THE NUCLEOLUS OF WHEAT STEM RUST UREDOSPORES

by

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ABSTRACT

A cytological study of sporogenesis, mature uredospores and hydrated uredospores of *Puccinia graminis tritici* was made to determine if a definitive nucleolus was present. Electron microscopy has shown that the nuclei in immature uredospores and associated fungal tissue of the uredosorus possess prominent nucleoli. Nucleoli whose average diameter was 1.7 microns were observed in 58 percent of the nuclear sections from immature uredospores. Presence of a nucleolus in ultrathin sections of mature uredospores is established. Nucleoli whose average diameter was 0.5 microns were observed in 6 percent of the nuclear sections from mature uredospores. Nucleoli were not observed in hydrated uredospores which had resumed active metabolism. The reduction in the size of the nucleoli in mature uredospores and absence of nucleoli in hydrated uredospores may indicate that ribosomal RNA synthesis is repressed as uredospores mature.

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INTRODUCTION

Rust fungi are dependent on host tissue for successful growth and reproduction. No biochemical lesions which may cause rusts to be obligate parasites have been defined and success in axenic culture of rusts has been limited.

Uredospores are the repeating spore form of the graminicolous rusts. These spores are produced on the grass host from a binucleate mycelium constituting a uredosorus beneath the epidermis of the host. The formation of uredospores ruptures the epidermis of the host and dissemination is accomplished by wind or rain. The ability of the spores to germinate at maturity makes the uredial stage the most destructive aspect in the rust life cycle and consequently has stimulated much investigation.

Uredospores will germinate on water at the expense of endogenous reserves. Until recently, differentiation of the germ tube of the sporeling could be achieved only on the host plant. Differentiation of germ tubes into infection structures has been induced with leaf cuticle hydrocarbons, and heat treatment. However, uredospore inoculation of defined media will not yield the luxuriant growth characteristic of saprophytic fungi.

The concept has emerged from biochemical studies of the utilization of exogenous carbon sources that germinating uredospores have a defective mechanism of protein synthesis. This was reinforced by the apparent absence of nucleoli in

the nuclei of mature uredospores as revealed by electron microscopy. The nucleolus-protein synthesis relationship of rust spores is further complicated by reports of extrusion of nucleoli from the nuclei of germinating uredospores. The present study was undertaken to determine whether the nuclei in immature, mature and hydrated uredospores of wheat stem rust, Puccinia graminis tritici, contain definitive nucleoli and to re-examine other earlier cytological findings. Electron microscopy was the primary tool for a cytological study of the above tissues.

LITERATURE REVIEW

Cytology

Cytological studies of rusts extend back to 1880 and in the interval from that date Savile (1939) recognized three phases of interest. The first was the recognition of a diploid stage, the teliospore, and subsequent reduction to haploidy. The second was initiated by the discovery of the origin of the dikaryotic state in the aecium. The third phase was ushered in by the discovery that the locus of nuclear mixing was the pycnium (Craigie 1927). Current research publications reveal that fungal cytologists and plant pathologists have embraced ultra-structural and biochemical studies and such studies may be considered to represent the fourth phase of rust cytology. Current investigations are being conducted at magnifications and with a degree of refinement unimaginable to the early cytologists.

Uredospores of Puccinia graminis tritici Erikss. and Henn. (wheat stem rust) are ovoid, single-cell structures 27μ in length and 16μ wide. The thick cell wall, 1.5μ - 2μ, is covered with spines which develop beneath the wall and move to the exterior as the spore develops (Thomas and Isaac 1967). Williams and Ledingham (1964), Strobel (1965) and Thomas and Isaac (1967) described the cell wall as consisting of three layers. The inner and outer layers were electron dense and the intermediate layer was electron transparent. The cell wall of flax rust uredospores has been described by Manocha and Shaw (1967) as a multi-layered structure. Thin areas in the spore

wall were termed germ pores (Williams and Ledingham 1964, Manocha and Shaw 1967). A reduction in cell wall thickness when spores were hydrated was reported by Manocha and Shaw (1967) but a similar change in spores of Puccinia striiformis (stripe rust) was not observed by Strobel (1965).

The plasma membrane of spore cytoplasm of flax rust uredospores has been described by Manocha and Shaw (1967) as a single membrane. The same zone in wheat stem rust uredospores was visualized by Williams and Ledingham (1964) as a layer of vesicles. Hyde and Walkinshaw (1966) described the plasma membrane in basidiospores of Lenzites as a single membrane with numerous invaginations.

Most cytoplasmic organelles and inclusions of fungi are common to other plants and animal tissues (Bracker 1967). Nuclei, mitochondria, endoplasmic reticulum, ribosomes and lipid bodies have been found in rust uredospores. Lipids in the form of large central droplets or small, rounded membrane-bound bodies were observed by Strobel (1965) to occupy a considerable volume of spore cytoplasm. Small lipid bodies in uredospores have also been described by Williams and Ledingham (1964) and Manocha and Shaw (1967). Lipid bodies have been described in basidiospores (Hyde and Walkinshaw 1966), aeciospores (Walkinshaw et al. 1967) and in spores of other fungal groups (Remsen et al. 1967, McKeen et al. 1967, Marchant 1966). It has been stated (Shaw 1964, Staples and Wynn 1965, Allen 1965) that lipids are an important source of metabolites for spore maintenance and germination. At the onset of germination of rust spores Strobel

(1965) observed that the mobilization of lipid reserves resulted in the reduction in size of large lipid bodies.

The very dense cytoplasm of spores with low water content (uredospores, aeciospores) has proved difficult to fix and embed (Williams and Ledingham 1964, Walkinshaw et al. 1967) and many features have remained obscure. Williams and Ledingham (1964) and Manocha and Shaw (1967) reported that the endoplasmic reticulum took the form of vesicles and ribbons in mature spores. The former authors observed increased amounts of the same form of endoplasmic reticulum in germ tubes whereas the latter observed elongated threads of endoplasmic reticulum in germ tubes. Walkinshaw et al. (1967) described the endoplasmic reticulum of aeciospores as irregular vesicles while germ tubes arising from these spores possessed elongate endoplasmic reticulum covered with ribosomes. Strobel (1965) did not describe endoplasmic reticulum in dormant uredospores but in hydrated spores he did observe a vesiculate endoplasmic reticulum the appearance of which was correlated with a decrease in soluble protein. Hyde and Walkinshaw (1966) did not find endoplasmic reticulum in basidiospores of Lenzites fixed in either permanganate or aldehyde although ribosome groupings were observed. The above condition was observed in germ tubes. Associations of endoplasmic reticulum with cytoplasmic elements other than ribosomes have been reported by Williams and Ledingham (1964) who observed endoplasmic reticulum closely associated with small lipid inclusions. McKeen et al. (1967) described an

apparent association of endoplasmic reticulum and the tonoplast or vacuolar membrane, in conidia of Erysiphe.

The mitochondria of mature uredospores have been described as rounded and about one micron in diameter (Williams and Ledingham 1964, Manocha and Shaw 1967). Williams and Ledingham noted that cristae within these mitochondria are few in number and lie in random directions. During germination cristae have been visualized as distinct parallel membranes (Williams and Ledingham 1964, Manocha and Shaw 1967). Walkinshaw et al. (1967) observed the aforementioned changes in cristae and the presence of tubules or ribosomes on the surface of mitochondria in dormant spores whereas the mitochondria in germ tubes exhibited smooth outer membranes.

Increased respiration associated with spore germination (Shaw 1964, Staples and Wynn 1965, Allen 1965) is consistent with reports of increased numbers of mitochondria (Marchant 1966, Remsen et al. 1967, Lowry and Sussman 1968, Buckley et al. 1968). Marchant (1966) described a reduction in size of mitochondria, suggesting division, whereas Lowry and Sussman (1968) suggested that a change in biochemical function might cause a reduction in size. Enlarged mitochondria have been reported in the germination of Rhizopus sporangiospores and it was suggested that this is a response to the requirement for membrane subunits (Buckley et al. 1968). An alignment of mitochondria along the plasma membrane when spores were

hydrated was noted by Strobel (1965) who suggested that this might facilitate gas exchange.

Metabolic activities within wheat stem rust uredospores are mediated by two haploid nuclei. The haploid chromosome number was determined to be six (McGinnis 1953). Savile (1939) presented an extensive study of nuclear behavior throughout the life cycle in the Uredinales. Savile (1939) described the uredospore nuclei as fully expanded with chromatin in an 'ectosphere' and a clear homogeneous inner area termed the 'endosphere'. The 'endosphere' was stated to be non-homologous to the nucleolus of higher plants. It was concluded that during uredospore germination, the 'endosphere' moved to the membrane of the 'ectosphere'. Subsequently the membrane broke down at the point of association and reformed behind the extruded 'endosphere' which disintegrated in the cytoplasm. Distorted nuclei, henceforth known as 'unexpanded' nuclei, were observed to round up and migrate into the germ tube. Similar observations were made at aeciospore germination. The presence of an unexpanded nucleus at various points within the rust life cycle prompted Savile to conclude that the transformation of an 'expanded' nucleus to an 'unexpanded' nucleus occurred at any point where a nucleus had to pass through a restricting pore. The existence of two nuclear forms was concluded to be functional.

Im a more recent study, Craigie (1959) observed nucleoli in the nuclei of immature uredospores but not in germ tube nuclei. It was concluded that extrusion of nucleoli from

the nuclei of germinating spores occurred in the manner described by Savile (1939).

Williams and Ledingham (1964) did not observe nucleoli in Puccinia graminis tritici uredospores in one of the first fine structure studies of rust spores. There was no indication that a nucleolus was present in basidiospores of Lenzites but prominent nucleoli were shown in germ tube nuclei (Hyde and Walkinshaw 1966). Manocha and Shaw (1967) did not report nucleoli in nuclei of mature hydrated uredospores and intercellular mycelium but structures interpreted as nucleoli occurred in immature uredospores. A study of the development of echinulations on spore walls (Thomas and Isaac 1967) revealed that nucleoli were present in immature uredospores of Puccinia graminis tritici. At the onset of sporogenesis in Plasmodiophora brassicae, nucleoli have been reported to disappear (Williams and McNabola 1967). Shaw (1967) has speculated that if the nucleolus is absent from mature uredospores or cannot be reformed after nuclear division during germination then subsequent proliferation of the fungus may depend on stimulation from the host directed at synthesis of ribosomes and protein synthesis in the fungus.

The conidia of Erysiphe cichoracearum exhibit a nucleolus adjacent to the nuclear membrane (McKeen et al. 1967). Neither nucleoli nor chromosome figures were found in dormant conidia or germinating conidia of Fusarium (Marchant 1966). In contrast, Heale et al. (1968) observed that a nucleolus was absent in the resting nucleus of Verticillium conidia. During

germination and nuclear division, a nucleolus appeared, increased in size and persisted until late division.

Nucleolus

Since the primary objective of the investigation was to determine if definitive nucleoli were present in uredospores it is desirable to discuss the nucleolus briefly. The nucleolus is a chromatin-associated organelle concerned with synthesis of ribosomal RNA (Waddington 1966). The major macromolecules of the nucleolus are nucleolar DNA, nucleolar RNA and proteins (Waddington 1966, Birnstiel 1967). Nucleolar DNA is that part of the genome containing the cistrons for precursors of ribosomal RNA. Nucleolar RNA exists as two species which are precursors of ribosome subunits. Nucleolar protein constitutes 80-90% of the dry matter in the nucleolus and exists in two classes, lysine-rich protein and residual proteins, the latter resembling ribosomal protein (Birnstiel 1967).

There is general agreement that the nucleolus has four structural components: granules, fibrils, protein matrix and chromatin fibrils (Birnstiel 1967). In plants, the fibrillar portion, termed the parsamorpha, is centrally located in the nucleolus while the granular portion, or nucleonema, is peripheral. Utilizing enzymatic digestions it has been shown that the granules, which resemble ribosomes, and the fibrils are composed of RNA and protein (Birnstiel 1967).

At mitosis the nucleolus begins to lose integrity in midprophase with the dispersion of the granular fraction

into the nucleoplasm (Lafontaine 1968). RNA synthesis stops at late prophase (Birnstiel 1967). Dissolution of the fibrillar material coincides with the breakdown of the nuclear membrane. RNA and protein synthesis are resumed during telophase and the structural elements of the nucleolus reappear in the reverse order of their dissolution (Chouinard 1966).

The behavior of the nucleolus in mature and germinating uredospores may be better understood when viewed within the context of the spore physiology and biochemistry.

Uredospore Physiology

The physiology of dormant and germinating rust uredospores has been reviewed by Shaw (1964) and Staples and Wynn (1965). Allen (1965) has reviewed fungal metabolism pertaining to spore germination.

The general composition of wheat stem rust uredospores is as follows: carbohydrate 22%, protein 26%, lipid 20%, chitin 1.4% and water 10 - 15% (Shaw 1964, Staples and Wynn 1965). Arabitol, mannitol and trehalose were found to be the major soluble carbohydrates (Wynn et al. 1966, Daly et al. 1967) and lesser amounts of arabinose, fructose, galactose, glucose, maltose, mannose, ribose, xylose and glycerol have been reported (Shaw 1964, Staples and Wynn 1965). A polysaccharide extracted from uredospores was shown (Klöker et al. 1965) to consist of D-mannose, D-galactose, and D-glucose, with pentoses and glucosamine as probable side-chain additions.

The nature of uredospore proteins remains open to

investigation. Many enzymes have been demonstrated in cell homogenates and cell-free breis (Shaw 1964, Staples and Wynn 1965). Acid hydrolysis of uredospore proteins yielded all the common amino acids (Klöker et al. 1965). Total protein content of uredospores may change under various environmental conditions and this may reflect the nutrient level in host tissue (Eyal et al. 1967).

Wynn et al. (1966) found that the predominant amino acids in uredospores were glutamate, alanine, cystine, aspartate and serine. With a different extraction procedure Stefanye and Bromfield (1965) isolated glutamine, glutathione, alanine, ammonia and glutamic acid as the main ninhydrin-positive substances.

Lipids comprise approximately 20% of uredospore fresh weight in wheat stem rust (Shaw 1964, Daly et al. 1967) and 14% in flax rust (Jackson and Frear 1967). The lipids have been found to be composed largely of glycerides, and a considerable fraction of unsaponifiable lipids (Staples and Wynn 1965) which are mainly sterols (Jackson and Frear 1968). Important fatty acids found were palmitic, stearic, oleic, linoleic, linolenic and cis-9,10-epoxyoctadecanoic acid (Jackson and Frear 1967, Daly et al. 1967). One sterol of wheat stem rust, and two sterols of flax rust have been reported (Jackson and Frear 1968). The unsaponifiable fraction of wheat stem rust uredospores has been found to contain β-carotene, γ-carotene, lycopene and phytoene which are partly responsible for the brownish color of spores (Shaw 1964, Staples and Wynn 1965). Bush (1967)

supported the above findings and showed that carotene content of spores changes with season and age of spores. He suggested that carotenes serve to protect the spore protoplast from ultraviolet radiation damage. The unsaponifiable lipid fraction from flax rust uredospores has been separated into β -carotene, γ -carotene and an unidentified pigment (Kelley 1965).

The many other substances which have been isolated from uredospores include organic acids, phenolics, vitamins, nucleotides and auxins (Shaw 1964, Staples and Wynn 1965).

Existence of the Embden-Meyerhof-Parnas pathway, hexose monophosphate pathway, tricarboxylic acid cycle and the glyoxylate pathway has been demonstrated. Detection of the appropriate enzymes forms part of the evidence for the presence of these pathways. Shaw (1964) and Staples and Wynn (1965) have comprehensively reviewed the respiratory metabolism of uredospores.

In order to determine the nature of respiratory pathways and substrates being utilized, uredospores were allowed to respire in the presence of C^{14}-labelled sugars, sugar alcohols, organic, amino and fatty acids. It was found that uredospores assimilate exogenous substrates very slowly and that the incorporation of labelled carbon into cell carbon was very low. Short chain fatty acids (C_2 to C_6) were utilized more rapidly than other substrates and increased chain length promoted higher oxygen consumption. Some features of uredospore respiration included (a) low oxygen consumption, (b) a low

C_6/C_1 ratio suggesting that much CO_2 is released through the pentose phosphate pathway and (c) no inhibition of oxygen uptake by malonate or sodium fluoride but (d) inhibition by cyanide and 2,4-dinitrophenol (Shaw 1964, Staples and Wynn 1965).

Inability of obligately parasitic fungi to grow on simple media prompted comparative studies of exogenous substrate utilization by saprophytic and obligately parasitic fungi (Staples et al. 1961, Staples et al. 1962). Non-germinating spores of the latter group of fungi did not synthesize net protein to a greater extent than the obligate fungi. It was concluded that the differences were due in part to the ability of the spores to remove the labelled precursors from the exogenous medium.

When the studies were extended to germinating spores, Staples et al. (1962) found no net synthesis of protein or nucleic acids by the germinating uredospores. In contrast to germinating spores of Aspergillus, Neurospora and Glomerella which assimilated more substrate and synthesized more intermediates, the uredospores incorporated C^{14} into protein in a manner suggesting that proteins were formed by a turnover process. The nature of the block in protein synthesis in uredospores was not known.

More recently Staples et al. (1966) demonstrated that ribosomes extracted from dormant and germinating uredospores were equally effective in incorporating phenylalanine in a polyuridylic acid directed system which included activating

enzymes prepared from rice embryos. Subsequently Staples and Bedigian (1967) described an amino acid incorporating system prepared from bean rust uredospores in which both the activating enzymes and ribosomes were derived from uredospores. From the evidence for polyribosomes in dormant uredospores Staples et al. (1968) suggested that an intact mechanism for protein synthesis in uredospores is under restraint. In a review of the above work and current research, Staples (1968) reported that cessation of germ tube growth correlated with decreasing template activity of spore messenger RNA and decreasing amino acid incorporation by ribosomes and suggested that interaction with a host may allow maintenance of these nucleic acid activities. In addition to the evidence presented for the existence of an intact protein synthesis mechanism, uredospores are also capable of incorporating ammonia nitrogen into amino acids which are in turn utilized in protein synthesis (McConnell and Underhill 1966).

Uredospores are able to fix carbon dioxide into organic acids (Staples and Weinstein 1959, Rick and Mirocha 1968). The former workers found that labelled carbon was distributed rapidly throughout intermediates of the Krebs cycle and postulated that phosphoenolpyruvate carboxylase was the mechanism of incorporation. Rick and Mirocha (1968) demonstrated that the malic enzyme present in uredospores catalyzed the formation of malate from pyruvate and CO_2 in cell-free preparations. The reverse reaction was also demonstrated and no evidence for the mechanism of CO_2 fixation postulated by

Staples and Weinstein (1959) was found. The significance of CO_2 fixation by rust spores is not clear, although it may enhance the Krebs cycle or engage in hydrogen transport by utilizing NADPH from the pentose cycle for the reductive carboxylation of pyruvate to malate. Oxidation of malate in the Krebs cycle would produce NADH which could participate in the electron transport system (Rick and Mirocha 1968).

Germination of rust spores and many other spores is preceded by hydration which may be effected by free water or high humidity (Staples and Wynn 1965). Hydration coincides with an abrupt increase in oxygen uptake, the QO_2 rising from 0.5 to 12 and declining to 5 after the first hour (Williams and Allen 1967). Bush (1968) observed an initial high rate of oxygen consumption by uredospores but detected the decline in oxygen uptake after ten minutes incubation. Germination and oxygen uptake have been stimulated by many exogenous substrates but short chain (C_1 - C_6) fatty acids have proven most effective (Shaw 1964) and probably reflect the utilization of endogenous lipid reserves. Similar findings have been obtained by Bush (1968) and Löesl (1967). In contrast, Walkinshaw (1968) observed that oxygen uptake by aeciospores was inhibited by short chain fatty acids but stimulated by C_{18} unsaturated fatty acids.

Considerable evidence has been obtained (Shaw 1964, Staples and Wynn 1965, Allen 1965) which suggests that uredospores germinate at the expense of lipid reserves. This evidence rests mainly on the low respiratory quotient of germinating spores and the disappearance of lipids. Jackson and Frear (1967) found that total lipids decreased but unsaponifiable lipids and lipid phosphorus (indicative of phopholipid

synthesis) increased during the germination of flax rust uredospores. There was also a rapid transformation of cis-9,10-dihydroxyoactadecanoic acid to threo-9,10-dihydroxyoctadecanoic acid. In an effort to obtain a better understanding of the role of lipids and carbohydrates in spore germination, Daly et al. (1967) utilized uredospores labelled with C^{14} fed to host plants during spore formation. An average of 7% of the spore carbon, which was mostly carbohydrate in nature, was found in the water on which the spores germinated. Soluble carbohydrates were reduced by 30% within the first hour and at seven hours approximately one-half the carbohydrate pool had been metabolized to other compounds. Free fatty acids changed markedly within one hour with a sharp reduction in the amount of cis-9,10-epoxyoctadecanoic acid. Total lipids did not change greatly during seven hours. It is noted that the carbohydrate derivatives, chitin and glucosamine, were found to increase during germination (Gottlieb 1966).

Protein synthesis in germinating uredospores is apparently very low compared to that in saprophytes according to the work from Staple's laboratory which has been mentioned earlier in this review. Dunkle et al. (1969) have shown by the use of inhibitors that completion of infection structure formation is dependent upon protein synthesis. According to Gottlieb (1966) increases in cell protein are equivalent to increases in enzymes. Increased enzyme activity, particularly respiratory enzymes, has been reported by Shaw (1964) and Allen (1965). De novo synthesis of enzymes during germination has

been detected in many fungi, but Ustilago is a notable example since enzymes of the EMP, PP, and TCA pathways were synthesized (Gottlieb 1966). Hemicellulase, cellulase and polygalacturonase were found to increase in concentration during uredospore germination (Shaw 1964). In contrast to Staples et al. (1966), Van Etten (1968) found that an in vitro amino acid incorporating system prepared from resting spores of a facultative parasite was inactive whereas the same system prepared from germinating spores was very active. It was concluded that the enzyme fraction was defective in ungerminated spores and subsequently, Van Etten and Brambl (1968) demonstrated that the specific activity of the aminoacyl-s RNA synthetases is low in ungerminated spores but increases during germination.

Net synthesis of nucleic acids in germinating spores has been documented in species of Aspergillus and Penicillium (Gottlieb 1966, Allen 1965). Staples et al. (1962) did not observe any net synthesis of nucleic acids in germinating uredospores supplied with acetate. However, the germ tube nuclei observed by Maheshwari et al. (1967) indicated a net synthesis of DNA and the arrest of infection structure differentiation by inhibitors of RNA synthesis has suggested that synthesis of a new RNA is a prerequisite to this differentiation (Dunkle et al. 1969). Gottlieb et al. (1968) have described a complexed RNA in teliospores of Ustilago which is enzymatically changed to normal RNA. The appearance of the normal RNA coincided with synthesis of missing enzymes.

Germination of uredospores is affected by self-inhibitors and many external factors. Allen (1955) discovered that a water soluble, volatile substance, synthesized de novo, inhibited germination of dense spore masses floating on buffer. Dinitrophenol, methyl naphthoquinone and coumarin were effective in counteracting the inhibition. In attempting to purify aqueous extracts of the inhibitor, Allen (1957) found a distillate fraction which stimulated germination and differentiation of germ tubes to infection structures. The stimulant was effective either as a solution in direct contact with spores or as a gas.

There are suggestions that a relationship between light and inhibitors may exist (Givan and Bromfield 1964, Wiese and Daly 1967). The depression in germination of spores exposed to 400 foot candles of light is temperature sensitive, suggesting photochemical and enzymatic controls (Givan and Bromfield 1964). Temperature optima for uredospore germination of Puccinia graminis tritici was 22°C (Wiese and Daly 1967), 7°C for Puccinia striiformis (Sharp 1965) and 20° - 30°C for Puccinia cynodontis (Vargas et al. 1967). Surface secretions by host tissues and factors derived from microflora have been implicated as other influences on spore germination (Sharp 1965).

MATERIALS AND METHODS

A. Uredospore culture

1. Plant materials

Uredospores of *Puccinia graminis* Pers. f. sp. *tritici* Erikss. and Henn., race 15B (wheat stem rust) were obtained from the Department of Biology, University of Saskatchewan, Saskatoon. These spores were increased on Little Club wheat, *Triticum aestivum* s. sp. *compactum* (Host) Mackey, grown in a sandy loam in ten-inch plastic pots. Healthy and rusted plants were grown in a Sherer growth cabinet at 22-24°C during the day and 17-19°C during the night. An illumination of approximately 700 ft-c 18 inches from the source (top of pot) and 1100 ft-c 6 inches from the source was available for 16 hours daily.

2. Spore inoculation and collection

Plants one week to one month old were inoculated by rubbing uredospores onto the leaves using a moistened thumb and forefinger. Leaves were then sprayed with distilled water from an atomizer. A plastic bag was placed over the plants in order to maintain high humidity and prior to sealing the bag around the pot, the plants were watered. Inoculated plants were left in a dark growth chamber overnight, after which the plastic covering was removed. Flecking in leaf tissues was evident at 5 days after inoculation and sporulation began at 7 to 10 days after inoculation. Mature spores were collected by tapping the leaves over a manila folder or by suction into a glass collector.

B. Cytology

1. Tissues

Immature uredospores *in situ* in leaf tissue, mature uredospores and hydrated uredospores were studied in thin sections in the electron microscope. Immature uredospores, sampled on two occasions, were obtained by excising unbroken pustules immersed in fixative. Hydrated uredospores were prepared by heat-shocking (5 min at 40°C) spores stored in liquid nitrogen for 7 days. Spores were then (a) floated on 20 ml distilled water in a 9 cm petri dish in darkness at room temperature for 1.25 hr or, (b) placed in aluminum foil boats floating on water in a saturated atmosphere for 24 hr at room temperature in darkness. In a third method, freshly collected spores were sprayed onto 2% agar in 9 cm petri dishes and incubated in darkness at room temperature for 25 min. Mature uredospores no more than 48 hr old were obtained directly from actively sporulating pustules on two occasions. Availability of fresh spores of approximately the same age was assured by collecting spores or shaking plants every two days.

2. Fixation

All tissues were fixed in a mixture of 5% acrolein and 5% glutaraldehyde (final concentrations) in 0.1M phosphate buffer pH 7.2. A trace of 1% Rexol (non-ionic wetting agent) was added to the fixative to ensure wetting of the spores. Excised pustules were fixed in 2 dram shell vials. Mature uredospores were stirred into the fixative in 10 x 75 mm culture tubes and sank within five minutes. Hydrated uredospores were fixed by

flooding the petri dishes with fixative and after one hour the spores were pipetted into a culture tube. Fixation was done at room temperature overnight but never exceeded 20 hours. After primary fixation, tissues were rinsed at least three times with equal parts of buffer and water. Postfixation was accomplished with 1 part 2% $KMnO_4$, 1 part 0.1M phosphate buffer pH 7.2 (Buckley et al. 1968) and 1 or 2 drops of 1% Rexol. Tissues were fixed for 1 hour at room temperature then rinsed with distilled water at least three times over 15 to 30 minutes. Mature spores and hydrated spores were embedded in 1% agar to facilitate handling during dehydration and embedding.

3. Dehydration and embedding

An ethanol series starting with 30% ethanol was employed for dehydration of tissues. Transition to the embedding medium was made with propylene oxide. Maraglas 605 used according to Bislputra and Weier (1963) and Epon-Araldite (Skvarla 1966) were the most satisfactory epoxy resin embedding media. Tissues were soaked overnight in a mixture consisting of 1 part propylene oxide and 1 part resin. The infiltration mixture was then adjusted to 3 parts resin and 1 part propylene oxide and left for 3-4 hours.

Leaf tissues were flat-embedded in pure resin medium in a miniature ice cube tray. Agar blocks containing spores were placed at the top of polyethylene (BEEM) capsules filled with resin. When the tissues had sunk in the resin, the containers were placed in a 60-65°C oven for 24 hours.

4. Sectioning, staining and microscopy

Polymerized blocks were trimmed to a pyramidal face less than 0.5 mm on a side. Sections were cut with glass knives on a Sorvall MT-1 ultramicrotome and were flattened and expanded with xylene vapor. Parlodion-carbon coated grids (3 mm, 200 mesh) were used to collect sections. Staining was done with 5% aqueous uranyl acetate (40 min) and Reynold's lead citrate (20 min).

Sections were viewed and photographed at 60KV in a Philips EM 100B electron microscope. Micrographs were made on a 35 mm film, Recordak Microfile. Measurements of organelles were made on negatives using an 8X Leitz lens.

Sections for light microscopy were cut 0.5 μ thick and stained for 5 min at 55°C with 1% toluidine blue in 1% sodium borate.

RESULTS

A. Preliminary Investigation

Investigation into the structure of uredospores commenced July 1966 at the Department of Biology, University of Saskatchewan, Saskatoon. The investigation began with a light microscope study of sections of mature uredospores embedded in epoxy resin. Adequate fixation, embedding, and stain differentiation were not achieved. Fixatives used were: $KMnO_4$, acrolein-glutaraldehyde plus OsO_4 (Hess 1966), and acrolein or glutaraldehyde alone followed by OsO_4. Epon 812 used according to Luft (1961) did not infiltrate spores adequately and extensive tearing during sectioning occurred. Richardson's stain (Azure 2 and methylene blue in borax solution), Azure B (Dodge 1964), crystal violet, and haematoxylin were tried in numerous attempts to achieve suitable staining and differentiation of spore cytoplasm. The use of light microscopy was eventually abandoned in favour of electron microscopy.

Preliminary ultrastructural investigations at Saskatoon were terminated in late summer 1967 when the author moved to the Division of Plant Science, Faculty of Agricultural Sciences, University of British Columbia.

The use of Maraglas epoxy resin was brought to the author's attention by T. Bisalputra, Department of Botany, U.B.C., and it has proven to be quite satisfactory. Sections of spores postfixed in OsO_4 were found to have a grey overcast which did

not allow stain differentiation of cytoplasm. The use of OsO_4 was discontinued in favour of $KMnO_4$ (Buckley et al. 1968) and all results presented in this thesis have been obtained with a double fixation using aldehydes and $KMnO_4$.

B. Cytology of spore development, maturation and hydration

1. Immature uredospore

Uredospores of Puccinia graminis tritici arise from a binucleate layer of mycelium beneath the epidermis of host tissues. The extent of the infection court and early pustule development on the abaxial leaf surface are shown in Figure 1. The nature of the sporulating tissue and its association with the host tissue can be seen in Figure 2. Nucleoli are evident in nuclei of the basal cells and sporogenous hyphae. Figure 3 delimits areas of increasing differentiation which were found in a study of thin sections made through a young pustule which had not ruptured the host epidermis.

Stages in the ontogeny of uredospores are represented by Figures 4-6. An enlargement (Fig. 7) of the apical cell in Figure 4 shows that prominent nucleoli occur in both nuclei. Increased cell volume is a feature of spore formation as shown by Figures 5 and 6. Thickness of the cell wall increased from 0.2μ in the apical cell to approximately 1μ in the immature spore. Protrusions of the cell wall into the cytoplasm are seen in Figure 5. The complexity of the spore wall increased, as shown in Figures 4-6, with the appearance of spines (Figure 5) and a layering as seen in the upper left part of Figure 6.

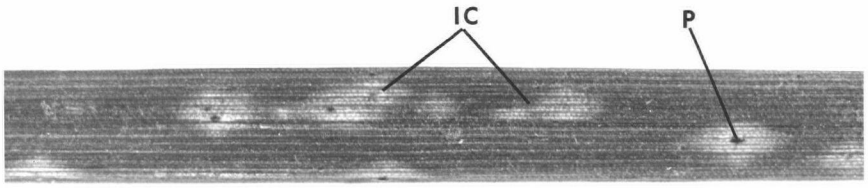

Figure 1. Abaxial surface of wheat leaf showing infection courts (IC) and pustules (P).

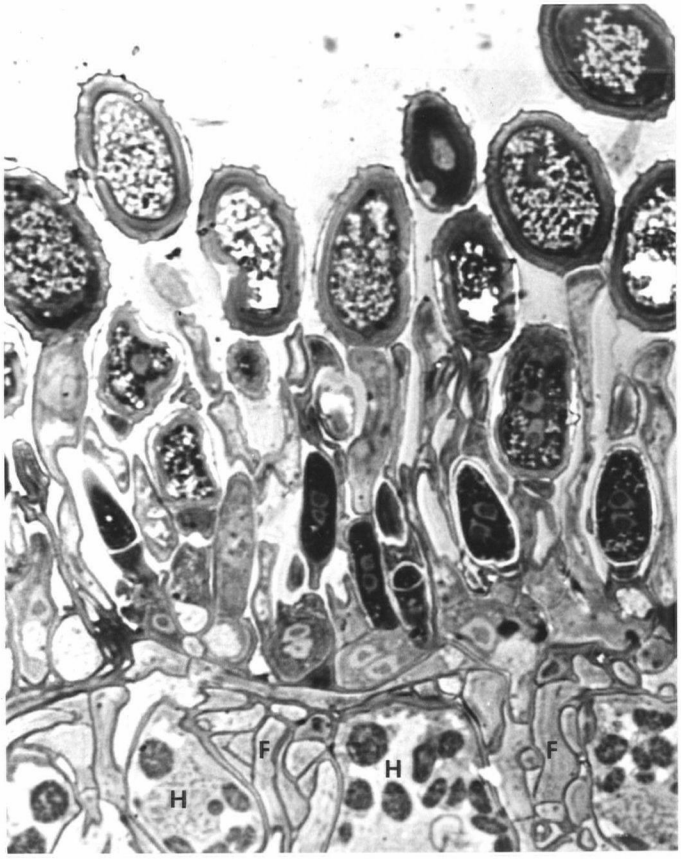

Figure 2. Sporulating rust fungus in pustule. Note association of fungus (F) and host (H). Maraglas section, toluidine blue, phase contrast. X940.

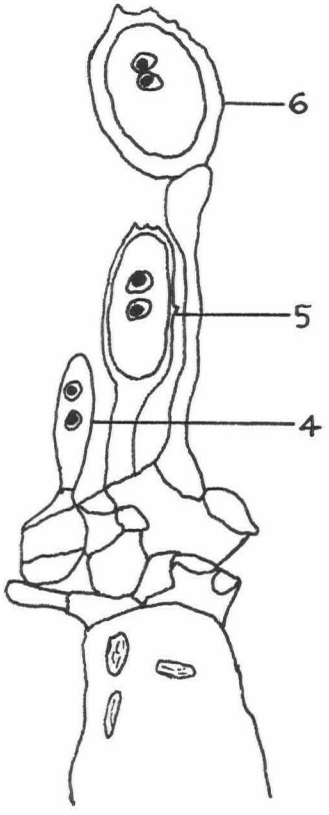

Figure 3. Stages in differentiation shown in Figures 4-6.

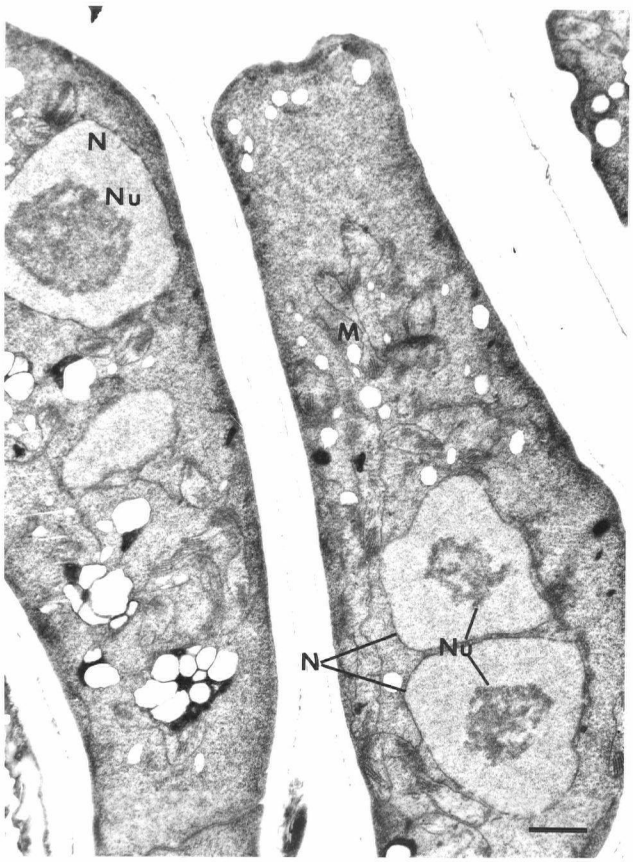

Figure 4. Apical cells in pustule. Note nucleoli (Nu) in nuclei (N) and dividing mitochondrion (M). X7600. *Scale line 1μ in length.

*Scale lines on all micrographs represent distance of 1μ.

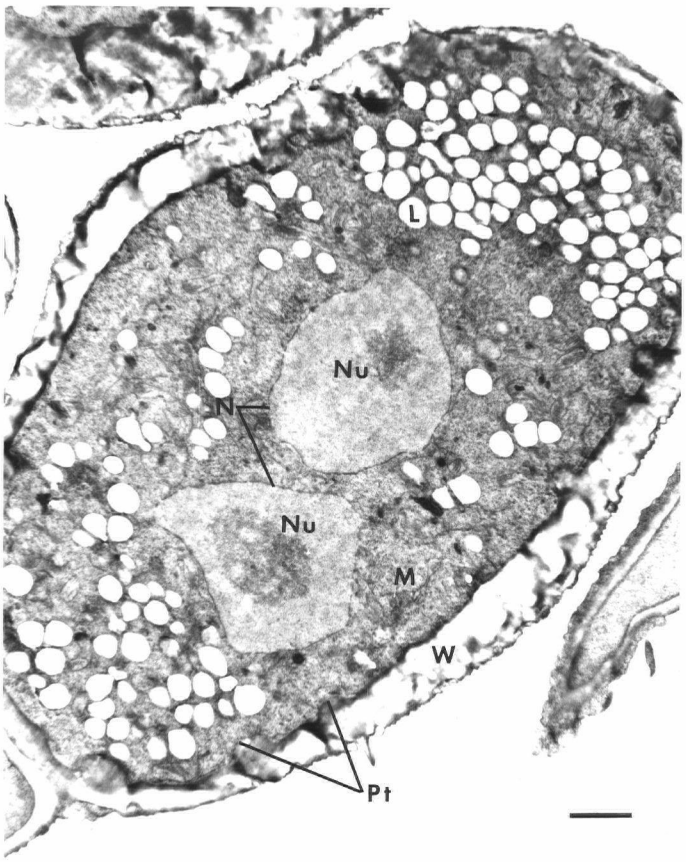

Figure 5. Incipient uredospore showing two nuclei (N) each with a nucleolus (Nu). Note protrusions (Pt) of cell wall (W), increasing number of lipid droplets (L) and dividing mitochondrion (M). X7800.

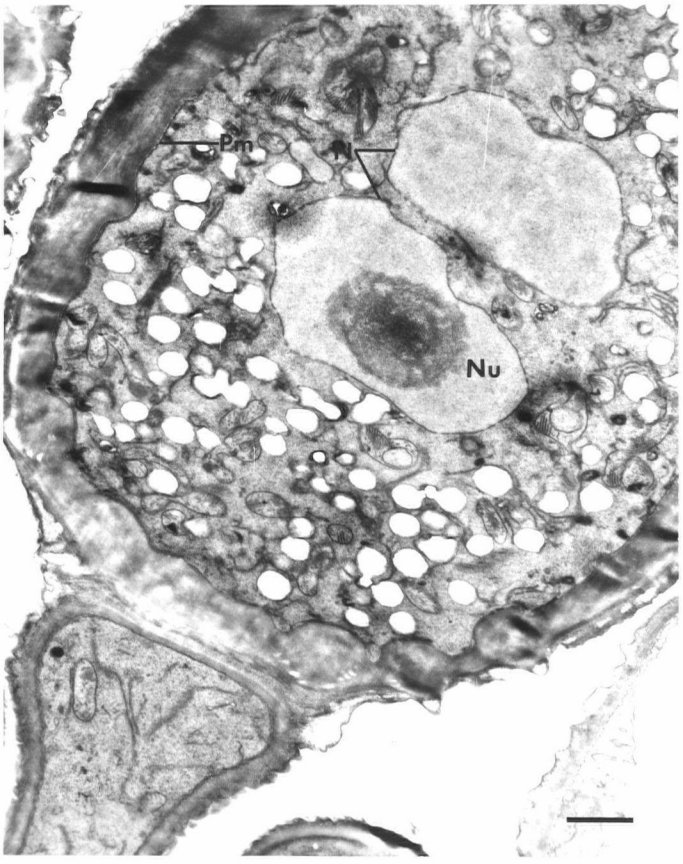

Figure 6. Immature uredospore with prominent nucleolus (Nu) in one nucleus (N). Note undulating plasmalemma (Pm). Compare cell volume and cell wall with previous figures. X8400.

Lipid droplets, visualized as clear white areas, increased markedly in number as spores developed. Some lipid droplets appeared to be membrane bound whereas others were bounded only by cytoplasm (Figs. 7-9). Mitochondria in the differentiating cells appeared elongate with distinct, parallel cristae. Dividing mitochondria appeared elongate but constricted near the middle (Figs. 4 and 5). Extensive variation in the length of mitochondria was found, the shortest being 0.4μ in length and 0.2μ wide and the longest being 1.5μ by 0.2μ. Dimensions of cell walls, lipid droplets and mitochondria of the three spore stages investigated are presented in Table II. Endoplasmic reticulum was present in the form of elongate ribbons. A close association was observed between the ER (Fig. 9) and one nucleus and the double membrane nature of the ER was evident. The plasma membrane appeared as a thin electron dense layer adjacent to the spore wall (Fig. 6) but did not appear smooth at some points. Prominent nucleoli (Figs. 9 and 10) were present in immature uredospores and each nucleus had a single nucleolus (Figs. 8-10). The nucleoli were granular and more dense at the center than the periphery or non-homogeneous. The mean diameter of a nucleus in an immature spore was 3.1μ and the mean diameter of the nucleolus was 1.7μ (Table I). Discontinuities in the nuclear envelope were approximately 500Å in length.

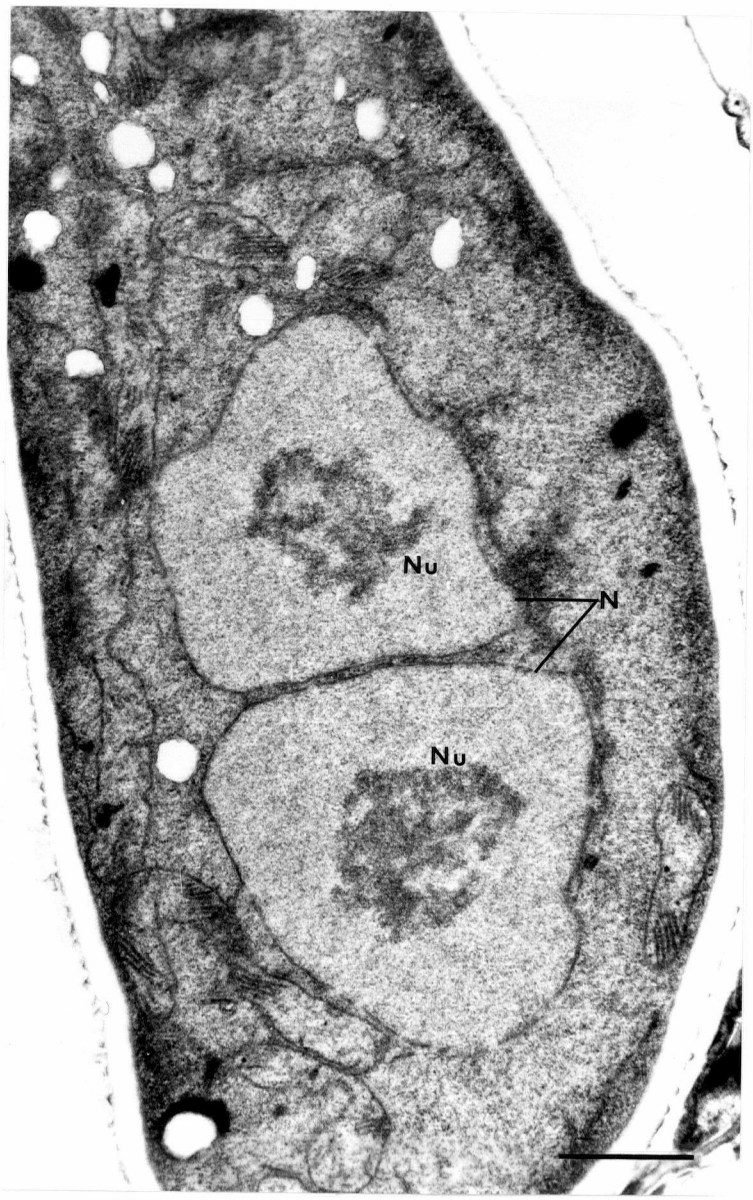

Figure 7. Enlargement of nuclei (N) in apical cell shown in Figure 4. Note prominent nucleoli (Nu). X17400.

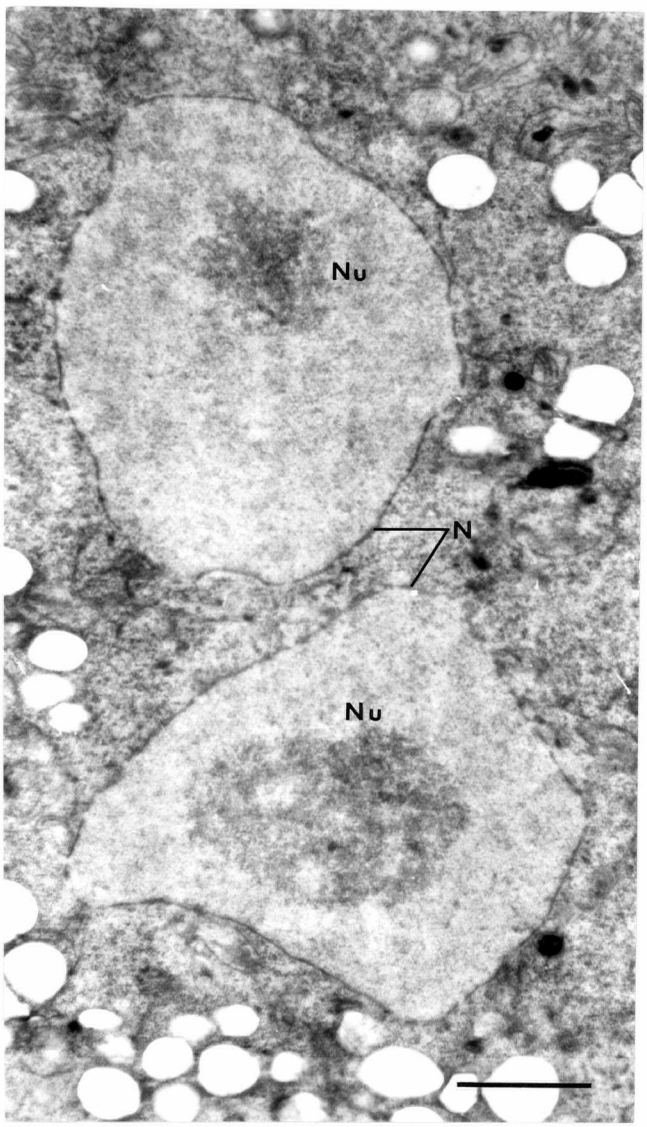

Figure 8. Enlargement of nuclei (N) in incipient uredospore shown in Figure 5. X17400.

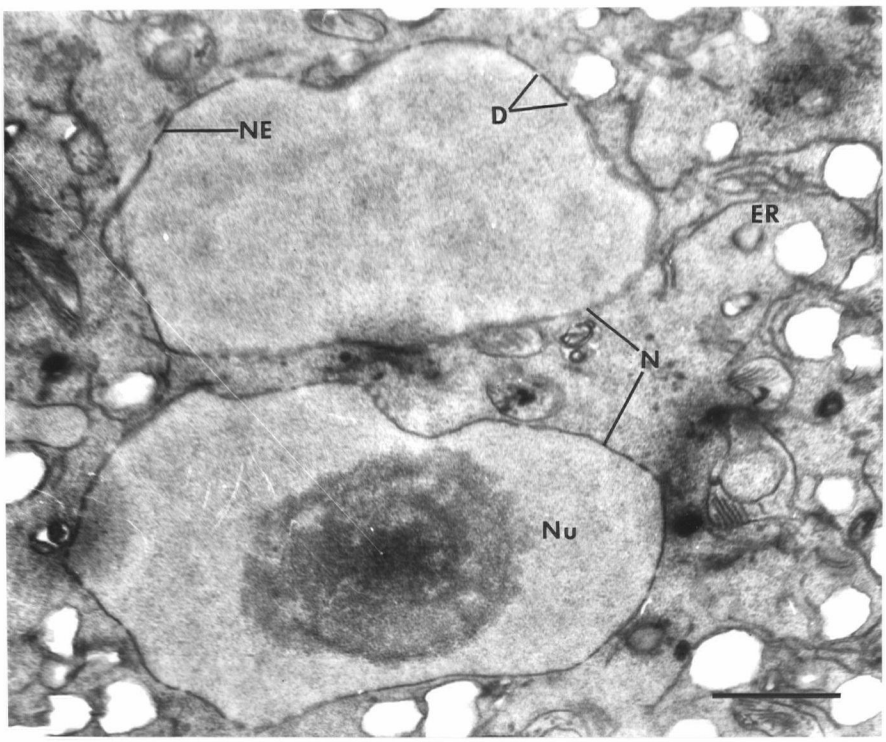

Figure 9. Enlargement of nuclei (N) in immature uredospore shown in Figure 6. Note prominent, nonhomogeneous nucleolus (Nu) in one nucleus, close association of endoplasmic reticulum (ER) and nucleus, and discontinuities (D) in nuclear envelope (NE). X16600.

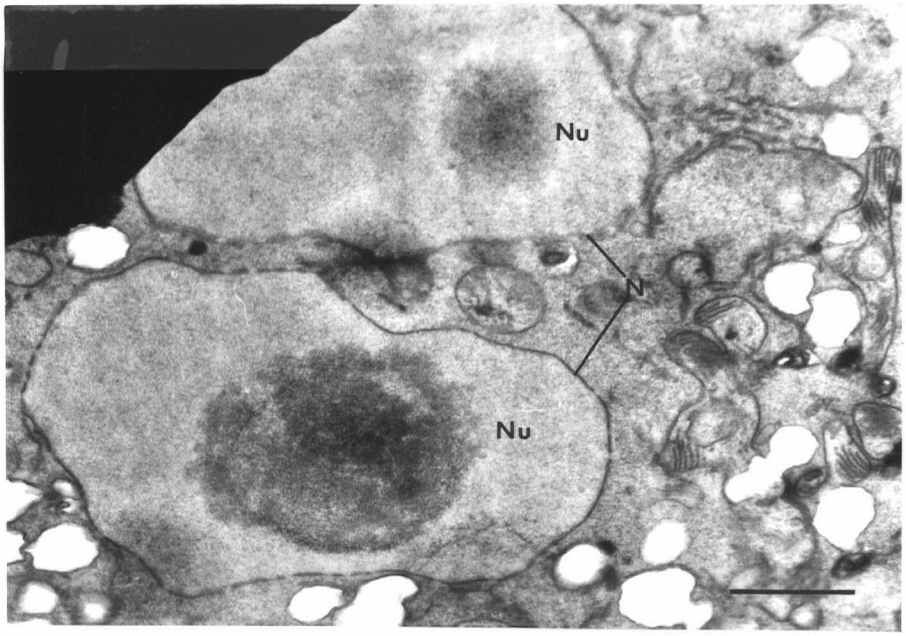

Figure 10. Section of immature uredospore which may be serial to section in Figure 9. Note nucleolus (Nu) in each nucleus (N). X16400.

2. Mature uredospores

Mature uredospores were very difficult to fix, embed, and section due to the impermeability of the thick spore wall. Lipid droplets occupied a considerable portion of the cytoplasm (Fig. 11). Endoplasmic reticulum was present in the form of short, thin or vesiculate ribbons. The double membrane nature of the ER was evident (Figs. 11, 12). In contrast to immature uredospores, the mitochondria of mature spores were nearly spherical. The cristae exhibited a parallel profile. The plasma membrane appeared as a thin electron dense layer adjacent to the spore wall but was separated from the wall at many points (Fig. 11).

Nucleoli occurred in the nuclei of mature uredospores but were much reduced in size. The mean diameter of the nuclei was 2.7μ and that of the nucleoli was 0.5μ (Table I). The nucleoli of mature uredospore nuclei were non-homogeneous (Fig. 12), certain areas being more electron dense than others, but the overall density was not as great as that of nucleoli in immature spores. One nucleolus had a doughnut-like configuration (Fig. 13).

The double membrane nature of the nuclear envelope is evident (Fig. 12). Discontinuities in the nuclear envelopes of the nuclei of mature uredospores had the same dimensions as those given for immature uredospores.

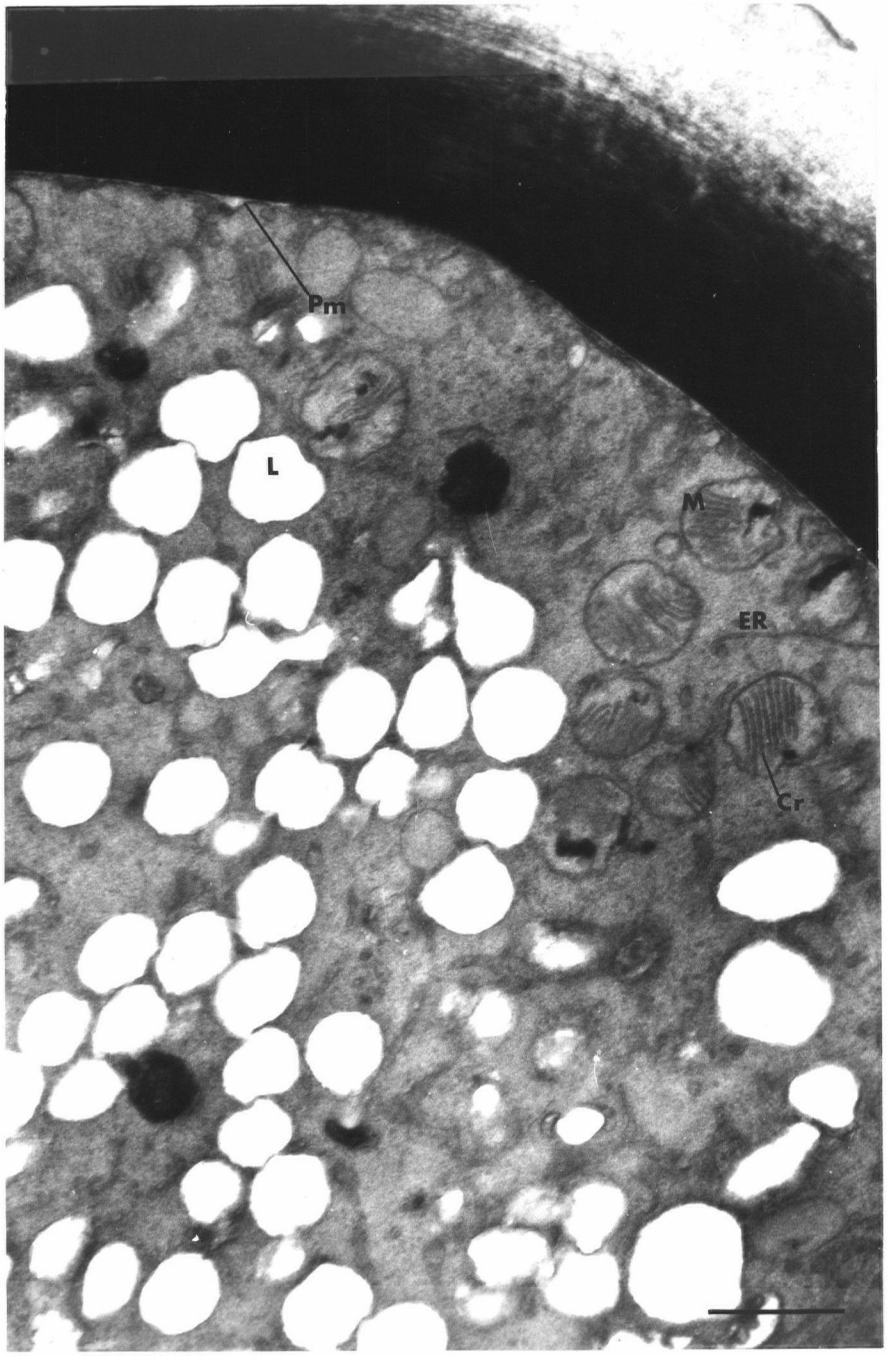

Figure 11. Cytoplasm of mature uredospore. Note abundance of lipid droplets (L), spherical mitochondria (M) with well developed cristae (Cr) and endoplasmic reticulum (ER). Plasmalemma (PM) has separated from cell wall at some points. X18000.

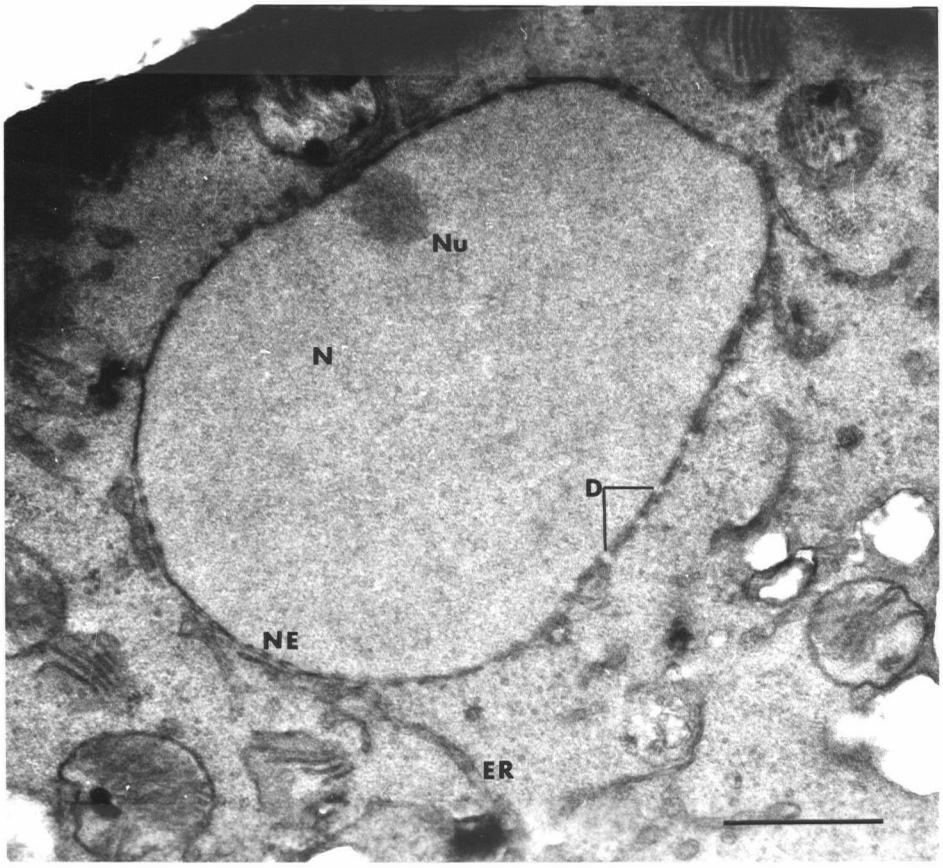

Figure 12. Nucleus (N) of mature uredospore showing nucleolus (Nu) close to nuclear envelope (NE). Note double membrane of nuclear envelope, discontinuities (D) in nuclear envelope and endoplasmic reticulum (ER). X20800.

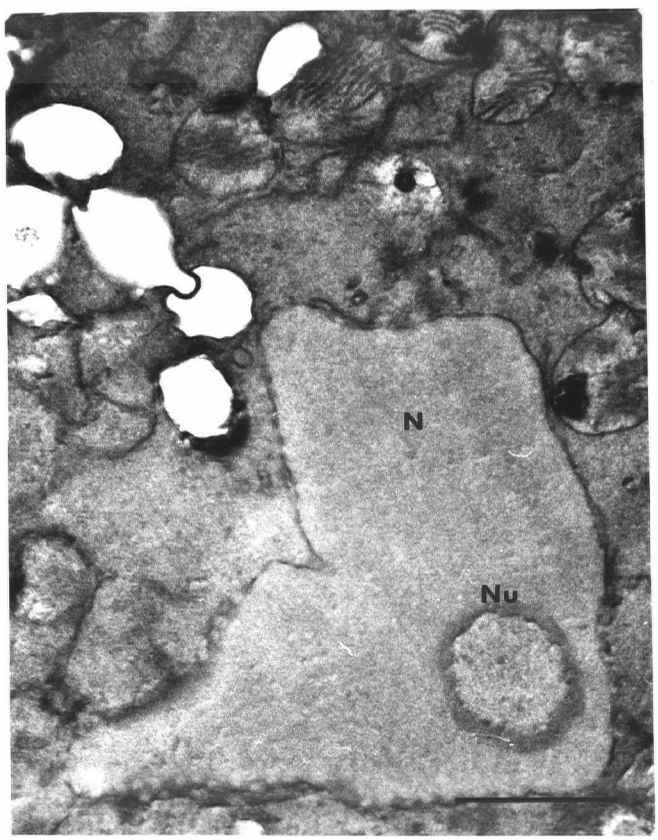

Figure 13. Nucleus (N) of mature uredospore showing doughnut-like nucleolus (Nu). X21,300.

3. Hydrated uredospores

Uredospores hydrated on 2% agar exhibited germ processes after 25 minutes incubation in darkness whereas spores floated on distilled water or in aluminum foil boats were apparently inhibited. In the spores which appeared to be inhibited mitochondrial elongation was the only indication of enhanced metabolic activity. In the spores which had been sprayed onto the agar plates, one or two germ processes developed in many spores and occasionally three in others. Figure 14 is representative of spores hydrated on agar and further description will be restricted to these spores.

The spore wall, which had two zones, did not appear to swell with hydration but loosening and dissolution of outer wall material prevented adequate measurements. Mitochondria assumed an elongate shape similar to that found in the immature spore. Extensive fusion of the lipid droplets occurred although a thin partition could be seen between many droplets. The plasma membrane was a thin, irregular, electron dense layer adjacent to the spore wall but separated from it. Continuity of the plasma membrane of the spore and of the germ process was very distinct. Vesicles occupied a considerable portion of the germ process cytoplasm.

In a total of 38 nuclei observed, no nucleoli were found. The mean diameter of the nuclei in hydrated spores was 3.1μ, the same as that of nuclei in immature spores (Table I).

Figure 14. Cytoplasm of hydrated uredospore. Plasmalemma (Pm) of germ process (GP) is continuous with plasmalemma of spore. Mitochondria (M) appear elongate. Note fusion of lipid droplets (L) and vesicles (V) in germ process. X16,600.

Table I. Diameters of Nuclei and Nucleoli

	Nuclei		Nucleoli	
	No.	Mean diameter (μ)	No.	Mean diameter (μ)
Immature uredospores	20	2.1 ± 0.6*	15	1.7 ± 0.4
Mature uredospores	30	2.7 ± 0.8	3	0.5
Hydrated uredospores	24	3.1 ± 0.5	0	0

*Standard deviation.

Table II. Dimensions of Uredospore Structures

	Spore Stage		
	Immature	Mature	Hydrated
Cell wall thickness (μ)	0.9 (30)*	2.0 (30)	1.8 (8)
Lipid droplet diameter (μ)	0.5 (30)	0.7 (30)	0.5 (30)
Mitochondria length x width	0.7x0.3(30)	0.7x0.6(30)	0.7x0.3(30)
Mitochondria length/width	2.64[a]**	1.15[b]	2.73[a]

*Number of observations.

**Means followed by the same letter are not significantly different (p = 0.1).

4. Observations of a nucleolus

Nucleoli were observed in immature uredospores in 33 out of 57 or 58% of nuclear sections and in 3 out of 55 or 6% of the nuclear sections from mature uredospores. An estimate of the probability of observing a nucleolus in any particular nucleus is given by the expression mean diameter nucleolus/mean diameter nucleus using the data in Table I. Thus, the expected relative frequency with which nucleoli were observed in nuclear sections was 55% for immature and 18% for mature uredospores. The apparent discrepancy between the estimate of 18% and the observed frequency of 6% for mature uredospores may be due partly to lack of sections which pass through the diameter of the nucleus.

DISCUSSION

Differentiation of an organism is reflected by a change in composition or structure, the latter being the most obvious (Baldwin and Busch 1965). The cytological and biochemical changes which occur in fungi during the differentiation of the vegetative state to a spore state have been widely studied in simple systems such as slime molds and yeasts. In these systems, manipulation of growth media is the tool for investigation of metabolic changes. Biochemical investigation of the formation of rust uredospores is hampered by the intimate association of the fungus and host. Uredospore germination, which is a form of differentiation, is not subject to the above limitation and numerous studies of dormant and germinating spores have been cited. The inability of germinating uredospores to synthesize net protein and nucleic acids has been implicated as a possible metabolic lesion (Shaw 1967, Brian 1967, Staples 1968). An ultrastructural study of sporogenesis, mature uredospores and hydrated uredospores was therefore undertaken to determine if definitive nucleoli were present.

The large nucleoli seen in stages of sporogenesis (Figures 4-9) are associated with the ribosomal RNA requirements of a differentiating and growing cell. The nucleoli in the immature uredospore, (Figs. 6, 9 and 10) support the finding of Thomas and Isaac (1967). The nucleolus in these developmental stages may be analogous to the nucleolus in maturing amphibian oocytes which exhibit intense synthesis of ribosomal RNA (Brown 1966). In primary root tissues of Plantago (Hyde

1967) nucleoli are small in the quiescent meristematic zone but increase ten to twelve times in diameter in the actively dividing cells of the cortex, epidermis and root cap. Demands for protein synthesis during spore formation are visualized in cell enlargement (Figs. 4-6), cell wall synthesis (Fig. 5), and the accumulation of lipid droplets (Figs. 4-6). The latter probably involves the conversion of carbohydrates to lipids. The protrusions of cell wall into the cytoplasm (Fig. 5) are possible sites of cell wall synthesis.

During maturation of the spore on the stalk, the spore wall increased in thickness. The average thickness of 2μ, given in Table II, may be high due to many spore walls being sectioned obliquely. The number of lipid droplets in mature spores appears to increase beyond that in immature spores. A layer of vesicles which Williams and Ledingham (1964) observed as the plasmalemma may have been a result of fixation damage. In the current study the plasmalemma of the mature spore (Fig. 11) was seen as a thin electron-dense layer which had pulled away from the spore wall at some points. This separation of the membrane and spore wall may have been fixation damage in part but Hawker (1965) states that the plasmalemma in the young fungal cells while normally in close contact with the cell wall may undulate or show invaginations in older cells.

Lipid droplets observed in mature spores have the same size as those described in earlier reports (Williams and

Ledingham 1964, Manocha and Shaw 1967). Unlike Williams and Ledingham (1964) the dense layer surrounding some lipid droplets (Figs. 9, 14) is not interpreted as a membrane. Fawcett (1966) states that the dense line (product of fixation at cytoplasm-lipid interface) around the periphery of the droplet is thicker and denser than ordinary membranes and does not have the trilaminar structure of lipoprotein membranes.

Endoplasmic reticulum (Fig. 12), visualized as thin ribbons, corresponds to that reported by other workers. No evidence of ribosome groupings on the ER was observed but the film magnification was low ($<$2500X) and the use of $KMnO_4$ may have disrupted ribosomes.

Mitochondria in mature uredospores tended to be spherical and in contrast to the observations of Williams and Ledingham (1964) and Manocha and Shaw (1967) the cristae appeared well developed and were arranged in parallel arrays.

The occurrence of reduced nucleoli in mature uredospores is the most significant finding of the study. Williams and Ledingham (1964) did not observe a nucleolus in any nucleus although they admitted that the number of sections viewed was too small to exclude the existence of a nucleolus. It is very doubtful however, that their preparations would have revealed nucleoli since $KMnO_4$ preserves little more than lipoprotein membranes. Manocha and Shaw (1967) were unable to detect nucleoli in mature flax rust uredospores but this may have resulted from the use of $KMnO_4$ as a primary fixative or an insufficient number of sections viewed. In the present

study a nucleolus was found in 3 out of 55 nuclear sections. The difficulty of sectioning mature spores with glass knives reduces the number of adequate sections. A reduced nucleolus, as shown in Figure 12, may be present in every nucleus. Alternatively, the observed nucleoli may signify degeneration of all nucleoli with increasing spore age. Decreased size of the nucleolus or complete absence of the nucleolus could result from repression of that part of the genome concerned with synthesis of ribosomal precursors. The nucleolus shown in Figure 13 had a doughnut-like configuration for which no explanation is available. There is no evidence to suggest that it is an intermediate stage in the reduction of the nucleolus from the size presented in Figure 9, to the size in Figure 12. Doughnut-like configurations of nucleoli have been reported in interphase and prophase nuclei in Vicia faba (Lafontaine and Chouinard 1963), cortex cells of Plantago (Hyde 1967) and in young and old leaves of Stellaria media (Weintraub et al. 1968). Nucleoli with two zones which were differentiated by cytochemical tests or staining methods have been described by Snoad (1956). Hyde (1967) suggests that the doughnut-like and angular configurations in Plantago may result from (a) synthetic activity of nucleolus, (b) mechanical relationship of nucleolus and chromosomes, and (c) rearrangement of nucleolus during duplication of chromosomes.

Hydration of uredospores in non-inhibiting conditions resulted in rapid initiation of germination as seen by the

presence of germ processes in approximately one-half hour (Fig. 14). Mitochondrial elongation and fusion of lipid droplets were obvious cytological changes.

The shape of mitochondria at sporogenesis and hydration is significantly different, at the 1% level of significance, from the mitochondria of mature spores (Table II). It is concluded that the elongate mitochondria were associated with actively respiring tissue.

Nucleoli were not found in any of the nuclei of hydrated spores in the present study and the germ tube nuclei described by Manocha and Shaw (1967) did not possess any nucleoli. This evidence suggests that nucleolar function is completely repressed through the early stages of germination. It is significant that dormant conidia of Verticillium albo-atrum, a facultative parasite, do not exhibit a nucleolus but that during germination a nucleolus appears and increases in size (Heale et al. 1968). This is consistent with the demand for protein synthesis in the germling.

Immediate enzymatic functions within the germinating uredospore would be the mobilization of lipids and carbohydrates for synthesis of membranes and cell wall of the germ tube, and dissolution of the germ pore. Vesicles in the germ processes may be transporting substrates for synthesis of cell components. Wynn et al. (1966) have suggested that the germinability of uredospores depends on the ability of the spores to mobilize food reserves. Hemicellulase, cellulase and polygalacturonase have been reported to increase during spore germination (Shaw

1964) and it is conceivable that they could be synthesized from existing protein. The potential for protein synthesis in germinating uredospores has been likened (Shaw 1967) to the anucleolate mutant of Xenopus (South African clawed toad), described by Brown (1964). The anucleolate mutant of Xenopus develops to the swimming stage, utilizing preformed ribosomes, then dies for lack of new ribosomes.

Amino acid incorporating systems derived wholly or in part from uredospores have been described by Staples and his co-workers and it has been concluded that uredospores possess a complete protein synthesizing apparatus which operates at very low rates. More recently (Dunkle et al. 1969), development of infection structures was shown, by use of appropriate inhibitors, to be dependent on RNA and protein synthesis, although the species of RNA that is affected is not known. The germ tube nuclei observed by Maheshwari et al. (1967) are evidence that a functional system of nucleic acid and protein synthesis exists. The presence of a diminished nucleolus in mature uredospores as described earlier suggests that ribosomal RNA may normally limit growth of this obligate parasite in the absence of a susceptible host tissue. It has been suggested (Shaw 1967) that if indeed the nucleolar apparatus of uredospores is repressed, interaction of the fungus with the host may provide the stimulus to reactivate synthesis of ribosomal RNA and protein. Presumably some component of the media used in the successful axenic culture of wheat and

flax rusts (Williams et al. 1966, Williams et al. 1967, Bushnell 1968, Scott 1968, Turel 1969, and Coffey and Bose 1969) fulfills the role normally played by a susceptible host.

The formation of uredospores and their subsequent germination is an intriguing problem in cell biology since normal nucleolar function in the mature spore appears to be repressed. Further studies of rust spore formation and spore germination could include a high resolution study of the nucleolus although improved fixation and embedding must be attained. Light microscope studies of nuclear behavior utilizing whole amounts of germ tubes and infection structures or half-micron thick glycol methacrylate sections would be fruitful. It is desirable to study spores germinating in a manner similar to that occuring in nature. Attention is drawn to the heat-shock induction of infection structures by Maheshwari et al. (1967). Studies of nucleic acid and protein synthesis in the germling, using inhibitors, should also provide insight into the nature of nuclear control. The axenic culture of rusts can provide tissues for cytological study and it is expected that improvements in culturing will yield sufficient quantities of tissue for biochemical study.

SUMMARY

1. An electron microscope study of immature uredospores, mature uredospores and hydrated uredospores of *Puccinia graminis tritici* (Race 15B) was undertaken to determine if definitive nucleoli were present.
2. Prominent nucleoli (1.7μ diam.) were present in the dikaryotic fungal tissue giving rise to and including immature uredospores.
3. Reduced nucleoli (0.5μ diam.), which were less dense than nucleoli found in immature uredospores, were found in 3 out of 55 nuclear sections from mature uredospores. Two nucleoli had a normal shape while the third had a doughnut-like configuration.
4. Nucleoli were not observed in nuclei of hydrated uredospores.
5. Increasing cell volume, increasing cell wall thickness and accumulation of lipid droplets were evident parameters of growth and development during sporogenesis.
6. Elongate mitochondria in immature and hydrated uredospores were distinct from the round mitochondria of mature uredospores.

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