

REGULATION OF NUCLEAR DIVISION AND ASCOSPOROGENESIS IN APOMICETIC STRAINS  
OF SACCHAROMYCES CEREVISIAE

By

CARL ANTHONY BILINSKI, B.Sc., M.Sc.

A Thesis Submitted to the School of Graduate Studies  
in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

© July, 1983

REGULATION OF NUCLEAR DIVISION  
IN APOMICTIC YEAST

DOCTOR OF PHILOSOPHY (1983)  
(Biology)

McMASTER UNIVERSITY  
Hamilton, Ontario

TITLE: Regulation of Nuclear Division and Ascosporeogenesis in Apomictic  
Strains of Saccharomyces cerevisiae.

AUTHOR: Carl Anthony Bilinski, B.Sc. (McMaster University)  
M.Sc. (McMaster University)

SUPERVISOR: Professor J.J. Miller

NUMBER OF PAGES: (Xi), 123 pages

## ABSTRACT

Effects of nutritional alterations (carbon source, zinc) on nuclear division and protein synthesis during apomictic and meiotic differentiation in Saccharomyces cerevisiae strain 19e1 were investigated. The approach taken has led to identification of: physiological prerequisites for both developmental routes, landmark cytological and molecular events controlling the manner of nuclear division and spore production, other environmental modifications suppressing apomixis, and the period of sporulation during which a decision is made concerning the manner of differentiation.

Unlike cells cultured under meiosis-promoting conditions, cells cultured under apomixis-promoting conditions exhibited extensive protein synthesis during the first 3 h of sporulation. Cycloheximide treatment of the latter cells induced meiosis and maximum yields of meiotic asci resulted when the treatment was given for the first 3 h in sporulation medium. Temperature shock treatments administered during early hours under sporulation-inducing conditions also increased the frequency of meiosis. Thus the data indicate that the decision concerning which developmental route, apomictic or meiotic, a given cell will follow is made shortly after transfer of cells to sporulation medium. Electrophoretic analysis of labeled proteins synthesized during sporulation revealed bands unique to both developmental routes.

A novel microcytochemical staining procedure was developed to determine sites of zinc accumulation in vegetative and sporulating

yeast. The procedure was used to monitor intracellular distribution of zinc during sporulation in an effort to integrate cytological with molecular data. The micronutrient translocated from vacuolar to nuclear compartments during the early critical period of sporulation. Possible roles of zinc were considered and several hypotheses were offered to explain the restoration of meiosis in apomictic mutants by environmental manipulation.

#### ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. J.J. Miller, Professor of Biology, McMaster University, for his incessant encouragement and kind assistance. The interest, assistance and input of the following colleagues is also greatly appreciated: Drs. R.E. Esposito and S. Klapholz (Department of Biology, University of Chicago), Dr. N. Marmioli and co-workers (Institute of Genetics, University of Parma) and Dr. P.B. Moens (Department of Biology, York University).

The financial assistance by the Natural Sciences and Engineering Research Council of Canada and McMaster University is gratefully acknowledged (Clifton W. Sherman Graduate Scholarship).

The assistance of Mr. D.P. Holmyard in photography and preparation of photographic prints is acknowledged. A special note of appreciation to my wife, Sharon, for her encouragement, thoughtfulness, and assistance in the typing of the original manuscript. A special thanks to Bev Bardy for her excellent preparation of the final typescript.

TABLE OF CONTENTS

	<u>PAGE</u>
INTRODUCTION.....	1
Life cycle of <u>Saccharomyces cerevisiae</u> .....	1
Nutritional requirements for the phases of the life cycle of <u>Saccharomyces</u> .....	2
Protein synthesis during yeast sporulation.....	5
Factors influencing the number of spores per ascus.....	8
Unusual strains of <u>Saccharomyces</u> with two-spored asci.....	13
Apomixis.....	18
Nutritional control of ascosporeogenesis in apomictic yeast..	21
Objectives.....	24
MATERIALS AND METHODS.....	27
Stock cultures.....	27
Presporulation phase.....	27
Sporulation phase.....	28
Cytological staining procedures.....	29
Giemsa nuclear stain.....	29
Microcytochemical detection of zinc in yeast.....	30
Determination of bud scar numbers by fluorescence analysis.....	31
Labeling of proteins during sporulation.....	31
Protein preparation.....	31
Polyacrylamide gel electrophoresis.....	32
Measurement of protein synthesis.....	33
Chemicals.....	34

TABLE OF CONTENTS (cont'd)	<u>PAGE</u>
RESULTS.....	36
Comparative response of three apomictic strains of <u>S. cerevisiae</u> to nutritional variation.....	36
Effects of nutrition on timing of nuclear events during sporulation.....	38
Protein synthesis during sporulation.....	41
Gel electrophoresis of proteins synthesized during sporulation.....	43
Effect of cycloheximide on nuclear division and spore formation.....	46
Temperature regulation of nuclear division in apomictic yeast.....	49
Effect of presporulation culture at high temperature on nuclear division and spore formation in SPM.....	55
Cell age dependency of apomictic and meiotic sporulation....	56
Relationship between cell division age and cell size.....	62
Sites of zinc accumulation during vegetative growth and sporulation.....	63
Timing of zinc translocation from vacuolar to nuclear compartments during sporulation.....	69
Dithizone as a cytological stain.....	71
DISCUSSION.....	75
Prerequisites for meiosis in apomictic <u>Saccharomyces</u> <u>cerevisiae</u> .....	75
Qualitative changes in protein synthesis during sporulation.	78
Temperature regulation of nuclear division during sporulation.....	79
Possible roles for zinc in restoration of meiosis in, apomictic <u>S. cerevisiae</u> .....	84



TABLE OF CONTENTS (cont'd)

PAGE

Timing of the decision, concerning the manner of development during sporulation.....	86
Hypotheses to account for restoration of meiosis in apomictic yeast.....	88
SUMMARY.....	100
APPENDIX I.....	102
REFERENCES.....	103

LIST OF TABLES

<u>TABLE NO.</u>		<u>PAGE</u>
1	Effect of glucose, acetate and zinc on sporulation of apomictic <u>S. cerevisiae</u> .....	37
2	Effect of growth at high temperature on sporulation of strain 19el.....	57
3	Relationship between cell division age and cell size....	64

LIST OF FIGURES

<u>FIGURE NO.</u>		<u>PAGE</u>
1	Life cycle of <u>Saccharomyces</u> showing alternation of haploid with diploid generations.....	3
2	Life cycle of unusual two-spored strains of <u>Saccharomyces cerevisiae</u> which do not undergo reduction division during sporulation.....	14
3	Alternative life cycles in <u>Saccharomyces cerevisiae</u> strain 19el.....	25
4	Sequence of cytological events during sporulation.....	40
5	Protein synthesis during sporulation.....	42
6	SDS-polyacrylamide gel electrophoresis of proteins synthesized during sporulation under A-P conditions and M-P conditions.....	44
7	Effect of cycloheximide on nuclear division and sporulation.....	48
8	Effect of sporulation culture at 36° on nuclear division and sporulation in strain 19el.....	51
9	Effect of sporulation culture at 18° on nuclear division and sporulation in strain 19el.....	52
10	Heat and cold induced trinucleate ascus formation.....	54
11	Relationship between cell age and manner of nuclear division during sporulation under M-P conditions.....	59
12	Relationship between cell age and manner of nuclear division during sporulation under A-P conditions.....	61
13	Specificity of dithizone for zinc in <u>S. cerevisiae</u> .....	66
14	Paired photomicrographs of strain 19el stained with dithizone and DAPI.....	68
15	Timing of translocation of zinc from vacuolar to nuclear compartments during sporulation.....	70

LIST OF FIGURES (cont'd)

<u>FIGURE NO.</u>		<u>PAGE</u>
16	Observations on dithizone-stained sporulating cells.....	72
17	Stages of intersperal body formation as revealed by dithizone staining.....	74
18	Summary of treatments which restored meiosis in <u>S. cerevisiae</u> strain 19el.....	87

## INTRODUCTION

### Life Cycle of *Saccharomyces cerevisiae*

With genetical and cytological evidence, Winge (1935) and Winge and Laustsen (1937, 1939) demonstrated the alternation of haploid and diploid generations in *Saccharomyces* yeasts. When the four spores from a single ascus were isolated and cultivated, genetic segregation for morphological characters was evident. It was concluded that segregation by reductional nuclear division preceded ascospore formation and that the cells of cultures derived from single spores were haploid. Furthermore, during or shortly after ascospore germination diploidization could occur through conjugation between haploid cells followed by nuclear fusion. Like haploid cells, diploid cells so formed could multiply asexually by budding. Lindegren and co-workers (1949) confirmed these observations and in addition, established the existence of a mating system in *Saccharomyces cerevisiae*. When the four spores from a single ascus were cultivated, four non-sporogenic haploid colonies developed, two of which were of one mating type (designated a) and the other two were of opposite mating type (designated α). The existence of such a mating system was indicated by the consistent observation that haploid cells of one mating type could fuse only with haploid cells of the other mating type. Diploid cells remained diploid under conditions favouring vegetative growth, but when transferred to a medium favouring sporulation, they differentiated into asci containing four haploid ascospores, two of which were a and two of α mating type.

The life cycle of a typical diploid strain of Saccharomyces cerevisiae is given in Figure 1.

The ability to undergo the two successive meiotic nuclear divisions and form uninucleate haploid spores per ascus requires the presence of both a and α mating type alleles (Roman and Sands, 1953). Using an a/α haploid disomic strain of S. cerevisiae, Roth and Fogel (1971) reported that cells require the simultaneous presence of both a and α alleles for premeiotic DNA synthesis. Although haploid strains of either a or α mating type and diploid strains homozygous (a/a or α/α) for the mating type alleles are capable of vegetative reproduction by mitotic nuclear division and budding, they do not undergo premeiotic DNA synthesis when transferred to a medium favouring sporulation and consequently, recombination, nuclear division by meiosis and ascospore formation do not occur (Roth and Lusnak, 1970). Diploid strains heterozygous (a/α) for the mating type alleles are competent to undergo premeiotic DNA synthesis, recombination, the two successive meiotic nuclear divisions and ascospore formation. Thus heterozygosity for the mating type alleles is a necessary prerequisite for meiosis and sporulation in S. cerevisiae.

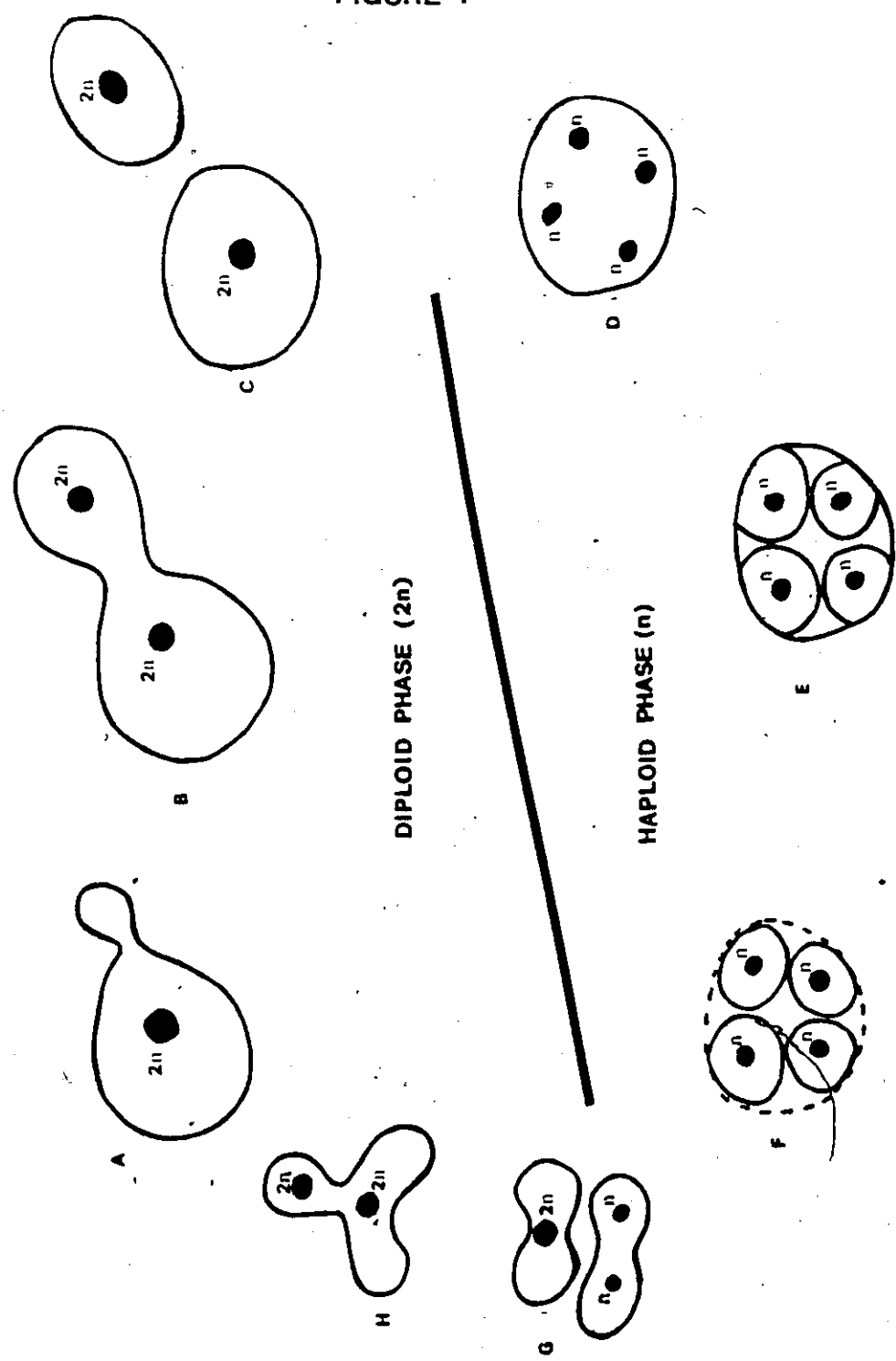
#### Nutritional Requirements for the Phases of the Life Cycle of Saccharomyces

The alternation of haploid and diploid phases in the life cycle of Saccharomyces yeasts is dependent upon the nutritional conditions to which cells are exposed. De Séynes (1868), who was the first to observe endospore formation in yeast, noted that it occurred when the growth

Figure 1: Life cycle of Saccharomyces showing alternation of haploid with diploid generations (after Grewal, 1972).

- 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, two of which are a mating type and two α mating type.
- F: Ascus after a few hours in germination medium; spores swell and the ascus wall degenerates (- - - - -).
- G: Plasmogamy and karyogamy between spores of opposite mating type.
- H: Mitotic division of diploid nucleus and production of first bud.

FIGURE 1





medium became depleted of nutrients. Subsequently others found that nutritional deprivation caused Saccharomyces cells to cease growth and differentiate into asci containing refractile spores (e.g. Engel, 1872; Stantial, 1935). In a recent study, Freese et al. (1982) demonstrated that partial deprivation of carbon, nitrogen or phosphate sources in the presence of all other nutrients necessary for vegetative reproduction can initiate sporulation of S. cerevisiae in growth medium. Generally for maximal yields of asci, sporulation-competent cells of Saccharomyces should be nourished in a nutrient rich growth medium consisting of sources of nitrogen, carbon, vitamins and inorganic salts, and then allowed to respire in a nitrogen-free sporulation medium containing a suitable carbon source such as acetate (Miller and Hoffmann-Ostenhof, 1964). In S. cerevisiae ascus production in acetate sporulation medium has been optimized by using glucose-grown cells harvested from early stationary phase during the shift from fermentation to respiratory metabolism (Croes, 1967a,b) or respiratory-competent, acetate-grown cells harvested during the exponential phase of growth (Roth and Halvorson, 1969). In the former procedure maximum yields of asci are observed between 36 - 48 hours in sporulation medium whereas in the latter procedure final yields are attained by 24 hours. It is not an absolute requirement that vegetative cells be transferred from such growth media to a sporulation medium in order for them to differentiate into asci. As noted above, yeast cells can sporulate when nutrients become depleted from the growth medium and depending on the strain and culture conditions, moderate yields of asci can be obtained from vegetative cultures in the stationary phase. This is particularly so if

glycerol or acetate is supplied as the carbon source for vegetative growth (Dawes, 1975; Fast, 1978). If asci are returned to nutrient rich conditions, the ascus wall degenerates and each of the ascospores swells and germinates. During germination diploidization usually occurs in the ascus through fusions between adjacent spores of opposite mating type.

#### Protein Synthesis during Yeast Sporulation

In S. cerevisiae protein synthesis is essential for sporulation (Sando, 1960; Croes, 1967a; Esposito et al., 1969; Magee and Hopper, 1974). For example, Magee and Hopper (1974) demonstrated an irreversible inhibition of ascus formation when the inhibitor of cytoplasmic protein synthesis, cycloheximide, was administered on transfer of cells from presporulation growth medium to sporulation medium. Addition of this antibiotic at any time during sporulation did not affect protein breakdown but did inhibit all other key events associated with sporulation - premeiotic DNA synthesis, recombination, nuclear division, RNA degradation, glycogen synthesis and breakdown, ascus formation and to some extent RNA synthesis.

Mitochondrial protein synthesis may also be required for sporulation since sporogenic diploid strains of S. cerevisiae cannot sporulate in the presence of erythromycin (Puglisi and Zennaro, 1971; Marmioli et al., 1981b), an antibiotic which inhibits mitochondrial but not cytoplasmic protein synthesis (Clark-Walker and Linnane, 1966; Lamb et al., 1968). Inhibition of mitochondrial protein synthesis by erythromycin causes cells to arrest in prophase of meiosis I at some point between intragenic and intergenic recombination, and consequently

the two successive meiotic nuclear divisions and ascospore formation do not occur (Marmioli et al., 1983). Ethidium bromide, a drug which inactivates mitochondrial DNA (Goldring et al., 1970; Perlmann and Mahler, 1971) and inhibits mitochondrial transcription and consequently mitochondrial protein synthesis (Fukuhara and Kujawa, 1970) greatly reduces ascus formation when administered during the first 4 hours of sporulation (Newlon and Hall, 1978), which for glucose-grown cells corresponds to the period during which cells complete respiratory adaptation (Marmioli et al., 1981b).

As in other developmental systems (e.g. Schultz and Wasserman, 1977), sporulating yeast cells would be expected to synthesize proteins required for differentiation from the vegetative to the sporulated state. Morphogenetic events in sporulating cells which are essentially absent from vegetative cells include formation of synaptonemal complexes, meiotic spindle plaques and ascospore walls (Moens and Rapport, 1971a,b; Zickler and Olson, 1975; Horesh et al., 1979). As noted above certain physiological and biochemical processes associated with meiosis and ascospore formation are dependent on protein synthesis. The occurrence of such morphogenetic, physiological and biochemical events implies synthesis of sporulation-specific proteins. Moreover the existence of temperature-sensitive mutants that are capable of vegetative reproduction but not of sporulation at their restrictive temperatures (Esposito and Esposito, 1969) suggests also that sporulation-specific proteins exist and in S. cerevisiae at least 50 loci have been identified which code for indispensable sporulation-specific functions (Esposito et al., 1972). However several studies

(Hopper et al., 1974; Petersen et al., 1979; Trew et al., 1979) using polyacrylamide gel electrophoresis and comparison of autoradiograms of [<sup>35</sup>S]-methionine-labelled proteins extracted from sporogenic (a/a diploid) and non-sporogenic (a or α haploid) strains have met with little success in the demonstration of meiosis-specific proteins. Although the pattern of proteins synthesized by sporulating (a/a) cells differed from vegetative (a/a) cells, the investigators were unable to conclude whether proteins present in the former and absent from the latter were meiosis-specific, since the same differences between vegetative and sporulation cultures were detected for meiosis-incapable, non-sporogenic diploid (a/a; α/α) and haploid (a or α) strains. In addition, qualitative changes in proteins synthesized during the course of sporulation in a/a strains were also detected in the non-sporogenic strains. Similar results were described by Friedmann and Egel (1978) with the fission yeast Schizosaccharomyces pombe. In a recent search for meiosis-specific proteins in Saccharomyces cerevisiae, Wright et al. (1981) identified qualitative protein changes unique to a/a diploids which were not evident in non-sporogenic a/a diploids. Instead of pulse-labelling cells with [<sup>35</sup>S]-methionine, they prelabelled proteins synthesized during vegetative growth by supplying [<sup>35</sup>S]-sulfate in presporulation medium and then extracted proteins for electrophoretic analysis at intervals during a period of 24 hours in unlabelled sporulation medium. In this way 21 sporulation-specific proteins unique to a/a cells were detected, some of which were due to the appearance of new polypeptides. They also provided evidence indicating the possibility that modifications to proteins synthesized during vegetative

growth may play an important role in the control of meiosis and sporulation.

Although electrophoretic studies prior to those of Wright et al. (1981) did not convincingly demonstrate induction of meiosis-specific proteins in S. cerevisiae, sporulation-specific increases in the activities of certain proteins have been reported and these are as follows: proteinase A (Chen and Miller, 1968; Klar and Halvorson, 1975; Betz and Weiser, 1976), proteinase B (Klar and Halvorson, 1975; Betz and Weiser, 1976), proteinase C (Klar and Halvorson, 1975) and ribonuclease (Tsuboi, 1976). Colonna and Magee (1978) detected an increase in  $\alpha$ -glucosidase activity in developing asci after completion of the meiotic nuclear divisions. This particular glycogenolytic enzyme activity was present in only trace amounts in non-sporogenic strains cultured under sporulation-inducing conditions and absent in vegetative cells, thus suggesting the enzyme to be sporulation-specific. In a recent study with temperature-sensitive sporulation-deficient mutants, Clancy et al. (1982) reported that the appearance of increased  $\alpha$ -glucosidase activity in sporulating (a/a) cells is developmentally regulated since it depended on the occurrence of premeiotic DNA synthesis and on some recombination events. However it did not require completion of the two successive meiotic nuclear divisions.

#### Factors Influencing the Number of Spores per Ascus

As noted earlier, vegetative cells of sporulation competent Saccharomyces can differentiate into tetranucleate asci, the nuclei of which constitute the haploid products of meiosis. But not each of the

haploid nuclei necessarily becomes enclosed within a spore wall. A maximum of four uninucleate spores per ascus can be expected but asci containing one, two or three uninucleate spores are often found. The cytological observations of Nagel (1946) and Pontefract and Miller (1962) demonstrated completion of two successive nuclear divisions in developing asci prior to the appearance of refractile ascospores. Mature asci with fewer than four spores contained unenclosed nuclei in the epiplasm between the spores and ascus walls.

Nutritional conditions may have a marked effect on the number of spores per ascus. Lindegren and Lindegren (1944) found that a sporulation medium prepared from potato favoured two-spored ascus formation, whereas on beet medium most asci were four-spored. Miller (1957) surveyed the effects of various carbon sources supplied in sporulation medium and reported that with acetate or pyruvate there were more spores per ascus than with glucose, fructose, mannose, galactose and dihydroxyacetone. Addition of assimilable nitrogen compounds to sporulation media may also influence the number of spores per ascus. Miller (1963) showed that some nitrogen sources (casein hydrolysate, glutamic acid, ammonium sulfate) decreased the average number of spores formed per ascus when supplied in an acetate sporulation medium, whereas others (valine, tyrosine, isoleucine, reduced glutathione) increased it. Fowell (1967) varied the cell density of S. cerevisiae in sporulation medium and observed a narrow optimum cell density range for maximum four-spored ascus formation. He also noted that the pH of the sporulation medium influenced four-spored ascus production: strains displayed either broad or narrow pH optima for maximum yields of four-

spored asci. Some of Fowell's strains produced no four-spored asci on sodium pyruvate agar medium but the further addition of potassium chloride made four-spored ascus development possible. With other strains which produced some four-spored asci on sodium pyruvate slants, the potassium chloride addition increased yields of such asci further.

The composition of the sporulation medium may also induce some strains of S. cerevisiae to form more than four spores per ascus. Santa María (1959) obtained 7 isolates that produced up to eight spores per ascus in a sporulation medium devised by Kleyn (1954) containing glucose, sodium acetate, sodium chloride and tryptose, but in sporulation medium with acetate alone the maximum was four per ascus. Patel and Miller (1972) observed a small proportion of asci with more than four spores in an acetate sporulation medium containing glycerol. Ashraf and Miller (1977) reported induction of asci containing five or more spores by the herbicide amitrole.

The composition of the presporulation medium in which cells are grown can affect their ability to form four-spored asci after transfer to sporulation medium. Tremaine and Miller (1954) reported an increase in yields of four-spored asci in acetate sporulation medium when an isolate of baker's yeast was grown in a defined presporulation medium supplemented with the vitamin inositol. Fowell and Moorse (1960) showed that increasing the glucose concentration in Lodder-Rij's presporulation medium increased the proportion of four-spored asci formed after transfer to acetate agar and that aeration of presporulation cultures gave a marked increase in yields of four-spored asci as compared with cells grown anaerobically. Halbach-Keup and Ehrenberg (1971) reported

that Saccharomyces cells transferred to sporulation medium during the transitory phase between fermentative and respiratory stages of the growth curve gave highest yields of four-spored asci.

It is possible to determine the number of buds a yeast cell has produced, and hence its age, by fluorescence staining of bud scars with calcofluor. Sando and Saito (1970) found that older cells which had completed a budding cycle produced three- and four-spored asci, whereas freshly formed cells which had not yet produced daughter cells differentiated into two-spored asci or did not sporulate at all. Haber and Halvorson (1972) noted increased levels of two-spored asci in small cells or buds that in general have a lower capacity to sporulate than larger mother cells. However even buds similar in volume to their mother cells sporulated much less frequently and with fewer spores per ascus.

It is well known that the extent of four-spored ascus production in any sporulation medium varies from strain to strain in S. cerevisiae (Kirsop, 1956). Furthermore, within a strain, single cell isolates may show differences in yields of four-spored asci in sporulation medium (Fowell and Moorse, 1960).

Under certain circumstances S. cerevisiae can be induced to differentiate into asci containing predominantly two spores. Kleyn (1954) added various nitrogen compounds to a dilute complex sporulation medium and noted that with aspartic acid 99% of the asci formed were two-spored. When yeast cells sporulate in distilled water or buffer in the absence of a carbon source, few asci develop and these are mostly two-spored (Miller and Halpern, 1956; Miller, 1957; Vezinhet, 1969).



Srivastava et al. (1981) interrupted the meiotic process by transferring cells to water after a brief exposure to acetate sporulation medium and obtained almost exclusively two-spored asci. Kuenzi et al. (1974) found that cells that had their mitochondrial genome destroyed by ethidium bromide during the last one or two doublings in acetate presporulation medium sporulated when transferred to sporulation medium but that most of the asci were two-spored. Sporulation culture at 36° followed by a downshift to 23° induces formation of two-spored asci at elevated levels (Davidow et al., 1980). Esposito et al. (1974) observed exclusive formation of two-spored asci at a semi-permissive temperature in *spo3*, a sporulation mutant.

As mentioned above, Nagel (1946) and Pontefract and Miller (1962) demonstrated that incomplete asci, i.e. those with less than four spores, have non-enclosed nuclei visible in the epiplasm. This cytological evidence suggests that since four nuclei were formed, the two successive meiotic nuclear divisions preceded spore formation and that the nuclei in all the ascospores were haploid. This has been confirmed by DNA determinations (Kuenzi et al., 1974) and by genetic analyses (Bevan, 1953; Magni, 1958; Takahashi, 1962; Takahashi and Akamatsu, 1963; Esposito et al., 1974; Davidow et al., 1980; Srivastava et al., 1981). However it does not follow that two successive meiotic nuclear divisions precede formation of asci containing less than four spores in all strains of *S. cerevisiae*.

Unusual Strains of Saccharomyces with Two-Spored Asci

Grewal and Miller (1972) examined cytologically 17 strains of S. cerevisiae which formed few or no asci with more than two spores.

Fourteen of the strains underwent apparently normal meiosis since the asci always contained four nuclei. The remaining three strains were unusual in that asci never contained more than two nuclei. From a detailed study they reported that these strains (19e1, ATCC-4117, ATCC-4098) were unlike typical S. cerevisiae yeasts in the following respects:

- (i) Asci containing only two spores were consistently produced in acetate sporulation medium.
- (ii) Sporulating cells never contained more than two nuclei.
- (iii) The nucleus within each ascospore was apparently diploid.
- (iv) Ascospores were never observed to conjugate during germination, which the haploid spores in four-spored asci usually do.
- (v) Clones derived from single germinated spores were competent to sporulate in the absence of prior mating between cells.
- (vi) Unlike meiosis I, the single nuclear division that preceded ascospore formation was not inhibited by glucose as it usually is in Saccharomyces (Miller, 1964).
- (vii) The two spores in each ascus were connected by a conspicuous intersporal body.\*

↓  
The life cycle of these unusual two-spored strains of Saccharomyces cerevisiae is shown in Figure 2.

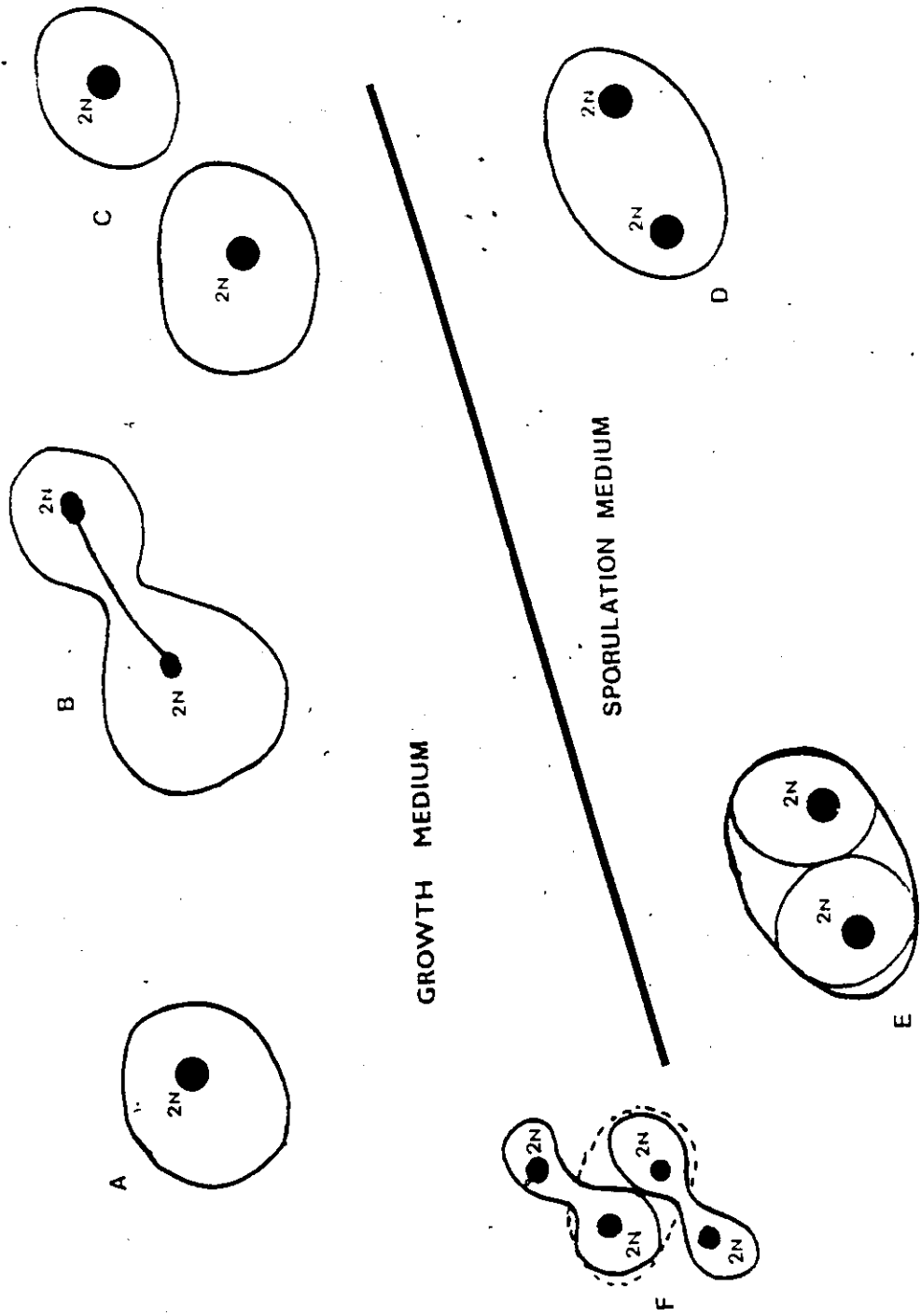
Figure 2: Life cycle of unusual two-spored strains of Saccharomyces cerevisiae which do not undergo reduction division during sporulation (after Grewal, 1972).

A,B,C: Vegetative nuclear division by budding during the growth phase.

D,E: A single equational nuclear division yields two diploid nuclei preceding ascospore formation. Consequently, two-spored asci are produced in the sporulation medium.

F: Germination of the ascospores without conjugation.

FIGURE 2



Grewal and Miller at first assumed that the single nuclear division in the ascus was mitosis. However, when an unpublished cytological observation by Dr. C. F. Robinow using the Giemsa stain indicated that the nuclear division had some of the morphological characteristics of meiosis I, they decided to refer to it by the non-committal term 'equational'.

Eighty years ago Guilliermond (1903, 1905) made cytological observations on nuclear division during sporulation of a two-spored strain of Saccharomyces pastorianus. Like the unusual two-spored strains of S. cerevisiae described above, sporulating cells never contained more than two nuclei and conjugations were not observed during ascospore germination.

In an ultrastructural study of strains 19e1 and ATCC-4117, Moens (1974) observed that the single nuclear division which preceded diploid spore formation resembled the nuclear behaviour of typical sporulating strains which undergo the two successive meiotic nuclear divisions and thus produce haploid spores. As in typical four-spored Saccharomyces, a round, granular body was observed in association with nucleoli of sporulating cells after 4 hours in sporulation medium. This structure is known to contain synaptonemal complex-like elements (Moens and Rapport, 1971a). After 8 hours had elapsed in sporulation medium, he noted that nuclear divisions in both strains began with a spindle which could have been either a mitotic or a first meiotic spindle, but suddenly the spindle, spindle pole bodies and nucleus took on features characteristic of meiosis II in four-spored Saccharomyces. In addition he noted that on completion of the single nuclear division and ascospore

formation, the parental nucleolus was discarded as in yeast meiosis (Moens, 1971) in the epiplasm surrounding the two ascospores and this does not occur with nuclear division by mitosis. Recently Marmioli et al. (1981a) confirmed the foregoing observations of Moens (1974) using a two-spored strain derived from ATCC-4117.

In a subsequent study, Moens et al. (1977) combined genetical and cytological approaches to study the manner of nuclear division during sporulation of strain ATCC-4117. Again the nucleolus-associated granular round body was observed in sporulating cells but synaptonemal complexes were rare. Using a force-mating technique devised by Morrison et al. (1975), they mated 4117 with a haploid adenine-requiring strain and sporulated the resulting triploids. Ascospores dissected from the resulting asci yielded diploid colonies which produced two-spored asci like the original 4117 strain. However, these synthesized diploid strains showed abundant synaptonemal complexes and meiotic levels of recombination. In their opinion this was evidence that: (1) a normal meiotic prophase was retained in the original 4117 strain, (2) following synapsis and recombination, desynapsis occurred, (3) duplicated homologues failed to segregate at anaphase of meiosis I and (4) instead, cells went into meiosis II; that is, there was segregation of sister centromeres, giving rise to two diploid nuclei per ascus.

Klapholz and Esposito (1980a) undertook a genetic analysis of strain ATCC-4117 in an effort to identify loci responsible for its unusual sporulation phenotype. They discovered that ATCC-4117 harbours two homozygous recessive mutations, designated *spol2-1* and *spol3-1*, which map to chromosome VIII either of which alone was capable of

causing production of asci containing two diploid spores. By complementation analysis strain 19e1 was found to be homozygous for *spol2-1* and strain ATCC-4098 was homozygous for both recessive alleles (personal communication, S. Klapholz). The segregation of centromere-linked markers in the two-spored products originating from *spol2-1* and *spol3-1* diploids indicated that segregation was generally equational (Klapholz and Esposito, 1980b), although a few percent of the dyads were the products of a single reductional division. The segregation patterns of markers at various distances from their centromeres and of several pairs of markers on the same chromosome indicated that recombination in either genetic background occurred at nearly standard meiotic levels. In their studies they also proposed several hypotheses to account for the single nuclear division which preceded spore formation:

- (i) replacement of meiosis I and meiosis II with mitosis
- (ii) an equational centromere division during meiosis I and subsequent failure of meiosis II
- (iii) failure of meiosis I reductional chromosome segregation followed by meiosis II

They argued against the first hypothesis because cells exhibited high levels of recombination indicating completion of a landmark meiotic event and dyads were occasionally recovered which were products of a single reductional nuclear division. The occasional recovery of products of a single reductional division made the second hypothesis untenable also. They did not consider *spol2-1* and *spol3-1* to be 'leaky' alleles because such a condition would predict occasional recovery of asci containing four haploid spores. Yet such asci were evidently

recovered at low frequency (less than 2%) but they considered such yields to be insignificant. The third hypothesis seemed most likely since it offered a reasonable explanation for the occasional occurrence of a single reductional division. If homologues managed to remain paired at the time of congression to the meiosis II metaphase plate, then a single reductional division might ensue. Also the third hypothesis is supported by data from the electron microscopic studies described above, which indicated that cells initiate aspects of meiosis I but then bypass reductional chromosome segregation and instead undergo the equational centromere disjunction of meiosis II to yield two diploid nuclei and consequently two diploid spores per ascus.

The term "apomictic" has been used to describe the developmental process which yields diploid rather than haploid spores (Moens et al., 1977), since, by analogy to apomictic plants, a sexual structure (ascus) is produced but gamete formation does not occur and instead diploid progeny (spores) result which give rise to clones that are competent to sporulate in the absence of prior mating.

#### Apomixis

Plants and animals were classified by Winkler in 1908 into 3 groups according to their mode of reproduction. Those organisms in which he believed sexual differentiation and fertilization had not yet arisen were called amictic (Gk. a-without, mictic-mingling). Sexually differentiated organisms were designated mictic. Within the mictic category, organisms occasionally arose in which reproduction was not associated with fertilization and in which the latter process was in



fact absent. These organisms were called apomictic (Gk. apo-from or away). Thus apomixis was a derived state in which the fertilizing mechanism had been lost.

In the plant kingdom seed formation without fertilization was first recorded by Smith in 1841 in Alchornea ilicifolia from Australia. Braun (1857) confirmed Smith's observation, and also reported an alga (Chara crinita) in which the egg cell developed without prior fertilization. Subsequently, egg cell development in the absence of fertilization was found in many higher plants. This subject has been extensively reviewed by Gustafsson (1946; 1947a,b) who described instances of apomixis in all major plant groups. His treatise is the basic botanical work on the subject.

Palmer (1971) described a form of apomictic parthenogenesis in an ameiotic mutant of Zea mays, in which presumptive meiotic cells underwent a single nuclear division that cytologically resembled mitosis in most respects. The single equational nuclear division occurring within meiocytes of sterile anthers however had some features common to the first meiotic division of cells from fertile anthers, but chromosome pairing and genetic exchange were absent.

Mittwoch (1978) briefly reviewed parthenogenesis or virgin birth in the animal kingdom. There are strains of parthenogenetic chickens and turkeys. The New Mexico whiptail lizard, Cnemidophorus neomexicanus is a species hybrid consisting exclusively of females which reproduce parthenogenetically. The presumptive sex cells undergo two rounds of DNA replication instead of one, and normal reductional nuclear division occurs, but the egg cell is diploid with the same chromosome complement

as the mother. No cases of virgin birth have been authenticated in mammals. According to Mittwoch, however, there is strong evidence that ovarian tumours known as dermoid cysts originate from diploid cells which undergo meiosis I but not meiosis II.

Apomictic parthenogenesis in insects has been reviewed by Suomalainen et al. (1976). Chromosome pairing is lacking in some of these insects although transient pairing has been observed in others. Only one "maturation" division occurs in the eggs of apomictic parthenogenetic insects and this division is equational.

In the fungi, Gäumann (1928) used the term "apomixis" to describe the development of sexual cells in the absence of copulation. He referred to the apomictic development of diploid cells as apogamy. Burnett (1956) modified Gäumann's definitions using the general term amixis to describe parthenogenesis in haploid organisms and apomixis to describe parthenogenesis in diploid organisms where certain events of sexual reproduction are lacking. In the present study, Burnett's definition of apomixis will be adopted.

Meiosis in the fungi occurs in a differentiated cell e.g. ascus in Ascomycetes, basidium in Basidiomycetes, zygosporangium in Zygomycetes. Yet certain members of these taxonomic groups produce fruiting structures but bypass gamete formation within them. Instead the nucleus divides equationally so that each of the resultant nuclei is identical to the original parental nucleus. Such is the case in multisporous strains of the yeast Lipomyces lipofer (Henninger et al., 1974; Henninger and Emeis, 1974). In apomictic strains of this species, the nucleus does not undergo reduction division during sporulation, and

instead, a series of equational nuclear divisions precedes ascospore formation within the ascus. In Saccobolus versicolor and Peziza quelepidotia (O'Donnell et al., 1976a,b), sexuality is apparently absent since they never observed male reproductive structures (antheridia) but only female reproductive structures (ascogonia). Pyronema domesticum undergoes apomictic production of ascogenous hyphae with formation of sterile apothecia following treatment with the herbicide 2,4-D (Moore-Landecker, 1972).

Thus the production of diploid progeny in sexual structures without fertilization is a well known phenomenon in plants, animals and fungi. The availability of 3 apomictic strains of S. cerevisiae (19e1, ATCC-4117, ATCC-4098) makes possible investigation of the induction and regulation of apomixis in a genetically-defined unicellular eukaryote.

#### Nutritional Control of Ascosporogenesis in Apomictic Yeast

As indicated earlier nutritional and other environmental factors may influence the number of spores per ascus Saccharomyces yeasts will produce in sporulation medium. Sporulation procedures with apomictic Saccharomyces cerevisiae have yielded exclusively two-spored asci (Grewal and Miller, 1972; Ho and Miller, 1978). However a few three- and four-spored asci have been observed in agar slant sporulation cultures (personal communication, K.H. Ho). In 1977, the writer sporulated one of the apomictic strains (19e1) in 2% potassium acetate sporulation medium following growth in a defined liquid presporulation medium (Yeast Nitrogen Base) supplemented with 2% instead of the usual 1% glucose, and a few 3- and 4-spored asci (approximately 5%) were

obtained. The previous workers (Grewal and Miller, 1972; Ho and Miller, 1978), using 1% glucose, had not observed such asci but only two-spored asci and the writer has since confirmed their observation.

The difference in the sporulation phenotype of strain 19e1, detected in response to an adjustment in carbon source levels, led the writer to undertake detailed studies of the effects of nutrition and other environmental factors on nuclear division and ascosporeogenesis in this apomictic strain (Bilinski, 1979; Bilinski and Miller, 1980). It was found possible to restore production of asci containing 3 or 4 spores by increasing carbon source and zinc levels in defined presporulation and sporulation media. An increase in the concentrations of glucose (from 1 to 6%) supplied in defined Yeast Nitrogen Base presporulation medium and of potassium acetate (from 2 to 3.5%) in sporulation medium increased yields of 3- and 4-spored asci from insignificant levels (less than 2%) to  $16 \pm 3\%$ . Inclusion of 25  $\mu\text{g/ml}$  zinc sulfate or zinc acetate in the sporulation medium gave a further increase in yields of 3- and 4-spored asci and maximum yields ( $48 \pm 1\%$ ) were obtained when zinc was added to both the presporulation and sporulation media.

The following evidence indicated that the above adjustments in carbon source and zinc levels which increased the number of spores per ascus in strain 19e1 from two to four did so by restoring the two successive meiotic nuclear divisions:

- (i) Giemsa nuclear staining demonstrated that a single nuclear division consistently preceded formation of asci containing 1 or 2 spores whereas two successive nuclear divisions preceded

formation of asci containing 3 or 4 ascospores. Thus two classes of asci were evident: binucleate (1- and 2-spored) and tetranucleate (3- and 4-spored). Formation of the latter class was blocked in the presence of the meiosis inhibitor, glucose. Inclusion of glucose in acetate sporulation medium is known to inhibit the two successive meiotic nuclear divisions in typical S. cerevisiae (Miller, 1964) but not the single equational nuclear division characteristic of apomictic S. cerevisiae (Grewal and Miller, 1972).

- (ii) Studies on the mating behaviour of cells originating from single isolated ascospores indicated that reductional chromosome segregation preceded formation of asci containing 3 or 4 ascospores whereas equational chromosome segregation preceded formation of asci containing 1 or 2 spores. During or shortly after germination of single spores isolated from 3- and 4-spored asci, conjugations were observed between cells in the mitotic progeny of the single isolated spores, a feature characteristic of germinating haploid spores produced by homothallic diploid S. cerevisiae (Herskowitz and Oshima, 1981). However such conjugations were not observed between cells in the mitotic progeny of single spores isolated from 1- and 2-spored asci. Nonetheless the progeny were able to sporulate. The ability to sporulate in the absence of prior matings indicated the spores in 1- and 2-spored asci to be diploid products of a single equational nuclear division.

(iii) Genetic analysis of spores from single asci indicated reductional segregation for mating type and morphological markers in 3- and 4-spored asci but equational segregation for the same markers in 1- and 2-spored asci.

Thus the number of spores per ascus related directly to the manner of development, apomictic or meiotic, a given cell followed during sporulation. The alternative routes that cells of the 19e1 strain may follow during sporulation are given in Figure 3.

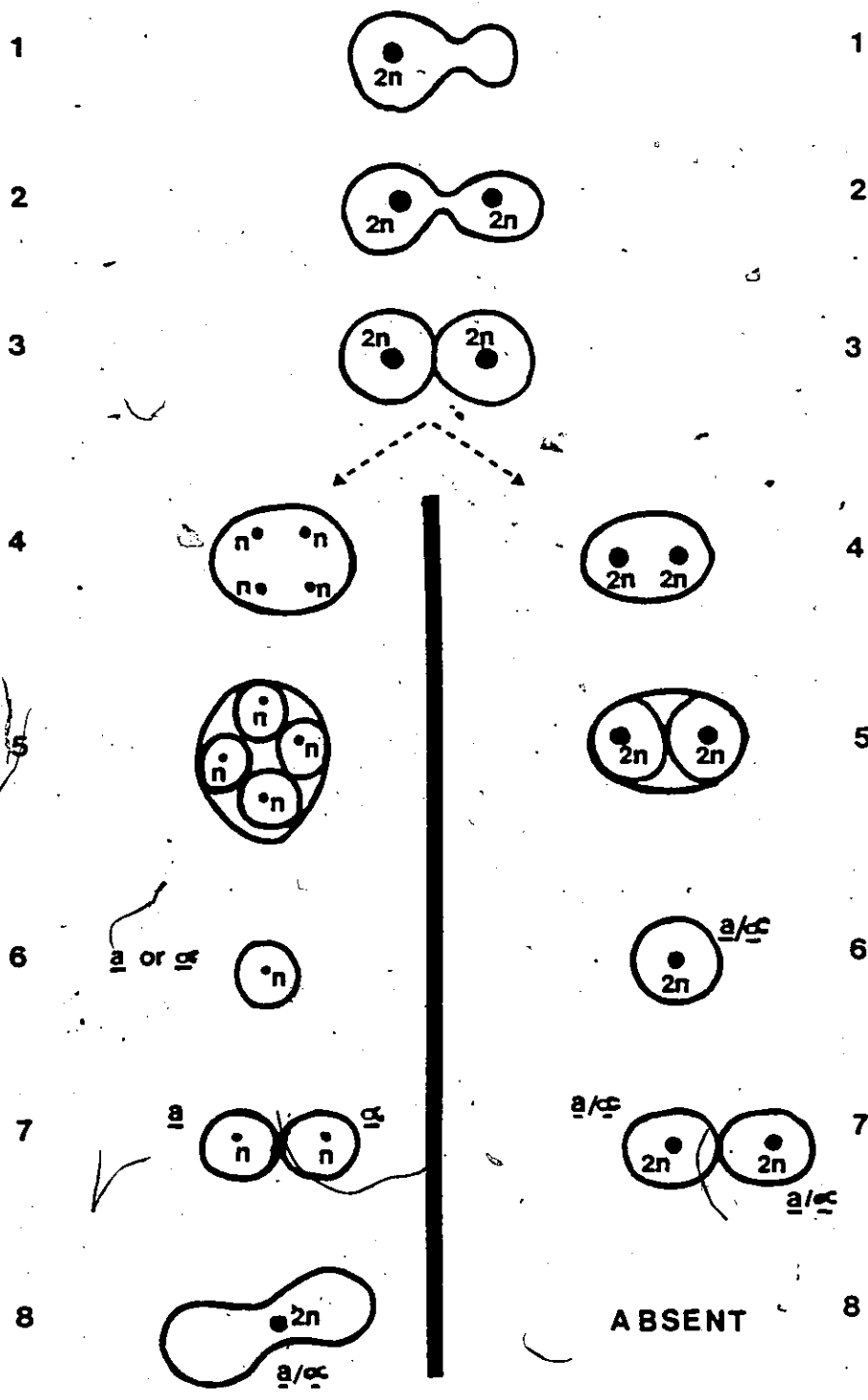
#### Objectives

It is evident that increased levels of carbon source and zinc supplied in defined culture media can condition cells of an apomictic strain of Saccharomyces cerevisiae (19e1) to undergo the two successive meiotic nuclear divisions and differentiate into tetranucleate asci containing 3 or 4 haploid spores. Without these increased levels the cells undergo only a single apomictic nuclear division and differentiate into binucleate asci containing 1 or 2 diploid spores. The ability to condition cells nutritionally for either meiotic or apomictic development has presented an opportunity to investigate cytological and macromolecular events associated with each developmental route. Therefore a study was undertaken to identify landmark events involved in the determination of meiosis and apomixis in this yeast system. Alternative nutritional conditions were used to monitor the sequence of nuclear events and changes in protein synthesis during the course of apomictic and meiotic sporulation. Clearly zinc plays an important morphogenic role in yeast meiosis. The zinc-specific stain, dithizone,

Figure 3: Alternative life cycles in *Saccharomyces cerevisiae* strain 19el (after Bilinski, 1979).

- 1,2: Mitotic nuclear division of diploid nucleus during budding.
- 3: Mature bud ready to separate from mother cell. (Between 3 and 4 the yeast is transferred from growth to sporulation medium).
- 4: Diploid nuclei of some cells undergo reduction division yielding four haploid nuclei in the ascus, two of which are a mating type and two α mating type (left). Diploid nuclei of other cells divide equationally yielding two diploid nuclei of a/α mating type (right).
- 5: A spore wall forms around each nucleus.
- 6: A single spore isolated from the four-spored ascus (left) and a single spore isolated from the two-spored ascus (right) are transferred to germination medium.
- 7: During or shortly after the germination of the single haploid ascospore derived from the four-spored ascus, there is a directed switch of the mating type allele to opposite mating type (left). This change of mating type does not occur with the ascospore derived from the two-spored ascus when it germinates since its nucleus is already a/α (right).
- 8: Plasmogamy followed by karyogamy between haploid sister cells of opposite mating type (left).

FIGURE 3





was used in an effort to detect histochemically sites of zinc accumulation in vegetative and sporulating yeast cells. A further objective was to investigate the cell age dependency of apomictic versus meiotic sporulation by fluorescence analysis of bud scar numbers.

## MATERIAL AND METHODS


### Stock Cultures

The three two-spored strains of Saccharomyces cerevisiae (19e1, ATCC-4117, ATCC-4098) described by Grewal and Miller (1972) were employed in this study. Stock cultures were maintained on slants of Wickerham's (1951) Yeast Nitrogen Base (YNB) containing 2% glucose. The slants were prepared as follows: Three grams Difco Bacto agar were dissolved in 100-ml deionized glass-distilled water and 9-ml volumes of the agar solution were transferred to 15 mm test tubes. One ml of a 10x solution of Difco Yeast Nitrogen Base containing 20% glucose was added to each of the test tubes and mixed thoroughly. The test tubes were autoclaved for 15 min at 121° and 15 psi pressure. After inoculation, the slants were incubated for 2 days at 27° and were then stored at 4°. Fresh stock cultures were prepared every 3 to 4 weeks.

### Presporulation Phase

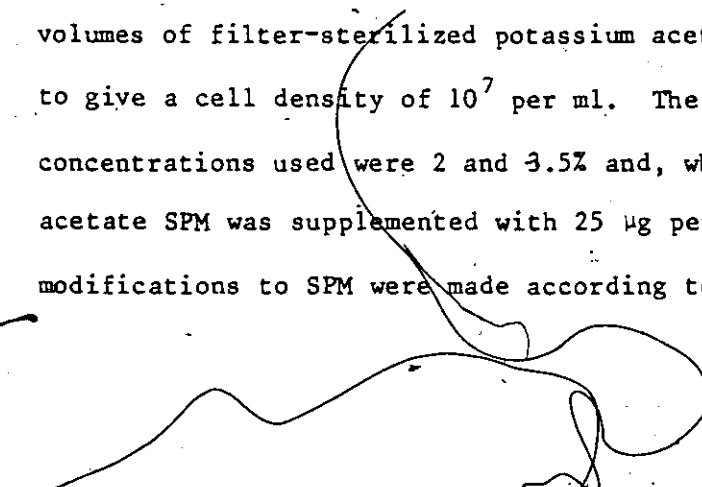
The medium used to grow cells for inoculation of sporulation cultures was Wickerham's (1951) defined Yeast Nitrogen Base (YNB) medium. This medium, which lacks a carbon source, contains vitamins, trace elements, 3 amino acids in trace amounts, ammonium sulfate and other salts in known quantities (Appendix I). The presporulation medium (PSM) was prepared directly in final strength by dissolving 0.67 g Difco Yeast Nitrogen Base in 100-ml deionized glass-distilled water containing 1 or 6% glucose. Where indicated, the  $ZnSO_4 \cdot 7H_2O$  concentration in PSM

was increased from 0.4  $\mu\text{g}$  to 25  $\mu\text{g}$  per ml. Medium was sterilized by filtration through Millipore Type HA membrane filters (47 mm, pore diameter 0.45  $\mu\text{m}$ ) and stored at 4° in sterile 1,000-ml erlenmeyer flasks. For experimental use, 50-ml volumes were dispensed aseptically into sterile 250-ml erlenmeyer flasks. All glassware was oven-sterilized.

Prior to each experiment an inoculum was transferred from a stock culture to a fresh 2% glucose YNB agar slant and incubated at 27° for 24 h. The 50-ml volumes of growth media in sterile 250-ml flasks were inoculated with cells from the 24 h slant at an initial cell density of  $10^5$  per ml of medium. Cell counts were made with Spencer "Bright-Line" hemocytometer counting chambers. The growth flasks were incubated at 27° for 21 h in a Warner-Chilcott Laboratories model 2156 water bath shaker operated at 100 oscillations per min. 

#### Sporulation Phase

After 21 h incubation, the vegetative cells were harvested from growth medium by centrifugation in a Beckman Model J-21 centrifuge at 3,020 g and 5° for 15 min. The cells were washed twice with sterilized deionized glass-distilled water and resuspended in the sterile water. An appropriate volume of cell suspension was then transferred to 50-ml volumes of filter-sterilized potassium acetate sporulation medium (SPM) to give a cell density of  $10^7$  per ml. The potassium acetate concentrations used were 2 and 3.5% and, where indicated, 3.5% potassium acetate SPM was supplemented with 25  $\mu\text{g}$  per ml  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ . Further modifications to SPM were made according to experimental requirements



and are indicated in the text. Sporulation cultures were incubated in the same manner as presporulation flasks but for a longer time: 48 h. The percentage of cells that sporulated and the percentage of cells containing 1, 2, 3, or 4 ascospores were determined by scoring 500 asci from each culture flask. Each experiment was done in quintuplicate and all experiments were repeated.

#### Cytological Staining Procedures

##### (i) Giemsa Nuclear Stain

To monitor the sequence of nuclear events during sporulation 1-ml aliquots were removed from sporulating cultures at hourly intervals from 0 - 16 h, harvested by centrifugation, washed twice and Giemsa nuclear stained using the simplified procedure of Miller (1968) but with gentle heat fixation instead of chemical fixation. A drop of cell suspension in distilled water was dried on the surface of a clean glass slide. The preparation was fixed by passing through a low bunsen flame three times, hydrolyzed 8-10 min at room temperature in 5N HCl, rinsed thoroughly in cold water, dipped in double strength Gurr pH 6.8 buffer and placed in a staining solution prepared by 10-fold dilution of Gurr R66 Giemsa stain with the buffer. After 15 min in the staining solution, preparations were washed and air-dried. Percent yields of first nuclear division asci, second nuclear division asci, spores in binucleate asci, spores in tetranucleate asci were determined by scoring 200 asci five times in different experiments. Thus each point in Figure 4 is based on scoring of 2,000 entities, 1,000 from each of two experiments.

(ii) Microcytochemical Detection of Zinc in Yeast

During the course of this work, the writer developed a novel staining procedure employing the UV fluorochrome DAPI (4, 6-diamidino-2-phenylindole.2HCl) and dithizone (diphenylthiocarbazon) for microcytochemical determination of sites of zinc localization in vegetative and sporulating yeast (Billinski and Miller, 1983). Cells from vegetative and sporulation cultures were harvested and washed by centrifugation before staining with dithizone and DAPI. Dithizone for use in staining procedures was prepared by diluting a freshly-prepared, filtered, saturated stock solution of dithizone in 60% ethanol with an equal volume of 0.02M sodium borate, pH 8.4. Cells ( $1 \times 10^7$ ) in Eppendorf vials were gently agitated at room temperature with 1-ml volumes of dithizone solution for 3 to 24 h in the dark. Yeast thus treated was then harvested by centrifugation, washed and stained 16 to 18 h with 1 ml of 0.5  $\mu\text{g/ml}$  DAPI solution prepared in deionized glass-distilled water. Cells were harvested by centrifugation and resuspended in 50  $\mu\text{l}$  deionized glass-distilled water. In the preparation of slides for microscopic examination, equal volumes (15- $\mu\text{l}$ ) of cell suspension and 5% gelatin were combined on the surface of a clean glass slide. A coverslip (no. 1, 18mm square) was then pressed firmly over the surface of the preparation and excess moisture was removed with filter paper. The edge of the coverslip was then sealed with lacquer to prevent evaporation. All observations were made with a Leitz Orthoplan microscope equipped with vertical UV illumination. DAPI fluorescence was excited by light from an HBO 50W Super Pressure mercury lamp using a Leitz H2 filter block.

(iii) Determination of Bud Scar Numbers by Fluorescence Analysis

Bud scars were stained with Calcofluor White ST Solution MR-1562 (American Cyanimid Co., Bound Brook, N.J.). Vegetative cells ( $1 \times 10^7$ ) from fresh 21 h growth cultures were treated for 30 min with 1 ml of a 2,000-fold diluted solution of the stain prepared in deionized glass-distilled water. Asci ( $1 \times 10^7$ ) from fresh 48 h sporulation cultures were treated for 90 min with 1 ml of a 3,000-fold diluted solution of the stain. Yanagita et al. (1970) recommended a higher concentration of the stain for vegetative than for sporulated yeast cells. Slides were prepared and examined under the UV fluorescence microscope as described above.

Labeling of Proteins during Sporulation

At hourly intervals from 1-10 h cells were removed from SPM and resuspended at a density of  $10^7$ /ml in 5-ml volumes fresh SPM containing 100  $\mu$ Ci: [ $^{35}$ S]-methionine (1212 Ci/mole, New England Nuclear). After 1 hour incubation in labeling medium at 27°, cells were pelleted by centrifugation at 3,020 g at 5° for 15 min, washed in deionized glass-distilled water, transferred to Eppendorf tubes for two further washings and then immediately frozen at -70°.

Protein Preparation

Whole cell protein was prepared as follows. Each pellet ( $5 \times 10^7$  cells) was suspended in 25  $\mu$ l of Solution A (0.0625 M tris (hydroxymethyl) aminoethane, pH 6.8, containing 0.3 mg/ml pepstatin A and 1 mg/ml O-phenanthroline). To each was added 0.15 g glass beads

(0.45-0.50 mm diameter, Braun Melsungen AG). The Eppendorf tubes were then placed into a pre-cooled adapter fitting into a Braun Model MSK cell homogenizer (Bronwill 2876) and cells were disrupted for 5 min. The tubes were then removed from the apparatus, placed on ice and to each was added 25  $\mu$ l of Solution B (Solution A with addition of 5%  $\beta$ -mercaptoethanol and 3% sodium dodecylsulfate). Tubes were then returned to the homogenizer for an additional 5 min disruption. Cell lysates were eluted from the glass beads with two 50- $\mu$ l washings with Solution A. Immediately after elution of cell lysates from glass beads, equivolumes (50- $\mu$ l) of cell lysate and 2x sample buffer (0.0625 M Tris, pH 6.8; 2% SDS; 10% glycerol; 0.002% bromophenol blue; 0.1 M dithiothreitol) were combined. Prepared samples were heated in a water bath at 100° for 3 min and kept frozen at -70° until electrophoretic analysis. The remaining volumes of cell lysates were used in measurement of protein synthesis (described below).

The preparation of cell lysates involving initial treatment with Solution A followed by abrupt denaturation with Solution B and several rinses with Solution A is in accordance with recommendations by Pringle (1975) to avoid generation of proteolytic artifacts. During the course of this study it was found that a two-step disruption procedure involving the addition of denaturing agents ( $\beta$ -mercaptoethanol, SDS) midway in the procedure further reduced proteolysis.

#### Polyacrylamide Gel Electrophoresis

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) with a 9 cm, 13%

acrylamide resolving gel, pH 8.8 and a 1.5 cm 4% acrylamide stacking gel, pH 6.8. Samples prepared for SDS-polyacrylamide gel electrophoresis were heated at 60° in a water bath for 1 min before loading. In each case a 20 µl aliquot was loaded into a gel slot and subjected to electrophoresis. All samples were electrophoresed simultaneously using a single gel box adapted to hold two gels at a time. Gels were electrophoresed at room temperature at 100V for approximately 6 h. After electrophoresis gels were fixed with 7% acetic acid in 40% methanol for 12-16 h and dried under vacuum before exposure to Kodak RPR medical X-ray film.

#### Measurement of Protein Synthesis

Cell lysates obtained by the two-step disruption procedure described above were assayed for [<sup>35</sup>S]-methionine incorporated into protein. Aliquots (5-µl) of sample were added to 5-ml volumes of cold 5% trichloroacetic acid (TCA) and left on ice for 30 min. TCA-treated samples were collected under suction onto 0.45 µm nitrocellulose membrane filters and extensively washed with 5% TCA. The filters were air dried, placed into glass scintillation vials and dissolved in 1 ml of ethyl acetate for 30 min at room temperature. A 10 ml volume of Aquasol 2 (New England Nuclear) was added to each vial and [<sup>35</sup>S]-methionine counts were assayed by liquid scintillation counting. All determinations were carried out in duplicate.



Chemicals

The following analytical grade reagents were obtained from the J.T. Baker Chemical Company:

dithizone  
ethyl acetate  
glucose (anhydrous dextrose)

potassium acetate  
zinc sulfate

The following analytical reagents were obtained from the Sigma Chemical Company:

bromophenol blue  
cycloheximide  
 $\beta$ -mercaptoethanol  
pepstatin A  
sodium dodecylsulfate  
tris (hydroxymethyl) aminoethane

The following reagents were obtained from the Fisher Scientific Company:

gelatin  
O-phenanthroline  
sodium borate

The following were obtained from BDH Chemicals:

dithiothreitol  
glycerol  
trichloroacetic acid

DAPI (4, 6-diamidino-2-phenylindole.2HCl) was obtained from Accurate Chemical and Scientific Corporation, Hicksville, N.Y. Gurr R66 Giemsa stain and Gurr pH 6.8 buffer tablets were obtained from Hopkins & Williams, Chadwell Heath, Essex, England.

## RESULTS

### Comparative Response of Three Apomictic Strains of *S. cerevisiae* to Nutritional Variation

As described in the Introduction, increases in carbon source and zinc levels supplied in presporulation medium (PSM) and/or sporulation medium (SPM) conditioned the 19el strain of *S. cerevisiae* to produce meiotic asci containing 3 or 4 haploid spores rather than only apomictic asci containing 1 or 2 diploid spores. There has been no previous work on effects of glucose, acetate and zinc on sporulation of the other two known apomictic strains of *S. cerevisiae* (ATCC-4117, ATCC-4098). This was investigated and the results were compared to those obtained with strain 19el (Table 1).

Strain 4117 differed from 19el in that an increase in the glucose concentration supplied in YNB PSM from 1 to 6% did not increase yields of 3- and 4-spored asci obtained in SPM but with zinc addition to 6% glucose YNB PSM yields increased significantly. Zinc supplementation of 0.5% acetate SPM after cultivation in 6% glucose YNB PSM containing 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  did not increase the frequency of asci undergoing meiosis. However zinc supplementation of 2% acetate SPM had a markedly stimulating effect and yields of 3- and 4-spored asci were further increased, from  $11.9 \pm 1.5\%$  to  $19.6 \pm 2.4\%$ .

With ATCC-4098 significant yields of meiotic asci in SPM occurred only after cultivation in PSM containing elevated glucose and

Table 1  
Effect of glucose, acetate and zinc on sporulation of  
*Saccharomyces cerevisiae* strains 19el, ATCC-4117 and ATCC-4098<sup>a</sup>.

Presporulation medium	Sporulation medium <sup>b</sup>	19el		ATCC-4117		ATCC-4098	
		Total sporulation (%)	3+4-spored asci (%)	Total sporulation (%)	3+4-spored asci (%)	Total sporulation (%)	3+4-spored asci (%)
1% Glucose-0.67%YNB <sup>c</sup>	0.5% acetate	79.3 ± 1.0	0.05 ± 0.1	86.9 ± 2.9	0.6 ± 0.5	39.6 ± 3.1	0
	2.0% acetate	80.7 ± 2.5	0.8 ± 0.3	73.8 ± 4.4	0.3 ± 0.5	37.1 ± 2.0	0
	3.5% acetate	77.4 ± 2.9	3.9 ± 0.5	0	0	0	0
	0.5% acetate-zinc sulfate(25)	79.1 ± 1.7	0.05 ± 0.2	82.9 ± 5.0	0.5 ± 0.2	21.5 ± 1.9	0
	2.0% acetate-zinc sulfate(25)	80.0 ± 1.6	1.7 ± 0.5	72.1 ± 5.1	1.4 ± 0.3	33.0 ± 3.5	0
	3.5% acetate-zinc sulfate(25)	74.7 ± 2.3	7.4 ± 1.4	0	0	0	0
	0.5% acetate	82.1 ± 1.5	1.3 ± 0.1	91.2 ± 2.0	0.6 ± 0.4	50.5 ± 2.1	0.2 ± 0.2
6% Glucose-0.67%YNB <sup>c</sup>	2.0% acetate	82.8 ± 1.7	5.3 ± 1.0	86.4 ± 1.1	0.7 ± 0.6	34.5 ± 2.8	0.6 ± 0.4
	3.5% acetate	68.9 ± 2.1	19.6 ± 1.3	0	0	0	0
	0.5% acetate-zinc sulfate(25)	82.1 ± 1.1	1.5 ± 0.5	86.2 ± 4.8	1.4 ± 0.5	26.8 ± 1.6	0.3 ± 0.6
	2.0% acetate-zinc sulfate(25)	84.0 ± 1.6	10.6 ± 0.7	71.4 ± 1.8	2.7 ± 0.8	33.8 ± 2.8	0.1 ± 0.3
	3.5% acetate-zinc sulfate(25)	66.8 ± 3.2	27.0 ± 2.3	0	0	1.0	0
	0.5% acetate	83.2 ± 1.6	3.3 ± 0.4	86.4 ± 1.7	7.8 ± 1.1	38.0 ± 2.0	3.0 ± 1.0
	2.0% acetate	80.5 ± 1.6	8.8 ± 1.3	48.0 ± 3.0	11.9 ± 1.5	11.3 ± 1.8	5.6 ± 1.8
6% Glucose-0.67%YNB-(25) of zinc sulfated per ml	3.5% acetate	46.2 ± 3.1	31.2 ± 2.4	0	0	0	0
	0.5% acetate-zinc sulfate(25)	79.3 ± 2.1	6.1 ± 0.9	73.6 ± 4.0	6.0 ± 1.3	14.1 ± 3.6	0.2 ± 0.5
	2.0% acetate-zinc sulfate(25)	81.6 ± 1.8	14.9 ± 1.5	43.5 ± 2.3	19.6 ± 2.4	8.1 ± 2.2	1.2 ± 1.0
	3.5% acetate-zinc sulfate(25)	48.4 ± 2.0	43.5 ± 2.4	0	0	0	0
	0.5% acetate	83.2 ± 1.6	3.3 ± 0.4	86.4 ± 1.7	7.8 ± 1.1	38.0 ± 2.0	3.0 ± 1.0
	2.0% acetate	80.5 ± 1.6	8.8 ± 1.3	48.0 ± 3.0	11.9 ± 1.5	11.3 ± 1.8	5.6 ± 1.8
	3.5% acetate	46.2 ± 3.1	31.2 ± 2.4	0	0	0	0

<sup>a</sup>Each value recorded represents the mean and standard deviation.  
<sup>b</sup>Sporulation media were sterilized by filtration. Concentration in parentheses was micrograms per milliliter.  
<sup>c</sup>The medium contained 0.4 µg zinc sulfate per ml. Final concentration of zinc sulfate in PSM.

zinc levels. Modifications to SPM evidently did not promote meiosis in ATCC-4098.

Hence meiotic sporulation in strains ATCC-4117 and ATCC-4098 depended more closely on addition of zinc to the nutritional environment than in strain 19e1. An increase in carbon source levels alone did not induce meiosis in strains 4117 and 4098. In fact neither strain sporulated in 3.5% acetate with or without zinc supplementation, unlike strain 19e1 which gave maximum yields ( $43.5 \pm 2.4\%$ ) of meiotic asci in 3.5% acetate containing 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ . These yields of meiotic asci were the highest obtained in this study. Of the three known apomictic strains, 19e1 is therefore most suitable for investigation of metabolic events that occur during sporulation and which may be causally related to sporulation, e.g. protein synthesis.

#### Effects of Nutrition on Timing of Nuclear Events during Sporulation

Although the effects of glucose, acetate and zinc on ascosporeogenesis in S. cerevisiae strain 19e1 have been demonstrated (Bilinski, 1979; Bilinski and Miller, 1980), the effects of these environmental modifications on the sequence of nuclear events during sporulation have not been investigated hitherto. Using a Giemsa nuclear staining procedure, a series of experiments was undertaken to monitor the sequence of landmark events in ascosporeogenesis under the following 3 nutritional conditions: (a) cultivation at low carbon source levels (1% glucose in PSM; 2% acetate SPM) (b) cultivation at high carbon source levels (6% glucose in PSM; 3.5% acetate SPM) (c) cultivation at high carbon source levels but with inclusion of zinc in SPM (6% glucose

in PSM; 3.5% acetate SPM containing 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ). In a, b and c respectively final yields of meiotic asci determined at 48 h in SPM were  $0.8 \pm 0.3\%$ ,  $19.6 \pm 1.3\%$  and  $27.0 \pm 2.3\%$  (Table 1).

Figure 4 summarizes effects of the foregoing conditions on the time of appearance of the following cytological landmark stages of sporulation: first nuclear division, second nuclear division, spores in binucleate asci, spores in tetranucleate asci. In (a) a few first or single nuclear division asci were evident at the time of transfer of cells from PSM to SPM and the next three stages were evident at 5, 6 and 9 h respectively in SPM (Fig. 4A). In (b) the four stages were detected at 1, 8, 9 and 12 h (Fig. 4B) and in (c) at 3, 8, 11 and 14 h (Fig. 4C) respectively. Hence cultivation at the higher carbon source levels delayed appearance of all four stages. Zinc supplementation of SPM delayed these stages further except for time of appearance of second nuclear division asci, which were evident at 8 h with or without the zinc addition. In this experimental series, the final yields of meiotic asci determined at 48 h in SPM were  $1.5 \pm 0.2\%$ ,  $19 \pm 2\%$  and  $30 \pm 3\%$  for a, b and c respectively. In each case the later the time of appearance of asci engaged in nuclear division, the higher the final yields of meiotic asci.

Under a fourth set of conditions (not shown in Fig. 4) that differed from (c) above in that the amount of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  added to PSM was increased from 0.4  $\mu\text{g}$  (control) to 25  $\mu\text{g/ml}$ , final yields of meiotic asci were even greater ( $44 \pm 2\%$ ) and first nuclear divisions were likewise not observed until 3 h in SPM.

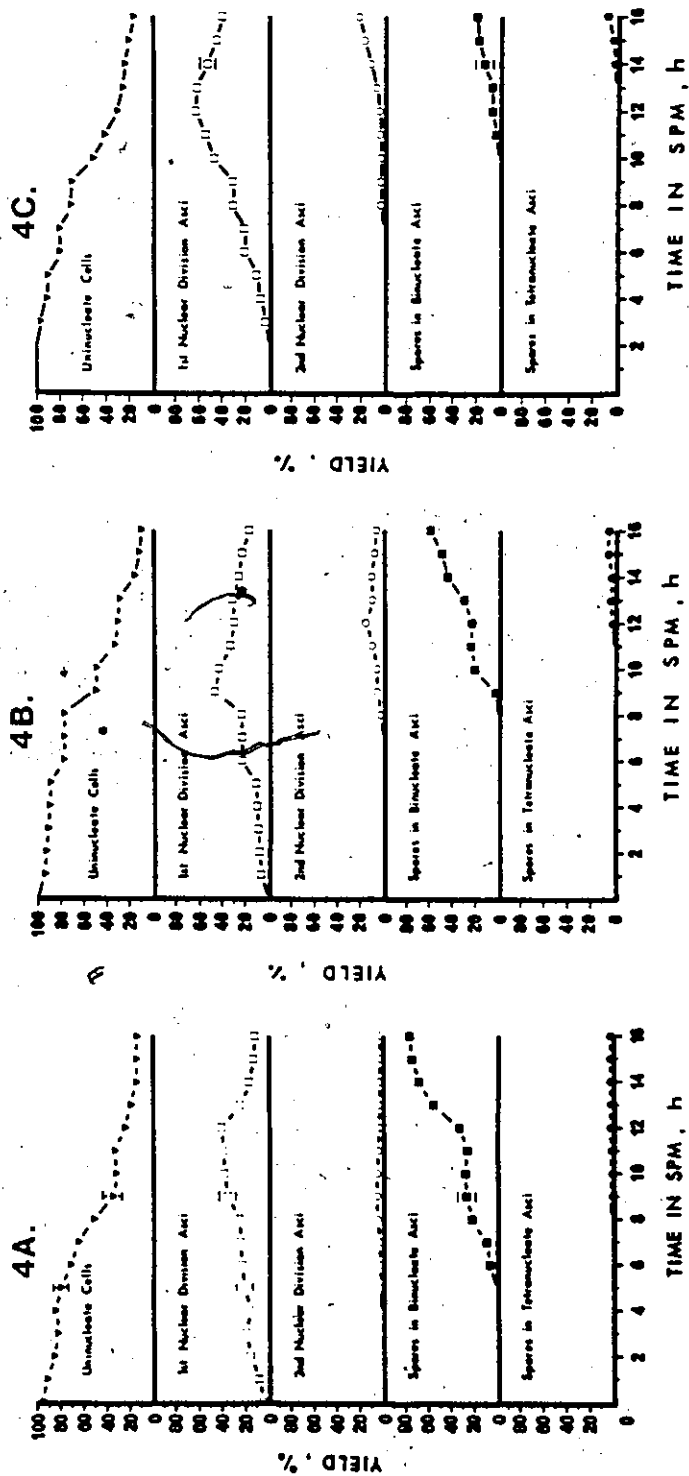
Figure 4: Sequence of cytological events during sporulation.

Aliquots (1 - ml) were removed from sporulation cultures at hourly intervals from 0-16 h, harvested by centrifugation, washed twice and Giemsa nuclear stained (see Materials and Methods). The graphs show the sequence of events in:

- A: 2% acetate SPM after cultivation in 1% glucose YNB PSM. Final yields of 3- and 4-spored asci (determined at 48 h) were less than 2%.
- B: 3.5% acetate SPM after cultivation in 6% glucose YNB PSM. Final yields of 3- and 4-spored asci were  $19 \pm 2\%$ .
- C: 3.5% acetate SPM supplemented with 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  after cultivation in 6% glucose YNB PSM. Final yields of 3- and 4-spored asci were  $30 \pm 3\%$ . Error bars indicate standard deviation.

AC

FIGURE 4



98



### Protein Synthesis during Sporulation

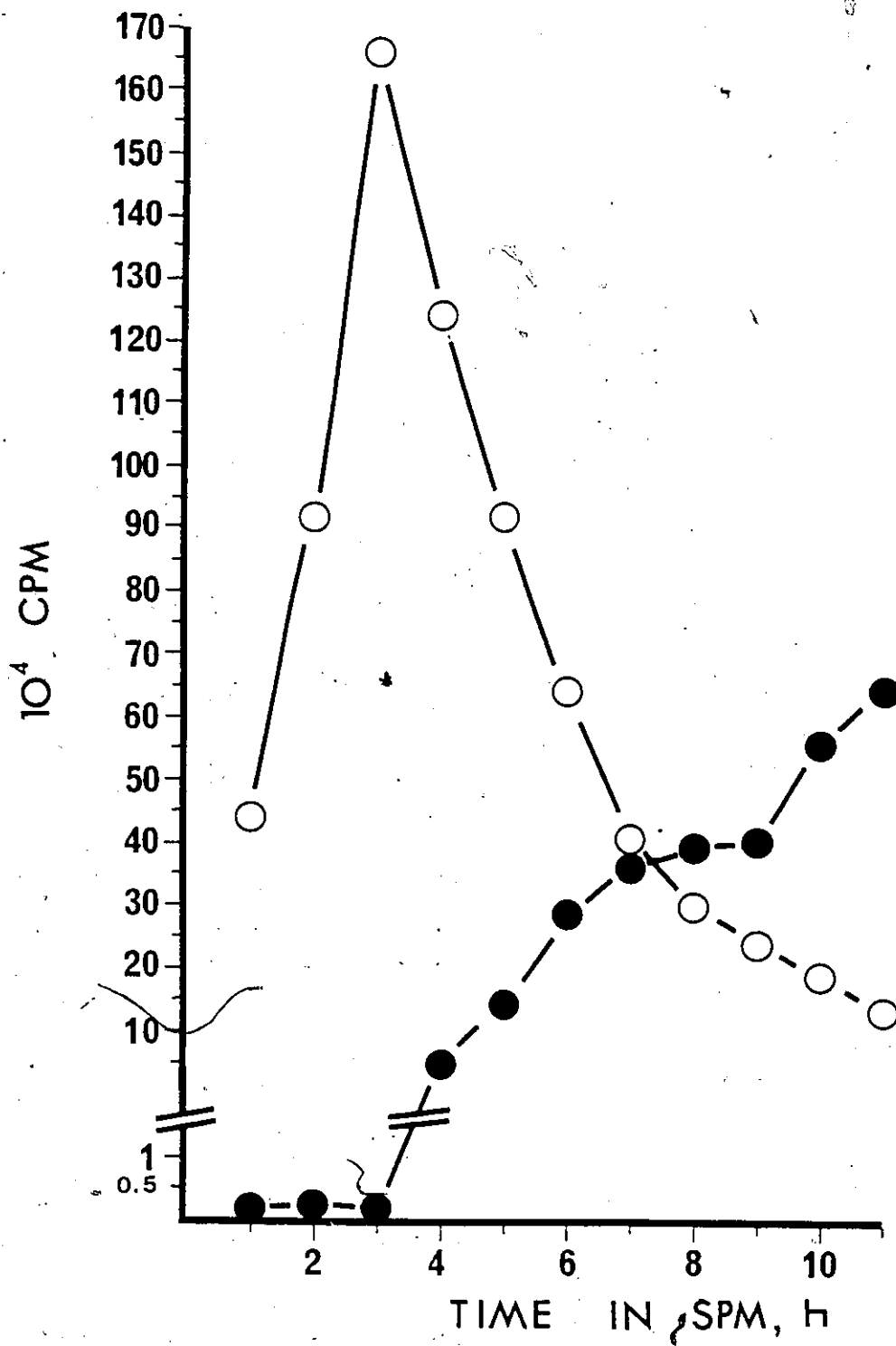
Protein synthesis was compared during the first 11 h of sporulation under conditions that gave lowest (1% glucose in PSM; 2% acetate SPM) and highest (6% glucose in PSM with 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 3.5% acetate SPM supplemented with 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) final yields of meiotic asci. Since final yields of such asci (at 48 h) in the former were insignificant (less than 2%), such conditions henceforth will be referred to as apomixis-promoting (A-P) because nearly all the sporulating cells undergo a single nuclear division and differentiate into binucleate asci containing 1 or 2 uninucleate diploid spores (Fig. 4A). The latter set of conditions, which gave highest final yields ( $44 \pm 2\%$ ) of meiotic asci, i.e., highest yields of tetranucleate asci containing 3 or 4 uninucleate haploid spores, will be referred to as meiosis-promoting (M-P). Cells in SPM were pulse-labeled with [ $^{35}\text{S}$ ]-methionine for 1 hour periods commencing every hour from 0-10 h. The results are shown in Figure 5.

It is apparent that cells in A-P conditions synthesized more protein than those cultivated under M-P conditions for the first 6 h in SPM and that after 7 h the reverse was true. Under A-P conditions protein synthesis attained a maximum at 3 h but under M-P conditions it was barely detectable during the first 3 h. The differences in incorporation of [ $^{35}\text{S}$ ]-methionine into acid-precipitable material cannot be attributed to an effect of pH on precursor uptake (Mills, 1972), since the pH of A-P SPM and M-P SPM changed little during the first 6 h. Under A-P conditions the pH increased from 7.5 to 8.1 and under M-P conditions from 7.6 to 7.9 during this 6 h period. During the first 4 h

Figure 5: Protein synthesis during sporulation.

At hourly intervals cells harvested from SPM were pulse-labeled with [ $^{35}\text{S}$ ]-methionine for 1 hour (see Materials and Methods). Protein synthesized in SPM under A-P (○-○) and M-P (●-●) conditions. Each point represents total protein determined as acid-precipitable [ $^{35}\text{S}$ ] counts per min (cpm) synthesized during the indicated hour in SPM. A-P conditions refer to sporulation in 2% acetate after cultivation in 1% glucose YNB PSM (final yield of meiotic asci at 48 h less than 2%). In this experiment the M-P condition used was sporulation in 3.5% acetate after cultivation in 6% glucose YNB PSM, both media containing 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (final yield of meiotic asci  $44 \pm 2\%$ ).

FIGURE 5



there was no pH change under M-P conditions and under A-P conditions pH changed slightly from 7.5 to 7.8.

A correlation is evident between protein synthesis during the early hours of sporulation and the sequence of nuclear events. Under M-P conditions protein synthesis was evidently limited during the first 3 h in SPM and nuclear divisions were not observed during this period. Conversely, under A-P conditions, protein synthesis was extensive during the first 3 h (Fig. 5) and nuclear divisions were observed (Fig. 4A).

#### Gel Electrophoresis of Proteins Synthesized during Sporulation

The availability of M-P and A-P conditions presented an opportunity to investigate qualitative changes in protein synthesis during the course of meiotic versus apomictic sporulation (Bilinski et al., 1983). An experimental series was therefore undertaken to monitor the sequence of qualitative protein changes during sporulation under M-P and A-P conditions. Sporulation cultures were pulse-labeled with [<sup>35</sup>S]-methionine for an hour at hourly intervals from 0-10 h in SPM and proteins were extracted for analysis by one-dimensional SDS-polyacrylamide gel electrophoresis. Autoradiograms showing the sequence of protein changes during the first 11 h of sporulation under M-P and A-P conditions are given in Figure 6.

Similar proteins were synthesized by cells under M-P and A-P conditions. However, some qualitative and quantitative differences were noted. Two band differences were evident between cells in apomictic and meiotic sporulation. A single band present under A-P conditions from 5 h (arrow, lane a<sub>5</sub>) onward was not detected under M-P conditions. A band

Figure 6: SDS - polyacrylamide gel electrophoresis of proteins synthesized during sporulation under A-P conditions (lanes a<sub>1</sub>-a<sub>11</sub>) and M-P conditions (lanes m<sub>1</sub>-m<sub>11</sub>).

The A-P and M-P conditions used were the same as given in Figure 5. Subscripts indicate the hour during which proteins were synthesized. For example, lanes a<sub>1</sub> and m<sub>1</sub> indicate proteins synthesized during the first hour in SPM under A-P and M-P conditions respectively. Cells were pulse-labeled at hourly intervals with [<sup>35</sup>S]-methionine and proteins extracted for analysis by gel electrophoresis (see Materials and Methods). [<sup>35</sup>S]-methionine-labeled proteins extracted from human KB cells infected with adenovirus type 2 were used as standard molecular weight markers (lane V). Autoradiograms were obtained by exposure of X-ray film to gels for 2 days at -70°. A band present in A-P conditions from 5 h onward (arrows, lanes a<sub>5</sub> and a<sub>6</sub>) was not detected in M-P conditions. A band detected in M-P conditions at 6 h onward (arrow, lane m<sub>6</sub>) was not detected in A-P conditions.

FIGURE 6

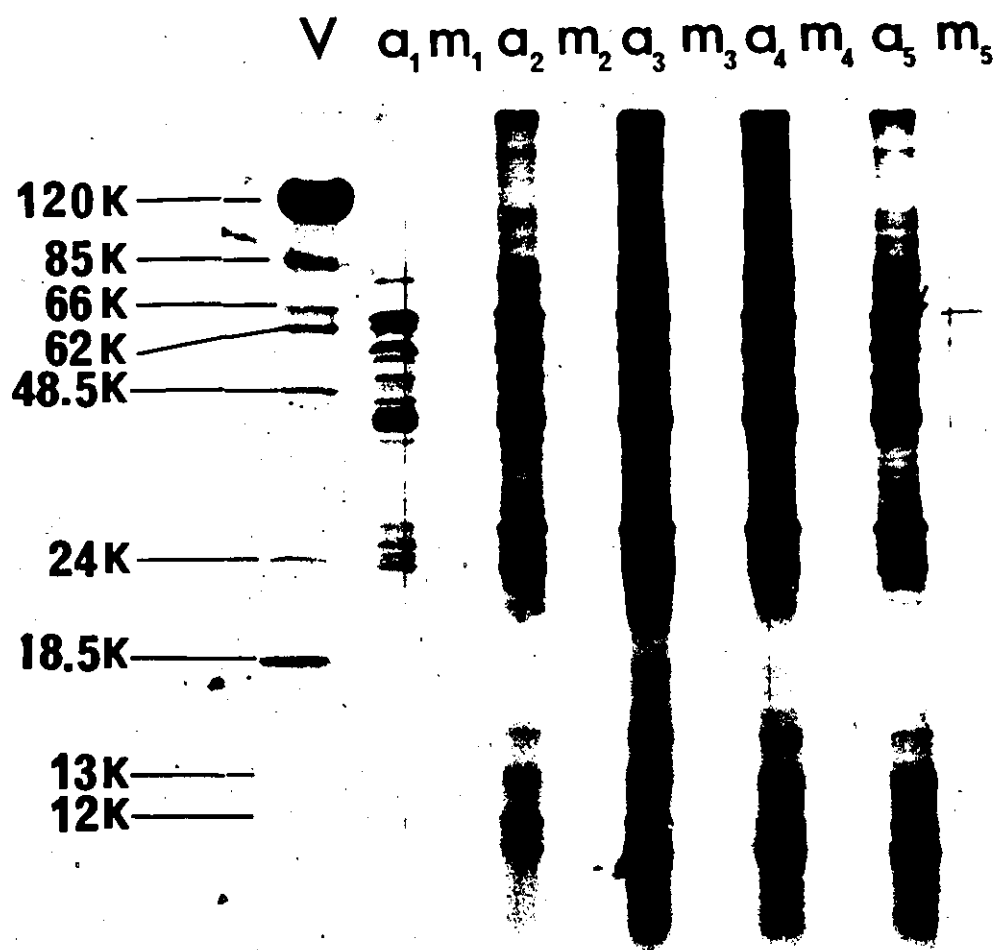


FIGURE 6



present under M-P conditions from 6 h (arrow, lane  $m_6$ ) onward was absent under A-P conditions. These observed differences were still apparent on other autoradiograms (not shown) prepared by increasing film exposure time to 1 month. The low levels of incorporation of [ $^{35}\text{S}$ ]-methionine into acid-precipitable material detected during early hours of sporulation in cells cultured under M-P conditions (Fig. 5) were also evident in Figure 6.

Thus a protein band detected during sporulation under M-P conditions was not evident under A-P conditions. Conversely, a band detected under A-P conditions was not observed under M-P conditions. The significance of these differences is at present unclear. Nonetheless it is of particular interest that a band was present under M-P conditions that was not detected under A-P conditions. Strain 19e1 harbours a single homozygous recessive mutation (*spol2-1*) inducing apomictic development (Klapholz and Esposito, 1980a). Perhaps the band evident under M-P but not under A-P conditions and vice versa reflects differences in gene expression between the two developmental sequences. It must be emphasized that other qualitative differences between apomictic and meiotic sporulation may exist because the procedure used in the present study resolved proteins according to only one criterion, molecular weight and not according to molecular weight and isoelectric point. A two-dimensional gel electrophoretic study would be useful.

#### Effect of Cycloheximide on Nuclear Division and Spore Formation

The detection of low levels of [ $^{35}\text{S}$ ]-methionine incorporation into acid-precipitable material during early hours of sporulation under



M-P conditions (Figs. 5 and 6) suggested that inhibition of protein synthesis during sporulation under A-P conditions might be a conditional requirement for meiotic development. To test this possibility, cells cultured under A-P conditions were treated with cycloheximide for 1, 2, ... 10 h periods in SPM, at a concentration (100 µg/ml) known to inhibit yeast protein synthesis markedly (Esposito *et al.*, 1969; Magee and Hopper, 1974). After each period of cycloheximide treatment the drug was washed out and cells were returned to cycloheximide-free SPM. In the present study this concentration was found to reduce protein synthesis to 0.1% of cycloheximide-untreated controls. The results are given in Figure 7.

Inhibition of protein synthesis by cycloheximide greatly increased yields of tetranucleate asci and maximum yields resulted when the drug treatment was given for the first 3 h (Fig. 7A). A similar result was obtained for final yields of 3- and 4-spored asci, as determined at 48 h (Fig. 7B, lower curve). There was no marked inhibition of total ascus formation by these periods of cycloheximide treatment (Fig. 7B, upper curve).

As seen by comparison of Figs. 7A and 7B, the frequency of tetranucleate asci was somewhat greater than that of 3- and 4-spored asci. This can be attributed in part to cycloheximide inhibition of events required for spore formation in tetranucleate asci subsequent to completion of the two meiotic nuclear divisions. Giemsa nuclear staining of 24 h sporulation cultures demonstrated that tetranucleate asci induced by cycloheximide treatment contained 0-4 uninucleate spores. Figure 7B (lower curve) does not include those tetranucleate

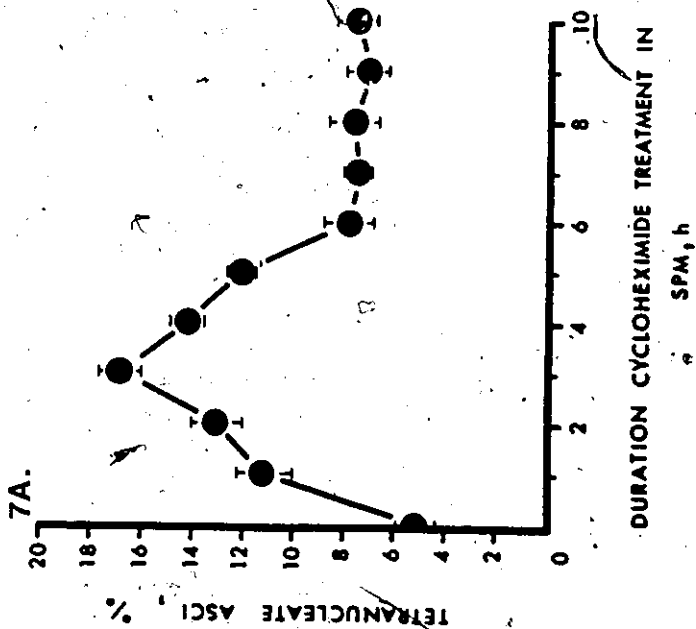
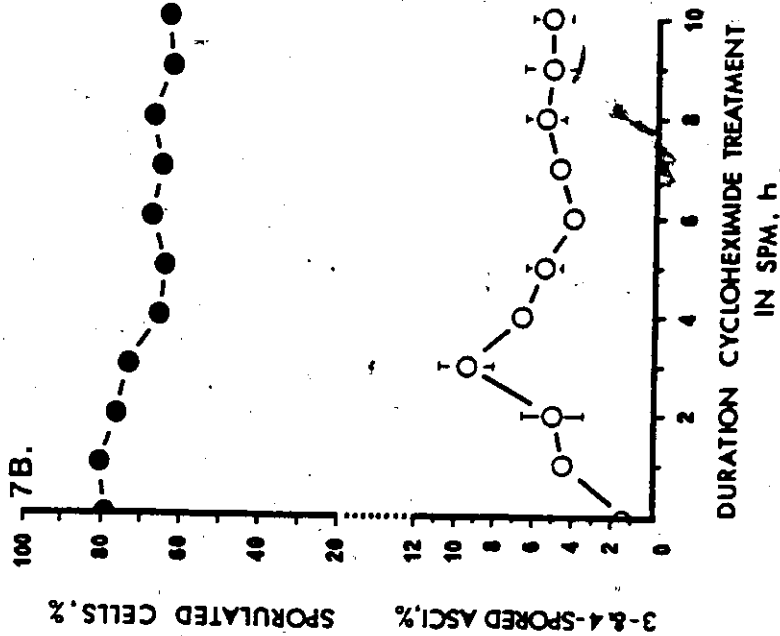
Figure 7: Effect of cycloheximide on nuclear division and sporulation.

Cells harvested from 1% glucose YNB PSM were transferred after washing to 300-ml 2% acetate SPM containing 100 µg cycloheximide (Sigma) per ml at a cell density of  $10^7$ /ml. Volumes (25-ml) were then dispensed in sterile 125-ml Erlenmeyer flasks and incubated in the usual manner. At hourly intervals for the first 10 h, a culture flask was removed from incubation, cells pelleted by centrifugation and after thorough washing resuspended in 25-ml cycloheximide-free 2% acetate SPM.

- 7A: yields of tetranucleate asci (those engaged in or having completed 2<sup>nd</sup> nuclear division) expressed as percent total cells determined by Giemsa nuclear staining 24 h after transfer from PSM to SPM.
- 7B: Percent yields of sporulated cells (●-●) and percentage of the asci that contained 3-4 spores (○-○) determined 48 h after transfer from PSM to SPM.

In Figure 7A yields were determined by scoring 200 Giemsa-stained asci five times in duplicate experiments, 24 h after the transfer from PSM to SPM. Thus each point in Figure 7A is based on a total of 2,000 asci. In Figure 7B yields were determined with hemocytometer counting chambers by scoring 500 asci five times in duplicate experiments, 48 h after the transfer. Thus each point in Figure 7B is based on a total of 5,000 asci. In both 7A and 7B, yields indicated at 0 h were for cycloheximide-untreated controls. Error bars indicate standard deviation.

FIGURE 7



asci containing fewer than 3 spores since the data were obtained by examination of unstained asci.

Cycloheximide, when administered for the early hours of sporulation under A-P conditions, restored meiotic development (Fig. 7) in cells which would have otherwise undergone apomictic development in the absence of treatment with the protein synthesis inhibitor. Thus the limitation of protein synthesis evident during the first 3 h of sporulation under M-P conditions (Figs. 5 and 6) appears to play a role in the induction of meiosis in apomictic yeast. However it does not follow that apomixis requires active protein synthesis during the first 3 h of sporulation because even under M-P conditions approximately half of the cells that sporulated did so apomictically.

#### Temperature Regulation of Nuclear Division in Apomictic Yeast

Perhaps other environmental modifications known to affect protein synthesis would also induce apomictic yeast to undergo two successive meiotic nuclear divisions in SPM. Since it is well known that temperature shock treatments can alter protein synthesis in many organisms (Ashburner and Bonner, 1979) including yeast (Miller *et al.*, 1979; McAlister *et al.*, 1979; Lindquist, 1981), an experimental series was undertaken to determine the effects of mild temperature stress treatments on nuclear division and spore formation in strain 19e1. Cells cultured under A-P conditions were heat treated by exposure to a temperature of 36° for 1, 2, ... 5 h periods upon transfer from PSM to SPM. For comparison, experiments were performed in parallel in which

sporulation cultures were exposed to a lower temperature (18°) for the same time periods. The results are given in Figures 8 and 9.

Giemsa nuclear staining revealed that both temperature treatments stimulated formation of tetranucleate asci. However, some trinucleate asci were also evident. Such asci were not observed under any other meiosis-promoting conditions including cycloheximide treatment. Maximum yields of trinucleate- and tetranucleate-asci resulted when sporulation cultures were incubated at 36° for the first 1 or 2 h in SPM and then shifted down to 27° (Fig. 8B). Yields declined when the heat treatment was given for the first 3 h. With low temperature treatments maximum yields were obtained when cells were incubated at 18° for the first 3 h in SPM and then shifted up to 27° (Fig. 9B).

As seen by comparison of Fig. 8A with 8B and Fig. 9A with 9B, the increase in yields of trinucleate- and tetranucleate-asci was not accompanied by a significant increase in yields of 3- and 4-spored asci. Giemsa nuclear staining demonstrated that both treatments induced formation of 3 classes of asci: binucleate, trinucleate and tetranucleate. Trinucleates contained one larger and two smaller nuclei and never more than one spore. Spore wall enclosure when it occurred was invariably around the larger nucleus (Figs. 10A and 10B). Tetranucleate asci contained 1-4 uninucleate spores and binucleate asci 1 or 2 uninucleate spores. Thus 1-spored asci were common to all 3 classes of asci. Two-spored asci were detected in both binucleate and tetranucleate classes and 3- and 4-spored asci were of course confined to the tetranucleate class. The observation of the foregoing classes of

Figure 8: Effect of heat shock on nuclear division and sporulation.

Cells were inoculated into 50-ml volumes 1% glucose YNB PSM at an initial cell density of  $10^5$ /ml and the growth culture flasks were incubated 21 h at 27° in a water bath shaker operated at 100 oscillations per min. Cells harvested from 21 h presporulation cultures were washed twice with sterile deionized glass-distilled water, transferred to 50-ml volumes 2% acetate SPM at a cell density of  $10^7$ /ml and incubated at 36° in a water bath shaker operated at 100 oscillations per min. SPM was prewarmed to 36° in the water bath shaker immediately prior to inoculation with cells. At hourly intervals commencing at 1 h for the first 5 h in SPM, culture flasks were removed from the 36° shaker and placed into a 27° shaker operated at 100 oscillations per min.

8A: Mean percentage of sporulated cells (□-□) and of total asci that were 3- and 4-spored (■-■).

8B: Mean percentages of trinucleate and tetranucleate asci (●-●).

In Figure 8A yields were determined with hemocytometer counting chambers by scoring 500 asci five times in duplicate experiments, 48 h after the transfer from PSM to SPM. Thus each point in Figure 8A is based on a total of 5000 asci. In Figure 8B yields were determined by scoring 200 Giemsa-stained asci five times in duplicate experiments, 24 h after the transfer. Thus each point in Figure 8B is based on a total of 2000 asci. In both 8A and 8B, yields indicated at 0 h are for heat-untreated controls incubated at 27° and yields indicated at 48 h are for cultures maintained at 36°. Error bars indicate standard deviation.

FIGURE 8

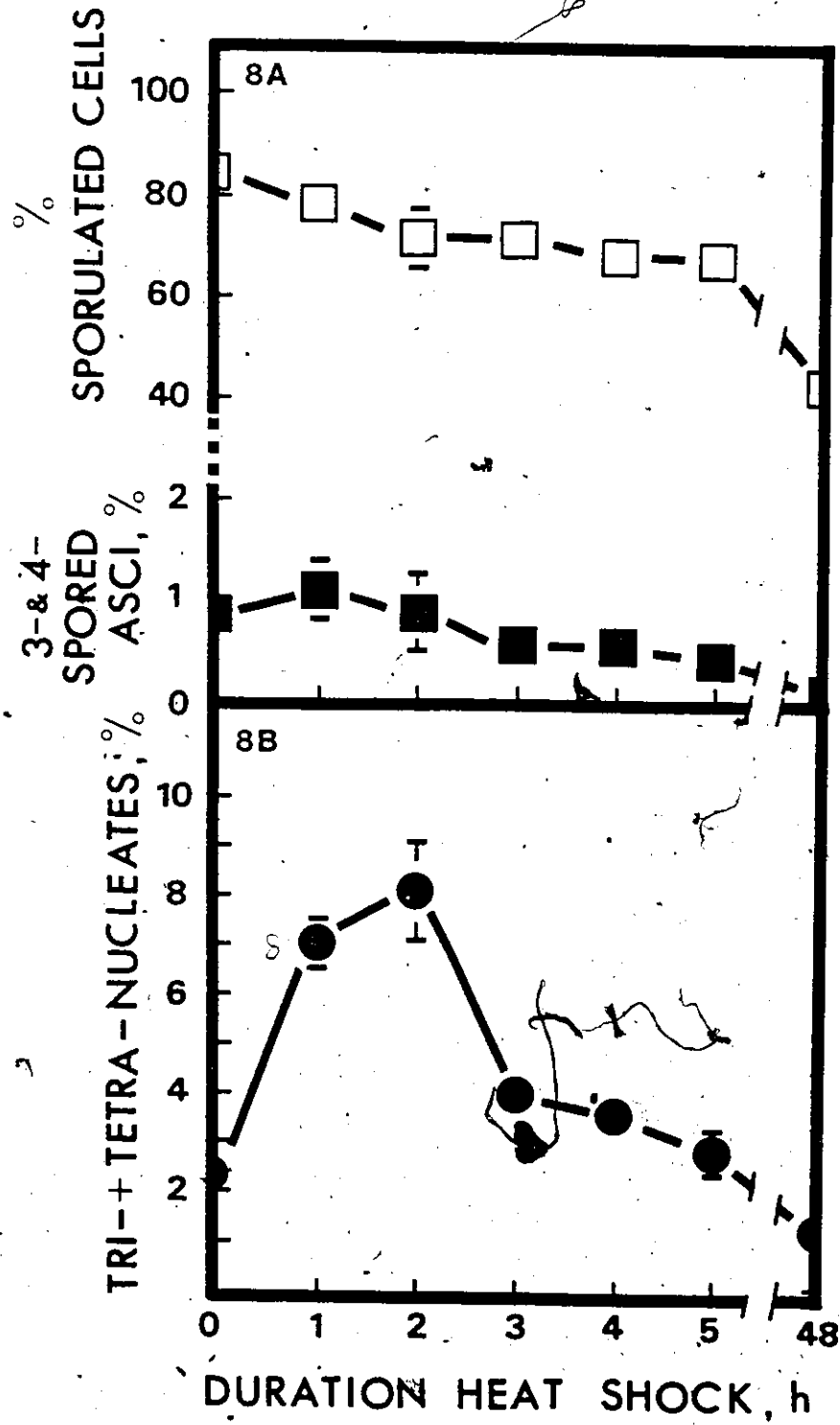


Figure 9: Effect of cold shock on nuclear division and sporulation.

The culture procedure used to investigate the effect of sporulation culture at a low temperature (18°) were the same as those described in the preceding figure for sporulation culture at a high temperature (36°) except SPM was precooled to 18° immediately prior to inoculation with cells. Sporulation cultures were incubated at 18° and at hourly intervals commencing at 1 h for the first 5 h in SPM culture flasks were shifted up from 18 to 27° by direct transfer from an 18° to a 27° water bath shaker.

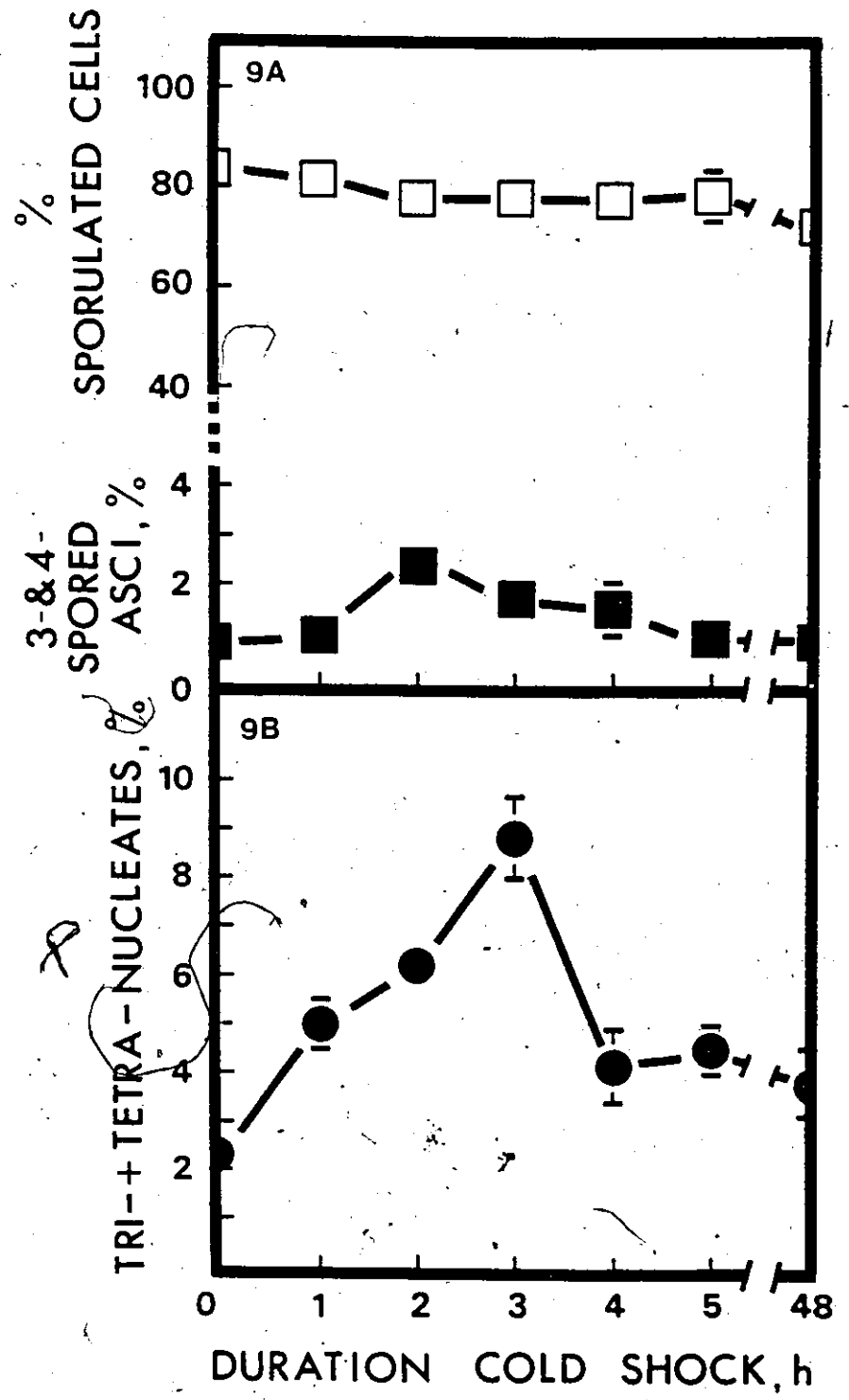
9A: Mean percentages of sporulated cells (□-□) and of total asci that were 3- and 4-spored (■-■).

9B: Mean percentages of trinucleate- and tetranucleate-asci (●-●)

In Figure 9A yields were determined by scoring 500 asci five times in duplicate experiments, 48 h after the transfer from PSM to SPM. Thus each point in Figure 9A is based on a total of 5000 asci. In Figure 9B yields were determined by scoring 200 Giemsa-stained asci five times in duplicate experiments, 24 h after the transfer. Thus each point in Figure 9B is based on a total of 2000 asci. In both 9A and 9B, yields indicated at 0 h were for cold-untreated controls incubated at 27° and yields indicated at 48 h for cultures maintained at 18°. Error bars indicate standard deviation.



FIGURE 9



asci can account for the lack of correspondence between yields of trinucleate- + tetranucleate-asci and yields of 3- + 4-spored asci. The latter yields were determined by examination of unstained asci which does not permit enumeration of nuclei in asci.

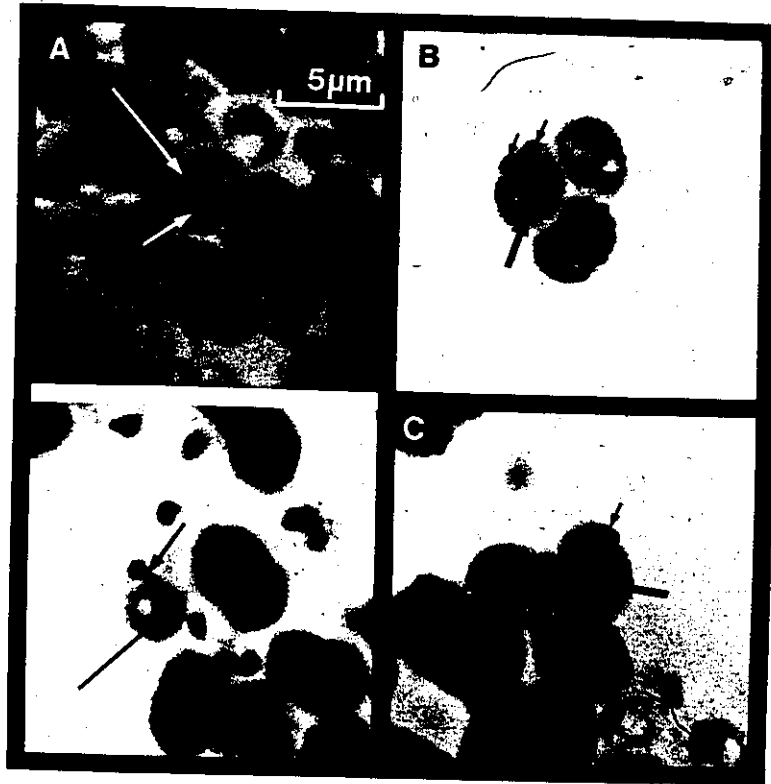
As noted in the Introduction, nuclear divisions are normally completed before the onset of spore formation in yeast (Nagel, 1946; Pontefract and Miller, 1962). In the present study this was true of spore formation in binucleate and tetranucleate asci. However in the case of heat- and cold-induced trinucleate asci, nuclear divisions were not always completed before the onset of spore formation. Figure 10A shows two examples of a single uninucleate spore in an ascus in which a second unenclosed nucleus was engaged in nuclear division. The consequence was formation of a trinucleate ascus containing a single nucleus enclosed within spore walls and two unenclosed nuclei (Fig. 10B). One possibility is that both high and low temperature treatments interfered with events that would normally prevent spore formation until nuclear divisions are completed. It is noteworthy that the two unenclosed nuclei in any given trinucleate ascus were consistently smaller than the enclosed nucleus. This is of particular interest since the manner of chromosome segregation in such asci is at present unclear and the consistent difference in size between enclosed and unenclosed nuclei might reflect differences in ploidy, the former being diploid and the latter haploid in DNA content. This possibility is supported by the observation that in apomictic asci containing 1 spore, enclosed and unenclosed (diploid) nuclei were similar in size to the single enclosed nucleus in trinucleate asci (Figs. 10B and 10C).


Figure 10: Heat and cold induced trinucleate ascus formation.

- A: Heat induced trinucleate ascus (upper photo) and cold induced trinucleate ascus (lower photo) as revealed by Giemsa nuclear staining showing a spore nucleus (long arrow), and a second nucleus (short arrow) engaged in nuclear division in the epiplasm.
- B: Mature trinucleate ascus showing one larger nucleus enclosed within a spore (large arrow) and two smaller unenclosed epiplasmic nuclei (small arrows).
- C: A one-spored apomictic ascus with one enclosed and one unenclosed nucleus.

All photographs were the same magnification. Scale bar: 5  $\mu$ m.

FIGURE 10





The limitation of protein synthesis evident during the first 3 h of sporulation under M-P conditions (Figs. 5 and 6) was correlated with induction of meiosis in experiments in which cells cultured under A-P conditions were administered cycloheximide for the early hours of sporulation (Fig. 7). Inhibition of protein synthesis by cycloheximide during the first few hours of sporulation was followed by a dramatic increase in yields of tetranucleate asci. Thus it is not surprising that treatments expected to reduce rates of macromolecular synthesis, such as incubation at low temperature or to alter synthesis of a number of polypeptide species, such as incubation at high temperature (McAlister et al., 1979; Lindquist, 1981), induce meiosis in apomictic yeast when administered for the early hours of sporulation under A-P conditions.

Effect of Presporulation Culture at High Temperature on Nuclear Division and Spore Formation in SPM

The effect of PSM modifications on meiotic ascus production in SPM (Table 1) suggested that events associated with vegetative reproduction might condition cells of the 19el strain for meiosis in SPM. This possibility was investigated further by determining the effects of presporulation culture at elevated temperature (36°) on nuclear division and ascospore formation in SPM. Presporulation growth cultures in 6% glucose YNB PSM with and without zinc addition were incubated at either 27° (control) or 36° for 21 h and transferred to 3.5% acetate SPM with and without zinc supplementation for 48 h at 27°.

The resultant yields of sporulated cells and of 3- and 4-spored asci are given in Table 2.

Yields of 3- and 4-spored asci in 3.5% acetate SPM increased from control values ( $19.6 \pm 1.2\%$ ) to  $26.2 \pm 3.0\%$  when 6% glucose YNB presporulation cultures were incubated at  $36^\circ$  rather than  $27^\circ$  (Table 2, upper half) and these increased yields were similar to those ( $27.6 \pm 3.0\%$ ) obtained in 3.5% acetate SPM supplemented with zinc sulfate after presporulation culture at  $27^\circ$ . However, there was no further increase in yields with zinc addition to SPM after presporulation culture at  $36^\circ$ . Zinc additions to PSM followed by incubation of the growth culture at  $36^\circ$  had a more striking effect on subsequent yields of meiotic asci obtained in SPM (Table 2, lower half). Yields increased from  $29.2 \pm 2.0\%$  (controls) to  $45.1 \pm 2.3\%$ . The yields of such asci induced were similar to those ( $42.3 \pm 3.3\%$ ) obtained with inclusion of zinc in SPM after presporulation culture at  $27^\circ$ . Again, inclusion of zinc in SPM did not increase yields further following presporulation culture at  $36^\circ$ . It is thus evident from the marked increase in yields of 3- and 4-spored asci obtained in SPM that presporulation culture at  $36^\circ$  rather than at  $27^\circ$  increased the ability of cells to undergo two meiotic nuclear divisions in SPM.

#### Cell Age Dependency of Apomictic and Meiotic Sporulation

Saccharomyces cerevisiae multiplies by budding leaving on the surface of mother cells bud scars which stain conspicuously with the UV fluorochrome calcofluor (M. Hayashibe, cited in Yanagita et al., 1970). Newborn daughter or infant cells however do not bear a stainable bud

Table 2

Effect of Growth at High Temperature on Sporulation of strain 19el.

Additions to PSM <sup>a</sup>	Temperature PSM	Additions to SPM <sup>b</sup>	Total Sporulation <sup>c</sup> (%)	3- + 4-spored Ascidi (%)
None	27° (Control)	None	68.9 ± 2.1	19.6 ± 1.2
None	27	Zinc sulfate (25) <sup>e</sup>	66.6 ± 2.9	27.6 ± 3.0
None	36	None	72.7 ± 2.0	26.2 ± 3.0
None	36	Zinc sulfate (25) <sup>e</sup>	70.4 ± 2.0	26.3 ± 1.4
Zinc sulfate (25) <sup>e</sup>	27° (Control)	None	58.9 ± 2.3	29.3 ± 2.0
Zinc sulfate (25) <sup>e</sup>	27	Zinc sulfate (25) <sup>e</sup>	52.9 ± 1.1	42.3 ± 3.3
Zinc sulfate (25) <sup>e</sup>	36	None	45.5 ± 1.0	45.1 ± 2.3
Zinc sulfate (25) <sup>e</sup>	36	Zinc sulfate (25) <sup>e</sup>	47.4 ± 1.2	45.9 ± 2.6

<sup>a</sup>PSM was 6% glucose YNB. This medium contains 0.4 µg per ml ZnSO<sub>4</sub>·7H<sub>2</sub>O. Cells for inoculation of sporulation cultures were grown at either 27 or 36° for 21 h in a waterbath shaker operated at 100 oscillations per min.

<sup>b</sup>SPM was 3.5% potassium acetate. Cells were incubated for 48 h in the 27° waterbath shaker.

<sup>c,d</sup>yields were determined at 48 h with hemocytometer counting chambers, by scoring 500 entities five times from each of two replicate flasks. Experiments were done twice. Each figure represents mean and standard deviation.

<sup>e</sup>The numbers in brackets are µg per ml ZnSO<sub>4</sub>·7H<sub>2</sub>O.

scar. Hence the number of bud scars on the surface of any given cell corresponds to its "cell division age". In this context age is understood in the physiological rather than chronological sense. Yanagita et al. (1970) found that most newborn cells fail to sporulate when transferred from PSM to SPM but that cells bearing 1 or more bud scars (age 1 or more) exhibit a much greater sporulation capacity. The availability of M-P nutritional conditions presented an opportunity to compare the cell division age dependency of apomictic and meiotic sporulation using an ideal cell population consisting of a mixture of apomictic and meiotic phenotypes derived from cells of the same genetic background. The cell division age distribution patterns obtained under M-P conditions can be related directly to the manner of nuclear division during sporulation, since, only two classes of asci are produced: binucleate (apomictic) asci which always contain 1 or 2 uninucleate spores and tetranucleate (meiotic) asci which always contain 3 or 4 uninucleate spores (Bilinski and Miller, 1980). Asci from 48 h sporulation cultures were stained with calcofluor and the age distribution frequencies for meiotic asci, apomictic asci and non-sporogenic cells were determined. The results are given in Figure 11. The age distribution frequencies of apomictic asci and non-sporogenic cells formed under A-P nutritional conditions are included for comparison (Fig. 12).

Under M-P conditions newly-formed daughter cells (age 0) failed to undergo meiotic sporulation (Fig. 11A) but such cells were able to sporulate apomictically (Fig. 11B). Of apomictic asci formed under M-P conditions, approximately 20% resulted from differentiation of age 0



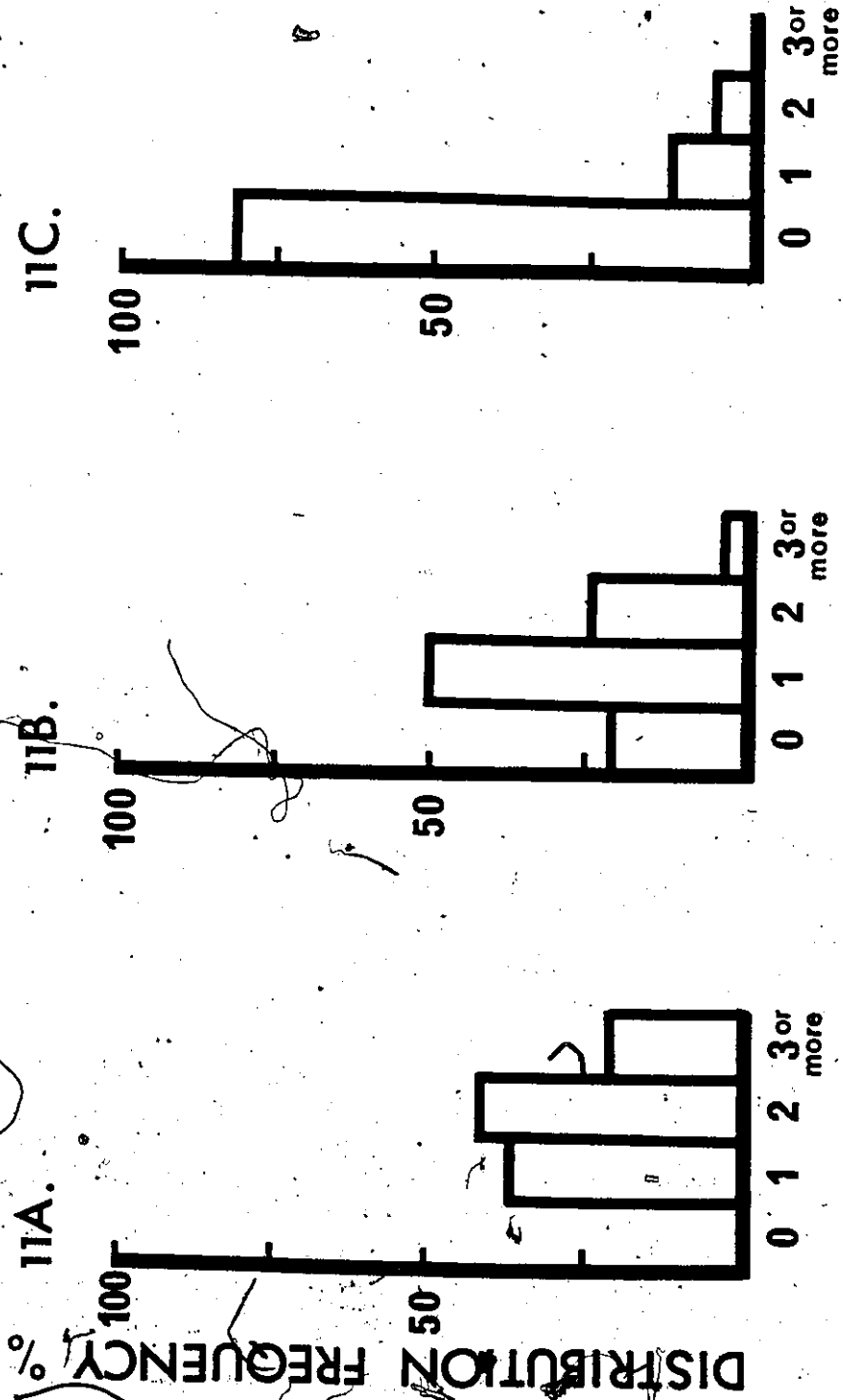
Figure 11: Relationship between cell age and manner of nuclear division during sporulation under M-P conditions.

Cells were inoculated into 50-ml volumes of 6% glucose YNB P6M containing 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  at an initial cell density of  $10^5/\text{ml}$  and growth culture flasks were incubated 21 h at 27° in a water bath shaker operated at 100 oscillations per min. Cells harvested from growth medium were transferred after washings to 3.5% acetate SPM containing 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  at a cell density of  $10^7/\text{ml}$ . Sporulation cultures were maintained in the same manner as presporulation growth cultures, except they were incubated 48 h. Aliquots (1-ml) were removed from sporulation cultures at 48 h, harvested, washed and stained with calcofluor (see Materials and Methods). By means of fluorescence microscopy the proportion of cells with 0, 1, 2 and 3 or more bud scars was determined by scoring 400 randomly-selected entities in each of the following classes of asci: (A) Meiotic asci; (B) Apomictic asci; (C) Nonsporogenic asci.

Division age (horizontal axis) refers to the number of bud scars on a given cell.



FIGURE 11



cells (Fig. 11B). Hence it is apparent that passage of cells through at least 1 complete mitotic cell division cycle is a prerequisite for meiotic but not for apomictic sporulation. But this is not sufficient for occurrence of meiosis, since some cells bearing 1 or more bud scars sporulated apomictically (Fig. 11B) while others with 1 and even 2 bud scars were non-sporogenic (Fig. 11C). Nonetheless older cells underwent meiosis more frequently than apomixis since the proportion of asci bearing 2 and 3 or more bud scars was much higher in the meiotic class (Fig. 11A) than in the apomictic class (Fig. 11B). That the cell age distribution pattern of meiotic asci differed significantly from apomictic asci was confirmed by comparing cumulative cell division age distributions (data not shown) using the Kalmogorov-Smirnov two sample statistical test (Sokal and Rohlf, 1981). The probability that such differences arose by chance was determined to be of the order of  $10^{-16}$ .

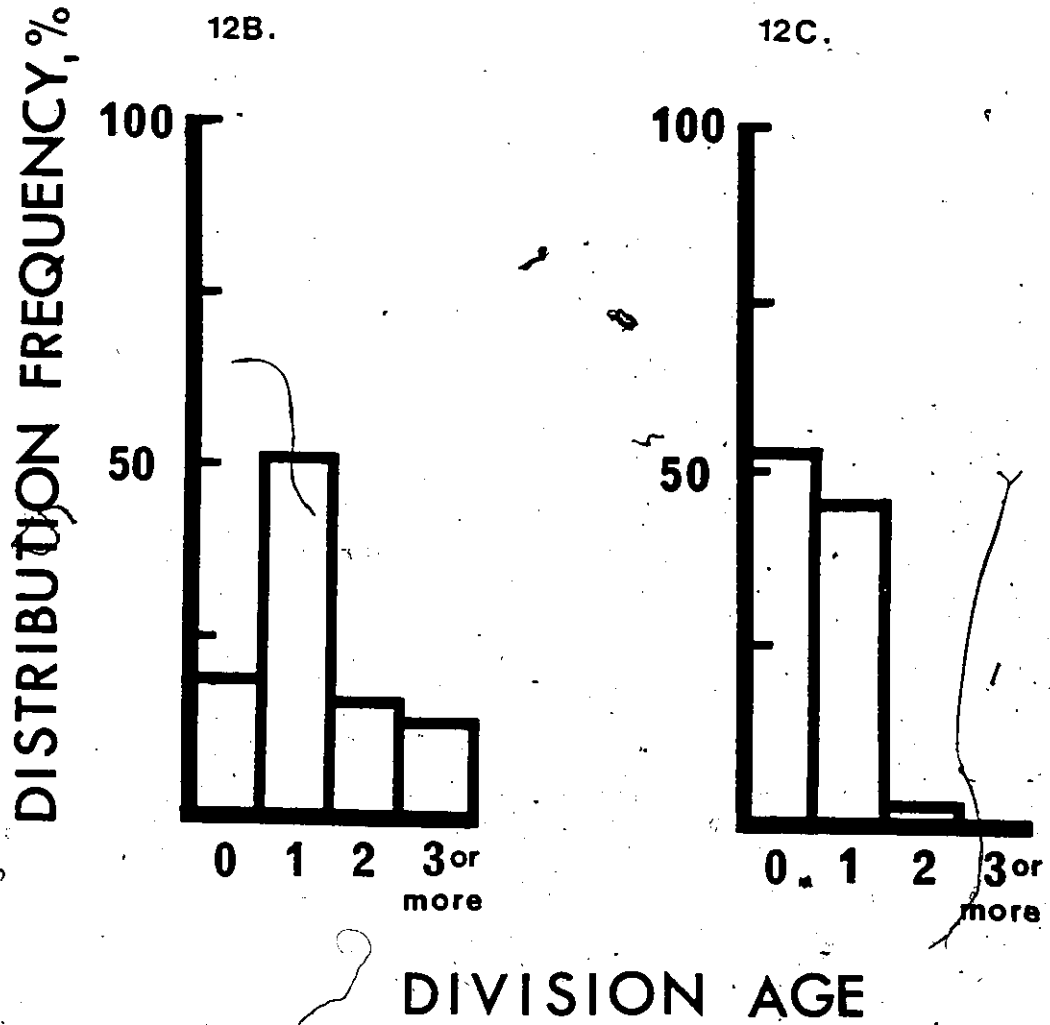
The cell division age distribution patterns for apomictic asci and non-sporogenic cells produced under A-P nutritional conditions are given in Figure 12. Approximately 20% of apomictic asci resulted from differentiation of cells lacking bud scars and approximately 50% resulted from differentiation of cells bearing a single bud scar (Fig. 12B). This was also observed under M-P conditions (Fig. 11B). However the proportion of apomictic asci obtained from differentiation of age 2 cells was higher under M-P than under A-P conditions and the opposite was true of age 3 cells. Incidentally, of non-sporogenic cells observed under A-P conditions the frequencies of age 0 and age 1 cells were similar (Fig. 12C), whereas, under M-P conditions most non-sporogenic cells were age 0 (Fig. 11C).

Figure 12: Relationship between cell age and manner of nuclear division during sporulation under A-P conditions.

Cells were inoculated into 50-ml volumes of 1% glucose YNB PSM at an initial cell density of  $10^5$ /ml and growth culture flasks were incubated 21 h at 27° in a water bath shaker operated at 100 oscillations per min. Cells harvested from growth medium were transferred after washings to 2.0% acetate SPM at a cell density of  $10^7$ /ml. Aliquots (1-ml) were removed from sporulation cultures at 48 h, harvested, washed and stained with calcofluor (see Materials and Methods). By means of fluorescence microscopy the proportion of cells with 0, 1, 2 and 3 or more bud scars was determined by scoring 400 randomly-selected entities in each of the following classes of asci: (B) Apomictic asci; (C) Nonsporogenic asci.

Division age (horizontal axis) refers to the number of bud scars on a given cell.

FIGURE 12



Yanagita et al. (1970) reported that most newborn (age 0) cells of S. cerevisiae lack the ability to sporulate when transferred from PSM to SPM, but that older cells bearing 1 or more bud scars exhibit a higher percentage of sporulation and data in the present study confirm their observation. Of cells which failed to sporulate under M-P and A-P conditions, none had 3 or more bud scars and most were newborn (age 0) cells (Figs. 11C and 12C). That age 0 cells were less sporogenic than older cells bearing 1 or more bud scars is also indicated in Figs. 11A, 11B and 12B.

Thus by fluorescence analysis of bud scar numbers it was found possible to define fundamental differences in cell division age dependency of meiotic versus apomictic sporulation. Older cells (ages 2 and up) underwent meiosis more often than apomixis and young cells (age 1) underwent apomixis more often than meiosis (Figs. 11A and 11B). Age 0 cells, on the other hand, were capable of apomictic but not of meiotic sporulation.

#### Relationship Between Cell Division Age and Cell Size

As noted in the Introduction, the sporogenic capacity of a given cell of S. cerevisiae is known to show some dependence on cell age (Yanigita, et al., 1970; Sando, 1977) and the data from the present study confirmed this (Figs. 11 and 12). But cell size may also be important since it has been reported that small cells exhibit poorer sporulation than larger mother cells (Haber and Halvorson, 1972). Perhaps the basis for the relationship between cell age and manner of nuclear division during sporulation is that there is a correlation between cell age and

cell size. In an effort to determine whether such a correlation exists the major and minor axes of calcofluor-stained vegetative cells were measured and used to calculate the average volumes of cells bearing 0, 1, 2, and 3 or more bud scars respectively. M-P nutritional conditions were used and volumes were determined on cells from 21 h pre-germination cultures. The results are given in Table 3.

The average volume of age 0 cells differed significantly from the average volume of older cells bearing 1, 2 and 3 or more bud scars (t-test, 98 degrees of freedom  $p < 0.001$ ). Therefore it is possible that cell size is correlated with the manner of nuclear division during sporulation.

#### Sites of Zinc Accumulation During Vegetative Growth and Sporulation

Fujii (1954, 1955) used dithizone (diphenylthiocarbazon) in an effort to detect zinc in nucleoli of starfish oocytes. By fractionation and analysis he related dithizone staining to the presence of zinc in the nucleoli. Others have developed this stain as a highly sensitive colorimetric method for zinc determination (Song et al., 1976). Since zinc was found to play an important morphogenic role in restoration of normal meiosis in apomictic yeast (Bilinski and Miller, 1980), the availability of dithizone as a heavy metal stain offered the possibility

Table 3

Relationship between Cell Division Age and Cell Volume<sup>a</sup>

Cell Division Age <sup>b</sup>	Mean Cell Volume, <sup>c</sup> $\mu\text{m}^3$
0	92 $\pm$ 28
1	125 $\pm$ 48
2	127 $\pm$ 34
3 or more	149 $\pm$ 64

<sup>a</sup>Cells of the 19el strain inoculated into 50-ml volumes 6% glucose YNB-PSM containing 25  $\mu\text{g}$  per ml  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  at an initial density of  $10^5/\text{ml}$  were incubated 21 h at 27° in a waterbath shaker operated at 100 oscillations per min. Volumes (10-ml) of vegetative cell suspension were harvested by centrifugation, washed twice with distilled water and stained with calcofluor (see Materials and Methods).

<sup>b</sup>Cell division age refers to the number of calcofluor-stained bud scars on a given cell surface.

<sup>c</sup>In order to calculate cell volumes the major (L) and minor (l) axes of 50 randomly-selected entities in each age group were measured in duplicate experiments. Cell volumes were then calculated using the formula for the volume of a spheroid,  $V = \frac{4}{3}\pi r^3$ , where  $r = \frac{L+l}{4}$ . Each figure represents mean and standard deviation.



of localizing sites of zinc accumulation in vegetative and sporulating yeast.

Initially the specificity of the stain for zinc was assessed. Cells were cultured 21 h in 1% glucose YNB PSM with and without  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  addition and in 6% glucose YNB PSM with and without  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  addition. Cells from each of the respective media were harvested and washed thoroughly with deionized glass-distilled water. The cells were then treated with dithizone staining solution (see Materials and Methods) and then collected onto membrane filters. As shown in Figure 13 only cells cultured in PSM supplemented with zinc displayed the characteristic red color of a positive dithizone test for zinc.

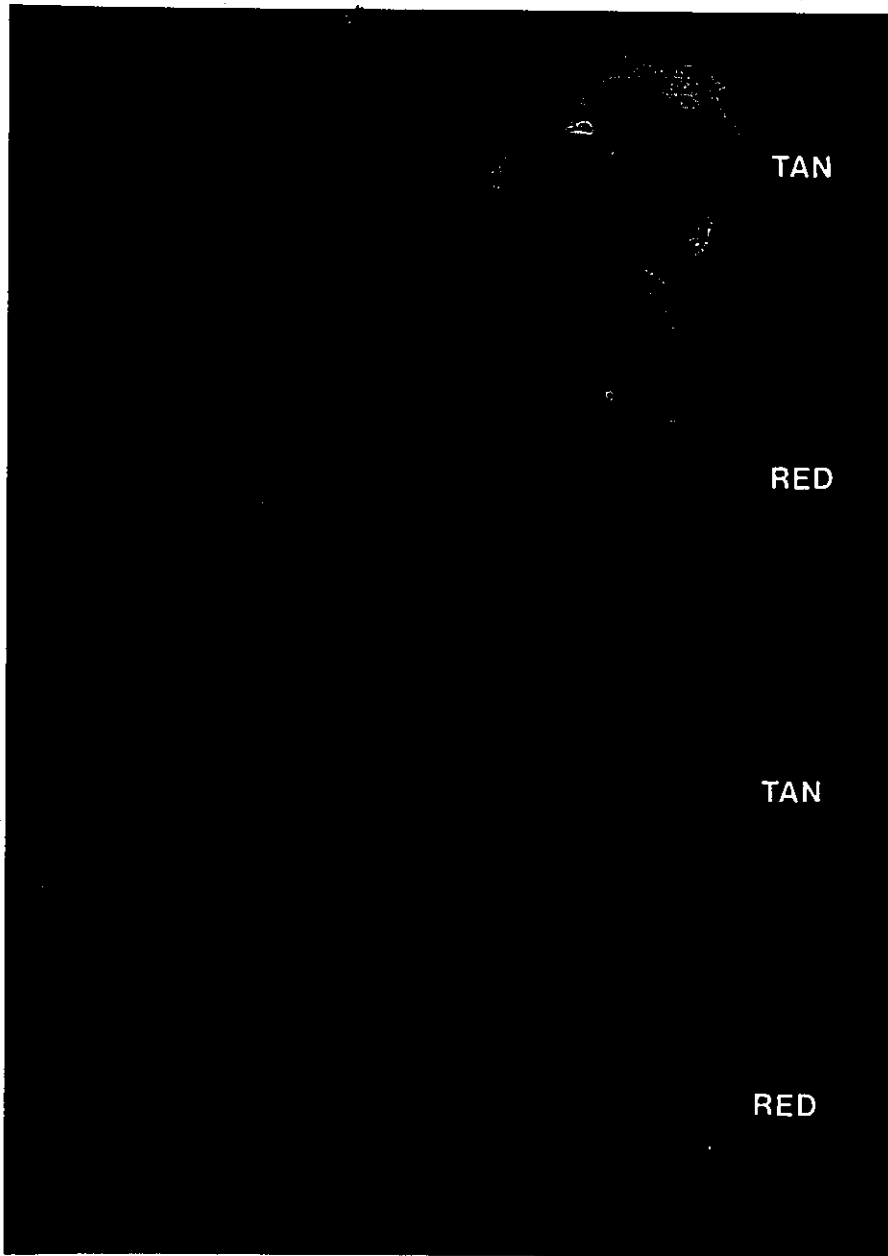
The specificity of dithizone for zinc was investigated by a further in vivo test. Cells were cultured 21 h in 6% glucose YNB presporulation media in which levels of each of the following constituents present in trace quantities (Wickerham, 1951) were tripled individually in amount:  $\text{H}_3\text{BO}_3$ ,  $\text{CuSO}_4$ ,  $\text{KI}$ ,  $\text{FeCl}_3$ ,  $\text{MnSO}_4$ ,  $\text{NaMoO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{NaCl}$  and  $\text{CaCl}_2$ . When cells harvested from each of the media were treated with dithizone, only cells from 6% glucose YNB supplemented with zinc sulfate gave the positive red colour reaction. The specificity of dithizone for this micronutrient was confirmed in vitro in experiments in which dithizone was added to saturated solutions of the foregoing constituents. Again only zinc sulfate gave the typical red colour reaction with dithizone.

Microscopic examination revealed dithizone to stain small spherical entities in vacuoles of vegetative cells and, in asci,

Figure 13: Specificity of dithizone for zinc in Saccharomyces cerevisiae.

Cells were inoculated into 50-ml volumes of PSM (indicated in Figure 13) at an initial density of  $10^5$ /ml and growth culture flasks were incubated 21 h at 27° in a water bath shaker operated at 100 oscillations per min. Cells harvested from growth medium were transferred after thorough washing to 1-ml volumes of dithizone staining solution (see Materials and Methods) for 3 h. Cells thus treated were collected by filtration onto membrane filters (Type HA Millipore, pore diameter 0.45  $\mu$ m) and washed with deionized glass-distilled water.

FIGURE 13



COLOURED PAPER  
PAPIER DE COULEUR

material between spores and spherical entities in spores (possibly in nuclei). To identify sites of zinc localization in the spores, a combined staining procedure involving use of both dithizone and the DNA-binding UV fluorochrome DAPI was developed (see Materials and Methods and Bilinski and Miller, 1983). DAPI also stains vacuolar polyphosphate or volutin bodies (Allan and Miller, 1980), which could be sites of zinc accumulation in vegetative cells.

The vegetative cell in Fig. 14 was stained with dithizone and DAPI. Examination under UV light (Fig. 14a) showed DAPI-induced fluorescence of the nucleus (Williamson and Fennell, 1975), and of the vacuolar polyphosphate body (Allan and Miller, 1980). Standard optics (Fig. 14b) showed the polyphosphate body to stain deep reddish-purple with dithizone and the vacuolar fluid pink. This indicated that zinc, like S-adenosylmethionine (Allan and Miller, 1980), may occur in polyphosphate bodies as a counterion component. The mature ascus containing four haploid ascospores showed DAPI-induced fluorescence of material between spores and of nuclei (Fig. 14c). With dithizone the material between spores stained purple and within spores nucleoli stained deep red (Fig. 14d). The location of the nucleolus in the spores was confirmed by acridine orange staining which induces differential fluorescence of nucleolus and nucleoplasm (Royan and Subramaniam, 1960). On examination of asci stained with toluidine blue (2 mM toluidine blue followed by destaining with 0.1 N HCl) the material between spores stained indicating presence of polyphosphate (Wiame, 1947), but nucleoli did not stain. That the material between spores fluoresced with DAPI also indicated the presence of polyphosphate in

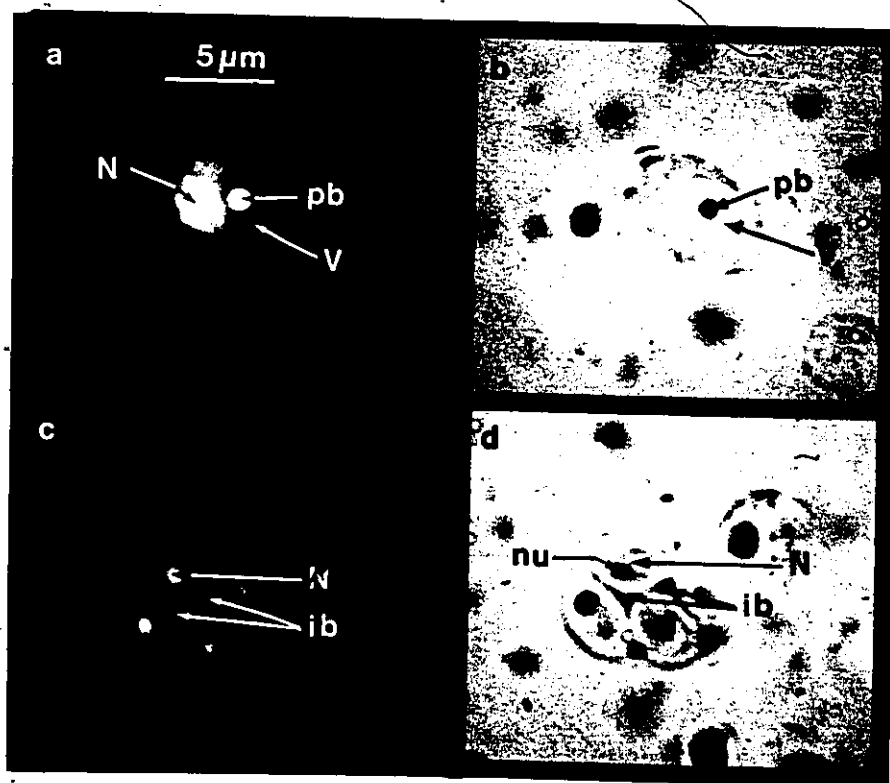
Figure 14: Paired photomicrographs of yeast stained with dithizone and the UV fluorochrome DAPI.

- a,b: Vegetative cell from a 21 h presporulation growth culture. The UV photomicrograph shows fluorescence of nucleus and vacuolar polyphosphate body. In the visible light photomicrograph of the same cell, the polyphosphate body is stained with dithizone.
- c,d: A mature ascus containing a tetrad of spores. Each spore nucleus and the intersporal body fluoresce. The nucleolus and nucleus of each spore and the intersporal body stain with dithizone.

Symbols: N, nucleus; pb, polyphosphate body; V, vacuole; nu, nucleolus; ib, intersporal body.

All photographs are the same magnification as given by the scale bar in photograph a.

FIGURE 14



this location (Fig. 14c). Thus use of fluorochromes (DAPI, acridine orange) made possible positive identification of the entities in spores which stained with dithizone as nucleoli.

Timing of Zinc Translocation from Vacuolar to Nuclear Compartments  
During Sporulation

The foregoing cytological observations were made on cells cultured in presporulation and sporulation media to which zinc sulfate was added. Cells cultured under the same conditions except without zinc sulfate addition to SPM, exhibited the same staining properties with dithizone as those described above for vegetative cells and mature asci, i.e., staining appeared in vacuolar polyphosphate bodies of vegetative cells but then in nucleoli of spores, indicating translocation of zinc from vacuolar to nuclear compartments during differentiation from the vegetative to sporulated state. The timing of this translocation event was investigated using cells cultured under M-P conditions. At hourly intervals for the first 10 h in SPM aliquots of cells were stained with dithizone and the proportion of cells with stained nucleoli was determined. The results are given in Figure 15. The proportion of cells with dithizone-stained nucleoli increased from 0% at 0 h to 20% after 1 h in SPM. Yields remained constant until 3 h and then began to decline at 4 h. Hence it is apparent that translocation of zinc from vacuoles to nucleoli commenced shortly after transfer of cells to sporulation conditions.

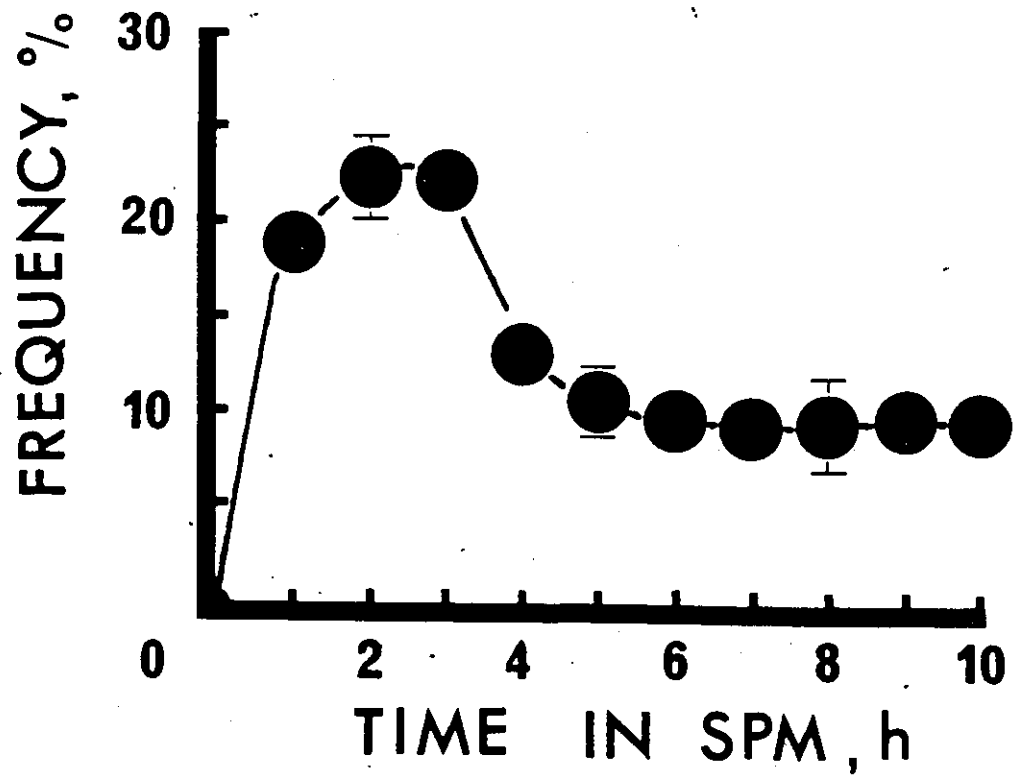
It is noteworthy that loss of zinc from nucleoli and apparent translocation to vacuolar polyphosphate bodies occurred within 30 min of

Figure 15: Timing of translocation of zinc to the nucleolus during sporulation.

Cells harvested from 6% glucose YNB PSM containing 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  were transferred after washing to 50-ml volumes 3.5% acetate SPM containing 25  $\mu\text{g/ml}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  at a cell density of  $10^7/\text{ml}$ . At hourly intervals for the first 10 h in SPM aliquots (1-ml) were withdrawn from SPM, washed with deionized glass-distilled water and treated with dithizone staining solution (see Materials and Methods). The percentage of cells with stained nucleoli was determined by scoring of 200 entities five times in duplicate experiments. Thus each point is based on scoring of 2000 entities, 1000 from each of two experiments. Error bars indicate standard deviation.



FIGURE 15



spore germination in PSM. Thus the translocation of zinc from nucleoli to vacuolar polyphosphate bodies occurred much more quickly during germination than translocation of the micronutrient in the reverse direction observed during the first few hours of sporulation under M-P conditions.

#### Dithizone as a Cytological Stain

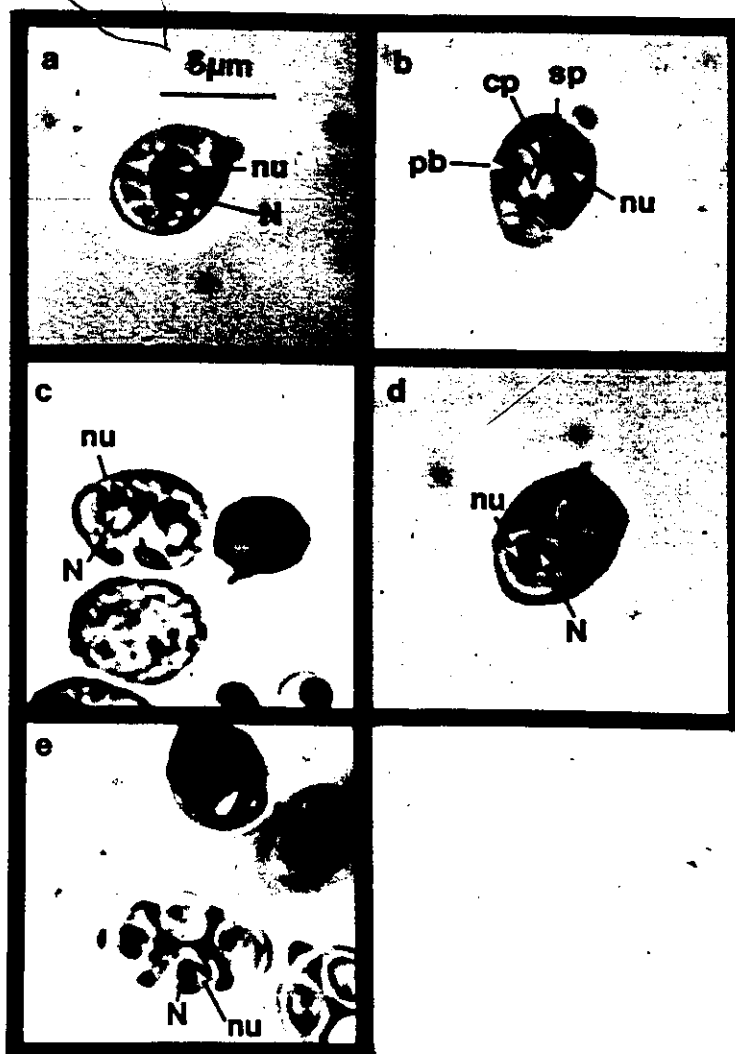
As noted above, an accumulation of zinc in nucleoli during differentiation of cells from the vegetative to the sporulated state was indicated by dithizone staining and in fact nucleoli became dithizone-reactive within the first few hours of incubation of cells under sporulation-inducing conditions (Fig. 15). Figure 16a shows a cell from a 3 h sporulation culture in which the nucleolus and nuclear envelope stained. Vacuolar fragmentation, an event commonly associated with the onset of yeast sporulation (Mundkur, 1961), was evident in this cell and was indicated by the presence of small vacuoles containing faintly stained polyphosphate bodies. Dithizone staining of cells from 8 h sporulation cultures (e.g. Fig. 16b) showed zinc accumulation in polyphosphate bodies and nucleoli but also in centriolar plaques and division spindles. The morphology of the spindle and associated plaques resembles that demonstrated by Robinow and Marak (1966) and Robinow (1980) with an acid fuchsin stain, which also demonstrates the nucleolus. In early stages of spore formation (Fig. 16c) the nucleoli and nuclear membranes stained with dithizone. In mature asci (Figs. 16d, 16e) the nucleoli of spore nuclei stained intensely but spindle pole bodies in nuclear envelopes were barely visible at this stage.

Figure 16: Observations on dithizone-stained sporulating cells.

- a: Cell from a 3 h sporulation culture. The nuclear membrane and nucleolus are stained and faintly stained polyphosphate bodies in small vacuoles are evident in the cytoplasm.
- b: Cell from an 8 h sporulation culture in which centriolar plaques, division spindle and nucleolus of the nucleus and vacuolar polyphosphate bodies stain.
- c: An ascus in early stages of apomictic spore formation from a 16 h culture in which nucleoli and nuclear envelopes are stained.
- d: A mature apomictic ascus containing two diploid spores. The nucleolus of each spore nucleus stains but spindle pole bodies are barely evident at this stage.
- e: A mature meiotic ascus containing four haploid spores. The staining properties are the same as those described in 16d.

Symbols: N, nucleus; pb, polyphosphate body; V, vacuole; nu, nucleolus; sp, spindle; cp, centriolar plaque.

FIGURE 16



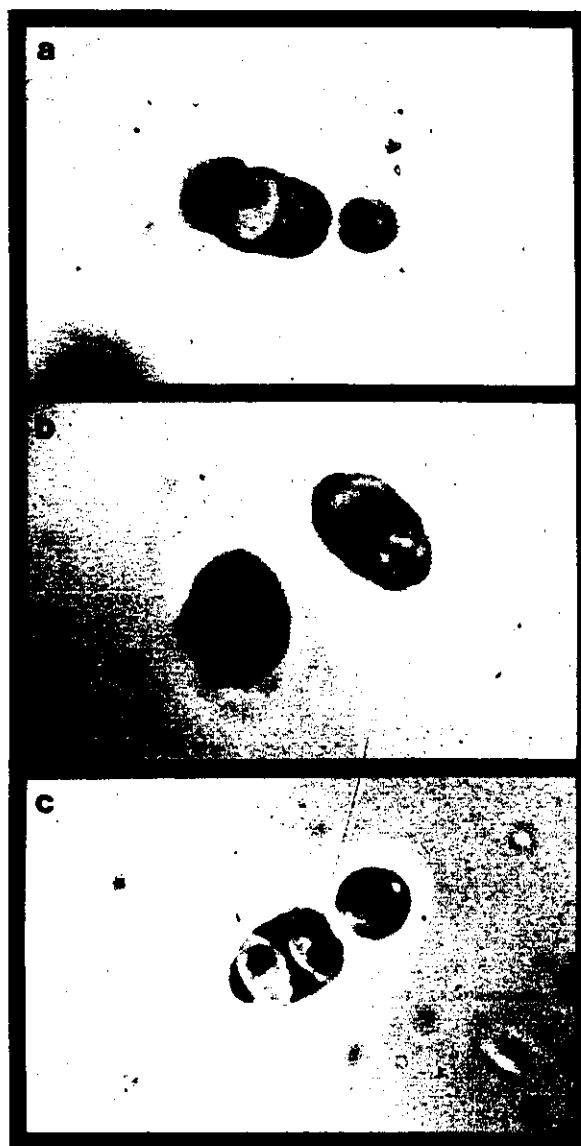
Grewal and Miller (1972) described the formation of a conspicuous structure, the intersporal body, connecting the two spores in apomictic asci. Dithizone staining made it possible to follow the stages of intersporal body formation (Fig. 17). Material accumulated initially outside the spores (Fig. 17a) and as spore development proceeded (Fig. 17b) it tended to move to a central position between the spores (Fig. 17c). In addition to zinc, this structure evidently contains polyphosphate as indicated by DAPI staining (Fig. 14c).

Thus by dithizone staining it was found possible not only to locate zinc microcytochemically in vegetative and sporulating yeast but also to demonstrate some details of yeast nuclear cytology.

Figure 17: Stages of intersporal body formation as revealed by dithizone staining.

- a: Immature ascus from a 24 h sporulation culture in which material outside the spores is stained.
- b: At a later stage (36 h) the stained material outside the spores appears to have migrated toward a central position between the spores.
- c: With ascus maturation (48 h) an intersporal body is well defined.

FIGURE 17



## DISCUSSION

### Prerequisites for Meiosis in Apomictic *Saccharomyces cerevisiae*

In investigations of factors controlling yeast meiosis an approach frequently used has been to compare cytological and metabolic events during sporulation of meiosis-capable and meiosis-incapable strains cultured under identical nutritional conditions (Hopper et al., 1974; Trew et al., 1979). However in the present study a strain of *Saccharomyces cerevisiae* (19el) known to have two alternative modes of nuclear division during sporulation (Bilinski and Miller, 1980; Klapholz and Esposito, 1980a) was employed. Under commonly used culture conditions ascospore formation in 19el is preceded by a single equational nuclear division and consequently only two spores form per ascus, each of which is uninucleate and diploid (Grewal and Miller, 1972). As mentioned in the Introduction, such a developmental process is apomictic, since, by analogy to plant apomixis (Gustafsson, 1946; 1947) a sexual structure (ascus) develops but gamete formation does not occur; instead, diploid progeny (spores) form which are each capable of giving rise to a vegetative clone of cells, competent to sporulate without prior mating. But cultivation of cells in defined presporulation and sporulation media containing elevated carbon source and zinc levels induces about one half of the sporulating cell population to undergo normal meiosis with production of tetranucleate asci containing 3 or 4 uninucleate haploid spores (Bilinski, 1979; Bilinski and Miller, 1980). The ability to condition apomictic yeast



and to induce them, by supplying appropriate nutritional factors, to undergo meiotic development formed the basis for the present study.

This deals with the sequence of certain cytological and biochemical events associated with restoration of meiosis in apomictic yeast (Bilinski et al., 1983), i.e., associated with the reversal of a genetic deficiency by nutritional supplements.

The most striking difference evident between cells cultured under meiosis-promoting (M-P) versus apomixis-promoting (A-P) conditions (as defined in Fig. 5) was that in the former protein synthesis was barely detectable during the first 3 h, whereas in the latter protein synthesis was very high during this period, attaining a maximum at 3 h (Fig. 5). This does not mean that apomictic sporulation requires active protein synthesis during this period because even under M-P conditions approximately half of the cells which sporulated did so apomictically. However, low levels of protein synthesis may be a prerequisite for meiotic differentiation in this yeast system, particularly since cycloheximide, a known inhibitor of protein synthesis in eukaryotes, induced meiosis in cells cultured under A-P conditions when administered for the early hours of sporulation (Fig. 7).

It is known that the capacity of a given cell to undergo meiosis in SPM is dependent on the phase of the cell cycle which it had attained at the time of transfer from PSM to SPM (Haber and Halvorson, 1972). The observation that in M-P conditions half of the cell population which sporulated did so apomictically may be attributed in part to asynchrony in the vegetative cell population. In addition data from the present study concerning the cell division age dependency of meiotic versus

apomictic sporulation can account for the persistent production of apomictic asci even under M-P conditions. Experiments in which bud scar numbers were counted on the surface of mature asci and related to the manner of nuclear division during sporulation revealed that newly-formed (age 0) daughter cells were capable of apomictic but not of meiotic sporulation (Figs. 11A and 11B). Unlike wild type strains of Saccharomyces cerevisiae in which age 0 cells have been observed to sporulate infrequently (Yanagita et al., 1970; Haber and Halvorson, 1972), under both A-P and M-P conditions approximately 20% of 19e1 cells gave apomictic asci that resulted from differentiation of age 0 cells (Figs. 11B and 12B). Thus the genetic deficiency in strain 19e1 prevents meiosis but allows age 0 cells to sporulate. Furthermore, the observation that newly-formed daughter cells were capable of apomictic but not of meiotic sporulation suggests that passage of cells through at least one complete mitotic cell division cycle is required for meiotic but not for apomictic sporulation. Since freshly-formed (age 0) daughter cells tend generally to be smaller than their mother cells at the time of cell division (Yanagita et al., 1970; Tsuboi and Yanagishima, 1974), the apparent requirement that a given cell pass through at least one complete mitotic cell division to undergo meiosis may reflect some minimum cell size prerequisite for entry into a complete meiotic program which does not apply for entry into an apomictic program. In fact experiments to relate the manner of development to cell volume suggest that such a cell size prerequisite may exist (Table 3). Haber and Halvorson (1972) noted that buds

similar in volume to their mother cells at the time of scission sporulate much less frequently.

Thus at least two events are indicated as prerequisites for meiotic tetrad development in strain 19e1: (i) low levels of protein synthesis during early hours of sporulation (Fig. 5) and (ii) passage through at least one complete mitotic cell division cycle. However these events are found not to be prerequisites for apomixis.

#### Qualitative Changes in Protein Synthesis during Sporulation

As described in the Introduction, investigators (Hopper et al., 1974; Petersen et al., 1979; Trew et al., 1979) have approached identification of meiosis-specific yeast proteins by comparing autoradiograms of labeled proteins synthesized by meiosis-capable (MAT a/MAT α) diploid strains to those synthesized during sporulation of meiosis-incapable (MAT a/MAT a), (MAT α/MAT α) diploid and haploid (MAT a or MAT α) strains. Although changes in the pattern of proteins synthesized during sporulation of these strains were detected, the same changes occurred in both meiosis-capable and meiosis-incapable strains. Wright et al. (1981), however, demonstrated recently 21 protein changes specific to MAT a/MAT α cells, some of which were due to the appearance of new polypeptides. In the present study the lower levels of protein synthesis apparent during the first 3 h of sporulation under M-P conditions (Fig. 5) were followed by a period of protein synthesis in which a band present in A-P conditions was absent (Fig. 6, lanes a<sub>5</sub> and m<sub>6</sub>). But in addition a new band appeared in M-P conditions at 6 h which was absent in A-P conditions. Although the observation of these and

possibly other protein band differences between cells in M-P and A-P conditions suggests a change in gene expression, the precise basis for any observed qualitative differences remains to be elucidated. In addition, the possibility that differences between M-P and A-P culture media contributed to these differences cannot be excluded.

#### Temperature Regulation of Nuclear Division during Sporulation

When eukaryotic cells are exposed to temperatures 5 - 15° above their optimum for growth, they respond by inducing synthesis of a small group of proteins, the so-called heat shock proteins (for a review, see Ashburner and Bonner, 1979). Saccharomyces cells respond to temperature elevation with a marked rapid increase in synthesis of heat shock proteins while synthesis of other cellular proteins decreases gradually or continues at normal heat-untreated control levels (Lindquist, 1981). This specific alteration in protein synthesis is accomplished through rapid degradation of pre-existing messenger ribonucleic acids (mRNAs) present prior to exposure of cells to elevated temperatures and through changes in transcription (Lindquist, 1981; Lindquist et al., 1982). The degree of induction of heat shock proteins in yeast has been shown to depend on the carbon source to which cells are exposed. The heat shock response of cells in dextrose-containing medium is transient (Miller et al., 1979; McAlister et al., 1979) whereas the heat shock response of cells in acetate-containing medium can be maintained indefinitely as long as there is continued incubation at elevated temperature and under this condition total protein synthesis declines (Lindquist et al., 1982).

In the present study incubation of the 19e1 strain at an elevated temperature (36°) for the first 1 or 2 h of sporulation in 2% acetate SPM induced an increase in yields of meiotic asci (Fig. 8). As noted in the Introduction, apomixis in strain 19e1 is the consequence of a homozygous recessive mutation designated *spo12-1* (Klapholz and Esposito, 1980a). The absence of a functional *SP012* gene does not appear to interfere with entry of cells into meiosis I as high levels of recombination approximating standard meiotic levels (Klapholz and Esposito, 1980b) are evident, indicating cells to be capable of completing at least one landmark event of prophase of meiosis I. However the cells subsequently fail to complete the first meiotic nuclear division and instead a single equational nuclear division (resembling a meiosis II division occurs to yield two diploid nuclei) instead of the usual four haploid nuclei per ascus (Moens, 1974; Moens *et al.*, 1977; Marmioli *et al.*, 1981a). Since transcription of genes coding for heat shock proteins occur in Saccharomyces cells never exposed to elevated temperatures, as a part of the sporulation process (personal communication, S. Lindquist) it is possible that the high temperature treatment administered to cells of the 19e1 strain compensated for the deficiency in gene function(s) conferred by *spo12-1* by activating transcription of specific genes required to ensure completion of meiosis I followed by meiosis II.

Davidow *et al.* (1980) observed that sporulation culture at 36° followed by a shift down to 23° induces abundant formation of asci containing two uninucleate haploid spores in typical S. cerevisiae. Although the asci were tetranucleate, the spores of the dyads were

furnished with nonsister haploid nuclei that were the products of two separate meiosis II divisions. On electron microscopic examination the two nonsister nuclei which failed to undergo spore enclosure, each harboured a spindle pole body (SPB) that did not exhibit certain characteristic modifications associated with initiation of prospore enclosure. But the other two nonsister nuclei did and spore wall enclosure ensued. These observations suggested a role for SPBs in initiation of enclosure of nuclei by spore walls after completion of the two successive meiotic nuclear divisions and also indicated high temperature treatment to interfere with aspects of their function required for initiation of prospore enclosure.

Although sporulation culture at 36° for the first 1 or 2 h under A-P conditions followed by a downshift to 27° induced formation of tetranucleate asci containing 1,2,3 and 4 uninucleate spores in strain 19el, one-spored asci that were trinucleate containing two smaller unenclosed nuclei and one larger enclosed nucleus were also observed (Fig. 10B). In the absence of genetic data the manner of chromosome segregation which precedes formation of these trinucleate asci is at present unclear. As mentioned in the Results, it is conceivable that the first meiotic nuclear division occurred in these asci but only one of the two meiosis I division products entered and completed meiosis II. Unlike meiosis in typical *S. cerevisiae* (Moens and Rapport, 1971b; Zickler and Olson, 1975), in apomixis the SPB undergoes only one duplication and the second duplication required for formation of the 3<sup>rd</sup> and 4<sup>th</sup> SPBs is either incomplete (Moens et al., 1977) or does not occur at all (Moens, 1974; Marmiroli et al., 1981a). Occasionally in the

sequence of events leading to formation of trinucleate asci in strain 19e1, after completion of one nuclear division, one of the two resultant nuclei initiated spore wall enclosure while the remaining nucleus divided again to yield two smaller nuclei (Fig. 10A) which consistently failed to become enclosed (Fig. 10B). It is possible that after completion of the first meiotic nuclear division, one of the two SPBs failed to duplicate completely and then underwent structural modifications associated with initiation of prospore enclosure, while the other duplicated completely facilitating occurrence of a meiosis II division to yield two haploid nuclei. Such a sequence is conceivable in a genotype that produces apomixis for two reasons:

- (i) the second round of duplication of the SPBs to yield the 3<sup>rd</sup> and 4<sup>th</sup> SPBs which in meiosis of wild type S. cerevisiae is required for the second meiotic nuclear division after completion of meiosis I has been observed to be incomplete in some of the developing asci in apomictic S. cerevisiae (Moens et al., 1977).
- (ii) a small proportion of dyads (approximately 5%) in apomictic S. cerevisiae result from enclosure of 'diploid' products of a single complete reductional nuclear division (Klapholz and Esposito, 1980b).

Spore wall enclosure of the 'diploid' products of a meiosis I division has been reported in two temperature-sensitive cell division cycle (cdc) mutants of S. cerevisiae that are defective in mitotic nuclear division in PSM and in meiosis in SPM at their restrictive temperature (Schild and Byers, 1980). When diploid strains homozygous for either of these two temperature-sensitive mutations, cdc 5 and cdc 14, are cultured

vegetatively at the permissive temperature and sporulated at a semi-restrictive temperature, meiosis I proceeds to completion and two meiosis II spindles develop but they fail to elongate. Consequently encapsulation of meiosis I division products occurs to yield two uninucleate 'diploid' spores per ascus. In strain 19e1, failure of one of two meiosis II division spindles to undergo elongation could result in formation of trinucleate asci.

Temperature shock treatments administered to flagellates of Naegleria stimulate duplication of "centriole-like" basal bodies, structures which share some structural and functional homology with centrioles of dividing nuclei (Dingle, 1970). Such an effect of temperature shock on subsequent SPB duplication during meiosis in strain 19e1 may have occurred at one of the meiosis I division poles, thereby satisfying a precondition for occurrence of a meiosis II division at one pole. This would yield a trinucleate ascus.

Combined electron microscopic and genetic studies should prove useful in analyzing the sequence of ultrastructural changes and the sequence of meiotic events that occur during the course of formation of temperature shock-induced trinucleate asci in strain 19e1. Although the role of the SPB in control of chromosome segregation during yeast meiosis is unknown, apomixis in strain 19e1 may be due to a defect in some aspect of spindle pole body duplication, separation and function. It is known from in vitro studies that SPBs serve as nucleation sites for the tubulin polymerization (Byers et al., 1978; Hyams and Borisy, 1978) necessary for formation of the spindle apparatus in vivo and as noted in the Introduction, it has been proposed that apomixis may be due



to formation of a defective meiosis I spindle apparatus (Klapholz and Esposito, 1980b).

Possible Roles for Zinc in Restoration of Meiosis in Apomictic *S.*

*cerevisiae*

Although significant increases in yields of meiotic tetrads occurred in SPM when strain 19el was cultivated at high carbon source levels, maximum yields occurred when both PSM and SPM were supplemented further by the addition of zinc sulfate (Table 1 and also see Bilinski and Miller, 1980). However in the other two known apomictic strains of *S. cerevisiae* (ATCC-4117 and ATCC-4098) increases in carbon source levels alone did not stimulate meiosis in SPM but the combination of increased carbon source and zinc levels did (Table 1). That meiotic sporulation in these two strains depended closely on addition of zinc to the nutritional environment indicates that this micronutrient plays a key morphogenic role in restoration of meiosis. The presence of higher carbon source levels in PSM and SPM may be required to enhance zinc uptake (Failla et al., 1976). The importance of this element in restoration of meiosis in apomictic *S. cerevisiae* is also indicated by the observation that addition of two salts known to inhibit zinc uptake in yeast,  $\text{CaCl}_2$  (Failla et al., 1976) and  $\text{KH}_2\text{PO}_4$  (Fuhrman and Rothstein, 1968) inhibited meiotic tetrad formation in strain 19el (Bilinski and Miller, 1980). Also, presporulation culture at a higher temperature ( $36^\circ$ ), a condition known to enhance zinc uptake in yeast (Failla et al., 1976), increased the frequency of meiotic tetrads produced in SPM (Table 2). The precise role of the micronutrient remains to be elucidated.

Zinc is known to interact with membranes, participate in polymeric organization of macromolecules (Chvapil, 1973), affect chromatin configuration (Kvist, 1980), promote tubulin polymerization (Crepeau and Fram, 1981), affect ribosome structure (Subcommittee on Zinc, 1979) and accumulate in nucleoli (Fujii, 1954, 1955). In fact, the dithizone staining procedure developed in the present study indicated translocation of zinc from vacuolar to nuclear compartments shortly after transfer of cells from PSM to SPM (Bilinski and Miller, 1983). Zinc accumulated in nucleoli in the first few hours of sporulation under M-P conditions (Fig. 15) during the period of protein synthesis limitation (Fig. 5). Yet once protein synthesis commenced under M-P conditions, the proportion of cells with dithizone-stained nucleoli began to decline indicating release of zinc from nucleoli. There may be a relation between the accumulation of zinc in nucleoli and lower levels of protein synthesis during the first few hours of sporulation under M-P conditions since nucleoli are the sites of synthesis of ribosomal RNA (Sillevis Smitt et al., 1972; 1973), an integral component of ribosomes needed for protein synthesis (for a review see Warner, 1981).

As mentioned previously, in apomixis only a single nuclear division occurs and this division initially resembles meiosis I but then takes on the appearance of meiosis II, particularly in the structure of the spindle plaque. It has been proposed that the single equational nuclear division which precedes spore formation may be due to a defective meiosis I spindle apparatus (Klapholz and Esposito, 1980b). Since zinc promoted occurrence of the two successive meiotic nuclear divisions in apomictic S. cerevisiae (Table 1), the microcytochemical

detection of zinc in centriolar plaques and division spindles of sporulating cells (Fig. 16B) is of particular interest. It is conceivable that zinc confers stability on the meiosis I spindle apparatus and thereby facilitates completion of the reductional chromosome segregation of meiosis I followed by meiosis II.





#### Timing of the Decision Concerning the Manner of Development during Sporulation

The approach taken in the present study has led to the identification of a number of treatments which restored to cells of the 19el strain the ability to undergo meiotic rather than apomictic development under sporulation-inducing conditions. These are summarized in Figure 18. Under M-P conditions low levels of protein synthesis during the first 3 h of sporulation were correlated with restoration of meiosis (Fig. 18B). Cycloheximide was similar to increased zinc in its effect on development since the drug limited protein synthesis and was most effective in stimulating occurrence of meiosis when administered to cells cultured under A-P conditions for the first 3 h of sporulation (Fig. 18A, ii). Heat shock, a treatment known to transiently decrease rates of precursor ribosomal RNA production (Johnston and Singer, 1980), inhibit synthesis of ribosomal proteins (Gorenstein and Warner, 1976; Warner and Gorenstein, 1977) and alter transcription (Lindquist, 1981) was effective when administered for the first 1 or 2 h in SPM (Fig. 18A, iii). Incubation of sporulation cultures at a low temperature (18°), a treatment not used previously in yeast sporulation studies, was effective when given for the first 3 h in SPM (Fig. 18A, iv). That all

Figure 18: Summary of treatments that restored meiosis in *S. cerevisiae* strain 19el.

A: Treatments administered under A-P nutritional conditions:

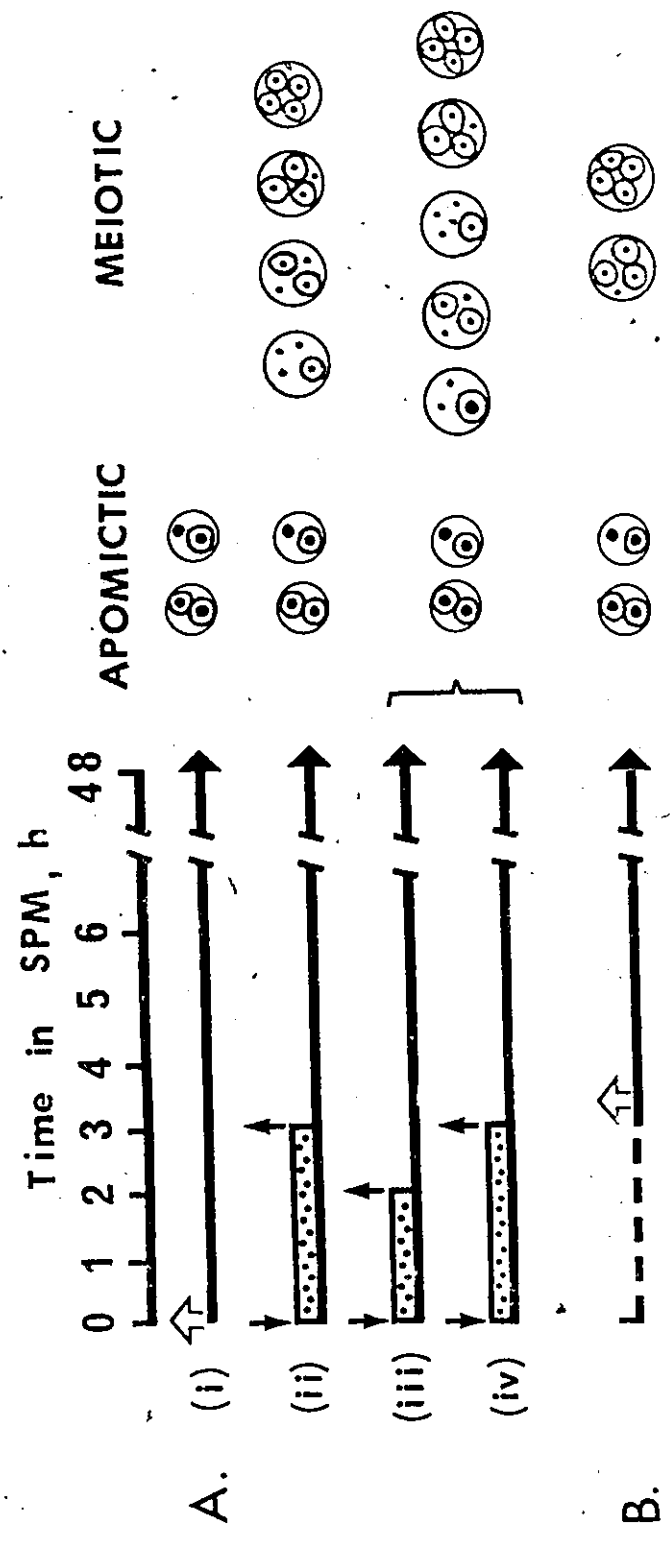
- (i) Untreated control cultures
- (ii) Cycloheximide treatment (based on Fig. 7)
- (iii) Heat shock (based on Fig. 8)
- (iv) Cold shock (based on Fig. 9)

Symbols: , time in SPM when protein synthesis commenced;  
, time in SPM when cells were administered treatment;  
, time in SPM when cells released from treatment;  
, optimum duration of treatment for induction of meiotic asci.

B: Cultivation under M-P nutritional conditions. -----, period during which protein synthesis is limited (based on Fig. 5) and during which cells accumulated zinc in nucleoli (based on Fig. 15).

In both A and B, the classes of asci resulting in SPM are indicated on the right.

FIGURE 18



treatments found to restore meiotic development were effective when administered for the early hours of sporulation indicate that the decision concerning the manner of development, apomictic or meiotic, a given cell will follow is made shortly after transfer of vegetative cells from PSM to SPM.

#### Hypotheses to Account for Restoration of Meiosis in Apomictic Yeast

As described in the Introduction, Klapholz and Esposito (1980a) determined that strain 19el has a homozygous recessive nuclear gene mutation, designated *spol2-1*, which caused apomixis. Electron microscopic (Moens et al., 1977; Marmioli et al., 1981a) and genetic (Klapholz and Esposito, 1980b) studies indicate that in apomictic development duplicated homologues synapse and recombine as in prophase of meiosis I. However, meiosis I does not proceed to completion, that is, homologous centromeres and of course chromosomes fail to segregate from each other to opposite division poles. Instead they desynapse completely and divide equationally as in meiosis II to yield two diploid nuclei and consequently two diploid spores per ascus. With restoration of meiosis and tetrad formation in strain 19el, it follows that the various treatments summarized in Fig. 18 conferred on cells an ability to overcome the deficiency in gene function(s) caused by *spol2-1*, such that they become competent to complete meiosis I chromosome segregation followed by meiosis II. From the data on which Fig. 18 is based it can be suggested that key metabolic events occur shortly after transfer of cells to sporulation-inducing conditions which restored the ability to undergo a complete meiotic programme. The nature of the underlying

molecular events involved in restoration of meiosis in strain 19e1 is at present unclear. However several testable hypotheses are offered and these are described below. These hypotheses attempt to integrate nutritional, cytological and biochemical data of the present study with the genetic and ultrastructural information on the apomictic strains detailed above and with information derived from studies of other systems.

I. One early landmark biochemical event associated with the onset of yeast sporulation is respiratory adaptation (Klapholz and Esposito, 1981), a process required for the shift from fermentative to respiratory metabolism that involves expression of both mitochondrial and nuclear genes (Dujon, 1981), which for glucose-grown cells is completed within the first 2-4 h in acetate SPM (Marmioli et al., 1981b). Zinc, a micronutrient shown to play a key role in restoration of meiosis in apomictic S. cerevisiae (Table 1) is known to stimulate cytochrome synthesis and respiration in fungi including yeast (Grimm and Allan, 1954; Ward and Nickerson, 1958). This may be of considerable importance in view of the fact that apomictic sporulation occurs in the presence of a known repressor of respiration, glucose (Eaton and Klein, 1954; Polakis and Bartley, 1965; Witt et al., 1966), added to acetate SPM (Grewal and Miller, 1972; Bilinski and Miller, 1980). Incidentally, an apparent absence of a dependency on respiratory adaptation to initiate apomixis can account for the observation that a few cells cultured under A-P conditions had initiated apomictic nuclear division at the time of transfer from PSM to SPM (Fig. 4A). Inclusion of glucose in acetate SPM

prevents occurrence of the two successive meiotic nuclear divisions by arresting development in meiosis I not only in typical (Miller, 1964) but also in apomictic S. cerevisiae cultured under meiosis-permissive conditions (Bilinski and Miller, 1980). This suggests apomictic unlike meiotic sporulation to be independent of mitochondrial function. In fact it has been recently demonstrated that meiosis in apomictic S. cerevisiae is dependent on mitochondrial protein synthesis whereas apomixis is not (personal communication, N. Marmioli). Using the nutritional regime which gave highest yields of meiotic tetrads in strain ATCC-4117 (Table 1), Marmioli and his co-workers showed that in a derivative strain, 4117-H2, inclusion in SPM of erythromycin, a specific inhibitor of mitochondrial protein synthesis (Lamb et al., 1968), blocks meiotic but not apomictic development (manuscript in preparation). This has also been confirmed in our laboratory in strain 19e1 (unpublished data, M. Gross and S. Mokrytzki). That certain yeast nuclear genes may mediate expression of mitochondrial genes and vice versa has been reported (Dujardin et al., 1980a,b; Algeri et al., 1981). Perhaps strain 19e1 is defective in some nucleo-mitochondrial interaction required for the two successive meiotic nuclear divisions.

As noted previously, low levels of protein synthesis during the early hours of sporulation under M-P conditions (Fig. 5) were correlated with restoration of meiosis in strain 19e1 in experiments in which cells cultured under A-P conditions were administered the protein synthesis inhibiting drug, cycloheximide (Fig. 7). It has been reported that cycloheximide specifically inhibits cytoplasmic protein synthesis and that residual protein synthesis resistant to cycloheximide constitutes



the fraction of total protein synthesis carried out in mitochondria (Clark-Walker and Linnane, 1966; Lamb et al., 1968). In fact, cycloheximide has been used in experiments in which labeling of proteins was being studied within the yeast mitochondrial compartment (Douglas and Butow, 1976; Douglas et al., 1979). Residual protein synthesis during cycloheximide treatment of cells cultured under A-P conditions and during the first 3 h of sporulation under M-P conditions may be attributed to mitochondrial protein synthesis. Thus it is conceivable that during the early period of protein synthesis limitation which was correlated with induction of meiosis in strain 19e1 that alterations in mitochondrial activity occur which play a role in the determination of a meiotic phenotype.

The foregoing hypothesis that mitochondrial functions may play a role in the restoration of meiosis could be experimentally tested by determining whether mitochondrial translation products are required for initiation or completion of the two successive meiotic nuclear divisions in strain 19e1.

II. Although this study does not deal with analysis of the cell cycle dependency of apomictic versus meiotic sporulation, cell cycle regulation may be involved in induction of meiosis in strain 19e1. The yeast mitotic cell cycle is conventionally divided into a  $G_1$  phase, which precedes initiation of nuclear DNA synthesis; an S phase, during which chromosomal DNA is replicated; a subsequent  $G_2$  phase; and an M phase during which mitosis and nuclear division occur (for a review, see Pringle and Hartwell, 1981).  $G_1$  is defined as the interval of time

between completion of nuclear division and initiation of DNA synthesis. Most yeast cells arrest in  $G_1$  before they embark upon a meiotic programme (Hirschberg and Simchen, 1977) and it is known that gene functions required for entry of cells into meiosis occur within the  $G_1$  interval of the cell division cycle (Shilo et al., 1978). But Marmioli et al., (1981c) doubt the existence of a true  $G_1$  period in the mitotic cell division cycle of apomictic *S. cerevisiae* - in some cases both mother and daughter cells were able to produce buds prior to their separation by cell membranes (cytokinesis) and cell walls. Although their suggestion of the absence of a typical  $G_1$  period is based on ultrastructural observations and not on determinations of the amount of time elapsed between completion of nuclear division and initiation of DNA synthesis, the various treatments which restored meiosis in strain 19e1 given in Fig. 18 may have done so by affecting passage of cells through the  $G_1$  phase of the cell division cycle after the transfer from PSM to SPM. Unger and Hartwell (1976) showed that conditions which limit the process of protein synthesis can serve as 'signals' for  $G_1$  arrest and clearly lower levels of protein synthesis during early hours of sporulation under M-P conditions (Fig. 18B) was correlated with induction of meiosis. Cycloheximide, a drug known to inhibit protein synthesis and to arrest yeast cells in  $G_1$  (Hartwell and Unger, 1977), stimulated occurrence of meiosis when administered for the early hours of sporulation under A-P conditions (Fig. 18A, ii). Johnston and Singer (1980) demonstrated a transient arrest of yeast cells in  $G_1$  by heat shock, a treatment which when administered shortly after transfer of cells to SPM increased the frequency of meiosis (Fig. 18A, iii). That

treatments known to arrest yeast cells in  $G_1$  restored meiotic tetrad formation in strain 19e1 may be of importance for the following reasons:

- (i) all treatments were found most effective in restoration of normal meiotic development when administered for the first few hours in SPM during which cells are known to make the transition from mitosis to meiosis (Esposito and Esposito, 1974; Hartwell, 1974; Shilo et al., 1978) and
- (ii) the single equational nuclear division which precedes apomictic dyad formation may be due to defects in 'signals' regulating the start of meiosis I (Klapholz and Esposito, 1980b). It would not be unreasonable that some controlling events for meiotic differentiation in yeast occur within the  $G_1$  interval of the cell division cycle as is indicated by the available genetic evidence (Hartwell, 1974; Hirschberg and Simchen, 1977; Shilo et al., 1978).

An analysis of the cell division cycle dependency of apomictic versus meiotic sporulation would give valuable information as to whether entry into apomixis occurs at the same or different point(s) in the cell division cycle as entry into a complete meiotic sequence.

Initiation of the mitotic cell division cycle, which commences at a point in the  $G_1$  phase, termed "start" (Hartwell, 1974), requires several gene functions including CDC28, TRA3, CDC25 and CDC35 (Hereford and Hartwell, 1974; Wolfner et al., 1975; Johnston et al., 1977). Shilo et al. (1978) demonstrated that two of the loci, CDC28 and TRA3, are

required for both initiation of the mitotic cell division cycle and for entry into meiosis.- In contrast, the other two loci, CDC25 and CDC35, were required for initiation of the mitotic cell division cycle but not for entry into meiosis. Diploid strains homozygous for temperature-sensitive mutations in either of these two genes were capable of meiosis in acetate SPM at both permissive and restrictive temperatures. Since these two gene functions were found essential for initiation of the mitotic cell division cycle but not for initiation of meiosis, the authors suggest that CDC25 and CDC35 regulate the choice between meiosis and mitosis. In the writer's opinion, CDC35 and CDC25 may be mitotic cell division cycle gene functions which are normally turned off when vegetative cells of S. cerevisiae are transferred from PSM to SPM, thereby allowing cells to enter a complete meiotic program. This would account for the ability of these two mutants to undergo meiosis in SPM at both permissive and restrictive temperatures. An important question which should be considered is the phenotypic consequence of failure to turn off certain mitotic cell division cycle gene functions such as CDC35 and CDC25 on meiosis and sporulation in S. cerevisiae (see hypothesis III below).

III. The occurrence of a single equational nuclear division in strain 19e1 may be the consequence of any of the following:

- (i) a failure to maintain synapsis between recombined homologues resulting in presence of univalents rather than ~~the~~ bivalents at the time of congression at metaphase of meiosis I which may in turn

- 'signal' occurrence of meiosis II with equational sister centromere disjunction,
- (ii) A precocious 'signal' for initiation of meiosis II during meiosis I which interrupts and terminates meiosis I resulting in failure to complete the first meiotic nuclear division, and
  - (iii) formation of a defective meiosis I spindle apparatus or a defect in centromere-spindle attachment sites on paired homologues.

The consequences of the above defects could be the formation of two diploid nuclei per ascus. It should be stressed, however, that only ultrastructural evidence based on a sudden change in spindle and spindle pole body morphology suggests occurrence of meiosis II in apomictic S. cerevisiae and this may be incorrect. Moens (1974), Moens et al. (1977) and Marmiroli et al. (1981a) observed that the two spindle bodies from which the spindle apparatus emanates initially are mitotic or meiosis I-like but then suddenly they adopt a modified appearance characteristic of meiosis II in typically four-spored Saccharomyces (Moens and Rapport, 1971b; Peterson, Gray and Ris, 1972). Although these ultrastructural observations may indicate that some functions associated with meiosis II are expressed, this does not necessarily mean that a single meiosis II division precedes apomictic dyad formation. In an ameiotic (am) mutant of Zea mays ultrastructural studies (Palmer, 1971) have shown the development of a spindle apparatus that is morphologically meiotic but meiosis does not occur. Instead a mitotic nuclear division takes place in the developing meiocytes. In an asynaptic mutant (as<sub>3</sub>) in Brassica campestris (Stringham, 1970) univalents congress at metaphase and divide equationally at anaphase of meiosis I. Meiosis II does not occur:

diploid nuclei are produced. Although apomictic S. cerevisiae are clearly not asynaptic, failure to maintain synapsis or some form of an association between duplicated homologues after completion of recombination may lead to occurrence of an equational division of univalents at anaphase of meiosis I to yield two diploid nuclei per ascus. The presence of univalents at metaphase of meiosis I rather than bivalents may have signalled the spindle pole bodies to adopt morphological characteristics of meiosis II. As to whether univalents or bivalents congress to metaphase of meiosis I in apomictic development is unknown since the available electron microscopic techniques have not shown electron opaque chromosomes at this stage of meiosis in Saccharomyces (Moens and Rapport, 1971b; Zickler and Olson, 1975).

Strain 19e1 harbours a homozygous recessive nuclear gene mutation, *spol2-1*, which confers an apomictic phenotype (Klapholz and Esposito, 1980a). However under appropriate culture conditions in which protein synthesis is inhibited by cycloheximide or reduced markedly for the first few hours of sporulation, a significant number of cells express a meiotic rather than an apomictic phenotype (Figs. 18A, ii and 18B). It is difficult to reconcile suppression of an apomictic phenotype by nutrition with its suppression by other environmental manipulations. Much of our understanding of the meiotic process in yeast has come from studies of conditional thermosensitive mutants and no report has been made hitherto of suppression of a conditional mutant of meiosis by not only one but by several different experimental manipulations.

It is well known that induction of gene expression by transcriptional activation is an important aspect of development and differentiation. Various genes are silent until a particular stimulus results in activation of transcription of the gene(s). Examples of such positive transcriptional control include various genes subject to hormonal control (Swanek et al., 1979; LeMeur et al., 1981) and the Drosophila (Ashburner and Bonner, 1979) and yeast (Lindquist, 1981; Lindquist et al., 1982) heat shock genes. When vegetative cells of the 19e1 strain are transferred from PSM to SPM, it is conceivable that the presence of certain cellular factor(s) exert positive control over transcription of certain mitotic or vegetative genes which must be turned off in a *spo12-1* mutant background to allow expression of certain meiosis gene functions. This hypothesis predicts all treatments which restored meiosis, regardless of whether they act via common or different mechanisms, lead to synthesis of gene products required for cells to undergo a complete meiotic sequence. Expression of these genes may be necessary for maintenance of synapsis after completion of recombination, stabilization of the kinetic apparatus or in preventing precocious 'signals' for equational sister centromere disjunction during meiosis I. As noted above, a defect in any of these aspects of meiosis could cause occurrence of a single equational nuclear division and apomictic dyad formation.

Proteolysis is a process which commences on transfer of vegetative Saccharomyces cerevisiae from PSM to SPM (Hopper et al., 1974; Klar and Halvorson, 1975; Betz and Weiser, 1976). Inhibition of protein synthesis by cycloheximide prevents initiation of premeiotic DNA

synthesis but has no apparent effect on rates of protein breakdown during sporulation of S. cerevisiae (Magee and Hopper, 1974). Such a condition in which protein synthesis is inhibited by cycloheximide would permit endogenous proteolytic enzyme activities to degrade sufficiently the cellular factor(s) thereby allowing transcriptional activation of genes required to ensure completion of meiosis I followed by meiosis II. That some change in gene expression may be involved is suggested by the observation that heat shock, a treatment known to alter transcription and protein synthesis in Saccharomyces (McAlister et al., 1979; Lindquist, 1981) induced a significant number of cells to express subsequently a meiotic phenotype (Fig. 18A, iii). As shown in Fig. 6, the low levels of protein synthesis during the early hours of sporulation under M-P conditions was followed by a period of protein synthesis in which a band appeared which was absent in A-P conditions. Conversely a band present in A-P conditions was absent in M-P conditions. Although the basis for these and possibly other qualitative differences remains to be elucidated, they may reflect a change in transcription.

In the adenovirus system, a region designated E1A, codes for proteins required for the transcriptional activation of early viral genes (Berk et al., 1979; Jones and Schenk, 1979; Nevins, 1981). However, when mammalian cells are infected with E1A-mutant virus harbouring a deletion in the E1A region under conditions in which protein synthesis has been inhibited by cycloheximide or other drugs, transcription of the early viral genes occurs in the E1A-deletion mutant background, suggesting the wild type E1A protein to affect cellular



control function so as to allow transcription of the early viral genes (Nevins, 1981). The E1A region of adenovirus and the SP012 gene of Saccharomyces may have analogous functions. In fact the similarity between this adenovirus system and the yeast system under study here is quite remarkable. E1A deletion-mutants are conditionally mutant for some unknown physiological factor at high multiplicity of infection (Nevins, 1981). The two known apomixis genes (spol2-1, spol3-1) in S. cerevisiae are also conditionally mutant for some unknown physiological factor since workers have observed production of a few percent tetrads by apomictic S. cerevisiae under commonly-used culture conditions (Moens et al., 1977; Bilinski and Miller, 1980; Klapholz and Esposito, 1980a). The E1A gene products are known to activate at least one cellular gene specifying a HeLa cell heat shock protein (Nevins, 1981). Heat shock treatments administered to cells of the 19el strain stimulated meiotic differentiation (Fig. 18A, iii) and the available evidence from heat shock studies in S. cerevisiae suggests heat shock proteins to play a role in meiosis and sporulation (Lindquist et al., 1982).

The foregoing three hypotheses are not the only possible explanations for restoration of meiosis in apomictic S. cerevisiae but to the writer they seem the most reasonable. With the alternative culture conditions developed in the present study for restoration of a meiotic phenotype in an apomictic strain of S. cerevisiae it will now be possible to analyze further the molecular basis for induction and regulation of certain key events in meiosis of S. cerevisiae.

5

SUMMARY

1. The sequence of nuclear events and of protein synthesis during the course of sporulation under meiosis-promoting and apomixis-promoting nutritional conditions was monitored. Comparison of the sequence of events between the two nutritional conditions indicated the following to be prerequisites for meiosis but not for apomixis in Saccharomyces cerevisiae strain 19el:

- (a) limitation of protein synthesis during early hours of sporulation, and
- (b) passage of a given cell through at least one complete mitotic cell division cycle.

2. Three environmental manipulations were identified which suppress apomixis and promote meiosis in strain 19el:

- (a) presporulation culture at an elevated temperature (36°),
- (b) cycloheximide inhibition of early protein synthesis in sporulation cultures, and
- (c) temperature shock.

3. It was concluded that the decision concerning the manner of development, apomictic or meiotic, a given cell will follow is made shortly after the transfer of cells to sporulation-inducing conditions for the following reasons:

- (a) low levels of protein synthesis detected during early hours of sporulation under meiosis-promoting conditions were correlated with induction of meiosis in experiments in which cells cultured under apomixis-promoting conditions were administered the protein synthesis inhibitor cycloheximide,

and

(b) treatments (cycloheximide, temperature shock) administered to sporulation cultures were found most effective in stimulating meiosis when given for the early hours of sporulation.

4. A comparative study of the response of the three known apomictic strains of S. cerevisiae (19e1, ATCC-4117, ATCC-4098) to adjustments in carbon source and zinc levels indicated zinc to be the key metabolic factor involved in restoration of meiosis in these yeasts. A novel microcytochemical staining procedure was developed to determine sites of zinc localization in vegetative and sporulating yeast. The procedure was used to monitor the distribution of zinc during sporulation under meiosis-promoting conditions. The micronutrilite translocated from vacuolar to nuclear compartments during the early critical period of sporulation. Possible roles of zinc in the meiotic process were considered.

APPENDIX I

Composition of Yeast Nitrogen Base+

	Concentration in a 0.67% solution YNB(µg/ml)
<b>Nitrogen source</b>	
Ammonium sulfate	5,000
<b>Amino Acids</b>	
l-Histidine Monohydrochloride	10
dl-Methionine	20
dl-Tryptophane	20
<b>Vitamins</b>	
Inositol	2
Calcium Pantothenate	0.4
Niacin	0.4
Pyridoxine Hydrochloride	0.4
Thiamine Hydrochloride	0.4
Biotin	0.002
Para-Aminobenzoic acid	0.2
Riboflavin	0.2
Folic Acid	0.002
<b>Salts</b>	
MgSO <sub>4</sub>	250
CaCl <sub>2</sub> ·2H <sub>2</sub> O	75
KH <sub>2</sub> PO <sub>4</sub>	1,000
NaCl	100
<b>Compounds supplying trace elements</b>	
Acid Boric	0.5
KI	0.1
FeCl <sub>3</sub>	0.2
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.4
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.2
MnSO <sub>4</sub>	0.4
CuSO <sub>4</sub>	0.025

+Wickerman (1951)

#### REFERENCES

- ALGERI, A.A., L. BIANCHI, A.M. VIOLA, P. P. PUGLISI, and N. MARMIROLI.  
1981. IMP1/impl: A gene involved in the nucleo-mitochondrial control of galactose fermentation in Saccharomyces cerevisiae. Genetics 97: 27-44.
- ALLAN, R.A., and J.J. MILLER. 1980. Influence of S-adenosylmethionine on DAPI-induced fluorescence of polyphosphate in the yeast vacuole. Can. J. Microbiol. 26: 912-920.
- ASHBURNER, M., and J.J. BONNER. 1979. The induction of gene activity in Drosophila by heat shock. Cell 17: 241-254.
- ASHRAF, M., and J.J. MILLER. 1977. Induction of multispored asci in two-spored strains of Saccharomyces cerevisiae. Can. J. Microbiol. 23: 690-694.
- BERK, A.J., F. LEE, T. HARRISON, J. WILLIAMS, and P.A. SHARP. 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell 17: 935-944.
- BETZ, H., and U. WEISER. 1976. Protein degradation and proteinases during yeast sporulation. Eur. J. Biochem. 62: 65-76.
- BEVAN, E.A. 1953. Genetic analysis of intact incomplete asci of yeast. Nature 171: 576-577.
- BILINSKI, C.A. 1979. "Nutritional control of ascosporeogenesis in an apomictic strain of Saccharomyces cerevisiae". M.Sc. Thesis, McMaster University, Hamilton, Ontario.

- BILINSKI, C.A., and J.J. MILLER. 1980. Induction of normal ascosporeogenesis in two-spored Saccharomyces cerevisiae by glucose, acetate, and zinc. *J. Bacteriol.* 143: 343-348.
- . 1983. Translocation of zinc from vacuole to nucleus during yeast meiosis. *Can. J. Genet. and Cytol.* (in press).
- BILINSKI, C.A., J.J. MILLER, and S.C. GIRVITZ. 1983. Events associated with restoration by zinc of meiosis in apomictic yeast. *J. Bacteriol.* (in press).
- BRAUN, A. 1857. Über Parthenogenesis bei Pflanzen. *Abh. Kgl. Akad. Wiss. Berlin* 1856: 311-376.
- BURNETT, J.H. 1956. The mating systems of fungi. I. *New Phytol.* 55: 50-90.
- BYERS, B., K. SHRIVER, and L. GOETSCH. 1978. The role of spindle pole bodies and modified microtubule ends in the initiation of microtubule assembly in Saccharomyces cerevisiae. *J. Cell Sci.* 30: 331-352.
- CHEN, A.W.C., and J.J. MILLER. 1968. Proteolytic activity of intact yeast cells. *Can. J. Microbiol.* 14: 957-963.
- CHVAPIL, M. 1973. New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes. *Life Sci.* 13: 1041-1049.
- CLANCY, M.J., L.M. SMITH, and P.T. MAGEE. 1982. Developmental regulation of a sporulation-specific enzyme activity in Saccharomyces cerevisiae. *Molec. Cell. Biol.* 2: 171-178.

- CLARK-WALKER, G.D., and A.W. LINNANE. 1966. In vivo differentiation of yeast cytoplasmic and mitochondrial protein synthesis with antibiotics. *Biochem. Biophys. Res. Commun.* 25: 8-13.
- COLONNA, W.J., and P.T. MAGEE. 1978. Glycogenolytic enzymes in sporulating yeast. *J. Bact.* 134: 844-853.
- CREPEAU, R.H., and E.K. FRAM. 1981. Reconstruction of imperfectly ordered zinc-induced tubulin sheets using cross-correlation and real space averaging. *Ultramicroscopy* 6: 7-18.
- CROES, A.F. 1967a. Induction of meiosis in yeast. I. Timing of cytological and biochemical events. *Planta (Berl.)* 76: 209-226.
- 1967b. Induction of meiosis in yeast. II. Metabolic factors leading to meiosis. *Planta (Berl.)* 76: 227-237.
- DAVIDOW, L.S., L. GOETSCH, and B. BYERS. 1980. Preferential occurrence of non-sister spores in two-spored asci of Saccharomyces cerevisiae: evidence of regulation of spore wall formation by the spindle pole body. *Genetics* 94: 581-595.
- DAWES, I.W. 1975. Study of cell development using derepressed mutations. *Nature* 255: 707-708.
- DE SEYNES, M.J. 1868. Sur le Mycoderma vini. *Compt. Rend. Acad. Sci.* 67: 105-109.
- DINGLE, A.D. 1970. Control of flagellum number in Naegleria: temperature shock induction of multiflagellate cells. *J. Cell Sci.* 7: 463-482.

- DOUGLAS, M.G., and R.A. BUTOW. 1976. Variant forms of mitochondrial translation products in yeasts: evidence for location of determinants on mitochondrial DNA. Proc. Natl. Acad. Sci. U.S.A. 73: 1083-1086.
- DOUGLAS, M.G., D. FINKELSTEIN, and R.A. BUTOW. 1979. Analysis of products of mitochondrial protein synthesis in yeast: genetic and biochemical aspects. Methods Enzymol. 56: 58-66.
- DUJARDIN, G., P. PAJOT, O. GROUDINSKY, and P.P. SLONIMSKI. 1980a. Long range control circuits within mitochondria and between nucleus and mitochondria. I. Methodology and phenomenology of suppressors. Mol. Gen. Genet. 179: 469-482.
- DUJARDIN, G., O. GROUDINSKY, A. KRUSZEWSKA, P. PAJOT, and P.P. SLONIMSKI. 1980b. Cytochrome b messenger RNA maturase encoded in an intron regulates the expression of the split gene. III. Genetic and phenotypic suppression of intron mutations. In: The organization and expression of the mitochondrial genome (eds. C. Saccone and A.M. Kroon), Elsevier/North-Holland, Amsterdam, p. 157.
- DUJON, B. 1981. Mitochondrial genetics and functions. In: The molecular biology of the yeast Saccharomyces I. Life cycle and inheritance (eds. J. Strathern et al.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 505-635.
- EATON, N.R., and H.P. KLEIN. 1954. The oxidation of glucose and acetate by Saccharomyces cerevisiae. Biochem. J. 67: 373-381.
- ENGEL, L. 1872. "Les ferments alcooliques." Thèse pour le Sc. nat. Univ., Paris.



- ESPOSITO, M.S., and R.E. ESPOSITO. 1969. Genes controlling meiosis and spore formation in yeast. *Genetics* 69: 79-89.
- 1974. Genetic recombination and commitment to meiosis in Saccharomyces. *Proc. Natl. Acad. Sci. U.S.A.* 71: 3172-3176.
- ESPOSITO, M.S., R.E. ESPOSITO, M. ARNAUD, and H.O. HALVORSON. 1969. Acetate utilization and macromolecular synthesis during sporulation of yeast. *J. Bacteriol.* 100: 180-186.
- ESPOSITO, M.S., R.E. ESPOSITO, and P.B. MOENS. 1974. Genetic analysis of two-spored asci produced by the *spo3* mutant of Saccharomyces. *Mol. Gen. Genet.* 135: 91-95.
- ESPOSITO, R.E., N. FRINK, P. BERNSTEIN, and M.S. ESPOSITO. 1972. The genetic control of sporulation in Saccharomyces. II. Dominance and complementation of mutants of meiosis and spore formation. *Mol. Gen. Genet.* 114: 241-248. 7
- ESPOSITO, R.E. and S. KLAPHOLZ: 1981. Meiosis and ascospore development. In: The molecular biology of the yeast Saccharomyces I. Life cycle and inheritance (eds. J. Strathern et al.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 211-287.
- FAILLA, M.L., C.D. BENEDICT, and E.D. WEINBERG. 1976. Accumulation and storage of  $Zn^{2+}$  by Candida utilis. *J. Gen. Microbiol.* 94: 23-36.
- FAST, D. 1978. "Premeiotic DNA synthesis and recombination (in the yeast Saccharomyces." Ph.D. thesis, University of Chicago, Illinois.
- FOWELL, R.R. 1967. Factors controlling the sporulation of yeasts. II. The sporulation phase. *J. appl. Bact.* 30: 450-474.

- FOWELL, R.R., and M.E. MOORSE. 1960. Factors controlling the sporulation of yeasts. I. The presporulation phase. J. appl. Bact. 23: 63-68.
- FREESE, E.B., M.I. CHU, and E. FREESE. 1982. Initiation of yeast sporulation by partial carbon, nitrogen, or phosphate deprivation. J. Bacteriol. 149: 840-851.
- FRIEDMANN, K.L., and R. EGEL. 1978. Protein patterns during sporulation in fission yeast. Z. Naturforsch. 33c: 84-91.
- FURHRMAN, G.F., and A. ROTSTEIN. 1968. The transport of  $Zn^{2+}$ ,  $Co^{2+}$ , and  $Ni^{2+}$  into yeast cells. Biochim. Biophys. Acta 163: 325-330.
- FUJII, T. 1954. Presence of zinc in nucleoli and its possible role in mitosis. Nature 174: 1108-1109.
- 1955. Presence of zinc in nucleoli and chromosomes and its possible role in mitosis. J. Fac. Sci. Tokyo Univ. Sec. IV 7: 313-325.
- FUKUHARA, H., and C. KUJAWA. 1970. Selective inhibition of the in vivo transcription of mitochondrial DNA by ethidium bromide and by acriflavine. Biochem. Biophys. Res. Commun. 41: 1002-1008.
- GAUMANN, E. 1928. Comparative Morphology of Fungi. 701 pages. McGraw-Hill Book Co., New York.
- GOLDRING, E.S., L.I. GROSSMAN, D. KRUPNICK, D. CRYER, and J. MARMUR. 1970. The petite mutation in yeast: loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide. J. Mol. Biol. 52: 323-335.

- GORENSTEIN, C.G., and J.R. WARNER. 1976. Coordinate regulation of the synthesis of eukaryotic ribosomal proteins. Proc. Natl. Acad. Sci. U.S.A. 73: 1547-1558.
- GREWAL, N. 1972. "A study of sporulation and spore germination in two-spored yeasts". M.Sc. Thesis, McMaster University, Hamilton, Ontario.
- GREWAL, N., and J.J. MILLER. 1972. Formation of asci with two diploid spores by diploid cells of Saccharomyces cerevisiae. Can. J. Microbiol. 18: 1897-1905.
- GRIMM, P.W., and P.J. ALLAN. 1954. Promotion by zinc of the formation of cytochromes in Ustilago sphaerogena. Plant Physiol. 29: 369-357.
- GUILLIERMOND, M.A. 1903. Recherches cytologiques sur les levures. Rev. Gén. Bot. 15: 49-185.
- 1905. Recherches sur la germination des spores et la conjugaison chez les levures. Rev. Gén. Bot. 17: 337-376.
- GUSTAFSSON, A. 1946. Apomixis in higher plants. I. The mechanism of apomixis. Lunds Univ. Arsskr. 42: 1-68.
- 1947a. Apomixis in higher plants. II. The causal aspect of apomixis. Lunds Univ. Arsskr. 43: 69-180.
- 1947b. Apomixis in higher plants. III. Biotype and species formation. Lunds Univ. Arsskr. 43: 181-370.
- HABER, J.E., and H.O. HALVORSON. 1972. Cell cycle dependency of sporulation in Saccharomyces cerevisiae. J. Bacteriol. 109: 1027-1033.

- HALBACH-KEUP, G., and M. EHRENBERG. 1971. Einfluss der Phasenstatus der Vorkulturzellen auf die Ascosporenbildung von Saccharomyces cerevisiae. Arch. Microbiol. 78: 17-24.
- HARTWELL, L.H. 1974. Saccharomyces cerevisiae cell cycle. Bacteriol. Rev. 38: 164-198.
- HARTWELL, L.H., and M.W. UNGER. 1977. Unequal division in Saccharomyces cerevisiae and its implications for the control of cell division. J. Cell. Biol. 75: 422-435.
- HENNINGER, W., AND C.C. EMEIS. 1974. Der apomiktische Entwicklungscyclus der vielsporigen Stämme der Lipomyces-Hefen. Arch. Microbiol. 101: 365-377.
- HENNINGER, W., C.C. EMEIS, and S. WINDISCH. 1974. The apomictic life cycle of the multispored strains of the yeast Lipomyces. Proc. Fourth Int. Symp. on Yeasts, Vienna, Austria, Part I: 171.
- HEREFORD, L., and L.H. HARTWELL. 1974. Sequential gene function in the initiation of S. cerevisiae DNA synthesis. J. Mol. Biol. 84: 445-461.
- HERSKOWITZ, I., and Y. OSHIMA. 1981. Control of cell type in Saccharomyces cerevisiae: mating type and mating-type interconversion. In: The molecular biology of the yeast Saccharomyces I. Life cycle and inheritance (eds. J. Strathern et al.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 181-209.
- HIRSCHBERG, J., and G. SIMCHEN. 1977. Commitment to the mitotic cell cycle in yeast in relation to meiosis. Exp. Cell. Res. 105: 245-252.

- HO, K.H., and J.J. MILLER. 1978. Free proline content and sensitivity to dessication and heat during yeast sporulation and spore germination. Can. J. Microbiol. 24: 312-320.
- HOPPER, A.K., P.T. MAGEE, S.K. WELCH, M. FRIEDMAN, and B.D. HALL. 1974. Macromolecule synthesis and breakdown in relation to sporulation and meiosis in yeast. J. Bact. 119: 619-628.
- HORESH, O., G. SIMCHEN, and A. FRIEDMANN. 1979. Morphogenesis of the synapton during yeast meiosis. Chromosoma 75: 101-115.
- HYAMS, J.S., and G.G. BORISY. 1978. Nucleation of microtubules in vitro by isolated spindle pole bodies of the yeast Saccharomyces cerevisiae. J. Cell Biol. 78: 401-414.
- JOHNSTON, G.C., J.R. PRINGLE, and L.H. HARTWELL. 1977. Coordination of growth with cell division in the yeast Saccharomyces cerevisiae. Exp. Cell Res. 105: 79-98.
- JOHNSTON, G.C., and R.A. SINGER. 1980. A model for cell cycle regulation in the yeast Saccharomyces cerevisiae. In: Current developments in yeast research. Advances in biotechnology (eds. G.G. Stewart and I. Russell). Pergamon Press, Toronto. pp. 555-560.
- JONES, N., and T. SHENK. 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. U.S.A. 76: 3665-3669.
- KIRSOP, B.H. 1956. "Studies of sporulation in the genus Saccharomyces." Ph.D. Thesis, University of London.

- KLAPHOLZ, S., and R.E. ESPOSITO. 1980a. Isolation of *spol2-1* and *spol3-1* from a natural variant of yeast that undergoes a single meiotic division. *Genetics* 96: 567-588.
- 1980b. Recombination and chromosome segregation during the single division meiosis in *spol2-1* and *spol3-1* diploids. *Genetics* 96: 589-611.
- KLAR, A.J.S., and H.O. HALVORSON. 1975. Proteinase activities of *Saccharomyces cerevisiae* during sporulation. *J. Bact.* 124: 863-869.
- KLEYN, J.G. 1954. A study of some environmental factors controlling sporulation of yeast using a new presporulation medium. *Wall. Labs. Commun.* Vol. XVII: 91-104.
- KUENZI, M.T., M.A. TINGLE, and H.O. HALVORSON. 1974. Sporulation of *Saccharomyces cerevisiae* in the absence of a functional mitochondrial genome. *J. Bacteriol.* 117: 80-88.
- KVIST, U. 1980. Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation ability in man. *Acta. Physiol. Scand.* 109: 79-84.
- LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature* 227: 680-685.
- LAMB, A.J., G.D. CLARK-WALKER, and A.W. LINNANE. 1968. The differentiation of mitochondrial and cytoplasmic synthesizing system in vitro by antibiotics. *Biochim. Biophys. Acta.* 161: 415-427.

- LEMEUR, M., N. GLANVILLE, J.L. MANDEL, P. GERLINGER, R. PALMITER, and P. CHAMBON. 1981. The ovalbumin gene family: hormonal control of X and Y gene transcription and mRNA accumulation. *Cell* 23: 561-571.
- LINDEGREN, C.C. 1949. *The Yeast Cell, Its Genetics and Cytology*. Saint Louis Educational Publishers Inc.
- LINDEGREN, C.C., and G. LINDEGREN. 1944. Sporulation in Saccharomyces cerevisiae. *Bot. Gaz.* 105: 304-316.
- LINDQUIST, S. 1981. Regulation of protein synthesis during heat shock. *Nature* 294: 311-314.
- LINDQUIST, S., B. DIDOMENICO, G. BUGAISKY, S. KURTZ, and L. PETKO. 1982. Regulation of the heat shock response in Drosophila and yeast. In Heat Shock: from bacteria to man (eds. M.J. Schlesinger et al.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 444 pp.
- MAGEE, P.T., and A.K. HOPPER. 1974. Protein synthesis in relation to sporulation and meiosis in yeast. *J. Bacteriol.* 119: 952-960.
- MAGNI, G.E. 1958. Changes in radiosensitivity during meiosis in Saccharomyces cerevisiae. II. U.N. Int. Conf. Peaceful Uses Atomic Energy, Geneva 22: 427-436.
- MARMIROLI, N., C. FERRARI, F. TEDESCHI, P.P. PUGLISI, and C. BRUSCHI. 1981a. Ultrastructural analysis of the life cycle of an apomictic (Apo) strain of Saccharomyces cerevisiae. I. Meiosis and ascospores development. *Biol. Cell* 41: 79-84.

- MARMIROLI, N., F. TASSI, L. BIANCHI, A.A. ALGERI, P.P. PUGLISI, and M.S. ESPOSITO. 1981b. Erythromycin and cycloheximide sensitivities of protein and RNA synthesis in sporulating cells of Saccharomyces cerevisiae: environmentally induced modifications controlled by chromosomal and mitochondrial genes. *Curr. Genet.* 4: 51-62.
- MARMIROLI, N., F. TEDESCHI, C. FERRARI, P.P. PUGLISI, and C. BRUSCHI. 1981c. Ultrastructural analysis of the life cycle of an apomictic (Apo) strain of Saccharomyces cerevisiae. II. Mitotic reproduction. *Biol. Cell* 41: 85-90.
- McALISTER, L., S. STRAUSBERG, A. KULAGA, and D.B. FINKELSTEIN. 1979. Altered patterns of protein synthesis induced by heat shock of yeast. *Curr. Genet.* 1: 63-74.
- MILLER, J.J. 1957. The metabolism of yeast sporulation. II. Stimulation and inhibition by monosaccharides. *Can. J. Microbiol.* 3: 81-90.
- 1963. The metabolism of yeast sporulation. V. Stimulation and inhibition of sporulation and growth by nitrogen compounds. *Can. J. Microbiol.* 9: 259-277.
- 1964. A comparison of the effects of several nutrients and inhibitors on yeast meiosis and mitosis. *Exp. Cell Res.* 33: 46-49.
- 1968. Some cultural and staining methods for the observation of meiosis in Saccharomyces cerevisiae. In: *Effects of Radiation on Meiotic Systems*, International Atomic Energy Agency, Vienna, pp. 177-184.



- MILLER, J.J., and C. HALPERN. 1956. The metabolism of yeast sporulation. I. Effect of certain metabolites and inhibitors. *Can. J. Microbiol.* 2: 519-537.
- MILLER, J.J., and O. HOFFMANN-OSTENHOF. 1964. Spore formation and germination in Saccharomyces. *Z. Allg. Mikrobiol.* 4: 273-294.
- MILLER, M.J., N.H. XUONG, and E.P. GEIDUSCHEK. 1979. A response of protein synthesis to temperature shift in the yeast Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U.S.A.* 76: 5222-5225.
- MILLS, D. 1972. Effect of pH on adenine and amino acid uptake during sporulation in Saccharomyces cerevisiae. *J. Bacteriol.* 112: 519-526.
- MITTWOCH, U. 1978. Virgin birth. *New Scientist* 78: 750-752.
- MOENS, P.B. 1971. Fine structure of ascospore development in the yeast Saccharomyces cerevisiae. *Can J. Microbiol.* 17: 507-510.
- 1974. Modification of sporulation in yeast strains with two-spored asci (Saccharomyces, Ascomycetes). *J. Cell Sci.* 16: 519-527.
- MOENS, P.B., M. MOWAT, M.S. ESPOSITO, and R.E. ESPOSITO. 1977. Meiosis in a temperature-sensitive DNA synthesis mutant and in an apomictic yeast strain (Saccharomyces cerevisiae). *Phil. Trans. R. Soc. Lond. B.* 277: 351-358.
- MOENS, P.B., and E. RAPPORT. 1971a. Synaptic structures in the nuclei of sporulating yeast, Saccharomyces cerevisiae (Hansen). *J. Cell Sci.* 9: 665-677.
- 1971b. Spindles, spindle plaques and intranuclear meiosis in the yeast Saccharomyces cerevisiae. *J. Cell Biol.* 50: 344-361.

- MOORE-LANDECKER, E. 1972. Abnormalities in the morphology of Pyronema domesticum induced by 2,4-D. *Mycologia* 64: 276-287.
- MORRISON, D., M.R.A. MOWAT, and P.J. HASTINGS. 1975. Genetics of a strain of S. cerevisiae which gives 2-spored asci. *Can. J. Genet. Cytol.* 17: 463.
- MUNDKUR, B. 1961. Electron microscopy of frozen-dried yeast. III. Formation of a tetrad. *Exp. Cell Res.* 25: 24-40.
- NAGEL, L. 1946. A cytological study of yeast. *Ann. Missouri Bot. Garden* 33: 249-281.
- NEVINS, J.R. 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. *Cell* 26: 213-220.
- 1982. Induction of the synthesis of a 70,000 Dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell* 29: 913-919.
- NEWLON, M.C., AND B.D. HALL. 1978. Inhibition of yeast sporulation by ethidium bromide. *Mol. Gen. Genet.* 165: 113-114.
- O'DONNELL, K.L., W.G. FIELDS, and G.R. HOOPER. 1976a. Apothecial ontogeny in Saccobolus versicolor (Pezizales: Ascomycetes). *Can. J. Bot.* 55: 2055-2060.
- O'DONNELL, K.L., G.R. HOOPER, W.G. FIELDS, and A.O. ACKERSON. 1976b. Apothecium morphogenesis in Peziza quelipidotia: scanning electron and light microscopy. *Can J. Bot.* 54: 2254-2267.
- PALMER, R.G. 1971. Cytological studies of aneuploid and normal maize with reference to premeiotic pairing. *Chromosoma (Berl.)* 35: 233-246.

- PATEL, P.V., and J.J. MILLER. 1972. Stimulation of yeast sporulation by glycerol. *J. appl. Bact.* 35: 63-69.
- PERLMANN, P.S., and H.R. MAHLER. 1971. Molecular consequences of ethidium bromide mutagenesis. *Nat. New Biol.* 231: 12-16.
- PETERSEN, J.G.L., M.C. KIELLAND-BRANDT, and T. NILSSON-TILGREN. 1979. Protein patterns of yeast during sporulation. *Carlsberg Res. Commun.* 44: 149-162.
- PETERSON, J.B., R.H. GRAY, and H. RIS. 1972. Meiotic spindle plaques in *Saccharomyces cerevisiae*. *J. Cell Biol.* 53: 837-841.
- POLAKIS, E.S., and W. BARTLEY. 1965. Changes in the enzyme activities of *Saccharomyces cerevisiae* during aerobic growth on different carbon sources. *Biochem. J.* 97: 284-297.
- PONTEFRACT, R., and J.J. MILLER. 1962. The metabolism of yeast sporulation. IV. Cytological and physiological changes in sporulating cells. *Can. J. Microbiol.* 8: 573-584.
- PRINGLE, J.R. 1975. Methods for avoiding proteolytic artefacts in studies of enzymes and other proteins from yeasts. *Methods Cell Biol.* 12: 149-184.
- PRINGLE, J.R., and L.H. HARTWELL. 1981. The *Saccharomyces cerevisiae* cell cycle. In: The molecular biology of the yeast *Saccharomyces* I. Life cycle and inheritance (eds. J. Strathern et al.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York pp. 97-142.
- PUGLISI, P.P., and E. ZENNARO. 1971. Erythromycin inhibition of sporulation in *Saccharomyces cerevisiae*. *Experientia* 27: 963-964.

- ROBINOW, C.F. 1980. The view through the microscope. In: Current developments in yeast research. Advances in biotechnology (eds. G.G. Stewart and I. Russell). Pergamon Press, Toronto. pp. 623-634.
- ROBINOW, C.F., and J. MARAK. 1966. A fiber apparatus in the nucleus of the yeast cell. J. Cell Biol. 29: 129-149.
- ROTH, R., and S. FOGEL. 1971. A system selective for yeast mutants deficient in meiotic recombination. Molec. Gen. Genetics 12: 295-305.
- ROTH, R., and H.O. HALVORSON. 1969. Sporulation of yeast harvested during logarithmic growth. J. Bacteriol. 98: 831-832.
- ROTH, R., and K. LUSNAK. 1970. DNA synthesis during yeast sporulation: genetic control of an early developmental event. Science 168: 493-494.
- ROMAN, H., and S.M. SANDS. 1953. Heterogeneity of clones of Saccharomyces derived from haploid ascospores. Proc. Natl. Acad. Sci. U.S.A. 39: 171-179.
- ROYAN, S., and M.K. SUBRAMANIAM. 1960. Differential fluorescence of the chromocenter and nucleolar equivalents of the yeast nucleus in acridine orange. Proc. Ind. Acad. Sci. B. 51: 205-210.
- SANDO, N. 1960. Ascospore formation of yeast. IV. Effects of metabolic inhibitors. Sci. Rep. Tohoku Univ. Fourth Ser. (Biol.) 26: 157-161.

- 1977. Aging and sporulating ability in yeast cells. In: Growth and differentiation in microorganisms (eds. T. Ishikawa, Y. Maruyama, and H. Matsumiya), University of Tokyo Press (Tokyo), pp. 151-163.
- SANDO, N., and T. SAITO. 1970. Sporogenic activities of mother and daughter cells in Saccharomyces cerevisiae. J. Gen. and Appl. Microbiol. 16: 347-350.
- SANTA MARÍA, J. 1959. Polyploidia en Saccharomyces. An. Inst. nac. Invest. agron. Madrid. 8: 679-801.
- SCHILD, D., and B. BYERS. 1980. Diploid spore formation and other meiotic effects of two cell-division-cycle mutations of Saccharomyces cerevisiae. Genetics 96: 859-876.
- SCHULTZ, R.M., and P.M. WASSERMAN. 1977. Specific changes in the pattern of protein synthesis during meiotic maturation of mammalian oocytes in vitro. Proc. Natl. Acad. Sci. U.S.A. 74: 538-541.
- SHILO, V., G. SIMCHEN, and B. SHILO. 1978. Initiation of meiosis in cell cycle initiation mutants of Saccharomyces cerevisiae. Exp. Cell Res. 112: 219-248.
- SILLEVIS SMITT, W.W., J.M. VLAK, I. MOLENAAR, and T.H. ROZIJN. 1973. Nucleolar function of the dense crescent in the yeast nucleus: a biochemical and ultrastructural study. Exp. Cell Res. 80: 313-321.
- SILLEVIS SMITT, W.W., J.M. VLAK, R. SCHIPHOF, and T.H. ROZIJN. 1972. Precursors of ribosomal RNA in yeast. Exp. Cell Res. 71: 33-40.

- SMITH, J. 1841. Notice of a plant which produces seeds without any apparent action of pollen. Trans. Linn. Soc. Lond 18 (cited after Braun, 1857).
- SOKAL, R.R., and F.J. ROHLF. 1981. Biometry, 2nd edition, W.H. Freeman and Co., San Francisco, pp. 440-445.
- SONG, M.K., N.F. ADHAM, and H. RINDERKNECHT. 1976. A simple, highly sensitive colorimetric method for the determination of zinc in serum. Amer. J. Clin. Path. 65: 229-233.
- SRIVASTAVA, P.K., S. HARASHIMA, and Y. OSHIMA. 1981. Formation of two-spored asci by interrupted sporulation in Saccharomyces cerevisiae. J. Gen. Microbiol. 121: 39-48.
- STANTIAL, H. 1935. The sporulation of yeast: second paper. Trans. Roy. Soc. Can. III. 29: 175-188.
- STRINGHAM, G.R. 1970. A cytochemical analysis of three asynaptic mutants in Brassica campestris. Can. J. Genet. Cytol. 12: 743-749.
- SUBCOMMITTEE ON ZINC. 1979. Committee on Medical and Biologic Effects of Environmental Pollutants, Division of Medical Sciences, Assembly of Life Sciences, National Research Council (Baltimore: University Park Press), pp. 222-223.
- SUOMALAINEN, E., A. SAURA, and J. LOKKI. 1976. Evolution of parthenogenetic insects. Evol. Biol. 9: 209-257.
- TAKAHASHI, T. 1962. Genetic segregation of 2-spored ascus in Saccharomyces. Bull. Brew. Sci. 8: 1-9.
- TAKAHASHI, T., and K. AKAMATSU. 1963. Genetic segregation of 3-spored ascus in Saccharomyces. Seiken Zihō 15: 54-58.

- TREMAINE, J.H., and J.J. MILLER. 1954. Effect of 6 vitamins on ascospore formation by an isolate of bakers' yeast. *Bot. Gaz.* 115: 311-322.
- TREW, B.J., J.D. FRIESEN, and P.B. MOENS. 1979. Two-dimensional protein patterns during growth and sporulation in Saccharomyces cerevisiae. *J. Bacteriol.* 138: 60-69.
- TSUBOI, M. 1976. Correlation among turnover of nucleic acids, ribonuclease activity, and sporulation ability of Saccharomyces cerevisiae. *Arch. Microbiol.* 111: 13-19.
- TSUBOI, M., and N. YANAGISHIMA. 1974. Changes in sporulation ability during the vegetative cell cycle in Saccharomyces cerevisiae. *Bot. Mag. Tokyo* 87: 183-193.
- UNGER, M.W., and L.H. HARTWELL. 1976. Control of the cell division cycle in Saccharomyces cerevisiae by methionyl-tRNA. *Proc. Natl. Acad. Sci. U.S.A.* 73: 1664-1668.
- VEZINHET, F. 1969. "Contribution à l'étude de la sporulation chez Saccharomyces cerevisiae Hansen." Faculté des Sciences de Montpellier. Thèse. 38 pages.
- WARD, J.M., and W.J. NICKERSON. 1958. Respiratory metabolism of normal and divisionless strains of Candida albicans. *J. Gen. Physiol.* 41: 703-724.
- WARNER, J.R. 1981. The yeast ribosome: structure, function, and synthesis. In: *The molecular biology of the yeast Saccharomyces II. Metabolism and gene expression* (eds. J. Strathern et al.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 529-560.

- WARNER, J.R., and C.G. GORENSTEIN. 1977. Synthesis of eukaryotic ribosomal proteins in vitro. Cell 11: 201-212.
- WIAME, J.M. 1947. Étude d'une substance polyphosphorée basophile et métachromatique chez les levures. Biochim. Biophys. Acta 1: 234-255.
- WICKERHAM, L.J. 1951. Taxonomy of yeasts. pp. 1-56. Technical bulletin 1029. U.S. Department of Agriculture, Washington, D.C.
- WILLIAMSON, D.H., and D.J. FENNELL. 1975. The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondria DNA. In: Methods in Cell Biology XII (ed. D.M. Prescott). Academic Press, New York, pp. 335-351.
- WINGE, Ö. 1935. On haplophase and diplophase in some Saccharomyces. Compt. rend. trav. lab. Carlsberg. Sér. Physiol. 21: 77-111.
- WINGE, Ö., and Ö LAUSTSEN. 1937. On two types of spore germination, and on genetic segregation in Saccharomyces, demonstrated through single spore cultures. Compt. rend. trav. lab. Carlsberg. Sér. Physiol. 22: 99-116.
- 1939. Artificial species hybridization in yeast. Compt. rend. trav. lab. Carlsberg. Sér. Physiol. 22: 235-244.
- WINKLER, H. 1908. Über Parthenogenesis und Apogamie im Pflanzreiche. Progr. rei Bot. 2: 293-454.
- WITT, I., R. KRONAU, and H. HOLZER. 1966. Repression von Alkoholdehydrogenase, Malatdehydrogenase, Isocitratlylase and Malatsynthese in Hefe durch Glucose. Biochim. Biophys. Acta (Amst.) 118: 522-537.



WOLFNER, M., D. YEP, F. MESSENGUY, and G.R. FINK. 1975. Integration of amino acid biosynthesis into the cell cycle of S. cerevisiae. J. Molec. Biol. 96: 273-290.

WRIGHT, J.F., N. AJAM, and I.W. DAWES. 1981. Nature and timing of some sporulation-specific protein changes in Saccharomyces cerevisiae. Molec. Cell. Biol. 1: 910-918.

YANAGITA, T.; M. YAGISHAWA, S. OISHI, N. SANDO, and T. SUTO. 1970. Sporogenic activities of mother and daughter cells in Saccharomyces cerevisiae. J. Gen. Appl Microbiol. 16: 347-350.

ZICKLER, D., and L.W. OLSON. 1975. The synaptonemal complex and the spindle plaque during meiosis in yeast. Chromosoma 50: 1-23.