant strain did not grow in the absence of glycerol or with 0.05 mM-glycerol, while 0.5 mM-glycerol permitted growth. By further increasing the glycerol concentration (in 10-fold steps) the lag phase of growth was shortened to become – as cultures were supplemented with 50 mM-glycerol – close to that seen for the wild-type grown in normal medium (Fig. 4). Interestingly, there was also a reduction in the length of the lag phase for the wild-type when provided with 50 mM-glycerol. The growth rates of the two strains were, however, not appreciably affected by the increased glycerol concentration. Since the smallest glycerol addition that permitted growth of the mutant was sufficient to provide only a small fraction of the amount needed for the expected glycerol adjustments, the intracellular glycerol content was examined for exponentially growing cells at 8% or 16% NaCl in cultures supplemented with 0.5 mM-glycerol. The levels obtained, 1.0–1.5 and 2.7–2.9 μmol glycerol (mg dry wt cells)\(^{-1}\) at 8% and 16% NaCl, respectively, were 20–40% lower than those for wild-type cells at these salinities, and the total glycerol content of the cultures was 2–8 times higher than what was originally added. These results indicate that the mutant requires a minimum level of external glycerol to initiate growth at high salinity but is thereafter capable of maintaining growth-promoting glycerol levels by intracellular production. The levels accumulated under steady-state growth were, however, lower than the corresponding wild-type levels.

The fact that the mutant was isolated as a glycerol non-utilizer and, for reasons that have not been clarified, unable to grow on glycerol, points to a strictly osmoregulatory role for the glycerol that is concentrated intracellularly. However, when the growth characteristics of the mutant were examined in glycerol media supplemented with NaCl, growth was observed at raised salinity and this growth was stimulated as the NaCl concentration increased from 2 to 8% (Fig. 5). A further increase to 12% NaCl caused slower growth and at 16% NaCl, a concentration at which the wild-type still grows on glycerol, no growth at all was observed. These results suggest that the mutant is salt-remedial and at least partially regains lost functions when exposed to 2–12% NaCl.
Previous studies have shown the content of glycerol in D. hansenii to be markedly increased in cells growing at high salinity (Gustafsson & Norkrans, 1976; Gustafsson, 1979; Adler & Gustafsson, 1980; Adler et al., 1985; Nobre & daCosta, 1985). The intracellular pools of Na⁺ and K⁺ (Norkrans & Kylin, 1969; Hobot & Jennings, 1981) and amino acids (Adler & Gustafsson, 1980) did not behave in a similar fashion and glycerol was suggested as a major compatible solute in this yeast. In the present study we have sought further evidence on the role of glycerol in salt- and osmotolerance of D. hansenii.

The NaCl concentration of the growth medium was clearly a major determinant for the glycerol accumulation of this organism as shown by the glycerol accumulation curves for exponentially growing cells subjected to increased salinity (Fig. 1). Both the rate of the glycerol accumulation and the glycerol levels reached were determined by the magnitude of the increase in NaCl concentration. The cells responded to all changes in salinity tested and no threshold in NaCl concentration was found below which glycerol accumulation did not occur. Within the salinity range 0.25%–8% NaCl, the glycerol adjustments were essentially complete within a period that had the magnitude of a generation time, which may imply a cell-cycle-phase-dependent phenomenon. Although seemingly stable plateau levels were established after this primary adjustment period, it should be pointed out that the cellular glycerol pool, as expressed on a dry weight basis, shows a dynamic behaviour during the growth cycle; the glycerol content decreases during the late exponential phase in a process that coincides with a less pronounced increase in cellular arabinitol (Adler & Gustafsson, 1980).

After transfer to a medium of high salinity (>4%), there was a temporary cessation of growth (Fig. 1b, inset) which gradually resumed during the period of glycerol production and accumulation. This correlation suggests that the glycerol accumulation following a severe salt shock may re-establish the turgor pressure and the biochemically functional internal milieu required to restore growth. The osmotic contribution of the glycerol accumulated at 8% NaCl can be estimated to equal approximately half (about 1.3 M-glycerol) the osmotic potential of the external medium, assuming a water soluble space in D. hansenii similar to that of Saccharomyces cerevisiae, i.e. 2 µl (mg dry wt yeast)⁻¹ (e.g. Meredith & Romano, 1977). Cell volume is, however, dependent on growth rate (Lord & Wheals, 1980) and if D. hansenii growing in a medium of 8% NaCl has an internal aqueous space similar to that of the osmotolerant yeast Zygosaccharomyces bailii growing at a comparable rate [about 1 µl (mg dry wt)⁻¹, Cole & Keenan, 1987] the intracellular glycerol would, per se, be sufficient to generate the appropriate osmotic potential.

If glycerol accumulation represents adaptation to osmotic stress, the cells must be capable of reversing the process in the face of varying osmolarity. Elimination of intracellular glycerol in response to a sudden decrease of medium salinity was achieved by loss of glycerol to the surrounding medium (Fig. 2). By such adjustments, which caused no loss in viability, lower and seemingly balanced levels of glycerol were reached in all cases but the most drastic down-shocks, where the final glycerol content was higher than expected. These results may indicate that part of the intracellular glycerol suffers restrictions in motional freedom and does not readily take part in osmotic equilibration (cf. Adler et al., 1981). However, the main conclusion from the down-shock experiments must be that the bulk of the glycerol is maintained in an osmotically responsive state within the cells.

The intracellular accumulation of glycerol appears to be osmotically controlled rather than specifically induced by NaCl, as judged from the fact that iso-osmotic concentrations of various stress solutes increased the intracellular glycerol content to similar extents (Table 2). The observations by Nobre & daCosta (1985) on glycerol accumulation during exponential growth of D. hansenii seem to indicate that osmotically controlled accumulation also occurs under more severe stress conditions.

Further evidence that the regulation of cell osmolarity entails control of the glycerol content comes from studies of the mutant strain 26-6. This strain showed poor glycerol production and was inhibited by NaCl at concentrations about half as high as those tolerated by the wild-type. However, the cells were capable of accumulating high intracellular glycerol levels by concentrating exogenously supplied glycerol (Fig. 3) and such additions of small quantities of
glycerol completely restored the colony forming ability at otherwise inhibitory concentrations of NaCl (Table 3). Since the mutant was unable to utilize glycerol for growth at high salinity (16% NaCl, Fig. 5) it is unlikely that the growth promotion caused by the addition of glycerol to the glucose medium (Fig. 4) was due to metabolism of the accumulated glycerol, leaving osmoregulation/osmoprotection as the most feasible alternative.

The ability of the mutant to accumulate exogenous glycerol is in agreement with the previously reported existence of a concentrative uptake of glycerol in *D. hansenii* (Adler et al., 1985). The *K_m* of this transport system was reported to be 0.9 mM, which is similar to that of the mutant (A. Blumberg, personal communication); the transport system is therefore far from saturated at glycerol concentrations below the growth promoting level (0.1-0.5 mM). Thus, at such glycerol concentrations, the mutant was probably unable to accumulate glycerol at a sufficient rate to reach the intracellular concentration required for growth. Increases of the glycerol concentration above the growth promoting level had little effect on the growth rate of the mutant, but caused substantial decrease of the lag phase of growth (Fig. 4). The fact that high external concentrations of glycerol also caused a shortening of the lag phase of the wild-type suggests that the establishment of an appropriate glycerol level is rate limiting at high salinity and that increased external glycerol concentrations facilitate the required glycerol adjustments via the uptake system. Acclimatized and growing cells seem, on the other hand, to be more or less independent of extracellular glycerol as indicated by the insignificant effect of glycerol on growth rates.

The results presented suggest that the intracellular glycerol levels of *D. hansenii* could be adjusted in response to increased osmotic stress by glycerol production (Fig. 1) and, as indicated by the behaviour of the mutant (Fig. 3b, c), also by glycerol uptake. However, the mechanisms by which the glycerol levels are adjusted remain obscure. To effect such regulation one could envisage a control mechanism where glycerol accumulation is stimulated in response to increased osmotic stress by glycerol production (Fig. 1) and, as indicated by increased pressure (Laimins et al., 1981) and potassium concentration (Sutherland et al., 1986) control the expression of transport systems involved in osmoregulation in *Escherichia coli* and *Salmonella typhimurium*. Studies of properties and regulation of enzymes in the glycerol metabolism of *D. hansenii* are presently in progress.

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REFERENCES


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