BIOCHEMISTRY OF THE ACTINOMYCETALES

II. A Comparison of the Cell Wall Composition of Species of the Genera *Streptomyces* and *Nocardia*

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In the previous paper of this series (Romano and Nickerson, 1956), it was reported that purified cell wall preparations of *Streptomyces fradiae* were solubilized by lysozyme, indicating the presence of a mucopolysaccharide. In addition chemical evidence was advanced to support this thesis. It was shown that on acid hydrolysis of the cell walls, there was a rapid liberation of reducing sugars, the major portion of which was accounted for by a hexosamine. This work has now been extended to include other members of the genus *Streptomyces* and representative members of the genus *Nocardia*. Some striking differences in the carbohydrate composition of the cell walls of these two genera have been found, and will be reported here.

MATERIALS AND METHODS

Organisms. Cell wall preparations of the following organisms were made: *Streptomyces fradiae* 3535, *Streptomyces griseus* 3492, *Streptomyces bobiliae* 3310, *Nocardia rubra* 3639, *Nocardia polychromogenes* 3409, and *Nocardia asteroides* 3573. The above culture numbers refer to the Institute of Microbiology collection.

Medium and culture conditions. The organisms were grown in shake flasks in a medium of the following composition: glucose, 10.0 g; sodium glutamate, 10.0 g; yeast extract, 3.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $ZnSO_4$, 0.05 g; $CaCl_2$, 0.05 g; $FeSO_4$, 0.05 g; and distilled water, 1000 g.

The cultures were incubated at 28 C on a rotary shaker at 250 rpm. The time of incubation varied with each organism because of differences in the rate of growth (table 1).

Preparation of cell walls. Streptomyces cells were harvested by filtration through Reeve Angel No. 802 filter paper; Nocardia cells were harvested by use of a Sharples centrifuge. The cells were washed twice with distilled water and once with 0.3 M sucrose; they were then resuspended in 0.3 M sucrose and broken by placing 35 ml of the suspension in a Raytheon 50-watt, 9-kc magnetostrictor oscillator, which was run at a plate voltage of 130 and an output voltage of 100. The breakage times varied with the organisms studied. In general, the time required to achieve extensive breakage was shorter in the case of species in the genus *Streptomyces* than in the case of species in the genus *Nocardia*. The degree of breakage was indicated by the gram stain; an estimate could be made on this basis, since whole cells were gram positive and the fragmented cell walls were gram negative. The breakage times are shown in table 1.

Following the breaking of the cells, the suspensions were centrifuged at 2000 rpm for 20 min to remove unbroken cells. The supernatant was then centrifuged at 10,000 rpm in an Aminco high speed angle centrifuge for 15 min to collect the cell wall material. This material was then washed twice with water, five times with M/15 phosphate buffer (pH 7.5), and finally, six to eight times with water. It was then suspended in water and lyophilized. Purity of the preparations was checked by electron microscopy, using an RCA model EMU-2 electron microscope.

Analytical procedures. Total reducing sugar was determined by the method of Folin and Malmros (1929). Hexosamine was determined by the Blix (1948) modification of the Elson and Morgan method (1933). Pentose was determined by the orcinol method of Mejbaum (1939).

Chromatographic procedures. Pentoses and hexoses were identified by descending paper chromatography on Whatman #1 filter paper, using the following solvent systems: ethyl acetate-pyridine-water (2:1:2), and *n*-butanol-acetic acid-water (4:1:5). The papers were irrigated for 24 hr when the first solvent was used, and for 72 hr when the second was used. Sugars were de-

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TABLE 1

Incubation periods and cell breakage times

Organism	Incubation Time	Breakage Time
	hr	*min*
Streptomyces fradiae	72	3
Streptomyces griseus	72	10
Streptomyces bobiliae	120	15
Nocardia rubra	48	45
Nocardia polychromogenes	120	15
Nocardia asteroides	48	45

per cent decrease in optical density of the cell wall suspensions is plotted as a function of time. It is apparent that the cell walls of the *Streptomyces* species were lysed by lysozyme, while the cell walls of the *Nocardia* species tested were resistant to the action of this enzyme. These differences in susceptibility to attack by lysozyme indicated a difference in the carbohydrate composition of the cell walls, since lysozyme has been shown to be a mucopolysaccharase (Epstein and Chain, 1940; Meyer and Hahnel, 1946), which

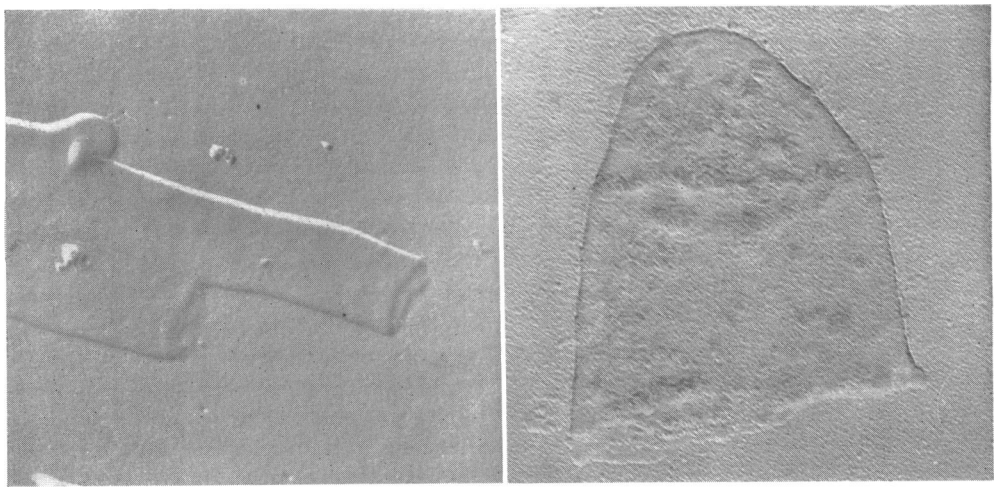

Figure 1. Electron photomicrographs of cell walls, shadowed with chromium at an angle of 25 degrees. *Left: Streptomyces griseus*, 19,000 ×. *Right: Nocardia rubra*, 33,000 ×.

tected by spraying the papers after drying with aniline acid phthalate, according to the method of Partridge (1949).

Susceptibility of the cell wall material to lysis by lysozyme was determined by suspending the cell walls in M/15 phosphate buffer, pH 6.6, containing 0.017 M NaCl, adding lysozyme, and incubating in a water bath at 37 C. Decrease in optical density was determined at suitable time intervals by the use of a Klett-Summerson photoelectric colorimeter.

RESULTS

Electron photomicrographs of cell wall fragments of *S. griseus* and *N. rubra* prepared as described above are shown in figure 1. These appear to be homogeneous and essentially free of intracellular material.

Effect of lysozyme. The effect of lysozyme on the cell wall material of three *Streptomyces* and three *Nocardia* species is shown in figure 2, where the

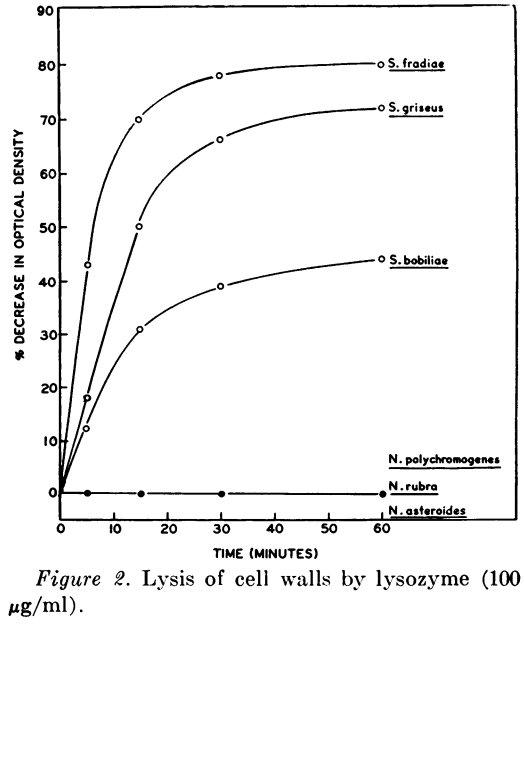

Figure 2. Lysis of cell walls by lysozyme (100 μg/ml).

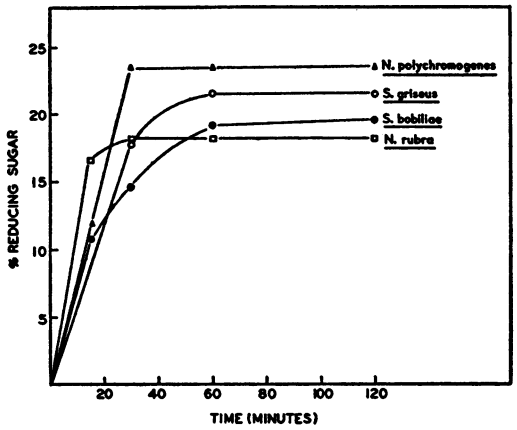

Figure 3. Liberation of reducing sugars (expressed as per cent of dry weight of cell walls) on 1 N HCl hydrolysis at 100 C.

acts by depolymerizing the mucopolysaccharide that makes up the fabric of the cell wall (Salton 1952). Studies of the carbohydrate composition of this cell wall material were accordingly carried out in order to account for these differences in lysozyme susceptibility.

Carbohydrate composition. The cell wall material was hydrolyzed with 2 N HCl at 100 C. There was a rapid liberation of reducing sugars on hydrolysis, as shown in figure 3. The hydrolysis in all cases appeared to be complete in 30 min. The results of carbohydrate analyses carried out on these hydrolyzates are shown in table 2. Some distinct differences between Nocardia and Streptomyces are apparent. The amount of hexosamine present was closely correlated with the degree of susceptibility to lysozyme. In Streptomyces, the major portion of the reducing sugar was accounted for by hexosamine; in Nocardia, relatively small amounts of hexosamine were present. A more striking difference was the presence in the cell wall of Nocardia of a large amount of pentose which was completely absent in the cell walls of Streptomyces tested. The differences between the pentose and hexosamine values reported appear to be accounted for by hexoses. Values for hexose are not included in table 2 because the hexose analyses suffered from interference by pentose and hexosamine.

The pentose and hexose occurring in the cell wall of Nocardia have been tentatively identified by paper chromatography as arabinose and galactose, respectively. A typical chromatogram is shown in figure 4.

TABLE 2

Carbohydrate composition of cell walls: reducing sugars liberated by 2 N HCl hydrolysis at 100 C for 2 hr

Organism	Per Cent Total Reducing Sugar	Per Cent Pentose	Per Cent Hexosamine
Streptomyces griseus 3492..	21.6	0	19.9
Streptomyces fradiae 3535..	22.0	0	19.8
Streptomyces bobiliae 3310..	19.6	0	12.0
Nocardia rubra 3639.....	18.1	10.5	2.6
Nocardia asteroides 3573...	17.6	12.2	2.6
Nocardia polychromogenes 3409.....	23.6	10.3	3.1

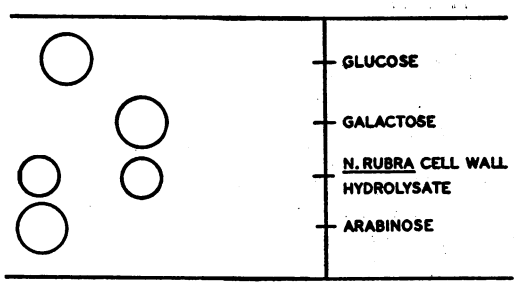

Figure 4. Paper chromatogram of *Nocardia rubra* hydrolyzate. Irrigated for 24 hr in descending direction with ethyl acetate-pyridine-water (2:1:2).

DISCUSSION

Striking differences have been found in the carbohydrate composition of the cell walls of *Streptomyces* and *Nocardia* species. While the data obtained so far is too scant to warrant the attachment of great taxonomic significance to these results, the extension of this approach may yield criteria which will be of value in the classification of the actinomycetales. The use of cell wall composition as a criterion in the determination of phylogenetic relationship in the plant kingdom was advocated by von Wettstein (1921) and carried further by Nabel (1939).

The differentiation of the genera *Nocardia* and *Streptomyces* has been particularly difficult, since morphological and colonial characteristics used in their classification (Waksman and Henrici, 1943) are subject to widespread variation. Two of the organisms used in this study present such a problem: *S. bobiliae* did not produce aerial mycelium, whereas *N. asteroides* produced a well-developed aerial mycelium with conidia. Fragmentation of

vegetative hyphae seldom occurred in the latter organism. If strict morphological criteria were applied, these organisms most resemble Nocardia and Streptomyces, respectively. However, by comparing the cell wall composition of these two organisms, they fall into their respective groups rather well, as shown in table 2.

This study is being pursued further to determine the value of this approach in the clarification of the taxonomy of the actinomycetales.

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SUMMARY

Cell wall preparations of representative species of the genera *Streptomyces* and *Nocardia* have been made. Differences in the carbohydrate composition of the cell walls of these two genera have been observed. The cell walls of the *Streptomyces* species have been found to be of a mucoid nature, lysed by lysozyme and containing considerable amounts of hexosamine. The cell walls of the *Nocardia* species were not susceptible to the action of lysozyme, contained much smaller amounts of hexosamine, but contained about 10 per cent pentose, tentatively identified as arabinose. The possibility of using cell wall composition as a taxonomic criterion of the differentiation of the actinomycetales is discussed.

REFERENCES

BLIX, G. 1948 The determination of hexosamines according to Elson and Morgan. Acta. Chem. Scand., **2**, 467–473.

ELSON, L. A. AND MORGAN, W. T. J. 1933 A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J. (London) **27**, 1824–1828.

EPSTEIN, L. A. AND CHAIN, E. 1940 Some observations on the preparation and properties of the substrate of lysozyme. Brit. J. Exptl. Pathol., **21**, 339–355.

FOLIN, O. AND MALMROS, H. 1929 An improved form of Folin's micromethod for blood sugar determinations. J. Biol. Chem., **83**, 115–120.

MEJBAUM, W. 1939 Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Hoppe-Seyler's Z. physiol. Chem., **258**, 117–120.

MEYER, K. AND HAHNEL, E. 1946 The estimation of lysozyme by a viscosimetric method. J. Biol. Chem., **163**, 723–732.

NABEL, K. 1939 Über die Membrane niederer Pilze, besonders von *Rhizidiomyces bivellatus* nov. spez. Arch. Mikrobiol., **10**, 515–541.

PARTRIDGE, S. M. 1949 Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature, **164**, 443.

ROMANO, A. H. AND NICKERSON, W. J. 1956 The biochemistry of the Actinomycetales. I. Studies on the cell wall of *Streptomyces fradiae*. J. Bacteriol., **72**, 478–482.

SALTON, M. R. J. 1952 Cell wall of *Micrococcus lysodeikticus* as the substrate of lysozyme. Nature, **170**, 746–747.

VON WETTSTEIN, F. 1921 Das Vorkommen von Chitin und seine Verwertung als systematisch-phylogenetisches Merkmal im Pflanzenreich. Sitzber. Akad. Wiss. Wien Math.-naturw. Kl. Abt. I, **130**, 3–20.

WAKSMAN, S. A. AND HENRICI, A. T. 1943 The nomenclature and classification of the actinomycetes. J. Bacteriol., **46**, 337–341.