The Composition of the Cell Wall of *Aspergillus niger*

By I. R. JOHNSTON
Department of Biochemistry, University College London, Gower Street, London, W.C. 1

(*Received* 25 *January* 1965)

1. The cell-wall composition of *Aspergillus niger* has been investigated. Analysis shows the presence of six sugars, glucose, galactose, mannose, arabinose, glucosamine and galactosamine, all in the D-configuration, except that a small amount of L-galactose may be present. Sixteen common amino acids are also present. 2. The wall consists chiefly of neutral carbohydrate (73–83%) and hexosamine (9–13%), with smaller amounts of lipid (2–7%), protein (0·5–2·5%) and phosphorus (less than 0·1%). The acetyl content (3·0–3·4%) corresponds to 1·0 mole/mole of hexosamine nitrogen. 3. A fractionation of the cell-wall complex was achieved, with or without a preliminary phenol extraction, by using N-sodium hydroxide. Though this caused some degradation, 30–60% of the wall could be solubilized (depending on the preparation). Analyses on several fractions suggest that fractionation procedures bring about some separation of components although not in a clear-cut fashion. 4. Cell-wall preparations were shown to yield a fraction having $[\alpha]_D$ approx. +240° (in N-sodium hydroxide) and consisting largely of glucose. This was separated into two subfractions, one of which had $[\alpha]_D$ +281° (in N-sodium hydroxide) and had properties resembling the polysaccharide nigeran; the other had $[\alpha]_D$ +231° (in N-sodium hydroxide). It is suggested that nigeran is a cell-wall component.

Previous studies on the chemical composition of the fungus cell wall related mainly to yeasts (see Crook & Johnston, 1962). More recently several papers have been concerned with the chemistry of the cell wall in filamentous species (Aronson & Machlis, 1959; Bartnicki-Garcia & Nickerson, 1962*a*,*b*,*c*,*d*; Hamilton & Knight, 1962; Horikoshi & Arima, 1962; Horikoshi & Iida, 1964; Parker, Preston & Fogg, 1963; Russell, Sturgeon & Ward, 1964; Sturgeon, 1964). In a previous paper (Crook & Johnston, 1962) it was shown that the qualitative composition of the cell wall of *Aspergillus niger* was representative of that found in several species of Ascomycetes. It was therefore decided to examine the walls of this species in more detail.

MATERIALS AND GENERAL METHODS

Growth of A. niger *and preparation of cell walls.* The batches of *A. niger* (no. 17454 of the Commonwealth Mycological Institute, Kew, Surrey) were grown and the cell walls isolated from them as described by Johnston (1963). From cultural records (see Table 2) and a comparison of wet yields of mycelium, preparations 3, 4, 5 and 6 (60 g.) were derived from physiologically similar cultures, preparations 1 and 2 (40–50 g.) represented younger cell walls, and preparation 7 (about 70 g.) older cell walls. Preparations 8 and 9 were from young mycelium. Preparations 1 and 2 were slow-growing, having been started from slopes, 8 and 7 weeks old respectively. By serial transfer, all other cultures were started from material that had been brought to a maximal rate of growth.

Paper chromatography and electrophoresis. These were by the methods given by Crook & Johnston (1962). Additional solvents were as follows: solvent *A*, butan-1-ol–ethanol–water (5:1:4, by vol.); solvent *B*, ethyl acetate–pyridine–water (8:2:1, by vol.); solvent *C*, butan-1-ol–pyridine–water (6:4:3, by vol.); solvent *D*, propan-2-ol–water (9:1, v/v); solvent *E*, pentan-1-ol–acetic acid–water (4:1:5, by vol.). The M_{Glc} value in paper electrophoresis is electrophoretic mobility relative to glucose (Foster, 1957) corrected for electroendosmosis.

Hydrolysis conditions. For amino acids heating was in 5–6 N-HCl at 105° for 18 hr.; amino sugars were released as described by Johnston (1963); neutral-sugar hydrolysates were prepared by soaking material in 70% (v/v) H_2SO_4 at 0° to −5° overnight, diluting to 2N-acid and heating for 4 hr. at 100° to produce maximum release of reducing power. Neutralization was with $Ba(OH)_2$, 5% (v/v) tri-*n*-octylamine in $CHCl_3$, or De-Acidite FF (CO_3^{2-} form).

Analytical methods. The following were used: total nitrogen was determined as described by Crook & Johnston (1962); protein was taken as total nitrogen minus hexosamine nitrogen multiplied by 6·25; total hexosamine was determined by the method of Boas (1953); lipid was 'free lipid' extracted in boiling ether (3 × 15 ml./50 mg. of walls) plus 'bound lipid' extracted at 21° with ether–ethanol–conc. HCl (100:100:1, by vol.); neutral carbohydrate was estimated by an anthrone method (Chen, 1959) or the orcinol–H_2SO_4 method (François, Marshall & Neuberger, 1962); acetyl was determined after total oxidation with 5N-chromic acid (Pregl, 1951) and distillation of the volatile

acids in a Markham still; *O*-acyl was determined as described by Abrams (1958); total phosphorus was determined by the method of Chen, Toribara & Warner (1956) after digestion with H_2SO_4–60% (w/w) $HClO_4$ (3:2, v/v); total nucleic acid was extracted and determined from its extinction at 260 mμ as described by Ogur & Rosen (1950); neutral-sugar composition of the cell walls and fractions was determined by the method of Wilson (1959); ash was the residue after heating, with 2 or 3 drops of conc. H_2SO_4 at red heat, to constant weight.

Isolation of sugars. For neutral sugars this was accomplished by using a cellulose column (4 cm. × 34 cm.) with solvent *D*, or, after removal of all but a trace of glucose, by chromatography on Whatman no. 17 paper with solvent *B*. Removal of glucose was achieved with glucose oxidase (EC 1.1.3.4) at room temperature. A 15 ml. portion of Fermcozyme (solution as supplied by Hughes and Hughes Ltd., London, W.1) was added to the sugars, from a hydrolysate of about 6·5 g. of cell walls, in 150 ml. of water, and 30% (w/v) H_2O_2 was added dropwise (to supply oxygen; Scott & Hammer, 1962). Then N-NaOH was added to maintain the pH near 5·2 until, as judged by a trial run, most of the glucose was oxidized (1·0–1·5 hr.). The solution was then boiled, deproteinized with 5% $ZnSO_4$ and 0·3 N-$Ba(OH)_2$ and deionized with Amberlite IR-120 (H^+ form) and De-Acidite FF (CO_3^{2-} form). The resulting solution was made 1 N with respect to H_2SO_4 and boiled for 3 hr. to complete hydrolysis of the gluconolactone. Neutralization was effected with De-Acidite FF (CO_3^{2-} form) and, when free from acid, the solution was concentrated and chromatographed.

Amino sugars were isolated on a Dowex 50 column (Gardell, 1953). Sugars were isolated as amorphous solids or as syrups, but glucosamine hydrochloride was obtained as the α-D-anomer.

For optical rotations, concentrations were estimated by weight for glucose and glucosamine; for the remaining sugars an appropriate estimation was used (see the 'Analytical methods' section).

EXPERIMENTAL AND RESULTS

Components of the cell wall

Amino acids. The 16 amino acids found were the same as those detected in another strain of *A. niger* (Crook & Johnston, 1962), namely aspartic acid, glutamic acid, glycine, alanine, serine, threonine, leucine, isoleucine, valine, tyrosine, phenylalanine, proline, cystine, arginine, lysine and histidine.

Amino sugars and neutral sugars. Paper chromatography with solvent *C* showed glucosamine and a small amount of galactosamine. These were confirmed by: (*a*) ion-exchange chromatography (Zeo-Karb 225; Gardell, 1953), the elution volumes of the only two peaks, 59 and 70 ml., being the same (±1 ml.) as those for authentic glucosamine and galactosamine; (*b*) ninhydrin oxidation (Stoffyn & Jeanloz, 1954) of isolated samples of glucosamine and galactosamine, which gave arabinose and lyxose respectively; (*c*) *N*-acetylation and electrophoresis (Crumpton, 1959), when the mobilities of the *N*-acetyl derivatives of the isolated hexosamines were the same as those of authentic *N*-acetyl-glucosamine and -galactosamine respectively.

Paper chromatography in solvents *A* and *B* showed the presence of glucose, galactose, mannose and traces of arabinose, as well as traces of two substances with R_F values 0·40 and 0·78 in solvent *A*. A small specimen of arabinose from 200 mg. of cell walls had M_{Glc} 0·97, similar to that of the authentic sugar. The substance with R_F 0·78 reacted only with aniline hydrogen phthalate and alkaline silver nitrate, had M_{Glc} less than 0·05, and $R_{tetramethylglucose}$ 0·97 in solvent *A*. The substance could be furfural or 5-hydroxymethylfurfural [Taufel & Reiss (1957) record R_F 0·9 for the latter in butan-1-ol–acetic acid–water]. The substance with R_F 0·4 reacted only with alkaline silver nitrate (negative for aldose, ketose and deoxy sugars) and had a mobility in solvent *A* identical with that of authentic laevoglucosan (β-1,6-anhydroglucopyranose, an acid reversion product of glucose; Peat, Whelan, Edwards & Owen, 1958). Since laevoglucosan has an R_F close to that of rhamnose in several solvents, it could be mistaken for rhamnose in cases where only alkaline silver nitrate was used as detecting reagent (Hamilton & Knight, 1962).

Chromatographically pure samples of all six sugars were obtained. These are listed below. For glucose and the small sample of galactose, 500 mg., and for the remaining sugars, 5–10 g. of cell walls were used. Amounts isolated are given in parentheses. Derivatives were usually recrystallized twice. Melting points are not corrected. Rotations were equilibrium values determined at 21–25°.

D-Glucose (134 mg.) had $[\alpha]_D + 52°$ (*c* 1·6 in water); Pigman (1957) gives +52·7°; *N*-*p*-nitrophenylglucopyranosylamine had m.p. and mixed m.p. 183–184° (decomp.) (Barker, Foster, Siddiqui & Stacey, 1958).

D-Mannose (45 mg.) had $[\alpha]_D + 14 \pm 2°$ (*c* 1·0 in water); Pigman (1957) gives +14·6°; *N*-*p*-nitrophenylmannopyranosylamine had m.p. and mixed m.p. 219° (decomp.) (Barker *et al.* 1958).

D-Arabinose (18 mg.) had $[\alpha]_D - 106 \pm 6°$ (*c* 0·7 in water); authentic L-arabinose had $[\alpha]_D + 104°$. The toluene-*p*-sulphonylhydrazone of the isolated arabinose had m.p. 154–155° (decomp.); the derivative of the authentic L-sugar had m.p. 154–155° (Easterby, Hough & Jones, 1951).

D-Glucosamine (577 mg. as hydrochloride) had $[\alpha]_D + 72·1°$ (*c* 1·8 in water), the value for the authentic material being +71·8°. The 2-hydroxy-1-naphthylmethylene derivative had m.p. and mixed m.p. 199–202° (decomp.) (Jolles & Morgan, 1940).

D-Galactosamine (16 mg. as hydrochloride) had $[\alpha]_D + 97°$ (*c* 0·9 in water), the authentic material showing +96°. The 2-hydroxy-1-naphthylmethylene derivative had m.p. 175–177° (decomp.); the

Table 1. *Percentage composition of* A. niger *cell walls*

Preparations 1, 2 and 7 were made in the Braun cell homogenizer and preparations 3, 4, 5 and 6 in the Mickle cell disintegrator. Analyses are based on material dried *in vacuo* over P_2O_5.

	Percentage composition	
Prep. no.	1, 2 and 7	3, 4, 5 and 6
Protein	0·56–2·5	0·45–1·10
Hexosamine	9·1–9·9	11·9–13·1
Neutral carbohydrate	73–78	78–83
Lipid	6·7–7·0	1·2–2·0
Acetyl	—	3·0–3·4
Total phosphorus	0·025–0·06	0·02–0·03
Ash	0·1–0·3	0·45–0·8
Nucleic acid	0·2–0·35	<0·1
Recovery	92·2–94·9	96·7–102·1

Table 2. *Percentage of mannose, galactose and glucose in the neutral-carbohydrate portion of* A. niger *cell walls.*

Analyses were by Wilson's (1959) method. In most cases four or five determinations were used to compute a value, standard deviations being within the range ±2–5%.

	Percentage composition			Growth conditions	
Prep. no.	Mannose	Galactose	Glucose	Duration of shaking (hr.)	Temp.
1	5·1	22·1	72·8	52	30°
3, 4, 5 and 6	3·1–4·7	13·6–16·3	80–82·4	54	30
7	3·1	5·9	91·0	54	33

authentic derivative had m.p. 175–178° (Jolles & Morgan, 1940).

Galactose (116mg.) had $[\alpha]_D + 66 \pm 2°$ (*c* 1·26 in water); the diethyldithioacetal had m.p. 140–142° (decomp.) (Wolfrom, 1930). A second sample (10mg.) also had $[\alpha]_D + 66 \pm 4°$. Authentic D-galactose had $[\alpha]_D + 80°$.

Quantitative analysis of the cell walls

General composition. This is shown in Table 1.

Acyl groups in the cell wall. The acyl content, calculated as acetyl, for four preparations was 3·0–3·4% of the wall, corresponding to 1·0 ± 0·04 mole of acetyl/mole of hexosamine nitrogen. Values for *O*-acyl in these same preparations (about 0·03 μmole/mg. of wall) were only 4–5% of the total acetyl values.

The acyl group was identified as acetyl by pooling titrated distillates and treating as described by Bergmann & Segal (1956), except that, after esterification with diazomethane, 2ml. of the supernatant solution of a mixture of 2N-hydroxylamine hydrochloride and 3·5N-sodium hydroxide (1:1, v/v), both in 95% (v/v) methanol, was added, and that, after hydroxamate formation, sodium hydroxide was precipitated by the addition of several pellets of solid carbon dioxide. After centrifugation, the alcoholic supernatant was removed and reduced in volume for chromatography. The removal of excess of alkali as sodium carbonate strikingly improved chromatographic separation over the original method (run against markers of formhydroxamic acid and acetohydroxamic acid; solvent *E*).

Composition of neutral-carbohydrate fraction. The molar percentages of mannose, galactose and glucose were determined within the neutral-carbohydrate portion of the cell wall, which did not include ash, lipid or hexosamine. (Arabinose, representing only about 0·3% of this portion of the wall, was not included.) The results for several preparations are given in Table 2.

It is evident that within the group of preparations 3–6, of similar physiological age, only minor variations in composition occur. By comparison, in preparation 1 (younger culture) and preparation 7 (older), the mannose content appears to fall but slightly with age, whereas galactose is diluted about fourfold and there is a relatively large increase in glucose. This suggests that polysaccharides rich in glucose are distinct from those containing galactose in the cell wall, and may eventually supersede the latter as the principal biosynthetic product. There is some preliminary indication that a fraction containing about 90% of glucose does increase in quantity in the cell wall with age.

Fractionation of the cell wall

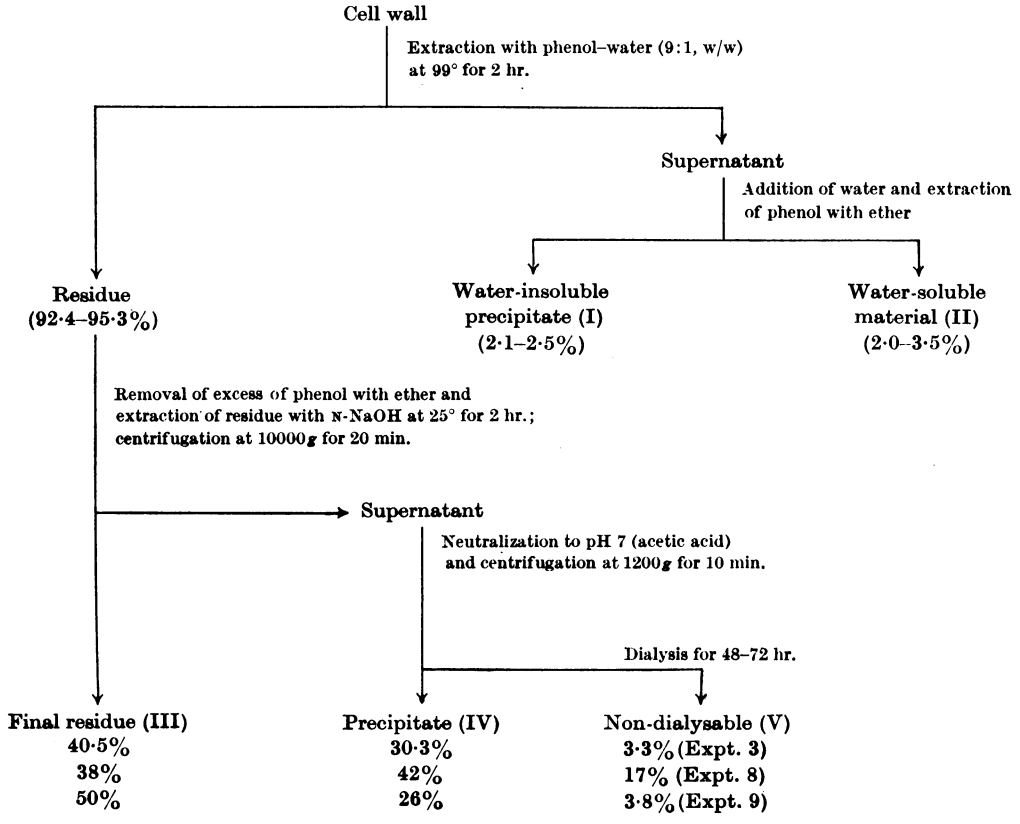
Before extraction, preparations were refluxed with ether or with ether–methanol (1:1, v/v) and dried. The effectiveness of various reagents in solubilizing the cell walls is shown in Table 3.

Whereas phenol, dimethyl sulphoxide and 0·01N-sodium hydroxide remove only a small portion of the wall, N-sodium hydroxide and, to a smaller extent, 5% (w/v) trichloroacetic acid were much more effective. The lower yields of extracted material obtained with N-sodium hydroxide in Expts. 3 and 9 (younger walls) compared with Expt. 8 (older) are probably related to the physiological age of the cultures used (see yields of fraction IV in Scheme 1 and the Discussion section) as well as, in Expts. 3 and 9 where 11g. and 28g. of walls were extracted, to the alkaline degradation of fraction V, discussed below. Further, the residual fraction appears smaller in older (38%; Expt. 8) compared with younger mycelium (50%; Expt. 9). The residue in Expt. 3 (40·5%) is, however, somewhat

Table 3. *Extraction of* A. niger *cell walls with various reagents*

Preparations were made in the Braun cell homogenizer. About 5–10 ml. of reagent was used/50–200 mg. of walls. For Expts. 1, 5, 6, 7 and 8, 50 mg., Expts. 2 and 4, about 500 mg., Expt. 3, 11 g., and Expt. 9, 28 g. of walls were used. Alkali extractions were done under O_2-free N_2. Conditions of extraction are included.

Solvent	Extracted (%)	Residue (%)	Recovery (%)	Expt. no.
Phenol–water (9:1, w/w): 70–99° for 2–4 hr.	3·0–5·6	92·4–95·3	95·4–101	1, 2 and 3
Dimethyl sulphoxide:				
21° for 16 hr.	6·1	84·1	90·2	4
99° for 8 hr.	9·0	—	—	5
Trichloroacetic acid (5%, w/w) 90° for 15 min.	24	57	81	6
0·01 N-NaOH: 25° for 3 hr.	5	93	98	7
N-NaOH: 25° for 2 hr.	59	38	97	8
N-NaOH: 25° for 2 hr.	30	50	80	9
N-NaOH: 25° for 2 hr.	33·6	40·5	78·1	3



Scheme 1. Values are based on three experiments; those for fractions III, IV and V are given separately. Dialysis, at 2°, was against several changes of distilled water. Extraction with N-NaOH was under O_2-free N_2. In Expts. 8 and 9, the cell walls were extracted directly with N-NaOH. Centrifugation of the alkali extract was done in securely capped tubes in an Angle 17 centrifuge (Measuring and Scientific Equipment Co. Ltd., Crawley, Sussex).

smaller than expected. Since, however, N-sodium hydroxide could solubilize up to 60% of the wall, its use was continued. A fractionation procedure that was used (omitting phenol extraction in some experiments) to obtain the various fractions I–V for further analysis is shown in Scheme 1.

Hot phenol was included in Scheme 1 since it removed RNA and protein impurities, unavoidably present to a small extent in large-scale extractions. The yield of fraction III (38–50%) appears to decrease as that of fraction IV (26–42%) increases, and together these account for 70–80% of the wall. Alkali does not appear to degrade fraction IV, since it could be obtained from the same cell-wall preparation in 42% yield both with a 2hr. and a 3·25hr. extraction in N-sodium hydroxide. Further extraction of fraction III (final residue) with N-sodium hydroxide yielded no more fraction IV, although during the process fraction III was degraded (material not precipitated on neutralization and not retained by the dialysis bag) to the extent of 5% of the total wall. Alkaline degradation probably also accounts for the variable yield of fraction V. Thus from 50mg. of walls, 17%, from 1·5g., 8·2%, and from 28g., 3·8% of fraction V were obtained, the more prolonged manipulation times with the larger samples clearly decreasing the yield. The degradation of fraction III (5%) taken with that of fraction V (approx. 14%) would almost account for the 80% recovery in some experiments.

The analysis of several fractions isolated as in Scheme 1 are given in Table 4. Though all six sugars of the wall occur in every fraction, suggesting that the fractionation procedure has 'cut through' several polymers, enrichment in some fractions has been achieved. Some concentration of the arabinose (fraction I), hexosamine (fraction III), glucose (fraction IV) and galactose (fraction VS) is apparent. Bound lipid has not been estimated, although its presence in fraction VS could account for the solubility of this small but reproducible fraction in 85% (v/v) ethanol. In fractions where protein is indicated, it was shown qualitatively that the amino acids of the cell wall were present. Extracts obtained with dimethyl sulphoxide showed, on hydrolysis, neutral and amino sugars and amino acids; trichloroacetic acid extracted a fragment containing neutral and amino sugars.

In an experiment where fraction VP was isolated in 6–7% yield, the approximate mannose:galactose:glucose molar proportions were 1·0:3·0:14·0, but when isolated (from a different wall preparation) in 3·1% yield the values were 1·0:6·0:4·0. This would indicate the lower yields of fraction V to be due to selective alkaline cleavage of the glucose, since for other fractions the glucose content did not vary by more than ±5% between different wall preparations.

Further study of fraction IV

Since fraction IV represented a large component of the cell wall (26–42%; Scheme 1), its nature was further examined. It was found to exhibit a high dextrorotation in N-sodium hydroxide (which, moreover, was still constant after 5 days). For two samples $[\alpha]_D^{24}$ was +238° and +244° (concentration determined by weight, on portions of aqueous suspension dried at 80°) respectively. It is already known that the polysaccharide nigeran occurs in the mycelium of certains strains of *A. niger* (Barker, Bourne & Stacey, 1952, 1953; Barker, Bourne, O'Mant & Stacey, 1957; Dox & Neidig, 1914; Yuill, 1952), and the fraction was tested for its presence by boiling with water and filtering while hot. On cooling there appeared a flocculent white precipitate with a pellicle typical of nigeran (Yuill, 1952). The flocculent material was given two further treatments (boiling in water and filtration) in this way. It then showed $[\alpha]_D^{24}$ +281° (*c* 0·184 in N-

Table 4. *Analyses of fractions obtained from* A. niger *cell walls*

Molar ratios (to the nearest 0·1 mole) and relative proportions (scored from a chromatogram) are from preparations 8 and 9. Under percentage composition is included the range of values found for the particular fraction in several experiments (except where a single value is stated). Fraction VP is the portion of fraction V that is insoluble, and VS the portion (about 25% of the fraction) that is soluble, in 85% (v/v) ethanol. Percentage values are not corrected for the destruction of neutral or amino sugars and acid hydrolysis (approx. 5 and 12·5% respectively). +, Present but not estimated.

	Molar proportions				Relative proportions		Percentage composition		
Fraction	Mannose	Galactose	Glucose	Arabinose	Glucosamine	Galactosamine	Neutral carbohydrate	Protein	Hexosamine
I	1·0	0·8	6·4	0·5	+	Trace	51*–98	—	1·8
II	1·0	1·3	3·4	Trace	+	Trace	40*–76	—	0·9
III	1·0	3·0	14·0	Trace	+++++	+	60–75·4	Nil	18·5–23·6
IV	1·0	3·0	96·0	Trace	+	++	89·3–94	0–0·9	0·9–3·4
VP	1·0	6·0	4·0	Trace	Trace	+	61·3–87	6·4–7·0	1·2–1·6
VS	1·0	4·0	0·4	Trace	Trace	Trace	40–47	31·5	0·2

* These values are low owing to protein impurity extracted from a large batch (11g.) of walls.

sodium hydroxide; concentration estimated by hydrolysis to glucose; also checked by dry weight). Barker *et al.* (1952) give $[\alpha]_D + 283°$ for nigeran in N-sodium hydroxide. With acetic anhydride and pyridine (Wurzburg, 1964) it gave an acetate (95%) having $[\alpha]_D^{19} + 156°$ (*c* 0·49 in chloroform) (Found C, 50·3%; H, 5·9%; a triacetyl glucan requires C, 50·0%; H, 5·6%). Barker *et al.* (1952) give $[\alpha]_D^{17} + 157°$ for the triacetate of nigeran. The polysaccharide on partial acid hydrolysis (N-sulphuric acid for 1hr. at 99°) showed the presence, on paper chromatography with solvent *C*, of approximately equal quantities of nigerose and maltose, the latter giving the typical blue colour (shown also by authentic maltose) with aniline–diphenylamine (Schwimmer & Bevenue, 1956). On complete hydrolysis glucose was present as the major sugar; with heavier loadings (1·0–1·5mg.) traces of galactose and mannose, estimated to occupy together less than 0·5% of the total hexose, were seen.

To the fraction (57·2mg., estimated by hydrolysis to glucose) with $\alpha_D + 281°$, suspended in 25ml. of water, 40mM-sodium metaperiodate (25ml.) was added, and the mixture gently shaken in the dark at room temperature. At intervals, portions (5ml.) were removed, centrifuged if necessary (the material went into solution in about 30hr.), and 2ml. of the supernatant was titrated with 35mN-sodium thiosulphate, after the addition of potassium iodide and 2N-sulphuric acid. The uptake of metaperiodate, expressed as moles/mole of $C_6H_{10}O_5$, was as follows: 6hr., 0·35; 24·5hr., 0·52; 49·5hr., 0·52; 121hr., 0·52. For three experiments, with up to 113mg. of material, $0·51 \pm 0·01$ mole of periodate was consumed/mole of $C_6H_{10}O_5$. This is consistent with the presence of 51% of (1→4)-linkages [see Peat, Whelan, Turvey & Morgan (1961) on isolichenin]. The evidence taken together indicates the polysaccharide to be nigeran.

The residue of fraction IV (referred to as IVR), after repeated removal of material soluble in boiling water until the extracts gave a negative reaction in the phenol–sulphuric acid test (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), was still alkali-soluble and precipitable on neutralization. It had $[\alpha]_D^{22} + 231°$ (*c* 0·106 in N-sodium hydroxide) and contained glucose, galactose and mannose in the approximate proportions 97:2:1 [Wilson's (1959) method].

There is no evidence of any other component in fraction IV. From the values of optical rotation, the sample of fraction IV having $[\alpha]_D + 238°$ contained about 14% of nigeran, and that of $[\alpha]_D + 244°$ 26% of nigeran. When nigeran and fraction IVR were extracted from whole mycelium (the same batch as that giving rise to fraction IV of $[\alpha]_D + 244°$), nigeran constituted approx. 23% of the total yield of the two fractions.

DISCUSSION

Excluding galactose, all the sugars of the wall are of the D-configuration; D-arabinose occurs rarely in Nature but has been found in polysaccharides of *Nocardia asteroides* (Bishop & Blank, 1958) and *Mycobacterium tuberculosis* (Haworth, Kent & Stacey, 1948) as well as in the aloins (Gibson & Simonsen, 1930). Galactose in two preparations appears to contain about 10% of the L-sugar. The point has not been further studied.

Carbohydrate is the chief component (73–83%) of the wall, and together with hexosamine (9·1–13·1%) accounts for the major part of the cell wall. Phosphorus (0·02–0·06%) and protein (0·45–2·5%) are of minor significance. It was found that washing preparations with acid (pH 2·4; preparation 5) or alkaline (pH 10·0; preparation 6) fluids had no evident effect on wall composition. In particular this indicates protein contamination to be minimal and its presence in the preparations may be of structural significance. Both the hexosamine (9·1–9·9%) and neutral-carbohydrate (72–78%) contents of Braun preparations are slightly lower than the corresponding values for Mickle preparations (11·9–13·1 and 78–83% respectively). The higher total lipid of Braun preparations will partly explain this dilution, but the values for hexosamine are believed to vary with the age of the culture used (this has been discussed by Johnston, 1963). Although a significantly higher protein content does not accompany the excess of lipid of the Braun preparations, nucleic acid content is relatively higher. Probably cytoplasmic contamination is greater with this machine.

Since one cell-wall preparation (preparation 8, grown for 47hr. at 30°) yielded 30% whereas an older preparation (preparation 7, grown for 54hr. at 33°) yielded 42% of fraction IV, it evidently increases with the age of the culture. As it consists of more than 90% of glucose, it could account for the relative increase in glucose in the neutral-sugar portion of the cell wall (Table 2).

With regard to the hexosamine content of the final insoluble residue (fraction III, which contains most of the cell-wall hexosamine), it has been established that when the neutral carbohydrate of the fraction was decreased to 55% (by extraction with trichloroacetic acid, then N-sodium hydroxide) the hexosamine content rose to 34·4%, suggesting the presence of a resistant polyhexosamine, probably chitin. X-ray evidence has been obtained for the presence of chitin in the cell walls of *A. niger* (Khouvine, 1932) and of *Aspergillus oryzae*. The origin of the galactosamine residues is unknown, although a galactosamine polymer, in degraded form, has been isolated from the cell walls of *Neurospora crassa* (Harold, 1962), which might be

expected to be similar to those of *A. niger* (Crook & Johnston, 1962).

Horikoshi & Arima (1962) have provided evidence for the existence of a β-(1→3)-glucan in *A. oryzae* cell walls. Such a polymer, with a free reducing group, could account for the alkali-lability of fraction VP (Whistler & BeMiller, 1958).

The most important feature of the cell wall revealed by the present study is the presence of highly dextrorotatory fraction (IV), which has been separated into two further fractions, one with $[\alpha]_D + 281°$ and the other with $[\alpha]_D + 231°$ (both in N-sodium hydroxide). That of higher rotation resembles and is assumed to be nigeran, which has unequivocally been shown to be a linear glucan in which α-(1→3)-links alternate with α-(1→4)-links (Barker *et al.* 1952, 1953, 1957). Evidence in the next paper (Johnston, 1965) shows that the fraction of lower rotation consists predominantly of α-(1→3)-linked glucose units. It seems probable that nigeran is a cell-wall component. It has been shown (Dox & Neidig, 1914; Yuill, 1952) that the amount of nigeran (called 'mycodextran' by Dox & Neidig, 1914) per culture flask remains constant when the mycelium of *A. niger* is starved. The fact that nigeran cannot readily be used as a source of glucose during starvation suggests a structural rather than a reserve function for this polysaccharide. In a shaken (submerged) culture of *A. niger*, the cell wall is the most evident structural feature. For a given batch of mycelium the ratio of nigeran to fraction IVR (26:74, determined from optical rotations in N-sodium hydroxide) extracted from a cell-wall preparation was almost the same as that (23:77, estimated by weight) for the substances isolated from the whole mycelium. This suggests that nigeran is intimately associated with the cell-wall complex during the isolation procedure. Further, the residual fraction IVR and nigeran have several features in common. Both fractions contain a high proportion of α-(1→3)-linked D-glucose units, and both also contain small amounts of galactose and mannose. In the nigeran fraction these were present even after three 'recrystallizations' from hot water. Further, although fraction IVR was repeatedly extracted with boiling water (autoclaving) until the supernatant yielded no more nigeran, it still contained α-(1→4)-linkages (isolated as maltose; Johnston, 1965) although in much smaller amounts than in nigeran. These facts taken together suggest that fraction IVR and nigeran are fractions usually associated with one another, Finally, the fact that nigeran is soluble in hot water and that autoclaving in water (rather than using extremes of pH) suffices to release it from cell-wall preparations cannot be held to indicate that it is not a cell-wall component, since Peat, Whelan & Edwards (1961) were able to solubilize the cell-wall mannan of yeast by heating in an autoclave at pH 7·0. The isolated mannan is known to be freely soluble in water.

By taking into account the nigeran contents of the two samples of fraction IV studied, 14 and 26% respectively, and the proportion of the cell wall that these represented (30·3 and 26% respectively), this corresponds to the presence of about 4–6% of nigeran in these cell-wall preparations. Although in some strains of *A. niger* nigeran can occupy as much as 30% of the mycelial dry weight, it is absent completely from others (Yuill, 1952). The proportion may also vary, particularly in the young mycelium, with the age of the culture (see, e.g., Dox & Neidig, 1914).

The cell wall of *A. niger*, like those of other organisms, is clearly one of great complexity. Chitin, a glucan consisting largely of (1→3)-linked residues, and probably nigeran are present. The possibility of a β-(1→3)-glucan and a galactosamine polymer may also have to be considered. Because of the fact that all six sugars of the wall occur in all the fractions shown in Table 4, it is evident that the regions of 'homogeneity' are intimately associated with one another (they may nevertheless exist in different layers of the wall), possibly linked by bridging units of galactose, mannose, arabinose and even protein.

I thank Professor E. M. Crook for advice and encouragement during the greater part of this work, Mr D. J. Thomas for technical assistance, and the Central Research Fund of London University for a grant for equipment.

REFERENCES

Abrams, A. (1958). *J. biol. Chem.* **230**, 949.
Aronson, J. M. & Machlis, L. (1959). *Amer. J. Bot.* **46**, 292.
Barker, S. A., Bourne, E. J., O'Mant, D. M. & Stacey, M. (1957). *J. chem. Soc.* p. 2448.
Barker, S. A., Bourne, E. J. & Stacey, M. (1952). *Chem. & Ind.* p. 756.
Barker, S. A., Bourne, E. J. & Stacey, M. (1953). *J. chem. Soc.* p. 3084.
Barker, S. A., Foster, A. B., Siddiqui, I. R. & Stacey, M. (1958). *J. chem. Soc.* p. 2358.
Bartnicki-Garcia, S. & Nickerson, W. J. (1962*a*). *Biochim. biophys. Acta*, **58**, 102.
Bartnicki-Garcia, S. & Nickerson, W. J. (1962*b*). *Biochim. biophys. Acta*, **64**, 548.
Bartnicki-Garcia, S. & Nickerson, W. J. (1962*c*). *J. Bact.* **84**, 829.
Bartnicki-Garcia, S. & Nickerson, W. J. (1962*d*). *J. Bact.* **84**, 841.
Bergmann, F. & Segal, R. (1956). *Biochem. J.* **62**, 542.
Bishop, C. T. & Blank, F. (1958). *Canad. J. Microbiol.* **4**, 35.
Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
Chen, S. L. (1959). *Biochim. biophys. Acta*, **32**, 480.
Crook, E. M. & Johnston, I. R. (1962). *Biochem. J.* **83**, 325.

Crumpton, M. J. (1959). *Biochem. J.* **72**, 479.
Dox, A. W. & Neidig, R. E. (1914). *J. biol. Chem.* **18**, 167.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Easterby, D. G., Hough, L. & Jones, J. K. N. (1951). *J. chem. Soc.* p. 3416.
Foster, A. B. (1957). *Advanc. Carbohyd. Chem.* **12**, 81.
François, C., Marshall, R. D. & Neuberger, A. (1962). *Biochem. J.* **83**, 335.
Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
Gibson, C. S. & Simonsen, J. T. (1930). *J. chem. Soc.* p. 553.
Hamilton, P. B. & Knight, S. G. (1962). *Arch. Biochem. Biophys.* **99**, 282.
Harold, F. M. (1962). *Biochim. biophys. Acta*, **57**, 59.
Haworth, W. N., Kent, P. W. & Stacey, M. (1948). *J. chem. Soc.* p. 1211.
Horikoshi, K. & Arima, K. (1962). *Biochim. biophys. Acta*, **57**, 392.
Horikoshi, K. & Iida, S. (1964). *Biochim. biophys. Acta*, **83**, 197.
Johnston, I. R. (1963). *Biochem. J.* **86**, 254.
Johnston, I. R. (1965). *Biochem. J.* **96**, 659.
Jolles, Z. E. & Morgan, W. T. J. (1940). *Biochem. J.* **34**, 1183.
Khouvine, Y. (1932). *C.R. Acad. Sci., Paris*, **195**, 396.
Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
Parker, B. C., Preston, R. D. & Fogg, G. E. (1963). *Proc. Roy. Soc.* B, **158**, 435.
Peat, S., Whelan, W. J. & Edwards, T. E. (1961). *J. chem. Soc.* p. 29.
Peat, S., Whelan, W. J., Edwards, T. E. & Owen, O. (1958). *J. chem. Soc.* p. 586.
Peat, S., Whelan, W. J., Turvey, J. R. & Morgan, K. (1961). *J. chem. Soc.* p. 623.
Pigman, W. (1957). *The Carbohydrates*, p. 80. New York: Academic Press Inc.
Pregl, F. (1951). *Quantitative Organic Micro-analysis*, 5th English ed., p. 199. Revised by Grant, J. London: J. and A. Churchill Ltd.
Russell, D. W., Sturgeon, R. J. & Ward, V. (1964). *J. gen. Microbiol.* **36**, 289.
Schwimmer, S. & Bevenue, A. (1956). *Science*, **123**, 543.
Scott, D. & Hammer, F. E. (1962). *Analyt. Biochem.* **3**, 13.
Stoffyn, P. J. & Jeanloz, R. W. (1954). *Arch. Biochem. Biophys.* **52**, 373.
Sturgeon, R. (1964). *Biochem. J.* **92**, 60P.
Taufel, K. & Reiss, R. (1951). *Z. analyt. Chem.* **134**, 252.
Whistler, R. L. & BeMiller, J. M. (1958). *Advanc. Carbohyd. Chem.* **13**, 289.
Wilson, C. M. (1959). *Analyt. Chem.* **31**, 1199.
Wolfrom, M. L. (1930). *J. Amer. chem. Soc.* **52**, 2466.
Wurzburg, O. (1964). In *Methods in Carbohydrate Chemistry*, vol. 4, p. 286. Ed. by Whistler, R. L. & Wolfrom, M. L. London: Academic Press (Inc.) Ltd.
Yuill, J. L. (1952). *Chem. & Ind.* p. 755.