SENSITIVITY TO DESICCATION

AND

HEAT DURING YEAST LIFE CYCLE

VARIATION IN SENSITIVITY TO DESICCATION AND HEAT STRESSES DURING YEAST SPORULATION AND SPORE GERMINATION

By

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ABSTRACT

A culture procedure was established to obtain uniformly high yields of yeast spores with high desiccation resistance, and this was used to study the following parameters during both the sporulation phase and spore germination process of a yeast strain: changes in heat resistance, desiccation resistance, cellular free proline content and alpha-amino acid content. The effect on desiccation resistance of supplying exogenous proline was studied, and this is the first work to report that exogenous proline can increase desiccation resistance of yeast vegetative cells or sporulating cells. Small-celled colonies found after vegetative or sporulating cells were desiccated or treated with proline solutions are concluded to be possibly a mutant or a symbiont of the yeast cells.

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INTRODUCTION

Yeasts have been widely used as experimental organisms in various aspects of biological research. The common commercial yeast, *Saccharomyces cerevisiae*, a single -celled organism, is the most popular. Yeasts can be cultured, maintained and manipulated with standard microbiological techniques, like many prokaryotic organisms. Their life cycle is relatively short and cell divisions are rapid. Different stages of their life cycle can be reproduced in the laboratory under given conditions and with known nutrient media. Although the yeasts are one of the simplest eukaryotes, their cells possess many structures common to higher eukaryotic cells, such as a definite membrane-bound nucleus, mitochondria, plasma membrane, vacuoles, ribosomes, centriole and mitotic spindle (Guth *et al.*, 1972). Macromolecular syntheses, chromosome replication and segregation in yeasts are homologous to those in higher eukaryotic cells. So, the yeast *Saccharomyces* is a useful experimental subject for developing models for differentiation and development in unicellular eukaryotes.

In many ecological niches, organisms may encounter adverse environmental conditions. Drying is a common

physical stress experienced by living cells. In the work presented here, the resistance to desiccation of a strain of the yeast *Saccharomyces cerevisiae* was studied. Its resistance to another physical stress, high temperature, which may not be so frequently experienced in the natural environment was also investigated.

The majority of the published work on heat resistance of living cells has been carried out with prokaryotes, especially the spore-forming and thermophilic bacteria. Among the most recent publications on this topic was the study of heat resistance of spores of *Clostridium sporogenes* by Anema and Geers (1973), of *Clostridium perfringens* by Nakamura and Nishida (1974), and of *Clostridium putrefaciens* by Roberts and Derrick (1975). Reports on heat resistance of germinated spores of *Bacillus cereus* (Dring and Gould, 1975), and on the thermal profile of an extremely heat resistant *Bacillus* species (Bond and Favero, 1975) represent some of the recent research on heat resistance of aerobic spore-formers. Investigations have been made on the nature of heat resistance of *Bacillus stearothermophilus*, which is a thermophilic bacterium (Ljunger, 1970; Ljunger, 1973). Papers have appeared on the heat resistance of bacteria related to food industry, such as *Salmonella* (Corry, 1974). Ebner and Frea (1970) investigated the heat resistance of the exospores and vegetative cells of the actinomycete *Streptomyces fradiae*.

The heat resistance of fungi has received much less attention. Warcup (1951) recorded some ascomycetes in soil including *Aspergillus fischeri, Penicillium luteum, Penicillium baarnense* which had spores resistant to a short partial sterilization treatment by steam applied to the soil. Changes in the heat resistance of ascospores of *Neurospora* upon germination were followed (Lingappa and Sussman, 1959). *Neurospora* ascospores were activated to germinate by a short heat-shock at 60°C, followed by incubation at 27°C. When activated, ascospores after 5 minutes incubation at 27°C were given a heat treatment at 65°C for 5 minutes; only 30% of the spores survived and germinated. If the same heat treatment was given after 25 minutes incubation at 27°C, all the ascospores were killed. So there was a rapid loss in heat resistance of *Neurospora* ascospores accompanying germination. Heat resistance of a number of xerophilic fungi, such as *Aspergillus chevalieri* was studied and it was found that some spores remained viable after 10 minutes at 80°C (Pitt and Christian, 1970). Heat resistant moulds including species of *Byssochlamys, Paecilomyces, Aspergillus, Penicillium* were isolated from New York orchards and vineyards, and their spores could survive 1 hour at 70°C (Splittstoesser *et al.*, 1971). *Byssochlamys* ascospores were chosen for heat resistance studies because the fungus causes spoilage of canned fruit and fruit juice,

and the decimal reduction time at 66°C of the spores was found to be 13 minutes (Michener and King, 1974). *Eleutherascus tuberculatus*, a new heat resistant ascomycete, was isolated from Dutch soil after moist heat treatment at 70°C for 30 minutes (Samson and Luiten, 1975).

Hansen (1883) was the first to demonstrate that spores and vegetative cells of *Saccharomyces* were different in their resistance to elevated temperatures. Young vegetative cells of *Saccharomyces cerevisiae* were killed by five minutes heating at 54°C, and the spores after five minutes at 62°C. Old vegetative cells were more heat resistant than young cells. Chudyk *et al.* (1969) investigated the survival of yeast ascospores and vegetative cells at high and low temperatures. They showed that the yeast ascospores were more heat resistant than vegetative cells, and found that with a 5 minute exposure the lethal temperature for vegetative cells was within the range of 51-52°C, and that for ascospores was within 57-58°C. Fowell (1966) described a selective heat treatment procedure for use in yeast genetics research to obtain populations of haploid spores free of the diploid vegetative stage. Heating aqueous suspensions of cells and spores at 58°C for 5 minutes killed about 95% of the vegetative cells but the spores survived.

The desiccation resistance of microorganisms has been less investigated than their response to heat. In

general, spores are more resistant than vegetative cells. *Pseudomonas aeruginosa* was tested for its sensitivity to desiccation and it was found that cells in the exponential growth phase were more susceptible than cells in the stationary phase (Skaliy and Eagon, 1972). Yamamoto (1975) found that akinetes of *Anabaena cylindrica*, a blue-green alga, were more tolerant to desiccation than vegetative cells. Desiccated akinetes were still able to germinate after 5 years of storage in dark, whereas desiccated vegetative cells lost their viability after storage of only 15 days.

There have been many records of survival of fungi under prolonged desiccation. As examples, air-dried conidia of *Aspergillus oryzae*, which had been kept in a sealed test tube at room temperature in the dark for 22 years, grew on every medium tried when plated (McCrea, 1923). McKay (1935) observed germination of oospores of the onion mildew, *Peronospora schleideni*, at the end of four years of storage in dry condition. Cultures of *Allomyces arbuscula* dried by evaporation on filter paper strips and stored in this way survived for more than 14 years (Fennell, 1960). Cultures of *Candida albicans* dried in a vacuum over calcium chloride at room temperature and stored at 10 °C survived after 21 years in this condition (Miller and Simons, 1962). Three fungus species pathogenic to insects were shown to retain viability and pathogenicity

after three years storage of their spores on silica gel crystals at -20°C (Bell and Hamalle, 1974). Rathaiah and Pavgi (1971) reported that germinated conidia of *Cercospora carthami* and *Ramularia carthami*, which were desiccation-tolerant fungi, could withstand adverse dry conditions prevalent in the field during host infection.

Reess (1870) noted that yeast spores could survive drying for months. Hansen (1898) maintained yeast vegetative cells and spores in a desiccator over concentrated sulfuric acid. It was found that desiccated spores remained viable much longer than desiccated vegetative cells. This also was true of spores and vegetative cells dried at ambient humidity. Miller (1974) in quantitative experiments reported that the percentage of yeast ascospores that survived drying was far greater than vegetative cells from growth medium and that some yeast spores germinated and produced normal colonies after ten years over silica gel at laboratory temperature.

There has been no report in the literature in which the variation in desiccation resistance or heat resistance during the successive stages of the life cycle of a fungus has been followed. Also little attention has been given to the mechanism of heat resistance and desiccation resistance in fungi. In this present work, the acquisition of heat resistance and desiccation resistance by yeast cells during sporulation and the loss of these

resistances during germination are traced. The phenomenon that some living cells are to a certain degree resistant to desiccation and heat presents interesting and fundamental problems for investigation. The present work represents an effort to contribute to an understanding of the resistance mechanism of yeast cells to these environmental stresses.

Heat and desiccation are two possible means to control yeasts. Since yeasts are present in many natural foods and food ingredients,and are microbial agents affecting food processing and fermentation industry, information about development and loss of heat and desiccation resistance in yeasts may have industrial application.

In sporulation, resistance to these stresses may be acquired through structural or biochemical changes. An electron microscopic study could attempt to correlate changes in structure with acquisition of resistance. In this present work, an attempt is made to relate resistance to internal chemical changes, in particular, to changes in proline content. The choice of proline as a subject to study was prompted by the previous finding of Ramirez and Miller (1963; 1964) that the amount of free proline in *Saccharomyces cerevisiae* cells increased greatly during sporulation and decreased during spore germination. All the other free amino acids, on the contrary, diminished during sporulation and increased during germination.

Rousseau and Halvorson (1973) confirmed this finding and they also observed that proline was excreted into the medium from the spores during germination.

Bathurst (1954) studied pollen from grass species and reported that of the free amino acids in the pollen the most abundant was proline. Britikov and Musatova (1964) analyzed the pollen and pistils of a large number of plants (about 200 species from 63 families) and found that the pollen differed from other plant organs in its unusually high content of free proline, reaching 1.5% or even more per fresh weight in many species. Britikov *et al.* (1964) concluded that the high content of free proline in pollen and the considerable excess over its content in pistil tissues is consistent fact which, apparently, has no exceptions. A number of other workers have reported high proline content in pollen (see: Stanley and Linskens, 1974). Pollen would be expected to have adaptations to desiccation stress, but it is interesting that there are many reports describing increases in proline content of other plant tissues when subjected to desiccation stress. When there was water deficiency in the soil, there was an increase in the level of proline in barley (Savitskaya, 1965). Under water stress, there was a ten-fold to a hundred-fold accumulation of free proline in shoots of Bermuda grass and the turnover of free proline was much slower than that of any other free amino acid (Barnett and Naylor, 1966).

Non-protein proline was also found to accumulate in wilted, excised turnip leaves while such accumulation was not apparent in unwilted leaves (Thompson *et al.*, 1966; Stewart *et al.*, 1966). Protsenko *et al.* (1968) compared the amino acid composition of drought resistant and less drought resistant varieties of winter wheat. It was demonstrated that during drought, proline accumulation was greater in the drought resistant varieties. Palfi (1969) showed that the proline content of the leaves increased several times after 2-3 days of water insufficiency. Even when there was enough water in the root medium, there was still a significant increase of proline in the leaves if the water uptake was hindered. Physiological drought could be induced artificially in the leaves by increasing the salt content of the irrigation water or by chilling the root system (Palfi and Juhasz, 1970). Water deficiency induced artificially in the leaves was also accompanied by great increase in the proline content. Routley (1971) worked with wilting detached Ladino clover leaves and similarly found accumulation of proline. According to Palfi *et al.* (1973) the content of free proline in plants optimally supplied with water was usually extremely low (0.2-0.6 mg/gm dry matter) and during slow dehydration of plant tissues, the quantity of free proline rose sharply and could reach 40-50 mg/gm. Waldren and Teare (1974) demonstrated that free proline did not accumulate markedly

in intact sorghum and soybean leaves until the plants were severely water-stressed and they suggested that proline accumulation might be an indicator of drought resistance or susceptibility. Dr. C. Ramirez (personal communication, 1975) found large accumulation of proline under severe conditions of dryness in the most drought-resistant species of the clover *Trifolium* in Spain. The less resistant species did not accumulate proline. Hence it seems well established that the content of free proline in plants increases under conditions of insufficient moisture. Palfi *et al.* (1973), based on this fact, even proposed the proline test as an index of water deficit in plant tissues.

The accumulation of proline under conditions of drought has been attributed to protective, adaptive reactions (Startseva, 1964). Britikov *et al.* (1965) suggested that for plants with a high content of proline in the pollen, the free proline could serve as a protective and stabilizing factor for the pollen. Slukhai and Opanasenko (1974) worked on winter wheat and also proposed that proline might play a protective role in water deficits.

Thus in many higher plants, proline content is increased by drying and there is a suggestion that it may have a protective function.

In bacteria, a striking chemical change associated with spore formation is the synthesis, in large amounts, of a low molecular weight compound, dipicolinic acid

(pyridine-2:6-dicarboxylic acid). This substance constituted approximately 15% of the dry weight of the *Bacillus megatherium* spore (Powell and Strange, 1953), but was undetectable in bacterial vegetative cells. It was shown that the acquisition by the *Bacillus* spore of heat resistance followed very closely the formation of dipicolinic acid during the maturation process (Hashimoto *et al.*, 1960). Day and Costilow (1964) did similar work on *Clostridium* and also found that dipicolinic acid synthesis paralleled the development of heat resistance. They determined that the spores of *Clostridium botulinum* contained dipicolinic acid at concentrations from 3-4% of their dry weight. Extreme resistances to heat and desiccation are two of the characteristic properties of the bacterial spores, and the appearance of these types of resistance at different stages during sporulation and their loss during germination makes it clear that they are due to the particular physicochemical composition of the dormant spore (Halvorson and Szulmajster, 1973).

Such information on dipicolinic acid renders it meaningful to investigate whether free proline may play a similar role in the yeast spore, since both substances increase greatly during sporulation and are excreted into the medium during spore germination.

The life cycle of a typical *Saccharomyces* yeast is shown in Figure 1a. The majority of the strains of *Saccharomyces* are four-spored, and undergo meiosis during sporulation and show conjugation at the time of germination.

The life cycle of the 2-spored yeast 19el studied here (Figure 1b) differs in that there is only one nuclear division in the developing ascus, and germinating spores do not conjugate. From this and other evidence, Grewal and Miller (1972) concluded that the 19el vegetative cells and spores were all diploid and that nuclear division in the ascus was not reductional. In an electron microscopic study of this strain, Moens (1974) found evidence that the first division of meiosis was absent and that the nuclear division in the ascus had the morphological characteristics of meiosis II.

There are several reasons why strain 19el was chosen as the experimental organism in this project: (1) plating was the method used to determine viability of spores. A germinating ascus will give rise to one visible colony irrespective of how many ascospores encased in it germinate. Killing by heating or by desiccation is assessed by colony counting. If there are more spores in an ascus, there will be more chance for one spore to survive the harsh treatment and hence for the ascus to give rise to a colony. A two-spored strain in this case

Figure 1. Life cycles of *Saccharomyces* yeasts.

(a) The typical life cycle of *Saccharomyces cerevisiae*:

(1) & (2) mitotic nuclear division of diploid nucleus during budding;

(3) mature bud ready to separate from parent cell; (between 3 and 4 the yeast is transferred from a growth to a sporulating medium;

(4) & (5) as a result of meiosis, the diploid nucleus of a vegetative cell divides into 4 haploid nuclei;

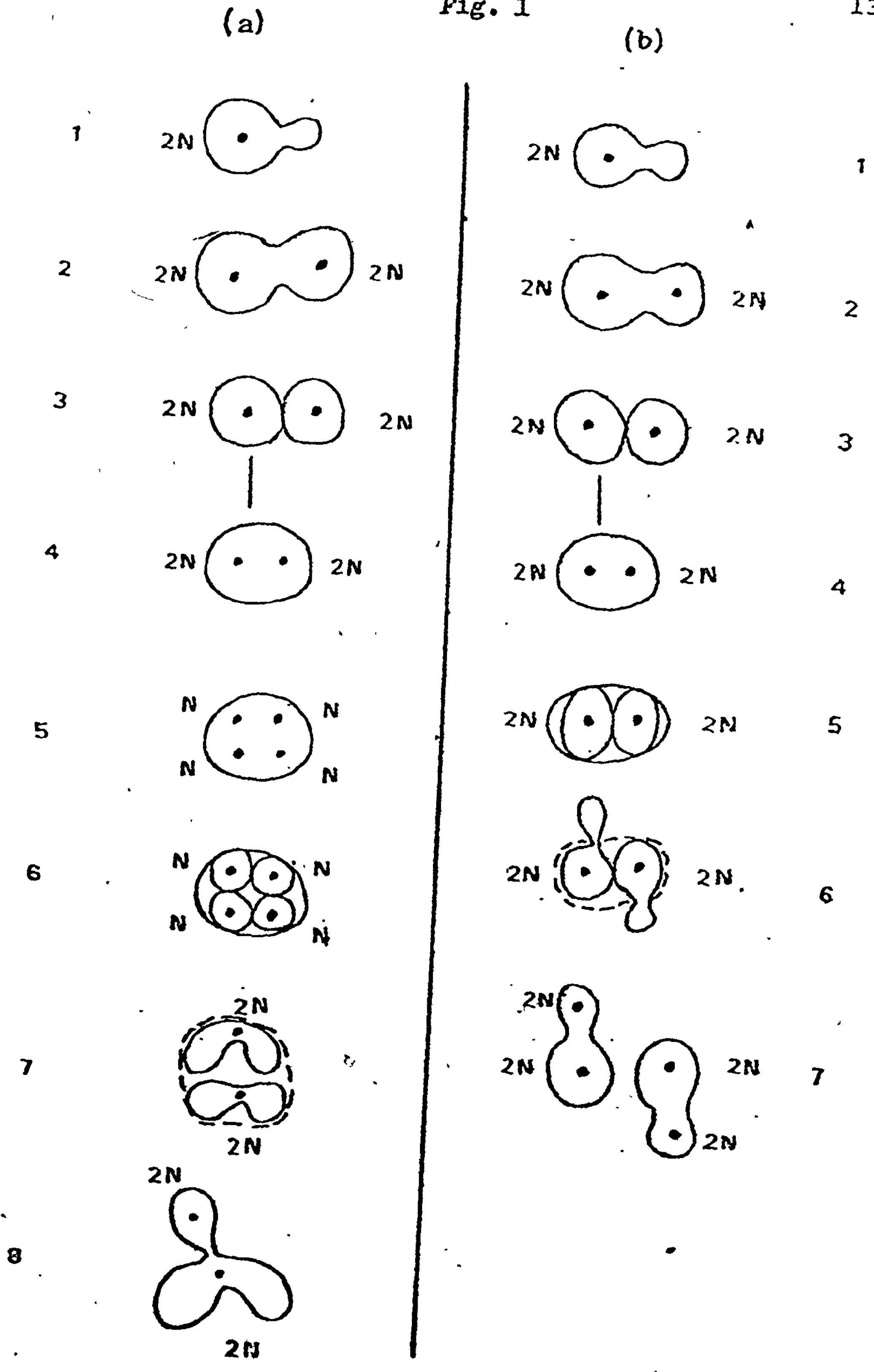
(6) a spore wall forms around each of the 4 haploid nuclei, and the cell becomes the ascus containing 4 haploid ascospores;

(7) & (8) the ascus wall usually disintegrates, the spores germinate, and pairs of germinating spores of opposite mating types conjugate to form diploid vegetative cells.

(b) The life cycle of strain 19el:

This life cycle differs from the one in (a) in that there is only one nuclear division in the developing ascus, and germinating spores do not conjugate.

Fig. 1



is clearly better than a four-spored strain; (2) a very high percentage of 19el vegetative cells sporulate when placed in an appropriate sporulating medium. The percentage of sporulation can be as high as 97%. The advantages of working with almost pure ascus populations for viability plating and chemical analyses are obvious.

The approach to the present study is in three phases. Firstly, the acquisition of heat resistance and desiccation resistance by 19el cells during sporulation and the loss of these two resistances during germination are followed. Secondly, an attempt is made to correlate the content of free proline of the cells or spores with resistance. Thirdly, the effect on desiccation resistance of exogenously-supplied proline is determined.

MATERIALS AND METHODS

Organism and Maintenance

The strain (19el) of *Saccharomyces cerevisiae* Hansen used in this work was obtained from Station Agronomique et Oenologique de Bordeaux, France.

Stock cultures of the yeast strain were maintained on slants of malt extract-yeast extract-peptone-glucose medium (MYPG) containing 2.5% agar. The composition of MYPG is as follows:

Difco malt extract	3 g
Difco yeast extract	3 g
Difco peptone	5 g
glucose	20 g
potassium dihydrogen phosphate (KH_2PO_4)	1 g
distilled water	1 liter

This medium was devised by Wickerham (1951) and modified by Patel and Miller (1972) who increased the glucose content to 2% and added KH_2PO_4 to adjust the pH to 5.4 ± 0.2.

Vegetative Growth

To obtain the growth phase, cells from a one day old slant culture on MYPG were inoculated into 50 ml liquid MYPG medium in a 250 Erlenmeyer flask at a density of 1 x 10^4 cells per ml. The cells were incubated with shaking (100 strokes per minute) at 27°C in a Warner-Chilcott Laboratories model 2156 water-bath shaker. Unless otherwise stated, the vegetative cells used for experiments in this work had been grown for 22 hrs in liquid MYPG.

Sporulation

The sporulation medium used was 2% potassium acetate (Steele and Miller, 1974a). Yeast sporulation can be initiated by transfer of cells to a nitrogen-free medium containing a non-fermentable carbon source (Miller and Hoffman-Ostenhof, 1964.

The vegetative cells were harvested from the growth (pre-sporulation) medium by centrifugation in a Sorvall RC-2 refrigerated centrifuge at 3,020 g and at 4°C for 15 mins. The cells harvested were washed twice with sterilized distilled water, and then resuspended in 50 ml of 2% potassium acetate at a cell density of 1 x 10^7 cells per ml in a 250 ml Erlenmeyer flask. The cells were incubated at 27°C in the water-bath shaker. The

spores used in this work were harvested after 48 hrs of incubation in the acetate sporulation medium, unless otherwise stated.

Spore Germination

The germination medium used was a chemically defined medium of the following composition:

Difco Yeast Nitrogen Base (without amino acids or ammonium sulfate)*	0.078 g
glucose	0.50 g
ammonium sulfate	0.05 g
glass-distilled water	50 ml

This medium was sterilized by filtration through an autoclaved Sartorius GmbH Sm 16510 membrane-filtration holder with a Gelman GN-6 0.45 μ pore diameter Metricel membrane filter (47 mm diameter).

The spores, harvested from the sporulation medium by centrifugation, were washed twice with sterilized distilled water, inoculated into 50 ml of germination medium in a 250 ml Erlenmeyer flask at a density of 1×10^7 asci per ml, and incubated at 27°C in the water-bath shaker.

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* see Appendix I

Determination of Heat Resistance

One ml samples were withdrawn from the cell suspension during the course of development of spores in sporulation medium or from the spore suspension during the spore germination process in germination medium.

Each one ml sample, containing approximately 1×10^7 cells, was mixed with 100 ml sterilized distilled water and then passed through a membrane filter. The cells retained on the membrane filter were washed twice by passing 50 ml sterilized distilled water through the filter. Then the whole filter was shaken in 7 ml sterilized distilled water in a 50 ml Erlenmeyer flask. The cell density of the resulting cell suspension was determined with an American Optical Co. Bright Line haemacytometer counting chamber. At least 8 counting chamber fields were counted. The cell suspension was diluted to 1.0×10^4 cells/ml and 1.0 ml of it was added to 49.0 ml of sterilized distilled water in a 250 ml Erlenmeyer flask preheated for at least 1 hr in a water-bath shaker at 55°C. The flask was immediately placed back in the 55°C water-bath shaker. After a heating of 5.0 mins, about 10 ml of the cell suspension was quickly transferred to a 125 ml Erlenmeyer flask at room temperature. One ml volumes of the cooled suspension were pipetted into plastic petri dishes (100 x 15 mm) and immediately approximately 20 ml

of MYPG agar (with 2% agar) at 52 ± 1°C was poured into each dish. After tilting and mixing the agar in each dish thoroughly, the dishes were incubated at 27°C. There were always six replicate plates for each sample. After three days of incubation, the colonies in each plate were scored using an American Optical Co. darkfield colony counter.

Determination of Desiccation Resistance

Two ml samples were withdrawn from the cell suspension during the course of development of spores in sporulation medium or from the spore suspension during the spore germination process in germination medium.

Each 2 ml sample, containing approximately 2×10^7 cells, was mixed with 100 ml sterilized distilled water and then passed through a membrane filter. It was calculated that 2×10^7 yeast cells, whether as vegetative cells or asci, when filtered through the membrane filter of 47 mm diameter would be spread so thinly on the membrane surface that seldom would any two be in contact, thus mutual protection against desiccation due to crowding of cells on the membrane surface was avoided. The cells retained on the membrane filter were washed twice by passing 50 ml sterilized distilled water through the filter. After washing, the membrane filter with cells was placed in a glass desiccator (11 cms in diameter) containing approximately

250 g anhydrous silica gel, with a Whatman number 3 filter paper (9.0 cm diameter) between the membrane filter and the silica gel. The membrane filter was handled with sterilized Millipore forceps. The desiccator, sealed with Fisher petrolatum, was kept in the dark in a 27°C incubator for 24 hrs. After 24 hrs in the desiccator, an approximately one-quarter sector of the membrane filter bearing the desiccated cells was cut out with a sterilized razor blade and the sector was shaken vigorously, using a Vortex mixer, for 2 mins in 2.5 ml sterilized distilled water in a 50 ml Erlenmeyer flask. The density of the resulting cell suspension was determined by counting at least 8 fields of the haemacytometer counting chamber. By appropriate dilutions, the density of the cell suspension was adjusted finally to 200 cells per ml and plated in MYPG agar as in the procedure for determining heat resistance. Six replicate petri dishes containing 1 ml of suspension were poured for each sample. The plates were scored after 3 days of incubation at 27°C.

Determination of Free Proline and Free Alpha Amino Acids in Yeast Cells

i) Extraction. Sporulating cells (or germinating spores) were separated from the sporulation medium (or germination medium) and then washed with water by centrifugation. A

50 ml cell suspension containing 5 x 10^8 cells was placed in two centrifuge tubes, 25 ml per tube, and centrifuged at 23,500 g at 4°C for 30 mins in a Beckman J-21 centrifuge. After centrifugation, about 12 ml of the supernatant was carefully pipetted out from the top portion of the liquid in each centrifuge tube, without disturbing the cell pellet at the bottom. The remaining supernatant and the cells of the two tubes were pooled and centrifuged at 23,500 g at 4°C for 60 mins. Immediately after this second centrifugation, all the supernatant was poured out very carefully. The cell pellet remaining was washed once with 30 ml distilled water, and the cells were separated from the water by centrifugation for 60 mins. The cell pellet obtained after the third centrifugation was suspended in 10 ml distilled water. The supernatants of each of the three centrifugations were saved and combined. The cell density of this combined supernatant was determined and its volume was measured, so that the amount of cell loss during these three centrifugations was known and hence a correction factor could be added to the final yield of amino acid.

The cells in the 10 ml suspension were extracted by adding 40 ml of 95% ethanol and warming at 70°C for 20 mins. The cell debris was then recovered by centrifugation at 9,750 g at 4°C for 40 mins, and the alcohol supernatant was kept. The cell debris recovered

was extracted with 40 ml of 95% ethanol at 70°C for 20 mins. This second volume of alcohol was separated from the cell debris by filtration through a membrane filter and the two alcohol extracts were pooled.

The pH value of the total alcohol extract was adjusted to 7.0 with 0.01 N hydrochloric acid and a Beckman Zeromatic pH meter. The total alcohol extract was then reduced in volume to less than 5 ml in a 125 ml round-bottomed Pyrex flask connected to partial vacuum and simultaneously heated in a boiling water bath.

The concentrated alcohol extract was extracted with 10 ml chloroform. After shaking vigorously for 5 mins, the mixture was centrifuged at 10,800 g at 4°C for 30 mins. The alcohol-water (top) layer was separated from the chloroform (bottom) layer, with a Pasteur pipette and kept. The chloroform layer was shaken with 3 ml distilled water for 5 mins and centrifuged at 10,800 for 30 mins. The aqueous layer separated was pooled with the alcohol-water layer obtained above. This pooled extract was finally extracted with 5 ml of chloroform for 5 mins. The alcohol-water and chloroform layers were again separated by centrifugation at 10,800 at 4°C for 30 mins. The final solution obtained was made up to 10.0 ml exactly with distilled water. (As a result of the chloroform extraction, proteins and lipid were removed from the alcohol-water extract.)

Five ml of the final alcohol-water extract was then treated with acidic ninhydrin (for proline determination), and 1.0 ml of the remaining alcohol-water extract was treated with acetate-cyanide-ninhydrin (for alpha amino acid determination).

The extraction procedure described above was that of Sutherland and Wilkinson (1971).

ii) Estimation of free proline. The ninhydrin method for determination of proline in this work was based on Troll and Lindsley (1955).

Acidic ninhydrin reagent was prepared as follows: 0.125 g of triketohydrindene hydrate (ninhydrin) was dissolved in 3 ml of glacial acetic acid and 2 ml of 6 M phosphoric acid (H_3PO_4) with heating to 70°C. This ninhydrin reagent was stable for at least 24 hours after preparation.

Five ml of the final alcohol-water extract was shaken with approximately one-tenth of its weight of Permutit for 5 mins. The Permutit served to remove the interfering amino acids lysine, hydroxyproline and ornithine.

The 5.0 ml of Permutit-treated extract were then heated with 5 ml of glacial acetic acid and 5 ml of acidic ninhydrin reagent in a boiling water bath for 1 hr in a 30 ml Kimax test tube with plastic screw cap. The

solution was cooled to room temperature and extracted with 5.0 ml of benzene by shaking the mixture vigorously for 5 mins. The phases were allowed to separate using a 60 ml separatory funnel, and the benzene phase was obtained and kept. The benzene phase was diluted appropriately with pure benzene, if necessary. About 5 ml of the diluted benzene phase, at the suitable range of absorbance, was transferred to a Spectronic 20 colorimeter tube and the optical density was determined at 515 nm with a Bausch and Lomb model 340 colorimeter. The standarization curve was prepared using L-proline (Calbiochem A grade; hydroxyproline-free; chromatographically homogeneous).

To test the reliability of the extraction and analytical procedure, 2.9×10^{-4} g of proline in 1 ml distilled water was added to a preparation of 5×10^{8} asci after the initial centrifugation step and prior to alcohol extraction. It was determined that the recovery of the exogenously added proline was 93%.

iii) Estimation of free alpha amino acids: The ninhydrin method for determination of free alpha amino acids in this project was based on Rosen (1957).

Acetate buffer (pH 5.3-5.4) was prepared by dissolving 135 g sodium acetate ($CH_3COONa.3H_2O$) in 100 ml distilled water and 25 ml glacial acetic acid, and then the solution was made up to 375 ml with distilled water.

The acetate-cyanide buffer used in the analysis was 2×10^{-4} M sodium cyanide in acetate buffer. Three percent ninhydrin solution was prepared by dissolving 3 g of ninhydrin in 100 ml Methyl Cellosolve (ethylene glycol monomethyl ether). Both the acetate-cyanide buffer and the 3% ninhydrin solution were stable indefinitely at room temperature.

One ml of the final alcohol-water extract was heated with 0.5 ml cyanide-acetate buffer and 0.5 ml 3% ninhydrin solution in Methyl Cellosolve in a boiling water bath for 15 mins in a screw-capped test tube. Immediately after removal from the water bath, 5.0 ml of diluent (1:1 isopropyl alcohol-water) was added rapidly to the boiling solution. The mixture was shaken vigorously. The resulting solution was allowed to cool to room temperature, and read in a colorimeter tube at 570 nm. If the color density was too high, more isopropanol-water diluent was added until the optical density was within the suitable range for measurement.

The standardization curve for determination of alpha amino acids was prepared with L-alanine (Calbiochem A grade; chromatographically homogeneous).

Materials

The following were analytical grade chemicals (unless otherwise stated) supplied by the Fisher Scientific Company:

ammonium sulfate

ammonium oxalate

crystal violet (bacteriological)

ethylene glycol monomethyl ether

glacial acetic acid

hydrochloric acid

iodine

methylene blue (bacteriological)

Permutit (Folin)

potassium acetate

potassium dihydrogen phosphate

potassium iodide

safranin O (bacteriological)

sodium acetate

triketohydrindene hydrate

The following were analytical grade chemicals (except silica gel) obtained from British Drug Houses Limited:

chloroform

phosphoric acid

propan-2-ol

silica gel (self-indicating, 6-20 mesh)

sodium cyanide

Basic fuchsin (91% dye content) was supplied by the Matheson Company; benzene (thiophene free; analytical grade) by Mallinckrodt Chemical Works Limited; glucose

(anhydrous dextrose, analytical grade) and phenol (analytical grade) by the J.T. Baker Chemical Company.

The alanine and proline used throughout this work were L-alanine (A grade; chromatographically homogeneous) and L-proline (A grade; hydroxyproline-free; chromatographically homogeneous) supplied by the Calbiochem Company.

The agar used was Difco Bacto-agar.

RESULTS

Relationship Between Length of Pre-sporulation Vegetative Growth, Subsequent Sporulation, and Acquisition of Desiccation Resistance

The growth curve of 19el vegetative cells in liquid MYPG is shown in Figure 2. The stationary phase was reached after about 24 hours of growth. Cell samples were withdrawn from the growing culture at four different times in the latter part of the exponential phase and transferred to sporulation medium. After 24 hours and 48 hours in sporulation medium, samples were withdrawn, examined for production of asci, and tested for desiccation resistance (Table 1).

There were striking differences in sporulation percentage and desiccation resistance (after both 24 hours and 48 hours in sporulation medium) between the cells taken out after 16 hours growth in liquid MYPG and after 18 hours. The latter had a much higher percentage of sporulation and more of them survived desiccation. Sporulation percentage and desiccation resistance were even greater with cells that had been grown 20 and 22 hours.

Twenty two hours in liquid MYPG was chosen as the standard pre-sporulation growth time. A longer time in

Figure 2: Growth curve of vegetative cells in liquid MYPG at 27°C.

One day old vegetative cells from MYPG slant were inoculated into 50 ml liquid MYPG to a density of 1.0×10^4 cells per ml. The cells were grown at 27°C in water-bath shaker. One ml samples were withdrawn at intervals from the growing liquid culture. Each sample was diluted appropriately before the cell density was determined with a haemacytometer counting chamber.

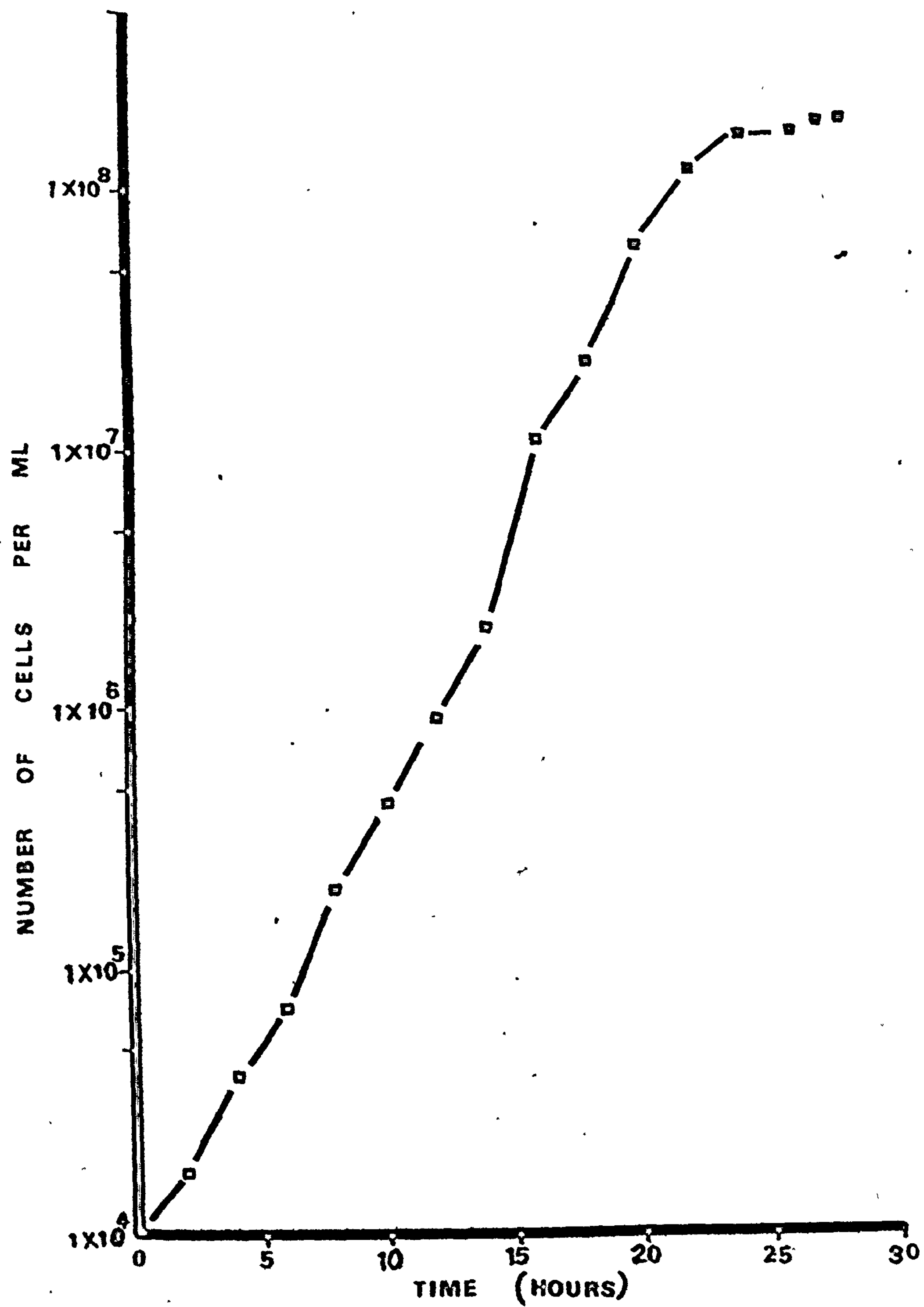

1X10^8
1X10^7
1X10^6
1X10^5
1X10^4
NUMBER OF CELLS PER ML
0
5
10
15
20
25
30
TIME (HOURS)

Table 1

Relationship Between Time in Growth Medium, Subsequent Sporulation in Sporulating Medium and Desiccation Resistance

Time in growth medium before vegetative cells transferred to sporulation medium (hours)	After 24 hrs in sporulation medium		After 48 hrs in sporulation medium	
	sporulation %*	colonies developed per petri dish after desiccation treatment**	sporulation %*	colonies developed per petri dish after desiccation treatment**
16	2.5	24 ± 2.8	7.5	45 ± 3.4
18	72.2	96 ± 4.5	80.0	134 ± 6.7
20	84.2	102 ± 6.8	87.5	166 ± 7.4
22	92.2	148 ± 9.1	97.5	193 ± 3.5

* Scored by haemacytometer counting; at least 400 entities counted.

** Out of 200 cells plated per petri dish; each number is the average of 6 replicate petri dishes, with one standard deviation shown.

pre-sporulation medium might give cells capable of even higher sporulation percentages than 22 hour cells, but the writer has noted that after 2 days in MYPG medium, some cells contain spores. That is, this strain can sporulate in growth medium, though not so frequently as in acetate sporulation medium. By choosing cells from the end of the logarithmic growth phase one should obtain a population which, unlike stationary phase cells, will not have begun to sporulate yet will be capable of high sporulation after transfer to sporulation medium.

Development of Spores and Germination of Spores

The acid-fastness,(i.e., the ability to retain stains such as basic fuchsin despite washing with 3% HCl in 95% ethanol) of the yeast spore provides a useful criterion for determining the production of spores in yeast cells (Phaff and Mrak, 1948). Similarly, Seigel and Miller (1971) found loss of acid-fastness to be a useful criterion when quantitating yeast spore germination. So, in this work, the property of acid-fastness was employed in estimating the percentage of spores formed during the course of sporulation (Figure 3) and that of spores not yet germinated in germination experiment (Figure 4). It was found that entities which morphologically looked like mature spores retained the red basic fuchsin stain, whereas

Figure 3: Development of spores in the acetate sporulation medium as determined by the acid-fast staining technique.

One ml samples were withdrawn at intervals from the sporulation medium. The cells of each sample were separated from the acetate medium and washed twice with distilled water by filtration with a 0.45 μ membrane filter before a smear was made on a slide. The dried smear was stained by acid-fast staining technique (see Appendix II) and the percentage of acid-fast entities in the stained smear was scored. In estimating the percentage acid-fastness of a given preparation, at least 400 randomly selected entities were counted.

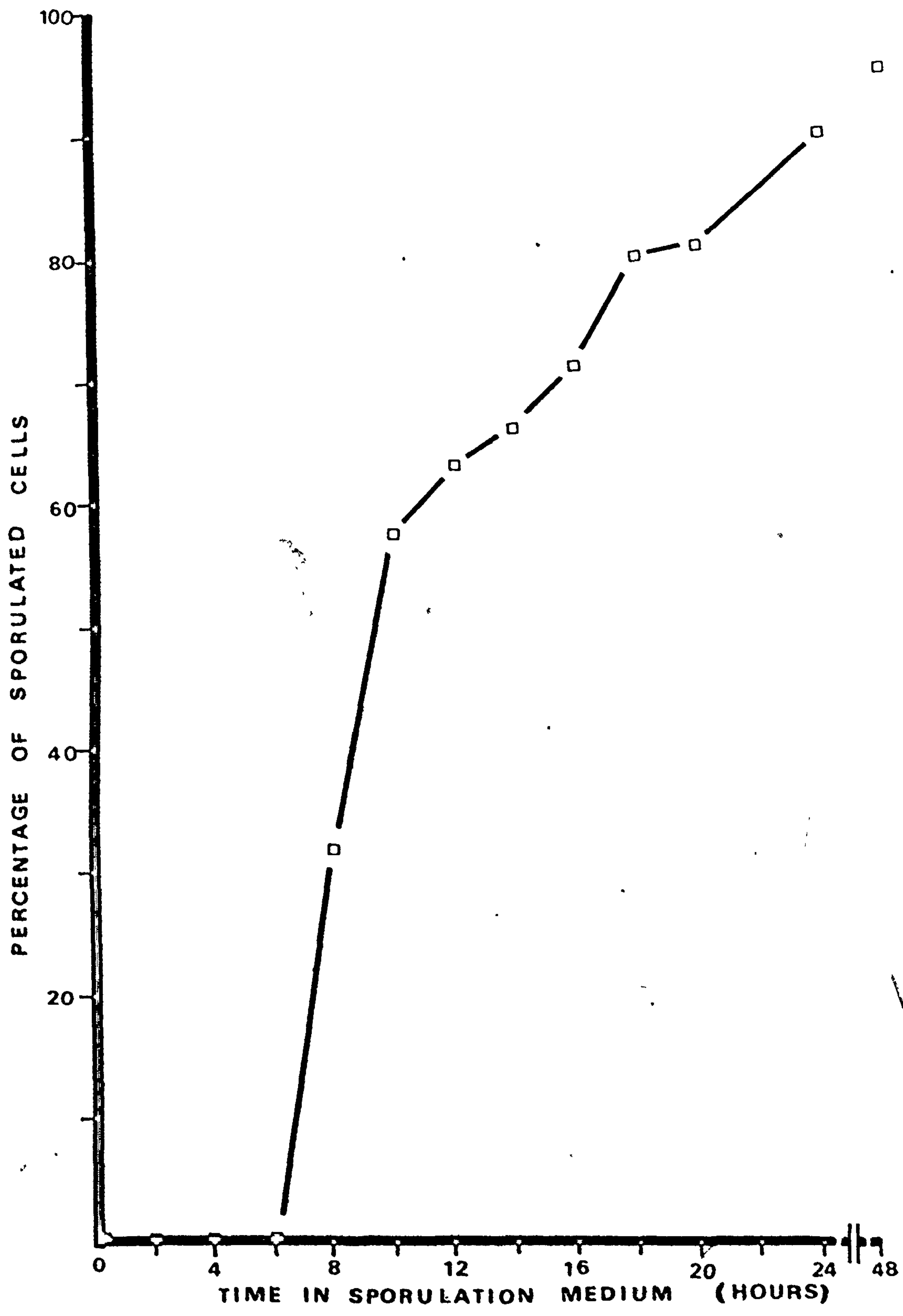
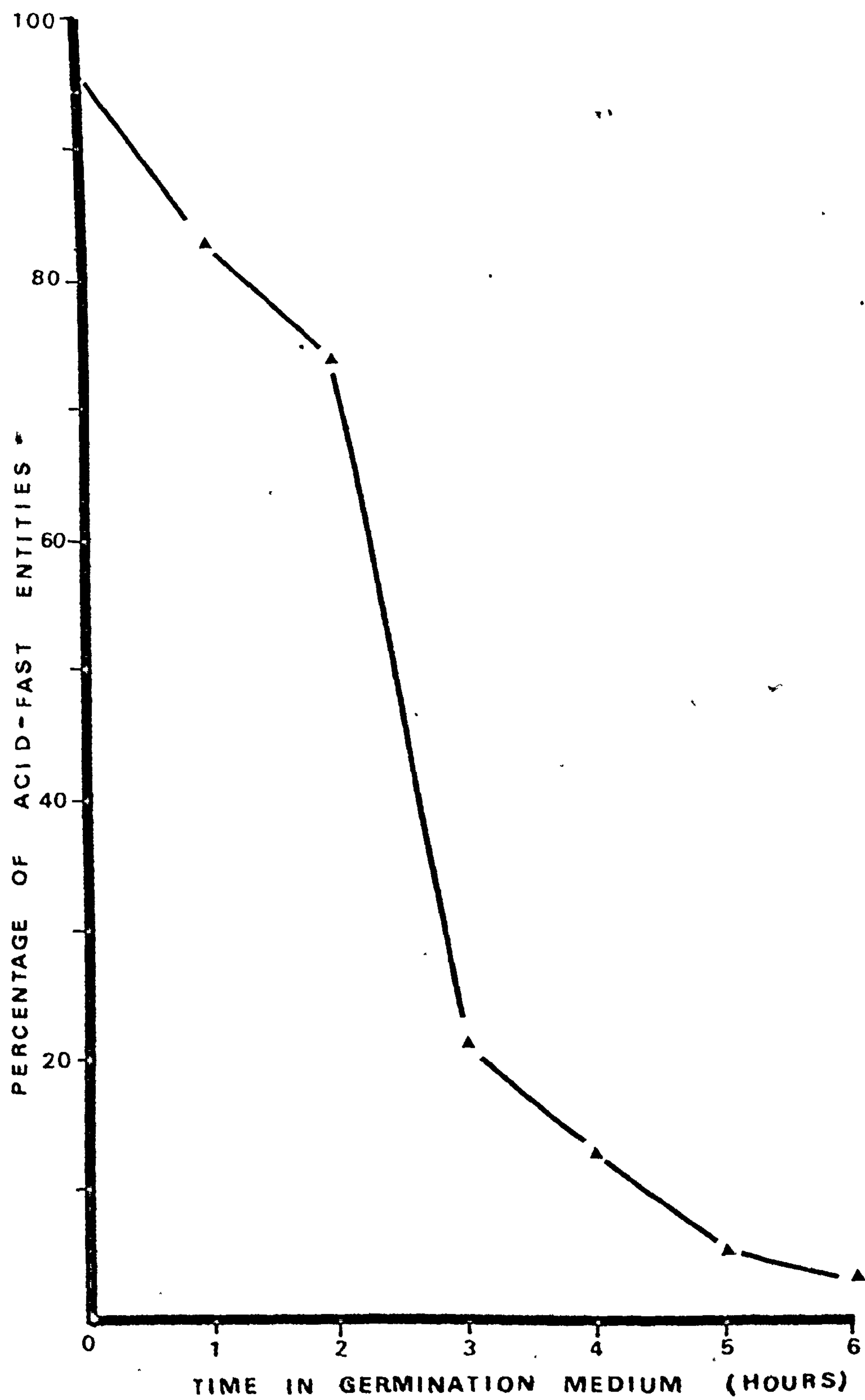

PERCENTAGE OF SPORULATED CELLS
100
80
60
40
20
0
4
8
12
16
20
24
48
TIME IN SPORULATION MEDIUM (HOURS)

Figure 4: Loss of acid-fastness by spores in germination medium.

One ml samples were withdrawn at intervals from the germination medium. The cells of each sample were separated from the germination medium and washed twice with distilled water by filtration with a 0.45 μ membrane filter before a smear was made on a slide. The dried smear was stained by acid-fast staining technique and the percentage of acid-fast entities in the stained smear was scored. In estimating the percentage acid-fastness of a given preparation, at least 400 randomly selected entities were counted.


PERCENTAGE OF ACID-FAST ENTITIES *
100
80
60
40
20
0
1
2
3
4
5
6
TIME IN GERMINATION MEDIUM (HOURS)

morphologically immature spores or vegetative cells showed the methylene blue counterstain. Unlike bacterial spores, yeast spores are large enough to observe in unstained mounts, e.g. in the haemacytometer counting chamber. But while these experiments were in progress there was usually not enough time to make counts, and the permanent slides obtained by the acid-fast staining procedure (Appendix II) could be counted when convenient. The sporulation percentages scored were similar, whether or not unstained or acid-fast preparations were examined. For example, the sporulation percentages after 12 hours and 24 hours in sporulation medium in one experiment were 64.0% and 92.2% as determined by haemacytometer counting, and 63.2% and 90.5% by acid-fast staining.

Figure 3 shows the results of a typical sporulation experiment. Spores first appeared between 6 and 8 hours in sporulation medium. They increased in abundance up to 24 hours, and a slight further increase in the following 24 hours. A very high percentage of the cells (95.8%) sporulated.

Figure 4 shows the results of a typical spore germination experiment. Some spores lost their acid-fastness during the first 2 hours, but the greatest rate of loss was between 2 and 3 hours. By the 6th hour almost all the spores had lost their acid-fastness. By this time also most of the spores had produced a germination bud.

It is obvious that when viability is to be estimated by a quantitative plating technique as in the experiments to follow, an increase in cell population density through release of mature buds by germinating spores would affect the results. Table 2 shows the cell counts obtained with a germinating culture during a 6 hour period. When each 2-spored ascus plus the spore bud was counted as a single entity, there was no increase in cell population density during 6 hours. However, it was observed that germinating spores began to clump at 5 hours of germination,and large clumps were detected at 6 hours. But by agitation of the germination culture with a Vortex mixer, for 2 minutes, all clumps could be separated into the single entities.

Comparison of Heat Resistance of Vegetative Cells and Spores

From the work of Hansen, Chudyk and others described in the Introduction, it is evident that exposure to a temperature of 55°C should give clear distinction in the heat resistance of yeast vegetative cells and spores, but this must, of course, be checked with the strain used here, and the best length of exposure determined. Figure 5 shows that spores of 19el survived a much longer exposure to this temperature than vegetative cells, and that a 5 minute exposure killed almost all vegetative cells but only about 10% of the spores. This time of exposure at 55°C was, therefore, adopted for the subsequent heat resistance experiments.

Table 2

Determination of Cell Population Density During the Course of Germination of Spores

Time in germination medium (hours)	Cell population density* (cells/ml) x 10^7
0	1.09 ± 0.14
1	1.08 ± 0.13
2	1.06 ± 0.13
3	1.08 ± 0.17
4	1.02 ± 0.08
5	1.04 ± 0.15
6	1.07 ± 0.15

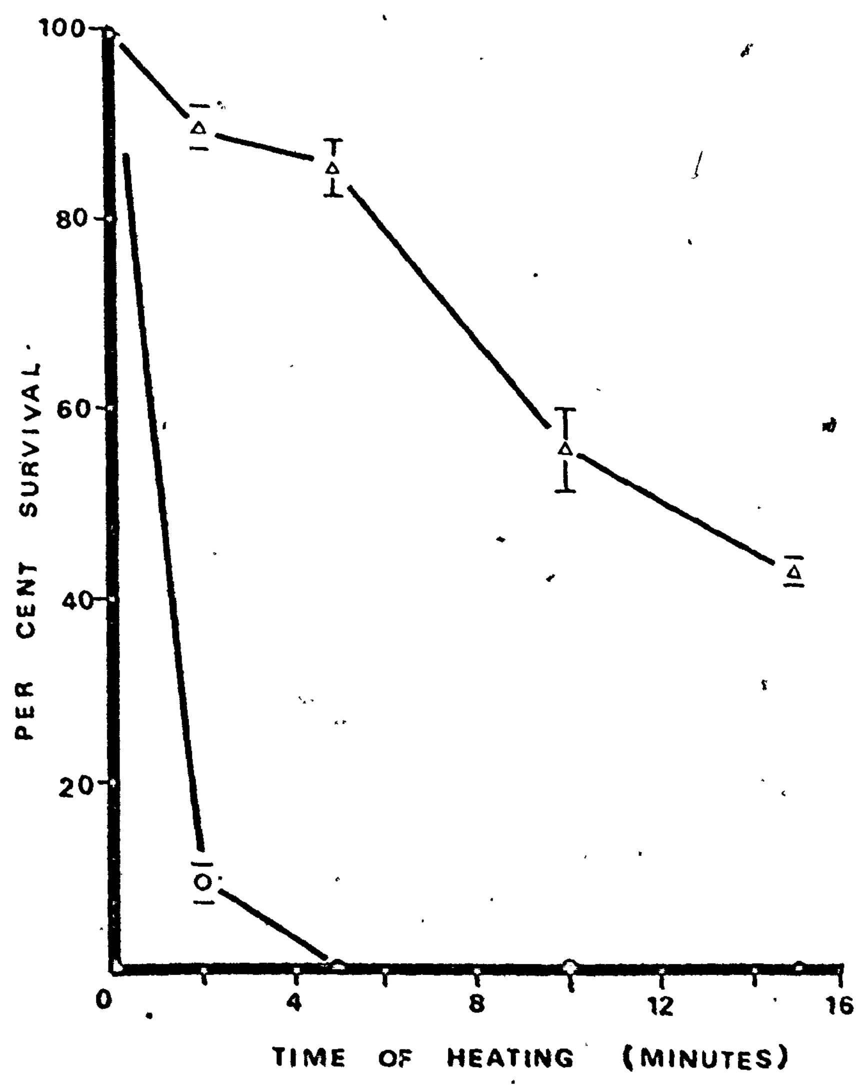
* Determined by haemacytometer counting; each number is an average based on 10 haemacytometer counting chamber fields counted in the determination, with one standard deviation shown. The figures represent cell groups, i.e., each 2-spored ascus plus the spore bud if present was counted as one entity.

Figure 5: Heat sensitivity of vegetative cells and spores determined by exposure to temperature of 55°C.

Vegetative cells (or asci) were inoculated at 1.0×10^4 cells per ml into 50 ml sterilized distilled water in a 250 ml Erlenmeyer flask preheated at 55°C. The cell suspension was shaken at 55°C and at each of the desired time intervals 7 ml of the cell suspension was withdrawn and allowed to cool to room temperature. The withdrawals were done as rapidly as possible and the flask was immediately placed back into the hot water bath after each withdrawal. Five 1.0 ml volumes (each containing 200 cells per ml) of each sample withdrawn were plated, using the pour plate method. The plates were scored after 3 days of incubation at 27°C.

o—o vegetative cells

Δ—Δ spores


100
80
60
40
20
0
4
8
12
16
PER CENT SURVIVAL
TIME OF HEATING (MINUTES)

Acquisition of Heat Resistance During Sporulation

Figure 6 shows the increase in ability of sporulating yeast cells to survive a temperature of 55°C for 5 minutes. The capacity to resist this treatment was not apparent until they had been in sporulation medium for 14 hours. There was a rapid increase in heat resistance from 20 to 24 hours. During the second 24 hours in the sporulation medium the proportion of resistant individuals in the population increased still more.

Loss of Heat Resistance During Germination

Figure 7 shows the loss of the ability, by the germinating spores, to stand 5 minutes heat treatment at 55°C. There was not much loss of heat resistance during the first hour of germination. But the loss was precipitous during the second and third hours. By the sixth hour, very few were still heat resistant.

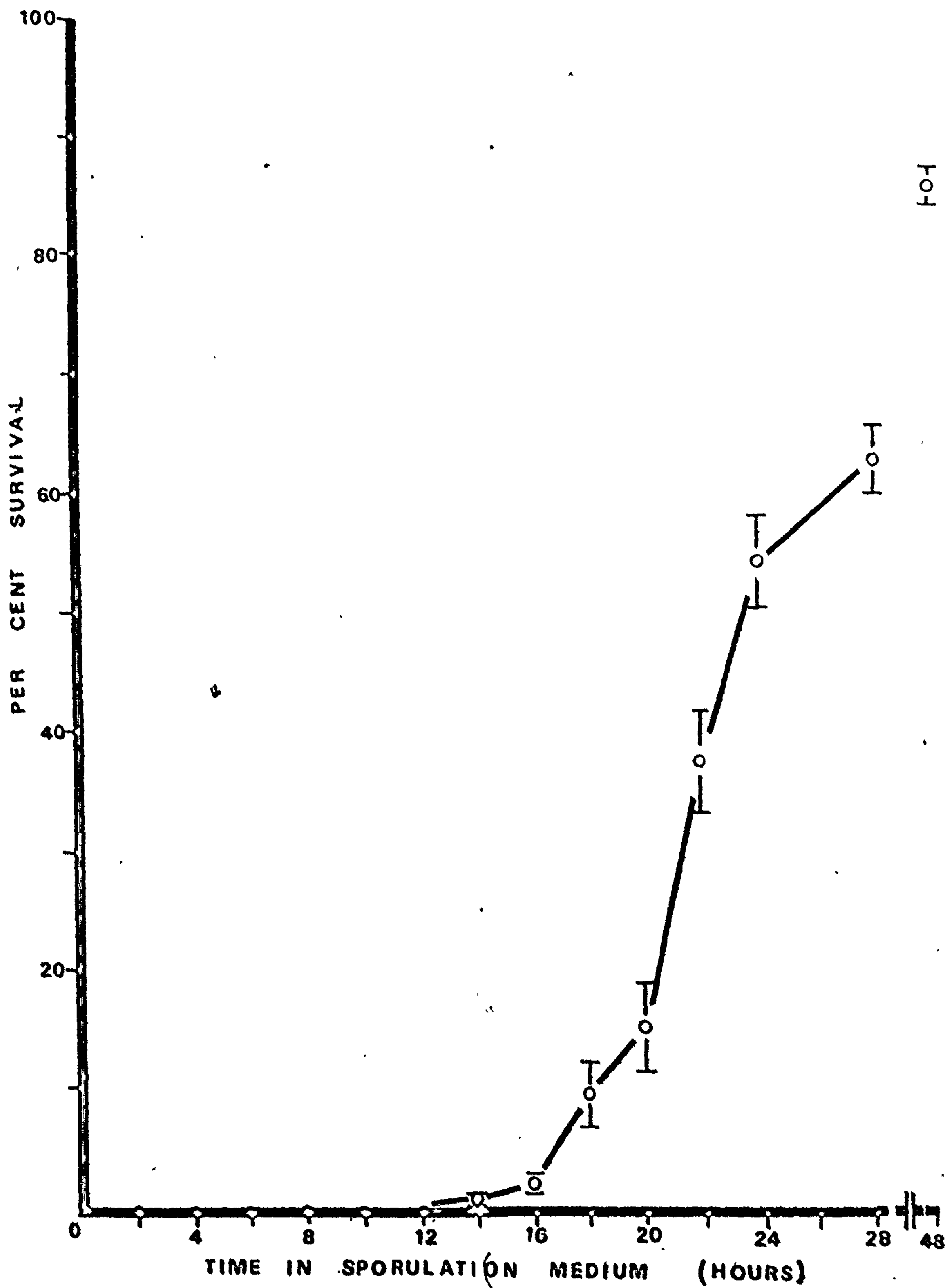
Comparison of Desiccation Resistance of Vegetative Cells and Spores

The relative sensitivity to desiccation stress of vegetative cells and spores was determined as described in Materials and Methods by exposing vegetative cells and spores

Figure 6: Acquisition of heat resistance during sporulation.

Sporulating cell samples, withdrawn at intervals from the sporulation medium, were washed by membrane-filtration. The cell density was determined and the cells were then inoculated at 1.0 x 10^4 cells per ml into 49.0 ml of preheated sterilized distilled water. After 5 minutes heating at 55°C, part of the cell suspension was withdrawn and six 1.0 ml volumes were plated. The petri dishes, each with 200 cells plated, were incubated for 3 days before they were scored for colonies.

The total length of each error bar represents the standard deviation.

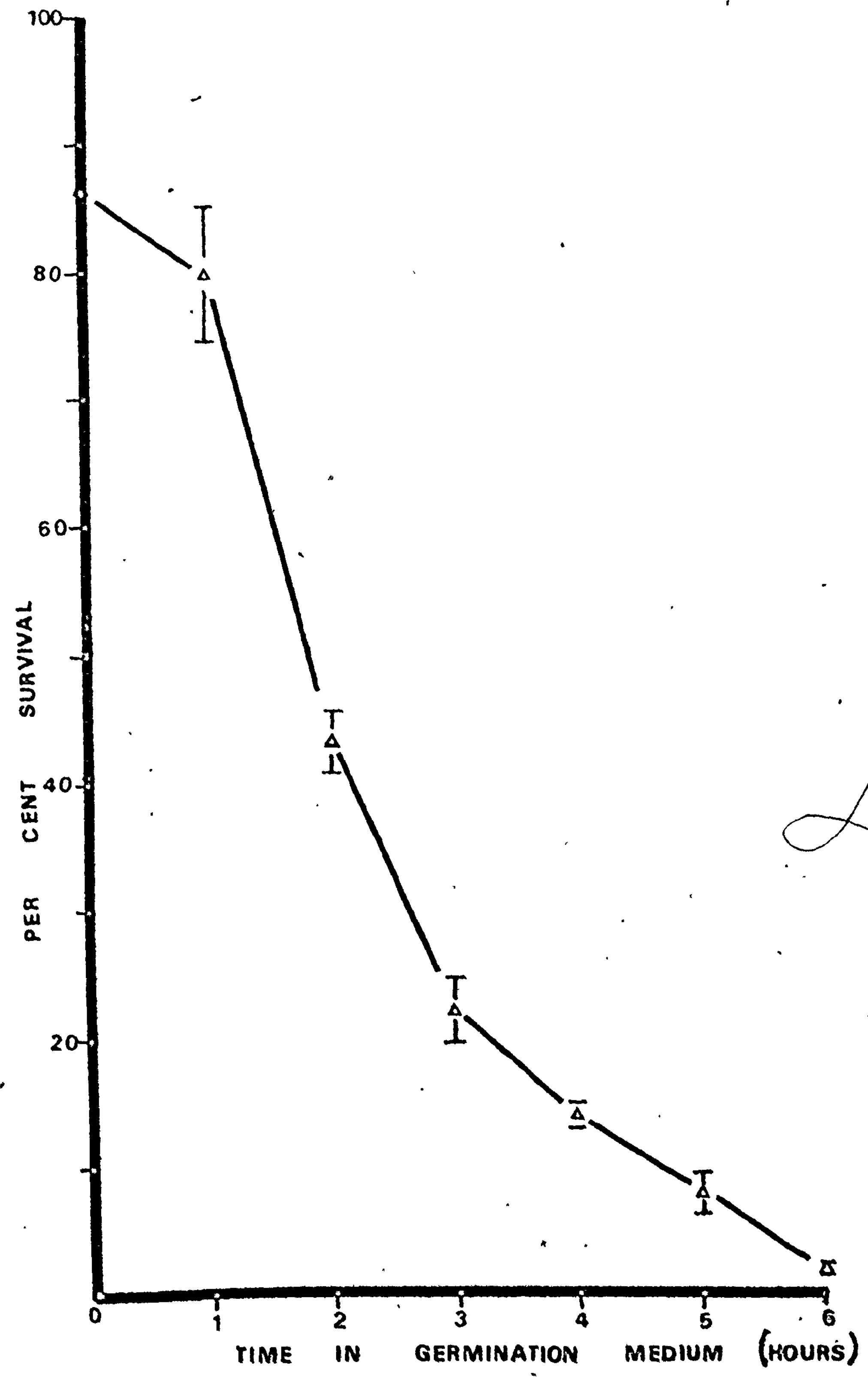


100
80
60
40
20
0
PER CENT SURVIVAL
4
8
12
16
20
24
28
48
TIME IN SPORULATION MEDIUM (HOURS)

Figure 7: Loss of heat resistance during spore germination.

Germinating cell samples, withdrawn at intervals from the germination medium, were washed by membrane-filtration. The cell density was determined and the cells were then inoculated at 1.0×10^4 cells per ml into 49.0 ml of preheated sterilized distilled water. After 5 minutes heating at 55°C, part of the cell suspension was withdrawn and six 1.0 ml volumes were plated. The petri dishes, each with 200 cells plated, were incubated for 3 days before they were scored for colonies.

The total length of each error bar represents the standard deviation.


100
80
60
40
20
PER CENT SURVIVAL
0
1
2
3
4
5
6
TIME IN GERMINATION MEDIUM (HOURS)

on the surface of membrane filters to the atmosphere in a desiccator containing anhydrous silica gel for periods of up to 24 hours. Figure 8 shows the percentage survival of yeast in these two states. The vegetative cells were killed within one hour, whereas the viability of the spores was not appreciably altered after 24 hours.

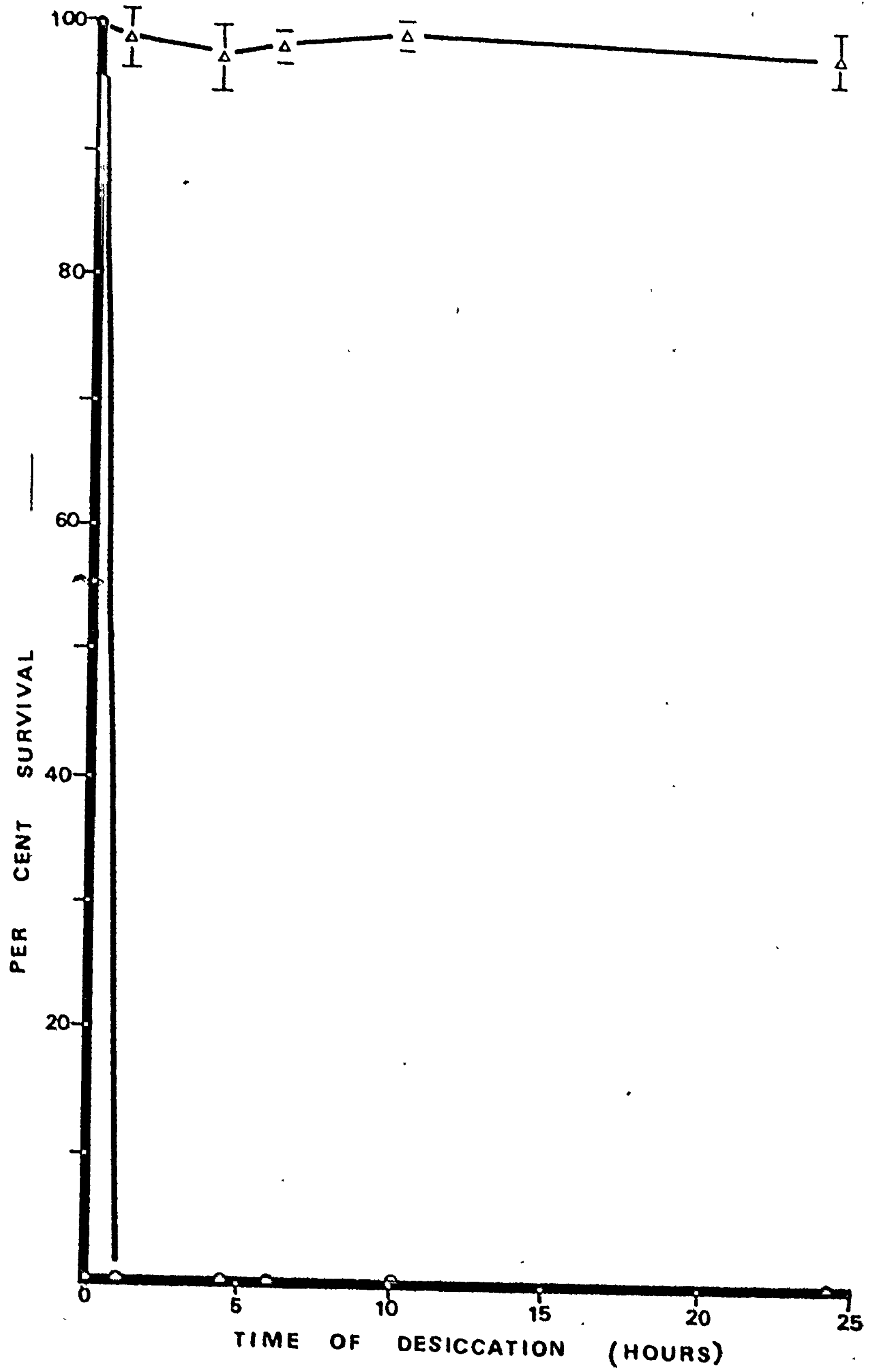
An important factor in selecting a standard exposure time was that the exposures with some treatments should be completed in the morning so that the rest of the day could be available for preparing suspensions of all the treated cells and plating them in MYPG agar. One hour or else a full day (24 hours) would meet this requirement. Another factor was that the yeast should be in equilibrium with the dry air for most of the period. An experiment was therefore done to determine how long it would take for the moist membrane filter bearing the cells to dry to constant weight in the presence of anhydrous silica gel. A Whatman number 1 filter paper 7.0 cm in diameter, bearing a membrane filter was dried in an oven at 100°C overnight and then transferred to the weighing pan of a Mettler analytical balance (type B6) for 2 hours. The balance chamber contained 250 g anhydrous silica gel in four watch glasses. The filter paper plus membrane filter were then weighed. The filter was removed and used to filter 2×10^7 vegetative cells from 100 ml distilled water in the filter holder. The membrane filter was removed from the

Figure 8: Survival of vegetative cells and spores in desiccator in presence of anhydrous silica gel at 27°C.

Vegetative cells (or asci) (2×10^7) were filtered with a 0.45 μ membrane filter. The membrane filter with the cells was put into a glass desiccator (containing about 250 g anhydrous silica gel) and desiccated in the dark at 27°C. After the desired time of desiccation, the membrane filter was taken out, and a quarter of the membrane was cut out. The desiccated cells on the cut-out sector were resuspended in 2.5 ml sterilized distilled water. The density of the resulting cell suspension was counted by haemacytometer, and the suspension was diluted appropriately to 200 cells per ml. Five 1.0 ml volumes of the final suspension were plated in MYPG agar. After 3 days incubation at 27°C, the plates were scored.

o–o vegetative cells

Δ–Δ spores



100
80
60
40
20
PER CENT SURVIVAL
0
5
10
15
20
25
TIME OF DESICCATION (HOURS)

holder and placed on the filter paper on the weighing pan. Weighings were made periodically for 135 minutes. The results, shown in Figure 9, indicate that at least 2 hours was required for the membrane filter bearing the cells to reach equilibrium with the dry air in the balance chamber.

On the basis of this experiment, 24 hours rather than one hour was adopted as the standard period of exposure to silica gel in the desiccator in the desiccation resistance experiments.

Acquisition of Desiccation Resistance During Sporulation

Figure 10 follows the acquisition of resistance to desiccation during sporulation. Some sporulating cells acquired desiccation resistance within 2 hours in sporulation medium, but large numbers of resistant cells were not present until 10 to 12 hours. At 24 hours, 76% of the population of sporulating cells had acquired desiccation resistance. Within 48 hours almost all had become resistant.

Loss of Desiccation Resistance During Germination

Figure 11 shows the decrease in the capacity of germinating spores to survive the desiccation treatment. There was a gradual loss of desiccation resistance during

Figure 9: Determination of rate of loss of moisture from vegetative cells and membrane filter in Mettler Balance weighing chamber.

About 250 g anhydrous silica gel in four watch glasses was placed in the weighing chamber for 2 hours. 2×10^7 vegetative cells in 100 ml water were filtered through a Gelman GN-6 0.45 μ membrane filter. The moist membrane filter bearing the cells was immediately placed on a dry Whatman number 1 filter paper (7.0 cms), laid on the weighing pan of the balance, and the slide doors of the weighing chamber were closed tightly. At intervals, the weight of the filter paper and the membrane with cells was read. The total weight of the dry membrane filter (without cells) + dry Whatman filter paper was determined to be 0.4049 g before the experiment.

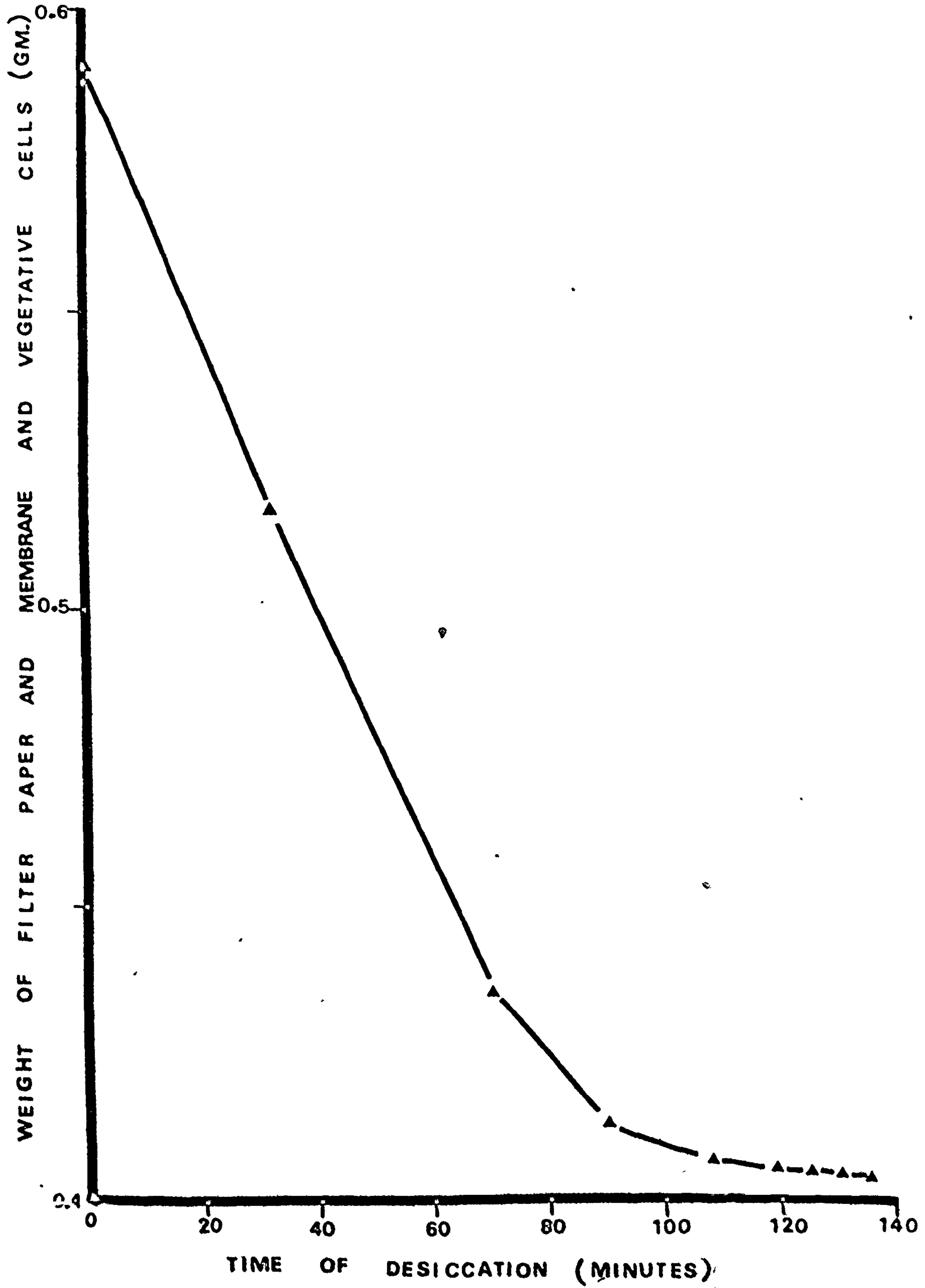

0.6
0.5
0.4
WEIGHT OF FILTER PAPER AND MEMBRANE AND VEGETATIVE CELLS (GM.)
0
20
40
60
80
100
120
140
TIME OF DESICCATION (MINUTES)

Figure 10: Acquisition of desiccation resistance during sporulation.

Two ml sporulating cell samples, withdrawn at intervals from the sporulating medium, were retained and washed by membrane filtration. The membrane filter with cells was put into desiccator with silica gel. After 24 hours of desiccation, the membrane filter was taken out, and the desiccated cells on the membrane were resuspended in sterilized distilled water. The cell density of the resulting cell suspension was counted, and the suspension was diluted to 200 cells per ml. Six 1.0 ml volumes of the final suspension were plated in MYPG agar. After 3 days incubation, the plates were scored.

The total length of each error bar represents the standard deviation.

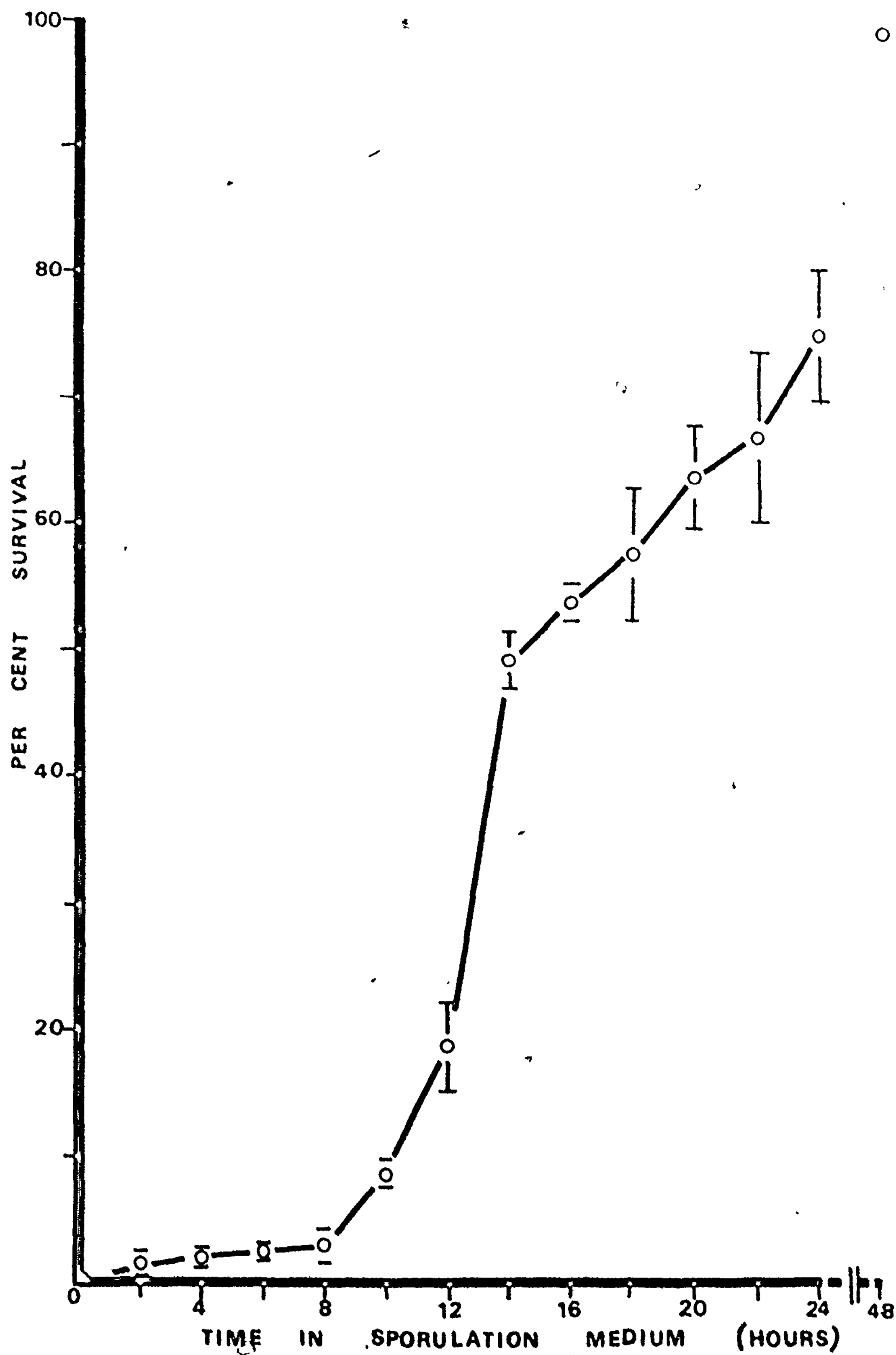
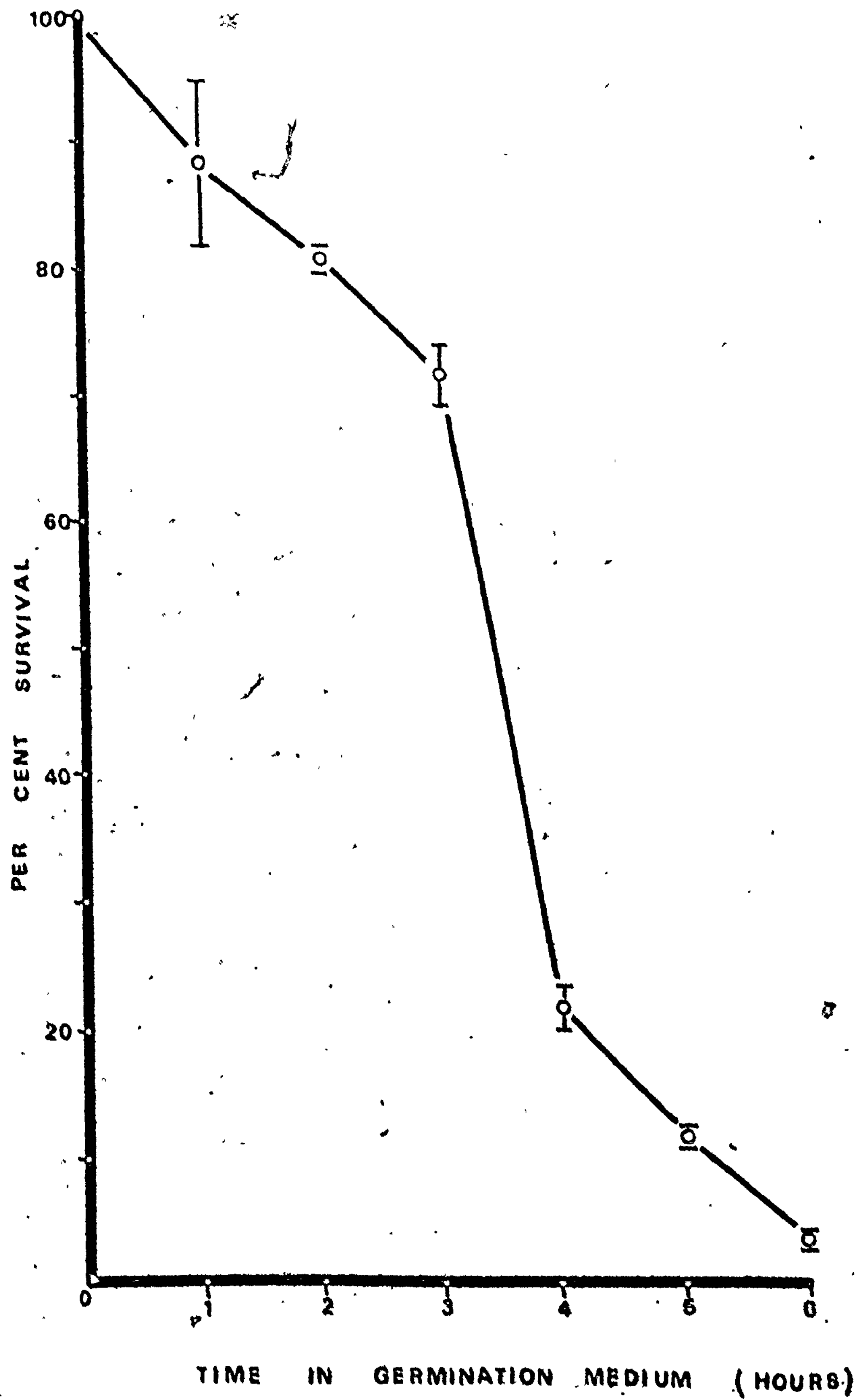

100
80
60
40
20
0
PER CENT SURVIVAL
4
8
12
16
20
24
48
TIME IN SPORULATION MEDIUM (HOURS)

Figure 11: Loss of desiccation resistance during spore germination.

Two ml germinating cell samples, withdrawn at intervals from the germination medium, were retained and washed by membrane-filtration. The membrane filter with cells was put into desiccator with silica gel. After 24 hours of desiccation, the membrane filter was taken out, and the desiccated cells on the membrane were resuspended in sterilized distilled water. The cell density of the resulting cell suspension was counted, and the suspension was diluted to 200 cells per ml. Six 1.0 ml volumes of the final suspension were plated in MYPG agar. After 3 days inoculation, the plates were scored.

The total length of each error bar represents the standard deviation.


100
80
60
40
20
0
1
2
3
4
5
6
PER CENT SURVIVAL
TIME IN GERMINATION MEDIUM (HOURS)

the first three hours of germination. However, there was a large decline in desiccation resistance in the population between the third and fourth hours in germination medium. At the end of 6 hours, almost the whole population of germinating spores were sensitive to the desiccation treatment.

Changes in Content of Free Proline During Sporulation and Spore Germination

The standardization curve (Figure 12) confirms that the method of Troll and Lindsley (1955) will measure proline concentrations of the order of 10^{-5} M.

The free proline content in vegetative cells grown in the MYPG medium was 6.1×10^{-5} g per 5×10^{8} cells. But when these vegetative cells were placed in sporulation medium, the proline content dropped, especially during the first hour (Figure 13) and reached a minimum at 4 hours. Later the proline content began to rise and the increase was more rapid after 16 hours. After 48 hours sporulation, the free proline content was 2.9×10^{-4} g per 5×10^{8} asci.

The change in free proline content with time during germination is shown in Figure 14. The proline content decreased continually and the loss was most rapid between one and two hours. By the sixth hour, intracellular proline was almost undetectable.

Figure 12: Standardization curve for absorbance by proline solutions at 515 nm.

A series of proline solutions of known molarities was prepared. Five ml of each of these was heated with 5 ml of glacial acetic acid and 5 ml of acidic ninhydrin in a boiling water bath for 1 hour in a 30 ml screw-capped test-tube. The solution was cooled and extracted with 5.0 ml of benzene for 5 minutes. The optical density of the benzene phase separated was determined at 515 nm with a Bausch and Lomb model 340 colorimeter. Pure benzene was used as the blank.

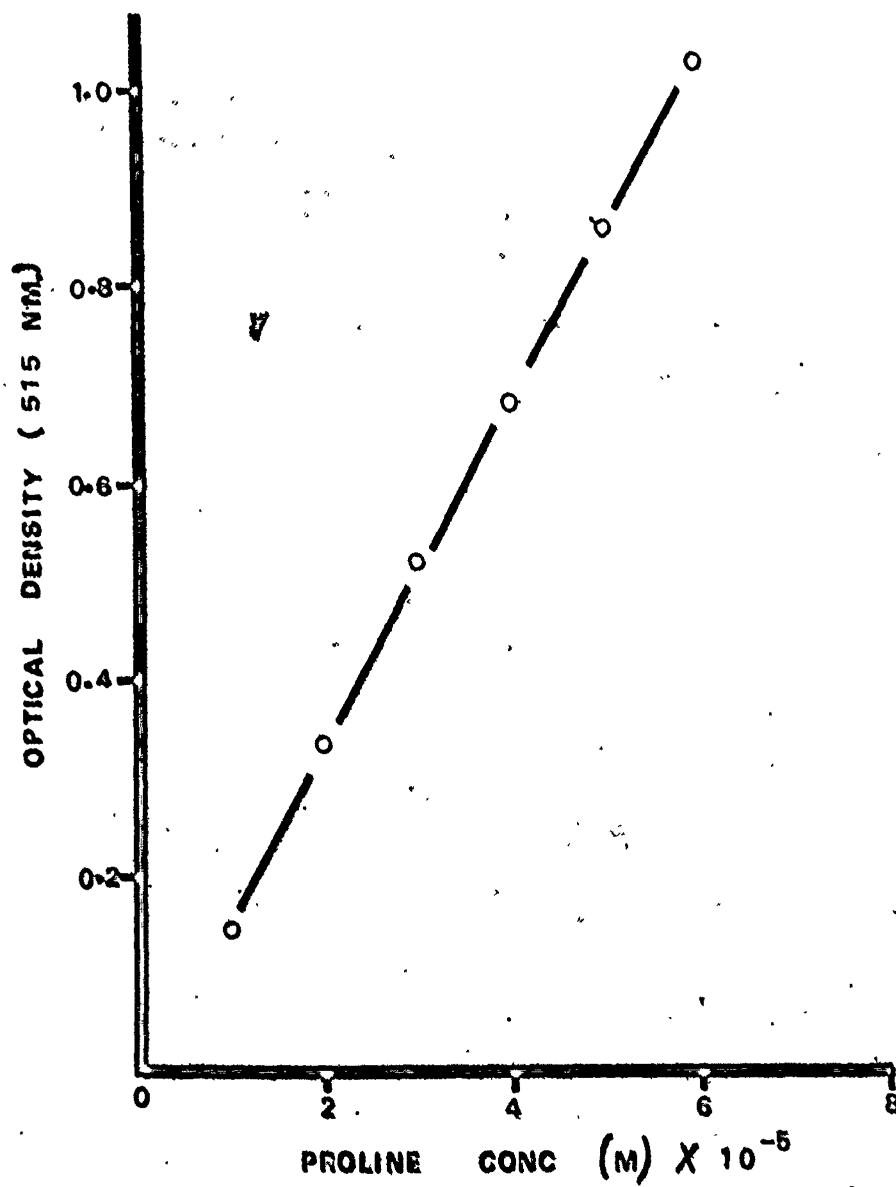
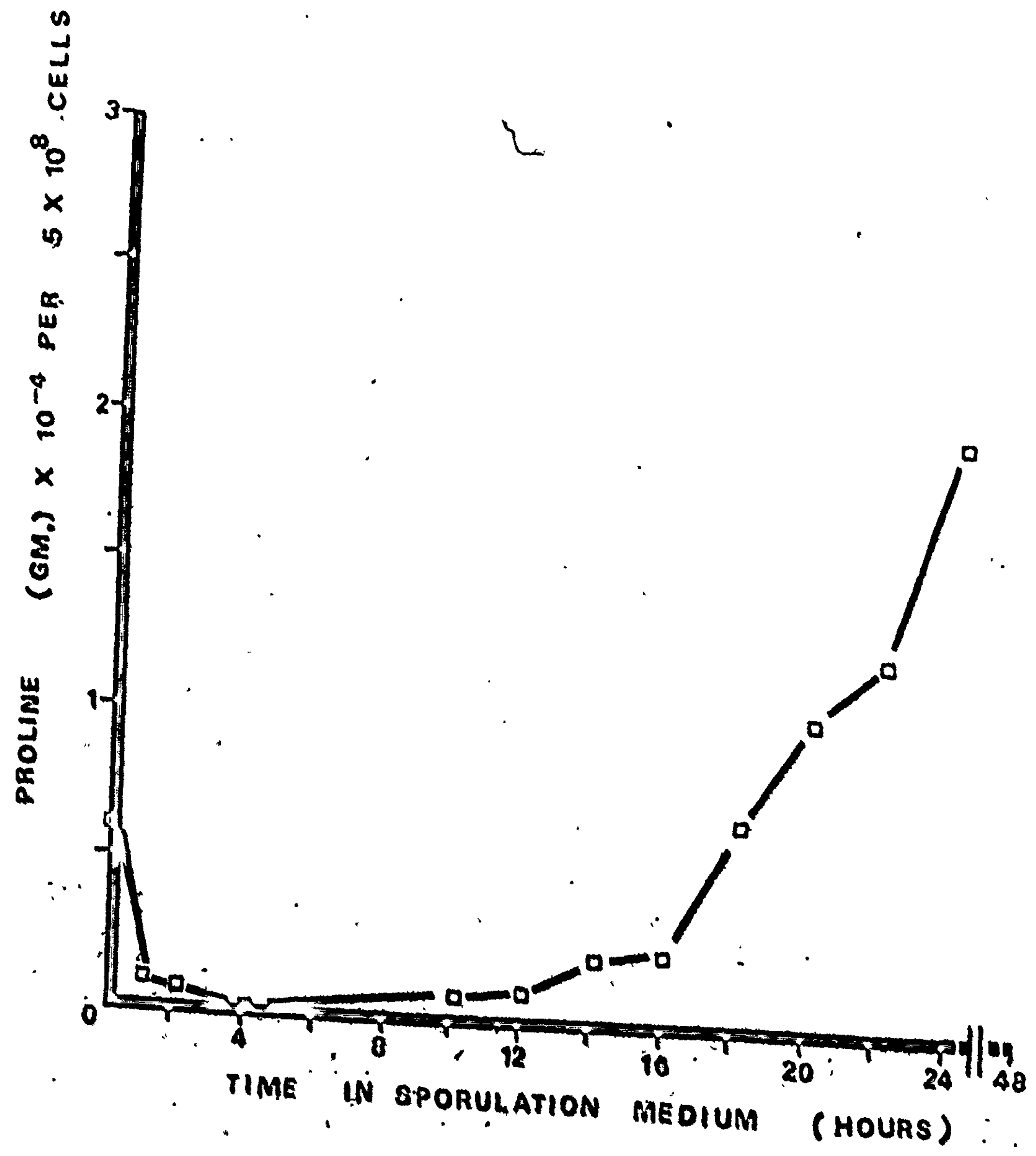

OPTICAL DENSITY (515 NM)
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
PROLINE CONC (M) X 10^-5

Figure 13: Change in amount of free proline in yeast cells during sporulation.

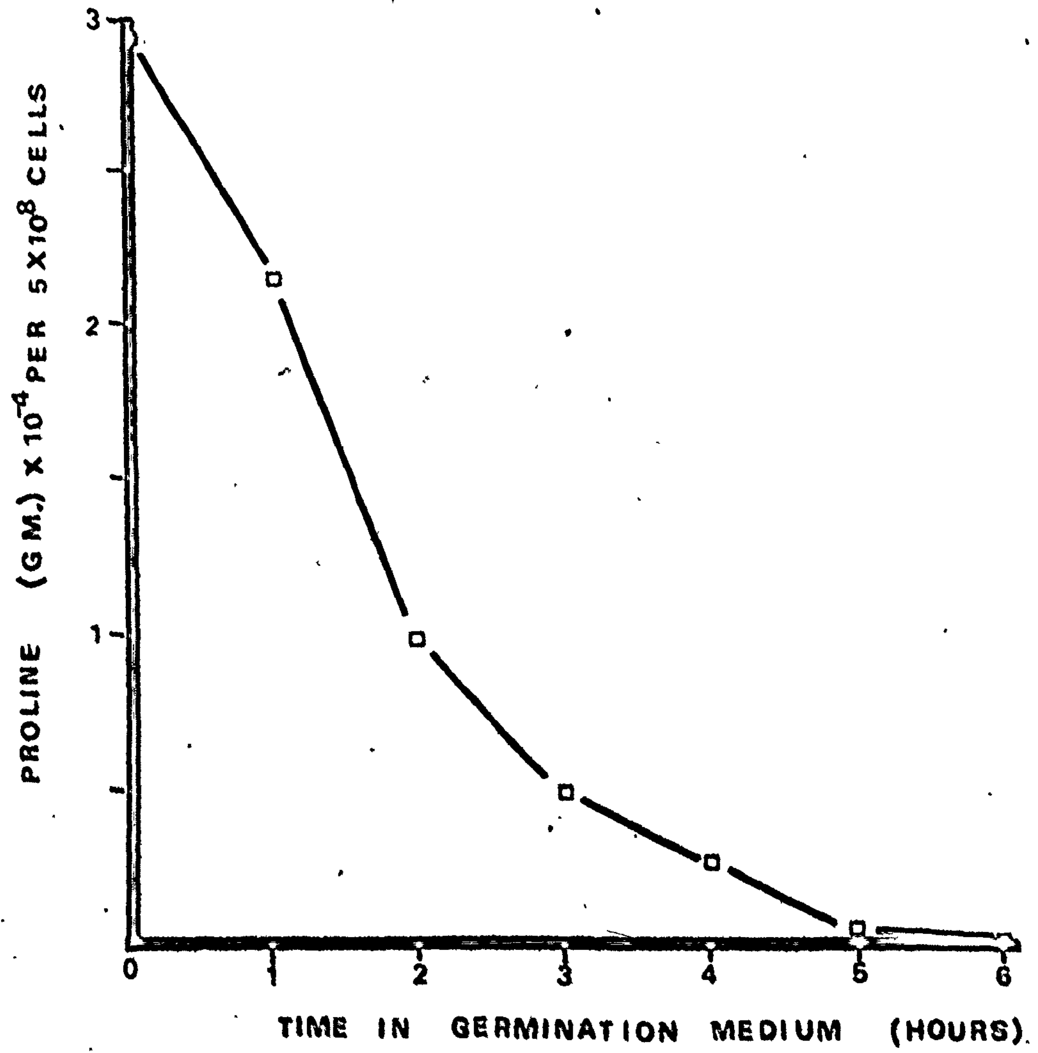
Fifty ml samples of sporulation medium each containing 5×10^8 cells, were subjected to centrifugation and washing. The cells separated were extracted twice with 95% ethanol at 70°C. The combined alcohol extract was concentrated by boiling under partial vacuum. The concentrated extract was then extracted twice with chloroform. The final aqueous extract, after chloroform extraction, was made up to 10.0 ml, with distilled water, 5.0 ml of which was treated with Permutit and then heated with 5 ml glacial acetic acid and 5 ml acidic ninhydrin for 1 hour at 100°C. The solution was then extracted with 5.0 ml benzene. The optical density of the benzene phase was measured at 515 nm.



PROLINE (GM.) X 10^{-4} PER 5 X 10^{8} CELLS
3
2
1
0
4
8
12
16
20
24
48
TIME IN SPORULATION MEDIUM (HOURS)

Figure 14: Change in amount of free proline in yeast cells during spore germination.

Fifty ml samples of germination medium, each containing 5×10^8 cells, were subjected to centrifugation and washing. The cells separated were extracted twice with 95% ethanol at 70°C. The combined alcohol extract was concentrated by boiling under partial vacuum. The concentrated extract was then extracted twice with chloroform. The final aqueous extract, after chloroform extraction, was made up to 10.0 ml with distilled water, 5.0 ml of which was treated with Permutit and then heated with 5 ml glacial acetic acid and 5 ml acidic ninhydrin for 1 hour at 100°C. The solution was then extracted with 5.0 ml benzene. The optical density of the benzene phase was measured at 515 nm.



PROLINE (GM.) X 10^{-4} PER 5×10^{8} CELLS
3
2
1
0
1
2
3
4
5
6
TIME IN GERMINATION MEDIUM (HOURS)

Detection of Proline in Germination Medium

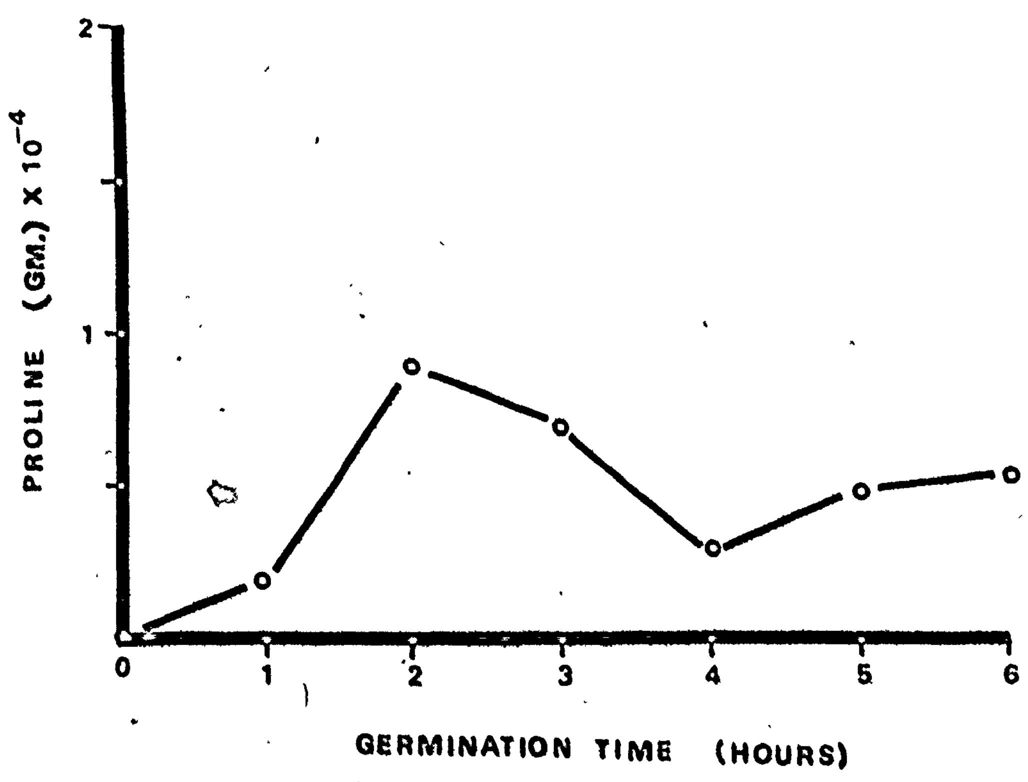
The decline in proline content in germinating spores could result from its excretion into the germination medium or through its metabolic conversion to other substances. Figure 15 shows that the germination medium, which initially contained only ammonium sulfate as source of nitrogen, acquired an appreciable content of proline in one hour. A comparison with Figure 14 indicates that not all the free proline originally in the spores was excreted and presumably some of it was metabolized by the germinating spores. The amount of proline in the medium increased to a maximum of 8.9×10^{-5} g in 2 hours and this was equivalent to 46% of the intracellular free proline lost. After 2 hours germination, the proline in the medium declines, which indicates that part of the proline which had been excreted into the medium was reabsorbed and utilized by the germinating spores.

Changes in Content of Alpha Amino Acids During Sporulation and Spore Germination

Rosen's method (1957) for amino acid determination was used to estimate changes in intracellular content of alpha-amino acids using L-alanine as the standard (standardization curve, Figure 16). Within the first hour

Figure 15: Change in amount of free proline in the germination medium during spore germination.

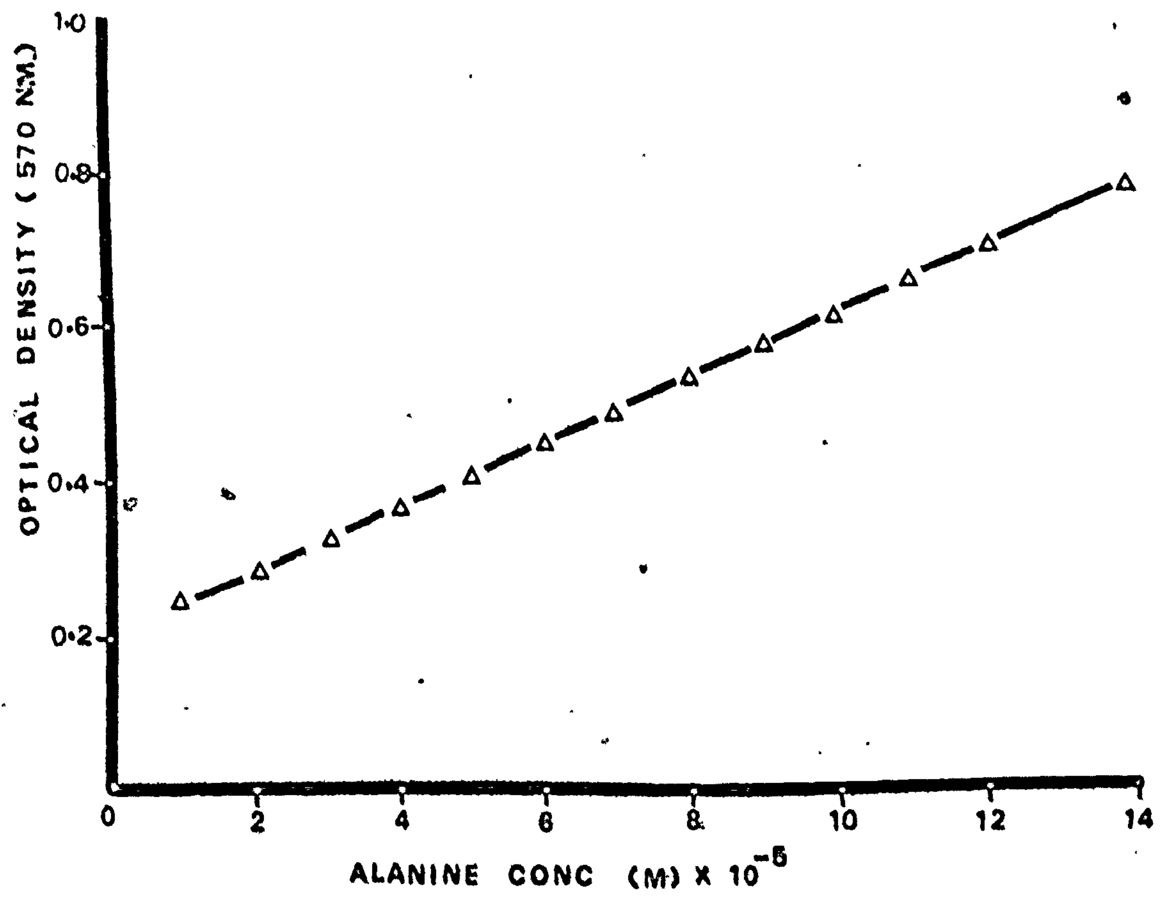
Spores (5×10^8) were allowed to germinate in 50 ml germination medium. After the desired time of germination, the cells were separated from the germination medium by filtration with a 0.45 μ membrane filter. The filtrate obtained was reduced in volume to 5 ml in a 125 ml round-bottomed Pyrex flask connected to a partial vacuum and simultaneously heated in a boiling bath. The concentrated germination medium was treated with Permutit and then heated with 5 ml glacial acetic acid and 5 ml acidic ninhydrin for 1 hour at 100°C. The solution was then extracted with 5.0 ml benzene. The optical density of the benzene phase was measured at 515 nm.



PROLINE (GM.) X 10^-4
2
1
0
1
2
3
4
5
6
GERMINATION TIME (HOURS)

Figure 16: Standardization curve for absorbance by alanine solutions at 570 nm.

A series of alanine solutions of known molarities were prepared. One ml of each of these was heated with 0.5 ml cyanide-acetate buffer and 0.5 ml 3% ninhydrin solution in Methyl Cellosolve in a screw-capped test-tube in a boiling water bath for 15 minutes. Immediately after removal from the water bath, 5.0 ml of 1:1 isopropyl alcohol-water was added rapidly to the boiling solution. The resulting solution was shaken, cooled and its optical density was read at 570 nm in a Bausch and Lomb model 340 colorimeter. 1:1 isopropanol-water was used as the blank.


1.0
0.8
0.6
0.4
0.2
OPTICAL DENSITY (570 N.M.)
0
2
4
6
8
10
12
14
ALANINE CONC (M) X 10⁻⁵

of sporulation, there was a sharp decrease in alpha-amino acids (Figure 17). After 10 hours sporulation, an increase was evident, which continued up to 24 hours, and decreased somewhat by 48 hours.

There was no marked change in alpha-amino acid content in the germinating spores during the first four hours of germination (Figure 18). By five hours, there was an obvious decline to about 20% of the initial content in the spores.

Proline Content of Vegetative Cells and Spores Expressed as Percentage of Dry Weight

The total weight of a membrane filter and a Whatman number 1 filter paper (7.0 cms in diameter) was determined by a Mettler analytical balance. The membrane and the filter paper were first dried overnight in the silica gel desiccator. The membrane filter was placed in a membrane-filtration holder and a known number of yeast cells were retained by the membrane filter. The membrane with cells was placed on the filter paper and put in the desiccator with silica gel for 24 hours. The total weight of membrane plus cells and filter paper after desiccation was determined. The membrane bearing the cells was then placed in an oven at 100°C overnight. Finally, the total weight of dried cells, membrane filter and filter paper was determined.

Figure 17: Change in content of free alpha amino acids in yeast cells during sporulation.

Fifty ml samples of sporulation medium, each containing 5×10^8 cells, were subjected to centrifugation and washing. The cells separated were extracted twice with 95% ethanol at 70°C. The combined alcohol extract was concentrated by boiling under partial vacuum. The concentrated extract was then extracted twice with chloroform. The final aqueous extract, after chloroform extraction, was made up to 10.0 ml with distilled water, 1.0 ml of which was heated with 0.5 ml cyanide-acetate buffer and 0.5 ml 3% ninhydrin solution in Methyl Cellosolve at 100°C. After 15 minutes boiling, 5.0 ml of 1:1 isopropyl alcohol-water was added to the solution, and the optical density of the resulting solution was determined at 570 nm.

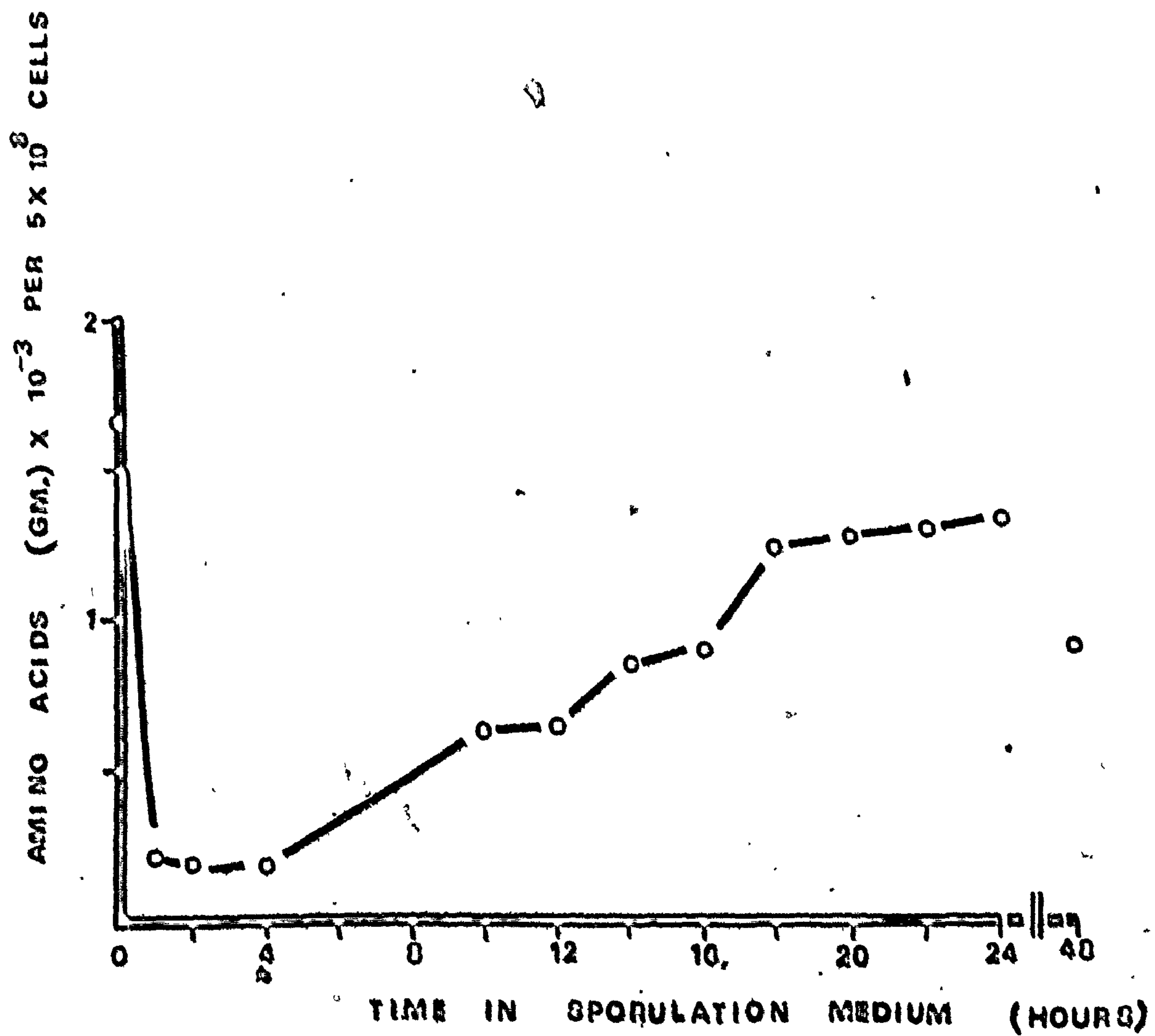
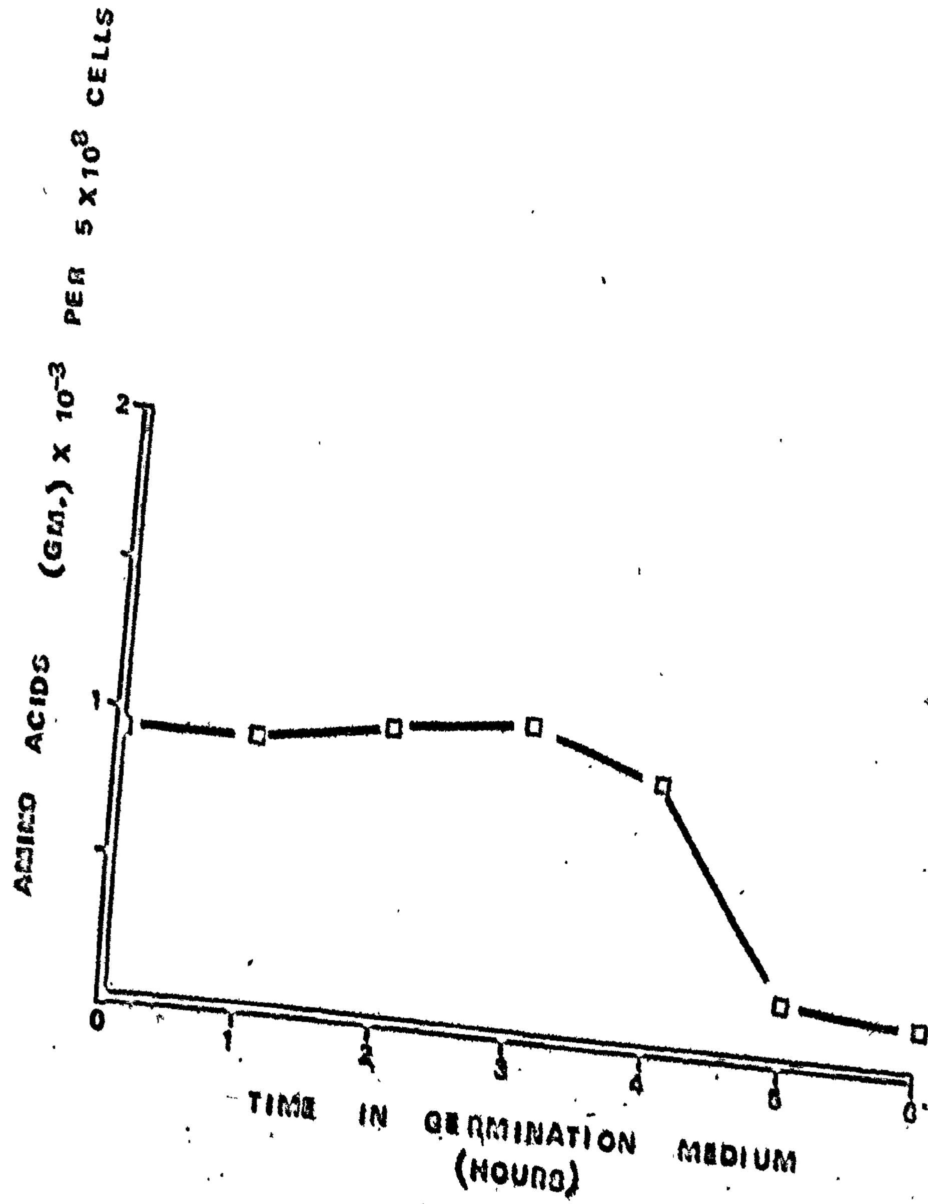

AMINO ACIDS (GM.) X 10^{-3} PER 5 X 10^{8} CELLS
2
1
0
4
8
12
16
20
24
40
TIME IN SPORULATION MEDIUM (HOURS)

Figure 18: Change in content of free alpha amino acids in yeast cells during spore germination.

Fifty ml samples of germination medium, each containing 5×10^8 cells, were subjected to centrifugation and washing. The cells separated were extracted twice with 95% ethanol at 70°C. The combined alcohol extract was concentrated by boiling under partial vacuum. The concentrated extract was then extracted twice with chloroform. The final aqueous extract, after chloroform extraction, was made up to 10.0 ml with distilled water, 1.0 ml of which was heated with 0.5 ml cyanide-acetate buffer and 0.5 ml 3% ninhydrin solution in Methyl Cellosolve at 100°C. After 15 minutes boiling, 5.0 ml of 1:1 isopropyl alcohol-water was added to the solution, and the optical density of the resulting solution was determined at 570 nm.


AMINO ACIDS (GM.) X 10-3 PER 5 X 108 CELLS
2
1
0
1
2
3
4
5
6
TIME IN GERMINATION MEDIUM
(HOURS)

The weight of cells after desiccation and the dry weight were determined for both vegetative cells and spores. The weight of 5 x 10^8 desiccated vegetative cells was 1.30 x 10^{-2} g and the oven-dried weight was 1.21 x 10^{-2} g. 5 x 10^8 asci weighed 2.00 x 10^{-2} g after desiccation and the oven dried weight was 1.91 x 10^{-2} g. Thus, the dry weight of an ascus is greater than that of the vegetative cell from which it developed, and this has been observed by others (e.g., Roth, 1970). It should also be noted that the "bound" water[1] content of asci (4.7% of the dry weight) was less than that of vegetative cells (7.4% of the dry weight). But on a cell to cell basis, vegetative cells and asci contained approximately the same amount of "bound" water.

It was calculated that free proline constituted 0.5% and 1.5% of the dry weight of vegetative cells and spores, respectively.

Effects of Exogenously Supplied Proline on Sensitivity of Yeast Cells to Desiccation

(A) Vegetative cells

As noted in the Introduction, there is evidence of a correlation between desiccation resistance of certain cells and their proline content. It is, therefore of interest to supply non-resistant yeast cells with exogenous

[1] The concept of "bound" water in microorganisms as discussed by Lamanna and Mallette (1965) is difficult to define. But in the sense used here it means the amount of water driven off by the dry heat less the amount removed by the silica gel desiccant.

proline and observe whether this affected their resistance to desiccation.

Vegetative cells were inoculated at a density of 4×10^5 cells/ml into 50 ml volumes of distilled water containing 0,0.1,0.5 and 1.0 M proline. These cell suspensions in 250 ml Erlenmeyer flasks were incubated in the water-bath shaker at 27°C for one hour. Then, the cells were recovered by filtration through membrane filters. Without washing, the membrane filters were put in the desiccator for 24 hours. Then approximately one-quarter sector of each membrane filter was cut out and then shaken vigorously for 2 minutes in 2.5 ml sterilized distilled water in a 50 ml Erlenmeyer flask. The density of the resulting cell suspension was determined with the haemacytometer counting chamber. Ten fields were counted for each treatment. The cell suspension was diluted to 4.0×10^3 cells/ml, and six 1.0 ml volumes of this final suspension were plated in MYPG medium. The plates were scored after 3 days of incubation at 27°C.

Table 3 shows the effect of these proline treatments on the desiccation resistance of vegetative cells. Proline at a concentration of 0.1 M increased the number of survivors, but nevertheless survival was low, 2.3 cells per 4,000, as compared with 0.6 per 4,000 in the absence of proline. This increase in desiccation resistance, although small, was significant (Table 4). Treatments

Table 3

Effect of Exogenously Supplied Proline on Desiccation Resistance of Vegetative Cells

Solution in which cells were suspended for 1 hour before desiccation	Average number of colonies developed per petri dish after desiccation treatment*		Frequency of small colonies among the colonies that developed	
	expt. i	expt. ii	expt. i	expt. ii
distilled water	0.66 ± 0.58	0.33 ± 0.38	25%	0%
0.1 M proline solution	2.3 ± 1.1	2.0 ± 0.5	7.1%	8.3%
0.5 M proline solution	0.16 ± 0.34	0.33 ± 0.68	0%	50%
1.0 M proline solution	0.16 ± 0.34	0	100%	-

* Out of 4000 cells plated per petri dish, each number is the average of 6 replicate petri dishes, with one standard deviation shown.

Table 4

One-way Analysis of Variance of the Results of the Experiments on the Effect of Exogenously Supplied Proline on Desiccation Resistance of Vegetative Cells

(refer Table 3)*

Samples compared	F value obtained	Difference between the means of the two samples
d. water treatment (expt. i) and 0.1 M proline treatment (expt. i).	7.8	not significant at 1% level of significance, but significant at 5% level.
d. water treatment (expt. ii) and 0.1 M proline treatment (expt. ii).	24.9	significant at 1% level of significance.
d. water treatment (expt. i) and d. water treatment (expt. ii).	0.70	not significant at 5% level of significance.
0.1 M proline treatment (expt. i) and 0.1 M proline treatment (expt. ii).	0.98	not significant at 5% level of significance.

* For comparison of two samples, each of 6 scores, the critical value of F at $\alpha = 0.05$ is 4.96

at $\alpha = 0.01$ is 10.04.

with 0.5 and 1.0 M proline did not increase the survival of the vegetative cells.

When the plates were scored, a few tiny, white, compact colonies, which appeared quite different from normal colonies were found. Cells from these tiny colonies were transferred to MYPG slants. After 2 days growth on the slants, examination under the light microscope revealed that the cells were very small and spherical (Plate 1). Their diameter was 0.81 ± 0.12 microns, based on 50 cells measured, whereas the dimensions of the normal, oval vegetative cells were 5.1 ± 0.8 microns (major axis) and 3.3 ± 0.3 microns (minor axis), based on 50 cells measured. The tiny cells had a strong tendency to remain together in small groups or tetrads, and often adhered in large clumps. Budding was not obvious among the cell populations. However, few details of the cellular structure could be seen by light microscopy. These cells were suspected to be mutant.

(B) Sporulating cells

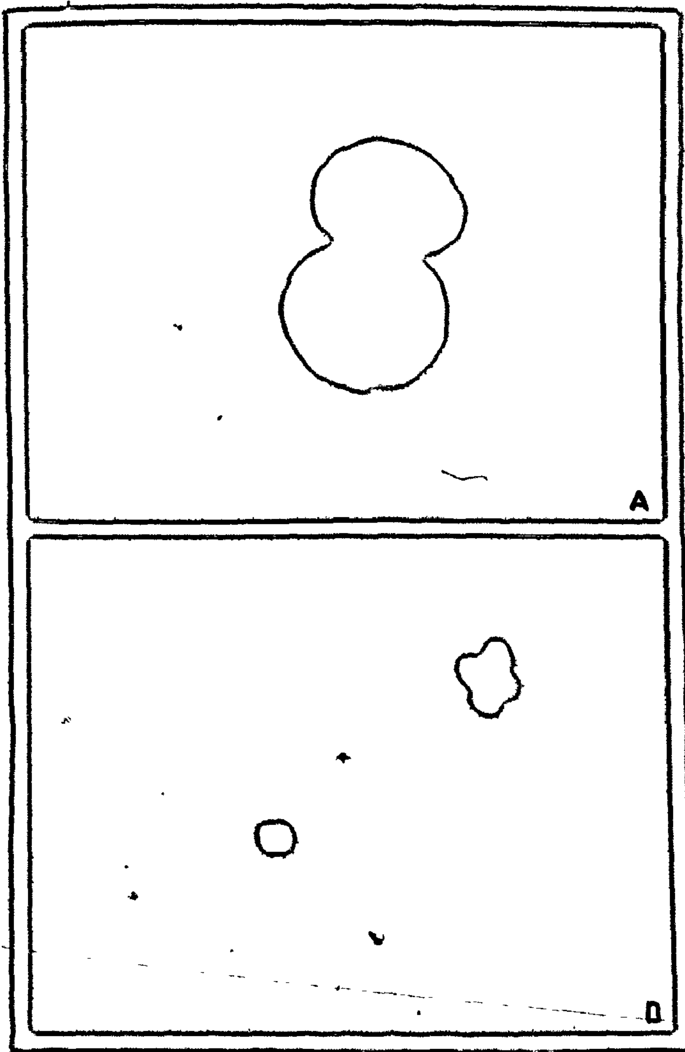
From the foregoing there was no strong increase in resistance induced by exogenous proline. To test whether cells later in the sporulation process are protected to a greater extent by exogenous proline, experiments were done in which cells were removed from the sporulation medium after 7 1/2 hours. From Figure 10, cells at this

Plate 1: <u>Photomicrographs of 19e1 normal vegetative cells and small cells derived.</u>

The cells had been stained with ammonium oxalate crystal violet, using the bacteriological dry smear technique. Magnification X 4000.

(A) normal vegetative cells.

(B) small cells.


A
B

stage would not be very resistant to desiccation but would be expected to increase considerably in resistance shortly afterward. The cells were freed of potassium acetate by washing with sterilized distilled water, and inoculated at a density of 4×10^5 cells/ml into 50 ml volumes distilled water containing 0,0.1,0.5 and 2.0 M proline, and 0.1 M alanine. These cell suspensions in 250 ml Erlenmeyer flasks were incubated in water-bath shaker at 27°C for one hour. The subsequent procedure was the same as that for the experiment on the effect of exogenously supplied proline on desiccation resistance of vegetative cells, except that the final cell suspension for plating was 500 cells/ml instead of 4,000 cells/ml. Fewer cells were plated here so that the expected greater survival would not overcrowd the plates.

There was an obvious increase in survival of the 7 1/2 hour sporulating cells, as a result of treatment with 0.1 M proline before desiccation (Tables 5 and 6). But treatments with 0.5 M and 2.0 M proline before desiccation reduced the resulting survival. Treatment with 0.1 M alanine, on the other hand, did not affect the survival after desiccation.

The same suspected mutant as in Table 3 was again found. They were fairly frequent after the treatment with 0.5 M proline (Table 5).

Table 5

Effect of Exogenously Supplied Proline and Alanine on Desiccation Resistance of Sporulating Cells (7 1/2 Hours in Sporulation Medium)

Solution in which the cells were suspended for 1 hour before desiccation	Average number of colonies developed per petri dish after desiccation treatment*		Frequency of small colonies among the colonies that developed	
	expt. i	expt. ii	expt. i	expt. ii
distilled water	32.0 ± 1.4	30.3 ± 1.9	0.5%	0%
0.1 M proline solution	73.8 ± 6.1	75.1 ± 2.8	0%	0%
0.5 M proline solution	1.3 ± 0.7	1.0 ± 0.9	12.5%	16.6%
2.0 M proline solution	0	0	-	-
0.1 M alanine solution	29.5 ± 2.0	23.8 ± 1.3	0%	0%

* Out of 500 cells plated per petri dish; each number is the average of 6 replicate petri dishes, with one standard deviation shown.

Table 6

One-way Analysis of Variance of the Results of the Experiments on the Effect of Exogenously Supplied Proline and Alanine on Desiccation Resistance of 7 1/2 Hour Sporulating Cells (Refer Table 5)*

Samples compared	F value obtained	Difference between the means of the two samples
d. water treatment (expt. i) and 0.1 M proline treatment (expt. i).	225	significant at 1% level of significance.
d. water treatment (expt. i) and 0.1 M alanine treatment (expt. i).	5.29	not significant at 1% level of significance, but significant at 5% level.
d. water treatment (expt. ii) and 0.1 M proline treatment (expt. ii).	861	significant at 1% level of significance.
d. water treatment (expt. ii) and 0.1 M alanine treatment (expt. ii).	1.98	not significant at 5% level of significance.
d. water treatment (expt. i) and d. water treatment (expt. ii).	4.96	not significant at 1% level of significance, but on borderline at 5% level.
0.1 M proline treatment (expt. i) and 0.1 M proline treatment (expt. ii).	0.19	not significant at 5% level of significance.
0.1 M alanine treatment (expt. i) and 0.1 M alanine treatment (expt. ii).	0.35	not significant at 5% level of significance.

* For comparison of two samples, each of 6 scores, the critical value of F at $\alpha = 0.05$ is 4.96
$\alpha = 0.01$ is 10.04.

(C) Effect of treatment with proline on viability of non-desiccated vegetative cells

The foregoing experiments indicate a toxic effect of the more concentrated proline solutions. To determine whether proline treatment alone would kill the cells, an experiment was therefore done in which vegetative cells were exposed to such concentrations for one hour and then plated without desiccation. The results, as shown in Table 7, indicate that 0.1 M proline solution did not affect the viability of the vegetative cells. About 20% of the cells were killed as a result of the one hour suspension in 0.5 M proline. 1.0 M and 2.0 M proline treatments were very harmful to the yeast cells, few of which survived the exposure to these concentrations.

The suspected mutant comprised 4.1% of the survivors after treatment with 1.0 M proline. Only one of the 200 cells plated grew after treatment with 2.0 M proline, and it gave a small colony.

(D) Are the tiny colonies contaminants?

The slowly-growing colonies found above were strikingly different from normal even for a mutant. This suggests that these tiny cells might be a contaminant. There were several possible sources of contamination in

Table 7

Effect of Treatment with Proline on Viability of Non-Desiccated Vegetative Cells

Solution in which the cells were suspended for 1 hour*	Average number of colonies developed per petri dish**	% of survival	Frequency of small colonies among the colonies that developed
distilled water	185 ± 3.6	100	0%
0.1 M proline solution	180 ± 9.7	97.2	0%
0.5 M proline solution	147 ± 6.9	79.4	0%
1.0 M proline solution	4 ± 1.6	2.1	4.1%
2.0 M proline solution	0.16 ± 0.34	0.05	100%

* The cells were plated directly (without desiccation) after suspension in the solution.

** Out of 200 cells plated per petri dish; each number is the average of 6 replicate petri dishes, with one standard deviation shown.

the experiments: (i) air, (ii) chemicals and (iii) stock culture.

It is highly improbable that the source is a random contamination from the air. The tiny cells appeared in several experiments and they were never found unless the cells were first subjected to treatment with proline or with desiccation or with both. If the cells that gave rise to the small colonies had come from the laboratory air, they should have appeared randomly in the earlier experiments, e.g. those illustrated in Tables 3, 5, 7. (Also, in Table 9 the dishes in which the vegetative cells plated were suspended in distilled water without other treatment did not yield small colonies.)

The second possibility is that it is a very heat-resistant organism present in one of the chemicals used, e.g. proline, which survived autoclaving. An experiment was therefore done to see if the suspected mutant could survive heat treatment in the autoclave and also to test if the chemicals might be the source of the organism. Substances or cells were added or inoculated into sterilized slants of MYPG agar and then these slants were either steamed or autoclaved for 15 minutes in an American Sterilizer Co. Type LS-1726 autoclave. Each treatment was done in duplicate. After the heat treatment, the tubes were sloped again until the agar had set and then incubated at 27°C. The slants were examined for signs of any

microbial growth after 2 days and 7 days incubation. Table 8 shows that no colonies grow on the slants with the added chemicals after steaming or autoclaving and that the suspected mutant, like the normal strain, was killed as a result of 15 minutes steaming, a less severe treatment than autoclaving. In all previous plating experiments, all media, solutions and glassware used had been sterilized by 15 minutes autoclaving at a temperature of at least 110°C and at 15 lbs per square inch pressure, as routine practices. Thus the possibility that the suspected mutant was a contaminant that originated from the proline or the ingredients of the MYPG agar and survived the routine heat sterilization treatment is ruled out. It should also be mentioned that plates or slants of this medium not inoculated could be kept for months at room temperature without development of contaminants.

An experiment was done to check the third possible source of contamination, i.e. that the organism was carried in the stock cultures of the 19el strain at a low population level and was selected out by the desiccation and proline treatments because it happened to be more resistant to these stresses. If this were so, then purification of the stock should yield a culture from which the tiny colonies could not be obtained. The 19el stock culture was purified as follows: A small loopful of culture was streaked on the surface of a plate of MYPG agar. The plate was

Table 8

To Determine the Heat Resistance of the Small Cells and to Test for the Presence of

Contaminants in Chemicals

Material added to MYPG slant	Heat treatment		Microbial growth on slant reset	
	Steamed for 15 mins at 110°C in autoclave	Autoclaved for 15 mins at 110°C and 15 lbs per sq in pressure in autoclave	after 2 days incubation at 27°C	after 7 days incubation at 27°C
normal vegetative cells*	−	−	+	+
normal vegetative cells**	−	+	−	−
normal vegetative cells**	+	−	−	−
small cells*	−	−	+	+
small cells**	−	+	−	−
small cells**	+	−	−	−
proline crystals (0.1 g)	−	+	−	−
proline crystals (0.1 g)	+	−	−	−
mixture of ingredients of MYPG (2.5 g)	−	+	−	−
mixture of ingredients of MYPG (2.5 g)	+	−	−	−
agar (1 g)	+	−	−	−
agar (1 g)	−	+	−	−
distilled water (0.5 ml)	+	−	−	−
distilled water (0.5 ml)	−	+	−	−

* A small inoculum at the end of inoculation needle was stabbed deep into the agar of the slants, which were also streaked on the surface.

** Three loopfuls of inoculum was streaked on the agar surface of the slant.

incubated at 27°C, for a day. Then a typical yeast colony growing well away from other colonies was picked off with a pointed inoculating needle and transferred to a MYPG agar slant. When the slant culture had grown for a day, it was streaked on a fresh MYPG agar plate. This streaking and transferring process was repeated two more times. An experiment was then done in which vegetative cells of the three times purified culture were exposed to three treatments that, from the data shown in Tables 3 and 7, caused small-celled colonies to appear among survivors of vegetative cells of the stock culture. Table 9 shows that treatment with 0.5 M proline followed by desiccation or treatment with 1.0 and 2.0 M proline without desiccation caused the small colonies to appear. The appearance of the tiny cells when purified 19el vegetative cells were subjected to proline treatment and desiccation treatment is strong evidence that these were not contaminants present in the stock culture of 19el. Conceivably, it could be a very unusual mutant or an organism living in or on the yeast cells. Microscopic examination shows no evidence of such cells on the surface of the normal cells.

Characteristics of the Small Cells

(A) Cell volume

From the measurement of cell diameters given on

Table 9

Effect of Treatment with Proline and Desiccation Treatment

on Purified Vegetative Cells

Solution in which the cells were suspended for 1 hr at 27°C	Initial inoculum into the solution (cells/ml)	Desiccation treatment (24 hrs) after treatment with proline	Total number of petri dishes plated	Number of cells plated per dish	Total number of colonies developed in all dishes after 3 days incubation at 27°C	Total number of small colonies developed in all dishes after 3 days incubation at 27°C
25 ml distilled water	5×10^4	-	12	50	578	0
50 ml 0.5 M proline solution	4×10^5	+	10	1×10^5	21	8
25 ml 1.0 M proline solution	5×10^4	-	8	5×10^2	97	1
25 ml 2.0 M proline solution	5×10^4	-	6	1×10^3	5	1

page 61, the average volume of the small cells was calculated to be 0.28 μ^3, whereas that of the normal vegetative cells was 29.7 μ^3. The small cells were taken to be spheres and thus the formula used for estimating volume is $\frac{4}{3}\pi r^3$, where r is the radius. The formula adopted for calculating the volume of normal vegetative cells is that of a prolate ellipsoid: $\frac{4}{3}\pi a b^2$, where a is the major axis and b is the minor axis (Weast and Selby, 1967). The difference in volume between the small cells and the normal vegetative cell is approximately a hundred-fold.

(B) Stability

The small cells were never observed to revert back to the normal during over 10 successive transfers on MYPG slants over a period of 6 months.

It was of interest to see if the small cells could be induced to revert back to the normal by mixing separate isolates. Cells from 6 different isolates were mixed thoroughly together and then mixed cells were streaked on a MYPG slant. No reversion could be observed after 6 days of incubation at 27°C.

(C) Gram staining properties

Smears of 1 day old cultures of small cells and of normal 19el vegetative cells were made. They were stained

with the Gram procedure (see Appendix III). The small cells acquired the pink counterstain after 10 seconds of alcohol destaining, whereas the normal vegetative cells still retained the initial crystal violet stain after 30 seconds of alcohol decolorization. Thus, the small cells are Gram negative, unlike normal yeast cells which are Gram positive.

(D) Respiration deficiency test

Growth on MYPG slants was slower than that of normal vegetative cells. This suggests the possibility that the small cells might be respiration-deficient. So the tetrazolium overlay technique of Ogur *et al.* (1957) was used to test for respiration deficiency.

The composition of the Fisher TTC overlay agar is:

triphenyl tetrazolium chloride (TTC)	0.5 g
glucose	5.0 g
agar	15.0 g
distilled water	1000 ml

The TTC overlay technique is based on the use of a relatively high concentration of TTC at a pH which yields deep red color development in respiration-sufficient yeast colonies in a test period sufficiently short so that respiration-deficient colonies are white. TTC is a redox dye which is red when reduced and colorless when oxidized.

One day old cultures of normal 19el vegetative cells and small cells on MYPG slants were streaked on the surface of MYPG plates (with 2.5% agar). Two duplicate plates were streaked with normal vegetative cells only. Two duplicate plates were streaked with small cells only. Four replicate plates were streaked with both normal and small cells, but the streaks of normal and small cells were well separated on the same plate. These 8 plates were incubated at 27°C for one day.

TTC overlay agar powder (4.1 g) was added to 200 ml of distilled water, mixed thoroughly and heated to boiling to dissolve completely. The agar solution was cooled to 50°C and then poured gently over the yeast colonies on the surface of the one day old plates. When the overlaid agar was set, the plates were incubated at 27°C for 2 hours. It was then found that the "mutant" streaks were all pale pink throughout in color, whereas the edges of the streaks of normal vegetative cells were distinctly deep red. This indicated that the normal vegetative cells were respiration-sufficient and the small cells were respiration deficient.

(E) Nutrition

The small cells grew well in MYPG, but did not show appreciable growth in the yeast nitrogen base

germination medium. MYPG contains yeast extract, malt extract and peptone but the defined germination medium must contain far fewer varieties of nutrients. This indicates nutritional deficiency in the small cells. That is, they require a substance or substances present in MYPG but absent in YNB. The composition of MYPG is given in the Materials and Methods, and that of YNB in Appendix I. The normal strain grew well on both media.

(F) Fermentation

The small cells were inoculated into a test-tube of liquid MYPG, with an inverted Durham tube immersed in the medium. There was no evolution of gas observed within 5 days. For normal vegetative cells, gas production could be detected after 1 day incubation. This indicates that the small cells do not have the typical yeast alcoholic fermentation.

(G) Acid production

Since small cells do not produce gas from glucose, they may be converting glucose to some other substance or substances, instead of to carbon dioxide and ethanol. This alternative was checked by measuring the pH of the MYPG medium after growth. It was found that after 1 day growth in liquid MYPG, normal vegetative cells lowered

the pH of the medium from 5.4 to approximately 5.1. On the other hand, the small cells lowered the pH to about 4.0 after 1 day growth. This indicates that the latter are producing an acid as a result of glucose utilization.

(H) Sporulation ability

It was of interest to test if the small cells could sporulate. They were inoculated onto 2% potassium acetate slants. Smears of the cells were made after 2 days and 7 days of incubation in sporulation slants. The smears were stained by acid-fast staining technique (Appendix II), but no acid-fast cells could be seen. This indicates that if they do sporulate, the spores are not acid fast.

(I) Desiccation resistance

Since the small celled colonies were found among the survivors after desiccation treatment, it is interesting to see whether these cells were desiccation-resistant. Cells grown two days on MYPG slants were suspended in 2.5 ml sterilized distilled water. The cell population density was very roughly determined by haemacytometer counting. Because clumping of these cells is very frequent, it was impossible to count accurately the number of cells in a haemacytometer counting chamber field.

About 5 x 10^7 cells were suspended in 50 ml sterilized distilled water and then filtered out with a membrane filter. The filter bearing the cells was placed in the desiccator for 24 hours. The initial small cell suspension was also diluted to about 5 x 10^6 cells/ml and one loopful of this diluted suspension was streaked on a MYPG plate (with 4% agar). Six pieces of autoclaved coverglass were put on the agar surface, covering some of the streaks. The agar plate, with its lid closed, was incubated at 27°C.

After 24 hours desiccation, the membrane filter was taken out from the desiccator and the desiccated cells were suspended in 2.5 ml sterilized distilled water. The cell concentration was approximately determined by haemacytometer counting, and the cell suspension was diluted to about 5 x 10^6 cells/ml. One loopful of this diluted suspension was streaked on a MYPG plate (with 4% agar) and then incubated as in the case of undesiccated cells.

The non-desiccated small cells were found to grow more rapidly than the desiccated ones on the agar plate. The plate streaked with non-desiccated cells was examined microscopically using the 40X objective after one day incubation, but the plate with desiccated cells was examined after 2 days incubation. At least 95% of the non-desiccated cells were seen to form colonies. About 21% of the desiccated cells developed colonies.

This is a very high survival percentage as compared with that of desiccated normal vegetative cells (see Table 3). Thus the small cells are much more resistant to desiccation than normal vegetative cells.

DISCUSSION

Relationship Between Length of Pre-Sporulation Vegetative Growth and Subsequent Sporulation

The state of vegetative cells in the pre-sporulation medium is important in determining the percentage of sporulation when the vegetative cells are transferred to sporulation medium (Table 1). Vegetative cells harvested at the end of the logarithmic growth phase produced far more spores when transferred to sporulation medium than those harvested a few hours earlier in the logarithmic phase.

Croes (1967b) pointed out that several factors are important in the induction of sporulation in yeasts. These factors include the exposure of vegetative cells to acetate, the change of carbohydrate metabolism from fermentation to respiration and hence the ability to utilize acetate. The acetate is oxidized and the major metabolic pathway of acetate oxidation is the tricarboxylic acid (TCA) cycle (DeMoss and Swim, 1957). Eaton and Klein (1954) noted that yeast cells harvested in the late logarithmic or stationary phase of growth can oxidize glucose, ethanol and acetate rapidly, but cells harvested

in the early logarithmic phase can only utilize glucose. Eaton and Klein accounted for the inability of young vegetative cells to utilize acetate by a deficiency of certain enzymes in the TCA cycle. The enzymes of the TCA pathway are repressed by glucose, but they are derepressed towards the end of the logarithmic phase when the glucose level is running low (Polakis and Bartley, 1965). Miyake *et al.* (1971) observed that the activities of the TCA cycle enzymes increase during early sporulation. Kuenzi *et al.* (1974) showed that extensive derepression of the respiratory system is required for sporulation. Therefore, cells in logarithmic phase are unable to metabolize acetate and, as a result, cannot undergo sporogenesis. The change over to oxidative metabolism which occurs at the end of the logarithmic phase is an important preparation for sporulation. Only those vegetative cells which are prepared can utilize acetate and sporulate in the acetate medium. Croes (1967a) proposed that the acetate is a trigger rather than an inducer of sporulation, and that it promotes a development already started during the late logarithmic growth phase.

Esposito *et al.* (1969) observed that vegetative cells grown at the appropriate physiological age for sporulation (at early stationary phase) readily take up acetate after transfer to the sporulation medium. They

found that during sporulation in acetate medium about 62% of the acetate consumed is respired, 22% remains in the soluble pool, and 16% is incorporated into proteins, nucleic acids, lipids and other cell components. Görts (1975) based on observations on incorporation of acetate, suggested that the acetate consumed is not only a source of energy but is also an important source of intermediates for biosynthetic processes occurring during sporulation. During sporulation, there is great turnover of cellular macromolecular components, and utilization of an extracellular carbon source is required for this purpose.

The writer has confirmed the observation of Esposito *et al.* that time in pre-sporulation medium has an important effect on subsequent sporulation in acetate medium, and has shown further that time in pre-sporulation medium affects the degree of desiccation resistance that develops in the sporulation medium.

Uptake of acetate during sporulation accounts for the greater dry weight of spores than vegetative cells observed in this present work. Roth (1970) estimated that 67% of the dry weight increase during sporulation is due to the increase in carbohydrate content and found that trehalose and insoluble carbohydrates were the cellular carbohydrates synthesized.

The Acquisition and Loss of Resistance to Heat and Desiccation

The acquisition and loss of heat resistance did not coincide with that of desiccation resistance (Figures 6 and 10, 7 and 11). Desiccation resistance was apparent even in the early phase of sporulation whereas heat resistance was undetectable in the first 12 hrs of the sporulation phase. After 14 hrs in sporulation medium, 50% of the sporulating cells survived the desiccation treatment, but it required approximately another 10 hrs for 50% of the sporulating cells to acquire ability to resist the heat treatment. During germination a large decline in heat resistance preceded the loss of desiccation resistance. Thus after nearly 2 hrs in germination medium, 50% of the germinating population lost their ability to survive the heat treatment, but it took about 3 1/2 hrs for the same proportion of the population to lose their ability to survive desiccation. Thus the population acquired heat resistance later than desiccation resistance during sporulation and lost heat resistance earlier during germination. These observations suggest that the mechanisms underlying heat resistance and desiccation resistance are different.

In correlating resistance to heat and desiccation with sporulation, it is obvious that spores are more resistant to these factors than vegetative cells. However, this correlation is not a direct one, because there are instances where sporulated cells are still not resistant.

For instance, comparing Figures 3 and 10,some cells became desiccation-resistant before spores appeared. Moreover, after 10 hrs, for example, approximately 60% of the cells had sporulated but less than one-fifth were resistant to desiccation, and none survived the heat treatment. As another example, in Table 1, the cells grown for 18 hrs in MYPG yielded 72.2% asci after 24 hrs in sporulation medium, but only about 50% of the population survived the drying. On the other hand, after 16 hrs in the MYPG and 48 hrs in sporulation medium only 7.5% of cells sporulated, but nearly one-quarter survived desiccation. That is, the cells can become desiccation-resistant without sporulating.

This lack of correlation between presence of visible spores and resistance indicates that the morphological structure of the spore is not the sole basis of resistance. It suggests that chemical composition may be important, and this makes the following studies of proline content relevant.

Changes in Content of Free Proline During Sporulation and Spore Germination

The difference in free proline content between the mature spores and the germinated spores at 6 hrs in germination medium was striking (Figure 14). This is

consistent with similar findings of Ramirez and Miller (1964) and Rousseau and Halvorson (1973). Ramirez and Miller (1964) noted that vegetative cells harvested from a pre-sporulation medium consisting of Yeast Nitrogen Base and glucose contained no detectable amount of free proline, whereas the cells after exposure to 1% sodium acetate for 6 days contained a high level of free proline. Rousseau and Halvorson (1973) also found that the spores were rich in free proline but during the course of germination the free proline level dropped dramatically. Ramirez and Miller (1964) showed that there was a considerable protein breakdown during sporogenesis and suggested that proline accumulated in the sporulating cells because it is not lost through the cell walls into the medium like many other amino acids and there is a metabolic block which limits the consumption of proline by the endogenous respiratory system during sporulation.

The vegetative cells harvested from MYPG pre-sporulation medium had an appreciable amount of free proline compared to the germinated cells at 6 hrs in Yeast Nitrogen Base germination medium (Figures 13 and 14). This may be due to the difference in nutrient content of the two media which may affect the free proline content in the growing cells. When the vegetative cells from MYPG were placed in acetate, the proline content dropped rather sharply during the first hour in sporulation medium

and then decreased to a minimum at about 4 hrs. This indicates that free proline can be utilized early in sporulation, a point that has not been noted by earlier investigators. However, between 4 and 10 hrs in sporulation medium, intracellular proline content started to rise.

From a comparison of Figures 14 and 15, it is evident that the amount of proline lost from the germinating spores was greater than that which accumulated in the medium, which indicates that some was utilized by the germinating spores.

Proline and Desiccation Resistance of the Yeast Ascospore

No close parallel was found between the acquisition and loss of desiccation resistance and the change in cellular free proline content (Figures 10 and 13, 11 and 14). However, desiccation-sensitive vegetative cells are low in proline and desiccation-resistant spores are high in proline, which suggests a protective role for proline. Moreover, the observation that suspension of vegetative cells and sporulating cells in 0.1 M proline solution before desiccation resulted in significant increase in the number of survivors after desiccation treatment (Tables 3 and 5) is strong evidence that proline is involved in increasing desiccation resistance of sporulating

yeast cells. A very specific transport system for uptake of exogenous proline has been reported for the yeast *Saccharomyces chevalieri* (Magana-Schwencke and Schwencke, 1969). So, presumably, the 19el cells could take up the proline from the exogenous solution, enabling the cells to resist desiccation better. Increased desiccation resistance was not conferred by exogenously added alanine, although alanine, like proline, is a small amino acid with a nonpolar R group and of relatively low molecular weight. Hubac and Guerrier (1972) reported that the drought-resistant plant species *Carex pachystylis* accumulated a higher proline content than the non-resistant species *C. setifolia*, and that they could increase the drought-resistance in *C. setifolia* by exogenous application of proline. Thus this similar result reported for plants gives support to the finding in this present work that enhancement in desiccation resistance by exogenous proline is possible. Hubac and Guerrier suggested that proline is a resistance factor and not a simple consequence of water stress.

Vegetative cells harvested from liquid MYPG contained an appreciable quantity of free proline and yet they were sensitive to desiccation stress (Figures 13 and 10). It seems that besides the amount of intracellular free proline, the physical state or the structure of the cell are important in determining the resistance of the yeast cell to desiccation. As a

vegetative cell sporulates, it becomes receptive to the increase of desiccation resistance by proline. The sporulating cells at 7 1/2 hrs in sporulation medium were more receptive to help by exogenous proline than the vegetative cells (Tables 3 and 5).

How does proline increase desiccation resistance of yeast spores and sporulating cells? As water is withdrawn from the cells during desiccation, the osmotic pressure in the cells is affected. Thus this hints a possibility that proline may protect the yeast spores by involvment in osmotic adjustment. There have been numerous papers published, reporting the accumulation of free proline in plants under water stress, examples of which have been referred to in the Introduction. But it has also been noted that free proline is accumulated in algae (Schobert, 1974), higher plants (Singh *et al.*, 1973; Hanower and Brzozwska, 1975), higher plant halophytes (Treichel, 1975; Stewart and Lee, 1974) when they are subjected to osmotic stress, e.g. treating the plants with sodium chloride solution. These observations suggest that there may be some effect on living cells common to desiccation stress and osmotic stress. Britikov *et al.* (1965) pointed out that the properties of proline seem to enable it to meet the requirements for being a cellular osmotic factor and physicochemical stabilizing factor under severe water stress. Besides being a compound of

comparatively low molecular weight (115.1), it is highly hydrophilic and extremely soluble. 1620 g L-proline is soluble in 1 liter of water at 25°C (Merck Index, 1960). Of the common amino acids encountered in proteins, the other very soluble ones are glycine, threonine, serine and alanine, but they are at least 6.5 times less soluble than proline. (The solubility of sodium chloride in water at 25°C is only 357 g/l.) Britikov *et al.* mentioned that in resting cells like pollen, free proline has only limited metabolic activity and that when demanded by appropriate circumstances, proline can be drawn into the main stream of metabolism through the actions of enzymes specific for proline. They showed that it can be utilized as a source of energy and as a source of nitrogen. Stewart and Lee (1974) found that in higher plant halophytes proline at high concentrations had little effect on enzyme activity. Some enzymes involved in amino acid metabolism or with their activity modulated by amino acids, and some enzymes not related to amino acid metabolism were studied, but in every case proline did not affect the enzyme activity. Palfi *et al.* (1973) mentioned the ability of proline to stabilize plant cell colloids. They also commented that the special properties of proline can be of considerable significance during unfavorable conditions of desiccation, as well as for the success of reparational processes on rehydration.

Applying the ideas of Britikov *et al.* (1965) as to how proline protects the pollen from desiccation stress to the present case of the yeast spore, possibly as water is extracted from the yeast spore during desiccation, the amount of cellular water diminishes, but since the solubility of proline is so high, it would remain in solution in the small amount of water remaining. This concentrated proline solution inside the cell then could act as an osmotic factor, preventing further loss of water from the cell, thereby preventing damage to the cellular structures. But the writer in this present work, on comparing the "bound" water content of asci with that of vegetative cells, showed that the asci are drier than vegetative cells after desiccation. So this indicates that Britikov's hypothetical mechanism of protection by proline is not applicable to the case of the yeast spore.

Proline is one of the amino acids in proteins. It is well known that when proline occurs in a peptide chain, it interrupts the α-helix and creates a kink in the helix. Possibly proline could protect the yeast spore from desiccation stress by modifying the conformation of proteins. But the writer thinks this possibility is not very likely. Ramirez and Miller (1964) found no difference in proline content of the protein of yeast spores and vegetative cells. Apparently all reports on proline in relation to desiccation are centred on free

(non-protein) proline, and there has been no mention of a high proline content in proteins in desiccation-resistant cells. On the contrary, Britikov and Musatova (1964) performed labelled-proline feeding experiments on plants and concluded that the selective accumulation of free proline in the pollen is a result of its relatively small incorporation into the proteins (about 50%) in comparison with the pistils, where over 90% of the proline entering is incorporated.

So the basis for the protection of the yeast spore against desiccation stress by proline still remains obscure.

The reason that concentrated exogenous proline solutions are harmful to yeast cells (Tables 3, 5 and 7) may be that the osmotic pressure of the concentrated proline solution is so great that a large amount of cellular water is suddenly extracted from the cells as soon as they are suspended in proline solution, before sufficient proline can enter the cells to reduce the osmotic gradient between the cell and the medium. The proline concentration in the spore may become very high, but since this concentration builds up gradually, no harm is done to the developing spore.

The finding in this present work that proline plays a role in conferring desiccation resistance on yeast spores and developing spores does not exclude the possibility that other factors are also involved in giving them resistance to desiccation. Desiccation resistance

in yeast spores may be rather complex. This present finding is a beginning effort to a thorough understanding of the mechanism behind desiccation resistance in the yeast spore. Besides proline, the structure of the spore is probably of importance. Protective substances against desiccation have been suggested for some other organisms. For example, mannitol and trehalose appear in high concentrations in some fungus spores and they may play a part in helping the spores to offset the detrimental effects of drying (Sussman and Halvorson, 1966). Trehalose is a major carbohydrate reserve in yeast cells and increases during sporulation (Roth, 1970; Banerjee, 1971) and it is quite possible that it may contribute to desiccation resistance.

To recapitulate, based on the data in this present work, it can be concluded that free proline plays at least two roles in the yeast spore. Firstly, proline in some manner gives desiccation resistance to sporulating cells and spores. Secondly, proline is utilized metabolically, at least during very early sporulation and during germination.

Changes in Alpha Amino Acid Content During Sporulation and Spore Germination

Ramirez and Miller (1964) noted that at the end of the sporulation phase the total free amino acid content

decreased to about one-third of that of vegetative cells and that transfer of sporulated cells to growth medium led to an increase in the pool of total free amino acids. Rousseau and Halvorson (1973) found that during germination, with the exception of proline and cysteine, most amino acid pool components significantly increased. But in this present work, the alpha amino acid content declined sharply within the first hour of suspension of vegetative cells in sporulation medium, then between 4 and 10 hours it started to rise, and it continued to increase up to 24 hrs and finally showed a decrease within the last 24 hrs in sporulation medium (Figure 17). At the end of the germination phase, the alpha amino acid content was only about one-fifth of that of the spores (Figure 18). So there is an apparent inconsistency between these data in this present work and that of Ramirez and Miller, and of Rousseau and Halvorson. Ramirez and Miller subjected the extract of free amino acids to separation by chromatography before the individual amino acids were assayed. The total free amino acid content they referred to is the sum of the common amino acids only. Rousseau and Halvorson used an amino acid analyzer for their work and again they only included the common amino acids as the components of the free amino acid pool. The ethanol extraction procedure (Sutherland and Wilkinson, 1971), adopted in this work, extracts free amino acids as well as other low molecular

weight intermediary metabolites from microbial cells. So in this present work, some small peptides may have been extracted which would give the ninhydrin reaction. This might explain why the data in Figures 17 and 18 were not in accord with the previous documented findings. Dr. S.D. Steele (personal communication, 1975) estimated the free amino acid composition in 19el vegetative cells and spores with a Beckman 120C amino acid analyzer. He found that the free common amino acids were less abundant in 19el spores than in vegetative cells, as previous workers had found. However, he also detected large amounts of some unknown ninhydrin-positive substances in spores not corresponding to any of the common amino acids known to occur in yeast cells. If, in this present work, the alcohol extract had been subjected to chromatographic separation and each of the common amino acids estimated individually, the combined result might be consistent with previously documented observations.

It is possible that the unknown ninhydrin-positive substances detected by Steele are responsible for the high content of α-amino nitrogen found in spores in this study. The writer's work also indicates that they accumulate steadily during sporulation and do not diminish until late in germination. Possibly they may be involved in giving the spores resistance to desiccation. This obviously opens up an important problem for further investigation.

The Small Cells

What is the origin of the small-celled colonies that were found in certain of the plating experiments? Strong evidence has been presented in the Results that these small cells are not contaminants. Two other possibilities can be suggested.

The first possibility is that the small cells originated through mutation of the 19el strain. The size difference between yeast cells and their mutants is not normally large. For example, the well-known respiratory deficient "petite" mutants, characterized by very slow growth rate and small colony size, which occur in natural populations of some *Saccharomyces cerevisiae* strains are not distinguishable in cell size from normal cells (Ephrussi and Hottinguer, 1951). The fact that the cell volume of the small cells found in this work is only about one-hundredth of that of a normal vegetative cell is not in favour of the idea of the small cell being a mutant. However, the possibility has not been excluded. If the small cell is indeed a mutant, it must be a very unusual mutant.

The second possibility is that the small cells are a symbiont of the 19el cells. The normal 19el cultures were examined under the light microscope as haemacytometer counting chamber preparations and as smears stained with crystal violet, and also with the acid-fast staining

procedure. No small cells were observed either in the culture among the normal cells or adhering to the side of normal 19el cells. Thus the possibility that the small cells are an ectosymbiont seems not very likely. In some of the electron micrographs presented by Grewal and Miller (1972; Figures 9 and 10), Steele and Miller (1974a; Figures 10, 1974b; Figures 1 and 3), there are seen spherical structures in the 19el cells and asci, whose shape and size are not unlike the small cells. These spherical structures were labelled as "globules", "vacuoles" or "cytoplasmic bodies". Is it possible that these bodies are in fact endosymbiotic bacteria inhabiting the yeast cells? Conceivably the desiccation or proline treatments might have killed the yeast cells and liberated the symbionts. Against this suggestion is the fact that no bacteria have been described inhabiting the cells of yeasts, or of any fungus. However, previous to 1962 when Stolp discovered *Bdellovibrio*, no bacteria endoparasitic on other bacteria had been described, yet *Bdellovibrio* is now known to be very common in soil, water and sewage (Stolp, 1973).

The writer believes that an interesting problem may have been opened up by the isolation of the small cells, and suggests the following approach for a future investigation. (1) The treatments of the 19el strain that yielded the small cells should be repeated and if the organism is again isolated the following program should be undertaken. (2) Other selective treatments should be

tried which may yield the small cells. As examples, some antibiotics which inhibit only fungi would be applied to the 19el cultures. Alkaline pH, which does not favor the growth of yeasts, may select out the small cells if they are bacteria. Sonication followed by filtration using membrane filters of 2.0 μ pore size may retain the yeast cells and allow the small cells to pass through. Separation of the yeast cells from the small cells by centrifugation may also be possible, as the yeast cells should sediment to the bottom of the centrifuge tube faster than the small cells. (3) Electron microscopic studies should be carried out on the small cells. This may solve the problem of whether the small cells are mutant or not. High resolution electron micrographs should decide whether the small cells are mutants or symbionts. If the small cells are mutants, then they should possess some eukaryotic characteristics. (4) Other yeast strains should be studied to see if they will yield the small cells.

SUMMARY

1. The length of time that vegetative yeast cells were grown in pre-sporulation medium had an important effect on both subsequent percentage of sporulation and the degree of desiccation resistance that developed in the sporulation medium.

2. The dry weight of sporulated cells was greater than that of vegetative cells but the spores contained a lower percentage of "bound" water.

3. The acquisition of heat resistance during sporulation and its loss during spore germination did not coincide with that of desiccation resistance. The population acquired heat resistance later than desiccation resistance during sporulation and lost heat resistance earlier during germination.

4. With the exception of the first few hours in sporulation medium when proline appeared to be utilized, the intracellular free proline content increased during sporulation phase. During germination the proline content decreased and evidence was obtained that the decline was partly due to excretion into the germination medium and partly to metabolic utilization.

5. Exogenous proline added to vegetative cells or sporulating cells before they were desiccated significantly increased their survival, indicating that the high proline content found in the spores has a protective function. However, a close parallel was not apparent between the changes in cellular free proline content during sporulation or germination and the acquisition or loss of desiccation resistance. This implies that other factors in addition to a high free proline content are involved in the ability of spores to survive desiccation.

6. Dormant spores had a higher free alpha-amino acid content than germinated spores. During the major part of the sporulation process, the free alpha-amino acid content gradually increased. An attempt was made to explain these data in terms of the results of previous workers.

7. Small-celled colonies were found after vegetative cells or sporulating cells were desiccated, or suspended in proline solutions or treated with both. These cells were very small when compared to normal 19el vegetative cells and some of their physiological properties appeared to be very different from those of 19el cells. It was suggested that these small cells might possibly be a mutant or a symbiont of the 19el cells.

Appendix I

Difco Yeast Nitrogen Base Medium

(without amino acids or ammonium sulfate)

biotin	2 mcg
calcium pantothenate	400 mcg
folic acid	2 mcg
inositol	2000 mcg
niacin	400 mcg
p-aminobenzoic acid	200 mcg
pyridoxine hydrochloride	400 mcg
riboflavin	200 mcg
thiamine hydrochloride	400 mcg
boric acid	500 mcg
copper sulfate	40 mcg
potassium iodide	100 mcg
ferric chloride	200 mcg
manganese sulfate	400 mcg
sodium molybdate	200 mcg
zinc sulfate	400 mcg
potassium phosphate monobasic	1 g
magnesium sulfate	0.5 g
sodium chloride	0.1 g
calcium chloride	0.1 g
distilled water	1000 ml

This is based on a medium devised by Wickerham (1951).

Appendix II

Ziehl-Neelsen acid-fast staining procedure

The procedure followed was that of the Society of American Bacteriologists, Manual of Methods (1957) as modified by Seigel and Miller (1971).

1. Stain dried smear for 4 mins with freshly filtered Ziehl's carbol fuchsin.
2. Rinse in tap water.
3. Decolorize for 10 secs in 95% ethanol containing 3% by volume of concentrated hydrochloric acid.
4. Immediately wash in tap water.
5. Counterstain with methylene blue for 2 mins.
6. Wash in tap water.
7. Air-dry the smear, and examine under oil immersion.

Acid-fast microbial cells are stained red; non-acid-fast cells are stained blue.

Ziehl's carbol fuchsin stain

basic fuchsin	0.3 g
ethanol (95%)	10 ml
phenol	5 g
distilled water	95 ml

Dissolve basic fuchsin in ethanol and phenol crystals in distilled water. Mix the two solutions.

Appendix II (cont'd)

Methylene blue staining solution

methylene blue	0.3 g
ethanol (95%)	30 ml
distilled water	100 ml

Appendix III

The Gram stain procedure

This procedure is based on Hucker's modification of the original Gram stain (Society of American Bacteriologists, Manual of Methods, 1957).

1. Stain smears for 1 min with ammonium oxalate crystal violet.
2. Wash in tap water.
3. Cover smear for 1 min with Gram's iodine.
4. Wash in tap water.
5. Decolorize with 95% ethanol for the desired destaining time.
6. Immediately wash in tap water.
7. Counterstain with safranin for 10 secs.
8. Wash in tap water.
9. Air-dry the smear, and examine under oil immersion.

Gram-positive microorganisms are stained purple; Gram-negative ones are stained red.

Ammonium oxalate crystal violet stain

crystal violet	2 g
ethanol (95%)	20 ml
ammonium oxalate	0.8 g
distilled water	80 ml

Appendix III (cont'd)

Dissolve crystal violet in ethanol and ammonium oxalate in distilled water. Mix the two solutions.

Gram's iodine

iodine	1 g
potassium ioide	2 g
distilled water	300 ml

Safranin stain

safranin O (2.5% solution in 95% ethanol)	10 ml
distilled water	100 ml

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