LOCALIZATION OF STRUCTURAL POLYMERS IN THE CELL WALL OF *NEUROSPORA CRASSA*

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ABSTRACT

The distribution and localization of structural polymers in the cell wall of *Neurospora crassa* has been studied by selective removal and light and electron microscope examination. Observations with the light microscope indicated that each polymer by itself can provide structural integrity to the cell wall. Examination by electron microscopy showed that the cell wall consists of an outer layer of thick fibrils, identified chemically as a glucan-peptide-galactosamine complex, and an inner layer made up of β-1,3 glucan and thin fibrils of chitin.

INTRODUCTION

That there is a relationship of cell wall structure and chemistry to colonial morphology and wild type growth in *Neurospora crassa* seems clear from the results of de Terra and Tatum (1963) and Mahadevan and Tatum (1965). Four major fractions of the cell wall were obtained, of which two homogeneous fractions were characterized as β-1,3 glucan and chitin. A third fraction was identified as a glucan-peptide-galactosamine complex, the carbohydrate portion of which contained mainly glucose. Glucan and chitin have also been identified as major constituents of *Neurospora* cell wall by Skujins and Potgieter (1964), and Potgieter and Alexander (1965).

The localization of these polymers in the cell wall may be important in morphology. For example, the extent of branching in *Neurospora* hyphae might be influenced by the amounts, quality, and location of the structural polymers in the lateral cell wall. Earlier studies have shown that significant alterations in the levels of structural polymers, glucan, chitin, and the glucan-peptide complex, accompany increased branching in colonial mutants (Mahadevan and Tatum, 1965).

This paper presents observations on the structure of the wild type cell wall after selective removal of one or more of the structural polymers from the cell wall, as examined by light and electron microscopy.

MATERIALS AND METHODS

The strains of *Neurospora crassa* used for the present studies were the wild types RL-3-8A and Perkins A. The strains were grown and the cell wall prepared essentially as described earlier (Mahadevan and Tatum, 1965). The cell wall was degraded by treating with chitinase (Nutritional Biochemicals Corporation) at a concentration of 2 mg/ml at pH 5.0 and also with a crude preparation of *Streptomyces C-3* filtrate containing β-1,3 glucanases. Different fractions of the cell wall were prepared by the chemical methods of Mahadevan and Tatum (1965). Glucose was estimated with the glucostat reagent (Worthington Biochemical Corporation), and glucosamine and acetylglucosamine by the methods of Rondle and Morgan (1955) and Tracey (1955), respectively. Total carbohydrates were estimated with the anthrone reagent (Trevelyn and Harrison, 1952) and protein by the biuret method (Gornall et al., 1949).

For electron microscope studies, freshly prepared

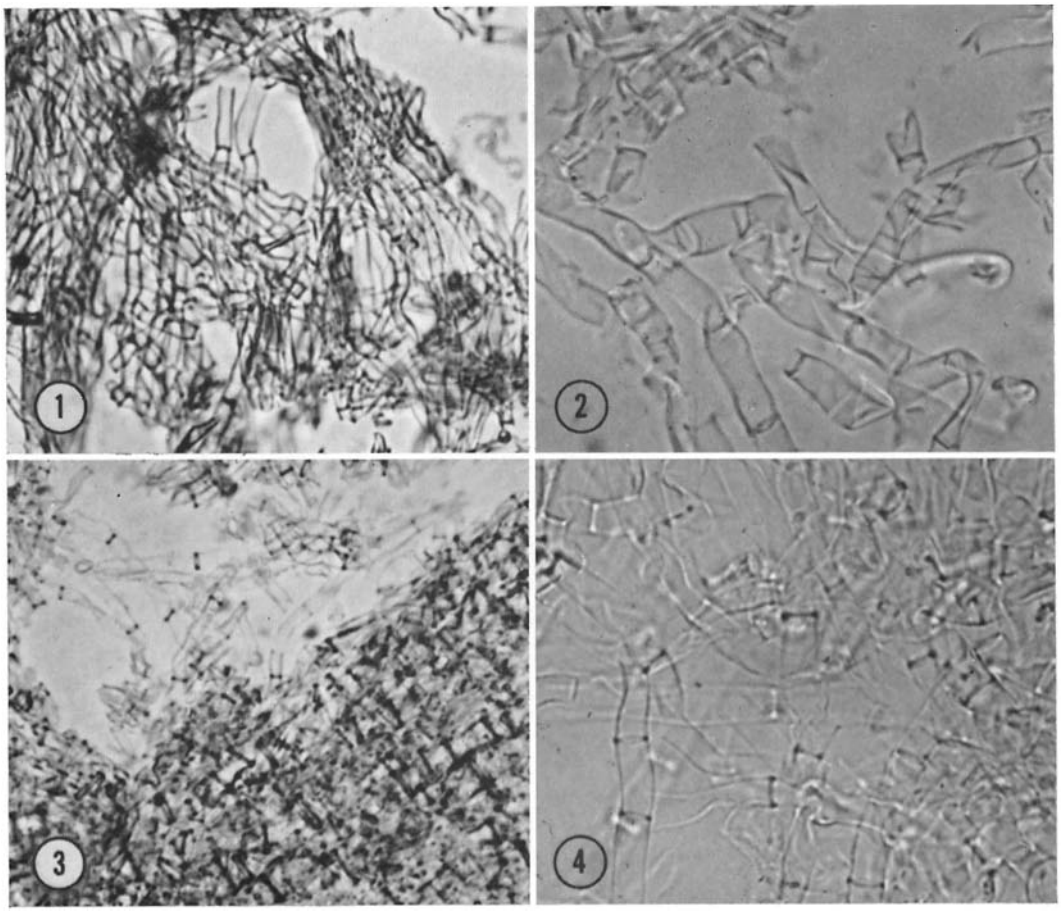

FIGURE 1 Cell wall preparation of *N. crassa* RL-3-8A. × 800.

FIGURE 2 RL cell wall treated with 2N NaOH. × 1600.

FIGURE 3 RL cell wall treated with *Streptomyces* enzyme. × 800.

FIGURE 4 RL cell wall treated with chitinase and *Streptomyces* enzyme. × 1600.

cell wall or cell wall fraction was fixed in 1% osmium tetroxide in acetate-Veronal buffer, pH 6.1 (Kellenberger et al., 1958), and embedded in Epon 812 (Luft, 1961). Sections were stained with uranyl nitrate for 10–30 min. Shadowed preparations were made with specimens dried on formvar-coated grids, by depositing uranium metal at an angle of 12°. Both sections and shadowed preparations were observed in the RCA EMU-2B microscope.

RESULTS

FRACTION I: The glucan-peptide-galactosamine complex, Fraction I, was removed by treatment of cell wall with 2 N sodium hydroxide (Mahadevan and Tatum, 1965). On microscopic examination of the cell walls before (Fig. 1) and after (Fig. 2) removal of Fraction I, no significant difference was observed. The integrity and characteristic outline of the cell wall were retained after this treatment although the wall appeared somewhat thinner.

By treatment with a crude enzyme preparation from *Streptomyces C-3*, cell wall preparations were obtained which consisted essentially of Fraction I. A crude culture filtrate of *Streptomyces* contains β-1,3 glucanases and chitinase as well as some proteinases. This enzyme complex has no activity

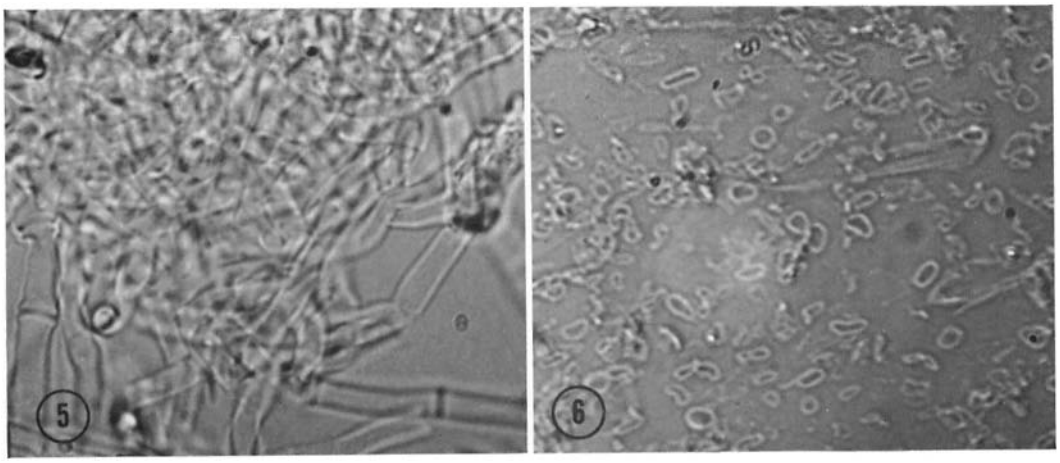

FIGURE 5 RL cell wall treated with chitinase and 2N NaOH. × 1600.

FIGURE 6 Cross wall rings obtained by treatment of RL cell wall with *Streptomyces* enzyme and 2N NaOH. × 1600.

towards cellulose or starch, and very little activity towards the glucan portion of Fraction I. These properties permitted the removal of Fractions II and III and most of the chitin (Fraction IV) from a cell wall preparation. Thus exposure to *Streptomyces* enzyme, for 72 hr, left primarily Fraction I. As shown in Fig. 3, the lateral cell walls appear thinner and more fragile, but the original cell outline is retained. The cross-wall areas, however, are more prominent. A similar result was obtained (Fig. 4) by removing most of the chitin with chitinase, followed by removal of Fractions II and III with *Streptomyces* enzymes.

That the residual materials in Figs. 3 and 4 consist largely of Fraction I is shown by their easy solubility in 2 N sodium hydroxide. The alkali-soluble portion from the material of Fig. 3 contained 12% carbohydrate and 4–6% protein in terms of weight of the original cell wall. From this soluble portion a complex was precipitated with ammonium sulfate. The weight of this precipitate, when dialyzed and lyophilized, was 12–13% of the weight of the original cell wall. This compares with the 13-14% weight of Fraction I obtained by direct fractionation of the cell wall (Mahadevan and Tatum, 1965).

FRACTION III: Similar studies were made after removal of all but Fraction III (β-1,3 glucan) and possibly Fraction II, from the cell wall. The wall sample was treated with chitinase to remove Fraction IV, and then with 2 N sodium hydroxide at room temperature for 16 hr, to remove Fraction I. The residual material, pictured in Fig. 5, showed the original cellular integrity and morphology. That this preparation consisted primarily of the β-1,3 glucan was shown by its susceptibility to digestion by *Streptomyces C-3* enzyme, with the liberation of glucose and glucose oligosaccharides.

OUTER AND INNER FIBRILS: Examination of cell wall preparations of *N. crassa* with the electron microscope before and after removal of some of the well characterized structural components made possible the tentative localization of these components.

The cell wall of the wild type strains of *Neurospora*, RL-3-8A and Perkins A, on examination of shadowed preparations, clearly shows fibrils arranged in a random manner. The fibrils are much clearer in Perkins wild type cell wall (Fig. 7), even under low magnification. Longitudinal sections of the cell wall (Figs. 8 and 9) showed these outer fibrils (*OF*), as well as inner fibrils (*IF*).

Treatment of the cell wall with 2 N sodium hydroxide for 16 hr removes Fraction I, the glucan-peptide-galactosamine complex (Mahadevan and Tatum, 1965), and does not alter the appearance of the cell wall as examined with the light microscope. However, electron microscopy of both shadowed preparations and sections (Fig. 10) of cell wall after removal of Fraction I showed

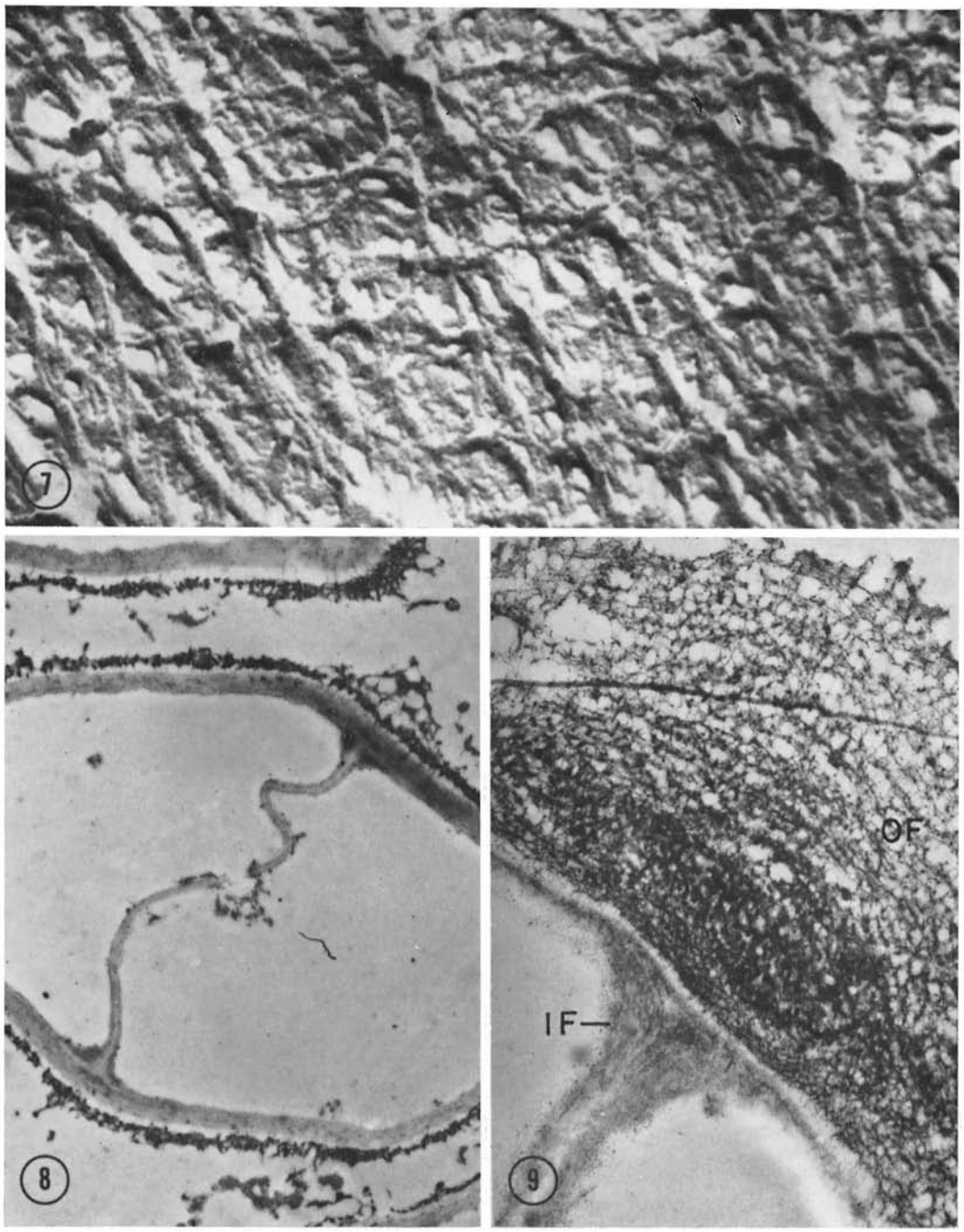

FIGURE 7 Cell wall surface of RL shadowed with uranium. × 130,000.

FIGURE 8 Longitudinal section of PA cell wall showing outer and inner layers, and cross-wall. × 45,000.

FIGURE 9 Longitudinal section of PA cell wall showing outer fibrils (*OF*) and inner fibrils (*IF*). × 80,000.

that the outer fibrils had been removed, exposing the inner fibrils. Compare Fig. 10 with Figs. 8 and 9.

The outer glucan-peptide fibrils cover the ring of the cross-wall and often pile up at the cross-wall region (Fig. 9). Some of the preparations also showed fibrils connecting the outer and inner layers. These observations support the conclusion that the glucan-peptide is concentrated primarily in an outer layer. That the thick fibrils do constitute the glucan-peptide complex was further shown by examining cell wall after treatment for 72 hr with *Streptomyces C-3* enzyme complex. This enzyme complex removes the glucan of Fractions II and III and most of the chitin. The residual material when shadowed and examined showed a network of thick fibrils resembling the outer fibrils of Fraction I. When embedded and sectioned, this residual material appeared to consist essentially of thick fibrils (Fig. 11).

The inner fibrils (*IF*) shown in Figs. 8–10 appear to consist primarily of Fraction II and III. They are seen most clearly after removal of the glucan-peptide complex of Fraction I (Fig. 10), and are almost completely absent after treatment with *Streptomyces* enzyme (Fig. 11). These inner fibrils appear to be oriented primarily in a parallel fashion, and apparently participate in cross-wall formation (see Fig. 10).

CHITIN: The distribution of chitin (Fraction IV) was next investigated. Treatment of cell wall with N.B.C. chitinase (2 mg/ml) releases only acetylglucosamine, and does not significantly alter the morphological appearance of the cell wall. The release of acetylglucosamine by this chitinase is incomplete, even after prolonged treatment. However, subsequent treatment with *Streptomyces* filtrate releases more acetylglucosamine. This suggests that the chitin is more accessible to chitinase in the presence of β-1,3 glucanase. Similar findings have been reported by Skujins et al. (1965). The chitin, therefore, appears to be located interior to the glucan, as a skeletal core in the cell wall, in part protected by glucan.

Removal of Fractions II and III (glucan) with 2 N sodium hydroxide followed by 1 N sulfuric acid and 2 N sodium hydroxide (Mahadevan and Tatum, 1965) leaves essentially only chitin. Shadowed preparations of this material clearly showed randomly arranged fibrils (Fig. 12). This preparation was made from the wild type RL 3-8A. Sections of the Perkins A Fraction IV clearly showed the localization of chitin fibrils throughout the cell wall (Fig. 13).

There is evidence that chitin is concentrated in the cross-wall region of the lateral wall, perhaps in a state more resistant to enzyme attack. As mentioned earlier, in cell wall treated with *Streptomyces* enzymes the cross-wall areas appear relatively denser than the lateral walls (Fig. 3). When this material is treated with sodium hydroxide to dissolve the glucan-peptide, the residual material consists largely of rings, which appear to represent undigested cross-wall areas (Fig. 6). This ring fraction on acid hydrolysis (3 N hydrochloric acid for 1 hr) released primarily glucosamine but very little carbohydrate and, therefore, consists primarily of chitin.

Similar preparations of residual chitinous rings were produced by other combinations of treatment. Digestion of the preparation shown in Fig. 5 with *Streptomyces* enzymes for 72 hr, with addition of fresh enzyme every 24 hr, left only a small amount of residue. This consisted essentially of rings. In another experiment, cell wall was first treated with 2 N sodium hydroxide to remove the glucan-peptide and then exposed to *Streptomyces* enzyme for 72 hr. This treatment removed most of the carbohydrate and the acetylglucosamine of chitin. The residue showed some cross-wall rings which were not susceptible to further digestion by snail enzyme complex (containing chitinase), but on acid hydrolysis liberated glucosamine and no glucose. At this time, it cannot be decided whether the resistant cross-wall rings consist of very compact and not easily digested chitin, or of deacetylated chitin.

DISCUSSION

The primary objective of this study was to investigate the localization and orientation of the cell-wall polymer fractions described by Mahadevan and Tatum (1965). Earlier studies by Tsuda (1955) and Shatkin and Tatum (1959) showed by electron microscopy the existence of two distinct layers in the cell wall of *Neurospora*. The outer layer of coarse fibrils, described by Shatkin and Tatum (1959) as frequently sloughed off at the outer surface of the wall, has now been identified as consisting primarily of Fraction I, the glucan-peptide-galactosamine complex, of Mahadevan and Tatum.

The inner layer is made up primarily of the β-1,3 glucan, Fraction III, and Fraction II, of

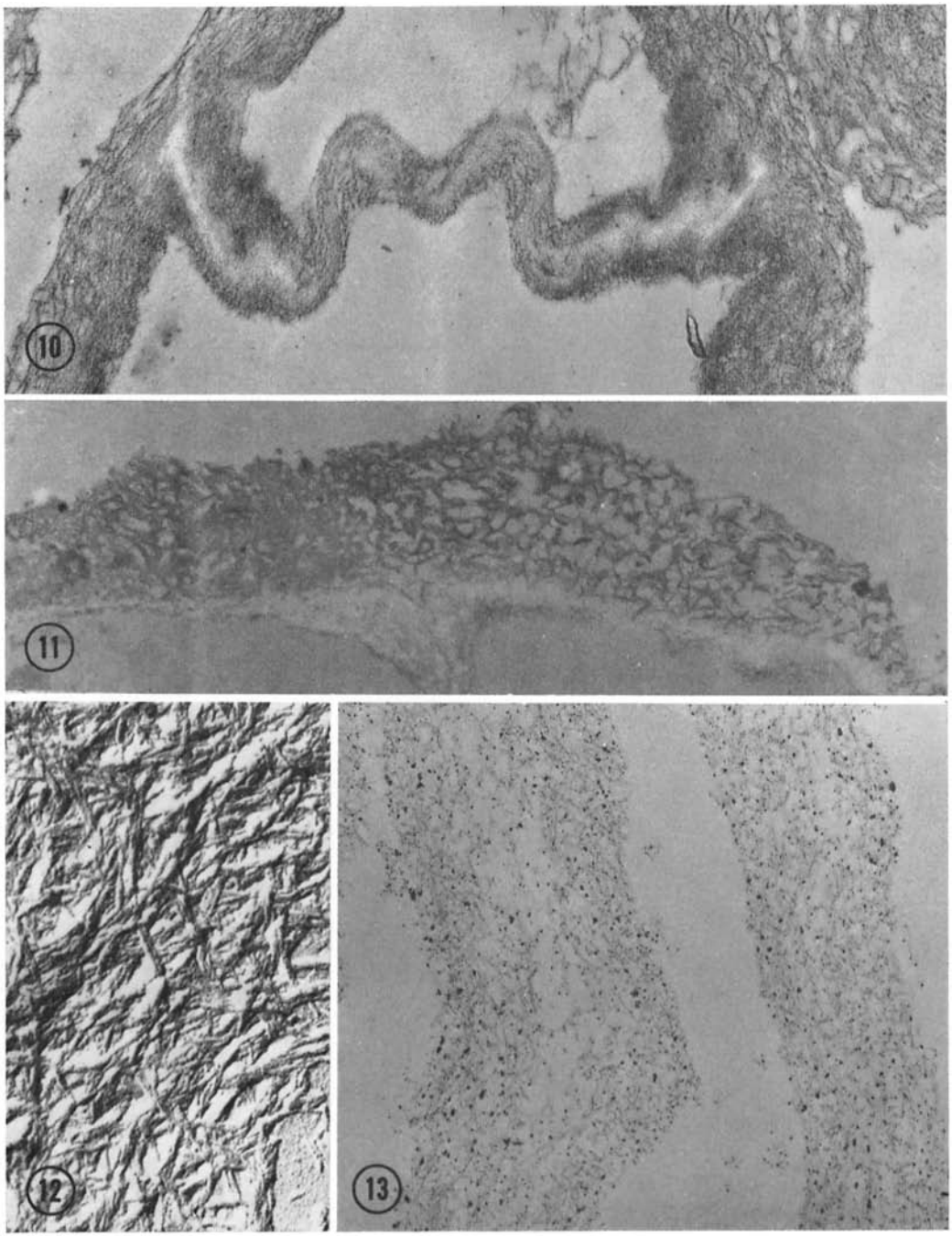

FIGURE 10 Longitudinal section of PA cell wall after removal of Fraction I. × 53,000.

FIGURE 11 Longitudinal section of PA cell wall after treatment with *Streptomyces* enzyme. × 35,000.

FIGURE 12 Shadowed chitin fibrils (Fraction IV) of RL cell wall. × 45,000.

FIGURE 13 Sectioned Fraction IV of PA cell wall. × 35,000.

Mahadevan and Tatum, with an imbedded internal core of chitin fibrils (Fraction IV).

The glucan-peptide becomes thicker at the cross-wall regions and may add additional support to those areas. However, it does not appear to enter significantly into the cross-walls themselves, which consist primarily of Fractions III and IV.

Chitin appears to be protected from enzyme attack by Fractions II and III to some extent, most strikingly in the cross-wall areas. In addition, chitin fibrils are clearly visible in shadowed preparations only after removal of Fractions II and III.

Chitin, Fraction IV of Mahadevan and Tatum, therefore, seems to be distributed throughout the inner cell-wall layer as fine fibrils, embedded in the β-1,3 glucan Fractions II and III.

No similarly direct evidence has yet been obtained for the less well characterized Fraction II, since suitable methods are not available to remove only this fraction from the cell wall or to remove all the others selectively. However, it seems most likely that Fraction II is likewise generally distributed. The treatment of cell wall with *Streptomyces* β-1,3 glucanases releases glucose equivalent to the total glucose of Fractions II and III together. Also, the removal of both Fraction I with sodium hydroxide and Fraction II with acid is necessary to permit the easy solution of Fraction III in sodium hydroxide. Fraction II, a heterogeneous fraction which consists primarily of free sugars and amino-sugars (Mahadevan and Tatum, 1965), may actually represent smaller molecules released from the Fraction III glucan by acid. The Fraction II constituents may, therefore, be integral components of the in vivo Fraction III glucan.

The general conclusions here reported on the ultrastructure of *Neurospora* cell-walls are consistent with findings with other fungi, such as *Aspergillus*, *Rhizopus*, and *Phycomyces* (Hawker, 1965). In most of these forms, the microfibrillar nature of the cell wall has been described. In *Saccharomyces cerevisiae* (Northcote and Horne, 1952; Nickerson et al., 1961) a microfibrillar network has been identified as β-1,3 glucan. Similar findings have been made for the *Aspergillus* spore coat (Horikoshi and Iida, 1964).

However, in addition to providing information on the ultrastructure of *Neurospora* cell-wall, the present study is of interest in relation to growth morphology. Earlier work has indicated a relationship of colonial growth with increased branching (de Terra and Tatum, 1962) and with alterations in cell-wall composition (Mahadevan and Tatum, 1965). The localization of the glucan-peptide in the outer cell-wall layer, and of the β-1,3 glucan in the inner layer centers interest on these components and their biosynthesis in regard to branching patterns and morphological differentiation.

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