

LIFE CYCLES OF TWO-SPORED YEASTS

A STUDY OF SPORULATION AND SPORE GERMINATION

IN

TWO-SPORED YEASTS

By

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Nuclear divisions during sporulation of 15 predominantly two-spored and 2 predominantly four-spored yeasts were followed by Giemsa staining and light microscopy. The number of nuclei present per ascus was related to the presence or absence of conjugation at the time of germination of the spores. Plating experiments and a modification of the Finder Slide technique were used to determine whether progeny cells that developed from single spores of 2 two-spored and one four-spored strain could sporulate. The ploidy of these yeasts at different stages of their life cycles was estimated by DNA extractions.

The spores of the two-spored yeasts were very difficult to separate following removal of their ascus walls, and electron microscopy was employed in an attempt to account for this.

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INTRODUCTION

The Ascomycetes are distinguished from all other fungi by the presence of the ascus, a sac-like cell containing usually a definite number of ascospores. Typically eight ascospores are formed within the ascus, but this number may vary from one to over a thousand according to the species (Alexopoulos, 1962). A Saccharomyces ascus normally contains four spores but fewer may be present. The nutritive conditions in the growth and sporulation media to some extent determine the number of spores in each ascus. Miller (1957) found that the number of spores per ascus increased as the concentration of carbon source in sporulation medium was increased. A recent report (Veziñhet 1969) showed that Saccharomyces cells produced two-spored asci in buffer, and three and four-spored asci when a carbon source was added to the buffer. Santa Maria (1959) often observed yeast asci with more than four spores. Dr. P.V. Patel (personal communication) was able to obtain more than four spores per ascus in Saccharomyces by using a solution of acetate + glycerol as the sporulation medium. But some strains of Saccharomyces produce predominantly two spores per ascus even in acetate sporulation medium. In this study a cytological explanation for the two-spored condition of a number of such strains was looked for.

In the remainder of this Introduction, certain aspects of yeast biology are considered, relevant to the results obtained with the two-spored yeasts.

Vegetative Growth

Cells of Saccharomyces yeasts grow vegetatively through the process of budding as long as a sufficient supply of essential nutrients is provided. During vegetative growth a yeast cell enlarges to its adult size, and develops a protruberance or bud, which remains attached to the mother cell until it reaches approximately $3/4$ of the adult size. Williamson and Scopes (1960) by means of DNA analysis of synchronously growing yeast, showed that the DNA content of a cell doubles at or just after the first appearance of the bud. Similar results were obtained by Williamson (1965) through autoradiography. After the bud has reached at least $1/4$ the size of the mother cell the nucleus moves to the isthmus between cell and bud and there divides into two. Ramirez and Miller (1962) demonstrated that the nucleus can divide entirely within the mother cell, but this is rare. One daughter nucleus remains in the mother cell and the other enters the bud. When each bud separates from the mother cell, it leaves behind a bud scar on the mother cell and carries a birth scar itself. A given cell may bud repeatedly, but never more than once at the same place. Barton (1950) recorded up to 23 bud scars on one cell.

The yeast nucleus, compared to that of higher organisms, is small and difficult to stain clearly. Yeast cytologists have reported many conflicting observations in the past. Janssens and Leblanc (1898) regarded the vacuole as the nucleus. Wager and Peniston (1910) thought the yeast nucleus was made up of a number

of different parts, e.g. the vacuole, chromatin threads in the vacuole, a body beside the vacuole called nucleolus. Lindegren (1949) described a darkly staining body (similar to the nucleolus of Wager and Peniston) as a centrosome surrounded by heterochromatin. He considered the vacuole to be part of the nucleus. Lindegren et al. as recently as 1956 reported that the "nucleolus" during the period of rapid division appeared in the vacuole and also that the spindle was intravacuolar, thus reaffirming their view that the vacuole was a nuclear structure. However, Mundkur (1954) claimed that the yeast nucleus was an extra-vacuolar structure. Later the electron microscopic observations of Agar and Douglas (1957), and Hashimoto et al. (1958) and (1959) supported Mundkur by showing that nucleus and vacuole were distinct, and each had its own membrane. Vitols et al. (1961) demonstrated that the nucleus, enclosed by a pair of unit membranes, was situated close to but separate from the vacuole which had a single unit membrane.

There has been a considerable amount of controversy over the number of chromosomes, if, indeed chromosomes are present in yeast. Using classical cytological methods, Beams et al. (1940) and Mundkur (1954) failed to detect structures corresponding to chromosomes within the yeast nucleus. On the other hand DeLamater (1950) claimed to have demonstrated a maximum of four chromosomes, Winge (1951) two to four, Lietz (1951) three, and Subramaniam (1952) two. The electron microscopic observations of Hashimoto et al. (1959) and Robinow and Marak (1966) did not demonstrate chromosomes in the

vegetatively growing yeast cells. Genetic evidence of Lindegren (1949) indicated at least four linkage groups in haploid cells of Saccharomyces cerevisiae. Mortimer and Hawthorne (1966) in their genetic maps of Saccharomyces showed at least 14 chromosomes. Robinow and Marak (1966) reported that chromosomes become visible under light microscopy at meiosis but they did not attempt a count.

The reports on nuclear division during budding in Saccharomyces have been as controversial as the nature of the nucleus and number of chromosomes. The early workers, Janssens and Leblanc (1898), Guilliermond (1903) and (1905) and Wager and Peniston (1910) did not see the typical phases of mitosis and therefore thought that the nuclear division in yeast was amitotic. Beams et al. (1940) from their work on budding Saccharomyces cerevisiae cells also concluded that the nucleus divided amitotically. On the other hand, Lindegren (1949), Kater (1951), Lindegren et al. (1956), Swaminathan and Ganesan (1958) and Ganesan (1959) claimed the existence of all phases of mitosis in yeast. Swaminathan and Ganesan (1958) proposed a scheme by which mitosis occurred inside the nuclear membrane which remains intact until it extends into the bud and separates by constriction. In a review article on fine structure of fungi, Hawker (1965) concluded that the nuclear envelope of yeasts remains intact during division. Robinow (1961) was able to show nuclear division equivalent to mitosis of higher organisms but without spindle apparatus in Lipomyces, a soil yeast with high lipid content. The nuclear division of yeast during

vegetative growth is now accepted to be mitotic even though all phases are not always visible, (Lindgren et al. 1956, Bautz 1960, Ramirez and Miller 1962, and Robinow and Marak 1966).

Spore Formation

Under certain environmental conditions, yeast cells may undergo a marked morphogenetic change and produce ascospores. De Seynes (1868) was the first to observe the formation of endospores in yeast. He also noticed that the yeast endospores germinated to give rise to budding cells again. Reess (1869) confirmed De Seynes' findings and described changes in the cytoplasm during sporulation. Reess recognized that the yeast endospores were ascospores and that yeasts belong to the ascomycete fungi.

The early workers regarded spore formation as a response to starvation conditions in their environment, (De Seynes 1868, and Reess 1869, 1870). On this basis a variety of techniques have been developed to produce spores. Engel (1872) used moistened gypsum blocks over the surface of which cells were thinly spread. This has been historically the most widely-used method. Beijerinck (1898) spread the cells on washed agar blocks. More recently, Stantial (1935) developed a liquid medium containing small amounts of acetate and sugar.

Considerable work has been done on the factors influencing the spore formation in yeast, and this has been reviewed by Miller and Hoffman-Ostenhof (1964). For good sporulation the following

prerequisites should be met:

- (1) the cells should be precultured in a well nourishing pre-sporulation medium.
- (2) the sporulation medium should contain an acetate as the carbon source;
- (3) sporulating cultures should be well aerated;
- (4) the temperature should be maintained between 25 and 30°C.

The nucleus during sporulation divides twice to give rise to four nuclei. The mode of division is considered to be equivalent to meiosis of higher organisms. Nagel (1946), McClary et al. (1957), and Pontefract and Miller (1962) in their cytological studies described the stages of meiosis in sporulating Saccharomyces. Engels and Croes (1968) reported the occurrence of a synaptonemal complex in the electron micrographs of sporulating Saccharomyces cerevisiae cells, during the interval between the maximum of DNA synthesis and the first nuclear division. Recently Moens and Rapport (1970) showed in sporulating cells of the same organism centriolar plaques, microtubules, and bodies with multiple synaptonemal complex-like structures. Each of the four haploid nuclei produced by meiosis becomes enclosed by a spore wall.

Alternation of Generations in Yeast

In 1870, Reess observed that some pairs of yeast spores fused during germination and realized that this might imply a sexual fusion, but discarded the idea because many other germinating

spores were in contact but did not fuse, and also there were no ascogonia in yeast and no sign of fertilization preceding spore formation as in other Ascomycetes. Hansen (1891) saw some evidence of conjugation in a strain of Saccharomyces cerevisiae in which walls between adjacent spores in asci disappeared. In the same publication he described typical conjugation in germinating spores of Saccharomyces ludwigii, now called Saccharomyces ludwigii (Phaff 1970). He attached no sexual significance to it but suggested that as a result of fusion the young spores were better able to germinate under adverse conditions.

The review articles of Guilliermond (1903 and 1905) clearly showed that the cells of most yeasts produced four nuclei before sporulation, but he failed to conclude that a reduction division was occurring. He believed that the majority of yeasts had lost all traces of sexuality since sporulation was not immediately preceded by fertilization. Cytological and genetical evidence of Winge (1935) and Winge and Laustsen (1937 and 1939) demonstrated the existence of alternation of haploid and diploid generations in the yeast life cycle. They showed that reduction division occurs immediately before sporulation and that in most instances karyogamy takes place at spore germination. As a result for most of the life cycle the yeasts are diploid. The diploidization may occur by the fusion of two haploid spores of opposite mating type, e.g. in Saccharomyces ludwigii (Winge and Laustsen, 1939; Lindegren, 1945; and Winge 1947). The fusion of

haploid cells has been demonstrated in Saccharomyces cerevisiae by Winge and Laustsen (1937) and Lindegren and Lindegren (1943), and in Schizosaccharomyces pombe by Leupold (1950).

The life cycle of a typical Saccharomyces yeast is shown in Figure 1.

Ultra-structural Studies on Yeast

Yeast cells are difficult to fix and embed properly for thin sectioning, possibly because the walls are thick in proportion to the cell size. Agar and Douglas (1955) fixed budding yeast cells with osmium. Their micrographs lacked internal structural details. Agar and Douglas (1957) concluded that the nature of the medium in which cells were grown was a critical factor for fixation. This later work showed vacuole, nucleus, mitochondria and storage granules in cells treated similarly to those in their former work but grown differently. Hashimoto et al. (1958, 1959, 1960) used potassium permanganate for fixation and achieved good cytoplasmic detail. The usual organelle and particles, e.g. vacuole, mitochondria, endoplasmic reticulum, nucleus, lipid globules and sometimes glycogen granules, can be identified in the sectioned and stained yeast cell.

The chemical fixation treatment of yeast cells results in somewhat distorted views of various organelles which possibly is due to shrinkage and washing out of material. To overcome this Mundkur (1961a) and (1961b) freeze-dried the yeast cells and then fixed them with

FIGURE I

Life cycle of Saccharomyces showing alternation of haploid with diploid generations.

- A, B, C: Budding of a vegetative cell with mitotic division of the diploid nucleus
- D : Four haploid nuclei resulting from meiotic division of the diploid nucleus
- E : Four haploid spores in an ascus
- F : Ascus after a few hours in germination medium; spores are swollen
- G : Plasmogamy and Karyogamy between spores of opposite mating type
- H : Mitotic division of diploid nucleus and production of first bud

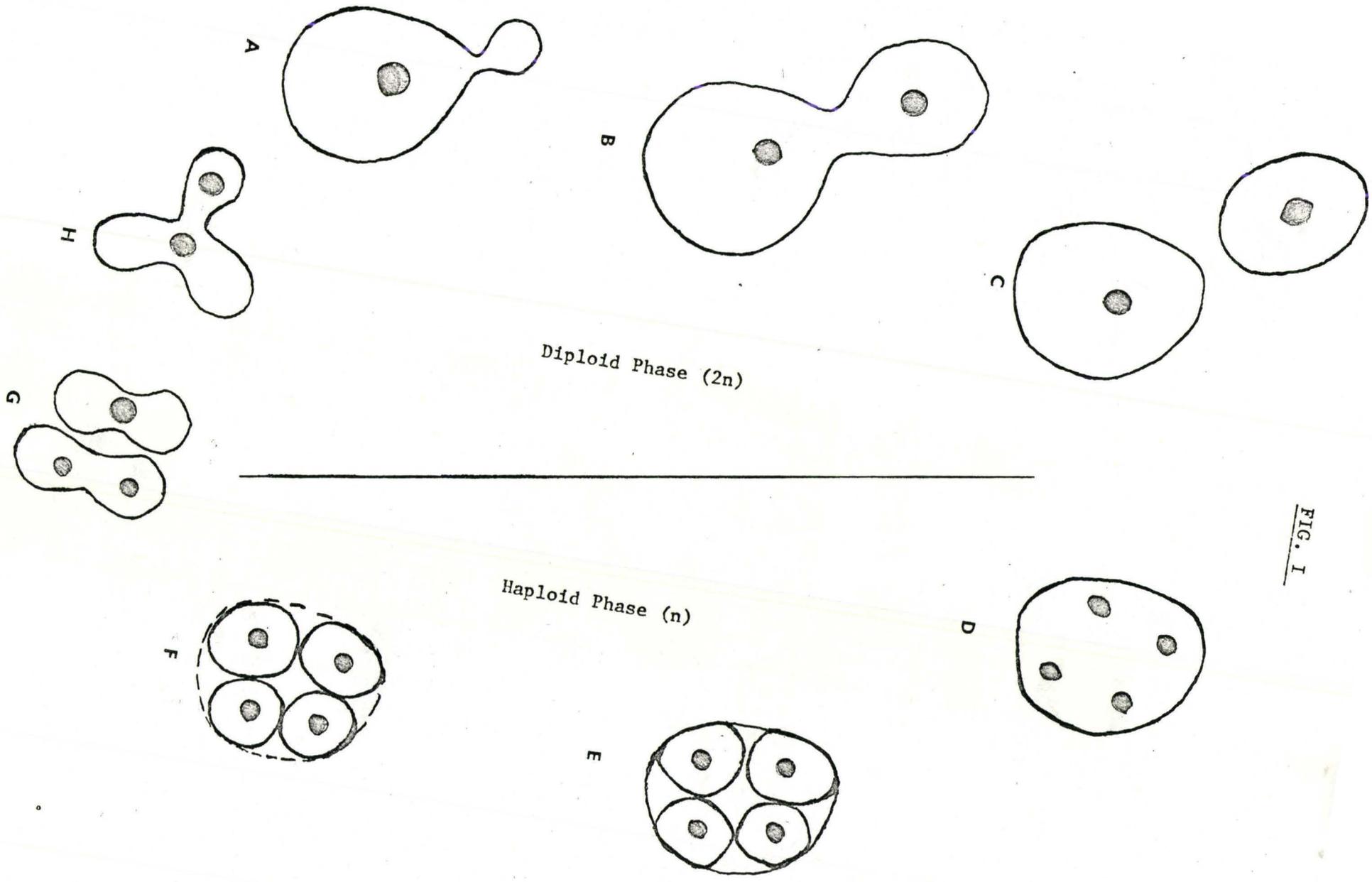


FIG. I

alcohol. Nucleus, vacuole and some macromolecules were seen better than before. But many organelles, especially their membranes, were not very clear.

Steere (1957) described a procedure whereby replicas could be obtained from frozen biological specimens. The frozen specimens could be cut and then etched by sublimation of ice from their surface prior to replica formation. Thus internal detail of the cells could be observed. Moor et al. (1961) reported improvements of the Steere technique. The device described by them was a combination of an ultramicrotome with freeze-drying and shadow-casting installations in the same vacuum container. It was a conveniently operable device and made freeze-etching popular.

Moor and Mühlethaler (1963) by using the freeze-etching technique described the Golgi apparatus, and invaginations of the plasmalemma of yeast cells. Since then the structure of yeast cell wall, plasmalemma and that of organelles has been studied by many workers.

Although the freeze-etching technique is a very valuable tool for studying surface structures, it is not without its drawbacks. One crucial problem arises with the formation of artifacts when the osmotic stabilizers get demixed and crystallized. This problem is discussed by Moor et al. (1961) and by Necas et al. (1969).

Invaginations of the Plasmalemma

Matile et al. (1965) reported the occurrence of invaginations of the plasmalemma of Neurospora during secretion of proteolytic enzymes. Sassen et al. (1967) saw invaginations in the plasmamembrane of Penicillium megasporum spores. These invaginations contained material similar to the inner spore wall. Conspicuous invaginations of the

plasmalemma during sporulation of Saccharomyces have been revealed in frozen-etched preparations by Bauer and in sections by Robinow and Marak (Robinow, February 1971. Personal communication).

Objectives

The nuclei of diploid Saccharomyces cells during sporulation undergo meiosis to form four haploid nuclei. Each of these may be surrounded by a spore wall and, therefore, a maximum of four spores per ascus are expected. But some strains of Saccharomyces produce predominantly two-spored asci. This study was undertaken to gain some insight into the causes leading to the two-spored condition.

During the course of this work, it was consistently observed that the pairs of spores of certain two-spored strains tended not to separate following removal of the ascus wall. An examination of these developing asci by electron microscopy was undertaken in an attempt to explain the failure of the spores to separate.

MATERIALS AND METHODS

Yeast Strains

The two-spored yeast strain used for most of the work in this study was Saccharomyces ellipsoideus, designated as 19el. A culture of this strain was obtained from Station Agronomique et Oenologique de Bordeaux, France.

Two strains of Saccharomyces cerevisiae, namely X-901-35C, a haploid, and X-190-T, a tetraploid, were received via Dr. P.V. Patel from Dr. J. R. Johnston's laboratory in Glasgow, Scotland. These two strains were used for comparison in DNA analyses.

The rest of the strains are listed below. ATCC refers to American Type Culture Collection, IFO refers to Institute for Fermentation, Osaka, Japan, NCYC refers to British National Collection of Yeast Cultures, and EVE refers to Estación de Viticultura y Enología, Jerez de la Frontera, Spain.

ATCC-11428	<u>Saccharomyces cerevisiae</u> var. <u>ellipsoideus</u>				
ATCC-4098	"	"	"	"	"
ATCC-4117	"	"	"	"	"
ATCC-4921	"	"	"	"	"
IFO-0553	"Beer yeast"	No species name given			
IFO-2000	"	"	"	"	"
IFO-2011	"	"	"	"	"
IFO-0556	"Bread yeast"	"	"	"	"

IFO-2040	"Bread yeast"	No species name given
IFO-2043	" "	" " " "
IFO-2045	" "	" " " "
IFO-2241	"Wine yeast"	" " " "
IFO-2244	" "	" " " "
NCYC-620	<u>Saccharomyces cerevisiae</u>	Hansen
NCYC-79	" "	" "
EVE-18	<u>Saccharomyces chevalieri</u>	Guilliermond

All these strains, like 19e1, produced predominantly two-spored asci, with the exception of NCYC-79 and EVE-18, which were 4-spored. According to the most recent revision of the yeast classification, (Lodder, 1970), ATCC-4098, ATCC-4117, ATCC-4921 and 19e1 would be included in Saccharomyces cerevisiae Hansen.

Stock Cultures

Stock cultures were maintained on slants prepared in the following manner: To 150 ml. distilled water 3 gm. Bacto agar were added in a 500 ml. flask. The agar was dissolved by heating the flask in a double boiler. To the liquid agar 1 gm. Difco Yeast Nitrogen Base (see Appendix I for composition) and 1.5 gm. glucose were added and dissolved by stirring. 5 ml. quantities of this medium were poured into 16 mm. test tubes which were capped and autoclaved for 20 minutes at 121°C and 15 p.s.i. pressure. After

inoculating from previous stock cultures, growth was allowed to take place for two days in a 27°C. incubator and the slants were then stored in a refrigerator at 4°C.

The haploid, X-901-35C, and the tetraploid, X-190-T, strains were maintained on slants of MYPG solidified with Bacto agar (2%). The constituents of MYPG medium are given below.

Pre-sporulation

Three types of media were used to grow cells for the inoculation of sporulation cultures:

(1) Malt extract, yeast extract, peptone, glucose medium (MYPG):

This is an undefined medium with the following constituents per litre of distilled water:

3 gms.	Bacto malt extract
3 gms.	Bacto yeast extract
5 gms.	Bacto peptone
1 gm.	$K H_2 P O_4$
20 gms.	glucose

30 ml. quantities of this medium were poured into 250 ml. erlenmeyer flasks. The flasks were stoppered with cotton plugs and autoclaved for 20 minutes. They were inoculated from stock culture slants and incubated for 24 hours at 27°C on a Warner-Chilcott Laboratories Model 2156 water bath shaker with a horizontal displacement of 5 cm. and at a rate of 80 oscillations per minute.

Modifications of this medium were made according to the experimental requirements. In some instances the glucose content was changed. For observing germination of spores directly under the microscope, the medium was solidified with Fisher Scientific Co. Agar S P (5%).

(2) Molasses, Phosphate and Urea medium (MPU):

5 ml. of Domolco Molasses were dissolved in 90 ml. of distilled water in a 500 ml. flask. To a 25 ml. flask containing 5 ml. distilled water, 0.2. gm. urea and 0.1 gm. KH_2PO_4 were added. The two flasks were autoclaved for 20 minutes. After cooling to room temperature, the contents of the 25 ml. flask were transferred to the larger flask. For use, 30 ml. volumes of the mixture were poured into 250 ml. sterile flasks.

(3) Yeast Nitrogen Base Medium (YNB):

This chemically defined medium developed by Wickerham (1946) was prepared by dissolving 0.67 gm. Bacto Yeast Nitrogen Base in 100 ml. distilled water. The composition is given in Appendix I. For normal use 2 gm. glucose was added before sterilizing in an autoclave. For some experiments the glucose content was varied.

Sporulation

Cells grown on any of the preceding media were sporulated in 2% potassium acetate in distilled water. The cell density of the suspension was adjusted to approximately 40 million per ml., with the

aid of a Spencer AO haemocytometer counting chamber, or by examining the turbidity. The flasks were incubated in the same manner as the pre-sporulation flasks but for longer periods of time, up to four days.

Solid sporulation medium was prepared by dissolving 2 gm. Difco agar and 2 gm. potassium acetate in 100 ml. distilled water. After autoclaving, Petri dishes were poured, each with 10-15 ml. of the medium. These dishes of sporulation medium were inoculated thinly with cells from pre-sporulation medium. Slight vegetative growth was observed to occur during the first 2 days and abundant spores were normally present by the fourth day.

Spore Germination

To obtain germinating spores for Giemsa staining and DNA analyses, liquid media were used. The yeast was separated from sporulation medium by centrifugation, washed with sterilized distilled water and suspended in MYPG medium at a density of approximately 40 million asci per ml. 250 ml. flasks each containing 30 ml. germination culture were incubated under the same conditions as the presporulation cultures.

For observing conjugation of germinating spores directly under the microscope, MYPG medium containing Agar SP (5%) was poured into 10 X 100 mm. petri dishes, 10-12 ml. per dish. A loopful of suspension (approximately 10 million asci per ml.)

was put on the surface of this agar and covered with a sterile cover glass. The details of this technique were described by Miller (1970). The stiff agar held the asci in one position and germination of all spores in an ascus took place in one plane, between glass and agar, facilitating the observation of conjugation.

Preparation of Spore Suspensions

Yeast ascus walls can be removed by Helix digestive enzymes (Johnston and Mortimer, 1959, and Snider and Miller, 1964). In the present work "Suc Digestif D'Helix pomatia", supplied by Industrie Biologique Francaise, was used. The contents of each vial (1 ml.) were diluted to 5 ml. with pH 6.8 phosphate buffer solution (Harleco, 1%). Then approximately 10 million asci were added per ml. of this mixture and shaken frequently to prevent settling, for 10 minutes at room temperature. Virtually all the spores were freed from their asci but unsporulated cells were not visibly affected. Vegetative cells in the logarithmic phase of growth treated similarly did not lose their walls and were found to remain viable.

Use of Finder Slides

The Finder slides were obtained from Graticules Ltd., London, England. These slides (which measure 28 x 75 mm.) are divided into minute areas which are marked with microletters and numbers easily identifiable under the microscope. These slides enable one to note the location of single cells or spores on the slide, and,

by re-examining at later times, to follow their progress in germination, growth, and sporulation. They have been used with success in algal morphogenesis studies by Wilfong (1969) and have been adapted here for use with yeast by:

- (1) burying the slides under a clear agar layer;
- (2) germinating and sporulating the yeast on top of the agar; and
- (3) covering the inoculum with a Teflon membrane (instead of a coverglass) which restricts water loss but is sufficiently permeable to oxygen to permit sporulation. Fig. 2A shows the appearance of a Finder slide under the microscope, and Fig. 2B shows the arrangement employed.

In practice 10-12 ml. volumes of pre-sporulation medium containing Agar SP(5%) were poured into 10 X 100 mm. petri dishes, each containing a Finder slide previously sterilized in 70% ethanol. After the medium had solidified, a loop of a spore suspension which had been treated with Helix enzymes, washed by centrifugation with sterile distilled water and agitated in a Waring Blendor, was put on the agar surface. The area was then covered with a piece of Du Pont 200-gauge Teflon F.E.P. type A membrane (Miller, 1970). The spores germinated and grew vegetatively to form microcolonies, and after several days some of the cells in these colonies sporulated. Growth and sporulation were much affected by the nature of the medium and this matter will be dealt with under Results.

FIGURE 2A: Drawing of a Finder Slide showing the coding system to locate spores under the low power of a microscope.

FIGURE 2B: The arrangement employed for the use of Finder Slide. (Out of proportion, for illustration only)

FIG 2A

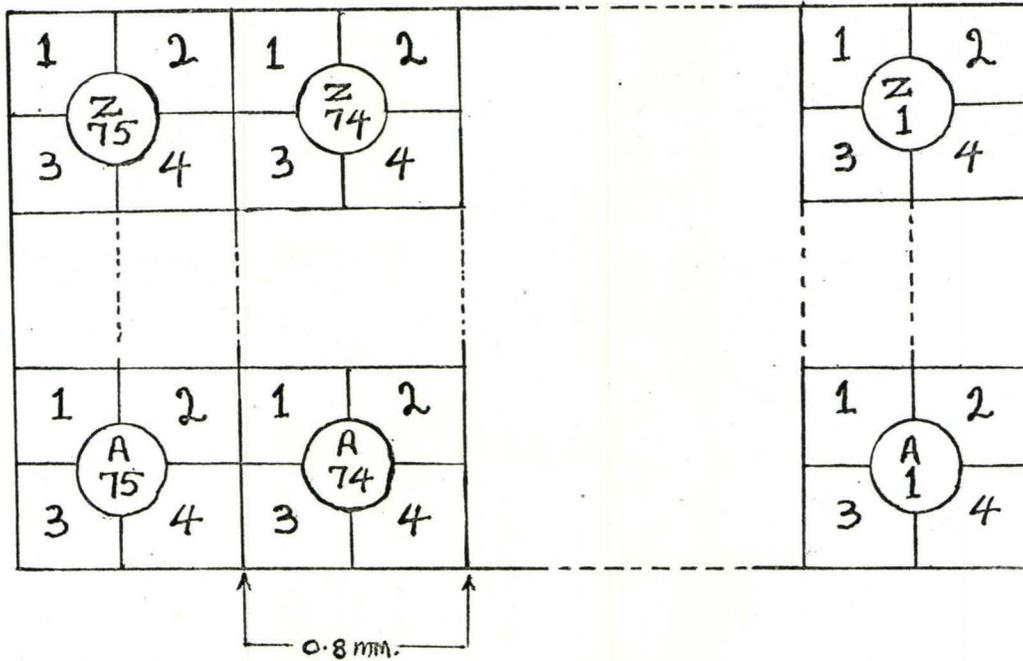
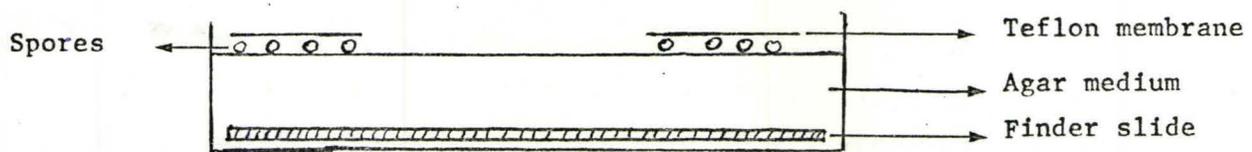


FIG 2B



Giemsa Stain Techniques

For preliminary studies Miller's (1968) abbreviated Giemsa method was followed. On a clean slide a drop of cell suspension in distilled water is spread and allowed to dry. For fixation the slide is placed in a Coplin jar containing ethanol and acetic acid (3:1) for eight minutes. The slide is washed with cold water, placed in a Coplin jar containing 5N HCl at room temperature for 6-7 minutes and washed again in cold water. The staining is done for five minutes in a Coplin jar containing Gurr's Improved Giemsa R66 stain diluted 1/10 with Gurr's pH7 buffer (ESBE Laboratory Supplies, Toronto, Ontario). After washing again in water the smear is allowed to dry and covered with a drop of immersion oil. The nuclei appear dark blue under oil immersion.

A second Giemsa technique obtained from Dr. C.F. Robinow (Personal Communication, 1971) was modified and used for Giemsa staining. A thin film of Mayer's albumin fixative (Fisher Scientific Co.) is spread on a 22 x 22 mm. cover glass. Cells are placed on albumin film and air dried. Fixation is done by placing the cover glass in a Columbia staining jar containing Helly's fixative (50 ml. 3% potassium dichromate + 50 ml. aqueous saturated mercuric chloride + 1 gm. sodium sulfate + 2.5 ml. formaldehyde; Gurr, 1957) for ten minutes. The cover glass is rinsed with 70% ethanol and placed in a Columbia staining jar containing 1N HCl for 8-10 minutes at 60°C. for hydrolysis. Vegetative cells are extracted with 1% sodium chloride at 60°C. for one hour before hydrolysis. The rinsed cover glass is placed in Gurr's

R66 Giemsa stain diluted to 1/10 with Gurr's pH 6.8 buffer for two hours in a staining jar. The cover glass is mounted on a slide carrying a drop of the diluted Giemsa stain and vigorously flattened between wooden blocks. Finally, the edges of the cover glass are sealed by applying petroleum jelly with a tooth pick to prevent evaporation. The nuclei appear bluish pink to blue.

Determination of DNA content of Yeast Cells

DNA was extracted from known numbers of cells or asci and estimated by the diphenylamine method. The steps of this method are:

Estimation of total number of cells:

The cells are counted with the aid of a Spencer AO haemocytometer counting chamber. All buds are counted as cells. In fact the slightest protuberance indicative of a bud is counted as a cell in view of the reports that the DNA of a yeast cell has replicated at or just before the time that a bud becomes visible (Beams et al., 1953; Ogur et al., 1953; Williamson and Scopes, 1960; and Williamson, 1965). The number of cells or asci used for each DNA determination was 3×10^9 .

DNA Extraction:

A modified form of Burton's (1956) method was followed.

Briefly the steps are:

(a) Cells are treated with 70% ethanol for 5 minutes at 4°C. before counting and then resuspended in sterilized distilled water. This treatment fixes the cells and hence counting can be done

at any convenient time. Also most of the carbohydrates, free amino acids and organic acids are removed.

(b) The counted cells are treated with 2:1 chloroform-methanol for 5 minutes at room temperature to remove most of the lipids.

(c) DNA is extracted with 2.5 ml. 0.5N perchloric acid at 70°C. for 20 minutes. The process is repeated to ensure the total recovery of DNA and the two extractions are combined.

Reagent and colour development:

Fresh reagent was prepared every time just before use as follows:

1.5 gm. diphenylamine (Fisher Scientific Co., indicator grade) is dissolved in 100 ml. glacial acetic acid. To this solution 1.5 ml. conc. sulfuric acid and 0.5 ml. aqueous acetaldehyde (16 mg./ml.) is added.

2.5 ml. of DNA extract is mixed with 5 ml. of the diphenylamine reagent. The control tube contains 2.5 ml. of 0.5N perchloric acid and 5 ml. of reagent. The tubes are incubated for colour development in the dark at 27-30°C. for 16 hours. The intensity of the colour is then measured at 600 m μ with a Bausch and Lomb Model 340 colorimeter.

To make the determinations quantitative, a standard curve was prepared using salmon sperm DNA (Calbiochem, grade A).

Electron Microscopy

Thin Sectioning: Cells were separated from the medium by centrifugation and rinsed with sterilized water. The first fixation

was done with 5% glutaraldehyde for 1/2 hour at 0°C. and the cells were then separated from glutaraldehyde fixative by centrifugation and washed with Millonig (1962) buffer pH 6.8. A second fixation was done in 2% osmium tetroxide for one hour at 27°C. Cells were again separated from fixative, rinsed with buffer and then dehydrated in an ethanol series. Ethanol was replaced with proplene oxide, and the cells were embedded in Spurr's (1969) medium. Sections of 550-650°A thickness were made with an LKB ultra-microtome using a glass knife. The staining was done on 100/400 mesh (Mason and Morton Ltd.) copper grids which were floated on drops of lead citrate (Reynolds, 1963) for one minute and the sections were examined with a Zeiss EM9 electron microscope.

Replica preparation: A suspension of spores was spread on a clean microscope slide. After air drying the slide was placed at an angle of 35° and 6 cm. away from the source of evaporation, in an Edward's model 4 vacuum evaporator. One pellet of carbon-platinum (Ladd Industries Ltd.) was completely evaporated to obtain good contrast. The specimen was subsequently reinforced by evaporating carbon (Johnson and Matthey Ltd.) 8 cm. above the slide. The amount of carbon deposited was checked by using a folded piece of filter paper near the slide.

The specimen was scored into 2 mm. squares with a razor and the slide was briefly dipped into a plastic petri dish containing 10% hydrofluoric acid to detach the squares from the slide. The

squares were then floated on chromic acid (saturated Cr_2O_3 solution) for two hours to remove the yeast. The replicas were washed with distilled water and picked up on R150A copper grids (Mason and Morton Ltd.) previously made sticky with 0.01% formvar. The grids were examined with the electron microscope.

Freeze-etching technique: The cells were washed with distilled water and then suspended in 20% glycerol solution. A small droplet of a very concentrated suspension was placed on a 3 mm. gold disc which was immediately immersed into freon 22 cooled with liquid nitrogen. After approximately five seconds in freon 22, the sample was stored in N_2 and was fractured, etched and then replicas were prepared in a Balzers freeze-etching device, model BA360M, by using Bishop's (1969) technique. For fracturing a Schick injector type razor blade was used. Etching was allowed to take place for five minutes and then replicas were prepared by shadowing first with platinum and then with carbon. The replicas were separated from the specimens by immersing the discs in distilled water. Adhering cells were removed by floating the replicas on chromic acid (saturated Cr_2O_3) for two hours. Further cleaning was accomplished by floating the replicas on sodium hypochlorite solution (4-6% NaOCl ; Fisher Scientific Company) for ten minutes. The replicas were then washed with distilled water and picked up on copper grids (Mason and Morton Ltd.) previously made sticky with 0.01% formvar, for examination with the electron microscope.

RESULTS

A. INVESTIGATIONS ON THE LIFE CYCLE OF TWO-SPORED YEASTS

The nuclei of Saccharomyces during vegetative growth undergo mitotic divisions each of which gives rise to two nuclei. But during sporulation the nucleus of a diploid cell undergoes meiosis to form four haploid nuclei each of which may be enclosed by a spore wall. The spores of Saccharomyces of opposite mating type at germination, conjugate to give rise to the diploid phase of the life cycle. It seemed of interest to determine whether a number of predominantly two-spored yeasts had life cycles of the established kind.

Nuclear Division During Sporulation and Conjugation During Germination

Samples of cells of fifteen strains of yeast producing predominantly two-spored asci and two strains (EVE-18 and NCYC-79) producing pre-dominantly four-spored asci were Geimsa stained at successive times during sporogenesis in potassium acetate (2%) to determine the maximum number of nuclei produced per cell before spore wall formation.

To observe germination of these spores, the yeast from sporulating medium was transferred to the surface of MYPG 5% SP agar medium with a sterile loop and the area was covered with a cover glass (Miller 1970). The germinating spores were observed directly by placing the plate under the microscope at successive times between five and thirteen hours. The results of these observations are listed in Table I.

TABLE I

Number of nuclei formed per ascus during sporulation of 17 yeast strains, and presence or absence of conjugation during spore germination.

<u>Strain</u>	<u>Maximum number of nuclei observed per ascus</u>	<u>Conjugation during spore germination</u>
19e1	2	-
ATCC-1142-B	4	+
ATCC-4098	2	-
ATCC-4117	2	-
ATCC-4921	4	+
IFO-0553	4	+
IFO-0556	4	+
IFO-2000	4	+
IFO-2011	4	+
IFO-2040	4	+
IFO-2043	4	+
IFO-2045	4	+
IFO-2241	4	+
IFO-2244	4	+
NCYC-620	4	+
EVE-18	4	+
NCYC-79	4	+

After the spore walls became visible, it was consistently observed that the asci of those strains containing a maximum of four nuclei, but only two spores, had two nuclei in the epiplasm of the ascus not enclosed within the walls of the two spores. With the other three strains, namely 19e1, ATCC-4098 and ATCC-4117, which produced only two nuclei before sporulation, no nuclei were observed in the epiplasm. However, with those asci that produced only one spore, the second nucleus was usually visible in the epiplasm (Fig. 7).

Conjugation during spore germination was observed only in those strains whose asci contained four nuclei during sporulation. Fig. 174 shows a typical conjugation in four-spored strain EVE 18.

The Giemsa stain of germinating 19e1, ATCC-4098, and ATCC-4117 spores showed that the nucleus of each spore divided at the time of bud formation so that the bud and the mother spore had one nucleus each (Figs. 11 and 12).

Effect of Presporulation Medium Composition of Cell Dimensions and on Number of Spores per Ascus.

It appeared from casual observations that the cells of the two-spored yeast strains were smaller than those of the two strains, EVE-18 and NCYC-79, which produced predominantly four-spored asci. The possibility was considered that the smaller size of asci was an important factor in determining the low number of spores produced. If, by some means the size of the cells could be made to

increase, these strains might then produce more spores per ascus.

Preliminary experiments indicated that cells of EVE-18 and NCYC-79 were larger when grown on MPU than when grown on MYPG. An experiment was then done in which all 17 of the strains were grown on those two media, and then sporulated in 2% potassium acetate solution. The cell sizes and number of spores per ascus are shown in Table II.

These results indicate that (a) with every strain but one (IFO-2045) cells grown in MPU were longer than when grown in MYPG, (b) with approximately half of the strains the percentage of cells that sporulated was greater when MPU was the pre-sporulation medium, (c) approximately half of the pre dominantly two-spored strains which produced four nuclei before spore wall formation showed an increase in the proportion of 3-4 spored asci, and (d) 19e1, ATCC-4098 and ATCC-4117 which never had more than two nuclei per ascus did not produce any 3-4 spored asci after growth in either pre-sporulation medium.

It would appear that increase in cell dimension does not necessarily lead to an increase in the number of spores per ascus.

TABLE II: Effect of two pre-sporulation media on cell sizes of 17 yeast strains, and on number of spores per ascus.

Strain	Cells grown 24 hr in MYPG					Cells grown 24 hr in MPU				
	Dimensions		% sporulation in 4 days			Dimensions		% sporulation in 4 days		
	Long axis μ	Short axis μ	Asci with 1-2 spores	Asci with 3-4 spores	Total asci	Long axis μ	Short axis μ	Asci with 1-2 spores	Asci with 3-4 spores	Total asci
19e1	6.0 \pm 0.3	3.1 \pm 0.3	90%	0%	90%	7.2 \pm 0.4	3.3 \pm 0.3	91%	0%	91%
ATCC-114218	5.7 \pm 0.5	3.7 \pm 0.4	31%	1%	32%	5.9 \pm 0.6	3.8 \pm 0.4	28%	0%	28%
ATCC-4098	5.9 \pm 0.5	3.9 \pm 0.5	38%	0%	38%	6.9 \pm 0.5	4.1 \pm 0.4	44%	0%	44%
ATCC-4117	4.3 \pm 0.3	3.4 \pm 0.3	71%	0%	71%	5.2 \pm 0.4	3.4 \pm 0.5	72%	0%	72%
ATCC-4921	5.1 \pm 0.6	2.9 \pm 0.5	50%	2%	52%	5.4 \pm 0.5	2.6 \pm 0.5	73%	1%	74%
IFO-0553	4.0 \pm 0.3	2.7 \pm 0.4	15%	1%	16%	4.3 \pm 0.3	3.3 \pm 0.4	74%	6%	80%
IFO-0556	6.4 \pm 0.5	2.8 \pm 0.5	59%	1%	60%	8.0 \pm 0.6	2.8 \pm 0.5	51%	4%	55%
IFO-2000	6.6 \pm 0.6	3.4 \pm 0.5	31%	13%	44%	8.4 \pm 0.7	3.3 \pm 0.5	61%	16%	77%
IFO-2011	4.9 \pm 0.5	3.1 \pm 0.3	21%	10%	31%	6.9 \pm 0.6	3.8 \pm 0.6	40%	6%	46%
IFO-2043	4.7 \pm 0.5	3.7 \pm 0.5	13%	10%	23%	6.7 \pm 0.6	3.8 \pm 0.5	28%	37%	65%
IFO-2040	5.6 \pm 0.6	3.4 \pm 0.4	18%	0%	18%	6.6 \pm 0.6	3.6 \pm 0.5	33%	11%	44%
IFO-2045	6.6 \pm 0.6	3.5 \pm 0.5	38%	0%	38%	6.4 \pm 0.7	3.6 \pm 0.4	70%	3%	73%
IFO-2241	5.9 \pm 0.4	3.4 \pm 0.3	70%	1%	71%	6.8 \pm 0.5	3.6 \pm 0.4	66%	6%	72%
IFO-2244	5.4 \pm 0.5	3.9 \pm 0.5	52%	1%	53%	5.9 \pm 0.6	3.4 \pm 0.7	62%	1%	63%
NCYC-620	6.2 \pm 0.6	3.4 \pm 0.4	85%	0%	85%	7.4 \pm 0.7	3.5 \pm 0.5	88%	0%	88%
NCYC-79	5.6 \pm 0.6	4.0 \pm 0.4	3%	81%	84%	10.1 \pm 0.8	3.9 \pm 0.5	2%	83%	85%
EVE-18	6.2 \pm 0.6	3.4 \pm 0.2	5%	61%	66%	9.8 \pm 0.9	4.7 \pm 0.2	5%	64%	69%

Effect of adding glycerol to acetate sporulation medium

Dr. P. V. Patel observed (personal communication 1971) that certain strains of Saccharomyces cerevisiae Hansen, produced more than the normal maximum of four spores per ascus when 2% glycerol was included in 1% potassium acetate sporulation medium. Cells of all 17 strains, after growth in MYPG medium, were shaken in a water bath at 27°C. for four days in Patel's sporulation medium. There was no evident increase in the number of spores per ascus.

Sporulation of Cells in Colonies Derived from Single Spores

Since the asci of three strains (19e1, ATCC-4098 and ATCC-4117) appear to lack reduction division, it is possible that their sporulation is preceded by mitosis instead. If so, the spores should, like their parent cells, be able to produce progeny capable of sporulation. To check this possibility, experiments were performed which involved isolation of single spores, the development of colonies from them, and the induction of sporulation in these colonies.

It was found possible to obtain spore suspensions by removal of the ascus walls with Helix enzymes, but most of the pairs of spores thus freed from their asci remained together even after vigorous agitation for 5-10 minutes on a Vortex mixer (Scientific Industries Inc. N.Y.) Ten minutes of agitation in a Waring Blendor was found to separate most of the spore pairs, producing suspensions consisting predominantly of single spores. These spore suspensions were used in two types of experiments: dilution plating, and Finder slide.

For the dilution plating experiments, the cells were separated by centrifugation from two 30 ml. sporulated cultures after four days, washed once in sterilized distilled water, treated with the contents of a vial of Helix preparation (diluted to one-tenth in the case of 19e1 and to one-fifth with ATCC-4117) until a microscopic check showed the ascus walls to be almost all removed. The cells were separated by centrifugation, washed, resuspended in 90-100 ml. water in the blendor jar, and agitated for ten minutes. After estimation of cell density with the counting chamber and appropriate dilutions, petri dishes containing MYPG agar were flooded evenly with 0.1 or 1.0 ml. volumes of suspension and incubated at 27°C. After 24 hours cells from isolated colonies were transferred with sterilized toothpicks to plates of potassium acetate sporulation agar. The cells were checked for spore formation after 1-4 days. In the few instances where no spores were found, the cells were transferred to plates of MYPG agar, grown for 24 hours, and again inoculated on sporulation

agar. The results are shown in Table III.

With strain 19e1, 403 colonies from the dilution plates and with ATCC-4117, 154 colonies, were examined for their sporulation ability, and every one was found to sporulate after transfer to sporulation medium to form two-spored asci. This test was not performed on strain 4098 because, owing to its poor sporulating ability, it was not found possible to obtain suspensions with a predominance of single spores.

Table III also demonstrates the effectiveness of the Waring Blendor treatment in separating spore pairs. It is of interest that with strain ATCC-4117 the Helix enzymes alone caused about one quarter of the spore pairs to separate.

The treatment with Helix enzymes and Waring Blendor had no observed effect on spore viability. The number of colonies that grew per dilution plate was not less than the number of cells or spores plated. Similarly, it was found that vegetative cells from growth medium did not have their walls removed by treatment with the Helix preparation, nor was their viability affected. This indicates a difference between the walls of the vegetative cell and of the ascus.

The Finder slide method, described in detail in Materials and Methods, requires a medium capable of supporting germination, growth and sporulation. Therefore six media of different nutritional composition were tried in hope of finding a satisfactory one. All

TABLE III

Sporulation of cells in colonies grown from single spores and spore pairs freed from asci by Helix enzymes and Waring Blendor treatment..

Yeast Strain	Cell Treatment	% Unsporulated cells	% Asci with one spore	% Asci with two spores	% Free Single Spores	% Free spore pairs	% Colonies sporulated
19e1	Sporulated	5	6	89	0	0	
	Sporulated, Helix enzymes treated for 20 min.	5	0	0	11	84	
	Sporulated, Helix enzymes treated for 20 min. Waring Blendor treated.	0	0	0	76	24	100 *
ATCC-4117	Sporulated	22	5	73	0	0	
	Sporulated, Helix enzymes treated for 10 min.	17	0	0	30	53	
	Sporulated, Helix enzymes treated for 10 min. Waring Blendor treated.	11	0	0	60	29	100 **

* 403 colonies were checked

** 154 " " "

contained glucose, shown by Seigel (1969) to be an effective germination stimulant, and usually other materials required for growth. In all but one (MYPG) the glucose content was low, as glucose inhibits sporulation at moderate concentration but stimulates at low (Miller and Hoffmann-Ostenhof, 1964).

The results obtained with Finder slides are summarized in Table IV. MYPG medium was unsatisfactory as the cells grew to form large colonies which merged. Spores of 19e1 formed in single spore colonies on all the other media, and of ATCC-4117 on only three media. In the latter strain the media on which no sporulation occurred in colonies grown from single spores did not support spore formation on the agar surface anywhere under the Teflon membranes where some colonies must have developed from pairs of spores and unsporulated cells. This implies that lack of sporulation was owing to the composition of the medium, and not to any need for conjugation. No cells in colonies that developed from single spores of EVE-18 were seen to sporulate, but asci were observed to develop elsewhere under the membrane on media 2, 4 and 6 in colonies which presumably grew from more than one spore or from unsporulated cells. This implies that these media could support sporulation of diploid EVE-18 cells, but not of its haploid cells derived from single spores. ATCC-4098, a third two-spored strain not included in the table, did not form spores in any colonies derived from single spores on any of the six media. But this strain sporulates poorly, even on potassium acetate sporulation medium.

The results of this experiment confirm that, at least for

TABLE IV

Observation of sporulation of cells in microcolonies grown from single spores of three yeast strains on six media, using the Finder slide technique.

Composition of medium	Yeast strain	Number of single spores marked and observed	Results
(1) MYPG 5% agar SP	19e1 ATCC-4117 EVE-18		Too much growth to see individual colonies " " " " " " " " " " " "
(2) 0.01% glucose 0.1% malt extract (Bacto) 5% agar SP	19e1 ATCC-4117 EVE-18	8 8 8	Spores in 18 days No spores in 28 days " " " " "
(3) 0.1% glucose 0.67% YNB 5% agar SP	19e1 ATCC-4117 EVE-18	8 8 8	Spores in 15 days No spores in 28 days " " " " "
(4) 0.01% glucose 0.67% YNB 5% agar SP	19e1 ATCC-4117 EVE-18	10 10 10	Spores in 9 days Spores in 12 days No spores in 28 days
(5) 0.01% glucose 0.34% YNB 5% agar SP	19e1 ATCC-4117	10 10	Spores in 4 days Spores in 6 days
(6) 0.01% glucose 5% agar SP	19e1 ATCC-4117 EVE-18	10 10 10	Spores in 7 days Spores in 7 days No spores in 28 days

19e1 and ATCC-4117, the progeny of single spores can sporulate. This contrasts with EVE-18, a typical four-spored strain, which undergoes meiosis to form four nuclei (Fig. 13) and four haploid spores during sporogenesis, and then shows conjugation at the time of spore germination (Fig. 14). Cells in colonies derived from single spores of this strain, as expected, did not sporulate.

DNA Content of Cells of strain 19e1 at Different stages of Life Cycle

It has been shown above by Giemsa staining that only two nuclei were formed per ascus during sporulation of yeast strains 19e1, ATCC-4117 and ATCC-4098. At the time of germination of spores of these three strains no conjugations were observed. The progeny of single spores of 19e1 and ATCC-4117 were found to be able to sporulate without coming in contact with cells derived from other spores. These observations are consistent with the hypothesis that nuclear division in the ascus of these strains is mitotic, not meiotic. To investigate further the type of nuclear division taking place prior to sporulation, a comparison was made of the DNA content of spores and vegetative cells. Strain 19e1 was used as it consistently produced high yields of asci (90 - 95%) in 30 hours in sporulation medium. Two strains of known ploidy, X-901-35C (haploid) and X-190-T (tetraploid), were included in the experiment for reference.

Vegetative cells of all the strains were grown in 30 ml. volumes of MYPG for 24 hours on the water bath shaker at 27°C. Ascii

of strain 19e1, were obtained by washing MYPG-grown cells with sterile distilled water and incubating for 30 hours in 30 ml. volumes of 2% potassium acetate at a density of approximately 40 million cells per ml. To obtain germinating spore cultures, the yeast was separated from a sporulated culture by centrifugation and resuspended in the same volume of MYPG. After incubation in the water bath shaker at 27°C for seven hours, 65 to 75% of the asci contained at least one germinated spore. The criterion for spore germination was the production of a visible bud.

Yeast separated from the culture liquids by centrifugation was washed with sterilized distilled water, treated with ethanol, chloroform-methanol, and perchloric acid as described in Materials and Methods. Cell counts were made after ethanol treatment using the haemocytometer counting chamber, and the final determinations were made on 3×10^9 cells. Four experiments were done and the mean values with standard deviations are shown in Table V.

The DNA content of vegetative cells of ATCC-4098, ATCC-4117, 19e1, and EVE-18 was approximately twice that of haploid, X-901-35C and approximately half that of tetraploid, X-190-T cells, indicating that all four of these strains are diploid. The asci of 19e1, which contain predominantly two spores per ascus with one nucleus in each spore, had a DNA content approximately twice that of the vegetative cells of this strain. That is each spore with one nucleus contained the same amount of DNA as a vegetative cell. This implies that the

TABLE V

Equivalents of *Ag.* salmon sperm DNA in yeast
vegetative cells, asci and germinating spores.

Strain	Ploidy	Stage in Life Cycle	DNA μ g. per 3×10^9 cells
X-901-35C	Haploid	Vegetative cells	51 \pm 8
X-190-T	Tetraploid	"	207 \pm 29
EVE-18	Presumed Diploid	"	104 \pm 14
ATCC-4098	?	"	107 \pm 18
ATCC-4117	?	"	108 \pm 11
19e1	?	"	118 \pm 25
19e1		Asci	211 \pm 29
19e1		Asci with germinating spores	428 \pm 54

nuclear division preceding sporulation was mitotic.

By Giemsa staining it has been shown (Figs. 10, 11 and 12) that the nucleus divides during spore germination as in the budding of vegetative cells. The DNA determinations of cultures with 65-75% germinated spores showed that DNA had become double that of the spores, indicating that this division was also mitotic. If it were the second division of a meiosis, the DNA content would not be expected to increase during spore germination.

Effect of Inhibitors of Meiosis on Sporulation of Yeast

Miller (1964) showed that when glucose or ammonium sulphate was present in an acetate sporulation medium, spore formation was inhibited. Giemsa staining demonstrated that the block to sporulation occurred at the stage of meiosis. As the ~~two~~ strains (19el, ATCC-4117, and ATCC-4098) appear to sporulate without meiosis, it therefore was thought to be of interest to study the effects of glucose and ammonium sulphate on their sporulation.

Cells growing in logarithmic phase in MYPG medium were separated by centrifugation. After washing with sterilized distilled water, they were suspended in various sporulation media and incubated in the usual manner. The cells were stained and examined for nuclear division and sporulation after 24 hours. The results of this experiment are shown in Table VI. They confirm Miller's (1964) finding that glucose and ammonium sulphate inhibited meiosis in a "normal" four spored yeast, but the two 2-spored strains

sporulated well in 2% acetate + 1% glucose sporulation medium, with no or comparatively small inhibition. However, ammonium sulphate when present in the acetate medium, inhibited sporulation completely with all strains. With ammonium sulphate alone (i.e. without acetate) sporulation was very similar to that in distilled water. On the other hand, 1% glucose alone supported sporulation of 19e1 but not of the other. It is of interest that when sporulation occurred in 19e1 in distilled water or ammonium sulphate alone, there was rarely more than one spore per ascus but two nuclei were found in these asci, only one of which was enclosed by the spore wall. The epiplasm of these one-spored asci broke down quite rapidly in comparison with cells sporulating in presence of acetate. After 48 hours the epiplasm, including the nucleus, was usually completely disintegrated and the asci seemed empty except for the single spore.

TABLE VI

Effect of 2 Inhibitors of yeast meiosis and sporulation (glucose and ammonium sulphate) on sporulation of three strains of yeast.

Sporulation medium	Yeast Strains								
	19e1			ATCC-4117			EVE-18		
	Nuclei per ascus	Spores per ascus	% spores	Nuclei per ascus	Spores per ascus	% spores	Nuclei per ascus	Spores per ascus	% spores
Distilled water	2	1	69	2	1	41			0
2% Potassium acetate	2	2	94	2	2	71	4	3-4	63
2% Potassium acetate + 0.1% Ammonium sulphate			0			0			0
2% Potassium acetate + 1% glucose	2	2	83	2	2	49			0
0.1% Ammonium sulphate	2	1	61	2	1	2			0
1% Glucose	2	2	75			0			0

B. ULTRASTRUCTURAL STUDIES ON A TWO-SPORED YEAST

When spore suspensions of 19c1, ATCC-4098 and ATCC-4117 were prepared by dissolving the ascus walls with Helix digestive enzymes, it was observed that the two spores of an ascus had a strong tendency to stay together. But when the walls of typical four-spored asci of EVE-18 were removed, in the same manner, the spores separated quite readily, indicating that these spores were held together only by the ascus wall. Similar behaviour to that of EVE-18 was observed by Johnston and Mortimer (1959) and Patel (Personal communication, 1970) with four-spored strains of Saccharomyces cerevisiae. The possibility was considered that some structure was holding the pairs of spores together in 19c1, ATCC-4098 and ATCC-4117 after ascus wall removal. No distinct connection between the spores of each pair could be seen by light microscope (oil immersion) examination of intact asci (Fig. 9) or of spore pairs freed from their asci. Electron microscope studies were therefore undertaken, using a variety of techniques, to investigate the nature of the linkage.

Presence of "Inter-sporal Body" between Sister Spores

For preliminary observations, carbon-platinum replicas were made of the pairs of spores of 19c1 with and without the ascus wall. In Fig. 15, in which the spores are still within the ascus wall, no structure can be observed between them. Fig. 16 is an electron micrograph of the replica prepared from a pair of spores after ascus wall removal by the Helix enzymes. The arrow indicates a continuity

between the two spores.

In further investigations, the material was fixed, embedded and sectioned. The sections were contrasted with lead citrate. Fig. 17 shows a structure between the two spores which appears distinct from the adjacent epiplasm. A similar structure is apparent in Fig. 18 in which the two sister spores appear slightly separated from each other but the structure appears to link them together. This electron micrograph indicates (arrows) the existence of a membrane which encloses the spores and contacts the structure, at least on one side. It was decided to designate this structure between the two spores of an ascus as "inter-sporal body".

To define the inter-sporal body more fully, further work was thought desirable using the freeze-etching technique. Fig. 19 shows that after ascus wall removal by fracturing and etching, the two spores (S, S) are only partly visible, as the central portion of each is concealed by the epiplasm. This material protrudes out and extends partly over the spores as revealed by Fig. 20 in which part of the covering has been removed by fracturing. Fracturing (and possibly etching) more deeply (Fig. 21) allows one to see that there is a membranous inner surface to the epiplasmic layer. It does not fill all the space between the spores, i.e., it does not have a double concave lens shape but rather, is belt-shaped. When the fracture is more median (Fig. 22) the inter-sporal body is revealed.

Development of Inter-sporal Body

In the sporulating cultures, spore development was not synchronous. With strain 19e1 the first spores appeared 7-10 hours after vegetatively

growing cells were suspended in potassium acetate medium and sporulation was not complete for all cells in less than 24 hours. After approximately 16 hours all stages of sporogenesis could be seen.

There is no doubt that the inter-sporal bodies appear early, e.g. Fig. 19 shows spores from a ten-hour sporulating culture with a well developed inter-sporal body. Fig. 23 shows what may be a stage in the development of the inter-sporal body. Globules of unknown composition have accumulated between the spores. Since this stage was more frequently seen in 16-hour sporulating cultures than in 24- and 36-hour cultures, it may precede the fully developed inter-sporal body. Fig. 24, taken from a replica, prepared from a 24 hour sporulating culture, suggests that the globules in the inter-sporal space are merging with one another, and it appears that a connection is thus being established between the two spores leading to the formation of the "inter-sporal body".

Composition of Inter-sporal Body

The enzymes present in the Helix preparation used for ascus wall removal are not defined by the supplier (Industrie Biologique Francaise). Holden and Tracey (1950) found that Helix digestive extract has 30 or more enzymes of which 20 are carbohydrases including cellulase and chitinase. However, they did not detect proteases. The fact that after ascus wall removal the members of spore pairs stay together indicates that the inter-sporal body is resistant to Helix enzymes and that its composition is different from that of the ascus wall.

Since it is possible that the Helix enzymes preparation used lacked active protease and lipase, the pairs of spores after removal of the ascus walls were treated with Calbiochem pronase (a protease) and lipase (1 mg. per 10ml. pH 8.6 Harleco buffer) consecutively for 1/2 hour each at 27°C. The spores still did not separate when agitated in suspension on the Vortex mixer for five minutes. This indicates that the inter-sporal body is not made up of lipids and proteins.

Definition of the Inter-sporal body

The inter-sporal body is here defined as an epiplasmic structure which occurs between the members of a pair of ascospores of some yeasts and is 1 to 2 μ in diameter. It may be separated from the remainder of the epiplasm by a membrane. Fig. 25 on the next page shows the location of the inter-sporal body in relation to the rest of the components of the ascus. In shape it resembles a biconcave lens. Its fine structure and chemical composition differs from that of the remainder of the epiplasm and the ascus wall.

Invaginations associated with the Plasmalemma of Sporulating Cells and Spores

The electron micrographs of sections of the asci (Figs. 17 and 18) of strain 19e1 show numerous distinct invaginations of the spore plasmalemma. From examination of other electron micrographs of actively growing vegetative cells (not shown), the plasmalemma had few or no invaginations, but they became more conspicuous in the ascus plasmalemma after about three hours in sporulation medium, and

tended to increase in number with time. They were always conspicuous in the spore plasmalemma. They were less frequently seen, however, when the asci matured. Their function in sporulation was not investigated in the present study.

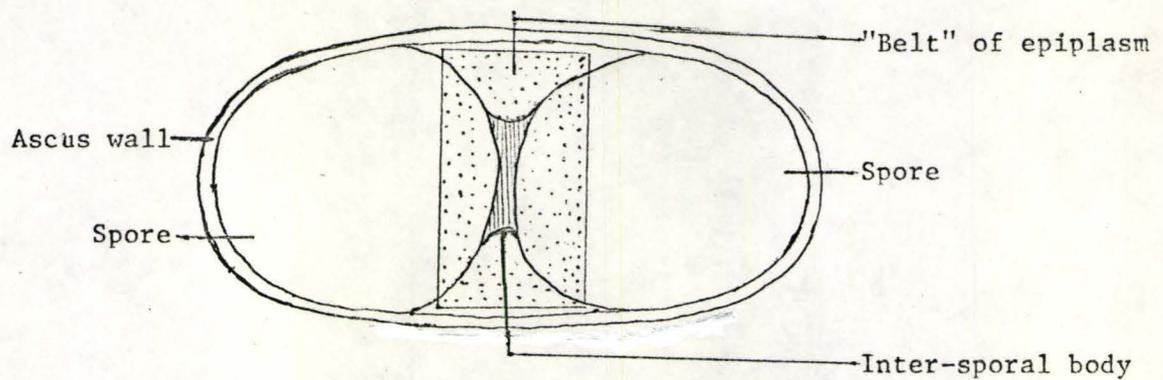


Figure 25: Diagram showing relationship of inter-sporal body to the other components of the ascus.

DISCUSSION

Life Cycle of Yeasts

Winge (1935) and Winge and Laustsen (1937 and 1939) established the existence of a life cycle in Saccharomyces involving a sexual process linking the haploid phase with the diploid phase. Cytological evidence was presented, and genetic segregations by spore formation in the asci were demonstrated. The first report of Mendelian segregation in yeast was by Winge and Laustsen (1939), working with Saccharomyces ludwigii. When diploid cells of genotype NnLl (N = normal cell growth; n = abnormal growth which was limited to the formation of one or two "hypha-like" cells; L = long cells and l = short cells), sporulated, the four spores produced per ascus were either two Nl and two nL, or two NL and two nl. The spores germinated to produce haploid vegetative cells which could, by mating, give rise to the diploid stage again. Mendelian segregation was shown for Saccharomyces cerevisiae by Lindegren and Lindegren (1943). Bevan (1953), Magni (1958) and Takahashi (1962) showed by genetical analyses that the spores of one, two, three or four spored yeast asci were haploid.

In the present study, out of 15 strains of yeast producing predominantly two-spored asci, 12 were found to have a normal-appearing meiosis during sporulation, but usually only two of the four resulting nuclei became enclosed within spore walls. With three strains, however, (19e1, ATCC-4117, and ATCC-4098) the cells during sporulation contained no more than two nuclei, each of which

became enclosed by a spore wall. At the time of germination no conjugation was observed between adjacent germinating spores. Cells in colonies derived from single spores of 19e1 and ATCC-4117 could sporulate, and determinations of DNA content indicated that both vegetative cells and spores were diploid. These observations could be accounted for by assuming that meiosis does not occur during sporulation of these strains, but that the nuclear division in the ascus is a mitosis. Other explanations, however, are possible and these will now be considered.

(1) During sporulation only the first division of meiosis takes place, and the second division is delayed until spore germination. But no such example of separation of the two divisions of meiosis in space and time, has been reported to my knowledge. Also, the results shown in Table V provide evidence that during spore germination, the DNA content of spore and bud became double that of the original spore, indicating that DNA is replicated for this nuclear division. This should not take place during the second division of a meiosis.

(2) Meiosis occurs but two of the four resulting haploid nuclei disintegrate and disappear. The remaining two become enclosed by spore walls. When these germinate the two haploid nuclei resulting from the mitotic division of the spore nucleus fuse to form a diploid nucleus. Dr. C. F. Robinow (personal communication, January 1971) reported that in Wickerhamia three of the four potential spore nuclei degenerate and the ascus invariably contains only one spore. Winge and Laustsen (1937) described a process in a Danish baking yeast which they called "direct diploidization" in which a haploid spore germinates and at the completion

of the first mitotic division the resulting two daughter nuclei fuse and as a result the diploid phase begins in the germinating spore. Winge and Roberts (1949) discovered a gene in Saccharomyces chevalieri which caused obligatory diploidization at the time of germination of single haploid spores or during subsequent growth of the colony. Such diploidized cells did not show conjugation, and gave rise to colonies which could sporulate.

There are strong arguments against this possibility. Firstly, disintegration of two of the four haploid nuclei is unlikely because thousands of Giemsa-stained sporulating cells were examined and none was ever seen that contained more than two nuclei. The assumption that nuclei not enclosed within a spore disintegrate is invalid, in any case, because sometimes only one spore was formed per ascus and the second nucleus was then consistently visible in the epiplasm outside the spore (Fig. 7). Also, in the other 12 predominantly two-spored strains which had normal meiosis in the ascus, the nuclei not enclosed within spore walls were still visible in the epiplasm of the ascus. Secondly, the two daughter nuclei formed at spore germination were never seen to fuse. Thirdly, the determinations of DNA content indicated the spore nucleus to be diploid.

(3) Similar to (2), but diploidization in this case results from "illegitimate" mating as described by Lindegren (1944). In this process haploid cells of the same mating type fuse to form diploids. This possibility is ruled out by the first and third arguments.

mentioned with reference to (2), above. Moreover, Lindegren's illegitimate diploids sporulated sparingly, and the spores were incapable of germination.

(4) Dr. C. F. Robinow (personal communication, April 1971) after Giemsa staining studies on two of the three strains (19e1 and ATCC-4117) confirmed that no more than two nuclei per ascus are produced during sporulation. On the basis of Giemsa staining and cytological observations, however, he concluded that the diploid state of the spore nucleus is not the result of its having arisen by an ordinary mitosis, but rather of its failure to carry out the second division of meiosis. This is, the spore walls develop around the products of meiosis I.

There is one observation which seems to contradict this proposal, i.e., glucose when present in acetate medium inhibits meiosis I, in the asci of "normal" four-spored yeasts (Table VI, strain EVE-18; and Miller 1964) but it had little or no effect on the nuclear division and sporulation of the two-spored strains. On the other hand, another inhibitor of yeast meiosis, ammonium sulphate, strongly inhibited the nuclear divisions in the ascus of these yeasts in the presence of acetate. The results with these two inhibitors therefore do not assist in resolving the problem.

If Robinow's proposal is correct, the end result of the nuclear division would be the same in so far as the ploidy is concerned.

6 Spore,

After consideration of these alternative explanations one must return to the assumption that all the nuclear divisions during vegetative growth, sporulation and germination of these unusual two-spored strains are non-reductional. Related to this are two interesting observations:

(a) A low, but fairly constant proportion (10-15%) of the sporulated cells of 19e1 and ATCC-4117 had, still attached, buds which lacked nuclei. Williamson and Scopes (1960) reported that the amount of DNA in synchronously growing yeast culture sharply doubled "at or just after" the appearance of visible buds. Possibly DNA synthesized in preparation for the mitotic division of budding was utilized instead for the division within the mother cell that preceded sporulation.

(b) Spores were first visible in strain 19e1 after only seven hours in sporulation medium. Other workers reported longer sporulation times, e.g. Pontefract and Miller (1962), 15-20 hours; Croes (1967), 14 hours. Possibly 19e1 can sporulate so quickly because mitosis (or meiosis I) can be completed more rapidly than meiosis. However, Chen and Miller (1968) observed similar sporulation times for 19e1 and EVE-18 (a strain with four spores per ascus). The other two strains which resembled 19e1, i.e. ATCC-4117 and ATCC-4098, required several hours longer than 19e1 to sporulate, but fewer of their cells completed the sporulation process.

Ganesan et al. (1958) removed single cells of yeast from 17 hour sporulating cultures to micro-droplets of nutrient medium, and

observed that up to 50% of the cells sporulated instead of growing vegetatively. They concluded that if the cells had initiated meiosis, as indicated by nuclear stains, the nutrient material could not prevent completion of sporulation. Obviously the final stage of sporulation, the formation of the spore walls around the nuclei is not inhibited by growth nutrients. It would be interesting to do similar experiments with two-spored yeasts.

The only known instances of mitosis in yeast asci were established genetically by Gutz. He found (1967) that in crosses of diploid and haploid cells of Schizosaccharomyces pombe, a small number of the resulting asci contained six haploid spores, four possessing markers of the diploid parent and the other two carrying markers of the haploid parent. It was concluded that during sporulation the haploid nucleus had divided mitotically and the diploid meiotically and, as a result the ascus contained two haploid spores from the haploid parent and four haploid spores from the diploid parent. More recently Gutz (personal communication, 1971) observed a similar situation in diploid x diploid crosses of the same yeast. One diploid nucleus divided mitotically and the other meiotically to produce an ascus containing two diploid spores and four haploid spores.

Guilliermond (1903) and (1905) published cytological observations on many yeasts. Included was a description of nuclear behavior during sporulation and spore germination of a two-spored strain of Saccharomyces pastorianus. In this strain during sporulation only two nuclei occurred

per ascus each of which became enclosed by spore walls, and there was no conjugation during spore germination. He did no further experimental work on this yeast, and, owing to the lack of understanding of yeast life cycles at the time, did not interpret his observations in terms of mitosis and meiosis.

From the observations made in this study the life cycle of three 2-spored strains of yeast can be depicted as in Fig. 26a. In Fig. 26b the life cycle of Saccharomyces yeasts, as normally accepted, is shown for comparison. The difference in the two life cycles is that no reduction division takes place in the two-spored strains before the spore production and thus no conjugation is required to achieve the diplophase again.

Inter-sporal Bodies

The electron micrographs of Bandoni et al. (1966) showed that the ascospores of Hansenula anomala were joined together with two projections arising on one or two sides of a spore and meeting similar projections from the adjacent spores. Thus the connections were visible in "dome to brim and brim to dome" arrangements. Kreger-van Rij and Ahearn (1968) and Kreger-van Rij (1968) saw similar bifurcate connections between ascospores of Hanseniaspora uvarum and Saccharomyces ludwigii.

Connections between adjacent spores of an ascus of Saccharomyces have never been reported. Johnston and Mortimer (1959) dissolved the ascus walls of Saccharomyces with digestive enzymes of Helix aspera

FIG 26 A

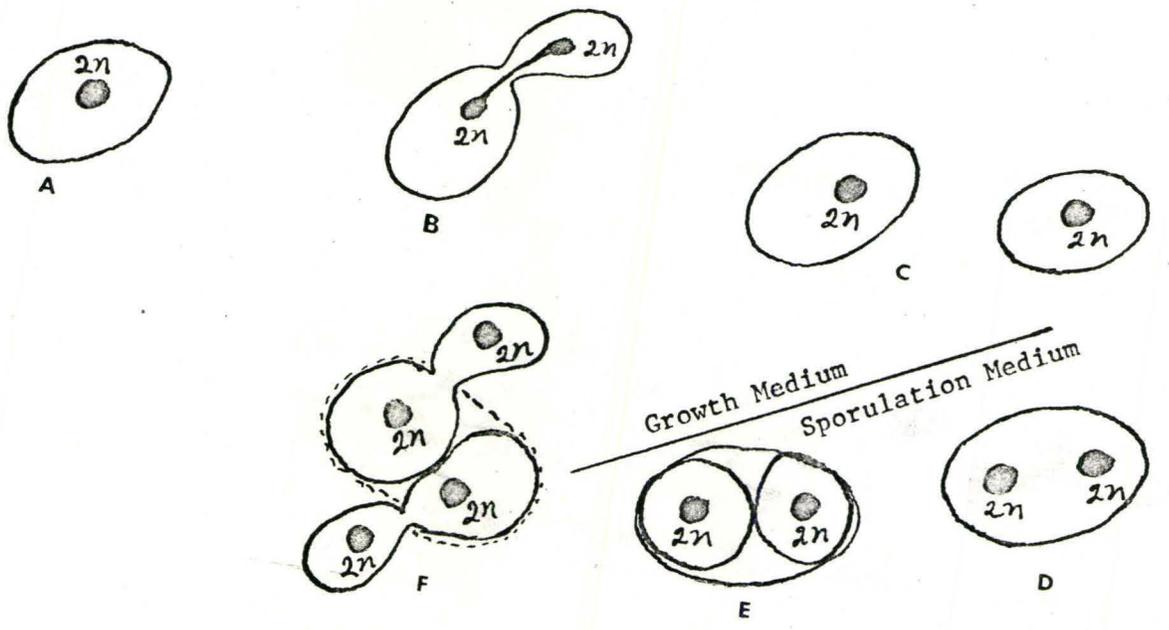


FIG 26 B

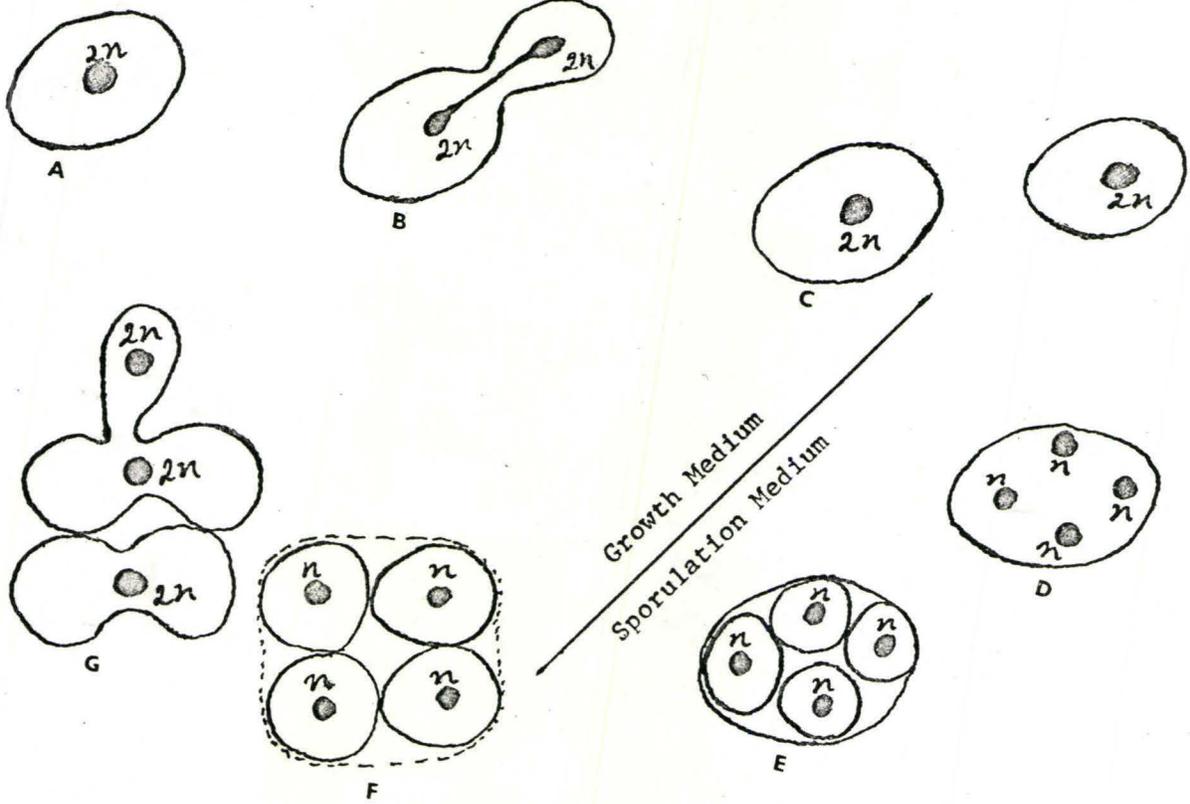


FIGURE 26A: Life cycle of a two-spored yeast which does not undergo reduction division.

A, B, C: Vegetative growth

D, E : Sporulation

F : Germination of spores (no conjugation)

FIGURE 26B: Life cycle of a normal four-spored Saccharomyces which undergoes meiosis and shows conjugation at the time of germination.

A, B, C: Vegetative growth

D, E : Sporulation

F, G : Germination of spores (conjugation)

and noted that the spores of a tetrad remained together, but their separation by micromanipulation was not difficult. Dr. P. V. Patel (personal communication, 1970) reported that he could routinely separate the naked tetrads by suspending them in 0.1% Tween 80 solution. The spores in this solution would swell and push one another apart. The spores of EVE-18, a diploid four-spored Saccharomyces, used for comparison in the present study, separated quite easily after ascus wall removal. But the pairs of spores of strains 19e1, ATCC-4117 and ATCC-4098 resisted efforts to separate them, after the Helix enzymes treatment, by exposure to Tween 80, followed by agitation in a Vortex mixer. Violent agitation in a Waring Blendor, however, separated most of the spore pairs.

EM examination of thin sections of two-spored strain of Saccharomyces (19e1) revealed a structure designated as the "inter-sporal body" between the two spores of an ascus (Figs. 17 and 18). The inter-sporal body appears quite different from the connections between adjacent spores of an ascus described by Bandoni et al. (1966), Kreger-van Rij and Ahearn (1968) and Kreger-van Rij (1968) in that here no projections of the spore walls extend from one spore toward the other, enclosing epiplasmic material in between. Instead epiplasmic material of unknown composition fills the inter-sporal space, the remainder of the epiplasm forms a "belt" as revealed by the freeze-etching technique (Figs. 19 and 21). When the belt is removed completely by fracturing (Fig. 22) the inter-sporal body is exposed. Since, after the Helix enzymes treatment, (Fig. 16),

only the inter-sporal body connection remains, one can conclude that the latter body alone is important in holding the two sister spores together following ascus wall removal.

The epiplasmic belt is etched more easily than the spore walls. This might indicate a difference in composition of the two. Spore walls are believed to be covered with a lipid layer (Miller and Hoffman-Ostenhof, 1964) which would make them relatively resistant to etching (Bishop, 1969).

There has been a great deal of freeze-etching work done on yeast, but none of the published reports deals with sporulated cells. An unpublished observation on Saccharomyces by Dr. C. F. Robinow and H. Bauer did not show an inter-sporal body or a belt partly covering the spores. In a freeze-etch attempt on the asci of the four-spored strain EVE-18 no inter-sporal body or belt was revealed. Perhaps the belt is unique to this strain (19e1). H. Pohl (Personal communication, 1971) has demonstrated an inter-sporal body in electron micrographs of sectioned material of another four-spored Saccharomyces strain.

SUMMARY

Fifteen strains of yeast producing predominantly two-spored asci were investigated in an attempt to explain this condition.

1. Nuclear stain studies showed that 12 strains produced four nuclei in the asci before spore formation and usually two nuclei could be seen in the epiplasm outside of the spores. The remaining three strains (19e1, ATCC-4117 and ATCC-4098) produced a maximum of two nuclei per ascus, each of which was usually surrounded by spore walls.
2. During spore germination, typical conjugations were observed in the 12 strains that produced four nuclei but the other three strains appeared to lack conjugation.
3. Cells of colonies derived from individual spores of strains 19e1 and ATCC-4117 sporulated without coming in contact with cells of other colonies.
4. The DNA content of the vegetative cells of the three strains was equivalent to that of diploid cells. The spores of 19e1 were found to be diploid. This indicates that nuclear division in the ascus of this strain is non-reductional.
5. Glucose, an effective inhibitor of yeast meiosis and sporulation in normal four-spored strains of yeast, was found to have little adverse effect on the nuclear division and sporulation of 19e1

and ATCC-4117. But ammonium sulphate, another inhibitor of yeast meiosis in presence of acetate, strongly inhibited nuclear division and sporulation of 19e1 and ATCC-4117.

6. EM studies showed a structure, the "inter-sporal body" between the spore pairs of 19e1. It is suggested that the inter-sporal body is responsible for the difficulty encountered in separating the sister spores from each ascus.
7. Conspicuous invaginations were noted in sporulating 19e1 cells. These structures were seen at the plasmalemmas of sporulating cells and of developing spores and were relatively inconspicuous in growing cells.

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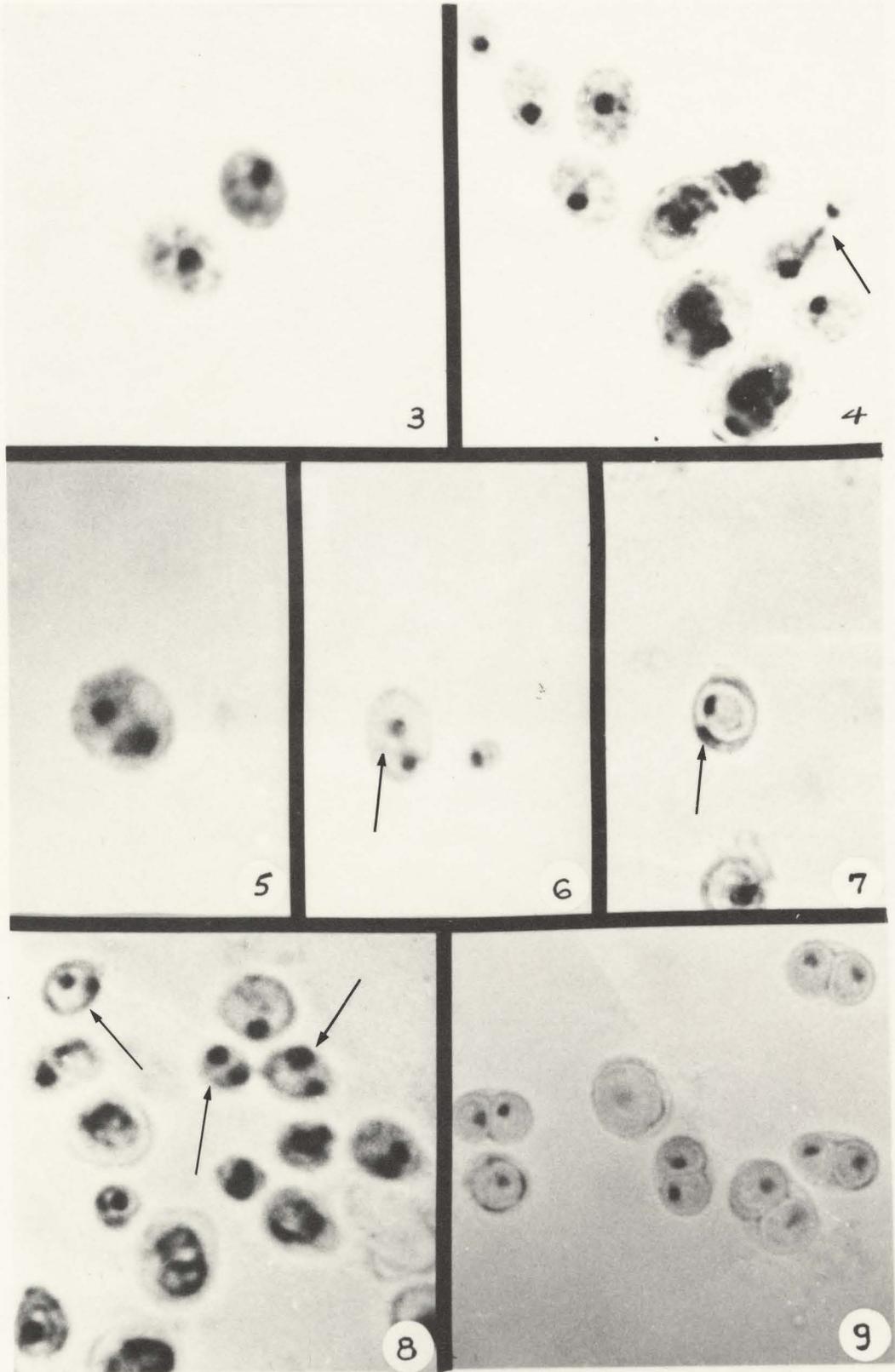
APPENDIX I

WICKERHAM'S (1951) YEAST NITROGEN BASE MEDIUM

ammonium sulphate	5g.
L-histidine monohydrochloride	10mg.
DL-methionine	20mg.
DL-tryptophan	20mg.
biotin	2mcg.
calcium pantothenate	400mcg.
folic acid	2mcg.
inositol	2000mcg.
niacin	400mcg.
p-aminobenzoic acid	200mcg.
pyridoxine hydrochloride	400mcg.
riboflavin	200mcg.
thiamine hydrochloride	400mcg.
boric acid	500mcg.
copper sulphate	40mcg.
potassium iodide	100mcg.
ferric chloride	200mcg.
manganese sulphate	400mcg.
sodium molybdate	200mcg.
zinc sulphate	400mcg.
potassium phosphate monobasic	1g.
magnesium sulphate	0.5g.
sodium chloride	0.1g.
calcium chloride	0.1g.
distilled water	1000ml.

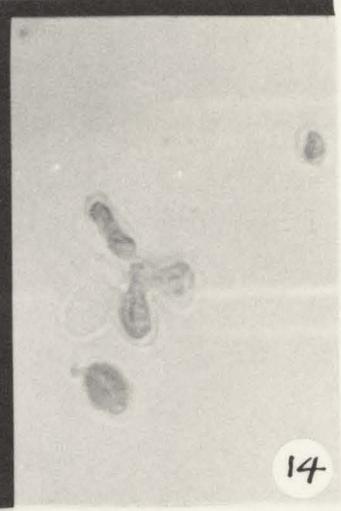
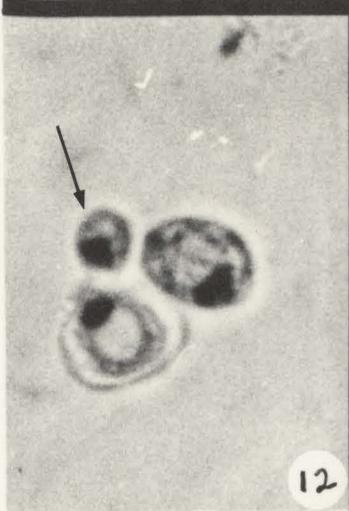
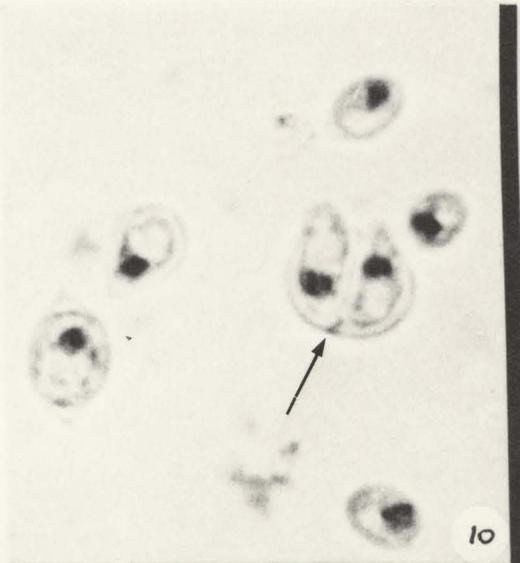
- PLATE I. : 19e1 cells Giemsa stained. X2500.
- FIGS. 3 and 4: Vegetative cells. Arrow in Fig. 4 points to the nucleus in the bud.
- FIGS. 5, 6, 7,
8 and 9: Cells from sporulating culture.
- Fig. 5 and arrows in Fig. 8 point to the asci containing two nuclei each before spore wall formation. Arrow in Fig. 6 points to a faint line which may be the beginning of spore wall formation around the upper nucleus. In Fig. 7, an ascus contains only one spore with a nucleus, and the second nucleus (arrow) which can be seen outside the spore walls in the epiplasm.
- In Fig. 9, several asci contain two spores each with one nucleus per spore.

PLATE I



- PLATE 2 : Spore germination and sporulation in two yeasts.
- FIGS. 10, 11 and 12: Giemsa stained germinating spores of 19e1. In Fig. 10 arrow points to an ascus in which spores are swollen. Arrow in Fig. 11 indicates a nucleus at the isthmus of spore and bud. Fig. 12 shows one spore with a bud. Both the bud (arrow) and the mother spore contain a nucleus. X2500
- FIG. 13 : Giemsa stained cell from a sporulating culture of EVE-18. Four nuclei can be seen. X2500
- FIG. 14 : Conjugation in germinating spores of EVE-18. Not Giemsa stained. X1250

PLATE 2



- PLATE 3: Electron micrographs of Carbon-platinum replicas of 19e1 ascus and spores. X26000
- FIG. 15: An ascus containing two spores.
- FIG. 16: Two spores without ascus walls after Helix enzymes treatment. The arrow points to the continuity of the two spores.

PLATE 3

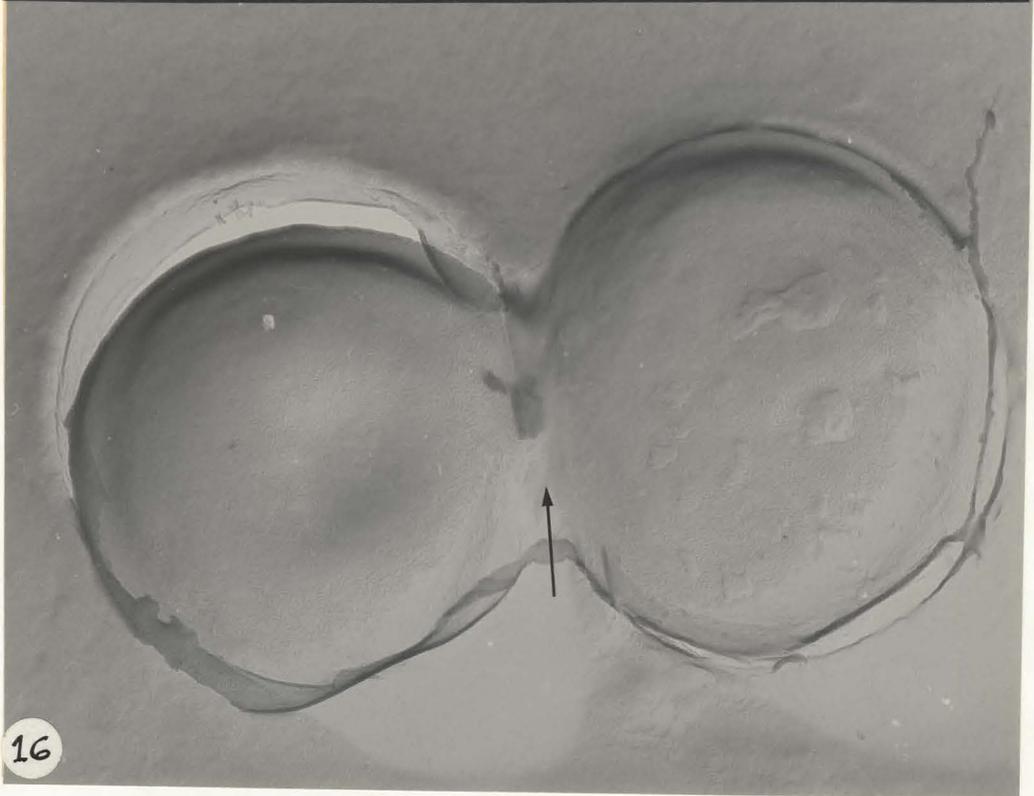
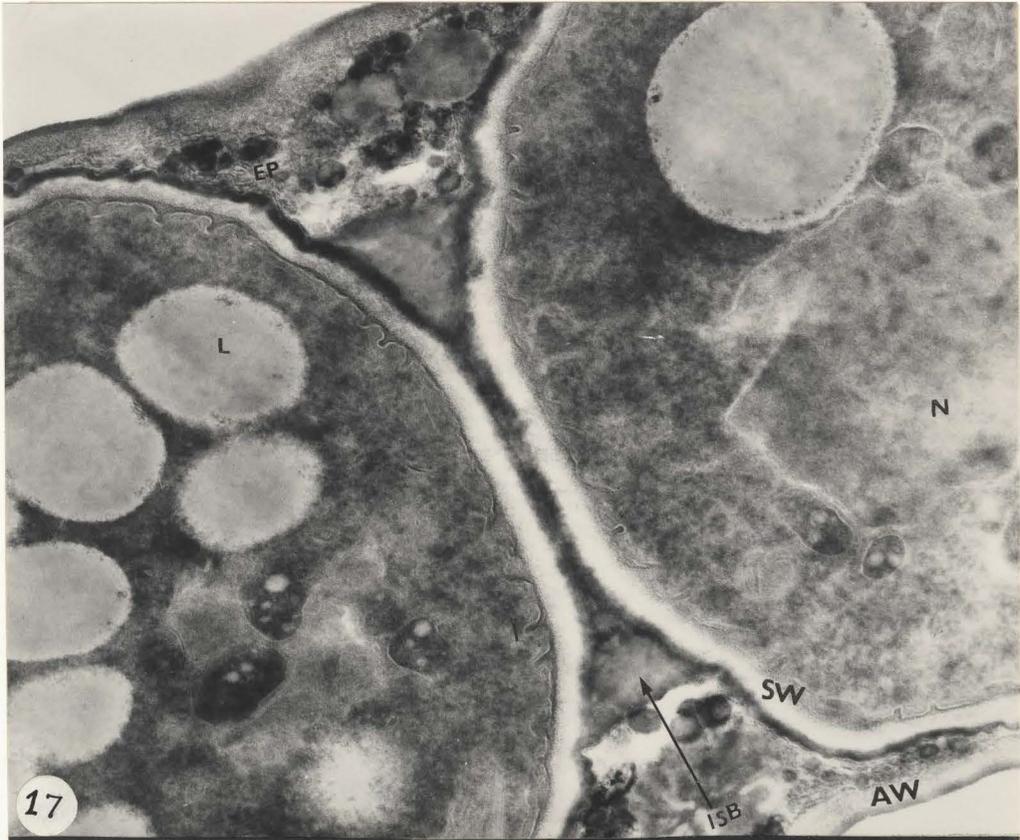


PLATE 4: Electron micrographs of sections of 19e1 asci.
AW, ascus walls; EP, epiplasm; I, invagination;
ISB, inter-sporal body; L, lipid; N, nucleus;
SW, spore walls.

FIG. 17: Ascus from a 10 hour sporulating culture. X50000
Inter-sporal body (arrow) is conspicuous and 1.5 μ
in diameter.

FIG. 18: Ascus from a 24 hour sporulating culture. X40000
Inter-sporal body is 1.2 μ in diameter. The two
arrows indicate membranous structures.

PLATE 4



17



18

- PLATE 5: Electron micrographs of replicas prepared following freeze-etching of 24 hour sporulating 19e1 cells. X28000
- AW, ascus wall; B, belt; EP, epiplasm; S, spore
- FIG. 19: Ascus wall is removed. The pair of spores are partly concealed by the belt.
- FIG. 20: Part of the belt is fractured away revealing the epiplasm underneath.

PLATE 5

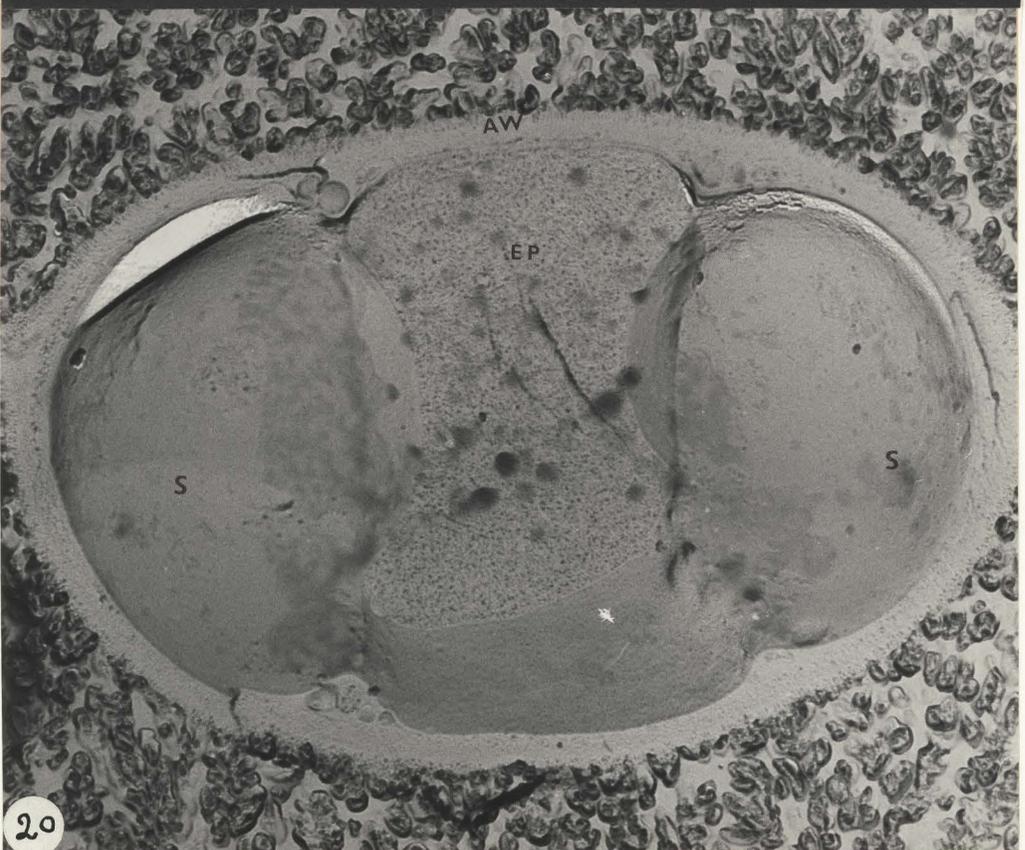
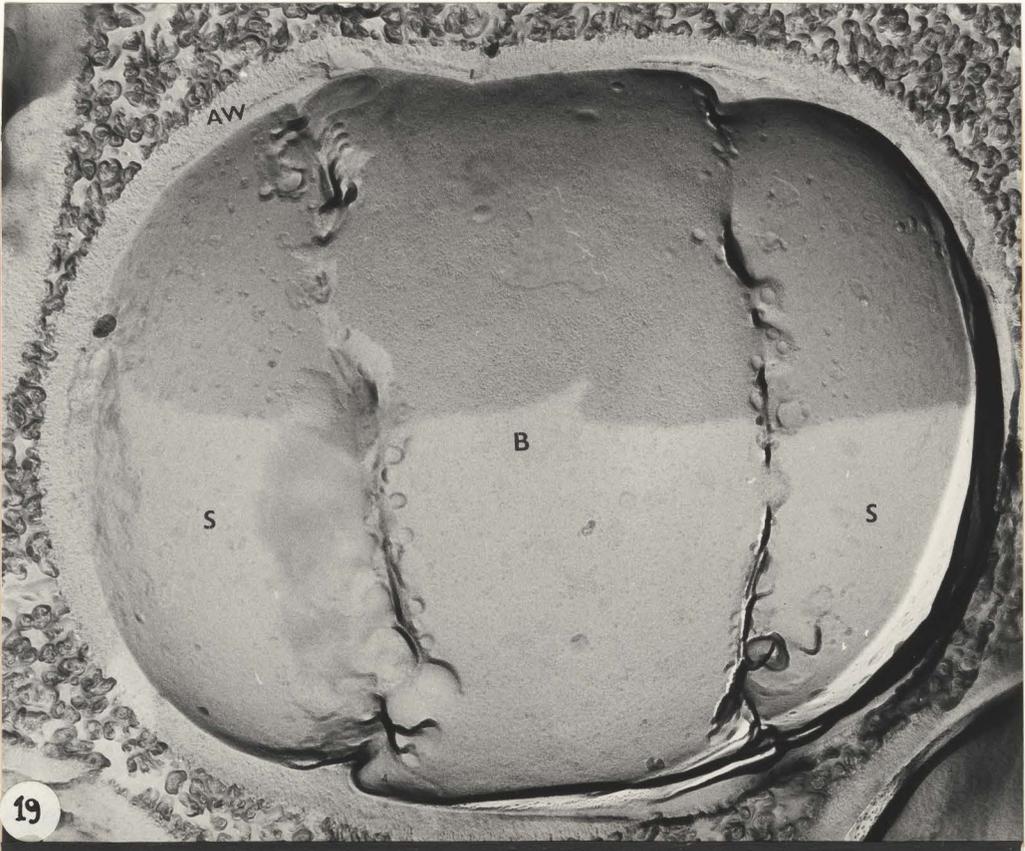


PLATE 6: Electron micrographs of replicas prepared following freeze-etching of 36 hour sporulating 19el cells. X26000.

AW, ascus wall; B, belt; EP, epiplasm; ISB, inter-sporal body; S, spore

FIG. 21: Part of the belt is etched away revealing epiplasm. The belt reaches the ascus wall (arrow).

FIG. 22: Belt is completely removed. Epiplasm and inter-sporal body can be seen. The inter-sporal body is 1.5 μ in diameter.

PLATE 6

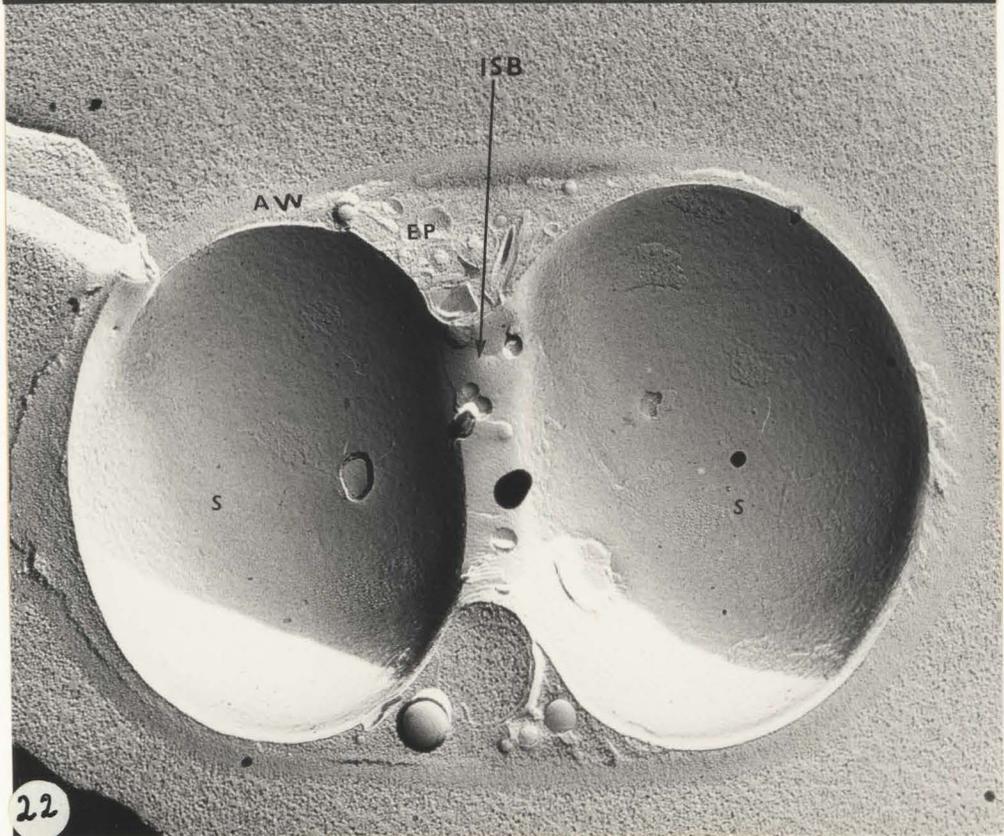


PLATE 7: Electron micrographs of replicas prepared following freeze-etching of 19e1 asci. X26000

AW, ascus wall; S, spore

FIG. 23: Ascus from a 16 hour sporulating culture. Arrow points to globular material in the inter-sporal space.

FIG. 24: Ascus from a 24 hour sporulating culture. Arrow points to developing inter-sporal body.

PLATE 7

