COMPOSITION OF THE SHEATH OF *SPHAEROTILUS NATANS*

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ABSTRACT

ROMANO, ANTONIO H. (University of Cincinnati, Cincinnati, Ohio) AND JOYCE P. PELOQUIN. Composition of the sheath of *Sphaerotilus natans*. J. Bacteriol. **86**:252–258. 1963.—The sheath of *Sphaerotilus natans* was isolated and subjected to chemical analysis. Isolation of the sheaths was accomplished by incubating cells in the presence of lysozyme and ethylenediaminetetraacetic acid in tris(hydroxymethyl)aminomethane buffer and adding sodium dodecyl sulfate subsequently. Under these conditions, there was complete dissolution of cells. The sheaths, which were left intact by this treatment, were recovered by centrifugation, washed exhaustively, lyophilized, and subjected to analysis. Hydrolysis of the sheath material with 2 N HCl at 100 C resulted in the liberation of reducing sugars amounting to 36% of the dry weight. Amino sugar accounted for 11% of the dry weight. Paper chromatography of hydrolysates showed the presence of glucose and hexosamine. Tests for muramic acid were negative. In addition to carbohydrate, 27% protein and 5.2% lipid were found to be present. Fractionation studies indicated that essentially all of the polysaccharide was associated with a trichloroacetic acid-soluble fraction. The sheath is therefore considered to be a protein-polysaccharide-lipid complex, which is chemically and anatomically distinct from the cell wall and the slime layer. It is hypothesized that this unique structure may be related to the microcapsule found in many gram-negative bacteria, and may represent a structural specialization of this more common structure.

Sphaerotilus natans is a typical sheath bacterium, belonging to the order *Chlamydobacteriales*. This group of organisms is characterized by the presence of a contiguous closely fitting sheath which encloses long chains of rod-shaped cells. This sheath is of ecological significance to the organism, since it allows a means of attachment to solid surfaces. The organism thus gains a selective advantage in streams with appreciable current flow, since it can obtain sufficient nutrient from a large volume of water to achieve massive growth even if nutrient concentration is extremely low. Accordingly, *S. natans* is of considerable importance as a nuisance organism in water supplies, since it can grow in such quantities in streams receiving organic enrichment that massive slime infestations can result.

In view of the problems caused by this organism, the ecological significance of the sheath, and the current interest in the chemical composition of surface structures of microbial cells, the present study was undertaken to find a method of isolating sheath material free from cells so that the chemical nature of this unique structure could be elucidated.

Little information on the chemical composition of the sheath has been reported since the early observations of Linde (1913), who found that the sheath was soluble in 50% H_2SO_4, 60% KOH, or ammoniacal copper sulfate but was insoluble in 5% H_2SO_4, and the observations of Zikes (1915), who stated that hemicellulose might be present but chitin was absent. Wuhrmann and Mechsner (1960) carried out an electron microscopic study on isolated sheaths and reported some optical and chemical properties. They found that the sheaths contained 6.8% N and, on the basis of qualitative tests, reported the presence of amino acids and glucosamine in hydrolysates. X-ray diffraction studies did not show patterns corresponding with known plant cell-wall substances.

This paper is an extension of preliminary studies (Romano and Lugannani, 1961) reported from this laboratory.

MATERIALS AND METHODS

Organism. The organism used in this study was isolated from a slime infestation of the Altamaha River below Jesup, Ga. It was isolated in pure cul-

ture by repeated streaking, using the methods and medium described by Stokes (1954). The organism was identified as *S. natans* by morphological and cultural properties, according to the descriptions of Pringsheim (1949) and Stokes (1954).

Growth conditions and harvesting. Cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of Stokes (1954) medium. Stock cultures were incubated at room temperature and transferred at weekly intervals. For purposes of making sheath preparations, 40 to 60 flasks of Stokes medium were inoculated with *S. natans* and incubated at room temperature for 72 hr; the cell mass was then harvested by centrifugation at 6,000 × *g* for 15 min and washed with distilled water.

Preparation of sheaths. The method that was developed to remove cells and cell walls and leave intact sheaths was based on the observations of Repaske (1956) that certain gram-negative bacteria could be lysed with lysozyme in the presence of ethylenediaminetetraacetic acid (EDTA) and tris(hydroxymethyl)aminomethane (tris) buffer, and those of Shafa and Salton (1960) that sodium dodecyl sulfate was capable of causing the complete disaggregation of cell walls of various gram-negative bacteria. A combination of these two methods was used to effect complete lysis of cells within the sheath.

The washed cell mass was suspended in 0.03 M tris buffer (pH 8.0) and agitated in a Waring Blendor for 2 min; this served to break up large clumps of filaments and to suspend the material sufficiently so that lysis could be followed photometrically. The density of the suspension was adjusted to an optical density of 0.7 to 1.0. Tetrasodium EDTA (Matheson, Coleman & Bell Div., Matheson Co., Inc.) and lysozyme (Mann Research Laboratory) were added at final concentrations of 500 and 125 μg/ml, respectively. This suspension was incubated at 37 C for 45 min. At this time, sodium dodecyl sulfate was added to give a final concentration of 0.01 M; incubation was continued for an additional 30 min, or until there was no further change in optical density.

Figure 1 shows the change in optical density which took place when *S. natans* was treated with sodium dodecyl sulfate alone and with the combination of lysozyme, EDTA, and sodium dodecyl sulfate in tris buffer. Lysozyme and EDTA caused a slow and incomplete lysis; when sodium dodecyl sulfate was added, there was a very rapid and complete lysis. Sodium dodecyl sulfate alone

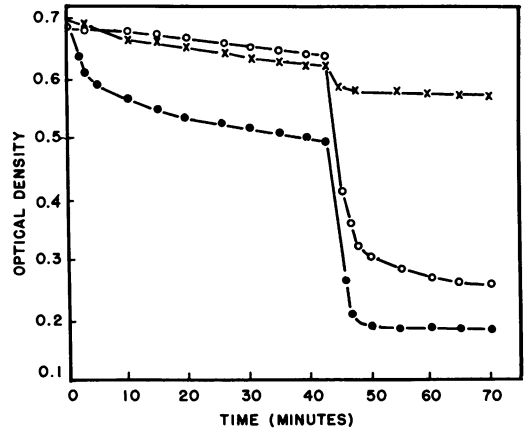

FIG. 1. *Comparison of lytic effects of sodium dodecyl sulfate alone and lysozyme with EDTA followed by sodium dodecyl sulfate.* × = *Control; cells suspended in 60 ml of 0.03* M *tris buffer (pH 8.0); 6 ml of buffer added at 45 min.* ○ = *Cells suspended in 60 ml of 0.03* M *tris buffer (pH 8.0); 6.0 ml of 0.1* M *sodium dodecyl sulfate in buffer added at 45 min.* ● = *Cells suspended in 60 ml of 0.03* M *tris buffer (pH 8.0) containing 500 μg of EDTA per ml and 125 μg of lysozyme per ml; 6.0 ml of 0.1* M *sodium dodecyl sulfate in buffer added at 45 min.*

caused lysis that was rapid and extensive, but not as complete as that caused by the combination. These results are interpreted as an indication that the cell wall is composed of lipoprotein and mucocomplex, both of which must be completely disaggregated before complete lysis can occur.

Microscopy of the suspension after the EDTA-lysozyme-detergent treatment described above revealed the presence of empty sheaths free from cells. This is shown in Fig. 2, where light and electron photomicrographs of untreated and treated cells are compared.

The sheath material was recovered by centrifugation at 15,000 × *g* for 20 min in a Servall Superspeed refrigerated centrifuge, and was washed five times with 0.03 M tris buffer (pH 8.0), eight times with 0.06 M phosphate buffer (pH 7.5), and eight times with distilled water. Preparations were then lyophilized from an aqueous suspension.

Analytical methods. Total reducing sugar (expressed as glucose) was determined by the method of Folin and Malmros (1929). Hexosamine was determined by the Palmer, Smythe, and Meyer

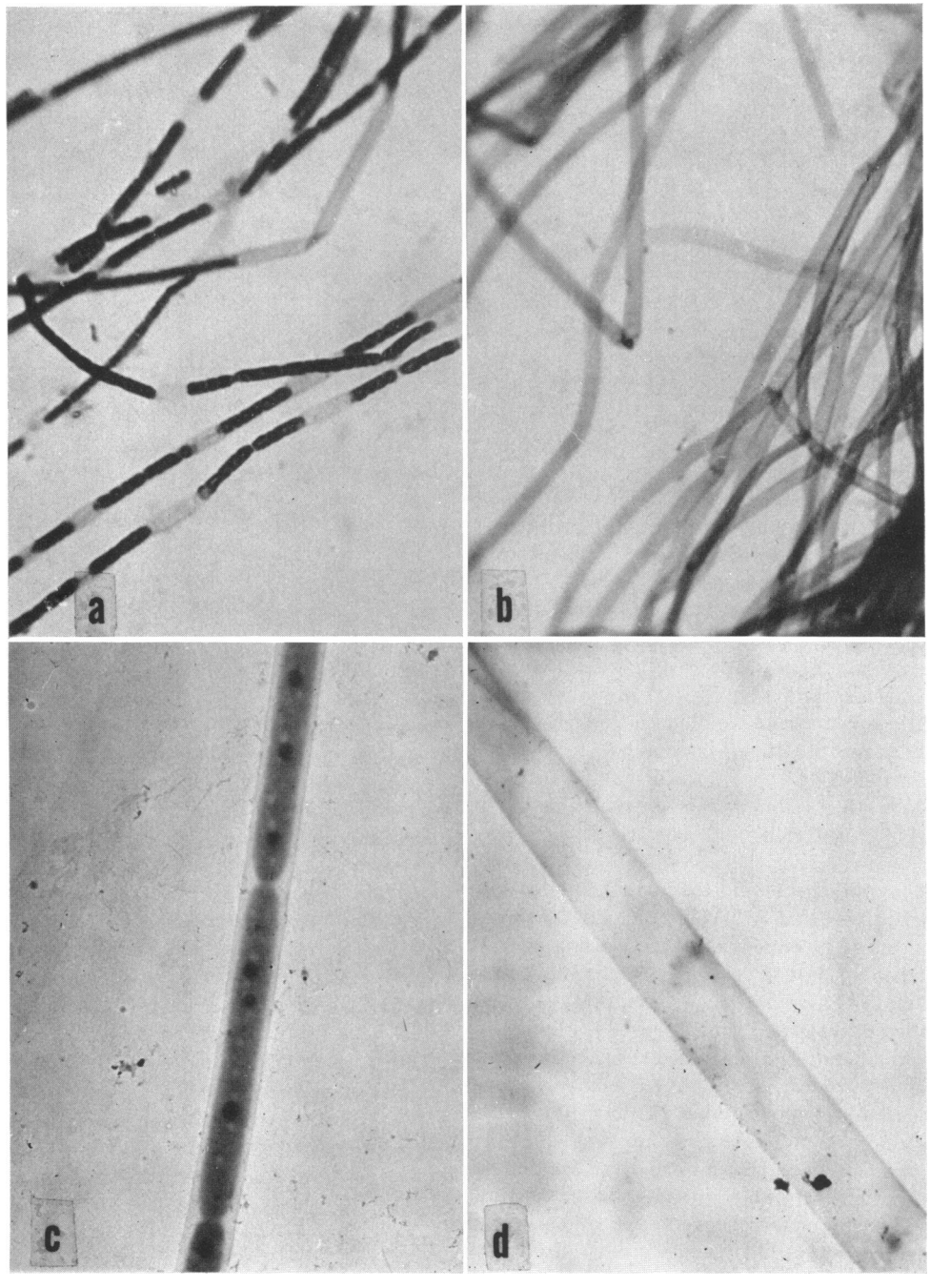

FIG. 2. *(a) Light photomicrograph of untreated filaments; stained with phenolic rose bengal (× 1500); (b) light photomicrograph of filaments after treatment with lysozyme, EDTA, and sodium dodecyl sulfate (× 1500); (c) electron photomicrograph of untreated filament (× 7000); (d) electron photomicrograph of filament after treatment with lysozyme, EDTA, and sodium dodecyl sulfate (× 9000).*

(1937) and the Schloss (1951) modifications of the Elson and Morgan (1933) procedure.

Protein was determined by the biuret method of Robinson and Hogden (1940) and by the procedure of Lowry et al. (1951). In the latter procedure, the sheath sample was solubilized by heating in 1 N NaOH at 100 C for 10 min, and then was diluted to 0.5 N NaOH for analysis. Crystalline bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) used as a standard was treated identically. Total nitrogen was determined by the micro-Kjeldahl procedure, and total phosphorus was determined by the method of Fiske and SubbaRow (1925) after hydrolysis with 10 N H_2SO_4 at 140 C and addition of H_2O_2 as described by Umbreit, Burris, and Stauffer (1957).

Chromatography. Sugars and amino acids present in hydrolysates were identified by paper chromatography with Whatman no. 1 filter paper. For sugars, chromatograms were irrigated with ethyl acetate-pyridine-water (2:1:2; v/v) in a descending direction. Ammoniacal silver nitrate (0.5 N $AgNO_3$ in 5 N NH_4OH) and triphenyl tetrazolium chloride (Wallenfels, 1950) were used as detection reagents. For amino acids, two-dimensional ascending chromatograms with butanol-acetic acid-water (250:60:250; v/v) and phenol-ammonia were done. Amino acids were detected by spraying with 0.2% ninhydrin in acetone containing 2% pyridine and heating at 100 C for 5 min.

Results

Carbohydrate composition. Samples of 5 mg of sheath material were hydrolyzed in 5 ml of 2 N HCl at 100 C for 2 hr. Portions were removed after 20, 40, 60, and 120 min; they were then neutralized and analyzed for total reducing sugars and hexosamine. The rate of release of reducing sugars and hexosamine from several different sheath preparations is shown in Fig. 3. There was a rapid release of reducing sugar during the first 20 min, and then a gradual increase to 120 min. The results indicate that 36% of the sheath is made up of a hexosamine-containing polysaccharide.

The reducing sugars liberated by hydrolysis have been identified by paper chromatography as glucose and glucosamine. A tracing of a typical chromatogram of a hydrolysate is shown in Fig. 4. A prominent spot corresponding to glucosamine

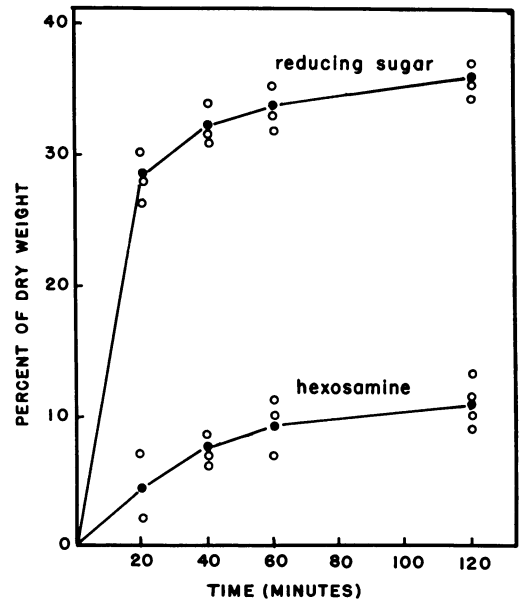

FIG. 3. *Rate of release of total reducing sugars and hexosamine from sheaths by hydrolysis with 2 N HCl at 100 C. Symbols:* ○ = *individual determinations on different sheath preparations;* ● = *average.*

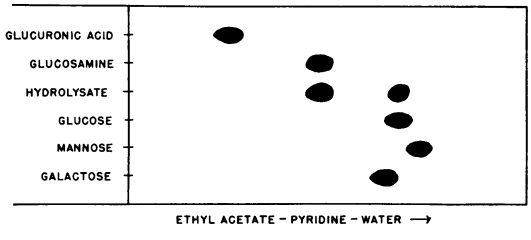

FIG. 4. *Tracing of paper chromatogram of sheath hydrolysate.*

was also found in subsequent amino acid chromatograms (Fig. 5).

It was of interest to determine whether muramic acid was present, in view of the universal occurrence of this substance in bacterial cell walls. All attempts to demonstrate muramic acid have been negative. Hence, it is considered to be absent on the basis of the following criteria. (i) No spot corresponding to muramic acid was detected on paper chromatograms. (ii) Hexosamine analyses of hydrolysate samples by the modifications of Palmer et al. (1937) and Schloss (1951) of the Elson and Morgan (1933) procedure

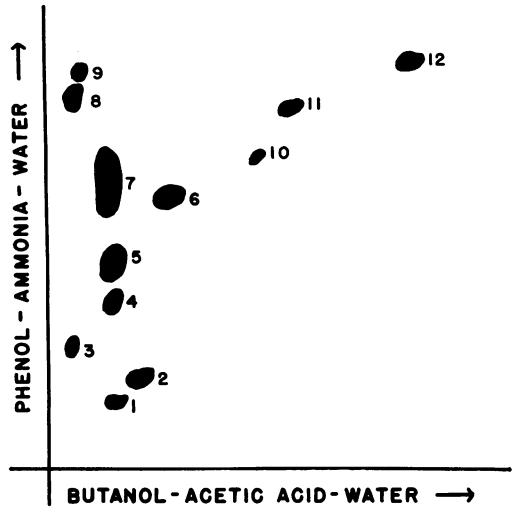

FIG. 5. *Tracing of two-dimensional amino acid chromatogram of sheath hydrolysate: 1, aspartic acid; 2, glutamic acid; 3, diaminopimelic acid; 4, serine; 5, glycine; 6, alanine; 7, glucosamine; 8, lysine; 9, arginine; 10, tyrosine; 11, valine; 12, leucine or isoleucine, or both.*

gave chromagens with absorption maxima identical to that of glucosamine. (iii) Hydrolysates gave a negative test for lactic acid by the Barker and Summerson (1941) procedure.

Protein and amino acid composition. Protein analysis by both the biuret procedure of Robinson and Hogden (1940) and the Lowry method (Lowry et al., 1951) gave values of 27%. Total nitrogen analysis gave a value of 7.6%.

Amino acids present in hydrolysates of the sheath were identified by paper chromatography. Sheath samples of 25 mg were hydrolyzed with 5 ml of 6 N HCl at 100 C for 18 hr in a sealed tube. Hydrolysates were dried in vacuo, redissolved in ethanol, and dried three times to remove acid. Finally, the hydrolysates were taken up in 0.5 ml of distilled water and subjected to chromatography (Fig. 5). A broad range of amino acids were found, including aspartic acid, glutamic acid, diaminopimelic acid, serine, glycine, alanine, lysine, arginine, tyrosine, valine, and leucine or isoleucine. A prominent spot corresponding to glucosamine also occurred on all chromatograms.

Lipid. An estimate of the lipid content of the sheath was made by carrying out a series of ether extractions before and after acid hydrolysis. Samples were extracted three times with absolute ether, and the pooled extracts were dried and weighed. This was considered to represent free lipid. The sheath residue was then hydrolyzed with 2 N HCl for 2 hr at 100 C and again extracted three times with absolute ether. These extracts were pooled, dried, and weighed. This was considered to be the bound lipid. The results are included in Table 1, which is a summary of the analyses carried out on whole-sheath preparations.

Fractionation of sheaths. A fractionation procedure has been designed by Park and Hancock (1960) to separate major classes of high molecular weight components of the cell. Of particular interest to this study was the ability of the fractionation procedure to separate polymers such as teichoic acid from cell-wall mucopeptide by extraction with 5% trichloroacetic acid at 90 to 100 C. A decision could thus be made as to whether the hexosamine-containing polymer of the sheath was associated with mucopeptide or some other polymer. Therefore, sheath samples were fractionated according to the Park and Hancock (1960) procedure; the fractions were then hydrolyzed with 2 N HCl at 100 C for 2 hr and were analyzed (Table 2). It is clear that essentially all of the carbohydrate constituents of the sheath were found in the hot trichloroacetic acid-extractable fraction. This would indicate that little or no cell-wall mucopeptide is present in the sheath, and that this structure is principally composed of a hot trichloroacetic acid-soluble polymer. It is unlikely that this polymer is a teichoic acid, in view of the following considerations. (i) The total phosphorus content of the sheath is below that which would be expected if a polyol phosphate were a major constituent (see Table 1). (ii) Ribitol, anhydroribitol, or ribitol phosphate could not be detected

TABLE 1 *Summary of analytical results*

Substance	Per cent of dry weight
Reducing sugar	36.0
Hexosamine	11.0
Protein	
Biuret method	27.5
Lowry method	27.2
Nitrogen	7.6
Phosphorus	0.5
Lipid	5.2
Free lipid	2.8
Bound lipid	2.4

TABLE 2. *Distribution of sugars in hydrolysates of fractions*

Fraction	Reducing sugar		Hexosamine	
	Amount*	Per cent of total	Amount*	Per cent of total
Hot trichloroacetic acid extract				
First	30.4	93.0	13.1	93.0
Second	1.8	5.5	0.64	5.8
Residue	0.54	1.6	0.18	1.3

* Expressed as mg/100-mg sample.

in sheath hydrolysates. Estimation of glycerol in sheath hydrolysates by the periodate oxidation method of Lambert and Neish (1950) gave negative results.

It has been possible to isolate a polymer from the hot trichloroacetic acid fraction by adding 2 volumes of acetone to the cooled extract and storing it at 5 C overnight. A gel-like precipitate was obtained which solidified to a glass on drying. Studies are in progress to characterize this substance fully.

Discussion

The sheath of *S. natans* is composed of a protein-polysaccharide-lipid complex. It is morphologically distinct from the cell wall, and chemically distinct from the capsule, which has been shown to be composed of a polysaccharide containing galactose, fucose, glucose, and glucuronic acid (Gaudy and Wolfe, 1962).

Although the sheath is a unique structure found only in members of the *Chlamydobacteriales*, it is of interest to seek relationships with common structures which are known to occur in other bacteria. With respect to its protein-polysaccharide-lipid nature and its anatomical position immediately outside the cell wall and within the capsule or slime layer, the sheath has much in common with the microcapsule which is known to occur in gram-negative bacteria. The microcapsule, as defined by Wilkinson (1958), is chemically and immunologically distinct from the cell wall, is not essential for the mechanical stability of the cell wall, and can be removed without impairing the viability of the cell. Microcapsular substances are protein-polysaccharide-lipid in nature, and are considered to constitute the somatic O antigens and endotoxins of gram-negative bacteria. The manner in which the microcapsule is attached to the cell wall is not clear.

While immunological and toxicity studies have not yet been carried out with sheaths of *S. natans*, it does not appear unreasonable to consider the sheath as a modified microcapsule on the basis of chemical composition and anatomical position. The hypothesis is put forth, therefore, that the sheath is a structure that is synthesized as a microcapsule immediately outside the cell wall, and that subsequently is separated from the cell wall at the poles of the cell upon cell division.

Acknowledgment

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Literature Cited

Barker, S. B., and W. H. Summerson. 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. **138**:535–554.

Elson, L. A., and W. T. J. Morgan. 1933. A colorimetric method for the determination of glucosamine and chondrosamine. Biochem. J. **27**:1824–1828.

Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375–400.

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

Gaudy, E., and R. S. Wolfe. 1962. Composition of an extracellular polysaccharide produced by *Sphaerotilus natans*. Appl. Microbiol. **10**:200–205.

Lambert, M., and A. C. Neish. 1950. Rapid method for estimation of glycerol in fermentation solutions. Can. J. Res. Sect. B **28**:83–89.

Linde, P. 1913. Zur Kenntnis von *Cladothrix dichotoma* Cohn. Zentr. Bakteriol. Parasitenk. Abt. II **39**:369–394.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

Palmer, J. W., E. M. Smythe, and K. Meyer. 1937. Glucoproteins. IV. Estimations of hexosamine. J. Biol. Chem. **119**:491–500.

Park, J. T., and R. Hancock. 1960. A fractionation procedure for studies of the synthesis of cell-wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. J. Gen. Microbiol. **22**:249–258.

PRINGSHEIM, E. G. 1949. The filamentous bacteria, *Sphaerotilus*, *Leptothrix*, *Cladothrix*, and their relation to iron and manganese. Phil. Trans. Roy. Soc. London, Ser. B **233**:453–482.

REPASKE, R. 1956. Lysis of gram-negative bacteria by lysozyme. Biochim. Biophys. Acta **22**:189–191.

ROBINSON, H. W., AND C. G. HOGDEN. 1940. The biuret reaction in the determination of serum proteins. I. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. **135**:707–725.

ROMANO, A. H., AND J. LUGANNANI. 1961. Preparation and properties of the sheath of *Sphaerotilus natans*. Bacteriol. Proc., p. 95.

SCHLOSS, B. 1951. Colorimetric determination of glucosamine. Anal. Chem. **23**:1321–1325.

SHAFA, F., AND M. R. J. SALTON. 1960. Disaggregation of bacterial cell walls by anionic detergents. J. Gen. Microbiol. **23**:137–141.

STOKES, J. L. 1954. Studies on the filamentous sheathed iron bacterium Sphaerotilus natans. J. Bacteriol. **67**:278–291.

UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1957. Manometric techniques and tissue metabolism, 3rd ed., p. 273. Burgess Publishing Co., Minneapolis.

WALLENFELS, K. 1950. Über einen neuen Nachweis reduzierender Zucker in Papierchromatogramm und dessen quantitative Auswertung. Naturwissenschaften **37**:491–492.

WILKINSON, J. F. 1958. The extracellular polysaccharides of bacteria. Bacteriol. Rev. **22**:46–73.

WUHRMANN, K., AND K. MECHSNER. 1960. Über den Bau der Scheiden des Bacteriums *Sphaerotilus natans* Kütz. Z. Wiss. Mikroskopie **64**:474–481.

ZIKES, H. 1915. Vergleichende Untersuchungen über *Sphaerotilus natans* (Kutzing) und *Cladothrix dichotoma* (Cohn) auf Grund von Reinkulturen. Zentr. Bakteriol. Parasitenk. Abt. II **43**:529–552.