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Chemical Composition of Wild-type and Mutant *Aspergillus nidulans* Cell Walls. The Nature of Polysaccharide and Melanin Constituents

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SUMMARY

Chitin and a β-linked glucan were the major chemical components of *Aspergillus nidulans* cell walls. Other monomeric residues identified in enzymic and acid hydrolyses of whole cell walls and cell-wall fractions included galactose, mannose, glucuronic acid and galactosamine. The β-glucan contained (1 → 3) and (1 → 6) linkages and was two-thirds digested by an exo-β-D-1,3 glucanase prepared from a cell-wall lysing Streptomyces species. An α-glucan was identified as a cell-wall component and it also contained (1 → 3) linkages. This latter polysaccharide was distinguishable from nigeran (an α-1,3; α-1,4 glucan present in other Aspergillus species) by infrared spectroscopy and by its low susceptibility to hydrolysis by an endo-α-1,3; α-1,4 glucan glucanohydrolase. Both glucans were alkali-soluble, but the β-glucan was completely solubilized only after acid extraction of the wall. The *N*-acetylglucosamine to galactosamine ratio in the *A. nidulans* cell wall was 1·32 and the two hexosamines were shown to be constituents of distinct polymers. The remaining cell wall was accounted for by protein, lipids, readily extractable and bound, and, in the wild-type, melanin.

The melanin was distributed throughout the cell wall but was associated particularly with the chitin fraction. The pigment has been partially characterized chemically and contains indolic residues; this result does not substantiate earlier views that indolic melanins are peculiar to the animal kingdom. Melanin appears to be a finite heteropolymer both in terms of its molecular size and its chemistry.

INTRODUCTION

A positive correlation has been demonstrated in several species between the presence of melanin, or melanin-like pigments, in the cell walls of fungi and resistance to microbial and enzymatic lysis (Potgieter & Alexander, 1966; Bloomfield & Alexander, 1967). Furthermore, studies on *Aspergillus nidulans* (Kuo & Alexander, 1967) established that the resistance to lysis was affected by the melanin concentration in the cell wall. The mechanism by which melanin may exert its antilytic effect in this fungus has recently been discussed (Bull, 1970*a*).

As an essential prelude to investigations of the melanin effect the cells walls of *Aspergillus nidulans* were examined chemically with reference to the nature of

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melanized and non-melanized walls. Extensive chemical analyses of cell walls of filamentous moulds remain few in comparison with bacteria and, in particular, only two fungal melanins have been studied in any detail (Nicolaus, Piattelli & Fattorusso, 1964).

METHODS

Microbiological techniques. Wild-type strain 13 and an albino mutant 13.1.OL of *Aspergillus nidulans* Eidam (Wint.) have been described previously (Bull & Faulkner, 1964, 1965). The two strains were distinguished by the presence and absence respectively of melanin in their cell walls. Stock cultures were maintained on a glucose–salts agar medium and 10 l. batch cultures for cell-wall preparation were grown in a liquid medium of similar composition (Carter & Bull, 1969; Bull, 1970*a*).

Cell walls of *Aspergillus nidulans* 13.1.OL were degraded extensively by a complex of lytic enzymes synthesized by a Streptomyces species. The lytic organism was isolated from soil enriched with *Phytophthora megasterium* mycelium (Wang, 1964). Cultures of the streptomycete were maintained on a mineral salts agar containing 2 % (w/v) wet wt of blended *Aspergillus oryzae* mycelium (supplied by Wallerstein Co., Division of Travenal Laboratories Inc., Staten Island, New York, U.S.A.) as the sole carbon source. Forty-litre cultures of the streptomycete were grown in a stainless-steel fermenter and the lytic enzymes were precipitated from the culture liquors by 70 % saturation with $(NH_4)_2SO_4$. Full details of these procedures, together with descriptions of β-1,3 glucanase and chitinase purification, have been reported previously (Bull, 1970*a*).

Cell-wall preparation. Two methods of cell-wall preparation were used. (i) Freshly harvested mycelium was washed thrice with Na/K phosphate buffer (0·033M, pH 6·1), its pH adjusted to 6·8 with 2N-NaOH and collected by centrifugation. The mycelium was blended for 2 min. in an Omni-Mixer (Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.), then treated with 1 % (w/v) sodium dodecyl sulphate (10 ml./g. wet wt mycelium) (Mahadevan & Tatum, 1965) and stirred overnight at 4°. Fivefold washing of the hyphal fragments in phosphate buffer was followed by treatment of the residue with increasing concentrations of aqueous ethanol, after which it was suspended in distilled water and freeze-dried. (ii) Phosphate-buffer washed mycelium (1·5 g. amounts in 50 ml. buffer) was macerated in a glass tissue homogenizer (Thomas, Philadelphia, Pennsylvania, U.S.A.) driven by an electric motor at maximum speed and then centrifuged at 800*g* for 10 min. Merthiolate (0·01 %, v/v) (Eli Lilly and Co., Indianapolis, Indiana, U.S.A.) was added to the buffer solution to prevent microbial growth. The supernatant containing cytoplasmic debris was discarded and the combined residues resuspended in buffer (50 ml.). The suspension held below 12° was ultrasonicated in 15 ml. amounts for 2 min. with the sonicator (Biosonik, Bronwill, Rochester, New York, U.S.A.) operating at full power. Cell-wall material was recovered by centrifugation (800*g*, for 10 min.) and the ultrasonication cycle repeated twice more. The residue, collected at 1400*g* for 10 min., was washed twice with chilled buffer and freeze-dried. Wall preparations were examined microscopically during the course of their isolation; the procedures detailed above removed all cytoplasmic material as shown by staining techniques, phase-contrast observation and lack of detectable extinctions at 260 and 280 nm.

Chemical analyses. Chemical fractionation of the cell wall was made according

to the method described by Mahadevan & Tatum (1965). At all times during the fractionation, reaction mixtures were flushed with nitrogen gas to minimize oxidative degradation. The four fractions were characterized as follows: (1) alkali-soluble; (2) acid-soluble; (3) acid-insoluble, alkali-soluble; (4) acid- and alkali-insoluble. Fractions 1, 3 and 4 were suspended in distilled water, neutralized, then dialysed against distilled water for 36 h. and freeze-dried; fraction 2 was neutralized with solid $Ba(OH)_2$ and the supernatant, after centrifugation, was freeze-dried.

In some experiments cell-wall fractions were extracted with Schweizer's cuprammonium reagent, 13·5 g. Cu/l. (Jayme & Lang, 1963). The sample (30 mg.) was twice extracted with the solvent (5 ml.) under N_2 for 3 h. at room temperature. The residue was centrifuged off (14,000*g*, 15 min.), washed exhaustively with distilled water and dried over NaOH pellets.

Carbon, hydrogen and nitrogen determinations were made with a model 185 C-H-N Analyser (Hewlett-Packard, Avondale, Pennsylvania, U.S.A.) using 2, 32 and 8 attenuation respectively for N_2, CO_2 and H_2O. The instrument was calibrated with cyclohexane-2,4-dinitrophenyl hydrazone (N = 20·14 %) and acetanilide (N = 10·36 %). Micro-Kjeldahl determinations of total N were made by the method of Bremner (1960). Phosphorus was determined with the *N*-phenyl-*p*-phenylenediamine hydrochloride chromogen (Dryer, Tammes & Roth, 1957).

Acid hydrolyses of cell walls were made by the procedures described by Bartnicki-Garcia (1966) and were carried out under N_2. After hydrolysis with H_2SO_4, hydrolysates were neutralized with $BaCO_3$ and the clarified supernatants deionized by passage through a bed of Dowex-50W-X8 (hydrogen form). Hydrochloric acid was removed by evaporation *in vacuo* over NaOH pellets. Cell walls were enzymically dissolved with a partially purified lytic complex (Bull, 1970*a*) in 0·05M-citrate–phosphate buffer, pH 5·0, containing 0·01 % (w/v) merthiolate. Reaction mixtures contained 353 units of endo-β-1,3 glucanase (β-1,3(4) glucan glucanohydrolase, E.C. 3.2.1.6), 2·62 units of chitinase (chitin glycanohydrolase, E.C. 3.2.1.14), 93 units of mycodextranase (α-1,3, α-1,4 glucan glucanohydrolase), and unspecified lipolytic and proteolytic activities. Highly purified hydrolases (Bull, 1970*a*) also were used to investigate the nature of various cell-wall polymers.

Cell-wall hydrolysis products were separated by descending paper chromatography (Whatman no. 1) or ascending cellulose thin-layer chromatography (Cellulose F, E. Merck AG, Darmstadt, Germany). Chromatograms were irrigated with either butan-1-ol+pyridine+water (6+4+3, v/v) or propan-2-ol+acetic acid+water (3+0·5+1, v/v) and developed with aniline hydrogen phthalate, ammoniacal $AgNO_3$ (reducing sugars), *p*-dimethylaminobenzaldehyde (amino sugars) or ninhydrin (amino acids). Sugars separated on paper chromatograms were estimated quantitatively by cutting out the sugar spots (located on parallel chromatograms developed with $AgNO_3$), eluting with distilled water and determining the sugar concentration of the clarified eluate with anthrone and *p*-dimethylaminobenzaldehyde.

Total carbohydrate was determined by the anthrone procedure of Loewus (1952) using a glucose standard. Reducing sugars were determined as glucose with a low-alkaline Cu reagent (Somogyi, 1952) and the arsenomolybdate chromagen of Nelson (1944). The method of Reissig, Strominger & Leloir (1955) was used to determine *N*-acetylamino sugars which were expressed as *N*-acetylglucosamine. Glucose and galactose were assayed enzymically with the appropriate sugar oxidases (Glucostat

Special and Galactostat, Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.). Galactosamine identity was confirmed by converting it to the corresponding pentose, i.e. lyxose (Stoffyn & Jeanloz, 1954). Presumed galactosamine spots were cut out from paper chromatograms and eluted with water. The clarified eluate was spotted (20 μl.) on to cellulose TLC plates which, after drying, were sprayed with 2 % (w/v) ninhydrin containing 4 % (v/v) pyridine and heated to 80° for 3 h. in an atmosphere of pyridine and water (1 + 1). The dried plates were irrigated with butan-1-ol + ethanol + water (4 + 1 + 1, v/v) and galactosamine, glucosamine, lyxose and arabinose co-chromatographed for reference. Uronic acids were determined with the Dische carbazole reagent (Gancedo, Gancedo & Asensio, 1968). Passage of hydrolysates through a bed of Amberlite IR-4B removed the mineral acid following which the uronic acids specifically were absorbed on to Dowex-1 (formate form) and recovered by elution with 0·5 N-formic acid. Protein and lipid contents were determined on cell-wall material prepared by method (ii) above. Protein was measured directly in untreated walls using the Folin–Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951), or in detergent extracts of cell walls (van Soest, 1963). Readily extractable and bound cell-wall lipids were measured by the procedure of Bartnicki-Garcia & Nickerson (1962). Nucleic acid content was estimated by measuring the extinction at 260 nm. of hydrochloric acid digests of cell walls; absorption readings were corrected for background. Ultraviolet and visible spectra were obtained with a Beckman DB-G Spectrophotometer (Beckman Instruments Inc., Fullerton, California, U.S.A.). Samples for infrared spectroscopy were powdered with i.r. quality potassium bromide, pressed under vacuum into discs, and the spectra recorded in a Beckman IR 10 infrared spectrophotometer.

Melanin investigations. Melanin was extracted from wild-type mycelia by the procedures described by Nicolaus *et al.* (1964). The fine amorphous black pigment could be solubilized completely at 60° by 0·5 N-NaOH. Melanin was determined gravimetrically or by measuring the extinction at 540 nm. of NaOH solutions. The molecular-size distribution of melanin samples was investigated by gel filtration on a column (255 × 20 nm.) of Sephadex G-200 (Pharmacia, Uppsala, Sweden). Samples were applied to the column and eluted with 1 M-NaCl. The column was calibrated with the following proteins of known molecular weight: urease ($4{\cdot}8 \times 10^5$), β-glucuronidase ($2{\cdot}8 \times 10^5$), phospholipase ($1{\cdot}0 \times 10^5$) and ribonuclease ($1{\cdot}4 \times 10^4$). Nitrogen was determined by the methods noted above. The preparation of [^{14}C]melanin specimens, specifications of radiochemicals, counting procedures and equipment have been reported previously (Bull, 1968). Attempts to characterize the melanin in terms of its degradation products (Nicolaus *et al.* 1964) were made by (1) fusing in a mixture of sodium hydroxide and sodium dithionate at 300°, and (2) oxidizing with 3 % (w/v) aqueous potassium permanganate. Chemisynthetic DOPA-melanin was prepared by the method described by Bull (1970*b*).

Materials. Insoluble laminaran was obtained from the Seaweed Research Institute, Musselburgh, Midlothian, Scotland; nigeran extracted from *Aspergillus japonicus* QM 332 and *A. luchuensis* QM 873 were gifts from D. E. Eveleigh of the Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada; chitin was prepared as a colloidal suspension from crustacean chitin (Kylan PC, Moretex Chemical Products, Spartanburg, South Carolina, U.S.A.). Ion exchange resins were purchased from J. T. Baker Chemical Co. (Phillipsburg, New Jersey, U.S.A.) and enzymes from Worthington

Biochemical Corp. A wide range of non-substituted and substituted indoles, catechols, pyrroles and benzoic acids were purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A.; Eastman Organic Chemicals, Rochester, New York, U.S.A.; and K. and K. Laboratories Inc., Plainview, New York, U.S.A.

RESULTS

The growth characteristics of *Aspergillus nidulans* wild-type and albino strains in 10 l. batch cultures were similar to those reported by Carter & Bull (1969). Both strains produced homogeneous, filamentous growth; pellet formation did not occur and only during the late stages of autolysis were abnormal cell morphologies observed. At a growth temperature of 32°, the maximum mycelial dry weight was produced after about 35 h. ($Y_{glucose}$ = 0·40 to 0·44 g. dry wt g.$^{-1}$). Intracellular melanin synthesis became noticeable after about 40 h., by which time the medium was exhausted of glucose. A few hours later the culture began to autolyse and the pH value progressively rose, accompanied by an accumulation of melanin in the medium. Cultures for cell-wall studies were harvested during the maximum population phase (strain 13.1.OL) or, when melanized material was required, after 25 % autolysis had occurred (strain 13). The cell walls of both strains comprised 18 % of the total dry weight of washed mycelia under these conditions.

Chemistry of wild-type and mutant cell walls

The basic chemical features of the cell walls of the wild-type and albino mutants are shown in Table 1. Elemental analyses suggested identical chemical composition of the two cell walls prior to the establishment of secondary metabolism and the subsequent large-scale synthesis of melanin. Control analyses made on cell walls of *Aspergillus oryzae* (N = 3·5; P = 1·0 %) and *Neurospora crassa* (N = 2·9 %; P = 0·7 %) gave results of the same order as the *A. nidulans* data and agreed well with previously reported values (e.g. Mahadevan & Tatum, 1965). Because melanin interfered with the assaying of cell-wall components, analyses of certain components were made on the albino cell walls only.

Neutral sugars. Acid hydrolysates (22·5N-H_2SO_4 for 3 h. at 30°; dilution to 0·85N and 4 h. at 97°) of both cell walls contained three neutral monosaccharides, glucose, galactose and mannose. The amounts in the cell wall of *Aspergillus nidulans* 13.1.OL were determined enzymically and by quantitative paper chromatography of acid hydrolysates (Table 2). The glucose content was considerably underestimated. Paper chromatograms of wall digests contained several reducing sugar spots (equivalent to 18·7 % of the total carbohydrate) which were identified, tentatively, as β-linked (Bull, 1962) and α-linked (see below) glucose oligosaccharides. The two assays of galactose agreed very closely and, because all of this monosaccharide was a substrate for galactose oxidase, a D-configuration was concluded.

Amino sugars. Three amino sugars, having R_{Glc} values of 0·60, 0·72 and 1·25 in the butan-1-ol+pyridine+water solvent, were detected in the acid hydrolysates (6N-HCl for 6 h. at 100°) of the mutant cell walls. The two most rapidly moving sugars had R_{Glc} values identical with those of authentic glucosamine and *N*-acetylglucosamine. The unknown component (R_{Glc} 0·60) had a mobility close to that reported for galactosamine. The identity of the non-acetylated hexosamines was

confirmed by their oxidation to pentoses; only arabinose and lyxose were detected as products from presumed glucosamine and galactosamine respectively. Glucosamine, *N*-acetylglucosamine and galactosamine were present in the cell-wall hydrolysate in the ratio 5·2:1:4·7 (see Table 2).

Uronic Acids. The cell walls of the wild type and the mutant contained significant amounts of a hexuronic acid residue (Table 1) identified on the basis of paper chromatography as glucuronic acid. The most reliable estimates of glucuronic acid were

Table 1. *Chemical composition of the cell walls of Aspergillus nidulans*

Component	Percentage of the cell wall dry weight: Wild type, 13	Mutant, 13.1.OL
C-, H-, N- C-H-N analyser	27·6 (45·5)*; 4·8 (7·7)*; 4·1 (3·4)*	41·4; 6·7; 3·3
N, Kjeldahl	3·7	3·0
P (as H_2PO_3)	0·82	0·86
Total carbohydrate	60·5	82·8
Total hexosamine	n.d.	25·1
Uronic acid residues	1·9† 1·0‡	3·5† 2·6‡
Protein	5·0§ 10·0–10·4‖ 8·9–12·7¶	8·0§ 8·6–10·6‖ 8·9–9·4¶
Nucleic acid	0·61	0·60
Lipid		
Free	2·3	2·8
Bound	7·2	6·2
Melanin		
Total	16·3–18·2	0
'Encrusting'	1·0–3·2	0
Sum**	91·5–101·1	101·1–102·7

n.d. = not determined.

* Values refer to pre-melanized cell walls.
† Values derived from paper chromatographically separated material.
‡ Method of Gancedo *et al.* (1968).
§ Method of Lowry *et al.* (1951).
‖ Detergent-extracted cell walls.
¶ Values calculated from total nitrogen data and corrected for total hexosamine and melanin contents of the samples.
** Total carbohydrate, protein, lipid, nucleic acid and melanin.

Table 2. *The neutral and amino sugars present in the cell wall of Aspergillus nidulans 13.1.OL*

Sugar	Percentage of cell wall dry weight
Glucose	28·9; 26·2*
Galactose	3·8; 3·9*
Mannose	2·8
Glucosamine	12·0
N-Acetylglucosamine	2·3 } 14·3
Galactosamine	10·8

Sugars determined by quantitative paper chromatography.
* Sugars determined enzymically.

obtained by using the method of Gancedo *et al.* (1968), which gave values 47 % (wild-type) and 25 % (mutant) less than those obtained by applying the carbazole assay directly to H_2SO_4 hydrolysates. The hexuronic acid contents of *Aspergillus oryzae* and *Neurospora crassa* cell walls were found to be 2·7 % and 3·4 % respectively when estimated by the latter method.

Table 3. *Infrared spectroscopy of purified cell walls: distinctive band assignments*

Strain/wave number ($cm.^{-1}$)		
13	13.1.OL	Interpretation
3270 w sh	3275 m sh	Chitin (N-H stretching)
3110 w sh	.	Chitin
2920 s p	2930 m p	β-Glucan (CH_2 and CH_3 stretching)
1725 m p	1725 m p	Undissociated carboxyl
1655 s p	1660 s sh	Chitin (amide I band)
1640 w sh	1640 w p	Protein (amide)
1630 w sh	1630 w p	Chitin (C = N)
1550 s p	1540 m sh	Chitin/protein (amide II band)
915 w sh	917 s sh	α-Glucan
889 s sh	890 s sh	β-Glucan
825 s sh	.	α-Glucan
Major spectral changes following partial enzymolysis*		
889 shoulder lost	890 shoulder lost	
850 appeared	850 appeared	α-Glucan (-1,3)
.	820 appeared	α-Glucan (-1,3)
805 appeared	810 appeared	Glucomannan

p, Peak; sh, shoulder; s, strong; m, medium; w, weak.
* Endo-β-D-1,3 glucanase+chitinase (Bull, 1970*a*: carboxymethyl cellulose fractionation, peak I).

Protein. The cell walls of the wild-type and mutant *Aspergillus nidulans* were similar in containing approximately 10 % of protein. Table 1 indicates efficiency of detergent extraction in the determination of cell-wall protein. Cell walls were extracted (×6) with anionic or cationic detergents and the amount of protein recovered agreed well with that calculated on the basis of total nitrogen.

Lipid. The contents of readily extractable and bound lipids in the two cell walls were not significantly different (Table 1).

Nucleic acid. Absorption spectra of cell-wall hydrolysates (6N-HCl for 8 h. at 100°) indicated the presence of nucleic acid bases. Using a molar extinction of 8800 for an equimolar mixture of adenine, cytosine, guanine and uracil (summed molecular weight, 509), the nucleic acid content (as RNA) of the cell walls was calculated to be less than 1 % of the dry weight (Table 1). Exhaustive washing of the cell-wall preparations with phosphate buffer or partial destruction with crude Streptomyces lytic enzymes failed to remove this nucleic acid component.

Melanin. The melanin content of wild-type *Aspergillus nidulans* was found to account for between 16·3 and 18·2 % of the cell wall on a dry weight basis. Of this melanin, a small proportion could be released from the cell wall by ultrasonication in the presence of detergents (Table 1) (Bull, 1970*a*).

Infrared spectroscopy. The salient absorption maxima in the i.r. spectra of wild-type and mutant *Aspergillus nidulans* cell walls are summarized in Table 3. Reference spectra were recorded also of representative β-glucans (laminaran, cellulose, pustulan),

α-glucans (nigeran, dextrin) and other polymers of relevance to the chemistry of fungal cell walls (yeast mannan, galactan, native and chemisynthesized melanins). Bands were assigned after comparisons of the cell wall and reference spectra and reference to appropriate literature data (Bacon, Jones, Farmer & Webley, 1968; Barker, Bourne, Stacey & Whiffen, 1954; Pearson, Marchessault & Liang, 1960; Marchessault, 1962; Mitchell & Scurfield, 1967). Infrared analyses indicated the presence of α- (915 and 825 cm.$^{-1}$) and β- (890 cm.$^{-1}$) linked glucans, chitin (3270, 1665, 1630 and 1550 cm.$^{-1}$) and protein (1640 and 1550 cm.$^{-1}$) in the cell walls of both strains. The 1550 cm.$^{-1}$ absorbance can be assigned to amide groups which could have arisen from protein and chitin. Similarly, the undissociated carboxyl absorbance at 1725 cm.$^{-1}$ may have comprised both protein and melanin signals. The presence of melanin in the cell wall of the wild-type strain was not indicated by i.r. spectroscopy. Partial degradation of the cell walls by a mixture of endo-β-D-1,3 glucanase and chitinase caused the 890 cm.$^{-1}$ absorbance to be lost while absorbance maxima characteristic of α-glucans (840 and 820 cm^{-1}) became discernible (Table 3). A shoulder at 805 to 810 cm^{-1} also was indicated after partial hydrolysis, a feature suggestive of a glucomannan component (Marchessault, 1962).

Chemical fractionation of the cell wall

The cell wall was extracted chemically with alkali and mineral acid in order to obtain information on the nature of the polymeric components. Such attempts at fractionation can resolve the polymers only partially, but use of the Mahadevan & Tatum protocol (Mahadevan & Tatum, 1965) with the *Aspergillus nidulans* cell wall enabled at least two polymers to be isolated and identified. Cell walls of the albino mutant or premelanized wild type were used to avoid complications due to melanin which is extracted by the alkali treatments.

Fraction 1. Cell walls (500 mg.) prepared from *Aspergillus nidulans* 13.1.OL were extracted with 2M-NaOH (250 ml.) at room temperature for 16 h. The 24% of the total wall material recovered by this treatment was largely insoluble in cold water, and the only identifiable soluble components were glucose and reducing sugars with R_{Glc} values referable to laminaridextrins (Bull, 1962). Elemental analysis of fraction 1 gave C = 35·8%, H = 6·30%, N = 0·31% and P = 1·11%. The P content represented approximately one-third of the total wall content. Acid hydrolysis produced glucose (62·5%), galactose (4·7%) and galactosamine. The latter was not estimated quantitatively, but paper chromatographic evidence suggested that the majority of the galactosamine was present in this fraction (see fraction 2 below). Fraction 1 material was not very susceptible to β-1,3 glucanase attack and only 7% of the material was solubilized (based on glucose and laminaritetraose recovery).

Infrared spectra of fraction 1 showed a strong shoulder at 917 to 915 cm.$^{-1}$, weak shoulders at 890 cm.$^{-1}$ and 800 cm.$^{-1}$, and a medium peak at 830 to 825 cm^{-1}. These absorbances are indicative of an α-glucan containing −1,3 glucosidic linkages and a small amount of a β-linked glucan. The absorbance at 800 cm.$^{-1}$ could have been due to the α-1,3 glucan or could have arisen from a glucomannan. Treatment of fraction 1 with β-1,3 glucanase produced sharper definition of the 920 to 910 cm.$^{-1}$ shoulder and resolved the 830 to 825 cm.$^{-1}$ absorbance into two small peaks (850 and 835 cm.$^{-1}$), which pointed to the α-glucan being of the α-1,3 type described by Bacon *et al.* (1968) and not of the nigeran (Glcpα1-4Glc; Glcpα1-3Glc) type (see

Fig. 1). Figure 1 shows also that NaOH (3 %, w/v) extractions of the cell wall for 5 h. at high temperature (75°) produced still greater resolution of the α-1,3 glucan absorbance peaks and gave further evidence of mannose-containing polysaccharides (870 cm.$^{-1}$ absorbance). When fraction 1 was incubated with a mycodextranase-containing preparation (Bull, 1970*a*), reducing sugars other than glucose and β-oligoglucosides were released. A preparation having 2·1 units of mycodextranase produced 62·0 %

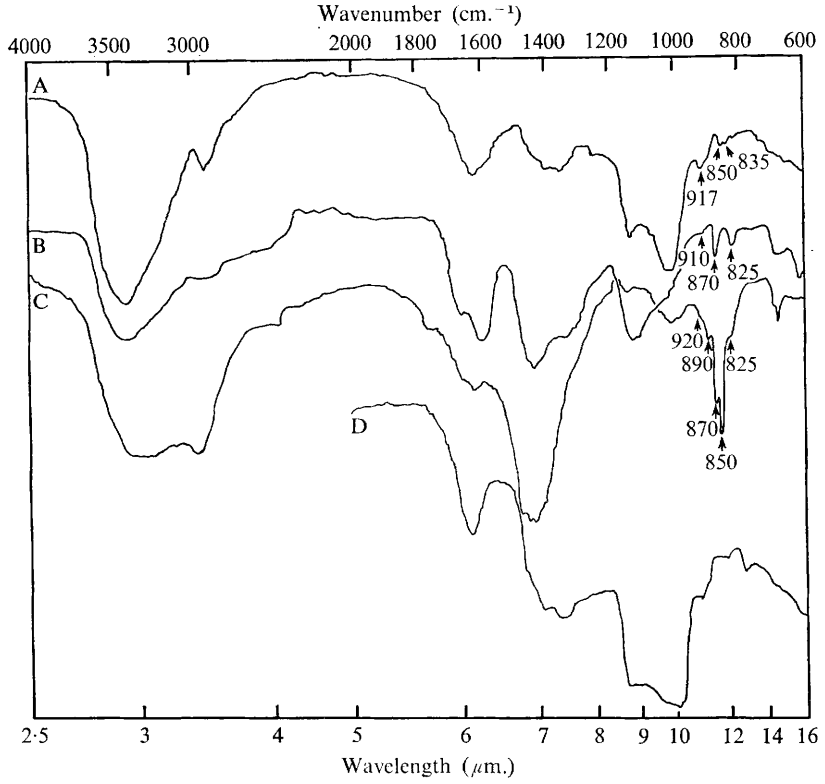

Fig. 1. Infrared spectra of *Aspergillus nidulans* cell walls: α-glucans. A, Cell-wall fraction 1 after treatment with β-D-1,3 glucanase. B, Cell-wall fraction 2. C, Hot alkali extract of the cell wall (see Results section for further experimental details). D, Nigeran (ex *Aspergillus japonicus* QM332). Tracings displaced vertically to facilitate comparison.

hydrolysis of the nigeran samples and 13·5 % of fraction 1. Two α-linked oligoglucosides detected in nigeran digests were identified as nigerose (Glcpα1-3Glc) and a tetrasaccharide (Glcpα1-3Glcpα1-4Glcpα-3Glc) (chromatographic criteria of Tung & Nordin, 1967). Unequivocal proof of the presence of these sugars in fraction 1 digests was not obtained. Following β-1,3 glucanase digestion of fraction 1, the absorbance characteristic of the β-glucan was lost and consequently the latter contained some -1,3 linked residues; simultaneously, the 800 cm.$^{-1}$ absorbance shifted to 810 to 800 cm.$^{-1}$ which was preliminary evidence of a glucomannan entity in the fraction. The effect of Schweizer's reagent on the i.r. spectrum of fraction 1 was slight; most significant was the retention of the absorbances at 917 and 890 cm.$^{-1}$. Spectroscopic analyses of the corresponding cell-wall fraction from *Aspergillus oryzae*

and *Neurospora crassa* gave evidence of α- and β-glucans and the *N. crassa* spectrum also had a weak absorbance at 770 cm.$^{-1}$, which was suggestive of a galactan.

Fraction 2. The residue from cold alkali treatment was extracted with hot H_2SO_4 (1 N, 96°) for 16 h., then neutralized; the freeze-dried supernatant accounted for 50 % of the total cell wall. The C, H, N and P contents were 23·1 %, 4·02 %, 1·93 % and 0·88 % respectively. The P amounted to 53 % of the total wall content, and when combined with that of fraction 1 gave a recovery of about 84 %. Fraction 2 was completely soluble in cold water, and paper chromatography indicated the presence of glucose, laminaridextrins, galactose, mannose, *N*-acetylglucosamine and glucosamine. The amino sugars were probably derived from chitin (see fraction 4) and, during the partial hydrolysis of the latter, deacetylation occurred. Acid hydrolysis produced glucose (34·0 %), galactose (3·4 %), galactosamine (trace) and mannose (4·1 %) and an uncharacterized mixture of amino acids. The collective data from fractions 1 and 2 were equivalent to galactose and mannose recoveries of about 70 % and 73 % respectively. Assuming the majority of fraction 2 nitrogen to be protein, the protein content was calculated as 6·1 %, a recovery of about 68 % of the total cell-wall protein. β-1,3 Glucanase digestion of fraction 2 caused little depolymerization (about 3 %).

The i.r. spectrum of fraction 2 indicated the presence of an α-1,3 glucan type polysaccharide (910 cm.$^{-1}$, 825 cm.$^{-1}$), a trace of a β-glucan (very weak absorbance at 890 cm^{-1}) and a mannan (870 cm.$^{-1}$) (Fig. 1).

Fraction 3. Fraction 3 comprised material from the second cold 2 N-NaOH extraction and accounted for 13·8 % of the total cell wall. This fraction lacked P, and C, H and N values were respectively 40·60 %, 6·86 %, 0·03 %. Solubility in cold water was slight and the only information derived from paper chromatography of the solubilized products was a reducing sugar reaction at the origin. The trace amount of nitrogen was not further identified. Glucose was released stoichiometrically from fraction 3 by mild acid hydrolysis (3 N-HCl). Digestion by an exo-β-1,3 glucanase released glucose, laminaribiose, laminaritriose, laminaritetraose and two reducing sugars characteristic of gentiobiose, and a trisaccharide containing a β-1,6 linkage (Chesters & Bull, 1964, for method of analysis and identity) (see Fig. 2). The maximum hydrolysis of fraction 3 produced by this enzyme was just over 68 % (reducing sugar: glucose, 1·03) and neither dialysis of the reaction mixture or replenishment of the enzyme induced further degradation. The elemental analysis of this fraction was in accord with its identity as a glucose polymer (theoretical values for a glucan are C, 44·4 %; H, 6·2 %). The collective data from fractions 1, 2 and 3 gave a glucose recovery of 94 %. The only notable feature of the i.r. spectrum was the strong shoulder at 890 to 880 cm^{-1} characteristic of the β-glucan (Fig. 3); this absorbance was not observed after β-1,3 glucanase digestion.

Fraction 4. This final fraction comprised a residue insoluble following the second alkali extraction and was equivalent to 12·2 % of the original wall material. The elemental composition (C, 43·5 %; H, 7·10 %; N, 5·67 %) was very similar to the theoretical analysis of chitin (C, 43·5 %; H, 5·4 %; N, 6·4 %), and when treated with a high purity chitinase from a Streptomyces species (Bull, 1970*a*) chitobiose and *N*-acetylglucosamine were released. Exhaustive incubation with this enzyme produced a maximum 80 % hydrolysis; digestion of the comparable *Neurospora crassa* cell-wall fraction caused 73 % solubilization. The *N*-acetylglucosamine plus glucosamine

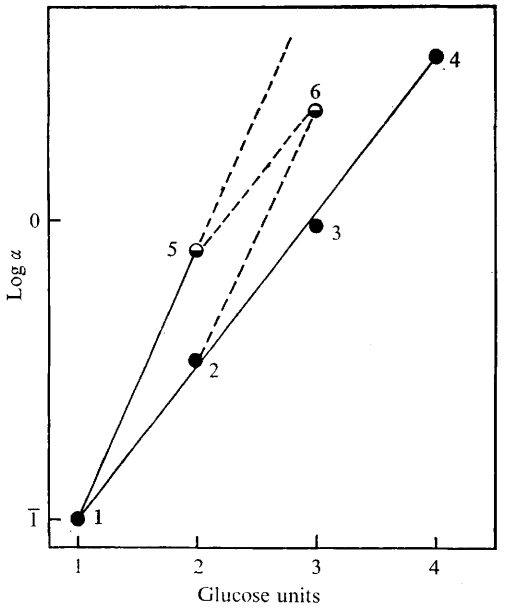

Fig. 2. Products of exo-β-D-1,3 glucanase digestion of cell-wall fraction 3. 1, Glucose; 2, laminaribiose; 3, laminaritriose; 4, laminaritetraose; 5, gentiobiose; 6, triglucoside (Glcpβ1-3Glcpβ1-6Glc). $\alpha = (1 - R_{Glc})R_{Glc}$, see Chesters & Bull (1964). Solvent: butan-1-ol+pyridine+water (6+4+3, by vol.)

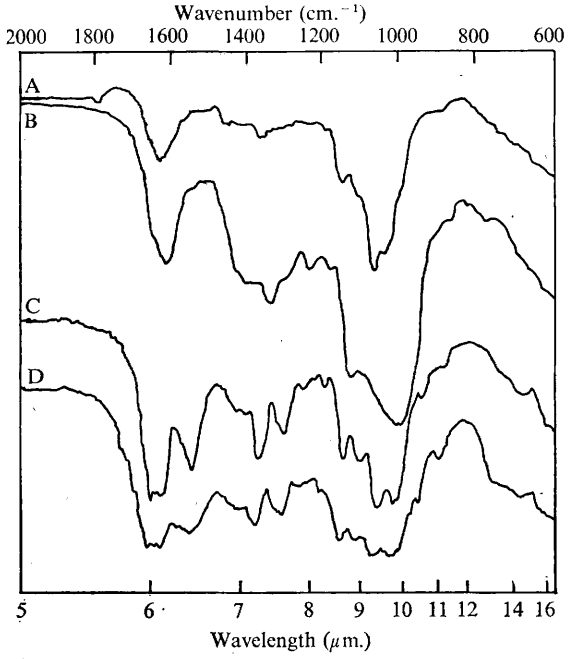

Fig. 3. Infrared spectra of *Aspergillus nidulans* cell walls: β-glycans. A, Laminaran. B, Cell-wall fraction 3. C, Cell-wall fraction 4. D, Purified crustacean chitin. Tracings displaced vertically to facilitate comparison.

content of the cell wall (see Table 2) agreed well with the amount of material recovered as fraction 4. The identity of fraction 4 was confirmed by i.r. spectroscopy (Fig. 2).

Extraction of the wild-type 13 cell wall by the Mahadevan & Tatum procedure revealed that a significant amount of melanin remained associated with the chitin fraction. Conversely, when melanin was being extracted and purified, chitin was the most intractable polysaccharide in the cell wall and was present until the final stages of melanin preparation.

The melanin pigment

The preparation of melanin involved the complete removal of all extraneous cell wall materials by solvent and acid extractions. Nicolaus *et al.* (1964) found that such treatment did not produce major chemical changes in melanins although

Table 4. *Elemental analyses of Aspergillus nidulans melanin: effect of growth conditions on composition*

Strain	Conditions of melanin synthesis	Percentage analysis C	H	N
13	Czapek-Dox medium* (batch culture)	56·40	6·55	3·92
13	Czapek-Dox plus DOPA (0·5 mg. ml.$^{-1}$)†	.	.	5·82
13	Czapek-Dox plus Catechol (0·5 mg. ml.$^{-1}$)†	.	.	1·78
224	DAN‡ (batch culture)	44·80	5·87	4·34
224	DAN (chemostat culture, D = 0·024 h.$^{-1}$)‡	59·52	6·74	3·25
.	Chemosynthetic DOPA-melanin	48·50	4·66	6·25
.	DOPA-melanin, theoretical ($C_9H_3O_4N$)	56·00	3·63	7·26

* Used in earlier investigations by Bull & Faulkner (1965).

† DOPA or catechol added to shake-flask cultures as sterile solutions during the declining growth rate phase.

‡ Defined *Aspergillus nidulans* medium; for medium composition and conditions of continuous culture see Carter & Bull (1969).

partial decarboxylation and deamination probably occurred. The final product accounted for about 16 to 18 % of the *Aspergillus nidulans* wild-type cell wall. The pigment was insoluble in all non-polar solvents tested and in mineral acids; hot NaOH (0·5N, 60°, 24 h.) produced complete solubilization. Precipitation resulted when alkaline solutions were acidified to pH 2 to 3. The *A. nidulans* pigment had properties in common with other melanins: bleached by H_2O_2 (30 %, v/v); colour intensity reduced by glutathione and dithionate and restored on reoxidation; reduced ammoniacal $AgNO_3$; produced characteristic blue-green ferrous sulphate–ferricyanide reaction; formed a flocculent iron-brown precipitate on addition of $FeCl_3$ to an alkaline solution. That the pigment varied in composition with changing growth conditions is indicated by the data in Table 4. The most significant finding was the widely varying N content of the melanin in response to the growth medium, especially when the latter was supplemented with *o*-dihydroxy phenols. Both L-[^{14}C]tyrosine and DL-[carboxy-^{14}C]3(3,4-dihydroxyphenyl)-alanine were incorporated into the cell-wall melanin and the extent of incorporation was dependent on the age of the culture at the time of feeding. Thus, when [^{14}C]tyrosine and [^{14}C]DOPA were supplied to the culture at zero time, 19 % and 28 % of the label was incorporated into cell-wall melanin respectively; the incorporation was 43 % and 69 % when the precursors were fed to the culture after 30 h., that is at the onset of declining growth rate.

Attempts to characterize the *Aspergillus nidulans* melanin on the basis of its degradation products were only partially successful. The pigment (100 mg.) was oxidized by 37·8 ml. $KMnO_4$ (3 %, w/v) but ether extraction of the solution did not reveal any identifiable products. In contrast, alkali fusion of the melanin (100 mg.) with NaOH (300 mg.) and sodium dithionite (50 mg.) at 300° for 10 min. produced

Table 5. *Degradation products from the alkali fusion of Aspergillus nidulans melanin*

Tentative identity	Chromatography R_F (BAW)*	Chromatography R_F (PAW)†	Co-electrophoresis (PyA)‡
5,6-Dihydroxyindole-2-COOH	0·57	.	.
Indole-2-COOH§	0·93	.	+
5-Hydroxyindole§	0·90	.	.
Pyrrole-2-COOH	0·87	0·76	+
Pyrrole-3-COOH§	0·84	0·49	.
Pyrrole-2,4-COOH, or pyrrole-2,5-COOH	.	0·27	.
3,4-Dihydroxybenzoic acid	.	.	+

\+ Present.
* Butan-1-ol+acetic acid+water (60+15+25, v/v).
† Propan-1-ol+ammonia+water (60+30+10, v/v).
‡ Pyridine+acetate, pH 6·1.
§ Spots of strongest intensity indicated.

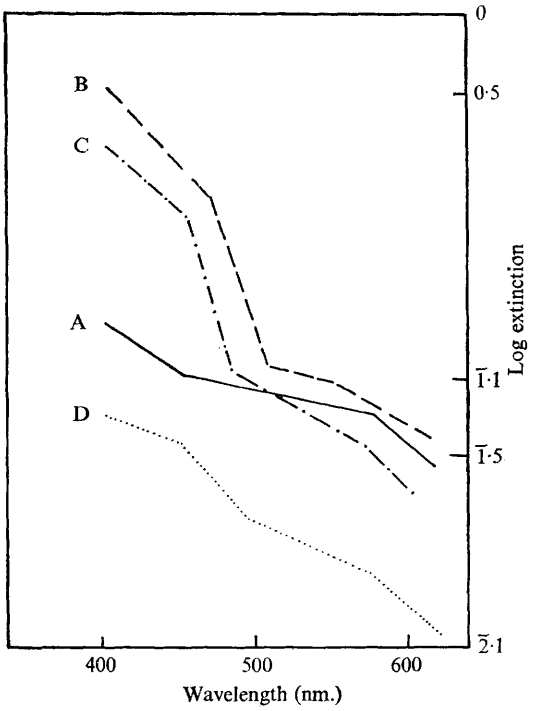

Fig. 4. Extinction of wild-type and mutant *Aspergillus nidulans* melanins. A, Wild-type 13 melanin. B, Purple mutant 13y.ma alkali-extracted pigment. C, Purple mutant 13y.ma×pink mutant 13y.pi alkali-extracted hybrid pigment. D, Chemisynthesized DOPA-melanin. Mutants 13y.ma and 13y.pi are described by Bull & Faulkner (1965).

23·8 % degradation; degradation of the chemisynthesized DOPA-melanin by similar treatment was 28·6 %. Of the 18 ether-extractable degradation products from the fungal melanin, half were identified tentatively (see Table 5) on the basis of co-chromatography and co-electrophoresis, colour reactions with ethanolic 3 % $FeCl_3$, diazotized sulphanilic acid and Ehrlich's reagent, and response to u.v. light. The chromatographic mobilities of authentic samples and published values (Nicolaus *et al.* 1964) often differed by as much as one or more R_F units.

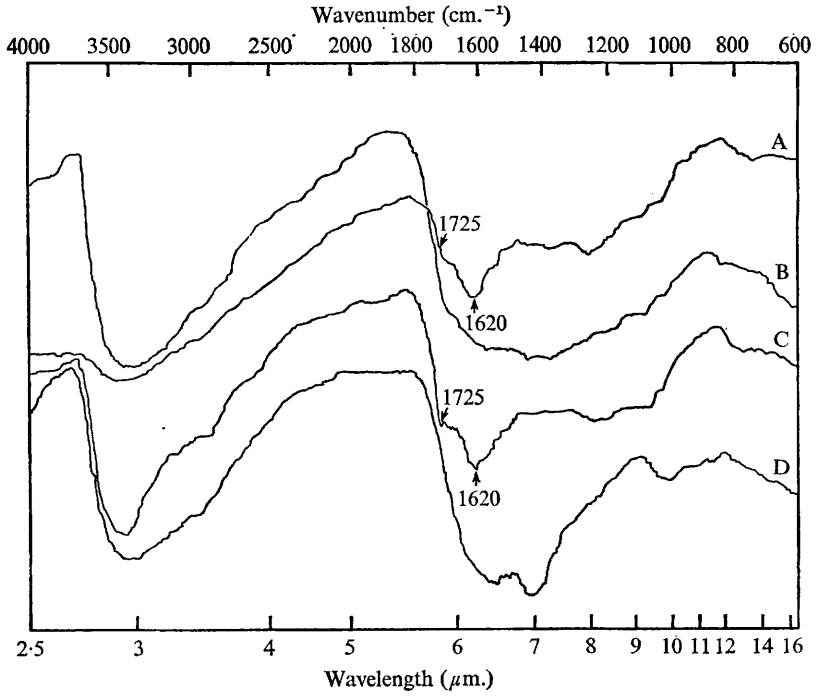

Fig. 5. Infrared spectra of native and synthetic melanins. A, Wild-type *Aspergillus nidulans* 13 melanin. B, As A but extracted with 0·5 N-NaOH. C, Chemisynthesized DOPA-melanin. D, As C but extracted with 0·5 N-NaOH. Tracings displaced vertically to facilitate comparison.

Logarithmic plots of absorbance in the visible range for the wild-type melanin and melanoid pigments from previously described mutants (Bull & Faulkner, 1964; 1965) produced a series of straight lines as shown in Fig. 4. The i.r. spectrum of the wild-type melanin was very similar to that of DOPA-melanin. Neither spectrum had features of significant diagnostic value but the absorbances at 1620 and 1725 cm.$^{-1}$ gave some indication of carboxyl groups. The latter absorbances disappeared after brief treatment of the pigments with dilute NaOH (Fig. 5).

Finally, the homogeneity of various samples of the *Aspergillus nidulans* melanin was investigated by gel filtration on a Sephadex G-200 column previously calibrated for molecular size determination. All samples were separable into a series of fractions having different molecular sizes equal to or greater than 2×10^6 (Fig. 6). Recovery of the melanin from this column was high in all experiments when based upon the extinction at 540 nm. The elution pattern of extracted melanins was composed of three commonly positioned peaks (P1, P2*b*, P4) and other minor peaks (P2, P3*a*, P3*b*)

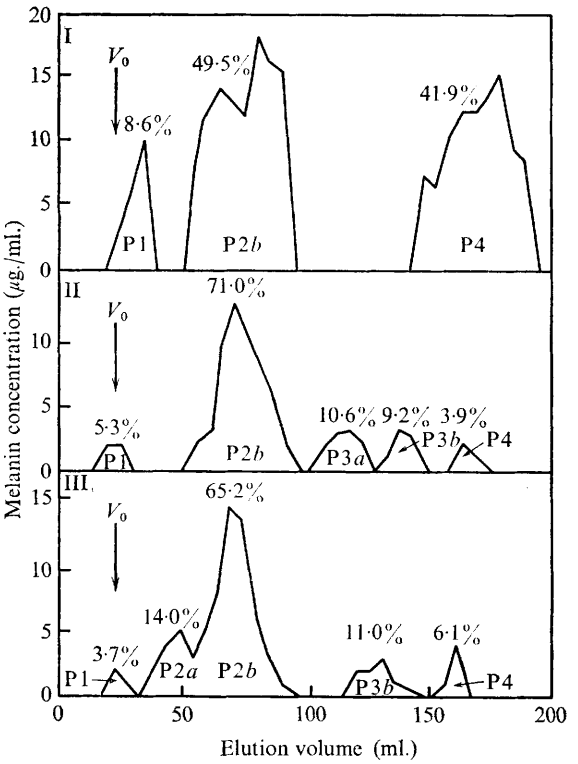

Fig. 6. Fractionation of *Aspergillus nidulans* melanins on Sephadex G-200 gel. I, Cell-wall melanin produced in unsupplemented cultures. II, Cell-wall melanin produced in L-[^{14}C]-tyrosine cultures. III, Cell-wall melanin produced in DL-[carboxy-^{14}C]DOPA cultures. Column size: 255 × 20 mm.; V_0 (void volume) measured with Dextran Blue (M.W. = 2·0 × 10^6). Elution with 1 M-NaCl at 10° in the dark. Fractions (10 ml.) collected automatically and melanin assayed spectroscopically. Recovery of melanin applied to the column: I (82·1 %); II (88·0 %); III (100·0 %).

Table 6. *Molecular size and specific activity of melanin fractions*

	Melanin sample†				
	I: Unsupplemented	II: [^{14}C]tyrosine		III: [^{14}C]DOPA	
Peak*	(mol. size‡)	Mol. size	Sp.act.§	Mol. size	Sp.act.
P1	⩾ 2·0 × 10^6	⩾ 2·0 × 10^6	161	⩾ 2·0 × 10^6	240
P2*a*	.	.	.	6·6 × 10^5	53
P2*b*	3·3 × 10^5	3·2 × 10^5	494	3·8 × 10^5	1278
P3*a*	.	4·7 × 10^4	80	.	.
P3*b*	.	1·0 × 10^4	39	1·3 × 10^4	104
P4	2·7 × 10^3	3·1 × 10^3	191	2·7 × 10^3	56

* See Fig. 5.

† Melanins extracted from wild-type *Aspergillus nidulans* 13. Growth in: I, defined medium (DAN); II and III, DAN supplemented with L-[^{14}C]tyrosine and DL-[^{14}C]DOPA during the declining growth rate phase.

‡ Molecular size estimated by gel filtration on Sephadex G-200.

§ mμCi mg.$^{-1}$

characterized the tyrosine- and DOPA-labelled melanins. Also, the data in Table 6 indicate that the specific activity of the ^{14}C-labelled melanin fractions varied markedly and was not related in a simple way to molecular size.

DISCUSSION

The hyphal walls of *Aspergillus nidulans* comprised largely polysaccharide with minor amounts of lipid and protein and, in the case of the pigmented wild-type strain, melanin. The high glucan plus chitin content of the *A. nidulans* cell wall (60 to 64 %) is typical of Ascomycetes, as is also the presence of galactose and galactosamine. Mannose also is a usual component of Asomycete cell walls (see Bartnicki-Garcia, 1968, for a critical review). Until recently, the evidence for uronic acids in fungal cell walls has been disputed, but the mild hydrolysis and separation procedures recommended by Gancedo *et al.* (1968) have enabled this question to be resolved. Application of these methods to *A. nidulans* revealed the presence of approximately 1 to 3 % of glucuronic acid in its cell wall, a value of the same order as reported for Alternaria, Fusarium and Penicillium (Gancedo *et al.* 1968). It must be stressed that the percentage composition data presented in this paper are not absolute and can be expected to vary significantly with the conditions of culture of the fungus. Moreover, the data on the recovery of various components are subject to the limitations of the extraction techniques used. For example, β-1,3 glucans are susceptible to degradation by β-alkoxyl elimination and hence the value of 13·8 % obtained for the β-glucan (fraction 3) was probably a serious underestimate; this interpretation seems warranted by the detection of laminaridextrins in cell-wall fractions 1 and 2. The low recovery (about 68 %) of protein from fractionated cell walls was because it was partially extracted with lauryl sulphate during the initial stage of the Mahadaven & Tatum (1965) fractionation procedure. Indeed, the present investigation has demonstrated the efficacy of detergent extraction for measuring cell-wall protein, especially when the conventional, direct methods such as biuret are subject to interference by materials such as melanin.

Chemical fractionation studies of *Aspergillus nidulans* cell walls allowed a number of conclusions to be made on their polymer composition. There appeared to be at least two alkali-soluble glucans, one of which, the β-glucan, was released only after the acid extraction, whilst the α-glucan was solubilized by the initial alkali treatment. The infrared spectral analyses of the α-glucan indicated its close relation to the α-1,3 glucan of Cryptococcus cell walls reported by Bacon *et al.* (1968) and distinguished it clearly from the mixed linkage α-glucan nigeran. Furthermore, the release of reducing sugars from fraction 1 and attributable to mycodextranase action amounted to only a very small portion of the total glucose present. The *A. nidulans* α-glucan resembled other strongly dextrorotatory fungal glucans in not giving a colour reaction with iodine (Gorin & Spencer, 1968); moreover, it was not attacked by amylases. The β-glucan, which was partially destroyed during acid and alkali extraction, was of a non-cellulosic type as demonstrated by its susceptibility to exo- and endo-β-1,3 glucanase hydrolysis and the lack of complex formation with Schweizer's reagent. On the basis of its insolubility in cuprammonium hydroxide and paper chromatography of enzyme hydrolysates, it was concluded that the β-glucan contained a preponderance of β-1,3 glucosidic linkages and a smaller

proportion of β-1,6 linkages. The 68 % limit to hydrolysis by exo-β-1,3 glucanase suggested that the β-glucan might have a 'block-type' structure in which a number of say -1,6 linkages are surrounded by -1,3 linkages.

Aspergillus nidulans cell walls contained two hexosamine polymers: chitin and one composed of, or containing, galactosamine. The galactosamine polymer was largely extracted as fraction 1 by 2N-NaOH whereas the chitin remained resistant to alkali and acid treatments; the two hexosamines are therefore components of different polymers. The *N*-acetylglucosamine to galactosamine ratio in the wall of the albino mutant was 1·32, a value similar to that found in the walls of *Helminthosporium sativum* (Applegarth & Bozoian, 1969). Much smaller galactosamine contents have been reported for other fungal cell walls, including a different strain of *A. nidulans* (Mahadevan & Tatum, 1965; Cohen, Katz & Rosenberger, 1969).

The polymers containing galactose, mannose, amino and glucuronic acids were not characterized. Most of the cell-wall mannose was found in fraction 2, and i.r. spectroscopy suggested the presence of a glucommanan. Galactose was extracted in almost equal proportions by cold alkali and acid (fractions 1 and 2). Protein was associated with fraction 2. Emiliani & Ucha de Davie (1962) claimed that such components were found as glucomannoprotein and galactomannoprotein complexes in *Aspergillus phoenicis*, while an alkali insoluble glucomannogalactan has been proposed as an entity in an unidentified *Aspergillus* species (Ruiz-Herrera, 1967).

The presence of melanin in the wild-type cell wall is clearly responsible for its resistance to lytic enzymes (Bull, 1970*a*). However, the respective limit hydrolyses of the β-glucan (68 %) and chitin (80 %), which represented 6 to 7 % of the cell wall not degraded, suggest that factors other than melanin may be involved in the prevention of total cell wall dissolution. Previously I have found (Bull, 1970*a*) that a resistant 'core' of chitin and α-glucan persisted after enzymic lysis of *Aspergillus nidulans* cell walls.

Histochemical data and evidence from enzymolysis experiments indicated that the melanin was located throughout the thickness of the wall and, probably, formed an especially intractable complex with chitin (Bull, 1970*a*). In gel filtration experiments of *Aspergillus nidulans* melanin preparations, all melanin samples analysed possessed three common peaks, corresponding to molecular weights of approximately 2,000,000, 350,000 and 2900. These data suggest that the melanin is a finite heteropolymer whose component molecules differ in molecular size, in contrast to an infinite homopolymer whose size is limited merely by the amount of available precursors. This size distribution of melanin may reflect a location of pigment molecules of specific molecular dimensions in different parts of the cell wall. Brown, Falkehag & Cowling (1967) favoured a similar explanation for the macromolecular structure of lignin in sweetgum wood.

On the basis of the degradation studies, the *Aspergillus nidulans* melanin appears to be, at least in part, indolic in nature. A number of degradation products have been tentatively identified but further interpretation of these results must be cautioned on the grounds of artefact production during melanin extraction: (1) strong acid treatment of the cell wall may have caused acid-catalysed rearrangements in the quinonoid structure; (2) the degradation products (note their low yields) may have been differentially susceptible to further decomposition by alkali fusion. Hence the relative intensities of products noted in Table 5 are not necessarily significant in any

definition of the melanin structure. Visible spectra of the pink and purple mutants' pigments invariably had broad extinction maxima at 520 to 550 nm. and, less frequently, a sharper extinction at 480 nm. These extinctions closely correspond to those of melanochromes and dopachrome (Bu'Lock, 1960) and add further support to the characterization of the *A. nidulans* melanin as indolic.

Few detailed analyses of fungal melanins are available with which to compare those from *Aspergillus nidulans*. Potgieter (1965) quotes elemental analyses of melanins extracted from *Rhizoctonia solani* (C, 62·0; H, 4·8; N, 3·3 %) and *Cladosporium herbarum* (C, 60·7; H, 4·0; N, 2·2 %) which are very similar to those of *A. nidulans*. Further information on the Rhizoctonia and Cladosporium pigments is not available, consequently reference of the nitrogen content to indolic residues cannot be made. Nicolaus *et al.* (1964), however, concluded that the melanins of *Ustilago maydis* spores and *Capnodium nerii* hyphae are non-indolic, the melanin from the former species being catecholic in composition.

During the present investigations, carboxyl groups were indicated in the *Aspergillus nidulans* melanin by i.r. spectroscopy and by the incorporation of [carboxy-^{14}C]DOPA into the pigment. Carboxyl groups in the fungal pigment were titrated against 0·2 N-Na_2CO_3 by the method of Piattelli & Nicolaus (1961) (A. T. Bull, unpublished experiments). A titration difference (0·2 N-HCl) of 133·3 ml. g.$^{-1}$ was observed for melanin synthesized in unsupplemented media (Table 4, first entry), while a chemisynthetic DOPA-melanin had a corresponding value of 99·2 ml. g.$^{-1}$. The origin of these carboxyl groups is open to doubt; they may reflect incorporation of DOPA itself into the melanin molecule, or may be derived from oxidative decomposition of quinones during the synthesis of melanin. The question of whether melanin is a *chemically* homogeneous polymer (single type of subunit and linkage group) or heterogeneous polymer (multiple types of subunit and bondings) also has been debated (see Mason, 1966). The data reported in this paper on the elemental composition of the pigment synthesized under different growth conditions and on the specific activities of [^{14}C]melanin fractions offer some support for the heterogeneous view of melanins.

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