Physicochemical factors affecting glycerol production in Saccharomyces Cerevisiae

Keshav Kumar Singh

University of Wollongong

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PHYSICOCHEMICAL FACTORS AFFECTING GLYCEROL PRODUCTION IN SACCHAROMYCES CEREVISIAE

A thesis submitted in fulfilment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

from

THE UNIVERSITY OF WOLLONGONG

by

Keshav Kumar Singh, B. Sc., M. Sc. (Microbiology)

DEPARTMENT OF BIOLOGY
1987
What is there to show for a voyage of exploration other than a scar and a few scrubbles on a map.

Bronowski (1978)
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<td>Adenosine diphosphate</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>aw</td>
<td>Water activity</td>
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<td>cAMP</td>
<td>Adenosine 3' 5' monophosphate</td>
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<tr>
<td>DDR</td>
<td>DNA damage regulation genes</td>
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<td>DHA</td>
<td>Dihydroxy acetone</td>
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<td>DHAP</td>
<td>Dihydroxy acetone phosphate</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<td>SHA</td>
<td>Synthetic honey agar</td>
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<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
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I dedicate this thesis to my creative, ever encouraging parents, brother, sisters and their families, with loving and affectionate regards.
ABSTRACT

Both Saccharomyces cerevisiae and S. rouxii respond to a water stress by accumulating the compatible solute glycerol yet only the latter is xerotolerant. When exponentially growing yeasts were transferred from a normal high water activity growth medium (aw 0.997) to synthetic medium containing 8% NaCl (low water activity growth medium aw 0.955) the pattern of glycerol accumulation during the first eight hours of the adaptation was markedly different in the two species. S. rouxii began to accumulate glycerol immediately after transfer whereas glycerol accumulation by S. cerevisiae was both retarded and greatly diminished in magnitude. S. cerevisiae did not recommence growth till 50 hours after transfer. Investigation of the underlying reasons for the slow onset of glycerol accumulation revealed that not only was overall glycerol production reduced by salt transfer but also the rate of ethanol production and glucose consumption were each reduced. A 31P NMR spectroscopic study of glycolytic intermediates revealed an accumulation of glucose-6-phosphate, fructose-6-phosphate, fructose 1,6 bisphosphate and phosphoenolpyruvate in S. cerevisiae three to four hours after transfer to salt, suggesting that one or more glycolytic enzymes were inhibited by salt. Potassium ions accumulated in S. cerevisiae after salt transfer and reached a maximum about six hours after transfer whereas the sodium ion content progressively increased during the adaptation period. The trehalose content also increased in adapting cells. It is suggested that inhibition of glycerol production during initial period of adaptation could be either due to the inhibition of glycerol-3-phosphate dehydrogenase by increased potassium ion content or to the inhibition of glycolysis, glycerol being produced glycolytically in S. cerevisiae. The mechanism whereby synthesis of trehalose is integrated with glycerol production and potassium uptake has not been identified. The increased production of glycerol towards the end of the adaptation period suggests that the osmoregulatory response of S. cerevisiae involves complex sets of adjustments in which inhibition of
glycerol-3-phosphate dehydrogenase must be relieved before glycerol functions as a major osmoregulator.

When *S. cerevisiae* was grown anaerobically in the presence of linoleic acid, under which the fatty acid was assumed to be incorporated into the plasma membrane, glycerol retention was increased. Growth in the presence of linolenic acid or oleic acid did not cause this effect. The higher intracellular glycerol content accompanying the apparent incorporation of linoleic acid into the plasma membrane of *S. cerevisiae* did not improve its tolerance of a sudden water stress by plating the cells on to low water activity agar.

A trehalose content of about 0.2 μmol/mg dry wt. of *S. cerevisiae* prior to the water stress was demonstrated to be indispensable for survival of *S. cerevisiae* to a sudden water stress. The trehalose content of *S. cerevisiae* was shown to be positively correlated with the viability of yeast on low water activity agar. However, glycerol did not show such a relation. When exposed to a nonlethal heat shock before subjected to a water stress, the proportion of the population able to form colonies on low water activity agar was 10 to 100 times higher than that obtained for cultures not exposed to a heat shock. Heat shock treatment also improved the water stress tolerance of a trehalose-negative *S. cerevisiae*. The heat shock did not effect the levels of trehalose or glycerol content in either strain. The inclusion in the medium of cycloheximide, an inhibitor of protein synthesis, nullified the effect of the heat shock on viability on low water activity agar without altering trehalose or glycerol content. When cultivated at 40°C instead of 30°C trehalose and glycerol contents differed little but tolerance of a water stress improved 10 fold. It is possible that 'heat shock proteins' may also have a role in conferring resistance on *S. cerevisiae* against the water stress.
AIMS

Cellular adaptation to a water stress is a biological process that protects organisms against the lethal effects of dehydration. Water stress may conceivably arise and be studied at two levels, a transient and a steady state level. A transient stress follows the transfer of a microorganism from one level of water activity to another. A steady state situation is concerned predominantly with the physiology of the organism when it has fully adapted to its new environment (Brown 1979).

Much information is accumulated about the response of yeast to water stress based on steady state experiments. The importance of glycerol in the growth of yeast at low water activities was recognised by Gustafsson and Norkrans (1976) and Brown (1978 a). Subsequent studies by Edgley (1980) and Edgley and Brown (1983) identified a major difference between the xerotolerant *Saccharomyces rouxii* and the nontolerant *S. cerevisiae* with regard to their glycerol production and the means by which they adjust glycerol contents in response to low water activity. The aim of the present investigation was to study glycerol production in *S. cerevisiae* with particular emphasis on the period of transition from high to low water activity and also the various factors affecting sensitivity of this yeast to a sudden water stress.
CHAPTER ONE:
GENERAL INTRODUCTION
1.1 GENERAL ASPECTS

Any environmental change potentially unfavourable to a living organism can be defined as a stress. The organism could be called stress tolerant if it has the ability to survive the unfavourable change and subsequently grow and multiply. In the face of change a living organism may show a physical strain or a chemical strain such as a shift in metabolism. If either strain is sufficiently severe the organism may suffer a permanent injury or death (Levitt 1980).

Environmental stresses are of two types, biotic and physicochemical. Biotic stresses include infection by, or competition with other organisms and are the subject of pathology and ecology, respectively. Physicochemical stresses are of several types, for example temperature, water, radiation, chemical, pressure, magnetic and electrical stresses. Since all biological systems have an absolute functional requirement for water, the ability to cope with a water stress is clearly of paramount importance and is the subject of this thesis.

1.1.1 Concept of water activity

The water relations of microorganisms have been discussed by many authors in terms of osmotic pressure (Pouncy-Summers 1939; Grover 1947), vapour pressure and relative humidity (Mossel and Westerrdijk 1955; Clyson 1955) and water activity (Scott 1953; Christian and Scott 1953; Christian 1955; Mossel and Ingram 1955; Scott 1956; Anand and Brown 1968; Brown 1976, 1980). The terms are inter related and under appropriate conditions, one defines the other. Water activity defines the water status of the medium or substrate (Scott 1956). It is a direct function of the mole fraction of the solvent, which in turn is determined by the concentration of any solute present. Water activity is the amount of thermodynamically available water in a solution:
\[ a_w = \frac{p}{P_0} = \frac{n_2}{n_1 + n_2} \]

where

- \( a_w \) = water activity
- \( p \) = vapour pressure of solution
- \( P_0 \) = vapour pressure of solvent
- \( n_1 \) = number of moles of solute
- \( n_2 \) = number of moles of solvent

and \( a_w \) is numerically equal to the equilibrium relative humidity of the solution expressed as a fraction. Osmotic pressure is related to water activity by the following equation

\[ \text{O.P.} = \frac{-RT \ln a_w}{V_w} \]

where

- \( \text{O.P.} \) = osmotic pressure
- \( R \) = universal gas constant
- \( T \) = temperature (°K)
- \( V_w \) = the partial molal volume of the solvent in the solution, which is equal to the volume occupied by one gram mole of the solvent in a solution.

Methods of measuring and controlling water activity have been reviewed by Smith (1971) and Corry (1973). As solutions rarely behave ideally, water activities of solutions must be experimentally determined.
1.1.2 Principles of adaptation

Environmental factors, both nutritional and physiological, invariably determine the survival and growth of microorganisms. A microbial cell must achieve thermodynamic equilibrium with its environment by the passage of solute molecules and by the movement of water across the cell membrane.

Recognizing the contribution of pool intermediary metabolites, proteins, nucleotides and salts to the water status of the cell, it follows that the water content of the cellular interior must be such that it allows access to required extracellular nutrients, as well as maintaining a satisfactory level of biological function. Depending on the growth conditions, a living cell contains approximately 60% water (Brown 1964 a). This fact, along with the semipermeable nature of the cellular membrane, places certain demands on a microorganism exposed to changes in environmental water activity, if it is to survive and subsequently grow and multiply. Brown (1976, 1978 a) indicated that there are two major stages in the response of a microorganism to a new physiological situation. In stage 1, the cell thermodynamically adjusts to the new conditions and this adjustment is comparatively rapid. In the case of a change in water activity the response of the organism in stage 1 involves a transient osmotic stress (Brown 1976). If the cell is exposed to a more dilute environment (i.e. increased water activity) then there will be a net flux of water into the cell. In stage 1, the microorganism will either attain a state of thermodynamic equilibrium with the environment otherwise it will lyse. Either way this stage has a time scale of seconds or at the most minutes (Brown 1978 a).

A decrease in the water activity of the environment will result in an immediate efflux of water from the microbial cell and possibly of an increase in the intracellular solute content. Brown (1978 b) indicated that an uncontrolled increase in the intracellular solute content could create biological instabilities and could result in the cell's death.
This would involve the diffusion of extracellular solutes into the cell in response to the water activity gradient. However, if the organism has a source of energy, it has the potential for retaining selectively a metabolite or selectively accumulating an extracellular substrate. The intracellular accumulation of a solute will lower internal water activity and water will reenter the cell to maintain thermodynamic equilibrium.

The second stage of adjustment to changes in water activity involves changes in the levels of enzymic activity, modification of biosynthesis and changes in the details of control of enzyme formation. These changes are more complex and much slower than those of stage 1 and can be expected to have a time scale of the order of one generation (Brown 1976). Brown (1978 a) stated that a great deal of energy can be used in this stage and an organism’s success is determined by the degree to which it can return to its original volume or state of turgor. At this point the organism becomes fully adapted to the new physicochemical situation. Survival, growth and multiplication of the fully adapted organism depends on its ability to function under the resulting new intracellular conditions and to maintain its osmoregulatory solutes at the required levels.

1.1.3 Concept of compatible solutes

Although the fluids which surround the cells of various organisms are diverse, the mechanisms which have evolved to protect a cell from changes in the osmolarity of those fluids are remarkably similar. The principal mechanism discovered to date functions through the intracellular accumulation of specific compounds called "compatible solutes" (Brown and Simpson 1972).

A compatible solute is usually a low molecular weight solute which is accumulated to high intracellular concentrations. These solutes function as osmoregulators and protect
enzymes of the cell against both inactivation and inhibition. Borowitzka (1981) and Yancey et al. (1982) have tabulated the different compatible solutes in various organisms. Four different classes of compatible solutes have been found in microorganisms, these being polyhydroxy alcohols, carbohydrate derivatives, potassium and free amino acids and their derivatives. The principal compatible solute discovered so far in unicellular eucaryotes under relatively severe conditions of stress is glycerol. Other polyols, such as arabinol, can also function as 'secondary' or 'backup' solutes (Brown 1978 a). In a review by Borowitzka (1981) the functional requirements of compatible solutes have been discussed. These important requirements are:

For effective function at low water activity:

a) They must be low molecular weight in order to be soluble (e.g. glycerol, mannitol and proline).

b) They must carry no net charge at neutral pH (exception being $K^+$ in halophilic bacteria).

c) They must be retained by the plasma membrane against a large concentration gradient.

For minimum alteration of enzyme structure and inhibition:

a) They should cause minimum alteration to water structure (e.g. glycerol and betaine are better than $K^+$).

b) If they do alter water structure, they should do so in a way that stabilizes the native active conformation ($K^+$ is better than $Na^+$ and $Li^+$).

c) They should have a low binding affinity, if they interact directly with enzyme macromolecules, in order to stabilize the native active structure ($K^+$ charge shielding in halobacterial proteins).

Biochemical requirements for rapid and fine control by the cell over compatible solute level:

a) For inorganic solutes e.g. $K^+$ there must be close metabolic regulation of transport into the cell.
b) For organic solutes they should be separated by several enzyme reaction steps from an intermediate in a major active biochemical pathway and be finely controlled, possibly through activation or inhibition of allosteric enzymes.

1.2 WATER STRESS

A water stress may conceivably arise either from insufficient or an excessive water activity in a microorganism's environment (Brown 1979) and can be subdivided into (a) a primary stress and (b) a secondary stress. Primary stresses involve specific toxic effects of the solute, such as the effect of salt on the external plasma membrane and its associated functions. Secondary stresses may arise as a result of a primary stress, for instance a loss in cell turgor which may lead to a subsequent shift in metabolic pattern (Levitt 1980).

1.2.1 Primary stress

1.2.1.1 Direct

The plasma membrane is the primary organelle to come in direct contact with changes in the composition of extracellular fluid and consequently changes in transport across this membrane may occur. In this section such changes are discussed.

1.2.1.1.1 Membrane

Phospholipids and other lipids like sterols are integral hydrophobic components of biomembranes and can be considered as the insulating material between membrane proteins and other charged molecules. The diversity in the types of fatty acids that have been detected in various living systems suggest that different lipid properties are required by each organism for survival. The difference is mainly in the chain lengths, saturation and substitution. In bacterial systems fatty acid chain lengths are usually
from C\textsubscript{10} to C\textsubscript{20} with chain lengths in the range of 15 to 19 predominating. In fungi there is an abundance of C\textsubscript{16} and C\textsubscript{18} fatty acids, with oleic and linoleic as the major unsaturated fatty acids (Chopra and Khuller 1984). Investigations of the yeast plasma membrane have been mostly concerned with describing the types of fatty acids and sterols present under a variety of conditions.

Andreasen and Stier (1953, 1954) discovered that under anaerobic conditions yeast requires an unsaturated fatty acid and ergosterol for proper growth. These requirements have provided a useful tool for manipulating membrane composition, since supplied compounds, even if not normally present in the membrane, are incorporated largely unchanged, and account for a major proportion of the fatty acid and sterol constituents (Alterthum and Rose 1973; Hossack and Rose 1976; Thomas et al. 1978). A \textit{Saccharomyces cerevisiae} sterol auxotroph with a defect in heme synthesis has been developed by Lorenz et al. (1986). The use of this auxotroph has allowed the discovery of multiple specific functions for sterol in \textit{S. cerevisiae} (Rodriguez et al. 1985).

A number of properties of yeasts are known to be influenced by the fatty acid and sterol content of the yeast plasma membrane. Longey et al. (1968) reported that 39% lipid was present in the plasma membrane of \textit{S. cerevisiae} which was mainly composed of triglycerides, phospholipid and sterol. Ergosterol was the predominant sterol. Similar observations were made in \textit{Candida utilis}, in the sugar tolerant yeast \textit{Hansenula anomala} (Ng and Laneelle 1977) and in the halotolerant \textit{Debaryomyces hansenii} (Merdinger and Devine 1965). Alterthum and Rose (1973) observed that sphaeroplasts enriched in linoleic or linolenic, rather than oleic acid, lysed in hypotonic solutions. They suggested that the higher degree of unsaturation of the fatty acids resulted in a more fragile membrane. Lysis of sphaeroplasts was reported to occur to a lesser degree under similar conditions when the membranes contained ergosterol or stigmasterol rather than cholesterol or campesterol (Hossak and Rose 1976). It was proposed that ergosterol and
stigmasterol were better able to restrict the mobility of the fatty acid chains, which increase when the membranes are stretched, thus conferring greater stability on membrane structure. Hunter and Rose (1971) gave evidence that cell wall composition and structure is influenced by lipid composition of the membrane. The lipid environment was shown to affect the activities of cell wall synthesizing enzymes located in the membrane. *S. cerevisiae* cells enriched in linoleic or linolenic acids were found to be less susceptible to the action of β-glucanase than those enriched with oleic acid (Alterthum and Rose 1973). Koh (1975) isolated mutants of xerotolerant *S. rouxii* which have a low hexoseamine content and lack an outer mannan-containing layer. He proposed that this could be due to the altered cell membrane composition which contained a higher proportion of unsaturated fatty acids.

The influence of culture age on the cellular fatty acid composition of four different yeasts was studied by Viljoen et al. (1986). They found that oleic acid and linoleic acid increased significantly in *Metchnikowia reukauftii* during exponential phase. Culture age has no significant effect on the relative amounts of other fatty acids present in other yeasts. These workers did not include *S. cerevisiae* in their study but Homann et al. (1987) reported maximal activities of enzymes associated with membrane phospholipid biosynthesis as cells entered stationary phase in *Saccharomyces cerevisiae*.

Combs et al. (1968) found that there were no changes in the fatty acid composition of *C. albicans* grown in the presence of various levels of NaCl, whereas significant quantitative changes in the relative proportion of the fatty acids were observed. Henry et al. (1981) described the mutants of yeast which are defective in phospholipid metabolism. Rao et al. (1985) reported that, as compared with normal *S. cerevisiae* cells, phospholipid-enriched cells acquired resistance towards different polyene antibiotics. The thermotolerant yeast *H. polymorpha* grown at 40°C was characterized by the presence of 42-43% linoleic acid, 25-27% oleic acid and 3-6% linoleic acids as its major fatty
acids (Chandrani et al. 1986). Thomas et al. (1978) found that enriching the membrane of *S. cerevisiae* with palmitoleic, oleic or linoleic acids increased the viability of the cells in ethanol and that the effectiveness of fatty acids increased with an increasing degree of unsaturation and decreasing chain length. Tolerance of ethanol was greater if the cells were enriched with ergosterol or stigmasterol rather than cholesterol or campesterol.

No significant changes in either fatty acid or sterol composition were observed when the nontolerant *S. cerevisiae* and xerotolerant *S. rouxii* were subjected to a water stress. However *S. rouxii* differed from *S. cerevisiae* in its linoleic acid content (Brown and Edgley 1980; Edgley 1980), irrespective of stress. Xerotolerant *S. rouxii* contained 25% of total fatty acid as linoleic acid whereas this fatty acid was absent in nontolerant *S. cerevisiae*. This difference possibly accounts for better glycerol retention by *S. rouxii* than by *S. cerevisiae*.

Interestingly, as noted above, yeasts thermotolerant and resistant to ethanol also contain high amounts of linoleic acid. Sheffer et al. (1986) reported that the halotolerant alga *Dunaliella salina* contained palmitic (31%) and, significantly, linoleic acid (20%) as major fatty acid whereas major lipids were diacylglyceroltrimethylhomoserine (23.5%) and sterol peroxides (22%). Effects of monovalent and divalent salts on the phospholipid and fatty acids composition of a halotolerant *Planococcus sp* were studied by Miller (1986). She found that for all conditions of growth the fatty acid of *Planococcus sp* was C_{15:0} and the relative percent content of this fatty acid increased as the osmolality of the growth medium was increased and did not appear to be significantly influenced by the specific nature of the cations or anions added to the growth medium. An increase in the cardiolipin/phosphotidylglycerol molar ratio was detected when the organism was grown in the presence of high concentrations of monovalent cations.
1.2.1.1.2 Transport

Transport studies have mainly concentrated on the differences in transport of different compounds in xerolerant and nontolerant yeasts rather than any salt induced changes in transport mechanisms. Brown (1974) suggested that the different kinetics of glycerol uptake in *Saccharomyces cerevisiae* and in *S. rouxii* indicated that entry of glycerol into the former yeast was by simple diffusion but for the latter an active process was involved. The presence of polyunsaturated fatty acids in *S. rouxii* but not in *S. cerevisiae*, as mentioned above, could be related to this difference in transport of glycerol. This is supported by the fact that several investigators (Cirillo 1968; Seaston et al. 1973; Kotyk and Michajancova 1974) reported that glucose and galactose transport in *S. cerevisiae* is by facilitated diffusion. However, the transport of glucose, galactose and several other monosaccharides in *Rhodotorula gracilis* and of galactose, L-sorbose and xylose in *Torulopsis candida* seems to be active processes. *Rhodotorula gracilis* and *T. candida* have a similar plasma membrane composition as *S. rouxii* (Kotyk and Hofer 1965; Haskovec and Kotyk 1973).

Lindman (1981) showed by using the glucose analogues, glucosamine and 2-deoxyglucose, that the halotolerant *D. hansenii* has an intrinsically halotolerant sugar transport mechanism, since this yeast maintained its transport capacity significantly better in increased salinity than did *S. cerevisiae*. The comparison between *D. hansenii* and *S. cerevisiae* revealed a high capacity of the former to transport sugar both in ordinary and water stress conditions.

Keenan et al. (1982) compared the kinetics of solute accumulation by *S. cerevisiae* grown anaerobically under conditions that lead to enrichment of the plasma membrane with ergosterol and either the oleyl or linoleyl residue. Values for $K_t$ and $V_{max}$ were identical for the accumulation of L-asparagine, L-glutamine, $H_2PO_4$, $Ca^{2+}$, $SO_4^{2-}$ and
glucose while the values for L-lysine decreased when oleyl residues were replaced by linoleyl residues.

Recently Walter et al. (1987) found that low water activity inhibits the glucose transport system called phosphotransferase system in *Clostridium pasteurianum*.

### 1.2.1.2 Indirect

A water stress induces an indirect inhibition of growth and multiplication in microorganisms. Even if it regains a cell volume or turgor compatible with growth by the process of osmoregulation, a microorganism must maintain a state of equilibrium with its environment in order to survive. Maintenance of this equilibrium requires the continuous expenditure of energy (Gale 1975). One other indirect effect of a water stress is that it causes several metabolic disturbances in the cell. These metabolic disturbances are being discussed below.

#### 1.2.1.2.1 Fermentation

A metabolic feature which seems to be common to xerotolerant yeasts is their low fermentation capacity. Spencer (1968) has reported that several such yeasts produced little or no ethanol. Edgley (1980) reported that xerotolerant yeasts, in general, have a highly active pentose phosphate pathway and a low fermentation capacity. Typical examples of such yeasts are *Saccharomyces rouxii*, *Debaryomyces hansenii* and *Rhodotorula glutinis*. Largely because of the interest in the commercial production of ethanol from yeast there is a wealth of literature on glycolysis but very few studies have been done regarding the effect of water stress on this phenomenon. Tajima et al. (1966) reported the inhibition of ethanol formation when *S. formensis* was grown on molasses in the presence of salts. Panchal and Stewart (1980) also found that ethanol production is decreased when *S. carlsbergensis* was grown in minimal media containing increasing
concentrations of sucrose. They also showed that the production of another fermentation product, glycerol, increased under the same growth conditions.

1.2.1.2.2 Respiration

The Crabtree effect, the inhibition of respiration in the presence of a rapidly fermentable substrate such as glucose, has been reported to occur in many yeasts of several genera (De Deken 1966 a; John and Brown 1972; Johnson et al. 1972). Brown (1975) reported the absence of the Crabtree effect in the xerotolerant yeast, *Saccharomyces rouxii*, when grown in high glucose concentrations (24%, 36%, w/v). In contrast there was a much greater decrease in O2 uptake in the nontolerant yeast *S. cerevisiae* when grown in high glucose media. This was accompanied by the almost complete loss of internal structure in the mitochondria and a large reduction in the NADH oxidase activity and cytochrome content. However, there was little change in mitochondrial morphology in the tolerant yeast, the decrease in NADH oxidase activity was much less and the cytochrome content increased. Almost similar observations were made when the yeasts were grown in media in which the water activity was lowered with PEG 200. A much greater decrease in respiratory activity was reported for *S. cerevisiae* exposed to low water activity medium (10% NaCl) than for the xerotolerant *Debaryomyces hansenii* (Norkrans 1968). Brown (1975) suggested that polyols could be involved in the much lower sensitivity of respiration in *S. rouxii* to high concentrations of glucose and also in apparent resistance of respiratory activity to low water activity in xerotolerant yeast.

Petite mutants are respiratory deficient mutants and are unable to grow on nonfermentable substrates such as glycerol, due to a loss of respiratory capacity (Nagley 1977). Several yeasts, *S. rouxii* and *S. melliis* (Lodder 1972), *S. baillii* (Pitt 1975), *Hansenula anomala* (Whalley and Scarr 1967), *Torulopsis magnoliae* (Spencer 1968) and *T. versatilis* (Onishi and Suzuki 1968 a) have been shown to be petite negative.
Nontolerant yeasts such as *S. cerevisiae* produce the petite mutation when subjected to a water stress (Petelo 1986).

### 1.2.1.2.3 Protein metabolism

Water stress decreases protein synthesis and increases its hydrolysis in many crop plants, for instance pea roots (Klyshev and Rakova 1964) and grape leaves (Saakyan and Petrosyn 1964). In pea and maize, water stress causes an accumulation of free amino acids, especially proline (Jaeger and Priebe 1975) which attains ten times the control value.

Amino acid accumulation in response to low environmental water activity has been observed in many bacteria (Tempest et al. 1970 and Measures 1975) and algae (Borowitzka 1981). In yeast, amino acids are reported to be the dominant group of soluble intermediates (Conway and Armstrong 1961). Changes in free L-lysine content of *Saccharomyces cerevisiae* growing in a medium of low water activity have been reported by Tanner et al. (1981). If cultivated anaerobically no increase in L-lysine content is observed with increase in NaCl levels (Wei et al. 1982). Adler and Gustafsson (1980) found no major changes in the composition of the total amino acid pool of *Debaryomyces hansenii* in response to increased medium salinity. They found a small rise in proline content. At low salinity the total amino acid pool increased during log phase and then declined in stationary phase. A similar pattern was observed for L-lysine in *S. cerevisiae*. Adler and Gustafsson (1980) showed that L-lysine probably functions as an osmoregulatory solute under mild water stress.

A stimulation of protein synthesis has been found in plants upon water stress and also upon other stresses (Boyer 1976). Recently heat shock and other environmental stresses have been found to induce a small set of proteins and to inhibit normal protein synthesis. These proteins have been described as heat shock proteins (hsp). Heat shock
proteins have been found to be present in numerous organisms, ranging from bacteria, yeast, other microorganisms to plants, insects and higher vertebrates such as chicken, mouse and man (Schlesinger 1982a and b). The functional significance of heat shock proteins is unknown. At the simplest level, there is little doubt that these proteins protect the cell against environmental challenge and ensure that a cell continues its normal life after the crisis has passed. Perhaps the best available evidence for this is seen from experiments studying what has been described as "acquired thermotolerance". Cells including yeast exposed to one heat shock are relatively protected against the effect of a second heat shock (Li and Hahn 1978; Henle and Dethlesfson 1978; Mitchell et al. 1979; Mc Alister and Finkelstein 1980).

In order to explore the possible functions of hsp, Hightower et al. (1985) recently did interesting experiments on chicken embryo cells. Using glycerol or erythritol or D$_2$O (all macromolecular stabilizing agents) in the medium they found that when chicken embryo cells were heat shocked no hsp were induced. Their experiments further showed that glycerol did not actually block gene activation or expression or a mechanism for sensing heat stress. It rather protected the heat sensitive cellular targets and hence no stress signal was generated.

Mc Alister et al. (1980) also reported that hsp in yeast bind to DNA. Four small hsp 22, 23, 26 and 27 in *Drosophila* are related to each other and to mammalian $\alpha$-crystallin (Ingolia and Craig 1982). It has been suggested by these authors that these hsp might similarly form large molecular aggregates such as $\alpha$-crystallin, that could, for example, protect DNA or other components of the nucleus from damage. Some other related functions of hsp have been discussed in a recent review by Burdon (1986).

One striking similarity between the heat shock response and osmoregulatory response is their conservation throughout evolution. In nearly all species heat shock induces the
synthesis of proteins in the size range of 80 to 90kd, 68 to 74kd and 18 to 30kd (see the review by Burdon 1986). Yancey et al. (1982) have reported that very few classes of osmoregulatory solutes are present in distinctly diverse organisms.

It has also been shown that two types of the hsp, hsp 70 in maize and hsp 27 in soyabean can be induced by water stress (Czarnecka et al. 1984; Heikkila et al. 1984). Singh et al. (1985) examined the protein profile in salt adapted and non-adapted cell lines of Nicotiana tabacum. Several proteins were found to be more abundant in salt adapted cells and a protein of 26 Kd was unique to salt-adapted cells.

1.2.1.2.4 Nucleic acid

Water stress reduces the rate of RNA synthesis in Phaseolus vulgaris and has no effect on DNA synthesis (Nieman 1965). Tsenov et al. (1973) reported that in tomato tissues the content of DNA actually increases in the presence of 0.8% of NaCl, but both RNA and DNA decreases at 1.6% NaCl because of inhibition and intensification of breakdown. In pea leaves it was found by Morozovski and Kabaniv (1968) that 0.2-0.4% NaCl interferes with nucleic acid synthesis, but 0.8% NaCl interferes with synthetic processes following the stages of replication and transcription (Kabanov et al. 1973).

Nucleotides have also been reported to change as a result of salinity. In moderately resistant barley, increased content of AMP, ADP, ATP, UTP and GTP are found upon a water stress. In pea root tips, NAD+ and NADH were decreased by sodium salt, while NADP+ and NADPH increase only in the presence of NaCl (Hason-Porath and Polykoff-Mayber 1970). An uridine sugar nucleotide, UDP-N-acetylglucosamine was reported to be abundantly accumulated in Saccharomyces rouxii when grown in low water activity medium (Tomita 1983). This nucleotide is known to participate in the biosynthesis of chitin in some fungi. Further study is required to assess the role of this nucleotide in yeast at low water activity.
Recently Saenger et al. (1986) reported conformational changes in DNA at low and high water activities. They found that under conditions of high water activity the "B" conformation prevails. If the water activity is reduced by addition of salt or organic solutes, transformation occurs to "A" DNA or to the left handed "Z" DNA.

### 1.2.1.2.5 Enzyme activities

Brown and Edgley reported (1980) the activities of several enzymes of carbohydrate metabolism and their response to water stress in xerotolerant *Saccharomyces rouxii* and nontolerant *S. cerevisiae*. They found that the enzyme activities were correlated with their respective methods of osmoregulation. When both yeasts were grown in 1.8 molal NaCl, phosphofructokinase activity was suppressed in *S. rouxii* but was enhanced in *S. cerevisiae*. The effect of NaCl was even greater on sn-glycerol-3-phosphate dehydrogenase, an enzyme responsible for reduction of dihydroxyacetone phosphate (DHAP) in *S. cerevisiae*, whereas dihydroxyacetone activity being negligible in this yeast. DHAP reducing activity was unaffected by salt in *S. rouxii*.

Brown and Edgley (1980) assayed the activities of some other important enzymes, for instance glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, transaldolase and transketolase. The activities of these enzymes were not high, neither were they enhanced by salt in *S. rouxii* while in *S. cerevisiae* these enzyme activities were significantly low under all conditions. These findings together with the known NADPH involvement in glycerol production in *S. rouxii*, led Brown and Edgley (1980) to propose that glycerol production in this organism is by the pentose phosphate pathway. This is in contrast to the documented glycolytic production of glycerol in *S. cerevisiae*. 

1.2.2 Secondary stress

Since addition of a salt to water lowers its water activity, the water stress must expose the organism to a secondary osmotic stress. Secondary stress requires some time to develop (see section 1.2).

1.2.2.1 Osmotic stress

Protection from osmotic stress is important for all organisms. A cell must maintain an internal environment different from the external milieu and it does so by means of varying the content of one or more specific solutes. At low water activity these solute concentrations are high and it is essential for the continuation of metabolism that these solutes are not inhibitory at the concentrations that prevail in the cell. For this reason the term "compatible solute" was introduced to describe those substances that function as osmoregulators at low water activities (Brown and Simpson 1972; Brown 1978).

The process of osmoregulation involves survival of the initial phase of dehydration, accumulation of a solute to appropriate levels and finally the resumption of cellular function at the new level of environmental water activity (Brown et al. 1986). Reed (1984) has put forward other terms which he suggests are more suitable in describing the response of a microorganism to a water stress. Turgor regulation and volume regulation have been suggested as more appropriate terms in the case of walled and wall-less cells, respectively, by Zimmermann (1978) and Bisson and Gutnecht (1980).

1.2.2.2 Methods of osmoregulation

Brown and Borowitzka (1979) have reviewed the three representative methods of controlling the contents of osmoregulatory solutes in eucaryotes. These are:
(a) A method used by *Saccharomyces rouxii* where the retention of the osmoregulator is controlled by permeation or transport and the total yield of the osmoregulator is largely independent of water activity.

(b) A method used by *S. cerevisiae* where the total yield of osmoregulator is dependent on water activity but a constant proportion of the osmoregulator is retained within the cell.

(c) A method used by *Dunaliella* where the total yield of osmoregulator is proportional to water activity and virtually all of the osmoregulator is retained within the cell.

Glycerol is the osmoregulator in all of these three methods of osmoregulation. Extensive amounts of glycerol are leaked into the medium as a result of the method adopted by *S. cerevisiae*. This partly explains why *S. cerevisiae* is nontolerant (Edgley and Brown 1983). Methods (a) and (c) are very conservative and effective and the most xerotolerant eucaryotes known use one or other of these methods to osmoregulate.

1.2.2.3 Types of osmoregulation

There is much speculation about the regulation of intracellular solute concentration by extracellular water activity. Various aspects of the problem have been discussed in reviews such as those by Cram (1976); Hellebust (1976); Brown (1976, 1978 a, 1978 b); Brown and Borowitzka (1979); Zimmerman (1978) and Brown et al. (1986). However, two broad types of osmoregulation have been recognised in microorganisms so far, which are ionic and metabolic osmoregulation.

1.2.2.3.1 Ionic osmoregulation

The typical example of ionic osmoregulation is found in the halophilic bacteria where accumulation of K+ and Cl- is itself sufficient to counter the osmotic stress (Brown 1976). Brown (1978 b) has suggested that in halophilic bacteria, regulation of intracellular K+ concentration involves both active uptake and passive diffusion of the ion from the cell to maintain the thermodynamic balance between internal and external water
and electrochemical potential. The active elimination of Na⁺ is known in many algal species, for example Chlamydomonas (Okamoto et al. 1964), Chlorella (Barber 1968) and Dunaliella (Pick et al. 1986; Avron et al. 1986). The cells of Dunaliella maintain an approximately constant intracellular concentration of Na⁺ at least over the range of external NaCl concentration that have been documented, 0.5 M to 3.0 M (Katz and Avron 1985). The other cation with significant involvement in osmoregulation in algae is K⁺. Eppley (1958) reported that K⁺ content in the marine alga Porphyra perfecta was forty times higher than in sea water, whereas the intracellular concentration of Na⁺ was ten times lower than sea water.

Norkrans and Kylin (1969) measured the K⁺ and Na⁺ content in yeast at low water activity. They found that the ratio of K⁺/Na⁺ was much higher in the cells than in the medium and higher in the xerotolerant Debaryomyces hansenii than nontolerant Saccharomyces cerevisiae. The difference between the two species was due to better Na⁺ extrusion and K⁺ uptake in D. hansenii than in S. cerevisiae. Norkrans and Kylin (1969) therefore concluded that xerotolerance is partly dependent on the yeast's ability to mobilize energy to extrude Na⁺ from the cells and to take up K⁺.

1.2.2.3.2 Metabolic osmoregulation

Unlike inorganic ions, such as K⁺, organic osmoregulatory solutes are metabolic products. However, different reaction sequences may be responsible for the synthesis of the same solute in related organisms, as is the case for glycerol in the xerotolerant Saccharomyces rouxii and the nontolerant S. cerevisiae (Brown and Edgley 1980).

In S. cerevisiae, increases in production and intracellular concentration of glycerol in response to decreased water activity are accompanied by increased levels of sn glycerol-3-phosphate dehydrogenase activity (Brown and Edgley 1980). This suggests that osmoregulation in this organism involves changes in the amount of sn glycerol-3-
phosphate dehydrogenase synthesized. In yeast and many other organisms there have been
many reports of increases or decreases in the levels of various enzyme activities in the
cells or protoplasts of various plant tissues subjected to water stress (see for example
Todd 1973; Lazer et al. 1973; Armstrong and Jones 1973; Dhinsa and Cleland 1975;
Premecz et al. 1977). Armstrong and Jones (1973) suggested that the observed
dissociation of polyribosomes could be responsible for decreases in enzyme levels in
barley aleurone cells. However, there is no evidence as yet that changes in protein
synthesis to account for the observed changes in enzyme activities accompanying a water
stress.

Premecz et al. (1977) reported that the proline concentration in tobacco leaf protoplasts
increased in response to decreased water activity but the enzymes which they
investigated, the levels of which were affected by water stress, were not directly
involved in the metabolism of this osmoregulatory solute. However, these observations
do indicate that decreased water activity can affect protein and so enzyme synthesis and
suggest at least one site at which the intracellular concentration of osmoregulatory solute
may be controlled.

In other organisms there is a good evidence that enzyme synthesis is not important in the
regulation of osmoregulatory solute content. Factors involved in the regulation of the
glycerol enzymes in Dunaliella including pH, substrate and product concentration have
been discussed by Brown and Borowitzka (1979). Since the accumulation of
isofloridoside in Ochromonas malhamensis and glycerol in D. tertiolecta and D. viridis is
not prevented by a protein synthesis inhibitor, Kauss and Schober (1971) and
Borowitzka et al. (1977) proposed that changes in the activities of existing enzymes
were responsible for regulation of the levels of respective solutes. Schoffeniels (1976)
suggested that increased level of amino acids in the tissues of marine invertebrates when
subjected to a water stress could be due to the effects of the resultant changes in the
intracellular ion concentration on enzyme activity. The activity of glutamate dehydrogenase increased, but the activities of lactate and glycerol-3-phosphate dehydrogenases decreased.

1.2.2.4 Osmoregulatory signals

Osmoregulation has mainly been studied at two levels: the biophysical and the biochemical level. Biophysical studies have generally concentrated on direct measurement of physical parameters of cells, such as changes in volume and turgor in an attempt to identify the nature of osmoregulatory signal.

Based on biophysical studies, Cram (1976), Hellebust (1976), Coster et al. (1977) and Zimmermann (1978) put forward an osmoregulatory signal model for plant and algal cells. They proposed that the effects on the cell membrane of turgor or volume changes (or both) resulting from the water efflux from the cell when the external water activity was lowered constituted the primary signal. Turgor changes would be more important in walled cells while volume changes are important in wall-less cells. The model describes an event probably occurring in all cells subjected to water stress, including yeast (Morris et al. 1986).

Coster et al. (1977) provided evidence that forces due to turgor pressure can compress or stretch a membrane. Berezin (1974) reported that compressive forces applied to gels containing enzymes altered the activities of the enzymes. Thus Zimmermann (1978) suggested that turgor induced changes in membrane conformation could affect enzymes and other activities associated with the membrane. Volume changes have similar effects.
Gutknecht (1968) showed that changes in intracellular hydrostatic pressure had marked effects on K⁺ fluxes in the marine alga *Valonia* while Zimmermann and Steudle (1974) reported that alterations in the electrical properties of the algal membrane accompanied such pressure changes. Racusen et al. (1977) described experiments showing that the membranes of plant protoplasts transferred to a more concentrated medium also have altered electrical properties. The decrease in volume of red blood cells when they were transferred to low water activity medium is accompanied by changes in Na⁺ and K⁺ fluxes (Elford and Soloman 1974). It was suggested that shrinkage of the cell membrane altered its conformation and hence its permeability to various substances.

Microorganisms have not been amenable to very precise biophysical studies, at least partly because their cells are generally too small for relevant physical measurements to be made with a satisfactory degree of precision and accuracy (Brown et al. 1986). Nevertheless it can not be denied that the accumulation of osmoregulatory solutes in microorganisms could be a direct consequence of biophysical changes in membrane properties.

Decreasing the external water activity alters the water content of cells, at least in the initial phases of adaptation and hence the concentration of intracellular constituents such as K⁺ and other ions as well as substrates and effectors of enzyme activities are also altered (this thesis). Haslo (1973) and Brown and Borowitzka (1979) have suggested such changes could also be the signals involved in the osmoregulatory response, acting by affecting enzyme activities.

The concept of the recognition of an extracellular messenger by a membrane or by internal molecules which subsequently translate ligand binding into cell activation, thus causing structural or functional changes has been dealt with by Strosberg (1984). Since different types of solutes elicit a similar osmoregulatory response, as seen in algae...
(Wegmann 1971; Kauss and Schober 1971; Kirst 1975; Borowitzka et al. 1977), protozoa (Kanesturo et al. 1969; Stoner and Durham 1970) yeast (Edgley 1980) and plants (Moore 1975), it seems highly unlikely that solute-specific receptors are involved in the osmoregulatory signal in these organisms.

Recently protein kinases have been recognised as playing a role in signal transduction in various animal tissues (Nishizuka 1984). Two main types of cAMP-dependent protein kinases have been reported. These are protein kinase A (Walsh et al. 1968) and protein kinase C (Nishizuka 1984). A cAMP-dependent phosphorylation cascade which regulates trehalase activity in yeast has been reported (Chvojka et al. 1981; Oritz et al 1983) as well as a cAMP-dependent activation of phosphofructokinase 2 (Francois et al. 1984).

1.3 GLYCEROL METABOLISM IN YEAST

1.3.1 Synthesis and degradation

Glycerol is produced by both yeasts, xerotolerant *S. rouxii* and nontolerant *S. cerevisiae* and algae *Dunaliella* (Brown 1972), fungi including *Penicillium chrysogenum* (Ballio et al. 1964) and *Aspergillus niger* (Barker et al. 1958) and the protozoan parasite *Trypanosoma rhodesiense* (Grant and Fulton 1957). Two reaction sequences have been suggested for the formation of glycerol from its triosephosphate precursors, dihydroxyacetone phosphate (DHAP) and glyceraldehyde phosphate. DHAP may be reduced by a sn glycerol-3-phosphate dehydrogenase (GPDH) catalysed reaction to glycerol-3-phosphate, which is then dephosphorylated to glycerol. This pathway seems to be responsible for glycerol production in *Saccharomyces cerevisiae* (Gancedo et al. 1968). Alternatively, DHAP may also be first dephosphorylated to dihydroxyacetone (DHA) or glyceraldehyde (GA) then reduced to glycerol. Activities preferentially reducing DHA
rather than GA with NADPH + H+ rather than NADH + H+ have been found in *S. rouxii* (Verachert and Dooms 1969) and in the fungus *Mucor javanicus* (Hochuli et al. 1977). Verachert and Dooms (1969) suggested that since they could not detect DHAP reducing activity in the yeasts *S. rouxii* and *Candida utilis*, reduction of nonphosphorylated triose, either DHA or GA, was probably responsible for glycerol production in these yeasts. Scher and Horecker (1966) suggested that GA reduction in *C. utilis* is involved in the conversion of the aldose to the corresponding ketose. Likewise, the enzyme activity in a species of *Rhodotorula* could have a similar function in the dissimilation of GA which can be used as a carbon source by this yeast (Watson et al. 1969; Sheys et al. 1971).

Most of the organisms able to reduce DHA or GA, or both, also oxidise glycerol. Enzyme from *Dunaliella* species (Ben Amotz and Avron 1974; Borowitzka and Brown 1974) have much higher pH optima for the oxidation of the polyols than the reduction of the triose. The situation in yeast has been controversial. Verachert and Dooms (1969) could not detect any glycerol-oxidising activities in extracts of *S. rouxii* and *S. cerevisiae*. This finding was supported by the results of Simpson (1976) but the latter was in conflict with the work of Uehara and Takeda (1964), who reported that GA reduction by *S. cerevisiae* was reversible.

Jeffery and Jornvall (1983) reported a pathway in animal tissues from glucose to triosephosphate via sorbitol which bypasses the recognised control points, hexokinase and phosphofructokinase, of glucose catabolism. It may also produce glycerol linking the bypass to lipid synthesis. Utilization of this bypass is favoured by a plentiful supply of glucose the conditions under which glycolysis is also active.

As is the case for glycerol synthesis, glycerol may be degraded by one of the many pathways. For instance, in comparative studies of several methylotrophic yeasts the pathway of glycerol metabolism were shown to be diverse (Tani and Yamada 1987).
Glycerol degradation may be involved not only in its utilization as a carbon source by many bacteria (Lin 1976) and yeast (Lodder 1970) but also in the regulation of its intracellular concentration in organisms such as Dunaliella species in which glycerol functions as an osmoregulatory solute (Brown and Borowitzka 1979).

Gancedo et al. (1968) presented evidence that glycerol utilization by Candida utilis involves phosphorylation followed by oxidation. Glycerol kinase could only be detected in S. cerevisiae when glycerol was present in the medium (Wireland and Suyter, 1957) and this yeast also contains glycerophosphate oxidase activity (Gancedo et al. 1968). A similar reaction is probably responsible for the catabolism of glycerol in S. cerevisiae. May et al. (1982) reported that the fission yeast, Schizosaccharomyces pombe, lacks glycerol kinase but possesses an NAD+ linked glycerol dehydrogenase. This yeast first oxidizes glycerol to DHA by an NAD+ linked glycerol dehydrogenase and then phosphorylates the triose by a specific DHA kinase. This alternative route of glycerol utilization has been reported to occur in several other yeasts (Babel and Hofmann 1982). Properties of different enzymes metabolizing glycerol and DHA in fission yeasts of the genus Schizosaccharomyces have been discussed by Vasiliadis et al. (1987). Existence of two different pathways were reported for glycerol dissimilation in Debaryomyces hansenii (Adler et al. 1985). One way involves glycerol kinase and a mitochondrial glycerol-3-phosphate dehydrogenase and the other involves glycerol dehydrogenase and DHA kinase. Tom et al. (1978) discovered that Neurospora crassa metabolizes glycerol in an unusual sequence of reactions. Glyceraldehyde, the product of glycerol oxidation is further oxidised to glycerate which can then be phosphorylated to a glycolytic intermediate. Recently Legisa and Mattey (1986) detected two different cytosolic NADP+-specific glycerol dehydrogenases and a mitochondrial NADP specific glycerol dehydrogenase in Aspergillus mycelium. They found that these enzymes were regulated by bicarbonate or CO2.
1.3.2 Glycerol-3-phosphate dehydrogenase

Glycerol-3-phosphate dehydrogenase (GPDH) is classified as glycerol-3-phosphate: NAD 2- oxidoreductase (E.C.1.1.1.8). This enzyme catalyzes the reaction:

\[
\text{L-glycerol-3-phosphate + NAD}^+ \rightarrow \text{DHAP + NADH + H}^+
\]

The reduction of DHAP by an extract from \textit{S. cerevisiae} was specific for NADH (Gancedo et al. 1968). In contrast, for the \textit{Escherichia coli} enzyme the rate of DHAP reduction with NADPH was twice that with NADH (Kito and Pizer 1969). Sols (1967) reported that DHAP reduction in \textit{S. cerevisiae} is reversible.

This enzyme has been isolated from many organisms and tissues. Its characteristics and properties have been studied in detail. Edgley (1980) has tabulated the various properties of this enzyme from different sources. Interestingly in most cases this enzyme has been shown to be a dimer. The presence of a nonprotein UV absorbing component is common to the enzyme from rabbit, monkey muscle and yeast (Eys et al. 1959) and this component could be removed from the rabbit muscle enzyme with no effect on its activity provided EDTA was present. GPDH from \textit{Saccharomyces cerevisiae} has been purified and some of its properties have been studied by Merkel et al. (1982). They reported that this enzyme has a molecular weight of approximately 31000, a pH optimum between 6.8 and 7.2 and is sensitive to high ionic strength salt solutions.

Gancedo et al. (1968) suggested roles for GPDH in \textit{S. cerevisiae} similar to those proposed by Baranowski (1949) for the enzyme in other organisms. GPDH seems to be involved in increased glycerol production by \textit{S. cerevisiae} under anaerobic conditions or when there is a blockage in acetaldehyde reduction, thus cytoplasmic NADH is oxidised by the reduction of DHAP. The glycerophosphate so formed may then be dephosphorylated by an L-glycerophosphate specific phosphatase (Gancedo et al. 1968). A respiratory-deficient mutant of \textit{S. cerevisiae} was reported by Watson (1969) to produce much more glycerol than wild type. It is significant that Gancedo et al. (1968) found that the levels
of GPDH activity were higher in extracts of *S. cerevisiae* grown anaerobically rather than in the presence of oxygen.

Lagunas and Gancedo (1973) proposed that the regeneration of NAD\(^+\) in the GPDH reaction is an important regulatory device in yeast, this reaction maintaining the cytoplasmic NAD\(^+\)/NADH + H\(^+\) ratio at a steady level. The ratio is maintained by the GPDH reaction not only in the conditions such as those described in the previous paragraph but also when *S. cerevisiae* is grown aerobically and in the presence of glucose, conditions under which respiration is inhibited due to the Crabtree effect. When this yeast was grown in 2% glucose Lagunas and Gancedo (1973) found that only 1-2% of the sugar consumed was catabolised by the respiratory pathway. Both Lagunas and Gancedo (1973) and Nordstrom (1966, 1968) have pointed out that, while the reduction of acetaldehyde to ethanol can account for the oxidation of the NADH formed in glycolysis, many biosynthetic reactions are operating resulting in further formation of the reduced nucleotide. It is in the regeneration of NAD\(^+\) from this NADH, that the GPDH reaction is important.

Regulation of GPDH may be achieved by modification of the activity of enzyme already present, or by changes in the levels of enzyme protein or both. Modification of enzyme activity was responsible for the higher levels of GPDH activity in rat mammary gland during lactation (Emery and Baldwin 1967). Whereas the increased levels of DHAP-reducing activity in bee flight muscle at that time in its life cycle when it begins to fly (Marquadt and Brosmer 1966 a) and in extracts of anaerobically grown *S. cerevisiae* are probably due to the increases in the amount of enzyme protein (Gancedo et al. 1968). Product or feed-back inhibition of DHAP reduction by glycerophosphate has been reported for the enzymes from *Escherichia coli* (Kito and Pizer 1969; Edgar and Bell 1978), honey bee flight muscle, squid mantle muscle, trout, turtle and tuna muscle (Guppy and Hochachka 1978), rabbit muscle (Blanchaer 1965; Black 1966) and liver (Lee and Craine 1971). Nader et al. (1979) reported that the enzyme from
**S. carlsbergensis** is strongly inhibited by inorganic phosphate, pyrophosphate and the nucleotides ATP and ADP. Activity of GPDH has been reported to be increased by up to 30 fold with NADH as coenzyme when *S. cerevisiae* was grown in media containing 10% w/v NaCl (Edgley and Brown 1983). Factors which have been shown to inhibit GPDH activity in vitro in *S. cerevisiae* are K+, Mg++ and ADP (Gancedo et al. 1968). Edgley (1980) also found that GPDH activity was inhibited by K+ in *S. cerevisiae*.

Marengo et al. (1985) studied the catalysis of the GPDH reaction in a chloroplast enriched fraction of *D. tertiolecta*. They found that the reaction was inhibited by inorganic orthophosphate and adenine nucleotides. Their results suggested that the regulatory potential of the reaction is probably more relevant to homeostatic control of glycerol content under steady state conditions than to controlling the response to water stress.
CHAPTER TWO:

METABOLIC AND IONIC CHANGES ASSOCIATED WITH GLYCEROL PRODUCTION DURING ADAPTATION OF SACCHAROMYCES CEREVISIAE TO A WATER STRESS.
2.1 INTRODUCTION

The study of yeast water relations has mainly been concentrated on three organisms, namely halotolerant *Debaryomyces hansenii* (Norkrans 1966; Adler 1978; Adler et al. 1985), xerotolerant *Saccharomyces rouxii* (Onishi 1963; Onishi and Siromaru 1984; Anand and Brown 1968; Edgley and Brown 1983) and nontolerant *S. cerevisiae* (Norkrans 1968; Brown 1975; Edgley and Brown 1983; Mackenzie et al. 1986; Brown et al. 1986). The areas of investigations have so far focussed mainly on the steady state situations, that is when an organism has fully adapted to a water stress. The interests have been in the study of the properties of enzymes (Anand and Brown 1968; Brown and Simpson 1972; Brown 1976; Adler 1978), energetics (Gustaffson 1979), glycerol metabolism (Adler et al. 1985), transport of ions (Norkrans and Kylin 1969), ultrastructure and sugar uptake (Lindman and Norkrans 1982; Lindman 1981), respiration and fermentation activity (Norkrans 1968; Brown 1975), comparative studies of osmoregulatory responses (Brown and Edgley 1980) and physical responses to osmotic stress (Rose 1975). However, very few studies have been done during the period of adaptation of organisms to the water stress.

Edgley and Brown (1983) have compared the physiological changes induced by a water stress in nontolerant *Saccharomyces cerevisiae* and xerotolerant *S. rouxii* when fully adapted and during the adaptation or transition period. Their results suggested that growth of *S. cerevisiae* at low water activity dramatically increased the activity of glycerol-3-phosphate dehydrogenase whereas this enzyme in *S. rouxii* remained unaffected. Their transition study showed that *S. rouxii* adapted to salt broth rapidly (6 hours) without any effect on viability on high or low water activity agar. The adaptation period for *S. cerevisiae* was very long (140 hours) and was divided into two stages. In stage 1 (about 40 hours), a large proportion of the population died whereas at the beginning of stage 2 (after 40 hours), the viability progressively increased and ultimately reached a level equal to the initial value. Both yeasts started accumulating glycerol immediately after transfer, but *S. cerevisiae* leaked a significant amount of
glycerol into the medium during the adaptation. Glycerol-3- phosphate dehydrogenase activity also increased in *S. cerevisiae* but not until stage 2 of the adaptation sequence. Onishi (1957a) reported that during adaptation of *S. rouxii* to 18% NaCl medium there was a drop in viability of this yeast when plated on plain and 18% NaCl agar. Onishi and Shiromaru (1984) have since shown that when *S. rouxii* is transferred to 18% NaCl medium a loss in viability occurred within the first 2 hours on both media, with or without 18% NaCl. They have called this phase stage 1. In stage 2, they found that there was no difference in viable counts for 24 hours between this value and the stage 1 value. After 24 hours *S. rouxii* grew normally. During a short exposure (15 minutes) of *S. cerevisiae* to low water activity ultrastructure and viability studies have also been made (Morris et al. 1986). These researchers found a positive correlation between reduction in cell volume and the loss of viability after water stress. They proposed that the plasma membrane does not seem to be the primary site of injury during a water stress. The cell wall thickness of shrunken cells increased while the outer surface remained unaffected.

Larsson and Gustafsson (1987) recently have studied the production of glycerol in relation to the ATP pool and heat production rate in *D. hansenii* and *S. cerevisiae* after stationary phase transfer of cells to a liquid medium containing salt. They found that, during a water stress, *D. hansenii* possesses the capacity to regulate the metabolism of glycerol to optimize growth while *S. cerevisiae* is not able to do so when exposed to different demands on its glycerol metabolism.

With a view to understanding further the biochemical adjustment that must be made when yeast is subjected to a water stress, in this study, the glycerol content of *S. cerevisiae* was monitored during the early stages of adaptation to liquid medium containing salt. It was found that the initial accumulation of glycerol that normally occurred when yeast was transferred to a fresh high water activity liquid medium, was both retarded and greatly diminished in magnitude by salt (Brown et al. 1986). Further, the yeast did not begin to accumulate glycerol until about 6 hours after transfer. Glycerol is produced
glycolytically in *S. cerevisiae* (see Brown and Edgley 1980), so the osmoregulatory response is likely to be closely integrated with glycolysis. The levels of phosphorylated glycolytic intermediates, nucleotides and ions were quantified in order to identify the intracellular event(s) limiting glycerol production during initial critical period of adaptation after transfer of yeast to liquid medium containing salt.

### 2.2 MATERIALS AND METHODS

#### 2.2.1 Yeast strains

*Saccharomyces cerevisiae* (strain Y41, ATCC 38531) and *S. rouxii* (strain YA, ATCC 38528), described by Anand and Brown (1968) were maintained at 4° C on slopes of malt extract agar (MA; 0.997) and synthetic honey agar (SHA; containing 48% w/v glucose; \( a_w \) 0.924; Anand and Brown, 1968) respectively. The cultures were transferred once in every three months.

#### 2.2.2 Growth conditions

Cultures were grown in Wickerham's synthetic medium (Barnett et al.1983; \( a_w \) 0.997) in Erlenmeyer flasks of a capacity twice the volume of the growth medium and incubated at 30° C with rotary agitation (200 rpm). This medium, without the carbon source, glucose, was stored at -10° C as a stock mineral salts solution at 10x final concentration. The medium and glucose solution were sterilized by filtration. Glucose was added at a final concentration of 0.5% (w/v). Neutral red was used, as required, to a final concentration of 0.1mM which was sterilized by filtration.

#### 2.2.3 Inoculation and transfer procedures

The inoculation and transfer procedures used in the adaptation of *S. cerevisiae* and *S. rouxii* from synthetic medium (\( a_w \) 0.997) described above to the same medium
containing 8% NaCl, w/v (aw 0.955) or otherwise specified concentration, was monitored as follows:

A loopful of yeast was inoculated into synthetic medium (50 ml) and grown to a stationary phase (48 hours, preinoculum). A new batch of synthetic medium (50 ml) was inoculated to a level of 5% v/v with preinoculum culture and grown to stationary phase (48 hours, inoculum). The experimental medium (125 ml normally but 500 ml for NMR studies) was inoculated to a level of 5% v/v with the inoculum and grown to mid-exponential phase (4.5 hours). The culture was aseptically filtered through nitrocellulose filter (1μm pore size) under vacuum and cells collected on the filter were transferred to the medium containing 8% NaCl unless otherwise specified (normally 250 ml but 1 litre for NMR measurements) at 30° C. Aseptic precautions were taken throughout the transfer.

2.2.4 Counting

Samples for plating and microscopical counting were first treated in a glass/teflon homogeniser to disaggregate clumps. A 3 ml sample was subjected to 75 double strokes of the teflon plunger. Aseptic conditions were maintained throughout the disaggregation procedure.

2.2.4.1 Total counts

Total counts were made conventionally in a counting chamber. A colony forming unit (cfu) was defined as any viable cell or aggregate of cells which if viable would produce a single colony. Any cfu that was not a single cell was scored as a budded cfu irrespective of the bud size. A minimum of 300 cfu was counted.

2.2.4.2 Viable counts

Disaggregated cell suspensions were serially diluted in sterile quarter-strength Ringer's solution. Pour plates were prepared by pipetting 0.1 ml of a cell suspension in a sterile disposable plastic petri dish and mixing with 10 ml of molten agar (40° C). Plates were
incubated at 30° C. All viable counts were done in duplicate on malt extract agar (MA; a normal high water activity medium, aw 0.997) and synthetic honey agar (SHA; a nutrient medium containing 48 % w/v glucose, aw 0.924). SHA plates were stored in plastic bags to prevent loss of water from the agar. A period of three days (MA) to seven days (SHA) was required before colonies were visible to the naked eye.

2.2.5 Analytical methods

2.2.5.1 Cell sampling
At various time intervals, samples (10 ml) were withdrawn and quickly filtered under vacuum through a cellulose acetate membrane (pore size 0.8 μm). The cells and filter were frozen in liquid nitrogen, freeze dried and stored at -80° C until extracted. The cells were not washed except when trehalose was estimated. For trehalose estimation the yeast was washed twice on the filter with isotonic phosphate buffer (pH 5.9) to remove residual extracellular hexose. This buffer contained the major salts present in Wickerham's medium. The filtrate was also frozen in liquid nitrogen and kept at -80° C until analysed.

2.2.5.2 Dry mass
Dry mass was determined on a separate sample. A sample (10 ml) was filtered onto a tared membrane filter (0.8 μm). The cells were washed on the filter with distilled water three times the volume of the sample. After drying at 105° C for 24 hours the filters were cooled in a desiccator and weighed.

2.2.5.3 Extraction
Freeze dried yeast was extracted with ethanol according to Edgley and Brown (1983). The extracts were dried under vacuum and then redissolved in 1 ml of water. These extracts were stored at -80° C until analysed.
2.2.5.4 Ethanol

The enzymic method described by Beutler (1984) was used to estimate ethanol.

2.2.5.5 Glucose

Glucose was estimated enzymically as described by Edgley and Brown (1983) using a commercial glucose oxidase kit (GOD-Perid, Boehringer).

2.2.5.6 Glycerol

The method described by Eggstein and Kuhlmann (1974) as modified by Edgley and Brown (1983) was used for glycerol determination.

2.2.5.7 Trehalose

Trehalose was estimated with anthrone (Stewart 1975) in ethanolic cell extracts. Trehalose is normally extracted from yeast using 0.5 M trichloroacetic acid. However, Mackenzie (1987) found no difference in trehalose values when cells were extracted with either trichloroacetic acid or ethanol.

2.2.5.8 K+ and Na+

K+ and Na+ were extracted and estimated according to Camacho et al. (1981) by flame photometry.

2.2.6 31P Nuclear magnetic resonance spectroscopy

Phosphorylated glycolytic intermediates and nucleotides were estimated by the method of 31P nuclear magnetic resonance (NMR) spectroscopy as described by den Hollander et al. (1986a).

2.2.6.1 Cell sampling

Cultures (approximately 800 ml) at different time intervals were harvested by filtration using nitrocellulose filters (1μm pore size, 47 mm diameter). Two Sartorius
glass filtration assemblies were used in order to collect the cells rapidly (1-3 min).

Filters were transferred to glass scintillation vials (kept in liquid nitrogen) and cells were immediately frozen and stored in liquid nitrogen until extracted. A third Sartorius glass filtration assembly was used for the determination of dry mass.

2.2.6.2 Preparation of cell extract

Cell extracts for analysis by NMR spectroscopy were prepared as described by Saez and Lagunas (1976) by adding 3M perchloric acid (prechilled to -20° C) to the cells, vigorously shaking the cell suspension, freezing and thawing three times and pelleting the cellular debris by centrifugation at 7000 rpm for 15 minutes. The extract was neutralized with 2M KHCO₃, the precipitate removed by centrifugation and then the extracts passed through a column of Chelex 100 (Sigma) to remove paramagnetic cations. Columns were washed several times and washing and extracts pooled together, frozen in liquid nitrogen and freeze dried.

2.2.6.3 Operating conditions

Freeze dried samples were dissolved in 20 mM Na₂EDTA containing 50% D₂O and adjusted to pH 8.0. All NMR measurements were performed with a Bruker CXP 300 spectrometer, operating at 121.46 MHz for the ³¹P nucleus. Spinning sample tubes (10 mm outside diameter) were used at a temperature of 296 K. One thousand to ten thousand scans were accumulated for different samples depending on the concentration of the samples, with a 90° pulse, 68 μs repetition time and a 5208.33 Hz sweep width. The phosphorus nuclei were proton decoupled using broad band irradiation with power approximately 1.5 watts. Chemical shifts were measured relative to 85% phosphoric acid but against a primary standard of 0.05% phosphoric acid contained in a capillary. The spectra of different concentrations of pure metabolites were acquired under identical conditions to those used for spectra of extracts. Pure metabolites were included in some
sample extracts in order to facilitate the identification of metabolites in samples. Compounds were estimated from this peak height in the NMR spectra.

2.2.7 Chemicals

Various glycolytic intermediates and other biochemicals used were of the highest purity available either from Boehringer Mannheim Corp or from Sigma Chemicals Company. Other chemicals were of reagent grade.

2.2.8 Analytical Error

Estimated maximum analytical error was not more than 20%.
2.3 RESULTS

2.3.1 Growth of *Saccharomyces cerevisiae* and *S. rouxii* during adaptation to liquid medium containing salt

The effects of 6% NaCl (aw 0.974) and 8% NaCl (aw 0.955) on adaptation of *S. cerevisiae* from a stationary phase transfer are shown in figure 2.1. At 0.974 water activity, *S. cerevisiae* growth was not inhibited as much as at a water activity of 0.955. A water activity of 0.955 stretched the growth of the yeast to its limit and caused a very long period lag phase (fig. 2.2). During the time shown in figure 2.1 buds did not separate. Similar effects of salt on growth were observed when exponentially growing cells were transferred to a liquid medium containing salt. No growth was evident in the 8% NaCl culture in the first 50 hours after transfer whereas in the 6% NaCl culture yeast was able to recommence growth after transfer (fig. 2.2). Judging from the length of the lag in the 6% NaCl transfer experiments stationary phase cells were able to adopt and recommence growth more easily than exponential phase cells. Unlike *S. cerevisiae* exponential phase *S. rouxii* was able to start growing shortly after being transferred to liquid medium containing 8% NaCl.

Exponentially growing *S. cerevisiae* was also transferred to the same but fresh medium (control) and growth was monitored by measuring total count and viability on MA and SHA (fig. 2.3). After a lag of less than two hours the cells began growing. While growth on MA was in agreement with the total counts, the SHA viability was extremely low throughout the duration of this experiment.

2.3.2 Glycerol and ethanol production during adaptation of *Saccharomyces cerevisiae* to liquid medium containing salt

Edgley and Brown (1983) measured the levels of glycerol during the adaptation of *S. cerevisiae* to complex medium containing 10% NaCl. They found that glycerol
accumulation apparently begins immediately after the transfer, but their observation was extended over a long period with the first few samples taken at 0, 2, and 20.5 hours after the transfer. Once it was determined that 8% NaCl was close to the limit of tolerance of *S. cerevisiae* in Wickerham's synthetic medium, subsequent adaptation experiments were carried out using this growth medium and at short sample intervals confined to first 8 hours of the adaptation.

Glycerol production in the control experiment (exponentially growing yeast transferred to Wickerham's synthetic medium without salt) is shown in figure 2.4. Intracellular glycerol showed an early peak and then gradually declined whereas extracellular glycerol progressively increased. In comparison to the control experiment total glycerol production by *S. cerevisiae* during adaptation to liquid medium containing 8% NaCl was greatly diminished (fig. 2.5). The intracellular glycerol peak which characterized the control transfer was also evident in the salt transfer but this peak was both retarded and greatly diminished in magnitude (fig. 2.6). It was only after about 6 hours in the salt that the intracellular glycerol content of *S. cerevisiae* began to increase. Extracellular glycerol rose after transfer, a pattern similar to the control experiment and began to decline 7 hours after transfer.

In contrast to *S. cerevisiae*, *S. rouxii* responded to being transferred to salt (8% NaCl) by accumulating intracellular glycerol from the onset of the adaptation (fig. 2.7). This yeast was able to recommence growth within 4 hours after transfer to salt medium at which point it contained approximately 1 μmole glycerol/mg dry weight. The glycerol content was low in *S. cerevisiae*. Even when this yeast had began to accumulate glycerol, it contained only about 0.15 μmole glycerol/mg dry weight while the glycerol content of *S. rouxii* was 10 fold higher at this stage of the adaptation. Interestingly, *S. rouxii* did not show a peak in intracellular glycerol, a peak that was characteristic of *S. cerevisiae* under both salt and control transfers.
Ethanol production was also monitored during adaptation of *S. cerevisiae* to salt medium. The yield of ethanol was more than halved in 8% NaCl medium (fig. 2.8) but the rate of ethanol production was not noticeably affected by salt for about 3 hours.

### 2.3.3 Trehalose production during adaptation of *Saccharomyces cerevisiae* to liquid medium containing salt

Measurement of trehalose in salt-adapting cells of *S. cerevisiae* showed that trehalose accumulation began immediately after transfer (fig. 2.9). At 4 hours the trehalose content was about ten times more than the control values and decline thereafter during adaptation.

### 2.3.4 Changes in levels of phosphorylated glycolytic intermediates during adaptation of *Saccharomyces cerevisiae* to liquid medium containing salt

The reduction in the glycerol and ethanol production by *S. cerevisiae* during adaptation to liquid medium containing salt was accompanied by changes in the levels of glycolytic intermediates. The amounts of hexose phosphates glucose-6-phosphate, fructose-6-phosphate and fructose 1,6 bisphosphate declined during the first hour of adaptation to salt as they did in the control indicating that the glycolytic capacity of the cells had not been significantly affected by salt during this period. The levels of these intermediates began to increase after 1 hour and peaked at 3 hours in the salt medium (figs. 2.10, 2.11 and 2.12). By 8 hours, when glycerol production had begun to increase (fig. 2.5), the levels were close to the levels of the control culture. The increasing levels of these intermediates after 1 hour in the salt medium suggests that by this time one or more glycolytic enzyme activities may be restricting the pathway.

A similar pattern to that of hexose phosphate occurred for phosphoenolpyruvate (fig. 2.14). However, 3-phosphoglycerate levels were similar in salt from those of the control culture (fig. 2.13). The levels of 3-phosphoglycerate in salt rapidly declined after transfer as did in the control. These observations suggest that the glycolytic
pathway was restricted in the early part. Thus those intermediates upstream from the blockage accumulated. This restriction was relieved by 8 hours. A second restriction in the pathway as a result of the accumulation of hexose intermediates may have also developed in the latter part of the pathway after salt transfer, which could explain the accumulation of phosphoenolpyruvate.

2.3.5 Changes in levels of nucleotides during adaptation of *Saccharomyces cerevisiae* to liquid medium containing salt

Since some of the glycolytic enzymes require nucleotides for activity, the availability of these cofactors could be responsible for the low glycerol and ethanol production after transfer to salt. Therefore NAD⁺, ATP and ADP were measured using ³¹P NMR.

2.3.5.1 NAD⁺

In figure 2.15, the content of NAD⁺ is shown during adaptation of *Saccharomyces cerevisiae* to the salt medium. The amount of NAD⁺ increased about five fold during the period when hexose phosphates were accumulated in the cells. Since only perchloric acid extracts were analysed, NADH levels could not be measured. Nevertheless, an increase in NAD⁺, may reflect changes in the availability of NADH, which in turn will affect glycerol and ethanol production.

2.3.5.2 ATP and ADP

Both ATP and ADP showed a pattern similar to the hexose phosphates during adaptation to the salt medium (figs. 2.16 and 2.17) that is the levels of both nucleotides declined in the first hour as in the control but rose to its maximum between 2 and 3 hours after transfer.
2.3.6 Glucose consumption by *Saccharomyces cerevisiae* during adaptation to liquid medium containing salt

During the first 4 hours of adaptation to the salt medium *Saccharomyces cerevisiae* consumed glucose at a similar rate to that of the control without salt. A divergence in glucose consumption was evident 4 hours after transfer (fig. 2.18) with salt-adapting yeast consuming less glucose than the control without salt.

2.3.7 Changes in levels of ions during adaptation of *Saccharomyces cerevisiae* to liquid medium containing salt

Accumulation of potassium seems to be a preferred method of osmoregulation in many microorganisms (Brown 1964; Epstein 1986; Wegmann 1986; Larsen 1987). When *Saccharomyces cerevisiae* was transferred to the salt medium, it accumulated potassium (fig. 2.19) while sodium progressively entered into the cells (fig. 2.20). The content of potassium remained high until 6 hour, then gradually declined, this decline coinciding with the time when intracellular glycerol production begins to increase. When *S. cerevisiae* was transferred to medium containing neutral red, a dye known to cause K⁺ depletion, (Pena et al. 1979) intracellular glycerol levels increased rapidly, reaching a maximum after 3 hour in this medium (fig. 2.21).
FIGURE 2.1 Growth of *Saccharomyces cerevisiae* during adaptation to Wickerham's medium containing 6 and 8% NaCl after transfer of stationary phase cells.

Top panel
- % budded cells 6%NaCl.
- % budded cells 8%NaCl.

Bottom panel
- Total counts (c.f.u/ml) 6%NaCl
- Total counts (c.f.u/ml) 8%NaCl

The points represent the means of two experiments.
FIGURE 2.2 Growth of *Saccharomyces cerevisiae* and *S. rouxii* during adaptation to Wickerham’s medium containing 6 and 8% NaCl after transfer of exponential phase cells.

Top panel

- % budded cells 6%NaCl
  
  *S. cerevisiae*.

- % budded cells 8%NaCl
  
  *S. cerevisiae*.

- % budded cells 8%NaCl
  
  *S. rouxii*.

Bottom panel

- Total counts (c.f.u/ml) 6%NaCl
  
  *S. cerevisiae*.

- Total counts (c.f.u/ml) 8%NaCl
  
  *S. cerevisiae*.

- Total counts (c.f.u/ml) 8%NaCl
  
  *S. rouxii*.

The points represent the means of two experiments.
FIGURE 2.3 Growth of *Saccharomyces cerevisiae* in Wickerham's medium without added salt after transfer of exponential phase cells.

Top panel proportion of budded c.f.u.

Bottom panel

- Total counts  (c.f.u./ml)
- ▲ MA counts  (c.f.u./ml)
- ■ SHA counts  (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts).
FIGURE 2.4 Glycerol production during growth of *Saccharomyces cerevisiae* in Wickerham's medium after transfer of exponential phase cells.

- Intracellular
- Extracellular

The points represent the means of two experiments.

Note the difference in scales of the two vertical axes.
Intracellular glycerol (μ mole /mg dry yeast)

Extracellular glycerol (μ mole /mg dry yeast)

Time after transfer (h)
FIGURE 2.5 Total glycerol production during growth of *Saccharomyces cerevisiae* in Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- □ 8% Salt

The points represent the means of two experiments.
Glycerol (mmol/mg dry yeast)

Time after transfer (h)

Glycerol

(μmol/mg dry yeast)

0  2  4  6  8

Time after transfer (h)
FIGURE 2.6 Glycerol production during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Intracellular
- Extracellular

The points represent the means of two experiments.
production

FIGURE 2.7 Intracellular glycerol during adaptation of *Saccharomyces rouxii* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

The points represent the means of two experiments.
Intracellular glycerol ($\mu$ mole / mg dry yeast)
FIGURE 2.8 Ethanol content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

The points represent the means of two experiments.
FIGURE 2.9 Trehalose content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

The points represent the means of two experiments.
Trehalose content (μ mole hexose/mg dry yeast)
FIGURE 2.10 Glucose-6-phosphate content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

Not every point was replicated due to high cost of running the NMR machine. Those which were replicated are shown with the vertical bars depicting the range of the values.

FIGURE 2.11 Fructose-6-phosphate content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

Not every point was replicated due to high cost of running the NMR machine. Those which were replicated are shown with the vertical bars depicting the range of the values.
Fructose-6-phosphate content
(μ mole /g dry yeast)

Time after transfer (h)
Glucose-6-phosphate content
(μ mole /g dry yeast)
FIGURE 2.12 Fructose 1,6 bisphosphate content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

Not every point was replicated due to high cost of running the NMR machine. Those which were replicated are shown with the vertical bars depicting the range of the values.

FIGURE 2.13 3-Phosphoglycerate content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

Not every point was replicated due to high cost of running the NMR machine. Those which were replicated are shown with the vertical bars depicting the range of the values.
3-Phosphoglycerate content
(μ mole /g dry yeast)

Time after transfer (h)
Fructose 1,6 bisphosphate content
(μ mole /g dry yeast)
FIGURE 2.14 Phosphoenol pyruvate content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

Not every point was replicated due to high cost of running the NMR machine. Those which were replicated are shown with the vertical bars depicting the range of the values.

FIGURE 2.15 NAD$^+$ content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

Not every point was replicated due to high cost of running the NMR machine. Those which were replicated are shown with the vertical bars depicting the range of the values.
NAD+ content (μ mole /g dry yeast)

Time after transfer (h)
Phosphoenolpyruvate content
(μ mole /g dry yeast)
FIGURE 2.16 ATP content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

Not every point was replicated due to high cost of running the NMR machine. Those which were replicated are shown with the vertical bars depicting the range of the values.

FIGURE 2.17 ADP content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

Not every point was replicated due to high cost of running the NMR machine. Those which were replicated are shown with the vertical bars depicting the range of the values.
FIGURE 2.18 Glucose consumption during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

The points represent the means of two experiments.
FIGURE 2.19 Potassium content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

- Control
- 8% salt

The points represent the means of two experiments.
Potassium content (μ mole /mg dry yeast)
FIGURE 2.20 Sodium content during adaptation of *Saccharomyces cerevisiae* to Wickerham's medium containing 8% NaCl after transfer of exponential phase cells.

■ Control

□ 8% salt

The points represent the means of two experiments.
Sodium content (μ mole/mg dry yeast)

Time after transfer (h)
FIGURE 2.21 Intracellular glycerol content during growth of *Saccharomyces cerevisiae* in Wickerham's medium containing neutral red (0.1 mM) after transfer of exponential phase cells.

The points represent the means of two experiments.
2.4 DISCUSSION

2.4.1 Growth of *Saccharomyces cerevisiae* and *S. rouxii* during adaptation to liquid medium containing salt

Anand and Brown (1968) and Edgley (1980) have demonstrated that the growth rate of *Saccharomyces cerevisiae* is severely affected at low water activities. Norkrans (1966) has also reported that 4% NaCl reduces the growth of *S. cerevisiae* by 10 to 15% compared to that in the absence of salt. The studies presented here also demonstrate the marked effect of salt on the growth of *S. cerevisiae* and as numerous others have shown (see for example Anand and Brown 1968), the remarkable ability of *S. rouxii* to grow at salt concentration totally inhibitory to *S. cerevisiae*. Transferring yeast to the salt medium has been used to impose a water stress in this study. Figure 2.2 shows that *S. rouxii* reproduced easily and rapidly to a water stress. In the salt medium the nontolerance of *S. cerevisiae* was shown by its inability to grow when either stationary or exponential phase cultures were transferred (figs. 2.1 and 2.2).

A second method of exposing yeast to a water stress was used in the preliminary stages of this study. Imposing a water stress by plating on SHA instead of transferring *S. cerevisiae* to the salt medium gave viable counts which were at least $10^{-3}$ less than on the nonstressing agar (fig. 2.3). This phenomenon has been described as "water stress plating hypersensitivity" (Mackenzie et al. 1986). Several aspects of this phenomenon have been described in chapters 3 and 4 of this thesis.

2.4.2 Metabolic and ionic changes during adaptation of *Saccharomyces cerevisiae* to liquid medium containing salt

When some yeasts are grown at low water activity they accumulate glycerol. Until a yeast has gained its original turgor, growth is not possible. Turgor is regained largely by glycerol accumulation although uptake of salts in yeast also has been reported (Norkrans...
and Kylin 1969). Therefore, an essential part of adapting to low water activity by yeast is the accumulation of glycerol.

*Saccharomyces rouxii* grew in the salt medium presumably because, among other things, it accumulated sufficient glycerol to regain a turgor pressure and volume compatible with growth. This process was initiated soon after transfer. *Saccharomyces cerevisiae* did not begin accumulating glycerol until it had been in the salt medium for several hours. When exponentially growing *S. cerevisiae* was transferred to a liquid medium containing 8% NaCl the accumulation of intracellular glycerol was both retarded and delayed in two respects (fig. 2.5). Firstly, when compared to the early glycerol peak in the control, the peak in the salt medium was delayed until 3 hours. In addition the magnitude of the peak was greatly diminished. Secondly, *S. cerevisiae* did not begin to accumulate significant amounts of glycerol until about 6 hours. Based on the response of *S. rouxii* to salt, one can assume that the best strategy for yeast when challenged by low water activity is to accumulate glycerol quickly. Yet *S. cerevisiae* did not adopt this strategy. Initially it seemed that the low levels of glycerol produced after salt transfer reflected the inhibitory effects of salt on the metabolic processes of *S. cerevisiae* in general. However, glucose consumption and ethanol production both testify that, at least for the first 3 hours after transfer, salt had no effect on the ability of *S. cerevisiae* to catabolize glucose. The yeast was also able to synthesize trehalose and take up K+ from the medium and largely extrude Na+ in the yeast few hours after transfer. So, it would seem that *S. cerevisiae* cells were able to function in some respect in salt medium for 3 hours but glycerol production and accumulation was not initiated in this period as it was in *S. rouxii*.

Several mechanisms could be operating that may result in low levels of glycerol. Firstly, NaCl could have a direct effect on the activity of the enzymes involved in glycerol synthesis. Glycerol is produced glycolytically in *S. cerevisiae* and is possible that the upper glycolytic enzymes as well as those involved only in glycerol synthesis may be
inhibited by NaCl. The initial levels of ethanol produced probably exclude salt inhibition of the glycolytic enzymes as being responsible for the low glycerol production. Of the two glycerol synthesis enzymes, only glycerol-3-phosphate dehydrogenase has been studied in any detail in yeast. Edgley and Brown (1983) reported that cell free extracts of *S. cerevisiae* can reduce dihydroxyacetone phosphate with NADH or NADPH although the activity with NADPH is low. The activity of this enzyme in *S. carlsbergensis* is known to be inhibited by NaCl in vitro (Nader et al. 1979) but the in vivo activity cannot be accurately predicted from the activity measured in vitro under substrate saturating conditions. Na⁺ ions certainly entered the cells (fig. 2.20) a finding in agreement with Norkrans and Kylin (1969) but it does seem unlikely that the glycerol synthesis enzymes would be more sensitive to Na⁺ than the glycolytic enzymes.

A second explanation for the low level of glycerol production could lie with the observation of Edgley (1980), Gancedo et al. (1968), Gancedo and Gancedo (1973) that the NADH dependent activity of glycerol-3-phosphate dehydrogenase in *S. cerevisiae* is severely inhibited by KCl at a concentration that is within the range of physiological concentrations. That K⁺ ion content of the cells increases after salt transfer has been demonstrated by this study (fig. 2.19) and also by Norkrans and Kylin (1969), Hobot and Jennings (1984). A comparison of glycerol content and K⁺ content after salt transfer reveals a negative relation between K⁺ content and glycerol content. The neutral red experiment also suggests that K⁺ may be inhibiting glycerol formation as the apparent K⁺ depletion caused by neutral red led to glycerol formation (fig. 2.21). Brown (1978a) suggested that a decrease in environmental water activity might affect the H⁺/K⁺ exchange across the yeast cell membrane resulting in increases in intracellular pH and K⁺ concentration. The physiological significance of K⁺ and glycerol production was alluded to by Brown and Edgley (1983) who concluded that the K⁺ inhibition of glycerol-3-phosphate dehydrogenase in *S. cerevisiae* must be overcome in vivo as evidenced by the high production of glycerol they observed in high salt medium. It would appear from the salt transfer experiments done in this thesis that substantial
amounts of glycerol could only be synthesized and accumulated about 6 hours after transfer because the K⁺ content of the cells was too high up to this time. Once K⁺ content was lowered glycerol synthesis and accumulation proceeded at a greater rate. However, the observation that *S. cerevisiae* had not begun budding after 50 hours in salt broth suggests that the amount of glycerol synthesized and accumulated was not sufficient even after 50 hours to permit the yeast to divide. It is possible that the K⁺ content remained at a sufficiently high level through out the 50 hours to continue inhibiting glycerol production. K⁺ measurements later than 8 hours after salt transfer would verify whether this is true or not.

While glycerol is recognized as the major osmoregulatory solute in yeast this study points to two other substances, K⁺ and trehalose, that are making major contributions to turgor after exposure to low water activities. When the yeast was transferred to the salt medium potassium uptake from the medium increased. Like other microorganisms, *S. cerevisiae* accumulates K⁺ from the external medium to fulfill cellular requirements. Short term increases in cell K⁺ content are also observed in response to a water stress in gas-vacuolated cyanobacterium *Microcystis* sp, indicating that regaining turgor may involve a turgor sensitive K⁺ uptake system (Reed and Walsby 1985). Estimation of internal K⁺ concentration in *Microcystis* cells transferred to 250 mosmol NaCl kg⁻¹ showed that changes in K⁺ may account for at least half of the observed turgor regain in one hour. In *S. cerevisiae* a dual system for K⁺ transport has been reported, one with high *Kₘ* and the other with low *Kₘ* value (Rodríguez-Navarro and Ramos 1984). It is tempting to suggest that in ordinary conditions high *Kₘ* system may be operative, but under conditions of water stress low *Kₘ* system should be operational to take up large amounts of K⁺ inside the cells to regain the lost turgor. Many organisms have more than one transport systems to ensure that they are able to accumulate sufficient quantities of the required ion. Typical examples are the transport of phosphate by yeast (Borst-Pauwels 1981) and *Neurospora* sp. (Lowendorf et al. 1975) and the transport of K⁺ by *Escherichia coli* (Epstein and Kim 1971) and *Anabaena variabilis*
In *E. coli* under most conditions a constitutive system with low affinity for K\(^+\) called the trk system is the only one present. When trk activity is reduced by the use of media with a very low K\(^+\) concentration the repressible high affinity kdp system is expressed. Both systems respond to low water activity in the same way leading to a stimulation of K\(^+\) influx without any change in efflux (Rhoads et al. 1978). An accumulation of K\(^+\) after a water stress has also been reported in yeast (Norkrans and Kylin 1969) in the alga *Dunaliella* (Pick et al. 1986) and in fungi (Luard 1982). A leakage of K\(^+\) occurs, however, in xerotolerant *S. rouxii* (Onishi and Shiromaru 1984) during adaptation to salt broth containing 18% NaCl. In the light of the suggested role of K\(^+\) in inhibiting glycerol-3-phosphate dehydrogenase in *S. cerevisiae* it would be important to know how glycerol-3-phosphate dehydrogenase in *S. rouxii* is affected by K\(^+\). A leakage of K\(^+\) in *S. rouxii* with simultaneous increase in glycerol synthesis is suggestive that glycerol-3-phosphate dehydrogenase from *S. rouxii* may also be inhibited by K\(^+\).

During adaptation to salt, trehalose was accumulated maximally between 3-4 hours and remained significantly high even after this period. In a semiquantitative analysis by paper chromatography of extracts of *S. cerevisiae* 20 hours after transfer to salt broth, Edgley and Brown (1983) provisionally identified trehalose. Trehalose has previously been reported as present in fully adapted cultures of *S. cerevisiae* at low water activity (Brown 1978a). Increase in trehalose content in response to reduced water activity has also been reported for *Candida tropicalis* grown in a pH stat. In medium containing 110 g/l of NaCl, trehalose content was twice that found in the absence of NaCl (Furyaeva et al. 1985). Ozawa and Iwamoto (1981) however, reported a decline in trehalose content in *Hansenula* with increasing salt concentration. They found that at low salt concentration trehalose was the major intracellular solute, but at higher salt concentration glycerol predominated and trehalose declined. A somewhat similar situation is encountered during the adaptation of *S. cerevisiae* to liquid medium containing salt in this study. As described in the results, the trehalose content first increased in response to low water
activity and then declined at the time when glycerol content began to predominate. Trehalose has also been reported as an osmoregulatory solute in cyanobacteria (Reed and Stewart 1983) and *Escherichia coli* (Strøm 1986). Trehalose is often accumulated during the periods of reduced growth in fungi (Mandels 1981).

While trehalose and K⁺ have been identified as osmoregulatory substances in other organisms and there is evidence to suggest that K⁺ is generally less inhibitory than other cations (Brown 1976) and trehalose is able to protect *S. cerevisiae* against the effects of low water activity (see chapter 3, section 3.4.2), the third substance accumulated in this yeast after salt transfer was NaCl. NaCl is known to be very inhibitory of cellular function. Na⁺ entered the yeast progressively over a period of 6 hours. It was mentioned earlier that glucose consumption and ethanol production were not reduced during the first few hours after salt transfer but coinciding with the higher Na⁺ levels from about 3 hours onwards glucose consumption and ethanol production slowed significantly. It is conceivable that the Na⁺ content was the reason for the decline in glucose consumption. Steady state studies have documented a decrease in ethanol production in the presence of salt (Umemoto et al. 1967; Tajima and Yoshizumi 1972). Furthermore in describing the "sugar defect" (this term meaning sugar not converted to alcohol) during alcoholic fermentation of Okinawa molasses by *Saccharomyces formensis*, Tajima and Yoshizumi (1973) reported the inhibition of alcohol dehydrogenase and an increase in the accumulation of acetaldehyde with increasing concentrations of salt in the medium. The results of Wei et al. (1982) on the effects of NaCl on yeast grown in gelatin have also indicated that alcohol dehydrogenase activity decreases as the NaCl concentration increases. To explain the inhibitory effects of electrolytes, Umemoto et al. (1967) suggested that yeast pyruvate decarboxylase (the enzyme catalyzing the decarboxylation of pyruvate to acetaldehyde) is inhibited. Once acetaldehyde production is diminished there should subsequently be a proportional reduction in ethanol formation. For fermentation to continue, a hydrogen acceptor other than acetaldehyde is required to oxidize the NADH formed earlier in the glycolytic pathway. Umemoto et al. (1967)
proposed that the substitute hydrogen acceptor was phosphoglyceraldehyde. Tajima and Yoshizumi (1973) also reported the formation of glycerol and an increase in glycerol-3-phosphate dehydrogenase activity in the yeast *S. formensis* during ethanol fermentation of molasses containing salt. For glycerol formation NADH is required in the reduction of DHAP to glycerol-3-phosphate and is supplied from the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase. So it seems that the formation of ethanol in salt medium was inhibited by a shortage of NADH which is needed for glycerol production and by a lowering of alcohol dehydrogenase activity. Edgley and Brown (1983) also reported a substantial increase in the activity of glycerol-3-phosphate dehydrogenase in yeast adapting to salt broth, this rise in activity coincided with stage 2 of the adaptation process. Curiously the yeast was producing large amounts of glycerol before the glycerol-3-phosphate dehydrogenase activity increased.

When exponentially growing *Saccharomyces cerevisiae* was transferred to the same but fresh medium of the same composition the intracellular glycerol showed an early peak after the transfer (fig. 2.4). presumably in direct response to the availability of glucose in the fresh medium. A similar peak in intracellular glycerol production was also observed when stationary phase cells were inoculated into fresh medium (see fig. 3.4). In describing the regulation of glycogen synthesis in *S. cerevisiae* Rothman and Cabib (1969) reported a consistent and reproducible occurrence of an early peak in glucose-6-phosphate, ATP and ADP. A more complex case of transition from a steady state into a periodic oscillation has been described for the situation where an enzyme is activated by its products under suitable turnover conditions (Hess and Markus 1987). Glycolysis is known to oscillate in intact cells and experiments with yeast extracts have shown that glycolytic oscillations can be caused by glucose input (Hess 1977).

The intracellular glycerol peak characteristic of *Saccharomyces cerevisiae* after transfer to fresh medium was both delayed and diminished in magnitude after transfer to salt. In high water activity medium the timing of the glycerol peak coincided with a well known
event in *S. cerevisiae*. cAMP has received considerable attention as a possible organizer of carbohydrate metabolism in yeast (Francois et al. 1984, 1987). An abrupt increase in intracellular cAMP levels immediately after glucose addition has been documented by several researchers (Van der Plaat and Van Solingen 1974; Tortara et al. 1982, Purwin et al. 1982, Mazon et al. 1982). This glucose-stimulated cAMP increase has been shown to be mediated by intracellular acidification in *S. cerevisiae* (Caspani et al. 1985, Valle et al. 1986). Valle et al. (1987) recently demonstrated that addition of K+ to the medium reversed glucose-induced internal acidification. When it is considered that glycerol-3-phosphate dehydrogenase has been shown to be activated by cAMP (Tajima and Yoshizumi 1973) it is not implausible that the glycerol peak in fresh medium may reflect this activation and that the retardation and diminution of the glycerol peak after salt transfer may be a result of the effect of K+ accumulation on cAMP levels.

With the advent of advances in 31P NMR spectroscopy it is now possible to measure phosphorylated glycolytic metabolites and nucleotides with confidence, the knowledge of which was thought to be of prime importance in understanding the metabolic changes occurring in *S. cerevisiae* after transfer to salt. The usefulness of high resolution 31P NMR in monitoring phosphorylated metabolites has previously been demonstrated in yeast by a number of authors (Nevon et al. 1979; Barton et al. 1980; Gillies et al. 1981; den Hollander et al. 1981; Nicolay et al. 1982; Brindle and Krikler 1985; den Hollander et al. 1986a and b). Undoubtedly NMR spectroscopy has also proved its usefulness in the study of osmoregulation in *Escherichia coli* (Strøm et al. 1986), cyanobacteria (Mackay et al. 1984), fungi (Hocking et al. 1983), and *Dunaliella* (Brown et al. 1982; Fontana and Haug 1982; Degani et al. 1985). It should be noted that metabolite levels were expressed as amounts rather than concentrations as no attempt was made to determine the changes in cell volumes that invariably occurred after the yeast was exposed to a water stress (Morris et al. 1986) and as it began to reestablish its original turgor pressure and volume. The assumption is implied in interpreting these results that the yeast contains a single pool of soluble metabolites, an assumption which evidently may not be
true for every metabolite since subcellular compartmentation plays an important role in eucaryotic microorganisms. However, as a first approximation values related to glycolytic intermediates will not be affected by this fact. Only in those cases where a metabolic pathway occurs primarily in an organelle would it be likely that metabolites are at a quite different concentration in the organelle from that in the whole cell sap.

Accumulation of certain glycolytic intermediates in *Saccharomyces cerevisiae* cells provides evidence for the physiological relevance of defects in enzyme functioning under water stress conditions. Previous research aimed at investigating the control of glycolysis in *S. cerevisiae* by measuring pools of glycolytic intermediates (den Hollander et al. 1986a, 1986b) have identified three probable sites of control: glucose entry into the pathway (glucose uptake and/or initial phosphorylation), phosphofructokinase and pyruvate kinase. Measurements of triose phosphates and hexose phosphates after transfer to salt revealed an accumulation of high levels of hexose phosphates and phosphoenolpyruvate after three hours in salt. Major shifts in the metabolic pools of these intermediates have been documented in *S. cerevisiae* strains having mutational block in glycolytic enzymes (Ciriacy and Breitenbach 1979; Navon et al. 1979). These strains may contain 2 to 30 fold more of intermediates than the parent strain. The increased levels of hexose phosphates during adaptation of yeast to liquid medium containing salt may reflect the inhibition of phosphofructokinase activity. Mutants defective in this enzyme are known to accumulate both glucose-6-phosphate and fructose-6-phosphate (Ciriacy and Breitenbach 1979). Phosphofructokinase synthesizes fructose1,6 bisphosphate. An inhibition of this enzyme should not lead to an accumulation of fructose1,6 bisphosphate and accumulation of fructose1,6 bisphosphate did occur it would be expected if salt inhibited one of the enzymes lower in the glycolytic pathway such as aldolase and/or triose phosphate isomerase. Triose phosphate isomerase inhibition may first lead to an accumulation of one of the triose phosphates and finally lead to a backup of fructose1,6 bisphosphate. Alternatively a block at the level of phosphoglucomokinase or glyceraldehyde-3-phosphate dehydrogenase
could also buildup fructose1,6 bisphosphate. This alternative, however, does not seem attractive as substrate level phosphorylation of ADP to ATP occurs at this stage and if the yeast is to survive, the water stress it needs to obtain energy.

A buildup of phosphoenolpyruvate during adaptation of yeast to the salt medium suggests that a second enzyme, probably pyruvate kinase activity may also be inhibited at 3 hours after transfer. In fact a mutant lacking the pyruvate kinase activity is known to accumulate 30 times more phosphoenolpyruvate than the wild type (Ciriacy and Breitenbach 1979). As mentioned earlier pyruvate kinase has been proposed as a principle determinant of glycolytic flux, not only in *S. cerevisiae* (Maitra and Lobo 1977) but also in *Streptococcus* sp.(Thompson and Torchia 1984), *Veillonella parvula* (Ng and Hamilton 1975) and *Propionibacterium shermanii* (Smartt and Pritchard 1982). This enzyme is known to be subject to allosteric control, being activated by fructose 1,6 bisphosphate, phosphoenolpyruvate and other glycolytic intermediates and inhibited by one of its products, ATP (Cooper 1978; Kotlarz et al. 1975; Thompson and Torchia 1984). So, pyruvate kinase is considered to be a key regulatory enzyme although the regulation of glycolysis is still not fully understood (Crabtree and Newsholme 1985).

During alcoholic fermentation of molasses containing 0.25 to 1M NaCl Tajima and Yoshizumi (1972) reported that *Saccharomyces formensis* Nakazawa converted much of the sugar substrate to glycerol, 2,3-butanediol, mannitol, erythritol, organic acids, vicinal diketone, acetaldehyde and carbon dioxide. They also reported an accumulation of glucose-6-phosphate, fructose-6-phosphate and fructose1,6 bisphosphate at an early stage of fermentation. In investigating the reasons for this abnormal fermentation they reported that the inhibition of alcoholic fermentation under these conditions was caused by inhibition of hexokinase, aldolase and triosephosphate isomerase activity.
Irrespective of which glycolytic enzymes are inhibited after 3 hours in salt it would seem that the inhibition of glycolysis results in the abrupt decline in ethanol production and glucose consumption. Whether inhibition of glycolysis is caused by the rising intracellular Na+ content is uncertain nor is it clear if the accumulation of ATP, ADP and NAD+ after 3 hours reflect the inhibitory effect of salt or alternatively are themselves causing the decline in glucose consumption and ethanol production. It would be expected that the allosteric effects of the low ATP/ADP ratios within cells after salt transfer would promote glycolysis (Fraenkel 1982). The potential significance of the NAD+ level cannot be assessed without also determining the level of NADH but it is possible that low amounts of glycerol and ethanol produced in salt reflect a high NAD+/NADH ratio.

Even though the metabolic pools of hexose phosphates and phosphoenolpyruvate returned to their basal levels after peaking at 3 to 4 hours after salt transfer, the sustained low rates of ethanol production and glucose consumption after these peaks indicate that the cells were unable to utilize glucose at the rate observed in the first few hours after transfer. Conceivably this could be due to a reduction in the rate of entry of glucose into the glycolytic pathway that is glucose uptake and/or its Initial phosphorylation and evidence from steady state salt growth experiments support this claim. A lowered sugar uptake in S. cerevisiae has been reported in medium containing NaCl when yeast was grown in gelatin (Wei et al. 1982) and Lindman (1981) demonstrated an intrinsically halotolerant sugar transport system in D. hansenii but this is not characteristic of S. cerevisiae. It remains to be determined whether the low utilization of glucose is due to the direct effect of the NaCl on the carrier mediated facilitated diffusion or on the activity of the three hexokinases known to be involved in glucose transport in S. cerevisiae (Lang and Cirillo 1987). It is also possible, of course, that glucose entry into glycolysis is reduced indirectly by NaCl. For instance AMP is known to be a competitive inhibitor of hexokinase with respect to ATP (Fromm 1969; Rudolph and Fromm 1971) so an increase in the AMP/ATP ratio would consequently lower the glucose uptake into the cells.
CHAPTER THREE:

PHYSICOCHEMICAL FACTORS INFLUENCING VIABILITY
ON LOW WATER ACTIVITY AGAR DURING A GROWTH CYCLE OF

*SACCHAROMYCES CEREVISIAE*
3.1 INTRODUCTION

In describing the processes of response and adaptation to a reduced water activity, Edgley and Brown (1983) encountered major physiological differences between the two species of yeast, xerotolerant *Saccharomyces rouxii* and nontolerant *S. cerevisiae*. The adaptation of *S. rouxii* to a growth medium containing NaCl (10% w/v) was simple whereas the response of *S. cerevisiae* was extremely complex. Edgley and Brown (1983) described two phases in the adaptation process. *S. cerevisiae* showed a catastrophic drop in apparent viability during the first phase which lasted for about 40 hours. After the adaptation process entered stage 2, there was a gradual recovery of viability to the levels before transfer over the next 140 hours. Other features of the adaptation response were synthesis of glycerol immediately after transfer to a salt broth and an increase in glycerol-3-phosphate dehydrogenase activity from the beginning of stage 2.

In an attempt to clarify the viability changes during the adaptation sequence, Mackenzie et al. (1986) compared plate counts on three plating media namely malt extract (MA; a normal high water activity medium, aw 0.997), salt agar (a nutrient medium containing 10% NaCl (w/v), aw 0.936) and synthetic honey agar (SHA; a nutrient medium containing 48% glucose (w/v), aw 0.924) during a normal growth cycle of *S. cerevisiae*. *Saccharomyces cerevisiae* produced a major discrepancy between counts on MA and the other two media in the exponential growth phase (salt agar and SHA). This discrepancy was at least of the order of $10^{-4}$ which means that during the critical period of the growth cycle, only one cell in $10^4$ can form a colony under conditions of the low water activity. Cells become resistant as they enter stationary phase. Once well into stationary phase there is no difference in counts on MA and SHA. Mackenzie et al. (1986) have called this phenomenon "water stress plating hypersensitivity".

In this chapter some physiological and biochemical explanations of sensitivity of *S. cerevisiae* are advanced. These experiments were done in a chemically defined
Wickerham's synthetic medium (Barnett et al. 1983) in contrast to a complex basal yeast medium used by Mackenzie et al. (1986).

3.2 MATERIALS AND METHODS

3.2.1 Yeast strains

Saccharomyces cerevisiae (strain Y41, ATCC 38531) was maintained on malt extract agar (MA; $a_w$ 0.997) as described in chapter 2, section 2.2.1. Strain 212-244-1A (genotype glc 1) was obtained from the Yeast Genetic Stock Center, University of California, Berkeley, USA. This strain does not accumulate each glycogen or trehalose.

3.2.2 Growth conditions

Experimental liquid batch cultures were grown at 30°C with rotary agitation at 200 rpm in Wickerham's synthetic medium (Barnett et al. 1983, 0.997 $a_w$). This medium was sterilized and stored as described earlier (chapter 2, section 2.2.2).

Heat shock experiments were performed by exposing cells to 40°C for 60 minutes. Since a 25 ml culture was needed for the biochemical analysis of trehalose and glycerol, that amount of exponentially growing cells (at 30°C normal temperature) was withdrawn and placed in a preheated (40°C) Erlenmeyer flask. This was immediately transferred to a 40°C shaking water bath for 60 minutes (heat shock). When a smaller size of culture (5 ml) was similarly heat shocked, essentially the same results were obtained as those with 25 ml culture. This implied that within the experimental limits, time to thermal equilibrium was not critical.

3.2.3 Inoculation procedure

A preinoculum culture was prepared by transferring a loopful of yeast from a slope to a 100 ml Erlenmeyer flask containing 50 ml of the growth medium. The culture was
grown to the stationary growth phase (48 hours). The inoculum was seeded with preinoculum culture in a proportion of 5% (v/v). The inoculum culture had the same medium composition as the preinoculum. A ratio of 2:1 was maintained between the size of flask and volume of culture medium. The inoculum culture was grown to stationary growth phase (48 hours). The experimental culture was inoculated from this culture.

3.2.4 Counting
Sample preparation for counting and procedures used for total and viable counts were essentially the same as described in chapter 2, section 2.2.4 except that a preheated (40°C) plunger was used for the heat shock experiment.

3.2.5 Analytical methods
Cell sampling, dry mass, cell extraction, ethanol, glucose, glycerol and trehalose estimation procedures used are described in chapter 2, section 2.2.5.

3.2.6 Analytical error
Estimated analytical error was not more than 20%.
3.3 RESULTS

3.3.1 Viability changes during a growth cycle of *Saccharomyces cerevisiae* at 30° C

During a growth cycle of *Saccharomyces cerevisiae* in Wickerham's synthetic medium its response to a sudden water stress was monitored by plating yeast onto SHA. Total microscopic counts and viability on MA were also measured. Figure 3.1 shows that during the exponential growth phase, the ability of yeast to form colonies was drastically reduced on SHA. Counts on MA, a non-stressing agar showed no such reduction in viability but had a similar pattern to that of total counts.

The plating discrepancy (log<sub>10</sub> MA count-log<sub>10</sub> SHA count) was at a maximum 5 hours after the culture was inoculated. At this stage 1 cfu in 10<sup>4</sup> was viable on SHA. Maximum water stress plating hypersensitivity coincided with the highest percentage of budded cfu (fig. 3.1).

The difference between these results in Wickerham's medium and those reported by Mackenzie et al. (1986) for *S. cerevisiae* cultures in basal yeast medium was the slower growth rate (0.45 generation/hour) and prolonged hypersensitive period observed in Wickerham's medium.

3.3.2 Biochemical changes during a growth cycle of *Saccharomyces cerevisiae* at 30° C

Figure 3.2 shows glucose consumption by the yeast throughout the growth cycle. The glucose supply was effectively exhausted after 5 hours and for the remainder of the incubation period the yeast consumed ethanol and glycerol from the medium (fig. 3.3).

Intracellular glycerol content peaked very early in the growth cycle (fig. 3.4), followed by a gradual decline to a low value. This decline in glycerol content correlated
approximately with the decline in viability on SHA for about the first 5 hours (fig. 3.1). After 5 hours, when the resistance to plating onto SHA began there was no correlation between the SHA viability and glycerol content.

Increase in trehalose content in response to lowered water activity has been reported for *Candida tropicalis* growing in a pH stat (Furyaeva et al. 1985). Thevelein (1984b) extensively reviewed the conditions leading to trehalose accumulation, degradation and also its regulation in yeast and fungi. Measurement of trehalose during the growth cycle (fig. 3.5) revealed a positive correlation between trehalose content and changes in the SHA plate count (fig. 3.1). When plating discrepancy was plotted against the trehalose content the relation between these two parameters was shown to be curvilinear (fig. 3.6).

3.3.3 Viability and biochemical changes after heat shock of exponential growing *Saccharomyces cerevisiae*

In virtually all organisms, resistance to thermal killing is a cellular property that is acquired by exposure to a nonlethal heat shock or other agents that induce the heat shock response (Schlesinger et al.1982a, 1982b). It was investigated how a nonlethal heat shock affected water stress plating hypersensitivity in *S.cerevisiae*. This was achieved by exposing yeast to a 60 minute 40°C heat shock. Heat shock treatment to exponentially growing cells of the Y41 strains and glc1 strain resulted in about 100 fold and 10 fold improvement in SHA viability, respectively (figs. 3.7 and 3.8). There were no significant changes in the levels of glycerol (figs. 3.9 and 3.11) or trehalose (figs. 3.10 and 3.12) in either strain.

Resistance to thermal killing or ionizing radiation upon heat shock treatment of *S. cerevisiae* is largely due to the production of heat shock proteins and the shutdown of normal protein synthesis (McAlister and Finkelstein 1980). Heat shock protein production upon exposure to a high temperature can be inhibited in *S. cerevisiae* if
cycloheximide is used in the medium (McAllister and Finkelstein 1980). An experiment was done to test whether the improved tolerance of a water stress after exposure to 40°C was dependent on protein synthesis. When 100 μg/ml of cycloheximide was used in the medium during heat shock, there was no improvement in yeast viability (fig. 3.13) on SHA. The glycerol and trehalose content in the cycloheximide heat shock experiment (figs. 3.14 and 3.15) was not significantly different from that obtained in the absence of cycloheximide.

3.3.4 Viability and biochemical changes during a growth cycle of *Saccharomyces cerevisiae* at 40°C

Viability changes throughout the growth cycle of *S. cerevisiae* grown at 40°C is illustrated in figure 3.16. There was about a 10 fold improvement in resistance to plating onto SHA during the exponential growth phase when compared to the 30°C viability values. In general, levels of intracellular glycerol were low (fig. 3.17) whereas trehalose content was high in the beginning of incubation at 40°C (fig. 3.18) in comparison to the corresponding values in the 30°C growth cycle (figs. 3.4 and 3.5).
FIGURE 3.1 Changes in viability during the growth cycle of

Saccharomyces cerevisiae in Wickerham's medium.

Top panel proportion of budded c.f.u.

Bottom panel

- Total counts (c.f.u./ml)
- ▲ MA counts (c.f.u./ml)
- ■ SHA counts (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts). This experiment was collaborated with Ms Kylie Mackenzie.
Logio (count/ml) ' Budded c.f.u. (%)

Incubation time (h)
FIGURE 3.2 Glucose consumption during the growth cycle of *Saccharomyces cerevisiae* shown in figure 3.1.

The points represent the means of two experiments.

FIGURE 3.3 Glycerol and ethanol content of the medium during the growth cycle of *Saccharomyces cerevisiae* shown in figure 3.1.

- Glycerol
- Ethanol

The points represent the means of two experiments.
Glucose concentration (mM)
FIGURE 3.4 Intracellular glycerol content during the growth cycle of
Saccharomyces cerevisiae shown in figure 3.1.

The points represent the means of two experiments.

FIGURE 3.5 Intracellular trehalose content during the growth cycle of
Saccharomyces cerevisiae shown in figure 3.1.

The points represent the means of two experiments.
Trehalose content
(μ mole hexose/mg dry yeast)

Incubation time (h)

Incubation time (h)
Glycerol content (μmol/mg dry yeast)
FIGURE 3.6  The relation between plating discrepancy and intracellular trehalose content during the growth cycle of Saccharomyces cerevisiae shown in figure 3.1.
Plating discrepancy (log_{10} MA counts - log_{10} SHA counts)

Trehalose content (μ mole hexose/mg dry yeast)
FIGURE 3.7 Changes in viability after a heat shock during the growth cycle of *Saccharomyces cerevisiae* in Wickerham’s medium.

Top panel proportion of budded c.f.u.

Bottom panel

- Total counts (c.f.u./ml)
- MA counts (c.f.u./ml)
- SHA counts (c.f.u./ml)
- SHA counts (c.f.u./ml) after heat shock.

The points represent the means of two experiments (means of log values for total and viable counts). As the MA and total counts were identical in both control and heat shock samples, the values are incorporated within the same symbol.

The culture was grown at 30° C. Samples were transferred to 40° C at 3 and 5 hours for 60 minutes (heat shock). They were counted at two separate occasions during the heat shock period.
FIGURE 3.8 Changes in viability after a heat shock during the growth cycle of *Saccharomyces cerevisiae* strain 212-244-1A (*gIC 1*) in Wickerham's medium.

Top panel proportion of budded c.f.u.

Bottom panel

- Total counts (c.f.u./ml)
- ▲ MA counts (c.f.u./ml)
- ■ SHA counts (c.f.u./ml)
- □ SHA counts (c.f.u./ml) after heat shock.

The experimental conditions were same as for figure 3.7.
FIGURE 3.9 The effect of heat shock on the intracellular glycerol content of *Saccharomyces cerevisiae* during the growth cycle in Wickerham's medium.

○ Control

□ Heat shock

The points represent the means of two experiments.

FIGURE 3.10 The effect of heat shock on the intracellular trehalose content of *Saccharomyces cerevisiae* during the growth cycle in Wickerham's medium.

○ Control

□ Heat shock

The points represent the means of two experiments.

These results refer to the experiment illustrated in figure 3.7.
Trehalose content
(μ mole hexose/mg dry yeast)
Intracellular glycerol
(μ mole /mg dry yeast)
FIGURE 3.11 The effect of heat shock on the intracellular glycerol content *Saccharomyces cerevisiae* strain 212-244-1A (*glc1*) during the growth cycle in Wickerham's medium.

- Control
- Heat shock

The points represent the means of two experiments.

FIGURE 3.12 The effect of heat shock on the intracellular trehalose content *Saccharomyces cerevisiae* strain 212-244-1A (*glc1*) during the growth cycle in Wickerham's medium.

- Control
- Heat shock

The points represent the means of two experiments.

These results refer to the experiment illustrated in figure 3.8.
Trehalose content

(μ mole hexose/mg dry yeast)
Intracellular glycerol
(μ mole /mg dry yeast)
FIGURE 3.13 Changes in viability after a heat shock during the growth cycle of *Saccharomyces cerevisiae* in Wickerham's medium containing cycloheximide.

Top panel

Bottom panel

- Total counts (c.f.u./ml)
- ▲ MA counts (c.f.u./ml)
- ■ SHA counts (c.f.u./ml)
- □ SHA counts (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts). As the MA and total counts were identical in both control and heat shock samples, the values are incorporated within the same symbol.

The culture was grown at 30° C. Samples were transferred to 40° C at 3 and 5 hours for 60 minutes (heat shock). Cycloheximide was added to a final concentration of 100 µg/ml at the start of heat shock treatment. Samples were counted at two different occasions during the heat shock period.
FIGURE 3.14 The effect of a heat shock on the intracellular glycerol content of *Saccharomyces cerevisiae* during the growth cycle in Wickerham's medium containing cycloheximide.

- Control
- Heat shock

The points represent the means of two experiments.

FIGURE 3.15 The effect of a heat shock on the intracellular trehalose content of *Saccharomyces cerevisiae* during the growth cycle in Wickerham's medium containing cycloheximide.

- Control
- Heat shock

The points represent the means of two experiments.

The results refer to the experiment illustrated in figure 3.13.
Incubation time (h)

Trehalose content (μ mole hexose/mg dry yeast)
Intracellular glycerol
(μ mole /mg dry yeast)
FIGURE 3.16 Changes in viability during the Growth cycle of *Saccharomyces cerevisiae* at 40° C in Wickerham's medium.

Top panel proportion of budded c.f.u.

Bottom panel
- Total counts (c.f.u./ml)
- MA counts (c.f.u./ml)
- SHA counts (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts).

See figure 3.1 for a comparison at 30° C.
FIGURE 3.17 Intracellular glycerol content of *Saccharomyces cerevisiae* during the growth cycle in Wickerham's medium at 40° C.

The points represent the means of two experiments.

See figure 3.4 for a comparison at 30° C.

FIGURE 3.18 Intracellular trehalose content of *Saccharomyces cerevisiae* during the growth cycle in Wickerham's at 40° C.

The points represent the means of two experiments.

See figure 3.5 for a comparison at 30° C.
Trehalose content

($\mu$ mole hexose/mg dry yeast)
Intracellular glycerol
(μ mole /mg dry yeast)

Incubation time (h)
**TABLE 3.1**

Protection conferred by metabolites against plating mid exponential phase cells of *Saccharomyces cerevisiae* to low water activity agar

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>Plating discrepancy (log$<em>{10}$ MA-log$</em>{10}$ SHA)</th>
<th>Metabolite content $\mu$ mole/mg dry yeast</th>
<th>Glycerol</th>
<th>Trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.5</td>
<td>0.12</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.8</td>
<td>0.02</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

Mackenzie et al. (1986) have reported that the complete protection against plating mid exponential phase yeast on to low water activity agar is conferred by glycerol at a content of about 2 $\mu$ mole /mg dry yeast.
3.4 DISCUSSION

3.4.1 The water stress plating hypersensitivity

*Saccharomyces cerevisiae* gave a relation between plate count and incubation time in which a major difference in counts on MA and SHA medium developed during the exponential growth phase. At the time of greatest plating discrepancy, counts on SHA were $10^{-4}$ of the corresponding counts on MA (fig. 3.1).

The period of water stress plating hypersensitivity was consistently shown to occur while the cells were budded (fig. 3.1). However, Mackenzie et al. (1986) found that when *S. cerevisiae* was grown in continuous culture, the plating discrepancy ($\log_{10} MA - \log_{10} SHA$) was about 1 for dilution rate in the range of 0.02-0.05 h$^{-1}$ and about 3 in the range of 0.01-0.13 h$^{-1}$. The result presented in figure 3.1 also shows that relation to budding is very weak. As a result of the inoculation procedure used in these experiments (see section 3.2.3), cell division in the experimental culture is likely to be synchronized. But to assign a role to a specific event in cell division to plating onto SHA, the culture has to be synchronized to such a degree that all but 1 cfu in 10$^4$ cells are at the same point in the cell division cycle at the time of maximum plating discrepancy. This is clearly not the case when compared to the proportion of unbudded cfu (fig. 3.1).

When the culture was inoculated, about two thirds of the population was in G1 phase. Therefore at the most two thirds of the population were dividing synchronously at the time of maximum plating discrepancy. Hence water stress plating hypersensitivity did not occur because of the sensitivity of a particular event in the cell cycle.

The ability of some cells to produce colonies on SHA during the period of hypersensitivity could not be attributed to genetic heterogeneity of the population. This is because cultures grown from single colonies on SHA plates taken at the time of maximum plating discrepancy were hypersensitive when plated onto low water activity agar during the exponential growth phase (Mackenzie et al. 1986).
The results do not distinguish between a reversible inhibition of growth or an irreversible process leading to death as the cause of plating hypersensitivity. This question was resolved by an experiment (Mackenzie et al. 1986) in which yeast exposed to low water activity were subsequently exposed to conditions that would release the cells from a dormant state (that is incubation at high water activities). They found that cells, not visible as colonies on SHA, were dead.

3.4.2 Role of trehalose in the water stress plating hypersensitivity

The reason why *Saccharomyces cerevisiae* displayed plating hypersensitivity when growing exponentially but recovered during the stationary phase is evident from figure 3.5. Changes in trehalose content approximately paralleled changes in the SHA plate count. When plating discrepancy was plotted against trehalose content during growth cycle, it gave a clear correlation between the magnitude of the plating discrepancy and trehalose content (fig. 3.7).

Another type of evidence for a protective role of trehalose in resistance to plating on SHA is provided by the 212-244-1A strain. This strain carries the glc1 mutation, a regulatory pleotropic mutation that diminishes the accumulation of both trehalose and glycogen (Pringle 1974). When heat shocked at 400°C for 30 and 60 minutes, there is only about 10 fold increase in viability on SHA (fig. 3.8) during the exponential phase as compared to the Y41 strain which showed at least a 100 fold improvement. This difference in recovery is at least partly attributable to the lower levels of trehalose in strain 212-244-1A (fig. 3.7). The role of trehalose is further supported by the observation of Mackenzie, Singh and Brown (submitted). It is reported that strain 212-244-1A does not recover from its hypersensitive state upon entering the stationary phase.
As shown in figure 3.1, in order to survive the stress of plating onto SHA, a yeast cfu should not be growing. In other words, from figure 3.5, a trehalose content of 0.2 \( \mu \text{mole/mg} \) dry yeast is probably sufficient to protect stationary phase yeast completely against the stress. Trehalose is more effective per mole than glycerol in protecting the cellular functions of \( S. \text{cerevisiae} \) against a sudden water stress (table 3.1). Water stress plating hypersensitivity is a binary phenomenon and the viability measurements divided the population into sensitive and resistant groups to the imposed water stress. Hence the values presented in table 3.1 are averaged measurements of the bulk population. No conclusion can be drawn about the distribution of trehalose in the portion of the population that is sensitive to the water stress. However, it is reasonable to say that those cfu which do not have the trehalose content equal to the critical value (approximately 0.2 \( \mu \text{moles/mg} \)) do not tolerate stress. With regards to glycerol values, Edgley and Brown (1983) reported that essentially the entire population, dead or alive produces glycerol when yeast was adapting to 10% NaCl medium.

An appropriate level of trehalose seems to be indispensable if \( S. \text{cerevisiae} \) is to survive a sudden water stress and it is a better compatible solute than glycerol in this situation. However, the mechanism by which trehalose improves the resistance of \( S. \text{cerevisiae} \) to a water stress is unknown. Trehalose has been reported to be an osmoregulatory solute in microorganisms such as \( E. \text{schierichia coli} \) and many freshwater and brackish cyanobacteria (Mackay et al. 1984; Larsen et al. 1987). It is clear that this disaccharide contributes to the overall osmotic status of yeast (this thesis, chapter 2; Edgley 1980). In stabilizing the structure of both isolated lipoprotein membranes and lipid monolayers trehalose has been found to be the most effective compound out of eleven polyhydroxy alcohols tested with glycerol being the least effective (Crowe et al. 1984a and 1984b). Thus \( S. \text{cerevisiae} \) is primarily protected at the plasma membrane level by trehalose with its protective effects perhaps on processes like ion transport. Glycerol seems to protect enzyme activity (Brown 1978) and is also known to stimulate
transcription in both prokaryotic (Nakanishi et al. 1974) and eukaryotic systems (Buss and Stalter 1978).

3.4.3 Adaptive processes

Microorganisms generally are more resistant to chemical and physical stresses when resting than when multiplying (Epifanora 1977). The exposure of cells from a wide variety of species including yeast to an increase in temperature results in the enhanced synthesis of several specific proteins. These have been referred to as heat shock proteins (hsp). This phenomenon has been called the heat shock response (Schlesinger et al. 1982a and 1982b). Because recovery from anoxia, ethanol, inhibitor of oxidative phosphorylation and a number of other chemicals have also been shown to induce the synthesis of similar proteins, perhaps it would be more appropriate to refer to this response in general as a 'stress response'.

McAlister and Finkelstein (1980) reported that a rapid shift in the cultivation temperature of *S. cerevisiae* from 23 to 36° C results in protection from death that normally occurs after exposure to extreme heat (52° C). The level of this acquired thermal resistance shows an excellent correlation with the cellular level of the hsp which are transiently induced by such a temperature shift. A similar heat treatment prior to exposure to a water stress also provided resistance to a water stress (fig. 3.7). Conceivably synthesis of hsp may be involved in this response specially in the light of reversal produced by cycloheximide (fig. 3.13) where there was no significant improvement in resistance to a water stress when the yeast was unable to synthesize proteins. The levels of intracellular glycerol and trehalose in these experiments did not alter significantly, thus their contribution to the improved tolerance of SHA after heat shock was probably minimal.
These results provide some evidence that the osmotic response of *S. cerevisiae* has elements in common with the heat shock response. Though it is clear that a water stress can influence gene expression significantly (Bewley and Oliver 1983) "water stress protein(s)" in yeast has not been demonstrated. While not documented in yeast a water stress, however, has been shown to induce a subset of the hsp in archaebacterium - *Halobacterium volcanii* (Daniels et al. 1984) and in *Escherichia coli* (Sherman 1987). Likewise in plants two of the hsp can be induced by a water stress, hsp 70 in maize (Heikkila et al. 1984) and hsp 27 in soyabean (Czarnecka et al. 1984). Iida and Yahara (1984a, 1984b) observed that *S. cerevisiae* synthesizes six of the hsp when it enters the resting state called G0. Since then Boucherie (1985) has shown that five hsp are synthesized by stationary phase yeast after glucose is exhausted from the medium. Stationary phase resistance to water stress plating hypersensitivity may perhaps be due to the presence of these hsp prior to exposure to a water stress.

Resistance to a water stress started after glucose was exhausted from the medium, the time when it is known that hsp synthesis begins. A regulation of hsp by glucose metabolism has been reported by Lanks (1983). Besides the role of trehalose, it is of some interest that a regulatory role of glucose in maintaining the hsp level might be involved in stationary phase resistance to a water stress. For example in yeast, constitutive levels of hsp synthesis are different, depending on whether the cells are grown on a fermentable or nonfermentable substrate (Kurtz et al. 1982). Interestingly hsp purified from murine cells are also known to be regulated by glucose (Kasambalides and Lanks 1979).

As is evident from a comparison of SHA viability (figs. 3.7 and 3.16), heat shock at 40°C for a brief period confers better resistance to a water stress than growing the yeast at 40°C. When grown at 40°C through a growth cycle there was about 10 fold improvement in the magnitude of the plating discrepancy in comparison to a 100 fold improvement in the heat shock experiment. Though there is no experimental evidence provided in this
thesis it is reasonable to assume that the smaller plating discrepancy in the 40°C growth cycle experiment may probably be due to low levels of hsp than the levels synthesized upon heat shock. Protection against the water stress plating hypersensitivity by giving *S. cerevisiae* a heat shock is an example of cross resistance. Such a cross resistance has been reported previously in yeast (Plesset et al. 1982).

A insight into the possible role of hsp in water stress may come from an experiment testing the water stress plating hypersensitivity in the hsr1 mutant of *S. cerevisiae* (Lida and Yahora 1984b). This mutant is resistant to a heat shock because it constitutively synthesizes hsp during exponential growth. If one or more "water stress proteins" are also hsp then a reduction in the exponential phase plating discrepancy would be expected in relation to that of the parent strain.
CHAPTER FOUR:

AN EFFECT OF FATTY ACID SUPPLEMENTATION DURING ANAEROBIC GROWTH OF SACCHAROMYCES CEREVISIAE ON THE GLYCEROL RETENTION AND WATER STRESS TOLERANCE
4.1 INTRODUCTION

In response to a change in water activity *Halobacterium* and presumably *Halococcus* alter their potassium content by what is sometimes called the "pump and leak" mechanism in which there is an active uptake or a passive efflux of the relevant solute (Brown 1978a). An analogous situation involving a metabolite is encountered in the two yeasts *Saccharomyces rouxii*, a xerotolerant and *S. cerevisiae* a nontolerant yeast. In spite of the fact that both species osmoregulate with glycerol, which also functions as a compatible solute at extremely low water activities, there are important physiological differences in their osmoregulatory mechanism. *Saccharomyces rouxii* regulates glycerol content in response to a reduced water activity by synthesizing an approximately constant amount of glycerol but controls the amount excreted into the medium. *Saccharomyces cerevisiae* responds to a water stress by varying its glycerol content. It achieves this by controlling glycerol synthesis, while glycerol efflux is apparently not controlled (Edgley and Brown 1983). The salt tolerant yeast *Debaryomyces hansenii* also osmoregulates with glycerol but by both synthesizing more glycerol and retaining a greater proportion of it in response to reduced water activity (Gustafsson and Norkrans 1976; Gustafsson 1979).

In investigating some of the fundamental differences associated with the osmoregulatory mechanism used by *S. cerevisiae* and *S. rouxii*, Brown and Edgley (1980) found that there is a major difference in the fatty acid composition of the two yeasts when grown in basal yeast medium (aw approximately 0.997). *Saccharomyces rouxii* is rich in linoleic acid (18:2) whereas none could be detected in *S. cerevisiae*. It has been observed that *S. cerevisiae* requires an unsaturated fatty acid and ergosterol when growing anaerobically (Andreasen and Stier 1953, 1954). Alterthum and Rose (1973), Hossack and Rose (1976) and Thomas et al. (1978) have discovered that this yeast incorporates fatty acids and sterol constituents largely unchanged under anaerobic conditions. The method described by Alterthum and Rose (1973) for incorporation of fatty acids and sterols into the plasma membrane of *S. cerevisiae* was utilized in this study to test whether plasma
membrane composition influences the glycerol retention of *S. cerevisiae* and, subsequently its ability to adapt to low water activities. It was of particular interest to know if the fatty acid composition of the plasma membrane resembled more closely to that of *S. rouxii*, namely rich in linoleic acid, whether glycerol retention and growth at low water activities would be improved in *S. cerevisiae*.

### 4.2 MATERIALS AND METHODS

#### 4.2.1 Yeast strain

*Saccharomyces cerevisiae* strain Y41 (ATCC 38531) was used throughout the study. This strain was maintained and subcultured as described previously (section 2.2.1).

#### 4.2.2 Inoculation procedure

An inoculum culture was prepared by inoculating 100 ml of growth medium (described below) in a 250 ml Erlenmeyer flask with yeast from a slope culture. This culture was incubated statically overnight at 30° C, after which time the density was about 1.0 mg dry wt / ml. The experimental culture, contained in the fermenter, was inoculated from the inoculum culture to the level of 5% (v/v).

#### 4.2.3 Growth conditions

The yeast was grown anaerobically in a medium originally described by Andreasen and Stier (1954) but later modified by Calderbank et al. (1984). The medium contained per liter: glucose 50g; (NH₄)₂SO₄ 3g; KH₂PO₄ 4.5g; yeast extract 1.0g; MgSO₄. 7H₂O 250mg; CaCl₂. 2H₂O 250mg; pH 4.5 and was sterilized by autoclaving at 110° C for 10 minutes. To each liter of medium anaerobically-induced essential nutrients were added, namely 5mg of sterol and 30mg of a fatty acid. A 0.2% w/v solution of sterol and 1% w/v solution of the fatty acids were prepared in 95% ethanol and prior to being added to the medium these solutions were filter sterilized.
The medium for experimental cultures was sterilised in a one liter (final volume) assembled fermenter (LH series 111, LH Fermentation, Stoke poges, Bucks England) without sterol and/or fatty acid. Sterile solutions of the sterol and fatty acid were added after autoclaving. Incoming air was passed through two membrane filters of 0.2μm pore size connected in series. Agitation was achieved by means of flat bladed impellers operating at 700 rpm. Anaerobic conditions were achieved (as determined by oxygen electrode readings) by purging the culture for two hours before inoculation and throughout the fermentation with oxygen free grade nitrogen (Commonwealth Industrial Gases) at the rate of 100ml/min. Samples of the culture were removed from the vessel via an air lock device into a sterile screw top bottle.

4.2.4 Counting
Sample preparation for counting and methods for total and viable counts are described in section 2.2.4.

4.2.5 Analytical methods
Cell sampling, dry mass, cell extraction, glycerol and trehalose estimation procedures were essentially the same as those described in section 2.2.5.

4.2.6 Analytical errors
Estimated maximum analytical error was within the limits of 20%.
4.3 RESULTS

4.3.1 Glycerol retention by *Saccharomyces cerevisiae*

Fig 4.1 shows the amounts of glycerol produced intracellularly and leaked into the medium without any supplementation of fatty acid or sterol (control). It is clear that the anaerobic production of glycerol by *Saccharomyces cerevisiae* was higher than that produced during aerobic conditions (see figs 3.3 and 3.4), showing thereby that anaerobic conditions favoured glycerol formation. An intracellular/extracellular quotient (retention factor) was calculated from this figure as a measure of the distribution of glycerol (table 4.1).

Evidently the growth phase influenced glycerol retention. Supplementation of the growth medium with linoleic acid enhanced the retention of glycerol by about two fold (fig. 4.2, table 4.1). However, no significant improvement in the retention ability of glycerol by *S. cerevisiae* was shown when the growth medium was supplemented with either ergosterol, linolenic or oleic acid (figs. 4.2, 4.5, 4.6, respectively and table 4.1). A similar retention of glycerol was achieved when linoleic acid and ergosterol were supplied together (fig 4.4 and table 4.1) as achieved when only linoleic acid was supplied to the yeast.

When both the inoculum and experimental culture were supplied with linoleic acid in Wickerham's medium, even better retention of glycerol was possible. At its maximum, glycerol retention was about seven times greater than the control as compared to two times more than the control when linoleic acid was not supplied in the inoculum (compare tables 4.1 and 4.2).

4.3.2 Response of *Saccharomyces cerevisiae* to water stress

When growing exponentially under anaerobic conditions, *Saccharomyces cerevisiae* was hypersensitive to being plated on SHA (fig. 4.7). The maximum plating discrepancy
(log$_{10}$ MA - log$_{10}$ SHA) under this condition was the same order of magnitude as documented for aerobic growth in Wickerham's medium (see chapter 3). When supplemented with linoleic acid (fig. 4.8), ergosterol (fig. 4.9), linoleic acid together with ergosterol (fig. 4.10), linolenic acid (fig. 4.11) or oleic acid (fig. 4.12), S. cerevisiae still displayed water stress plating hypersensitivity. The maximum plating discrepancies in all these growth cycles were within the range of 2.8 to 3.5.

4.3.3 Trehalose content of Saccharomyces cerevisiae

In chapter 3 the relation between water stress plating hypersensitivity and trehalose content was demonstrated. Likewise, when growing anaerobically in the presence (figs. 4.14, 4.15, 4.16, 4.17, and 4.18) or absence (fig. 4.13) of fatty acid and ergosterol, trehalose content correlated with the SHA viability. The trehalose content at the end of the anaerobic incubations was higher, however, than that found when Saccharomyces cervisiae was grown aerobically (see fig. 3.5).
Table 4.1

Glycerol retention* of *Saccharomyces cerevisiae* under anaerobic conditions in the presence and absence of fatty acids and ergosterol

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control</th>
<th>Linoleic acid</th>
<th>Ergosterol</th>
<th>Linoleic acid and ergosterol</th>
<th>Linolenic acid</th>
<th>Oleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.127</td>
<td>0.117</td>
<td>0.108</td>
<td>0.184</td>
<td>0.155</td>
<td>0.128</td>
</tr>
<tr>
<td>4</td>
<td>0.081</td>
<td>0.220</td>
<td>0.043</td>
<td>0.273</td>
<td>0.044</td>
<td>0.040</td>
</tr>
<tr>
<td>6</td>
<td>0.045</td>
<td>0.082</td>
<td>0.046</td>
<td>0.124</td>
<td>0.025</td>
<td>0.037</td>
</tr>
<tr>
<td>8</td>
<td>0.048</td>
<td>0.103</td>
<td>0.030</td>
<td>0.114</td>
<td>0.033</td>
<td>0.030</td>
</tr>
<tr>
<td>10</td>
<td>0.036</td>
<td>0.095</td>
<td>0.037</td>
<td>0.076</td>
<td>0.022</td>
<td>0.044</td>
</tr>
<tr>
<td>12</td>
<td>0.027</td>
<td>0.102</td>
<td>0.036</td>
<td>0.023</td>
<td>0.039</td>
<td>0.032</td>
</tr>
</tbody>
</table>

*Expressed as a 'retention factor' i.e. the ratio of intracellular glycerol content to extracellular glycerol yield (as μmole/mg dry yeast)
Table 4.2

Glycerol retention* of *Saccharomyces cervisiae* in Wickerham's medium under anaerobic conditions with linoleic acid present in both inoculum and experimental cultures.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control</th>
<th>Linoleic acid</th>
<th>Linoleic acid/ control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.894</td>
<td>0.804</td>
<td>0.90</td>
</tr>
<tr>
<td>3</td>
<td>0.048</td>
<td>0.369</td>
<td>7.68</td>
</tr>
<tr>
<td>4</td>
<td>0.036</td>
<td>0.261</td>
<td>7.25</td>
</tr>
<tr>
<td>5</td>
<td>0.033</td>
<td>0.175</td>
<td>5.30</td>
</tr>
<tr>
<td>18</td>
<td>0.047</td>
<td>0.148</td>
<td>3.14</td>
</tr>
<tr>
<td>20</td>
<td>0.086</td>
<td>0.102</td>
<td>1.54</td>
</tr>
<tr>
<td>24</td>
<td>0.054</td>
<td>0.093</td>
<td>1.71</td>
</tr>
</tbody>
</table>

*Expressed as a 'retention factor' i.e. the ratio of intracellular glycerol content to extracellular glycerol yield (as µmole/mg dry yeast)
FIGURE 4.1 Glycerol production by *Saccharomyces cerevisiae* during anaerobic growth.

- Intracellular
- Extracellular

The points represent the means of two experiments.

This figure depicts the results for table 4.1.
Intracellular glycerol (μ mole /mg dry yeast)

Extracellular glycerol (μ mole /mg dry yeast)

Incubation time (h)
FIGURE 4.2 Glycerol production by *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with linoleic acid.

- Intracellular
- Extracellular

The points represent the means of two experiments.

This figure depicts the results for table 4.1.
Intracellular glycerol ($\mu$ mole /mg dry yeast)

Extracellular glycerol ($\mu$ mole /mg dry yeast)

Incubation time (h)
FIGURE 4.3  Glycerol production by *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with ergosterol.

- Intracellular
- Extracellular

The points represent the means of two experiments.

This figure depicts the results for table 4.1.
Extracellular glycerol
(μ mole / mg dry yeast)

Incubation time (h)

Intracellular glycerol
(μ mole / mg dry yeast)
FIGURE 4.4 Glycerol production by *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with linoleic acid and ergosterol.

- Intracellular
- Extracellular

The points represent the means of two experiments.

This figure depicts the results for table 4.1.
Intracellular glycerol (μ mole /mg dry yeast)

Extracellular glycerol (μ mole /mg dry yeast)

Incubation time (h)
FIGURE 4.5 Glycerol production by *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with linolenic acid.

- Intracellular
- Extracellular

The points represent the means of two experiments.

This figure depicts the results for table 4.1.
FIGURE 4.6 Glycerol production by *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with oleic acid.

- Intracellular
- Extracellular

The points represent the means of two experiments.

This figure depicts the results for table 4.1.
FIGURE 4.7 Anaerobic growth of *Saccharomyces cerevisiae*.

Top panel proportion of budded c.f.u.

Bottom panel

- Total counts (c.f.u./ml)
- MA counts (c.f.u./ml)
- SHA counts (c.f.u/ml)

The points represent the means of two experiments (means of log values for total and viable counts).
FIGURE 4.8 Anaerobic growth of *Saccharomyces cerevisiae* in medium supplemented with linoleic acid.

Top panel proportion of budded c.f.u.

Bottom panel

- Total counts (c.f.u./ml)
- MA counts (c.f.u./ml)
- SHA counts (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts).
FIGURE 4.9 Anaerobic growth of *Saccharomyces cerevisiae* in medium supplemented with ergosterol.

Top panel

proportion of budded c.f.u.

Bottom panel

- Total counts  (c.f.u./ml)
- MA counts  (c.f.u./ml)
- SHA counts  (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts).
FIGURE 4.10 Growth of *Saccharomyces cerevisiae* in medium supplemented with linoleic acid and ergosterol.

Top panel

proportion of budded c.f.u.

Bottom panel

- Total counts  (c.f.u./ml)
- MA counts  (c.f.u./ml)
- SHA counts  (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts).
FIGURE 4.11 Anaerobic growth of *Saccharomyces cerevisiae* in medium supplemented with linolenic acid.

Top panel

proportion of budded c.f.u.

Bottom panel

- Total counts (c.f.u./ml)
- MA counts (c.f.u./ml)
- SHA counts (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts).
FIGURE 4.12 Anaerobic growth of *Saccharomyces cerevisiae* in medium supplemented with oleic acid.

Top panel proportion of budded c.f.u.

Bottom panel
- Total counts (c.f.u./ml)
- ▲ MA counts (c.f.u./ml)
- ■ SHA counts (c.f.u./ml)

The points represent the means of two experiments (means of log values for total and viable counts).
FIGURE 4.13 Intracellular trehalose content of *Saccharomyces cerevisiae* during anaerobic growth.

The points represent the means of two experiments.

FIGURE 4.14 Intracellular trehalose content of *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with linoleic acid.

The points represent the means of two experiments.
Trehalose content
(μ mole hexose/mg dry yeast)
Trehalose content
(μ mole hexose/mg dry yeast)
FIGURE 4.15 Intracellular trehalose content of *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with ergosterol.

The points represent the means of two experiments.

FIGURE 4.16 Intracellular trehalose content of *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with linoleic acid and ergosterol.

The points represent the means of two experiments.
Incubation time (h)

Trehalose content
(μ mole hexose/mg dry yeast)
Incubation time (h)

Trehalose content
(μ mole hexose/mg dry yeast)
FIGURE 4.17 Intracellular trehalose content of *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with linolenic acid. The points represent the means of two experiments.

FIGURE 4.18 Intracellular trehalose content of *Saccharomyces cerevisiae* during anaerobic growth in medium supplemented with oleic acid. The points represent the means of two experiments.
Trehalose content
(μ mole hexose/mg dry yeast)

Incubation time (h)

0 4 8 12 24

0 0.1 0.2 0.3 0.4
Trehalose content
(μ mole hexose/mg dry yeast)

Incubation time (h)
4.4 DISCUSSION

4.4.1 Glycerol retention by *Saccharomyces cerevisiae*

Rose (1981) has suggested three methodological approaches that can be utilized for altering the membrane lipid composition. The first involves changes in membrane composition by altering environmental conditions, such as temperature. It has been shown that lowering the growth temperature of *Saccharomyces cerevisiae*, causes an increase in fatty acyl unsaturation which is accompanied by alteration in proportion of sterols synthesized (Hunter and Rose 1972). This method was not sufficiently specific to be exploited in this study.

The second and the most effective method to bring about desired alterations in the composition of membrane lipids is to use mutants of *S. cerevisiae* that are auxotrophic for lipids which are incorporated into the plasma membrane. Mutants of *S. cerevisiae* that are auxotrophic for saturated (Henry and Keith 1971) or unsaturated fatty acids (Resnick and Mortimer 1966) or for sterol (Karst and Lacroute 1974; Taylor and Parks 1980) have been isolated. The use of these mutants has been limited since they are known to be quite unstable and the requirement of the mutants for unsaturated fatty acid is mostly induced by surface active agents. These agents render the mutants of questionable value in any study of the plasma membrane (Rose 1981).

The third approach which can bring about specific alterations in the lipid composition of *S. cerevisiae* is to grow the yeast anaerobically. Anaerobic conditions induce a requirement for a sterol (Andreasen and Stier 1953) and an unsaturated fatty acid (Andreasen and Stier 1954) in *S. cerevisiae*. Since these requirements for a sterol and a fatty acid are quite broad (Light et al. 1962, Proudlock et al. 1968), this approach offers more flexibility in effecting a sufficiently specific enrichment of *S. cerevisiae*’s plasma membrane with the desired fatty acid or sterol. This flexibility has been demonstrated by Hossack and Rose (1976) and Thomas et al. (1978). When *S. cerevisiae*
is grown anaerobically in the presence of sterol and an unsaturated fatty acid the plasma membrane is enriched to the extent of about 70% of the sterol supplied in the medium (Hossack and Rose 1976) and approximately 55% of the fatty acyl residues in the plasma membrane phospholipid contains residues of unsaturated fatty acids included in the medium (Thomas et al. 1978).

The plasma membrane is the first sensitive organelle to come in contact with solutions when cells are exposed to a water stress. Linoleic acid is absent in the plasma membrane of nontolerant *S. cerevisiae* as compared to xerotolerant *S. rouxii* (Brown and Edgley 1980). Experiments were conducted to study the glycerol retention and water stress tolerance *S. cerevisiae* when grown anaerobically in media supplemented with linoleic or other fatty acids. It was found that glycerol was retained to a greater extent when the plasma membrane is known to be enriched with linoleic acid or linoleic acid and ergosterol rather than ergosterol only or linolenic or oleic acid (table 4.1). So it can be concluded that improved glycerol retention during anaerobic growth is an effect specific for linoleic acid.

Several other studies have also described the influence of linoleic acid content on cell permeability. Walker and Kummerow (1964) reported an inverse relationship between the linoleic acid content and the glycerol permeability of human erythrocytes. Kogl et al. (1960) surveyed the permeability properties of the erythrocytes of several mammalian species. It was indicated that the membrane of those least permeable to glycerol contained the highest proportion of eighteen carbon fatty acids and had the greatest degree of fatty acid unsaturation. The results of Ishikawa and Yoshizawa (1979) suggest that there is a similar relationship between the linoleic acid content of sake yeast (a strain of *S. cerevisiae*) and its permeability to a flavour ester isoamyl acetate. The linoleic acid content of the yeast was increased when grown in Wickerham's medium supplemented with fatty acids. Johnson and Brown (1972) reported that yeasts containing linoleic acid were much less permeable to a mutagen, acriflavin, than those which did not contain
linoleic acid. Interestingly yeast grown in medium containing linoleic acid has also been found to be less susceptible to sphaeroplast formation by digestion of the wall with a basidiomycete glucanase than when grown in presence of oleic acid or other fatty acids or ergosterol (Alterthum and Rose 1973). Burr and Burr (1930) described the development of a number of disease symptoms which appeared when linoleic acid was excluded from the diet of growing rats and which disappeared when linoleic acid was supplied. One of these disease symptoms, increased water consumption, was later found to be due to an abnormal increase in trans-epidermal water loss (Basnayake and Sinclair 1956).

Though multiple functions for sterol in yeast has been proposed (Rodriguez et al. 1985) ergosterol did not alter the glycerol permeability of \textit{S. cerevisiae} when supplied in the medium (Table 4.1). However, when supplied in combination with linoleic acid it appears to contribute to the effect of linoleic acid on glycerol retention.

The osmoregulatory response of \textit{S. cerevisiae} to reduced water activity involves huge increases in glycerol production but little or no change in its retention (Brown 1978). As the water activity is reduced, more and more of the yeast's metabolic efforts must be directed to glycerol production at the expense of those processes necessary for the maintenance of viability and growth. This is a wasteful and extravagant use of the yeast's resources, which makes no contribution to its energy or normal biosynthetic requirements. This is also a relatively inefficient method of raising the intracellular levels of glycerol since only a minor proportion of that produced remains in the cell. It is clear that since \textit{S. cerevisiae} can not synthesize linoleic acid (Brown and Edgley 1980), it has to adopt other means to tolerate the reduced water activity of the medium, that is synthesize more glycerol in order to survive. This limits the ability of the yeast to tolerate reduced water activity. In this respect it would be interesting to know what determines the maximum intracellular levels of glycerol which can be attained in \textit{S. rouxii}, specifically if there is a limit to the value of retention factor or whether the
proportion of glycerol retained increases until virtually all that is being produced remains in the yeast. Nevertheless the ability of this yeast to accumulate glycerol without making any additional biosynthetic demands on the cell's metabolism gives it a decisive advantage over *S. cerevisiae*.

4.4.2 Water stress plating hypersensitivity of *Saccharomyces cerevisiae*

The phenomenon of water stress plating hypersensitivity has been described in chapter 3, section 3.4.1. Kappeli (1987) has discussed the regulation of glucose metabolism in terms of the limited respiratory capacity concept. He suggests three types of glucose metabolism in *Saccharomyces cerevisiae*, fermentative, respiro-fermentative and respiratory. The absence of water stress plating hypersensitivity in *S. rouxii* (Mackenzie et al. 1986) a yeast that metabolizes glucose respiratively (Brown 1975) suggested that type of glucose metabolism could be significant in water stress tolerance. The ability to select growth conditions in which glucose is metabolized only fermentatively by *S. cerevisiae* enabled the importance of the type of glucose metabolism in the tolerance of water stress to be determined. The period of water stress plating hypersensitivity was associated with respiro-fermentative glucose metabolism (fig. 3.1). During anaerobic conditions (fig. 4.7) glucose was used fermentatively, hence a greater amount of glycerol was produced (fig. 4.1, cf 3.3 and 3.4). Anaerobically grown yeast lack both an operational tricarboxylic acid cycle and a functional respiratory chain and are deprived of molecular oxygen, essential for lipid biosynthesis. The maximum plating discrepancy \( \log_{10} MA - \log_{10} SHA \) was approximately the same whether glucose was being utilized exclusively fermentatively (fig. 4.7) or respiro-fermentatively (fig. 3.1). It is clear from this comparison of the plating discrepancy values that the operation of the respiratory metabolism of glucose does not significantly affect water stress plating hypersensitivity in *S. cerevisiae*.

The values of trehalose were found to be somewhat higher in stationary phase anaerobic cultures as opposed to aerobic culture. This may be attributed to the higher amount of
glucose supplied in the medium for anaerobic experiments, anaerobic conditions or both. In fact, higher contents of trehalose have been reported in yeast during anaerobic conditions (Wilson and Mcleod 1976).

A better retention of glycerol by S. rouxii is one of the important factors which makes this yeast xerotolerant while S. cerevisiae is nontolerant (Edgley and Brown 1983). However, when grown under anaerobic condition in presence of linoleic acid in the medium, S. cerevisiae showed water stress plating hypersensitivity in spite of the fact that it retained more glycerol intracellularly. These results provide evidence that under conditions which are known to enrich the plasma membrane of S. cerevisiae with linoleic acid with concomitant improvement in glycerol retention, shown in this thesis, is not in itself sufficient to eliminate water stress plating hypersensitivity. These results are in contrast to those of Mackenzie et al. (1986). They reported that about 2μmole/mg dry wt of glycerol achieved by incubating the yeast in 2% NaCl prior to plating is sufficient for elimination of water stress plating hypersensitivity. Approximately equal amounts of glycerol to those reported by Mackenzie et al. (1986) were found in exponential yeast growing anaerobically in the presence of linoleic acid (fig. 4.2) but the yeast still displayed plating hypersensitivity. These finding are also different from those showing improved ethanol tolerance of S. cerevisiae enriched with linoleic acid (Thomas et al. 1978).

A possible factor restricting the ability of S. cerevisiae to tolerate plating onto SHA during exponential phase even may be the ratio of lipid to protein. Dombek and Ingram (1984) suggest that in Escherichia coli a decrease in the lipid/protein ratio is essential for ethanol tolerance. In Saccharomyces sp. it has been shown that the lipid/protein ratio in ethanol-containing medium falls as the concentration of ethanol rises (Aguilera and Benitez 1986). Since proteins are barriers to ion movement (Hayshida and Ohta 1978) exposing yeast to reduced water activity may induce changes in the protein composition of the plasma membrane. In fact, it has been reported that during adaptation
presence of a protein inhibitor (Jimenez and Benitez 1987). Membrane proteins that
confer permeability to \textit{E. coli} cells has been reported (Rosenbusch 1974) and are termed
porins (Nakae 1976). These proteins are regulated by several environmental factors
such as phosphate limitation, low temperature, mutagens, low cAMP and changes in
osmolarity. It would not be surprising that similar proteins are also regulated by
environmental factors in yeast.

The ultrastructure of \textit{S. rouxii} is for the most part comparable to that of other
\textit{Saccharomyces} species (Matile et al. 1969) but a novel feature of this yeast is the
occurrence of periplasmic bodies (Arnold et al. 1974). These periplasmic bodies are
probably a manifestation of the high sugar or salt tolerance of this yeast (Onishi 1963)
and this yeast's ability to accommodate changes in water activity may be due to an unusual
plasma membrane. In support of this argument there are evidences of independent lysis
(Arnold and Garrison 1979) and variations in the size (Arnold et al. 1974) of
periplasmic bodies in osmotically fortified media. The regulation of the biosynthesis of
cyclic (1-2) \( \beta \)-D-glucan by \textit{Agrobacterium tumefaciens} is known to parallel the osmotic
regulation of membrane-derived oligosaccharide biosynthesis in \textit{E. coli} (Miller et al.
1986). These results together with the presence of periplasmic bodies in \textit{S. rouxii},
suggest a general role for periplasmic oligosaccharides in the osmotic adaptation of
microorganisms as ecologically diverse as enteric bacteria and soil bacteria and
xerotolerant yeast.
CHAPTER FIVE:

GENERAL CONCLUSION
The transition experiments highlighted a complexity of the osmoregulatory response of *Saccharomyces cerevisiae* which has not been previously recognised. Although osmoregulation in both the xerotolerant yeast *S. rouxii* and the nontolerant *S. cerevisiae* involves accumulation of the compatible solute, glycerol, the immediate response of *S. cerevisiae* was such that the glycerol accumulation in this yeast was both retarded and greatly diminished in magnitude by salt. However, glycerol does accumulate at the later stages. During the initial period of adaptation, *S. cerevisiae* both accumulated potassium, energetically the cheapest form of osmoregulator, and synthesized trehalose. Turgor recovery in *S. cerevisiae* is thus complex and apparently well coordinated involving intracellular levels of potassium, trehalose and glycerol. Since *S. cerevisiae* accumulates high amounts of potassium in response to water stress and in view of an observed inhibition of glycerol-3-phosphate dehydrogenase by potassium at its physiological concentration (Edgley 1980) this seems to be a potential inhibitory agent for glycerol formation during the initial period of adaptation. One further explanation why initial glycerol production was low may rest with the conclusion that glycolysis seemed to be severely inhibited after salt transfer. The inhibition of glycolysis by a water stress was evidenced by the low rates of ethanol production and glucose consumption measured in the adaptation period. Glucose-6-phosphate and fructose-6-phosphate were also accumulated which suggest an inhibition of phosphofructokinase activity. An accumulation of fructose 1,6 bisphosphate and phosphoenolpyruvate indicates the inhibition of aldolase and/or triosephosphate isomerase and pyruvate kinase activity. The understanding of salt compatibility of metabolic functions in yeast is limited to in vitro study of enzymes. An in vivo enzymic study of glycolysis should provide an answer further to the question concerning the mechanism of this apparent inhibition and its amelioration at later stages of adaptation. There is evidence that an elevation of substrate levels can to a considerable extent relieve salt inhibition of enzyme activities (Ahmad et al. 1979, Greenway et al. 1974)
The marked increase in the intracellular accumulation of glycerol in *S. cerevisiae* after about 7 hours in salt occurred at a much earlier point in the adaptation process of *S. rouxii*. As the osmoregulatory response is characterized in both yeasts by the accumulation of glycerol, the question is raised, when does the osmoregulatory signal act in *S. cerevisiae*, immediately or after several hours in salt? As yet a unique osmoregulatory signal has not been identified in yeast although Brown and Edgley (1980) have advocated an efflux of K+ as a possible signal. Regardless of the nature or timing of the signal it is evident that the mechanism responsible for the increased proportion of glycerol found intracellularly in *S. cerevisiae* that occurred after about 7 hours in salt needs to be further investigated.

As the salt transfer experiments demonstrated, glycerol accumulation is slow when *S. cerevisiae* is challenged by a severe water stress. This slow response may partly explain why *S.cerevisiae* is a nontolerant yeast but as the imposition of a water stress by plating on low water activity agar indicated, retardation in glycerol accumulation is not the only factor. The physiological status of the cell prior to the stress is also important. Those cells least able to survive a water stress are in an active biosynthetic mode (exponential phase) while in those cells resistant to a water stress the cellular machinery is that suited for survival and cell maintenance (stationary phase). Of the numerous physiological differences between these different cell types two factors have so far been identified as being important in governing whether the cells survive a water stress. This study has illustrated that the trehalose content is always correlated with the viability on low water activity agar. It was of some interest that a heat shock increased resistance of *S. cerevisiae* to a sudden water stress and, in the absence of a direct demonstration it is reasonable to assume that the so called 'heat shock' proteins might have contributed in the development of resistance.
Previously Brown (1978 a) proposed that the wasteful method employed by *S. cerevisiae* to control glycerol content largely explained its nontolerance. *Saccharomyces rouxii*, in contrast, controls its content efficiently, altering permeation and/or transport of glycerol in response to water activity. The putative fatty acid incorporation experiments supported a role for linoleic acid in glycerol retention by *S. cerevisiae*. The increased glycerol retention by yeast grown in the presence of linoleic acid did not, however, enhance the xerotolerance of *S. cerevisiae*. 
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PUBLICATIONS
Selected aspects of microbial osmoregulation

(Osmoregulation; compatible solutes; water stress; eukaryotes; cell physiology; homeostasis)

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1. SUMMARY

Some salient characteristics of microbial osmoregulation are reviewed, with specific examples drawn from eukaryotes. As well as the need for an osmoregulatory solute to be ‘compatible’ with cellular processes under all conditions, the importance of the physiological method of regulating the content of the solute as a factor determining xerotolerance is emphasized. The significance of turgor/volume homeostasis is discussed and examples are cited in which, during exponential growth, there is apparently no homeostatic control of the cellular content of the major osmoregulatory solute. Some implications of this for the overall mechanism of osmoregulation are considered.

A recent experiment is described which raises questions about the timing of an osmoregulatory ‘signal’ in Saccharomyces cerevisiae. Other experiments are summarized which distinguish between osmoregulatory and compatible solutes in yeast. These experiments implicate trehalose as a non-osmoregulatory compatible solute in certain circumstances.

2. INTRODUCTION

Our task in this review is to give an ‘overview’ of the phenomenon of osmoregulation in microorganisms. It is a formidable task. Studies in osmoregulation in general can be broadly divided into two distinct areas. One is biophysical, in which attention is focused on direct measurement of physical parameters such as membrane potential and turgor pressure. The second is more descriptive, and is concerned with the biochemistry and broader aspects of the cell physiology of osmoregulation. Microorganisms have generally not been amenable to very precise biophysical studies, at least partly because microbial cells for the most part are too small for relevant physical measurements to be made with a satisfactory degree of precision and accuracy. Recently, however, Walsby [1] has exploited the pressure/volume relations of gas vacuoles to measure directly turgor pressure in cyanobacteria. A more precise approach to the biophysics of microbial osmoregulation might thus be possible in the future, at least in those organisms with gas vacuoles.

Until now, however, most of the relevant work has been at the levels of biochemistry and cell physiology. Since this is also the area of our own experience, our comments will be restricted to that aspect. Moreover, because of space limitations, examples will be confined to eukaryotes although

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principles are equally applicable to prokaryotes.

Specifically, we wish to do 3 things, namely: (1) emphasize the importance of the physiological method of osmoregulation in determining microbial water relations; (2) question some basic assumptions about the physiology of osmoregulation; (3) distinguish between osmoregulatory and compatible solutes.

3. DEFINITION

In a previous review [2] we defined osmoregulation as 'the maintenance of approximately constant cell volume and turgor pressure in the face of changing water potential'. For reasons that should become apparent in due course, we now wish to use a somewhat different working definition, namely the maintenance of turgor pressure and/or cell volume within limits necessary for growth and multiplication of an organism. (Whether or not 'osmoregulation' is itself a satisfactory term is a matter on which we do not wish to comment at this stage. The questions raised by Reed [3] about terminology, however, deserve serious consideration.)

4. METHODS OF OSMOREGULATION

Under appropriate conditions, microorganisms of various kinds can thrive in environments from those that are very close to distilled water (water activity $a_w 1.00$) down to about $a_w 0.62$ and at salinities from effectively zero to saturated NaCl (6.2 molal at 30°C, $a_w 0.75$) [4]. In order to tolerate the lower levels of $a_w$, a microorganism must accumulate a compatible solute. In order to adapt to a change from one water activity to another, however, it must osmoregulate, that is it must control its volume or turgor pressure or both. It does this by regulating the content of one or more osmoregulatory solutes.

At high water activity, the concentration of an osmoregulatory solute is low enough for intermediary metabolites or the inorganic ions that commonly accumulate to fill this role without exerting any generalized inhibition or toxicity. Thus, in a dilute environment, a relatively wide spectrum of osmoregulatory solutes is encountered. As water activity or water potential is lowered, however, the environment becomes progressively more selective of osmoregulatory solutes just as it does of organisms. Under severe conditions, an osmoregulatory solute attains a high intracellular concentration (several molal) and then toxicity is a potential problem. If an organism is to thrive in this situation, an osmoregulatory solute must be 'compatible' with the entire range of essential cellular functions. At extremely low levels of water activity the only osmoregulatory solute so far identified in eukaryotic microorganisms is glycerol [5].

Accumulation of glycerol is not in itself sufficient to ensure growth at very low water activity although it does seem to be an essential prerequisite. For example, the two yeasts $S. cerevisiae$ and Saccharomyces rouxii, when grown under optimal conditions, will respectively tolerate about $a_w 0.90$ and 0.62 (each is much less tolerant of a saline environment). Both yeasts osmoregulate with glycerol as do the salt-tolerant yeast Debaryomyces hansenii and the uniquely salt-tolerant unicellular alga Dunaliella [5-7].

Brown and Borowitzka [7] have listed some of the more representative methods of controlling the content of an osmoregulatory solute of which the following three are of immediate relevance:

1. An approximately constant total yield (per cell) of osmoregulator is produced, more or less independently of water activity, but the proportion retained within the cell is controlled by permeation/transport in response to water activity (e.g., glycerol and $S. rouxii$).

2. The total yield (per cell) of osmoregulator is controlled in response to water activity and a constant proportion of it is retained within the cell (e.g., glycerol and $S. cerevisiae$).

3. The total yield (per cell) of osmoregulator is controlled in response to salinity (or water activity) and virtually all of it is retained within the cell (e.g, glycerol and Dunaliella).

Mechanisms (1) and (2) involve extensive leakage to the medium. Nevertheless, Method (1) is conservative. Method (2) is extremely wasteful
wastefulness is a sufficient reason why \textit{S. cerevisiae} is substantially less xerotolerant than \textit{S. rouxii}. Method 3 is very conservative and effective (see also [4,7]).

There are many biochemical characteristics that are obviously related to the differences in methods of glycerol production by the two exemplar yeasts. For example, when grown in 10\% (w/v) NaCl, \textit{S. cerevisiae} produces about 40 times as much glycerol-3-phosphate dehydrogenase (NAD-linked) as it does in a conventional high water activity growth medium. No such response is given by \textit{S. rouxii} [8].

In theory there should be two levels at which an organism can regulate its volume or turgor pressure. One is homeostatic, that is the maintenance of turgor or volume within the limits necessary for growth. The other is adjustment to changing water potential or water activity. Perhaps osmoregulation is most refined at the level of homeostasis, inasmuch as the other aspect, the adjustment to change, might simply involve the removal of a control—in some cases. It might even be that, in a strict sense, the homeostatic mechanism is the only valid example of osmoregulation, adaptation perhaps being essentially the removal of a control with its reposition at the end of the adaptation sequence. It might also be, of course, that in phrasing the question in this way we are merely playing with semantics.

Whether or not this suggestion is valid, however, we wish to assert that osmoregulation as a phenomenon cannot be understood until the homeostatic aspect is understood and, in turn, this cannot by understood in a vacuum. Osmoregulation with metabolites is a special case of general metabolic regulation and can ultimately be interpreted only in that light. Moreover, the situation is complicated because many organic osmoregulatory solutes function also as reserve fuels and probably all can participate in ‘other’ non-osmoregulatory metabolic sequences.

If an osmoregulatory solute is a metabolite, the ramifications of controlling its content can be quite different from one metabolic sequence to another. For example, the implications of regulating the content of glycerol produced predominantly by glycolysis (\textit{S. cerevisiae}), via the pentose phosphate cycle (\textit{S. rouxii}) or by the short sequences between dihydroxyacetone phosphate and glycerol sometimes called the ‘glycerol cycle’ (\textit{Dunaliella}) [9] are quite different in each case [8]. Moreover, whether or not there is one unique signal that triggers an osmoregulatory response, to expect a single transduction site of that signal is probably as naive as an expectation of a unique rate-limiting step in a metabolic sequence (for example, see [10]).

Let us now return to the question of homeostasis. Most microbiological experimentation is conducted with batch cultures. In batch culture, no nutrient is at a rate-limiting concentration while the population is growing exponentially but the composition of the growth medium changes progressively during a growth cycle. Nevertheless, the assumption is usually made that, while a population grows exponentially, it is in a steady state and the cells are of constant average chemical composition, biochemical activity and size (with some qualifications about size for budding yeasts). A logical extrapolation from these assumptions is that turgor and hence the concentration of the main osmoregulatory solute are also constant.

These assumptions are demonstrably false, at least in some cases. For example, the salt-tolerant yeast, \textit{D. hansenii} accumulates both arabitol and glycerol. The arabitol content responds slightly to salinity but the major osmoregulator is glycerol. Neither polyol is maintained at a constant intracellular concentration during exponential growth but the actual pattern of accumulation is dependent on the salinity of the medium [6]. In 2.7 M NaCl, for example, the glycerol content ranges between effectively zero and 3 \(\mu\)mol·mg\(^{-1}\) (approx. 2 molal) dry yeast during a growth cycle: during the actual period of exponential growth, the range is approx. 1.6–3.0 \(\mu\)mol·mg\(^{-1}\) dry yeast with a sharp peak in mid-exponential phase. On the other hand, the content of arabitol changes only slightly but progressively during exponential growth and reaches its highest level well within the stationary phase [6]. Its accumulation in no way compensates osmotically for the substantial variation in glycerol content during exponential growth. We also find that intracellular glycerol in \textit{S. cerevisiae} is far from constant during exponential
growth (Singh et al., unpublished results).

These yeasts thus are clearly not biochemically homeostatic during exponential growth. If, in spite of this, they are strictly osmotically homeostatic then their osmoregulatory mechanisms are much more complex and sophisticated than previously suspected. If, on the other hand, they do not compensate at all, then their osmoregulation is much cruder than we have believed and they tolerate substantial fluctuations in turgor pressure during a growth cycle. If the yeasts do have a capacity for strict biochemical homeostasis, it is likely to be recognizable only in continuous culture. Even here, however, there is reason for uncertainty since glycolysis is classically an oscillating system and, in addition, oscillations are common in microbial populations, including those of yeasts, in continuous culture [11].

It should not be difficult to answer all of these questions with more comprehensive analyses and there are good reasons why this should be done. For the present, however, we can only guess at what happens. Since, in the example quoted for D. hansenii, glycerol was well short of the concentration needed for osmotic balance, our guess is that the changes in glycerol content are partly compensated by changes in other solutes (perhaps ions, or trehalose).

The immediate response of S. cerevisiae to a salt stress has a more complex pattern than we had previously recognized or suspected. We reported previously [8] that glycerol accumulation begins immediately after transfer of exponential phase S. cerevisiae from a conventional broth culture to one containing NaCl (10% w/v) and continues progressively thereafter. This result was obtained over an extended period with the first samples taken at 0, 2.0 and 20.5 h after transfer. More recent experiments using a synthetic medium and a shorter time scale, however, gave a very different impression (Fig. 1). In this case the initial accumulation of glycerol that normally occurred when the yeast was transferred to a fresh medium was both retarded and greatly diminished in magnitude by salt and the ‘true’ osmoregulatory response did not begin for about 6 h. So when does the ‘signal’ act? Immediately with a delayed response or not for 6 h? As yet we do not know.

5. DISTINCTION BETWEEN OSMOREGULATORY AND COMPATIBLE SOLUTES

In the organisms discussed so far, glycerol has both of these functions. S. rouxii, however, also accumulates arabitol, which necessarily contributes to the total osmotic status of the cell but which does not respond appreciably to water stress [5,8]. A somewhat similar situation exists with D. hansenii [6]. We have always assumed that the chemical characteristics of arabitol enable it to act as a reserve or supplementary compatible solute if needed and the experiments of Edgley and Brown [8] support this assumption. Some recent experiments of a different type have clearly demonstrated the compatible or protective nature of some non-osmoregulatory solutes and their importance in sustaining viability in a sudden solute stress.

When S. cerevisiae is cultivated through a growth cycle in a conventional liquid medium and

![Fig. 1. The intracellular glycerol content of S. cerevisiae grown to mid-exponential phase and transferred (A) to fresh medium and (B) to fresh medium containing NaCl (8%, w/v). A synthetic medium with glucose (0.5% w/v) was used throughout. The unit of glycerol content is μmol·mg⁻¹ dry yeast. Note the different scales for parts A and B.](image-url)
plated out at intervals onto malt-extract agar (MA, a conventional high aw plating medium) and also onto any of several stressing (low aw) plating media, a response of the kind shown in Fig. 2 is obtained. Depending on the details of the experimental conditions, one cell in 10^3–10^6 can form colonies on the stressing agar when transferred from mid-exponential phase. The culture becomes more resistant as it ages and, when fully into stationary phase, there is little or no difference between plate counts on MA and on 'synthetic honey agar' (SHA, see Fig. 2). We have called this phenomenon 'water stress plating hypersensitivity' and it will be discussed in detail elsewhere (Mackenzie et al., in preparation).

S. rouxii is not sensitive in this way, nor indeed are some 13 other species we tested. It was encountered only in strains of S. cerevisiae and in Candida krusei.

Other important characteristics of the phenomenon are: (i) the low plate count on the stressing medium is attributable to death, not dormancy; (ii) the heterogeneity in the population that produces this result is physiological, not genetic; (iii) the 'plating discrepancy' (log MA count – log count on stressing agar) has a complex relation with aw of the stressing medium. For example, in plating media adjusted with glucose, such as SHA, there is no discrepancy below 30% (w/v) glucose and there is approximate proportionality between plating discrepancy and glucose concentration above that; (iv) in continuous culture the plating discrepancy is less at low than at high dilution rates; (v) the culture can readily adapt to effectively complete resistance to the plating stress. For example, growth in 2% (w/v) NaCl will confer resistance to plating on 8–10% (w/v) NaCl or on SHA. (It is of considerable interest to us that, as early as 1940, Doudoroff [12] reported a somewhat similar overall phenomenon in bacteria).

The explanation of these observations seems to be as follows: The resistant species, when growing exponentially in a conventional medium, all accumulate intracellularly one or more polyols (up to 6 C-atoms) to a level above about 0.02 mmol·g\(^{-1}\) dry yeast. The sensitive strains do not. The adaptation of S. cerevisiae by growing in a low concentration of NaCl is accompanied by the accumulation of glycerol. This is an osmoregulatory response, but the protection so conferred is effective at a glycerol content substantially less than that needed for complete osmotic balance. The resistance acquired by S. cerevisiae in stationary phase (and presumably at low dilution rates in continuous culture) is associated with accumulation of the storage carbohydrate, trehalose, which reaches 12–13% of the mass of the dry yeast. This is not an osmoregulatory phenomenon but a biochemical consequence of the changing dynamics of carbohydrate metabolism [e.g., 13–15]. Fig. 3 shows the behaviour under relevant conditions of a trehalose-negative mutant of S. cerevisiae. Other biochemical evidence for the role of trehalose will be presented elsewhere (Mackenzie et al., in preparation), although we suspect that there is an additional factor(s) that might also contribute to some extent to stationary phase resistance.

To summarize, then, the roles of the various
polyhydroxylic compounds in protecting yeast against the potentially lethal effects of a sudden solute stress are as follows:

1. protection is conferred by an inherent (i.e., non-osmoregulatory) accumulation of a polyol (we assume that any naturally-occurring polyol will do);
2. protection is conferred by the adaptive (i.e., osmoregulatory) accumulation of glycerol to a level substantially less than that needed for complete osmotic balance;
3. protection is conferred by the (non-osmoregulatory) accumulation of the storage polysaccharide, trehalose.

In each of these circumstances the polyhydroxylic compound is a compatible solute. Only in (2) is it also an osmoregulatory solute.

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REFERENCES

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Short contribution

Clumping characteristics and hydrophobic behaviour of an isolated bacterial strain from sewage sludge

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Summary. A clump-forming bacterial strain was isolated from a sludge community derived from a waste water treatment plant. The Gram-negative bacterium is hydrophobic and forms an extensive capsule while clumping in dilute medium (0.1% bacto peptone). Emulsan, a capsule inhibitor, does not affect the clumping ability of this bacterium. Clumps are not dispersed with high and low pH, detergents or chelators. Bacterial clumping selected by the waste water treatment processes appears to be a complex set of interactions within and between the strains of bacteria. This study reveals some of the complexities.

Introduction

For the great majority of microorganisms the business of adhering to some kind of surface, animate or inanimate, is an essential prerequisite to normal life (Lewin 1984). The mechanism of bacterial adhesion to surfaces ranging from human teeth, lungs and the intestine of a cow, to a rock submerged in fast moving streams, have been described (Costerton et al. 1978). However, very little is known about the mechanism by which bacteria attach to other bacteria. We describe here some of the biological characteristics of a clump-forming bacterial strain isolated from waste water sewage sludge.

Materials and methods

Isolation

A strain of bacteria tentatively identified as *Pseudomonas* sp. designated KEWA-1 was isolated from sewage sludge by a conventional streaking method. The identification was based on oxidase and catalase tests, growth factor requirements, growth at different pH and arrangement of flagella (Palleroni 1984). The sewage sludge was obtained from Air Products and Chemicals Inc., Allentown, Pa., USA from their 5700 m³/day, anaerobicoxic plant.

Media

Three media were used: (1) minimal medium; (2) dilute medium (0.1% bacto peptone); (3) rich medium (1.0% bacto peptone).

Minimal medium consisted of the following constituents (mg per liter): glucose 100, glutamic acid 40.6, sodium citrate 72.4, sodium acetate 37.6, sodium succinate 26.9, sodium bicarbonate 337.5, magnesium sulphate 30.2, potassium sulphate 30.2, calcium chloride 34.4, ferric chloride 6 H₂O 4.96, ammonium chloride 56.0, potassium phosphate 27.9, thiamine 0.01, riboflavin 0.01, niacin 0.01. pH was adjusted to 7.0. The medium was filter sterilized.

Culture conditions

One milliliter of stationary phase culture was inoculated into 50 ml of minimal, dilute or rich medium. Optical density was measured at 600 nm after homogenizing the cells for 30 s.

Hydrophobicity test

The method described by Rosenberg et al. (1980) was used.

Transmission electronmicroscopy

Specimen to be prepared was washed and resuspended in distilled water. The cells were allowed to settle onto nitrocellulose coated 300 mesh copper grids for 5—10 min, then drained dry with absorbent tissue. The dried grids were shadowed with Pt: Pd 80:20 at an angle of 27 degrees (Poin Dexter and Hagenzieker 1981).

Dissociation of clumps

The settling characteristic of a preparation was used as a measure of clump dissociation. Settling was measured on expo-
nationally growing cells by transferring a 4 ml sample to a 5 ml cuvette and suspending it by inverting several times. Initial O.D. was made at 600 nm in a Beckman KBGT spectrophotometer. The sample was allowed to settle in the cuvette for 2 h and the final O.D. was measured. Settling was expressed as the settling index (S.I.):

\[ S.I. = \frac{\text{O.D. initial} - \text{O.D. final}}{\text{O.D. initial}} \]

High values indicate the presence of clumps whereas low values show clumps have dissociated somewhat.

Results and discussion

**Clumping characteristics**

The isolate designated KEWA-1 is a Gram-negative, motile rod and has a single polar flagellum (Fig. 1). Growth in dilute medium showed that this organism grew slowly (doubling time 2.5 h) and clumped whereas in rich medium the growth was fast (doubling time 1.4 h) but the cells became free swimming after 6—8 h of growth. At this stage microscopic examination revealed that the cells were either single or in very small chains. It seems that the clumping is a well responded adaptation to the two main features of the waste water treatment plants. Under these conditions only those organisms can survive which have the ability to rapidly take up organic carbon from the nutritionally dilute environment and to form clumps in order to sediment rapidly under normal gravity. After staining the cells with India ink, microscopic observation revealed that in dilute medium the cells produce extensive capsules, which fused together with other cells to form large clumps. Cells grown in rich medium did not exhibit a capsule. The extensive capsular material may confer a selective survival advantage under waste water treatment environment.

Furthermore, the aggregation of cells within such a capsule may confer an advantage in allowing such cells or other cells to benefit from the metabolic activity of other individuals within the consortium (i.e. cross feeding). This type of phenomenon has been observed (Vincent et al., unpublished results) among the organisms isolated from sewage sludge. In addition, the biochemical remains of dead cells within this encapsulated population may serve to supply precursors for macromolecular synthesis.

**Hydrophobicity**

The sorption of bacteria to hydrocarbons and their partitioning in a hydrocarbon-aqueous biphasic system has been suggested as a method for measuring cell surface hydrophobicity (Rosenberg 1980). Using this technique it became clear that the cells growing as clumps in dilute medium were hydrophobic (Fig. 2). The free swimming cells in rich medium do not demonstrate hydrophobicity. This observation is correlated with the presence and absence of capsules in clumping and free swimming phases of bacteria. Adhesion by cell surfaces has been known to play an important role in biological processes such as contact inhibition, cell differentiation and motility (Le touneau et al. 1980), interaction between pathogenic bacteria and various target cells (Halt 1982) and phagocytosis of bacteria (Van Oss 1978). Each of these involves highly specialized mechanisms of recognition by lectins and specific receptors on the cell surface. Many microorganisms, such as pathogens and rhizobia on plant roots (Dazzo 1980), adhere to surfaces in this way. Whether these bacteria attach to each other by one of the above sophisticated mechanisms or attach nonspecifically is not known.

Recently hydrophobicity has been shown as a nonspecific adhesion mechanism in cyanobacteria (Ali and Shilo 1984). Cations, such as Ca\(^{2+}\) and Mg\(^{2+}\), are reported to be important in such a case for expression of hydrophobicity (Ali and Shilo 1984). We have found that the expression of
hydrophobicity of clumping bacteria does not require the presence of cations.

**Effect of emulsan**

Emulsan (a galactosamine polysaccharide complex) is a known inhibitor of capsule formation in bacteria (Rosenberg 1980) which has been isolated from *Acinetobacter calcoaceticus* RAG-1. When various concentrations ranging from 0.05 to 1 mg/ml of emulsan were used in minimal medium, the organisms behaved as if grown in rich medium, i.e. the cells went into a free swimming stage. Experiments with minimal medium without glucose but supplemented with emulsan showed that the cells did produce capsules and clumped. It seems emulsan supplies a carbon and energy source to the bacterium.

**Dissociation of clumps**

The settling index of about 0.85 remained unchanged when Tween 80 or DMSO was used at different concentrations. Although the optimal growth was at pH 7.0, the clumps were not dispersed with high (10.0) and low (4.0) pH, other detergents or chelators.

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APPENDIX
A typical $^{31}$P spectrum from the experiments conducted in this thesis is shown in figure 1 where chemical shifts are presented in ppm expressed to 85% phosphoric acid but were measured against a primary standard of 0.05% phosphoric acid contained in a capillary. Peak heights (or intensities) reflect the amount of metabolite present. It was not possible to distinguish between the peaks of the different nucleotides triphosphates. The levels of nucleotides triphosphate in *Saccharomyces cerevisiae* cell has been previously determined to be 4.8, 1.5, 1.5 and 1.1 nmol/mg dry wt for ATP, GTP, UTP and CTP respectively (Kundra R and Edlin G (1975) J Bacteriol 121: 740). Thus ATP was presumed to be the predominant nucleotide triphosphate present in the cell.

a) Glucose-6-phosphate  
b) 3-phosphoglycerate  
c) Fructose1,6 bisphosphate  
d) Fructose-6-phosphate  
e) Inorganic phosphate  
f) Phosphoenolpyruvate  
g) γ ATP  
h) β ADP  
i) α ADP  
j) α ATP  
k) NAD+  
l) β ATP  
m) Polyphosphate
FIGURE 1