A STRATEGY FOR THE QUANTITATIVE

ANALYSIS OF FUNGAL CELL WALLS

by

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ABSTRACT

Cultures of *Tremella mesenterica* grown as haploid yeast-like unicells and *Saprolegnia diclina* were grown on incompletely defined media and the procedures for isolating cell wall preparations from each, free of cytoplasmic and capsular material, are described. A strategy for quantitative analysis of these cell wall preparations was devised and tested, selecting from the numerous procedures available: extraction and gravimetry for lipids; hydrolysis followed by gas-liquid chromatography for neutral sugars (as trimethylsilyl derivatives) and automatic amino acid analysis for amino acids and amino sugars.

Problems of degradation as a result of hydrolysis have been considered. The extent to which degradation occurs is difficult to estimate because each constituent polymer is 'contaminated' with the others. This may accelerate degradation, particularly of amino acids.

Analytical procedures were reproducible but only 90% of the weight of the cell wall was recovered. The remaining 10% is probably the result of degradation losses that have not been accounted for, and further studies are required to improve the estimation of this error.

Even under carefully standardized conditions, cell wall preparations show variable composition. A complete analysis was therefore performed on a single cell wall preparation of each of the two species. Analyses were performed on other cell wall preparations of the two species and they showed general similarities; the ratios of components were similar, although, for example one preparation of *S. diclina* contained more than twice as much total protein as another.

Similar recoveries of constituents suggest that the strategy is appropriate for quantitative analysis. However, alternative
methods for lipid analysis that provide more specific information are available and should be adapted for cell wall analysis. Quantitative recovery of uronic acids provides substantial difficulties, and improved methods are required.
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INTRODUCTION

Plant cells are characteristically enclosed by a cell wall that is semi-rigid, yet capable of growing. There has been considerable interest in the mechanism of the cell wall extension that accompanies cell growth and several experimental approaches applied to a wide range of organisms have yielded useful information. The problem is of interest from several quite different viewpoints: genetic control systems of cell wall assembly (Katz and Rosenberger 1971; Davies 1972), molecular control of morphogenesis and differentiation (Cabib and Farkas 1971; Bartnicki-Garcia and Lippman 1972), integration of components (Steward, Israel and Salpeter 1967; Sadava and Chrispeels 1969; Northcote 1969), properties of structural polymers using the electron microscope and X-ray diffraction (Mahadevan and Tatum 1967; Aronson and Fuller 1969), and chemical constituents (Novaes-Ledieu and Jiménez-Martínez 1968; Wang and Bartnicki-Garcia 1970; Marks, Keller and Guarino 1971). Only by a synthesis of results from all of these approaches will the processes of cell wall extension be completely understood.

Investigators of cell wall extension ultimately must determine the chemical constituents of the cell wall, the chemistry and geometry of the linkages between them, and a description of how these change as the cell grows. The fundamental requirements for the initial investigation of the whole cell wall are a qualitative and a quantitative analysis of its constituents. These studies may be followed by fractionation of cell wall macromolecules, analysis of their composition, determination of their structure and attempts to reassemble these molecules into a working model of cell wall structure. At each step in fractionation of the cell wall, quantitative recovery of components from each fraction is essential.
This aspect of the problem is essentially chemical in nature and at least in initial studies plant material should be selected to present the minimum of analytical problems. Although secondary deposition products are important cell wall constituents, they provide both qualitative and quantitative analytical problems that may preclude satisfactory resolution of primary cell wall components. Vascular plants are an example of tissues that present numerous problems. With many different cell types present, the resulting cell wall preparation is heterogeneous. As the cell walls may be lignified to varying extents, analysis becomes far more difficult. Tissue culture procedures can provide more uniform, less lignified cells, but with these procedures there is the problem of producing sufficient quantities of cells. Furthermore, since the cells from either source tend to grow attached together in masses, the preparation of cell walls free from both cytoplasm and the middle lamella becomes more difficult.

Certain other groups of organisms do not present these problems. Bacteria have already been extensively and comprehensively studied (for a general review see Rogers and Perkins 1968). Algae and fungi do not generally produce lignin-like compounds and many species grow as simple or branched filaments or as unicells. A number of algal species have been studied (Aaronson 1970; Parker 1970). Fungi are in general easier to culture than algae and they grow rapidly so that large amounts are relatively easy to produce. Yeasts, primarily *Saccharomyces*, have been widely studied (Phaff 1971) and the advantages of fungi as source material have led to investigation of an increasing number of other fungal types.

The first problem associated with any cell wall analysis is that of obtaining the cell wall free from cytoplasmic and intracellular contamination. In general this involves breaking the cell wall and
washing it free of contaminants. Depending on the species and the component under investigation fungal cell walls have been disrupted with a French press (Kanetsuna, Carbonell, Moreno and Rodriguez 1969) or broken by mechanical agitation with glass beads in a Braun homogenizer (Sietsma, Eveleigh and Haskins 1969), a Mickle disintegrator (Griffin and MacWilliams 1969), or a Sorvall omnimixer (Aronson and Fuller 1969). The resultant suspensions are often further treated by sonic oscillation and then variously washed with water, glycerol and aqueous solutions of NaCl, NaCl/NaHCO₃, EDTA and sucrose. Physical wall fragmentation followed by vigorous washing with aqueous solutions near pH 7 causes minimal degradation of the cell wall, whereas treatment with strong acids or bases, alcohol, or heat may cause denaturation of protein components or oxidation of polysaccharides. The result of any of these treatments is a cell wall preparation which "cannot sensibly be equated with the functional wall of the organism from which it was isolated" (Crook and Johnston 1962).

Cell wall polymers are most frequently hydrolyzed at temperatures near 100 C with aqueous acids; this releases the monomers but may also degrade them after (and perhaps before) their intermolecular bonds are broken. The extent of the degradation varies for individual monomers depending on the type of linkage between them and on the conditions of hydrolysis. Proteins and their component amino acids are much more stable to acid hydrolysis than polysaccharides and their constituent monosaccharides. Proteins are generally hydrolyzed with 6 N HCl in vacuo for 10 to 70 hr (Tristram and Smith 1963); under these conditions polysaccharides are significantly degraded. Polysaccharides may be hydrolyzed with 0.1 to 2 N H₂SO₄, 24 N HCOOH, or 2 N CF₃COOH for periods up to 8 hr to release neutral sugars. Glycosidic bonds involving amino sugars are much more stable to acid hydrolysis, and are generally
hydrolyzed with 2 N to 6 N HCl for 8 to 32 hr (Dutton 1972). Quantitative recovery of uronic acids under conditions of acid hydrolysis has not been accomplished (Nordstedt and Samuelson 1966; Stacey 1970). Although the development of paper chromatography provided a valuable tool for qualitative analysis modern technology has surpassed its capacities for resolution and sensitivity. Compounds more similar in structure can now be detected and separated in exceedingly small amounts. There are many analytical procedures in common use that can yield quantitative information from cell walls, provided that precautions are observed.

The procedures developed by Spackman, Stein and Moore (1958) have been widely used where precise quantitative amino acid data are required. Tristram and Smith (1963) recommended serial hydrolysis to determine the corrections which must be applied for amino acids that are labile under hydrolytic conditions and those that are difficult to hydrolyze. They emphasized the need for independent analysis of cysteine/cystine and tryptophan. The ninhydrin reaction with hydroxyproline is not satisfactory for determination of microgram amounts (Mitchell and Taylor 1970). Separate determination is preferred. In the literature of cell wall analysis there have been no reports that satisfy these requirements. Only a few authors (Wang and Bartnicki-Garcia 1970; Buck and Obaidah 1971) have used serial hydrolysis or attempted to determine tryptophan (Korn and Northcote 1960; Russell, Sturgeon and Ward 1964; Ballesta and Villanueva 1971; Marks, Keller and Guarino 1971) or hydroxyproline (Crook and Johnston 1962; Dyke 1964; Bartnicki-Garcia 1966; Novaes-Ledieu, Jiménez-Martínez and Villanueva 1967; Aronson and Fuller 1969; Reuvers, Tacoronte, García Mendoza and Novaes-Ledieu 1969; Pao and Aronson 1970). The recent development of procedures for analyzing amino acids by gas-liquid chromatography as trimethylsilyl derivatives (Gehrke,
Nakamoto and Zumwalt 1969) or as N-trifluoroacetyl n-butyl esters (Gehrke, Kuo and Zumwalt 1971) may provide a rapid and sensitive alternative to the ninhydrin reaction but corrections for hydrolytic degradation must still be applied.

The procedures for monosaccharide estimation are not as standardized as those for amino acids and considerable variation in methodology is evident. Stacey (1970) has discussed the advantages of gas-liquid chromatography in quantitative analysis of sugars, yet few investigations of fungal cell walls have made use of this technique. Sugars have been estimated as alditol acetate derivatives (Albersheim, Nevins, English and Karr 1967; Wang and Bartnicki-Garcia 1970), or trimethylsilyl derivatives (Namba and Kuroda 1971; Siki, Masler and Bauer 1970). Lloyd (1970) and Lloyd and Bitoon (1971) used both procedures. Only a few authors (Albersheim et al. 1967; Applegarth 1967; Applegarth and Bozoian 1968) have applied corrections derived from serial hydrolysis to sugar analysis.

Amino sugars are resolved on an amino acid analyzer using standard procedures and those investigators who used the analyzer for amino acid estimations determined amino sugars as well. There are reports of the estimation of amino sugars as trimethylsilyl derivatives but there have been problems in obtaining quantitative recoveries or resolution (Dutton 1972).

Chattaway, Holmes and Barlow (1963) observed that "losses [of sugars] during hydrolysis were found to be considerable and variable", yet only they, Marks, Koller and Guarino (1969) and Lloyd and Bitoon (1971) have examined the degradation of sugars under hydrolyzing conditions in conjunction with cell wall studies.

Uronic acids have proved difficult to estimate by gas-liquid chromatography (Blake and Richards 1970). They are usually estimated as
neutral sugars after reduction of the carboxyl group in the intact polysaccharide before hydrolysis (Dutton and Kabir 1971) or by reduction of the carboxyl group of the free uronic acids or aldobiouronic acids after hydrolysis (Bartnicki-Garcia and Reyes 1968; Dutton and Kabir 1972; Jones and Albersheim 1972).

Lipids have been widely determined by the gravimetric method of Bartnicki-Garcia and Nickerson (1962). Dyke (1964), Russell, Sturgeon and Ward (1964) and Sietsma, Eveleigh and Haskins (1969) saponified the lipids and estimated the methyl esters of the fatty acids by gas-liquid chromatography. Suomalainen and Nurminen (1970) determined cell wall lipids (fatty acids and phospholipids) in baker's yeast and found monosaccharides associated with some of the phospholipid components.

Errors in quantitative estimation due to incomplete cleavage of linkages and degradation of components under conditions of hydrolysis must be taken into account in any quantitative analysis. The reproducibility of the analysis may also depend on the availability of cellular material whose molecular content is uniform, and this in turn depends on factors such as morphology, state of maturity and nutritional conditions that may differ from one source of material to another.

The purpose of the present study was to select and test appropriate procedures for obtaining quantitative and reproducible information relating to the structure of the fungal cell wall. These procedures in themselves are not intended to provide any structural information about cell walls. They do, however, form the basis for all future structural studies which are planned in this laboratory. In order to elucidate the complete structure of cell walls, the structural units must be identified chemically and the method of assembly determined. One of the most profitable approaches to this sort of study has been electron
microscopic examination of the cell wall before and after selective removal of structurally distinct units by differential solubilization in various reagents and enzymes (Mahadevan and Tatum 1965, 1967). There is the possibility of extensive degradation as a result of these procedures, especially with NaOH extraction; quantitative recoveries at each stage must be achieved. The analytical methods described in this thesis must be applied and extended to achieve this end. Only by quantitating the entire analysis can accurate models of the cell wall be constructed.

This investigation concerns two fungal species from quite unrelated groups that have not previously been examined - *Saprolegnia diclina*, a mycelial Oomycete, and *Tremella mesenterica*, a Basidiomycete that can be grown in a yeast-like form.

The analytical regime includes:

1) serial hydrolysis of cell wall polysaccharides to release neutral sugars (estimated as trimethylsilyl derivatives by gas-liquid chromatography).

2) serial hydrolysis of cell wall polysaccharides to release amino sugars (estimated by ninhydrin reaction on the amino acid analyzer).

3) estimation of uronic acids as neutral sugars by reduction of the carboxyl groups in cell wall polysaccharides followed by analysis as for neutral sugars.

4) serial hydrolysis of cell wall proteins to release amino acids (estimated by ninhydrin reaction on the amino acid analyzer), including separate analysis for hydroxyproline, tryptophan and cysteine/cystine.

5) degradation studies of free neutral sugars, free amino sugars, and free amino acids under the conditions for hydrolysis of cell wall polymers.

6) gravimetric determination of lipids.
7) analysis for C, H, O, N, S, P and ash.

The success of this strategy can be measured as the percentage of total cell wall starting material which is recovered by the selected analytical procedures.
MATERIALS AND METHODS

*Tremella mesenterica* and *Saprolegnia diclina* were from the UBC mycological culture collection. Culture media were obtained from Difco Laboratories, Detroit, Michigan.

Reagents were obtained from the suppliers as indicated: Beckman Amino Acid Calibration Mixture Type 1 (Beckman Instruments, Inc., Spinco Division, Palo Alto, California); thiodiglycol (Bio-Rad Laboratories, Richmond, California); D-galactose, D-galactosamine hydrochloride, LiBH₄, pyridine AnalR ACS (British Drug Houses Ltd., Poole, England); all amino acids except methylhistidine (Calbiochem, Los Angeles, California); CF₃COOH, L-rhamnose monohydrate (Eastman Kodak Company, Rochester, New York); D-mannose, cyclohexane certified ACS spectranaIyzed (Fisher Scientific Company, Fair Lawn, New Jersey); L-1-methylhistidine monohydrate, L-3-methylhistidine puriss. (Koch-Light Laboratories Ltd., Colnbrook England); erythritol, all neutral monosaccharides except D-galactose, D-mannose, L-rhamnose (Nutritional Biochemicals Corporation, Cleveland, Ohio); methyl cellosolve, ninhydrin, hexamethyldisilazane, trimethylchlorosilane (Pierce Chemical Company, Rockford, Illinois); D-glucosamine hydrochloride, myo-inositol (Sigma Chemical Company, St. Louis, Missouri). All other chemicals were obtained locally. "Baker Analyzed" grade (J. T. Baker Chemical Company, Phillipsburg, New Jersey) was used when available.

**Cell Wall Preparation**

Cultures of *Tremella mesenterica* Fries (UBC collection #2259-6) were maintained at 20 C on nutrient agar (15.0 g bacto-agar, 7.5 g bacto-malt extract, 0.5 g bacto-yeast extract and 1.0 g bacto-soytone per litre).
Inoculum cultures were prepared in 50 ml of liquid medium (that described above without bacto-agar) and shaken at 20 C for 48 hr. Aliquots of the inoculum (5 ml) were transferred to a 2800 ml Fernbach flask containing 1 litre of the liquid medium and shaken at 20 C for 48 hr. Under these conditions the fungus grew as haploid, yeast-like unicells.

Cells were harvested by centrifugation at 9000 \( \times \) g for 10 min, washed once with water, and cooled by stirring for 30 min in an ice bath. Cells were broken in 50 ml portions using a Blackstone Ultrasonic Probe at 200 watts for 2 min, cooled for 30 min and fragmented for a further 2 min. The cells were kept chilled in an ice bath throughout and the probe was immersed in ice between treatments. The resulting suspension of whole and broken cells was centrifuged at 750 \( \times \) g for 5 min at 2 C. The supernatant suspension consisted of broken cells. The pellet, consisting largely of whole cells, was treated again with the ultrasonic probe and centrifuged; the supernatant suspensions were combined. As a result of this treatment approximately 95% of the cells were broken. Any remaining whole cells were removed by centrifugation during the course of the washing procedure.

Cultures of *Saprolegnia diclina* Humphrey (UBC collection #145) were maintained at 4 C on slants of the same medium used for *Tremella*. Inoculum cultures prepared on this medium in petri plates were grown at 20 C for 96 hr. The medium and mycelium were homogenized in a sterile blender containing 100 ml distilled water; 20 ml of the inoculum were transferred to a 2800 ml Fernbach flask containing 1 litre of liquid medium (10.0 g dextrose, 5.0 g bacto-peptone, 0.5 g bacto-yeast extract per litre). Cultures were grown at 20 C for 60 hr without shaking. Examination of samples from each of the culture flasks with the light
microscope showed no spores to be present.

The mycelium was harvested by filtration on a Büchner funnel and washed once with water. The pad of mycelium was ground to a fine powder in liquid \( \text{N}_2 \) with a mortar and pestle. When the temperature rose above 0°C, 50 ml of water were added to make a thick slurry which was treated with the ultrasonic probe (twice for 2 min) as described above. As a result of this treatment virtually all of the cells were broken.

The washing procedure is based on that of Mitchell and Taylor (1969). The suspension of broken cells was centrifuged at 2°C (27000 \( \times \) g for 10 min with *Tremella*, 3000 \( \times \) g for 5 min with *Saprolegnia*) to recover cell wall fragments. The supernatant solution was discarded. This procedure was repeated 4 times. The cell wall fragments were then washed 5 times with ice-cold 1.0 M NaCl solution (centrifugation at 12000 \( \times \) g for 10 min with *Tremella*, 3000 \( \times \) g for 5 min with *Saprolegnia*), 5 times with cold water and twice with 8.0 M urea solution (centrifugation at 27000 \( \times \) g for 10 min with *Tremella*, 3000 \( \times \) g for 5 min with *Saprolegnia*). The fragments were suspended for 12 hr in 8 M urea solution at 4°C, then centrifuged and washed twice more with 8 M urea solution, 5 times with water, 5 times with 1.0 N NH\(_4\)OH solution (centrifugation at 12000 \( \times \) g for 10 min with *Tremella*, 3000 \( \times \) g for 5 min with *Saprolegnia*) and 5 times with water. The cell walls recovered from this treatment were freeze-dried and stored at -20°C.

**Analytical Procedures**

Lipids were estimated by the procedures of Bartnicki-Garcia and Nickerson (1962). Elemental analysis for C, H, N, O, P, S, and ash
were performed by Organic Microanalysis, Montréal, Québec.

Cell walls (approximately 5 mg) were hydrolyzed in vacuo with 0.5 ml of 6 N HCl containing 5% (v/v) CH$_2$SH-COOH (Matsubara and Sasaki 1969) at 110°C for 8, 24, 48, 72 and 144 hr (Tristram and Smith 1963). Hydrolysates were dried in vacuo over concentrated H$_2$SO$_4$ and KOH pellets, then redissolved in 1.0 ml water. Aliquots of this solution, 50 μl HCl (pH 2.2) and 200 μl of the internal standard solution (α-amino-β-guanido-propionic acid for basic amino acids and norleucine for neutral and acidic amino acids) in pH 2.2 buffer were applied to the appropriate column of a Beckman Amino Acid Analyzer Model 120C. The analysis is based on the method of Spackman, Stein and Moore (1958). Basic amino acids were separated on a 13 cm column; this resolved glucosamine from galactosamine, and 1-methylhistidine and 3-methylhistidine (these were not resolved from each other) from histidine (Figure 1). Tryptophan and galactosamine were not resolved by this system. Since the alkaline hydrolysis procedure to release tryptophan degrades galactosamine, and the acidic hydrolysis procedure to release galactosamine generally degrades tryptophan, this presented no problem. Acidic and neutral amino acids were resolved on a 58 cm column, glucosamine was separated from phenylalanine and thus did not interfere with the phenylalanine determination. The length of the column is critical to this resolution. Hydroxyproline was determined by the spectrophotometric method of Bergman and Loxley (1970) using p-dimethylaminobenzaldehyde. Tryptophan was determined using the amino acid analyzer after hydrolysis of cell walls with 4.2 N NaOH (Hugli and Moore 1972).

Analyses were performed in triplicate on a single cell wall.
FIGURE 1 RESOLUTION OF BASIC COMPOUNDS FROM THE 13 CM COLUMN OF THE AMINO ACID ANALYZER

GlcN

1-MeHis
3-MeHis

Lys

His

GluN

NH₃

Qpa

Arg

Absorbance

Time (min)
preparation of *Tremella*, and once on each of 2 cell wall preparations of *Saprolegnia*.

A synthetic mixture of amino acids present in the cell walls (except hydroxyproline and tryptophan) and glucosamine hydrochloride was prepared for degradation studies; it was treated with 6 N HCl by the same procedure as the cell wall preparations. Degradation of both hydroxyproline and tryptophan was determined independently.

Cell walls (approximately 2 mg) were hydrolyzed in sealed tubes at 110 C with 0.5 ml 2 N CF3COOH for 15, 30, 60, 120, 240 and 480 min (Albersheim et al 1967) to release neutral sugars. Hydrolysates were dried in vacuo over KOH pellets.

Uronic acids were estimated as neutral sugars by reducing the carboxyl groups of the polysaccharides with LiBH4 (Dutton and Kabir 1971). The procedure was adapted for micro analysis by reducing the proportions of reagents. The cell wall preparation (20 to 35 mg) was dissolved in 8.0 ml formamide and treated with 6.0 ml pyridine and 4.0 ml propionic anhydride. After storage for 2 days the solution was precipitated by adding it to 150 ml 2% HCl. The total recovered precipitate, filtered and dried in vacuo, was retreated with 10.0 ml pyridine and 1.5 ml propionic anhydride. The total recovered propionated acid was dissolved in 15.0 ml tetrahydrofuran, and 10.0 ml diethyl ether containing diazomethane (cooled to -73 C) were added; the mixture was allowed to stand at -73 C for 6 hr. Diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide according to Vogel (1956). Subsequent reactions up to the recovery of carboxyl-reduced polysaccharides were identical with those described by Dutton and Kabir. The reduced polysaccharides
were then hydrolyzed with 2 N CF₃COOH for 2 hr to release neutral sugars.

Neutral sugars were analyzed by gas-liquid chromatography as trimethylsilyl (TMS) derivatives. Hydrolysates were dissolved in 1 ml pyridine and treated successively with 0.1 ml hexamethyldisilazane and 0.05 ml trimethylchlorosilane (Sweeley et al. 1963). The mixture was shaken for a few seconds, and after 30 min was freeze-dried to remove the pyridine. The TMS derivatives were redissolved in 100 µl cyclohexane and aliquots of this mixture or suitable dilutions of it were injected into a Varian Aerograph dual column gas chromatograph Model 1740, equipped with flame ionization detectors. The flow rates of N₂ and H₂ were 25 ml / min and air 250 ml / min. Columns (4.9 m × 3 mm) of 10% silicone fluid SF 96 on acid washed DMCS treated 60/80 mesh flux-calcined diatomite (Chromosorb W, Chromatographic Specialities, Brockville, Ontario) were temperature programmed linearly from 130 C (at injection) to 230 C at 2 degrees / min. Figure 2 shows the resolution obtained by this system.

A synthetic mixture of free neutral sugars was prepared for degradation studies; aliquots were treated with 2 N CF₃COOH by the same procedures as the cell wall preparations. It also served as a calibration mixture.

Cell walls (approximately 2 mg) were hydrolyzed in vacuo with 0.5 ml 2 N HCl (Oates and Schrager 1967) for 8, 16, 32 and 72 hr to release amino sugars. Hydrolysates were dried in vacuo over concentrated H₂SO₄ and KOH pellets, dissolved in pH 2.2 buffer, and estimated on the amino acid analyzer using the 13 cm column.

The mixture of glucosamine hydrochloride and amino acids used for degradation studies was treated with 2 N HCl by the same procedures
FIGURE 2  RESOLUTION OF NEUTRAL SUGARS (AS TMS DERIVATIVES) ON THE GAS-LIQUID CHROMATOGRAPH
as the cell wall preparations to determine the degradation of glucosamine under these conditions.

**Calculation of Results**

Peak areas were estimated by the product of peak height \( \times \) peak width at the half-height which Ball, Harris and Habgood (1967) concluded is the most reliable manual method. The estimates, particularly of the width at the half-height are more accurate on amino acid chromatograms where the number of dots printed per time (\( \propto \) width) can be counted; the measurement of the width on gas-liquid chromatograms is less accurate because of the width of the ink trace.

Internal standards are necessary in both monosaccharide and amino acid estimations. For gas-liquid chromatography the internal standards were used to check detector response and dilution errors. The great disparity of sugar concentrations in the cell walls examined meant that 1 to 2 \( \mu l \) of the initial silylated preparation (ca. 2 mg dissolved in 100 \( \mu l \) cyclohexane) had to be injected onto the gas-liquid chromatograph in order to detect minor constituents. Cyclohexane is volatile at room temperature, and even though the preparations were immediately stoppered and placed at -20 C, evaporation losses were possible. Furthermore, the silylation procedure produces \( \text{NH}_4\text{Cl} \), which is insoluble in cyclohexane. With as little as 1 to 2 \( \mu l \) in the syringe, particles of \( \text{NH}_4\text{Cl} \) may cause significant volume errors that are difficult to estimate. To detect the major constituents 20 \( \mu l \) of the concentrated solution were diluted with 500 \( \mu l \) cyclohexane; a similar error from \( \text{NH}_4\text{Cl} \) could result. This is the dilution error that was corrected by the use of internal standards. Both erythritol and myo-inositol were used; the concentration of erythritol
was approximately 1% that of inositol in order to obtain measurable peaks on scale at the different concentrations. All traces of CF$_3$COOH were removed after hydrolysis and before 50 µl of each internal standard solution were added.

The ratio of area per weight was determined for each internal standard (on calibration runs). For each sample analyzed, the area of the internal standard calculated from the dilution of the sample, was compared with the actual area measured on the chromatogram. The weight of anhydro sugar in the samples was calculated by comparison of its peak area with the area of a known weight of anhydro equivalent in the calibration mixture. Where there were several peaks for a sugar, the best resolved one from the sample was compared with the equivalent peak of the calibration analysis.

For amino acid analysis the internal standard was used to compensate for the deterioration of the ninhydrin reagent. For every run on the amino acid analyzer 20 nmoles of internal standard were added to the appropriate column with the sample or calibration mixture (α-amino-β-guanidino-propionic acid on the basic amino acid column and norleucine on the neutral and acidic amino acid column). The area of each amino peak was compared with the area of the internal standard for that analysis. The weight of the anhydro amino acid was calculated by comparison with the similar ratio in a calibration analysis containing 20 nmoles of the amino acids in the Beckman Amino Acid Calibration Mixture Type 1.
RESULTS

The cell walls after washing showed no evidence of cytoplasmic contamination by phase contrast light microscopy, no evidence of capsular material with India ink under the light microscope and no visible ribosomes, membranes or capsular material with electron microscopy.

The yield of cell walls from 6 l of medium was 100 to 150 mg from *Tremella mesenterica* and 200 mg from *Saprolegnia diclina*. These preparations were stored at -20 C and dried in vacuo for 12 hr before weighing.

Degradation under Hydrolyzing Conditions

An aliquot of the synthetic mixture of amino acids and glucosamine hydrochloride was subjected to the hydrolyzing conditions used for cell wall proteins (6 N HCl in vacuo) for each of the time periods (8 to 145 hr) as the cell wall preparations. A solution of hydroxyproline was similarly treated. Aliquots of a solution of tryptophan were treated (with 4.2 N NaOH in vacuo) like the cell wall preparations for 8 to 96 hr. The recoveries of the amino acids are shown in Figure 3.

Aliquots of the mixture of amino acids and glucosamine hydrochloride were also subjected to the hydrolysis conditions for releasing amino sugars from cell wall polysaccharides (2 N HCl in vacuo) for 8 to 96 hr. The recoveries of glucosamine from the two treatments are compared in Figure 4. Glucosamine is degraded to a far greater extent in 6 N HCl.

Aliquots of the calibration mixture of free neutral sugars were subjected to 2 N CF₃COOH for 15 min to 8 hr (the same treatment used to release neutral sugars from cell wall polysaccharides). The recoveries
FIGURE 3 RECOVERY OF FREE AMINO ACIDS UNDER HYDROLYZING CONDITIONS

(6 N HCl in vacuo)
FIGURE 4 RECOVERY OF GLUCOSAMINE UNDER HYDROLYZING CONDITIONS

A Comparison of 2 N and 6 N HCl
are shown in Figure 5.

Protein Analysis.

All of the usual protein amino acids except cysteine/cystine were found in the cell walls of both species. Application of larger samples to the 58 cm column failed to show any trace of cysteine/cystine or cysteic acid. Hydroxyproline and tryptophan were detected as protein constituents in both species. It was not possible to determine amide in the cell wall preparations because of residual NH$_3$ from the washing procedure and degradation of amino compounds. The results are presented in Tables I and II, and in Figures 6 and 7.

The amount of each monomer recovered after hydrolysis is determined primarily by two reactions: the rate at which the monomer is released from the polymer and the rate at which the free monomer is degraded under the hydrolyzing conditions. Curves such as those in Figures 6 and 7 represent the amount of monomer released during hydrolysis minus the amount of free monomer degraded.

Polysaccharide Analysis

Neutral Sugars

Arabinose, xylose, fucose, mannose, galactose and glucose were identified in the cell walls of both organisms. Rhamnose was detected in T. mesenterica but not in S. dilcina; traces of ribose were detected in S. dilcina but not in T. mesenterica. The results are presented in Tables III and IV, and in Figures 8 and 9.

Amino Sugars

Glucosamine was found in the cell walls of both organisms;
FIGURE 5  RECOVERY OF FREE NEUTRAL SUGARS UNDER HYDROLYZING CONDITIONS

(2 N CF3COOH)
TABLE I  AMINO ACIDS IN THE CELL WALL OF *Tremella mesenterica*

(μg anhydro amino acid recovered / mg cell wall preparation)

<table>
<thead>
<tr>
<th>Duration of Hydrolysis with 6 N HCl</th>
<th>8 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>145 hr</th>
<th>best estimate&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3</td>
<td>0.1</td>
<td>&lt;0.1</td>
<td>0.1&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>0.1</td>
</tr>
<tr>
<td>Lys</td>
<td>2.2</td>
<td>3.3</td>
<td>2.9</td>
<td>2.3</td>
<td>2.0</td>
<td>3.3</td>
</tr>
<tr>
<td>His</td>
<td>0.8</td>
<td>1.2</td>
<td>1.1</td>
<td>0.9</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Arg</td>
<td>2.2</td>
<td>3.5</td>
<td>3.2</td>
<td>2.5</td>
<td>2.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Asx</td>
<td>2.6</td>
<td>5.0</td>
<td>4.7</td>
<td>3.3</td>
<td>2.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Thr</td>
<td>1.3</td>
<td>2.3</td>
<td>2.3</td>
<td>1.7</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Ser</td>
<td>1.1</td>
<td>1.5</td>
<td>1.8</td>
<td>1.4</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Glx</td>
<td>4.3</td>
<td>7.5</td>
<td>7.2</td>
<td>5.2</td>
<td>3.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Pro</td>
<td>1.8</td>
<td>2.6</td>
<td>2.4</td>
<td>2.0</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Gly</td>
<td>1.6</td>
<td>2.1</td>
<td>2.3</td>
<td>1.6</td>
<td>1.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Ala</td>
<td>2.1</td>
<td>3.0</td>
<td>3.3</td>
<td>2.1</td>
<td>1.7</td>
<td>3.3</td>
</tr>
<tr>
<td>Cys</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Val</td>
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<td>2.3</td>
<td>2.8</td>
<td>2.1</td>
<td>1.8</td>
<td>2.8</td>
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<tr>
<td>Met</td>
<td>1.1</td>
<td>1.2</td>
<td>1.2</td>
<td>0.7</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Ile</td>
<td>0.9</td>
<td>2.1</td>
<td>2.5</td>
<td>1.9</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Leu</td>
<td>2.5</td>
<td>3.6</td>
<td>4.3</td>
<td>3.7</td>
<td>2.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.4</td>
<td>2.0</td>
<td>1.9</td>
<td>1.4</td>
<td>1.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Phe</td>
<td>1.1</td>
<td>1.6</td>
<td>2.2</td>
<td>1.7</td>
<td>1.5</td>
<td>2.2</td>
</tr>
<tr>
<td>Hyp&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Total Recovery: 48.9

<sup>1</sup> maximum value

<sup>2</sup> cell wall preparation hydrolyzed in 4.2 N NaOH (Hugli and Moore 1972)

<sup>3</sup> not determined

<sup>4</sup> hydrolyzed for 96 hr

<sup>5</sup> determined spectrophotometrically (Bergman and Loxley 1970)
TABLE II  AMINO ACIDS IN THE CELL WALL OF Saprolegnia diclina
(μg anhydro amino acid recovered / mg cell wall preparation)

<table>
<thead>
<tr>
<th>Duration of Hydrolysis with 6 N HCl</th>
<th>8 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>145 hr</th>
<th>best estimate¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp²</td>
<td>3</td>
<td>0.2</td>
<td>&lt;0.1</td>
<td>&lt;0.1⁴</td>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>Lys</td>
<td>0.8</td>
<td>1.2</td>
<td>0.8</td>
<td>0.7</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>His</td>
<td>0.3</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Arg</td>
<td>0.6</td>
<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Asx</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.2</td>
<td>⁵</td>
<td>1.2</td>
</tr>
<tr>
<td>Thr</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
<td>1.1</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Ser</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Glx</td>
<td>1.0</td>
<td>1.2</td>
<td>1.3</td>
<td>1.4</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Pro</td>
<td>0.6</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Gly</td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ala</td>
<td>0.7</td>
<td>0.9</td>
<td>0.9</td>
<td>0.7</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Cys</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Val</td>
<td>0.4</td>
<td>0.6</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Met</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Ile</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Leu</td>
<td>0.6</td>
<td>0.8</td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Phe</td>
<td>0.4</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Hyp⁶</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Total Recovery                     13.3

¹ maximum value
² cell wall preparation hydrolyzed in 4.2 N NaOH (Hugli and Moore 1972)
³ not determined
⁴ hydrolyzed for 96 hr
⁵ not recovered
⁶ determined spectrophotometrically (Bergman and Loxley 1970)
FIGURE 6
RECOVERY OF AMINO ACIDS FROM CELL WALLS OF Thermus thermophilus

Hydrolysed in vacuo with 6 N HCl.
FIGURE 7  RECOVERY OF AMINO ACIDS FROM CELL WALLS OF *Saprolegnia diclina*

(Hydrolysis in vacuo with 6 N HCl)
### TABLE III  NEUTRAL SUGARS IN THE CELL WALL OF *Tremella mesenterica*

(μg anhydro sugar / mg cell wall preparation)

<table>
<thead>
<tr>
<th>Duration of Hydrolysis with 2 N CF₃COOH</th>
<th>best estimate¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Rha</td>
<td>13.0</td>
</tr>
<tr>
<td>Ara</td>
<td>1.0</td>
</tr>
<tr>
<td>Fuc*</td>
<td>1.0</td>
</tr>
<tr>
<td>Xyl</td>
<td>156.5</td>
</tr>
<tr>
<td>Man</td>
<td>21.5</td>
</tr>
<tr>
<td>Gal</td>
<td>1.5</td>
</tr>
<tr>
<td>Glc</td>
<td>225.0</td>
</tr>
</tbody>
</table>

Total Recovery 703.0

¹ maximum value
² not detected
³ spurious value
⁴ not confirmed, identified by chromatographic position only
TABLE IV  NEUTRAL SUGARS IN THE CELL WALL OF *Saprolegnia diclina*  
(μg anhydro sugar / mg cell wall preparation)

<table>
<thead>
<tr>
<th>Duration of Hydrolysis with 2 N CF$_3$COOH</th>
<th>best estimate$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Ara</td>
<td>0.5</td>
</tr>
<tr>
<td>Rib</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Fuc$^2$</td>
<td>1.0</td>
</tr>
<tr>
<td>Xyl</td>
<td>0.5</td>
</tr>
<tr>
<td>Man</td>
<td>2.0</td>
</tr>
<tr>
<td>Gal</td>
<td>22.5</td>
</tr>
<tr>
<td>Glc</td>
<td>187.0</td>
</tr>
</tbody>
</table>

Total Recovery

725.5

$^1$ maximum value

$^2$ not confirmed, identified by chromatographic position only
FIGURE 8 RECOVERY OF NEUTRAL SUGARS FROM CELL WALLS OF *Tremella mesenterica* -

(Hydrolysis with 2 N CF₃COOH)
FIGURE 9  RECOVERY OF NEUTRAL SUGARS FROM CELL WALLS OF Saprolegnia diclina

(Hydrolysis with 2 N CF$_3$COOH)
galactosamine was not detected in either. The extent of $N$-acetylation was not determined. The recoveries of glucosamine after hydrolysis of the cell wall preparations with $2\,N\,HCl$ are presented in Table V. The recoveries of glucosamine after hydrolysis with $2\,N\,HCl$ and $6\,N\,HCl$ are compared in Figure 10. The massive degradation of glucosamine with $6\,N\,HCl$ precludes its use in any study that attempts to be quantitative.

Uronic Acids

For reduction of carboxyl groups in the cell wall polysaccharides of *T. mesenterica* 35.29 mg of cell wall preparation were reduced by the method of Dutton and Kabir (1971); 10.55 mg of reduced cell wall preparation were recovered. For *S. diclina* the amounts were 20.93 mg and 3.87 mg respectively. After hydrolysis in $2\,N\,CF_3COOH$ the mole ratios of the sugars recovered were compared with those of the unreduced cell wall preparation hydrolysates. The differences were well within the experimental error so that uronic acids are present in very small amounts, if at all, in the cell walls of the two species.

Lipid Analysis

Individual lipid components were not determined; lipids were separated as two fractions (Bartnicki-Garcia and Nickerson 1962); those extractable in ethanol:diethyl ether and chloroform (readily extractable lipids) and those released after treatment with $1\%\,HCl$ in ethanol:ether (bound lipids). The results are present in Table VI.

Elemental and Ash Analysis

Two different cell wall preparations of each species were analyzed; the results are presented in Table VII.
### TABLE V  AMINO SUGARS IN THE CELL WALL OF Tremella mesenterica AND Saprolegnia diclina

(μg anhydro GlcNx / mg cell wall preparation)

<table>
<thead>
<tr>
<th></th>
<th>Duration of Hydrolysis with 2 N HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 hr</td>
</tr>
<tr>
<td><strong>T. mesenterica</strong></td>
<td>6.1</td>
</tr>
<tr>
<td><strong>S. diclina</strong></td>
<td>4.7</td>
</tr>
</tbody>
</table>

¹ maximum value
² not determined

### TABLE VI  LIPIDS IN THE CELL WALL OF Tremella mesenterica AND Saprolegnia diclina

(μg / mg cell wall preparation)

<table>
<thead>
<tr>
<th></th>
<th>Readily Extractable Lipids</th>
<th>Bound Lipids</th>
<th>Total Lipids Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T. mesenterica</strong></td>
<td>33.5</td>
<td>43.0</td>
<td>76.5</td>
</tr>
<tr>
<td><strong>S. diclina</strong></td>
<td>82.0</td>
<td>37.5</td>
<td>119.5</td>
</tr>
</tbody>
</table>
FIGURE 10 - RECOVERY OF GLUCOSAMINE FROM CELL WALLS OF Trametes versicolor AND Saprolegnum declina

Hydrolysis in vacuo with 2 N or 6 N HCI.
TABLE VII  ELEMENTAL AND ASH ANALYSIS OF CELL WALL PREPARATIONS OF
Tremella mesenterica AND Saprolegnia diclina ¹

(μg / mg cell wall preparation)

<table>
<thead>
<tr>
<th></th>
<th>Tremella mesenterica</th>
<th>Saprolegnia diclina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#4 ²</td>
<td>#5</td>
</tr>
<tr>
<td>C</td>
<td>377.1</td>
<td>404.4</td>
</tr>
<tr>
<td>H</td>
<td>66.1</td>
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<td>451.7</td>
</tr>
<tr>
<td>N</td>
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<td>18.4</td>
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<tr>
<td>S</td>
<td>69.0</td>
<td>29.7</td>
</tr>
<tr>
<td>P</td>
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</tr>
<tr>
<td>Total</td>
<td>1005.1</td>
<td>1002.2</td>
</tr>
</tbody>
</table>

¹ analysis performed by Organic Microanalysis, Montréal, Québec, on cell wall preparations that were dialyzed for 72 hr

² cell wall preparation used for complete analysis

³ not determined
Complete Cell Wall Analysis

The total recovery of cell wall components for each species is summarized in Table VIII. Approximately 90% of the weight of each cell wall preparation was recovered after the analysis in two quite dissimilar fungi. The remaining 10% is not accounted for but the similar recoveries obtained from two such different fungal species suggest that the strategy is reproducible.

Results presented are derived from one cell wall preparation for each species. Confirmation was obtained from two other preparations of each species, including some prepared by other workers in the laboratory. The cell wall preparations differed in total elemental, protein and polysaccharide compositions but mole ratios among neutral sugars and among amino acids indicated that monomeric compositions for each of these components were similar.

Analytical accuracy was established from repeat analysis of amino acid and sugar calibration mixtures. Reproducibility for amino acid analysis was ±3% (lower limit of detection ca. 0.5 nmole) and for sugar analysis ±5% (lower limit of detection ca. 50 ng).
<table>
<thead>
<tr>
<th></th>
<th><strong>Tremella mesenterica</strong></th>
<th><strong>Saprolegnia diclina</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polysaccharide</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anhydro neutral sugars</td>
<td>703.0</td>
<td>725.5</td>
</tr>
<tr>
<td>anhydro amino sugars</td>
<td>23.9</td>
<td>8.9</td>
</tr>
<tr>
<td>anhydro uronic acids</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anhydro amino acids</td>
<td>48.9</td>
<td>13.3</td>
</tr>
<tr>
<td><strong>Lipid</strong></td>
<td>76.5</td>
<td>119.5</td>
</tr>
<tr>
<td><strong>Ash</strong></td>
<td>32.7</td>
<td>24.8</td>
</tr>
<tr>
<td><strong>Total Recovery</strong></td>
<td>885.0</td>
<td>892.0</td>
</tr>
</tbody>
</table>
DISCUSSION

Although the cell walls of neither species have been studied previously, *Saprolegnia ferax* has been investigated (Crook and Johnston 1962; Parker, Preston and Fogg 1963; Novaes-Ledieu, Jiménez-Martínez and Villanueva 1967; Sietsma, Eveleigh and Haskins 1969). None of these studies were specifically quantitative but they did show that the cell wall contained glucose as the predominant polysaccharide constituent with ribose, mannose and glucosamine present as minor constituents. Novaes-Ledieu et al. (1967) reported the presence of rhamnose in addition to the other sugars. Rhamnose is not a constituent of the cell walls of *S. diclina*; the TMS ethers of rhamnose and ribose are completely resolved by the gas-liquid chromatography system employed (see Figure 2). Only Novaes-Ledieu et al. (1967) reported the presence of galactose, which in *S. diclina* is the second most abundant sugar. Arabinose, fucose and xylose were detected in trace amounts in *S. diclina* but not in *S. ferax*. Parker et al. (1963) studied only sugar constituents of the cell walls; they examined two additional species of *Saprolegnia*. The composition of *S. monoica* was similar to that of *S. ferax*; that of *S. litoralis* had the same constituents and uronic acids were detected as well.

Crook and Johnston (1962) and Novaes-Ledieu et al. (1967) estimated quantitative amino acid composition from relative intensities of ninhydrin-positive spots on paper chromatograms. The protein amino acids, including hydroxyproline were present, although neither tested for tryptophan; Crook and Johnston (1962) reported the absence of methionine. The quantitative results of the cell wall analyses of *S. ferax* and *S. diclina* are compared below.
<table>
<thead>
<tr>
<th></th>
<th><em>Saprolegnia ferax</em></th>
<th><em>S. diclina</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Novaes-Ledieu et al.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>neutral sugars</td>
<td>93 %</td>
<td>84 %</td>
</tr>
<tr>
<td>amino sugars</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>uronic acids</td>
<td>not detected</td>
<td>not determined</td>
</tr>
<tr>
<td>protein</td>
<td>1.2</td>
<td>3.0</td>
</tr>
<tr>
<td>lipid</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>ash</td>
<td>not determined</td>
<td>3.2</td>
</tr>
<tr>
<td>Sietsma et al.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>neutral sugars</td>
<td>72.5%</td>
<td></td>
</tr>
<tr>
<td>amino sugars</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>uronic acids</td>
<td>not detected</td>
<td></td>
</tr>
<tr>
<td>protein</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>lipid</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>ash</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

There is general agreement about the composition of the cell walls of the two species; the differences observed between species and between two analyses of the same species may be the result of several factors. Both Novaes-Ledieu et al. (1967) and Sietsma et al. (1969) estimated total carbohydrates with the anthrone reagent. Aminoff (1970) has stated that "quantitative determination is possible only when the identity of sugar components to be assayed is known. Several other substances, including tryptophan..., interfere in the [anthrone] reaction." The fact that the cell walls contain glucose as the predominant sugar reduces the magnitude of the first problem but the substances that cause interference (and tryptophan was determined in neither of the studies) may result in analytical determinations that are higher than the true carbohydrate content. The cells were grown in different media. Sietsma et al. (1969) did not report the age of their culture or the temperature at which they were grown; such factors may affect the composition of cell walls. The culture medium used by Sietsma et al. (1969) contained cholesterol; this may have affected the lipid content of the cell walls or the lipid determination.

There have been no reports of cell wall composition of *Tremella*, and *Ustilago maydis* growing in yeast-like form appears to be the only other
Heterobasidiomycete investigated (Crook and Johnston 1962). They found glucose and glucosamine to be the major monosaccharides of the cell wall with less galactose and traces of mannose. They detected all the protein amino acids except hydroxyproline and methionine (they did not test for tryptophan). O'Brien and Ralph (1966) examined the sugar components of cell walls of several Basidiomycetes and found glucose and glucosamine to be the major constituents, with less mannose and xylose, and traces of fucose present. Galactose was absent in all species examined except Coniophora cerebella. Tremella mesenterica (in the haploid yeast-like form) shows two striking differences from these Basidiomycetes; the glucosamine content was found to be very low (Table V), and hydroxyproline was detected (Table 1). In addition to galactose, both rhamnose and arabinose were detected.

Polysaccharides have been studied in a large number of fungi; Gorin and Spencer (1968) discussed the types and linkages of monosaccharide constituents in the fungi and how they might be useful in taxonomic studies. Bartnicki-Garcia (1968) considered the polysaccharides of fungal cell walls and devised a scheme based on the close correlation that can be established between chemical composition of the cell wall and major taxonomic groupings elaborated on morphological criteria. The data to extend this scheme will come from careful qualitative and quantitative studies not only of the sugar components present but of the types of linkages between them in many more fungal species than have been examined to this time.

The protein constituents in fungal cell walls have not been as widely studied as the polysaccharides. At present too few species have been examined to make more than very general statements. All the protein amino acids are present except cysteine/cystine which appears to be absent in many fungi (Roy and Landau 1972). Either aspartate and glutamate, or
threonine and serine are frequently the most abundant amino acids. Hydroxy-
proline and tryptophan have not been widely reported but they have not been
widely sought. Hydroxyproline has been found in species of the Oomycetes
(it constitutes 20.4% of the total amino acid component of *Atkinsiella
dubia*, Aronson and Fuller 1969) and in *Candida albicans* (Chattaway et al.
1968). It had not been previously reported in fungi with chitinous cell
walls and has been said to be characteristic of cellulosic cell walls of
fungi, algae, or higher plants (Bartnicki-Garcia 1968). The discovery of
hydroxyproline is *Tremella mesenterica* appears to be the first report in a
Basidiomycete. Further studies will reveal whether the cell wall of *T. mes-
enterica* lacks chitin, whether some of the glucan is cellulosic, or whether
hydroxyproline is not restricted in its distribution in cell wall proteins.

**Analytical Procedures**

The estimation of most amino acids is based on the procedures
of Moore, Spackman and Stein (1958) and Spackman, Stein and Moore (1958).
Acid hydrolysis of proteins is usually performed with 6 N HCl; serial
hydrolysis to estimate incomplete cleavage and degradation is recommended
(Tristram and Smith 1963).

Hydrolysis under such conditions generally brings about complete
destruction of tryptophan, with the rate accelerated in the presence of
carbohydrate (Spencer 1963). Matsubara and Sasaki (1969) attempted to
minimize the destruction of tryptophan by addition of CH$_2$SH-COOH to the
protein before hydrolysis. However this treatment improves the recovery
of tryptophan only in the absence of carbohydrate (Hugli and Moore 1972;
James 1972). Alkaline hydrolysis has proved a successful means of
achieving quantitative recovery of tryptophan. Hugli and Moore (1972)
have discussed the limitations of methods that have been employed and
proposed a simple quantitative procedure using the amino acid analyzer: NaOH is used for hydrolysis of the protein rather than Ba(OH)_2 to avoid adsorption of tryptophan on BaSO_4 or BaCO_3, starch is added as an antioxidant (for samples rich in carbohydrate such as cell walls, starch is not required), and the hydrolysate is dissolved in pH 4.25 buffer to avoid degradation of tryptophan in a highly acidic medium (amino acids are routinely dissolved in pH 2.2 buffer for estimation on the amino acid analyzer).

Hydroxyproline has been estimated using the amino acid analyzer but under the standard conditions used for protein hydrolysates, it co-chromatographs with aspartate. It can be detected by peak enhancement of the absorbance at 440 nm or estimated by alternative programming of the analyzer so that the two amino acids are resolved. However the procedure is not satisfactory because of the low sensitivity of the ninhydrin. Mitchell and Taylor (1970) examined a number of alternative procedures using p-dimethylaminobenzaldehyde as the spectrophotometric agent and found that of Bergman and Loxley (1970) to be the most suitable and the most sensitive for determination of hydroxyproline in cell walls.

There are some problems in quantitative recovery of cysteine/cystine after acid hydrolysis (e.g. oxidation to cysteic acid may occur). Even when a large excess of hydrolysates of cell walls of _T. mesenterica_ and _S. diclina_ were applied to the 58 cm column of the amino acid analyzer, no discernable peaks for either cysteine or cysteic acid were observed. Under these conditions cysteine, if present at all, was below the limits of detection and thus is considered to be absent.

James (1972) has reported that when CH_2SH-COOH is added to proteins before hydrolysis, recovery of proline is greater than from hydrolysates that do not contain mercaptans and no cysteine is recovered.
In the present studies, cell walls of *T. mesenterica* were hydrolyzed with and without \( \text{CH}_2\text{SH-COOH} \); there was no significant difference in the recovery of proline and no cysteine was detected with either treatment. Ross (personal communication) has examined the cell walls of *Cryptococcus laurentii* and *C. neoformans*, which may be related to *Tremella* (Slodki, Wickerham and Bandoni 1966); hydrolysis without \( \text{CH}_2\text{SH-COOH} \) showed no cysteine to be present in either species.

Cell wall polysaccharides have been hydrolyzed with various acids. \( \text{H}_2\text{SO}_4 \) has been widely used but neutralization with \( \text{BaCO}_3 \) produces a precipitate of \( \text{BaSO}_4 \) that may adsorb monosaccharide constituents, especially uronic acids. \( \text{HCl} \) is volatile, but it is generally agreed that it causes more degradation than \( \text{H}_2\text{SO}_4 \) (Dutton 1972). Albersheim et al. (1967) used \( \text{CF}_3\text{COOH} \) as the hydrolyzing acid because of its volatility and thus neutralization of the excess hydrolyzing acid was not required. The sugars released by hydrolysis have been estimated by gas-liquid chromatography as TMS derivatives or as alditol acetates. Alditol acetates have been more widely used in cell wall investigations but TMS derivatives may be superior in two respects: (i) they can be prepared directly without the intervention of a reduction step, and (ii) they produce multiple peaks (usually two to four) with the \( \alpha \) and \( \beta \) pyranosides as the major components. The number and proportion of the peaks for each sugar depend on the solvent in which the sugar comes to equilibrium, the solvent in which the derivative is injected onto the column, and the stationary phase of the column. Since the number of peaks and their relative proportions are found to be constant for each sugar under given conditions of derivative preparation, it is possible from appropriate data on expected peak proportions to calculate the total amounts of sugar from
any completely resolved peak (Holligan 1971). Since in biological material there is usually some degree of background contamination, the positive identification of a monosaccharide from the appearance of a single peak may often be impossible. The presence of a characteristic multiple peak pattern with known retention and peak area proportions allows identification of most monosaccharides with confidence (Bhatti, Chambers and Clamp 1970).

Amino sugars are customarily released from polysaccharides with HCl. The concentration of HCl used is critical for the recovery of liberated amino sugars. Figure 10 shows the massive degradation of glucosamine during hydrolysis of cell walls with 6 N HCl compared with 2 N HCl. This matter apparently is not widely appreciated as many recent papers (including Pao and Aronson 1970; Wang and Bartnicki-Garcia 1970; Gratzner 1972) report glucosamine composition data obtained after hydrolysis with 6 N HCl. The quantitative significance of such reports may be in doubt for they may represent as little as 5 to 10% of the real glucosamine content. There are conflicting reports on the resolution and the quantitative recovery of TMS derivatives of amino sugars (Dutton 1972). Moore and Stein (1948) showed that glucosamine reacts quantitatively with ninhydrin under conditions of assay for amino acids. Glucosamine and galactosamine can be resolved on the 13 cm column of the amino acid analyzer (Figure 1). At present this seems to be the preferable method of analysis for amino sugars.

Glycosiduronic acid linkages are resistant to acid hydrolysis and under conditions that release neutral sugars only partial cleavage of such bonds may occur (Dutton 1972). The resulting aldobiouronic acids can be hydrolyzed by prolonged acid treatment that degrades neutral sugars (Adams 1965). Free uronic acids are labile in acid media and
readily undergo decarboxylation to give products of unknown composition (Aminoff 1970). 

Jones and Albersheim (1972) hydrolyzed cell wall polysaccharides with dilute acid (0.2 N CF$_3$COOH), then treated the partially depolymerized preparation with a mixture of extracellular polysaccharide-degrading enzymes from Sclerotium rolfsii. The liberated sugars and uronic acids were reduced with NaBH$_4$ to alditols and aldonic acids respectively, which were separated using anion exchange resin. The aldonic acids were reduced with NaBH$_4$ to alditols. The alditols were estimated by gas-liquid chromatography as acetate derivatives.

Dutton and Kabir (1972) methylated polysaccharides from corn leaves and stalks, then hydrolyzed them to release methylated neutral sugars and methylated aldobiouronic acids, which were separated using ion exchange resins. The methylated aldobiouronic acids were hydrolyzed and the neutral sugars analyzed to reveal the linkages.

Dutton and Kabir (1971) also published a procedure for reducing the carboxyl groups of the uronic acids in polysaccharides with LiBH$_4$ before hydrolysis. This was the method chosen to estimate uronic acids in the present studies. However the method was found to be unsuitable for the information required in the present studies. After reduction of the carboxyl groups the polysaccharides were hydrolyzed to release neutral sugars. The amount of uronic acids could be estimated only by difference from the hydrolysis products of the unreduced polysaccharide. Aldobiouronic acids unhydrolyzed in the unreduced polysaccharide should represent an increase in the neutral sugar components when the reduced polysaccharide is hydrolyzed. If the aldobiouronic acids (from the unreduced polysaccharide) are hydrolyzed under the conditions chosen, the uronic acid estimate will be low. Particularly if the uronic acid
content of the polysaccharide is low, there may be considerable error in
this estimate as the reproducibility of analysis is only about 5%.
Furthermore the analysis does not reveal upon which component the carboxyl
group was located. The method is useful, however, in checking the
uronic acid composition of a polysaccharide for which an estimate is
already available.

While each of these three methods is useful in qualitative
analysis, they present problems of quantitative recovery viz. incomplete
hydrolysis, losses on ion exchange resins, incomplete derivatization
reactions. At present, the problem of quantitative recovery of specific
uronic acids has not been completely solved (Norstedt and Samuelson 1966;
Blake and Richards 1970). On a milligram scale quantitative decarboxylation
is the method of choice (Aminoff 1970).

The present studies have been particularly concerned with
quantitative analysis of protein and polysaccharide constituents of the
cell wall. A study of cell wall lipids of baker's yeast (Suomalainen
and Nurminen 1970) showed quantitative differences between the cell wall
and the whole cell. Procedures exist for the resolution of lipid
constituents. The extraction method of Folch, Lees and Sloane-Stanley
(1957) has been recommended. Dyke (1964) and Sietsma, Eveleigh and
Haskins (1969) quantitatively estimated lipids and methyl esters of fatty
extended the investigation to include phospholipids. Kuksis (1966) has
published an excellent review of quantitative procedures designed primarily
for animal tissues that might be adapted for cell wall investigations.
The results of the elemental analysis (Table VII) show reasonable agreement between the different cell wall preparations that were analyzed. However they do show that cell walls from different preparations are not identical. The amount of N is rather high in both species examined. In *T. mesenterica* the amounts of N derived from amino groups and NH₃ account for practically all of the N found. The high NH₃ levels apparently indicate that NH₄OH from the washing procedure is not completely removed even after the 72 hr dialysis period. In *S. diclina* the sum of N from amino groups and NH₃ accounts for only 33% of the total N. Even in preparation #1, which contained significantly more protein, the recovery of N from amino groups and NH₃ was of the same order. Evidently there are other N-containing substances present in *S. diclina*. There is no basis for speculation as to their identity.

The presence of large amounts of S cannot be explained in terms of S-containing amino acids (cysteine is absent and methionine accounts for only a small proportion of the total S found).

Phosphodiester linkages have been reported in fungal mannans (Cawley and Letters 1968), and Lloyd (1970b) has examined a peptido-phosphogonalactomannan in *Cladosporium werneckii* which contains 3.2% phosphate. This might also account for the P levels in *T. mesenterica* and *S. diclina*.

**Hydrolysis and Degradation**

The C-N peptide bond exhibits considerable double bond character and thus is stabilized by resonance (Spencer 1963). Although all of the peptide bonds in a protein are susceptible to hydrolysis by acid, the rate of hydrolysis will depend on factors affecting the approach of H⁺. Electrostatic and steric properties greatly influence the stability of each bond.
the most important factors influencing rate are the effective size of the amino acid side chains on either side of the peptide bond and their positions relative to the bond. Peptide bonds involving valine, with a bulky isopropyl group, are most stable; those with the group farther removed, as in isoleucine are less stable; those with steric factors at a minimum, like glycine and alanine, are still more labile. When the side chain is part of the amino acid contributing the carboxyl to the peptide link it has a greater effect of the rate of hydrolysis than when it is part of the amino acid that contributes the amino group. In acid solutions carboxyl groups are uncharged and basic groups tend to repel H\(^+\). When the basic group is in the side chain it has less effect on hydrolysis than a free \(\alpha\)-amino group adjacent to the peptide bond. This is indicated by an accumulation of dipeptides in partial hydrolysis of proteins (Harris, Cole and Pon 1956; Spencer 1963).

Peptide bonds involving the amino groups of serine and threonine are among the most labile in acid solution. Spencer (1963) presents hypotheses that have been advanced to explain this lability; they involve participation of the \(\beta\)-O in an oxazoline ring. In addition he discusses hypotheses to explain the preferential release of aspartate from protein with dilute acid.

Except under special conditions of hydrolysis for tryptophan, alkaline hydrolysis of proteins is not used because of extensive destruction of liberated amino acids and production of artifacts (Spencer 1963).

The generally accepted mechanism of hydrolysis of glycopyranosidic bonds involves a rapid, equilibrium-controlled protonation of the glycosidic oxygen (although the protonation of the ring oxygen cannot be entirely
excluded, available evidence favors the glycosidic oxygen) to give the conjugate acid. The conjugate acid decomposes to a glycosyl carbonium-oxonium ion, which then adds water (De Bruyne and Wouters-Leysen 1971). The carbonium-oxonium ion most probably exists in the half-chair configuration (BeMiller 1967). The mechanism is illustrated with a 1,4-β-D-glucopyranose polymer in Figure 11. De Bruyne and Wouters-Leysen (1971) showed that in HCl the reaction proceeds via carbonium-oxonium ions generated unimolecularly from the conjugate acid. In H₂SO₄ some of the criteria were not in accordance with the unimolecular mechanism but the authors attributed this not to a change in the mechanism but to the failure of acidity functions as general mechanistic criteria. Few experiments of this type have been performed with furanosides and the mechanism of hydrolysis of glycofuranosidic bonds has not been established.

It has been observed that α-D-glycopyranosidic linkages are usually more readily hydrolyzed than β-D linkages; furanosidic linkages are hydrolyzed under very mild conditions. When the glycosidic linkage involves the reducing group of a 2-amino-2-deoxy aldose the NH₃⁺ formed in acid solution electrostatically shields the neighboring glycosidic constituents from attack by H⁺; such bonds are much more stable to acid hydrolysis (Jones and Perry 1963). The glycosidic linkage involving the reducing group of the glyuronic acid shows strong resistance to acid hydrolysis; BeMiller (1967) discusses theories postulated to explain the stability of this linkage.

In alkaline medium polysaccharides are easily oxidized by atmospheric oxygen, and, even when the reactions are carried out under oxygen-free nitrogen, some degree of degradation, probably occasioned by traces of oxygen, is difficult to avoid (Bouveng and Lindberg 1960).
FIGURE II MECHANISM OF GLUCOPYRANOSIDE HYDROLYSIS

after BeMiller (1967)
Hydrolysis is the predominant reaction under conditions where both the acid and polymer are in dilute solutions at temperatures near 100 C. With polysaccharides acids also catalyze epimerization reactions and dehydration reactions that result in the formation of anhydro sugars and furfural derivatives (BeMiller 1967). Condensation products may also be formed with acid hydrolysis of proteins, especially from the degradation of tryptophan (James 1972). The complexity and composition of the reaction mixture depends on a number of variables, such as concentration of reagents, temperature, and time of heating (Aminoff 1970).

The black precipitates and highly colored soluble condensation products that are collectively called humin cause interference in automatic amino acid analysis procedures. The use of CH$_2$SH-COOH in protein hydrolysates (Matsubara and Sasaki 1969) to prevent tryptophan destruction also serves to reduce humin production. In the presence of polysaccharides tryptophan is degraded even with CH$_2$SH-COOH and humin production is increased. The effects of CH$_2$SH-COOH on amino acid recoveries have already been discussed. James (1972) does not recommend the addition of CH$_2$SH-COOH or CH$_3$-CHSH-COOH to protein hydrolysates containing tryptophan. For accurate amino acid estimation he suggests that samples be hydrolyzed in quadruplicate: addition of oxalic acid and mercaptosuccinic acid before hydrolysis, treatment with ion exchange resin and Norit after hydrolysis, and oxidation with performic acid before hydrolysis.

Nevertheless in samples that contain significant amounts of polysaccharides, some humin will be produced with acid hydrolysis. Some furfural derivatives (including those derived from hexoses) are converted to levulinic acid (Zacharius and Talley 1962; Anet 1972). Levulinic acid reacts with ninhydrin to produce a colored product which is eluted from
the 58 cm column of the amino acid analyzer between cysteic acid and aspartate (Zacharius and Talley 1962; Sentandreu and Northcote 1968). Taylor (1970) has detected a similar peak, which is characterized by a higher absorbance at 440 nm than at 570 nm, in hydrolysates of a mixture of hydroxyproline and sucrose. Zacharius and Porter (1967) have examined a number of other non-nitrogenous compounds (mainly monosaccharides, disaccharides and related compounds) that produce ninhydrin-positive derivatives. These peaks are almost all eluted before aspartate on the 58 cm column of the amino acid analyzer and have higher absorbance at 440 nm than at 570 nm. They all show much lower color intensities than amino acids. Taylor (1970) remarked that the hydrolysates were pale yellow before analysis but that there was no absorbance at 440 nm without reaction with ninhydrin. In the present studies, there were numerous very large peaks most of which were eluted from the 13 cm column before glucosamine, and from the 58 cm column before aspartate. However the elution was not sharp and the peaks often tailed into the amino acid peaks, rendering their area estimations difficult. It was found that these effects could be significantly reduced by delaying the addition of ninhydrin to the column eluates until just before the first amino acids were eluted. For the 13 cm column ninhydrin was added to the reaction coil 10 min after the elution started; for the 58 cm column it was added at 33 min. This procedure produced stable baselines.

In addition to these artifacts of hydrolysis, there were several ninhydrin-positive products with absorbances that resemble those of amino acid-ninhydrin peaks (higher absorbance at 570 nm than at 440 nm). Five of these peaks were regularly observed in amino acid analysis of all hydrolysates from *T. mesenterica* and *S. diclina*. Their positions do not correspond to any protein amino acids, 1- or 3-methylhistidine,
glucosamine or galactosamine. The size of these peaks tended to decrease during the course of hydrolysis but four were still detected after hydrolysis for 145 hr. A similar artifact has been reported from hydrolysis of cell walls that contain amino sugars (Applegarth and Bozoian 1967; Kanetsuna et al. 1969). The synthetic mixture of amino acids and glucosamine showed only the expected peaks on an amino acid analyzer chromatogram. When this mixture was subjected to hydrolyzing conditions in 6 N HCl, the same five additional peaks were observed. Since the recoveries of the amino acids were generally high (Figure 3) and that of glucosamine very low (Figure 4) these would appear to be degradation products of glucosamine hydrolysis. This view is supported by the fact that amino acid analyzer chromatograms of cell wall hydrolysates of *Phaseolus vulgaris* hypocotyls (Chang, personal communication) and *Avena sativa* coleoptiles (O'Sullivan, personal communication), which do not contain amino sugars, did not show any of these peaks.

All of these artifacts of hydrolysis are presumably derived from degradation or interaction of the monomers for estimates of which the analyses were performed. The aim of the analysis, however, is to determine the amounts of these monomers. Robel and Crane (1972) have examined the question of degradation of amino acids during protein hydrolysis. They observe that the true amino acid composition of a protein is determined ideally by quantitatively determining the amino acids when their peptide bonds have been broken and before degradation occurs. Corrections for amino acid destruction during hydrolysis are discussed and a method of extrapolation for determining true or original amounts at zero time with data involving simultaneous yield and decay. These principles can be applied to hydrolysis of any polymer. The derivation of equations assumes that the monomers must be in one of three states.
**State A.** The monomers are bound in the polymer and not observable by analysis.

**State B.** The monomers are hydrolyzed from the polymer (State A) and observable by analysis.

**State C.** Hydrolyzed anhydro monomers from State B are degraded and no longer observable by analysis.

As the monomers proceed through the different states, analytical observations are taken in State B. The problem rests, therefore, in finding the number of monomers initially in State A using the observations taken in State B.

The rate of hydrolysis from State A to State B is assumed to be constant for each monomer of a given type. The rate for each type is then proportional to its number remaining in State A. This assumption is expressed by the following differential equation

\[
dA / dt = -hA
\]

where \( A \) = the number of molecules remaining in State A, and \( h \) = hydrolysis constant in units of fraction of State A per hour (\( h \) is assumed to constant throughout the experiment for any given monomer).

The rate of change of the number of hydrolyzed (State B) molecules equals the rate they are coming out State A minus the rate they are being lost to State C. If \( I \) is defined as the loss constant, and it is assumed that the loss rate is proportional to the number in State B, then the loss rate is \( IB \), thus

\[
 dB / dt = (-dA / dt) - IB.
\]

The authors develop a method of non-linear least-squares to estimate the original monomer composition of the polymer. A computer is required to execute the program. A similar approach to the problem based on polysaccharide studies is discussed by Gheorghiu, Oette and Baumann (1970). It provides approximations for the correction factors discussed by Robel.
and Crane (1972) that do not require computer assistance.

Available data suggest that any restriction of flexibility of a polysaccharide chain reduces the rate of hydrolysis (BeMiller 1967). This may be related to the conformational changes necessary for forming the half-chair carbonium-oxonium ion (Figure 11) in more highly ordered systems. Sentandreu and Northcote (1968) have found O-glycosyl linkages to serine and threonine in yeast cell walls. They also suggested the existence of a N-glycosyl bond between N-acetylglucosamine and asparagine. Lamport (1967) has reported the presence of O-glycosyl linkages to hydroxyproline in the cell walls of higher plants. Such linkages are likely to exist between protein and polysaccharide in fungal cell walls such as T. mesenterica and S. diclina. This situation could cause polysaccharides in cell walls to be more resistant to hydrolysis if the proteins held the polysaccharides in configurations that were less flexible than those in pure polysaccharides. The hydrolysis conditions that release monosaccharides would not significantly affect peptide bonds, and if the glycosyl-amino acid linkages were even moderately stable the stability of the linkages between the monosaccharide units might well be altered. Bonds that were labile in the free polysaccharide might become less so in the cell wall unit.

This sort of linkage is implied by the recovery curves of serine and threonine (Figures 6 and 7). Characteristically the recovery curves of threonine and serine in purified proteins show a negative slope throughout the course of hydrolysis (for example, Robel and Crane 1972). The increased stability of the threonine and serine peptide bonds might be explained thus: if the β-0 were involved in a glycosyl bond, then it could not form the oxazoline ring which is thought to labilize the
peptide bond. This would leave the peptide bond more resistant to acid hydrolysis than in a pure protein.

Such complications arise in cell wall chemistry because the protein analysis is carried out on a protein that is contaminated with approximately 90% of other components, mostly polysaccharides. The polysaccharide is only 80% 'pure'. Thus appropriate correction factors for degradation are difficult to devise.

It is relatively easy to determine the rate of degradation of free amino acids and free monosaccharides under the hydrolyzing conditions used for cell wall preparations. Neutral and acidic amino acids are relatively stable to acid hydrolysis; basic amino acids are much less so (Figure 3). About 75% of the neutral sugars was recovered after 8 hr in hydrolyzing conditions (Figure 5); mannose is the most stable (85% recovery) and xylose the least (50%). Glucosamine is as stable after 8 hr in 2 N HCl as mannose in 2 N CF₃COOH, but only 30% is recovered after 97 hr. Degradation is much more extensive in 6 N HCl (even after 8 hr only 15% is recovered) and degradation is virtually complete after 97 hr (Figure 5). These curves show the effect of the loss rate, IB, defined by Robel and Crane (1972).

Figures 6 to 10 show curves involving simultaneous yield and decay. In the case of neutral sugars the relative ratios of degradation after maximal release in Figures 8 and 9 are of the same order as those in Figure 5. For the amino acids released from cell walls (Figures 6 and 7) the curves generally have negative slopes that are steeper than those in Figure 3 (free amino acids). This suggests that the amino acids in the cell wall preparations are being degraded to a greater extent than free amino acids under the same conditions of hydrolysis. Hydrolysis in 6 N HCl causes destruction of monosaccharides; humin degrades and forms
complexes with amino acids.

Thus the assumption that hydrolysis constants do not change throughout an experiment may introduce a serious error into studies of cell wall hydrolysis. For this reason the correction of extrapolation to zero time has not been used. The study of hydrolysis of cell walls in 2 N HCl and 6 N HCl show the gross inaccuracy of such a plot if degradation is extensive. The degradation of most amino acids in the cell walls seems extensive and although the total estimate of amino acids may be low, it does represent a real figure, based on actual recovery of each individual amino acid. The problem requires further study.

The 10% of the cell walls not recovered may be accounted for when such problems are solved.

Analysis of fungal cell walls is further complicated by the fact that reproducible cell wall preparations are often difficult to obtain. Biological diversity complicates quantitative analysis. In studying the cell wall components in fungi, this must be taken into account, especially when making comparisons.

The Cell Wall Preparation

The components that a fungus assembles to produce the cell wall are derived through the action of its metabolic pathways on nutrients from the medium on which it is growing. Different strains of the same fungus growing on the same medium may produce different cell wall components or the same components in different proportions. The same strain grown on different media may also produce this sort of difference. The question of whether a fungus synthesized cell wall from components that are already available in abundance or whether it requires certain components
for a specific cell wall assembly pattern cannot at present be properly answered. Furthermore, many fungi will grow only on media that cannot be completely chemically defined. It therefore becomes more difficult to relate specific structural elements in the medium with those ultimately incorporated into the cell wall.

For example, from the present studies, *S. diclina* was grown in a medium containing peptone, yeast extract, and \( \text{D-glucose} \). If cellulose made up part of the polysaccharide would \( \beta-\text{D-xylose} \), \( \beta-\text{D-mannose} \) or \( \alpha-\text{L-fucose} \) replace or even partly replace \( \beta-\text{D-glucose} \) since they have the same configuration at C-1 and C-4, or would other sugars be converted to \( \text{D-glucose} \) and assembled into cellulose? If the latter alternative occurs, would there be changes in the morphology of the fungus?

There have been few investigations of this sort. Bulmer and Sans (1968) cultured *Cryptococcus neoformans* on completely defined media differing only in the sugar provided. Their study was not of cell wall structure but they did report different responses in capsule development. Angluster and Travassos (1972) grew *Torulopsis pintolopesii* in defined media with choline or methionine and found quantitative differences in the carbohydrates and amino acids in the cell walls. Nevins, English and Albersheim (1967) cultured higher plant cells (*sycamore*) in media containing different sugars and found differences in the proportions of the sugar components of the cell walls.

Ideally all studies of a fungus should be performed on the same strain or culture, in the same medium (preferably completely defined) and grown for the same length of time under identical conditions. Yeast-like cells will be more uniform when grown in synchronous culture. When analysis of the same species are compared, the strain, age of culture and medium must be taken into account.
Both *T. mesenterica* and *S. diclina* were grown on undefined media, and even under careful standardization of other conditions there was variation among cell wall preparations.

The procedures for breaking fungal cells are dependent on the species, the culture conditions and the form of the fungus. Various methods for cell breakage have been described. Specific information about the size of glass beads (in theory they should be of such a diameter that the space formed when four beads come together tetrahedrally is slightly smaller than the diameter of the cells being broken), the proportion of cells and liquid, the speed of rotation of the breaking device (where adjustable) and the duration of the treatment(s) is essential. In the present studies, sonic oscillation completely broke yeast-like cells of *T. mesenterica* but had little effect on yeast cells (*Saccharomyces cerevisiae*) or filaments of *S. diclina*. For each set of circumstances the optimal conditions for breaking cells must usually be determined by trial and error. With some treatments complete cell breakage produces such fine cell wall fragments that they are difficult to collect and wash. In such cases it is preferable to reduce the breakage treatment so that cell wall fragments are larger, and to separate the intact cells from this suspension by centrifugation.

Once a successful cell-breaking procedure has been found, there are only a few precautions to observe. The cells should be kept cold at all times. Aqueous solutions near pH 7 should be used. The cell walls should be washed free of cytoplasmic contamination immediately after breakage to minimize enzymatic degradation. Where glass beads are used in breaking the cells, care must be taken to assure the corrections for glass fragments are applied (Bartnicki-Garcia and Nickerson 1962;
Horikoshi and Iida 1964). Kanetsuna et al. (1969) used a high-density medium (85% sucrose) to separate cell walls from whole cells and glass debris.

The purpose of washing the broken cells is to remove all traces of cytoplasm and any components within the cell wall that are not a part of it. Generally the broken cells are washed with water and dilute aqueous solutions of NaCl, sucrose or both. This procedure removes large amounts of cytoplasm. The cell wall fragments are recovered by centrifugation or filtration. Care must be exercised in filtration procedures to avoid polysaccharide contamination from the filter paper. Further washings are required to assure that other components are completely removed from the cell walls. Mitchell and Taylor (1969) washed the cell walls successively with 8.0 M urea, 1.0 M NH₄OH and 0.5 N HCOOH. The urea solution was chosen because it dissolves many proteins and is unlikely to cleave covalent bonds. NH₄OH and HCOOH were chosen to remove ionizable components compartmentalized in the cytoplasm of the intact cell. When these compounds are released they may become associated with charged groups (-COOH in uronic acids and dicarboxylic amino acids, -NH₂ in basic amino acids and amino sugars) of the cell wall. Both treatments could cause hydrolysis of very weak bonds. In the present studies the HCOOH treatment was omitted because it caused flocculation of cell wall fragments.

Other treatments have been used but they may affect the cell wall. Lipids have been widely reported as cell wall constituents and any washings that involve ethanol, ether (Moreno, Kanetsuna and Carbonell 1969), glycerol (Troy and Koffler 1969) or detergents (Troy and Koffler 1969; Zevenhuizen and Bartnicki-Garcia 1969) may also remove some of the lipid components. Proteolytic enzymes have been used to remove adsorbed proteins (Shah and
Knight 1968) but they may also digest the cell wall itself (Mitchell and Taylor 1969); in general they should not be used in the primary preparation of cell walls.

Crook and Johnston (1962) have discussed the differences between the cell wall preparation and the functional cell wall. Preparation of a reproducible cell wall fraction from different cell preparations is the aim. Removing contaminant material may also remove certain weakly bound cell wall components. At present the reproducibility of any cell wall preparation (even though it may not include all of the functional constituents) is preferable so that a body of basic structural information can be obtained. When the fundamental structure is established, more refined procedures can be developed to investigate those components that are associated with the cell wall by labile linkages.

The 'purity' of the cell wall preparation is usually defined as the absence of cytoplasm (and capsule if the cells possess one). This is widely determined by phase or dark field light microscopy; for rigorous quantitative analysis the electron microscope should be used to confirm the absence of cytoplasmic (and capsular) contamination. Most investigators accept the electron microscope assay as sufficient evidence for 'purified' cell wall preparations. Chemical tests for 'purity' are not widely used. Absence of nucleic acids or their purine and pyrimidine bases is usually taken to show lack of cytoplasm, yet Bartnicki-Garcia and Nickerson (1962) and Moreno et al. (1969) found traces of these substances in cell wall preparations that they accepted as 'pure'.

The present studies have confirmed that quantitative analysis of fungal cell walls is possible with certain limitations. The methods
chosen for estimation of neutral sugars (plus other complementary procedures that are required in many cases), amino sugars and amino acids are suitable for microanalysis of cell wall preparations. Reliable quantitative uronic acid recovery is not at present possible; the method selected is not suitable to provide the required information. Several possible alternatives are available. The procedures used for lipid and elemental analysis provided gross information; more refined techniques which are already available must be adapted for cell wall studies.

The procedures have been assembled and tested to provide the foundation for a comprehensive long-range project in this laboratory to build a model of the cell wall and to explain the mechanisms of its growth. They have been selected to provide basic quantitative information about cell wall monomers. This information is essential to complete elucidation of cell wall structure at the level of fundamental constituents and at each succeeding level of structural analysis. Their application is intended to be broad so the techniques and subsequent improvements can be applied to the study of cell wall structure in fungi and other organisms. In this respect the study may represent the first comprehensive attempt to completely quantitize cell wall analysis.


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### APPENDIX

Symbols Used for Monomers

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<tr>
<th>Symbol</th>
<th>Name</th>
<th>Abbreviation</th>
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<tr>
<td>Ala</td>
<td>alanine</td>
<td>Ile</td>
<td>isoleucine</td>
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<td>Ara</td>
<td>arabinose</td>
<td>Ino</td>
<td>myo-inositol</td>
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<td>arginine</td>
<td>Leu</td>
<td>leucine</td>
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<td>Asp</td>
<td>aspartate</td>
<td>Lys</td>
<td>lysine</td>
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<tr>
<td>Asx</td>
<td>aspartate or asparagine</td>
<td>Man</td>
<td>mannose</td>
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<td>cysteine</td>
<td>MeHis</td>
<td>methylhistidine</td>
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<td>Met</td>
<td>methionine</td>
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<td>fucose</td>
<td>Phe</td>
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<td>galactose</td>
<td>Pro</td>
<td>proline</td>
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<tr>
<td>GalN</td>
<td>galactosamine (2-amino-2-deoxy-galactose)</td>
<td>Qpa</td>
<td>α-amino-β-guanidino propionate</td>
</tr>
<tr>
<td>Glc</td>
<td>glucose</td>
<td>Rha</td>
<td>rhamnose</td>
</tr>
<tr>
<td>GlcN</td>
<td>glucosamine (2-amino-2-deoxy-glucose)</td>
<td>Rib</td>
<td>ribose</td>
</tr>
<tr>
<td>GlcNx</td>
<td>glucosamine or N-acetyl-glucosamine (undefined)</td>
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<td>serine</td>
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<tr>
<td>Glu</td>
<td>glutamate</td>
<td>Thr</td>
<td>threonine</td>
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<tr>
<td>Glx</td>
<td>glutamate or glutamine</td>
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<td>tryptophan</td>
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<td>glycine</td>
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<tr>
<td>Hyp</td>
<td>4-hydroxyproline</td>
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