EFFECTS OF GRISEOFULVIN ON DERMATOPHYTES

by

William Pattison Ronald

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Department of **Bacteriology and Immunology**

The University of British Columbia, Vancouver 8, Canada

Date **May 3, 1964**
ABSTRACT

The site of action of griseofulvin, a drug which is reported to be fungistatic in nature, has been under study for some time. As yet though, there is still a great deal of uncertainty as to which observed effects are primary and which are secondary.

In the study reported here, initial experiments were involved with effects of griseofulvin on oxygen uptake, in correlation with cell starvation. The effects of the drug on glucose oxidation also were studied. Definite alterations were noted in both of these areas. Further investigations were carried out, utilizing cell-free extracts and techniques for measurement of dehydrogenases, but these proved unsuccessful. Amino acid metabolism also was surveyed but no evidence of any alteration was observed.

Attempts to produce protoplasts from dermatophytes were successful, and utilizing these structures, investigations into the effects of griseofulvin on cytoplasmic membrane permeability and on cell wall resynthesis, were carried out. In both cases the alterations were small and appeared to be secondary in nature.

In the final study, purified cell walls of organisms grown in the presence and absence of griseofulvin, were compared on the basis of amino acid, amino sugar, and sugar content. No differences were observed in these preparations.
In addition, no evidence was found to show that griseofulvin was incorporated into cell walls.

It was concluded that griseofulvin may possibly affect the enzymes involved in the synthesis of the substrates of endogenous respiration, or the mechanisms controlling these enzymes. It was also concluded that the drug's site of action is probably on or near the cytoplasmic membrane, and by inference that the biochemical site may be in the area of purine and pyrimidine metabolism.
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INTRODUCTION

Griseofulvin is a fungistatic drug which is commonly isolated from the culture media of several *Penicillium* species. It was first recognized by its characteristic effect of causing the hyphal tips of many fungal species to become stunted and distorted. Early workers thus referred to this compound as "curling factor" (17, 18). In 1947, it was discovered that "curling factor" was a previously isolated compound known as griseofulvin (36).

Following the discovery of the antifungal properties of griseofulvin, many studies were carried out to determine its physiological site of action in susceptible fungi. These attempts met with little success and thus experimental work in this field slowed considerably. The discovery, in 1958, that griseofulvin was an effective agent for curing superficial skin infections caused by the dermatophytic fungi (26), caused an immediate revival of interest in this area. Studies were again initiated to determine the site of action of this drug, but up to the present, these efforts have been almost completely unsuccessful. In fact, much of the literature consists of conflicting reports and personal opinions rather than definite experimental findings.

One area of investigation which shows some promise is that of the oxidative pathways. Results have shown that uptake of phosphorus is decreased (12) and that assimilation of nitrogen
is lowered (71) in the presence of griseofulvin. It has been suggested that an oxidative phosphorylation step is being inhibited (71), but conflicting results in oxygen uptake investigations (15,44,63) have resulted in a lack of confirmation.

It is the purpose of this study to repeat these oxygen uptake investigations and in addition to compare the effects of the drug on the respiration of cells which have been starved for various lengths of time. It appears that a partial depletion of endogenous reserves in the cells may be responsible for conflicting results.

It is also the purpose of this study to carry out investigations into facets of this problem which have not been previously explored. For example, individual enzyme systems, cell-free extracts, membranes, and cell walls. If any effects of this agent are demonstrated using these materials, more detailed experiments will be undertaken.
REVIEW OF THE LITERATURE

Historical review. Griseofulvin is a fungistatic drug produced by several species of Penicillium. It was found by Brian et al. (17) in 1945, as a substance produced by Penicillium janczewskii, which caused abnormal development of young hyphae of Botrytis allii. A year later these workers reported isolating this compound, which they referred to as "curling factor" (18). They demonstrated no antibacterial properties. Just after this, McGowan investigating the chemical properties of "curling factor", proposed the formula C_{20}H_{20}O_{9} (45). In 1947, Grove et al. (36) reported the presence of chlorine in the compound and put forward a new formula C_{17}H_{17}O_{6}Cl. They showed that "curling factor" was in effect griseofulvin, a compound isolated from Penicillium griseofulvin in 1939, by Oxford et al. (53). Oxford had proposed a structure for griseofulvin and this was supported at first by Grove and McGowan (36). On the basis of infrared and ultraviolet absorption spectral data however, they were forced to postulate a new structure. This structure put forward by Grove et al. in 1951 (35) is shown in FIGURE 1.

FIGURE 1. The Structure of Griseofulvin.
The chemistry of griseofulvin has been under study for some time by fellow workers of Grove at the Butterwick Research Laboratories and also by other workers at the Glaxo Laboratories Ltd.

The preceding paragraph has primarily been an account of the problems of defining griseofulvin as a chemical entity, but there was also a considerable lag before its importance as an antifungal agent was realized. In 1949, Brian, the original discoverer of the antifungal effects of griseofulvin, carried out inhibition studies with the drug on a large number of representative fungi (15). He did not include a representative dermatophyte in his test series, thus there was still no indication of the medical importance of the drug. The emphasis was rather on plant protection and for some years it was put to this use (16). The medical value of the drug was realized in 1958, when Gentles (26) published his paper on the treatment of ringworm in guinea pigs by oral administration of griseofulvin.

**Dermatophytes.** Dermatophytes are usually defined as those fungi causing superficial diseases of the skin and hair. They are further limited as being in the genera; *Microsporum*, *Trichophyton*, and *Epidermophyton*. It must be remembered though, that only those members which will actively invade the skin or hair are dermatophytes. Other non-pathogenic fungi are found in these genera.

The dermatophytes are commonly classed as Fungi Imperfecti but recently, Benedek has demonstrated the formation of cleistothecia, that is perfect fructifications, by two members of Sabouraud's form genus Microsporon by means of symbiosis with
Bacillus weidmaniensis Benedek, 1938 (8). Other workers carrying out soil studies, have described the perfect stages of Microsporum gypseum (65), Microsporum nunum, and Keratinomyces ajelloi (22). The last organism was classed with the dermatophytes by Georg et al. in 1959 (34). This does not necessarily mean that all dermatophytes will be shown to form perfect stages but it may mean that they have evolved from Ascomycetes.

The invasion of tissues by dermatophytes has been termed "superficial". This means invasion of non-living tissue. These organisms then, grow mainly in the dead keratinized areas of the host. Dermatophytes have been found growing in the tissues, causing "deep mycoses". In these cases they have been found as a yeast-like form rather than the typical hyphal form. A similar growth form has been caused using cysteine as the inducing agent (57). Fortunately such deep infections are very rare and it is as yet uncertain how the induction occurs in the body.

Medical aspects of griseofulvin. On the basis of his initial studies, Gentles (26) felt that this drug probably fulfilled Wilson's definition of an ideal antifungal agent, at least as far as the dermatophytes are concerned. Wilson (70) stated that, "the ideal antifungal drug even for superficial mycoses would seem to be one which could be safely administered internally in amounts sufficient to endow the cells eventually destined to produce keratin with the power to resist fungi completely, this power persisting as they become keratinized,
and the drug thus exerting its effects from within outward". Through the work of Gentles (26,27,29,30,31,32) and others (54,58), griseofulvin has been shown to do exactly what Wilson's definition requires. It is incorporated into those cells which later form the keratin, thus endowing the patient with a tissue which is inhibitory to the fungus. Such a mode of action appears mandatory, as penetration of keratin by topical application, even of griseofulvin, has been found almost impossible. The greatest weight of literature at this time is on the clinical experiences with griseofulvin. As this is not the subject of this thesis, I will not mention the clinical cases and side effects involved in the use of this drug.

**Chemical properties of griseofulvin.** Griseofulvin is a colorless, odorless, white, crystalline, neutral compound. It has a melting point of 218-219°C and is exceedingly thermostable. It readily forms a crystalline mono-oxime, and it also reacts with phenylhydrazine and with 2,4-dinitrophenylhydrazine although crystalline compounds can not be readily obtained (53). This compound has absorption maxima at 296μμ and 326μμ(in water) (2), and at 289μμ (in butyl acetate) (59). Its characteristic ultraviolet and infrared absorption spectra were shown by Grove and McGowan (37). It is sparingly soluble in water which makes it difficult to handle under physiological conditions. It is soluble in ethanol, acetone, acetic acid, butyl acçetate, and N,N-dimethylformamide to name a few of the more common solvents.
Antifungal properties of griseofulvin. A report by Brian et al. in 1946 (17) was the first recorded observation that griseofulvin was antifungal in its properties. They noted that, in the presence of the drug, hyphae of *Botrytis allii* were stunted, distorted, and showed increased branching (in low dilutions of griseofulvin) or "waving" (in greater dilutions). They found that higher concentrations of the compound stopped the growth of *Botrytis allii* conidia, but only after some growth had occurred. It was also of interest to them that stunting and distortion of the hyphae occurred at much lower concentrations of griseofulvin than were required to stop conidial germination. Brian continued his studies by surveying the effects of this agent on a large group of representative fungi, actinomycetes, bacteria, and even some seeds of higher plants (15). He found that, "all Basidiomycetes, Ascomycetes, Fungi Imperfecti, and Zygomycetes, with the exception of two yeasts, were sensitive to griseofulvin", and also that, "it has no apparent effect on bacteria, actinomycetes, or on certain groups of fungi (Oomycetes and yeasts); it inhibits the germination of seeds of higher plants and retards root extension". His final conclusion was that griseofulvin acts on only those fungi which have chitinous cell walls while fungi with cellulose-containing walls are not affected. This is a very broad statement and there appear to be some exceptions (2).

Further visual observations have been carried out more recently using electron microscope techniques (10,66). On close
observation of the cell wall, it was seen that the drug caused a loss of integrity. The wall became much thicker, and splits appeared until it became separated into frayed and irregular layers. The cytoplasm was seen to become reduced, leaving remnants of cytoplasmic membrane and large lipid storage granules. Younger, actively metabolizing cells showed more drastic alteration than older cells.

The greater susceptibility of young hyphae to griseofulvin has frequently been mentioned in the literature (10,15). This was demonstrated more fully by Banbury (7). He applied the drug to growing portions of Phycomyces blakesleanus sporangio-ophores and noted a curvature. He noted no such reaction when the application was made to the non-growing area several millimeters below it. Other work has shown that no translocation of griseofulvin occurs within fungal hyphae and therefore intimate contact between the growing area and the drug is required (2). It has also been suggested that griseofulvin is fungistatic to older hyphae, but fungicidal to young growing hyphae (23). That it is fungistatic has been demonstrated (17,56).

Biochemical studies on the action of griseofulvin on fungi are as yet very limited. Oxygen uptake in the presence of the drug has given conflicting results (15,44,63) although it does seem likely that a reduction in respiratory rate occurs (63). Coupling this latter suggestion with other results, showing a decrease in phosphate uptake and a decrease in nitrogen
assimilation, it is felt that griseofulvin uncouples a phosphorylation in a respiratory pathway (12,71). However, according to Gentles (33), Rhodes feels that the compound may be incorporated into cell wall, giving a modified chitin. McNall, on the other hand has proposed interference with nucleic acid synthesis. He demonstrated partial reversal of the effect of griseofulvin with purines, pyrimidines, and their nucleotides (47).

Resistance by dermatophytes to griseofulvin. One of the early questions asked by medical workers in relation to a new drug, is; how easily and therefore how frequently may the susceptible organism become resistant to the drug? This of course was also the case with griseofulvin. In May 1960, four separate publications appeared, dealing with the problem of "in vitro" resistance by dermatophytes (4,60,62,64). The general conclusions were that all dermatophytes tested, acquired some "in vitro" resistance, but that on return to "in vivo" conditions, this resistance was lost. Isolates from patients under griseofulvin therapy were found to have no "in vitro" resistance. For a short period the situation looked extremely hopeful but then two cases of "in vivo" resistance were reported (41,50). One was experimentally induced, the other occurred naturally.

It was suggested that griseofulvin resistance was due to an enzymatic system which degraded the drug (4). This has been shown to occur with several species of fungi including Microsporum canis (13). The mechanism of deactivation was a demethylation, in all cases tested, but the particular methyl
group removed varied with different fungal species. *Microsporum canis* produced 4-demethylgriseofulvin, from griseofulvin.

**Griseofulvin analogues.** The structure of griseofulvin in relation to its activity is an important property. However, until recently few reports have appeared on the inhibitory action of griseofulvin analogues. Chemists at the Butterwick Research Laboratories in England have reported the preparation and isolation of many compounds closely related in structure to griseofulvin, but they have given little indication of their inhibitory activities. In one paper they did briefly mention the antifungal effects of dechlorogriseofulvin and of the bromo analogue of griseofulvin (46). In both compounds the chlorine of griseofulvin has been replaced. In one instance it is by hydrogen and in the other it is by bromine. They tested the analogues with *Botrytis allii* and found that to produce the effect caused by 0.1 μg./ml. of griseofulvin, they required 0.75 μg./ml. of the bromo analogue and 6.25 μg./ml. with dechlorogriseofulvin.

A report by Abbot and Grove in 1959 (1,2) refers to a "diol" (7-chloro-4':6'-dihydroxy-4:6-dimethoxy-2'-methyl grisan-3-one) which they tested with a strain of *Botrytis allii*. They found the "diol" to be less active than griseofulvin.

Finally, a paper by Boothroyd et al. in 1961 (13) reports demethylated products of griseofulvin. These compounds were found as inactive products of fungal metabolism of griseofulvin. They are 4-demethylgriseofulvin, 6-demethylgriseofulvin, and 2'-demethylgriseofulvin. These compounds although very similar in
structure to griseofulvin demonstrated no inhibitory activity.

**Cell wall constituents of dermatophytes.** The earliest studies on dermatophytic cell walls were carried out by Blank (9) on cell wall residues of alkali extractions. He concluded that the framework of the walls was composed completely of chitin. His methods were rather drastic, so that many wall components were lost. More recently, mechanical breakage and analysis of walls by McNall has yielded a different picture (48). He showed them to contain large quantities of polysaccharides of glucose and glucosamine, and in addition some protein and lipid. The protein reported by McNall is possibly a peptide, as a peptide linked to glucosamine has also been reported by Carlson and Knight (19) in a crude chitinase resistant fraction of a dermatophyte cell wall.

**Fungal protoplasts.** Perhaps the term "protoplast" is not entirely correct in referring to the fungi, but when fungal hyphae are attacked by mixtures of such enzymes as Streptomyces chitinase, $\beta$-1,3-glucanase, and digestive juice of *Helix pomatia*, "protoplast-like" structures are formed. Of course, this must be done under conditions of sufficient osmotic strength to prevent osmotic shock or disruption of these fragile structures. Crude digestive juice of *Helix pomatia* is probably the most commonly used enzyme for the production of fungal "protoplasts". It contains several enzymes which will attack various fungal wall components, so that it may be employed to produce "protoplasts" of yeasts as well as of filamentous fungi (40).
techniques normally used with filamentous fungi are modifications (3,43) of those methods described by Bachman and Bonner in their work with *Neurospora crassa* (5). As yet, no reports have been published on the production of "protoplasts" of dermatophytes, although purified cell walls of *Trichophyton mentagrophytes* have been reported to be partially digested by crude snail gut chitinase (19). A good criterion for the definition of a "bacterial protoplast" has been put forward by Brenner et al. (14). This could also be used for defining fungal protoplasts.
MATERIALS

I. Glassware

All glassware used throughout the course of these experiments was soaked or boiled in a 2% Haemo-sol solution for at least one hour. It was then washed and rinsed with warm water, followed by rinsings with cold tap water and finally with distilled water.

II. Reagents

A. Griseofulvin Solutions

The griseofulvin used in this study was a highly purified sample, which was kindly donated by Schering Corporation Limited, Montreal, Quebec. The following griseofulvin solutions were used in this study:

1. 2,000 μgm./ml. in redistilled butyl acetate (b.p. 124° to 126°C).

2. 2,000 μgm./ml. in 50% ethanol. The griseofulvin was dissolved in a minimum volume of 95% ethanol and then gradually diluted with distilled water. During the addition of the water, the solution was slowly agitated, using a magnetic stirrer. If precipitation occurred while the water was being introduced, a small volume of 95% ethanol was titrated in. When the required volume of water
had been added, the solution was brought to its final volume with 95% ethanol.

3. 600 µgm./ml. in redistilled N,N-dimethyl formamide (DMF) (b.p. 151° to 153°C).

B. Chromatographic Reagents

1. Solvents
   a. Butanol-acetic acid-water, 2:1:1, (v/v).
   b. Butanol-methyl ethyl ketone-water, 2:2:1, (v/v).
   c. Ethyl acetate-pyridine-water, 8:2:1, (v/v).

2. Developers
   a. 0.5% (w/v) ninhydrin in n-butanol.
   b. 0.5 ml. saturated silver nitrate solution in 100 ml. acetone, add distilled water until precipitate disappears.
   0.5 N sodium hydroxide in 80% ethanol.
   5% (w/v) Na₂S₂O₃·5H₂O in distilled water.

3. Standards
   a. Amino acids: 15 µM/ml. (0.01 ml. applied to chromatogram).
   b. Sugars and amino sugars: 2.0 mgm./ml. (0.01 ml. applied to chromatogram).
C. Hexosamine Assay Reagents

1. Glucosamine-HCl stock solution: 50 µgm. glucosamine-HCl /ml. in distilled water. Diluted to the following standards: 5.0 µgm./ml., 10 µgm./ml., 20 µgm./ml., 30 µgm./ml., and 40 µgm./ml.
2. 0.5% (w/v) phenolphthalein in 95% ethanol.
3. 2% (v/v) acetyl acetone in 1.0 N sodium carbonate. The acetyl acetone was redistilled and the fraction boiling at 140° to 141°C was used.
4. Ehrlich's reagent: 2.67% (w/v) p-dimethylaminobenzaldehyde in 95% ethanol and 12 N HCl, 1:1 (v/v)

III. Enzymes

A. Partially Purified Chitinase

A partially-purified sample of chitinase, isolated from the intestinal juices of the snail Helix pomatia, was kindly supplied by Dr. G. Strasdine (National Research Council, Ottawa, Canada).

B. Crude Snail Gut Juice

"Suc digestif d'Helix pomatia" was obtained from L'Industrie Biologique Française, 35 à 49, Quai du Moulin de Cage, Gennevilliers (Seine), France. This material contains a mixture of enzymes, including chitinase and cellulase.
C. Trypsin

This proteolytic enzyme was obtained from Nutritional Biochemical Co. It was used in the concentration of 0.5 mgm./ml., in 0.066 M phosphate buffer pH 7.6.

D. Ribonuclease

This enzyme was obtained from Nutritional Biochemical Co. It was used in the concentration of 0.5 mgm./ml., in 0.066 M phosphate buffer pH 7.6.

IV. Media

A. Sabouraud's Cerelose Broth

Cerelose ............... 40 gm.
Neopeptone (Difco) ...... 10 gm.
Tap water ............... 1,000 ml.

(Adjust pH to 5.8 - 6.0)

B. Vogel's Medium

Sodium citrate ............ 150 gm.
$\text{KH}_2\text{PO}_4$ ............. 250 gm.
$\text{NH}_4\text{NO}_3$ ............. 100 gm.
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ ....... 10 gm.
$\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ ....... 5.0 gm. (stir)

Trace elements solution ... 5.0 ml.
Biotin solution ........... 2.5 ml.

Successively dissolve in 750 ml. distilled water, then bring volume to 1,000 ml.
For Neurospora species supplement with: glucose (20 gm./l.), N.F. casein (5.0 gm./l.), and yeast extract (5.0 gm./l.)

**Trace Elements Solution**

- Citric acid ........... 5.0 gm.
- ZnSO$_4$·7H$_2$O ........... 5.0 gm.
- Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O ........... 1.0 gm.
- CuSO$_4$·5H$_2$O ........... 0.25 gm.
- MnSO$_4$·1H$_2$O ........... 0.05 gm.
- H$_3$BO$_3$ ........... 0.05 gm.
- Na$_2$MnO$_4$·2H$_2$O ........... 0.05 gm.
- Distilled water ........... 100 ml.

**Biotin Solution**

5.0 mgm. biotin in 50 ml. distilled water.

**C. Synthetic Medium for Amino Acid Studies**

- Glucose ........... 14 gm.
- (NH$_4$)$_2$SO$_4$ ........... 1.2 gm.
- FeCl$_2$ ........... 0.002 gm.
- CaCl$_2$·2H$_2$O ........... 0.002 gm.
- MgSO$_4$·7H$_2$O ........... 0.002 gm.
- Phosphate buffer, 0.066 M, pH 6.8 ........... 20 ml.
- Distilled water ........... 380 ml.

(The phosphate buffer was autoclaved separately).
V. Equipment

A. Warburg Respirometer

This instrument was used in all oxygen uptake studies. The water bath temperature was 30°C. It was manufactured by Gilson Medical Electronics, Middleton, Wisconsin.

B. Thunberg Tubes

Standard tubes as described (68) were employed.

C. Spectrophotometers

1. Spectronic 20, manufactured by Bausch and Lomb, was used for the hexosamine assay. Two tubes were used throughout the study. They were matched to give the same absorbance.

2. Beckman DU, was employed in measuring changes in absorbance in the protoplast experiments. It was also used for determining concentrations of griseofulvin in reaction mixtures.

D. Homogenizer

A VirTis "23" homogenizer was utilized for the breakage of long hyphal elements into shorter units.

E. Cell Disintegrator

Cells were disrupted, to isolate purified cell wall material, with a Nossal cell disintegrator. This was manufactured by McDonald Engineering Company, Cleveland, Ohio.
F. **Ultra-violet Viewer**

Observation of chromatograms for compounds which absorb or fluoresce in ultra-violet light was carried out with a "chromato-vue", (Ultra-violet Products, Inc., San Gabriel, California). This viewer contains two separate lamps and filters, giving wavelengths of 366 μm and 253.7 μm.
METHODS

I. Preparation of Cells

A. Fresh Whole Cells

Stock cultures of dermatophytes were maintained on Sabouraud's cerelose agar. The species used were;

Microsporum quinckeanum strain #8 - Dr. F. Blank,
McGill University.

Microsporum quinckeanum strain #7 - Dr. F. Blank,
McGill University.

Trichophyton asteroides - Dr. F. Blank,
McGill University.

Trichophyton mentagrophytes strain #666
- Dr. C. W. Emmons,
Bethesda, Md.

A culture of Neurospora tetrasperma was also used. It was obtained from Dr. R. J. Bandoni, University of B.C. Fungi were grown in shake culture in 500 ml. Erlenmeyer flasks containing 50 ml. of liquid medium. In this case, 0.2 ml. of a sterile 1.0% solution of triton X-100 was added.

Static fungal cultures were grown in 500 ml. Erlenmeyer flasks containing 100 ml. of liquid medium.

Shake cultures were grown (at room temperature) on a Burrell shaker, at 225 cycles per minute with a 4.0 cm. stroke. Static cultures were grown in a 25°C. incubator.
All cultures were inoculated with either hyphal fragments from liquid growth or with hyphae and conidia from fresh surface growth.

Cells were harvested by one of three methods. In the first method they were centrifuged at 2,000 revolutions per minute (rpm) for thirty minutes at room temperature. The second two methods involved filtration. The cells were collected either on cheese cloth, or on filter paper supported by a Buchner funnel, under suction. Depending on the requirements of the experiment, the cells were washed with 0.066 M phosphate buffer pH 7.0, 0.05 M NaCl, 20% sucrose, or distilled water.

B. Starved Cells

Using aseptic precautions, whole cells from four flasks were washed by centrifuging at 2,000 rpm for thirty minutes at room temperature, in sterile centrifuge cups with two volumes of sterile 0.85% NaCl. This was followed by one washing with sterile 0.066 M phosphate buffer pH 7.0. The cells were then resuspended in 200 ml. of the phosphate buffer. This suspension was divided into four 50 ml. fractions, each of which was placed in a sterile 500 ml. Erlenmeyer flask, to which had been added 0.2 ml. of a sterile 1.0% solution of triton X-100. The flasks were placed on the Burrell shaker at room temperature and the cells were starved for two, three, four or five days. The starvation medium was replaced after three days by centrifuging down the cells and resuspending them in fresh phosphate buffer and triton X-100.
C. **Cell Suspensions for O₂ Uptake Studies**

Whole cells or starved cells were washed by centrifuging at 2,000 rpm for thirty minutes at room temperature, with two volumes of 0.85% NaCl. The cell material from two flasks was resuspended in 20 ml. of 0.066 M phosphate buffer pH 7.0, to which had been added triton X-100 (0.4 ml. triton X-100 per 100 ml. buffer) and 20 International Units of penicillin per ml. of buffer. This suspension was transferred to a sterile 250 ml. macro VirTis homogenizing flask. The flask was then placed in an ice bath and allowed to cool for ten minutes. The mycelial pellets were homogenized at 23,000 rpm in a Vir-Tis "23" homogenizer for a total of three minutes. This was done by running the homogenizer for three intervals of one minute with a cooling period of two minutes between each. The resulting suspension contained short hyphal elements of approximately four cellular units. Such a suspension permitted easier and more accurate measurement of hyphal material being added to Warburg vessels. It also resulted in more uniform oxygen uptake values as variations in measurement occur with larger hyphal elements which tend to form clumps when being shaken.

D. **Cell-free Extracts**

Whole cells or starved cells were centrifuged at 2,000 rpm for forty-five minutes at room temperature. The wet, packed cell material was partially dried on filter paper, then weighed. To this material was added fine glass beads (Glass Homogenizing Beads, VirTis Company), which had previously been cleaned with sulphuric acid-dichromate solution and then rinsed ten times with 0.066 M
phosphate buffer pH 7.0. The glass beads were added in the ratio of 9.0 gm. to 1.0 gm. of wet, packed cell material. The beads and pellets were mixed as thoroughly as possible, then were frozen. 10 gm. of this frozen mixture was placed in a Wedgewood mortar which had previously been chilled to 0°C. The mixture was vigorously ground with a pestle until thawing was observed. The mortar was immediately returned to the refrigerator to refreeze the mixture. This procedure was continued until a total of thirty minutes of grinding had been achieved. The mixture was placed in a 50 ml. centrifuge tube at 4°C to thaw, then 5.0 ml. of 0.066 M phosphate buffer pH 7.0 which had been chilled to 0°C was added. The buffer was allowed to extract the cell material for several hours, then the tube was centrifuged at 2,500 rpm for thirty minutes at 4°C. The liquid phase was decanted into another 50 ml. centrifuge tube, a second 5.0 ml. aliquot of chilled phosphate buffer was added to the cell residue and this was allowed to extract for several more hours. The tube was again centrifuged and the liquid phase pooled with the first extract. The pooled sample was centrifuged at 2,500 rpm for sixty minutes at 4°C to remove any residual cell material. The cell-free supernatant was stored frozen at -8°C. The cell residue was separated from the glass beads by successive sedimentation and decanting with distilled water. These washings were centrifuged at 2,500 rpm for thirty minutes at 4°C and the packed residue was stored frozen.

E. Acetone Dried Preparations

Two flasks of cells, were grown for four days in shake
culture. They were harvested by filtration and the pellets were washed on the filter with 500 ml. of 0.85% NaCl. They were then quickly transferred to a 50 ml. aliquot of acetone at -8°C, and mixed well by stirring. The cell material was allowed to settle and the acetone was decanted off. This was followed by washing with three 50 ml. aliquots of acetone at -8°C. After the last decanting, the cells were spread on a watch glass and the acetone allowed to evaporate for several minutes. The watch glass was next transferred to a vacuum desiccator and the drying continued under vacuum at 4°C. The dried cell material was ground with a mortar and pestle and the dry powder stored in the deep freeze at -8°C.
II. **Preparation of Protoplasts**

A. **Preparation of Protoplasts of Neurospora Species**

Protoplasts of *Neurospora tetrasperma* were studied in order to become familiar with their appearance and with the techniques for their formation. The method of Colvin (20) was used. It is a modification of the original method of Bachmann and Bonner (5). Colvin's modification is described in the following section.

100 ml. of Vogel's medium in a 500 ml. Erlenmeyer flask was inoculated with a piece of vegetative growth about 0.5 cm. in diameter. This was incubated in static culture at 25°C for forty-eight hours, yielding a large, fuzzy, ball-like mass of submerged growth. This material was transferred to a mixture of nine parts 22% sucrose to one part "suc digestif d'Helix pomatia" (L'Industrie Biologique Française). The mycelial pellet was incubated with the enzyme mixture at 35°C for a total of four hours. A small sample was removed every fifteen minutes and observed with a phase contrast microscope. Formation of protoplasts started after approximately one hour and continued for the remaining three hours. To demonstrate the loss of cell wall, distilled water was slowly added under the cover slip to dilute the sucrose and thus cause bursting of the protoplasts due to "osmotic shock".

Protoplasts were partially purified by filtration through glass wool. Further purification was accomplished by passing the suspension through a "C" grade Pyrex sintered glass filter.
Concentration of the protoplasts was achieved by centrifuging at 12,100XG. for five minutes at 0°C. The pellet was resuspended in a small volume of the supernatant solution. All determinations of protoplast numbers were carried out using a Petroff-Hausser counting chamber. Protoplast diameters were determined by use of a calibrated ocular micrometer.

B. Preparation of Dermatophyte Protoplasts

The method of protoplast preparation used previously with *Neospora tetrasperma* was found to be less effective with dermatophytes. After some experimentation, Kinsky's modification (43) of the technique of Bachmann and Bonner, (5) was used as a basis for forming protoplasts of dermatophytes. The following is the modified method which was finally employed.

Pieces of vegetative growth or hyphal pellets approximately 0.5 cm. in diameter were inoculated into 100 ml. of Sabouraud's cerelose broth, in a 500 ml. Erlenmeyer flask, and the cells were grown for three to four days in static culture at 25°C. Ball-like masses of submerged growth similar to those of *Neurospora* were formed. Several of these were collected by filtering through a perforated aluminum foil cup. These were then washed on the aluminum cup with about 50 ml. of 20% sucrose in 0.066 M phosphate buffer pH 6.8. The washed hyphal elements were transferred to a mixture of 20% sucrose and 0.1 M glutathione in 0.066 M phosphate buffer pH 6.8 (9.0 ml.), sux digestif d'Helix pomatia (1.0 ml.), and penicillin solution containing 2,000 International Units per ml. (0.2 ml.). This enzymic mixture had been previously sterilized and partially
purified by passage through a sterile 0.45 μ millipore filter using a syringe with a Swinny filter adaptor. The final mixture was incubated at 35°C for fifteen hours. The resulting protoplasts were clarified by filtration through a "C" grade Pyrex sintered glass filter with a prefilter of Whatman No. 1 paper. The protoplasts were concentrated by centrifuging at 7,700XG for ten minutes at 0°C.
III. Paper Chromatography

A. General Methods

Amino acids, amino sugars, sugars, and griseofulvin hydrolysis products were separated by paper chromatography. In all cases, descending chromatograms were used, and run at room temperature.

The paper used for single dimensional chromatography was prepared as follows. A line was drawn 6.0 cm. from one end of the paper. A fold was made here to hang the paper over the antisiphon bar. Another line was drawn, 3.0 cm. below the first. This marked the "origin" or point where the samples were applied. The distances between the samples were from 2.0 to 3.0 cm., depending on the numbers and types of samples employed. The two dimensional chromatograms were marked with two extra lines. These were drawn at 6.0 cm. and 9.0 cm. from one edge of the paper. A single spot was applied at the point of intersection of the two 9.0 cm. lines.

Samples were applied to the paper using glass capillaries. The liquid sample was allowed to run onto the paper until a spot of about 1.0 cm. in diameter was obtained. This was dried and then more sample added to the center of the spot. All spotting of samples was done in a stream of warm air to facilitate drying.

Before addition of the paper to the chromatography tank, a volume of the solvent to be employed was placed in the bottom of the tank to allow saturation of the atmosphere. After about
thirty minutes, the paper was placed in the tank and allowed to equilibrate for at least another thirty minutes. At the end of the equilibration period, the solvent was quickly added to the trough and the system was sealed to prevent evaporation. At the end of the run, the paper was removed from the tank, the position of the solvent front was marked, and then the solvent was driven from the paper with a stream of warm air. When the paper was completely dry, the positions of the compounds were determined with the appropriate developing reagents. Developed spots were immediately outlined with a pencil line and their colours noted.

B. Amino Compounds

Amino acids and amino sugars were separated by one dimensional and two dimensional chromatography. The samples were placed in small test tubes and sufficient 1.0 N HCl was added to insure that all amino compounds were in the hydrochloride salt form. In the case of HCl hydrolysates this was not necessary.

Those samples which contained inorganic salts were previously purified by passing them through columns of DOWEX 50 ionized resin. The columns were in the H⁺ ion state and were eluted with 50 ml. of 1.0 N HCl after a preliminary washing of the sample in the column with 20 ml. of distilled water.

With all amino compounds in the hydrochloride salt form, samples were evaporated to dryness with a stream of warm air. This served to remove most of the excess hydrochloric acid. The samples were redissolved in a small volume of distilled water and spotted onto chromatograms. When the concentration of amino
compounds in a sample was uncertain, two or more volumes, containing different amounts of the sample, were spotted onto the chromatogram.

Amino acid and amino sugar standards were run parallel to the unknowns. They were prepared in a similar manner. That is, placed in the hydrochloride salt form, evaporated to dryness, and redissolved in distilled water. A quantity of 0.15 M of each amino compound was spotted onto the chromatogram. The twenty common amino acids were employed as standards and in addition, glucosamine and L-galactosamine were used. The solvent system used for amino compound separation was: butanol-acetic acid-water, 2:1:1, (v/v)(6). In the two dimensional system the second solvent was: butanol-methyl ethyl ketone-water, 2:2:1, (v/v) in the presence of cyclohexylamine vapours (51). In all cases Whatman No. 4 chromatography paper was used. All amino compounds were detected by spraying the chromatograms with 0.5% ninhydrin in n-butanol and drying in the oven at 85°C to 95°C. Characteristic colours developed, although different colours than are normally found were obtained in the presence of cyclohexylamine.

C. Sugars and Amino Sugars

Sugars and amino sugars were also separated on Whatman No. 4 chromatography paper. The sugar standards which were run parallel to the unknown samples were; glucose, galactose, mannose, ribose, deoxyribose, glucosamine, and galactosamine. Standards consisted of 0.01 ml. of a solution of 2.0 mgm. of the sugar per ml. of distilled water.
The solvent system used to separate these compounds consisted of ethyl acetate-pyridine-water, 8:2:1, (v/v). The developing agent used (67) was silver nitrate in acetone, followed by sodium hydroxide in ethanol. The background was removed with an aqueous solution of sodium thiosulphate. The spots were brown at first, but with drying their colour turned to black. All sugar chromatograms were dipped rather than sprayed. Care was taken when dipping through the silver nitrate solution to avoid streaking of the spots. Chromatograms utilizing this solvent system were run for twenty-four to thirty-six hours.

D. Products of Griseofulvin Hydrolysis

A small sample of griseofulvin or of material containing griseofulvin was hydrolyzed with 2.0 ml. of 2.0 N HCl at 100°C for eighteen hours, in a sealed glass ampoule. The supernate from this hydrolysate was collected and stored. The remaining solid residue was hydrolyzed with 2.0 ml. of 6.0 N HCl at 100°C for a further twelve hours. The supernant solution and residual pellet were separated and stored.

A 1.0 ml. volume of each of the supernates was evaporated to dryness with a stream of warm air and then redissolved in 0.1 ml. of distilled water. These samples were then spotted on to Whatman No. 4 chromatography paper.

The residual pellet and the remaining 1.0 ml. volumes of the supernates were each extracted with 2.0 ml. of butyl acetate. These extracts were evaporated to dryness, redissolved in 0.1 ml. of butyl acetate, and spotted on to the chromatogram.
The solvent used for this separation was butanol-methyl ethyl ketone-water, 2:2:1, (v/v). A sample of griseofulvin in butyl acetate was applied as a control. Spots were detected by viewing with an appropriately filtered ultra-violet lamp.
IV. Oxygen Uptake Studies

Oxygen uptake by dermatophytes was followed using a Warburg manometric apparatus. The preparation of cell material for these studies has been described in a previous section. The volume of liquid in the cups in all cases was 2.7 ml. The general protocol for these studies was as follows:

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>PROTOCOL FOR WARBURG RESPIROMETER CUPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endogenous</td>
</tr>
<tr>
<td>Cell Material in buffer</td>
<td>2.0 ml.</td>
</tr>
<tr>
<td>Buffer * (M/15, pH 7.0)</td>
<td>0.28 ml.</td>
</tr>
<tr>
<td>Substrate (M/50)</td>
<td>-</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>0.2 ml.</td>
</tr>
<tr>
<td>Griseofulvin 2,000µg/ml. in 50% Ethanol</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol 50%</td>
<td>0.02 ml.</td>
</tr>
<tr>
<td>KOH 20%</td>
<td>0.2 ml.</td>
</tr>
<tr>
<td>Total</td>
<td>2.7 ml.</td>
</tr>
</tbody>
</table>

* Buffer is 0.066 M phosphate buffer pH 7.0 containing triton X-100 (0.4 ml. 1.0% triton per 100 ml. buffer) and 20 International Units of penicillin per ml. of buffer.

The cell material, buffer, griseofulvin, and ethanol were placed in the Warburg cup. The substrate or distilled water was placed in the side arm. The KOH was placed in the center well.
of the cup with a folded piece of filter paper approximately 2.0 cm. square. The filter paper was used to increase the surface area of the KOH solution.

When the contents had been placed in the cups, these flasks were attached to their matching manometers and the plugs placed in the side arms. In both cases the ground glass connections were sealed with "vaspar" (1 part vaseline: 1 part paraffin wax) and secured with small springs. Vaspar has a higher melting point than vaseline, so that there is less chance of it allowing leakage into or out of the cup.

The cups were allowed to shake in the water bath for ten minutes to allow them and their contents to reach water bath temperature. All tests were carried out at 30°C. At the end of this time the seals on the ground glass joints were checked and the manometers were closed. Endogenous respiration of the cell material was measured for fifteen minutes before the substrate was tipped in from the side arm. This was to insure that all flasks were giving similar values and that no leakage was occurring. All readings were determined by returning the fluid in the closed arm of the manometer to the 15 cm. mark and noting the level of the fluid in the open arm of the manometer. The differences in values due to alterations in atmospheric pressure were corrected by comparison to a "thermobarometer". Since the temperature is held constant by the water bath, the only thing which can alter the levels of the fluid in the manometer is a change in atmospheric pressure. The oxygen uptake values are initially recorded in millimeters (mm.) on the manometer column.
The volumes of the flasks had been measured previously and a "flask constant" determined for each. When an oxygen uptake value in mm. is multiplied by the flask constant the result is the oxygen uptake value in microliters (µl).
V. **Dry Weight Determinations**

A method of cell standardization was required in order to have a basis for comparison of oxygen uptake values. Turbidimetric determinations were attempted but were found unsatisfactory with hyphal material. Wet packed weight was used but still gave a low degree of accuracy. Finally, dry weight of washed cell material was used and this gave good correlation of oxygen uptake values between cultures grown under similar conditions for the same length of time.

The cell material which was added to the Warburg flasks had been washed and resuspended in phosphate buffer containing triton X-100 and penicillin. Two 10 ml. volumes of this suspension were delivered into two dry, tared aluminum cups. Similarly two 10 ml. volumes of the phosphate buffer (containing triton X-100 and penicillin) were also delivered into two aluminum cups. All four cups were placed in an oven at 100°C for twenty-four hours.

At the end of the drying period, the cups were transferred to a desiccator containing anhydrous CaCl₂, where they were allowed to cool. The dried cells plus cups were then weighed and the weight of the cell material per cup determined. The weighing was done as quickly as possible in order to minimize any error due to uptake of water by the protein in the sample. The weight of the buffer was subtracted from the combined weight of cells and buffer to give the weight of cells alone.
VI. Quantitative Analysis of Griseofulvin in a Reaction Mixture

The method of analysis used in this study was based on that given by Robinson and fellow workers in 1960 (59). A sample of cell material and supernate containing griseofulvin was placed in a test tube and 1.0 ml. of butyl acetate was added. The tube was shaken well and then placed in the deep freeze for thirty minutes to allow separation of the two phases. The butyl acetate was removed from the tube with a Pasteur pipette. A second 1.0 ml. volume of butyl acetate was added and the process was repeated. The second extract was combined with the first and then the total volume was brought to 3.0 ml. The absorbance (A) of this solution was measured at 289.5 µm in a Beckman DU spectrophotometer. A control sample containing cells and supernate but no griseofulvin was treated in the same way and its absorbance subtracted from that of the test sample. A standard curve for the absorbance of griseofulvin in butyl acetate at 289.5 µm was plotted (FIGURE 2). The following concentrations of griseofulvin were measured against a butyl acetate blank: 2.5 µgm/ml., 5.0 µgm/ml., 10 µgm/ml., and 20 µgm/ml.
FIGURE 2

Standard Griseofulvin Curve

Absorbance 289.5 μm

Griseofulvin Concentration (μg/ml.)
VII. Dehydrogenase Studies

The method of Thunberg was utilized for the determination of dehydrogenase activity of cells and cell free extracts (68). Standard Thunberg tubes were employed. The contents of the tubes are given in the following protocol.

**TABLE II**

PROTOCOL FOR THUNBERG TUBES

<table>
<thead>
<tr>
<th></th>
<th>Endogenous</th>
<th>Endogenous &amp; Griseofulvin</th>
<th>Substrate</th>
<th>Substrate &amp; Griseofulvin</th>
<th>Substrate Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Source in buffer</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Substrate (M/50)</td>
<td>-</td>
<td>-</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
</tr>
<tr>
<td>Phosphate buffer (M/15, pH 7.0)</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>3.0 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.0 ml.</td>
<td>2.0 ml.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methylene Blue (1/10,000)</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
<td>1.0 ml.</td>
</tr>
<tr>
<td>Griseofulvin in 50% ethanol (2,000 ngm/ml)</td>
<td>-</td>
<td>0.05 ml.</td>
<td>-</td>
<td>0.05 ml.</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol 50%</td>
<td>0.05 ml.</td>
<td>-</td>
<td>0.05 ml.</td>
<td>-</td>
<td>0.05 ml.</td>
</tr>
<tr>
<td>Total</td>
<td>6.05 ml.</td>
<td>6.05 ml.</td>
<td>6.05 ml.</td>
<td>6.05 ml.</td>
<td>6.05 ml.</td>
</tr>
</tbody>
</table>
The enzyme source was placed in the side arm, the remainder of the contents in the tube. The ground glass joint of the Thunberg tube was sealed with "vaspar" (1 part vaseline: 1 part paraffin wax). The evacuation outlet was connected to a vacuum pump and air was removed from the tube. Evacuation was continued for three minutes. During this time the tube was tapped to release dissolved gases. The effects of "bumping" of the solutions as gases were released, were kept to a minimum by tilting the tube. When evacuation was complete, the tube was sealed by turning the cap.

The sealed tubes were placed in a water bath at 30°C for ten minutes, to come to temperature. Each tube was then tipped, mixing the enzyme with the other contents. The exact time of enzyme addition was noted, and then the time required for reduction of methylene blue was measured. The end point was taken as a faint blue solution rather than a colourless one. Tubes were shaken frequently to reduce errors due to precipitation of cell material.
VIII. **Amino Compound Studies**

A large amount of cell material from Sabouraud's cerelose broth was washed several times with sterile 0.85% saline using aseptic precautions. This cell material was resuspended in two 200 ml. volumes of a synthetic medium, one of which contained 25 μgm. of griseofulvin per ml. Each suspension was then divided into four equal volumes which were transferred to sterile 500 ml. Erlenmeyer flasks. One flask of each series was immediately removed for analysis as a zero time control. A 50 ml. portion of each medium, containing no cell material, was also analyzed as a control. The remaining six flasks were grown in shake culture at room temperature. One flask from each series was removed for analysis at two days, four days, and six days.

The supernatant solutions of the previously mentioned samples were analyzed by the following method. The contents of a flask were transferred to a 50 ml. centrifuge tube and centrifuged at 2,000 rpm for thirty minutes at 4°C. The supernate was decanted into a small beaker and an equal volume of 95% ethanol was added. This mixture was then placed in a stream of warm air and evaporated to a volume of 5.0 ml. This concentrate was centrifuged at 2,500 rpm for fifteen minutes at 4°C, to remove denatured protein. The supernate was then evaporated to dryness and resuspended in 1.0 ml. of distilled water.

The concentrated sample was passed through a Dowex 50 ionized resin column to remove anions which interfere with paper chromatographic separation of amino compounds. The column was
6.0 cm. long by 0.8 cm. in diameter. Before addition of the sample, the resin was placed in the hydrogen ion form by washing it with 50 ml. of 1.0 N HCl. It was then rinsed with freshly distilled water until no chloride ions could be detected, using a silver nitrate solution as an indicator.

After addition of the concentrated amino acid sample to the top of the Dowex 50 H⁺ column, anions and replaced H ions were washed from the column with 50 ml. of freshly distilled water. Next, cations were eluted from the column by the passage of 50 ml. of freshly prepared 1.0 N HCl. The HCl eluate was collected and evaporated to dryness in a stream of warm air. When residual HCl had been removed by evaporation, the sample was resuspended in 1.0 ml. of distilled water. Volumes of 0.05 ml., 0.1 ml., and 0.2 ml. were spotted onto chromatography paper for separation and identification of amino compounds.
IX. **Quantitative Assay for Hexosamines**

There are several assays for hexosamines and more specifically for glucosamine. Most of these assays depend on the acetylation of glucosamine so that N-acetyl glucosamine will also be measured. The method used in this study was one employing Ehrlich's reagent, and is the technique described in 1953 by Boas (11).

Glucosamine or N-acetyl glucosamine was produced either by acid or enzyme hydrolysis of chitin. Acid hydrolysis was carried out in 2.0 N HCl for eighteen hours at 100°C. Higher concentrations of acid result in breakdown of glucosamine. Breakdown of N-acetyl glucosamine to glucosamine and acetic acid does occur but the glucosamine is reacetylated during the assay so that this breakdown does not matter. Enzymic hydrolysis produces N-acetyl glucosamine. This enzymic reaction was stopped by using 12% trichloracetic acid (TCA) to precipitate protein.

Both samples were diluted with distilled water and then small volumes were applied to Dowex 50 columns. These columns had previously been prepared by the addition of 10 ml. of 1.0 N NaOH, followed by 10 ml. of freshly boiled distilled water. Next, 12 ml. of 1.0 N HCl was passed through each column. Finally, freshly distilled water was passed through the columns until no chloride ions could be detected, using silver nitrate as an indicator.

After the amines had been applied to the columns,
undesired anions were removed by the addition of 10 ml. of distilled water. Amines were then eluted with 5.0 ml. of 2.0 N HCl. Usually, 1.0 ml. of this eluate was assayed for glucosamine content. If the content was low, a larger sample could then be used.

An example of a typical glucosamine assay now will be provided. 1.0 ml. of a Dowex 50 eluate, 1.0 ml. each of three glucosamine standards (containing 5.0 µgm., 10 µgm., and 20 µgm. per ml.), and 1.0 ml. of distilled water were each placed in separate graduated test tubes. To each tube was added a drop of phenolphthalein (0.5%). 4.0 N NaOH was titrated into each tube until the indicator turned red. The amount of base added to each tube had been noted, and the difference between each tube and that requiring the most base was calculated. As the assay values are influenced by salt concentrations, all differences in volume of alkali added were made up by the addition of that volume of 4.0 N NaCl. All samples were back-titrated to a colourless end-point with 0.5 N HCl. The volumes in all test tubes were brought to 5.0 ml. with distilled water. 1.0 ml. of acetyl acetone reagent was added to each tube. All tubes were stoppered and placed in a water bath at 89° to 92°C for forty-five minutes.

When acetylation was completed, the samples were cooled and 2.5 ml. of 95% ethanol was added to each tube. Next, 1.0 ml. of Ehrlich's reagent was added to each, mixed well, and then the volume brought to 10 ml. with 95% ethanol. These mixtures were allowed to stand for one hour, and then were read at 530 µ
on a Bausch and Lomb "Spectronic 20" spectrophotometer.

The distilled water sample was used as a blank. The standards were used to plot a standard curve (FIGURE 3), and the hexosamine content of each unknown was determined from this curve. The samples were measured as glucosamine HCl, both in the test sample and in the standards. The weights of glucosamine or N-acetyl glucosamine were calculated from these values.
FIGURE 3

Standard Glucosamine-HCl Curve

Absorbance 530 mp

Glucosamine-HCl (µgm.)
X. Spectrophotometric Studies on the Effects of Griseofulvin on Protoplast Membranes

In 1962, Stephen Kinsky reported the use of the spectrophotometer in measuring changes in size of Neurospora protoplasts (43). The method used in this study was essentially the same as that described by Kinsky.

Protoplasts of dermatophytes were prepared by the previously described method. After centrifuging they were resuspended in 2.8 ml. of the protoplast preparation medium (containing snail digestive enzyme, buffer, sucrose, glutathione and penicillin). This medium had been previously clarified by passage through a 0.45 μ millipore filter.

The protoplast suspension was transferred to a cuvette and placed in a Beckman DU spectrophotometer. Absorbance was measured at 600 μm for several minutes. 2.8 ml. of the preparation medium was used as a blank.

After these initial readings, which were to insure that no alterations in absorbance were occurring, 0.2 ml. of N,N-dimethyl formamide (DMF), containing 600 μg.m. of griseofulvin per ml., was added. The addition of the griseofulvin was made very slowly with constant stirring, to minimize breakage of protoplasts. A similar 0.2 ml. of griseofulvin solution was added to the blank cuvette. Readings of absorbance at 600 μm were taken every two minutes. A control, containing protoplasts and DMF but no griseofulvin, was also measured.
XI. **Visual and Spectrophotometric Studies on Cell Wall Synthesis by Dermatophyte Protoplasts**

When fungal protoplasts are resuspended in the previously described preparatory medium, from which snail digestive enzyme has been deleted, they soon begin to resynthesize cell wall. Two methods were used to investigate this process, visual observations and spectrophotometric measurements.

Protoplasts were prepared as previously described. After centrifuging, the pellet was resuspended in 0.066 M phosphate buffer pH 6.8, containing sucrose (200 mgm/ml.) and penicillin (40 International Units/ml.). This suspension was again centrifuged at 7,700XG for ten minutes at 0°C, and the washed pellet was resuspended in 7.2 ml. of the washing medium. 2.8 ml. was transferred to each of two cuvettes, and 0.7 ml. was placed in each of two test tubes. To one of the cuvettes, 0.2 ml. of griseofulvin in N,N-dimethyl formamide (DMF) was slowly added with stirring. The concentration of griseofulvin in the solution was 600 µgm. per ml. DMF. Similarly, 0.05 ml. of griseofulvin in DMF was added to one test tube. Next, 0.2 ml. of pure DMF was added to the other cuvette and 0.05 ml. was added to the other test tube.

The cuvettes were placed in the Beckman DU spectrophotometer and their absorbances measured at 600 µm over a period of seven hours. A volume of the solution in which the protoplasts were suspended was used as a blank.

The samples in the test tubes were used for the visual
observations utilizing phase contrast microscopy. A small drop of sample was placed on a clean dry slide and then covered with a cover slip. While observing the protoplasts microscopically, a drop of distilled water was added to one edge of the cover slip. Protoplasts were tested in this way to see if they would rupture due to osmotic shock.
XII. **Formation of Griseofulvin Resistant Mutants**

The following methods were used as reported in a paper published by Aytoun, et al. in 1960 (4). The mutants eventually employed for further study were obtained using a selection method referred to as a "layer plate method".

This procedure consisted of layering a Petri plate with 5.0 m. of Sabouraud's cereolose agar. Before solidification, this agar was seeded with 1.0 ml. of a heavy suspension of dermatophyte spores or hyphal fragments. When the agar had solidified, it was dried for several hours at 37°C. A second thicker layer (10 ml.) of Sabouraud's agar, containing a known concentration of griseofulvin now was added. The mycelia which first appeared on the surface of the upper agar layer were transferred to slopes containing the same concentration of griseofulvin as was present in the agar layer from which they were isolated. These isolates were used as source of inocula for plates containing higher concentrations of griseofulvin.

An attempt was made to increase the mutation rate by irradiation with ultra-violet light. This was done by taking the seeded, first layer of agar and placing it one foot below a General Electric 15 watt, germicidal, ultra-violet lamp for forty seconds. The griseofulvin agar layer was added as before. The results obtained using this additional step were no better than without it.

A second selective method was also employed. This gave a quick, easy method for spot checks on the level of drug
resistance of strains, but was not as useful in the original isolation of variants. It consisted of layering Sabouraud's agar, containing a known concentration of griseofulvin, in a Petri plate, and then inoculating its center. Growth away from the point of the inoculation usually was fairly uniform. If, though, one part of the periphery grew faster than the remainder, this edge was assumed to contain resistant hyphal elements and was removed. The rate of growth away from the point of inoculation also indicated the inhibitory effect of the concentration of griseofulvin present, on the organism tested. This was done by comparison with the growth rate on a plate containing no griseofulvin.
XIII. **Cell Wall Preparation and Purification**

The method of cell wall preparation used in this study was based on the method reported by Carlson and Knight in 1962 (19), which was a modification of the method of Cummins and Harris (21). Cell material was grown in Sabouraud's cereolose broth in shake culture. The mycelial pellets were collected on a Buchner funnel under suction, and were washed three times with 100 ml. volumes of 0.85% NaCl. Excess moisture was drawn through the filter by negative pressure. Next, wet, packed cell material was transferred to a tared watch glass and weighed.

This cellular material was placed in a Nossal disintegrator capsule with glass beads and phosphate buffer. The ratio used was 2.0 gm. of cell material, to 8.0 gm. of fine glass beads (Glass Homogenizing Beads, VirTis Company), to 60 ml. 0.066 M phosphate buffer pH 7.0. The total volume of the capsule was 18 ml. The lid was placed on the capsule and sealed with a thin layer of silica grease. To insure purity of the sample, the capsule had been cleaned before use by shaking with a small volume of a warm, detergent solution in the Nossal apparatus. This wash was followed by several rinsings with tap water and finally distilled water.

When the capsule had been sealed, it was cooled in the deep freeze until the contents reached a temperature of approximately 0°C. This took a period of about ten minutes. It was then placed in the Nossal apparatus and shaken for a total of fifteen minutes. This was done by a series of one and one-half
minute bursts with cooling periods of ten minutes between each. The Nossal apparatus (McDonald Engineering Company, Cleveland, Ohio) reciprocates at 12,000 complete cycles per minute. The capsule is constantly cooled during operation by the release of carbon dioxide under pressure, through a nozzle.

At the end of the fifteen minutes of shaking, the capsule contents were transferred to a centrifuge cup and sedimented at 8,000 XG for fifteen minutes at 0°C. The supernate was discarded and the pellet of beads and impure cell wall material was transferred to a 100 ml. beaker. By successive decanting and resuspension in 0.05 M NaCl, the cell wall material was separated from the heavier glass beads. The separated material now was washed five times by centrifuging and resuspending in 0.05 M NaCl. After the last centrifuging, the pellet was resuspended in 10 ml. of 0.066 M phosphate buffer pH 7.6, containing trypsin (0.5 mgm/ml.) and ribonuclease (0.5 mgm/ml.). This digestion mixture was placed in a 37°C water bath and allowed to stand, with occasional mixing, for four hours. At the end of the digestion period, the remaining particulate material was centrifuged down at 8,000 XG for thirty minutes at 0°C. The pellet was then washed five times with 0.05 M NaCl and once with distilled water, by centrifuging and resuspending. The remaining pellet was dried over anhydrous CaCl₂, under negative pressure in a vacuum desiccator, at room temperature. The dried pellet of purified cell wall material then was ground with a small mortar and pestle to allow more accurate weighing of samples. This powder was stored in a sealed vial in the refrigerator, for later analysis.
XIV. Cell Wall Analysis

The purified cell wall material was qualitatively analyzed for amino acids, amino sugars, and sugars. To obtain these small molecules it was necessary to first hydrolyze the large polymers of which they were components. This was accomplished by placing 10 mgm. of cell wall powder in a glass ampoule, adding 2.0 ml. of 2.0 N HCl, and sealing the ampoule by heating. This ampoule was then placed in an oven at 100°C for eighteen hours, after which time it was cooled and opened. Next, it was centrifuged at 2,000 rpm for ten minutes at room temperature and the supernate removed and stored. The pellet was resuspended in 2.0 ml. of 6.0 N HCl, and the ampoule was resealed and heated for a further twelve hours at 100°C. This ampoule was again opened, centrifuged, and the supernate removed and stored. The pellet was extracted with 2.0 ml. of butyl acetate and then discarded. This pellet is believed to have contained metal or metallic salts resulting from contamination by the Nossal capsule during disintegration.

The 2.0 N and 6.0 N HCl hydrolysates were evaporated to dryness with a stream of warm air, thus driving off excess HCl. The dried residues were each resuspended in 1.0 ml. of distilled water. The butyl acetate extract was similarly evaporated and then resuspended in 1.0 ml. of butyl acetate. These samples then were analyzed using paper chromatographic techniques.
EXPERIMENTAL

I. Oxygen Uptake Studies

A. Preliminary Investigations

Preliminary experiments into the effects of griseofulvin on dermatophytes were involved with oxygen uptake for several reasons. The first was that there were conflicting results in this area (15,44,63). The second reason was that other workers had shown that nitrogen and phosphorus assimilation by cells, were decreased in the presence of the fungistat (28,29). Such results could indicate that oxidative phosphorylation was being inhibited. The third reason was that previous work in this laboratory had indicated that dermatophytes have extremely large quantities of endogenous reserves (67a). The resulting high rate of endogenous respiration could be lowered by "starving" the cells in the presence of phosphate buffer, thereby depleting the endogenous reserves and lowering the respiratory rate. Without first starving this cell material, oxidation of substrates could not be demonstrated by oxygen uptake methods. It should be mentioned here that Nickerson and Chadwick (52) report the oxidation of substrates by dermatophytes without involving starvation. The methods reported by these authors did not seem suitable in this study. It was the intention of this investigator then, to repeat the former experiments and to compare these results with those obtained with starved cell material.
The form of growth used throughout these experiments was the fungal pellet which results from shake culture methods. The morphology of this form of growth is in many ways similar to mycelial mat growth (72), but there are certain differences. The reasons for choosing growth obtained using the shake culture cultivation method for this study, were the shorter incubation time and the greater ease of handling.

A variety of methods of measuring out constant amounts of cell material were investigated. These were, separating pellets by size as described by Roth, et al. (63), weighing wet, packed cell material, and finally homogenizing for quantitative transfer by pipette. The first two methods were found to be highly unreliable. The results obtained from duplicate Warburg cups were similar only by chance. Errors were perhaps due to variations within the hyphae and possibly to variations in the handling techniques. Homogenized mycelial preparations did not give the former problems if the mycelia were broken sufficiently to give hyphal fragments of approximately four cell units in length. This material is more analogous to bacterial preparations. Unlike bacteria though, the large hyphae have the property of sedimenting faster when left standing. To offset this, the mycelial homogenate was constantly agitated when being pipetted into the Warburg vessels. All determinations were carried out using duplicate cups. The experimental data was plotted by averaging duplicate cup values. All experiments were repeated once or twice and compared on the basis of the dry weight of cell material.
Before carrying out any oxygen uptake investigations, it was necessary to determine the concentration of griseofulvin which would inhibit the growth of the organism under study. A single organism, *Microsporum quinckeianum* strain #8 (MQ8), was used for all initial investigations because it had the shortest growth and starvation periods (67a). This organism was inoculated onto Sabouraud's cerelose agar plates, containing known concentrations of griseofulvin. It was found that 4.0 μgm. of griseofulvin per ml. of medium would completely inhibit the growth of MQ8. Thus it was decided that 4.0 μgm./ml. would be the concentration used in the Warburg cups.

**B. Whole Cell Studies**

The first experiments were carried out on unstarved, whole pellets as described by Roth, et al. (63). The results are given in FIGURE 4. The decrease in oxygen uptake in the presence of glucose, agrees with the findings of Roth, et al., but their reported finding of decreased values in the presence of griseofulvin was not noted. Because these authors had used a higher concentration of griseofulvin (10 μgm./ml. in the flask), it was decided to test the effects of increasing concentrations of this drug, on cell respiration. Difficulties in handling pellets led to the decision to employ washed, homogenized suspensions.

Three concentrations of griseofulvin (8.0μgm./ml., 16 μgm./ml., and 32 μgm./ml.) were tested on three cellular
FIGURE 4

Oxygen Uptake by Unstarved MQ8 Pellets

Endogenous [Glucose (2.0 μM)]

Endogenous + Gris. (4.0 μg/ml.)

Glucose + Gris.
preparations (unstarved cell material, cell material starved for three days, and material starved for five days). Since the drug was dissolved in 50% ethanol, a duplicate series containing ethanol but no griseofulvin, was also tested in each case. The final concentrations of ethanol in each series were: 0.2%, 0.4% and 0.8%. Both of these test series were compared to the values from reaction vessels which contained no ethanol or griseofulvin. The increasing ethanol and griseofulvin concentrations caused no differences within each series, but variations were noted between cultures of different starvation periods. As an example, representative results for cell material which had been starved for five days are shown in FIGURES 5 and 6.

Several conclusions were drawn at this point. First, the concentrations of ethanol up to 0.8% were not inhibitory to cell respiration. Second, the presence of griseofulvin did cause differences in oxygen uptake values of cell material which had been starved for various lengths of time, but little or no alterations resulted from increasing drug concentrations. Finally, the changes in oxygen uptake values were different for each starvation time, as shown in FIGURES 7, 9, and 11.

At this time it was felt necessary to determine whether griseofulvin was present in the same concentrations at the end of a run as it had been at the start. This was accomplished by extracting the contents of each Warburg cup with butyl acetate and measuring the absorbance of the extract at 289.5 μ.μ
(see Methods). No alteration in griseofulvin concentration was observed. This meant that the drug was not being destroyed by the cells during the course of the experiment.

A study was now undertaken to compare the oxygen uptake values of cells which had been starved for various lengths of time. The purpose was to determine whether there was any correlation between starvation time and the effects of griseofulvin on endogenous respiration or glucose oxidation. To insure that sufficient griseofulvin was present, with the concentrated cell homogenate, the concentration was raised to 16 μgm./ml. Typical results from this study are shown in FIGURES 7 to 11. These results indicate that griseofulvin had a very small inhibitory effect on endogenous respiration, decreasing gradually throughout the starvation period. An effect was also observed with glucose oxidation values. Initially, oxygen uptake due to glucose oxidation was decreased, but with continued starvation, a greater percentage of the glucose was oxidized in the presence of the drug. This continued all through the starvation period, until after four days more glucose was oxidized in the presence of griseofulvin than in its absence. As an example, the percent alterations of total glucose and endogenous oxidation values (from FIGURES 7 to 11) are shown graphically in FIGURE 12. Three values are plotted against time: the percent reduction due to griseofulvin, of the total endogenous oxygen uptake; the percent reduction or increase due to griseofulvin, of the total oxygen uptake resulting from glucose oxidation, and the percent of total
theoretical oxygen uptake due to glucose oxidation (assuming that 134.4 μl. of oxygen would be taken up by the total oxidation of 1.0 μM of glucose). The calculations used to determine these values, are given in the Appendix. These experiments were repeated with *Microsporum quinckealum* strain #7, *Trichophyton asteroides*, and *Trichophyton mentagrophytes* strain #666. A similar pattern was observed in all cases, although the starvation times were longer with these organisms.

A question arose at this point in the study, as to whether glucose oxidation was being inhibited at the start of the starvation period or whether glucose was simply not being taken up by the cells. To answer this question, Warburg cups were removed from the shaker at the end of the run, and their contents extracted with 1.0 N HCl for ten minutes at 80°C. Denatured protein was removed by centrifuging for ten minutes at 8,000XG. The supernates were dried, resuspended in distilled water, and spotted onto paper chromatograms for sugar analysis. No residual glucose was found, thus indicating that it was being taken up and utilized by the cells.

The interpretation of these results is not conclusive. Oxidation values, both for endogenous and for glucose, do indicate an effect of griseofulvin on respiratory pathways. The continuous low inhibitory effect observed with endogenous metabolism suggests that perhaps the site of action of this drug is in a normal synthesis pathway. This might mean that at least part of this pathway was reversed during endogenous respiration. Another possibility is that the resynthesis of
endogenous substrates is being blocked. The latter explanation seems possible as it would result in only a small observable reduction in comparison to total endogenous respiration. This hypothesis is also supported by the glucose oxidation results. Assuming that as the endogenous reserves are depleted, the requirements of the cells for a source of energy increase, the addition of a substrate such as glucose, in the presence of an inhibitor of synthesis, should result in increased oxygen uptake values. Such an increase was observed in this study. The actual site of action of griseofulvin cannot be determined as yet, but from these results it may be related to the enzymes involved in synthesis, to the cofactors associated with them, or to the energy-transferring systems of the cells.

C. Cell-free Extract Studies

In an attempt to learn more about the alterations in oxidation values caused by griseofulvin, cell-free extracts were tested by manometric methods. Several substrates (glucose, fructose-1, 6-diphosphate, glucose-1-phosphate, glucose-6-phosphate, and pyruvate) were utilized, but no oxygen uptake could be measured using these preparations. An effort was made to replace cofactors by adding residual cell material which had been heated to 80°C for ten minutes, but this attempt also was unsuccessful. Because of the lack of results with manometric methods using cell-free extracts, it was decided to employ Thunberg techniques to follow dehydrogenase reactions.
Effect of Increasing Ethanol Concentrations on Oxygen Uptake by MQ 8 Starved for 5 Days
FIGURE 6
Oxygen Uptake by MQ8 Cells Starved for 5 Days (Homogenized) Effects of Increasing Griseofulvin Concentrations

[Graph showing the oxygen uptake over time in different conditions involving MQ8 cells starved for 5 days. The conditions include Endog. + 8.0 μg/ml. Gris. and Glucose + 6.0 μg/ml. Gris. with additional unspecified concentrations.]
FIGURE 7

Oxygen Uptake by Unstarved MQ8 Cells (Homogenized)

![Graph showing oxygen uptake over time for endogenous glucose and glucose with and without griseofulvin.]
Figure 8

Oxygen Uptake by MQ8 Cells
Starved for 2 1/2 Days (Homogenized)
Figure 9

Oxygen Uptake by MQB Cells
Starved for 3 Days (Homogenized)
**FIGURE 10**

Oxygen Uptake by MQ8 Cells

Starved for 4 Days (Homogenized)
FIGURE II

Oxygen Uptake by MQ8 Cells
Starved for 5 Days (Homogenized)
FIGURE 12

Effect of Griseofulvin on the Total Endogenous and Glucose Oxidation Values

% Increase of Endogenous

% Reduction of Endogenous

% Increase of Glucose Oxidation

% Reduction of Glucose Oxidation

Endogenous (left scale) ——— Glucose (right scale)

---% of total theoretical oxidation of Glucose (right scale)
D. Dehydrogenase Studies

The first dehydrogenase experiments were carried out on whole cell material, which had been starved for four days. The protocol was that given in TABLE III. Several substrates (glucose, fructose-1, 6-diphosphate, glucose-1-phosphate, glucose-6-phosphate, and pyruvate) were employed. With these substrates, the cells reduced the methylene blue within ten to fifteen minutes, while in the absence of substrate the reduction time was over twenty-five minutes. No appreciable difference was observed in the presence of added griseofulvin.

Cell-free extracts then were used as the enzyme source, and the previously mentioned substrates were tested. The only substrate which allowed methylene blue reduction was pyruvate. It required approximately thirty minutes for complete reduction, while the remainder of the substrates and the endogenous showed no reduction after two hours. It was shown that this was not autoxidation of pyruvate, as the substrate control did not become reduced. The presence of griseofulvin had no effect on this reaction. As results in this area were not satisfactory, it was decided to investigate other areas of cellular metabolism for griseofulvin effects.
II. Amino Compound Studies

The effects of griseofulvin appear to be very widespread. Electron micrographic studies by Blank et al. (10) show alterations in cell wall structure and also indicate an increase in concentration of lipid materials inside the cell. It was considered possible that in addition, amino compounds might be excreted by the organism when grown in the presence of griseofulvin. The medium used in this study was synthetic in nature, having glucose as the source of carbon and ammonium sulphate as the nitrogen source (see Materials). The organism studied was Microsporum quinckeanean strain #8 (MQ8).

The methods of extraction and analysis of amino compounds have been described in a previous section. Observations also were made during cell growth and during extraction of the supernates. The first question of course, was whether any growth would occur in the medium employed. From daily observations it was seen that after one day a small amount of growth occurred in all flasks. From the first day to the sixth day when the contents of the last flasks were analyzed, the control cultures containing only the basal medium showed a continuous increase in weight of hyphae. Those flasks containing griseofulvin though, showed no growth after the first day. It was possible in fact, that a slight decrease in cell weight occurred.

A second fact was noted during analysis of the supernates. After removing cell material by centrifugation, the remaining soluble protein was precipitated by the addition of
ethanol and then heating. A definite pattern was seen in the amount of protein precipitated in each flask. The zero time cultures both gave very little soluble protein. The griseofulvin culture at two days though, contained approximately three to four times the weight of protein as compared to the control. At four days and six days, the protein content of the controls increased steadily. The griseofulvin cultures showed no increase in soluble protein over the amount which had been observed after two days. The amount of soluble protein in each of the cultures containing griseofulvin was in all cases higher than that found in any of the controls.

The amino compound extracts for each day were run on one chromatogram. Three different concentrations of each sample were applied. The zero time samples both showed the presence of about the same quantity of amino compounds in the highest concentration applied. An equivalent increase was observed in all two-day old cultures. The concentrations of amino compounds continued to increase in the control flasks for the remaining four days. The concentrations in the cultures containing griseofulvin did not increase after two days.

The qualitative assay of the amino compounds showed no differences between the cells grown in the presence or absence of griseofulvin. The following spots were recorded and tentatively identified by their Rf values: lysine, arginine, histidine, aspartic acid, glycine (and/or serine), hydroxyproline, glutamic acid, alanine, proline, methionine, valine,
phenylalanine and leucine (and/or isoleucine). Traces of cystine and glucosamine were also detected. Both media containing no cell material were found to be free of ninhydrin-reacting compounds. Thus all spots appearing on the chromatograms originated from the cell material.

A partial interpretation of these results was attempted. First, the controls gave results which would be expected of an average culture growing slowly in a simple medium. The amounts of soluble protein and amino acids in the supernate slowly increased over the whole growth period. The medium containing griseofulvin, on the other hand, yielded what seemed to indicate a dead or non-metabolizing culture. The lack of growth and of amino acid release after one or two days would support this. The great increase in soluble protein which was observed after two days, in all cultures containing griseofulvin, might indicate auto-degradation and breakage of the cells.

The lack of qualitative difference in the amino compounds of the supernate does not conclusively prove that amino acid metabolism is not affected, as the simpler amino acids have many possible interlocking pathways. It may indicate though, that formation of the more complex amino acids is not inhibited, and that the growth inhibition is not due to amino acid metabolism being blocked.
III. **Chitinase Studies Using Whole Cells**

The investigation into the effects of chitinase on the cell walls of dermatophytes was initiated for two reasons. First, the thick cell walls possessed by these organisms made it very difficult to obtain cell free extracts. It was felt that the method being used might be destroying many cellular systems, and that by first weakening the cell walls breakage could be caused more easily. The second reason was to determine whether the formation of protoplasts might be possible with dermatophytes as it was with Neurospora species (5).

Two 500 ml. Erlenmeyer flasks, each containing 50 ml. of Sabouraud's cerelose medium, were inoculated with a fine suspension of *Microsporum quinckeanum* strain #8 (MQ8) mycelia. These cultures were grown for six days in shake culture, at room temperature. The resulting fine pellets were collected on Whatman No. 1 filter paper on a Buchner filter. The pellets were next washed on the filter, with 500 ml. of 0.85% NaCl. The washed cell material was then resuspended in 10 ml. of 0.033 M phosphate buffer pH 6.8. This suspension then was treated as described in TABLE III.

The sample used for dry weight determinations was weighed in comparison to 1.0 ml. of 0.033 M phosphate buffer pH 6.8. The weight of the buffer was subtracted from the weight of the cell suspension to give the weight of the cell material. The results are given in TABLE IV.

The samples of cell material were hydrolyzed with acid
in order to determine the total amount of N-acetyl glucosamine which might be expected from enzymic hydrolysis of the chitin. Several hydrolysis periods were used to determine the optimum conditions for hexosamine measurement. The results of these tests may be seen in TABLE V.
TABLE III

Procedure for Chitinase Studies
Using Whole Cell Material

MQ8 CELL SUSPENSION (10 ml.)

\[
\begin{align*}
\text{Cell Susp.} & \quad \text{Cell Susp.} & \quad \text{Cell Susp.} & \quad \text{Cell Susp.} & \quad \text{Cell Susp.} & \quad \text{Cell Susp.} \\
(1.0 \text{ ml.}) & \quad (1.0 \text{ ml.}) & \quad (1.0 \text{ ml.}) & \quad (1.0 \text{ ml.}) & \quad (1.0 \text{ ml.}) & \quad (5.0 \text{ ml.}) \\
\downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow \\
\text{Dry Weight} & \quad 2.0 \text{ ml.} & \quad 2.0 \text{ ml.} & \quad 2.0 \text{ ml.} & \quad 3.0 \text{ ml.} & \quad 50 \text{ mg. Chitinase}^* \text{ in 15 ml. of 0.2 M} \\
\text{Determination} & \quad 3.0 \text{ N HCl} & \quad 3.0 \text{ N HCl} & \quad 3.0 \text{ N HCl} & \quad 2.0 \text{ N HCl} & \quad \text{Hydrolysis} \\
\downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow \\
\text{Hydrolysis} & \quad \text{at } 100^\circ \text{C} & \quad \text{at } 100^\circ \text{C} & \quad \text{at } 100^\circ \text{C} & \quad \text{at } 100^\circ \text{C} & \quad \text{citrate} \\
\text{for 14 hr.} & \quad \text{for 17 hr.} & \quad \text{for 20 hr.} & \quad \text{for 33 hr.} & \quad \text{buffer pH 5.8} \\
\end{align*}
\]

Dilute each to 10 ml. with distilled water
Centrifuge
Hexosamine assay

\[
\begin{align*}
1.0 \text{ ml.} & \quad 0.5 \text{ ml.} \\
\text{samples} & \quad \text{samples for microscopic observations of 12\% TCA} \\
\downarrow & \quad \downarrow \\
\text{Each sample was} & \quad \text{Each sample was} \\
diluted to 5.0 ml. & \quad \text{diluted to 5.0 ml.} \\
\text{with dist. water} & \quad \text{with dist. water} \\
\downarrow & \quad \downarrow \\
\text{Dowex 50 columns} & \quad \text{Dowex 50 columns} \\
\downarrow & \quad \downarrow \\
2.0 \text{ N HCl eluate} (5.0 \text{ ml.}) & \quad 2.0 \text{ N HCl eluate} (5.0 \text{ ml.}) \\
\end{align*}
\]

\[
\begin{align*}
2.0 \text{ ml.} & \quad 3.0 \text{ ml.} \\
\text{eluate} & \quad \text{eluate} \\
\downarrow & \quad \downarrow \\
\text{Hydrolysis at } 100^\circ \text{C for 18 hr} & \quad \text{Hexosamine assay} \\
\downarrow & \quad \downarrow \\
\text{Dilute to 10 ml.} & \quad \text{Dilute to 10 ml.} \\
\downarrow & \quad \downarrow \\
\text{Centrifuge} & \quad \text{Centrifuge} \\
\downarrow & \quad \downarrow \\
\text{Hexosamine assay} & \quad \text{Hexosamine assay} \\
\end{align*}
\]

(*The chitinase was a purified preparation isolated from the intestine of the snail Helix pomatia. It was kindly supplied by Dr. G. Strasdinie, National Research Council, Ottawa, Canada.*)
TABLE IV
Dry Weight Determination

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cup Weight</th>
<th>Cup Sample Weight</th>
<th>Sample Weight</th>
<th>Minus Buffer</th>
<th>Weight in mgm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Material</td>
<td>1.3652 gm.</td>
<td>1.3728 gm.</td>
<td>0.0076 gm.</td>
<td>0.0026 gm.</td>
<td>2.6 mgm.</td>
</tr>
<tr>
<td>Buffer</td>
<td>1.3728 gm.</td>
<td>1.3778 gm.</td>
<td>0.0050 gm.</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

TABLE V
Glucosamine-HCl Content of Hydrolyzed Cell Samples

<table>
<thead>
<tr>
<th>Final Normality</th>
<th>Hydrolysis Period</th>
<th>Absorbance 530 μm</th>
<th>Glucosamine-HCl (μgm.)</th>
<th>Glucosamine-HCl (μgm.) in 1.0 ml. cell susp.</th>
<th>% Weight Glucosamine-HCl in cell susp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 N</td>
<td>14 hours</td>
<td>0.055</td>
<td>22.9 μgm.</td>
<td>229 μgm.</td>
<td>8.8%</td>
</tr>
<tr>
<td>2 N</td>
<td>17 hours</td>
<td>0.058</td>
<td>24.2 μgm.</td>
<td>242 μgm.</td>
<td>9.3%</td>
</tr>
<tr>
<td>2 N</td>
<td>20 hours</td>
<td>0.050</td>
<td>20.8 μgm.</td>
<td>208 μgm.</td>
<td>8.0%</td>
</tr>
<tr>
<td>1.5 N</td>
<td>33 hours</td>
<td>0.057</td>
<td>23.8 μgm.</td>
<td>238 μgm.</td>
<td>9.2%</td>
</tr>
</tbody>
</table>

It was concluded from the similarity of these results to those obtained by Boas (51), that samples for hexosamine assay should be hydrolyzed at 100°C for eighteen hours. When the assayed weight of glucosamine-HCl is compared with the dry weight of cell material, it can be seen that glucosamine-HCl would comprise between 9% and 10% of the total cell weight. The percentage weight using glucosamine-HCl is, of course, an artificial value, but the standards used were glucosamine-HCl. Using these values it was easy to determine the weights of free glucosamine and free N-acetyl glucosamine on a molar basis.
The enzymic hydrolysis of chitin was carried out on a shaker with the flask suspended in a 30°C water bath. Samples for hexosamine assay and microscopic examination were taken at: zero time (time when chitinase was added), 15 min., 30 min., 60 min., 90 min., 120 min., and 150 min. The hexosamine samples were all taken in duplicate.

The microscopic examination was carried out using an ordinary light microscope. This is not as satisfactory as a phase contrast microscope, for observation of cell wall alteration. It was noted though, that after ninety minutes the tips of hyphae began to swell. This was taken as an indication that the enzyme was hydrolyzing at least part of the hyphal wall, although the older portions of the hyphae showed no observable effects.

The samples for hexosamine assay were treated with 12% trichloracetic acid (TCA) to stop the reaction and to remove the protein from solution. The first step in the assay was purification of the sample by passage through Dowex 50 H+ionized resin. The presence of the amino group on the glucosamine causes it to bind to the column. The column was washed and then the glucosamine was eluted with 5.0 ml. of 2.0 N HCl. 3.0 ml. volumes of the eluate were assayed for hexosamines, but none of the samples showed their presence.

This result did not mean that there was no chitinase activity. It was considered quite possible that the enzyme was hydrolyzing the chitin into smaller units consisting of several N-acetyl sub-units. Such units should be soluble,
although larger ones might not bind to the Dowex column. To try to test this hypothesis, some of the remaining 2.0 ml. volumes of the 2.0 N HCl eluate were sealed in ampoules and heated at 100°C for eighteen hours. The samples tested were: zero time, 90 min., and 150 min. The values obtained for these samples, when assayed for hexosamines, are given in TABLE VI.

### TABLE VI

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance 530 μm</th>
<th>Average</th>
<th>Glucosamine-HCl in sample (μg.m.)</th>
<th>Glucosamine-HCl in 1.0 ml. of cell susp.</th>
<th>% of Glucosamine-HCl by HCl hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>zero time</td>
<td>0</td>
<td>0.001</td>
<td>0.4 μg.m.</td>
<td>4 μg.m.</td>
<td>1.7%</td>
</tr>
<tr>
<td>zero time</td>
<td>0.002</td>
<td>0.001</td>
<td>0.4 μg.m.</td>
<td>4 μg.m.</td>
<td>1.7%</td>
</tr>
<tr>
<td>90 min.</td>
<td>0.003</td>
<td>0.003</td>
<td>1.3 μg.m.</td>
<td>13 μg.m.</td>
<td>5.4%</td>
</tr>
<tr>
<td>90 min.</td>
<td>0.003</td>
<td>0.003</td>
<td>1.3 μg.m.</td>
<td>13 μg.m.</td>
<td>5.4%</td>
</tr>
<tr>
<td>150 min.</td>
<td>0.005</td>
<td>0.004</td>
<td>1.7 μg.m.</td>
<td>17 μg.m.</td>
<td>7.3%</td>
</tr>
<tr>
<td>150 min.</td>
<td>0.003</td>
<td>0.004</td>
<td>1.7 μg.m.</td>
<td>17 μg.m.</td>
<td>7.3%</td>
</tr>
</tbody>
</table>

The results shown here indicate that a small amount of chitin was hydrolyzed, but that it was only a small percentage of the amount obtained by acid hydrolysis. These results are not entirely conclusive though, as they are at the limit of the sensitivity of the assay.

The results do coincide with microscopic observations.
If only the chitin at the hyphal tips is affected then the percent hydrolysis would be extremely low. The fact that only growing tips showed any change, may mean that the chitin in the cell wall is shielded from the enzyme. This is supported by investigations carried out by Prince in 1960 (55). He found that the chitinase produced by a streptomycete failed to affect Trichophyton mentagrophytes. He suggested that something in or near the cell wall was acting as a shield. Other support is found in the work of Horikoshi and Iida (38,39), who showed that Aspergillus oryzae cells were not altered by chitinase until they had been previously treated with \( \beta-1,3 \)-glucanase.

Because of the problems involved in obtaining cell wall hydrolysis with purified chitinase preparations, it was considered fruitful to investigate the effects of a mixture of enzymes on dermatophyte cell walls. Some preliminary attempts were made to isolate a bacterial strain which would attack the cell walls, but these were unsuccessful. On further investigation it was found that the crude intestinal juices of Helix pomatia would cause at least partial weakening of Neurospora crassa cell walls (5).

A short study was carried out similar to that just described, but with two variations. First, crude intestinal juice of Helix pomatia (L'Industrie Biologique Française) was utilized. Secondly, an acetone-dried cell preparation was used rather than whole cells. The results in this experiment were very good, a yield of approximately 25% of the expected
total yield of glucosamine-HCl was obtained in two hours. This result was obtained from the acid hydrolyzed sample after Dowex 50 separation. The unhydrolyzed sample gave a yield of approximately 6% for the same time period. At this time it was decided to make attempts to obtain protoplasts of dermatophytes for studies on the effects of griseofulvin on membrane permeability, and on cell wall resynthesis.
IV. Preparation of Protoplasts

A. Preliminary Investigation with Neurospora tetrasperma

Before attempting to obtain protoplasts of dermatophytes it was considered more useful to carry out the procedure of Colvin (20) using Neurospora tetrasperma. The hyphae of this species are larger than those of Microsporum quinckeanum strain #8. Thus protoplasts and structures are more easily observed. All microscopic examinations were carried out with a phase contrast microscope.

The protoplast suspension was prepared as described in a former section. Samples were removed at fifteen minute intervals during the incubation period and observed under the microscope. The general pattern of protoplast extrusion may be seen in FIGURE 13. This is similar to the photographs shown by Bachmann and Bonner (5). The "blebs" mentioned by these authors were also observed. These protoplasts were from 10 to 30 μ in diameter.

The protoplasts were next purified, concentrated, and resuspended in a 20% sucrose solution. Small portions were removed at thirty minute intervals and challenged with distilled water. The results of this study are shown by diagram in TABLE VII. The synthesis of cell wall is seen after thirty minutes, but the new structures remain fragile until four hours. The times of course are not absolute. The observations are those for about 80% of the protoplast population at the given time. Upon osmotic shocking, "blebs" as described (5) were released.
The use of Neurospora protoplasts to determine the effects of griseofulvin was now considered. This was not possible though, since *Neurospora tetrasperma* grew in the presence of 3.0 µgm. of griseofulvin per ml. with very slight inhibition. This was in the form of slower growth rather than distortion of the hyphae. It now became necessary to investigate the possibility of forming protoplasts of ringworm fungi.
FIGURE 13

Diagramatic Representation of the Formation of Protoplasts of *Neurospora tetrasperma*

Zero time

One to two hours

Two to four hours
TABLE VII

Cell Wall Regeneration by *Neurospora tetrasperma* Protoplasts in 20% Sucrose

<table>
<thead>
<tr>
<th>Time</th>
<th>Protoplast before challenge with distilled water</th>
<th>Protoplast after challenge with distilled water</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero Time</td>
<td><img src="image" alt="Protoplast before challenge with distilled water" /></td>
<td><img src="image" alt="Protoplast after challenge with distilled water" /></td>
<td>In the presence of distilled water, the protoplast swells until the membrane ruptures, and the internal contents are released.</td>
</tr>
<tr>
<td>30 min. to 60 min.</td>
<td><img src="image" alt="Protoplast before challenge with distilled water" /></td>
<td><img src="image" alt="Protoplast after challenge with distilled water" /></td>
<td>A thin wall appears to form but when the cell is challenged, the inner protoplasm emerges and bursts as before.</td>
</tr>
<tr>
<td>after 4 hours</td>
<td><img src="image" alt="Protoplast before challenge with distilled water" /></td>
<td><img src="image" alt="Protoplast after challenge with distilled water" /></td>
<td>At this time the challenge with water has no effect. The wall around the cell becomes quite rigid.</td>
</tr>
<tr>
<td>after 12 hours</td>
<td><img src="image" alt="Protoplast before challenge with distilled water" /></td>
<td><img src="image" alt="Protoplast after challenge with distilled water" /></td>
<td>After the cell wall formation was complete, the cells usually altered in shape and appeared to grow. This growth did not last for very long, probably because of the lack of a nitrogen source in the medium.</td>
</tr>
</tbody>
</table>
B. Investigations on the Formation of Dermatophyte Protoplasts

The formation of protoplasts by the ringworm fungi, through the action of snail digestive enzymes, was considered extremely likely on the basis of the final results of the hexosamine assays. This was further supported by work carried out by Carlson and Knight on purified cell wall material of *Trichophyton mentagrophytes* (19). They demonstrated the formation of glucose and N-acetyl glucosamine from the cell wall material in the presence of "crude snail gut chitinase". The fact that the acetone-dried cells and purified cell wall preparations were attacked did not of course mean that living cells would be vulnerable. The chitin and glucose polymer layer could be protected by a glucosamine and peptide layer. Carlson and Knight reported such compounds when they acid-hydrolyzed their chitinase resistant, non-dialyzable fraction.

The first attempt to produce dermatophyte protoplasts was with a four day static culture of *Microsporum quinckeanum* strain #8 (MQ8). The organism was grown in Sabouraud's cere­lose broth. Colvin's modification of the method of Bachmann and Bonner was used. The observation procedure was the same as that used for Neurospora studies.

The observations using MQ8 almost paralleled those for Neurospora species as described in FIGURE 13. The only variations were that the protoplasts were smaller (from 5.0 μ to 20 μ in diameter) and that they took a shorter time to form. They started to appear after fifteen minutes and continued to
form for several hours. The first ones formed seemed to come from the hyphal tips, while those which formed later came from older sections of the hyphae.

The procedure of resuspending in 20% sucrose and then challenging at thirty minute intervals with distilled water was also followed. The results were similar to those of Neurospora species as shown in TABLE VII. The "blebs" were again observed after osmotic rupture.

Because of the low yields of protoplasts, variations of Colvin's method were tried. It was finally found that a modification of Kinsky's method (43) gave the greatest numbers. Attempts were made to obtain even greater yields by lowering the sucrose concentration from 18% to as low as 10%. This provided more protoplasts, but when the sucrose concentration was brought back to 20% sucrose, they did not appear normal. The lowered osmotic strength of the solution causes swelling of the protoplast. This aids in its extrusion from the hyphae, but it also appears to cause rupture and stretching of internal structures. Therefore, the concentration of sucrose finally used was 18%.

It was felt at this time, that further confirmation of the identity of these structures as protoplasts was necessary. Through the kindness of Dr. W. Chase, Department of Pathology, U.B.C., electron micrographs of MQ8 protoplasts were obtained. The following is a description of the method employed by Dr. Chase.

Protoplasts used in preparation for electron
micrographs were separated from the residual hyphal elements in the aforementioned manner, and concentrated by centrifugation. This material was fixed in veronal-buffered OsO₄, 1%, pH 7.4, for twenty minutes. The pellet was dehydrated in alcohol and fragments were embedded in Maraglass, according to the treatment of Freeman and Spurlock (25). Ultrathin sections were cut with glass knives on a Porter-Blum ultramicrotome and mounted on unsupported grids. Preparations were stained with lead hydroxide according to the method of Karnovsky (42). Electron micrographs were taken with a Siemens electron microscope at accelerating voltages of 60 kv. The resulting micrographs are shown in FIGURE 14.
14 a. Protoplast showing cytoplasmic membrane (cm) and mitochondria (mt).

14 b. Protoplast demonstrating cytoplasmic membrane (cm), nucleus (n), and nucleolus (nu).
14 c. Portion of a protoplast demonstrating the distinct limiting membrane (cm).
Brenner, et al. (14) have suggested that a "protoplast" should be defined as, "a structure in which the cell wall is known to be absent" or "that part of the cell which lies within the cell wall". They give several criteria upon which a protoplast may be tested. First, the structure must be osmotically sensitive and observations with an electron microscope must show no indications of a cell wall. These authors also suggest chemical and immunochemical tests for known cell wall compounds and antigens. In addition, they feel that interaction with phage would be a good test for the presence of cell wall. Finally, knowledge of what compounds are released from cell walls by the action of the enzyme employed, is mentioned as an indicator of the possibility of protoplast formation.

The protoplasts of dermatophytes which have been described in this study fulfill several of these standards given by these authors. The action of crude snail gut chitinase on isolated cell walls of dermatophytes has been demonstrated to release glucose and glucosamine (19). These compounds do not account for all of the cell wall material, but they do account for a large portion of it. Next, phase contrast microscopic observations in this study have shown that the membrane bound contents of one hyphal unit are extruded, leaving a cell wall "skeleton" behind. These membrane bound spheroids were proven to be osmotically fragile. Finally, the electron micrographs (FIGURE 14) show that the structures are membrane bound and that no cell wall is present. An excellent contrast to these micrographs are those taken by
Blank, et al. (10) of normal and griseofulvin treated cells. In the pictures taken by these authors, the thick, rigid cell walls are very evident.
V. Studies on the Effect of Griseofulvin on Cell Membranes

The paper by Kinsky in 1962 (43) gave not only an excellent method of mold protoplast preparation but also a technique for measuring alterations in protoplast size. He used this procedure to show the effects of certain drugs on the permeability of the cell membrane. In this investigation, similar techniques were used to determine whether griseofulvin had any such effects on the membranes of dermatophyte cells.

The protocol and techniques were as already described, and the organism was MQ8. Observations were made over a period of one hour. The results of these studies are shown graphically in FIGURE 15. According to Kinsky, increase in absorbancy results from shrinkage of protoplasts, while decreases are due to swelling, lysis, and precipitation.

The first experiments, as seen in FIGURE 15a, did not show any definite alterations in the size of the protoplasts. Microscopic observations at the beginning and the end of the reaction periods showed no great alteration in protoplast numbers. Similarly protoplast size remained almost the same although a slight increase may have occurred. The numbers of the protoplasts in the cuvettes were approximately $3.3 \times 10^5$ per ml. When these concentrations were compared with those used by Kinsky, it was realized that they were extremely low and that therefore small alterations in protoplast size could not be measured.

A second series of experiments was carried using concentrations of approximately $3.2 \times 10^6$ protoplasts per ml.
The results are described in FIGURE 15b. It is first observed that both test and control samples give slopes of decreasing absorbancy during the run. The initial steep slope is partially due to the dilution factor of the brown coloured background, resulting when the DMF was added. It is also partially due to a slight increase in protoplast size, as the sucrose concentration is reduced. The slow continuous character of the remainder of the slope is caused by slow precipitation of larger protoplasts. This was demonstrated by stirring the contents of the cuvettes at the end of the experiment. An immediate increase in absorbancy resulted in both cuvettes as the precipitated protoplasts were resuspended.

The major observation in this series was that the decrease of absorbancy in the test sample was greater than that in the control sample. As was previously stated, absorbancy decreases may be due to swelling, lysis or precipitation. Microscopic counts before and after the readings indicate that there was no decrease in numbers. These examinations did indicate a slight increase in average protoplast size in the test sample. In other words a small amount of swelling had been caused by the griseofulvin. An increased amount of precipitation in the test sample is also indicated by the values recorded after stirring.

The interpretation of these observations is not very conclusive. Griseofulvin appears to have some effect on the permeability of the cytoplasmic membrane. From the small
FIGURE 15
Effect of Griseofulvin on Dermatophyte Protoplast Membranes

15a. Low Protoplast Concentrations

15b. High Protoplast Concentrations

N,N-dimethylformamide (DMF)
Griseofulvin in DMF
effect of such a high concentration of drug it seems likely that the membrane is not the main object of the drug's attack. It is much more probable that the primary target is simply located on or near the membrane. This is supported by the effect observed in the oxygen uptake values with glucose as the substrate. The enzymes involved in the oxidative assimilation of glucose are probably associated with the cell membrane. Thus small alterations in the membrane might simply be an indication of disruption of these enzymes attached to it. A better possible locus for the site of action might be the cellular energy mechanisms. These are involved in maintaining osmotic equilibrium of the cell and are also involved in the glycolytic and assimilative pathways. If this was the case, the swelling of the protoplasts in the presence of 18% sucrose would be very slow, so that only a small difference would be observed during the course of this experiment. Supporting evidence for this hypothesis is found in the report by McNall on the partial reversal of the action of griseofulvin, by the addition of purines and pyrimidines (47), which are central figures in cellular energy systems.
VI. Studies on the Effect of Griseofulvin on Cell Wall Resynthesis

Electron micrographic observations of cells grown in the presence of griseofulvin (10,66) show a thickening and layering of cell walls. When it was found that dermatophyte protoplasts would resynthesize cell wall in the absence of crude chitinase, a study was initiated to determine whether griseofulvin would inhibit this process. Experiments were carried out, tracing the cell wall formation of MQ8 by measurement of absorbancy at 600 μm, over a period of seven hours. Microscopic observations were made simultaneously. Spectrophotometric data is given graphically in FIGURE 16, while microscopic observations are listed in TABLE VIII.

It can be seen that both samples show an initial lag period, the griseofulvin sample showing a slightly longer one than the control. Following this is a short period during which there is a rapid increase in the absorbancies of both samples. This slows in both samples and continues at a more moderate rate until six hours at which time they begin to level. At all times the test sample lags just behind the control. The rates of synthesis in both samples are the same. The total increase in absorbancy is less in the test sample, than in the control, which could indicate the formation of a less dense or thinner cell wall.

It is concluded that griseofulvin causes a slight inhibition of initial cell wall resynthesis, but that after the
first thin wall has been formed the drug then may cease to exert any effect. The lower, total absorbance increase observed, may indicate the formation of an irregular cell wall, as reported by Blank, et al. (10). The site of this action is again still uncertain. Combined with the results of the cell membrane studies it is probable that the site of action of the drug is on or near the membrane. The resulting minor disruption could cause the slight inhibition of cell wall resynthesis observed here.
### TABLE VIII
Microscopic Observations of Cell Wall Resynthesis by Protoplasts

<table>
<thead>
<tr>
<th>Time</th>
<th>Control Sample Containing DMF*</th>
<th>Test Sample Containing Griseofulvin in DMF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>30min.</td>
<td>Protoplasts appeared normal. Ruptured when challenged with distilled water.</td>
<td>As in control.</td>
</tr>
<tr>
<td>1 hr.</td>
<td>Protoplasts still appeared normal and ruptured when challenged.</td>
<td>As in control.</td>
</tr>
<tr>
<td>2 hr.</td>
<td>Between 20% and 30% of protoplasts showed a very thin cell wall. When the sucrose was replaced with distilled water the inner protoplast usually burst out of this thin wall and ruptured. Most still burst upon challenging.</td>
<td>Less than 10% of protoplasts exhibited a thin cell wall. Most still ruptured immediately upon challenging.</td>
</tr>
<tr>
<td>3 hr.</td>
<td>Between 60% and 70% showed the presence of a thin cell wall. About 10% showed a thicker cell wall which would not rupture even when all sucrose was replaced. The remainder burst upon challenging.</td>
<td>Between 40% and 50% showed thin cell walls. The remainder burst upon challenging.</td>
</tr>
<tr>
<td>4 hr.</td>
<td>All protoplasts exhibited thick cell walls. No bursts were observed upon challenging.</td>
<td>Approximately 80% showed thick cell walls. Most of the remainder had thin walls although the occasional free protoplast was observed.</td>
</tr>
<tr>
<td>5 hr.</td>
<td>As at 4 hours.</td>
<td>All protoplasts exhibited thick walls. No bursts were observed upon challenging.</td>
</tr>
<tr>
<td>6 hr.</td>
<td>As at 4 hours.</td>
<td>As at 5 hours.</td>
</tr>
<tr>
<td>7 hr.</td>
<td>As at 4 hours.</td>
<td>As at 5 hours.</td>
</tr>
</tbody>
</table>

* N,N-dimethylformamide
FIGURE 16

Effect of Griseofulvin on Cell Wall Resynthesis
VII Cell Wall Composition Studies

The results obtained in the resynthesis study indicate that a cell wall is formed in the presence of griseofulvin. They do not indicate though, whether this wall has the same composition as a normal cell. On the basis of the electron micrographs taken by Blank et al. (10), it would appear quite possible that the composition was not the same. An investigation was therefore undertaken to qualitatively analyze the cell walls of MQ8 cells, which had been grown in the presence and absence of griseofulvin. To increase the scope of the experiment, it was decided that a mutant of MQ8, which had developed resistance to the drug under "in vitro" conditions, should be isolated and analyzed at the same time.

A. Isolation of a Griseofulvin Resistant Mutant

As a first step in the isolation of a mutant, the stock MQ8 culture was again tested for griseofulvin sensitivity. It was inoculated onto a series of plates containing griseofulvin in the following concentrations: 1.0 µgm./ml., 2.0 µgm./ml., 3.0 µgm./ml., 4.0 µgm./ml., 8.0 µgm./ml., and 16 µgm./ml. The results are shown in TABLE IX. Observations were made at three and six days.
### TABLE IX

**Sensitivity of MQ8 to Griseofulvin**

<table>
<thead>
<tr>
<th>Griseofulvin Concentration (μgm./ml.)</th>
<th>Hyphal &quot;curling&quot;</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days</td>
<td>6 days</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>2.0</td>
<td>+</td>
<td>+2</td>
</tr>
<tr>
<td>3.0</td>
<td>+2</td>
<td>+4</td>
</tr>
<tr>
<td>3.0</td>
<td>+2</td>
<td>+4</td>
</tr>
<tr>
<td>4.0</td>
<td>+4*</td>
<td>-</td>
</tr>
<tr>
<td>4.0</td>
<td>+4*</td>
<td>-</td>
</tr>
<tr>
<td>8.0</td>
<td>+4*</td>
<td>-</td>
</tr>
<tr>
<td>8.0</td>
<td>+4*</td>
<td>-</td>
</tr>
<tr>
<td>16.0</td>
<td>+4*</td>
<td>-</td>
</tr>
<tr>
<td>16.0</td>
<td>+4*</td>
<td>-</td>
</tr>
</tbody>
</table>

* Inocula showed extreme distortion.
These results agree generally with those found previously for MQ8. This strain which was sensitive to 4.0 \( \mu \text{gm.} \) of griseofulvin (G.) per ml. was now subjected to a "layer plate" containing 2.0 \( \mu \text{gm.} \) G./ml. The first hyphae to appear on the surface were transferred to two "layer plates" containing 4.0 \( \mu \text{gm.} \) G./ml. Growth appeared on the surface of the plate after six days. This was immediately transferred to two "layer plates" containing 8.0 \( \mu \text{gm.} \) G./ml. Growth appeared on the surface of one plate after ten days and on the other after twelve days. The growth from the ten day plate was transferred to two "layer plates" containing 16 \( \mu \text{gm.} \) G./ml. No growth appeared on the surface by fourteen days. This step was repeated, but with no success even after twenty days. The MQ8 mutant, resistant to 8.0 \( \mu \text{gm.} \) G./ml. (referred to as MQ8R), was maintained on Sabouraud's cerelose agar containing this concentration of drug.

B. Analysis of Cell Walls

The mutant strain (MQ8R) and the parent (MQ8) were separately inoculated into Sabouraud's cerelose broth containing griseofulvin. The media contained: no griseofulvin (G.), 8.0 \( \mu \text{gm.} \) G./ml., and 16 \( \mu \text{gm.} \) G./ml. This original inoculation pattern was not satisfactory as the parent (MQ8) would not grow in the presence of 8.0 \( \mu \text{gm.} \) G./ml. and 16 \( \mu \text{gm.} \) G./ml. These two flasks of media then were replaced with a medium containing 3.0 \( \mu \text{gm.} \) G./ml. The growth periods for these cultures varied greatly as may be seen in TABLE X.
The cell walls of these cultures were purified and analyzed according to the procedure given in Methods. Chromatographic analysis of the crude cell wall hydrolysates was also carried out during the purification process, prior to the trypsin and ribonuclease treatment. The results of this preliminary analysis indicated the presence of the sugars, glucose and ribose, and possible mannose and galactose. The amino sugar, glucosamine, was also found in high concentrations. The amino acid chromatogram contained at least 16 spots and appeared to be a protein hydrolysate. These results were the same for all of the cell wall preparations.

The analysis of the purified cell wall preparations showed the presence of the sugars; glucose, mannose, and galactose and the amino sugar, glucosamine. Galactosamine was not observed in any of these preparations. An unidentified compound which ran approximately half-way between glucosamine and galactose was observed in all cases. No differences in sugar or amino sugar content were noted among the various cell
wall preparations, either qualitatively or in the relative quantitative observations. The densest spot appeared to be glucose, followed by glucosamine, mannose, galactose, and finally, the unidentified spot.

The amino acid analysis of the purified walls did not indicate any qualitative differences among the five cell wall hydrolysates. The following eleven amino acids were tentatively identified in all samples: lysine, histidine, arginine, aspartic acid, glycine, glutamic acid, alanine, proline, valine, phenylalanine, and leucine (and/or isoleucine). Traces of an amino compound which was possibly cystine were also observed.

In the analysis for griseofulvin products, the griseofulvin 2.0 N and 6.0 N HCl hydrolysates and the precipitate extract, were run as standards. A pure griseofulvin sample was also run as a standard, and had an Rf of 0.87. A single, unidentified, light-blue fluorescent spot was observed in trace amounts in the 2.0 N HCl hydrolysate and in high concentrations in the 6.0 N NCl hydrolysate. It was not observed in the butyl acetate extract of the precipitate. This compound had an Rf of approximately 0.73. No griseofulvin was observed in any of the hydrolysates.

Cell wall preparations were chromatographed and observed under ultra-violet light. No fluorescing or absorbing spots were observed in the hydrolysates. A spot having a yellow fluorescence was observed in all extracts of the precipitates. This spot may be disregarded at this time, as
it is obviously not a griseofulvin hydrolysis product.

These results indicate that although cells grown in the presence of griseofulvin show a structural alteration microscopically (10), they do not alter appreciably in the chemical content of the assayed compounds. Qualitative differences in amino acid, amino sugar, and sugar content could not be demonstrated. Similarly, no differences in the relative quantitative values of these compounds could be observed. Finally, the absence of griseofulvin products in the hydrolysates is a good indication that griseofulvin is not incorporated to form a modified chitin, as suggested by Rhodes (33).
GENERAL DISCUSSION

The initial review of the literature, with respect to the antifungal properties of griseofulvin, indicated a variety of unexplained facts about its mode of action. First, it causes stunting and distortion of growing hyphal tips (18). A wide survey of sensitive and resistant fungal species has shown also, that except for one or two notable cases (2), all fungi which are sensitive to griseofulvin, have chitinous cell walls (15). These walls are definitely altered, becoming frayed and forming thick irregular layers, when sensitive strains are grown in the presence of the drug. At the same time, large lipid granules are observed in the cytoplasm (10). The fact that only growing areas of the hyphae became deformed, was confirmed by studies involving the direct application of griseofulvin to various parts of the hyphal elements (7). It was shown also that griseofulvin was not translocated within the hyphae, thus it affects only the area of contact (2).

Various biochemical studies also have been reported. First, several workers have carried out studies on oxygen uptake by fungi, in the presence of griseofulvin. Two of these (15,44) have reported that griseofulvin has no effect on endogenous respiration, while a third reports that there is a very small percent decrease (63). One of the authors who reported that no alterations occurred, listed the QO\textsubscript{2} values obtained in his experiments (15). Closer observation of these values
indicated that in almost all cases, there was a decrease of approximately 2.0% in the presence of the drug. It was surprising at first, that none of these authors investigated the effects of griseofulvin on the oxidation of a substrate, such as glucose. The reason became clear though, when it was found that the addition of glucose to unstarved cell material, resulted in a decrease in the endogenous respiration rate (63).

The results of the oxygen uptake studies, which were reported in this thesis, confirm that a small reduction of endogenous respiration is caused by griseofulvin. They further show that glucose oxidation is initially reduced by the drug, but that with continued starvation of the cell material, an increased amount of glucose is oxidized. In fact, the amount of glucose oxidized in the presence of the drug is greater than the amount oxidized in its absence.

These oxygen uptake results are difficult to interpret without further correlation with other areas of cellular metabolism. For example, it was found that glucose was taken up by unstarved cell material, but that little or no oxygen uptake could be measured. With starvation of the cell material, a greater percentage of the glucose was oxidized. Also, it was seen that the effect of griseofulvin on the respiration of unstarved cell material was extremely different from its effect on the respiration of material which had been starved for five days. The reasons for this change can only be conjectured, because not enough is known about endogenous respiration in dermatophytes. In other words, how would
depletion of "endogenous reserves" alter the cell, and what are the "endogenous reserves"? The final conclusions of these experiments were that griseofulvin may inhibit enzymes involved in synthesis of "endogenous reserves", the cofactors associated with them, or the energy-transport mechanisms which drive these synthetic reactions. Some confirmation of these conclusions is found in the report that the assimilation of phosphate (12) and of nitrogen (71) are inhibited by the action of griseofulvin. The suggestion was made that griseofulvin uncouples a phosphorylation in a respiratory pathway (71).

A report by McNall (47) may help to clarify the previously mentioned results. His investigation involved the addition of compounds to culture media which also contained griseofulvin. He reported the partial reversal of the inhibitory effect of the drug, by the addition of purines, pyrimidines, and their nucleotides. The most effective compounds in this partial reversal of inhibition, were guanylic acid and to a lesser degree, adenylic acid. This possibly indicates that the purine nucleotides are closely related to the site of action of the drug. McNall, in fact, believes that nucleic acid synthesis is blocked. It seems likely though, that the cellular, energy-transport systems, in which these compounds play an important role, are also involved. It is even possible that griseofulvin is acting competitively with precursors of these compounds in energy-transport pathways.
A good case can be presented to show that griseofulvin could be a structural analogue of purine nucleosides. The bonding properties of griseofulvin (24) are in many ways similar to those of the nucleosides. Thus, this drug could enter the reaction sites of these compounds, but could not carry out their reactions. On the basis of the phosphate assimilation studies (71), one reaction which might be blocked is the phosphorylation of the purine nucleosides.

The results obtained in the amino compound studies, proved to be of little use in clarifying the previously obtained results. They simply indicated that, due to the influence of griseofulvin, growth was completely inhibited, after one or two days. No evidence was found in this investigation to indicate any barrier in amino acid metabolism.

Investigations into the action of the drug on membrane permeability, showed that there is a slight effect. In considering the small alterations observed, it appears likely that the site of action is on or near the membrane but is not the membrane itself. Suggested areas are, enzyme systems associated with the membrane, or energy mechanisms involved in maintaining osmotic equilibrium of the cell.

A large portion of the literature suggests that cell wall synthesis is the site of griseofulvin action. This is certainly supported by electron microscopic observations (10). The use of protoplasts to follow complete cell wall synthesis showed that, although there was an initial lag in the formation of this structure, a wall of some type was formed. This
does not necessarily mean that the synthesis of cell wall was inhibited. It appears quite possible that some locus on or near the membrane is the object of this compound's activity. The enzymes involved in cell wall synthesis also are probably associated with the membrane, thus they might show the effects of alterations on it. On the other hand, the synthesis of one cell wall component could be blocked without inhibiting all cell wall formation.

This study did not prove that all constituents of the cell wall were unchanged. To confirm or disprove the hypothesis that cell wall synthesis was the site of the drug's action, a qualitative analysis of cell wall was undertaken. The results showed that no qualitative or relative quantitative differences in amino acid, amino sugar, and sugar content, occurred between cells grown in the presence and the absence of griseofulvin. Finally, attempts to demonstrate griseofulvin breakdown products in cell wall hydrolysates, point to the fact that griseofulvin is not incorporated into cell walls to give a modified chitin, as has been suggested (33).

The general conclusions of this study are that the site of griseofulvin action is related to enzymes involved in synthesis of endogenous substrates, or to mechanisms controlling these enzymes and their activities. It is further concluded that these systems are located on or near the cytoplasmic membrane.
From the results obtained in these investigations, it appears that further work should be carried out to confirm the results of McNall (47), on the partial reversal of griseofulvin inhibition, using purines, pyrimidines, and their nucleotides. It also is suggested that such results be correlated with oxygen uptake studies.
SUMMARY

Several series of experiments were carried out to determine the effects of the fungistatic drug, griseofulvin, on dermatophytes. The purpose of the first experiments was to determine whether starvation of cell material would show more clearly if griseofulvin had any effect on cell respiration, both endogenous and glucose oxidation. The results demonstrated that the drug caused a slight reduction of endogenous respiration throughout the starvation period. It also was shown that griseofulvin affected glucose oxidation, inhibiting it initially. With continued starvation though, the effect was reversed, and increased oxidation of glucose occurred in the presence of the drug.

To further study these physiological alterations, attempts were made to utilize cell-free extracts. These tests were without success, not only with manometric methods, but also with Thunberg techniques for dehydrogenase measurements.

A short investigation next was undertaken, to determine whether any amino compounds were excreted by the cells, while under the influence of the drug. No such result was demonstrated. It was observed though, that growth was inhibited after one or two days, in the presence of griseofulvin, thus confirming earlier reports that some growth occurs before inhibition. It also was noted that this fungistat caused a great increase in soluble protein in the medium.

The lack of results with cell-free extracts was
considered due to the rather rigorous conditions necessary to obtain them. A study was undertaken to determine whether enzymes, more specifically chitinase and related enzymes, would so weaken cell walls as to make cell breakage a very simple procedure. It was also the purpose of this study to find out if the formation of protoplasts from dermatophytes would be possible. Results here, indicated that partially purified chitinase was not effective, but that crude snail gut chitinase provided a better chance for both cell wall weakening and protoplast formation.

A study now was initiated to form protoplasts of dermatophytes. As a preparatory step, protoplasts of Neurospora species were formed and studied. Further attempts with dermatophytes also were successful, and a modified procedure which would give higher yields of protoplasts was discovered.

Having obtained protoplasts, an experiment was carried out to determine the effects of griseofulvin directly on the protoplast cytoplasmic membrane. It was found that this membrane was altered only slightly by the drug's action. In addition, protoplasts were employed to study the influence of griseofulvin on cell wall synthesis. A small effect, in the form of a time lag in cell wall production, was observed, but a wall of some type was formed.

There was some question as to whether the cell wall which formed in the presence of griseofulvin, was the same as that which was formed in its absence. In other words, were
any cell wall components deleted or was griseofulvin possibly incorporated into the cell wall structure. It was concluded from experimental results that no alterations in amino acid, amino sugar, or sugar content could be observed. It was further demonstrated that griseofulvin was not incorporated into the cell wall.

A general conclusion was drawn that the biochemical site of griseofulvin action is related to the enzymes involved in synthesis of endogenous substrates, or to the mechanisms controlling these enzymes. It also was concluded that the morphological site of action of the drug was located on or near the cytoplasmic membrane.

The suggestion was made by this author, that the reported partial reversal of griseofulvin activity by purines, pyrimidines, and their nucleotides, be confirmed and correlated with oxygen uptake results.
I. Percent reduction of Endogenous Respiration

The calculation used to determine the percent reduction of the endogenous oxygen uptake by griseofulvin action is as follows:

\[
\frac{(E - E_{Gr}) \times 100}{E} \% \text{ Re}
\]

E - Endogenous oxygen uptake.
E_{Gr} - Endogenous plus griseofulvin oxygen uptake.
\% Re - Percent reduction of endogenous respiration.

II. Percent Reduction or Increase of Glucose Oxidation

The calculation used to determine the percent reduction or increase of the glucose oxygen uptake due to griseofulvin activity, is as follows:

\[
\frac{(G - E) - G_{Ox} ; (G_{Gr} - E_{Gr}) - G_{OxGr}}{(G_{Ox} - G_{OxGr}) \times 100} - % \text{ Rg*}
\]

G - Glucose oxygen uptake.
E - Endogenous oxygen uptake.
G_{Gr} - Glucose oxygen uptake in the presence of griseofulvin.
E_{Gr} - Endogenous oxygen uptake in the presence of griseofulvin.
G_{Ox} - Oxygen uptake due to the oxidation of glucose.

* (If this value was a negative one, it was described as percent increase).
GOxGr - Oxygen uptake due to the oxidation of Glucose in the presence of griseofulvin.

% Rg - Percent reduction of glucose oxidation.

III. Percent of Total Theoretical Glucose Oxidation

The calculation used to determine the percent of total theoretical oxygen uptake due to oxidation of glucose is as follows:

\[
\frac{(GOx)}{(No. \ of \ \mu M \ glucose \ in \ cup) \times (134.4\text{*})} \times 100 - \% TG0x
\]

GOx - Oxygen uptake due to the oxidation of glucose.

\%TG0x - Percent of total oxygen uptake expected if glucose was totally oxidized.

*(This value is the number of ul. of oxygen required to totally oxidize 1.0 \mu M of glucose to CO\text{2} and H\text{2}O).
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