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# Relationship of the Major Constituents of the *Neurospora crassa* Cell Wall to Wild-Type and Colonial Morphology

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ABSTRACT

MAHADEVAN, P. R. (The Rockefeller Institute, New York, N.Y.), AND E. L. TATUM. Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. J. Bacteriol. **90**:1073-1081. 1965.—The relationship of cell wall to morphology in *Neurospora crassa* was studied by correlating the levels of structural polymers of the cell wall with wild-type and colonial morphology. The cell wall of *N. crassa* contains at least four major complexes: a peptide-polysaccharide complex; two glucose polymers, one of which was found to be a laminarinlike β-1,3-glucan; and, lastly, chitin. The levels of one or more of these structural polymers are consistently altered in single-gene mutants with colonial growth, and in sorbose-induced colonial growth. The proportions of these polymers, particularly of the peptide-polysaccharide complex and the β-1,3-glucan, appear to be important to morphology.

Earlier work (de Terra and Tatum, 1961, 1963) has indicated that the study of cell-wall structure and chemistry offers an interesting and useful approach to the understanding of colonial morphology in *Neurospora crassa*. Colonial growth in *Neurospora* is characterized by tightly restricted growth on solid medium, and growth in pellets in liquid, in contrast to the loose, spreading, filamentous growth of the wild type (de Terra and Tatum, 1963). Colonial growth can result from single-gene mutations (Lindegren and Lindegren, 1941) or can be induced by the addition of sorbose to the medium (Tatum, Barratt, and Cutter, 1949). Glucose and glucosamine are the principal basic constituents of the *Neurospora* cell wall, and together account for approximately 60% of the cell wall. Differences from the normal wild-type strain in the ratio of total glucosamine to glucose have been observed in the cell walls of the sorbose-induced colonial form of the wild type (de Terra and Tatum, 1961) and of some colonial mutants (de Terra and Tatum, 1963). These results strongly suggested that both the relative and absolute amounts of structural polymers of the cell wall might be correlated with the morphological characteristics of *Neurospora*.

For a closer examination of the relationships between morphology and alterations in these cell-wall constituents, detailed information on the nature and characteristics of the structural polymers and other components of the *Neurospora* cell wall is essential. This paper reports the results of studies in this direction on wild-type and on certain colonial mutants.

## MATERIALS AND METHODS

The different strains of *Neurospora* used for the present study (listed in Table 2) were routinely grown for 3 to 4 days at 25 or 30 C with constant aeration in 15 liters of minimal medium (Vogel, *unpublished data*) containing 2% sucrose. In certain instances, 4% sorbose was added to the minimal-2% sucrose medium. Each 15-liter culture was then filtered, and the mycelial mat was washed with water and then lyophilized. Cell walls were obtained from lyophilized material by the following procedure. The dry mycelial powder (1 g) was treated with 100 ml of an aqueous solution of 1% sodium dodecylsulfate. Considerable lysis of the mycelial mass took place as early as 3 hr, but usually the treatment was prolonged for 15 to 16 hr at 4 C with continous stirring. The sedimentable cell-wall preparation was washed with water several times, and then with increasing concentrations of ethyl alcohol-absolute alcohol, and dried at 37 C. By microscopic examination this material appeared to consist of clean cell walls free from cytoplasmic contamination (Fig. 1).

Identification and estimation of cell-wall components were carried out on acid hydrolysates. Sugars and amino sugars were released from the cell-wall preparation by hydrolysis with 3 N hycrochloric acid for 3 hr at 100 C. The hydrolysate was dried in vacuo over potassium hydroxide pellets in a desiccator. The suspended material was

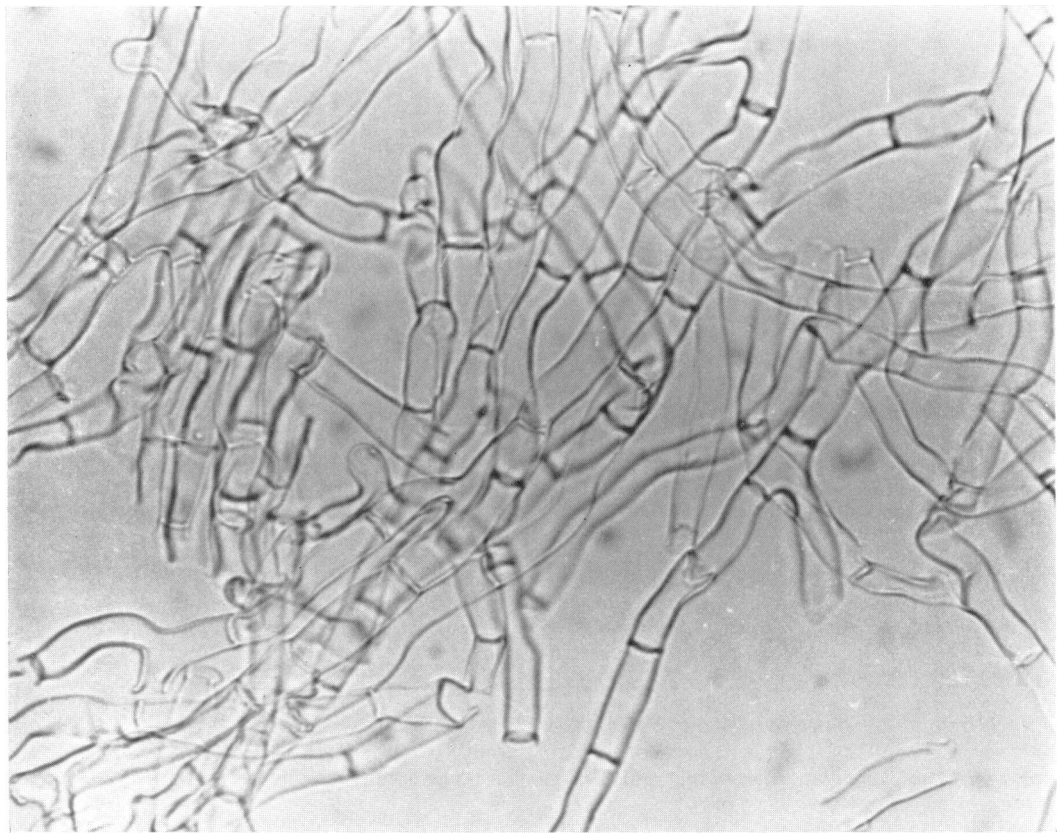

FIG. 1. *Cell wall of Neurospora crassa wild type RL-3-8A.* × *2,000.*

centrifuged, and the supernatant solution was analyzed for total hexoses and hexosamines. The cell-wall preparation was also hydrolyzed with 4 N sulfuric acid at 100 C for 4 hr, and total glucose was estimated after neutralization of the solution with concentrated barium hydroxide and removal of barium sulfate by centrifugation.

For the isolation of various cell-wall polymers and fractions, a stepwise degradation of the cell wall was developed by use of 2 N sodium hydroxide and 1 N sulfuric acid followed by precipitation of cell-wall components from the alkali extracts with ethyl alcohol or ammonium sulfate. This procedure is detailed in Fig. 2. The various fractions of the cell wall thus obtained were lyophilized, and were characterized by identifying their constituents after hydrolysis. The total hexose content of the acid hydrolysates was estimated by means of the diphenylamine reaction of Ashwell (1957), and the total hexosamine content, by the indole reaction of Dische and Borenfreund (1950). Total glucose was estimated by use of the Glucostat (Worthington Biochemical Corp., Freehold, N.J.) reagent (Meyers, Prindle, and Reynolds, 1960). Glucosamine and galactosamine were estimated by the method of Rondle and Morgan (1955), and acetylglucosamine, by the method of Tracey (1955).

Enzymatic digestion of cell wall and the various fractions of cell wall were performed with snail digestive juice (Industrie Biologique Française, Gennevilliers, Seine, France), Pancreatin (Mann Research Laboratories, New York, N.Y.), emulsin (Nutritional Biochemicals Corp., Cleveland, Ohio), and *Aspergillus niger* crude cellulase (Mann Chemical Co., New York, N.Y.). A 1-ml amount of snail digestive juice was diluted with 9 ml of 0.1 M citrate-phosphate buffer (*p*H 5.0), and was incubated with cell wall or cell-wall fractions at 37 C for 24 to 72 hr. Components released by enzyme digestion were identified colorimetrically and by paper chromatography. Pancreatin, emulsin, and cellulase were prepared in 0.1 M citrate-phosphate buffer (*p*H 5.0), and the cell-wall fractions were incubated for various periods at 37 C with these enzyme preparations. The amount of glucose released was estimated with Glucostat reagent. Laminarinase ($\beta$-1,3-glucanase) was obtained from a crude culture filtrate of *Streptomyces* C-3, according to the method suggested by J. J. Skujins of Cornell University (*personal communication*). This enzyme complex was incubated with cell wall

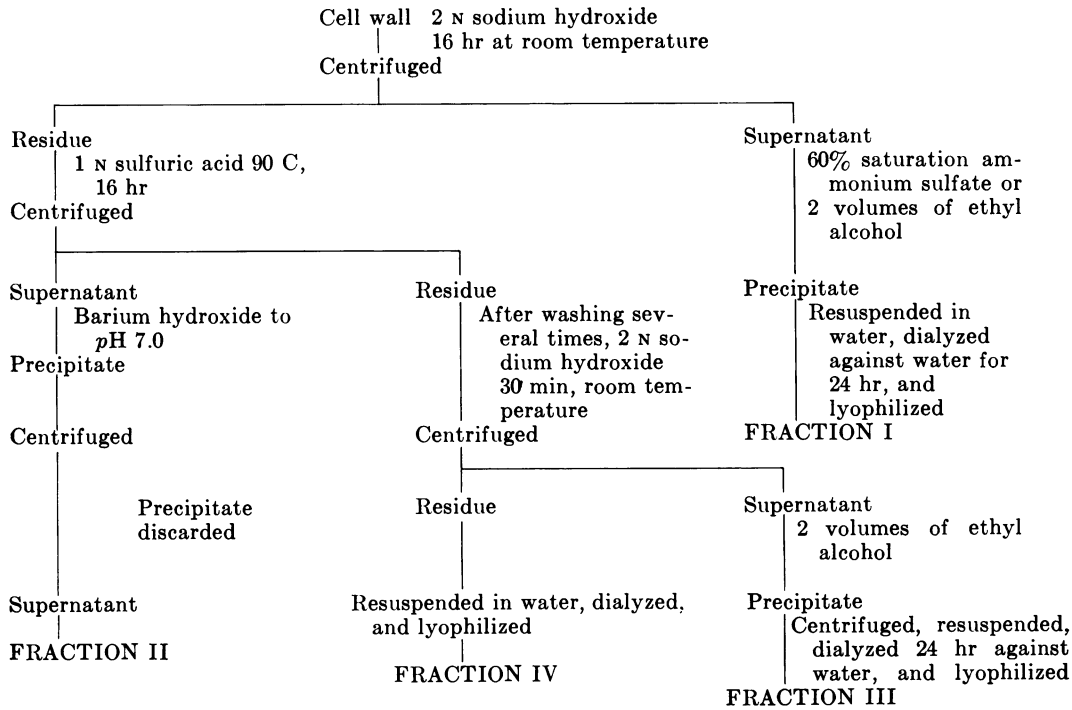


FIG. 2. *Fractionation procedure used to obtain four major components from the cell wall of Neurospora crassa.*

and cell-wall fractions in 0.1 M phosphate buffer (*p*H 5.8) at 37 C for various periods. The glucose and total carbohydrate in the enzyme digest were estimated by use of Glucostat and anthrone reagents (Trevelyan and Harrison, 1952), respectively.

Sugars and amino sugars from acid and enzymatic hydrolysates were separated by paper chromatography with Whatman no. 4 paper and a solvent system of ethyl acetate-pyridine-water (12:5:4). The substances were located on the paper by dipping it into alkaline silver nitrate (Smith, 1960). Amino sugars were also separated on paper by use of butanol-acetic acid-water (12:3:5), and were identified by spraying with ninhydrin reagent (Sigma Chemical Co., St. Louis, Mo.). Other ninhydrin-positive materials were also separated in the same chromatographic system.

Microanalyses for ash, C, H, N, and P were done by S. T. Bella of The Rockefeller Institute.

## RESULTS

*Fractionation of cell wall.* Preliminary experiments indicated that some glucose and hexosamine could be removed from the cell wall by treatment with 2 N sodium hydroxide. A precipitate was obtained by addition of 2 volumes of ethyl alcohol to the alkali extract. An acid hydrolysate of this extract contained no acetylglucosamine, but only hexosamine. Based upon this observation, the method of fractionation mentioned earlier was developed. This method, although not completely satisfactory, permits the separation of the cell wall into four principal fractions, one of which represents a major glucose-containing and another a major glucosamine-containing polymer of *Neurospora* cell wall.

*Fraction I.* The major constituent of the first alkali-soluble fraction of the cell wall can be precipitated either by alcohol or by ammonium sulfate (60% saturation). Both the total weight and the products of hydrolysis of the isolated fraction, obtained by either method, were the same. Hydolysis of the precipitate with 3 N hydrochloric acid released glucose, galactosamine, and small amounts of glucuronic acid and glucuronolactone. Hydrolysis with 6 N hydrochloric acid released some amino acids. These components were separated and identified by paper and column chromatography. The amino acids identified are glycine, alanine, leucine, isoleucine, valine, aspartic acid, and glutamic acid.

Harold (1962) isolated a galactosamine polymer from *Neurospora* cell wall and showed that it was the site of polyphosphate binding. Using his procedure, we have been able to isolate the same type of polymer from the cell wall of *Neurospora*. Such a polymer was isolated both from the cell wall

TABLE 1. *Microchemical data on the cell wall and various fractions from the cell wall of Neurospora crassa wild type (RL-3-8A)**

| Analysis for | Cell wall | Fraction I | Fraction III | Theoretical for glucan | Fraction IV | Theoretical for chitin |
|---|---|---|---|---|---|---|
| | % | % | % | % | % | % |
| C | 44.10 | 42.88 | 44.07 | 44.4 | 45.6 | 43.8 |
| H | 6.41 | 6.34 | 6.37 | 6.17 | 6.36 | 5.44 |
| N | 2.06 | 2.03 | 0.16 | | 5.67 | 6.4 |
| P | 0.31 | 0.18 | 0.0 | | 0.0 | |

* Values are corrected for ash content and moisture.

and from fraction I; in fact, all of the galactosamine of the cell wall has been found in fraction I. The relationship of this polymer to the other components of this fraction is not clear. However, estimation of galactosamine has shown that this polymer represents only 1 to 2% of the cell wall. The glucose content of fraction I represents about 50% of the fraction. Studies on the amino acids and other minor components, and on the homogeneity of fraction I, are being continued.

*Fraction II.* The second major fraction was the portion of the cell wall that was soluble in 1 N sulfuric acid after the first 2 N alkali treatment (Fig. 2). The free glucose in this fraction was estimated by use of the Glucostat reagent. Paper chromatographic separation of this fraction showed other minor components, which were tentatively identified as mannose, mannitol, and glucosamine. The glucosamine may in part have come from chitin. (See discussion of fraction IV.)

*Fraction III.* The second sodium hydroxide (2 N)-soluble portion of the cell wall was precipitated by the addition of 2 volumes of ethyl alcohol. This precipitate was insoluble in water and, on hydrolysis with 3 N hydrochloric acid or 4 N sulfuric acid, showed only glucose. Hydrolysis of 1 mg of this fraction with 3 N hydrochloric acid for 1 hr released 1.08 mg of glucose.

Microanalysis of fraction III gave values for C and H (Table 1) similar to those of a polysaccharide. It also showed a N content of 0.16%. The nature of this N is not known.

No glucose was released from this fraction by treatment with Pancreatin (mixture of amylase and β-glucosidase) or emulsin. The fraction is indicated as a long-chain polymer by its retention in dialysis tubing. Since amylase released no glucose, fraction III appears not to contain α-1,4 bonds. Crude cellulase from *A. niger* released glucose from fraction III, but did not act on methylcellulose. Purified laminarin (obtained from the Institute of Seaweed Research, Midlothian, Scotland) was hydrolyzed by this cellulase, and glucose was released, indicating a β-1,3-glucanase activity. This crude enzyme released 80 to 90% of the glucose from fraction III, and for this reason might be useful if further purified.

A crude culture filtrate of *Streptomyces* C-3 contains a lytic enzyme complex which released glucose from laminarin (β-1,3-glucan), but not from starch or cellulose. Such a crude extract released glucose from both whole cell wall and from fraction III. A more purified preparation of this enzyme complex (see Materials and Methods), on incubation with *Neurospora* cell wall or fraction III, released glucose and oligosaccharides, as evidenced by paper chromatography. The types of oligosaccharides produced from the cell wall and from fraction III by this enzyme complex appear to be similar in their mobility on paper. Incubation of cell wall with the enzyme complex for 72 hr showed that 30% of the cell wall can be liberated as carbohydrate by this enzyme. Glucose released under these conditions has been estimated to be 11.5% of the cell wall. The rest of the carbohydrates appear as oligosaccharides and short-chain polymers, as indicated by their disappearance after acid hydrolysis.

Incubation of 3 mg of fraction III for 48 hr

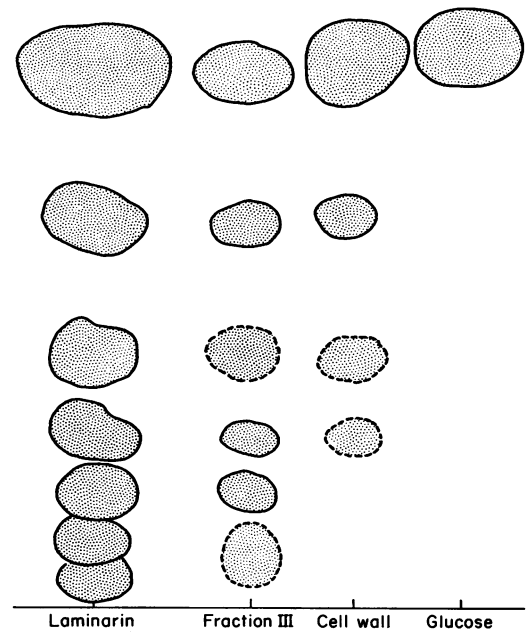


FIG. 3. *Glucose and oligosaccharides from laminarin, fraction III, and cell wall obtained after hydrolysis with 0.5 N HCl.*

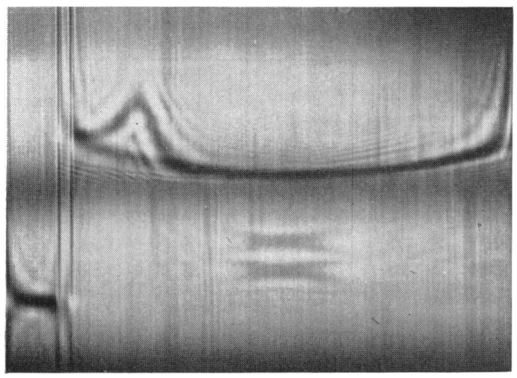

FIG. 4. *Sedimentation pattern of fraction III, in 1 N sodium hydroxide, after centrifugation for 34 min at 56,000 rev/min.*

with *Streptomyces* C-3 lytic complex released 1 mg of glucose and 1.4 mg of oligosaccharides, leaving some unhydrolyzed residue. This is nearly 80% recovery of the fraction as glucose. Lastly, 0.5 N hydrochloric acid hydrolysis of laminarin at 100 C for 20 min released oligosaccharides very similar to those of fraction III and of *Neurospora* cell wall hydrolyzed under the same conditions (Fig. 3). These results indicate that fraction III has $\beta$-1,3 linkages. Skujins and Potgieter (1964) recently showed that the crude extract of *Streptomyces* C-3 contained three $\beta$-1,3-glucanases and chitinase. They concluded that the cell wall of *Neurospora* contains a $\beta$-1,3-linked glucose polymer, based upon the susceptibility of the cell wall to the enzyme complex of *Streptomyces* C-3; however, they noted that all of the glucose from the cell wall was not removed by this enzyme complex. Our results point in the same direction, as indicated by enzymatic release of only 30% of cell wall as carbohydrate, whereas the cell wall has been estimated to contain 45 to 50% glucose (our results and de Terra and Tatum, 1963).

The sedimentation pattern of fraction III was examined in the ultracentrifuge. As shown in Fig. 4 the fraction appears homogeneous.

*Fraction IV.* The last fraction was that portion of the cell wall resistant to 2 N sodium hydroxide and 1 N sulphuric acid (Fig. 2). This fraction, referred to as IV, on hydrolysis with 6 N hydrochloric acid for 1 hr at 100 C released only glucosamine. Microanalysis of this fraction (Table 1) showed that it had a ratio of C, H, and N quite similar to that of the theoretical values for chitin. Digestion of this fraction with snail enzyme complex released only acetylglucosamine. In such an experiment, 85% of the fraction was obtained as acetylglucosamine in 72 hr of incubation. Snail enzyme complex is known to contain chitinase, carbohydrases, and proteases (Holden and Tracey, 1950). In another experiment, digestion of 2 mg of fraction IV with chitinase (Nutritional Biochemicals Corp.) released 2.1 mg of acetylglucosamine after 48 hr of incubation at 37 C in 0.1 M citrate-phosphate buffer (*p*H 5.0).

Control experiments indicated that separate treatment of cell wall with 2 N sodium hydroxide and 1 N sulfuric acid deacetylated some cell-wall chitin. These experiments were done by treating cell wall with alkali or acid and then digesting the residual material with snail enzyme for 24 hr at 37 C in 0.1 M citrate-phosphate buffer (*p*H 5.0). The amount of acetylglucosamine released by digestion of 10 mg of untreated cell wall was 1.02 mg. This compares with acetylglucosamine values of 0.78 and 0.72 mg recovered from 10 mg of cell wall treated with alkali and acid, respectively. It would appear, therefore, that the amounts of fraction IV actually isolated by the procedure of Fig. 2 may be lower than the true values.

*Cell-wall composition and morphology.* The second major aspect of the studies on the cell wall deals with the estimation of the amounts of the four principal components of the cell wall in several colonial mutants and in sorbose-induced colonial growth of wild-type *Neurospora crassa.* The mutants were B233, B28, B54, and B110, all colonial in growth except B110 which is considered as semicolonial (de Terra and Tatum 1963). These mutants have no nutritional deficiency. Others were the temperature sensitive mutants, R1006t and C102t (*cot*). These are colonial at a higher temperature (34 C), but appear similar to the wild type when grown at a lower temperature (25 C). The wild-type strains RL-3-8A and Perkins A were also grown in 4% sorbose medium, and cell-wall preparations were examined. The mutant *patch* (21863a), which was shown to be somewhat resistant to the sorbose effect (Stadler, 1959), was grown in the presence and absence of sorbose, and the cell-wall preparations were studied. Another mutant which requires glutamine (E. Reich, *personal communication*) was examined for its chitin level when grown in media containing minimal-2% sucrose and various amounts of glutamine. This strain was grown for 20 hr, and then the cell wall was prepared as described above.

The amounts of fraction I, of glucose in fraction II, and the total amount of fractions III and IV (see Fig. 2) in the various strains under different culture conditions are shown in Table 2.

The relative amounts of these fractions were determined and reported as per cent dry weight of cell wall, except for glucose in fraction II. The total amount recovered as fractions from most of

TABLE 2. *Amounts of cell-wall fractions isolated from wild-type and colonial mutants of Neurospora crassa*[a]

| Strain | Morphology | Wild-type background | Cell wall[b] | Fraction I[c] | Fraction II[d] | Fraction III[c] | Fraction IV[c] | Ratio[e] of glucosamine-glucose |
|---|---|---|---|---|---|---|---|---|
| RL-3-8A | Normal | Lindegren | 14.0 | 13.8 | 11.8 | 20.2 | 9.4 | 0.12(0.13) |
| Perkins A | Normal | St. Lawrence | 16.0 | 11.8 | 13.2 | 16.2 | 6.3 | 0.11(0.11) |
| RL-3-8A (34C) | Normal | | 13.5 | 16.0 | 9.0 | 15.0 | 11.5 | |
| RL-3-8A (sorbose) | Colonial | | 15.7 | 23.6 | 13.7 | 6.8 | 9.1 | 0.19(0.32) |
| Perkins A (sorbose) | Colonial | | 12.0 | 20.8 | 12.5 | 15.8 | 5.8 | 0.18 |
| B223 | Colonial | Perkins | 21.0 | 8.5 | 15.6 | 30.0 | 10.3 | 0.19 |
| B233 (sorbose) | Colonial | Perkins | 19.5 | 7.0 | 16.5 | 26.0 | 15.5 | |
| B54 | Colonial | Perkins | 19.0 | 24.0 | 15.5 | 18.0 | 7.8 | 0.27(0.11) |
| B28 | Colonial | Perkins | 17.0 | 14.0 | 13.5 | 24.3 | 7.5 | 0.23(0.24) |
| B110 | Semicolonial | Perkins | 21.0 | 13.5 | 16.0 | 22.3 | 9.8 | 0.19(0.12) |
| R1006t (25 C) | Normal | RL-3-8A | 15.0 | 20.5 | 13.0 | 11.3 | 7.1 | 0.12 |
| R1006t (34 C) | Colonial | RL-3-8A | 12.8 | 23.8 | 16.3 | 5.5 | 10.5 | 0.16 |
| R1006t (25 C, sorbose) | Colonial | RL-3-8A | 8.0 | 29.8 | 9.9 | 14.8 | 7.3 | |
| C102t (25 C) | Normal | RL-3-8A | 14.0 | 16.5 | 17.3 | 18.5 | 6.5 | 0.16 |
| C102t (34 C) | Colonial | RL-3-8A | 10.0 | 14.5 | 13.9 | 16.5 | 9.5 | 0.21 |
| C102t (25 C, sorbose) | Colonial | RL-3-8A | 20.0 | 29.5 | 11.3 | 11.5 | 10.0 | |
| Patch | Normal | St. Lawrence | 6.0 | 11.3 | 21.6 | 8.8 | 5.0 | |
| Patch (sorbose) | Normal | St. Lawrence | 4.3 | 10.8 | 21.2 | 7.5 | 5.5 | |

[a] Values are averages of at least two determinations.
[b] Percentage of lyophilized mycelium.
[c] Percentage of cell wall.
[d] Glucose only, milligrams per 100 mg of cell wall.
[e] Figures in parentheses are from de Terra and Tatum (1961, 1963).

TABLE 3. *Effect of glutamine level on cell-wall composition of glutamine mutant*

| Strain | Glutamine added | Fraction I* | Fraction II† | Fraction III* | Fraction IV* | Glucosamine in fraction II* | Acetyl glucosamine in cell wall‡ |
|---|---|---|---|---|---|---|---|
| | *mg/liter* | | | | | | |
| Wild type RL-3-8A | None | 13.8 | 11.8 | 20.2 | 9.4 | 1.0 | 14.4 |
| Glutamine mutant | 100 | 16.0 | 13.8 | 21.0 | 6.0 | 0.5 | 7.5 |
| | 300 | 17.0 | — | 22.3 | 6.0 | — | — |
| | 500 | 18.5 | — | 20.3 | 7.5 | — | — |

* Percentage of cell wall.
† Glucose only, milligrams per 100 mg of cell wall.
‡ Released by snail enzyme. Percentage of cell wall.

the strains was 55 to 60% of the dry weight of the cell wall. Recently, when using other methods, a higher recovery has been obtained. Comparisons between strains have been made on the amounts of these fractions obtained with the same fractionation procedure. The amounts of these fractions as percentage of total recovery showed no great discrepancy from the results presented as dry weight of cell wall.

The levels of the four fractions in the cell wall of the two wild types are quite similar. This is also true of the ratio of glucosamine to glucose. Each colonial mutant is compared with its respective wild type (Table 2).

The cell walls isolated from the wild-type strains RL-3-8A and Perkins A, when they were grown in 4% sorbose, are referred to in this paper as RLS and PAS, respectively. The data (Table 2) show clearly that the amounts of some fractions of the cell walls from the strains grown with and without sorbose were different. However, the two wild types showed some differences in the effects of sorbose. Whereas the levels of chitin were the same with or without sorbose in both wild types, fraction I was increased almost twofold in the presence of sorbose. The level of glucan (fraction III) was reduced to one-third in RLS, whereas it remained the same in PAS. Thus,

the common effect of sorbose in the two wild types was an increase in fraction I. The morphology of both wild types was colonial in the presence of sorbose.

The colonial mutant B233 showed considerable alterations in the amounts of most of the fractions of the cell wall. Compared with its wild type, Perkins A, this mutant had only 72% of fraction I, a twofold increase in fraction III, and a slight increase in chitin. Sorbose added to the medium for B233 did not alter the levels of the fractions further, except for an increase in chitin. B28 also showed an increase in fraction III without significant changes in other fractions. The mutant B54 showed an increase only in fraction I, and other fractions were quantitively the same as in the wild type. The colonial temperature-sensitive mutant R1006t, when grown at 34 C, showed a decrease in the amounts of glucan and an increase in chitin in comparison with the amounts found when it was grown at 25 C. When the same mutant was grown colonially in the presence of sorbose at 25 C, the component altered was fraction I, the amount of which increased. The other colonial temperature mutant, C102t, showed only a slight increase in chitin level when grown colonially at 34 C, but a greater increase in the level of fraction I when grown colonially at 25 C in the presence of 4% sorbose.

The results of the analysis of the cell wall from the mutant *patch* were interesting. This mutant, which has the property of "escaping" from the sorbose effect (up to a level of 10%), was grown with and without sorbose, and the cell walls obtained were analyzed. The data (Table 2) show that the cell-wall composition of this mutant is not significantly altered by sorbose.

Glutamine is the specific amino group donor in the biosynthesis of glucosamine, which is then acetylated and eventually incorporated into chitin (Leloir and Cardini, 1953). The glutamine mutant, when grown on different levels of glutamine, provided a potential method of altering the amount of chitin in *Neurospora* cell wall, and thus the possibility of examining the role of chitin in morphology. The minimal level of glutamine needed for the growth of the mutant was found to be 100 mg per liter of medium. With amounts lower than this, the growth was poor, but not colonial. The results presented in Table 3 show very little change in the levels of chitin in the cell wall when the glutamine levels in the medium varied fivefold. A concentration of 500 mg of glutamine per liter was enough to result in a rate of growth and morphology of the mutant similar to those of the wild type. At all three levels studied, however, the amount of chitin was altered very little. The level of glucosamine in fraction II of the mutant (grown in 100 mg per liter) was 0.48% and of the wild type, 1% of the cell wall (Table 3). Furthermore, the acetylglucosamine released from 10 mg of cell wall of the wild type by snail enzyme digest was 1.44 mg, and from the glutamine mutant it was 0.754 mg. These data clearly indicate that the level of chitin and glucosamine in the cell wall of the glutamine mutant is lower than the wild type. However, the chitin level in the mutant cannot be increased by higher levels of glutamine in the medium.

## DISCUSSION

Chitin and glucan have been identified in the cell walls of fungi, such as phycomycetes (Frey, 1950; Aronson and Machlis, 1959; Fuller and Barshad, 1960), and yeast (Roelofsen and Hoette, 1951). Chitin and glucan have also been found in the cell walls of *Aspergillus* (Horikoshi and Arima, 1962). These studies involved microchemical localization, X-ray diffraction of the acid- and alkali-resistant portion of the cell wall, and enzymatic hydrolysis of isolated polysaccharides. In *Neurospora*, the presence of chitin was shown by Blumenthal and Roseman (1957) when they estimated total glucosamine in mycelium. They found chitin to an extent of 2.4% of the mycelium. Crook and Johnston (1962), while studying the cell-wall components of several fungi, found glucose, glucosamine, and amino acids in *N. sitophila*. de Terra and Tatum (1963), besides identifying glucose and glucosamine in the cell walls of *Neurospora*, mentioned the possibility of two nonpolysaccharide components, ash and protein, amounting to 14% of the cell wall.

Our results have shown that, by the fractionation procedure outlined in Fig. 2, it is possible to isolate chitin (fraction IV) from the cell wall of *Neurospora*. The amount of chitin isolated was 6 to 7% and 9 to 10% of the cell wall in the wild types Perkins A and RL-3-8A, respectively. This value is similar to that reported by Potgieter and Alexander (1965).

The other polysaccharide isolated by the present method is the glucan (fraction III). The microanalysis of this fraction showed values characteristic of a polysaccharide, and the polymer yielded only glucose on hydrolysis. The nondialyzable nature and resistance to attack by pancreatic amylase indicate that it is a long-chain polymer not containing $\alpha$-1,4 bonds. The susceptibility to the *Streptomyces*-C3 $\beta$-1,3 glucanase complex suggests that this polymer has $\beta$-1,3 linkages, and its relative purity is shown by its homogeneity on ultracentrifugation. Fraction III appears to represent the laminarinlike polysaccharide reported in *Neurospora* cell wall by Skujins and Potgieter (1964) and Potgieter and Alex-

ander (1965). The similarity of oligosaccharides produced by enzymatic and acid hydrolyses of fraction III to those from laminarin further supports this conclusion.

Fraction I has not yet been fully characterized. It appears to be a complex of glucose, glucuronic acid and galactosamine, along with some amino acids. Our preliminary results indicate that the fraction is not homogeneous; it contains at least a polypeptide or protein portion, a glucosan, and a galactosamine polymer. All of the galactosamine of the cell wall is present in fraction I and appears to be present as the galactosamine polymer described by Harold (1962). The relationships of these various components of fraction I are not fully understood.

Fraction II appears to contain only simple sugars and sugar derivatives, including glucose, mannose, mannitol, and glucosamine, which are present as monomers only in this fraction. Possibly they were derived from polymers of the cell wall by the alkali and acid treatment.

The comparative studies of the levels of the four major cell-wall fractions, summarized in Table 3, provide some suggestions as to the significance of the various components in relation to morphological differentiation in *Neurospora*. As was noted by de Terra and Tatum (1963), the ratio of total glucosamine to glucose was higher in colonial mutants as compared with their wild types. The amount of cell wall per unit weight of lyophilized mycelia in the strains studied varied from 10 to 20%. The mutant *patch* showed much less cell wall than other strains.

Generally considered, there appear to be three principal changes in the cell-wall components of the colonial mutants as compared with their respective wild types: (i) an alteration in the amount of the glucan, (ii) alteration in the amount of fraction I, and (iii) an increase in the amount of chitin. The first change involves either an increase or a decrease of the glucan, increases being shown in B233, B110, and B28, and a decrease being shown in R1006t at 34 C. Change in fraction I in the cell walls of the colonials, such as B54, R1006t, and C102t in sorbose at 25 C, generally involved an increase as with the wild types grown with sorbose. The mutant B233 is the only one that showed a decrease in fraction I. Lastly, chitin levels tend to increase slightly in most of the colonial mutants but not in B54, B28, and the sorbose colonials of the wild types. Quantitatively it has been shown by this study that (i) the levels of chitin vary within a narrow range of 5 to 10% of the cell wall among strains, and (ii) the other polymer complexes (fractions I and III) vary from 8 to 30% and 5 to 35%, respectively.

It would be important to determine which of these changes in the major cell-wall component is most significant in colonial morphology. Some colonial mutants show no significant changes in chitin level compared with their normal wild-type strains. Sorbose-induced colonial growth of wild type, and of R1006t at 25 C, is not associated with alterations in chitin level. Interestingly, chitin in B233, and in C102t in sorbose at 25 C, shows increased levels. The two temperature-sensitive mutants have higher chitin levels at 34 C than at 25 C. In this connection, it should be pointed out that although the level of chitin in the glutamine mutant is not affected by the amount of glutamine supplied, the chitin level is somewhat less than in the corresponding wild type. In spite of this lower level, growth was normal in all cultures tested. Insofar as the role of chitin is concerned, there is no apparent consistent relationship between chitin level and morphology.

The most significant change associated with development of colonial morphology would appear to involve fraction I. Both wild-type strains showed appreciable changes in this fraction, an increase of nearly 100%, in sorbose-induced colonial growth. In RLS there was a decrease in fraction III, but this was not true of PAS. R1006t and C102t grown at 25 C with sorbose showed an increased amount of fraction I. In all these cases sorbose seems to increase the levels of fraction I.

In this connection, it is of interest that *patch*, which shows a resistance to the effects of sorbose, showed no morphological response to sorbose under the conditions of culture employed here. It likewise showed no changes in any of the fractions of the cell wall in the presence of sorbose. The sorbose resistance of *patch* may be related to the insensitivity of fraction I to alteration, rather than to the inherently high level of fraction II or the lower level of III.

Although only two temperature-sensitive mutants have been examined, it is interesting that both differ from the wild type in their cell-wall composition, even when grown at 25 C, where their morphology is relatively normal. R1006t has an elevated level of fraction I, a decreased III, and an unchanged IV. C102 has I and II somewhat elevated, with little change in III and IV. Colonial growth at 34 C is associated in R1006t with a slight increase in I and IV and a considerable decrease in III. C102t at 34 C shows a slight decrease in I, II, and III, with an increase in IV. Thus, the temperature effects on morphology have in common decreased levels of III and an increase in IV.

In conclusion, the estimation of the four major fractions of the cell wall from several single-gene mutants of *Neurospora* with colonial morphology

showed that colonial growth is associated with changes in the levels of one or more of these structural polymers of the cell wall as compared with their respective wild-type strains. The consistent increase in fraction I in the wild types and in two colonial temperature-sensitive strains in the presence of sorbose clearly indicates that sorbose alters the level of this fraction. In the mutant *patch*, the presence of sorbose does not significantly alter the level of any of the fractions, and growth is not changed. These facts lead to the conclusion that cell-wall composition is closely associated with morphology in *Neurospora*, that the change from wild-type growth to colonial growth may be due to changes in the relative levels of some of the structural components of the cell wall, and that fraction I is one of the most critical constituents. The genetic control of cell-wall biochemistry in additional single colonial mutants is being studied further.

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