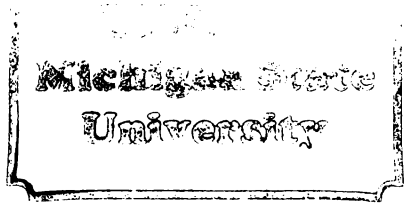

This is to certify that the

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EFFECT OF THERMAL STRESS ON

THE RESPIRATION OF YEAST

presented by

Apinya Assavanig

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Science and Human
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Robert B. Brennan
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EFFECT OF THERMAL STRESS ON THE RESPIRATION OF YEAST

By

Apinya Assavanig

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ABSTRACT

EFFECT OF THERMAL STRESS ON THE RESPIRATION OF YEAST

By

Apinya Assavanig

The purpose of this study was to investigate further the effects of thermal stress on the recovery and respiration of intact yeast cells of Saccharomyces cerevisiae and isolated preparations of mitochondria, as well as on the characteristics of the cell membrane of yeasts.

Viability and endogenous respiration were measured in actively growing cells and resting cells of S. cerevisiae subjected to heat at 56, 60, 62, and 65 C for 0 to 20 minutes. Actively growing cells were less heat resistant than resting cells, as demonstrated by lowered recovery on plate count agar (PCA) and greater reduction in respiration rates. Thermal destruction rates of the cells were greater with increasing temperatures. Complete loss of viability occurred with prolonged heating. Storage at 4 C did not affect the recovery and respiration of heat-stressed cells.

Due to the high rate of endogenous O_2 uptake in heat-stressed cells, the anticipated difference in oxygen uptake between heat-stressed and non-heated cells in the presence of glucose, succinate, pyruvate, and/or malate were not apparent.

Enzymatic digestion, hand-shaking the cells with glass beads, and mechanical disruption methods provided low yields of mitochondria. Oxidative and phosphorylative activities measured in heated and non-heated mitochondria isolated by hand-shaking did not indicate that thermal stress affected respiratory activity of mitochondria.

The fluorescence studies showed that phase transitions in the lipid components of cell membranes of S. cerevisiae were altered after heat stress. This presumed alteration in cell membranes of heat-stressed cells could be responsible for the leakage of cellular constituents, and in particular UV-absorbing materials, which is often associated with thermal injury. Storage of heat-stressed cells in water at 25 C for 5 hours allowed reorientation of lipids in the membrane, presumably, restoring the membrane to its normal state.

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INTRODUCTION

Yeast- and mold-contaminated fruit juice, fruit concentrate, and fruit drink is a major problem in industry. The internal tissues of sound fruit generally contains no yeasts before being processed. However, the great majority of yeasts occurring in the juice originate from the skin and from processing equipment. In general, carbonated and non-carbonated fruit drinks are pasteurized at temperatures designated to destroy pathogenic organisms. The presence of viable yeasts in the products is indicative of insufficient heat processing or inadequate plant sanitation. Several investigations have been conducted on the thermal resistance as well as the thermal injury of yeasts. Exposure of yeasts to sublethal temperature results in decreased viability, increased sensitivity to environmental conditions, and altered respiratory activity (Nash and Sinclair, 1968; Fries, 1969; Tsuchido et al., 1972a; Gibson, 1973; Meyer, 1975; Put et al., 1976; Stevenson and Graumlich, 1978).

A previous study by Graumlich (1978) on the thermal injury and recovery of Saccharomyces cerevisiae revealed that resting cells heated at 56 C exhibited an increased endogenous respiration rate and an altered respiratory

quotient. Prolonged high rates of endogenous respiration were related to evidence of differential recovery of heat-stressed cells on media plant count agar (PCA) and potato dextrose agar (PDA). After 20 hours of storage, the plate counts in the two media were similar and rates of oxygen uptake declined to levels approximating or lower than the oxygen uptake of non-heated cells. Most of the oxidative and respiratory activities of yeasts occur in mitochondria (De Moss and Swim, 1957; Lukin et al., 1968; Rose and Harrison, 1971; Lehninger, 1975). Yeast, particularly S. cerevisiae, is one of the simplest organisms from which mitochondria can be isolated (Ohnishi et al., 1966; Linnane and Lukins, 1975). It would be of interest to know whether the mitochondria have a specific lesion that is affected by thermal stress and results in abnormal yeast respiration. Thus, the purpose of this study was to investigate further the effects of thermal stress on the recovery and respiration of intact yeast cells; and the isolated preparations of mitochondria. Several researchers (Tsuchido et al., 1972a,b; Shibasaki and Tsuchido, 1973; Meyer, 1975; Graumlich, 1978) reported leakage of intracellular materials in many genera of yeasts after thermal injury. Also, the effect of heat stress on characteristics of the cell membrane of S. cerevisiae will be investigated.

LITERATURE REVIEW

Injury and recovery of yeasts have been observed after yeasts were subjected to supraoptimal or sublethal temperatures. Effects of heat stress on yeasts have been studied by many investigators (Nash and Sinclair, 1968; Fries, 1969; Tsuchido et al., 1972a; Nelson, 1972; Gibson, 1973; Hagler and Lewis, 1974; Meyer, 1975; Put et al., 1976). Later in 1978, Stevenson and Graumlich concluded that manifestations of thermal injury of yeasts fell into three main categories. These were increased sensitivity of yeasts to environmental conditions, i.e. Cl^- or Br^- salts, water activity, pH and temperature; alterations in growth and cell characteristics; and subcellular alterations. The objective of this study was to further investigate the effects of thermal stress on intact yeast cell, its mitochondria and membranes. Therefore, a review of the pertinent subjects will be discussed herein.

Effect of Heat on Subcellular Level of Thermally-Stressed Yeasts

Alterations in metabolic activity, of cell membranes and cellular organization have been reported to occur in

yeasts after exposure to elevated temperatures.

Metabolic Activity

Decreased fermentative activity (Sinclair and Stoke, 1965; Sinclair and Grant, 1967; Grant et al., 1968) as well as decreased respiratory activity (Baxter and Gibbons, 1962; Sinclair and Stokes, 1965; Shibasaki and Tsuchido, 1973; Meyer, 1975) were found in fungi which had experienced thermal stress. Growth of yeast at elevated temperature or heat shock also induced increased proportions of respiratory-deficient cells or petite mutants of Saccharomyces cerevisiae (Sherman, 1956, 1959).

During the last decade, intensive studies of thermal injury and recovery of yeasts were conducted by Meyer (1975) and Graumlich (1978). Meyer (1975) discovered decreased respiratory activity in Candida P 25, an obligate psychrophile, after exposure to 30 C. Regardless of their physiological state, organisms respiring exogenous substrates were less resistant to heat-induced injury than those respiring endogenous substrates. Heat-injury to exogenous respiration was irreversible when the organisms were heated in the presence of glucose but was reversible when heated in the absence of glucose. Graumlich (1978) reported that respiratory activity of S. cerevisiae reflected the severity of the heat stress. Cells heated at 56 C had increased rates of endogenous respiration along with

alteration of respiratory quotients. The addition of glucose resulted in no change but prolonged the presence of the high rates of respiration. The addition of 2,4-dinitrophenol stimulated O_2 uptake in non-heated cells but decreased O_2 uptake of heated cells.

In addition, Baxter and Gibbons (1962) noted decreased alcohol dehydrogenase activity and reduced uptake of glucosamine in a psychrophilic Candida sp. after exposure to supramaximal temperatures. Hagen and Rose (1961, 1962) revealed synthesis and uptake of amino acids, and synthesis of α-oxoglutarate decreased after thermal stress. Sinclair and Grant (1967) reported inhibition of glucose fermentation in an obligate psychrophile Candida sp. was due to heat-inactivated enzyme and protein syntheses. Other thermosensitivity of protein synthesis correlated with the thermolability of aminoacyl-tRNA synthetases and soluble enzyme involved in formation of ribosomal bound polypeptide chains, as well as with thermal instability of ribosomes (Nash et al., 1969; Nash and Grant, 1969; Spencer, 1972). Temperature-sensitive inhibition of DNA synthesis and RNA synthesis was observed in an obligately psychrophilic yeast, Leucosporidium stokesii (Silver et al., 1977).

Disruption of Cell Membranes and Cellular Organization

Thermal injury to fungal cell membranes resulting in the leakage of intracellular constituents was reported in

Candida utilis (Tsuchido et al., 1972a,b; Shibasaki and Tsuchido, 1973; Rudenok and Konev, 1973), S. cerevisiae (Rudenok and Konev, 1973; Hagler and Lewis, 1974), Candida sp. (Spencer, 1972; Meyer, 1975), C. nivalis (Nash and Sinclair, 1968), Leucosporidium frigidum and L. stokesii (Spencer, 1972).

Thermally-induced membrane damage was considered to be responsible for increased sensitivity of yeast to environmental conditions, i.e. altered nutritional requirements of thermally stressed C. nivalis (Nash and Sinclair, 1968), sensitivity to Cl^- and Br^- in heat-shocked cells of Rhodotorula glutinus (Fries, 1969, 1970). Rudenok and Konev (1973) studied thermal injury of S. cerevisiae and C. utilis. High concentrations of cells released sufficient intracellular materials during heat treatment to provide increased thermal resistance, presumably due to protection of cell membranes. Low concentrations (0.01 mM) of nonpolar aromatic and heterocyclic amino acids also provided a similar protection from thermal stress. In contrast to the results of Fries (1969, 1970), histidine did not provide significant protection during heating.

Hagler and Lewis (1974) reported heat stress of S. cerevisiae in the presence of glucose resulted in membrane damage as demonstrated by extracellular ATPase activity and loss of maintenance of sorbose gradients. The effect was noted uniquely with utilizable sugar and was inhibited

by Ca^{++} or inhibitors of sugar utilization. In addition, Meyer (1975) found thermally-stressed Candida P 25 had extensive ultrastructural changes including aggregation, alteration, and loss of mitochondria along with the appearance of numerous large vacuoles. Arnold and Lacy (1977) reported extensive membrane damage in heat-killed cells of S. cerevisiae. Leakage of intracellular UV-absorbing materials at 260 and 280 nm was found in C. utilis (Shibasaki and Tsuchido, 1973) and S. cerevisiae (Graumlich, 1978) after heat stress.

Yeast Respiration

Yeasts are heterotrophic organisms that can use sugars and a variety of other organic compounds as nutrient sources. From these compounds they derive the carbon skeletons needed to synthesize cellular constituents and the energy necessary to allow biosynthetic reactions to proceed (Rose and Harrison, 1971). Glucose enters the intact yeasts by a catalysed transport across the membrane since the plasma membrane of the yeast cell is highly impermeable to compounds possessing a particle size as large as the sugars (Cirillo, 1961). However, the constitutive hexose transport in S. cerevisiae can take glucose across the membrane about a million times faster than it would otherwise enter.

Yeasts employ primarily the Embden-Meyerhof (E-M, or glycolysis) pathway in the first stages of glucose breakdown, and utilize the Tricarboxylic Acid (TCA, or Krebs') cycle (De Moss and Swim, 1957) and a "Classic" cytochrome system (Cochran, 1958) in terminal oxidation of substrates. To link the E-M pathway to the TCA cycle and other pathways, acetyl-coenzyme A (acetyl-CoA) must be formed. Two pathways exist in yeast to form acetyl-CoA from pyruvate. The first is the enzyme complex pyruvate oxidase which is located in mitochondria only. The second is an enzyme system occurring only in the soluble fraction of yeast and which consists of carboxylase, acetaldehyde dehydrogenase and aceto-CoA-kinase (Holzer and Goedde, 1957). Reduced diphosphopyridine nucleotide (DPNH or NADH) is generated during the operation of these pathways and is reoxidized via the electron transport system (Anderson, 1963).

An alternate pathway of catabolism employed by yeasts is the oxidative pentose phosphate cycle (Blumenthal et al., 1954; Wood, 1955; Korkes, 1956). In this pathway energy is channelled, not to the formation of ATP, but to the reduction of NADP to form $NADPH_2$. In aerobic conditions $NADPH_2$ can be partly oxidized through transdehydrogenation and the respiratory chain, thus contributing to the generation of ATP (Rose and Harrison, 1971; Lehninger, 1975).

Under aerobic conditions, the TCA cycle coupled to the respiratory chain acts as the main energy producer of the

cell. Carbohydrate, amino acids, and lipid derivatives are oxidized to carbon dioxide and water. In yeasts, the different enzymes that catalyse the various steps of the cycle are localized in the mitochondria. The principal components of the respiratory chain and the direction of the electron flow are presented in Figure 1 (Crane and Glenn, 1957; Green, 1959; Rose and Harrison, 1971; Lehninger, 1975).

The transfer of the displaced electrons, through the respiratory chain to molecular oxygen is coupled to the synthesis of ATP from ADP and phosphate (so called "oxidative phosphorylation"). In animal tissues and in some genera of yeasts phosphorylation can take place at three sites, between pyridine nucleotide (NAD) and flavoprotein, cytochrome b and cytochrome c, and cytochrome c and cytochrome a (Morrison, 1961; Rose and Harrison, 1971; Lehninger, 1975). However, in yeasts of the genus Saccharomyces, site I is not coupled to phosphorylation (Ohnishi et al., 1966), with the consequent synthesis of only two instead of three ATP molecules per $NADH_2$ re-oxidized.

In intact yeast in the absence of an external nutrient, endogenous respiration occurs and cell reserves are used only sparingly. Although this phenomenon has not been completely defined, it appears to be nonidentical to metabolism resulting from exogenous substrate (Kotyk, 1961; Anderson, 1963). Earlier studies established that yeast

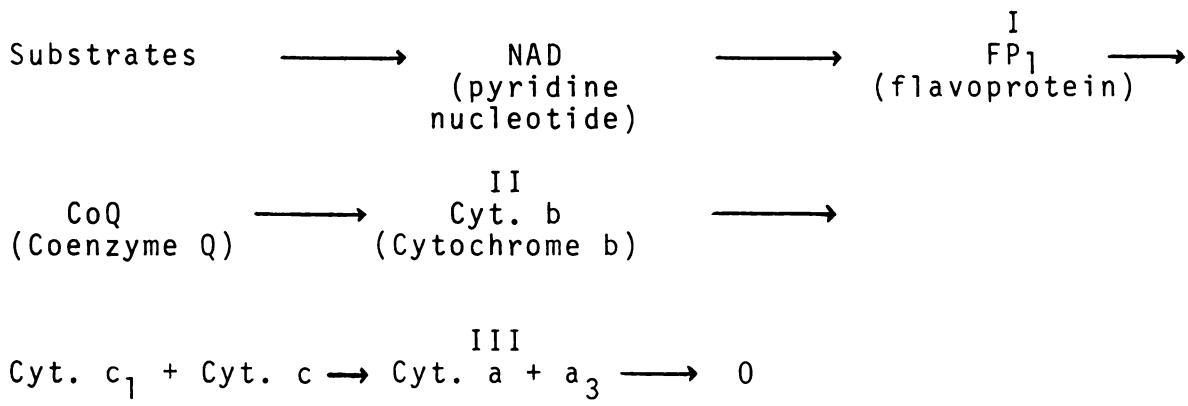


Figure 1. Schematic chart of the respiratory chain and the direction of electron flow (⟶). Sites I, II, and III are the phosphorylation sites.

cells are able to respire endogenously (Stier and Stannard, 1936; Spiegelman and Nozawa, 1945), but that endogenous anaerobic fermentation could not be observed unless certain "inducer" compounds (dinitrophenol or sodium azide) were present (Stickland, 1956; Brady et al., 1961). Additional reports (Chester, 1959a,b; Eaton, 1960) showed that yeast cells can both respire and ferment their endogenous reserves even in the absence of inducers.

The nature of the substance(s) utilized during endogenous respiration of yeast has been the subject of controversy (Dawes and Ribbons, 1962; Rose and Harrison, 1971). The endogenous respiratory quotient (R.Q.) values determined by different workers varying from 3.1 to 0.72 (Stier and Stannard, 1936; Spiegelman and Nozawa, 1945; Stickland, 1956; Chester 1959 a, 1964; Eaton, 1960) suggested that lipids, amino acids and carbohydrates are possible substrates. However, kinetic and chemical studies performed by Eaton (1960) showed the presence of three endogenous substrates in a strain of S. cerevisiae. These consist of two metabolically distinct glycogen pools and the disaccharide, trehalose. Lipids do not serve as a substrate for endogenous respiration. In addition, utilization of one glycogen pool requires the presence of oxygen, while the other can be metabolized either aerobically or anaerobically. Trehalose is used only anaerobically.

Structure, Function and Isolation of Mitochondria

General Features of Mitochondrial Structure

The general features of mitochondrial organization revealed in thin sections of osmium-fixed tissue, consist of an outer membrane which limits the organelle and an inner membrane which is periodically invaginated to form the characteristic cristae mitochondriales (Palade, 1953). The intracristal spaces communicate with the intermembrane space between inner and outer membranes, and these spaces together constitute the outer compartment. The inner membrane and cristae form the boundary layer of the inner compartment or matrix (Lloyd, 1974). Estimates of mitochondrial numbers per cell in yeast vary from 15-29 in a diploid strain and 7-17 in a haploid strain. About 12% of the total volume of the cell is occupied by mitochondria in both diploid and haploid strains (Mahler, 1974). In freeze-etched cells of S. cerevisiae mitochondria appear to be 1-2 μm long and 0.5-1.0 μm thick, shaped like sausages or dumb-bells, and lie near the cytoplasmic membrane (Moor and Muhlethaler, 1963; Moor, 1964). Thread-like mitochondria can be seen protruding into the buds of growing organisms (Matile et al., 1969) where they divide (Thyagarajan et al., 1961). However, extremely marked changes of mitochondrial features can occur during life cycle of yeast as a result of genetic or environmental

modifications (Lloyd, 1974).

Mitochondria from S. cerevisiae resemble mammalian mitochondria in their oxidative activity (Lukin et al., 1968) but differ in lipid composition. Yeast mitochondria contain a lower proportion of phospholipids and a higher proportion of triacylglycerols as well as a higher proportion of mono-unsaturated fatty acids in place of the polyunsaturated fatty acids found in mammalian mitochondria (Rose and Harrison, 1971).

Functions and Properties

The organization of respiratory chain components and the energy-coupling reactions of oxidative phosphorylation are present in the inner mitochondrial membrane (Chance, 1972). The boundary membranes contain the enzymes of the TCA cycle, the enzymes which carry out fatty acid oxidation and elongation, and various auxiliary enzymes such as the transaminases and transferases.

The mitochondrion under physiological conditions is largely an impermeable unit. Small molecules like water, oxygen and carbon dioxide and also ions, such as protons, Na^+, Cl^-, acetate, do indeed penetrate the outer membranes rather rapidly. However, in the absence of special facilitating conditions, larger molecules such as citrate and oxaloacetate, particularly when they have multiple charges, penetrate the outer membranes more slowly. Molecules which

are even larger, such as ATP or NAD, do not penetrate at all (Green and Baum, 1970).

Methods for Isolation of Mitochondria

Isolation of mitochondria from yeast cells is complicated by the presence of a refractory polysaccharide-peptide wall surrounding the cells. The principal components of the cell wall of Saccharomyces are several types of glucan- and a mannan-protein complex. These highly insoluble polysaccharides have predominantly β-1, 3 linkages between the glucose moieties, but β-1, 6 linkages have also been found. The cell wall represents about 15% of the dry weight of the yeast and consists of 20-40% mannan, 5-10% protein 1% chitin and 30-60% glucan (Bacon et al., 1965; Fleet and Phaff, 1974).

Rupture of the cell wall for the release of mitochondria can be achieved by mechanical means (Vitols and Linnane, 1961; Schatz, 1967; Mattoon and Balcavage, 1967), or the cell wall can be removed by digestion with the enzymes present in snail gut juice to form yeast protoplasts (Duell et al., 1964; Ohnishi et al., 1966; Schatz and Kovac, 1974).

Mechanical Means. Rapid mechanical methods are based upon direct agitation of yeast cells in the presence of fine glass beads (Lamanna and Mallette, 1954). Breakage of the yeast cell in the Braun (or Bronwill) glass-bead

shaker was found to be effective and inexpensive to operate but accommodates only 20-30 g of yeast cells per shaking vessel (Tzagoloff, 1971; Mason et al., 1973; Salton, 1974). Disintegration of yeast cells in a Waring blendor in the presence of liquid nitrogen (Tzagoloff, 1969) or disruption in a Manton-Gaulin homogenizer (Mason et al., 1973) was more applicable to large-scale preparation of mitochondria. However, the reliability of both methods was dependent upon different yeast strains or commercial preparations of yeast. Improvements in the yield of yeast mitochondria was achieved by grinding cells in a Dyno-Mill KDL continuous-flow cell disintegrator (Rehacek et al., 1969; Deters et al., 1976). These mechanical methods provided mitochondria with low content of cytochrome c, no respiratory control, and incapable of phosphorylating ADP (Labbe and Chambon, 1977; Lang et al., 1977). Labbe and Chambon (1977) used an osmotic stabilizer in the "breaking" buffer to retain good respiratory controls and ADP/O ratios of mitochondria.

Enzymatic Digestion. Preparation of protoplasts by dissolving the cell wall of yeast cells with the gut juice of the snail Helix pomatia has been reported (Eddy and Williamson, 1957, 1959; Bacon et al., 1965; Anderson and Millbank, 1966; Wiley, 1974). The efficiency of enzyme digestion was influenced by certain genera of yeasts, phase of growth of harvested cells, incubation at 30 C,

and the nature of sugar used in the incubation mixture (Eddy and Williamson, 1957, 1959; Duell et al., 1964; Ohnishi et al., 1966; Brown, 1971; Mann et al., 1972; Schwencke et al., 1977). Preincubation of yeast cells with ethylenediaminetetraacetate (EDTA) (Mann et al., 1972), sulfhydryl or thiol reagents (Schwencke et al., 1977) such as 2-mercaptoethanol (Duell et al., 1964; Bacon et al., 1965; Anderson and Millbank, 1966) and sodium thioglycollate (Kovac et al., 1968) enhanced the enzymic activity and improved yields of protoplasts prepared from cells harvested at the stationary phase of growth. Supplementation of sucrose, mannitol or sorbitol, and sometimes bovine serum albumin (BSA) in reaction mixture at certain concentration helped maintain stability in the protoplast.

The release of mitochondria from the protoplasts is achieved by osmotic shock (Duell et al., 1964), or low pressure treatment in a French pressure cell (Linnane and Lukins, 1975), or homogenization of protoplasts in a Waring Blendor at low speeds (Ohnishi et al., 1966; Schatz et al., 1967; Kovac et al., 1968; Grivell et al., 1971). Mitochondria released from gently ruptured protoplasts maintain adequate respiratory control, oxidative phosphorylation and respiratory activity (Linnane and Lukins, 1975).

MATERIALS AND METHODS

Organism Used

The yeast, Saccharomyces cerevisiae (coded Y 25), was obtained from the culture collection of the Food Microbiology Laboratory, Michigan State University. A stock culture was maintained on YMPG agar slant (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose, 2.5% Bacto agar; pH 5.25) at 4 C. The culture was transferred monthly and incubated overnight at 25 C to retain viability.

In some experiments, baker's yeast powder purchased from the Michigan State University Food Store was utilized. It was air dried and kept in the freezer until used.

Determination of Growth Cycle

Preparation of a starting culture was performed according to the procedure of Graumlich (1978). A loopful of stock culture of S. cerevisiae was incubated on a YMPG agar slant. After incubation for 24 hours at 25 C, the ensuing growth was used to inoculate 500 ml of YMPG broth in a 1-liter Erlenmeyer flask. The liquid culture was incubated at 25 C with rotation at 200 rpm on a Model G-25 gyratory

shaker (New Brunswick Scientific Co., New Brunswick, New Jersey). The samples were withdrawn by pipetting at time intervals ranging from 0 to 30 days. Each sample was serially diluted in water and duplicate pour-plates of the appropriate dilutions were prepared. The medium used was plate count agar (PCA) (Difco Laboratories, Detroit, Michigan) which was tempered to 45 C befour pouring. The colony-forming units (CFU) were counted after incubation for 5 days at 25 C. A growth curve was plotted as logarithm of CFU per milliliter of cell suspension vs days.

Preparation of the Intact Yeast Cell Suspensions

Suspensions of intact cells of S. cerevisiae were prepared as outlined by Graumlich (1978). As described previously, a 24-hour growth from a YMPG agar slant was used to inoculate 500 ml of YMPG broth in a 1-liter Erlenmeyer flask. After incubation for 0.5, 1 and 4 days at 25 C with a rotation at 200 rpm on a shaker, the cells were harvested by centrifugation for 10 minutes at 1,500 x g. The precipitate was washed 3 times by centrifugation with 200 ml of cold distilled water. The final cell pellets were suspended in 50 ml of cold distilled water. The cells were counted with a Petroff-Hausser Counting Chamber (C.A. Hausser and Son, Philadelphia, Pennsylvania), and were

adjusted to be at a concentration of 3.5×10^8 cells per ml. The resulting cell suspension was held at 4 C until used.

Thermal Stress of Yeasts

Thermal stress experiments were performed by following the method of Graumlich (1978) which utilized the flask method described by the National Canners Association (1968). The heating temperatures were 56, 60, 62, and 65 C. Each temperature was maintained in a water bath heated by a Bronwill 20 constant-temperature circulator (Bronwill Scientific Co., Rochester, New York). In one experiment, five ml of the yeast cell suspension was added to 245 ml of water preheated to 56 C in a 500-ml screw-capped Erlenmeyer flask. The original cell concentration was adjusted to a final cell concentration of 3.5×10^7 cells per ml. For the rest of the experiments, 11 ml of the yeast cell suspension was added to 99 ml of water preheated to a desired temperature in a 250-ml screw-capped Erlenmeyer flask. The flask was stabilized by large metal washers and the water in the bath was maintained at a level to within one inch of the screw cap. The content of the flask was mixed with a magnetic stirring bar to help provide a uniform temperature and to maintain suspension of the yeast cells.

Heated yeast cell suspensions were withdrawn at appropriate times, ranging from 0 to 20 minutes by pipetting,

and placed in precooled test tubes immersed in an ice bath. Unheated yeast cell suspension was withdrawn at 0 time of heating and treated as above.

Non-heated yeast cell suspension was prepared by adding 1 ml of the cell suspension (3.5×10^8 cells) to 9 ml water in a screw-capped test tube. This specimen was not heated and was held in an ice bath.

Samples of unheated and heated yeast cells were serially diluted in water. Duplicate pour-plates of the appropriate dilutions were prepared immediately. Sometimes, if the pour-plates were delayed, the samples were held in an ice bath. The media used were PCA and/or potato dextrose agar (PDA) (Difco Laboratories, Detroit, Michigan). The colony-forming units were counted after incubation at 25 C for 5 days.

Respiration of Intact Yeast Cells

Endogenous and exogenous respirations of non-heated and heated intact yeast cells were measured at 30 C by means of a Clark-type polarographic electrode used with a YSI model 53 Biological Oxygen Monitor (Scientific Division, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Appropriate temperatures were maintained in a water bath heated by a Model MDL 73 Heated Circulator (Poly-Science Corporation, Niles, Illinois). Oxygen uptake was

measured by placing 3 ml of air-saturated distilled water, prewarmed at 30 C, in a glass sample chamber of the 5301 Standard Bath Assembly equipped with a magnetic stirrer. The probe (electrode) was inserted. All excess air was expelled through a slot in the probe plunger by slightly twisting the plunger to gather the bubbles at the slot. After 3 minutes for temperature equilibrium, a tenth of a milliliter of non-heated or heated yeast cell suspensions in a one-milliliter syringe was added by inserting the needle through a slot in the plunger. For measuring exogenous respiration, substrate at a final concentration of 1 mM was added. Substrates used were glucose (Mallinckrodt, Inc., Paris, Kentucky), sodium succinate (Fisher Scientific Company, Fairlawn, New Jersey), potassium pyruvate (Pfaltz & Bauer, Inc., Stanford, Connecticut), and sodium malate (Sigma Chemical Company, St. Louis, Missouri).

The percentage of oxygen uptake was recorded for 15 minutes with a ten-inch potentiometric recorder (Beckman Instruments, Inc., Scientific and Process Instruments Division, Fullerton, California) which was connected to the oxygen monitor. Both the oxygen monitor and the recorder were calibrated at 100% O_2 reading with 3 ml of air-saturated distilled. For the zero percent O_2 reading, 3 ml of air-saturated distilled water, containing a trace amount of sodium hydrosulfite, was employed.

Dry weights of yeast cells were determined on one milliliter portions of cell suspensions added to pre-weighed aluminum-foil cups which were heated at 110 C for 1-2 days. The dry weight ranged between 2.0 to 4.5 mg/ml of cell suspension.

The O_2 uptake was calculated according to Estabrook (1967) and expressed as microliters (µl) of O_2 per mg dry weight of yeast cell. The respiration rate (µl O_2/mg dry weight/hour) was calculated by drawing the least square straight regression line and determining its slope.

Isolation of Yeast Mitochondria

Enzymatic digestion and mechanical breakdown or both were used in an attempt to lyse the cell wall of yeast cells as an approach to the isolation of crude mitochondria.

Method I: Using Enzymatic Digestion and/or Mechanical Breakdown

The enzyme glusulase, purchased from Endo Laboratories, Garden City, New York, was used. Protoplasts and mitochondria were prepared by a modification of several procedures (Duell et al., 1964; Ohnishi et al., 1966; Kovac et al., 1968; Lamb et al., 1968; Linnane and Lukins, 1975; Guerin et al., 1979). The yeast culture, grown as previously described, was harvested by centrifugation either after 0.5 or 1 day or 4 days of incubation. To

remove the spent medium, the cells were washed once and resuspended in Tris-ethylenediaminetetraacetic acid solution (Tris-EDTA), pH 9.3. β-Mercaptoethanol (β-M.E.) was added to reduce disulfide bonds which stabilize the cell wall against enzyme digestion. The suspension was incubated with occasional shaking in the water bath at 30 C for 15 minutes. After removal of the excess β-M.E. by centrifugation and washing with citrate buffer (0.6 M mannitol, 0.3 M sorbitol, 0.2 M potassium hydrogen phosphate, 0.1 M citric acid, and 0.001 M EDTA, pH 5.8), a solution of the glusulase enzyme was added (1-3 ml/10 ml packed cells). The mixture was incubated with occasional shaking at 30 C. The formation of protoplasts was followed by diluting a sample of the incubation mixture 80-fold in 0.9 M sorbitol. The protoplasts and whole cells were counted with a Petroff-Hausser Chamber and a phase microscope. When a similar dilution of a sample was made in water, only whole cells were counted. The difference in the two counts indicated the number of protoplasts.

After incubation with the glusulase enzyme, the cells and protoplasts were centrifuged and washed twice with citrate buffer. The protoplasts were lysed by either suspension in 0.05 M sorbitol solution containing 0.02 M magnesium chloride, 0.01 M potassium chloride, and 0.005 M potassium phosphate (pH 6.8) or homogenized in a Waring blendor (Waring Products Division, New Hartford,

Connecticut). Detailed procedures are outlined in Figures 2, 3a, and 3b. The crude mitochondrial precipitates thus prepared were suspended in buffer solution and kept in an ice bath until used.

Method II: Disruption of Cells with Glass Beads

This procedure, modified from the method of Lang et al. (1977), is outlined in Figure 4. A series of ice-cold, 180-gram acid-washed glass beads (0.25-0.3 mm in diameter, VWR Scientific, VWR United Company, Allen Park, Michigan) in 150-ml centrifuge bottles was prepared. An appropriate volume of breakage buffer (B.B.) was added to glass beads until the miniscus level was seen. About 5 ml each of the cell suspension was layered on the surface of the glass beads (about 2-3 mm thick). The bottle was held vertically and vigorously shaken up and down in a vertical motion for 4 minutes (150-200 times). The broken cell suspension was decanted from the glass bead, and the beads were washed repeatedly with small volumes of buffer which were added to the decantant which was centrifuged to yield pellet and supernatant. The pellet was redispersed by shaking in a small volume of buffer. Differential centrifugation was employed to separate crude mitochondria (pellet) from whole cells and cell debris. The white fat portion along the side of the centrifuge tube was wiped off. Finally, crude mitochondria were washed and resuspended in buffer and held

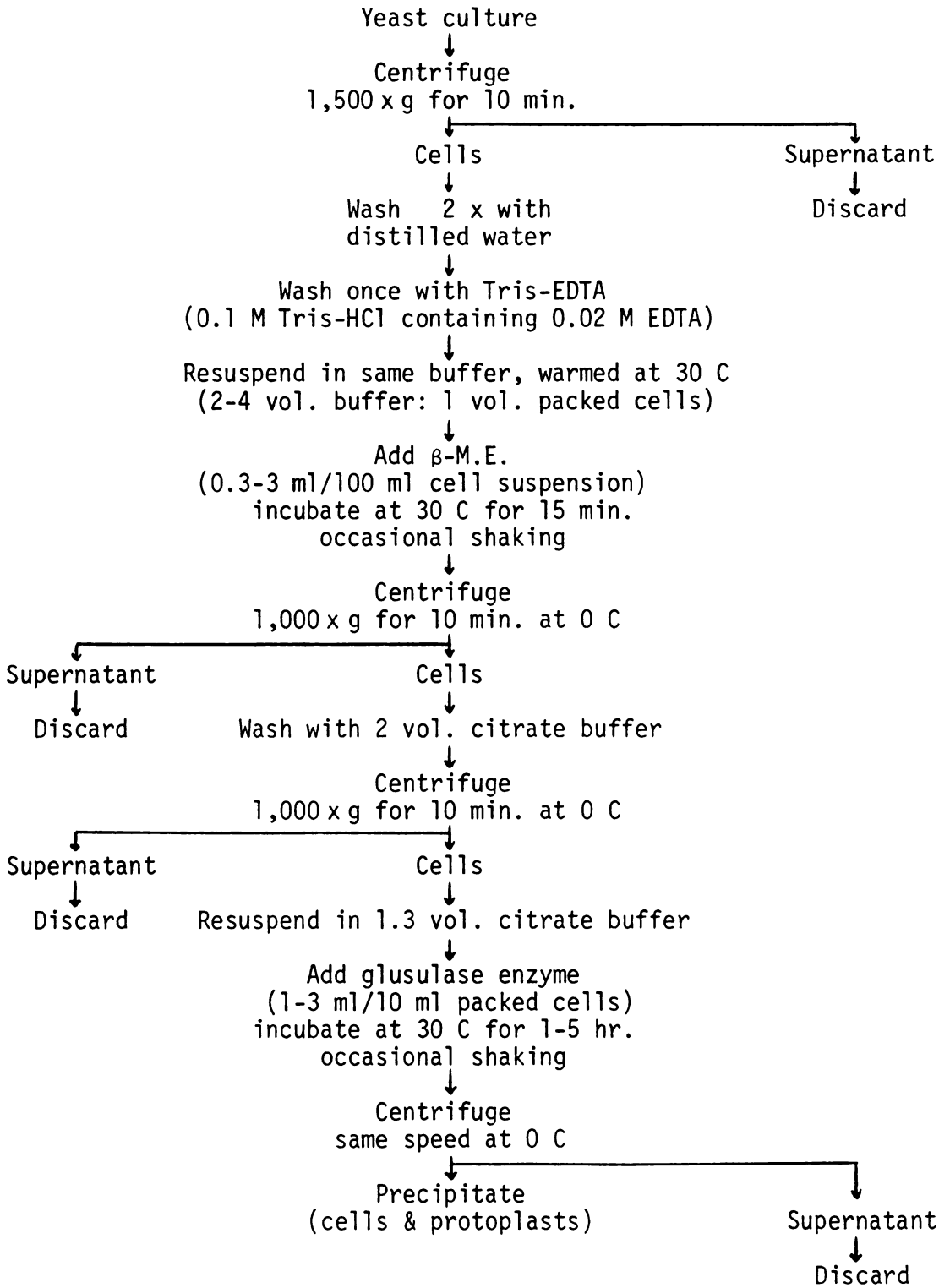


Figure 2. Flow chart for the preparation of protoplast from yeast cells.

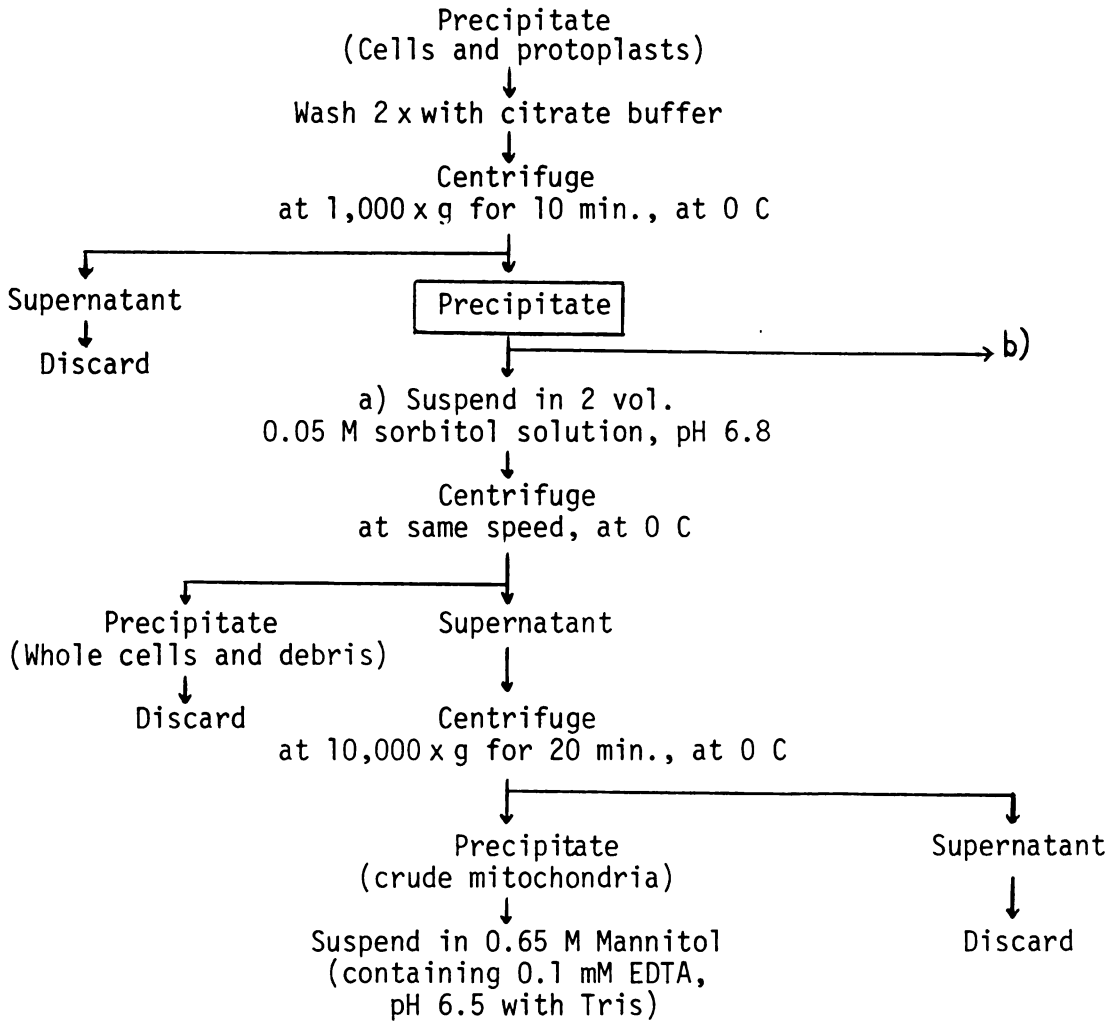


Figure 3a. Flow chart for the preparation of mitochondria from protoplast by lysis.

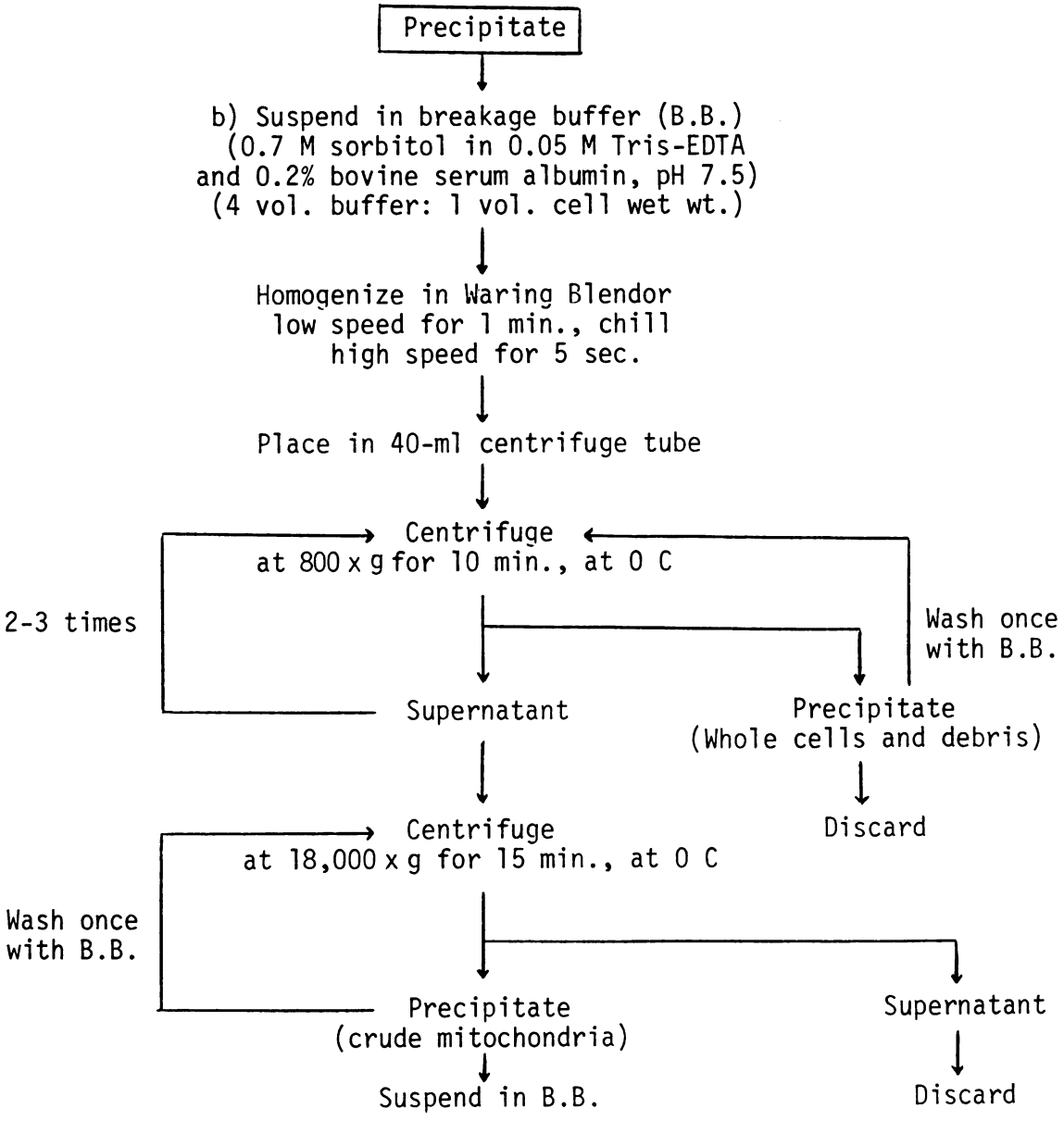


Figure 3b. Flow chart for the preparation of mitochondria from protoplast by homogenization.

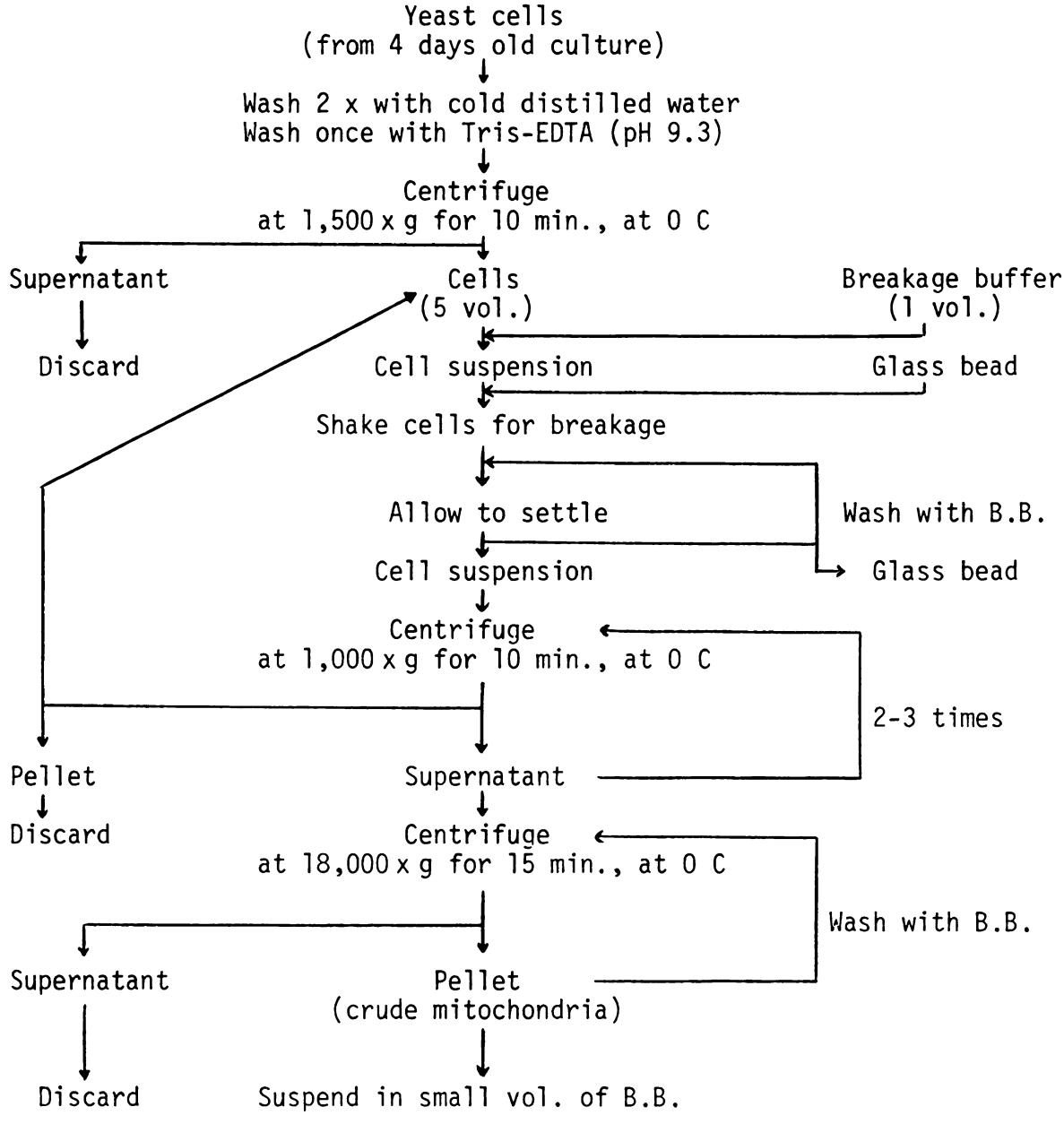


Figure 4. Flow chart for the preparation of mitochondria by disruption of yeast cells with glass beads.

at 0 C.

Method III: Mechanical Disruption of Cells

Crude suspensions of mitochondria were prepared from baker's yeast by following the methods of Gottlieb and Ramachandran (1961) and Anderson (1963) as outlined in Figure 5. Either a 1-liter Waring Blendor or a VirTis "23" Homogenizer (The VirTis Company, Inc., Gardiner, New York) was employed to break yeast cells in the presence of acid-washed glass beads (0.25-0.3 mm diameter). The choice of instrument is dependent upon the sample volume. Normally, 200 g wet weight of yeast and 300 g of glass beads in 50 µmole of NAD (nicotinamide adenine dinucleotide) and 75 ml of 0.4 M lactose containing 0.02 M potassium phosphate buffer (pH 7.0) were placed in 1-L Waring Blendor (metal container). For smaller lots, the VirTis Homogenizer was used; 4 g wet weight of yeast and 4 g of glass beads in 10 µmoles of NAD, 600 µmoles of lactose, and 30 µmoles of potassium phosphate at pH 7.0 (total volume excluding beads of 5 ml) constituted the charge. The cell suspension and container was cooled (not exceeding 4 C) by surrounding with a dry-ice packing or a dry ice-alcohol bath during the breakage period. As in method II, the crude mitochondrial suspension was separated from the glass beads; the remaining whole cells and cell wall debris was separated by low and high speed centrifugation and stored in ice bath.

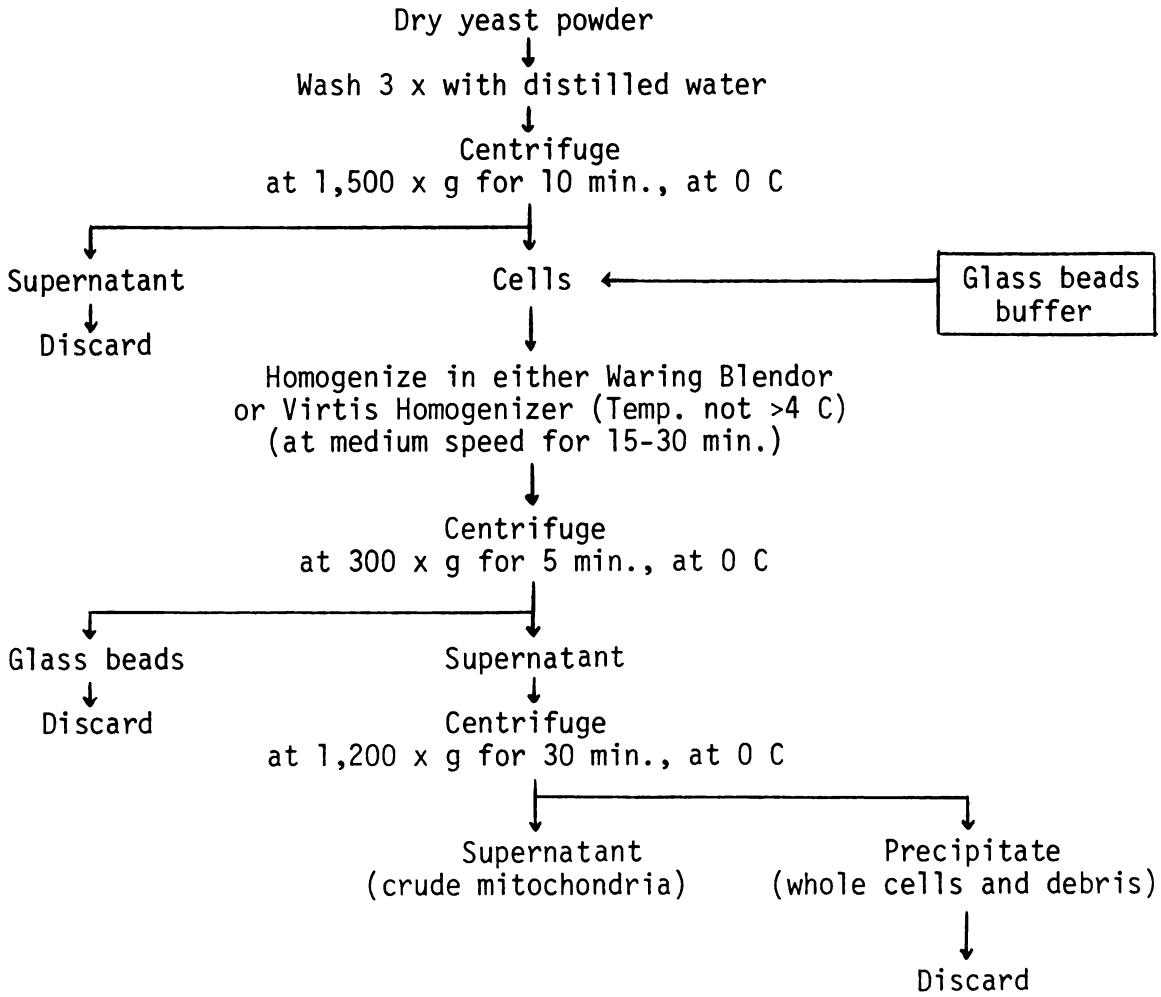


Figure 5. Flow chart for the preparation of mitochondria by mechanical disruption of yeast cells.

Determination of Protein Concentration

The concentration of mitochondria was expressed as mg protein/ml of suspension. The protein concentration was determined by the Bio-Rad Protein Assay. Absorbance at 595 nm was measured in a Spectronic 20 spectrophotometer (Bausch & Lomb Inc., Rochester, New York). A standard curve was developed, using bovine serum albumin (BSA, Sigma Chemical Company, St. Louis, Missouri) as the standard protein.

Thermal Stress of Yeast Mitochondria

The effect of heat stress on yeast mitochondria at 56 C was assayed similarly to the assay on intact yeast cells as described previously. However, instead of employing the flask method, a tube method was utilized. One ml of mitochondrial suspension was transferred to 13 x 100 mm screw-capped test tubes, held in a 56 C water bath. After 1 or 2 minutes of heating, the tubes were placed in an ice bath.

Respiration of Yeast Mitochondria

Determinations of the exogenous respiration rate for non-heated and heat-stressed mitochondria were conducted at 30 C by procedures similar to those employed for intact yeast cells. The reaction mixture was 0.4 M lactose/0.02 M potassium phosphate buffer, pH 7.0. Buffer volume used was varied so that the final volume in the glass sample chamber was between 3.0 to 3.02 ml. After addition of a small volume of mitochondrial suspension (0.5-2.5 mg protein), approximately 0.04-0.06 µl of various substrates were added. These consisted of sodium succinate, sodium citrate (Mallinckrost, Inc., Paris, Kentucky) (at final concentration of 6 mM) and NADH (reduced form of nicotinamide adenine dinucleotide) (Calbiochem, Los Angeles) at a final concentration of 375 and 576 µM. Adenosine, 5'-diphosphate (ADP) (Sigma Chemical Company, St. Louis, Missouri) and 2,4-dinitrophenol (DNP) (United States Biochemical Corporation, Cleveland, Ohio) were also used at a final concentration of 333 µM and 0.1 mM, respectively.

Oxygen uptakes were measured from polarographic tracings recorded in response to the addition of substrates. The oxidation rates were determined as µmoles of O_2/mg of protein/minute. Respiratory control ratios (RC ratios) were calculated by the method of Chance and Williams (1956) as described by Ohnishi et al. (1966), according to the equation:

$$\text{RC ratio} = \frac{\text{respiration rate in the presence of ADP}}{\text{respiration rate after exhaustion of ADP}}$$

The efficiency of phosphorylation in term of ADP:O ratios were calculated from the polarographic respiratory tracings in response to the addition of a definite amount of ADP (Chance and Williams, 1956; Ohnishi et al., 1966). The ADP/O ratio (P/O ratio) is defined as total amount of ADP added/amount of oxygen consumed from the addition of ADP until the respiration returns to a low rate (Nedergaard and Cannon, 1979).

Spectrophotometric assays of NADH oxidation by non-heated and heat-stressed yeast mitochondria were also conducted by following the decrease in optical density (O.D.) at 340 nm on a Beckman DB-G Grating Spectrophotometer (Beckman Instruments, Inc., Scientific and Process Instruments Division, Fullerton, California) (Anderson, 1963). Various concentrations of mitochondria, ranging from 1.0 to 3.4 mg of protein, were added to an appropriate volume of 0.4 M lactose/0.02 M potassium phosphate buffer, pH 7.0 in a glass cuvette. The reaction was initiated by the addition of NADH (0.1 and 0.5 µmoles). The total volume of the mixture was 3 ml. The absorbance (A_{340}) was recorded at different time intervals.

Phase Transitions in Cell Membranes of the Intact Yeast Cells

The effect of thermal stress on the cell membrane of S. cerevisiae was studied. The suspension of this organism was prepared as previously described from 4-day old culture. Phase transitions in cell membranes of non-heated cells and cells heated at 56 C for 1 and 2 minutes were observed by means of fluorescence studies (Overath and Trauble, 1973). The fluorescence measurements were performed immediately (at 0 hour) after the cells were heated, and again, at 5 hours after heating and storing the cells in water at 25 C. Approximately 1.0×10^8 cells in 2.9 ml aliquot of each specimen were centrifuged at 12,520 x g for 5 minutes in a Sorvall type SS-1 rotor (Ivan Sorvall, Inc., Norwalk, Connecticut) and the pellet was resuspended in 3.5 ml of CR buffer. CR buffer consisted of 12 g of K_2HPO_4, 3 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, and 0.2 g of $MgSO_4 \cdot 7H_2O$ (Mallinckrodt, Inc., Paris, Kentucky) per 1 liter of water. A 10 µl methanolic solution of 10^{-3} M N-phenyl-1-naphthylamine (PhNap) (Sigma Chemical Company, St. Louis, Missouri) was added. All reagents were used at room temperature.

Then, the mixture of cell suspension and dye was transferred into a quartz fluorimeter cuvette and cooled slowly to 2 C. Fluorescence was measured between 2 and 70 C with an Aminco SPF 125 spectrofluorimeter (American Instrument Co., Inc., Silver Spring, Maryland) connected to

a YSI Model 42 SC Tele-Thermometer (Scientific Division, Yellow Springs Instrument Co., Yellow Springs, Ohio). The excitation and emission wavelengths were at 360 and 410 nm, respectively. The temperature in the cuvette was changed at a rate of about 2-3 C/minute. Continuous recording of the fluorescence intensity vs temperature was accomplished by using a Sargent Recorder Model SR (E.H. Sargent & Co., Chicago, Illinois).

RESULTS

Growth Cycle of *Saccharomyces cerevisiae*

Figure 6 represents the growth cycle of aerobically cultured *S. cerevisiae* at 25 C with 200 rpm rotation. When 24-hour harvested cells were transferred into a large volume of YMPG broth, the initial cell concentration (at 0 day of incubation) was 10^6/ml. Without the lag phase, the organism started to grow exponentially and reached a stationary phase within 1 day. The concentration of the cells in the stationary phase was about 320 times greater than the initial concentration. With aeration in 500 ml broth, the organism maintained stationary population lever up to 30 days.

In this investigation, the cells were harvested after 0.5, 1 and 4 days of incubation. Thus, the effects of heat stress on the growing and resting cells of *S. cerevisiae* were studied.

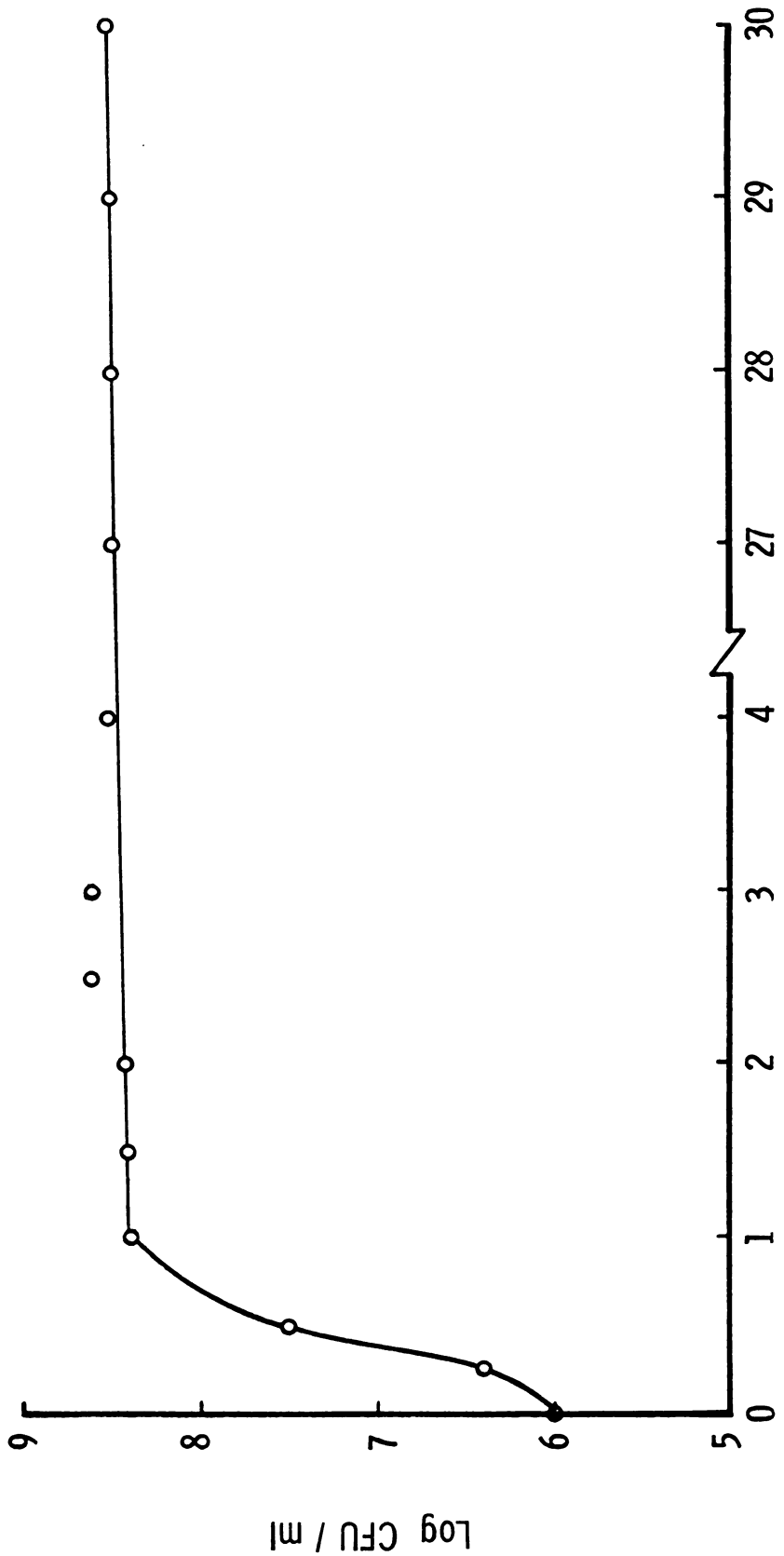


Figure 6. Growth curve of Saccharomyces cerevisiae, plated on plate count agar (PCA) and incubated at 25 C for 5 days.

Effect of Physiological State on Recovery of Thermally-Stressed Yeasts

4-Day Resting Cell Suspension

Experiments were conducted on the effect of heating suspensions of resting cells of S. cerevisiae at 56 C in water. The numbers of survivor cells were determined on PCA plates at sampling times of 0, 1.5, 3.0, and 4.5 minutes. The results (Figure 7), which were similar to those reported by Graumlich (1978), indicated that the plate counts of unheated cells on PCA and PDA media were equal, whereas the counts of cells heated for 1.5, 3.0, and 4.5 minutes were significantly different. The recoveries of heated cells on PCA were approximately 3.2, 10, and 100 times greater than those counted on PDA for cells heating at 1.5, 3.0, and 4.5 minutes, respectively.

Additional experiments involving the heating of yeast cell suspensions at 56 C for longer periods of time were conducted. As shown in Figure 8, cell populations were substantially decreased during the first 2 minutes. Approximately 10,000-fold reduction of cells occurred after 6 minutes (>99% reduction). The numbers of heat-stressed cells which remained were gradually decreased during the next 12 minutes and were destroyed completely after heating for 20 minutes.

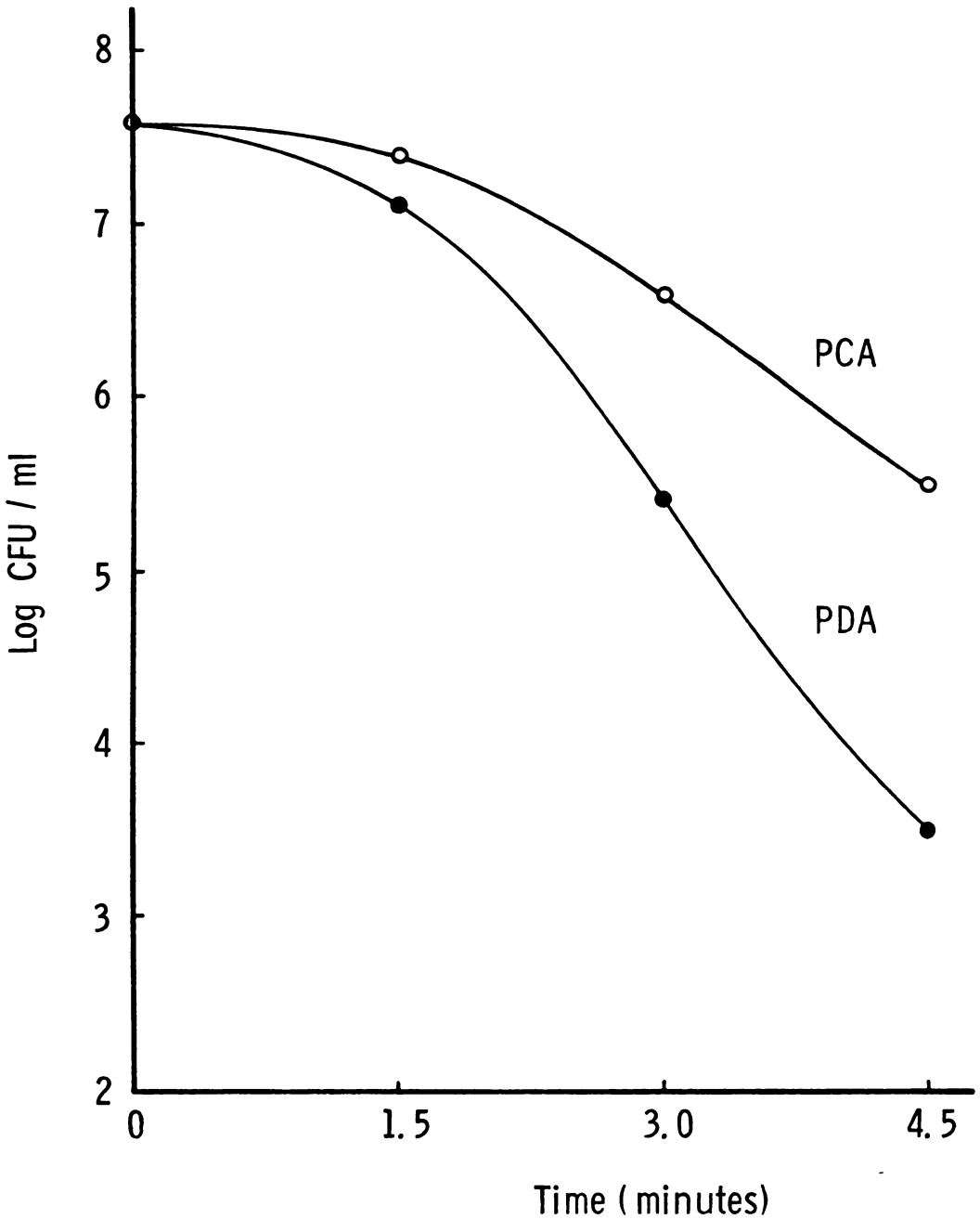


Figure 7. Plate counts of heat-stressed Saccharomyces cerevisiae on plate count agar (PCA) and potato dextrose agar (PDA). Cells were heated at 56 C. The colony-forming units (CFU) were counted after incubation at 25 C for 5 days.

Differences in the recovery of S. cerevisiae when heated at temperatures higher than 56 C were noted. These results (Figure 8) indicate that as the heating temperature was increased the plate count decreased. During the first 2 minutes of heating, cells were destroyed rapidly at 65 C > at 62 C > at 60 C. More than 4 to 6 orders of magnitude of cell populations were destroyed at these three heating temperatures. The remaining cells died completely within 4 to 5 minutes.

1-Day Yeast Cell Suspension

The organism started to enter the stationary phase after incubation at 25 C for 1 day. The heat resistance of the cells in this state was less than that for cells already in the stationary period (4-day cells). When the suspension of 1-day cells was heated at 56 C in water, a slight plateau in the curve was observed on the PCA plate count (Figure 9) for cells heated for 1 minute. After 2 minutes, more than 4 orders of magnitude of the heat-stressed cells were destroyed. The cells were completely killed within 4 minutes.

0.5-Day Yeast Cell Suspension

Organisms were harvested during the exponential growth phase. Suspension of the cells was heated at 56 C in water. Plate counts on PCA, shown in Figure 10, indicated that cells harvested in their exponential phase were much

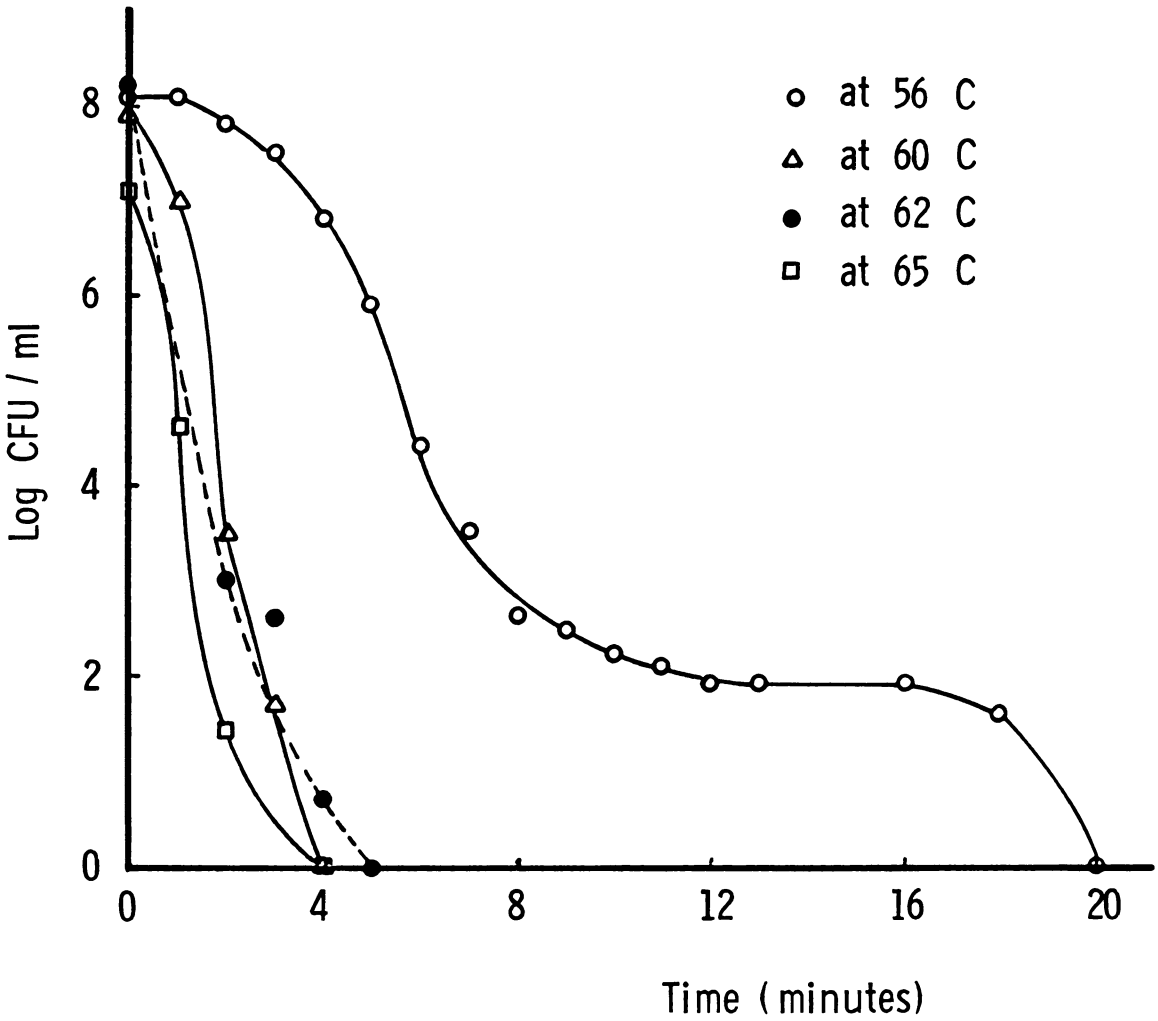


Figure 8. Plate counts on PCA of heat-stressed cells prepared from 4-day old culture of Saccharomyces cerevisiae. Cells were heated at 56, 60, 62, and 65 C. The colony-forming units (CFU) were counted after incubation at 25 C for 5 days.

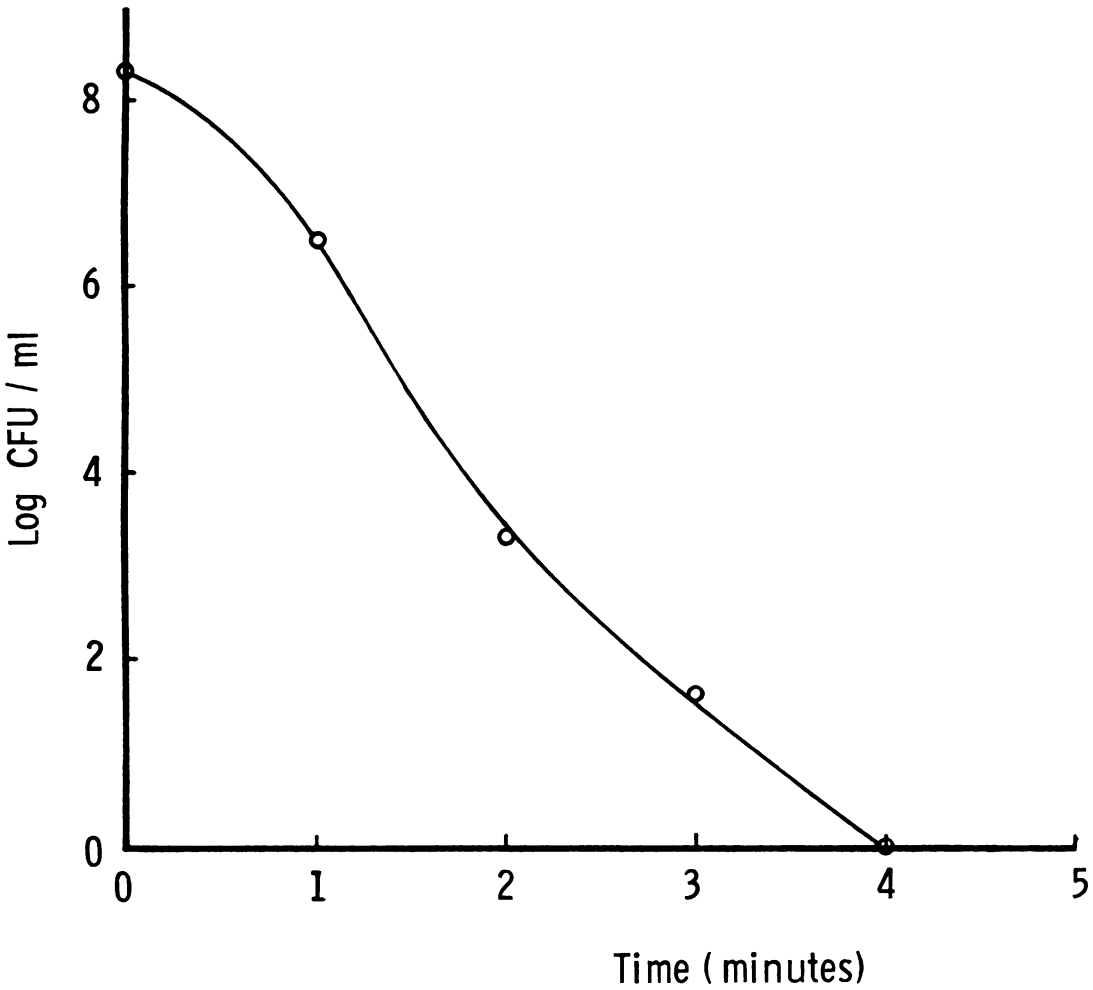


Figure 9. Plate count on PCA of heat-stressed cells prepared from 1-day old culture of Saccharomyces cerevisiae. Cells were heated at 56 C in water.

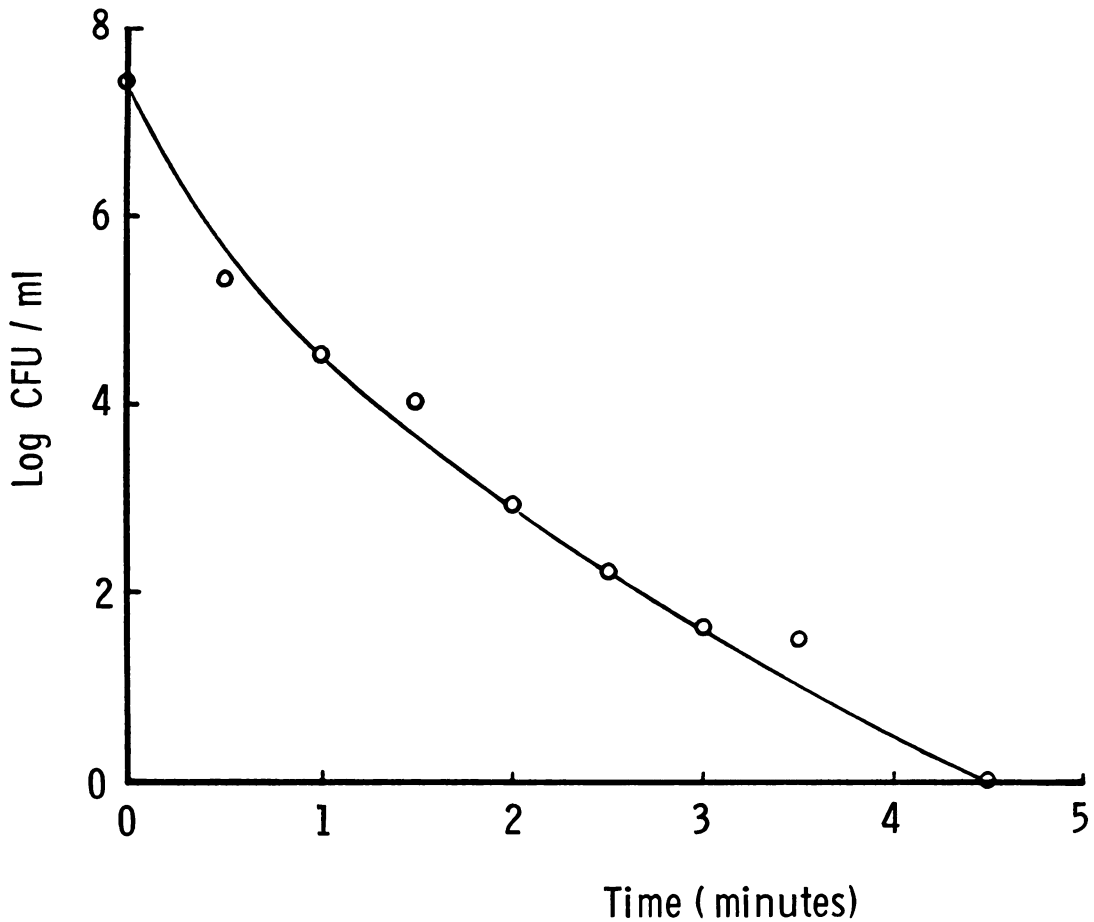


Figure 10. Plate count on PCA of heat-stressed cells prepared from 0.5-day old culture of Saccharomyces cerevisiae. Cells were heated at 56 C in water.

more susceptible than 1-day stationary growth phase cells when heated at 56 C for 1 minute. However, more than 4 orders of magnitude of exponential-phase cells were destroyed within 2 minutes of heating. The cells were completely inactivated after approximately 4.5 minutes at this temperature.

Effect of Cold Storage on Recovery of Thermally-Stressed Yeasts

Attempts were made to determine whether a delay in making plate counts had an effect on recovery of thermally-stressed yeasts. Following a thermal-stress treatment, samples from a suspension of S. cerevisiae were withdrawn at selected time intervals and stored in ice bath. Plate counts were performed on PCA following storage of unheated and heat-stressed cells at 4 C for 1, 2, 3, and 24 hours. The results (Figure 11) indicated that storage of thermally-stressed yeasts in water at 4 C did not affect cell recovery from thermal injury. The PCA plate counts were not significantly different during 24 hours of storage.

Respiration of Thermally-Stressed Intact Yeast Cells

4-Day Resting Cell Suspension

Figure 12 presents data representing endogenous respirations of non-heated and heat-stressed cells of

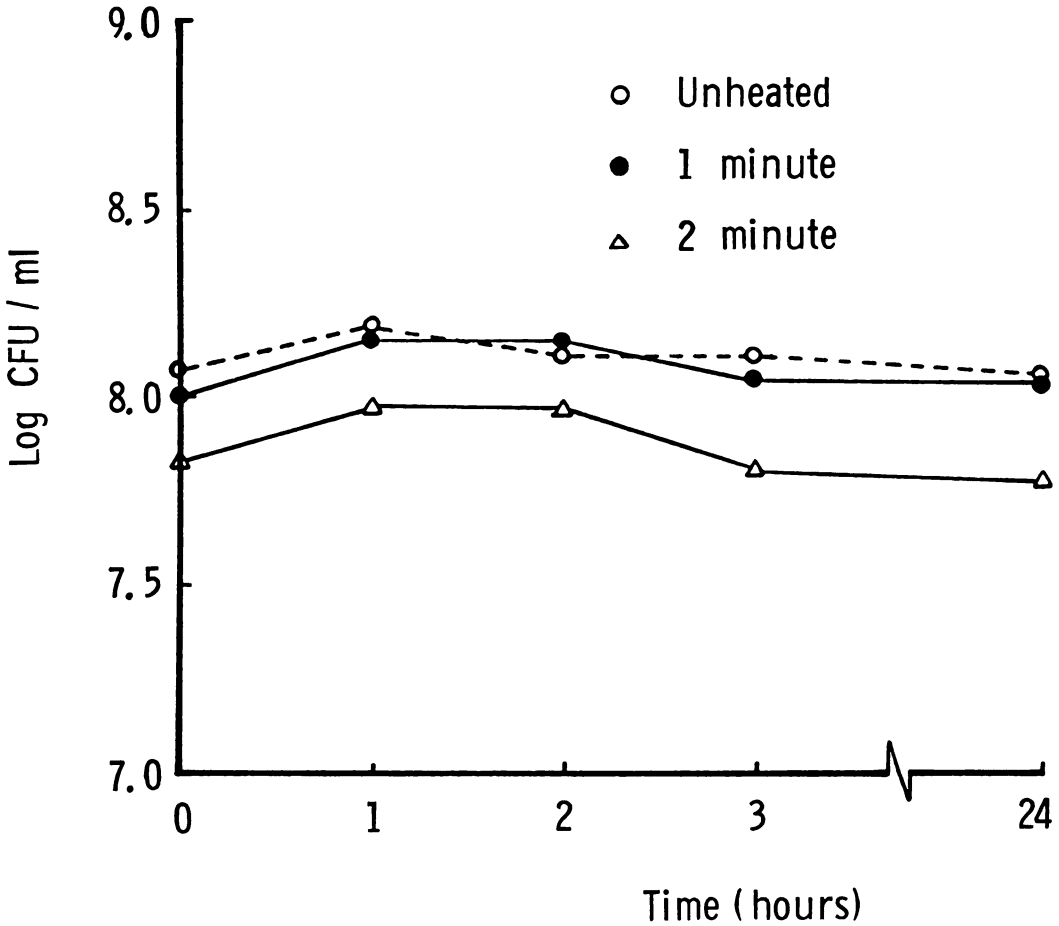


Figure 11. Effect of storage in water at 4 C on plate counts of heat-stressed Saccharomyces cerevisiae. Cells were heated at 56 C for 0, 1, and 2 minutes and plated on PCA.

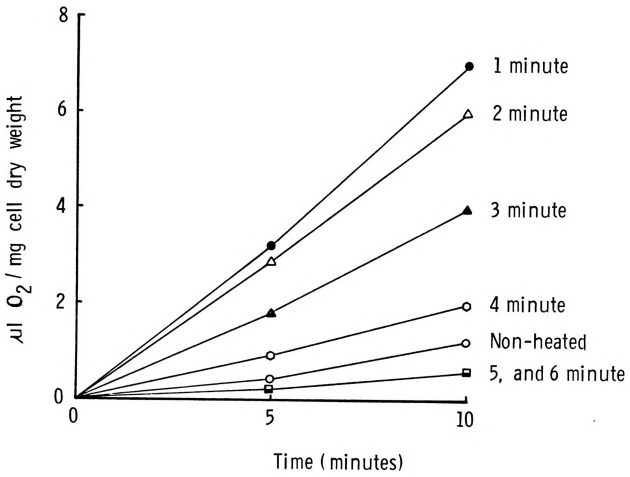


Figure 12. Endogenous respiration at 30 C in water of non-heated and heat-stressed cells prepared from 4-day old culture of Saccharomyces cerevisiae. Cells were heated at 56 C for various times.

S. cerevisiae measured in water at 30 C for 10 minutes. Oxygen uptakes by non-heated cells were 0.4 and 1.2 µl/mg cell dry weight at 5 and 10 minutes, respectively. Cells heated at 56 C for 1 through 4 minutes respired considerably more oxygen than did non-heated cells. The rate of O_2 uptake increased initially and continued through the first 10 minutes of measurement. However, cells heated for 5 and 6 minutes exhibited less uptake of O_2 than that of non-heated cells. No detection of O_2 uptake of cells heated for more than 6 minutes was noted during 10 minutes of measurement.

Differences in endogenous O_2 uptake among heat-stressed cells were associated with the severity of thermal stress. As illustrated in Figures 8 and 12, the longer cells of S. cerevisiae were exposed to heat stress, the lower the plate count on PCA and the lower the O_2 uptake. Lower populations remained after the cells were heated at 56 C for longer periods of time.

The rates of endogenous O_2 uptake of non-heated and heat-stressed cells, calculated as Q_{O_2} (µl O_2/mg dried yeast/hour), are summarized in Table 1. In a similar order from high to low, cells heated for 1 through 4 minutes had average Q_{O_2} of 40.9, 35.1, 22.2, and 11.8, respectively, while non-heated cells had average Q_{O_2} of 5.0. The Q_{O_2} of cells heated for 5 and 6 minutes were 3.4 and 1.1, respectively.

Table 1. Rates of endogenous O_2-uptake in water at 30 C for non-heated and heat-stressed cells of 4-day old cultured Saccharomyces cerevisiae.

Temp. (C)	Heat Stress (minute)	Q_{O_2} (μl O_2/mg dried yeast/hour)
56	none	5.0
	1	40.9
	2	35.1
	3	22.2
	4	11.8
	5	3.4
	6	1.1
60	none	5.0
	1	12.5
	2	1.5

Oxygen uptake in response to the presence of added substrates was also measured in non-heated and heat-stressed cells. The addition of exogenous substrates, like glucose, sodium succinate, potassium pyruvate and/or malate, stimulated oxygen uptake in non-heated cells and cells heated for 1 and 2 minutes. As shown in Figure 13, glucose provided the highest stimulation in rate of O_2 uptake in non-heated cells. In the presence of pyruvate and malate, non-heated cells had a higher O_2 uptake than with either pyruvate or malate alone. However, less stimulation in the rate of O_2 uptake was observed for non-heated cells in the presence of succinate.

Figure 14 shows that the addition of glucose to cells heated for 1 minute stimulated O_2 uptake greater than the addition of succinate but less than the addition of malate and/or pyruvate. In contrast, addition of glucose to cells heated for 2 minutes stimulated O_2 uptake to a greater extent than the addition of pyruvate or/and malate but less than succinate (Figure 15).

The values of exogenous Q_{O_2} in non-heated cells and cells heated for 1 and 2 minutes are summarized in Table 2. The addition of glucose stimulated the highest Q_{O_2} value in non-heated cells, whereas the addition of pyruvate, malate, and succinate stimulated the highest Q_{O_2} values in 1 and 2 minutes heat-stressed cells.

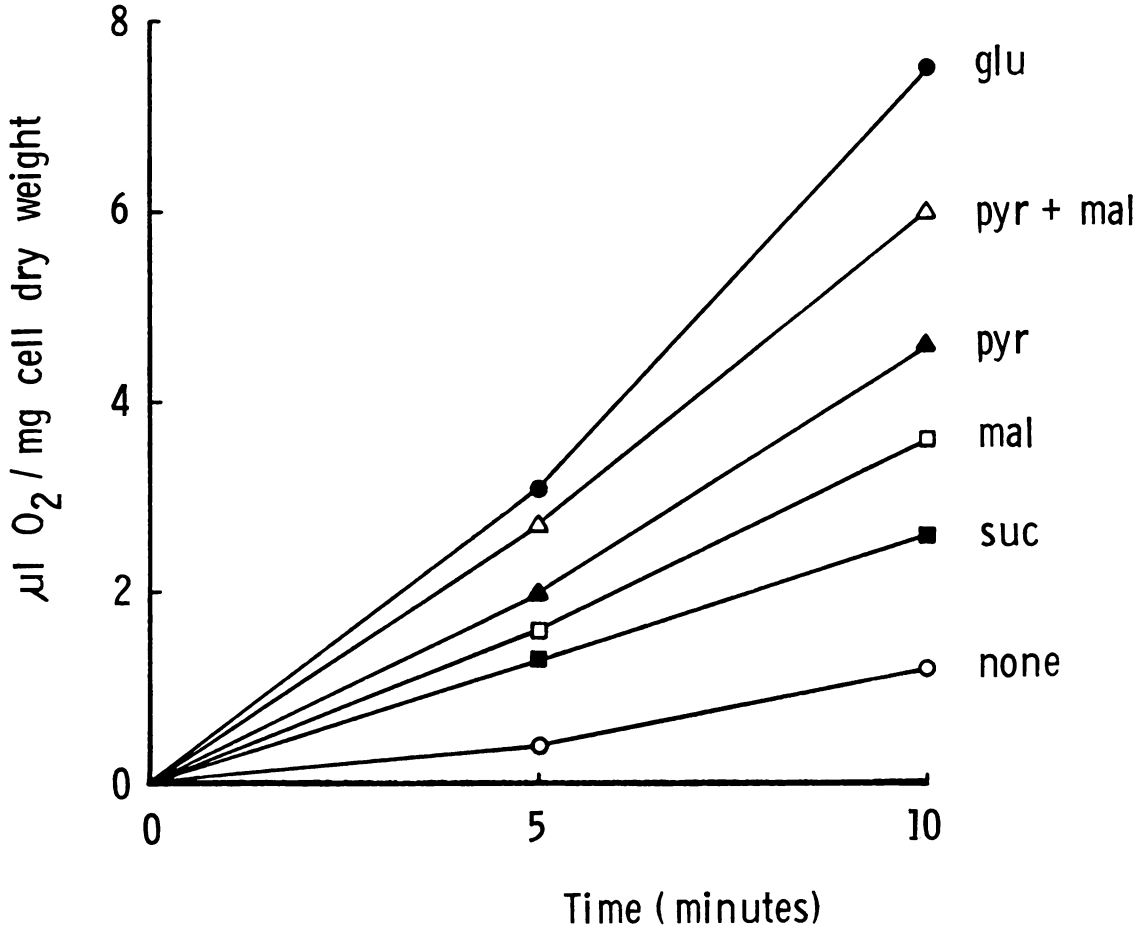


Figure 13. Respiration of non-heated cells of 4-day resting Saccharomyces cerevisiae. Oxygen uptake was measured at 30 C in water with or without substrates.

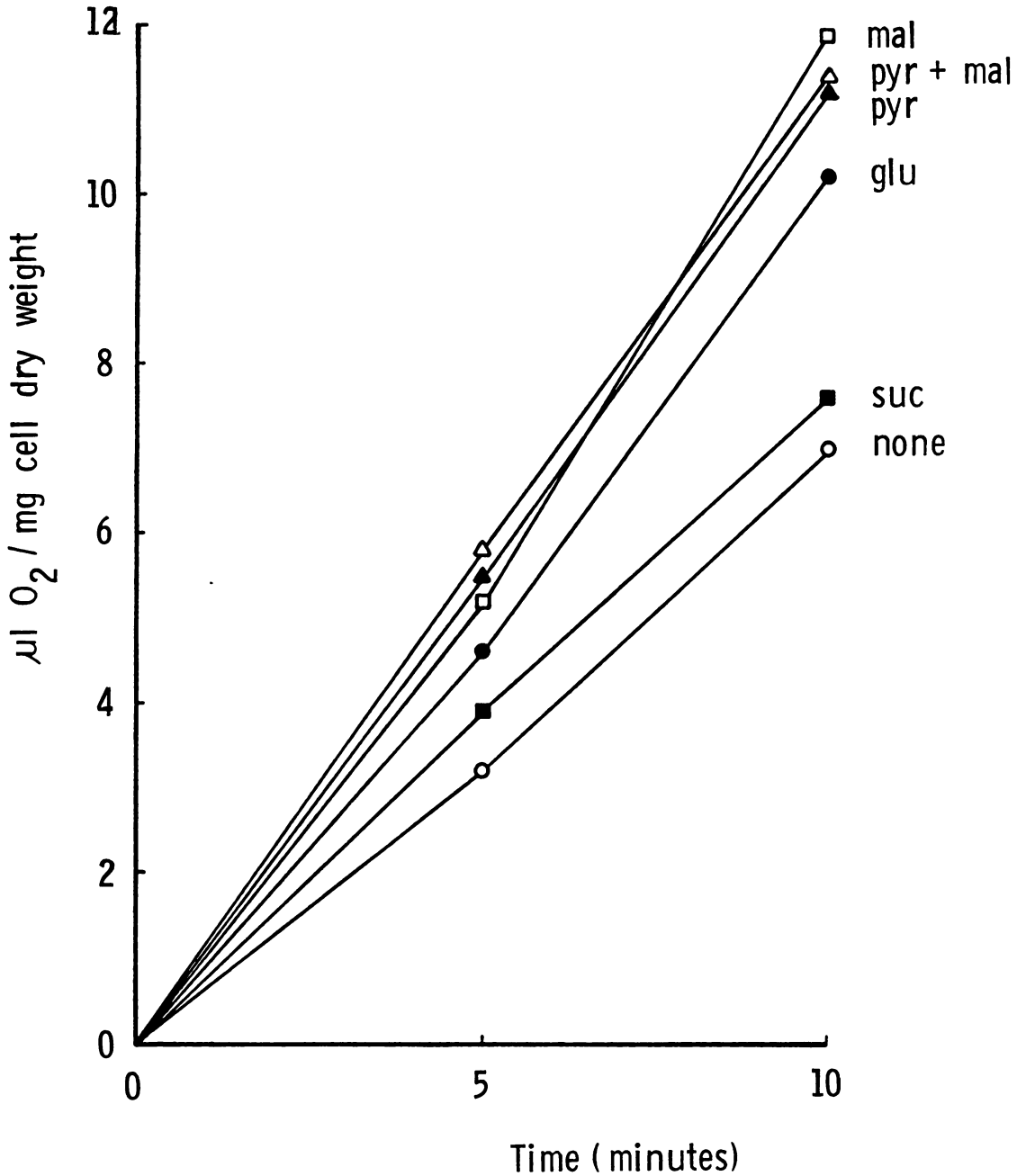


Figure 14. Respiration of 4-day resting cells of Saccharomyces cerevisiae heated at 56 C for 1 minute. Oxygen uptake was measured at 30 C in water with or without substrates.

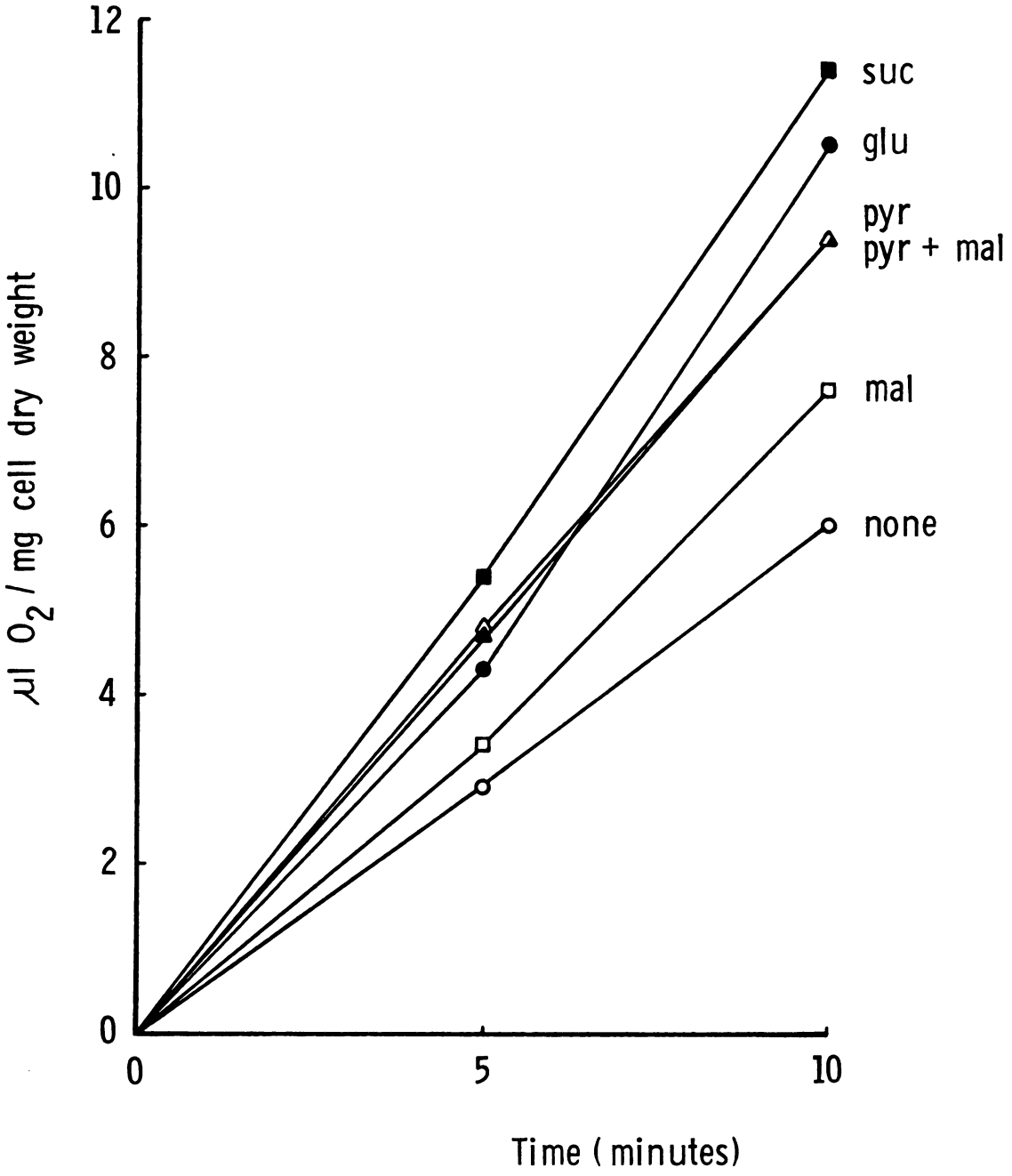


Figure 15. Respiration of 4-day resting cells of Saccharomyces cerevisiae heated at 56 C for 2 minutes. Oxygen uptake was measured at 30 C in water with or without substrates.

Table 2. Rates of O_2 uptake in water at 30 C for non-heated and heat-stressed cells of 4-day Saccharomyces cerevisiae in the presence or absence of substrates.

Heat Stress at 56 C (minute)	Q_{O_2} (µl O_2/mg dried yeast/hour)					
	none	glucose	succinate	pyruvate	malate	pyruvate + malate
none	5.0	41.5	15.4	25.8	19.2	34.1
1	40.9	58.6	46.8	66.0	42.2	71.0
2	35.1	53.2	64.0	56.0	44.6	51.6

When resting cells (4-day) of S. cerevisiae were heated at 60, 62, and 65 C, the endogenous O_2 uptake was also determined. Data in Figure 16 show that cells heated at 60 C for 1 minute had an O_2 uptake higher than that for non-heated cells and cells heated for 2 minutes. The Q_{O_2} of cells heated for 1 and 2 minutes were 12.5 and 1.5, respectively (Table 1). Slight or no O_2 uptake was detected in cells heated at 60 C for more than 2 minutes, or in cells heated at 62 and 65 C (data not shown).

1-Day Yeast Cell Suspension

Endogenous respirations of non-heated and heat-stressed cells of 1-day S. cerevisiae were measured at 30 C in water and the results are shown in Figure 17. Without heat stress, cells at this age consumed O_2 up to 5 µl/mg cell dry weight/10 minutes which was actually higher than that for the 4-day cell suspensions (1.2 µl O_2/mg cell dry weight). However, when cells were heated at 56 C for 1 minute, increased O_2 uptake was observed. Decreased O_2 uptake, compared to non-heated cells, was observed for cells heated for 2, 3, and 4 minutes. The average Q_{O_2} values of non-heated and heat-stressed cells are reported in Table 3. It was noted that the high rate of Q_{O_2} of cells heated at 56 C for 1 minute approximated that for 4-day cells heated at the same temperature and time.

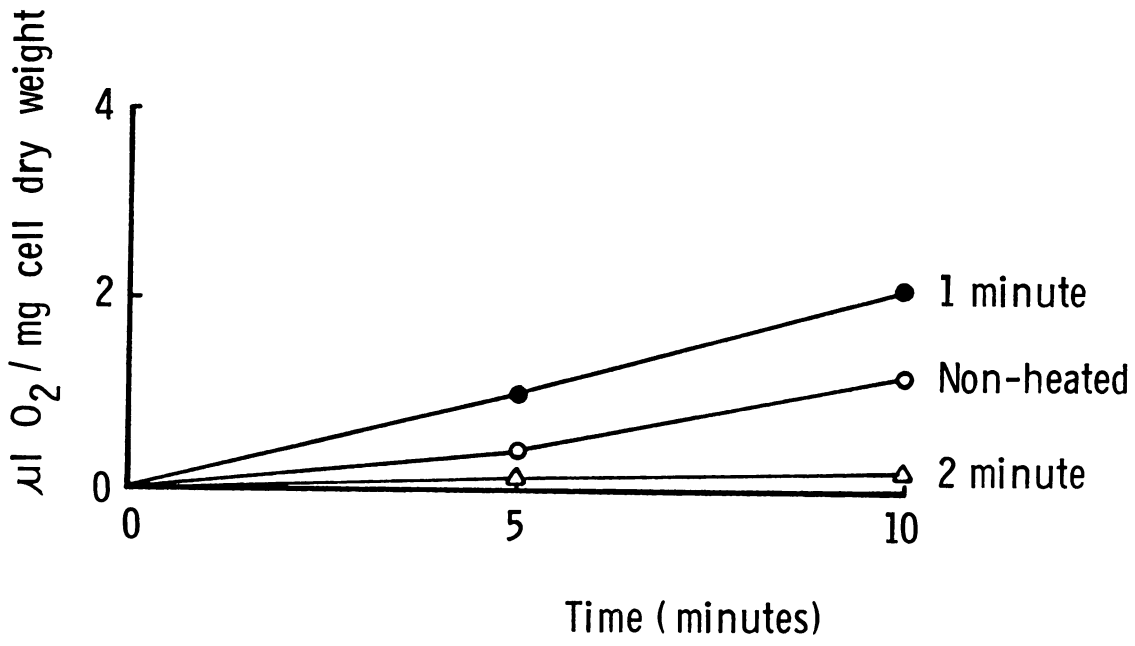


Figure 16. Endogenous respiration at 30 C for non-heated and heat-stressed cells of 4-day Saccharomyces cerevisiae in water. Cells were heated at 60 C for various times.

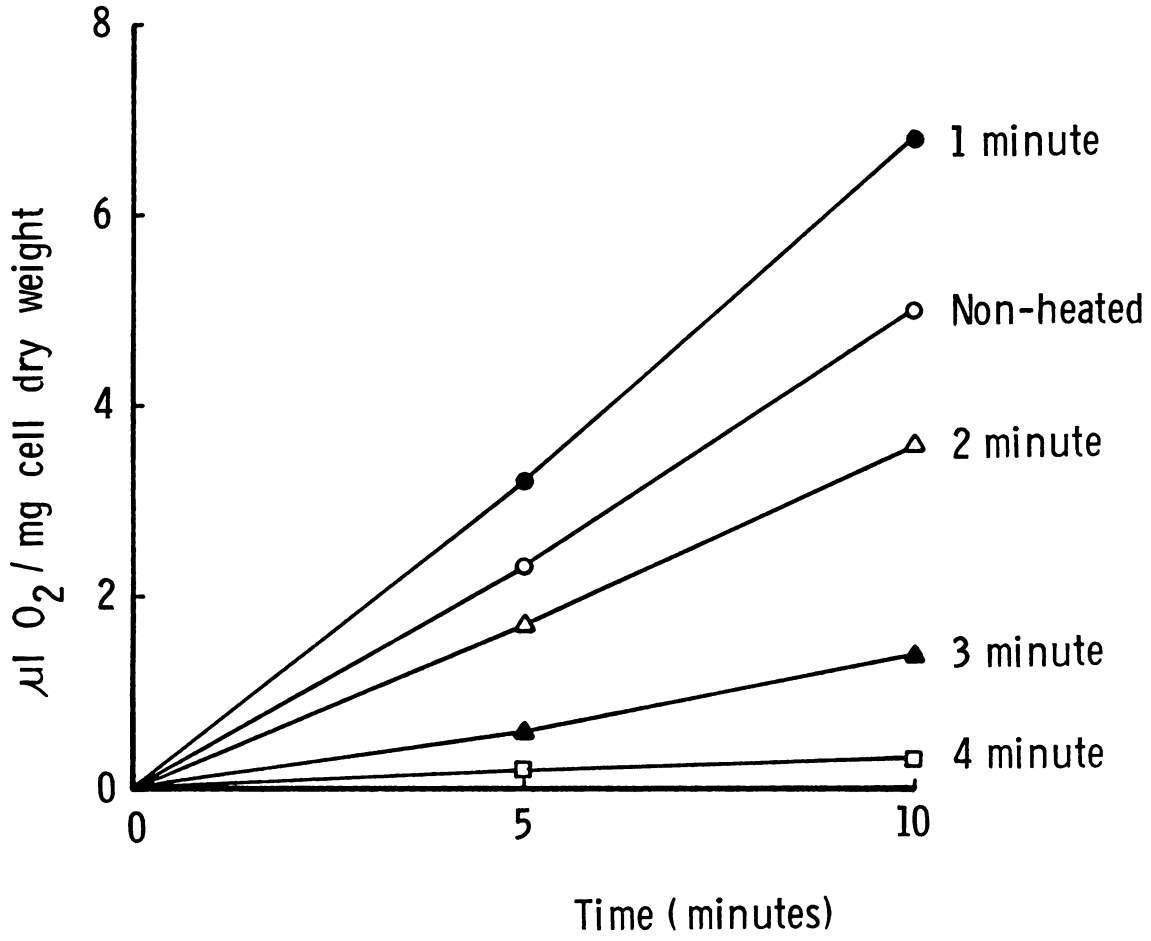


Figure 17. Endogenous respiration at 30 C for non-heated and heat-stressed cells of 1-day Saccharomyces cerevisiae in water. Cells were heated at 56 C for various times.

Table 3. Rates of endogenous O_2 uptake in water at 30 C for non-heated and heat-stressed cells of 1-day Saccharomyces cerevisiae.

Heat stress at 56 C (minute)	Q_{O_2} (µl O_2/mg dried yeast/hour)
none	29.1
1	40.3
2	23.0
3	7.3
4	3.5

0.5-Day Yeast Cell Suspension

Endogenous respirations of non-heated and heat-stressed cells of 0.5-day S. cerevisiae measured in water at 30 C are plotted in Figure 18. Actively growing cells required approximately 7.8 µl of O_2/mg cell dry weight/10 minutes. Oxygen uptake was diminished in cells heated at 56 C for 1 minute and was increased in cells heated for 2 minutes. The average Q_{O_2} values were 46.3 for non-heated cells, 15.9 and 29.5 for cells heated for 1 and 2 minutes, respectively (Table 4).

Effect of Cold Storage on Respiration of Thermally-Stressed Yeasts

The effect of a delayed measurement of endogenous respiration was determined on thermally-stressed yeast in parallel with plating on PCA. After an exposure to heat stress at 56 C for 1 and 2 minutes, a suspension of S. cerevisiae was sampled and held in an ice bath. Endogenous oxygen uptake was measured at 30 C for 5 and 10 minutes in water. As shown in Figure 19, there were no significant differences in O_2 uptake for both 5 and 10 minutes measurements for cells which had been heated for 1 and 2 minutes, following 0, 1, 2, and 3 hours of storage. Endogenous O_2 uptake for non-heated cells held at 4 C remained constant throughout the storage period.

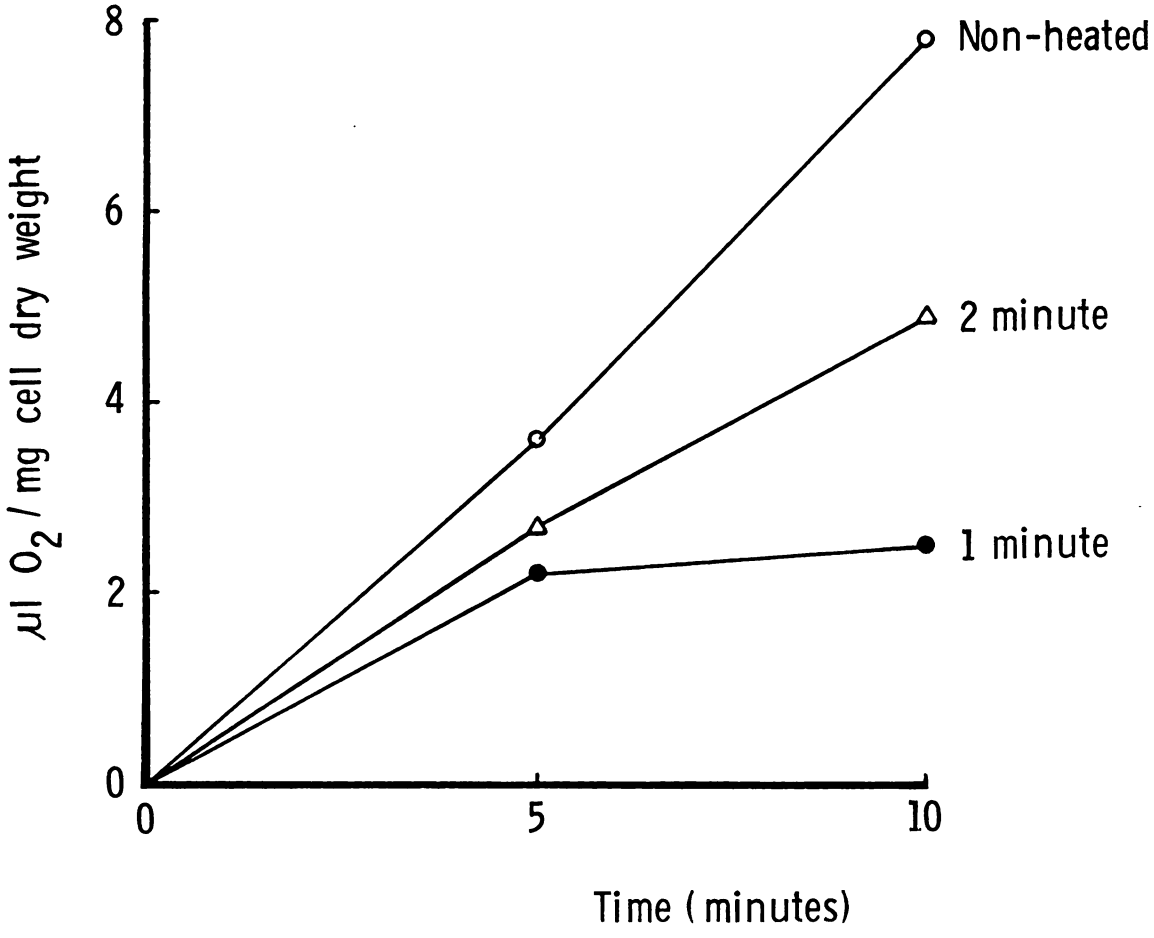


Figure 18. Endogenous respirations at 30 C for non-heated and heat-stressed cells of 0.5-day Saccharomyces cerevisiae in water. Cells were heated at 56 C for various times.

Table 4. Rates of endogenous O_2 uptake in water at 30 C for non-heated and heat-stressed cells of 0.5-day *Saccharomyces cerevisiae*.

Heat stress at 56 C (minute)	Q_{O_2} (µl O_2/mg dried yeast/hour)
none	46.3
1	15.9
2	29.5

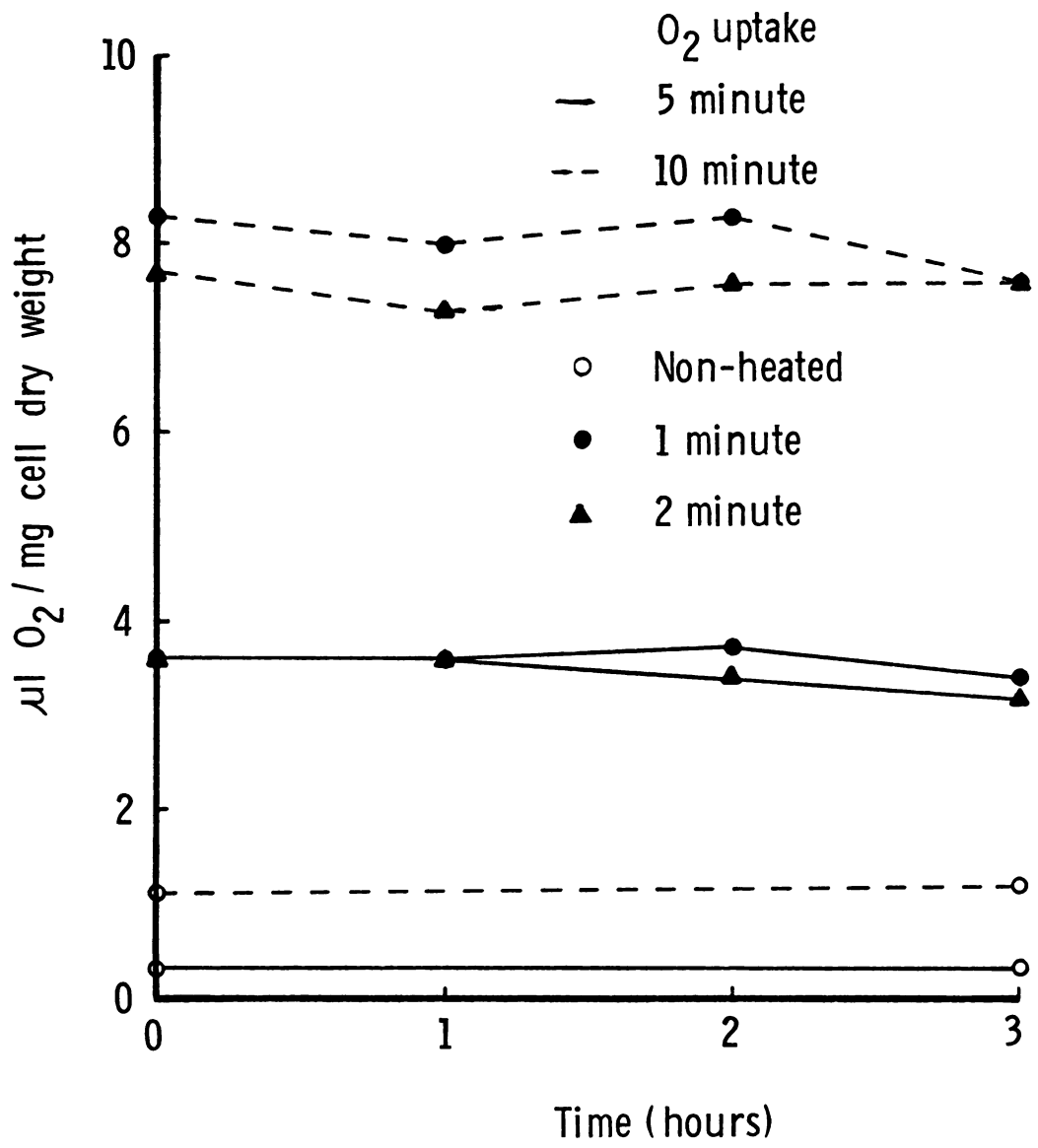


Figure 19. Effect of storage in water at 4 C on endogenous respirations of non-heated and heat-stressed Saccharomyces cerevisiae. Cells were heated at 56 C for 1 and 2 minutes. Oxygen uptake was measured at 30 C for 5 and 10 minutes.

Isolation of Yeast Mitochondria

Conventional and modified methods were employed to isolate mitochondria from the yeast cells, S. cerevisiae (coded Y 25).

Method I: Enzymatic Digestion and/or Mechanical Breakdown

Glusulase enzyme was used to lyse cell walls of the yeast cells, releasing protoplasts. To enhance the enzyme digestion, β-M.E. was added to the cells before the addition of the enzyme to reduce disulfide bonds which stabilize the cell wall. The concentrations of β-M.E. and the enzyme were varied to provide optimal yields of protoplasts. By counting the cells under phase contrast microscope, it was shown that only 30-35% of the cells yielded protoplasts after 5 hours of enzyme digestion. Lysis of yeast protoplasts by suspension in a low osmotic solution of mannitol, followed by differential centrifugation led to low recovery of mitochondria. Protein assays on these preparations indicated recoveries of 0.5 to 1.0 mg of protein/10 to 25 ml packed cell volume.

The preparation of protoplasts from cells in their log phase of growth instead of those from stationary-phase cells did not result in increased yields of mitochondria.

Method II: Disruption of Cells with Glass Beads

By vertically hand shaking the yeast cells from 4-day old culture with the glass beads, the cell breakage obtained was about 35-56%. The brown pellets of mitochondria were separated by differential centrifugation. This procedure provided 0.3 to 1.0 mg of protein/1 g of cell wet weight.

Method III: Mechanical Disruption of Cells

This method was carried out by utilizing commercial baker's yeast powder according to Gottlieb and Ramachandran (1961). Carefully mixing, washing with cold distilled water, and maintaining steady cold temperature procedures were employed with a large quantity of yeast powder. However, using a modification of Gottlieb and Ramachandran's method (Anderson, 1963), which reduced the proportion of the yeast cells and glass beads and disrupted the cells with the VirTis homogenizer, proved to be more convenient, less time-consuming, and easier to control. The concentration of mitochondria obtained from 1 g of wet cells was approximately 1.5 to 2.0 mg of protein when cells were broken in a Waring Blendor and 3.0 to 4.0 mg of protein when cells were broken in the homogenizer.

Effect of Heat on Respiratory Activity of Yeast Mitochondria

Preliminary studies involving measurements of oxygen uptake of mitochondria isolated from S. cerevisiae (Y 25) in the presence of ADP indicated that mitochondria isolated by method I had no respiratory response to the addition of ADP, while those obtained by methods II and III showed some uptake of oxygen when 333 μM of ADP was added. These results suggested that there were mitochondria present, although their protein concentrations were very low.

To determine the effect of heat stress on respiration of yeast mitochondria, oxygen uptakes of non-heated mitochondria and those that were heated at 56 C for 1 and 2 minutes were measured in the presence of various substrates, with and without ADP. The data are shown in Table 5. Non-heated mitochondria isolated by method II (Disruption of the cells with glass beads) were able to oxidize NADH, sodium succinate and sodium citrate. The oxidation rates were 0.23, 0.07, and 0.004 μmoles O_2/mg mitochondrial protein/minute, respectively. With NADH, the respiration rate was independent on the presence and absence of ADP. There was no increase of O_2 uptake in non-heated mitochondria with NADH when ADP was added. Neither was it possible to determine the ADP/O ratio. With sodium succinate and sodium citrate, the RC ratios were 1.2 and 1.1 and the

Table 5. Oxidation rates (O.R.), respiratory control ratios (R.C.R.), and ADP/O ratios for non-heated and heat-stressed yeast mitochondria (Mt.) measured at 30 C in 0.4 M lactose/0.02 M potassium phosphate buffer, pH 7.0. Mitochondria were heated at 56 C for 1 and 2 minutes.

Isolation Method	Yield of Mt.	Substrate	Non-heated Mt.			1-min. heated Mt.			2-min. heated Mt.		
			O.R.[a]	R.C.R.	ADP/O	O.R.	R.C.R.	ADP/O	O.R.	R.C.R.	ADP/O
I	0.5-1.0 mg/ 15-30 g wet cells	NADH	--[b]	--	--	-[c]	-	-	-	-	-
		Suc	--	--	--	-	-	-	-	-	-
		Cit	--	--	--	-	-	-	-	-	-
II	0.3-1.0 mg/ 1 g wet cell	NADH	0.23	--	--	-	-	-	0.56	-	-
		Suc	0.07	1.2	1.2	-	-	-	0.04	1.1	0.7
		Cit	0.004	1.1	3.4	-	-	-	0.05	1.3	4.4
III W.B.	1.5-2.0 mg/ 1 g wet cell	Suc	0.002	--	--	-	-	-	-	-	-
III V.H.	3.0-4.0 mg/ 1 g wet cell	Suc	0.002	--	--	0.001	-	-	trace	-	-
		Suc + DNP	0.002	--	--	-	-	-	-	-	-
			0.02	--	--	-	-	-	-	-	-

[a] μmoles O_2/mg protein/minute.

[b] not detectable.

[c] not determined.

ADP/O ratios were 1.2 and 3.4, respectively.

When mitochondria were heated at 56 C for 2 minutes, the oxidation rate with NADH was increased twice that of non-heated mitochondria. The oxidation rate, RC ratio and ADP/O ratio of 2-minute heated mitochondria were increased with sodium citrate but were decreased with sodium succinate.

Mitochondria isolated from baker's yeast by method III (Mechanical disruption of cells) seemed to lose respiratory activity with all substrates. As shown in Table 5, the oxidation rates of non-heated mitochondria with succinate, or succinate plus DNP, or NADH were similar at 0.002 μmoles O_2/mg of protein/minute. Lower or trace values for oxidation rates were observed for heated mitochondria. Neither an RC ratio or an ADP/O ratio could be calculated for mitochondria obtained by this method.

Spectrophotometric assays of NADH oxidation by non-heated and heat-stressed yeast mitochondria showed no significant decrease in optical density at 340 nm for various concentrations of mitochondria and NADH.

These data allow us to suggest that the mitochondria preparations lost their activity during the isolation process. The results obtained in these experiments provided indefinite information relative to the effect of thermal stress on the respiratory activity of yeast mitochondria.

Effect of Heat on Phase Transitions in Cell Membranes of the Intact Yeast Cells

Figure 20 presents data showing fluorescence studies of phase transitions in cell membranes of non-heated and heat-stressed intact cells of S. cerevisiae. When cells were heated at 56 C for 1 and 2 minutes, there were some observable changes in the physical state and the organization of the cell membranes especially in the lipid constituents. Crystalline lipids of heat-stressed cells underwent phase transition to a liquid crystalline state. This transition enhanced the freedom of rotation and some quenching of the fluorescent molecule. In the range 50-65 C, an event occurs which produces extreme quenching of fluorescence as demonstrated by the sharp decrease in its intensity. In previously non-heated cells, the quenching occurs over the temperature range 50-58 C with cells heated at 56 C for either 1 or 2 minutes, the quenching occurs over the temperature 50-67 C. The change in membrane which produces quenching does not appear to be readily reversible and has no further impact on the fluorescence above 67 C.

Storage of heat-stressed cells in water at 25 C for 5 hours allowed redistribution of lipids in the membrane and, as shown in Figure 21, the membrane was restored to approximately their normal state. The difference in values of fluorescence intensity between Figures 20 and 21 was an apparent difference arising from instrument adjustment.

Figure 20. Phase transitions in cell membranes of non-heated and heat-stressed intact yeast cells (4-day culture of S. cerevisiae). Cells were heated at 56 C for 1 and 2 minutes and resuspended in CR buffer. Fluorescence was measured at 0 hour after heating with PhNap dye.

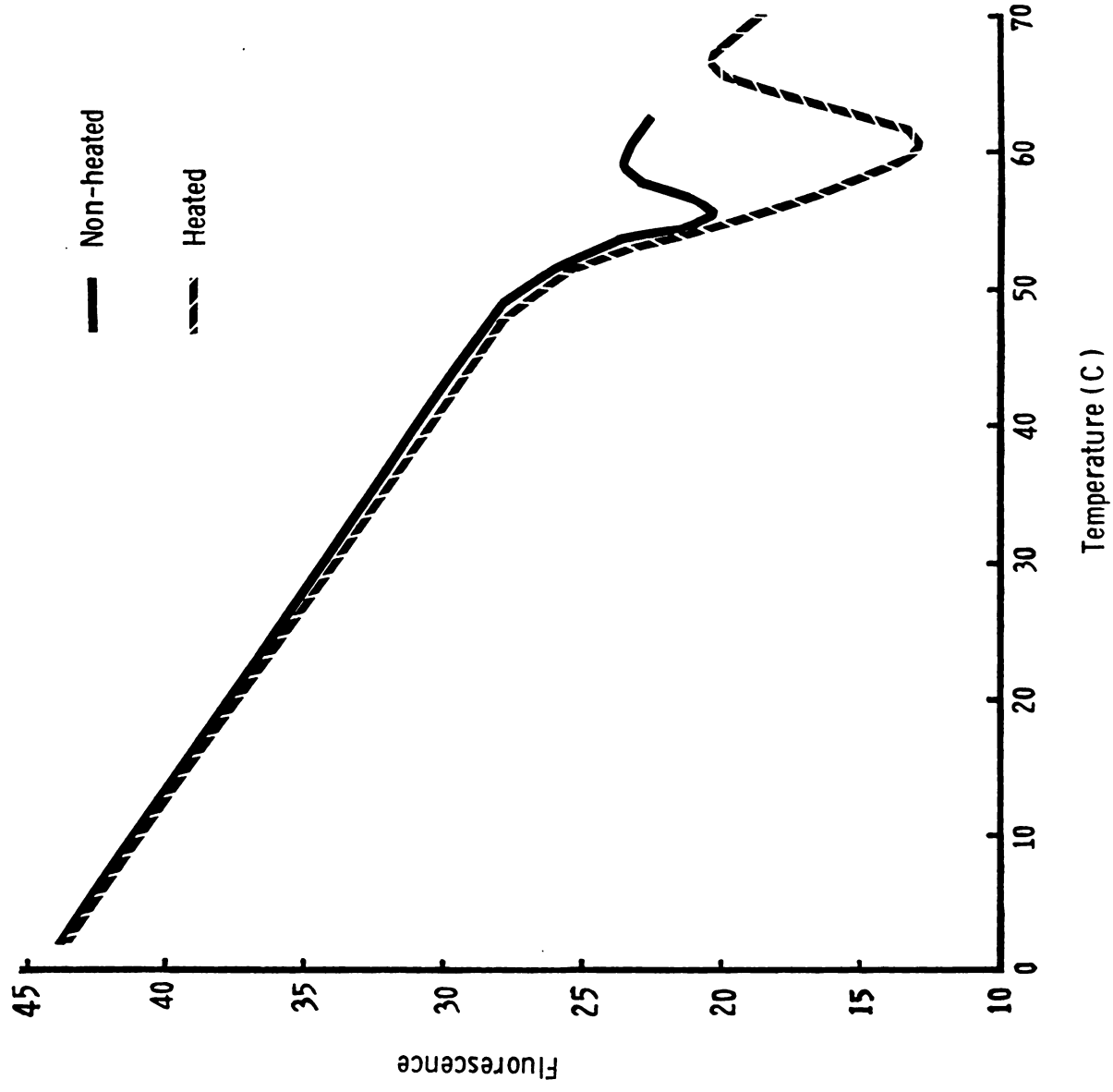
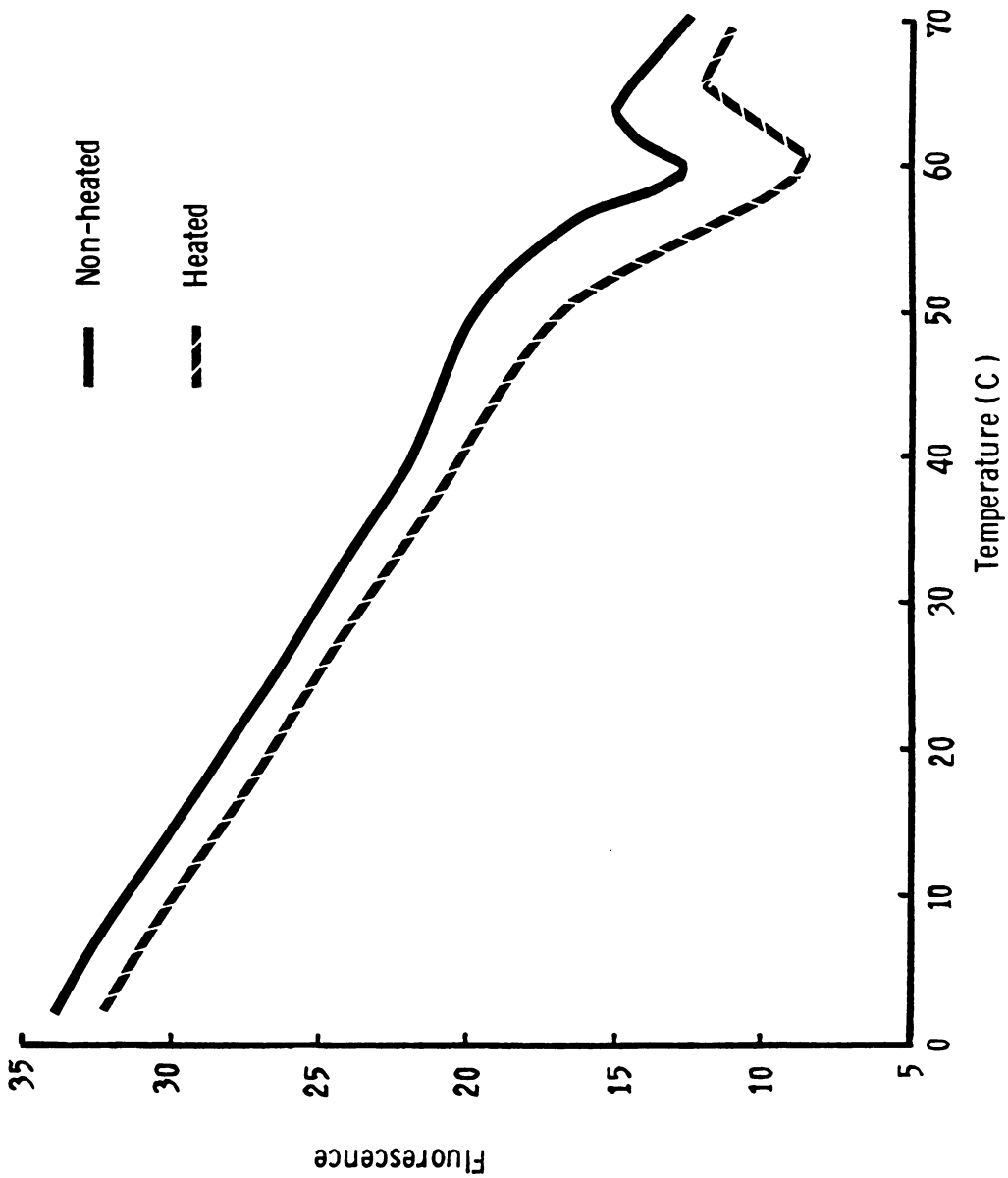

Non-heated
Heated
Fluorescence
45
40
35
30
25
20
15
10
0
10
20
30
40
50
60
70
Temperature (C)

Figure 21. Phase transitions in cell membranes of non-heated and 1-minute heat-stressed intact yeast cells. Fluorescence was measured at 5 hours after heating and storing the cells in water at 25 C.


Non-heated
Heated
Fluorescence
35
30
25
20
15
10
5
0
10
20
30
40
50
60
70
Temperature (C)

DISCUSSION

Effect of Physiological State and Temperature on Recovery of Thermally-Stressed Yeasts

Cell suspensions of S. cerevisiae (coded Y 25), with careful regard to physiological states, were used for the investigations of their survival, injury, and recovery from thermal stress at selected temperatures in water. However, media had some influence on recovery of heat-stressed cells. Plate counts of unheated yeast cells on PDA and PCA media were similar, while plate counts of heat-stressed cells at 56 C were reduced on PDA as compared to PCA. The reduction in colony-forming on PDA has been attributed to pH sensitivity of thermally-stressed fungi (Skidmore and Koburger, 1966; Mace and Koburger, 1967; Koburger, 1970, 1971, 1972, 1973; Nelson, 1972; Jarvis, 1973; Ladiges et al., 1974). To the contrary, Stevenson and Richards (1976) and Graumlich (1978) demonstrated that pH was not the major reason. Neutralization of PDA (pH 5.6) to the same pH as PCA (pH 6.6) or vice versa did not alter the differences in recovery observed between the two media. Graumlich (1978) showed that the glucose concentration (2-6%) in the medium affected the recovery of the

thermally-stressed cells. In addition, steam sterilization of the medium containing glucose produced the product that was responsible for the reduced recovery of heat-stressed cells (Tanner, 1974; Graumlich, 1978).

The severity of thermal destruction was dependent upon the physiological state of the cells. Resting cells of S. cerevisiae were much more resistant to thermal destruction than actively growing cells. The results of this investigation confirm and extend the observations of others (Sherman, 1956; Rosenberg and Wood, 1957; Schenberg-Frascino and Moustacchi, 1972; Stevenson and Richards, 1976). Heat stress at 56 C approximately reduced populations of resting cells (4-day old cultures) 10,000 fold within 6 minutes, while killed the same population of growing cells (0.5-day old culture) within 2 minutes. The 1 day old cells, even though their concentration indicated that they were entering the stationary phase, were less heat resistant than 4-day old resting cells, but were somewhat more heat resistant than 0.5-day old actively growing cells. Stevenson and Richards (1976) found that actively growing cells of S. cerevisiae from 4 hour-culture were destroyed much more rapidly than resting cells from 3 day-culture when they were heated at 56 C for 2 minutes. Rosenberg and Wood (1957) reported that yeasts from 1- and 2 day old cultures were approximately 10 times as sensitive to thermal inactivation as those from 14 day-old cultures.

However, thermal resistance decreased when the cultures were grown for 48 days. The increased thermal sensitivity in the very young cultures appeared to be related to a highly active metabolism. Decreased metabolic activity in the old culture resulted in a progressive decrease in the thermal sensitivity of the cells. Changes in the water content of the cell with aging were speculated to be responsible for the increased thermal sensitivity of the cultures over a month old (Rosenberg and Wood, 1975).

In addition to the nature of the injury, the ability to repair the drastic thermal injury was also highly dependent upon the physiological state of the cells. Unlike the resting cells, repair of heat-injured cells was observed in the actively growing yeasts during storage in buffer at room temperature. The ability to repair was suggested to be associated with the utilization of endogenous reserves present in the actively growing cells (Stevenson and Richards, 1976). In contrast to the finding of Graumlich (1978), recovery from thermal injury in the resting cells within the first 6 hours of storage in water at 22 C was noted in this study.

Despite the physiological state of the cells, the thermal destruction rate increased with an increase in the heating temperature. The cells were destroyed rapidly at 65 C > at 62 C > at 60 C > at 56 C during the first 2 minutes of heating. Complete loss of viability occurred

within 4 to 5 minutes when cells were heated at 65, 62, and 60 C whereas it took 20 minutes when heated at 56 C. In general, vegetative cells of yeasts are destroyed at temperatures and times similar to those which are lethal for bacterial cells. Injury as well as death may occur when cells are heated. Yeast cells are usually inactivated by exposure to 50-60 C for less than 30 minutes (Rose and Harrison, 1971). Obligately psychrophilic yeasts are much more thermolabile than other yeasts. Baxter and Gibbons (1962) found that the obligately psychrophilic Candida sp. was rapidly killed at 30 C. Likewise, cells of Rhodotorula infirmo-miniata were destroyed by exposure to 30 C for 24 hours (Rose and Harrison, 1971). Fries (1963) noted that cells of Rhodotorula glutinis heated at 48 C for 1-4 minutes grew when subsequently incubated at 20 C but not at 28 C. Mesophilic strains of S. cerevisiae exhibited extensive death only when exposed to 45 and 50 C for 24 hours (Sinclair and Stokes, 1965).

Effect of Cold Storage on Recovery of Thermally-Stressed Yeasts

Storage of thermally-stressed yeasts in water at 4 C did not induce recovery from thermal injury. The PCA plate counts of unheated and heat-stressed yeasts were not significantly different during 24 hours of storage. Similar

results were reported by Baldy et al. (1970), Schenberg-Frascino (1972) and Graumlich (1978). Storage at 4 C prevented the metabolic activity which was required for repair of injury. Some inhibitors, i.e. cycloheximide, chloramphenicol, actinomycin D, and hydroxyurea, had no effect on the recovery of colony-forming ability of heat-stressed cells. These results indicated that protein, RNA, and DNA syntheses were not required for the repair of thermal injury (Baldy et al., 1970; Graumlich, 1978). However, Schenberg-Frascino (1972) and Tsuchido et al. (1972 a,b) reported that inhibition of protein and RNA syntheses by cycloheximide or fluorophenylalanine or 8-azaadenine prevented recovery of thermally-injured cells.

Recovery from thermal injury resulted in increased PCA plate counts of heat-stressed cells was observed by Stevenson and Richards (1976) and Graumlich (1978) when cells were stored in either buffer or water at room temperature (20-22 C). Schenberg-Frascino (1972) found that enhanced recovery of yeast, heat-stressed at 52 C, occurred when yeast cells were held in water at 28 C with aeration for 24 hours to 5 days.

Respiration of Thermally-Stressed Intact Yeasts

The severity of thermal stress, as well as the physiological state of the cells affected endogenous respiration

of S. cerevisiae.

Effect of Temperature and Time of Heating

A high rate of endogenous O_2 uptake was observed in heat-stressed cells as compared to non-heated cells. Prolonged thermal stress as well as increasing the heating temperatures appeared to reduce the rate of O_2 uptake which was related to a decrease in viability of heat-stressed cells. Resting cells of S. cerevisiae from a 4 day old culture heated at 56 C for 1, 2, 3, and 4 minutes exhibited pronounced higher rates of O_2 uptake than non-heated cells. Declines in endogenous O_2 uptake of cells heated for more than 4 minutes were correlated with lowered recovery of heat-stressed cells on PCA media. Similar results were found in cells heated at 60 C for 1 and 2 minutes. High endogenous O_2 uptake in the heat-stressed cells of S. cerevisiae had been described by others. Brandt (1941) reported a high endogenous O_2 uptake concurrently with trehalose utilization in cells of S. cerevisiae heat-stressed at 50 C. Graumlich (1978) found similar results when S. cerevisiae was heated at 56 C for 1 and 2 minutes. However, there was a distinct decrease in respiration after 1 to 3 hours.

Exposure to temperatures of 62 and 65 C caused a great loss of respiratory activity in yeasts although there was some detectable recovery of thermally-injured cells cultured on PCA. Some vital cell functions may be impaired at higher

temperatures or longer heating periods (Farrell and Rose, 1967). Baldy et al. (1970) reported that endogenous O_2 uptake of non-heated and heated spores were similar while the viability of heated spores was less than that of non-heated spores. Evison and Rose (1965) reported a rapid decline in the viability and in the rates of respiration of endogenous reserves when a Candida culture was subjected to supramaximal temperature. Meyer (1975) also revealed decreases in endogenous O_2 uptake and viability of heat-stressed Candida P 25 after exposure to supramaximal temperatures.

Storage at 4 C did not affect respiration rates of either non-heated or heat-stressed cells of S. cerevisiae. Indeed, the respiration rate of non-heated cells remained constant throughout 3 hours of storage. Also, there was no recovery from thermal injury of heat-stressed cells during this time. These results were in agreement with the absence of significant changes in plate counts on PCA media of heat-stressed cells even after 24 hours at 4 C. Graumlich (1978) found that high rates of endogenous O_2 uptakes of heat-stressed cells were lessened to levels approximating or lower than those of non-heated cells after 20 hours of storage at 22 C.

Effect of Physiological State of Cells

Cells of S. cerevisiae harvested from different phases of growth had different specific oxygen uptake rates. Due

to the highly active metabolism in young cultures, endogenous respiration of actively growing cells (0.5 day old culture) was greater than those of resting cells (1-day and 4-day cultures). However, these respiration rates were decreased when cells were heated at 56 C. The increased susceptibility of actively growing cells to thermal destruction, as demonstrated by lowered recovery on PCA media, was also ramified in reduced respiration rates as compared to those of resting cells. Ward (1966) reported that total respiration was lowest and endogenous respiration was highest in old cells. During starvation, endogenous respiration decreased but did so most rapidly in young cells.

Effect of Exogenous Substrates

Exogenous respiration of resting cells of S. cerevisiae was also affected by thermal stress at 56 C. Glucose, pyruvate and some other intermediates of the TCA cycle, e.g., malate and succinate, enhanced the oxygen uptakes in both non-heated and heat-stressed cells. However, glucose stimulated the highest Q_{O_2} value in non-heated cells whereas pyruvate, malate and succinate stimulated the highest Q_{O_2} values in cells heated for 1 and 2 minutes, respectively.

The increases in O_2 uptake due to the additions of these exogenous substrates was not appreciably different in heat-stressed cells when compared to non-heated cells. The high rate of O_2 uptake in heat-stressed cells may have

masked their effects. Graumlich (1978) found that the increased O_2 uptake induced by glucose in cells heated at 56 C for 2 minutes compared to those in non-heated cells and cells heat-stressed for 1 minute. The increased consumption of oxygen may reflect the utilization of added substrates to meet increased energy demands of heat-stressed cells. Possible damage of the cytoplasmic membrane may occur during thermal stress, resulting in an alteration of the substrate-transport systems of heat-stressed cells. This modification may enhance the entrance of these substrates into the cells. Hagler and Lewis (1974) reported damage of the cytoplasmic membrane during thermal stress in the presence of glucose. Hagen and Rose (1962) reported psychrophilic Cryptococcus sp. required α-ketoglutarate, citrate or isocitrate since many enzymes of the TCA cycle were inactivated during exposure to supraoptimal temperature. Likewise, Evison and Rose (1965) found reduced respiration rates of exogenous glucose and pyruvate in heat-stressed Candida due to the same cause. Meyer (1975) suggested that heat stress affected the cells' respiratory complex, especially a heat-sensitive component of either the TCA cycle or electron transport chain.

Isolation of Yeast Mitochondria

A summary of the methods employed by several workers for the preparation of mitochondria from Saccharomyces sp. is presented in Table 6. The common features of these methods summarized by Lloyd (1977) are as followed: (1) cell breakage by the gentlest method consistent with efficient release of the organelles; (2) rapid removal of the mitochondria from the extract at 0-4 C to minimize the metabolic activity and the action of hydrolytic enzyme; (3) the use of isolation media buffered to 0.25-0.6 osmolarity with sucrose, mannitol, or sorbitol and kept in the pH range 6.8-7.6; and (4) the use of minimal centrifugation speeds for the sedimentation of the mitochondria.

Method I: Enzymatic Digestion and/or Mechanical Breakdown

The preparation of protoplasts by enzyme digestion has many advantages over mechanical methods in that the protoplast produced may be more gently disrupted than the cells from which they are derived. Duell et al. (1964) and Onhishi et al. (1966) reported that the osmotic lysis of protoplasts prepared by using the digestive enzymes of Helix pomatia provided satisfactory preparation of yeast mitochondria. However, very gentle mechanical breakage of protoplasts is still the method of choice (Cartledge and Lloyd, 1972 a,b).

Table 6. Methods for the preparation of mitochondria.

Organism	Method of disruption	Yield of mitochondria
S. cerevisiae Harden and young strain (near log phase and stationary phase) Duell et al., 1964	Protoplasts prepared using snail enzymes after pretreatment with mercaptoethylamine and EDTA. Disrupted in a Vortex mixer.	20-25 mg protein/1 g wet cells
S. carlsbergenesis (log phase) Ohnishi et al., 1966	Protoplasts prepared using snail enzymes and broken by osmotic shock	5-7 mg protein/1 g wet cells
S. cerevisiae strain DTX II (stationary phase) Kovac et al., 1968	Protoplasts prepared using snail enzymes after preincubation with 0.5 M thioglycollate (pH 9.3 with tris). Disruption by homogenization in blender	
S. cerevisiae strain DTX II (late log phase) Kovac et al., 1972	Same as above but preincubation with 0.5 M-mercaptoethanol (pH 9.3 with tris). Disruption by homogenization in blender.	15-20 mg protein/1 g dried yeast cells
S. cerevisiae (late log phase) Linnane and Lukins, 1975	Protoplasts prepared using enzymes after pretreatment with B-mercaptoethanol and EDTA. Disrupted in French pressure cell.	300 mg protein/100 ml packed cells
S. cerevisiae commercial baker's yeast Utter et al., 1958	Shaking with glass beads	-

Table 6. (cont'd)

S. cerevisiae Vitols and Linnane, 1961	Shaking with glass beads in a Waring Blendor	300 mg protein/100 ml packed cells
S. cerevisiae Gottlieb and Ramachandran, 1961	Shaking with glass beads in a Waring Blendor	90-95 mg protein/ml wet cells
S. cerevisiae commercial baker's yeast Tzagoloff, 1969	Homogenization in a Waring Blendor	9-11 g protein/8 lb yeast
S. cerevisiae PS 194 (late log phase) Labbe and Chambon, 1977	Grinding with a Dyno-Mill KDL continuous grinder	15 g protein/kg wet cells
S. cerevisiae commercial baker's yeast Labbe and Chambon, 1977	Same as above	8 g protein/kg wet cells
S. cerevisiae Lang et al., 1977	Hand-shaking with glass beads	0.5-1.0 g purified protein/ 100 g wet cells

Cells treated according to method I, failed to provide even theoretically optimal yields of protoplasts. Only 30-35% of approximately 15-30 g of wet cells of S. cerevisiae Y 25 (about 10-25 ml packed cells) yielded protoplasts after 5 hours of enzyme digestion. In contrast, Duell et al. (1964) found 32% of the yeast cells (1 g wet weight) formed protoplasts after incubation with the enzyme at 30 C for 30 minutes. Ohnishi et al. (1966) reported 80-90% of the yeast was converted to protoplasts within 1-1.5 hours of incubation. Lysis of yeast protoplasts by low osmotic shock in mannitol provided only 0.5-1.0 mg of mitochondrial protein which was much less than those obtained by other investigators. Duell et al. (1964), Ohnishi et al. (1966), and Kovac et al. (1968) isolated mitochondria ranged from 5-7 mg to 20-25 mg of protein/1 g of cells (as shown in Table 6). Alternatively, preparation of protoplasts from cells in their log phase of growth or additional mechanical rupture of protoplasts did not result in increased yields of mitochondria. The results suggested that the wall of S. cerevisiae Y 25 was not readily susceptible to degradation by the glusulase enzyme.

Eddy and Williamson (1957) indicated that cell walls of certain strains of S. carlsbergensis and S. cerevisiae were susceptible to the enzyme (1 mg/ml) in the presence of 0.5 M rhamnose at 25 C. However, the digestion of the cell wall was rather slow and only proceeded to completion when

young cells were used. Later, they found that increasing the amount of enzyme up to 5 mg/ml in the presence of mannitol at 30 C improved the conversion with more than 90% of the cells forming protoplasts within 2-3 hours (Eddy and Williamson, 1959). The cell walls of log phase cells were more susceptible to the enzyme digestion than that of stationary phase cells (Duell et al., 1964; Brown, 1971; Mann et al., 1972) possibly due to an incomplete formation of disulfide linkages and some degradation of glucan (Anderson and Millbank, 1966). An increased production of phosphomannans as well as the formation of additional glycosidic linkages which results in more highly branched glucan and mannan polymers were suggested as reasons for the loss of susceptibility to the enzyme in the stationary phase cells (Brown, 1971). Duell et al. (1964) reported that the yield of protoplasts from the log phase cells was sometimes rather poor, and this effect became more pronounced as cells were harvested at the stationary phase. Preincubation of yeasts with thioglycollate, ethylenediaminetetraacetate, or 2-mercaptoethanol facilitated protoplast formation by the snail gut enzyme from cells of all phases of the growth cycle (Bacon et al., 1965; Schwencke et al., 1969).

The main disadvantage to the use of enzyme methods besides its high cost and time consumption is the unpredictable variation of the organism. Differences in cell

wall composition in different species or strains, or of the organism grown under different conditions frequently led to low efficiency of cell wall degradation (Mann et al., 1972; Lloyd, 1974). Although several modifications of the original methods have been introduced to overcome these problems (Duell et al., 1964; Ohnishi et al., 1966; Kovac et al., 1968; Lebeault et al., 1969), the yield of mitochondria obtained in this research was still too low to permit planned experiments involving the effects of heat stress on their function.

Method II: Disruption of Cells with Glass Beads

Because of the low susceptibility of S. cerevisiae Y 25 cells to enzyme digestion, hand-shaking shaking with glass beads constituted an alternate means for preparing their mitochondria. Approximately 35-56% of resting cells were broken by this method which yielded 0.3-1.0 mg of mitochondrial protein/g of wet cells. This method provided better yields of mitochondria than method I. Lang et al. (1977) reported that a cell breakage of from 70 to 95%, yielding 0.5-1.0 g of purified mitochondria/100 g wet cells, was normally obtained with S. cerevisiae. This procedure is especially applicable to some mutants and to cells grown under selected conditions, as well as to organisms having tough cell walls (Lang et al., 1977; Vincent et al., 1980). The procedure has been applied to both small- and large-scale

preparations of mitochondria. The low yield of mitochondria in the present studies was attributed to the insufficient disintegration of the yeast cells or the loss of mitochondrial recovery during washing and centrifugation (Lang et al., 1977).

Method III: Mechanical Disruption of Cells

Mattoon and Balcavage (1967) utilized a mechanical rupturing method for the preparation of yeast mitochondria from commercial baker' yeast. Unlike the enzyme digestion method, the critical problems of detrimental exposure of mitochondria to the hydrolytic enzyme or dependence on physiological condition were eliminated. Several devices, as represented in Table 6, have been employed for breaking yeast cells. Deters et al. (1976) and Labbe and Chambon (1977) used a Dyno-Mill KDL continuous grinder to produce 5-8 g of mitochondrial protein/kg of wet cells from commercial baker's yeast. Gottlieb and Ramachandran (1961) prepared 90-95 mg of mitochondrial protein/ml of wet cells by utilizing a Waring Blendor to rupture the cells. Anderson (1963) employed a VirTis homogenizer for small-scale preparations of yeast mitochondria.

The mitochondrial yields obtained in this research ranged from about 1.5-2.0 and 3.0-4.0 mg of protein/g of wet cells when baker's yeasts were broken in a Waring Blendor and VirTis homogenizer, respectively. However, the

results appeared to be unsatisfactory. The employment of glass beads enhances the effective disintegration of the organism, but the relative amounts of beads and cell suspension affected the efficiency of disruption (Lamanna and Mallette, 1954). Mattoon and Balcavage (1967) reported that the main disadvantages of the mechanical rupture method were poor yields and the requirement for an empirical selection of optimum breakage conditions. Tzagoloff (1969) found that the yield of mitochondria obtained from baker's yeast depended upon the length of time the frozen cells were homogenized. Homogenization for a period of 2.5 to 3.0 minutes provided mitochondrial protein approximating 0.5% of the total wet weight of the cells. More prolonged blending resulted in some thawing of the frozen powder.

Respiratory Activity of Yeast Mitochondria

A great enhancement in both oxidative rates and respiratory ratios was reported for mitochondria prepared by controlled osmotic lysis of protoplasts of S. cerevisiae over those prepared by use of the Nossal shaker (Duell et al., 1964). Ohnishi et al. (1966) also reported that mitochondria of S. carlsbergensis were able to oxidize various intermediates of the TCA cycle. Externally added NADH, D- and L-lactate and ethanol were also actively oxidized and respiratory control was obtained with all

these substrates. High RC ratios were obtained especially with NADH and α-ketoglutarate. Exogenous ADP was phosphorylated faster than endogenous ADP even at low temperatures which was in contrast to the action of mammalian mitochondria. Carefully prepared yeast mitochondria still showed traces of respiratory control after storage at 4 C for 3 days and only a slight decrease in P/O ratios was observed over this period (Lloyd, 1974). Lloyd (1974) stated that satisfactory isolated mitochondria are those which possess negligible respiration in the absence of added respiratory substrate or are low in the absence of added ADP. Well prepared mitochondria undergo an increasing respiration rate on adding ADP accompanied by an abrupt return to normal rate, or have a high ADP/O ratio.

Mitochondria obtained from S. cerevisiae harvested in the stationary phase (Kovac et al., 1968) resembled those obtained by Ohnishi et al. (1966) from exponentially grown S. carlsbergensis in their oxidative activities, phosphorylation efficiencies, osmotic stability, and reactions to the presence of dinitrophenol and oligomycin. Table 7 presents examples of respiratory activities of yeast mitochondria investigated by other researchers. In the present study, enzymatically isolated mitochondria from S. cerevisiae showed no oxidative or phosphorylative activities. Possibly the loss of structural integrity or intactness of mitochondria contributed to those observations as well as

Table 7. Oxidative and phosphorylative activities of yeast mitochondria isolated by enzymical and mechanical methods. Substrates used were NADH, succinate, and citrate.

Researcher	Substrate	O.R.	R.C.R.	ADP/O
Duell *et al.*, 1964	NADH	0.15[a]	2.1	-
	SUC	0.15	2.9	-
Ohnishi *et al.*, 1966	NADH	0.6 -1.1[b]	3.0-3.6	1.5-1.8
	Suc	0.25-0.38	1.4-1.7	1.6-1.8
	Cit	0.20-0.45	1.5-1.7	1.7-1.8
Kovac *et al.*, 1968	Suc	0.28[c]	1.1	1.4
	Cit	0.49	1.6	1.5
Vitols and Linnane, 1961	NADH	0.19-0.23[b]	-	1.1-1.6
	Suc	0.10-0.18	-	1.0-1.6
Labbe and Chambon, 1977	Suc	-	1.6	1.5
	Cit	30[d]	2.1	1.6
Lang *et al.*, 1977	NADH	0.28-0.38[a]	1.2-1.9	0.4-1.2

[a] µmole O/mg protein/minute

[b] µatom O/mg protein/minute

[c] µg atom O/mg protein/minute

[d] nmole O/mg protein/minute

the low recovery of mitochondria.

Mechanical methods of disruption were reported to yield a large quantity of mitochondria with a great degree of functional integrity. However, these isolated mitochondria functioned variably in their oxidative and phosphorylative activities (Gottlieb and Ramachandran, 1961; Vitols and and Linnane, 1961; Deters et al., 1976; Labbe and Chambon, 1977). In this investigation, mitochondria isolated by the hand-shaking method represented the only mitochondrial preparation possessing oxidative and phosphorylative activities with the following substrates: NADH, succinate, and citrate. The values obtained appeared to be low as compared to those observed for mitochondria isolated by other mechanical or enzymical methods (see Table 7). For example, values for O.R. of only 2.1 and for the ADP/O ratio of only 1.6 were obtained with isolated mitochondria, employing NADH (567 μm) as substrate. Lang et al. (1977) reported an O.R. value of 0.28-0.38, a R.C.R. value of 1.2-1.9, and a P/O ratio of 0.4-1.2 (1 mM NADH as substrate) for mitochondria isolated by the same method. He stated that the hand-shaking method was preferred for obtaining intact mitochondria by mechanical disintegration but was not considered to be useful for the studies of energy conservation. Tzagoloff (1969) and Mason et al. (1973) reported that the disruption of yeast cells with either a Waring Blendor or homogenizer provided low yields of mitochondria

without respiratory control.

Effect of Heat on Phase Transitions in the Cell Membranes of Intact Yeast Cells

Fluorescence studies employed in this investigation indicated that phase transitions in cell membranes of S. cerevisiae were altered after heat stress at 56 C for either 1 or 2 minutes. This alteration was attributed to changes in the physical state and the organization of the membrane components, particularly in the lipid components. Moreover, the sharpness of the phase transitions suggested that the change was a co-operative phenomenon. It would also appear that this alteration in cell membranes of heat-stressed cells was responsible for the leakage of cellular constituents in particular UV-absorbing materials, which is often associated with thermal injury as reported by other investigators (Nash and Sinclair, 1968; Tsuchido et al., 1972a; Shibasaki and Tsuchido, 1973; Hagler and Lewis, 1974; Meyer, 1975; Graumlich, 1978). Storage of heat-stressed cells in water at 25 C for 5 hours allowed re-ordering of lipids in the membrane and the membrane was restored to its normal state.

CONCLUSIONS

Thermal stress affected the recovery and respiration of yeast cells. The severity of thermal stress was influenced by the physiological status of the cells and the time and temperatures of heating. Resting cells of S. cerevisiae were much more resistant to thermal destruction at 56 C than actively growing cells. Prolonged heating or increased heating temperature resulted in increased thermal destruction rates of the cells, as well as greater reduction in respiration rates. Endogenous and exogenous O_2 uptakes increased in heat-stressed cells when compared to non-heated cells. Storage at 4 C did not affect the recovery and respiration of heat-stressed cells.

Enzymatic digestion and mechanical disruption provided low yields of mitochondria. Only mitochondria isolated by hand-shaking yeast cells with glass beads exhibited respiratory response. However, the oxidative and phosphorylative activities measured in non-heated and mitochondria heated at 56 C were such that it was not definitely indicated that thermal stress affected the respiratory activity of mitochondria.

Fluorescence studies showed that phase transitions in cell membranes of S. cerevisiae were altered after heat

stress at 56 C for either 1 or 2 minutes, reflected changes in the physical state and the organization of the membrane, especially in the lipid components. However, storage of heat-stressed cells in water at 25 C for 5 hours allowed reorientation of lipids in the membrane. Thus, the membrane was restored to its normal state. Possibly this alteration of phase transitions in cell membranes of heat-stressed cells was responsible for the leakage of cellular constituents, and in particular UV-absorbing materials, which is often associated with thermal injury.

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