ULTRASTRUCTURE STUDIES

IN

USTILAGO HORDEI (PERS.) LAGERH.

BY

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

Botany

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

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Department of Botany

The University of British Columbia
Vancouver 8, Canada

Date September 2, 1971
For out of olde feldes, as men seyth,
Cometh al this newe corn from yer to yer,
And out of olde books, in good feyth,
Cometh al this newe science that men lere.

The Parlement of Foules
Geoffrey Chaucer
ABSTRACT

A comparative light and electron microscope technique has been used to study the cytological changes accompanying teliospore (i.e. probasidium) germination in *Ustilago hordei* (Pers.) Lagerh. Special emphasis has been placed on determining the ultrastructural events involved in karyokinesis, especially meiosis, and cytokinesis.

The thesis is divided into five parts, of which the first is concerned with pre-germinal differentiation. The great increase in microanatomical complexity which occurs during the pre-germinal stages is due largely to an increase in the amount of endoplasmic reticulum (ER) and to the formation of "primary hydration vacuoles." Evidently the nuclear envelope gives rise to the new ER which in turn dilates to form the vacuoles. This is accompanied by an increase in mitochondrial size and the development of patches of patches of "flocculent cytoplasm."

Part II concerns the initiation and subsequent extension of the metabasidium (i.e. promycelium). Initiation involves the localized degradation of the inner spore wall, and deposition of new wall material. The ER and spherosome-like bodies seem to be associated with these activities. Once spore wall rupture has occurred the structural basis of promycelial extension is unknown but changes in the number, size, and distribution of the spherosome-like organelles appear to have profound effects on the differentiation of the organism.
Septation, knee-joint formation, and budding are discussed in part III. Elaborate membrane complexes are associated with cross wall initiation. A membranous plate is completed across the cell before septal wall thickening begins. The initiation of sporidia (i.e. basidiospores) involves a localized plasticization of the promycelial wall followed by degradation of the old wall and subsequent synthesis of new wall material. Bridge-formation results when two adjacent cells give rise to bud-like processes which grow together and subsequently fuse to produce a protoplasmic bridge.

The structure and activities of the metabasidial nuclei and their associated structures are discussed in part IV. Both meiosis and mitosis are unusual in that the two chromatin bodies apparently remain attached to the centriolar-kinetochore-equivalent and at least one of the chromatin bodies in attached to the nucleolus throughout the division cycle. The results are compatible with Brown and Stack's (1971) model for somatic nuclear division in some fungi.

Membrane complexes, resembling those which initiate septa, form in association with prophase nuclei and maintain a specific relation with the nucleus throughout division. In part V the suggestion is made that these complexes form part of a mechanism controlling the positional relationships of nuclear and cell divisions in the promycelium.
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<tr>
<td>ch</td>
<td>chromatin</td>
</tr>
<tr>
<td>CKE</td>
<td>centriolar-kinetochore-equivalent</td>
</tr>
<tr>
<td>cl</td>
<td>central lamella (septum)</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>f</td>
<td>flocculent cytoplasm</td>
</tr>
<tr>
<td>L</td>
<td>lipid body</td>
</tr>
<tr>
<td>M</td>
<td>mitochondrion</td>
</tr>
<tr>
<td>mc</td>
<td>membrane complex</td>
</tr>
<tr>
<td>mt</td>
<td>microtubule</td>
</tr>
<tr>
<td>mtoC</td>
<td>microtubule organizing centre</td>
</tr>
<tr>
<td>N</td>
<td>nucleus (dN - diploid nucleus, hN - haploid nucleus)</td>
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<tr>
<td>NE</td>
<td>nuclear envelope</td>
</tr>
<tr>
<td>NP</td>
<td>nuclear pore</td>
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<td>Nu</td>
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<tr>
<td>P</td>
<td>perforations</td>
</tr>
<tr>
<td>pl</td>
<td>plate (septum)</td>
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<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>pW</td>
<td>promycelial wall</td>
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<tr>
<td>S</td>
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<td>V</td>
<td>vacuole</td>
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<td>ve</td>
<td>vesicle</td>
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**Magnifications** - The black scale represents 0.5 µ on electron micrographs and 1.0 µ on light microscope pictures unless otherwise indicated.
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I particularly wish to thank my supervisor, Professor Clayton Person for his encouragement of independent research and for his guidance and valuable suggestions in the preparation of this thesis. Thanks are also due to Dr. T. Bisalputra and other members of my thesis committee for invaluable discussions concerning the preparation of the manuscript. The author also wishes to acknowledge Dr. C. Robinow (University of Western Ontario, Canada) and Dr. R. M. Brown (University of Texas at Austin, U.S.A.) for their helpful criticism and advice in the preparation of part IV.

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Lastly, and most importantly, I wish to thank my husband, Dave, whose encouragement, help, understanding, and infinite patience enabled me to complete this investigation and manuscript.
GENERAL INTRODUCTION

The historical development of scientific interest in the smut fungi centres on one of Man's most pressing problems, the World's food supply. Many of these tiny plant pathogens which constitute the order Ustilaginales (subclass Heterobasidiomycetidae) are parasitic on cereal grains. The crop diseases which they cause greatly reduce not only crop yield but also quality; as vital agents of crop damage they are second only to rusts (Uredinales). Since cereals constitute approximately one-quarter of the total food consumed by Man, the economic importance of this group of fungi is clear (Fischer and Holton, 1958; Christensen, 1963), and, not surprisingly, much research has been devoted to its control. During the last 20 to 30 years this pursuit has led to the opening up of new and complex realms of study bent upon elucidating the physiological and genetical interrelationships between host and parasite (Flor, 1942 and 1954; Halisky, 1965; Person, 1959). Such studies, while of potential economic value, hold a theoretical fascination of their own. In addition smut fungi, particularly members of the genus Ustilago, have found favour as purely genetic tools being used in studies of mutation (Hood, 1968), recombination (Holliday, 1961 and 1964; Kozar, 1969), haplodization (Day and Jones, 1969), and genetic complementation (Dinoor and Person, 1969). The species most extensively used are Ustilago maydis, Ustilago violacea,
and *Ustilago hordei*.

Considering the importance of the smuts both economically and as a research tool, and considering the energies which have been devoted to certain aspects of smut physiology and genetics, amazingly little cytological information is available concerning any one species. Fischer and Holton have reviewed the cytology of the smuts up to 1957. Almost all knowledge of the structure, and life cycle of these organisms has been obtained by light microscopy; in general very little is known about the ultrastructure of the Ustilaginales. Even more amazing and annoying from the geneticists' point of view, is the paucity of information concerning the nuclei of these fungi, especially at meiosis. The major purpose of the work presented in this thesis was to obtain information on chromosome number, nuclear structure, and mechanism of division during the meiotic and post-meiotic stages in *Ustilago hordei* (Pers.) Lagerh. A comparative light and electron microscope technique has been employed for this purpose. In *Ustilago hordei*, as in other smuts, the meiotic process is inextricably associated with other cellular events pertaining to the germination of the sexual spore - the teliospore. The scope of the work was, therefore, broadened to include a cytological analysis of teliospore activation and germination, of germ tube extension, and of basidiospore formation.

A detailed description of the variations in the life cycles of different species of the Ustilaginales can be found in *The Biology and Control of the Smut Fungi* (Fischer and Holton, 1957). For convenience, the life cycle of *Ustilago hordei* is pre-
DIAGRAM I

Life cycle of *Ustilago hordei*

A schematic representation of the life cycle of *Ustilago hordei*. Haploid nuclei are denoted by $n$ and diploid nuclei by $2n$.

Erratum: diakaryon should read dikaryon.
sented schematically in Diagram I. *Ustilago hordei* is parasitic on barley during the major part of its life cycle. The parasitic stage is initiated only after the fusion of two compatible haploid cells (sporidia) of different mating type (+ and -) to form a dikaryon. The dikaryotic state is maintained until the parasitic stage culminates with the fusion in pairs, of compatible haploid nuclei and subsequent production of diploid teliospores. On teliospore germination, the diploid fusion nucleus undergoes meiosis within the promycelium, each teliospore giving rise to four haploid cells. Each cell then usually begins to bud in yeast-like fashion to form a clone of haploid cells (basidiospores or sporidia). There are two clones of each mating-type per promycelium.

The term "teliospores" is commonly applied to the sexual spores of rusts and smuts; however, notice should be taken that these structures are now more properly referred to as "pro-basidia". Germinating teliospores give rise to specialized tub-like cells of limited growth which are commonly called "promycelia" (Hawker, 1966). In keeping with the new terminology the term "promycelium" should be replaced by "metabasidium". Throughout this thesis these terms will be used interchangeably.

The most complete study to date on teliospore germination and basidiospore production in *Ustilago hordei* is that of D. T. Wang (1934). In her studies Wang observed most of the fundamental events which characterize this stage. The resting spore contains a single large nucleus which undergoes division shortly after the metabasidium is formed. In the particular strain observed by Wang this first nuclear division usually takes place
INTRODUCTION. PLATE 1

Figures 1a - 0. Serial photographs of teliospores of Ustilago hordei germinating in a thin layer of complete broth between a glass slide and a cover slip. 1a and 1b represent resting spores and spores after one hour of hydration respectively. Figures 1c - 0 were taken at half-hour intervals (Total = 6½ hr.). The arrow in 1i indicates the first septum; the arrows in 1j indicate the second and third septa. In the promycelium on the left the initiation of the first two sporidia can be detected in 1j, the third sporidia in 1l and the fourth sporidia in 1n. The promycelium on the right is undergoing Knee-joint formation. ca. X 1,000.

Note: each scale division represents one micron (i.e. total scale length is 10 microns).
within the spore, one of the daughter nuclei subsequently migrating into the promycelium, but she noted that sometimes the single nucleus will first migrate into the germ tube and then divide. The latter is almost always the case in the strain of *Ustilago hordei* studied in this thesis. The two daughter nuclei subsequently divide again, either simultaneously or independently. According to Wang, three septa are laid down separating the four nuclei. In the stock culture used in this thesis the three septa are not laid down together. The first septum is laid down immediately after the first nuclear division dividing the promycelium in two. The other two septa are formed immediately following the second nuclear division, one on either side of the first. The promycelium itself contains three of the chambers, the fourth chamber lying in the spore. Almost immediately each cell gives rise to a sporidium, and each of the nuclei divides again, one of the daughter nuclei passing into the bud, the other remaining in the parent cell. As an alternative to sporidial production Wang observed that two of the chambers sometimes anastamose via a bridge which bypasses a septum. The bridge then extends apically to form a branch. The two undivided nuclei of the joined cells pass into the branch to initiate a dikaryon. These events which were previously observed by Wang and which constitute the major portion of this thesis are summarized photographically in the introductory Figures 1a-o and 2-4. In addition to her observations on the nucleus Wang also studied the "cytome" which is now referred to as the mitochondria, the "ergastome" consisting of
osmiophilic lipid bodies, and formation of vacuoles in the germinating spore. Variations on the pattern of teliospore germination and basidiospore production in the smut fungi are described in The Biology and Control of the Smut Fungi by Fischer and Holton (1957).

At present very little ultrastructural information is available pertaining to the heterobasidiomycetes in general, and to the Ustilaginales in particular. Several workers have used scanning electron microscopy (Hille and Brandes, 1956) or surface replica techniques (Khanna et al., 1966) to observe the surface features of smut teliospores. Kukkonen and Vaissalo (1964) made a very preliminary attempt to investigate teliospore formation in Anthracoidea aspersa. Freeze-etching has revealed some of the fine structural features of the resting teliospores of Tilletia controversa (Hess and Weber, 1970) and Tilletia caries (Allen et al., 1971), and of the sporidia of Ustilago hordei (Higham, personal communication). The cytology of mycelial mutant of Ustilago hordei has been studied in some detail by Stein (1970). Fullerton (1970) has investigated several aspects of the intracellular hyphae of eleven species of smut in their corresponding hosts. Among other heterobasidiomycetes studies include those on basidial development in the tremellaceous fungus Exidia nucleata (Wells, 1964a and 1964b), budding in Tremella mesenterica (Bandoni and Bisalputra, 1971), and those on uredospore germination (Ehrlich and Ehrlich, 1969; Manocha and Shaw, 1967; Sussman et al., 1969; Williams and Legingham, 1964) and various aspects of the host-parasite
relationship among rusts (Bracker, 1967; Ehrlich and Ehrlich, 1963; Van Dyke and Hooker, 1969). Some information is also available concerning the Rhodotorulas (Marchant and Smith, 1967) which have now been reclassified as heterobasidiomycetes (Banno, 1967). Fungal ultrastructure has recently been reviewed by Bracker (1967); in many respects the cytology of the heterobasidiomycetes is very similar to that of other fungi.

The literature pertaining to the metabolism (Allen, 1965) and cytology (Bracker, 1967) of fungal spores is now quite voluminous. Excellent reviews of many aspects of quiescent and germinating fungus spores are to be found in Spores: Their Dormancy and Germination (Sussman and Halvorson, 1966), The Fungi, An Advanced Treatise (ed. Ainsworth, G. C. and Sussman, A. S., 1965), and The Fungus Spore (ed. Madelin, 1966). However, even though cytological investigations have been extensive, almost all studies have considered only quiescent spores and spores in which germination has occurred. Throughout this study the term "germination" will be applied to the formation of the metabasidium. Little information is available concerning the very important events which occur in the activating pre-germinal spore and which lead up to the process of germination itself. An attempt is made in this study to determine the sequential morphological changes which take place in the teliospore and metabasidium before and after germination. Such studies give rise to certain interesting conclusions concerning the changing numbers and distribution of organelles. Particular attention has been paid to the nature and origins of the
Figure 2. The one-celled state as seen with phase optics in a living promycelium growing in a thin layer of complete broth between a glass slide and a cover slip. The large diploid nucleus (dN) and a "spherosome-like body" is indicated. ca. X 4,600.

Figure 3. The two-celled state as seen with phase optics in a promycelium fixed in 2% glutaraldehyde in 0.01 M cacodylate buffer. The first septum (arrow) and one of the haploid nuclei (hN) are indicated. Note the condensed state of the nuclear chromatin. ca. X 4,600.

Figure 4. The four-celled state as seen with phase optics in a living promycelium grown as in figure 2. Two of the three septa are visible (arrows). A refractile membrane complex (mc) is associated with the first septum. Three of the four haploid nuclei are obvious. ca. X 4,600.

Note: each scale division represents one micron (i.e. total scale length is 10 microns).
endoplasmic reticulum and the vacuoles in the germinating sys-
tem, and to the possible functions of the "spherosome-like
bodies" (S) which are readily observable in the light microscope
(Fig. 2).

Several excellent reviews of meiosis and mitosis in fungi
are available (Burnett, 1968; Olive, 1953; Olive, 1965; Robinow
and Bakerspiegel, 1965). At best the study of fungal nuclei
is difficult and with the exception of yeast nuclei, those of
the smuts are perhaps the most difficult to observe and inter-
pret. The initial problem is the small size of the nuclei
(1.5-2.5 μ). This is compounded by the practically indestruc-
tible fungal wall, and the general unresponsiveness of the
protoplast to the usual methods of fixing and staining. Further-
more, the early meiotic stages occur within the forming
thick-walled teliospore which is usually embedded in dead host
tissue - material which is impossible to squash, and difficult
to section.

Chromosomes and spindles were first described in the nuclei
of a species of *Ustilago* by Harper (1898). Rawitscher (1922)
subsequently confirmed the presence of a spindle in dividing
nuclei and described its intranuclear nature. The teliospore
is the sexual spore, and presumably represents the only diploid
cell in the life cycle. It is commonly assumed that meiosis
occurs during the first two (or three) nuclear divisions after
the teliospore germinates, but the details of these nuclear di-
visions are so unclear that there is still some doubt as to which
represents the reduction division (Sampson, 1939; Hirshhorn,
1945). Wang (1934) and Sampson (1939) suggest that the first division is reductional. This has been supported genetically and is now commonly assumed to be the case; but Hirschhorn (1945), Das (1949), and even Fischer and Holton (1957) support the idea that the second division is the reductional one. Kharbush (1927), Wang (1934), and Hirschhorn (1945) collectively studied five different genera including eleven species of Ustilago. From their chromosome counts at meiosis and mitosis they concluded that \( n = 2 \). With the exception of Harper (1898) and Dickson (1931) this count has been confirmed by most workers (Rawitscher, 1922; Wang, 1943; Das, 1949; Person and Wighton, 1964).

To the best of the author's knowledge this is the first detailed ultrastructural study of nuclear activity in a smut fungus. Investigation of the metabasidial cell and nuclear divisions indicates that certain interesting relationships exist between the two. Special consideration has been given to the problems of chromosome number and mechanism of nuclear division in *Ustilago hordei*, and the data have been reanalysed in the light of the electron microscope observations and the current trends in fungal cytology.


PART I
Pregermination Development in Hydrating Teliospores

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PART I

Pregermination Development in Hydrating Teliospores

ABSTRACT

An attempt has been made to determine the sequence of cytological changes occurring during pregermination imbibition of *Ustilago hordei* teliospores. The first detectable changes are an increase in the amount of endoplasmic reticulum, the development of patches of "flocculent cytoplasm", and a peculiar amoeboid activity of the nucleus. Shortly after, vacuoles begin to appear in the central regions of the protoplast. The mitochondria increase in size throughout the pregermination period but show no evidence of division until shortly before germination. Lipid does not seem to decrease substantially. Hypotheses are put forward to account for the origin of the endoplasmic reticulum and of "primary vacuoles".

INTRODUCTION

In a recent review of fungal ultrastructure, Bracker (1967) states the need for further information concerning the cytology of spore germination, and particularly the need for studies in material which is amenable to specific structure-function analysis. With this in mind an attempt has been made to study, in some detail, the sequential cytological changes occurring during the pre-germination development of imbibing teliospores of the smut fungus, *Ustilago hordei*. 
Hopefully more information will be forthcoming. Most fine structure studies of the germination of fungus spores have included only observations of spores in the dormant or quiescent state, and spores during and after germination. Very little attention has been paid to the important events which lead up to germination itself (Corfman, 1966; Hyde and Walkinshaw, 1966).

The teliospores of *Ustilago hordei* belong to the category of resting spores which Allen (1965) refers to as "environmental", that is, they will resume development immediately when the environment permits. In this case only the presence of free water is required for germinations to proceed. The teliospore is also the sexual spore. It contains the only diploid nucleus in the entire life cycle (Intro. Diagram I).

At present very little is known about the ultrastructure of spores among the Ustilaginales. Kukkonen and Vaissalo (1964) have studied teliospore formation in *Anthracocidea aspersa*, and several workers have investigated the surface features of mature spores (Hille and Brandes, 1956). Recently, freeze-etching has been used to study the teliospores of *Tilletia controversa* (Hess and Weber, 1970) and *Tilletia caries* (Allen et al., 1971).

MATERIALS AND METHODS

CULTURES AND CULTURING

*Ustilago hordei* (Pers.) Lagerh. - All teliospores used in these studies were the product of crosses between the two
wild-type haploid mating strains $I_4^+$ and $E_3^-$, isolated by Thomas (1964). All were produced in the field. None of the samples had been stored longer than twelve months.

**Culturing.** — It is known that when spore samples are germinated on complete medium a greater amount of synchrony can be obtained than on distilled water (Bech-Hansen, unpublished). Hence, all teliospore samples were hydrated in a shake culture consisting of a modified complete Vogel's broth prepared according to Hood (1966) (see also Appendix A). The temperature was maintained at $22^\circ$ C.

**SAMPLE TAKING**

These studies show that the greater the initial spore concentration, the faster is the germination rate. The initial spore concentrations employed were not controlled. During the early studies the first promycelia were detected at approximately five hours after the initiation of hydration. In subsequent experiments where greater initial concentrations were used, the first signs of germination began after two and a half hours. However, the sequence of stages seen in all cases appeared to be identical. Only the period of time spent in each stage was longer in the former case. Each of the events described is, therefore, a composite of a number of observations, each observation being made in the context of the individual experiment.

During the early studies pre-germination samples were taken at 0, $\frac{1}{2}$, $2\frac{1}{2}$, and 5 hours; in later studies at 0, $\frac{1}{2}$, 1, and 2 hours. Each sample consisted of 3 millitres of spore suspen-
sion.

PREPARATION FOR ELECTRON MICROSCOPY (also see Appendix B)

During the first steps in the preparation procedure the spores were recovered by centrifugation. The material was fix-
ed for electron microscopy, at room temperature, according to one of the following procedures:

1. 1.5% KMnO₄ (aqueous) for 20 minutes.

2. 2.0% glutaraldehyde in 0.01 M cacodylate buffer at
pH 7.0 or 7.2 for 12 to 16 hours (the longer time was
required by the resting spores), followed by washing
in buffer and post-fixation in 1.0 or 2.0% OsO₄ in the
same buffer for 3-3½ hours.

The material was then washed in either distilled water (pro-
cedure 1) or buffer (procedure 2). Glutaraldehyde-osmium fix-
ed spores were subsequently stained in 0.5% aqueous uranyl
acetate for 2-4 hours. After pelleting in 2% water-agar the
spores were dyhydrated through a standard ethanol series. The
material was then either passed through a standard propylene
oxide series and embedded in Epon 812, or was directly embedded
in Spurr's plastic (Spurr, 1969). The three basic preparatory
methods are outlined in Table I.

Sections were cut our on a Sorvall Porter-Blum MT-2 ultra-
microtome using glass or diamond knives, and were post-stained
in a saturated solution of uranyl acetate in 70% ethanol follow-
ed by lead citrate (Reynolds, 1963). All sections were viewed
with an Hitachi HS-75 microscope operating at 50 KV.
<table>
<thead>
<tr>
<th>Method</th>
<th>Fixation</th>
<th>Post Fixation</th>
<th>Dehydration</th>
<th>Embedding</th>
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<tbody>
<tr>
<td>A</td>
<td>1.5% KMnO₄ aqueous</td>
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<td>Ethanol–propylene oxide</td>
<td>Epon</td>
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<td>B</td>
<td>2.0% buffered gluteraldehyde</td>
<td>1-2% buffered OsO₄</td>
<td>Ethanol–propylene oxide</td>
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<tr>
<td>C</td>
<td>2.0% buffered gluteraldehyde</td>
<td>1-2% buffered OsO₄</td>
<td>Ethanol</td>
<td>Spurr's</td>
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LIGHT MICROSCOPY (see also Appendix C)

Thick sections (0.25 μ) were made of resting spores and spores after one hour of hydration, which had been prepared according to electron microscope method A (KMnO₄). They were stained with 1.0% Toluidine blue in 1.0% corax. A Zeiss photomicroscope, equipped with 546 μm interference filter, was used in all studies.

OBSERVATIONS

PREPARATORY METHODS

Methods A, B, and C each provide a slightly different image of the internal contents of ungerminated spores. No doubt, each reflects some of the properties of the living organism. This is a strong argument for the necessity of employing more than one technique before attempting to visualize what the living state of the cell might have been. Unless otherwise stated all the events here described have been seen with all three procedures.

Method A appears to produce the highest level of artefact. As has often been noted, permanganate causes both nucleic acid and lipid to leach out; the chromatin, nucleolus, ribosomes, and lipid bodies being conspicuously absent. Distortion of the cytological contents also occurs. The protoplast and many of the organelles appear to shrink in total size; the intracisternal spaces of the ER and nuclear envelope, and the intermembranous space of the mitochondria are usually expanded. No doubt, the grotesque shapes of the mitochondria seen in the
dormant spores in Figure 1 are artefacts caused by KMnO₄ fixation.

Such distortions never occur in glutaraldehyde-osmium fixed material. Nevertheless, permanganate fixation consistently gives good membrane contrast and provides sufficient morphological information. It also allows rapid and even penetration of the embedding plastic. As has been noted by a number of authors (Sussman, et al., 1969), this latter property is of considerable importance in dealing with thick-walled spores. Because of these aspects, much of the information contained in this report has come from tissue prepared according to method A.

Glutaraldehyde-osmium fixation tends to preserve the cells in a more natural state, but the application is more problematic. Both pH and osmolarity appear to be important factors (Appendix B, Table I). Method B, in which material is embedded in Epon 812, appears to result in the leaching out of some proteinaceous material, causing an increase in membrane visibility. The major difficulty with this method as suggested by Susman et al. (1969), is improper penetration of the plastic (Figs. 6 and 15). Low viscosity Spurr's embedding plastic retains a greater proportion of the ground substance and infiltrates the tissue evenly. However, there is a general tendency towards negative staining of the membranes. This problem is unique to ungerminated spores and does not occur once a promycelium has been formed. Whether this effect is caused by physiological inactivity of the membranes or by masking of the membranes by dense cytoplasm (Walkinshaw et al., 1967; Weiss, 1963) is unknown.
RESTING SPORES

Spore Wall (W). - Considering that a resting spore has a total diameter of 8-10 μ, the spore wall, which is approximately 1 μ thick (Range: 0.63 - 1.24 μ), is its most prominent constituent. It is composed of three distinct layers (Figs. 1 to 8). The outermost layer (W1), which is the thinnest (Average width = 80 nm), is of medium electron density and of constant width around the entire surface. Its external face is slightly irregular but there is no characteristic pattern of organization. This layer does not seem to be visible after preparation with method B. In Figure 8, patches of amorphous electron dense material can be seen adhering to the outer surface.

The middle layer (W2) of the spore wall is exceedingly electron dense and structurally amorphous. The thickness of this layer is very variable; on one side of the spore it may be little wider than the outermost layer (Range: 0.12 - 0.16 μ), while on the opposite side it may be as thick as 0.62 - 0.70 μ.

Usually the boundary between the middle layer and the inner-most layer (W3) is abrupt. This third layer is the least dense of the three, and also the most structured. It consists of fibrils oriented parallel to the circumference of the spore and embedded in a homogeneous matrix. After gluteraldehyde-osmium fixation, a staining gradient apparently exists in this layer and the density of fibers decreases towards the protoplast. These fibrils are most distinct with methods A and C. This layer is 0.33 to 0.53 μ thick. Its internal surface is uneven and ridges periodically project for distances of approximately
60 μm into the protoplast (Figs. 5b and 17).

**Plasma Membrane (PM).**—When teliospores are fixed in KMnO₄ the protoplast pulls away from the spore wall and the plasma membrane is clearly visible (Fig. 1a), but with glutaraldehyde and osmium, the plasmalemma is rarely seen at this stage. The average unit membrane thickness is 124 Å. The plasmalemma is relatively smooth and the results with methods B and C indicate that in the living cell it is probably closely applied to the spore wall except where "paramural bodies" are present.

In the resting spore, small quantities of amorphous material are sometimes situated between the plasma membrane and the spore wall (Figs. 1, 11, and 17). These simple "paramural bodies" do not appear to be membrane bound and show none of the complex tubular or vesicular forms commonly associated with such structures (Marchant and Rabards, 1968). In addition, where the plasma membrane has pulled away from the wall, distinct fibers with an average diameter of 22 Å are visible between the protoplast and the spore wall (Figs. 1a, 1b, and 15).

**Endoplasmic Reticulum (ER) and Ribosomes.**—In resting spores, the ER is sparse. Usually a series of short cisternal elements lie just beneath, and parallel with, the plasma membrane, while in the central regions a few short fragments of indeterminate morphology are scattered at random. Although the amount of data on glutaraldehyde-osmium fixed tissue is limited, in this respect, the ER membranes appear to be smooth.
The ribosomes of *U. hordei* measure 100 to 150 Å in diameter. In the resting protoplast these are very densely packed. Most, if not all, of them appear to be free.

**Nucleus (N).** - Diploid resting nuclei (dN) are positioned centrally in the spore and appear to be spherical to ovoid in shape. Often the nucleus appears to be beaked on one side. The diameter ranges from 2.0 to 2.7 µ (Figs. 1 and 5). Occasionally a resting spore has two haploid nuclei (hN), each having a diameter ranging from 1.5 to 1.8 µ (Fig. 6). Light microscope observations on resting spores and spores after one hour of hydration indicate that nuclear fusion can occur during hydration (Figs. 2, 3 and 4), but no information is available at the ultrastructure level.

In the resting spore the nuclear envelope (NE) tends to be poorly defined. This lack of definition may be due to inadequate fixation, or to a real difference in the physiological state of the membrane, or to both. Only a hint of the familiar double membrane is observed in Figure 5 and few nuclear pores can be seen. In glutaraldehyde-osmium fixed tissue the nucleolus (Nu), appears to be uniformly granular and electron dense. In agreement with the results obtained from hyphal nuclei (Stein, 1970) the nucleolar diameter measures approximately 1 µ (Range 0.93 - 1.19 µ) in both haploid and diploid nuclei.

**Mitochondria (M).** - The mitochondria generally are located randomly in the resting teliospore although there may be a tendency to aggregate in the more central regions (Fig. 5).
At this stage their cross-sectional shape is ovoid to round. No evidence has been found for the presence of elongate mitochondria. As was previously mentioned, the distorted shapes observed after permanganate fixation are believed to be artefactual. The average maximum length of the resting mitochondria as determined after glutaraldehyde-osmium fixation, is \( 0.38 \mu \) (Range: \( 0.2^\circ - 0.60 \mu \)). The cristae, which are fairly well-developed, are plate-like, and tend to be arranged in parallel arrays which may or may not lie in the long axis of the mitochondrion.

**Lipid Bodies (L).** - The resting teliospore contains a large number of lipid bodies randomly scattered throughout the protoplast. After permanganate fixation they appear as conspicuous electron transparent bodies which sometimes contain traces of semi-electron dense material (Fig.1). They are bounded by an electron-dense line about \( 40 \text{ A}^\circ \) thick, which cannot be shown to have unit membrane structure even at high magnifications. No analogous membrane-like structure is present after preparatory methods B and C. After glutaraldehyde-osmium fixation the lipid bodies are usually slightly electron dense and homogeneous. In the spore these bodies range in size from 0.1 to 0.5 \( \mu \).

**Spherosome-like Bodies (S).** - After permanganate fixation semi-dense, membrane-bound bodies are often observed which range in size from 0.1 to 0.6 \( \mu \) (diameter). They are usually associated with fragments of endoplasmic reticulum. Evidence is presented in part II that these bodies are identical with
those membrane-bound bodies which contain very electron-dense material after glutaraldehyde-osmium fixation (Figs. 5 and 7). In spores these latter bodies range in size from 0.2 to 1.9 μ.

STAGE ONE

Approximately one-half to one hour after the beginning of hydration the nucleus of the teliospore undergoes a radical change in shape. It becomes lobed. The extent of this condition varies from a mild distortion (Fig. 8) to an extreme state in which the nucleus is deeply indented and sends out long attenuated arms that bulge into nuclear lobes (Fig. 7). This extreme state has only been seen after KMnO₄ fixation; however, the amount of data for glutaraldehyde-osmium fixed material is limited.

When the nucleus is in the lobed condition lipid bodies appear to become closely associated with the nuclear envelope. Figure 7 shows a lipid body, within a protoplasmic arm, extending deep into a nuclear cleft. At this time lipid bodies are also occasionally seen in close association with the membrane-bound bodies containing electron dense material (S). In Figure 8 a lipid body appears to be fused with two such organelles (Fig. 8, arrow).

Another early cytological change found to occur in the activating teliospore is an increase in the amount of smooth ER in the inner regions of the protoplast, particularly surrounding and parallel with the nuclear envelope. The ER appears to be connected with the nuclear envelope at some points (Fig. 7, arrow).
Shortly after hydration begins, well-developed, electron transparent zones develop in the cytoplasm (Figs. 7 and 8). These zones, referred to as "flocculent cytoplasm" (f), are relatively large and appear to have structure. Occasionally, after permanganate fixation, large semielectron dense granules (Average diameter = 0.18 μ) are located in, or around the edges of, the flocculent areas (Fig. 16). Similar regions have also been reported in aging hyphae of U. hordei, but only after glutaraldehyde-osmium fixation (Stein, 1970).

STAGE TWO

At approximately half way through the pregermination interval, complex whorls of ER develop in the cytoplasm (Fig. 9). All the information pertaining to these whorls has been derived from permanganate-fixed material; the density of the protoplast after glutaraldehyde-osmium fixation renders any observation of these membranes difficult. They are centrally located. Whether their proximity to the nucleus is fortuitous, or whether the proximity reflects some real relationship between the two is not yet clear.

About the same time, vacuoles with flocculent contents appear in the cytoplasm (Figs. 10 and 11). They are bounded by a unit membrane, the tonoplast, which has an average thickness of 96 Å (Range: 80-120 Å). After permanganate fixation they are very irregular in shape and project outwards sharply, especially at points of contact with what appears to be ER (Fig. 11, arrow). In Figure 10 several vacuoles are apparently interconnected and are partially surrounded by a complex system
of ER. One end of these early vacuolar systems always lies in the vicinity of the nucleus in the observed material (Figs. 10 and 11).

Spherosome-like bodies are often present in the vicinity of developing vacuolar complexes. At this stage they do not always appear to be associated with ER (Fig. 10). Well-developed spherosomes commonly occur, as well, aligned along portions of the ER which appear to be associated with developing vacuoles (Fig. 14).

As the spores approach germination a large proportion of the nuclei are associated with long ER cisternae (Fig. 17). Sometimes a nucleus is almost completely surrounded by several layers of cisternae which lie parallel with the nuclear envelope (Fig. 13). In Figures 12 and 13 two such cisternae clearly connect with the nuclear envelope (Arrows).

FULLY ACTIVATED SPORE (STAGE THREE)

Figures 15, 16, and 17 illustrate the maximum state of differentiation that the spore attains before the metabasidium begins to form. Certain of its components remain essentially unchanged from the dormant state (Figs. 1, 5 and 6). Although the spore increases slightly in volume during imbibition, the spore wall does not show any noticeable change in thickness, nor is there any evidence of stretching. Simple paramural bodies are still present (Fig. 17). Apparently the number, size, and position of both the lipid bodies, and the spherosome-like organelles remain constant. However, a comparison of Figure 1 with Figure 17 (method A) or of Figures 5 and 6
with Figure 15 (method B) indicates a much greater degree of
cytophological complexity in the fully activated spores. This
increase in complexity has been achieved via the stages al-
ready discussed. The sum total of these changes, plus the
final steps in the preparation for germination, can be sum-
marized as follows;

Endoplasmic Reticulum. - In the fully activated spore,
both the total quantity, and the length of individual cisternae
that can be traced in any one section increase considerably,
particularly in the perinuclear region (Fig 17). Rather late
in the pregermination period a further change occurs in the
distribution of ER. The cisternae tend to stack (Fig. 16,
arrows).

Vacuoles. - Vacuoles are not present in dormant spores.
By the time the hydrated spore reaches the stage of germination
it contains at least one large vacuole plus a variable number
of smaller vacuoles. In Figure 17 there are a number of pairs
of these small vacuoles joined together by what appears to be
short ER segments.

Nuclei. - The nucleus which is once again more regular
in shape has become eccentrically situated in the spore (Figs.
15 and 17). In Figure 17 the double nature of the nuclear en-
velope is clearly visible. Several figures also indicate the
presence of many simple nuclear pores (NP) (Figs. 9, 10, 11,
13, 15, and 17). Further details concerning the meiotic
nucleus will be discussed in part IV.

Mitochondria. - Figure 6 and Figure 15 illustrate material
prepared by method B and are at the same magnification. A comparison of these two figures clearly shows the dramatic increase in the size of the mitochondria during hydration. The average maximum length of the mitochondria in fully activated spores, after glutaraldehyde-osmium fixation, is 0.75 µ (Range: 0.7 - 1.2 µ), a figure which is almost twice that of the resting mitochondria (0.38 µ). A comparison of Figure 5 with Figure 15 indicates that there is no significant change in the shape of mature mitochondria, or in the number, length, and arrangement of cristae.

The number of mitochondria does not appear to increase significantly throughout pregermination development. Only in the very latest stages depicted is there evidence that the mitochondria are beginning to divide. In Figure 17 the arrows indicate a mitochondrion apparently in the process of constriction, and a double membrane-bound organelle which is interpreted as a cross-section through an immature mitochondrion. The most conspicuous feature of these dividing and immature organelles is the poor development of the cristae.

**Flocculent Cytoplasm.** - The patches of "flocculent cytoplasm" which develop very early in the hydration process are still present (Fig. 16). They do not seem to be localized in any particular area of the spore.

**DISCUSSION**

**THE SPORE WALL**

Teliospores have very complex walls - a fact which is pro-
bably important in their ability to survive (Allen, 1965). No doubt this same complexity, compounded by an astonishing thickness, is responsible for the lack of cytological information concerning the development and germination of these spores.

In agreement with previous studies on smut fungi (Graham, 1960; Hess and Weber, 1970) the teliospore wall of *Ustilago hordei* consists of three major layers. Graham (1960) also reported an intermediate cementing layer in *Tilletia contraversa* Kuhn. The amorphous electron dense material adhering to the outer surface may be the collapsed mucus-like jelly which surrounds the developing spore initials (Kukkonen and Vaissalo, 1964).

In agreement with the studies of Hille and Brandes (1956) the teliospore of *Ustilago hordei* appears to be exceptional among smuts in possessing a smooth outer surface.

All non-motile fungal spores possess at least one electron dense layer, and this layer is never the most proximal to the cytoplasm. Its dense nature is due to the presence of melanin which is postulated to give resistance to radiation damage and to enzyme lysis (Bartnicki-Garcia, 1969). The innermost wall layer of *Ustilago hordei* is the only fibrillar layer. This is in agreement with biochemical studies in *Tilletia contraversa* which show that chitin is predominant in the inner layers but is lacking in the outer (Graham, 1960). The density of the "chitin" fibrils decreases in the vicinity of the protoplast. A similar gradient has been noted in the inner wall layers of *Neurospora* ascospores (Lowry and Sussman, 1964). The ridges which project into the protoplast are similar
to those demonstrated by freeze-etching in vegetative fungal cells (Bauer, 1970; Hess, 1968), and conidia (Campbell, 1969b; Sassen et al., 1967).

THE PROTOPLAST

The plasma membrane of *Ustilago hordei* appears to be wider (approximately 120 Å) than that usually reported for fungi (60-80 Å). Corfman (1966), however, gives similar dimensions for the spores of a myxomycete. Unlike the plasma membrane of the hyphae of *Ustilago* (Stein, 1970) that of ungerminated spores is relatively smooth. A crenulate plasma membrane is often associated with active growth (Marchant et al., 1967) or with aging (Hawker, 1965) in fungi. During the development of conidia in *Alternaria brassicola* the plasma membrane, which is convoluted during the period of wall formation, becomes smooth in the mature spore (Campbell, 1969a). The tendency for the plasma membrane to pull away from the spore wall after permanganate fixation is probably artefactual.

Usually paramural bodies are not found in mature spores (Marchant and Robards, 1968). The amorphous structures that are sometimes seen lying between the plasma membrane and spore wall (Figs. 1 and 17) do not appear to be either tubular or vesicular and it is questionable whether they actually constitute paramural bodies. However, the possibility exists that these simple inclusions may be residual breakdown products of paramural bodies which were active during spore wall deposition (Campbell, 1969a; Carroll, 1969; Wilsenach and Kessel, 1965). The nature of the fibers which connect the
protoplast with the spore wall is unknown, but presumably their function is to maintain close adherence of these two parts of the spore during prolonged storage and dessication. Griffiths (1971) has described similar fibers in the basidiospores of *Panaeolus compumulatus* (L.) Fr.

The paucity of endoplasmic reticulum and the parietal position of the ER are features which are common to a number of resting spores (Buckley et al., 1966; Ehrlich and Ehrlich, 1969). The short parietal fragments are postulated to be the remanent of an ER-net which was active in the thickening of the immature spore wall (Reeves, 1967). Kukkonen and Vaissalo (1964) claim that ER is pressed against the mucous walls of the sporogenous hyphae shortly after spore wall formation begins in a smut fungus, *Anthracoidea aspersa*.

An increase in the amount of endoplasmic reticulum during germination appears to be almost ubiquitous among fungi (Bracker, 1967; for exception see Sussman et al., 1969) and is also characteristic of the dormant seed of higher plants. This increase reflects a generalized acceleration of the metabolic rate which accompanies activation and germination (Allen, 1965). With *Ustilago hordei* one of the first indications of increased metabolic activity in the spore is the appearance of short ER elements in the interior, particularly in the vicinity of, and parallel with the nuclear envelope. During the pregermination period this nuclear-associated ER continues to develop, and may form several parallel layers of cisternae almost completely surrounding the nucleus (Fig. 13). Several other reports of similar nuclear-ER associations
exist in the literature of fungi (Buckley et al., 1966; Gay and Greenwood, 1966; Peat and Banbury, 1967; Wells, 1964a; and Wells, 1965) and higher plants (Esau and Gill, 1971). Figures 12 and 13 indicate that during the period of maximal development of this association, clear connections exist between the ER and the nuclear envelope. Such connections are well-documented in fungal hyphae although rarely in spores (Namboodiri, 1966).

The origin of the abundant ER which forms in spores during the first few hours of hydration appears to have previously been a mystery! Linnane et al. (1962) suggested that in yeast the reticular system sometimes seems to originate from the nuclear envelope. In view of the fact that in Ustilago hordei the first ER to be formed lies in the vicinity of the nuclear envelope, and that nuclear envelope-ER connections can be demonstrated, this would appear to be probable. Abundant evidence indicates that in animal cells and more particularly in highly active cells such as eggs, the nuclear envelope does serve as a source of rapidly proliferating ER membranes (Wischnitzer, 1970).

As germination becomes imminent, smooth entoplasmic reticulum tends to form stacks in the cytoplasm — particularly in a parietal position (Fig. 16). Some authors have suggested that these stacks of membranes, frequently seen in fungi, resemble simple Golgi (Campbell, 1969b). However, the results of this work tend to support the view of Wells (1964a and 1964b) that since these stacks are not always associated with the nucleus or with vesicle production, they cannot justifi-
ably be referred to as Golgi. Such aggregations of ER are not restricted to fungi, and their possible significance is still debatable. Esau and Gill (1971) review the literature on this stacking phenomenon in plant and animal cells, with particular emphasis on the evidence linking it with either an active or a passive state. In most fungi the development of stacks of smooth membranes appears to be associated with highly active states such as ascospore wall formation (Carroll, 1969), and nuclear division in mature basidia (Manocha, 1965; Wells, 1964). In Ustilago hordei they form just prior to the first ultrastructural signs that germination has begun.

Vacuoles are not present in most resting or dormant spores (with the exception of the powdery mildews) but are formed during the processes of activation and germination (Hyde and Walkinshaw, 1966; Hawker et al., 1970; Hawker and Abbott, 1963; Niederpruem and Wessels, 1969; Voelz and Niederpruem, 1964). Such vacuolation appears to be a necessary adjunct of hydration and germination - so much so that if the vacuoles are damaged, or prevented from expansion, the spore ceases to germinate (Mitchell and McKeen, 1970). In spite of the obvious importance of the development of these organelles in the spore no previous attempt has been made to determine their origin.

Several theories exist to account for the origins of plant vacuoles. Vacuoles may arise via dilation of the intramembranous cisternae of either the endoplasmic reticulum (Buvat, 1961) or the Golgi apparatus (Marinos, 1963). From her studies
on the meristems of the higher plant *Anthoceros*, Manton (1962) proposed that vacuoles always arise from pre-existing vacuoles. Bell and Muhlethaler (1964) concluded that they could arise from the degeneration of mitochondria and plastids. Matile and Spichiger (1968) have more recently proposed that vacuoles are formed by the development and coalescence of lysosome-like bodies. The last four suggestions can be eliminated in the consideration of vacuole development in the ungerminated spore of *Ustilago hordei* because of the following facts:

1. No organelles which can be identified as Golgi are present in *U. hordei*.
2. Resting spores of this fungus possess no vacuoles.
3. Evidence for mitochondrial degeneration has been observed in aging hyphae but never in hydrating spores.

To the contrary, the mitochondria during pregermination development are seen to increase in size and number (i.e. only in the final pregermination stages).

4. Those membrane-bound dense bodies which, during and after germination, appear to act as lysosomal equivalents (Pt. II), do not coalesce or change in number or size throughout pregerminal development.

At about the time when vacuoles begin to appear in some spores, "Whorls" of endoplasmic reticulum occur in other spores. Such whorls have not been seen in spores which could be shown to possess a vacuole. Often, a whorl has one or more inflated cisternae (Fig. 9, arrow), the contents of which are flocculent after permanganate fixation, and resemble the flocculent
contents detectable in developing vacuoles. The evidence, therefore, seems to be most consistent with the hypothesis of Buvat. It is suggested that in *Ustilago hordei* the "primary vacuoles," which are responsible for the initial swelling of the teliospore, arise from dilation of the cisternae in whorls of ER. Figures 9, 10, and 11 are representative of the postulated developmental sequence. As germination approaches, this process seems to become more generalized, so that, as seen in Figure 17, small fragments of ER throughout the spore appear to give rise to similar dilations. The hypothesis is further supported by the fact that the tonoplast membrane (Average width = 96 Å) is the same width as the unit membranes of the ER (Average width = 94 Å). Robinson, Park and McClure (1969) have presented evidence that vacuoles, induced in the hyphae of *Aspergillus niger* by the vacuolation factor, also form by expansion of the ER lumen. What is now required is the application of the appropriate histochemical tests i.e., for acid phosphatase) to determine first whether vacuoles show lysosomal activity and second whether they are in fact derived from the ER.

Similar hypotheses have recently been suggested for the origin and development of vacuoles in the meristematic regions of the shoot and adventitious roots of *Glechoma hederacea* L. (Bowes, 1965), and in the root meristems of *Lupinus albus* (Mesquita, 1969). Mesquita was able to show that the rough ER, which gives rise to the vacuoles, is continuous with the nuclear envelope, and that there is direct communication be-
tween the intravacuolar space and the perinuclear cisterna. Such a direct connection has not been observed in *Ustilago hordei* but it has been mentioned that the whorls of ER are usually in close proximity with the nuclear envelope, and that one end of the first primary vacuoles is usually also very near the nucleus.

A detailed discussion of the meiotic nucleus of *Ustilago hordei* is presented in part IV, but a few points, which perhaps have special relevance to the germination process, deserve mention here. Normally, the teliospores of smut fungi are considered to contain a single diploid nucleus. To the best of the author's knowledge this is the first study to suggest that this is not always the case. The fact that the mature spore may sometimes have two haploid nuclei (Figs. 2, 3, 4, and 6) suggests that the synchronization of karyogamy with spore formation is loose. It is unknown whether such spores, in which karyogamy has evidently been delayed, are capable of undergoing germination and meiosis. A second unusual feature is the apparent plasticity of nuclear shape which develops very early in hydration. Corfman (1966) reported a similar occurrence shortly after the beginning of hydration in the spore of a myxomycete, *Fuligo septica* (L.) Weber, and suggested that its function was to maximize the potential for nucleocytoplasmic interaction. In *Ustilago hordei* the lobed state seems to be associated with the movement of the nucleus from a central to a peripheral position in the spore. This suggests that it may be
a manifestation of true nuclear amoeboïd motion. Whether the apparent association of lipid bodies with the lobate nucleus is fortuitous, or functional is unknown. Lipid bodies are also associated with the nuclei of some phycomycetes during gamete formation (Blondel and Turian, 1960).

The observations reported here on the development of the mitochondrial population during germination seem to be at variance with the commonly accepted views. In all cases so far reported the quiescent mitochondria are much larger than those seen after germination (Hawker and Abbott, 1963; Hawker, 1966; Lowry and Sussman, 1968; Marchant, 1966). Bracker (1967) noted that the decrease in mitochondrial size is often accompanied by an increase in the total number, suggesting that the large mitochondria divide to form smaller ones. During teliospore germination in Ustilago hordei the exact opposite seems to occur. The mitochondria, which are originally quite small, double their average maximum length during hydration. The population does not increase noticeably in number and figures which have been interpreted as division figures (Fig. 17, arrows) occur only prior to germination. During the last stage discussed here, and in subsequent stages, very few such figures occur at any one time, and the average size of the mitochondria in the population, in general, continues to increase until well into metabasidia extension. It would be interesting to know whether this unusual behaviour reflects some difference in the developing respiratory pattern of Ustilago hordei teliospores. On the other hand it may simply
result from the fact that the protoplast of the teliospore does not actually increase greatly in mass during the formation of the promycelium. The resting mitochondria of this fungus also seem to be unique in the fact that their cristae are well-developed and retain the parallel grouping previously reported for the hyphae (Stein, 1970).

Lipid is probably the most common storage material in fungus spores, particularly in the spores of rusts and smuts which germinate at the expense of their own reserves (Allen, 1965). A decrease in the number of lipid or oil bodies during germination is almost ubiquitous among fungi (Hawker et al., 1970; Manocha and Shaw, 1967; Walkinshaw et al., 1967; Williams and Ledingham, 1964; McKeen, 1970). In this respect Ustilago hordei also appears to be unique, since no decrease in the number or size of the lipid bodies is detectable. However, the occasional association of lipid bodies with the membrane-bound, lysosome-like organelles (Fig. 8) may be indicative that some utilization does occur. It seems likely that, because the spores were germinated in a comparatively rich medium, they may be less dependent on endogenous reserves.

Flocculent, or foamy, cytoplasm occurs in the spores of several fungi, and various functions have been attributed to it. Campbell (1969a) suggests that it represents dextran accumulations; Hyde and Walkinshaw (1966) and Voelz and Niederpruem (1964) suggest that they are regions in which glycogen, or some other storage compound, has been leached out. Sussman et al. (1969) postulate that such regions serve
the function of vacuoles. The observation that in Ustilago hordei such regions are often associated with clusters of large granules (Fig. 16) supports the idea that "flocculent" cytoplasm results from the leaching out of some component. No histochemical identification was attempted; the size and appearance of the granules are compatible with suggestions that they could be glycogen, dextran, or perhaps glucan. Wynn and Gajdusek (1968) have shown that during the germination of uredospores of the bean rust, Uromyces phaseoli, a portion of the endogenous reserves are transferred to a temporary reservoir of polysaccharide (probably glucan), which is utilized in the rapid elongation of germ tubes. With the electron microscope, this storage form is said to be represented by particles which are similar in appearance to glycogen.

**CONCLUSION**

During the period of pregermination hydration, the teliospore of Ustilago hordei undergoes a number of ultrastructural changes commonly associated with germination; the amount of endoplasmic reticulum increases, temporary storage material accumulates, and vacuoles form. However, the fungus appears to be unusual in the fact that the mitochondria do not decrease in size or increase notably in number, and the fact that the number and size of lipid bodies does not decrease. The feasibility of sequential pregermination studies is clear. The importance of similar studies in other types of fungus spores is made manifest by the fact that many, if not most, of the
metabolic changes which result in germination occur within the spore long before the event itself. Any attempt to correlate such metabolic changes with post-germinal cytological observations is of limited value, and may be misleading. In the author's opinion the previous "mystery" concerning the origin of the endoplasmic reticulum and the "primary hydration vacuoles" is indefensible. It has, tentatively, been suggested that the ER is derived in some manner from the nuclear envelope, and that the primary vacuoles form by dilation of ER cisternae. These suggestions are certainly stimulating, as well as being of critical importance to any understanding of fungal spore germination, and are worthy of further study.
I. PLATE 1

Figure 1a. General view of a quiescent teliospore (i.e. 0 hr. of germination), showing the three wall layers (W1, W2, and W3), the plasma membrane (PM), and the structured distribution of lipid bodies (L), spherosome-like bodies (S), mitochondria (M), endoplasmic reticulum (ER) and the diploid fusion nucleus (dN). Method A. ca. X 24,400.

Figure 1b. Part of figure 1a showing the relationship between the ER and a spherosome-like body. The plasma membrane (PM) is shown clearly. Note the fine filaments passing from the plasma membrane to the spore wall. Method A. ca. X 24,000.

Figures 2-4 Light microscope pictures of thick sections prepared by method A and stained with Toluidine blue (Appendix C). The pictures depict the steps in the fusion of haploid nuclei in the germinating teliospore. ca. X 5,040.
I. PLATE 2

Figure 5a. General view of a quiescent teliospore (i.e. 0 hr. of germination). Indicated are mitochondria (M), electron transparent lipid bodies (L), and spherosome-like bodies (S). Note the beaked appearance of the diploid nucleus (dN), the nucleolus (Nu) and the nuclear envelope (NE). Method B. ca. X 21,000.

Figure 5b. Part of figure 5a showing ridges of inner spore wall protruding into protoplast (arrows). ca. X 42,000.

Figure 6. Part of a quiescent teliospore (i.e. 0 hr. of germination) showing the presence of two haploid nuclei (hN). Mitochondria (M) and ER are also present. Note the size of the mitochondria. Method B. ca. X 29,500.
Figure 7. Part of a stage-one teliospore (approximately ½ hr. of hydration) showing a diploid nucleus (dN) in the extremely lobed state. Note the intimate relationship between the lipid bodies L and the nucleus during this stage. The arrow indicates a possible NE - ER connection. A large patch of flocculent cytoplasm (f) is visible. Method A. ca. X 38,500.

Figure 8. A general view of a stage-one teliospore. The nucleolus (Nu) is very conspicuous at one side of the gently lobed diploid nucleus. The three wall layers W1, W2, and W3 are very distinct. Note the fibrillar nature of the innermost wall layer. Two patches of flocculent cytoplasm (f) are present. The arrow indicates the point of fusion between a large lipid body (L) and one of two spherosome-like body (S). Method C. ca. X 18,400.
I. PLATE 4

Figure 9. Part of a stage two teliospore illustrating a whorl of endoplasmic reticulum (ER) with a swollen cisterna containing flocculent material (arrow). The whorl lies in close proximity to the diploid nucleus (dN). Note that after KMnO₄ fixation the nuclear pores (NP) appear simple. Method A. ca. X 39000.

Figure 10. Part of a stage two teliospore illustrating a more advanced stage in the formation of the primary hydration vacuoles. A system of small vacuoles (V), interconnected by short ER segments, lies near the diploid nucleus (dN). A number of free spherosome-like bodies (S) are present. Method A. ca. X 30,000.

Figure 11. A well formed vacuole (V) lies with one end adjacent to the diploid nucleus (dN). Note the vacuolar - ER connection (arrow). Method A. ca. X 34,500.

Figure 12. During stage one and two complex system of ER develop in association with the diploid nucleus (dN). The arrow indicates a prominent nuclear envelope-ER connection. Method A. ca. X 26,000.
I. PLATE 5

Figure 13. Part of a stage two teliospore illustrating several layers of ER surrounding the diploid nucleus (dN). Two nuclear pores (NP) and a nuclear envelope-ER connection (arrow) are indicated. Method A. ca. X 30,000.

Figure 14. Spherosome-like bodies (s) often lie along elements of ER which lead to the primary hydration vacuoles (V). Method A. ca. X 28,500.

Figure 15. General view of a teliospore just prior to germination showing the spherical to ovoid mitochondria (M) with well-developed parallel, plate-like cristae lying in the longitudinal axis of these organelles. Note that the nuclear pores (NP) appear simple after this method of preparation. Well developed ER is visible. Method B. ca. X 29,500.
Figure 16. General view of a teliospore just prior to germination. Note the presence of the large mitochondria (M), lipid bodies (L), many small vacuoles (V). Patches of electron dense granules are associated with a region of flocculent cytoplasm (f). The parietal endoplasmic reticulum (ER) tends to stack (arrows). Method A. ca. X 30,000.

Figure 17. General view of a teliospore just prior to germination. Note the eccentrically positioned diploid nucleus (dN) with a well-defined nuclear envelope (NE) and nuclear pores (NP). Long segments of ER are associated with the nucleus. Throughout the spore short ER-elements appear to be giving rise to small vacuoles by dilation. Numerous lipid bodies (L) are still present. The arrows indicate a dividing mitochondrion and a young mitochondrial element. Note the elementary state of the cristae in dividing and young mitochondria. Method A. Ca. X 18,000.
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PART II
Initiation of the Promycelium
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PART II
Initiation of the Promycelium
and Promycelial Extension

ABSTRACT

Initiation of the promycelium of *Ustilago hordei* involves the localized degradation of the inner spore wall, the localized deposition of a new wall layer, and the swelling of the protoplasm. During this period of development the endoplasmic reticulum and spherosome-like organelles (Pt.I) undergo changes in distribution. The significance of these changes is discussed.

Most of the spore contents flow into the promycelium as it extends. The structural basis for the extension of the promycelial wall is unknown. Mitochondria, lipid bodies, and endoplasmic reticulum are randomly distributed and do not increase substantially in quantity. Apart from the nucleus the only organelles which apparently change in number and distribution are those bodies that appear to be the ultrastructural equivalent of the light microscopists' spherosomes and vacuoles. In discussing the significance of these changes it is suggested that the spherosomal-vacuolar system in this fungus is the functional equivalent of the animal lysosomal system.

INTRODUCTION

On germination the teliospores (probasidian) of *Ustilago hordei* give rise to tube-like cells of limited growth known as
Promyelia or metabasidia. These structures are superficially similar to germ tubes produced by other nonsexual spores. In *Ustilago hordei* the promyelia is approximately 2.3 μ wide (Range: 1.40-2.8 μ). Once the spore wall has ruptured the promyelia extends at a maximum rate of approximately 0.25 μ per minute, slowing down as it attains its maximum length (Range: 20-30 μ). When growth has stopped, the cell divides twice in rapid succession giving rise to a four-celled promycelium containing the four meiotic products. There is very little terminal growth once division has occurred.

This study is concerned with those cytological events which occur during spore germination - that is, during promycelium development. In spite of the fact that spores (or sporangia in some Oomycetes) are notoriously difficult to prepare for electron microscopy prior to germination a number of attempts have been made to describe the early stages of germ tube initiation which occur within the intact spore (sporangial) wall (Elsner et al., 1970; Hawker and Abbott, 1963b; Hawker, 1966; Hawker et al., 1970; Lowery and Sussman, 1964; Marchant, 1966; Mitchell and McKeen, 1970; Walkinshaw et al., 1967). Although the literature relating to germ tube ultrastructure is quite extensive, to the best of the author's knowledge no literature is available on the ultrastructure of metabasidia. For the most part germ tubes seem to possess few features that distinguish them cytologically from hyphane. In most respects the young extending promycelium of *Ustilago hordei* also resembles the apical hyphal region of the mycelial mutant described by Stein (1970).
MATERIALS AND METHODS

The cultures and the methods of culturing and sampling were identical to those described previously (Pt. I), except that the samples were collected after 5 hours, and after 7 to 9½ hours of hydration. During the latter period, approximately 80 % to 95% of the spores have begun metabasidial extension; of these, 15% to 50% have formed the first septum, and 5% to 40% have formed the second septa. For example, after 8 hours of hydration the per centage germination (i.e., the number of spores with promycelia visible in the light microscope) is 90%, the per centage of spores that have formed at least one septum is 35% and the per centage of spores that have formed three septa is 25% (Bech-Hansen, 1968).

The material was prepared for electron microscopy according to methods A, B and C (Pt. I, Table I), with the exception that none of the material was pre-stained in aqueous uranyl acetate prior to embedding. Figure 4b was photographed using a Zeiss Photomicroscope, from thick sections prepared according to method A and stained with 1 % Toluidine blue in 1% borax. Figure 14b depicts a living cell photographed in a thin layer of complete liquid medium between a coverslip and a glass slide.

OBSERVATIONS

STAGE FOUR: INITIATION OF THE PROMYCELIUM

The first cytological indication that germination has begun is a change in the shape of the protoplast. The previously symmetrical, circular or ovoid cross-section acquires a
"beaked" appearance (Figs. 1, 3 4a, or 4b). Closer observation reveals that a very slight change in the shape of the actual protoplast is accentuated by the presence of a "cap" of material which covers the beaked portion of the cytoplasm and protrudes into the spore wall (Figs. 1, 3 and 4a).

The Wall. - As described in part I, the spore wall (W) of Ustilago hordei consists of three major layers (W1, W2, and W3). When the beak of the protoplast first becomes noticeable, and before the appearance of the "cap", perforations (p) develop in the innermost wall layer at a point opposite the beak (Fig. 2). After preparatory methods A and B, these spaces are usually empty, or occasionally they contain faintly granular material (Figs. 1 and 4a). However, after method C, they contain electron dense fibrils (Fig. 2).

Shortly after the first "perforations" appear in the inner wall, the new material (pW) begins to accumulate around the protoplast beak (Fig. 2). The new material is more electron translucent than is the inner wall layer. When it is first formed it is quite homogeneous but, as the layer thickens and extends laterally, the region most proximal to the spore wall becomes increasingly fibrillar. However, at this period of development, the orientation of these fibers never approaches the regularity shown by those of the inner spore wall (Fig. 6). Consequently, although the old and new layers are closely appressed the boundary between them is abrupt. The new wall layer is also closely appressed to the protoplast of the beak, but the boundary here is often indistinct (Figs. 1 and 3).
Once this pattern of development has been initiated, the processes involved proceed rapidly and simultaneously. As the region of perforation increases and extends around the sides of the beak, the protoplast seems to push forward displacing the perforated inner spore wall layer and expanding in length and in width (Fig. 4a). The new wall layer thickens and extends laterally at a rate sufficient to ensure that the increasing surface area of the beak is encased by it. As the thickness of the fibrillar spore wall layer is diminished by the advancing protoplast, the outer electron dense wall layers begin to show signs of disorganization (Fig. 4a). Finally, the spore wall ruptures. The spore illustrated in Figure 6 was caught at this exact moment. The beak of the protoplast, which can now be considered as a true promycelium, has barely extended beyond the spore wall, and is entirely encased by a well-defined portion of the newly synthesized wall layer. Between the young promycelium and the old spore wall the outer part of the newly synthesized wall material has formed a short collar. Remaining cell debris has sloughed off into the medium accompanied by spore wall debris.

**Plasma Membrane (PM) and Paramural Bodies.** - During development of the protoplasmic beak, the plasma membrane often appears crenulated (Figs. 1 and 3). In Figures 1 and 3, relatively large areas of the plasmalemma are separated from the spore wall. This separation later extends to the entire surface of the protoplast except for the beak region. In this localized area the protoplast and the new wall layer are
very closely apposed; so much so that the plasma membrane boundary between the two is indistinct in the region of most intimate contact (Figs. 1, 2, 3, and 4a).

**Endoplasmic Reticulum (ER).** - Throughout the pre-germination period, there is a consistent increase in the amount, and length of endoplasmic reticulum in the interior of the spore. ER elements toward the periphery are short and scattered (Pt. I, Fig. 17). Just prior to, and during, the initiation of the promycelium, there is a noticeable increase in the number and length of these peripheral elements, particularly in the region proximal to the developing beak (Figs. 1 and 4a). In Figure I, several ER cisternae have formed a loose association just beneath the incipient metabasidium. As has been previously noted in part I, it is very difficult to obtain information on these membrane systems after glutaraldehyde-osmium fixation because of the density of the cytoplasm in ungerminated spores (Figs. 2, 4a, and 6).

Just before the rupture of the spore wall, the peripheral ER elements undergo a further change in distribution. After potassium permanganate fixation a number of spores are observed in which the shorter membrane fragments have "disappeared" and most of the organelles seem to be encased, collectively, in a continuous "sac" composed of a single continuous ER cisterna (Fig. 3). The sac may lie directly beneath and parallel to the plasma membrane, or it may be "pulled away" on one or more sides leaving a fairly large portion of the cytoplasm between the sac and the plasmalemma. In this situation the nucleus
and all the mitochondria, as well as most of the lipid, vacuo­les, and remaining ER, lie within it. A few lipid bodies, small vacuoles, spherosome-like organelles and odd fragments of ER are excluded. Figure 8 illustrates a portion of a developing sac in which the individual ER elements composing it have not yet completely fused (arrows). The cytoplasm outside the sac is slightly less dense than that within (Fig. 8). An additional feature of such formations is that at various points the membranes are elaborated into more or less complex knots (Fig. 3, arrow).

**Spherosome-like Organelles (S).** - Frey-Wyssling et al. (1963) first described spherosomes ultrastructurally in the higher plant *Zea mays*. After permanganate fixation these round to angular bodies are characterized by the presence of a uniform semi-dense, granular matrix bounded by a unit membrane, and secondly, by a constant, though poorly defined, association with the endoplasmic reticulum. Bodies, ranging in diameter from 0.1 to 0.6 μ and satisfying these criteria, have previously been noted in KMnO₄-fixed teliospores of *Ustilago hordei* (Pt. I), and have accordingly been designated "spherosome-like."

As might be anticipated from the association of the spherosome-like organelles with the ER, these bodies tend to localize following the redistribution of membrane systems that accompanies beak formation. Clusters of these spherosome-like bodies are present in the region of beak development early in this stage of differentiation (Figs. 1 and 2). After the for-
mation of the ER-sac, they come to lie either inside or outside the sac-membrane (Fig. 3). They tend to accumulate peripherally in the vicinity of the two triangular areas of cytoplasm formed when the sac membrane pulls away from the plasmalemma (Figs. 3 and 8). The smallest of the spherosome-like bodies are roughly circular in cross-section and the membranes tend to be clearly defined although unit membrane structure is difficult to demonstrate. The long axis of each of these organelles aligns in parallel with an ER element, with one of the longer sides closely following the contours of the ER membrane. Often the boundary between the ER and a spherosome-like organelle is vague - the membranes in the region of association being indistinct. Probably this effect is an artefact caused by the angle of sectioning. Where the boundaries between them are well-defined, the membranes are separated from each other by a relatively constant distance of 55 to 80 Å (Fig. 8).

The most prominent organelles present in glutaraldehyde-osmium fixed tissue are those membrane-bound bodies with very electron dense contents. They are particularly conspicuous in tissue prepared according to method C. In the ungerminated spore, the electron dense material may be either scattered throughout an electron transparent matrix in the form of dense entwined strands (Fig. 2; Pt. I, Fig. 5), or it may be "condensed" into a solid core leaving the rest of the matrix absolutely clear (Pt. I, Fig. 8). These are the only organelles in glutaraldehyde-osmium fixed tissue which have not been assigned a function. They are also the only organelles whose
size range, and pattern of distribution can be said to correspond to those of the spherosome-like organelles described in KMnO₄-fixed material. Within the spore these bodies range in diameter from 0.24 to 0.36 μ. Like the spherosome-like bodies, their numbers appear to remain constant during the early stages of hydration and their distribution is random. Too little information is available on the distribution of the endoplasmic reticulum in glutaraldehyde-osmium fixed spores to verify their association with the ER-sac, but they do cluster in the region of the developing beak (Fig. 2). On this basis, it is tentatively suggested that these organelles are identical with the spherosome-like organelles found in permanganate fixed material.

STAGE FIVE: GERM TUBE EXTENSION

Promycelial Wall (pW). - Oddly enough when the promycelium first bursts through the spore wall, the new promycelial wall which surrounds the tip is relatively thick (approximately 30 μ), and quite compact and fibrillar (Fig. 6). During subsequent stages of elongation the promycelial tip is almost naked (Fig. 10 and 20b). A true wall can only be distinguished at a distance of about 2 μ behind the tip. At this point, it is approximately 10 μ thick but proceeding basipetally, it thickens rapidly over a distance of less than 2 μ (i.e. 4 μ from the tip) to a mature width of approximately 65 μ (Range: 33 - 75 μ).

At the apex, the promycelial wall is poorly defined (Fig. 20b) and little information has been obtained concerning its substructure. However, throughout the rest of its length, it is uniformly semi-dense and appears to be granular or fibrillar
(Figs. 18a, 18b, and 20). A fine fibrillar material extends from its surface (Figs. 18 and 20). Although it is not well illustrated in these pictures, this material is often extensive, sometimes attaining lengths of almost a micron (Pt. III, Fig. 7).

In Figures 16a-c large pores open from the cytoplasm through the wall into the external medium. Such pores occur infrequently and have been observed only after preparation by method C. When they are present, they occur in large numbers along the length of the promycelium. For example, at least 10 pores were visible in a single section through the germ tube illustrated by Figures 16a-c. The diameter of these hyphal pores is approximately 47 μ (Range: 38-53 μ) at the internal surface of the wall and narrows to approximately 25 μ (Range: 23-31 μ) towards the external surface. They are lined by plasma membrane.

**Plasma Membrane (PM).** - Throughout the period of metabasidial extension, the plasma membrane appears to be uniformly smooth and closely appressed to the hyphal wall. It is indistinct at the apex but, in outline, the tip is remarkably smooth (Figs. 10 and 20). No paramural bodies are present.

**Endoplasmic Reticulum (ER) and Ribosomes.** - The decrease in cytoplasmic density which accompanies spore germination allows more critical observation of the ER after glutaraldehyde-osmium fixation. The average width of the unit membranes after all three preparatory techniques is 94.0 Å (Range: 70-110 Å) but the width of the inter-membrane space is quite variable, ranging from 60 to 160 Å after permanganate and from 60 to 100 Å after gluteraldehyde-osmium fixation. Single lamellae
vary in total width from 210 to 375 Å.

Most of the endoplasmic reticulum flows from the spore with the rest of the organelles, and becomes scattered throughout the length of the germ tube except in the extreme apex. It does not seem to increase in quantity and is very sparsely distributed, consisting mainly of single, short, flattened cisternae lying just beneath and parallel to the plasma membrane (Figs. 5, 18a and 19). Occasionally two or three lamellae in the peripheral cytoplasm will form stacks parallel to the wall, and these stacks are often associated with small, membrane-bound vesicles (ve) (Fig. 15). ER elements which are situated away from the cell wall are usually associated with the spherosome-like organelles (Figs. 9 and 18a) or with the nucleus (Figs. 17 and 19). Nuclear envelope-ER connections are rarely seen during this stage.

Ribosomes are very distinct in tissue which has been fixed in glutaraldehyde-osmium and embedded in Spurr's plastic (Figs. 7, 9, 17, and 20a-c). Their density is uniform throughout the length of the promycelium but tends to decrease within the spore as the germ tube extends. Figure 7 illustrates the density gradient across the neck of the spore. Ribosomes appear to be the only material present within the 0.3 μ directly behind the cell apex. Most of the ribosomes are free; however, a small amount of rough ER occurs near the nuclear envelope (Figs. 17 and 19, arrows). The elements of the endoplasmic reticulum which are associated with the plasma membrane (Fig. 15) or with spherosome-like organelles (Fig. 9) are
smooth.

Nucleus (N). - Normally the nucleus is in the diploid state when it migrates (General Introduction). In Figures 4a and 4b the spore nucleus lies just posterior to the developing beak, "anticipating" as it were, its passage into the metabasidium. Usually, although not always, the nucleus is one of the first organelles to migrate and it does so in a distinctive and characteristic manner.

The neck by which the promycelium leaves the spore is the narrowest part of the cell (width = approximately 1 μ) (Figs. 5 and 7). In order to pass through this narrow region the spherical diploid nucleus (Average diameter = 2.6 μ) elongates, altering its shape apparently spontaneously. The nucleus in Figure 5 is just emerging from the neck into the promycelium. Its posterior end is still very narrow, while the remainder has broadened out leaving only a narrow cytoplasmic strip on either side. The width of the promycelium (Average width = 2.3 μ) is not sufficient to allow the diploid nucleus to return to its spherical shape and it continues to retain an elongate form (approximately 1.5 by 3.5 μ) (Figs. 5 and 10). During the passage of the nucleus up the metabasidium, the nucleus (Nu), is always at the extreme posterior (i.e. towards the spore) and a distinctive structure, the "centriolar-kinetochore-equivalent (CKE)" is always at the anterior end and to one side (Fig. 5). Further details concerning the nucleus and its associated structure will be discussed in part IV.

The nucleus does not continue to move with the flow of
cytoplasm and organelles along the extending tube. It comes to rest in such a position that when the cell has elongated to its total length the nucleus will be located in the posterior half. In the cell shown in Figure 10 the nucleus is at a position approximately mid-way along the extending promycelium. Since the diploid nucleus almost fills the entire diameter of the tube, larger organelles (i.e. mitochondria and spherosome-like bodies) which have been caught in the cytoplasmic stream cause a distortion in the shape of the nucleus as they pass by it on their way to the extending zone between the stationary nucleus and the apex (Fig. 10). When the nucleus has ceased to move and when apical extension has begun to slow down, or has stopped, the nucleus undergoes two rounds of nuclear division (meiosis) and cell division. Details concerning the mechanisms involved in these divisions will be discussed in parts III and IV. The consequence of these activities is that a one-celled diploid promycelium, such as that depicted in Figures 10 and 20a, is converted to a four-celled promycelium in which each cell contains a single haploid nucleus (hN) (Fig. 18a).

Cytoplasmic Microtubules (Mt). - Cytoplasmic microtubules are present in the developing promycelium of Ustilago hordei. In Figure 7 a number of them occur both in cross-section and longitudinal section. Within the promycelium they are often associated with the nucleus and more will be said about these structures in part IV.

Mitochondria (M). - Like the nucleus, the spherical and ovoid spore mitochondria undergo a spontaneous elongation as
they enter the promycelium (Figs 5, and 7). They become randomly distributed. Longitudinal sections through the germ tube reveal many circular and oval cross-sections of these organelles. These seem to cut tangentially and it is likely that they represent sections through longer mitochondria whose irregular shapes wander back and forth across the section. The maximum length measured in any one section is approximately 3.8 μ. In width they range from 0.2 to 0.5 μ. The numerous long, plate-like cristae seem to lie roughly parallel to the long axis of the mitochondrion and to extend from one side of the organelle to the other, effectively dividing it into compartments (Fig. 20). After preparation by method C the matrix is more electron dense than the cytoplasm, giving these organelles a distinctive appearance. After glutaraldehyde-osmium fixation the outer membrane averages 73 Å (Range: 61-98 Å) and the inner membrane averages 90 Å (Range: 73-109 Å).

**Lipid (L).** - Lipid bodies have been described in part I. A few bodies, with about the same size range as those in the spore, are scattered in the germ tube (Figs. 10, 18a, and 20a).

**Spherosome-like Bodies (S).** - Apart from the nuclei the spherosome-like bodies are the only organelles which undergo significant changes in number, appearance, and distribution during the development of the promycelium. In Figure 6, several are seen just posterior to the apex of the newly formed metabasidium. In the most anterior of these a small knot of finely fibrillar material lies on one side while the rest of the matrix is filled by dispersed fibrils. Some of the
spherosome-like bodies are always among the first organelles to flow into the metabasidium.

Early during the period of extension large numbers of these spherosome-like bodies, both large and small, are distributed randomly throughout the cytoplasm. In the light microscope they appear as conspicuous, refractile bodies of variable size (Fig. 14b) which, in the living organism, move quickly in all directions. They have been observed to move through distances at least half as long as the promycelium. In tissues prepared for electron microscopy by method C, the larger ones all appear to be in the conspicuous solid core form (Figs 9, 10, 14, and 20a), and to be surrounded by a membrane which is approximately 90 Å wide (Range: 74-111 Å). Figure 9 illustrates a number of spherosome-like organelles lying along a short segment of smooth endoplasmic reticulum. At the upper end of the membrane and to the left (arrow) is a small body with a tenuous "membrane", a granular semi-dense matrix and a small dark core region. This type of body is reminiscent of the smaller organelles with an indefinite membrane previously noted in Figure 8 (Method A). Their appearance, relationship with the ER, and association with well-developed spherosome-like bodies suggests that these smaller bodies are the formative stages of the larger.

Very large numbers of what have been designated spherosome-like bodies are present in glutaraldehyde-osmium fixed promycelia (Fig. 20a-c). They range in size from 0.2 to 2.3 μ. The numbers and diameters of these organelles are greater in promycelia that have almost attained their
maximum length (Fig. 20a) than in those which are still extending rapidly (Fig. 10). Progressing basipetally, the contents of the electron-transparent portion of the spherosome-like bodies become increasingly fibrillar (Fig. 20a). Toward the base of the promycelium shown in Figure 20 two large spherosome-like organelles appear to be in the process of fusing (Fig. 20c). The result of such fusions is the formation of large bodies with multiple electron dense cores. The latter structures are common in budding promycelia and in emptying spores (Fig. 12, V). Two other phenomena are apparently associated with aging tissue. First is the occurrence of pseudo-myelin figures within the spherosome-like organelles (Fig. 11, arrow). Second, when the organism has ceased to extend, what appears to be spherosome-like bodies occasionally begin to bleb vesicles in the direction of the septum (Fig. 18b) or the cell wall (Fig. 19).

Figure 18a illustrates a portion of a four-celled permanganate fixed promycelium. Contrary to what might be expected, the number of bodies with typical spherosome-like structure has decreased, and the size range remains 0.2 to 0.6 \( \mu \). In Figure 18a, one of these bodies has fused with a vacuole, and appears to be releasing its contents (arrow).

**Vacuoles (V).** - Promycelia that have been fixed in permanganate contain a distinctive class of organelles which, because of their similarity to the vacuoles described in part I, have temporarily been designated by the same name (Fig. 18a). Although not as irregular in shape as the spore vacuoles they possess a bounding membrane of approximately
the same width as the tonoplast (Average width = 96 Å) and occasionally contain flocculent material (Fig. 13). The number of these bodies increases with promycelial age. A very large vacuole with the more irregular shape described in part I develops within the aging spore as other cytoplasmic contents move into the promycelium (Fig. 13). In glutaraldehyde-osmium fixed tissue, there is very little evidence for the existence of unique corresponding structures. The closest equivalent is the large vacuole-like bodies with fibrillar contents situated in the spore and at the base of the four-celled promycelium in Figure 14. The extent of the increasing vacuolation which occurs at the base of the promycelium is readily demonstrated in living material (Fig. 14).

DISCUSSION

INITIATION OF THE GERM TUBE

Fungal germ tube walls can be initiated either by extension of one of the pre-existing layers of the spore wall, or by synthesis of a new wall (Hawker, 1966). Teliospores of Ustilago hordei resemble the latter case (Hawker and Abbott, 1963b; Hawker, 1966; Hawker et al., 1970; Marchant, 1966; Walkinshaw et al., 1967). Like the wall layer synthesized by germinating sporangia of Phytophthora infestans (Elsner et al., 1970), the new material in this smut forms a cap over the developing beak and tapers abruptly to the sides (Figs. 1, 3, 4, 5, and 7). It never surrounds the spore completely as reported for germinating sporangia of Phytophthora para-
sativa (Hemmes, 1969) and conidia of Cunninghamella elegans (Hawker et al., 1970). Although difficult to prove because of the impossibility of marking the extending wall layer, the general impression is that growth of the wall is not apical prior to spore wall rupture. The first material, which is synthesized at the apex (Fig. 2) rapidly becomes fibrillar and reasonably rigid. Apparently it is then pushed forward, forcibly, by the expanding protoplast. Meanwhile, synthesis continues down the sides of the lengthening beak so that it is completely encased. This hypothesis would account for the fact that when the promycelium first bursts through the spore wall the apex is encased by a wall layer which is almost 10 μ thick. Such a wall is not again seen at the tip until the germ tube has ceased apical extension.

Much of the spore's capacity for survival resides in the thick spore wall which keeps alien factors out, but equally well keeps the protoplast in. Even with the swelling of the protoplast and the synthesis of a hard "cap" over the beak, the act of penetration must be difficult. Evidently the fungus "prepares" its path by weakening the innermost spore wall layer over a localized area before it. The chitinous region does not show signs of mechanical stress but large spaces filled by electron dense fibrillar material develop (Fig. 2 and 4a). Such formations suggest that the wall is weakened enzymatically. Figure 2 illustrates that this process is already well-advanced before the new wall layer is laid down. The lateral extension of the region of perforation seems to pre-
cede extension of the new wall layer, and it seems likely that the length of the region of perforation determines the ultimate width of the neck region.

Many of the previous studies on spore germination have suggested that the spore wall is ruptured mechanically (Hawker and Abbott, 1963b; Hawker et al., 1970; Hashimoto et al., 1958). During a study of conidial germination in *Fusarium culmorum* Marchant (1966) noted that as the spore wall begins to bulge prior to its ultimate rupture, it becomes diffuse and on this basis he suggested that enzymatic degradation was involved. The results in *Ustilago hordei* support this hypothesis. However, it is uncertain whether the area of degradation extends into the outer spore walls. Figures 4a and 6 are perhaps more suggestive of a mechanical stretching and rupturing of the outer electron dense layers. The observations of Stocks and Hess (1970) in germinating *Psilocybe* basidiospores also suggest that both enzymatic dissolution and physical pressure may be involved in rupturing the spore wall.

What is the source of the enzymes and material responsible for degradation of the spore wall and synthesis of the new layer? The most prominent organelles in the beak region are endoplasmic reticulum which is known to be involved in protein synthesis and spherosome-like bodies which, as will be discussed later, are believed to sequester hydrolytic enzymes. To what extent each may be involved in which function is unknown. In Figure 2 the fibrillar contents of the spaces forming in the innermost wall layer resemble the contents of
the spherosome-like bodies and particularly the more finely fibrillar contents of the large spherosome-like body illustrated in Figure 6. This suggests that the spherosome-like bodies may be the source of the fibrillar material seen in the perforations. In their elegant study of hyphal tip growth in *Pythium ultimum*, Grove et al. (1970) postulated that vesicles, similar in appearance to what has been termed spherosome-like bodies in this study, are responsible for wall synthesis at the hyphal tip and perhaps also for wall plasticization. The hypothesis is particularly attractive because, in order for these vesicles to release their contents outside the cell, they must first fuse with the plasma membrane and hence serve the additional function of generating the new membrane necessary to cover the increasing surface area of the protoplast. In this respect it must be mentioned that the ER in the beak region has not been observed to give rise to a significant number of vesicles; no spherosome-like bodies have been seen to fuse with the plasma membrane, and the average width of the spherosome-like membranes (90 Å) is considerably less than that of the plasma membrane (124 Å). At the beak apex the plasma membrane is indistinct but does not appear to be crenulated or to undergo any noticeable activity; so, unless the plasma membrane itself synthesizes the enzymes, the manner in which they pass out of the protoplast remains obscure.

The significance and frequency of the formation of an ER-sac (Fig. 3) prior to germination is unknown. Presum-
ably it may be a mechanism of mobilizing organelles in an appropriate position to pass into the incipient promycelium. Moor (1967) has described the similar formation of an ER-sac prior to the initiation of a bud in vegetative cells of *Saccharomyces cerevisiae*. According to Moor's study the sac is open at one end and vesicles are produced from the opening edge which pass to the cell wall. At this point the wall is plasticized.

During various studies in fungi, Berliner and Duff (1965) and Lowry and Sussman (1968) have noted knots of endoplasmic reticulum similar to those which are associated with the ER-sac in *Ustilago hordei* and have suggested that they function as membrane generators (Robertson, 1961). However, from their appearance in this fungus they might equally well serve as a mechanism of membrane storage.

**PROMYCELIAL EXTENSION**

When the promycelium first emerges it is encased in a rigid wall for a very brief period. Evidently before extension can occur, the apical wall becomes plasticized in some manner and from this time until extension has ceased the first two microns of the promycelium are surrounded by a thin and indefinite boundary, which gradually merges into the typical hyphal-type wall (Average thickness = 65 μ) surrounding the rest of the tube. Obviously the key to either hyphal or promycelial extension lies in the structure and function of the apex. Elongation of the wall of such tube-like cells requires, first, that the tip remain plastic, second, that new wall material be
accreted continuously and third, that new plasma membrane be
generated continuously. Lastly, and most importantly, a
mechanism must exist by which these three activities are con-
trolled and co-ordinated. As mentionned previously, Grove,
Bracker, and Morré (1970) have suggested a system of apical
extension wherein vesicles containing the enzymes and materials
necessary for wall plasticization and synthesis would fuse
with the plasma membrane of the hyphal tip releasing their
contents to the outside. Such a mechanism would, at once sa-
tisfy all three requirements for hyphal tip extension. Sever-
al other studies have indicated an abundance of vesicles in
hyphal apices (McClure et al., 1968; Brenner and Carroll,
1968; and Hemmes and Hohl, 1969) and the authors have impli-
cated these vesicles in tip extension.

Unfortunately, in Ustilago hordei the same problem of
determining the mode of germ tube initiation also exists in
determining the mode of germ tube extension. The promycelial
tip is singularly "uninteresting" cytologically (Fig. 20b)! Its surface is evidently relatively smooth. No lomasomes or
apical corpuscles (Bartnicki-Garcia et al., 1968) are evident.
The cytoplasm at the tip is apparently devoid of endoplasmic
reticulum, vesicles and of other cell organelles with the ex-
ception of ribosomes. In short, the organelles which one might
expect to find at the apex are not there. Four possibilities
exist:

1. The density of free ribosomes at the apex obscures
the relevant structures.
2. The preparatory techniques are not sufficiently refined to demonstrate the relevant structures.

3. No apices have been seen at an appropriate stage; for example, the promycelium in Figure 20 may have stopped extending although the wall has not yet thickened.

4. The lack of cytoplasmic structures at the extreme promycelial apex reflects the true living state during extension.

The problem merits further study.

For the most part, with the exception of the appearance and activities of the spherosome-like organelles, an extending promycelium of *Ustilago hordei* resembles the hyphal apical region. The mature promycelial wall is similar in appearance and in width to the hyphal wall (Stein, 1970). To the best of the author's knowledge this is the first report of the presence of pores in hyphal walls. The fact that they are rarely present, but when present occur in large numbers, indicates that the pores are connected with a particular physiological state of the cell. When grown on artificial medium *Ustilago hordei* secretes large quantities of exoenzymes (Bech-Hansen, 1970). Possibly the pores are involved in secretion of exoenzymes, or of the mucous coat which is represented cytologically by the fibrous, material that covers the external surface of the promycelium. Similar mucous coats have been reported for a number of dimorphic fungi (O'Hern and Henry, 1956; Marchant and Smith, 1967).

As a consequence of the decrease in cytoplasmic density
during germination (Barer and Joseph 1958) more detailed studies can be made of membranous organelles. As in the hyphal apical region, described by Stein (1970), the endoplasmic reticulum of the promycelium is sparsely distributed and lies mainly beneath and parallel to the plasma membrane (Fig. 18a). In appearance they are identical except that a small quantity of rough ER which has not been reported in the hyphal apices, seems to develop in the promycelium. Gorfman (1966) reported a similar increase in the amount of rough ER after germination of spores of *Puligo septica*. It is commonly accepted that the ER, which is sparse or absent in resting spores, increases greatly during the early stages of germination (Bracker, 1967). In *Ustilago hordei* the increase in ER seems to be confined to the pre-germinal stage of hydration.

Stein (1970) reported that the outer mitochondrial membrane was wider than the inner in hyphae of *Ustilago hordei*. This has not been substantiated in this study. But in most other respects, including the parallel grouping and longitudinal orientation of the cristae, the promycelial mitochondria are similar to the hyphal ones. The tendency for the cristae to extend across the entire organelle, effectively dividing it into compartments, has also been described for *Microsporum canis* (Werner et al., 1966) and for the tip region of young sporangia of *Phycomyces* (Peat and Banbury, 1967). There is no evidence that the mitochondria of the promycelium ever undergo the complex changes which have been reported in differentiated regions of the hyphae.
An interesting aspect of the migration of organelles from spore to germ tube is the spontaneous alteration of shape which some of them undergo (Figs. 5 and 7). For the nucleus such a change is necessitated by the narrow dimensions of the neck and germ tube, but most if not all of the mitochondria could apparently proceed in either form. Thus, an elongate shape must possess other physiological advantages.

Once germination has begun in *Ustilago hordei* the amount of endoplasmic reticulum does not increase significantly, nor does the number and size of lipid bodies and mitochondria. With the exception of the extreme apical region which contains only ribosomes, the above organelles are distributed randomly throughout the promycelium thus ensuring that, following septation and bud formation, the sporidia share equally. The only organelles which significantly alter in number, size and appearance are the spherosome-like and vacuolar organelles.

In the promycelia the number of "permanganate-type" spherosomal bodies decreases, and their size range remains the same, while the number and size range of the "glutaraldehyde-osmium-type" bodies both increase. With the light microscope vacuoles can be observed forming at the base of the germ tube as it extends (Fig. 14b). Organelles which resemble the classical "vacuole" in permanganate fixed material are present after promycelia have been prepared by method A (Fig. 18a) but no "distinctive" organ-
elles satisfying the necessary criteria are observably present after methods B and C. What is observed at the base of the promycelium are large spherosome-like bodies apparently in the process of fusion (Fig. 20c). This suggests that the large spherosome-like organelles and fusion products observed in glutaraldehyde-osmium fixed tissue are identical to the "vacuoles" of permanganate fixed tissue. If this is true one might expect to find transitional bodies in KMnO₄ fixed material which might illustrate the manner in which the spherosome-like bodies give rise to vacuole-like bodies. At the top right of the promycelium depicted in Figure 18a the three organelles marked i, ii, and iii suggest such a sequence. The arrow in Figure 18a also indicates a point at which a vacuole is possibly engulfing a smaller spherosome-like body.

In part I the "primary hydration vacuoles" seem to originate from dilations of the ER. Subsequent extension of the primary vacuoles during promycelial extension undoubtedly occurs through fusion of these with the spherosome-like organelles. The latter also seem to be derived in some manner from the ER. Thus the mature spore vacuoles inevitably contain several electron dense cores (Figs. 12 and 13). The widths of the ER membranes, the tonoplast, and the spherosomal membranes are almost identical (Average widths = 94 Å, 96 Å, and 90 Å respectively).

The vacuoles which develop in the promycelium are formed by expansion and fusion of the spherosomal bodies (Figs. 20a and 20c). Formation of vacuoles by the expansion and fusion
of smaller elements, has been suggested for a large number of fungi (Buckley et al., 1966; Hyde and Walkinshaw, 1966; Hawker and Abbott, 1963a; Hawker and Abbott, 1963c; Smith and Marchant, 1968). Such vacuoles are autolytic and often contain fragments of other cell organelles. In *Ustilago hordei* their lytic nature is also demonstrated through the observation that where their membranes have been damaged during fixation the surrounding cytoplasm is digested (Fig. 20a).

During fungal spore germination large vacuoles commonly develop, first in the emptying spore case and later at the base of the extending germ tube or promycelium (Hyde and Walkinshaw, 1966; Hawker and Abbott, 1963a). A similar effect has also been noted during the extension of pollen tubes (Jensen et al., 1968). That the expansion of vacuoles at the bases of such tube-like cells actually generates the forward motion of the cytoplasm which results in apical extension has been suggested by Buller (1933) and Corner (1948). The observations in *Ustilago hordei* support this hypothesis. First, the mass of the protoplast does not appear to increase significantly during germination and budding. Second although the promycelium ceases to extend apically in a manner which indicates that the "pressure" for forward movement has temporarily ceased, the subsequent production of buds occurs almost explosively in a matter of minutes. This suggests that some form of pressure is generated within the tube after it has ceased to extend. The promycelium is undergoing extensive vacuolation during this period. However, although this hypo-
thesis is tempting, vacuoles may, in some or all such systems, be the consequence, rather than the cause, of protoplasmic movement (Hyde and Walkinshaw, 1966; Robertson, 1968).

THE SPHEROSOMAL VACUOLAR SYSTEM

During this study the term "spherosome-like" has been applied somewhat arbitrarily to two groups of organelles: one group occurring in KMnO₄ fixed tissue, and the other group, with a quite different appearance and size range (i.e. in the promycelium), occurring in glutaraldehyde-osmium fixed tissue. This term has been applied for historical reasons rather than from adherence to any particular school of thought concerning the structure and function of these organelles. For many years light microscopists have referred to the motile, spherical, refractile droplets which are common in higher plants and fungi as spherosomes (Armentrout et al., 1968; Sorokin and Sorokin, 1966). Frey-Wyssling (1963) first described bodies similar to those found in KMnO₄ fixed spores and promycelium of Ustilago hordei and named them "spherosomes." However, according to the various structural and functional features which these organelles exhibit at various stages in development they might equally well have been called microbodies (Frederick et al., 1968; Mollenhauer et al., 1966), or cytosomes (Frederick and Newcomb, 1969), or lysosomes (Matile, 1968; Matilé and Spichiger 1968; Wilson et al., 1970). Wilson et al. (1970) have recently reviewed the usage and significance of these terms as applied to fungi and have justly pointed out that what is now required are exhaustive studies on the structure, distribution,
and biochemistry of one or more of these bodies in the same higher plant or fungus.

The literature pertaining to the function of these spherosome-like bodies is at best confusing. The confusion arises largely from the fact that they are common to two systems which seem diametrically opposed. On the one hand, they are very numerous in degenerating systems such as higher plant coleoptiles and free cell cultures (Cronshaw, 1964), and aging and dying fungal tissues (Wilson et al., 1970). On the other hand they are also numerous in plant meristematic and differentiating tissues (Frederick et al., 1963) and fungal tip cells (Wilson et al., 1970). From this observation the conspicuous presence of such organelles in the spores and promycelia of Ustilago hordei is not surprising. Such a system is not only a young differentiating system but, in another sense, an aging system.

Biochemically oriented studies have indicated that spherosome-like bodies in plants contain protein and more specifically hydrolytic enzymes. Most of these studies have been carried out in higher plant systems (Matile, 1969). Typical lysosomal hydrolases have been detected in spherosomes of tobacco (Balz, 1966; Matile and Spichiger, 1968) and of corn (Semadeni, 1967; Matile, 1968). Matile (1968) also found a transaminase in larger spherosomes of corn and two oxido-reductases in the smaller ones. Although there have been fewer studies of this type in fungi several histochemical studies have indicated that fungal spherosomes contain acid
phosphatases (Armentrout et al., 1968; Buckley et al., 1968). Wilson et al. (1970) surveyed the "spherosomes" of seven different fungi and concluded, on a purely cytological basis, that the activities and distribution of these organelles are compatible with their equivalence to animal lysosomes. The yeast cell does not contain spherosomes and what has been classically referred to as the "vacuole" has the appearance of being the lysosomal equivalent. A number of studies have indicated that in other fungi, bodies similar to the spherosome-like bodies of *Ustilago hordei* also contain lipid (Allen et al., 1971; Matile and Spichiger, 1968; McKeen, 1970), and more particularly phospholipid (Buckley et al., 1966; Sorokin and Sorokin, 1966). Frey-Wyssling et al. (1963) and Semadeni (1967) have suggested that spherosomes give rise to lipid bodies in higher plants. Gay and Greenwood (1964) and Buckley et al. (1968) have associated fungal spherosomes with the mobilization of lipid to form membranes. In addition to the above functions vesicles which resemble the smaller "spherosomes" of *Ustilago hordei* seem to be involved in hyphal tip extension in *Pythium ultimum* (Grove et al., 1970); it has been suggested that they may also transport the enzymes and other materials required for wall synthesis and wall plasticization. The multiplicity of functions in which fungal spherosomes are tentatively implicated is at first disturbing; however, obviously in organisms as cytologically "simple" as most fungi, some of the organelles must perform more than one function.
In *Ustilago hordei* the structure and function of the organelles which we have termed spherosome-like bodies and vacuoles suggest that these structures actually represent stages in the differentiation and development of a single spherosomal-vacuolar system. The differences in the structure of these organelles after various methods of fixation, and the changes in their appearance and distribution during germination can be accommodated by the following hypothesis.

1. The spherosome-like bodies in *Ustilago hordei* contain proteins and lipids (perhaps in the form of a lipoprotein complex). The proteins are, at least in part, enzymes.

2. The permanganate-type spherosome-like bodies (Diameter = 0.11 - 0.56 μ) are identical to the small sized glutaraldehyde-osmium fixed ones (Diameter = 0.24 - 0.86μ). The vacuoles observed in promycelia fixed by method A are the permanganate equivalent of the larger glutaraldehyde-osmium fixed spherosome-like bodies (Diameter more than 1 μ).

3. Either the contents of the spherosome-like bodies change throughout development or else different contents are activated at different points in time. Consequently these organelles are involved in a variety of activities, some of which may be autolytic, synthetic or both. Simultaneously controlled alteration in the contents and localization of these bodies have profound effect on the differentiation of the organism.
4. Alteration in the structure of the cytologically observable contents can be correlated with changes in enzyme activity.

These four points are elaborated in the following discussion.

As has been previously indicated, ample evidence exists in the literature to support the concept of a lipid-protein matrix in spherosome-like bodies (Sorokin and Sorokin, 1966). That the "spherosomes" of *Ustilago hordei* contain lipid, at least in some stages of their development, is evident from the fact they often contain pseudomyelin-like figures (Fig. 11). Buckley et al. (1966) and Gay and Greenwood (1966) described such figures in the "spherosomes" of other fungi and interpreted them as a stage in the mobilization of lipid for membrane synthesis. Figures 18b and 19 illustrate more clearly that spherosome-like organelles in *Ustilago hordei* can evidently give rise to membranes which are spontaneously blebbed off in the form of vesicles in the direction of the plasmalemma. These membranes appear to have a regular tripartite structure and their width is similar to the width of the plasma membrane. During a recent study in which a freeze-etch technique was applied to the dormant teliospores of the smut *Tilletia caries* Allen et al. (1971) described a body (i.e. unidentified organelle) which resembles the spherosome-like bodies of *Ustilago hordei*. Lipid was demonstrated to be present in these organelles. As no cytochemistry has been attempted, the presence of protein has been inferred first from the apparently hydrolytic (and therefore enzymatic) activities.
of these bodies and secondly from known reactions of the differ­
ent preparatory techniques on various biochemical components.
The final identification of the contents of the spherosome-
like bodies will depend upon the use of appropriate biochemical
or histochemical techniques.

One of the problems in the literature on "spherosomes"
is that the bodies which are usually designated as spherosomes
in permanganate fixed material are quite different in appear­
ance from those so designated in glutaraldehyde-osmium fixed
material. Unfortunately few worker have simultaneously em­
ployed both techniques, causing doubt that the conclusions
drawn from different studies actually apply to the same organ­
elles. The following factors suggest that in Ustilago hordei
the bodies labelled spherosome-like after the different pre­
paratory methods are the same:

1. The appearance of these bodies after methods A, B,
and C is compatible with the expected effects of each
technique on a lipid-protein containing body.
2. Their numbers, distribution, and size range in hy­
drating spores is the same (Pt. I).
3. They cluster in an identical manner to one side of
the developing beak in germinating spores.
4. The alteration in number, size, and appearance in
promycelia is compatible with the hypothesis that the
permanganate-type spherosomal body is identical with
the small-sized glutaraldehyde-osmium ones and that the
permanganate-type vacuoles which develop in aging pro-
mycelia are identical with the large-sized glutaraldehyde-osmium spherosome-like bodies which also develop in aging promycelia.

Assuming that these various manifestations actually represent stages in the differentiation of a single system the activities in which the system has so far been implicated can be summarized, in the order of occurrence, as follows:

1. degradation of lipid bodies during pre-germinal development (Pt. I, Fig. 8.)
2. spore wall degradation and possible synthesis of the new promycelial wall (Figs. 1 and 2).
3. cytoplasmic degradation occurring outside the ER-sac in a germinating spore (Fig. 8).
4. promycelial wall plasticization and possibly synthesis.
5. formation of "vacuoles" by organelle expansion and/or fusion.

All of these activities seem to involve a hydrolytic component and on this basis it is tempting to suggest that *Ustilago hordei* the spherosomal-vacuolar system is also the lysosomal system. The actual identification of these organelles as lysosomal equivalents awaits future biochemical and histochemical evidence. Functions 1, 2, 3, and 4, mainly involve the smaller class of these bodies (i.e. those which appear as spherosome-like after KMnO₄ fixation); function 5 is confined to the larger class (i.e. those which appear as vacuole-like after KMnO₄). An interesting observation is that during studies on the lysosomes of root tip cells of corn
seedlings Matile (1968) has identified two classes of lysosomes which correspond in size-range and activities to the two classes of spherosome-like bodies discussed in Ustilago hordei. The heavy lysosomes which contain hydrolases, transaminases, and oxidoreductases are small spheres 0.1 to 0.3 μ in diameter, with membranes resembling the ER membranes. The "light lysosomes" or "small vacuoles" which contain hydrolases and one transaminase range in size from 0.3 to 1.5 μ in diameter.

The contents of the spherosome-like bodies condense into dense cores, thick fibrils (i.e. in pre-germinál spores), or fine fibrils. The contents of the large "spherosome" posterior to the apex of the newly formed promycelium in Figure 6 are of the fibrillar type. The larger spherosomes have dense cores, but they also contain a higher proportion of fibrillar material than the smaller ones. In fusing spherosomal bodies (Fig. 20) and large vacuoles (Figs. 12, 13 and 14) the contents are usually also present. The cytologically fibrillar state may reflect the biochemically state of the enzymatic contents.

**CONCLUSION**

Initiation of promycelial development in Ustilago hordei involves the controlled integration of three separate processes: localized degradation of the spore wall, swelling of the protoplast, and synthesis of a new layer of wall material. Further extension of the promycelium requires a balanced system of wall plasticization, wall accretion, and plasma membrane
synthesis in the region of the apex. No cytological evidence has been obtained in this organism to indicate what the structural basis for this system might be.

During the promycelium extension most of the cytoplasm flows into the tube; the remaining spore cytoplasm becomes highly vacuolate. The endoplasmic reticulum, mitochondria and lipid bodies are distributed randomly and do not increase noticeably in total quantity. One of the major functions of the ER in hydrating and germinating spores seems to be the formation, directly or indirectly, of bodies satisfying the classical definition of the fungus vacuole. In part I, a suggestion was made that the "primary hydration vacuole" is formed directly via dilation of the ER intermembrane space. Subsequent "vacuole" formation occurs by the expansion and fusion of smaller "spherosome-like" organelles, which also may be derived in some manner from the endoplasmic reticulum. During the transition from the spherosome-like state to the vacuole-like state these bodies evidently perform a number of functions all of which involve a hydrolytic component, and all of which have profound effects on the differentiation of the organism. The cytological evidence is compatible with the hypothesis that the spherosomal-vacuolar system in Ustilago hordei is the functional equivalent of the lysosomal system in animal cells (DeDuve and Wattiaux, 1966). However, as some evidence indicates that fungal spherosome-like bodies may perform anabolic functions (i.e. wall synthesis) as well as catabolic the adoption of a more "restrictive" nomenclature
does not seem to be justified at present.
II. PLATE 1

Figure 1. A general view of a teliospore at germ tube initiation. Note the large perforations (p) in the inner spore wall opposite the protoplastic beak. The new promycelial wall (pW) has begun to form over the beak. ER and spherosome-like bodies (S) cluster in the beak region. The protoplast contains many large matochondrial and primary vacuoles. Note that the protoplast has pulled away from the inner spore wall over most of the surface except in the beak region. Method A. ca. X 25,2000.

Figure 2. The beak at the earliest stages of germ tube initiation. The first promycelial wall (pW) material is present at the extreme tip of the beak. Large perforations (p) with fibrillar electron dense contents are already present on the inner spore wall opposite the beak. Note the characteristic presence of the spherosome-like bodies (S). Method C. ca. X 44,600.
II. PLATE 2

Figure 3. A general view of a teliospore at a slightly more advanced stage of promycelium formation. Note the presence of the continuous ER-sac with its associated spherosome-like bodies (S) and knots of endoplasmic reticulum (arrows). Most of lipid bodies (L) vacuoles (V) and mitochondria lie inside of the sac. Method A. ca. X 19,000.

Figure 4a. The beak region just prior to spore wall rupture. Note that the thin layer of new wall material (pW) completely surrounds the beak. Also note the extensive perforations (p) in the inner spore wall opposite the beak and the indications of stress in the outer spore wall layers opposite the beak. Peripheral ER elements are present. The diploid nucleus (dN) has moved into position behind the beak. Method B. ca. X 22,500.

Figure 4b. A thick section prepared by method A and stained in Toluidine blue. Note the characteristic position of the nucleus. ca. X 4,400.

Figure 5. Migration of the diploid nucleus (dN) and mitochondria (M) into the young promycelium. Note the elongate forms. Also note the characteristic positions of the nuclealus (Nu) and the centriolar-kinetochore-equivalent (CKE). The promycelial wall (pW) extends through the neck of the ruptured spore wall but does not completely encase the spore. Method B. ca. X 17,800.
II. PLATE 3

Figure 6. Spore wall rupture. A well-formed wall layer (pW) is present about the new promycelial tip. Note the "exploded" spore wall debris. Several large spherosome-like bodies (S) containing electron dense cores and fine fibrillar material are present just behind the promycelial apex. Lipid (L) and the inner spore wall perforations (p) are indicated. Method C. ca. X 29,750.

Figure 7. Migration into the promycelium. Note the large spore vacuole (V), the elongated mitochondria (M) and the presence of microtubules (mt). The promycelial wall (pW) and a young spherosome-like body are also indicated. Method C. ca. X 35,550.
II. PLATE 4

Figure 8. Part of a germinating spore showing the fusion of ER elements to form the ER-sac and showing the relationship of the sac membranes to the spherosome-like bodies (S) and vacuoles (V). Method A. ca. X 35,700.

Figure 9. A section through a promycelium illustrating the positional relationship between smooth ER and the young spherosome-like bodies (arrow) and the smooth ER and the mature spherosome-like bodies (S). Method C. ca. X 35,700.

Figure 10. A longitudinal section through a part of a promycelium showing a diploid nucleus (dN), spherosome-like bodies (S), lipid bodies (L), and mitochondria (M), and a large number of unidentified vesicles. Method C. ca. X 23,700.
II. PLATE 5

Figure 11. Large spherosome-like bodies with dense cores and pseudomyelinate figures (arrow) in an aging germinated spore. Method C. ca. X 29,475.

Figure 12. Large vacuole (V) containing multiple electron dense cores and large quantities of electron dense fibrillar material in an aging germinated spore. Method C. ca. X 15,750.

Figure 13. Large spore vacuole (V) containing electron dense fibrillar material and large electron transparent patches. Free spherosome-like bodies (S) are numerous in the vicinity of the vacuole. Method A. ca. X 19,950.

Figure 14a. Basal cell of a four-celled promycelium showing the presence of a large vacuole (V) with electron dense fibrillar contents. Method C. ca. X 14,000.

Figure 14b. A living two-celled promycelium viewed with phase optics. Note the presence of the basal vacuoles and the refractile appearance of the spherosome-like bodies. ca. X 2,500.
II. PLATE 6

Figure 15. A longitudinal section through a promycelium showing stacked ER lying near to and in parallel with the promycelial wall. Vesicles (ve) are often located near such ER stacks. Method C. ca. X 48,000.

Figure 16a. A longitudinal section through a one-celled promycelium in which many pores can be seen opening from the protoplast through the cell wall to the outside. Method C. ca. X 32,750.

Figure 16b. An enlarged view of one of the pores depicted in Figure 16a. Method C. ca. X 52,400.

Figure 16c. Two other pores from the same promycelium as in 16a. Method C. ca. X 52,400.

Figure 17. A haploid nucleus (hN) within the spore after germination. The arrow indicated the chromatin-nucleolar connection. Note that on the side of the connection opposite the nucleolus the chromatin strands are clearly visible. A short segment of rough ER lies close to the nuclear envelope. Method C. ca. X 53,550.
II. PLATE 7

Figure 18a. A general view of one of the cells in a four-celled promycelium. Note the haploid nucleus (hN) with simple nuclear pores (NP) and the mitochondria. ER and lipid (L) is scant. On the right a spherosome-like body (S) is being engulfed by a large vacuolar-structure (V). The bodies on the upper right labelled i, ii, and iii suggest a possible sequence of conversion of spherosome-like bodies (i) to vacuole-like bodies (iii). Vesicles (ve) are often conspicuous between the vacuoles (V) or the spherosome-like bodies (S) and the cell wall. Method A. ca. X 53,500.

Figure 18b. An enlarged view of one of the spherosome-like bodies (S) in Figure 18a which seems to be giving rise to vesicles which pass towards the septum. Method A, ca. X 95,200.

Figure 19. Part of a promycelium showing membrane formation within a spherosome-like body and the blebbing of vesicles (ve) from the spherosome-like body towards the cell wall. Short segments of rough ER (arrow) are present near the haploid nucleus (hN). ER is often also associated with the spherosome-like bodies (i.e., lower right). Method B. ca. X 41,000.
II. PLATE 8

Figure 20a. A longitudinal section through a single-celled promycelium showing the appearance and distribution of the mitochondria (M), lipid bodies (L) and spherosome-like bodies (S). Method C. ca. X 13,800.

Figure 20b. The apex of the promycelium depicted in Figure 20a. Note the lack of cytologically distinct structures in the extreme tip. Method C. ca. X 32,200.

Figure 20c. Fusion of two spherosome-like bodies to form a large vacuolar structure. Note the two electron dense cores and the large amount of fibillar material in these organelles (s). Part of the bounding membranes between the two fusing organelles (s) is still present. Method C. ca X 32,200.
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PART III

Initiation of the Promycelium and Promycelial Extension

ABSTRACT

In the promycelia of Ustilago hordei cross walls are complete. The pattern of septation resembles that found in actinomycetes, in Coccioidoides immitis, and in Exidia nucleata. Elaborate membrane complexes have been associated with the initiation of the cross wall. Two variations of the normal septation pattern are described and the results of these variations are discussed.

The initiation of sporidia involves a localized plasticization of the promycelial wall followed by degradation of the old wall and subsequent synthesis of new wall material. At present no information is available to indicate the manner in which the mature sporidium is separated from the parent cell.

INTRODUCTION

When the promycelium of Ustilago hordei has almost attained its maximum length the diploid nucleus undergoes meiosis. The first nuclear division (i.e. reduction division) is followed immediately by the formation of a single septum giving rise to a two-celled promycelium. Each of the haploid nuclei again divides (i.e. equational division) and a second round of cell division occurs with the almost
simultaneous establishment of two more septa. Once the four-celled state has been attained each cell gives rise to an additional daughter cell formed by a process of yeast-like budding. During production of the bud (i.e. sporidium or basidiospore) the parent cell nucleus divides again, mitotically, one of the daughter nuclei becoming situated in the sporidium and the other in the parent cell. This study is concerned with the ultrastructural cytoplasmic events which occur during septation and budding.

A sporidium is of one of two mating types designated $+\,$ and $-\,$. Generally a promycelium gives rise to equal numbers of each type. The infective dikaryon in *Ustilago hordei* is usually formed by the fusion of two sporidia of opposite mating type in the presence of a suitable host. However, among smuts an alternative mechanism exists whereby a dikaryon may be established (Dickinson, 1927; Duran and Safeeulla, 1965). A cytoplasmic bridge forms directly between a plus and a minus promycelial cell. In this particular smut species such bridges only form between immediately adjacent cells, and are often referred to as "knee-joints". The apex of the bridge then assumes the properties of a hyphal apex extending to produce a branch. The undivided nuclei of the confluent cells migrate into the branch region to establish a dikaryon. During this study the ultrastructural basis of bridge formation has been established.
MATERIALS AND METHODS

The materials and techniques are identical to those previously described (Pts. I and II), the tissue being fixed after 5 to 7½ hours of incubation. The promycelium in Figure 10 was fixed for 12 hours in 2.0% glutaraldehyde buffered at pH 7.2 with 0.01 M cacodylate buffer and observed directly using phase contrast optics as described in part II.

OBSERVATIONS

SEPTATION:

In Ustilago hordel a simple invagination of the plasma membrane initiates septum formation (Fig. 1a, arrows). Figure 1b clearly demonstrates the unit membrane delimiting the septum initial. Throughout development of the septum this membrane seems to be somewhat narrower (Average width = 100 Å) than the plasma membrane lining the lateral promycelial walls or the mature septum (Average width = 124 Å). At the frontal edge of the early invagination the membranes are closely appressed for a distance of 50 to 100 μm and then are separated by an electron transparent lamella 4 to 10 μm in diameter which merges with the lateral wall. An electron dense amorphous substance surrounds the advancing edge from its earliest inception (Fig. 1a, upper right).

Ingrowth of the septum initial occurs by what is commonly known as "centripetal invagination", somewhat after the fashion of a closing iris diaphragm. Amorphous electron dense material continues to encase the advancing edge (Fig. 3)
and once the membranes have met and fused centrally remnants of this substance can be seen lying beside the fused plate (Fig. 4). The septa within the promycelia of *Ustilago hordei* are complete (i.e. septal pores are absent).

Just prior to, or during, plate completion synthesis of the septal wall begins at the lateral edges and is formed in such a way as to be continuous with the inner layers of the promycelial wall. In appearance they are identical. The new wall material is laid down simultaneously all around the inside edge of the invaginated plasma membrane, thus forming a tube of wall material inside the invagination. The electron transparent lamella within the tube is not occluded. During septal development it retains the constant diameter (4 - 10 μm) first defined by the septum initial. Once the plate is complete this central region is continuous throughout the cross wall. Subsequent thickening of the wall at any point results from the accretion of new material along the plasma membrane on either side of the plate. Soon after plate completion, synthesis of the septal wall material spreads rapidly along the inner surface (Fig. 4). From this point on new material must be deposited synchronously throughout the septum until it has attained its maximum thickness (130 - 150 μm). In cross-section the total width of the cross wall is relatively constant across the promycelium, (with the exception of the triangular thickening (Bracker and Butler, 1963) at the extreme lateral edges. The thickness of the material on either side of the central
lamella is equal. Figures 4, 5 and 6 demonstrate sequentially the thickening of the septal wall.

In *Ustilago hordei* the plasma membrane of the invaginating septum initial is frequently extended into an elaborate complex (Figs. 2, 8, and 9). Although serial sections across an entire promycelium have not been obtained such complexes always seem to lie against one of the lateral walls (Figs. 2 and 7), and there is apparently at least one complex per septum. They are visible after both glutaraldehyde-osmium and permanganate fixations (Figs. 2, 8, 8, and 9, and Fig. 7, respectively). With the light microscope prominent bodies of similar size and shape occur in association with septa in glutaraldehyde-fixed material (Fig. 10). In living tissue conspicuous stationary refractile bodies clearly indicate the point at which a septum will later be visible (Introduction, Figs. li-j). The ultimate fate of the septum-associated membrane complexes is unknown but there is some evidence that they normally degenerate once the septal wall material begins to form. Not all the membrane complexes in the promycelium are associated with the septum (Fig. 7) and more will be said about the relationship of these unusual structures to other cell organelles in part V.

Figure 9 clearly demonstrates the unit structure of the membranes composing the complex. These membranes have an average width of 96Å (Range: 60 - 140 Å). After glutaraldehyde-osmium fixation they are arranged in concentric whorls (Fig. 2) or folds (Figs. 8 and 9) but after KMnO₄-fixation they follow a more random, but still compact, orientation (Fig. 7).
Aside from the complexes no other organelles are conspicuously associated with initiation or thickening of the septum. Spherosome-like bodies and lipid bodies often occur in the vicinity of the cross-walls but their occurrence does not seem to be any more frequent than in other parts of the cytoplasm. Short segments of endoplasmic reticulum sometimes lie just beneath and parallel with the plasma membrane of a well-developed septum in much the same fashion that the ER borders on the lateral walls (Pt. II). Sometimes the plasma-lemma is relatively smooth (Fig. 6) and sometimes it is quite crenulate even around mature septa which have presumably attained their maximum thickness (Figs. 15 and 16).

Two variations to the normal septation pattern have been observed. The first, depicted in Figure 1a, is a definitely abnormal event in which twin septa are laid down side by side. The second variation is involved in the production of "knee-joints". This latter phenomenon is initiated by the plasticization of the lateral wall over a localized area on both sides of and adjacent to a septum. The protoplasts of the two cells involved are then "blown out" as two bulbous extensions in a manner similar to the normal budding process. As the protoplasts bulge the lateral promycelial wall is extended around the outer surface. On the inner surface where the two expanding regions are growing together the septal wall resumes growth unidirectionally and so continues to separate the two protoplasts (Fig. 11). Spherosome-like bodies and long elements of endoplasmic
reticulum are very numerous and prominent on both sides of the bulbous bridge-initial during this early period of formation. What then seems to occur might best be described as a metabolic race between those elements engaged in synthesizing the walls separating the two cells and another set of elements engaged in breaking down the partition (Fig. 12). In Figure 12 a portion of the wall can still be seen between the two bulbous cell extensions while the regions on either side of it have become electron transparent and contain cell debris and vesicles which seem to be derived from the ER. Finally the degradatory elements pre-dominate and the partitioning wall in the bulbous region is eliminated allowing the protoplasts to fuse completely (Fig. 13). The plasma membrane reforms in some manner about the free end of the remaining portion of the septum. The net result is an incomplete septum partially separating two cells which are now joined by a cytoplasmic bridge. In Figure 13 the nucleus of one cell has moved into a position preparatory to entering the bridge region.

SPORIDIUM FORMATION:

Under the light microscope the first sign that a sporidium is about to form is the appearance of a bright refractile spot on the surface of the promycelium. After five to ten minutes the wall begins to bulge at this point, and to extend rapidly. During the first phase of elongation the bud is narrow, but after several milli-microns of growth (i.e. as measured under the electron microscope it undergoes a spontaneous ballooning of the diameter to give rise to the elongate ovoid form of the mature sporidium
(Introduction, Figs. 9 - 15). The total development of the bud from the appearance of the refractile spot to the mature sporidium requires 20 to 40 minutes.

Under the electron microscope the first sign that a bud is about to form is the disappearance of wall material from a small region of the lateral promycelial surface. The actual disappearance of the wall substance is preceded by a change in its structure. The semi-electron dense, fibrillar wall thickens and becomes more electron-translucent and apparently homogeneous, with the exception of the thin electron dense line which has been shown to separate the wall into two layers. In Figure 14 a short wallless zone is shown to be bounded by altered wall material which is, no doubt, in the process of being degraded, and the protoplast has already begun to bulge in the direction of the wallless region. Long elements of endoplasmic reticulum lying just beneath and parallel to the lateral wall seem to end in the vicinity of the electron translucent area.

Figures 15, 16, and 17 illustrate sequentially the development of a sporidium. In Figure 15 a thin amorphous layer of material covers the bud initial, and the fractured edges of the old promycelial wall are clearly visible. Beneath the new layer of wall material a large spherosome-like body is conspicuously present. Since the lateral wall of the adjacent cell on the other side of the septum also seems to be thinner than the usual promycelial wall the cells depicted in Figure 15 may actually represent a stage in bridge-formation rather than budding. Early in this devel-
opmental stage it is impossible to predict what course of
differentiation the cells will follow. As the bud increases
in size part of the protoplast of the parent cell moves into
it. The parental nucleus divides, mitotically, in the promy­
celium, in the bud, or in the neck between the two and the
daughter nuclei separate in such a way that the parent cell
and the basidiospore obtain one nucleus each. As was noted
during the passage of the diploid nucleus up the promycelium
(Pt. II) the nucleolus always lies at the posterior end of
a migrating nucleus. The mature sporidium (Fig. 18), then,
contains a single haploid nucleus, a number of mitochondria,
lipid bodies, and spherosome-like bodies, plus a small amount
of endoplasmic reticulum and unidentified vesicles. When the
bud is fully formed it is cut off from the mother cell to be­
come an autonomous individual. No evidence has been obtained
to indicate the manner in which this separation occurs or in
which the proximal end wall of the sporidium is completed.

DISCUSSION

SEPTATION:

The hyphal cells of most higher fungi maintain some sort
of cytoplasmic continuity with their neighbours by virtue of
one or more pores in the cross-walls separating the cells.
In general, the pores of the ascomycetes are represented by
"simple holes" while the pores of the basidiomycetes are
characterized by elaborate structures known as the "dolipore­
parenthosome" (Moore and McAlear, 1962; Bracker, 1967). A
major exception to this rule occurs among the heterobasidio-
mycetes (Bracker, 1967; Ehrlich et al., 1968). In this
group dolipore-septa have been found among the Tremellales
(Moore, 1965; Moore and McAlear, 1962; Wells, 1964), but
simple pores among the Uredinales (Ehrlich et al., 1968;
Manocha and Shaw 1967, Moore, 1963, Moore, 1965). It is
commonly accepted that dolipore-septa do not occur in the
Ustilaginales (Moore, 1965; Bracker, 1967; Ehrlich et al.,
1968) although to the best of the author's knowledge there
is only one report on smut septa in existence (Stein, 1970).
Complete septa, such as those observed in the promycelium
of Ustilago hordei, have occasionally been noted in phyco-
mycetes, hemiascomycetes, and deuteromycetes (Bracker, 1967)
as well as in a mycelial mutant of Ustilago hordei (Stein,
1970) and in a basidiomycete, Cryptococcus neoformis (Cutler
and Erke, 1971). Septa without pores, are often associated
with specialized situations such as sealing off injured or
evacuated cells (Wells, 1964) and delimiting reproductive
structures (Hawker and Gooday, 1967). The four-celled
metabasidium of Ustilago hordei constitutes a highly special-
ized system in which each of the cells contains a different
set of genetic information (i.e. as a result of meiosis). Of
course the cytoplasm of these cells is derived from a single
protoplast, and it is unknown how long it takes for the new
nucleus to exert its effect on the individual cell, but cer-
tainly an influence is manifest by the time of bridge forma-
tion and sporidial production. However, since complete septa
also occur in a mycelial mutant of this fungus (Stein, 1970)
the possibility exists that such septa may be a characteristic form in this species.

Moore (1965) described three patterns of somatic cell division in mycota. The mature septum of *Ustilago hordei* is of classifications type B, represented by *Streptomyces violaceoruber* and *Coccidioides immitis*. It consists of two layers of septal wall (i.e., two plates) each of which is continuous with the inner lateral wall and an electron transparent lamella lying between the two plates (Figs. 5, 6, 15 and 16). Cross walls of similar structure occur among the phycomycetes (Akai et al., 1968; Hawker and Gooday, 1967), the ascomycetes (Brenner and Carroll, 1968; Kreger and Veehuis, 1969; Moore, 1962), the basidiomycetes (Bracker and Butler, 1963; Jersild et al., 1967; O'Hern and Henry, 1956; Wells, 1964), and a number of imperfect human pathogens (Carbonelle and Rodriguez, 1968). They also occur among the actinomycetes (Glouert and Hopwood, 1961; Moore, 1965). The dimensions of the mature septa of *Ustilago hordei* are almost identical to those given for the cross-walls of *Rhizoctonia solani* (Bracker and Butler, 1963). In the former the newly-formed cross wall width is 6 to 10 μm (Fig. 4), the mature cross wall width is 130 to 150 μm. (Figures 6, 13, 15, and 16), and the diameter of the electron transparent lamella is 4 to 10 μm (Figs. 4, 5, and 6). In the latter the respective measurements are 7 to 8 μm, 120 μm, and 10 μm. Wells (1964b) has noted that the septa which cleave the hypobasidium of *Exidia nucleata* (a heterobasidiomycete) into four hypobasidial segments have a similar structure.
During septum formation in this smut fungus the plasma-lemma invaginates first, and septal wall material is not laid down until the membranes have fused, or have almost fused across the centre of the promycelium. This mode of formation most closely resembles that of *Coccidioides immitis* (O'Hern and Henry, 1956), certain species of *Streptomyces* (Moore, 1965), and some bacteria (Chapman, 1959), all of which form type B septa according to Moore's classification. All of these species also form complete septa. Moore (1963) and Wells (1964b) observed a similar mechanism of complete septation during thallus formation in the aecia of *Puccinia podophylli* and during hypobasidium segmentation in *Exidia nucleata*, respectively. The fact that a similar structure and developmental pattern have been observed for complete cross-walls in a rust (Moore, 1963), a tremellaceous fungus (Wells, 1963b) and a smut suggests that this form of septation may be common among heterobasidiomycetes. In most other cases where septal development has been observed (ie. exception—Hawker and Gooday, 1967) the synthesis of wall material keeps pace with the extension of the plasma membrane so that the septum initial, at all stages is a wedge of wall material closely surrounded by membrane rather than a flat membranous plate (Brenner and Carroll, 1968; Campbell, 1969; Conti and Naylor, 1959; Manocha and Shaw, 1967; Marchant and Smith, 1968; Moore, 1962; Wells, 1964).

The advancing lip of the septum initial of *Ustilago hordei* is closely associated with a rim of electron dense
material (Figs. 3 and 1a). A similar substance surrounds the advancing edge or the pore rim of the cross walls of Ascodemis sphaerospora (Brenner and Carroll, 1968), and Sordaria fimbicola (Furtado, 1971). In both the latter studies endoplasmic reticulum is associated with septum formation and/or the electron dense substance. Hawker and Gooday (1967) have suggested that the ER surrounding the septum initial of Rhizopus sexualis (Smith) Callen gives rise to vesicles which contain the new wall material, and which release this material by fusing with the septal membrane. Lomasomes may also be involved in the formation of cross walls (Brenner and Carroll, 1968; Hawker and Gooday, 1967). The source of septal wall material in Ustilago hordei is as obscure as the mechanism of apical extension (Pt. II). As noted by Wells for Exidia nucleata (1964b), vesicles, endoplasmic reticulum, and lomasomes are not conspicuously associated with the invaginating membrane or thickening plate.

In Ustilago hordei large membrane complexes are often associated with the invaginating septum initial. What are now required are serial sections through promycelia which are under-going cell division. Hopefully such studies will prove or disprove the hypothesis that there is always one or more of these structures present in the region of cell division. According to the current data a septal membrane complex is an "extension" of the invaginating plasma membrane which lies against one of the lateral germ tube walls (Figs. 2 and 8). Whether or not there is any relationship
between these complexes and the knots of membrane which form part of the ER-sac in a germinating cell (Pt. II) is unknown at present, but the former seems to be associated with the plasma membrane, and the latter with the endoplasmic reticulum. A number of researchers have described similar membrane complexes among fungi. In the basidiomycetes Armillaria meleae (Berliner and Duff), Coprinus micaceous (Edwards, 1969), Coprinus lagopus (Lu, 1965; Lu, 1966), and Lycoperdon perlatum (Marchant, 1969), the ascomycetes Aspergillus fumiculus (Edwards, 1969), Neurospora tetrasperma (Lowry and Sussman, 1968), and Neurospora crassa (Kozar and Weijer, 1969), and the imperfect fungi Paracoccidioides lobo (Furtado et al. 1967) and Verticillium dahliae (Griffiths, 1970) the respective workers have not noted any relationship of the complexes to the septum. The specific association of similar membrane systems with septa occurs in the imperfect fungi Paracoccidioides brasiliensis and Blastomyces dermatitidis (Carbonelle, 1967, and Carbonelle and Rodriguez, 1968) and in the basidiomycete Lenzites saepiaria (Hyde and Walkinshaw, 1966). Membrane systems derived from the plasma membrane have long been associated with septum formation among the actinomycetes (Edwards, 1970; Ellar et al., 1967; Glauert and Hopwood, 1960; Imaida and Ogura, 1963) where such structures have usually been referred to as "mesosomes" because of the striking structural similarity to the bacterial organelles of that name. One of the postulated functions of the bacterial mesosome is to assist in septum formation (Rogers, 1970; Ryter, 1968).
The membrane complex-cross wall association has suggested a number of possible functions for these elaborate membrane systems: synthesis of septal wall material between the cell and its environment during periods of increased metabolic activity (Carbonelle, 1967), and initiation of cell division (Edwards, 1970). In *Ustilago hordei* it is unlikely that the complex acts directly in deposition of the wall material since such systems are most prominent in the vicinity of the invaginating septal initial before wall thickening has begun. However, one of the ways in which a membrane complex might initiate septum formation would be to provide a localized source of pre-synthesized membrane which would then be incorporated into the developing membranous plate. This hypothesis is supported by the fact that the membrane of the septal initial is continuous with that of the complex and by the fact that the width of the invagination membrane (approximately 100Å) is closer to the mean width of the unit membranes of the complex (96Å) than to the mean width of the plasmalemma on the sides of the promycelium (124Å). Berliner and Duff (1965), and Lu (1965 and 1966) have also postulated that similar membrane complexes may act as membrane generators in connection with other activities in fungi. Further discussion of the smut membrane complex, its origin and possible functions will be reserved until part V.

The literature pertaining to membrane complexes in fungi has frequently been confused by the failure to distinguish these structures from artefacts produced in tissue
by glutaraldehyde fixation (Fawcett and Susuma, 1958; Ohal and Rohlich, 1966; Palade and Claude, 1949a; Palade and Claude, 1949b; Revel et al., 1958). The fact that stationary bodies of similar size to the membrane complexes can be observed in living germ tubes at points where septa will subsequently form indicates that such structures are not fixation artefacts. However, this does not rule out the possibility that these membranous whorls are formed by hyperactive membrane synthesis which results from growth in a rich medium. A comparison should be made with metabasidia produced in minimal medium or distilled water. Fungal membrane complexes have also been confused with pseudo-myelin figures which are frequently observed in vacuoles or lysosomes (Smith and Marchant, 1968; Thomas and Isaac, 1967). Intravacuolar pseudomyelinate figures also occur in Ustilago hordei (Pt. II); however, the membrane complexes per se are clearly not associated in any way with lysosome-like structures.

Two variations on the normal septation pattern have been observed. The formation of twin septa results in a promycelium with five compartments one of which contains no nucleus and rapidly becomes vacuolated. Knee-joints result from the partial degradation of a mature, complete septum and the formation of a cytoplasmic bridge between two cells of opposite mating type (♀ and ♂). These appear to be similar in structure and probably also in origin to the pseudo-septa, or incomplete transverse septa de-
scribed in other basidiomycetes (Ehrlich et al., 1968; Jersild et al., 1967; Koltin and Flexer, 1969). In part II long elements of endoplasmic reticulum and spherosome-like bodies were associated with wall synthesis and degradation in the region surrounding the beak during germination. Interestingly, a similar association exists in the region of the bridge formation (Figs. 11 and 12). Vesicles which seem to be derived from the ER accumulate in the region of degradation (Fig. 12) suggesting that the ER is more likely involved in catabolic activities (i.e. wall degradation) than in anabolic ones (i.e. wall synthesis).

SPORIDIUM FORMATION:

Bartnicki-Garcia and Lippman (1969) hypothesized that bud formation in dimorphic fungi might be based on localized resumption of growth of the parent cell, followed by a uniformly dispersed pattern of wall synthesis in the bud. This would result in spherical, yeast-like daughter cells as opposed to apical hyphal extension. The observations in *Ustilago hordei* at least support the first part of this hypothesis. The initial thickening of the parent wall material which occurs in this fungus prior to the production of a bud has been observed during budding of the dimorphic fungi, *Paracoccidioides brasiliensis* and * Blastomyces dermatitidis* (Carbonelle, 1967; Carbonelle and Rodriguez, 1968), as well as several species of *Histoplasma* (Edwards et al., 1959). However, in *Ustilago hordei* the thickened wall region is more electron translucent than
the mature promycelial wall, while in the human pathogens
the thickened region is more electron dense. The increased
homogeneity and decreased electron density in the former
is presumed to indicate an increase in plasticity. Un-
doubtedly the refractile spot which appears in the light
microscope before a sporidium becomes visible represents
this period of wall plasticization and the subsequent period
of wall degradation.

In the promycelium of *Ustilago hordei*, a localized
portion of the lateral wall is evidently completely re-
moved before the new bud wall is formed (Fig. 14). As
the new material thickens about the neck it again becomes
structurally continuous with the promycelial wall. This
mechanism of bud formation varies from the mechanisms
previously described. The bud wall in *Saccharomyces
cerevisiae* is a direct continuation of the parental cell
wall (Marchant and Smith, 1968; McClary and Bowers, 1965;
Moor, 1967). A similar mode of bud initiation has also
been suggested in several species of *Histoplasma* (Edwards
et al., 1959). Sporobolomyces-type budding involves the
rupture of an outer wall layer—the bud wall being an
extension of an inner wall layer of the parent (Calonge,
1969; Prusso and Wells, 1967). In *Rhodotorula glutinis*
a new wall layer is synthesized beneath the old and dur-
ing budding the parental wall layer ruptures, the bud
being encased by completely new material (Marchant and
Smith, 1967). Rhodotorula-type budding seems to be the
most similar to sporidial formation in *Ustilago hordei*. Notice should be made that the yeast-like *Rhodotorulas* have recently been reclassified as a new genus of the heterobasidiomycetes, the *Rhodosporidium* (Banno, 1967).

Long elements of endoplasmic reticulum are prominent beneath the bud initial during the earliest observation that during knee-joint formation vesicles which are apparently derived from the ER are prominent in the region of breakdown. Moor (1967) has suggested that in *Saccharomyces cerevisiae* vesicles derived from the ER carry enzymes which induce a localized plasticization of the wall of the mother cell, and hence initiate bud formation. On the other hand, Marchant and Smith (1967) have associated large amounts of ER and ER-derived vesicles with active wall synthesis during budding. In *Ustilago hordei* spherosome-like bodies are quite prominent once synthesis of the new wall has begun (Fig. 15). This observation helps to support the hypothesis previously discussed in part II, that in this species of smut fungus the spherosome-like organelles function in wall synthesis.

**CONCLUSION**

Septation in the promycelium of *Ustilago hordei* is initiated by the "centripetal invagination" of membranes which are continuous with the plasma membrane. The source of the membranes forming the initial membranous plate seems to be a large membrane complex. Further studies are
required to test the hypothesis that at least one such complex is associated with every developing septum initial. Septal wall material is not deposited in any quantity until the initial plate has been completed. The source of the septal wall material is unknown. No pores occur in the mature cross walls of the metabasidium. Two variations of the regular septation pattern occur, one of which gives rise to "knee-joints".

Budding is initiated by a sequential plasticization and degradation of the wall of the parent cell. This stage is followed by an explosive protrusion of the sporidium accompanied by synthesis of new wall material. Further studies are required to determine the manner in which the mature sporidium is separated from the promycelium.

Evidence is presented which supports the hypothesis that in *Ustilago hordei* the endoplasmic reticulum functions in the break down of mature wall material. The spherosome-like organelles, which may also be involved in wall degradation and plasticization, probably have an additional role in the biosynthesis of new wall material.
III. PLATE 1

Figure 1a. Septum initiation. An abnormal side-by-side septation. Each septum is formed normally. Three points at which the septum initials can be observed are indicated (i.e., arrows and insert). Each initial consists of an invagination of the plasma membrane bounding an electron transparent central lamella and ending in an amorphous electron dense material. Method C. ca. X 26,200.

Figure 1b. An enlarged view of one side of one of the septum initials in figure 1a. Note the well defined plasma membrane (PM), the central lamella, and the electron dense material surrounding the frontal edge. Method C. ca. X 45,850.

Figure 2. A more advanced septum initial showing a clear continuity between the plasma membrane delimiting the septal initial and the membranes composing a membrane complex (mc). The two sides of the septum initial are indicated by arrows. Method C. ca. X 39,300.
III. PLATE 2

Figure 3. A septum initial which is almost complete. The arrow indicates the electron dense frontal edge material. Note septal wall material is beginning to be deposited at the lateral edges and is continuous with the inner promycelial wall. Lipid bodies (L), mitochondria (M) and spherosome-like organelles (S) are indicated. None of these organelles are specifically associated with the forming cross-wall. Method C. ca. X 32,750.

Figure 4. A newly completed cross-wall. Note the very thin layer of septal wall material, the central lamella, and the remains of the electron dense frontal edge material (arrow). Method C. ca. X 45,850.

Figure 5. A more advanced stage in septal wall thickening. The two wall plates (pl) and the central lamella (cl) are indicated. Method C. ca. X 45,850.

Figure 6. A mature septum. The two wall plates (pl) and the central lamella (cl) are indicated. Method C. ca. X 45,850.
III. PLATE 3

Figure 7. A membrane complex (mc) which is not clearly associated with septum formation. This complex seems to be continuous with the ER as well as the plasma membrane. Note the distinctive fibrils of the mucous coat in the upper left. Method A. ca. X 35,700.

Figure 8. A membrane complex (mc) associated with the forming septum. Method C. ca. X 35,700.

Figure 9. An enlarged view of the membrane complex seen in Figure 8 showing the connection with the plasma membrane delimiting the septum and showing the structure of the complex membranes. Method C. ca. X 116,600.

Figure 10. A light microscope view of a refractile membrane complex (mc) associated with the formation of the first septum. The material was fixed in 2% glutaraldehyde and photographed unstained with phase optics. ca. X 3,000.
III. PLATE 4

Figure 11. Knee-joint formation. Note the protruberance on either side of the septum and the poorly defined walls regions in the zone between the two protruberances. Note the clustering of ER and spherosome-like bodies (S) beneath the wall of the larger protuberance. A lipid body (L) is also indicated. Method A. ca. X 22,500.

Figure 12. A more advanced stage in knee-joint formation. Note the clustering of ER and spherosome-like bodies (S) in the bridge region, the apparent degradation of material in the zone between the two bulges which are still separate. Vesicles (ve) are present in the region of degradation and can also be seen in the adjacent protoplast. Method A. ca. X 15,900.

Figure 13. A completed knee-joint. Note that the plasma membrane has been reformed around the end of the now incomplete septum. The wall surrounding the bridge is still thinner than the septal wall. Note the position of the haploid nucleus (hN). Method C. ca. X 26,200.
III. PLATE 5

Figure 14a. The earliest visible indication of bud formation (bracketed region). A central, wall-less zone is bounded by a modified zone of promycelial wall. Long ER elements end beneath the modified wall zone. Method C. ca. X 59,500.

Figure 14b. An enlarged view of the bracketed wall region in Figure 14a. Method C. ca. X 84,600.

Figure 15. A more advanced stage in bud formation. Note the appearance of new amorphous wall material covering the now protruding bud. A spherosome-like body (S) lies just behind the bud apex. Method C. ca. X 31,875.

Figure 16. A developing bud showing the position of the haploid nucleus (hN). The bud now has a well developed wall which thins towards the apex. The plasma membrane (PM) is very distinct. Method C. ca. X 31,875.
Figure 17. A sporidium which has almost attained maximum length. Note the narrow neck region. Numerous mitochondria (M) and spherosome-like organelles (S) are present in young sporidia. Method C. ca. X 19,500.

Figure 18. A mature sporidium. Each sporidium contains a single haploid nucleus (hN), lipid bodies (L), ER, and numerous unidentified vesicles (vc). The electron transparent zones at either end are probably regions in which the spherosomal-vacuolar bodies have lysed and degraded the surrounding cytoplasm. Note the well defined nuclear envelope (NE). Method B. ca. X 27,600.
BIBLIOGRAPHY


# PART IV

Nuclear Division with Special Emphasis on Meiosis

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PART IV
Nuclear Division With Special Emphasis on Meiosis

ABSTRACT
A study has been made of the ultrastructure of the nucleus and associated structures of the smut fungus, Ustilago hordei, with special emphasis on the position and structure of the nuclear pores, the centriolar-kinetochore-equivalent, and cytoplasmic microtubules. Electron microscope observations of the meiotic and mitotic promycelial divisions have been compared with light microscope observations of nuclear division in both Ustilago hordei and Ustilago killeri. Evidently certain aspects of the meiotic and mitotic divisions are unusual and the results have been interpreted according to Brown and Stack's (1971) model for somatic nuclear division in some fungi. The problem of chromosome number is discussed.

INTRODUCTION
The teliospore of Ustilago hordei is the sexual spore, and contains the only diploid nucleus in the life cycle. When the spore germinates the nucleus usually migrates into promycelium (metabasidium) in the diploid state (Pt. II). Three cycles of nuclear division occur in quick succession: the first two division are the meiotic reduction division and the meiotic equational division, respectively, and the last is the mitotic division which gives rise to the first
haploid sporidal nuclei. Chromosomes and spindles in the nuclei of a smut species were first described by Harper in 1893, and his results have since been substantiated by other workers. Most researchers have reported that the haploid chromosome number in smuts is two. Because of the difficulties in preparation and interpretation the state of knowledge concerning the nuclear cytology of the smut fungi rests precisely where it did in 1945. No photographic record exists, whether at the light or electron-microscope level and none of the details of nuclear division is known. Contrary to the stand-still in cytological progress, genetic information has been accumulating rapidly. Recently various *Ustilago* species have been used in studies of recombination (Holliday, 1961; Holliday 1964; Kozar 1969), of haploidization (Day and Jones, 1969), of genetic complementation (Dinoor and Person, 1969) and of parasitism (Thomas and Person 1965; Halisky, 1965). Furthermore several of these studies indicate that the haploid chromosome number of at least some smut species may be greater than two (Holliday, 1964; Day and Jones, 1969). Such studies lead to a renewed demand for more information concerning the nuclei of the smut fungi. In her presidential address to the British Mycological Society, in 1939, Kathleen Sampson stated the case most succinctly when she said,

... it would be unwise to become dazzled by the fascinations of smut genetics and to forget how few are the established facts concerning chromosome behaviour in this group of fungi. To obtain a convincing cytological picture of meiosis in this group is no easy task ...
MATERIALS AND METHODS

CULTURES AND CULTURING

The wild-type strains of *Ustilago hordei* are the same as those previously described in part I. Included in the light-microscope photographs, by way of comparison, are observations on the nuclei of a mycelial mutant of *Ustilago hordei* (Stein, 1970) (Figs. Pl-4), and on the meiotic and post-meiotic divisions in *Ustilago kollerii* (Willie) (Figs. Rows G-L). The mycelial mutant was prepared and photographed by Dr. Jean Mayo, and the *U. kollerii* samples by D. C. Wighton. All the material was cultured as described in part I with the exception of the mycelial mutant which was grown on the surface of agar plates (Appendix B).

LIGHT MICROSCOPY (see also Appendix C)

**Squash Preparations.** - To prepare stained squash preparations a drop of medium containing the material was air-dried briefly on cover-slips, and fixed in one of the following ways:

a) for one hour in acetic-alcohol (1:3) to which was added a few drops of chloroform.

b) for 24 hours in BAC-fixative (Lu 1962).

After fixation, the material was stained by one of the following procedures:

a) Feulgen (Darlington and La Cour 1962); *U. hordei* was hydrolyzed in 1 N HCl at 60° for 10-12 minutes; *U. kollerii* was hydrolyzed in 5 N HCl at room temperature for 1 hour. The material was then stained for 30-40 minutes
### TABLE I: Summary of Light Microscope Techniques

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<td><strong>U. Koller</strong></td>
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<td>acetic – alcohol</td>
<td>Feulgen</td>
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<tr>
<td></td>
<td>teliospores</td>
<td>acetic – alcohol</td>
<td>Feulgen, Haematoxylin</td>
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<td><strong>U. hordel</strong></td>
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and squashed in 45% acetic acid.

b) Propiono-haematoxylin (Henderson and Lu, Method A, 1968): the material was hydrolyzed in 1 N HCl at 60°C for 10-12 minutes and stained for 1-2 minutes.

All photographs were made from freshly prepared slides. Slides were subsequently made permanent with Euparol where desired.

The procedures used for each cell type are outlined in Table I.

The cell depicted in Figure 33a was fixed in glutaraldehyde and observed unstained with phase optics as described in part III.

Sections. - The material (U. hordei) was prepared according to electron microscope preparation B (Part I). Thick sections (0.25-0.50 μ) were cut on a Sorval Porter-Blum MT-2 ultramicrotome and were stained with 1% Toluidine blue in 1% borax.

Spheroplasts. - Spheroplasts were prepared by Mr. M. Holmwood as follows: a monosporidial line of U. hordei was cultured in modified complete broth containing 15% dextrose and glurolase (Endolaboratories Inc.). At 22°C 18-24 hours were required for spheroplasts to form. The material was then fixed in acetic-alcohol and stained with Henderson and Lu's haematoxylin.

Microscopy. - All the U. kolleri material was observed with a Leitz 3½" by 4½" bellows camera at full extension.
A Zeiss photomicroscope was used in all studies of *U. hordei*. Both microscopes were equipped with a 546 μm interference filter.

**ELECTRON MICROSCOPY**

Germinating teliospores of *Ustilago hordei* were prepared for electron microscopy according to methods A, B and C as described in part I.

**OBSERVATIONS**

**LIGHT MICROSCOPY**

Different stages of the life cycle apparently react quite differently to the various methods of staining. For studies of the promycelial divisions, fixation in acetic-alcohol followed by Feulgen staining (Figs. Rows A-L) gave greater chromosome contrast than did haematoxylin (Figs. M1, N1 and O1) with the techniques used. However, several haematoxylin preparations of meiosis in *Ustilago hordei* are included to show that similar configurations have been observed (Figs. M1, N1 and O1). On the other hand, fixation in acetic-alcohol followed by haematoxylin produced much superior results in vegetative tissue, particularly with the mycelial mutant. Haematoxylin, unlike Feulgen, also stains the nucleolus densely (Row P), allowing visualization of the chromatin-nucleolar relationship.

Little is known about the early stages of meiotic prophase I which presumably occur within the forming, thick-walled teliospore. When the nucleus first passes into the
promycelium the chromatin is already highly contracted (Figs. Rows A, B, and G). Normally this late prophase chromatin takes the form of two elongate densely-stained bodies lying side by side and parallel with the long axis of the promycelium. Occasionally, when the diploid nucleus is just entering the promycelium, ring-like configurations occur (Figs. A1, and G). Such configurations probably represent the earliest meiotic stages which can be seen in the promycelium. One characteristic of late prophase I nuclei is that the two chromatin bodies frequently appear to be closely associated at one or both ends. (Figs. A2, A3, A4, B3, B4, C4, E1, G2, G4). A second common feature is that one or both bodies may be joined by a fine Feulgen-positive thread to a small knob-like structure lying against one of the lateral promycelial walls (Figs. A2, B1, G4, M).

In *Ustilago hordei* and *Ustilago kolleri* no stage exists which visually compares with the metaphase plate seen at meiosis I in higher plants and many fungi. The chromatin bodies contract as nuclear division approaches (i.e. compare A2, B2, and P2; also G2 and H2). As they attain their minimum diploid length (approximately 1.6 μ) they begin to rotate and come to lie at an angle to the longitudinal axis of the metabasidium (Fig. E2). Following this there is a poorly defined stage in which little detail is discernible (Fig. E3). These last two stages are infrequently seen and are probably short. When the chromatin bodies again become distinct they lie in a well-defined anaphase
configuration (Figs. E4, H3-4, J1, and J0). Viewed with planar optics the Feulgen staining reaction is considerably less intense, supporting the common assumption that reduction division has occurred (i.e., compare H2 and H3). The daughter haploid nuclei pull apart rapidly and in so doing the two chromatin bodies within each nucleus continue the contraction which was initiated in the diploid state. The minimum length attained by the chromatin bodies of haploid nuclei is 1.0 μ (Figs. F2, and J1-2). Occasionally the pair of anaphase chromatin bodies in each of the separating nuclei appear to be joined at the "poleward" end (Figs. E4, and I2). In Figures F1 and I3 a chromatin tail is lagging behind each of the chromatin pairs and projecting in the direction of its homologue. The tails each terminate in a conspicuous knob. During the anaphase migration the two chromatin bodies generally lie parallel with the longitudinal promycelial axis.

Good light microscope pictures were rarely obtained of the second and third promycelial divisions, which are essentially mitotic in nature. Amazingly enough, with the exception of the reduced length of the two chromatin bodies, both these divisions progress in a manner essentially identical with the reduction division. The meiotic equational division is represented here by Figures F4 and L (note the arrow marking the first septum) and the set of serial sections (M3-4, N3-4, and O3-4). The first post-meiotic division is represented by Figure J3-4 where the nucleus
lies in the neck of the forming bud. Decondensed prophase II and III configurations (Fig. J3-4) are infrequent, suggesting either that chromatin decondensation between divisions is "optional" or that it occurs rapidly. During prophase II and III the condensed chromatin bodies rarely lie parallel to the long promycelial axis; usually they are angled steeply across it (Figs. F4 and N3-4). Little detail is discernible in the tiny anaphase II and III nuclei but, in general, they resemble anaphase I configuration (Fig. L).

By way of comparison a few photographs of somatic mitotic divisions have been included (Rows P, Z and R). Figures Q1-4 and R1-2 represent sporidial divisions. Again very little detail can be seen in haploid sporidial nuclei. Where division-like figures do occur they are located either in the parental cell or, more often, in the neck between the parent cell and the bud (R1-2). Two prophase-like configurations (Q1-2 and Q3-4) and an anaphase (R1-2) are shown. In all three cells the two chromatin bodies are clearly visible. Several attempts have been made to produce sporidial spheroplasts of *Ustilago hordei* since removal of the wall affords considerable advantages for fixing, staining and adequately squashing the preparations. Unfortunately most of the nuclei seen thus far in spheroplast cultures seem to be in a resting state. R3 and R4 are photographs of two spheroplasts containing prophase-like figures. Again there are two distinct chromatin bodies (Length = 1.0 μ).

All preparations of the mycelial mutant were made with
haematoxylin. The nuclei and chromatin bodies of the mycelial mutant are exceptionally large, being comparable to those of the meiotic diploid state. At present the ploidy of the mycelial mutant is unknown. The possibility exists that it may be a true diploid (J. Mayo, personal communication). In any case the large size of the nuclei does allow clear observation of the two chromatin bodies. Haematoxylin has the advantage of staining the nucleolus as well as the chromatin; in row P the conspicuous nucleolus, which lies at the end of the nucleus, is indicated by arrows. One or both of the chromatin masses are attached to the nucleolus. Figures P1, P2, and P3 are considered to be in late mitotic prophase. A number of anomalous figures like those shown in Figure P4 occur, and these have been interpreted as early somatic anaphase; the daughter chromatin bodies have begun to separate but the nucleolus which is persistent, is yet undivided.

ELECTRON MICROSCOPY

**Ultrastructure of the Nucleus and Associated Structures.** -

The observations concerning the ultrastructure and activities of the nucleus and its associated structures are based on glutaraldehyde-osmium fixed material (Methods B and C) unless otherwise stated. Potassium permanganate leaches out and destroys both nucleic acids and microtubular structures which mainly compose the nuclear apparatus.

Figure 5 illustrates a haploid, early prophase II nucleus
in a promycelium prepared by method C. The nuclear envelope is rarely distinct after glutaraldehyde-osmium fixation but when measurements are possible the average double membrane width is 300 Å (Range 210-400 Å) with each of the unit membranes averaging 83 Å (Range 50-120 Å). After KMnO₄ - fixation the respective measurements are 275 Å (Range 220-340 Å) and 80 Å. In spite of the tenuous nature of the nuclear membranes after glutaraldehyde-osmium fixation the nucleus is never difficult to distinguish. First, the outer surface of the envelope is often accentuated by a conspicuous attachment of ribosomes (Fig. 5). Second, the nucleoplasm is less dense than the cytoplasm (Figs. 7, 14, and 28).

The nuclear envelope of *Ustilago hordei* is frequently interrupted by nuclear pores. In material prepared by methods A and B, these pores appear as simple "holes", allowing direct continuity between the nucleoplasm and cytoplasm. However, after glutaraldehyde-osmium fixation and embedding in Spurr's plastic, it is clear that the pores are occluded by electron-dense material. In cross-section, this material is rather amorphous (Fig. 23), but in face-view the circular pore region, which has an average diameter of 1,060 Å (Range: 950-1100 Å), is highly structured (Fig. 6a-c). A ring of granular or microtubular elements lies just inside the pore periphery and is apparently embedded in the electron-dense substance. One or two large granules (or microtubules) lie in the centre (Average diameter 190-214 Å).
fibrillar material (Average diameter = 42 Å) radiates from the central element in all directions towards the pore periphery. Pores of this description are most prominent in nuclei which are close to division (Fig. 23 and Pt. V, Fig. 3a) and cytoplasmic microtubules are inevitably present in the peripheral cytoplasm near such pores (Fig. 6a–b, and 23).

The nucleolus is usually the most conspicuous structure in the nucleoplasm (Figs. 5, 20, 22, 23, 24, 27–9, and 31). In shape it is spherical to ovoid; its average diameter is 1.06 μ in both diploid and haploid nuclei. In structure it consists of loosely organized, electron-dense, granular material interspersed by patches of less dense nucleoplasm (Figs. 22 and 23). One often has the impression that it is divided into two separate sections by a thin, less-granular zone (Fig. 5, 23, and 27, arrows). The nucleolus is always closely appressed on one side to the inner surface of the nuclear envelope. As has been noted previously (Pts. II and III), the nucleolus always lies at the posterior end of the migrating nucleus (i.e. during migration up the promycelium or into the bud, or during anaphase repulsion).

At the anterior end of the migrating nucleus is another cytologically conspicuous body which will be referred to in this paper as the centriolar–kennetochores–equivalent, (CKE). (Figs. 7 and 22). The CKE is, strictly speaking, extra-nuclear (i.e. the main body of the CKE lies outside
the nuclear envelope); however, its structure and activities are intimately associated with nuclear events. Migrating nuclei are slightly elongate (Fig. 7). When the migration has ceased, the nuclei round-up insofar as is possible (Fig. 23), and the CKE assume a position closely appressed to the outer surface of the nuclear envelope and directly opposite to the nucleolus. Usually in non-dividing nuclei, the CKE lies in a cytoplasmic well protruding into the nucleoplasm. During prophase I, this well is shallow (Fig. 3, 22, and 23), but it increases in depth during prophase II and III (Fig. 7, and 20) corresponding with an increase in the size of the CKE. The well seems to be a relatively stable part of prophase nuclear structure whether or not the CKE lies within it. Figures 17a-b and 18a-b, illustrate respectively a nuclear well without a CKE and a nuclear well with a CKE. Usually the CKE is surrounded by a halo of low density cytoplasm from which other cytoplasmic organelles including ribosomes are excluded. The adjacent nuclear membrane remains intact (Fig. 8 and 11). On its inside surface is a cap of electron dense nucleoplasmic material (i.e. chromatin) which is associated with the presence of the centriolar-kinetochore-equivalent (Figs. 7, 9, 18b, and 20). In Figure 7, the chromatin cap is clearly bipartite. Notably, this is the only point on the nuclear envelope to which the chromatin can be shown to attach.

Structures resembling the CKE have never been observed in ungerminated spores but whether this is due to the actual
absence of the body or to the fact that it is masked by the
density of the cytoplasm is unknown. A single centriolar-
kinetochore-equivalent is located at the anterior of the
diploid nucleus when it first begins to migrate up the promy-
celium. (Pt. II, Fig. 5) In the cross-section, the CKE is
oval, (i.e. particularly in prophase I), circular, or
slightly triangular except when dividing. Its maximum
size in prophase I is approximately 165 by 95 μm (Fig. 8)
but during prophase II and III, it increases to as much as
370 by 370 μm (Fig. 9). This increase is readily observed
by comparing Figures 8 and 9 which are at approximately the
same magnification. Structurally, the centriolar-kinetochore-
equivalent consists of a tangle of fine fibrils with an
average diameter of 28.4 Å (Range: 18-49 Å) embedded in
an amorphous electron-dense matrix (Figs 9-13). In prophase
II and III, it often develops what appears to be a dense
elongated core that follows the contours of the outer sur-
face of the body on the side opposite to the nucleus (Figs.
9 and 12). Occasionally, as in Figures 10 and 11, there
are two distinct zones in the CKE, a dense fibrillar zone
on one side and a region in which the fibrils are "spun-out"
on the other. This effect may be produced when the section
passes through, and is in the same plane as the dense core.

In promycelia of Ustilago hordei, each prophase I, II,
and III nucleus is associated with a single small centriolar-
kinetochore-equivalent. Obviously for this to be so the
centriolar-kinetochore-equivalent must replicate once per
nuclear division. Occasionally elongate fibrillar structures
have been observed lying parallel with the nuclear envelope (Figs. 14-16) and these have been interpreted as dividing centriolar-kinetochore-equivalents. In Figure 15, fine fibrils, similar in structure to those constituting the CKE, seem to connect the dividing CKE to the nuclear envelope.

During late prophase, cytoplasmic microtubules develop in association with the nucleus and the CKE. The tubules have an average diameter of 244 Å (Range: 200-250 Å) with an electron transparent central core (Average diameter = 102 Å). For the most part these microtubules lie in the longitudinal axis of the promycelium or at a slight angle to it (Figs. 19, 20 and 23), and radiate from the vicinity of the CKE (Figs. 19 and 20). The tubules do not originate directly from the CKE itself but from the globular electron-dense structures (mtoc) in the vicinity of the CKE (Figs. 21). Other microtubules end in similar electron dense structures (Fig. 20).

Nuclear Division. - Figures 22 to 33 are arranged sequentially to demonstrate the main stages of the promycelial divisions according to the author's interpretation. Figures 22 to 26 illustrate meiosis I (i.e. reduction division) and Figures 27 to 33, meiosis II (i.e. equational division). The sequence of 27 to 33 is a composite of observations made from division II and III nuclei. These divisions are essentially identical in appearance with the exception that when division III occurs in the parent cell the nuclear axis (i.e. an imaginary line drawn through the two poles)
lies at an angle of about $45^\circ$ to the longitudinal promycelial axis while the nuclear axis of division II lies roughly parallel to the longitudinal cell axis. Unless the nuclear axis can be determined, septa counted, or a bud is visible it is impossible to distinguish division II and III nuclei.

The diploid fusion nucleus in Figure 22 is about 4.25 $\mu$ by 1.70 $\mu$. The arrow indicates the direction of the promycelial apex. At the posterior end of the nucleus lies the prominent nucleolus and directly opposite to the nucleolus and about one-third of the nuclear length behind the anterior nuclear tip is the centriolar-kinetochore-equivalent. Between the nucleolus and the CKE is a band of nucleoplasm of increased density (i.e. chromatin) about 0.7 $\mu$ wide and 1.3 $\mu$ long. This nucleolar-chromatin-CKE configuration is characteristic of the migrating prophase I nucleus. It is represented schematically in Figure 35a. When the nucleus has ceased its forward motion it shortens and rounds-up insofar as is possible. In Figure 23 the CKE has shifted its position posteriorly with respect to the nucleus so that it now lies midway along the nuclear length and at the same time the nucleolus has begun to move from a posterior to a lateral position. The impression is that the entire nucleus is rotating. The nucleolar-CKE distance is shortened to 0.6 $\mu$.

Such late prophase I nuclei are associated with microtubules lying parallel with the length of the nucleus (i.e. with the promycelial axis), on the side occupied by the CKE. Figure 23 is represented schematically in Figure 35b.
No data are available on the division of the centriolar-kinetochore-equivalent at reduction division. In the next characteristic stage that is observed the nucleus elongates and constricts in the center, becoming dumbbell shaped (Fig. 24). In Figure 23 the two bulbous ends of the nucleus have virtually separated. The nucleolus has divided and one of the CKE's is visible on the right side of the lower daughter nucleus. Figure 24 is represented diagrammatically in Figure 35d. As the two daughter nuclei pull apart the nuclear membrane undergoes a very short period of partial breakdown (Figures 25 and 26). In Figure 26 the centriolar-kinetochore-equivalent has moved from a lateral to an apical position.

Note the condensation of the chromatin-nucleolar material in Figures 25 and 26. After KMnO4 fixation the nucleic acid containing material (i.e. nucleolus and chromatin) is represented by electron-transparent patches (Figs. 25 and 33). Figure 7 illustrates a haploid daughter nucleus in repulsion. The nuclear envelope has been reconstituted and the centriolar-kinetochore-equivalent is in the lead position.

In Figure 27 the haploid daughter nucleus (Average diameter - 1.8 μ) has come to rest and has presumably entered prophase II. The CKE lies in a characteristic position directly opposite the nucleolus. A line drawn through the CKE and bisecting the nucleolus would cross the longitudinal promycelial axis almost perpendicularly. Figure 28 illustrates a late prophase II nucleus just prior to CKE division. The section has passed directly through the centre of the nucleus.
in the same plane as the chromatin which is condensed and clearly visible. One chromatin body directly joins the CKE to the nucleolus, and the second coils vertically upwards in Figure 28 (arrows). Both of the chromatin bodies are joined directly to the CKE by a pair of fibrils which have an average diameter of 80 Å (Range: 78-83 Å). Figure 28 is represented schematically in Figure 35e. Shortly after this stage the centriolar-kinetochore-equivalent moves out of the nuclear well, elongates and divides as described previously (Figs. 29 and 35f). In Figure 30 the daughter CKE's are migrating around the nuclear envelope which is outlined by the presence of nuclear pores, and the nucleus has already begun to elongate in the direction of the promycelial axis. Figure 30 is represented schematically in Figure 35g. Figure 31 shows the two CKE's at the poles of a division III nucleus (i.e. note the position of the nuclear axis with respect to the promycelial axis). The nucleolus lies in a characteristic position to one side of the nuclear axis and midway between the poles (Fig. 35h). As in Meiosis I the nuclear envelope partially breaks down and the daughter nuclei move apart (Figs. 32 and 35k). In Figure 32 microtubules can be seen passing into the open daughter nucleus. Presumably the microtubules represent part of the division spindle which in inadequately preserved by the techniques employed. Under the light microscope using phase contrast optics, this spindle appears in glutaraldehyde fixed material as a thin dark line in the
nuclear axis (Fig. 33b).

DISCUSSION

NUCLEAR PORES

Since Callan and Tomlin (1950) first discovered "pores" or "holes" in the nuclear envelope of amphibian oöcyte nuclei it has become increasingly clear that these regions are not just simple "spaces" allowing the free diffusion of substances between the nucleus and cytoplasm (Feldherr, 1965). When properly prepared the nuclear pores are filled with a variety of structural components embedded in an electron opaque substance. These pores seem to be ubiquitous among eukaryotic organisms and a large volume of literature pertaining to their chemical and structural properties now exists (Abelson and Smith 1970; Frank and Scheer, 1970; Koshiba et al., 1970; Yoo and Hayley, 1967). There are many reports of nuclear pores in fungi (Bracker, 1967) but in most cases they appear as simple "holes" in the nuclear envelope. In Ustilago hordei preparatory methods A and B (Pt I) evidently destroy the complex pore apparatus which is so prominent in tissue fixed in glutaraldehyde-osmium and embedded in Spurr's plastic (Figs. 6a-c). The observations on the nuclear pores of this fungus agree with the findings in other plants and animals in respect of the presence, appearance, and size of the pore regions, peripheral granules, central grânules, and radiating filaments. A complex pore structure of this type has also been described in Coprinus lagopus (Lu, 1965) and Ascobolus stercorarius (Wells, 1970).
In *Ustilago hordei* there is an increase in the number and prominence of nuclear pores as the nuclei approach division.

**CENTRIOLAR–KENETOCHORE–EQUIVALENT**

Although true centrioles have been found in phycomycetes with a motile stage in the life cycle (Bracker 1967) they do not occur among ascomycetes and basidiomycetes. Yet, when appropriately stained tissue is observed in the light microscope tiny spherical, elongate, or rectangular bodies frequently lie at the poles of dividing fungal nuclei and if these bodies are observed at various stages of the division cycle they behave in a "centriolar" manner. Under the electron microscope these centriole-like structures consist of semi-electron dense, usually amorphous, material. During division they lie at the poles of the spindle microtubules and are the hub of the astral rays. Wells (1970) has recently reviewed the literature pertaining to these bodies and nuclear division in the Ascomycetes: notable among these studies are those of Beckett and Wilson (1968) in *Podospora anserina*, Robinow and Marak (1966) in *Saccharomyces cerevisiae*, Schrantz (1967) in *Pustularia cupularis*, Wells (1970) in *Ascobolus stercorarius* and Zickler (1970) in two species of *Ascobolus* and two species of *Podospora*. Probably because of the difficulty of fixing basidiomycetous fungi with appropriate E. M. techniques (i. e. glutaraldehyde–sodium) little information is available concerning the "centriolar–equivalent" in these fungi. Girbardt (1968),
Lu (1965 and 1967b), and Motta (1967 and 1969) have described structures which appear to be the "centriolar-equivalent" in Polystictus versicolor, Coprinus lagopus, and Armillaria mellea respectively.

In Ustilago hordei the body which lies at the poles of the division spindle has been termed the centriolar-kinetochore-equivalent (CKE) for reasons which will be subsequently discussed. The CKE of Ustilago hordei closely resembles in shape and size the "centriolar-equivalent" in Coprinus lagopus and Armillaria mellea; that of Polystictus versicolor seems to be slightly different. In both Ustilago hordei and Coprinus lagopus (Lu, 1967b) this body has a fibrillar substructure. The diameter of the fibrils (Average diameter = 28.4 Å) and the probable Feulgen-positive nature of the CKE in this smut fungus suggest that the centriolar-kinetochore-equivalent may be composed partly of DNA. Why the CKE should increase in size during prophase of the second meiotic division and first post-meiotic mitotic division is, at present, unknown; but the phenomenon is widespread among fungi (Berkson, 1970; Lu, 1967a; Olive, 1965; Singleton, 1953; Zickler, 1970). As in most other basidiomycetes and ascomycetes studied ultrastructurally, the "centriolar-kinetochore-equivalent" of Ustilago hordei is closely associated with the outer surface of the nuclear envelope at all times, and except during the very brief period of division itself, it lies within a cytoplasmic well protruding into the nucleus (Girbardt, 1968; Lu, 1965; Motta, 1969; Robinow and Marak, 1966; Wells, 1970; Zickler, 1970).
In *Ustilago* an organelle-free zone surrounds the CKE. This is also the case in *Polystictus versicolor* (Girbardt, 1968) and *Ascobolus stercorarius* (Wells, 1970) and, as pointed out by Wells is reminiscent of the robosome-free zone which frequently surrounds true centrioles.

The large variety of names which have been applied to the bodies that act as centriolar equivalents during fungal nuclear division is at best confusing. Wells (1970) has recently reviewed the terminology as it has been applied to ascomycetes. The most commonly used terms are "centriole" (Lu, 1967a), "centrosome" (Lu, 1965; Beckett, and Wilson, 1968; Zickler, 1970) and "centriolar plaque" (Robinow and Marak, 1966; Wells, 1970). However, as pointed out by Pickett-Heaps (1969c) in an article on the evolution of the mitotic apparatus, the use of terms that suggest true centrioles when applied to these fungal structures may be misleading. Recently Girbardt (1968) has coined the phrase "kinetochore-equivalent" as a more appropriate term in *Polystictus versicolor*. In this fungus the chromatin appears to be directly attached to this body which is apparently involved in directing independent motions of both the nucleus as a whole, and the chromatin within. The "chromosomes" of *Polystictus* do not act as independent entities but pass through the division cycle as if they are collectively attached to this structure which acts as a common kinetochore. Hence the term "kinetochore-equivalent." This is not a new concept. It has long been observed with
the light microscope that in many fungi the chromatin is
attached by a fine filament to a small knob at the edge
of the nucleus in resting nuclei and throughout some or all
stages in nuclear division, either meiotic (Berkson and
Britton, 1969; Berkson, 1970; Olive, 1949) or mitotic (Girbardt,
1968; Lu, 1967a, Marks, 1965; Mitchell and McKeen, 1970).
During division, as in Polystictus, the "chromosomes" of
many of these fungi do not form a classical metaphase plate
but behave as if controlled solely by the dividing knob to
which they are joined; the knob acting as both a collective
kinetochore and a centriolar-equivalent. The meiotic
"chromosomes" of Ustilago hordei are also attached to a small
Feulgen-positive knob lying against the lateral promycelial
wall (Figs. A2, B1, G4, and M1) and this association persists
up to and perhaps during division (Figs. M3-4, N3-4, and
O3-4). Presumably the knob is either the centriolar-kine-
tochore-equivalent itself or the condensed chromatin cap that
lies just beneath the CKE (Fig. 7). This association has been
observed ultrastructurally at telophase (Fig. 7), prophase
(Fig. 22), and late prophase (Fig. 28), but not yet at
metaphase.

The possibility that a single structure may at once
fulfil the functions of both centrieole and kinetochore is
not as unusual as it may at first appear. First both
structures are known to give rise directly or indirectly
to microtubules. Second, some evidence exists that during
spermatogenesis in viviparid snails centromeres are trans-
formed directly into centrioles (Pollister and Pollister,
1943). Third, the "kinetochore-equivalent" in Ustilago hordei resembles very closely in structure the "kinetochore" of some animal cells (Brinkley, 1966) since it is composed of an electron dense axial filament (Figs. 9, 12, and 15) surrounded by fine fibrillar material.

**CYTOPLASMIC MICROTUBULES**

In size and appearance the microtubules of Ustilago hordei are similar to those of higher plants (Newcombe, 1968). The position and late prophase development of these aggregations are reminiscent of the nuclear-associated "prophase bands" of the cytoplasmic microtubules which herald the onset of nuclear division in many plants (Burgess and Northcote, 1967; Burgess, 1970a and b, Pickett-Heaps, 1969a and b). They may serve as "direction markers" which predetermine the direction of the mitotic spindle (Burgess, 1970a) or they may be a reservoir of presynthesized microtubules which move intact into the spindle (Pickett-Heaps, 1969a).

Most of the cytoplasmic microtubules of Ustilago do not radiate directly from the CKE (Figs. 19 and 20) but seem to terminate in globular, electron-dense regions of cytoplasm (Fig. 21). Similar densities (mtoc) often occur at the distal ends of other tubules (Fig. 20). Recent evidence suggests that these dense regions of cytoplasm which occur in association with microtubules in many plants (Burgess, 1970b) and animals (Tilney and Goddard, 1970) are the actual sites of microtubules synthesis (i.e. microtubule-organizing centres).
CHROMOSOME NUMBER:

When Harper (1898) first described chromosomes and spindles in nuclei of *Ustilago scabiosa*, he stated that the chromosome number was eight to ten for this species. With the exception of Dickinson (1931), however, subsequent cytological studies among the Ustilaginales have not supported this high chromosome count. Kharbush (1927), Wang, (1934) and Hirschhorn (1945) whose investigations collectively dealt with five different genera including eleven *Ustilago* species concluded that the haploid number is two. These chromosome counts were made mainly at meiosis, but in five species at mitosis as well. This count has also been confirmed by Rawitscher (1922), Wang (1943), Das (1949), and Person and Wighton (1964). Our observations in *Ustilago hordei* and *Ustilago kollerii* indicate that two elongate chromatin bodies are present in meiotic and mitotic prophase nuclei up to, and including division, and that during anaphase two chromatin bodies constitute each daughter complement. However, considering current investigations of fungal cytology, the presence of two chromatin bodies does not necessarily indicate that \( n = 2 \).

Undoubtedly in the majority of fungi, meiosis occurs classically (Olive, 1965; Lu, 1965; Lu, 1967a and b; Singleton, 1953; Westergaard and von Wettstein, 1965). The chromosomes pass through the leptotene, zygotene, pachytene, diplotene, and diakinesis prophase stages, line up on a typical metaphase plate, pull apart on a normal
spindle and regroup into daughter haploid nuclei at telophase. However, in *Ustilago hordei*, and several other fungi, meiosis apparently does not conform to this traditional pattern. In these species meiosis and mitosis look identical except that the quantity of prophase chromatin is greater in the former than in the latter. Furthermore, the division figures in these species strongly resemble the division figures in the many fungi (ascomycetes and basidiomycetes) which demonstrate a normal meiotic pattern but an abnormal mitotic one. Robinow and Caten (1969) have recently reviewed the literature pertaining to this latter class of fungi which includes such well-studied species as *Aspergillus nidulans* (Robinow and Caten, 1969), *Schizophyllum commune* (Bakerspiegel, 1959), *Neurospora crassa* (Namboodiri and Lowry, 1967), and *Fusarium oxysporum* (Robinow, personal communication).

These apparently unusual division figures all have the following characteristics in common when observed in the light microscope:

1. The chromatin takes the form of two elongate chromosome-like bodies lying parallel with or at a slight angle to the longitudinal cell axis in prophase nuclei, anaphase and telophase nuclei.

2. At all observable stages, each of the two chromosome-like bodies is joined by a fine filament to a knob-like structure which seems to precede the nucleus. This knob-like structure divides, gives
rise to the division spindle, and generally behaves in a manner suggesting that it is the centriolar-equivalent.

Significantly, in all cases where meiosis is normal but mitosis is not, the meiotic complement consists of more than two chromosome pairs. For example, in *Aspergillus nidulans* eight bivalents can be demonstrated cytologically at meiosis (Elliot, 1960) and this number agrees with the number of linkage groups established genetically (Kafer, 1958). To emphasize the point even more dramatically, somatic nuclei in a diploid variety of *A. nidulans* which must have sixteen chromosomes, only two chromatin bodies are observable and these look just like those of the haploid (Robinow and Caten, 1968). Clearly the presence of two chromatin bodies in this type of nucleus is not a sufficient reason to conclude that $n = 2$.

That unusual nuclear configurations occur in many fungi is a fact. The problem that confronts cytologists is to determine the significance of this fact. Two possibilities exist: either these configurations reflect mere-the inadequacy of available cytological techniques as applied to fungal nuclei or else they result from genuine behavioral variation. Unfortunately, fungal nuclei are for the most part very small; they are usually constrained in very narrow tubes with thick walls that defy squashing, and the chromatin frequently fails to stain with the usual chromosome stains (i.e. Feulgen, haematoxylin, methyl-green pyronine). These factors have, very justifiably,
led fungal cytologists to be highly skeptical of these unusual configurations. However, recent evidence suggests that the interpretational problem may have another basis:

1. Among fungi in which unusual chromosomal configurations are observed these configurations occur consistently and they have their own equally consistent characteristics.

2. These configurations are the same in both sectioned material (Figs. M3-4, N3-4, and O3-4) and squashed material and therefore probably do not result from squashing the material in a confined space.

3. The yeast, *Saccharomyces cerevisiae*, has at least eighteen linkage groups and presumably, therefore, eighteen chromosomes. Protoplasts of yeast are readily produced, eliminating the problem of the wall and yet no amount of squashing will further resolve the two chromatin bodies commonly seen at anaphase. A similar situation exists in *Ustilago hordei* (Figs. R3, R4). Therefore the problem does not seem to be entirely one of constraint.

4. As has been discussed previously, considerable, light microscope evidence indicates that in these fungi the two elongate chromosomal bodies are attached permanently to the "centriolar equivalent". Although still inconclusive, electron microscopic observations in *Schizophyllum commune* (Girbardt, 1968), *Saccharomyces pombe* (Robinow, personal communication) and
Ustilago hordei (Figs. 7, 22, and 28) support this hypothesis. Clearly, if it is true that the "chromosomes" are permanently attached to the centriolar-kinetochore-equivalent division could not conceivably occur in the classical fashion.

If the usual chromosome configuration in species like Aspergillus nidulans, Neurospora crassa, Schizophyllum commune, and Fusarium oxysporum are not due to technical inadequacies then one is forced to conclude that the chromosomes visible at meiosis become linked together in some manner in two groups in somatic nuclei.

The class of fungi which demonstrate the double chromatin-body configuration at meiosis as well as mitosis is quite small to date. Included in the class are certain heterobasidiomycetes such as the smuts Ustilago hordei and Ustilago kolleri, the rusts Puccinia lobata (Berkson, 1970), and Coleosporium vernonia (Olive, 1949) and the ceratobasidiaceous heterobasidiomycete Ceratobasidium practiculum (Saksena, 1961). Also included are several species of the homobasidiomycete Marasmius (Duncan and MacDonald, 1965) and the ascomycete Saccharomyces cerevisiae (Robinow, personal communication). At present there does not seem to be any conclusive way in which to determine cytologically the true chromosome number of these fungi, particularly in species like Ustilago hordei and Ustilago kolleri where the early meiotic prophase stages have not been seen.
Recent genetic studies indicate that some *Ustilago* species may have more than two linkage groups. Holliday (1964) has presented evidence for the existence of five chromosomal arms in *Ustilago maydis* and Day and Jones (1969) have concluded from haploidization studies that *Ustilago violacea* has at least ten chromosome per genome. Unfortunately the cytological data in these species is not available for comparison with *Ustilago hordei*. In spite of the analysis of a relatively large volume of recombination data arising out of many different crosses in *Ustilago hordei* only one linkage group has been found (Person, unpublished), supporting the suggestion that *Ustilago hordei* may have a low chromosome number. Interestingly only one centromere has been mapped in both *Ustilago hordei* and *Ustilago violacea*. This is in agreement with the possibility that the "chromosomes" share a common kinetochore.

**THE MODEL**

Brown and Stack (1971) have recently formulated an alternative theory of somatic division on fungi, (specifically *Aspergillus nidulans*), starting from the assumption that the double chromatin body configuration reflects the actual chromosomal behaviour pattern. Their model, depicted in Figure 34 with the kind permission of the authors, is designed to satisfy two criteria:

1. It must incorporate the characteristic features of the unusual nuclear configurations as they have been observed in the light and electron microscope:
DIAGRAM I (FIGURE 3k)

Brown and Stack's Model for Somatic Nuclear Division in Some Fungi
Diagram II (Figure 35)

Model for Meiosis in *Ustilago hordei*

- (a) SEPTUM
- (b) MT
- (c) Nu
- (d) one chromosome or more than one?
i) the chromosomes are joined end-to-end in two groups.

ii) the two chromatin bodies are permanently joined to a common kinetochore-equivalent.

iii) the kinetochore also acts as the centriolar-equivalent and gives rise to the spindle.

iv) at division the chromosomes do not form a metaphase plate but remain stretched out in two lines.

v) the chromosomes reach their maximum degree of contraction at telephase.

2. The model must account for the fact that during somatic division in the hyphae of *Aspergillus nidulans* sister chromatid segregation and nuclear migration are non-random (Rosenberger and Kessel, 1968). In their most intriguing experiment Rosenberger and Kessel found their results were compatible with the view that chromatids containing DNA strands of the same age segregate as a unit during mitosis.

Figure 35 depicts schematically meiosis in *Ustilago hordei* as it would be interpreted according to Brown and Stack's model. The original model has been modified only insofar as necessary to make it compatible with a meiotic situation and with the details of structure and function in the particular fungus. For example Figure 35 takes into account both nuclear rotation and the fact that at least one of the chromatin bodies of *Ustilago hordei* directly links the
centriolar-kinetochore-equivalent to the nucleolus. However neither of these situations is likely to be unique to Ustilago. Nuclear rotation is a well-known concomitant of nuclear division of many fungi (Aist and Wilson, 1968) and evidence in both Schizophyllum commune (Girbardt, 1968) and Saccharomyces pombe (Robinow, personal communication) suggests that the centriolar-kinetochore-equivalent and nucleolus are joined via at least one of the chromatin bodies. The diagrammatic representation (Fig. 35) is supported by Figures 22 and 33a which depict the major stages in the division process. At present the author considers the data in Ustilago hordei to be more compatible with Brown and Stack's model for fungal nuclear division that with the classical pattern. But all stages have not been seen in sufficient detail and the material is certainly subject to reinterpretation.

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Meiotic divisions following teliospore germination in Ustilago hordei. Feulgen stained. Phase optics.

Figures A1 - 4, B1 - 4, C1. Mid-prophase I.

Figures C2 - 4, D1 - 4, E1. Late prophase I.

Figure E2. Late prophase I.

Figures E3 - 4, F1 - 3. Anaphase I.

Figure F4. Metaphase II.

Note: in plates 1 - 3 (a) the longitudinal axes of the promycelium are horizontal.

(b) one scale division represents 1 micron. (Total = 10 u.) ca. X 5,000.
IV. PLATE 2

Meiotic and mitotic divisions succeeding teliospore germination in *Ustilago kolleri*. Feulgen stained. Planar optics.

Figures Gl - 4. Mid-prophase I.

Figures H1 - 2. Late prophase I.

Figures H3 - 4, II - 4, J1 - 2, K. Anaphase I.

Figure L. Anaphase II. (arrow indicates first septum).

Figure J3 - 4. Mitosis III.

Note: ca. X 5,000.
IV. PLATE 3


Group a: Haematoxylin.

Figure M1. Mid-prophase

Figure N1. Late prophase I

Figure O1. Anaphase I

Group b: serial sections stained in T. blue.

Figures M3 - 4, N3 - 4 and O3 - 4. Metaphase II.

Row P. Meiotic mycelial divisions (arrows indicate nucleolus). Planar optics.

Figures P1 - 3. Mid-prophase.

Figure P4. Metaphase.

Rows Q and R. Mitotic sporidial and spheroplast division. Planar optics.

Figure Q1 - 2. Prophase.

Figure Q3 - 4. Metaphase.

Figure R1 - 2. Anaphase.

Figure R3 - 4. Metaphase.

Note: ca. X 5,000.
IV. PLATE 4

Figure 5. A general view of a haploid nucleus (hN) showing the nucleolus (Nu) in its characteristic position to one side of the nucleus and against the nuclear envelope (NE). The arrow indicates a thin electron transparent zone apparently dividing the nucleolus into two. Note the attachment of ribosomes to the outer surface of the nuclear envelope. Method C. ca. X 40,600.

Figure 6a. Nuclear pores in cross-section. Note the microtubule in the upper left. Method C. ca. X 37,650.

Figure 6b. A longitudinal section through a promycelium showing the position of the nuclear pore region depicted in Figure 6a. Method C. ca. X 40,600.

Figure 6c. An enlarged view of two of the nuclear pores from figure 6a. Note the central granules and the fine filaments radiating from the central granule to a ring of peripheral granules. Method C. ca. X 95,200.

Note: the black scale represents only 0.1 μ.
A migrating haploid nucleus (hN). Note the elongate form of the nucleus and the characteristic position of the centriolar-kinetochore-equivalent (CKE) at the narrow end. The chromatin cap lying on the inner side of the nuclear envelope (NE) opposite the CKE is clearly bipartite. Method C. ca. X 47,600.

The centriolar-kinetochore-equivalent at prophase I. Note the small size of the CKE and its position within a shallow cytoplasmic well. The nuclear envelope (NE) appears to be intact. This is an enlarged view of the CKE seen in Figure 22. Method B. ca. X 60,400.

The centriolar-kinetochore-equivalent at prophase II. Note the size of the CKE in comparison with the CKE in Figure 8. Also note the narrow electron dense zone within the CKE and the chromatin cap. Method C. ca. X 60,700.

The CKE at prophase II. Note the fibrillar appearance. Method C. ca. X 129,900.

The CKE at prophase II. Note the fibrillar appearance and the two zones of varying electron density. Method C. ca. X 90,000.

The CKE at prophase II. Note the fibrillar appearance of the CKE and the thin electron dense zone within the CKE. Method C. ca. X 188,250.

The CKE at prophase II. Note the surrounding less electron dense halo. Method C. ca. X 126,900.

Note: The black scale on Figures 8 to 13 represents 0.1 μm only.
IV. PLATE 6

Figure 14. The division of the centriolar-kinetochore-equivalent (CKE) during the third promyelial nuclear division which gives rise to the first sporidial nucleus. Note that the CKE has left the nuclear well and is now elongate and parallel with the nuclear envelope. Method C. ca. X 13,000.

Figure 15. An enlarged view of the elongate CKE in Figure 14. Note the fibrillar nature of the CKE, the nuclear envelope (NE) and the fine fibrils passing between the CKE and the nuclear envelope. Method C. ca. X 127,500.

Figure 16. An elongate CKE which has been interpreted as a stage in CKE-division. Note the fibrillar nature of the CKE. Method C. ca. 127,500.

Figure 17a. A section through the nuclear region showing an enlarged nuclear pore which may be a permanent CKE-well. Method C. ca. X 53,550.

Figure 17b. An enlarged view of the well-region seen in Figure 17a. Method C. ca. X 158,600.

Figure 18a. A section through the haploid nucleus showing the CKE within the well. Method C. ca. X 29,750.

Figure 18b. An enlarged view of the CKE within the nuclear well seen in Figure 18a. Note the appearance of the chromatin cap in cross-section. Method C. ca. X 159,000.

Note: the black scale on Figures 17b and 18b represents 0.1 μ only.
IV. PLATE 7

Figure 19. Microtubules (mt) which often originate from the vicinity of the centriolar-kinetochore-equivalent (CKE) appear to end in dense amorphous structures resembling the microtubule-organizing centres (mtoc) of higher animals and plants. Note that the CKE lies in its characteristic position on the side of the haploid nucleus (hN) directly opposite the nucleolus (Nu). Method C. ca. X 36,000.

Figure 20. Microtubules (mt) radiating from the vicinity of the centriolar-kinetochore-equivalent (CKE). Method C. ca. X 51,250.

Figure 21. Microtubules (mt) ending in the vicinity of the centriolar-kinetochore-equivalent (CKE) do not terminate at the CKE itself but rather end in electron dense bodies (mtoc) surrounding the CKE. Method C. ca. X 96,200.

Figure 22. Diploid prophase I nucleus (dN) lying in the metabasidium. The arrow indicates the direction of the promycelial apex. Note the posterior position of the nucleolus and the position of the centriolar-kinetochore-equivalent (CKE) on the side of the nucleus opposite the nucleolus. The CKE lies in a shallow cytoplasmic well approximately one-third of the nuclear length behind the nuclear apex. A band of electron dense chromatin connects the nucleolus to the nuclear envelope just beneath the CKE. Method B. ca. X 34,000.
IV. PLATE 8

Figure 23. Diploid nucleus (dN) in late prophase I. The CKE has moved to a position midway along the nuclear length and the nucleolus (Nu) has shifted laterally to the side opposite the CKE. The nuclear envelope has become very indistinct and is mainly outlined by the position of the nuclear pores (NP). Microtubules (mt) lie parallel with the longitudinal cell axis on the CKE-side of the nucleus. Method C. ca. X 51,000.

Figure 24. Early anaphase I. Note the dumb-bell shaped nuclear profile, and the apparently intact nuclear envelope. A nucleolus (Nu) is distinct in each haploid nucleus (hN). One of the CKE's is visible on the right side of the lower nucleus. Method C. ca. X 19,125.

Figure 25. Early anaphase I suggesting a period of partial breakdown of the nuclear envelope (NE). Note that the electron-transparent regions represent nucleic acid containing material. Method A. ca. X 20,7000.

Figure 26. Telophase I nucleus. The CKE has moved to the leading end of the migrating nucleus. Note the density of the chromatin and partial breakdown of the nuclear envelope on the right side of the figure. Method C. ca. X 35,700.
Figure 27. Early prophase II nucleus. (hN). Note the characteristic positions of the CKE and the nucleolus (nu). Arrow indicates a thin electron transparent zone apparently dividing the nucleolus in two. Method C. ca. X 10,950.

Figure 28. Late prophase II nucleus (hN). Microtubule-like structures seem to be radiating from the enlarged CKE. One very condensed chromatin body joins the CKE directly to the nucleolus (Nu) and a second chromatin body is visible coiling upwards in the figure. The two arrows indicate the positions of the chromatin bodies. Each chromatin body is joined directly to the CKE by at least one pair of chromatin strands. Method C. ca. X 47,600.

Figure 29. CKE-replication during nuclear division II. The nucleolus (Nu) also seems to be bipartite. Method C. X ca. 47,600.

Figure 30. Migration of the daughter CKE's during division II. The nuclear region is demarcated by the position of the nuclear pores (NP). Note that the nucleus is becoming elongate. Method c. ca. X 40,650.
IV. PLATE 10

Figure 31. The metaphase or early anaphase equivalent at division III. The two enlarged GKE's die at the poles of the nucleus. Note that the nuclear axis lies at an angle of about 45° to the promycelial axis. The nucleolus (Nu) lies in a characteristic position to one side of the nuclear axis and midway between the poles. Method c. ca. X 42,000.

Figure 32. Anaphase II. Microtubules (mt) penetrate the daughter nucleus on the side opposite the lead end during nuclear migration. The nuclear envelope is broken on the side through which the microtubules pass into the nucleus. Method c. ca. X 32,750.

Figure 33a. Anaphase II. Note the incomplete nuclear envelope. Part of the first septum is visible in the upper right. Method A. X ca. 17,700.

Figure 33b. A light microscope view of anaphase II in material fixed in GA and viewed with phase optics. The second round of nuclear division is not always synchronous. The single arrow indicates a nucleus in prophase II; the double arrow indicates a nucleus in anaphase II. The thin dense line in the longitudinal axis of the dividing nucleus represents the spindle. ca. X 2,800.
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PART V

The Positional Regulation of Cell and Nuclear Division

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PART V
The Positional Regulation of Cell and Nuclear Division

ABSTRACT
Elaborate membrane complexes have previously been associated with septum initiation in *Ustilago hordei*. Evidently similar complexes are formed in the vicinity of the nucleus during meiotic prophases I and II. The hypothesis is presented that the complexes are part of a system governing the positional regulation of cell and nuclear division in the promycelium. A review is presented of the literature pertaining to fungal membrane complexes, and their relationship to mesosomes of bacteria and the Golgi apparatus of higher plants and animals.

INTRODUCTION
In part III membrane complexes were described in association with the septum initial of *Ustilago hordei* and the suggestion was made that these membranous structures are involved in the initiation of cross wall formation. The observations presented in this paper indicate that the membrane complexes form from the plasma membrane in association with prophase nuclei. Their subsequent activities are compatible with the hypothesis that they are an important part of the mechanism which regulates
the positioning of cell and nuclear division with respect to each other.

In higher plants the cell wall delimiting two daughter cells forms as a direct telophasic continuation of the nuclear division which gave rise to the two new nuclei—the microtubular spindle apparatus of the parent nucleus being reorganized to participate in the formation of the cell plate (Newcombe, 1969). The microtubular apparatus associated with many fungal nuclei is of very limited duration and disappears completely as soon as the daughter nuclei have separated. In septate fungi cross walls subsequently form, usually via centripetal invagination of the lateral wall and plasma membrane. Microtubules do not seem to be involved and there are no obvious connections between this process and nuclear division. Yet some method of ensuring that the septum separates the daughter nuclei must exist. Such a mechanism is particularly important in a metabasidium, like that of *Ustilago hordei* where failure to maintain a one-to-one relationship between the nucleus and the cell would greatly decrease the meiotic efficiency.

### MATERIALS AND METHODS

The materials and techniques used are the same as those previously described (Pt. I). Methods A, B, and C, for preparing tissue for electron microscopy are outlined in part I, Table I.
OBSERVATIONS

Membrane complexes, similar in appearance to those associated with septum initiation (Pt. III), also occur contiguous to the nuclei of Ustilago hordei. Figure 1 shows a cross-section through a very prominent complex which seems to be in direct connection with the nuclear envelope. The nucleus to which this complex is attached is a diploid nucleus which fixed while migrating into the promycelium. Figure 2 illustrates a sequential section through the same cell and about 0.2 μ removed from the first. In the promycelium, whenever similar membranous structures are observed in association with the undivided fusion nucleus, they are found in a characteristic position. The complex is always pressed close to the promycelial wall at the posterior end of the nucleus (i.e. the end closest to the spore: Figs. 3a and 4), and consequently is adjacent to the nucleolus (Fig. 4). It also lies on the side of the nucleus opposite to the single centriolar-kinetochore-equivalent (Fig. 4). A group of vesicles of various dimensions is inevitably located in the vicinity of the complex and also at the posterior end of the nucleus (Figs. 3a, 3b, and 4). These vesicles may be associated with microtubules.

Figure 5 is interpreted as a section through a dumbbell shaped nucleus which is in the process of reduction division and which has probably proceeded to a physiological state equivalent to early anaphase I. Presumably the haploid chromosome complements and the daughter nucleoli have
separated (Pt. VI). A membrane complex appears in cross-section in the middle of the constricting region between the daughter nuclei. At a slightly later stage when the daughter nuclei of the first meiotic division have virtually separated the membrane complex with its accompanying vesicles is again visible in the internuclear zone (Fig. 6).

Although membranous systems have not yet been observed in close proximity to metaphase II or anaphase II nuclei they do occur in the perinuclear region during prophase II (Figs. 7a and 7b), and prophase III (Fig. 8). Figure 7a illustrates the rare case in which two separate complexes seem to be associated with a single nucleus.

The origin of the membrane complexes is still uncertain. However, in several cases where nuclei have been observed close to the plasmalemma, the plasma membrane invaginates in the direction of the nucleus. The invaginating membrane does not travel in a straight line but coils on itself in double concentric layers (Figs. 9a and 9b). It seems reasonable that continuation of this kind of activity would give rise to structures resembling membrane complexes.

**DISCUSSION**

Membranous structures, similar in appearance to the membrane complexes of *Ustilago hordei*, have been observed in at least a dozen different fungi (Pt. III) including phycomycetes, basidiomycetes, ascomycetes, and imperfect forms (Table I). The complexes seem to be associated with
septa in two human pathogens, *Paracoccidioides brasiiliensis* and *Blastomyces dermatitidis* (Carbonelle, 1967; Carbonelle and Rodriguez, 1968) and in two basidiomycetes *Lenzites saepiaira* (Hyde and Walkinshaw, 1966) and *Ustilago hordei* (Pt. III). In both of the preceding basidiomycetes, membrane complexes also occur in proximity to the nuclei; a similar relationship has been noted in *Paracoccidioides loboi* (Furtado et al., 1967) and *Coprinus lagopus* (Lu, 1965; Lu, 1966). To date, the only studies which have considered this association in any detail are those in *Coprinus*. According to Lu (1965 and 1966) a single membrane complex forms in the basidium of *Coprinus lagopus* during prophase I of meiosis. At this time the complex which is 0.9 to 1.0 μ in diameter lies close to the diploid fusion nucleus and gives rise to large numbers of vesicles. The *Ustilago* complex described in this paper resembles the *Coprinus* complex both in time and place of origin and in its association with vesicles (Figs. 3a, 3b, and 4). In *Ustilago hordei* it has not been proven that the complex actually produces the vesicles. However, at metaphase I the *Coprinus* complex proliferates rapidly to almost five times its prophase size while that of *Ustilago* remains unchanged. Whether this is due to a difference in function or perhaps only in the degree of function is unknown.

Considering the relatively wide distribution of this type of membranous body among fungi, one wonders why so little attention has been paid to it. There are probably three
basic reasons for this neglect. First, as discussed in part III, there has been a general tendency to avoid these bodies because of the possibility that they may simply be fixation artefacts or else that they may result from the autolytic degradation of other "normal" organelles. Evidence has previously been presented which indicated that neither of these possibilities is likely to be the case in *Ustilago hordei*. Second, stressful conditions are known to cause the rolling up of ER-elements in higher plants (Whaley et al., 1964) and animals (Fawcett and Susuma, 1958). This possibility should be examined critically in fungal studies because most of the tissue prepared for electron microscopy has been artificially cultured. However whorls of endoplasmic reticulum also occur in some cases during apparently normal development; this is particularly true in embryonic tissues (Fawcett and Susuma, 1958; Robertson, 1961). In *Ustilago hordei* a further point to consider is that the complex seems to arise from a rolling up of the plasma membrane and not of the endoplasmic reticulum. A third reason that little information is available pertaining to fungal membrane complexes is that no one, with the exception of Lu (1965), appears to have obtained sufficient information indicating that these unusual structures might perform some physiological function. I submit that membrane complexes in *Ustilago hordei* do serve a definite and important function,—namely the positional regulation of cell and nuclear divisions.
The observations in the promycelia of this smut fungus suggest the following hypothesis:

1. During late meiotic prophase there is a localized proliferation of the plasma membrane opposite the basal portion of the diploid nucleus.

2. The proliferating membrane invaginates and coils on itself (Figs. 9a and 9b) to give rise to a reasonably large structure consisting of concentric unit membrane layers (Figs. 3a, 3b and 4).

3. The membrane complex, in some way, establishes firm connection with the nucleus. The nature of this contact is unknown. Possibly the nuclear envelope is involved in formation of the complex but there is no direct evidence for this. In Figure 1 the unit membrane of the complex, and the nuclear envelope seem to be in direct connection.

4. The nuclear rotation described in part IV which brings the centriolar-kinetochore-equivalent and the nucleolus into a medial position with respect to the length of the diploid nucleus also centres the membrane complex.

5. During reduction division the daughter haploid nuclei separate and the membrane complex, having now lost contact with the nuclear envelope, remains in the middle of the lengthening internuclear zone (Figs. 5 and 6).
6. The free membrane complex then begins to "unroll" providing the membrane to form the initial septal plate (Pt. III, Figs. 2 and 5).

7. Having completed its function the membrane complex is destroyed.

8. New complexes form in association with the prophase II nuclei and the cycle begins again.

This tentative hypothesis will demand considerable testing. One of the immediate requirements is to obtain serial sections since there is no other way to accurately judge the closeness of the complex-nucleus relationship or the one-to-one-to-one relationship of the nucleus, membrane complex, and septum. Should this hypothesis prove correct there is no reason to assume that the same mechanism is necessarily active during somatic mitotic divisions although limited evidence indicates that it may be. Possibly this mechanism has evolved as a specialized adaptation to the peculiar problems of undergoing meiosis in a metabasidium.

Other authors have proposed different functions which such membranous structures might fulfill in fungi and the complexes in *Ustilago hordei* may carry out one or more of these in addition to that of positioning. In *Coprinus lagopus*, Lu (1965) has postulated that since the meiotic divisions occur in rapid succession the membrane complex, which proliferates enormously at metaphase I, might be the generator of additional nuclear envelope. This does not seem to be the case in *Ustilago* since the complex-associated
vesicles have not been observed to fuse with the nuclear membrane, and most of the vesicles remain in the internuclear zone following nuclear division (Fig. 6). A second possibility is that the complex acts as an anchor which brings the nucleus to a stationary position prior to division. In part IV it was suggested that Brown and Stack's theory (1971) of fungal nuclear division is at present the most likely model to account for the phenomena which occur in *Ustilago hordei* during meiosis. This theory is partly based on the earlier work by Rosenberger and Kessel (1968) which showed non-random sister chromatid segregation and nuclear migration in the hyphae of *Aspergillus nidulans*. Interestingly the latter authors postulated that in order to obtain the results which they did the oldest of the segregating units should be anchored to some stationary part of the cellular membrane system.

The *Ustilago* complex resembles strikingly the mesosomal system of bacteria (Rogers, 1970; Ryter, 1968) and actinomycetes (Edwards, 1970; and Fitz-James, 1960). Structurally both the complex and the mesosome consist of coiled membranes which are derived from the plasma membrane. They both occur in association with the nuclear material and with the forming septa; consequently they have both been implicated in maintaining certain structural and functional relationships between cell and nuclear division. The obvious similarity between the two structures has prompted some workers to refer to these structures as "fungal mesosomes" (Edwards, 1969;
Kozar and Weijer, 1969). This is hazardous because it tends to imply some sort of evolutionary significance which may not exist at all. According to the current data there is reason to assume that the undeniable resemblance of the two systems is not by way of identity but rather by way of analogy. First the current model of the bacterial mesosome indicates that it is composed of tubular or vesicular membranes (Ryter, 1968) although some proponents of the lamellar membrane theory still exist (Highton, 1970). Of course the problem in bacterial cytology has long been that different fixation procedures result in a different appearance of the mesosomes (Burdett and Rogers, 1970; Ryter, 1968), and one must admit that the issue is not yet closed. Obviously the same situation exists in Ustilago hordei (Figs. 1, 3b and 10). A further complication is that most of the ultrastructural studies have described gram-positive bacteria, and there is good evidence that the lamellar conformation probably occurs among gram-negative bacteria (Kakefuda et al., 1967; Ryter, 1968) and actinomycetes. Second, while both systems are derived from the plasma membrane they are probably not formed in the same manner (Imaeda and Ogura, 1963; Rogers, 1970). Third, the bacterial mesosome carries out a variety of other important cellular functions such as cellular respiration (Ryter, 1968), wall secretion (Rogers, 1970) and exoenzyme secretion (Beaton, 1968). Whether the fungal complex carries out any of these additional functions will remain unknown until the appropriate biochemical and/or histochemical infor-
mation is available. However, the first two possibilities seem to be unlikely.

Lu (1965 and 1966) has noted that fungal membrane complexes also resemble the Golgi apparatus in some aspects of structure and function, and has hence referred to these structures as Golgi. Undoubtedly the membrane complex formed in *Coprinus lagopus* during meiotic prophase I bears a greater resemblance to a Golgi than does the complex in *Ustilago hordei* in its structure, its production of vesicles, and its probable functional significance as a nuclear membrane generator. The structure of the fungal membrane complex is particularly reminiscent of the Golgi apparatus described in certain types of algae (Bouck, 1965; Brown, 1969; Millington and Gawlik, 1970). Another feature which the two systems share in common is a tendency to be situated in the perinuclear region. Beams and Kessel (1968) and Mollenhauer and Morre (1966) have reviewed the various functions of the Golgi apparatus—the latter with particular reference to plants. Notably, functions which Golgi serve in animals, higher plants, and some fungi are also functions which have been suggested for fungal membrane complexes, for example enzyme secretion (Griffiths, 1970) and membrane generation and transformation (Berliner and Duff, 1965; Lu, 1965 and 1966). In further support of the hypothesis that fungal membrane complexes are the Golgi-equivalent is the observation that membrane complexes are usually not observed in fungi which possess the usual plant dictyosomes (Table II).
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<tr>
<th>Membrane Complexes</th>
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<td><strong>Phycomycetes:</strong></td>
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<td>Nowakowskia profusa</td>
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<td>Phytophthora parasitica</td>
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<td><strong>Ascomycetes:</strong></td>
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<tr>
<td>Neurospora tetrasperma</td>
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<td><strong>Basidiomycetes:</strong></td>
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<tr>
<td>Armillaria mellea</td>
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<tr>
<td>Coprinus lagopus</td>
<td>Lu (1965 &amp; 1966)</td>
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<td>Lenzites saepiaria</td>
<td>Hyde &amp; Walkinshaw (1966)</td>
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<td>Marchant (1969)</td>
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<tr>
<td>Blastomyces dermatitidis</td>
<td>Carbonelle &amp; Rodriguez (1968)</td>
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<td>Verticillium dahliae</td>
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At present too little information is available concerning fungal membrane complexes to attempt to identify them with either the mesosomal system or the Golgi apparatus. Consequently we should perhaps retain the simple term "fungal membrane complex" for these structures and so avoid a nomenclature which might result in misleading implications. Certainly the complexes resemble mesosomes and some types of Golgi in certain aspects of structure and function. However, the ontogeny of the three systems is different, indicating a separate evolutionary origin. What seems most likely to be the case is that coiled membrane systems of this type have optimal properties for certain kinds of physiological functions relating to secretion, and membrane generation and storage, and that these properties have been "utilized" independently in different classes of organisms.
V. PLATE 1

Figure 1  A large membrane complex (mc) in direct connection with the nuclear envelope of a prophase I nucleus (dN). Method A. ca. X 24,750.

Figure 2  A consecutive section about 1000 Å removed from figure 1 showing the position of the whole nucleus with respect to the membrane complex. Method A. ca. X 13,500.
V. PLATE 2

Figure 3a. A prophase I nucleus (dN) in the promyelium showing the posterior position of the membrane complex (mc) and the positional relationship between the complex and the vesicles (ve). The CKE lies in a position opposite the complex. The nuclear boundary is demarcated by nuclear pores (NP). Method A. ca. X 37,200.

Figure 3b. An enlarged view of the membrane complex and vesicle region seen in figure 3a. Method A. ca. X 74,400.

Figure 4. A prophase I nucleus (dN) showing the characteristic positions of the membrane complex (mc) vesicles (ve) and nucleolus (Nu). The arrow indicates one of the chromatin-nucleolar connections. Method C. ca. X 47,500.

Figure 5. Division I nucleus in the dumb-bell stage. The nucleus is the same as that shown in part IV figure 24. Note the position of the membrane complex (mc). Method C. ca. X 18,000.
V. PLATE 3

Figure 6. As the nucleus (N) elongates, constricts centrally and divides the membrane complexes (mc) comes to lie in a central position between the two separating daughter nuclei. Also note the vesicles in the internuclear zone. Method B. ca. X 18,700.

Figure 7a. A haploid nucleus associated with two membrane complexes (mc's). The membrane complex on the lower left is associated with vesicles. Method C. ca. X 30,000.

Figure 7b. An enlarged view of the membrane complex (mc) and vesicles seen in figure 7a. Method C. ca. X 64,400.

Figure 8. A haploid nucleus showing the intimate relationship between the membranes of the complex and the nuclear envelope. Method B. ca. X 60,600.
V. PLATE 4

Figure 9a. Formation of a membrane complex in vagination of the plasma membrane. Method B. ca. X 31,000.

Figure 9b. An enlarged view of the forming complex seen in Figure 9a. Method B. ca. X 81,200.

Figure 10. A longitudinal section illustrating the attachment between a membrane complex and the plasma membrane bounding a thickening septum. Method B. ca. X 37,000.
BIBLIOGRAPHY


GENERAL CONCLUSION

"The things that are not yet done."
(Isaiah, 46.10)

A generalized cytological study of an entire stage in the life cycle of an organism has certain advantages and certain disadvantages; however, in approaching an organism as little known as Ustilago hordei, this step is both necessary and valuable. Casual observation of organelles at miscellaneous stages in the life cycle of an organism reveals the remarkable variety of fascinating structures to be found. Unfortunately, this approach does little to establish the equally interesting and perhaps more relevant interrelations between organelles and organelle systems. In most cases, as in Ustilago hordei, the essential organelles are always present. It is the changes in the size, contents, distribution and interrelationships which direct the course of differentiation in the organism. Clearly a generalized study encourages the observation of development in its entirety, thus allowing one to perceive exactly which organelles and systems may be important at certain developmental stages. Perhaps the most important results of such a study are, first, the formulation of hypotheses concerning exactly what is important and when and where it is important; and, second, the formulation of methods by which these hypotheses might be tested.
This study indicates the feasibility of studying pregerminal microanatomical changes in hydrating spores. Considering that many of the fundamental events which lead up to metabasidium formation begin long before germination itself, the paucity of information in this developmental stage is astounding! What is now required are extensive studies on a variety of spores, coupled with the appropriate biochemical and histochemical techniques. In *Ustilago hordei*, two important aspects to study are the composition of the "floculent cytoplasm" and the development of the respiratory pattern as reflected in the peculiarities of the mitochondrial population. Another general area deserving of further study is that of promycelial extension.

In part I, the evidence suggests that the nuclear envelope gives rise to the endoplasmic reticulum, and the endoplasmic reticulum itself gives rise to the "primary hydration" vacuoles by dilation of the intermembranous space. More study is required to determine the exact manner in which this happens; information from glutaraldehyde-osmium fixed material is necessary. Certainly one of the next steps in pursuing this question should be the application of the Gomori reaction (or some equivalent technique) to determine whether the primary vacuoles have acid-phosphate activity, (i. e. are lytic in nature).

Vacuoles in *Ustilago hordei* are formed by several mechanisms. In the metabasidium, spherosome-like bodies seem to give rise to vacuolar structures by dilation and/or fusion. This would imply that they are equivalent in function
to the animal lysosome. However, this cannot be decided for
certainly without appropriate histochemical tests for lyso-
somal activity (i.e. the Gomori reaction). The evidence in
_Ustilago hordei_ suggests that these organelles may be in-
volved in other functions as well as lytic ones; thus, even
if the spherosomal bodies should prove to have lysosomal
activities, the author feels that one would be well advised
not to conclude that that is their only function.

Much yet remains to be learned about the nuclei of smut
fungi. To date, the evidence suggests that the meiotic divi-
sion figures are most compatible with Brown and Stack's model
for somatic nuclear division in some fungi. However, more
information is required on all stages of nuclear division
particularly at the electron microscope level, and serial
sectioning through entire nuclei is requisite. Studies of
the somatic sporidial nuclear divisions should also be made.
One also suspects that the techniques could be improved suf-
ficiently to demonstrate microtubules more clearly and so
establish their relationship to the dividing nucleus. Some
effort should be made to determine the extent to which this
mechanism of division occurs among fungi and any genetic im-
plications should be investigated (especially at meiosis).

There now seems to be little doubt that membrane com-
plexes are common among fungi, and it seems unreasonable
that these complexes should not be studied more seriously.
In _Ustilago hordei_, these complexes appear to play some part
in establishing the positional relationship between a septum and the preceding nuclear division. Serial sectioning is required to establish the one-to-one-to-one relationship between membrane complex, nucleus, and septum. Studies on cell division mutants may prove useful in determining the significance of the complex.

The disadvantage in a generalized study of this type is that although a multitude of hypotheses suggest themselves, few definite conclusions can be drawn. Such a study merely points the way. The author hopes that the observations and hypotheses presented are sufficiently well-documented and of sufficient interest to encourage others, as well to pursue answers to these problems.
APPENDIX A

Culture Medium

(A) COMPLETE BROTH

Vogel's solution (dilute) 20 ml.
Distilled water 1 l.
Tryptophane 50 mg.
Casein hydrolysate 5 gm.
Yeast extract (Difco) 55 gm.
Sucrose (20 gm.
or Dextrose (10 gm.
Vitamin solution 10 ml.

Note: 1. Vitamin solution to be added after autoclaving.
2. To make complete plates add 20 gm. Bacto-agar before autoclaving.

(B) VOGEL'S SOLUTION (concentrate)

Na$_3$ citrate . 2H$_2$O 123 gm.
KH$_2$PO$_4$ anhydrous 250 gm.
NH$_4$NO$_3$ anhydrous 100 gm.
MgSO$_4$ . 7H$_2$O 10 gm.
CaCl$_2$ . 2H$_2$O 5 gm.
Trace element solution 5 ml.
Distilled water 750 ml.
Chloroform 2 ml.

Note: 1. Add chemicals successively with stirring.
2. Store at room temperature.
3. Dilute 50-fold with distilled water before use.

(C) VITAMIN SOLUTION

Thiamin 100 mg.
Riboflavin 50 mg.
Pyridoxin 50 mg.
Calcium pantothenate 200 mg.
Benzoic acid 50 mg.
Nicotinic acid 200 mg.
Choline chloride 200 mg.
Inositol 400 mg.
Polic acid 50 mg.
Distilled water to a total of 1 l.

Note: 1. Store at 4°C.
2. Use 10 ml. of vitamin solution per litre of sterile medium.
(D) TRACE ELEMENT SOLUTION

Citric acid \cdot \text{H}_2\text{O} & 5 \text{ gm.} \\
\text{ZnSO}_4 \cdot \text{H}_2\text{O} & 5 \text{ gm.} \\
\text{F}_2\text{(NH}_4\text{)}_2\text{SO}_4 \cdot \text{H}_2\text{O} & 1 \text{ gm.} \\
\text{CuSO}_4 \cdot \text{H}_2\text{O} & 0.25 \text{ gm.} \\
\text{MnSO}_4 \cdot \text{H}_2\text{O} & 0.05 \text{ gm.} \\
\text{H}_3\text{PO}_4 \text{ anhydrous} & 0.05 \text{ gm.} \\
\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} & 0.05 \text{ gm.} \\
\text{Chloroform} & 1 \text{ ml.} \\
\text{H}_2\text{O distilled} & 95 \text{ ml.}

Note: 1. Store at room temperature.
NOTE: The entire procedure is carried out at room temperature.

1. Collection. - Resting spores are obtained by splitting open the kernels of smutted heads and shaking the spores into a test tube containing a few milli-litres of distilled water. These are shaken violently for several seconds to wet the spores, and are centrifuged immediately at low speeds for 1-2 minutes on a Phillips Drucker Combination centrifuge L-708. The water is decanted off and the spores resuspended in the desired fixative. The time in which the resting spores are in water must be kept to a minimum in order to avoid the possibility of activation.

For spores which have been hydrated half-an-hour or more in broth, a suitable aliquot of the spore suspension (this depends on the concentration of spores - usually 10-20 ml. is sufficient) is centrifuged down. The broth is decanted off, and the pellet washed once in water (before KMnO₄-fixation) or in the appropriate buffer, for 1-2 minutes. After repelleting, the liquid is poured off and the material resuspended in the desired fixative.

2. Fixation. - The two fixation procedures used are:

1) 1.5% KMnO₄ (aqueous) - Potassium permanganate is dissolved in distilled water over-night and filtered before use. Fixation time is 10 - 20 minutes.

2) 2.0% Glutaraldehyde - 1 part with 3½ parts 0.01 M cacodylate buffer (Sabatini et al., 1961; Bracker
and Grove, personal communication). The pH of the buffer is adjusted to 7.0-7.2 with HCl before use. Fixation time is 12-16 hours (the longer time is required for the resting spores).

When using glutaraldehyde as a fixative the osmolarity of the fixative is important in obtaining optimal results for Ustilago hordei. The osmolarity of the growth medium, the growth medium plus the material, and the final 2% glutaraldehyde solution were determined on an Advance Osmometer (Model 3W). The results (Table I) indicated that the osmolarities of all three are within 50 milli-osmoles of each other.

It is advisable to carry out the first hour of fixation under vacuum. This is particularly true of resting spores which tend to float. The material is agitated continuously throughout fixation.

3. Washing. — The fixed material is washed for a minimum of 1½ hour in water or buffer (whichever is appropriate) changing the washing fluid 6-10 times. The material is always recovered by centrifugation.

4. Post-fixation. — Only the glutaraldehyde-fixed material is post-fixed in OsO₄. OsO₄ is prepared by mixing equal parts of either 2% or 4% OsO₄ in distilled water with the appropriate buffer. Post-fixation time is 3 to 3½ hours.

5. Washing. — Post-fixed material is washed as in step 3.

6. Uranyl acetate staining. — Glutaraldehyde-osmium fixed material may be pre-stained with 0.5% Ur(Ac)₂ in distilled water for 2-4 hours. Note this step is OPTIONAL and is
usually not desirable for germinated material (Pt. II). If this step is used the material should be rewashed as in steps 3 and 5.

7. **Agar embedding.** - At this stage, the spores are collected on a millipore filter. The material is coated on the free side by dropping 2% water agar (47° C) onto the surface. The filter is stripped off once the agar has solidified and the under surface is similarly coated. The pellet is cut into small pieces for embedding.

8. **Alcohol dehydration.** - All material (KMnO₄ fixed and GA-Os fixed) is dehydrated through a standard ethanol series: 30%, 50%, 70%, 85%, 95%, 100%. The agar-embedded tissue is passed through the first 5 solutions in approximately 1 hour, and given 3 changes in absolute alcohol of 30 minutes each (Total = 1 ½ hr.).

**NOTE:** In the text, 3 basic preparatory methods are distinguished, noted A, B and C. Method A involves KMnO₄ fixation. Methods B and C involve GA-Os fixation and remain the same until the end of the alcohol dehydration. B and C are distinguished on the basis of the embedding plastic. Methods A and B are completed by following steps 9 and 10; method C by step 11 only.

9. **Propylene oxide dehydration.** - Propylene oxide is added drop-by-drop to the final absolute ethanol change until the solution is half-and-half (Time = ½ hour). Three changes are made in Propylene oxide (Time = 1 hour).

10. **Embedding in Epon 812.** - The material is infiltrated by Epon 812 (Ladd or Shell Oil); (Luft, 1961; 7A:3B). Plastic minus accelerator is added drop-wise to the last propylene oxide change over a period of 1 ½-2 hours to a final half-and-half
solution. The material is left overnight, uncovered and fresh plastic is made (including the accelerator, 1.5%-DMP 30). The fresh plastic is changed 3 times (½ hour each) before embedding. The Epon 812 is hardened by polymerizing the blocks 12 hours at 37° C and 28-32 hours at 60° C.

11. Embedding in Spurr's media. - The material is infiltrated by Spurr's standard embedding medium (Polysciences Incorporated) (Spurr, 1969; Standard medium A firm). It is brought to the plastic through immersion is five standard solutions of increasing plastic concentration: Spurr's : ETOH = 1 : 3, 1 : 2, 1 : 1, 2 : 1, 3 : 1. It is left in the last solution overnight; then is put through 3 changes of plastic (½ hr. each) and embedded in blocks. Harden Spurr's by polymerizing the blocks 6-7 hr. at 70° C.

12. Post-embedding technique. - Silver to grey sections are cut on a Sorvall Porter-Blum MT-2 ultramicrotome using glass of diamond knives (Dupont) knives, and are picked up on carbon-coated grids. The material is post-stained in a saturated solution of uranyl acetate in 70% ethanol followed by lead citrate (Reynolds, 1963). After methods A and B the staining times are 30-40 minutes and 20-30 minutes respectively; while after method C the times are 3-4 hours (37° C) and 30-40 minutes respectively.
<table>
<thead>
<tr>
<th>Material</th>
<th>Measurement (milli-osmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete broth</td>
<td>290.5, 289.8, 291.0</td>
</tr>
<tr>
<td>Broth plus 0 hr. spores</td>
<td>312.5, 312.5, 314.5</td>
</tr>
<tr>
<td>Broth plus 7 hr. spores</td>
<td>313.3, 315.3, 320.5</td>
</tr>
<tr>
<td>2% G.A. (cacodylate buffer)</td>
<td>336.0, 335.5, 339.5</td>
</tr>
</tbody>
</table>

**NOTE:** Measurements were made on an Advance Instrument Osmometer (Model 3W).

**BIBLIOGRAPHY**


APPENDIX C

Light Microscope Fixing and Staining Procedures

SQUASH PREPARATIONS

In all cases a drop of medium containing the material is air-dried briefly on coverslips before being treated by one of the following methods.

a) (Acetic-alcohol)-Feulgen

Acetic-alcohol fixative: Mix: 1 pt. glacial acetic acid with 3 pt. absolute alcohol.

Add: a few drops of chloroform to every 10 ml. of above solution.

Feulgen Stain (Darlington and La Cour, 1962): Pour 200 cc. boiling distilled water over 1 gram of Basic Fuchsin and shake. Cool to 50°C; filter into brown or darkened stock bottle with ground glass stopper and add 20 cc. 1 N HCl (Hydrochloric acid). Cool to 25°C and add either 1 gm. anhydrous sodium bisulfite (NaHSO₄) or 1-3 gm. potassium metabisulfite (K₂S₂O₄). Keep in the dark. After 24 hr. it will decolourize. If it does not decolorize properly add 0.5-1.0 gm of Activated charcoal, shake, and filter.

SO₂ water: Mix: 200 ml. of distilled water with 1 gm. potassium metabisulphite and 10 cc. 1 N HCl.

Method: 1. Wash two times in distilled H₂O - 5 min. each.
       2. Fix in acetic-alcohol - 1 hr.
       3. Wash six times in distilled H₂O - 5 min. each.
       4. Hydrolyse in 1 N HCl @ 60°C - 8 min. or in
$ \text{HCl @ } 22^\circ \text{C. } - 1 \text{ hr.}$

5. Wash 2 times in tap water - 5 min. each.

6. Wash four times in distilled water - 5 min. each

7. Feulgen - 1-2 hr.

8. Rinse twice in $\text{SO}_2$ water - 5 min. each.

9. Wash 6 times in distilled water - 5 min. each

10. Store cold until use.

To squash the coverslip is inverted (i.e. material down) on a glass slide on which a drop of 45% acetic acid has been placed, and pressure applied. All material was photographed from freshly prepared slides. After observation the slide is floated off in absolute alcohol and remounted in Euparol.

b) **BAC-Propionic-haematoxylin**


Use: fresh.

Propionic-haematoxylin (Henderson and Lu, 1968):

Stock solutions: A) 2% haematoxylin in 50% propiacid.

B) 0.5% iron alum in 50% propiacid.

Mix: equal volumes of A and B. If stock solutions are fresh mix together one day before use. Aged stock solutions (i.e. 3 mon. or more) can be mixed and used immediately.
Method: 1. Wash two timed in distilled water - 5 min. each.
   2. Fix in BAC-fixative @ room temperature - 24 hr.
   3. Wash six times in distilled water (last change at 60° C.) - 5 min. each.
   4. Hydrolyze in 1 N HCl @ 60° C. - 12 min.
   5. Wash two times in tap water - 5 min. each.
   6. Wash four times in distilled water - 5 min. each.
   7. Drain coverslip and apply a few drops of stain macerating the tissue with an iron needle.
   8. Invert onto a glass slide and apply pressure.

Material is photographed from freshly prepared slides and is made permanent as described in procedure a.

c) (Acetic-alcohol)-(Propiono-haematoxylin)

Preparation of the fixative (i.e. acetic-alcohol) is described in part a and preparation of the stain in part b.

Method: The method is the same as that described in part b substituting acetic-alcohol for BAC-fixative in step 2, and shortening the fixation time to 1 hr.

SECTIONS

Fixative: Method A, B, or C as described for electron microscopy (Appendix B).

Toluidine blue: Mix 1 gm. Toluidine blue in 100 ml. 1% borax.

Method: 1. Thick sections (0.25 - 0.50 u) are picked up with a small copper loop (1-2 mm diameter), and transferred to a drop of water on a clean glass slide.
   2. Evaporate water by heating gently over a flame.
   3. Place a drop of stain directly on sections.
4. Heat until the edge of the drop turn yellow.
5. Wash the stain off in tap water.
6. Dehydrate 1 min. in 70% ethanol followed by 1-2 min. in absolute alcohol.
7. Pass slide through xylene for 1 min.
8. Mount in immersion oil and seal with nail polish.

BIBLIOGRAPHY

