

GERMINATION
OF
YEAST ASCOSPORES

THE ROLE OF CARBOHYDRATE
IN THE
GERMINATION OF YEAST ASCOSPORES

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SCOPE AND CONTENTS:

The content and utilization of carbohydrate during the yeast life cycle were studied with special reference to spore germination. The experiments were designed to investigate the nutritional requirements, the changes in the carbohydrate content and dry weight, the respiratory activities, the possible substitution of exogenous glucose by other carbon sources, the effect of temperature treatments, the uptake and distribution of exogenous glucose and the effect of inhibitors of carbohydrate metabolism on germination.

The experiments showed the relative importance of endogenous and exogenous carbohydrates, the kind of respiratory activity and the fate of exogenous glucose carbon during germination. The pathway of carbon metabolism essential for germination was also indicated.

The present work is the first attempt to compare qualitatively and quantitatively the carbohydrate content of yeast during the three major phases of life cycle, viz., growth, sporulation and germination. Quantitative data on uptake and distribution of exogenous glucose during germination of yeast ascospores are provided. From an analysis of the

results an attempt is made to assess the role of carbohydrate in the germination of yeast ascospores.

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INTRODUCTION

Life cycles of yeast

The yeasts, a heterogenous group of organisms, exhibit more than one life cycle pattern. Their life cycles differ mainly in the relative length of the haploid and diploid generations. Three different types of yeast life cycle were recognized by Guilliermond (1940).

In the first of these, represented by Schizosaccharomyces octosporus Beijerinck, the cells are predominantly haploid and the diploid phase is very short. The haploid cells multiply vegetatively by transverse division. The diploid stage is achieved by fusion of haploid cells but reduction division follows karyogamy thus restoring the haploid phase again.

The second life cycle pattern in yeast, represented by Saccharomyces ludwigii Hansen, has a long diploid phase and a very short haploid phase. The diploid cells multiply vegetatively by budding. The nuclei in these cells may undergo meiotic division forming four haploid nuclei per cell around each of which walls are formed to produce ascospores. The ascospores normally fuse in pairs, while still within the ascus, bringing an end to the haploid stage.

In the third life cycle pattern, represented by many strains of Saccharomyces cerevisiae Hansen, both diploid and haploid cells produce buds, though the budding stage with haploid cells is normally

shorter. When diploid vegetative cells multiplying in a nutrient medium are transferred to an environment of poor nutrition (e.g. having only a source of carbon), the nuclei undergo meiosis forming four haploid nuclei per cell. Around all these nuclei (or 1, 2 or 3 of them) ascospores are formed by the method of free cell formation. On transfer to a nutrient medium the ascospores germinate to produce haploid cells which multiply by budding. Some of these conjugate in pairs to restore the diploid vegetative stage. Often, however, buds produced on adjacent spores in an ascus will fuse and in this case, the situation resembles the second life cycle pattern. The strain used in the present investigation is assumed to possess the third type of life cycle because no conjugations between the spores were observed by the writer.

Budding and sporulation are two mutually exclusive processes in yeast. A sporulating cell does not, at the same time, produce buds. The two processes differ cytologically and genetically in that mitotic division of the nucleus is associated with budding, whereas, sporulation follows a meiotic division. A diploid cell of a sporogenic strain of Saccharomyces is capable of following either one of these two paths. The path followed is determined by the physiological condition of the cell, which in turn, is influenced by the composition of the medium and other environmental factors.

Sporulation of fungi

Sporulation is the production of a special dormant structure which, in general, has the ability to survive over periods when

conditions are not favourable for growth. The ecological significance of spore production lies in providing the organism with increased survivability and better dissemination. Many environmental and nutritional factors are known to affect sporulation.

Fungal spores, in spite of their great diversity in their structures and functions, can be classified broadly into two kinds, namely, asexual spores and sexual spores. Asexual spores include the non-motile sporangiospores of lower fungi (Phycomycetes) and the conidia of Ascomycetes and Fungi Imperfecti. Sexual spores, on the other hand, are represented by the zygospores and oospores of Phycomycetes, ascospores of Ascomycetes and the basidiospores of Basidiomycetes. Zygospores and oospores, which are the product of fusion of like or unlike gametes or gametangia, are diploid structures, whereas, ascospores and basidiospores which form around the products of meiosis are haploid structures.

The early study of sporulation in fungi has been more concerned with morphogenetic and anatomical aspects than with physiology. Nutrition, however, has been known for a long time to be an important single factor controlling growth and reproduction. It is a very common observation that the induction of sporulation follows a fall in the level of food material. Thus, strains of the water mould Saprolegnia remained in the vegetative stage if kept supplied with fresh nutrients but produced sporangia when transferred to water (Klebs, 1898; Kauffman, 1908; Kanouse, 1932). This is also true of endospore production in bacteria. Knaysi (1948) observed that "endospores are formed in an aerobic environment by healthy cells facing starvation".

Since organic compounds serve as the only source of energy for the fungi, the carbon source would be expected to play a prominent role in controlling the production of spores. The effect of carbon source on the type of growth in Melanospore destruens and some other Ascomycetes was investigated by Hawker (1939, 1947) and Hawker and Chaudhury (1946). It was shown that the number of fruiting bodies produced was greatly influenced by the nature as well as by the concentration of carbohydrates or other carbon compounds. A decrease in fruiting was noticed with an increase of glucose above 0.5% and at 2% glucose, fruiting was inhibited completely. Sucrose, however, had a different effect when supplied as carbon source, in that, fruiting increased in amount with increasing concentration upto about 10% of this sugar. At this concentration, the number of perithecia was greater than that with optimum glucose concentration. This beneficial effect of sucrose on fruiting was suggested to be related to the rate of hydrolysis or phosphorylation of this sugar. A rate of hydrolysis that would maintain a concentration of hexose favourable for fruiting would stimulate the process. The higher rate of respiration observed with sucrose (thus providing more energy) might also have a stimulating effect.

Results similar to those with M. destruens were obtained with Coprinus lagopus in which an increase in glucose concentration resulted in delayed fruiting (Madelin, 1956). The inhibitory effect of increased glucose concentration on sporulation has been reported also in bacteria (Foster, 1956). In Bacillus subtilis sporulation was reduced by 60-80% due to the presence of an excess of glucose and glutamate (Hanson et al., 1964). Similar results were obtained by Donnellan et al., (1964) who

observed optimal sporulation of B. subtilis when glucose and glutamic acid concentration was 10 mM. With further increase in concentration sporulation decreased. The inhibitory effect of glucose and glutamate on sporulation was exerted through the repression of aconitase synthesis which is required for sporulation. A block in aconitase synthesis in aconitase minus mutants prevented bacterial cells from sporulating (Szulmajster and Hanson, 1964).

Oxidative metabolism of carbohydrate is known to be associated with sporulation. Hawker and Hepden (1962) found that intense respiration preceded development of perithecia in Sordaria and zygosporangia in Rhizopus, Mucor and Phycomyces. In Neurospora non-conidiating cultures had greater glycolytic activity than conidiating cultures (Weiss and Turian, 1966). When glycolysis was inhibited by metabolic poisons, conidiation took place. When the oxidative metabolism of the normally conidiating culture was limited, conidiation was suppressed. From these results it was suggested that the relative activities of fermentative and oxidative pathways regulated the production of conidia in Neurospora. At this point, asexual sporulation in Neurospora shows similarity to sporulation in aerobic bacteria where oxidative metabolism of carbon is a requirement. Hanson et al. (1963) reported that the vegetative cells of B. cereus were lacking a functional TCA cycle, but the enzymes required for the operation of the cycle were synthesized during sporulation. The inhibition of induction of a functional TCA cycle during early sporulation by chloramphenicol and α -picolinic acid prevented the morphological and physiological changes during the transition. These observations indicated that energy-providing reactions

during growth and sporulation were different. For growth, energy was derived from glucose oxidation while for sporulation energy was supplied by acetate oxidation. During growth in glucose, acetate accumulated, and its utilization started with the onset of sporulation. The enzymes of the TCA cycle were repressed during growth in a complex medium containing glucose, and the acetate utilization via TCA cycle marked the beginning of the sporulation process.

Carbon dioxide, a frequently used source of carbon, is known to affect sporulation in several fungi. In some cases the effect is stimulating, in some others, inhibitory. This gas has been shown to induce the production of resistant sporangia in Blastocladiella. Incubation with bicarbonate resulted in the development of thick walled resistant sporangia as opposed to the ordinary thin walled sporangia obtained without bicarbonate (Cantino, 1952, 1956). Once the production of resistant sporangia was initiated, it was sustained so long as bicarbonate was maintained for a certain duration. Having passed the critical stage the process was completed irrespective of presence or absence of bicarbonate. Cantino suggested a shift in metabolism due to the presence of bicarbonate. Increase in the concentration of bicarbonate tends to decrease oxidative decarboxylation of α -keto-glutarate and accelerates the condensation of CO_2 with pyruvate. This would result in the accumulation of intermediates between α -ketoglutarate and citrate within the organism leading to the shift towards an alternate pathway involving accelerated synthesis of lipid and cell wall material. Perithecial development in Chaetomium was shown by Bustin et al. (1966) to be promoted by carbon dioxide.

Due to nutritional and metabolic diversities in the sporulation of different groups of organisms, generalizations are difficult. From the above considerations it seems clear that changes in carbohydrate metabolism are associated with sporulation. The vegetative cells in a medium containing glucose show mainly glycolytic activities. An increased respiratory activity involving in many cases the utilization of acetate via the TCA cycle marks the shift in carbon metabolism and the beginning of the sporulation process. It is, however, remarkable in this connection that prokaryotic cells bear some similarities to eukaryotic cells, such as response to dilution of medium, oxidative metabolism and CO_2 requirement during sporulation.

Sporulation in yeast

The study of sporulation in yeast dates back to the 19th century. De Seynes (1868) published the first recognizable description of yeast ascospores. Hansen (1902) realized that budding and sporulation were two different routes that yeast cells can enter. Sporulation in yeast, like that in bacteria (Foster, 1956) is an irreversible process. Once the cells are committed to sporulation, they could not be converted back to vegetative growth. The point of irreversibility was found to lie between 13-17 hours after an exposure to the sporulation medium (Ganesan et al., 1958).

The early studies of yeast sporulation were mainly concerned with the conditions that affected the production of ascospores, their morphological development and their use in classification. A number of presporulation and sporulation media were used to induce sporulation.

These media which were undefined or ill-defined, contained fruits, vegetables and yeast extracts (Mrak et al., 1942; Lindegren and Lindegren, 1944; Wickerham et al., 1946). Simple defined media for sporulation were, however, used by Stantial (1935), Adams (1949), Fowell (1952) and Kirsop (1954). The important ingredient of the latter media was acetate.

The optimum physical and chemical conditions required for sporulation were studied by many workers. The results obtained on the effect of pH on sporulation vary considerably. Kleyn (1954) reported S. cerevisiae to form spores in the range of 6.8 - 8.7. Kirsop (1954), also with S. cerevisiae, observed an optimum range of pH for sporulation between 6.4 and 6.8. Fowell (1967), on the otherhand, obtained best sporulation with several yeast at pH 8.4 - 10.6. Adams and Miller (1954) found the optimum range of temperature for sporulation to be between 24°C - 27.5°C. Hansen (1883, 1896) noted that air was required for sporulation. This finding was later supported by Slator (1921), Stantial (1935), Kirsop (1954) and Miller et al. (1957). An inhibitory effect of CO₂, which could nullify the stimulating effect of oxygen on sporulation was observed by Adams and Miller (1954). On the otherhand, the removal of CO₂ from the environment had a limiting effect on sporulation (in acetate medium) which could partially be overcome by the addition of glyoxylate to the medium (Bettelheim and Gay, 1963; Banerjee, 1965). The effect of glyoxylate was attributed to its facilitation of acetate utilization (Bettelheim and Gay, 1963).

The importance of vitamins in sporulation was studied by Tremaine and Miller (1954). They found that the cells supplied with 8 vitamins in the presporulation medium required no vitamins for sporulation.

The effect of the source of carbon on sporulation of yeast was studied by several workers (Kirsop, 1954; Kleyn, 1954; Wagner, 1928; Miller and Halpern, 1956). There is ample evidence that the sporulation response is influenced by the concentration of utilizable carbon source in the medium. Miller and Halpern (1956) compared yeast sporulation in glucose and acetate media. Though low concentrations of glucose stimulated sporulation, higher concentrations (0.33%, 1.0%) of this substance were very inhibitory. Such inhibitory effects were not observed with acetate. The number of 3 or 4 spored asci in glucose medium was very small, whereas, in acetate medium half of the asci contained 3 or 4 spores. Considering these points, it appears that acetate is a better carbon source than glucose for yeast sporulation. From these studies it was suggested that "while glucose can support growth without being metabolized via the TCA cycle, the latter pathway must be followed for sporulation to result". With high glucose concentration, aerobic fermentation was high and a repression of the formation of respiratory enzymes took place (Ephrussi et al., 1956). With low concentration of glucose the capacity for aerobic glycolysis was lowered. Preliminary to sporogenesis in a dilute glucose medium or in acetate medium, a regulatory mechanism comes into action (Miller and Hoffmann-Ostenhof, 1964) involving the derepression of some enzymes. which participate in biosynthesis for sporulation, through removal of catabolite repression.

A decline in the value of respiratory quotient (CO_2/O_2) was observed during sporulation, but when the spores were transferred to growth medium, the high RQ value that characterizes growing cells was

obtained (Miller et al., 1959).

Recently the induction of meiosis, the first step towards sporogenesis in yeast, was studied by Croes (1967). He suggested that the preparation for meiosis started during the pre-meiotic mitosis. A change from fermentation to respiration during the later part of growth period when glucose in the medium was exhausted and ethanol respiration commenced, marked the beginning of this preparation. An exposure of the prepared cells to the sporulation medium containing acetate induced sporulation. An increase in the protein content was observed during the first two hours after the cells were suspended in the sporulation medium. At the onset of DNA synthesis, the protein content decreased to a slightly lower level. When the asci attained maturity, there was a steady decline in protein and RNA content. A low rate of metabolic activity during the latter stages of sporulation was suggested by a decline in the respiratory rate.

Utilization of acetate and macromolecular synthesis during sporulation was studied by Esposito et al. (1969). By using C^{14} -acetate it was shown that the respiration of acetate in the sporulation medium started without a lag. By the time sporulation was complete, 62% of acetate consumed was respired, 22% entered into the soluble pool and 16% entered lipid, protein, nucleic acid and other cell components. Using labelled lysine he found that there were two periods of protein synthesis required for the initiation and completion of ascospore development respectively.

Changes in carbohydrate content of the cells during sporulation of yeast was studied by several workers. Pazonyi and Markus (1955)

observed an increase in the trehalose content while glycogen content was unchanged. The present writer (Banerjee, 1965) found an increase in trehalose content of sporulating yeast in different sporulation media. Vezinhet (1969) and Roth (1970) also observed an increase in carbohydrate content of the yeast cells during sporulation. Though an increase in trehalose content is a common observation associated with sporulation, this substance is not unique to the spores. Vegetative yeast cells also contain trehalose, though the amount in this case is less.

Spore germination in fungi

Germination of spores in fungi involves a morphogenetic change accompanied by physiological and metabolic alterations. This process is generally characterized by three morphologically distinct stages (Allen, 1965). The first recognizable stage after a fungal spore is transferred to a germination medium is swelling, which, though commonly observed during germination, is not universal in fungi (Mandels and Darby, 1953). The second stage is the emergence of a germ tube. The third or the last stage is the early development of the germ tube, after its emergence, leading to the vegetative stage proper.

Spore germination in fungi, however, shows variation from organism to organism and from one kind of spore to another. Some fungal spores may follow alternate modes of germination depending on the environmental conditions. The basidiospores of Rhizoctonia solani form usually germ tubes in nutrient agar but form secondary spores on water agar (Hawn and Vanterpool, 1953). The sporangiospores of Phytophthora infestans produce zoospores when water is present, but produce germ tubes in

absence of water. The chlamydospores of Ustilago hordei form sporidia when germinating in water, but germ tubes are formed when the spores are exposed to 95-100% humidity (Clayton, 1942).

Those spores which germinate without any special treatment on transfer to a medium that supports growth are considered to have an "environmental" dormancy (Sussman and Halvorson, 1966). Other spores, which possess a "constitutive" dormancy, require a special activating treatment to initiate the process of germination. Many physical and chemical factors serve as activators for germination in different fungi. Of the physical factors serving as germination inducers, temperature treatments are the most common. A heat shock is a common requirement for germination of spores in many Ascomycetes, whereas, Basidiomycetes often need a cold treatment for germination. (Sussman and Halvorson, 1966). Thus, the ascospores of Neurospora tetrasperma require a heat shock for germination (Goddard, 1935; Lingappa and Sussman, 1959) and a cold shock is needed for the germination of spores in Tilletia tritici (Holton, 1943) and other Tilletia species (Meiners and Waldher, 1954). The mechanism of action of temperature treatments in fungal spore germination is not clearly known. Sussman (1965) suggested that heat treatment might bring trehalose and trehalase in Neurospora ascospores into contiguity. Many chemical factors are also known to induce germination in fungi, e.g., L-alanine (Yanagita, 1957) and different solvents (Sussman et al., 1959).

As regards their nutritional requirements, fungal spores vary widely. An example of one extreme is provided by the ascospores of N. tetrasperma which can germinate, after a heat shock, in distilled water without any added nutrient (Sussman, 1954). At the other extreme are the

spores of Glomerella cingulata which require carbon, nitrogen, phosphorus and sulphur for germination (Lin, 1945). Many other spores require one or more exogenous substances for germination. The conidia of Fusarium solani, Myrothecium verrucaria and Aspergillus niger (Cochrane *et al.*, 1963a, b; J. Cochrane *et al.*, 1963; Mandels, 1963; Yanagita, 1957) can be cited as examples. Cochrane (1960) has suggested an ecological advantage on the part of the fungi that require external nutrients for spore germination. The spores of these fungi will be protected from germinating in an environment that does not support mycelial growth.

Both oxygen and carbon dioxide have been reported to influence germination in fungi. With few exceptions, fungal spores do not germinate in complete anaerobiosis. Examples of the normal situation include the ascospores of Neurospora crassa (Goddard, 1935) and the uredospores of rust fungi (Allen, 1955). However, the oospores of Phytophthora infestans germinate anaerobically (Uppal, 1926). The influence of CO₂ on germination of Aspergillus niger conidia was studied by Yanagita (1957). This gas was found to be essential for the initiation of germination. Using C¹⁴O₂, this author studied the incorporation of CO₂ in germinating conidia (Yanagita, 1963). Most of the radioactivity was found in protein and acid soluble (of which ATP was the most heavily labelled) substances. Germination of resting sporangia of Physoderma maydis can, however, take place in an atmosphere lacking CO₂ (Hebert and Kelman, 1958).

Some of the common physiological changes observed during germination of fungal spores include changes in respiratory activities and endogenous metabolism as well as in the pattern of carbohydrate metabolism.

An increased respiratory activity during germination is exhibited by many fungi. Neurospora tetrasperma (Goddard, 1935; Goddard and Smith, 1938), Phycomyces blakesleeana (Halbgsuth and Rudolph, 1959; Rudolph, 1960), Myrothecium verrucaria (Mandels and Norton, 1948) and Aspergillus niger (Yanagita, 1957) furnish examples.

A change in the endogenous metabolism has been observed during the germination of N. tetrasperma ascospores. The dormant spores of this fungus utilize lipid as their endogenous substrate, whereas, trehalose serves as the major substrate for the germinating spores (Lingappa and Sussman, 1959). Spores of M. verrucaria, which contain sufficient reserve substances to support germination (20% of dry weight), required exogenous substances for rapid germination of a majority of the spores. However, some of the spores could germinate in absence of external substrate indicating that germination was possible utilizing only endogenous reserves. This view was further supported by the fact that germination took place utilizing the reserve substances if endogenous metabolism was stimulated by azide (Mandels, 1963).

Changes in the carbohydrate metabolism associated with germination have also been found in some fungi. These changes are proved to be quantitative rather than qualitative. Cochrane and Cochrane (1966) studied spore germination and carbon metabolism in Fusarium solani. They found that the spores had no detectable capacity to respire glucose anaerobically but the germinated spores fermented this sugar, though slowly. Data from enzymatic studies suggested that the appearance of fermentative capacity during germination was not due to the de novo synthesis of enzymes but was due to the acceleration of the preexisting

metabolic system. Glucose-C¹⁴ metabolism was studied in dormant and activated ascospores of N. tetrasperma (Budd et al., 1966). From the distribution of radioactivity in the same substances, whether the spores were dormant or activated, it appeared that the principle pathways of glucose metabolism in both cases were basically identical though some quantitative differences did exist.

Spore germination in yeast

Metabolic aspects of the germination of yeast ascospores have received little attention and have not yet been studied in much detail. There are few reports on the germination of yeast ascospores available in the early literature, and these are mainly descriptive in nature (Hansen, 1894; Guilliermond, 1920). Some scattered reports are found in the late fifties of the present century. Hashimoto et al. (1958) in the course of their electron microscopic studies on the fine structure of microorganisms observed that lipoidal vacuoles were present in both resting and germinating ascospores. They also noted that the inner spore coat gave rise to the vegetative cell wall during germination. Miller et al. (1959), while studying the respiration of sporulating and growing cells of yeast, noticed that the RQ value, which decreased during sporulation, returned to the high values characteristic of growing cells within 2-4 hours of transfer of the cells from sporulated culture to growth medium.

Ogur and St. John (1958) made the first attempt to study biochemical changes during germination of yeast. Using a tetraploid strain they found that germination occurred in nitrogen as well as in air.

Fermentation reappeared before respiration and before any detectable budding when the asci were incubated in glucose nutrient broth. A heat shock speeded the reappearance of fermentation.

Observations on the nutritional requirements for the germination of yeast ascospores were made by Nagashima (1959). His results indicate the importance of glucose in this process. No germination took place on plain agar without the addition of glucose or tomato juice. Ammonium sulphate alone did not cause germination but promoted it when used in addition to glucose. The presence of vitamins and salts had no effect. Sodium salts of pyruvic, malic, citric and acetic acids did not substitute for glucose. L-aspartic acid and L-glutamic acid had some stimulating effect when used with glucose, but alone, these amino acids did not cause germination.

The next report on the nutritional requirements for germination of ascospores in yeast was by Palleroni (1961) who also found that the spores do not germinate without a carbon source like glucose, fructose or galactose. Glucose alone can cause germination, and the spores germinated in a very wide optimum zone of pH between 5 and 9, though best value was obtained between 6 and 7. Acetate had no stimulating effect on germination.

Ramirez and Miller (1964) observed an increase in the pool of free amino acids, except for proline which showed a decrease, when asci were transferred to growth medium.

Very recently Seigel (1970) has studied the germination of ascospores of yeast. He found germination of yeast ascospores to be predominantly an anaerobic process. A carbon source was needed for

germination and most of the nitrogen sources alone did not cause germination, though many of them were stimulating when used in presence of glucose. Peptone and casein hydrolysate were exceptional in this regard as these materials induced some germination, as measured by the loss of acid-fastness, even in the absence of glucose.

The foregoing are the only reports in the literature on the physiological study of yeast spore germination.

Carbohydrate in yeast: content and metabolism

The importance of carbohydrate as a major component of the yeast cell is well known. Depending on the strain and the medium used, these substances in yeast can vary widely, accounting for as much as half of the dry weight in some cases. The occurrence of glycogen as a reserve substance in yeast was discovered by Errera (1885). The other reserve carbohydrate, trehalose, was isolated from yeast by Koch and Koch (1925). Although these two substances are known to serve as reserve material, their precise function and the conditions under which they are accumulated or utilized are not clearly understood.

In course of their studies on yeast metabolism, Trevelyan et al. (1952) noted an increase in cellular carbohydrate during fermentation. In the same series of studies, breakdown and resynthesis of trehalose was observed during fermentation of glucose, sucrose, fructose and mannose (Trevelyan and Harrison, 1956a). These authors (1956b) also observed a loss of 1% of the content of glycogen and trehalose each day from stored yeast.

Panek (1962) investigated the synthesis of trehalose in S. cerevisiae, and found that it occurred during non-proliferation of the cells.

An accumulation of the reserve carbohydrates was observed during the single cell phase between two buddings (Küenzi and Fiechter, 1969). A rapid degradation of part of these reserves began shortly before the swelling of the bud.

The metabolism of exogenous carbohydrate, especially of glucose, in yeast has been studied by many workers and the main features of the pathways involved seem to be known.

Chen (1969) studied the metabolic pathways for glucose utilization in yeast by using C^{14} glucose. The results showed that 58-60% of glucose carbon is assimilated into cell constituents and 40-42% metabolised into CO_2 . Of the CO_2 produced, 6% was produced via the pentose phosphate pathway, 51% by decarboxylation of pyruvate and 43% from the TCA cycle. From all the experimental results it appears that different oxidative patterns may be operative in yeast (Eaton and Klein, 1954), since late log phase cells can oxidize only glucose. It is generally known that the presence of sugar in the medium induces anaerobic metabolism. In this respect glucose is more effective than galactose. The enzymes of the TCA cycle and the glyoxylic acid cycle are almost completely repressed by glucose. The disappearance of sugar from the medium results in the appearance of activity of these enzymes (Polakis and Bartley, 1965). The apparent contradiction between the results obtained by Chen (which shows that 43% of CO_2 was derived from TCA cycle) and by Polakis and Bartley (that presence of sugar induces anaerobic fermentation) is probably due

to the different concentrations of sugar used. In Chen's experiments, care was taken to maintain low concentration of sugar in the medium so that no excess sugar should accumulate at any time during the course of aerobic fermentation. Maltose-grown cells have greater oxidative activity and unlike glucose-grown cells, they adapt to ethanol and acetate easily (Görts, 1967).

The ability of yeast cells to adapt to environmental change by changing their metabolism has been known since the time of Pasteur. The presence of oxygen resulted in an inhibition of anaerobic fermentation (Pasteur effect). Ephrussi and Slonimski (1950) observed that yeast cells grown anaerobically for a short time lose their ability to respire glucose aerobically. Cytochromes a, b and c were lost and two analogous cytochromes a₁ and b₁ were acquired. The synthesis of normal cytochrome accompanied by the disappearance of the new cytochromes, and the re-establishment of the ability to respire could be induced by oxygen (Ephrussi and Slonimski, 1950). On the other hand, Tustanoff and Bartley (1964) found that when the yeast cells were grown anaerobically on galactose, the respiratory activity was retained and the cells could immediately respire glucose, fructose, galactose or pyruvate; but cells grown similarly on fructose oxidized none of these substances immediately, though they could adapt to oxidize glucose or fructose.

A variation in the ultra-structure of mitochondria has been observed during aerobic growth of yeast cells on 5% glucose (Yotsuyanagi, 1962). During exponential growth when energy was derived from glycolysis, the number of mitochondria were few and their cristae were not clearly developed. During the transition from exponential to stationary phase many well

developed mitochondria with cristae appeared. There seems to be a parallelism between the development of the mitochondria and changes in metabolism from fermentative to oxidative.

Unlike yeast cells grown in glucose, cells grown in galactose, aerobically or anaerobically, as well as cells grown in very low concentration of glucose are found to possess mitochondria (Polakis et al., 1965). From the electron microscopic studies of the changes in mitochondrial structure and from enzyme activities of S. cerevisiae when the environment is changed, it seems that it is the nature of the carbon source present in the medium rather than the presence or absence of oxygen, that determines the metabolic capability of the cell. Complex mitochondrial structures are produced only when the substrate present requires them (Polakis et al., 1964).

Questions concerning carbohydrate metabolism during germination of yeast ascospores and the objective of the present study.

From the above survey it is clear that the study of yeast ascospore germination is still in an early stage. Also it is apparent that knowledge of carbohydrate content and metabolism in yeast concerns mostly the vegetative cells. Similar studies during yeast sporulation are comparatively few; with germinating yeast there has been little study of carbohydrate metabolism and no work on variation in carbohydrate content during germination. There has been no comparative study of the kinds and amounts of different carbohydrates in vegetative, sporulated and germinating yeast. Though it is generally known that carbohydrate content of yeast cells increases during sporulation the following questions concerning carbohydrate

metabolism during germination still remain to be answered: 1. Does the increased carbohydrate content of the sporulated cells have any special function in germination? 2. What happens to this increased reserve carbohydrate during germination? 3. What role, if any, do the reserve carbohydrates play in supplying energy and intermediates during germination? 4. Is exogenous carbon required for germination, and if so, how much, and for how long must it be supplied? 5. How does exogenous carbohydrate induce or support germination? 6. Can it be substituted for by other known activators of germination such as temperature treatments? 7. What is the major pathway of carbohydrate metabolism during transformation of a spore to a vegetative cell?

The present work is an attempt to provide answers to these questions. The objective of this study is to establish first the nutritional requirements for ascospore germination of a strain of Saccharomyces chevalieri. Then, experiments are undertaken to follow the changes in carbohydrate content and dry weight during sporulation and germination; to measure carbon dioxide production and oxygen uptake; to investigate the need for an uptake of exogenous carbon and its distribution; and to study the effects of some metabolic inhibitors on germination. Results are analyzed in search of possible interrelationships between the changes in endogenous carbohydrate content, exogenous carbohydrate uptake and respiratory activity. It is hoped thus to ascertain the major role or roles of carbohydrate in germination of yeast ascospores.

MATERIALS AND METHODS

Yeast strain

A strain of the yeast Saccharomyces chevalieri Guilliermond was used for the experiments. This strain was obtained (as their strain number 46) from the Instituto Nacional de Investigaciones Agronómicas (Ministerio de Agriculture); Jerez de la Frontera, Spain, via Professor J. Santa Maria. This strain was chosen because of its high and reliable sporulation with an average of at least 3 spores per ascus.

Cultures and media

a. Stock culture:

Stock cultures, which supplied inocula for growth cultures, were maintained in slants of yeast nitrogen base (Wickerham, 1951) plus 2% Bacto agar and 1% glucose. Yeast nitrogen base is a chemically defined medium that contains $(\text{NH}_4)_2\text{SO}_4$, three amino acids (histidine, methionine and tryptophan), vitamins and salts. The composition of this medium is given in Appendix I. Stock cultures were transferred once a week, incubated for two days at 27°C and stored in the refrigerator (5°C).

b. Growth culture:

The vegetative cells were grown for two days in malt extract-yeast extract-peptone-glucose (MYPG) medium (Wickerham, 1951) with the addition of 0.1% KH_2PO_4 . The composition of this medium is as follows:

1000 ml distilled water

3 g malt extract

3 g yeast extract

5 g peptone

20 g glucose

1 g KH_2PO_4

c. Sporulation culture:

Growth cells were centrifuged out of the medium, washed twice with sterile distilled water and resuspended in sporulation medium. The sporulation medium consisted of M/20 phthalate buffer of pH 5 containing 1% potassium acetate. The cells were allowed to sporulate for two days on a Warner Chilcott water bath shaker at 27°C with a speed of approximately 100 shakes/min. The horizontal displacement due to shaking was 5 cm. The cell population density (CPD) was 50 millions per ml.

d. Germination culture:

Two germination media, liquid and solid, were used. Most of the germination experiments were done in a defined liquid medium. This medium comprised 0.67% yeast nitrogen base (YNB) and 1% glucose in sterile distilled water. The cell population density for germination in liquid medium, unless stated otherwise, was 50 millions per ml. The sporulated cells were centrifuged out of the sporulation medium, washed twice with sterile distilled water, suspended in germination medium and incubated at 27°C in the water bath shaker when germination in liquid medium was desired. When a solid medium was used for germination, discs (12 mm X 3 mm) of the medium cut from a petri dish containing YNB, glucose and 5% Agar, SP.

(Fisher Scientific Co.) were used. The discs were seeded with the washed sporulated cells with the help of an inoculating needle and incubated at 27°C.

Determination of cell population density (CPD) and dry weight

The cell population density of a culture was determined with a haemocytometer counting chamber (Spencer AO). For each dry weight determination an aliquot of culture was filtered through a pre-weighed Millipore filter (Pore size 0.45 μ) of diameter 47 mm, air-dried for 15 minutes and then dried at 80°C until the weight of the filter plus the cells was constant. The final weight of the membrane filter plus the dried cells, minus the original weight of the filter, gave the dry weight of the cells.

Measurement of germination

A change in the staining property of the spores, namely, the loss of acid-fastness, was used as a criterion of germination. The extent of germination is indicated by the decline in acid-fastness (Seigel, 1970). The Ziehl-Neelsen carbol fuchsin method was used for determination of acid-fastness (Society of American Bacteriologists Manual of Microbiological Methods, 1957). An outline of this method and the composition of the stains are given in Appendix II. In addition, observations of the outgrowth of buds from the spores after 6 or 8 hours, and of the disappearance of the asci from the culture after 24 hours were made to be certain that all the spores produced vegetative cells.

Germination on solid medium was determined by observing the spores

directly under the high power objective for 8 hours after the medium was seeded with the sporulated cells, at 2 hour intervals.

Removal of CO₂ from the environment of the spores germinating in a solid medium

For the removal of CO₂ from the culture environment, the method of Bettelheim and Gay (1963) was used. An aqueous solution (15-20 ml) of NaOH (5.5% w/v) was put in the bottom of a petri dish, then the lid of the dish containing the discs of solid medium seeded with sporulated cells was added and the entire dish was incubated at 27°C. The control dish contained an aqueous solution of calcium chloride (9.3% w/v). These two solutions provided the conditions for absorption and non-absorption of CO₂ and ensured that the relative humidity (95%) should be the same in each dish (Stokes and Robinson, 1949).

Extraction of carbohydrates

Carbohydrates were extracted in several steps by following the method of Chung and Mickerson (1954) with slight modifications. Extraction with distilled water at room temperature for 30 minutes was omitted and the time of trichloro acetic acid extraction was increased from 10 minutes to overnight. The cells from a suspension of 40 ml for vegetative cells and 200 ml for sporulated and germinating cells were used for each extraction. The cells were centrifuged out of the medium, washed twice with sterile distilled water and then subjected to the following four step

extraction procedure.

1. The first extraction was made with 2 ml of 10% TCA at 5°C overnight. This step was repeated to ensure complete extraction of TCA-soluble carbohydrates which were later separated by thin layer chromatography.
2. The residue after the first extraction was treated with 2 ml of 30% KOH at 100°C for 30 minutes. This extract contained some glycogen and mannan which were separated later.
3. After the extraction with KOH, the residue was treated with 2 ml of 2N acetic acid at 100°C for 30 minutes. This extract contained a small amount of glycogen.
4. The last extraction in the series was made by 2 ml of H₂SO₄: water in the proportion of 3:1 and diluted with distilled water. The final volumes of all the extracts were brought to 10 ml by adding distilled water.

Separation and identification of carbohydrates from TCA extract

The carbohydrates in the TCA extract were separated by thin layer chromatography. Chromatograms were made on thin layers (0.25 mm) of silica gel G produced on 20 cm X 20 cm glass plates (Desaga) using 30 g of silica gel suspended in 60 ml of distilled water. The plates with the layers on them were heated in an oven at 100°C - 110°C for 30 minutes. The streaks were made on the plate by using 10 volumes of extract applied with a micro syringe. More than one streak was made on a plate with the same material so that one streak could be sprayed with the colouring reagent while the other streaks were covered and later used for elution and measurement of the separated carbohydrates. Two different solvent systems as shown below were used.

I

ethyl acetate	39 ml	
isopropanol	14 ml	Block, Durrum and Zweig (1958)
water	7 ml	

II

butanol	24 ml	
acetic acid	6 ml	Randerath (1963)
water	30 ml	

Chromatograms were removed from the tank when the solvent front had advanced a distance of 15 cm. They were dried in air for 3-5 minutes to evaporate the excess solvent and then sprayed with a solution of 0.5 ml of anisaldehyde (Eastman Kodak Co.) and 0.5 ml of concentrated H_2SO_4 (reagent grade) in 9 ml of 95% alcohol (Randerath, 1963).

The portion of the layer of silica gel where the streaks representing the separated carbohydrates should occur (as indicated by comparison with the sprayed streak) was scraped off, allowing a margin surrounding the streak, transferred to a 15 ml centrifuge tube and eluted with 1 ml of 5.0% TCA.

The identification of the separated carbohydrate from the TCA extract was first approximated by comparison with the R_f values of the reference spots of authentic substances on the same chromatogram (using both solvent systems as described above). This procedure indicated the separated spots to be glycogen and trehalose. This conclusion was then confirmed on the basis of the following criteria:-

Identification of trehalose: - (1) A mixture of the extract and authentic

trehalose did not separate but gave a single spot with Rf value corresponding to that of authentic trehalose.

(2) Eluates from the suspected trehalose spot did not reduce Fehling's solution but did so after hydrolysis with diluted H_2SO_4 showing that the carbohydrate in question was non-reducing but released reducing component(s) on hydrolysis.

(3) The spot made with the eluate of the suspected trehalose after hydrolysis gave a single spot with an Rf value that corresponded to the Rf value of glucose on the same chromatogram, thus, indicating that the yeast carbohydrate had no component other than glucose.

Identification of glycogen:- (1) An eluate of the suspected glycogen was chromatographed with authentic glycogen, but the two substances did not separate.

(2) An eluate of the suspected glycogen was treated with iodine-potassium iodide reagent (Appendix III) and a pale brown colour, characteristic of glycogen, developed.

Separation of mannan from glycogen in KOH extract

Mannan and glycogen were separated by precipitating mannan with Fehling's solution (Chung and Nickerson, 1954). A 5 ml portion of the alkali extract was treated with 2 ml of Fehling's solution. The mixture was put in the refrigerator overnight, centrifuged, and the precipitate was washed 3 times with 0.1 N KOH. The washed precipitate was dissolved in 1 ml of 2 N H_2SO_4 and made up to 5 ml with distilled water. The amount of glycogen was calculated by difference as described below under "Quantitative determinations of carbohydrates".

Quantitative determination of carbohydrates

For quantitative determination of the eluted carbohydrates the phenol-sulphuric acid reagent of Dubois et al. (1951, 1956) was used. One ml of the eluted carbohydrate in a colorimeter tube was treated with 1 ml of 5.0% phenol. To this mixture was added 5 ml. of concentrated H_2SO_4 (reagent grade) and an orange-yellow colour developed. The contents of the tube were mixed thoroughly and allowed to stand in a hot water bath for about 30 minutes to ensure the complete development of the colour. The intensity of the colour developed in each tube was measured with a Spectronic 20 colorimeter (Bausch and Lomb) at 490 $m\mu$, and the amount of carbohydrate was determined by comparing with a glucose standard curve for trehalose, glycogen and glucan and with a mannose standard curve for mannan.

The amount of glycogen in the KOH extract was calculated as follows:

$$\begin{aligned} \text{glycogen in KOH extract} &= \text{total carbohydrate in KOH extract} \\ &\quad \text{as glucose minus mannan in KOH extract} \\ &\quad \text{as glucose (Trevelyan and Harrison, 1952,} \\ &\quad \text{1956b)..} \end{aligned}$$

Temperature treatment of the sporulated cells before germination

For exposure of sporulated cells to high temperatures, the cells were separated from 40 ml of sporulation medium by centrifuging, washed twice with sterile distilled water, resuspended in 10 ml sterile distilled water in 16 mm test tubes and incubated at temperatures ranging from 50°C

to 90°C for the desired time periods. After these treatments, the cells were transferred to germination medium. For low temperature treatment, the sporulated cells were centrifuged, washed, suspended in distilled water and stored at 4°C and -4°C for 1, 2 and 6 months after which the cells were separated from the distilled water by centrifugation and re-suspended in germination medium.

Manometric techniques

For the measurement of CO₂ evolution and O₂ uptake during germination, the standard manometric techniques of Umbreit, Burris and Stauffer (1949) were followed. The respirometer used for this purpose was a Bronwill Series 5 UV, Warburg apparatus. Brodie's solution served as the manometer fluid.

The sporulated cells were separated from sporulation medium by centrifugation, washed as usual, and then suspended in the germination medium (0.67% yeast nitrogen base) without the added glucose. Two ml of this suspension were introduced in the Warburg flask. Glucose (1 ml of 3%) was put in the side arm and was tipped into the main vessel only after the manometer was attached to the Warburg apparatus and the flasks were allowed to equilibrate at 27°C for 10 minutes. The final cell population density in the flask was 15 millions per ml and the final glucose concentration was 1%. Two manometers were used simultaneously, one of which had 0.2 ml of 20% KOH in the central well for absorbing CO₂. A small filter paper wick was introduced into the central wall to facilitate CO₂ absorption. The calculation of O₂ uptake and CO₂ evolution, was done by the methods of Umbreit, Burris and Stauffer (1949).

Readings were taken at 2 hour intervals upto 6 hours after glucose was tipped into the Warburg flask.

Use of D-glucose, C¹⁴-U as tracer

The absorption of exogenous glucose and its incorporation in CO₂ and in different components during germination was studied by the use of uniformly labelled D-glucose-C¹⁴ (New England Nuclear Corporation). Labelled glucose (sp. act. 15-20 mc/mM), dissolved in 0.5 ml of 90% ethanol, was added to 20 ml of cell suspension in the germination medium (0.67% yeast nitrogen base + 1% glucose) during the periods 0-2, 2-4 and 4-6 hours. Each flask contained 50 μ c of labelled glucose (2.6×10^7 counts per minute - CPM) and 200 mg of glucose. Three similar flasks containing aliquots of the same culture were used simultaneously and one of the flasks received C¹⁴-glucose during each 2 hour time period. Samples of medium were taken out at "0" time and after 2 hours to calculate the proportion of exogenous glucose that was lost from the medium in two hours by the difference in the radioactivity of the two samples. This procedure was repeated for all the three flasks. At the end of every two hours, a sample of cells from the corresponding flask was taken out, centrifuged, washed and suspended in equal volume of distilled water. This suspension was used for measuring the radioactivity in the whole cells. A sample of the washings of the cells was also saved for the measurement of C¹⁴ present in that fraction. The CO₂ evolved by the cells from each flask during each 2 hour period was absorbed by sealing the flasks with corks carrying 4 pieces of Whatman Number 1 filter paper moistened with 0.2 ml of 20% aqueous KOH (Budd, et al.,

1966). These filter papers were used directly for the measurement of C^{14} in CO_2 .

For measuring the radioactivity in different cell components, the germinating cells were separated by centrifugation from medium containing C^{14} -glucose, washed with sterile distilled water and extracted for different components by following the methods of Esposito et al. (1969) with some modifications. The lipids were extracted with chloroform and methanol in the proportion 2:1 instead of with ethyl alcohol-ether 3:1. An additional step (extraction with cold TCA) was introduced to ensure the complete extraction of carbohydrates other than glycogen II and mannan. After each extraction the material was washed with the same solvent. The following is an outline of the procedure:

- (i) Extraction with 3 ml of distilled water at $100^{\circ}C$ for 10 minutes and washed with 2 ml distilled water (Water soluble fraction.).
- (ii) Extraction with 3 ml chloroform: methanol::2:1 at room temperature for 24 hours and wash with the same solvent (lipid fraction).
- (iii) Extraction with 3 ml cold TCA (10%) overnight and wash with 2 ml of the same solvent (TCA soluble carbohydrate fraction not extracted by hot water.).
- (iv) Extraction with 3 ml of 5% TCA at $100^{\circ}C$ for 10 minutes and wash with 2 ml of the same substance (nucleic acids).
- (v) Extraction with 5 ml of 0.1 M NaOH at $37^{\circ}C$ for 2 days and wash with 5 ml of the same substance (Protein + some carbohydrate).

Fractionation of hot water and NaOH extracts:

The water soluble fraction was further sub-fractionated into acid (mainly organic acids) basic (mainly amino acids) and neutral (mainly carbohydrates) substances by passing it consecutively through different ion exchange resin columns (Carvin and Beevers, 1961). A 2 ml portion of the aqueous extract was passed through a 6 X 1 cm (approximately) column of Dowex 50 (H^+). The column was washed with 60 ml of distilled water, which was then passed through a 6 X 1 cm (approximately) column of Dowex 1 formate, generated from the chloride form with 60 ml of 1M sodium formate and 30 ml of 0.1 N formic acid. The effluent from this fraction represented the neutral substances. Basic substances were eluted from the Dowex 50 with 60 ml of 1N NH_4OH and the acid part was eluted from the Dowex 1 by 4 M formic acid. Acid, basic and neutral substances were then evaporated to dryness on a sand bath at 60°-70°C and eluted with 2 ml of the respective solvents. These samples were later used for the measurement of the radioactivity.

The neutral portion was further analyzed by thin layer chromatography using the method described above. The acid portion was chromatographed using ethanol (96%)/water/ammonia solution (25%): 100/12/16 as a solvent (Braun and Seenan, 1962) followed by spraying with methyl red (Lynes, 1964).

Protein and carbohydrate from the NaOH extract were separated by using Pronase to hydrolyze the protein followed by the precipitation of the carbohydrate by 95% ethanol. Pronase was added to the NaOH extract (1 mg/10 ml) and was allowed to act for 48 hours at room temperature after which the carbohydrate was precipitated by the addition of 95% ethanol.

The tube containing the precipitate and the ethanol was kept overnight in the refrigerator. The precipitate was finally separated by centrifugation and dissolved in the same volume of water as that of the original NaOH extract. The supernatant was evaporated to dryness and redissolved also in the same volume of 0.1 M NaOH. The glycogen fraction thus separated was tested for the presence of protein by using the Folin phenol reagent (Lowry et al., 1951) to ensure that separation of protein and carbohydrate was complete. Similarly, the protein fraction was tested for the presence of carbohydrate by the phenol sulphuric acid reagent as described above in connection with the quantitative measurements of carbohydrate.

Samples from all the extracts, media washings and whole cells were applied to pieces of Whatman No. 1 filter paper and dried in air for the measurement of radioactivity. Samples of 50 λ were used with media, washings and whole cells, whereas, samples of 100 λ were used with the different extracts. A blank filter paper with no radioactive substances added to it was always included to record the radioactivity of the background. The dried filter papers carrying the radioactive substances were put in scintillation vials to which 15 ml of scintillation fluid (42 ml of liquifluor and 1000 ml of toluene) was added. The radioactivity was measured in a liquid scintillation counter (Unilux II, Nuclear Chicago).

Use of sodium acetate-2-C¹⁴ as tracer

Sodium acetate-2-C¹⁴ was used as a tracer when the spores were germinating in yeast nitrogen base + 1% glucose medium. For comparison, a parallel experiment was done in which the medium consisted of only yeast

nitrogen base with no glucose. To a 20 ml volume of medium, 6.25 μ c (0.26 mg, total CPM = 4.2×10^6) of C¹⁴-acetate (specific activity = 2 mc/ml) was added. As in the experiments with C¹⁴-glucose, here also the cells received radioisotope during 0-2 hours, 2-4 hours or 4-6 hours. The amount of radioactivity lost from the medium in each 2-hour period was determined, as in the case of C¹⁴-glucose, by the difference in the amount of radioactivity in the medium at '0' hour and after the 2 hours. The amount of C¹⁴ present in the medium, CO₂, whole cells, hot water soluble fraction and residue (only after hot water extraction) was determined as in the C¹⁴-glucose experiments.

Use of metabolic inhibitors

Sodium fluoro acetate, potassium cyanide, malonic acid, iodo acetic acid and 2-deoxy-D-glucose were added in different concentrations in the germination medium. The inhibitors were added to the medium 15-20 minutes before the carbon source was added, to favour maximum action, and the yeast was exposed to the inhibitors continuously for 24 hours. Sodium fluoro acetate and potassium cyanide were also added to sporulation cultures. As with germination medium, the inhibitors were added 15-20 minutes before the carbon source and 48 hours were allowed for sporulation.

RESULTS

Nutritional requirements for germination

a. Effect of removal of different components from germination medium.

The importance of different components of germination medium was tested by eliminating one or more of them, in turn, from the medium. In all instances, germination was measured by removing samples at 2-hour intervals for observation. The loss of acid-fastness by the spores and ultimately the production of buds by them were used as criteria of germination. When germination did not take place in 8 hours, a final observation was made after 24 hours to allow enough time for the ascospores to germinate in media which might only slow down the process without stopping it completely. The results presented in Table 1 show the mean value of 3 experiments.

It is apparent that glucose and $(\text{NH}_4)_2\text{SO}_4$ had to be present for germination to occur. The omission of vitamins or of histidine, methionine and tryptophan from the medium did not restrict germination, implying that the latter substances are not essential. Germination, however, did not take place in a medium consisting of only glucose and $(\text{NH}_4)_2\text{SO}_4$. It is clear, therefore, that one or more of the salts present in yeast nitrogen base are required for germination and that the vitamins and the 3 amino acids are not essential.

The time needed for the buds from the ascospores to appear was between 4-6 hours after the sporulated yeast was suspended in one of

Table 1

Effect of removal of different components from germination medium

Media	% non-acid-fast spores at	% Decline in acid-fastness after			% spores with buds at			Observation after 24 hours
	0 hr.	2 hrs.	4 hrs.	6 hrs.	4 hrs.	6 hrs.	8 hrs.	
YNB + 1% glucose	10	0	26	55	0	10	95	Complete germination
[YNB-aa]* + 1% glucose	8	0	23	52	0	10	90	Complete germination
YNB-aa.- (NH ₄) ₂ SO ₄ ** + 1% glucose	8	0	0	2	0	0	0	No visible germination
(NH ₄) ₂ SO ₄ + 1% glucose	10	2	2	2	0	0	0	No visible germination
1% glucose	10	0	0	2	0	0	0	No visible germination
YNB	10	2	0	0	0	0	0	No visible germination
Vitamin free yeast base***				55				Complete germination

YNB = yeast nitrogen base (Difco)

*, **, *** - Details in appendix 1.

the media that supported germination. No loss of acid-fastness by the spores was noted during 0-2 hours. During 2-4 hours some of the ascospores lost their acid-fastness and this trend continued during 4-6 hours. By 6 hours, 65% of the ascospores had lost their acid-fastness and 10% of them had produced buds. By 8 hours almost all the ascospores (95%) had buds and the asci were no longer visible. By this time the germination culture had become a growth culture consisting mostly of budding cells and no acid-fast spores were detectable.

A point to note from Table 1 is that in a suitable medium almost all the ascospores produced buds by 8 hours, whereas, in the media that did not support germination no sign of germination (as indicated by loss of acid-fastness or by outgrowth) was visible even after 24 hours.

b. Germination in media containing different carbon sources.

The possibility that some other sugars or potassium acetate would support germination was investigated. These substances were added in 1% concentration to YNB medium, one at a time. The percentage of non-acid-fast spores before exposure to germination medium was 10. Examinations for the occurrence of germination were made after 6 and 24 hours. The results (average of 3 experiments) are shown in Table 2.

Fructose, mannose and sucrose, like glucose, supported both germination and growth. The other sources of carbon failed to support germination and no growth was evident in 24 hours. Some increase in the number of non-acid-fast spores was, however, noticed after 6 hours

Table 2

Germination of yeast ascospores in yeast
nitrogen base with 11 carbon sources

Carbon source (1%)	Decline in acid- fastness, %, after 6 hours	Observation after 24 hours	
		Germination*	Growth, increase of CPD, %
glucose	53	+	206
fructose	35	+	246
mannose	37	+	319
sucrose	65	+	176
galactose	12	-	Nil
lactose	5	-	Nil
cellobiose	8	-	Nil
maltose	6	-	Nil
D-arabinose	6	-	Nil
L-arabinose	8	-	Nil
Potassium acetate	3	-	Nil

* + = all the spores germinated
- = no germination.

in most cases. Since the growth rates with different carbon sources were not compared, the data presented in column 4 of Table 2 are only semi quantitative.

c. Germination at different glucose concentrations.

Germination of yeast ascospores in different concentrations of glucose was investigated. Five concentrations from 0.01% to 1.0% were used in YNB medium and an initial determination of the percentage of acid-fast spores was made at 0-hour. Samples were removed for examination after 6 and 24 hours. The increase in the cell population density was also noted after 24 hours. The results (average of 2 experiments) are shown in Table 3.

About 10% of the spores were non-acid-fast at 0-hour. After 6-hour, the percentage of acid-fast spores were 12, 22, 29, 50 and 55 in glucose concentrations of 0.01, 0.03, 0.10, 0.30 and 1.0% respectively. It is clear from Table 3 that there was practically no germination in 0.01% glucose. The cell population density also remained unchanged even after 24 hours at this concentration of glucose. In 0.03% glucose concentration, some loss of acid-fastness ($22 - 10 = 12\%$) was observed but no further development towards germination took place, nor did the cell population density show any increase in 24 hours. In the glucose concentration 0.1%, there was a considerable loss ($29 - 10 = 19\%$) of acid-fastness after 6 hours and a decrease in the proportion of asci present in the culture along with some increase (41%) in cell population density after 24 hours. A glucose concentration of 0.30% was sufficient to cause germination and growth comparable to that found with 1.0% glucose.

Table 3

Germination of yeast spores in yeast nitrogen base
medium containing different concentrations of glucose

Glucose conc. %	Decline in acid-fastness, %, after 6 hours	Observation after 24 hours	
		Germination*	Increase in CPD, %
1.00	55	+	203
0.30	50	+	223
0.10	19	+ (incomplete)	41
0.03	12	-	No change
0.01	2	-	No change

* + = all the spores germinated
- = no germination

d. Effect of removal of CO_2 from the environment during germination.

Discs of solid germination medium placed inside the tops of petri dishes were seeded with washed sporulated cells as described in Materials and Methods. The bottom of the petri dish contained the NaOH solution, and a similar dish with CaCl_2 solution instead of NaOH solution served as the control. In the latter dish, CO_2 was not removed but the relative humidity should have been the same as in that containing NaOH. Sporulated cells from 2- and 5-day old cultures were used for these experiments. Germination was determined by direct microscopic examination at 2, 4, 6 and 8 hours after the medium was seeded, taking as criterion of germination the production of buds by the ascospores. Two experiments were done and the results are averaged in Table 4.

The removal of CO_2 from the environment had no evident effect on germination. About 30% of the asci from 2-day sporulation cultures and 60% of the asci from 5-day cultures contained at least one ascospore with a bud by the sixth hour, whether CO_2 was removed from the medium or not. By the eighth hour, 90% of the asci showed germination in either case. Though the amount of germination in 8 hours was the same for both 2- and 5-day asci, it was higher for older asci at 6 hours.

Comparing Table 1 and 4, it is noted that the germination on solid medium (Table 4) was somewhat faster than that in liquid medium (Table 1). On the former medium 30% of the asci from the 2-day sporulation culture contained spores with buds after 6 hours, whereas, in liquid medium, only 10% of the asci contained budded ascospores by that time.

Table 4

Effect of removal of CO₂ from the environment
during germination in solid medium

Environment	Age of sporulation culture	Germination after			
		2 hours	4 hours	6 hours	8 hours
CO ₂ not absorbed by NaOH	2 days	none	most asci swollen, no buds	29% of asci have budding spores	91% of asci have budding spores
	5 days	none	most asci swollen, no buds	60% of asci have budding spores	93% of asci have budding spores
CO ₂ absorbed by NaOH	2 days	none	most asci swollen, no buds	30% of asci have budding spores	89% of asci have budding spores
	5 days	none	most asci swollen, no buds	58% of asci have budding spores	90% of asci have budding spores

Changes in chemical composition and respiratory activity during germination

a. Changes in carbohydrate content and in dry weight during sporulation and germination.

The amount of carbohydrates in, and the dry weight of the vegetative, sporulated and germinating cells were measured. Fifty ml of cell suspension was removed from a 48-hour-old vegetative culture grown in MYPG medium. The cells were separated from 40 ml of this by centrifugation, washed twice with sterile distilled water, and used for extraction, separation, identification and measurement of carbohydrates, following the procedures described in the Materials and Methods. The cells from the remaining 10 ml were washed, separated by millipore filtration, and used for dry weight determination.

Next, cells from the same growth culture as above were sporulated for 48 hours by which time 72-75% of the cells had produced spores. From the sporulated culture a sample of 250 ml was taken out, of which 200 ml was used for the extraction and measurement of carbohydrates in the same way as with the vegetative cells, and 50 ml for dry weight determination. The reason for using a larger volume of cell suspension in the case of the sporulated cells was that the cell population density in these cultures was much lower (about 1/3) than that of the vegetative culture.

Using the same methods as with the sporulated cells, the changes in carbohydrate content and in dry weight during germination were measured at 2, 4 and 6 hours after the cells were suspended in

germination medium. Also a final measurement of carbohydrates was made after 24 hours to determine whether the carbohydrate content of the cells of the resulting vegetative culture resembled that of the original growth culture used for sporulation. The medium used to obtain the growth culture (MYPG) and that used for germination (YNB + 1% glucose) differed in their glucose content as well as in other constituents.

Carbohydrate determinations similar to the foregoing were performed, for comparison, using cells from culture medium consisting of yeast nitrogen base with no added glucose. This provided the sporulated yeast with all required nutrients except glucose, and the spores did not germinate.

The results obtained from these experiments are summarised in Table 5 and Fig. 1. Carbohydrates, which were a major component of the yeast cells, comprised 46% of the dry weight in the vegetative cells and 60% of the dry weight in the sporulated cells. Trehalose and glycogen were the most abundant carbohydrates in all stages (vegetative, sporulated and germinating) of the yeast life cycle. Trehalose and glycogen comprised 30% and 44%, respectively, of total carbohydrate in vegetative cells. In sporulated cells trehalose comprised 50% and glycogen 35% of the total carbohydrate. Though the amount of glycogen was higher than that of trehalose in the vegetative yeast, in sporulated yeast the situation was reversed (Table 5).

Total carbohydrates per yeast cell increased by $17 \mu\text{g}/10^6$ cells during sporulation. Both trehalose and glycogen contributed to this increase, though to a different extent. A higher increase ($11.6 \mu\text{g}/10^6$

Table 5

Changes in carbohydrate content and dry weight during
sporulation and germination of yeast ascospores

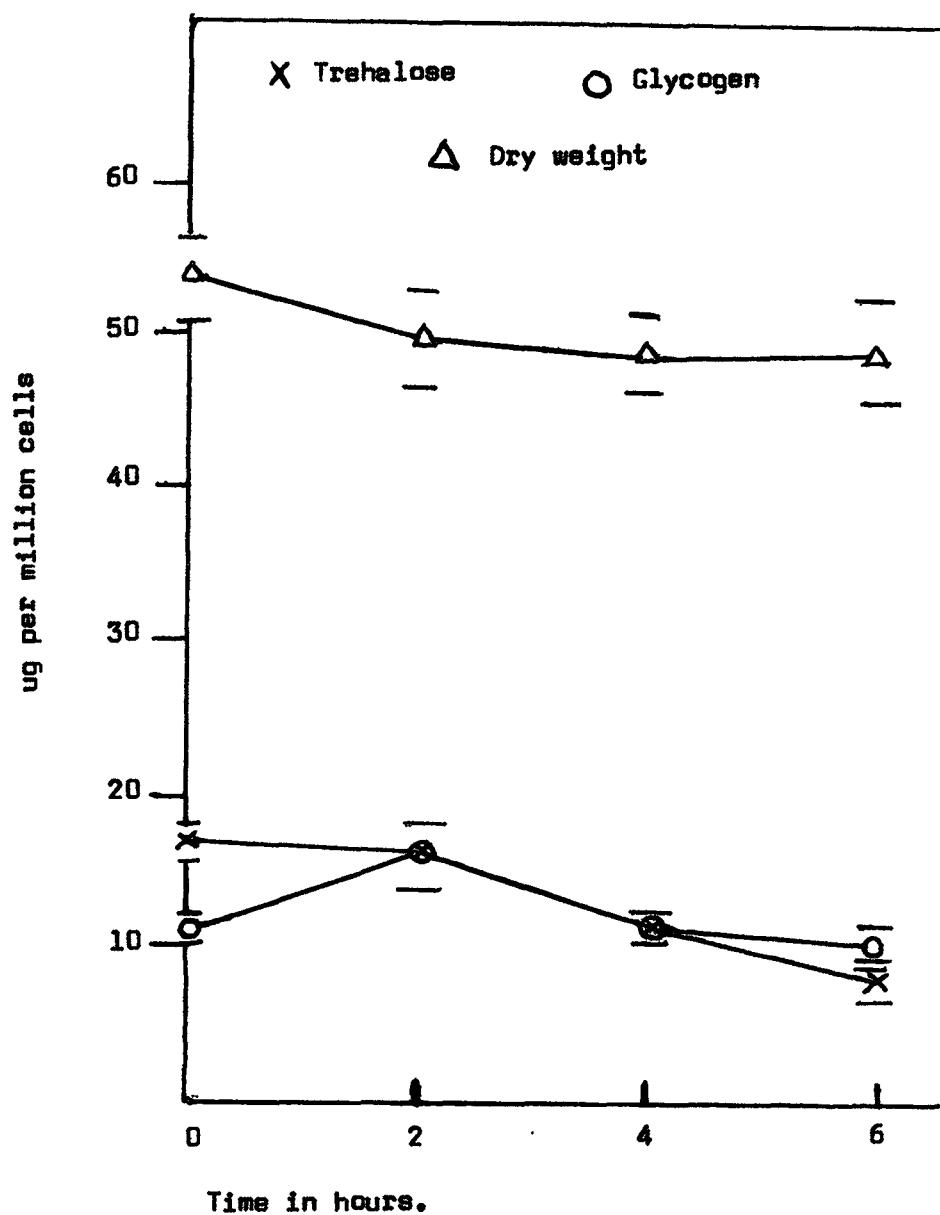
Carbohydrate content and dry weight in $\mu\text{g}/10^6$ cells							Changes during Sporula- tion	Changes during Germina- tion (6 hours)
Items	Vegetative yeast	Sporulated yeast	Germinating yeast (6 hours)	Veg. Yeast resulting from germ- ination (24 hours)	Sporulated yeast in medium (YNB) without glu- cose (6 hrs.)	Sporulated yeast in medium (YNB) without glu- cose (24 hrs.)		
Dry weight**	32.7 ± 0.5	53.7 ± 2.5	50.5 ± 3.1				Increase	No change
Trehalose**	4.6 ± 0.4	16.2 ± 0.9	8.6 ± 0.7	5.7 ± 1.2	18.1 ± 1.6	19.0 ± 0.9	"	Decrease
Glycogen I	3.4 ± 0.2	5.6 ± 0.8	5.7 ± 1.2	3.9 ± 0.6	5.0 ± 0.4	5.3 ± 0.5	"	No change
Glycogen II	3.0 ± 0.3	5.1 ± 0.5	4.0 ± 0.3	3.4 ± 0.8	5.8 ± 0.4	6.9 ± 0.3	"	Slight Decrease
Glycogen III	0.4 ± 0.1	0.7 ± 0.2	$*0.2 \pm 0.2$	0.2 ± 0.1	0.8 ± 0.3	1.3 ± 0.1	No change	"
Total Glycogen**	6.8 ± 0.5	11.4 ± 1.1	9.9 ± 1.5	7.5 ± 0.6	11.6 ± 0.7	13.5 ± 0.7	Increase	No change
Mannan	2.9 ± 0.3	3.3 ± 0.5	1.9 ± 0.3	1.3 ± 0.2	1.9 ± 0.5	1.6 ± 0.2	No change	Decrease
Glucan	0.9 ± 0.1	1.4 ± 0.2	$*0.4 \pm 0.4$	0.9 ± 0.3	1.4 ± 0.1	1.8 ± 0.1	Slight Increase	"
Total Carbo- hydrate**	15.2 ± 0.6	32.3 ± 2.3	20.8 ± 2.6	15.4 ± 1.1	33.1 ± 1.2	35.9 ± 1.2	Increase	"

* In 3 of 4 experiments '0' value was obtained.

** Significance test in Appendix VIII.

Fig. 1

Changes in trehalose, glycogen and dry weight
at 2-hour intervals during germination



cells) was shown by trehalose which changed from $4.6 \mu\text{g}/10^6$ cells in the vegetative cells to $16.2 \mu\text{g}/10^6$ cells in the sporulated cells. In other words, the amount of trehalose increased more than three fold during sporulation. The increase in this reserve carbohydrate accounted for about 70% of the total increase in carbohydrate content during sporulation. Glycogen showed a smaller increase in amount. This carbohydrate increased by $4.6 \mu\text{g}/10^6$ cells which comprised about 27% of total increase in carbohydrate (Table 5).

There was an increase in the dry weight of the yeast cells during sporulation. During 48 hours in the sporulation medium, the dry weight increased from $32.7 \mu\text{g}/10^6$ cells to $53.7 \mu\text{g}/10^6$ cells, i.e. to 165% of that of the vegetative yeast. Most of the increase in dry weight (80%) was due to the increase in carbohydrate. There was no appreciable change in dry weight in 6 hours of germination (Table 5).

A considerable decrease ($7.6 \mu\text{g}/10^6$ cells) in the amount of trehalose was observed during 6 hours of germination. Other carbohydrates, on the otherhand, showed no appreciable change during this time period. After 24 hours in the complete germination medium, the levels of trehalose, glycogen and total carbohydrate reached values comparable to those of the original vegetative cells (Table 5).

When germination was restricted owing to lack of glucose in the medium (Table 5), no significant change in carbohydrate content took place during the 6 hour period. Even after 24 hours no definite change in carbohydrate content was apparent. Stains made after 6 hours showed no loss of acid-fastness of the spores in this medium. After 24 hours, the asci appeared unchanged; they were still acid-fast and showed no evidence

of budding.

The foregoing compares the initial carbohydrate content and dry weight of the sporulated cells with the content after 6 hours in germination medium. These data will now be compared with the results of analyses made after 2 and 4 hours in germination medium (Fig. 1).

The reserve carbohydrate showed some change in abundance during germination. In the first 2 hours, there was no change detected in the amount of trehalose, but after that time it gradually decreased. During 2-6 hours this carbohydrate decreased from 16.3 to 8.6 $\mu\text{g}/10^6$ cells. In other words, by 6 hours half of trehalose was lost. Glycogen, on the other hand, showed some increase in the first 2 hours, after which time a slow decrease was noticed. During the first 2 hours, the amount of glycogen increased from 11.4 to 15.9 $\mu\text{g}/10^6$ cells, but after 4 hours, glycogen showed a decrease to 11.5 $\mu\text{g}/10^6$ cells. After 6 hours, the amount of glycogen was more or less the same as that in the sporulated cells. There was no appreciable change in dry weight during the entire 6 hour period of germination.

b. Carbon dioxide evolution and oxygen uptake during germination

The respiratory activity of the germinating yeast was studied by measuring CO_2 production and oxygen uptake during a 6-hour period at 2-hour intervals. The experiments were done under aerobic conditions. The results of the eight experiments and the mean value are presented in Table 6.

The respiratory activity during germination was mainly fermentative, i.e., having a high rate of CO_2 production with a low rate of oxygen

Table 6

Carbon dioxide production and oxygen uptake during
germination of yeast ascospores

Experiment number	CO ₂ production in μ l/million cells				Oxygen uptake in μ l/million cells				R.Q.
	0-2 hrs.	2-4 hrs.	4-6 hrs.	Total	0-2 hrs.	2-4 hrs.	4-6 hrs.	Total	
1	6.9	8.6	21.5	37.0	0.0	1.8	3.7	5.5	6.7
2	7.3	8.7	21.7	37.7	0.0	2.0	3.9	5.9	6.4
3	4.2	6.4	16.8	27.4	0.0	0.5	2.2	2.7	10.1
4	4.4	6.6	17.3	28.3	0.0	0.5	2.2	2.7	10.5
5	6.5	8.9	21.0	36.4	1.5	2.3	4.0	7.8	4.7
6	6.7	9.1	20.6	36.4	1.5	2.3	3.6	7.4	4.9
7	4.4	8.6	17.0	30.0	0.0	1.4	1.3	2.7	11.1
8	4.5	9.1	16.5	30.1	0.0	1.5	1.5	3.0	10.0
Mean	5.6	8.3	19.0	32.9	0.4	1.5	2.8	4.7	8.1

uptake. The rate of CO_2 production increased gradually during the 6 hours of the experiment. Oxygen uptake was almost nil (in 6 experiments out of 8 the value was 0) during the first 2 hours, after which a small amount ($1.5 \mu\text{l}/10^6$ cells/2 hour) of oxygen uptake was observed. In the final 2 hours the oxygen uptake was double that of the preceding 2 hours but still was quite low in comparison with the CO_2 production during that time. The total amount of CO_2 evolved during the 6 hour period (aerobic plus anaerobic) was equivalent to a loss of $115 \mu\text{g}$ of glucose.

Role of carbohydrate in germination

- a. Effect of removal of glucose from the germination medium at different times.

The two likely roles for glucose in the induction of germination are ^{as} triggering agent or as a germinating agent. If glucose is a triggering agent, its removal from the medium after the spores are briefly exposed should not prevent germination. On the otherhand, if glucose is a germinating agent (i.e. essentially a substrate), its continued presence in the medium would be needed for germination to take place (Sussman and Halvorson, 1966).

Cells were removed from the germination medium after 0, 1, 2, 3, 4 and 6 hours. They were separated by centrifugation, washed and re-suspended in a medium containing only yeast nitrogen base, but no glucose. With the "0" hour sample the cells were not placed in the complete germination medium at all. The original sporulated culture contained 4%

non-acid-fast spores. The spores were examined for germination in each case after 8 and 24 hours. The results from 3 experiments are given in Table 7.

In the cultures where glucose was absent throughout (0 hour) or where glucose was removed after 1, 2 or 3 hours, no sign of germination was observed. When glucose was removed after 4 hours, 36% of the spores showed loss of acid-fastness. Similarly, when glucose was removed after 6 hours, 61% of the spores showed loss of acid-fastness and 14% of the asci contained at least one spore which had produced a bud. It was apparent that germination did not proceed after the removal of glucose from the medium. This suggests that glucose is a germinating agent and not merely a triggering agent.

b. Possible substitution of glucose by trehalose.

As the endogenous trehalose content was found to decrease during germination, experiments were done to determine whether this substance, when supplied exogenously, could substitute for glucose as the carbon source in germination medium.

Cells from normal germination cultures were removed at successive times, centrifuged, washed and resuspended in a medium consisting of 0.67% yeast nitrogen base and 1% trehalose. The sporulated culture before exposure to germination medium contained 12% non-acid-fast spores. The spores were examined for germination after 6 and 24 hours. The results (average of 3 experiments) are presented in Table 8.

It is apparent that trehalose could not support germination when this substance was substituted for glucose at 0, 2 and 4 hours after the

Table 7

Effect of removal of glucose from the medium at different
times during germination of yeast ascospores

Glucose in YNB medium	Observation after 8 hours		Observation after 24 hours
	Decline in acid- fast spores, %	Presence of buds, %	
Present	96	96	complete germination
Absent	0	0	No germination
Removed at 1 hr.	0	0	"
Removed at 2 hrs.	0	0	"
Removed at 3 hrs.	0	0	"
Removed at 4 hrs.	36	0	"
Removed at 6 hrs.	61	14	Incomplete germination (14% buds)

Table 8

Possible substitution of glucose by trehalose at different
times during germination of yeast ascospores

Carbon source in the medium	Decline in acid-fast spores after 6 hrs., %	Germination* after 24 hrs.
Glucose	46	+
Trehalose	17	-
Trehalose at 2 hours	15	-
Trehalose at 4 hours	33	-
Trehalose at 6 hours	48	+

* + = complete germination
- = no germination

sporulated cells were transferred to the germination medium. Some loss of acid-fastness was, however, noticed. Germination was completed when trehalose was substituted for glucose after 6 hours exposure of the spores to the latter carbohydrate. In this case a normal appearing growth culture with increased cell population density developed within 24 hours and no asci were found.

It should be mentioned that 6 hours was the approximate time at which buds first became visible on the ascospores, marking the beginning of growth, in the medium containing glucose. It follows then that exogenous trehalose, though it could not substitute for glucose during germination, could support subsequent growth.

c. Possible substitution of glucose by temperature treatment.

Many Ascomycetes require a heat shock for germination of their ascospores (Sussman and Halvorson, 1966) and in some, for instance Neurospora, ascospores can germinate entirely on their endogenous reserves after such a treatment. On the otherhand, many fungi require a cold treatment for the activation of germination. The possibility of using a temperature treatment to replace the requirement for an exogenous source of carbon for germination of yeast ascospores was explored by exposing them to high and low temperature.

The sporulated cells were washed, suspended in water and incubated at 50°C, 60°C, 70°C, 80°C and 90°C for 2, 5, 10, 15, 30, 60 and 120 minutes for high temperature treatment. For cold treatment, the washed cells were suspended in distilled water and stored at 4°C and -4°C for 1, 2 and 6 months. Examinations for the occurrence of germination were made

24 hours after the sporulated cells were exposed to germination medium. The results (3 experiments) are shown in Tables 9a and 9b.

Neither high nor low temperature treatment induced germination of yeast ascospores in medium lacking a carbon source. The viability of ascospores was lost after the more extreme high temperature treatments (60°C for 60 minutes or 70°C for 15 minutes), whereas, after a cold treatment of 6 months at -4°C the ascospores were still viable.

It is of interest that the lethal effect of high temperature treatments was sharp. For example, all the spores germinated in medium containing glucose after 10 minutes at 70°C , but no germination took place after 15 minutes at 70°C . This indicates the spores to be fairly uniform in temperature sensitivity.

d. Effect of 2-deoxy-D-glucose on germination.

The effect of 2-deoxy-D-glucose, a glucose analogue and a specific inhibitor of glycolysis (Cramer and Woodward, 1952; Sols et al, 1960 and Webb, 1966) on germination was investigated by including this substance in the medium with and without glucose. This experiment had a dual purpose. The presence of 2-deoxy-D-glucose in a medium lacking glucose would test its possible substitution for glucose as a germination inducer. When included with glucose, its ability to inhibit glucose-induced germination would be tested.

Two-deoxy-D-glucose was added to the medium in the same concentration (0.3%) as that of glucose and the yeast was examined for germination after 6 and 24 hours. Growth was also measured after 24 hours by noting the increase in cell population density. The initial percentage of non-

Table 9a

Effect of high temperature treatments on germination of yeast ascospores

Duration of Treatment	Germination* after temperature treatment at									
	50°C		60°C		70°C		80°C		90°C	
	Glucose	No Glucose	Glucose	No Glucose	Glucose	No Glucose	Glucose	No Glucose	Glucose	No Glucose
2 minutes	+	-	+	-	+	-	+	-	+	-
5 minutes	+	-	+	-	+	-	-	-	-	-
10 minutes	+	-	+	-	+	-	-	-	-	-
15 minutes	+	-	+	-	-	-				
30 minutes	+	-	+	-	-	-				
60 minutes	+	-	-	-	-	-				
120 minutes	+	-	-	-	-	-				

* + = Complete germination
 - = No germination

Table 9b

Effect of low temperature treatments
on germination of yeast ascospores

Duration of treatment	Germination medium containing	*Germination after treatment at	
		4°C	-4°C
1 month	glucose	+	+
	no glucose	-	-
2 months	glucose	+	+
	no glucose	-	-
6 months	glucose	+	+
	no glucose	-	-

* + = Complete germination
- = No germination

acid-fast spores in the culture was 10. The results of 2 experiments, each in duplicate, are presented in Table 10.

It is apparent that 2-deoxy-D-glucose did not support germination of ascospores and thus failed to substitute for glucose. Some loss of acid-fastness was, however, observed. When used in addition to glucose, it strongly inhibited both germination and growth. These processes were reduced by 67% and 97% respectively.

e. Glucose uptake during germination.

The evidence presented above indicates that glucose must be present at a substrate level to induce germination. It thus becomes of importance to investigate the kinetics of glucose uptake by the cells during this period and relate it to the extent of germination. This should give some information about the role of exogenous glucose.

Glucose uptake was determined in germination medium by measuring the amount of glucose lost from the medium at the end of each 2 hour period. It was determined colorimetrically following separation of the medium from the yeast by centrifugation, using the phenol-sulphuric acid reagent for colour development. Samples of medium harvested at different times were diluted with equal volumes of distilled water to keep the measurements in the central part of the optical density scale of the colorimeter. Glucose uptake as related to the extent of germination (from Table 1) is shown in Fig. 2.

Glucose uptake started during the first 2 hours and continued throughout the period allowed for germination (6 hours). By this time about 110 μ g of glucose were taken up by 10^6 cells, that is, about 45% of

Table 10

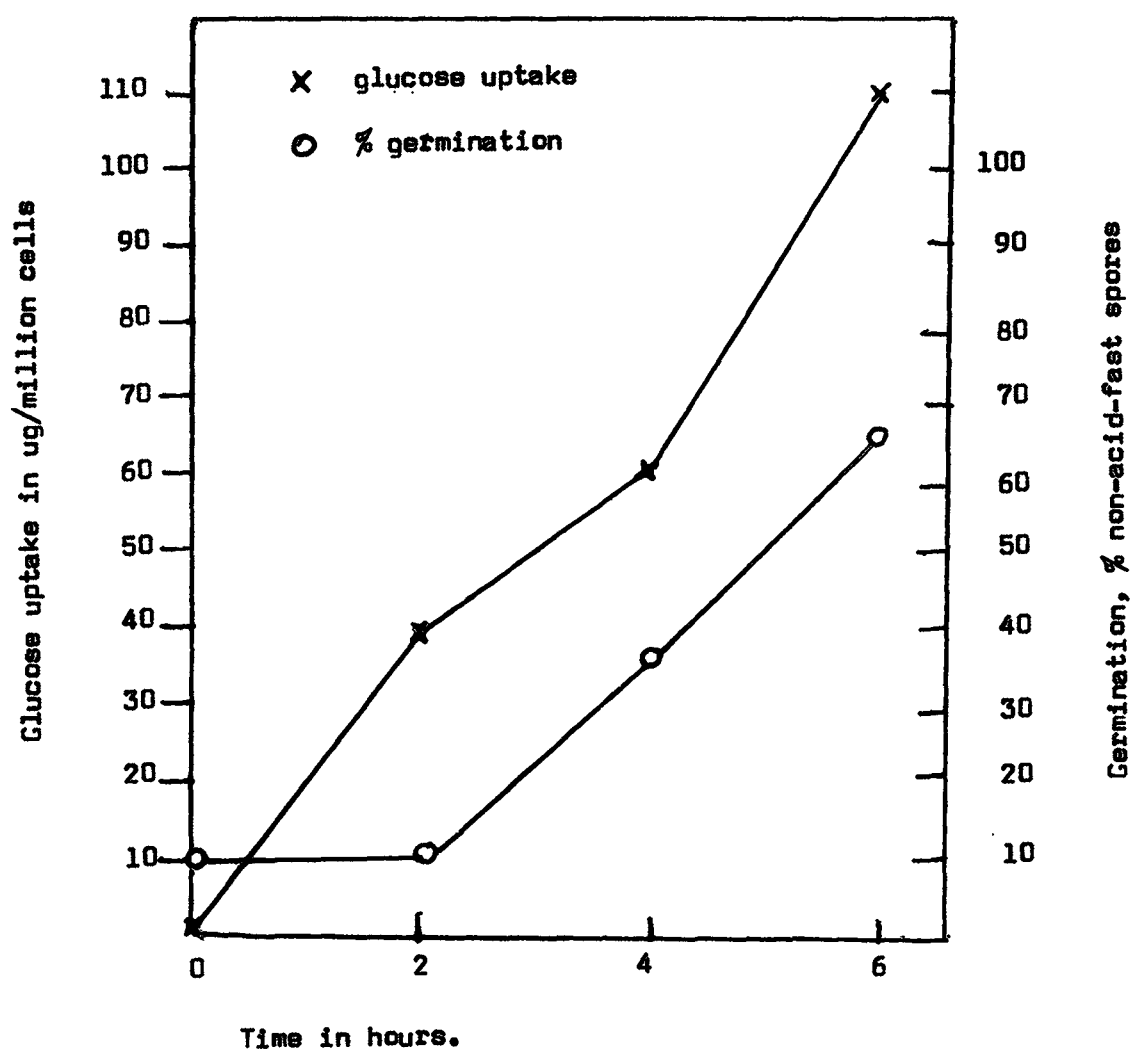
Effect of 2-deoxy-D-glucose on
germination of yeast ascospores

Source of Carbon	Decline in acid- fast spores after 6 hours, %	After 24 hours	
		Germination*	Growth in- crease in CPD %
0.3% Glucose	45	+	273
0.3% 2- deoxy-D- glucose	35	-	Nil
0.3% glu- cose + 0.3% 2- deoxy-D- glucose	15	-	7

*+ = Complete germination
- = No germination

Fig. 2

Glucose uptake during germination
of yeast ascospores



the total glucose ($239 \mu\text{g}/10^6$ cells) present in the medium originally. From Fig. 1 it is apparent that the dry weight of this quantity of yeast was $49 - 53 \mu\text{g}$. Thus, the amount of glucose taken up by the germinating yeast during 6 hours was approximately double its dry weight. The rate of glucose uptake was similar during the periods 0-2 hours and 4-6 hours but was somewhat lower during 2-4 hours. It would seem that during the first 2 hours, though there is glucose uptake, acid-fastness does not change.

f. Uptake of C^{14} -glucose during germination.

The radioactive glucose was made available to the germinating yeast at 0, 2 and 4 hours and the radioactivity in the cells as well as in the CO_2 produced by them were measured at 2, 4 and 6 hours respectively. The difference in the radioactivity in the medium initially and after 2 hours represented the uptake of labelled glucose during that period. The cells were first centrifuged out of the medium and then washed with an equal volume of distilled water. These washings were also tested for radioactivity. The results are summarised in Table 11* in which the radioactivity is shown as counts per minute (CPM)/ 10^6 cells.

The rate of uptake of C^{14} -glucose was more or less uniform during the whole period of germination. A major part of the radioactivity lost from the medium was recovered from the washing of the cells with distilled water after centrifugation from the medium. The radioactivity in this fraction decreased towards the end of the 6 hour duration of the experiments.

* Original data and calculation for total glucose uptake in Appendices IV and V.

Table 11

Uptake of C^{14} -glucose during germination of yeast ascospores (CPM/million cells)

Duration	Loss of C^{14} from the medium	Amount of C^{14} in wash	Amount of C^{14} in the cells	Amount of C^{14} in CO_2	Recovery of C^{14} Percent
0-2 hours (5 expt.)	2400	1458	262	139	77
2-4 hours (2 rxpt.)	2171	1000	274	124	65
4-6 hours (2 expt.)	2472	943	533	182	67

The amount of radioactivity in the cells showed no conspicuous change during first 4 hours, but increased to some extent during the last 2 hours. A small increase was noted also in the amount of C^{14} in CO_2 during the latter time interval. The amount of radioactivity in CO_2 was, however, only about half of that retained in the cells.

g. Distribution of radioactivity from exogenous C^{14} -glucose in different cell components.

The distribution of radioactivity from the C^{14} -glucose, in different cell components, was investigated during 0-2, 2-4 and 4-6 hours of germination. The cells were extracted and the extractions fractionated by following the procedure described in Material and Methods. The amounts of radioactivity in the different extracts and their sub-fractions are shown in Tables 12* and 13.

The hot water soluble fraction had the largest amount of radioactivity. This fraction plus the cold TCA extract together accounted for 45-52% of the total radioactivity recovered from the different components of the germinating yeast. The second largest amount of radioactivity was present in the NaOH extract. The radioactivity in this fraction accounted for 27-36% of the total radioactivity recovered from the cell components. The remainder (about 20%) of the radioactivity was found in the nucleic acid fraction (hot TCA extract) and in the residue. The lipid fraction (chloroform-methanol extract) had very little radioactivity during the first 4 hours, after which a slight increase was noted. The amount of radioactivity in hot water soluble fraction, NaOH extract and residue increased during the final 2 hours, whereas, that in other

* Original data and counting efficiency in Appendices VI and VII.

Table 12

Amount of radioactivity (C^{14} -glucose-u) in different
cell fractions (CPM/million cells)*

	1	2	3	2 + 3	4	5	6	7	8	9
Duration	Whole cell**	Hot Water Soluble	Cold TCA***	H.W.S. + cold TCA***	NaOH extract	Nucleic Acids	Lipid	Residue	Total	% of C^{14} recover- ed
0-2 hours 5 expt.	262	78	14	92	74	14	4	16	200	76
2-4 hours 2 expt.	274	96	10	106	60	15	8	15	204	75
4-6 hours 2 expt.	533	152	19	171	101	19	11	96	398	75

* Extracts from at least 10 million cells were used for counting
** 2.5 million cells were counted. *** TCA = Trichloroacetic acid.

Table 13

Radioactivity in different components of hot water soluble
fraction, NaOH extract and neutral part
(% of total radioactivity recovered)

Exposure of the cells to C ¹⁴ - glucose	Hot water soluble fraction			NaOH extract		Neutral part of hot water soluble fraction	
	Acid	Basic	Neutral	Glycogen	Protein	Glycogen	Trehalose
0-2 hours	17	6	77	30	70	37	63
2-4 hours	32	11	57	23	77	56	44
4-6 hours	46	5	49	32	68	15	85

components showed no considerable change with time (Table 12).

Most of the radioactivity in the hot water soluble fraction was recovered from the neutral portion. This portion represented about 80%, 60% and 50% of total radioactivity recovered from the hot water soluble fractions during 0-2, 2-4 and 4-6 hours respectively. The acid portion showed a gradual increase in its C^{14} content from the 0-2 hour to the 4-6 hour period. On the otherhand, the C^{14} content in the basic portion was low during the entire 6 hours (Table 13).

When the neutral portion was analyzed finally by TLC, trehalose and glycogen were separated. These substances shared the radioactivity of the neutral portion in approximately equal quantity up to 4 hours, but during 4-6 hours trehalose had about 85% and glycogen had about 15% of the radioactivity recovered in these two substances (Table 13).

A major portion of the radioactivity in the NaOH extract was found in the protein portion. This part represented approximately 70% of the radioactivity in the NaOH extract while the carbohydrate portion of this extract contained only 30%. The proportion of radioactivity in these two substances remained more or less constant during the 6 hours (Table 13). An analysis of tables 12 and 13 are shown in Table 14.

h. Distribution of radioactivity from C^{14} -acetate in CO_2 and cell components.

Labelled acetate was added to the germination medium to follow the distribution of acetate carbon in different cell components and in CO_2 when the sporulated yeast in YNB medium was metabolising glucose. For comparison, a parallel experiment was performed with no glucose in

Table 14

Distribution of Radioactivity in different cell components, %

Cell component	During 0-2 hours	During 2-4 hours	During 4-6 hours
Trehalose	23	14	19
Glycogen	25	23	12
Protein	25	24	19
Organic acids	7	16	20
Amino acids	3	5	2
Nucleic acids	7	7	5
Lipid	2	4	3
Residue	8	7	20

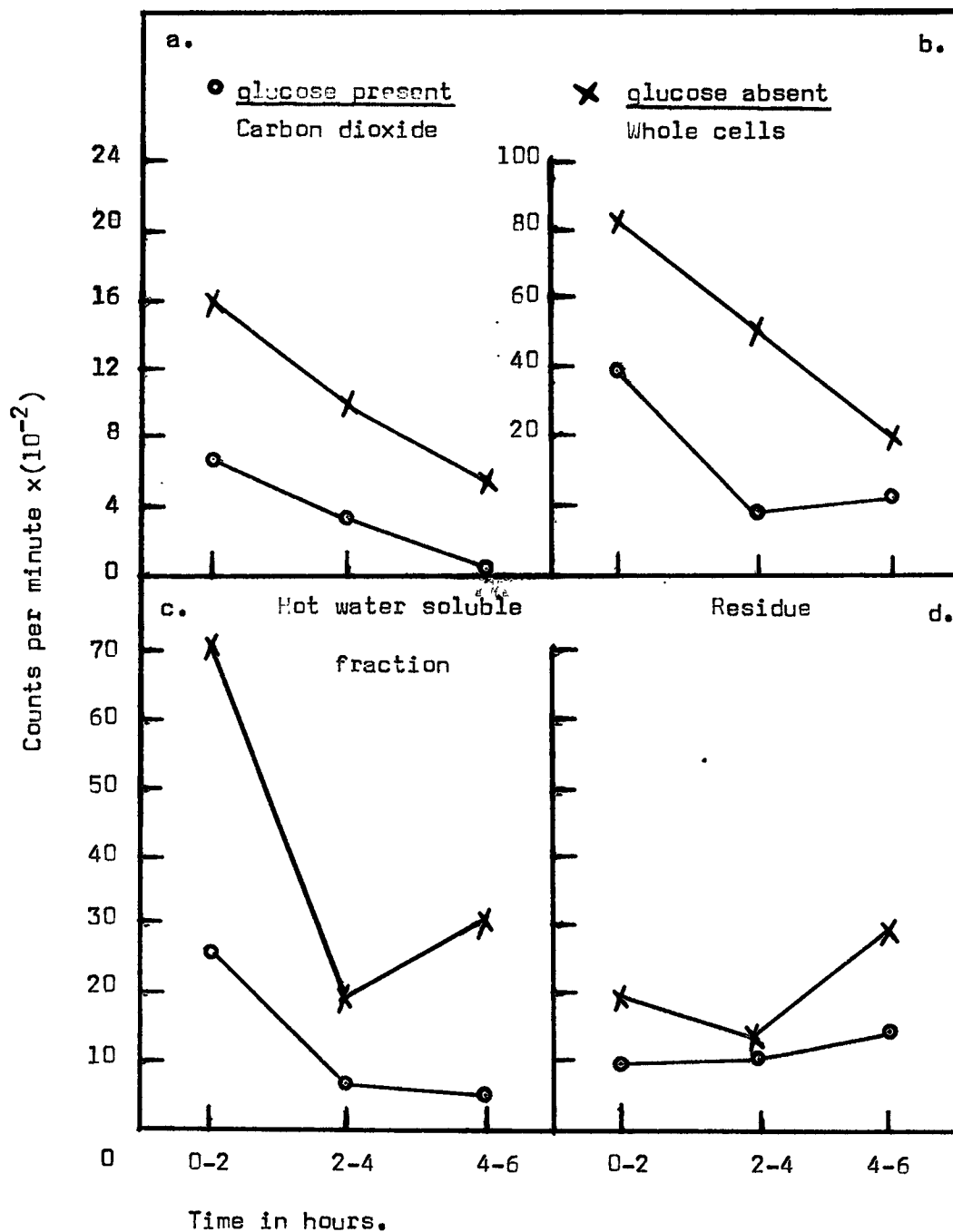
the medium. Acetate (C^{14}) was added to the medium at 0, 2 and 4 hours and the radioactivity in the cells and in CO_2 was measured at 2, 4 and 6 hours respectively. The cells exposed to C^{14} -acetate for each two hour period were extracted with hot water, and the radioactivity in the hot water soluble fraction and in the residue was determined. The hot water extract was further fractionated by passage through resin columns into acid, basic and neutral portions, and the radioactivity in each portion was measured. The acid portion was chromatogrammed for possible separation of different components. The mean values of the results from two experiments are shown in Figs. 3a, 3b, 3c and 3d.

It can be seen that both CO_2 and the cells become labelled with C^{14} -acetate. The amounts of radioactivity in CO_2 and in the cells, when glucose was present in the medium, were about half of those found in the absence of glucose. There was a decrease in the amount of radioactivity with time in both CO_2 and in the cells. This decreasing trend was observed with both media. The proportion of radioactivity in CO_2 to that in the cells was about 1:8 in both the media. It can be pointed out here that in the C^{14} -D-glucose experiments this proportion was approximately 1:2.

A major part of radioactivity in the cell was recovered from the hot water soluble fraction. The level of radioactivity in this fraction decreased with time and there was a simultaneous increase in the radioactivity of the residues indicating incorporation of C^{14} -acetate in the macromolecules. About 80% of radioactivity in the hot water soluble fraction was found in the acid portion, about 18% in the neutral and only about 2% in the basic portion. The acid portion, when chromatogrammed

Fig. 3

Incorporation of C^{14} -acetate in CO_2 and in
different cell fractions during germination



on TLC, showed a single spot with a very low Rf value (almost at the base). This acid was not identified. The Rf values of citric acid, succinic acid and α -ketoglutaric acid chromatogrammed in the same plate did not correspond to the Rf value of the unknown acid.

1. Effect of metabolic inhibitors on germination and sporulation.

Inhibitors known to affect carbohydrate metabolism at the levels of glycolysis, TCA cycle and terminal oxidation were added to germination medium. First the effects of sodium fluoroacetate and potassium cyanide on germination, growth (in YNB medium) and sporulation were compared. The glucose concentration in the germination medium in these experiments was 1%. The percentage of germination was determined at 4, 6 and 24 hours after the sporulated yeast was suspended in the medium. Growth was measured as the percentage increase in the cell population density after 24 hours, while sporulation was measured by counting the percentage of cells that had become asci after 48 hours. In presenting the results, germination, growth and sporulation without inhibitor (control) are set at 100. The results with inhibitors are expressed relative to this value. Two experiments were performed, each in duplicate, and the mean values are presented in Table 15.

It is evident that germination and growth were not affected by a millimolar concentration of fluoroacetate, but sporulation was inhibited considerably (45%). With a higher concentration (10 mM) of this substance no inhibition of germination was observed, though growth was inhibited to a certain extent (16%) and sporulation was strongly inhibited. Cyanide

Table 15

Inhibition of germination, growth and sporulation
by sodium fluoroacetate and potassium cyanide

Inhibitors added	Germination %			Growth % after 24 hour	Sporulation % after 48 hours
	after 4 hours	after 6 hours	after 24 hours		
Nil (Control)	100*	100*	100*	100*	100*
10^{-3} M sodium fluoroacetate	100	100	100	100	55
10^{-2} M sodium fluoroacetate	100	100	100	84	30
10^{-3} M potassium cyanide	98	90	100	20	50
10^{-2} M potassium cyanide	66	53	65		

* Germination, growth and sporulation in control were set at 100 and those with inhibitors were expressed relative to this value.

also had no appreciable effect on germination at mM concentration but with a higher concentration (10 mM) germination was reduced by 65%. Both growth and sporulation were cyanide sensitive even at millimolar concentration. .

The effect of malonate and iodoacetate on germination and growth was studied in another set of experiments. YNB medium with 0.3% glucose was used for germination and growth. The pH of the medium was adjusted to 4 by adding 0.1 N HCl, drop by drop, to facilitate entrance of malonate and iodoacetate into the cells. Germination at 6 and 24 hours, and growth after 24 hours were measured in the same manner as with fluoroacetate and cyanide. The results from 2 experiments, each done in duplicate, are shown in Table 16.

Malonate in 10 mM concentration had no inhibitory effect on germination, though it inhibited growth by about 35%. At the 100 mM concentration, however, malonate strongly inhibited both germination and growth. Iodoacetate in concentrations as low as 0.1 and 0.2 mM severely inhibited germination and growth. With the 0.05 mM concentration, though a considerable (63%) inhibition was evident at 6 hours, after 24 hours germination was complete. However, growth was reduced by 67% even at this low concentration of iodoacetate.

Table 16

Inhibition of germination and growth by malonate and iodoacetate

Inhibitors added	pH of the medium	Germination %		Growth % after 24 hours
		after 6 hours	after 24 hours	
Nil (Control)	5.5	100*	100*	100*
10^{-2} M malonate	5.5	100	100	65
10^{-2} M malonate	4.0	100	100	67
10^{-1} M malonate	4.0	20	12	0
0.05×10^{-3} M iodoacetate	4.0	37	100	33
0.1×10^{-3} M iodoacetate	4.0	25	15	4
0.2×10^{-3} M iodoacetate	4.0	28	17	0

* Germination and growth in control were set at 100 and those with inhibitors were expressed relative to this value.

DISCUSSION

Dormancy and germination

Spore dormancy may be either "environmental" or "constitutive" (Sussman and Halvorson, 1966). Those spores which need for germination a return to the conditions that permit growth have environmental dormancy. Constitutive dormancy, on the other hand, can be overcome only by a special treatment such as exposure to high or low temperature, light, etc., that is not required for vegetative growth. The yeast ascospores, evidently, have an environmental dormancy, since these spores germinate with no special treatment when transferred to a medium that supports growth. High and low temperature treatments had no observed effect on germination of these spores.

Spore germination is characterized by several physiological stages. In Neurospora, during the first two hours after activation, the respiration of ascospores increased with a concomitant rise in respiratory quotient (Sussman et al., 1956). After this time the fermentation products no longer accumulated in large amounts and the respiratory quotient decreased. A full complement of tricarboxylic acid cycle enzymes appeared, suggesting a change to oxidative metabolism from fermentation.

During germination of yeast ascospores three different steps may be recognized. After suspension of the asci in germination medium, there was a high carbon dioxide output with no or very little oxygen uptake. This fermentative activity could be observed within 2 hours after the

exposure of the asci to germination medium. The next step was the loss of acid-fastness of the spores, which could be detected between 2-4 hours and later. Finally, between 4-6 hours, buds from ascospores were visible. This stage represented the beginning of outgrowth which completes germination.

Germination of yeast ascospores differs from the germination of Neurospora ascospores in that there is no change over from fermentation to oxidative metabolism in the former case.

Metabolic patterns

a. Respiratory activity during germination.

A rapid rise in respiratory activity is one of the most common physiological changes observed during spore germination. The glucose oxidizing activity in the endospores of aerobic bacteria was found to increase during germination. An initial activation of dormant enzyme systems took place which led to the raised respiratory activity (Halvorson and Church, 1957). Vegetative cells of Bacillus cereus strain T had a QO_2 value of 60-100 whereas the QO_2 value of the dormant spores of this bacteria was less than 0.05 and there were a number of stages observable in the respiratory changes which corresponded to the successive phases of germination, viz., swelling, emergence, elongation and cell division (Levinson and Hyatt, 1956). The respiratory activity of the microcysts of fruiting myxobacterium Myxococcus xanthus was studied by Dworkin and Neiderpruem (1964). It was found that the QO_2 value of the microcysts was zero whereas that of the vegetative cells was 12.

Studies on the metabolic changes during germination of anaerobic bacterial spores are few. In one case (Costilow, 1962) an increased rate of amino acid fermentation was observed in germinating spores of Clostridium botulinum. Simmons and Costilow (1962) compared the activities of several enzymes (phosphohexoisomerase, phosphofructokinase, aldolase, etc.) of glucose and pyruvate catabolism in vegetative cells, spores and germinated spores of these bacteria. They found that, in general, spores had lower enzyme levels than did the vegetative cells, and germination led to an increased enzyme activity.

The ascospores of Neurospora, like endospores of aerobic bacteria, show an increased respiratory activity during germination. Thus, Goddard (1935) and Goddard and Smith (1938) observed an increase in the respiratory activity during ascospore germination of Neurospora tetrasperma almost immediately after activation with heat shock. The increase attained a level 20-30 times that of the dormant spores. When dormant spores were activated by chemicals such as furfurals, a similar rise was noted (Sussman, 1953). Increase in respiratory activity during ascospore germination had also been observed in Neurospora crassa (Emerson, 1954).

The same phenomenon (increased respiration during spore germination) is exhibited by several other fungi. An increase in the respiratory activity during germination was described for the conidia of Myrothecium verrucaria (Mandels and Norton, 1948; Mandels and Darby, 1953) and Aspergillus niger (Yanagita, 1957). The work of Halbsguth and Rudolph (1959) and Rudolph (1960) with the spores of Phycomyces blakesleeaunus has revealed an increase in O₂ uptake and CO₂ production during germination.

Some apparent exceptions to this general observation of increased respiration during germination are, however, available in the literature. Shu et al. (1954) observed that both respiratory activity and R.Q. of ungerminated and germinating uredospores of Puccinia graminis were equivalent. In their study the respiratory activity of germinating spores was measured 3 days after the beginning of incubation; this might have resulted in missing a transitory increase in respiration. Later studies (Allen, 1963), in fact, suggested that there was a rapid increase in respiration followed by a decrease when these spores were activated by pelargonaldehyde.

The macroconidia of Fusarium solani provide another interesting instance of increased respiratory activity during germination. Whether the respiratory rate of these spores would increase or not during germination depended on the source of carbon provided. Spores supplied with ethanol or mannose showed no increase in their rate of respiration but did show a slight increase when fructose or trehalose were supplied and a more than 2-fold increase with glucose (Cochrane et al., 1963; Cochrane et al., 1963a, 1963b).

The respiratory activities of vegetative and sporulated yeast cells were compared by Miller et al. (1959). They found that a decline in R.Q. took place during sporulation but, when the spores were transferred to the growth medium, the high R.Q. value characteristic of growth cells was restored.

Ogur and St. John (1958) reported the appearance of fermentation before respiration when ascal preparations were incubated in germination medium.

Seigel (1970) found that when spores were placed in germination medium, active respiration, aerobic and especially anaerobic occurred in 4 hours, resulting in an R.Q. value of approximately 4. Germination took place in completely anaerobic conditions. From the high R.Q. value and from the large amount of CO₂ production under anaerobic conditions, this author concluded that fermentation was more active than respiration in the early stages of F 493 (a strain of S. cerevisiae) ascospore germination.

The present work, which shows a high respiratory activity with an increasing rate of CO₂ production and a high R.Q. value, confirms the earlier observations (increased respiration of spores during germination) in general and those of Ogur and St. John, and Seigel (relatively more active fermentation) in particular.

Though respiratory activity increases during germination, yeast ascospores differ from other fungus spores in that the O₂ uptake is comparatively low and fermentation ~~serves as~~ the major route of CO₂ production.

b. Removal of CO₂ from the environment.

Carbon dioxide has been reported to have both stimulating and inhibitory effects on germination, depending on the kind of spores. Thus, the initiation of germination in Clostridium botulinum was delayed by the absence of CO₂ (Wynne and Foster, 1948). Among the fungi, the conidia of A. niger were found to have a CO₂ requirement for germination (Yanagita, 1957). When this gas was eliminated from the environment, even swelling, the first visible step towards germination, failed to

occur. The conidia incubated in a complete germination medium incorporated $C^{14}O_2$ very actively without a lag period. A large amount of radioactivity was found in the protein fraction as well as in the acid soluble fraction. Nucleic acid was also labelled quickly (Yanagita, 1963). In the absence of CO_2 basidiospores of Schizophyllum commune did not germinate (Niederpruem, 1964). A different kind of result was obtained with the conidia of F. solani (Cochrane, 1963c). Though the final germination of these conidia was unaffected by removal of CO_2 , the early stages were stimulated.

On the other hand, a deleterious effect of CO_2 was observed with spores of Coccomyces hiemalis (Magie, 1935), germination of which was inhibited by the small amount of CO_2 evolved by cherry fruits. A CO_2 concentration as low as 1% could inhibit germination of certain rusts (Stock, 1931) whereas higher concentrations were needed to inhibit that of the conidia of Botrytis cinerea (Brown, 1922) and Erysiphe graminis (Brodie and Neufeld, 1942).

The effect of removal of CO_2 from the environment of sporulating yeast was studied by Bettelheim and Gay (1963). They observed a strong reduction of sporulation on removal of CO_2 when acetate was the only carbon source in the sporulation medium, but less reduction when glyoxylate as well as acetate were supplied. These findings were later confirmed by Banerjee (1965).

In the present work, removal of CO_2 from the environment of the germinating yeast had no observable effect. Comparing with the results of sporulating yeast it appears that while sporulating yeasts require part of their carbon supply in the form of CO_2 , germinating yeast spores

had no such requirement. All the carbon requirements for the latter are met by the endogenous reserve and the exogenous carbohydrate added to the medium. This indicates an important metabolic difference between the formation and germination of yeast ascospores. It is in conformity with the conclusion that the fermentative, not the oxidative, pathway of carbon dissimilation is of primary importance in spore germination.

It should be made clear at this point that complete removal of CO_2 from the environment is very difficult to attain as this gas is a metabolic product of respiring cells. The concentration of CO_2 about the cells should, however, be reduced to a very low level by the experimental method used, i.e., exposure to the NaOH solution. The possibility, remains, therefore, that some CO_2 is consumed during germination but probably is less than in sporulation.

c. Inhibitors and spore germination.

Inhibitors are often used to investigate the importance of a metabolic pathway in different phases of the life cycle of an organism. An appropriate inhibitor will block the desired pathway at a specific site and the effect on the physiological, metabolic and morphological changes can be observed. Interpretation of results obtained from such experiments are sometimes difficult, because, quite often an inhibitor may influence more than one enzyme. In such cases the specificity for a particular enzyme may sometimes be obtained by using the minimum effective concentration of the inhibitor. Again, the concentration of the inhibitor outside and inside the cell may differ. Considering these points, the results obtained can be of value only when distinct inhibitions

are obtained and the data are analyzed in conjunction with data from other experiments, provided care is taken in the choice of appropriate concentration and, if possible, facilitating its entrance into the cells.

Inhibitors of carbohydrate metabolism have been used quite frequently in the study of yeast metabolism and morphogenesis. Barron and Ghiretti (1953) observed that acetate oxidation in bakers' yeast was arrested by malonic acid. Bartley and Tustanoff (1966) found that iodoacetate completely inhibited fermentation but allowed the development of respiration in anaerobically grown yeast. Respiration was found to be cyanide sensitive.

Miller and Halpern (1956) studied the effect of sodium fluoroacetate, potassium cyanide, malonic acid and iodoacetate on sporulation and growth of S. cerevisiae. Both sporulation and growth in acetate media were depressed by $10^{-3}M$ fluoroacetate. Both processes were more sensitive to the inhibitor when a low concentration of acetate was used. Growth in glucose was not much affected by fluoroacetate. Potassium cyanide at $10^{-4}M$ concentration strongly inhibited sporulation and growth in acetate (0.3%) medium. A low concentration ($10^{-5}M$) of cyanide partially inhibited growth but had little effect on sporulation. Malonic acid at a concentration of $5 \times 10^{-3}M$ inhibited sporulation and growth in media containing 0.3% acetate. Low concentrations of iodoacetate did not affect sporulation very much.

Two-deoxy-D-glucose (2DG), a glucose analogue, is a strong inhibitor of anaerobic fermentation, aerobic fermentation and growth in yeast (Cramer and Woodward, 1952; Woodward et al., 1953). This substance

competitively inhibited the utilization of fructose as well as that of glucose by intact yeast (Sols, 1956). Neurospora crassa and Aspergillus niger, could live on 2DG as sole source of carbon, but their growth on fructose or mannose was inhibited by 2DG. The effect of this substance on yeast life cycle phases, other than growth, has not previously been studied.

In the present work, 2DG and iodoacetate served as inhibitors of glycolysis, fluoroacetate and malonate as inhibitors of TCA cycle and cyanide as an inhibitor of cytochrome system. Concentrations considered to be appropriate were chosen. The entrance of malonate and iodoacetate into the cells was facilitated by lowering the pH of the medium to 4. The inhibitors of glycolysis reduced germination and subsequent growth. The inhibitors of the TCA cycle had no observed effect on germination though they had some effect on growth and sporulation. Potassium cyanide had no inhibitory effect on germination in mM concentration, but had a severe effect on growth and some effect on sporulation.

Judging the above observations in the light of the data of other experiments which observed that the respiratory activity during germination was mainly fermentative and that potassium acetate could not support germination, it appears that the glycolytic portion of the pathway of carbon catabolism is essential for germination of yeast ascospores. It would seem that the energy demand during germination of yeast spores can be met by glycolysis, despite the fact that this is a less efficient method for energy production than oxidative respiration. It can be pointed out here that the germinating yeast spores, though appearing mainly dependent on glycolysis, retain at least some capability to

metabolize acetate to CO_2 as indicated by the C^{14} -acetate experiments.

Carbohydrate: content and metabolism

a. Changes in the carbohydrate content and dry weight during sporulation and germination.

From the difference in structure and function of spores and vegetative cells it is obvious that important metabolic changes must take place during the transformation from resting spores to vegetative cells. There is considerable evidence that the composition of yeast spores differs chemically from that of vegetative cells.

The carbohydrate content of yeast cells has been reported by a number of authors to increase during sporulation. Pazonyi and Markus (1955) measured the carbohydrate content of vegetative and sporulated cells of a Hungarian wine yeast; they found that the sporulated cells were somewhat richer in trehalose, while the glycogen content was about equal in both. The changes in the ethanol-soluble carbohydrate (trehalose) content of a strain of S. cerevisiae were examined in a previous study by the writer (Banerjee, 1965) using several sporulation media. In every instance an increase in the trehalose content was noted when the cells were transferred from growth to sporulation medium. It was also evident that the trehalose content of the yeast was affected by the nutritional conditions in the sporulation medium. No definite correlation between the increase in trehalose content and the percentage of sporulation, however, could be established. Another observation was an increase in dry weight when the cells were transferred from growth to

sporulation medium. The increase in dry weight appeared related to the trehalose content. Cross (1967) found that the dry weight of cells of S. cerevisiae increased during the first 8 hours of sporogenesis and reached 146% of the original value. Vezinhet (1969) studied sporulation of a diploid, thermophilic strain of S. cerevisiae. She observed an accumulation of carbohydrate and an increase in dry weight during sporulation. A four-fold increase in trehalose and slightly more than a doubling of the glycogen content was observed, whereas, glucan and mannan remained unchanged. Carbohydrate accumulation during sporulation of S. cerevisiae was investigated by Roth (1970). He found that sporulation was characterised by an increase in dry weight, at least 67% of which was due to the synthesis of cellular carbohydrates consisting of trehalose and TCA-insoluble components.

The present work with a strain of S. chevalieri adds another example to the general experience that during yeast sporulation the dry weight increases and that a major part of the increase is contributed by an increase in carbohydrate content. However, the findings are not in complete agreement with those of previous authors. Pazonyi and Markus (1955) noticed no appreciable change in glycogen content, from which they suggested that glycogen was probably playing a less important role in sporulation than trehalose, but in the present work glycogen showed a considerable increase in amount during sporulation, though increase in trehalose was more prominent. Roth's data on dry weight of yeast (Z113) agree quite well with those of the present work, but the trehalose content of his strain was lower. Also the degree of increase in trehalose (over that of vegetative cells) in his strain was much greater (18-fold)

than that observed by this writer (3-fold). Another dissimilarity is observed in the content of TCA-soluble fraction. This fraction of Z113 included only trehalose, according to Roth, whereas, in the present work, it contained two distinct carbohydrates identified as trehalose and glycogen, separable by thin layer chromatography.

Two explanations can be offered for the discrepancies in the literature in the amount of increase in trehalose during yeast sporulation. Firstly, as indicated above, the writer's preceding study using yeast strain F.493 (Banerjee, 1965) showed that trehalose content of sporulated yeast was influenced by nutritional conditions in the sporulation medium. Secondly, in F.493, the trehalose content of the vegetative cells varied from 0.8-3.6% of the dry weight, whereas, in the present study it averaged 14% of the dry weight. The corresponding figure in Roth's work was 1.5% and in Vezinhet's work was 14.4%. Strain difference may, therefore, be very important.

What metabolic role does the increased carbohydrate reserve of yeast spores play during germination? In Neurospora tetrasperma (Sussman, 1961) endogenous trehalose serves as a source of carbon and energy during germination. In the present work, the amount of trehalose and glycogen decreased during germination and simultaneously synthesis of these substances continued from exogenous glucose. This would mean that both trehalose and glycogen were utilized at a high rate during germination. It seems likely, therefore, that the reserve carbohydrates of yeast spores are metabolized during germination, serving as a source of carbon and energy as in Neurospora. The endogenous reserve alone, however, is not competent to initiate and support germination in yeast

and thus an exogenous source of carbon, at least some of which is temporarily converted to reserve substances, is needed. This view finds support from results with other fungi. The spores of Blastocladiella emersonii consumed glucose in the early stage of germination and converted it to glycogen-like polysaccharide which was used in the later stages of development (Cantino and Goldstein, 1961).

An increase in the dry weight of fungus spores during germination, at least during the later part of the process, is a general observation made by different workers. Mandels and Norton (1948) and Mandels and Darby (1953) noted an increase in dry weight of more than 2-fold during incubation of Myrothecium verrucaria spores in a nutrient medium. Similar observations have been reported for Aspergillus oryzae (Horikoshi et al., 1965) and Fusarium solani (Cochrane et al., 1963b). No appreciable increase in dry weight was observed in conidia of Aspergillus niger during the first two hours of incubation (Takebe and Shimizu, 1959). There was an initial small decrease in dry weight in 3 of the 4 species studied by Ohmeri and Gottlieb (1965) during the first 2 hours of incubation, after which a steady increase was noted.

Yeast ascospores in the present work showed no evidence of change in dry weight during 6 hours in germination medium. Though some synthetic activities occurred during this time, we may recall that a

large volume of CO_2 was being produced representing degradative activities. It appears, therefore, that during ascospore germination in yeast, synthetic and degradative activities were taking place.

b. Utilization of endogenous substrate during germination.

The endogenous carbohydrate reserve (trehalose and glycogen) of the sporulated cells of Saccharomyces chevalieri decreased in amount during germination of the ascospores. There are two possible explanations for this decrease, namely, the reserve substances were utilized during germination, or they were leaked into the medium at that time. The second possibility can be ruled out because no trehalose or glycogen was detected from a chromatogram of the germination medium after 4 hours. In favour of the first possibility is the observation that there was an increase in the production of CO_2 during 2-4 and 4-6 hours. From the tracer experiments, the amount of CO_2 produced from exogenous glucose was found to remain more or less the same during the whole period of germination. Therefore, it is clear that an endogenous substrate must be consumed to account for the increased CO_2 production. Trehalose and glycogen seem to be the most likely substances for this purpose because carbohydrate is already known to serve as endogenous substrate during germination for several fungi. The ascospores of Neurospora tetrasperma can germinate on endogenous trehalose after a heat shock (Lingappa and Sussman, 1959). Besides, trehalose and glycogen were found to be the substrates for endogenous respiration of vegetative yeast (Eaton, 1960).

Eaton suggested that there are 3 sources of endogenous substrate in S. cerevisiae, trehalose and two metabolically distinct pools of glycogen. Lipid was not found to serve as an endogenous substrate in Eaton's yeast. Utilization of one pool of glycogen was aerobic, of the other aerobic or anaerobic, whereas, utilization of trehalose was only anaerobic. It can be noted here that, in the present work, some oxygen uptake was observed during the periods 2-4 and 4-6 hours but not during 0-2 hours. Another point suggesting trehalose and glycogen to be the endogenous substrates contributing to the increased CO₂ production is that the time period (2-4 hours) when these substances started to decrease coincided with that for beginning of increased CO₂ production.

The utilization of the endogenous carbohydrate reserve, from the present data, seems to be related to the occurrence of germination which depends on assimilation of exogenous carbohydrate. When the ascospores were suspended in a medium containing all the nutrients required for germination except a source of carbon, thus preventing germination, the amount of trehalose and glycogen showed no change. The former observation finds a parallel in the spores of Myrothecium verrucaria in which a rapid change in trehalose content took place prior to emergence of germ tubes in a glucose-yeast extract medium. Decrease in trehalose was not due to leakage into the medium. These spores could utilize exogenous trehalose with no lag, but required a stimulus such as heat, incubation with azide or germination on exogenous substrate to effect utilization of trehalose reserve (Mandels, 1963).

It may be concluded that the statement made by Cochrane (1966) that some putative endogenous reserve materials, e.g., trehalose

(Mandels et al., 1965) and lipids (Cochrane et al., 1963) are broken down by spores during germination or during early phases of germination, finds another example in the present work.

c. Distribution of exogenous glucose carbon in different cell components.

A knowledge of the distribution of exogenous carbon in cellular components and in CO_2 might help in understanding the role of the external source of carbon in germination. An examination of the present data with C^{14} -glucose experiments reveals that exogenous carbon plays a very important role in yeast ascospore germination, as it is involved in both biosynthesis and energy production. More of the exogenous carbon metabolized was used in biosynthesis than for energy production.

This observation is similar to that obtained by Budd et al. (1965) with N. tetrasperma. Dormant spores of this fungus converted 75% of the exogenous glucose absorbed to CO_2 in one hour, but the activated spores evolved only 35% of the absorbed glucose carbon as CO_2 during that time. These observations led the authors to suggest that though both dormant and activated spores converted glucose to CO_2 and to cell material, activation probably caused a change in the proportion of glucose carbon channeled into the 2 products.

Data on the distribution of exogenous carbon in cell components of dormant and germinating ascospores directly comparable to those of Neurospora are not available with yeast, but related observations of Esposito et al. (1969) with sporulating yeast are of interest. Using C^{14} -acetate these authors observed that 62% of the acetate consumed was given off as CO_2

by the time sporulation was complete, whereas, only 22% was present in the soluble pool and 16% in the macromolecules or cellular debris. From a direct comparison with present data it seems likely that in yeast the proportion of exogenous carbon incorporated in CO_2 and in cell material is different during sporulation and during germination, suggesting important changes in carbohydrate metabolism.

In the present work, the incorporation of radioactivity in carbohydrate, protein, nucleic acids, and residue indicates that biosynthesis of macromolecules took place, using exogenous carbon, during germination. A major part of the radioactivity was incorporated into carbohydrate, yet the amount of reserve carbohydrate per cell decreased during this time. It seems apparent, therefore, that a turnover of carbohydrate is associated with germination of yeast ascospores. Whether or not this is essential for germination can not be determined from the results of this study.

d. Carbon balance during germination.

From the different experiments involved in the present work the following rough calculation concerning the input and output of carbon may be provided. This would suggest possible sources of endogenous substrate.

(1) From the manometric experiments the glucose equivalent of CO_2 produced in 6 hours is $115 \mu\text{g}/10^6$ cells.

(2) From the glucose uptake experiments, the total glucose taken up by the cells in 6 hours is $110 \mu\text{g}/10^6$ cells.

(3) From the C^{14} glucose experiment the proportion of C^{14} retained in the cell to that given off as CO_2 is about 2:1. Suppose that all glucose taken up is either incorporated into cell components or given off as CO_2 (i.e. ignoring any loss of C^{14} in the wash). Then one third of the total glucose taken up goes to CO_2 formation. Therefore, $110/3 = 36.6 = 37 \mu g/10^6$ cell of exogenous glucose is used to produce CO_2 .

(4) Endogenous carbohydrate reserve in the sporulated cells:

Trehalose	$16.2 \mu g/10^6$ cells
Glycogen	<u>$11.4 \mu g/10^6$ cells</u>
Total	$27.6 = 28 \mu g/10^6$ cells.

Therefore, total exogenous and endogenous carbohydrate that may be available for CO_2 formation amounts to $37 + 28 = 65 \mu g/10^6$ cells.

But glucose equivalent of CO_2 lost = $115 \mu g/10^6$ cells, therefore, $(115-65) = 50 \mu g/10^6$ cells of glucose equivalent must come from other endogenous sources to produce CO_2 .

Or, only about 60% of CO_2 produced can be accounted for by exogenous and endogenous carbohydrate, leaving about 40% of CO_2 to be contributed by other sources.

In this connection mention can be made of the suggestion made by Gronlund and Campbell (1961) that the amino acid pool served as the major initial endogenous substrate in *S. cerevisiae*. Another possibility could be lipids, which were found to increase during yeast sporulation by Ramirez and Miller (1964). Lipid is known to serve as endogenous substrate for germination in some other fungi (Barash et al., 1967).

Role of glucose in germination

a. Exogenous nutritional requirements for germination.

Though sporulated yeast contains a large amount of reserve carbohydrate which decreased during germination, a suitable source of carbon, along with other requirements, had to be provided in the medium for germination to take place. To support germination, glucose was required to be present at substrate level and continuously.

That an exogenous carbon source is needed for germination of yeast spores was observed by several previous authors. Nagashima (1959), with a strain of wine yeast, found that no germination took place in plain agar; glucose or tomato juice was needed. A similar observation was made by Palleroni (1961) with a strain of S. cerevisiae. Ascospores of this yeast did not germinate without a carbon source like glucose, fructose or galactose. These findings were confirmed by Seigel (1970) using fresh and dried ascospores of a strain of S. cerevisiae. The present strain of yeast deviates from those previously studied in that it requires, in addition to glucose, the presence of ammonium sulphate and some other factor/s of yeast nitrogen base in the medium for ascospore germination. Glucose alone, therefore, does not suffice for germination of all fermentative yeasts.

With respect to the need of an exogenous carbon source for germination, yeast ascospores differ from those of Neurospora tetrasperma and from the macroconidia of Microsporium gypseum. Neurospora tetrasperma ascospores germinate in distilled water, after a heat shock, using a pool of alcohol soluble carbohydrate (trehalose) amounting to about 14-15% of the spore dry weight (Lingappa and Sussman, 1959). In conidia of M.

gypseum the spore reserves (fatty acids and soluble carbohydrates) were adequate to supply energy and carbon for germination though exogenous long chain fatty acids and leucine stimulated the process. In these spores preferential utilization of endogenous substrate occurred (Barash et al., 1967).

On the other hand, in their nutritional requirements for germination, the ascospores of the yeast strain studied here resemble closely the conidia of Fusarium solani which need a carbon source, a nitrogen source and an unknown factor in yeast extract (Cochrane et al., 1963). These conidia consumed 1.6 mg of glucose or equivalent carbon source per mg dry weight to complete germination. In the yeast ascospores glucose taken up by a million sporulated cells (dry weight = 49-53 μ g) amounted to about 100 μ g in 6 hours. The amount of exogenous glucose consumed by Fusarium conidia and yeast ascospores during germination is very large in comparison to the endogenous carbohydrate reserve present in Neurospora ascospores.

It has been suggested by Mandels (1963) that the apparent need for nutrients is an artifact as the nutrients serve initially to activate the spores. Recently, Lingappa and Lingappa (1965) presented evidence that in Glomerella cingulate the nutrients served to reduce self-inhibition of germination. Garret (1971, personal communication) found that active metabolism reduced the effect of self-inhibitors. In yeast, however, no evidence for the presence of auto-inhibitors for germination has yet been found. The fact that glucose had to be supplied continuously to complete yeast germination indicates that the need for this substance is not an artifact.

From the point of view of nutritional requirements for germination, yeast ascospores are "heterophagous" a term proposed by Cochrane (1966) to designate spores with an apparent requirement for exogenous nutrients. "Autophagous" spores, eg. Neurospora ascospores, do not require exogenous nutrients for germination.

b. Germination with different carbon sources.

As discussed above, several workers have shown that a source of exogenous carbon is required for the germination of yeast ascospores. An examination of the present data concerning the ability of different carbon sources to support germination reveals that fructose, mannose or sucrose, like glucose, can support germination when used as a sole source of carbon in yeast nitrogen base medium. On the other hand, galactose, lactose, cellobiose, maltose, arabinose (both D- and L- form), 2-deoxy-D-glucose, trehalose and potassium acetate failed to support germination. Ability of fructose and mannose to replace glucose is not unexpected because the first step in metabolism of these three sugars is phosphorylation by hexokinase.

Palleroni (1961) observed germination of a limited number of ascospores of a strain of S. cerevisiae on galactose. No germination was obtained with this strain when maltose was used as the source of carbon. This finding might be explained in light of observations by Sutton and Lampen (1962) who could not detect maltase activity in the cell wall fraction of log phase cells of S. cerevisiae. This enzyme was apparently only intracellular and could be released by osmotic lysis of the

protoplasts. Maltase in this yeast was not accessible to the external substrate and an active transport system was involved in maltose uptake. Seigel (1970) working with F.493, a strain of S. cerevisiae, also observed that the greatest decline in acid-fastness (used as a measure of germination) occurred when glucose, fructose, mannose or sucrose were used as sole source of carbon. However, he did note a moderate amount of germination (as evidenced by loss of acid-fastness) with maltose and galactose as well. It may be mentioned here that in the present work also some increase in the number of non-acid-fast spores took place though these spores did not produce buds in 24 hours with galactose and maltose.

Trehalose, when supplied exogenously as a sole source of carbon, did not support germination of S. chevalieri ascospores. A similar result was obtained by Seigel (1970) with his strain of yeast. It may be noted here that endogenous trehalose was found to decrease in the present work during germination; in other words, this strain of yeast is capable of catabolizing trehalose. It appears, therefore, that the failure of exogenous trehalose to serve as a substrate for germination might be due to difficulty of this sugar to enter into the cell. Souya and Panek (1968) studied the localization of trehalase and trehalose in yeast cells. They found that both trehalose and trehalase were in the protoplast, that is, not in the cell wall. Under such conditions, exogenous trehalose will not be readily accessible to trehalase. Trehalose, however, has been found to serve as an exogenous source of carbon in Fusarium solani during germination of conidia (Cochrane et al., 1963). In these spores normal germination took place with trehalose as a carbon source.

The fact that sucrose can support germination indicates the presence of invertase. In the vegetative yeast cells invertase was found at the periphery of the cell (Demis et al., 1954). In this location the enzyme hydrolyzes extracellular, non-penetrating substrate sucrose (Preiss, 1958). In the log phase cells of a strain of S. cerevisiae, the major portion of invertase activity was found in the cell wall preparation (Sutton and Lampen, 1962). The present work indicates that the invertase activity of yeast is retained even after sporulation since the spores can use sucrose during germination.

Potassium acetate, which served as the sole source of carbon during sporulation, failed to support germination. Palleroni (1961) and Seigel (1970) also could not obtain germination of yeast ascospores using potassium acetate as a sole source of exogenous carbon. It seems well established, therefore, that acetate, which is metabolized oxidatively, does not serve as a carbon source for germination. This indicates an important metabolic difference between sporulating and germinating yeast cells since acetate can serve as sole source of carbon for sporulation. The failure of acetate to support germination is in accord with the data from the manometric experiments which indicate that ascospore germination in yeast is predominantly a fermentative process. It can also be mentioned in this connection that growing yeast cells can respire acetate (Pontefract and Miller, 1962). The writer and others (Miller and Halpern, 1956) have observed slow growth of yeast with acetate as sole source of carbon. Carbon metabolism during germination, therefore, must also differ in some respects from carbon metabolism during growth.

c. Exogenous glucose as a germination stimulant.

The importance of exogenous glucose in inducing germination of yeast ascospores has been discussed above. There are 2 possible roles for glucose in stimulating germination, viz., as a trigger or as a substrate. Before attempting to decide which of these 2 roles exogenous glucose plays in causing germination we must first consider the nature of activators for germination. Many physical and chemical factors may serve as activators for germination. Chemical activators may be subdivided according to their mode of action in inducing germination as "triggering agents" and "germination agents". Triggering agents are those chemicals that induce germination but whose continued presence is not necessary. Germination agents are chemicals, the continued presence of which is required for the completion of germination (Sussman and Halvorson, 1966).

The yeast ascospores were unable to complete germination unless glucose was present in the medium continuously. The extent of germination varied with the concentration of glucose. Germination of a population of 30 million/ml was complete with an initial glucose concentration of 0.3%. With an initial glucose content of 0.1% or less for the same number of cells as above, no buds were formed even after 24 hours, though some loss in acid-fastness proportional to glucose concentration took place. These observations strongly suggest a metabolic role for glucose in inducing germination. If glucose were functioning only as a triggering agent, complete germination would be expected even if this substance was removed from the medium after a brief exposure of the spores to it. Variation in the glucose concentration would also

probably not be expected to affect markedly the extent of germination. That exogenous glucose was metabolized during germination is also indicated by the C^{14} -glucose experiments. Labelled glucose was dissimilated by the sporulated population to produce CO_2 and different cell components.

It is possible, however, that in addition to a definite metabolic role glucose might have a triggering action during germination of yeast ascospores. If a primary triggering action is operative, followed by a rapid metabolism of the germination stimulant, it would not be detected under the present experimental conditions.

Some other factors affecting germination

a. Germination on a solid medium.

Germination of yeast ascospores on a solid medium was found to be somewhat faster than that in liquid medium, though the final germination was same with both. The only ingredient that the solid medium had in addition to the constituents of the liquid medium was SP agar. As yeast ascospores can germinate under strictly anaerobic conditions (Seigel, 1970), better oxygen supply when the asci are spread on the surface of solid medium does not seem to be the factor accelerating germination. One possibility appears to be the presence of some germination stimulating substance or substances in agar as impurity. In this connection mention can be made of the fact that Miller (1959) has shown that "purified" agar may contain sufficient nutritives to support limited growth of yeast cells. Grewal (1971 - personal communication)

also noted that a strain of S. cerevisiae showed some growth before sporulation when transferred to a sporulation medium solidified with 5% SP agar, but produced spores directly in a liquid sporulation medium. Another possibility could be the diffusion of a self-inhibitor of germination into the solid medium.

b. Effect of age on germination.

Spore germination in yeast, as indicated by loss of acid-fastness in 6 hours in germination medium was faster with 5-day-old spores than with 2-day-old spores. The percentage of final germination after 8 hours, however, showed no correlation with age of the spores. The faster rate of germination associated with ageing might be attributed to the maturation of the ascospores. A similar phenomenon has been observed in other spores. Thus, the time required for germination has been found to be a function of age in Plasmopara viticola oospores (Arens, 1929). Gilbert (1928) observed that some spores of myxomycetes required $2\frac{1}{2}$ days to germinate when freshly harvested, but required only 12 hours to germinate when allowed to age.

Some observations opposite to the above findings have also been reported. Gäumann (1946) claimed an increase in time required for germination when the spores were aged. In A. niger, on the other hand, the spores germinated most rapidly when they were harvested from 15-day old cultures, but took longer to germinate if harvested from either younger or older cultures (Maruyama et al., 1961).

Some notes on Materials and Methods

a. Choice of time period for germination study

Germination is a process characterized by several stages and completed by the production of an outgrowth. It is very difficult to decide when germination is completed and growth has begun. One of the commonly used criteria for measuring germination in fungi is the emergence of outgrowth. Observation of an outgrowth (bud) can be taken to indicate that germination of a spore has ended and the next phase, viz., growth, has commenced.

In the present work 10-14% of the spores produced buds after 6 hours exposure to germination medium. By 8 hours, all the spores were with buds and the culture consisted mainly of newly produced vegetative cells, and thus resembled a growth culture. As the purpose of the present work was to study germination physiology, the 6 hour period was the time chosen when measurement for germination should be made using decline in acid-fastness as a criterion for germination. The later examinations were made for comparison with the observations made during the first 6 hours. The situation at 6 hours after the sporulated cells were exposed to the germination medium represented at least a well-advanced stage of germination without much accompanying growth. Another factor that indicated the choice of 6 hour over 8 hour period was the difficulty in determining the cell population density at the later time. All the spores in an ascus would bear one or more buds at this time, producing small groups of cells which were very difficult to count accurately.

b. Acid-fastness as a criterion for germination.

The loss of acid-fastness as a criterion for ascospore germination in yeast was studied by Seigel (1970). Using both fresh and dried ascospores of S. cerevisiae, he observed a loss of acid-fastness with the progress of germination. In the present work, also, decline in acid-fastness was used as one of the criteria for germination. This criterion was very useful in the early phases of germination when morphogenetic changes were not detectable under the light microscope. The proportion of non-acid-fast spores increased with time after the spores (in the asci) were exposed to the germination medium, and the criterion proved to be satisfactory in general; but there were always some non-acid-fast spores (4-12%) observed in the sporulation culture before the cells were exposed to germination medium. Whether germination was already induced in those spores or whether they had lost their acid-fastness due to other unknown causes is not clear. In experiments using different carbon sources in germination medium a decline in acid-fastness was observed in some spores by 6 hours, but they did not produce buds in 24 hours and hence did not complete germination. The loss of acid-fastness alone, therefore, does not necessarily guarantee that germination will be completed. Considering these points, decline in acid-fastness does not seem to be satisfactory as a single criterion for germination.

c. Glycogen I, II and III.

The extraction of glycogen from yeast has been found quite difficult and it can not all be extracted in one step. A part of glycogen is alkali-soluble and another part acid-soluble (Chung and Nickerson,

1954). Trevelyan and Harrison (1956) extracted yeast glycogen in 3 different fractions, one alkali-soluble fraction and two acid-soluble fractions. They suggested that glycogen in yeast was physiologically heterogenous. Eaton (1960) also observed 2 metabolically different glycogen pools in yeast. Northcote (1953) on the other hand, could not find evidence of more than one kind of glycogen in yeast chemically. The matter of whether different glycogen fractions of yeast are different physiologically or not remains unclear. In the present study glycogen was extracted in 3 different fractions, viz., TCA-soluble fraction, KOH-soluble fraction and acetic-acid-soluble fraction. These have been designated, purely for the sake of convenience, as glycogen I, II and III respectively.

Biological considerations

The information obtained from the present work and from the existing literature allows a comparison of the three major phases (vegetative, sporulated and germinating) of yeast life cycle as to content and metabolism of carbohydrate. The content was high all throughout the life cycle of yeast, but it became higher during sporulation. During germination the increased reserve of the sporulated cells declined until on resumption of growth, it reached a value comparable to that of the original vegetative cells.

The question arises now as to what biological function does the increased reserve carbohydrate in sporulated yeast serve in the life cycle. Several possible functions may be suggested.

Firstly, the reserve carbohydrate may serve as a substrate for germination, as with Neurospora ascospores. But from the evidence presented in this work, the endogenous reserve carbohydrate alone could not support germination. Exogenous carbohydrate was required. This failure to germinate without exogenous carbohydrate could not be attributed to a lack of activation of the enzyme systems responsible for breakdown of endogenous reserve carbohydrate. Under such circumstances, germination would be expected when glucose was removed from the germination medium after a short exposure. It appears that the endogenous reserve carbohydrate in yeast is incompetent to serve as a substrate for germination. It cannot be claimed that this is because the reserve is insufficient in amount, because none of it disappears without exogenous carbohydrate.

Secondly, the reserve carbohydrate may serve to maintain endogenous respiration in the resting stage. This view gets some support from a decrease observed in the ethanol-soluble carbohydrate (trehalose) in sporulated cells which were kept in sporulation medium for a long period (five days) in an earlier investigation by the writer (Banerjee, 1965). These spores showed no evidence of germination after five days indicating that the reserve carbohydrate can be utilized in the absence of germination. The ascospores of Neurospora are known to utilize their reserve lipid in the dormant stage (Lingappa and Sussman, 1959).

Thirdly, the increased carbohydrate reserve may improve resistance to the harmful effects of dessication, thus making the spores more capable of survival under natural conditions. It was observed by Pollock and Holmstrom (1951) that dried yeast with 16% trehalose and 40% total

carbohydrate content survived better in storage than yeast with 11% trehalose and 36% total carbohydrate content. The survival of bacteria was found to be increased greatly when 5-10% glucose was included in the suspending medium for drying (Fry and Greaves, 1951). Greaves (1964) found that the presence of sucrose in freeze-drying medium gave a high survival value, that was well maintained even at high temperature, to bacteria and some other living cells.. .

The inability of yeast ascospores to germinate on their endogenous reserves is of ecological interest. The resistant ascospores will germinate to develop the less resistant vegetative cells only when the conditions are favourable for growth and the species can flourish. The statement by Sussman and Holvorson (1966) that "the enhanced requirement for other nutrient materials may confer a selective advantage upon spore formers" finds an example in yeast. The yeast strain used by the writer has more exacting (glucose, ammonium sulphate and a factor/s of yeast nitrogen base) nutritional requirements than the strains used by previous students of yeast spore germination (Nagashima, 1959; Palleroni, 1961; Seigel, 1970) which required only a fermentable sugar.

SUMMARY AND CONCLUSIONS

1. Trehalose and glycogen, the two reserve carbohydrates that form a major part of the total carbohydrate content changed in abundance at different stages of yeast life cycle. The structural carbohydrates (mannan and glucan) which occur in less quantity showed no significant change.
2. The reserve carbohydrate increased during sporulation and decreased during germination of yeast ascospores. The amount of increase during sporulation and of decrease during germination was higher with trehalose than with glycogen. The dry weight of yeast increased during sporulation but showed no change during 6 hours in germination medium.
3. In spite of the high content of reserve carbohydrate, an exogenous source of carbon was required to induce and support this process. Some of the reserve was also utilized.
4. Germination, which took place in approximately 6 hours, required, in addition to glucose, the presence of $(\text{NH}_4)_2\text{SO}_4$ and some other factor or factors of yeast nitrogen base. Glucose had to be present in the medium continuously and at substrate level.
5. Fructose, mannose and sucrose, like glucose, supported germination but nine other carbon sources, namely, galactose, maltose, lactose, cellobiose, L-arabinose, D-arabinose, trehalose, 2-deoxy-D-glucose or acetate failed to do so.
6. Exposures to high or low temperatures did not induce germination.

7. When supplied with a suitable source of exogenous carbon, the yeast ascospores did not require exogenous CO_2 for germination, since the removal of this gas from the environment had no inhibitory effect.
8. The respiratory pattern during germination was mainly fermentative having an average respiratory quotient equal to 8.4 over 6 hours. Even at the period of maximum oxygen consumption, a high respiratory quotient equal to 6.2 was observed. Carbon dioxide evolution was high from the beginning and increased further during later periods.
9. Exogenous glucose was metabolized into CO_2 and different cell components. About two-thirds of the exogenous glucose assimilated by the cell was recovered from different cell components and about one-third from CO_2 .
10. Trehalose and glycogen were simultaneously utilized and synthesized during germination. The rate of utilization was higher than that of synthesis thus resulting in a decrease in the amount of these carbohydrates.
11. Though the sporulated yeast apparently retained some ability to metabolize acetate in germination medium, glycolytic part of carbohydrate metabolism predominated and seemed essential for germination, as was indicated by carbon source, respiration and inhibitor experiments.

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APPENDIX

APPENDIX I

Wickerham's (1951) yeast nitrogen base

ammonium sulphate	5 g.
L-histidine monohydrochloride	10 mg.
DL-methionine	20 "
DL-Tryptophan	20 "
biotin	2 mcg.
calcium pantothenate	400 "
folic acid	2 "
inositol	2000 "
niacin	400 "
p-aminobenzoic acid	200 "
pyridoxine hydrochloride	400 "
riboflavin	200 "
thiamine hydrochloride	400 "
boric acid	500 "
copper sulphate	40 "
potassium iodide	100 "
ferric chloride	200 "
manganese sulphate	400 "
sodium molybdate	200 "
zinc sulphate	400 "
potassium phosphate manobasic	1 g.

magnesium sulphate	0.5 g.
sodium chloride	0.1 "
calcium chloride	0.1 "
distilled water	1000 ml.

Yeast nitrogen base minus amino acids

Same as yeast nitrogen base except that L-histidine monohydrochloride, DL-methionine and DL-tryptophan have been excluded.

Yeast nitrogen base minus amino acids and ammonium sulphate

Same as yeast nitrogen base except that ammonium sulphate in addition to the three amino acids have been excluded.

Vitamin free yeast base

Same as yeast nitrogen base except that the nine B vitamins have been excluded and glucose (1%) has been added.

APPENDIX II

Ziehl Neelsen acid-fast staining procedure. (Society of the American Bacteriologists, Manual of Microbiological Methods, 1957, McGraw-Hill.)

1. Prepare a smear of the yeast by adding a drop on a slide and air-dry.
2. Cover the film with Ziehl-Neelsen's carbol-fuchsin stain and heat gently until the stain just steams for about three minutes.
3. Wash the slide in tap water and decolourize the film with acid alcohol until only a suggestion of pink remains.
4. Wash the slide in tap water, cover the film with methylene blue stain and allow to act for one minute.
5. Wash the slide again in tap water, drain, blot and air-dry.

Acid-fast spores stain red. Non-acid-fast spores stain blue.

Ziehl-Neelsen's carbol-fuchsin stain

Basic fuchsin	0.3 g.
Ethanol (95%)	10 ml.
Phenol crystal	5 g.
Distilled water	95 ml.

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

Methylene blue staining solution.

Methylene blue	0.3 g.
Ethanol (95%)	30 ml.
Distilled water	100 ml.

Dissolve methylene blue in ethanol. Add distilled water and filter the solution.

Acid alcohol

Hydrochloric acid (37%)	30 ml.
Ethanol (95%), sufficient to make 1000 ml.	

APPENDIX .III

Lugol's iodine solution

Iodine	50 g.
Potassium iodide	100 g.
Distilled water sufficient to make 1000 ml.	

APPENDIX IV

Original data for Table 11

Uptake of C¹⁴-glucose during germination of yeast ascospores (CPM/million cells)

Expt. No.	Duration	Initial medium	Final medium	Loss of C ¹⁴ from medium	Wash	Cell	CO ₂	Wash + cell + CO ₂	Recovery of C ¹⁴ , %
1	0-2 hours	37179	35042	2137	1367	276	165	1808	84.5
2		30079	27152	2927	2184	345	163	2692	92.0
3		27777	25641	2136	824	164	88	1076	52.7
Mean		31678.3	29278.3	2400	1458.3	261.6	138.6	1856.6	
1	2-4 hours	27027	25000	2027	947	250	109	1306	64.3
2		20833	18518	2315	1053	298	138	1489	64.5
Mean		23930	21759	2171	1000	274	123.5	1397.5	
1	4-6 hours	25216	22886	2330	903	461	109	1473	63.3
2		22222	19608	2614	982	605	255	1842	70.8
Mean		23719	21247	2472	942.5	533	182	1657.5	

APPENDIX V

Calculation of amount of glucose removed from the medium during 6 hours using the data from loss of radioactivity from the medium during that time.

From Appendix IV, the initial medium contained 26442 CPM/million cells (average of readings from 0-2, 2-4 and 4-6 hours), that is, 26000 CPM/million cells, approximately.

Therefore: Total count in the culture flask = 26,000,000 CPM

$$= 26 \times 10^6 \text{ CPM} \quad (\text{Since the flask contains 20 ml culture of } 50 \times 10^6 \text{ cells/ml.})$$

Now, $26 \times 10^6 \text{ CPM} = .6 \text{ mg} = 600 \mu\text{g}$ of C^{14} -glucose (the amount given).

From Table 11 and Appendix IV, radioactivity removed from the medium in 6 hours = 7043 CPM

= 7000 CPM, approximately.

This will represent $\frac{600 \times 7000}{26 \times 10^6} \mu\text{g}$ of C^{14} -glucose.

Therefore: Total glucose removed in 6 hours

$$= \frac{600 \times 7000 \times 333}{26 \times 10^6} \mu\text{g}. \quad (\text{Since the ratio of glucose to } \text{C}^{14} \text{ glucose is } 2000:6)$$

$$= 53.8 \mu\text{g} = 55 \mu\text{g (approx).}$$

From glucose uptake experiments (pp. 59) this value is $110 \mu\text{g}$.

Therefore, the amount of glucose removed from the medium as calculated from the C^{14} experiment accounts for only 50% (approximately) of that obtained from the glucose uptake experiment.

A suggestion can be made for this apparent discrepancy. Since metabolism is active in the germinating cell, some substances will be produced from C^{14} -glucose and be released into the medium, thus lowering the amount of C^{14} removed from the medium. In support to this is the observation that when cells are removed from the germination medium containing C^{14} -glucose and washed with distilled water, a considerable amount of radioactivity passes from the cells into the wash water. This is not in glucose because when a similar experiment was done using micro-chemical determinations, very little carbohydrate was found to be lost from the cells. This interesting observation on yeast spore metabolism was not investigated further.

APPENDIX VI

Original data for Table 12

Radioactivity in different cell fractions (CPM/million cells)*

Expt. No.	Duration	Whole cells**	Hot water soluble	Cold TCA*** soluble	Hot water + cold TCA	NaOH soluble	Nucleic acids	Lipid	Residue	Total	C ¹⁴ recovered, %
1	0-2 hours	276.0	74.8	15.3	90.1	92.7	18.1	2.7	7.4	211.0	76
2		345.0	90.0	19.0	109.0	79.0	10.0	5.0	27.0	230.0	67
3		164.0	70.0	6.2	76.2	50.0	12.3	4.3	12.5	155.3	95
Mean		261.6	78.3	13.5	91.8	73.9	13.5	4.0	15.6	198.8	
1	2-4 hours	250.0	87.0	5.0	92.0	43.0	13.0	9.0	12.0	169.0	68
2		298.0	105.2	14.1	119.3	77.0	16.0	6.0	17.0	235.3	79
Mean		274.0	96.2	9.5	105.7	60.0	14.5	7.5	14.5	202.1	
1	4-6 hours	461.0	151.0	7.9	158.9	78.0	8.0	10.2	60.7	315.8	69
2		605.0	152.3	29.7	182.0	122.9	29.6	12.2	131.5	478.2	79
Mean		533.0	151.7	18.8	170.5	100.5	18.8	11.2	96.1	397	

* Extracts from at least 10 million cells were used for counting.

** 2.5 million cells were counted. *** TCA = Trichloroacetic acid.

APPENDIX VII

Counting Efficiency

For comparing the efficiency of counting for different cell components, a curve obtained by the method of Baillie (1961)* was used. "It has been reported that such a plot is essentially independent of the nature of the quenching agent" (Wang, 1965, pp. 133).** Channel ratios of counts from different samples were found to be the same and corresponded to an efficiency of 80%. However, the actual efficiency was presumably somewhat lower because the samples were applied on filter paper in the present work. It has been reported by Bartley and Abraham (1965)*** that a sample which gave 222 CPM in solution gave 203 CPM on paper. The writer has made a comparison of efficiency in solution and on filter paper. It was found that a sample which gave 1400 CPM in solution gave 833 CPM on paper. Therefore on filter paper the efficiency was 60% (approximately) of that in solution.

The geometry of counting of different samples from different cell constituents was identical in all cases, since the filter paper was located in the same position. Though the absolute efficiency for each sample was not computed in the present work, the results with different extracts are comparable because these were counted in an identical manner.

* Baillie, L.A. (1961). Atom light, 19, pp. 1-11. New England Nuclear Corporation.

** Wang, C.H. (1965). Radiotracer methodology in Biological science, pp. 133. Prentice-Hall, Inc.

*** Bartley, J.C. and Abraham, S. (1965). Atom light, 49, pp. 1-8. New England Nuclear Corporation.

APPENDIX VIII

Significance test (Fisher's T. test)* for dry weight
trehalose, total glycogen and total carbohydrate
during sporulation and germination

d.f. = 6

	veg./sp.	sp./germi.
Dry weight	T = 7, highly significant	T = 0.7, not significant
Trehalose	T = 10.1, highly significant	T = 5.9, significant at P = .002
Total glycogen	T = 3.5, significant at P = .02	T = 0.79, not significant
Total carbohydrate	T = 6.1, highly significant	T = 2.9, significant at P = .05

* T was determined by using the formula

$$T = \frac{(\bar{X}_a - \bar{X}_b) \sqrt{\frac{N_a N_b}{N_a + N_b}}}{\sqrt{\frac{N_a^2 (SE_a^2) + N_b^2 (SE_b^2)}{N_a + N_b - 2}}}$$

(In, "The Essence of Biometry" by J. Stanley, 1963, McGill University Press, p. 42.)