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Purification and Chemical Composition of the Protective Slime Antigen of *Pseudomonas aeruginosa*

PASQUALE F. BARTELL, THOMAS E. ORR, AND BOHDANNA CHUDIO
Department of Microbiology, New Jersey College of Medicine and Dentistry, Newark, New Jersey 07103

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The slime obtained from *Pseudomonas aeruginosa* strain BI was purified by a system of ethanol precipitation, gel filtration, and ion-exchange chromatography. The slime polysaccharide was eluted as a single peak at a potassium chloride molarity of 0.30 to 0.40. The purification procedure was monitored by immunodiffusion techniques, and the number of bands was reduced from four to one, indicating the elimination of antigenic impurities that were present in the crude extracts of slime. The purified slime behaved as a homogeneous antigen, stimulating the production of a single species of antibody in rabbits. Hydrolyzed preparations of purified slime contained rhamnose, glucose, mannose, glucosamine, galactosamine, and glucuronic acid, as well as *N*-acetyl and *O*-acetyl groups. Only trace amounts of nucleic acids were detectable. A significant amount of protein was found to be associated with the carbohydrate moiety. The substrate characteristic of the slime was reaffirmed by measuring the release of hexosamines in the presence of the *Pseudomonas* phage 2 depolymerase PDB_2, and its activity as a protective antigen was demonstrated in passive-protection tests of mice.

The production of slime by *Pseudomonas aeruginosa* is considered to be a characteristic of the species (17). The clinical importance of the slime layer has been emphasized by the work of several investigators, and has been implicated in resistance to chemotherapy (12), toxicity, and pathogenicity (22), and as a protective antigen in experimental infection (1, 2, 22). More recently, it has been considered as a factor influencing the life cycle of bacteriophage (5–7).

In examining the roles of various cellular fractions in experimental infection, Liu et al. (22) found extracts of slime to be toxic and capable of stimulating a protective response in mice. The protective activity of slime was also demonstrated by Alms and Bass (2) and Alexander et al. (1), and Alms and Bass (3–4) subsequently reported that this activity was associated with an ethanol-precipitated carbohydrate fraction of the slime.

Although studies regarding the chemical composition of the slime of *P. aeruginosa* appear to be confusing and contradictory (11), evidence has been presented to indicate that variations in the composition of slime may exist from one strain to another (6, 7, 22). Eagon (16) reported that the slime polysaccharide was composed chiefly of mannose and nucleic acids, mostly deoxyribonucleic acid (DNA), and small amounts of protein. Uronic acids or amino sugars were not detected. The slime layer of *P. aeruginosa* was reported to be composed of DNA in a highly polymerized state (F. E. Halleck, M. A. Durkin, and W. Guschlbauer, Bacteriol. Proc., p. 176, 1960). Doggett et al. (15) identified fucose, glucose, mannose, galactose, glucosamine, galactosamine, and an unidentified hexosamine, and two other unidentified compounds in a "mucus-producing" strain of *P. aeruginosa*. The results of Carlson and Matthews (13) confirmed and extended the findings of Linker and Jones (21) in that uronic acids were the only detectable components of polysaccharides elaborated by *P. aeruginosa*, and the presence of *O*-acetyl groups in these polyuronic acids was later observed (A. Linker and R. S. Jones, Fed. Proc. p. 410, 1966). However, hexosamines were not detected. More recently, the slime composition reported by Brown et al. (11) was predominately polysaccharide containing 20% nucleic acids, DNA and ribonucleic acid (RNA), and a small amount of protein. The hydrolyzed slime consisted mainly of glucose with smaller amounts of mannose. In addition,

they found 5% hyaluronic acid. Minor components were protein, rhamnose, and glucosamine, the protein being less than 5% of the total.

The slime of *P. aeruginosa* strain BI has been observed to be hydrolyzed by the phage 2 depolymerase PDB_2, resulting in a decreased viscosity of 20 to 25% and a measurable increase in the levels of hexosamines, hexoses, and reducing substances (7). Recently, five other *Pseudomonas* phage-associated depolymerases were described (6). However, the chemical composition of the slime substrates has not been defined. In this connection, the hydrolytic activity of the phage-associated depolymerases may be useful in obtaining structurally informative fragments that might otherwise be lost with harsher chemical treatments (19, 28).

This communication describes the purification and chemical composition of the slime of *P. aeruginosa* strain BI as a prelude to determining the mode of action of the polysaccharide depolymerases and their use as an adjunct to chemical methods of fractionation in studying the chemical and biological characteristics of the slime polysaccharides.

MATERIALS AND METHODS

Microorganisms. *P. aeruginosa* strain BI was used in this study and has been described in a previous communication (7). The slime produced by this bacterium is known to serve as an excellent substrate, designated polysaccharide B, for polysaccharide depolymerase PDB_2 which is associated with *Pseudomonas* phage 2 infections (6). The depolymerase employed in this study was obtained from phage 2 lysates as previously described (7).

Extraction of slime. Slime was obtained from 3- to 4-day cultures of strain BI grown on sheets of cellophane overlaying Trypticase Soy agar (7). The slime was extracted in 0.15 M NaCl by gentle agitation in the presence of glass beads. After centrifugation at 12,000 × *g* for 30 min, the supernatant fluids were collected, dialyzed with three changes of distilled water in 48 hr, and treated with 3 volumes of ethanol in the presence of sodium acetate (10%) and glacial acetic acid (1%) at 4 C. The resulting precipitate was collected, suspended in distilled water, and again dialyzed. Trichloroacetic acid was added to the dialyzed material to a final concentration of 10%, and the precipitate that formed was discarded. After dialysis of the supernatant, the slime was reprecipitated with ethanol in the presence of sodium acetate and glacial acetic acid, as described above, then suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.2, and dialyzed against the same buffer.

Column chromatography. Three-milliliter samples of slime were placed on a Sephadex G-100 (Pharmacia Fine Chemicals) column (1.5 by 77.5 cm) with a bed volume of approximately 125 ml and a void volume of 25 ml. The column was equilibrated with 0.05 M Tris buffer, *p*H 7.2, and elution was accomplished at a flow rate of 8 drops/min in the same buffer. Five-milliliter fractions were collected. After gel filtration, selected fractions, identified by carbohydrate and protein tests, were further subjected to anion-exchange diethylaminoethyl (DEAE) cellulose (Cellex D, BioRad Laboratories). Columns (1.5 by 25 cm) were prepared and equilibrated with 0.05 M Tris buffer, *p*H 7.2. A 3-ml sample was added to the column, and 500 ml of buffer was then passed through the column, collecting 5-ml fractions at the rate of 7 drops/min. Linear gradient elution with increasing molarities of potassium chloride was established in a closed system using a magnetic stirrer to assure thorough mixing. The concentration of chlorides in the various fractions was estimated by titration with silver nitrate, with potassium chromate used as an indicator. Total hexoses were determined colorimetrically as glucose by the anthrone reaction (27). Determination of protein was based on the method of Lowry et al. (23), and the more highly purified fractions were assayed by ultraviolet-light absorption at 280 nm (20).

Identification and quantitation of sugars. Hydrolysates were prepared by suspending 2 mg of purified slime in 5 ml of 2 N HCl, by incubating at 110 C for 18 hr, and then by drying in vacuo over P_2O_5 and NaOH pellets.

The following chromatographic solvent systems were used: no. 1, *n*-butanol-95% ethanol-water (52:32:16); no. 2, ethyl acetate-acetic acid-water (3:1:3, upper phase only); no. 3, *n*-butanol-isopropanol-water (5:3:1); no. 4, ethyl acetate-pyridine-acetic acid-water (5:5:1:3). Descending chromatography was performed by using Whatman no. 1 paper. Sugars were detected by means of alkaline silver nitrate reagent (30).

Glucosamine was identified in hydrolysates prepared as described above after 24 hr of development in solvent no. 1 or no. 2 against a D-glucosamine-hydrochloride marker. Glucosamine and galactosamine were also identified by using a Technicon amino acid analyzer as described below. Neutral sugars were isolated by the method of Spiro (27). R_{glc} values were determined on the isolated sugars and compared to values of authentic samples in solvents no. 1 and no. 2. The individual sugars were quantitated by the anthrone method (27) from standards of authentic rhamnose, mannose, and glucose.

For the identification of the uronic acid, purified slime was hydrolyzed and dried as described above. The hydrolysate was developed on a cellulose thin-layer plate in solvent no. 3. The material remaining on the origin was eluted in deionized water and lyophilized. This material was hydrolyzed for a second time in 88% formic acid for 8 hr at 110 C and dried in vacuo over P_2O_5 and NaOH. Glucuronic acid was identified in this hydrolysate after 96 hr of development in solvent no. 1 against markers of D-glucuronic acid, as well as a hydrolysate of alginic acid [Matheson, Coleman, and Bell Co.] containing D-mannuronic and L-guluronic acids by the method of Linker and Jones (21). The presence of glucuronic

acid was also confirmed under similar conditions after development in solvent no. 4 for 24 hr.

Identification and quantitation of amino acids and amino sugars. Samples of purified slime for amino acid analysis were hydrolyzed in 6 N HCl for 20 hr at 110 C and dried in vacuo over P_2O_5 and NaOH pellets. Amino acids were identified on two-dimensional cellulose thin-layer chromatograms by using solvent systems of *n*-butanol-acetic acid-water (60:15:15) or 96% ethanol-34% ammonium hydroxide (7:3) for the first dimension and phenol-water (75:25 containing 25 mg of sodium cyanide per 100 ml) for the second dimension. Ninhydrin was employed for visualization. The amino acids and hexosamines were also analyzed by using a Technicon amino acid analyzer according to Piez and Morris (24). For this purpose, slime was hydrolyzed as described above to yield free amino acids and for 6 hr in 2 N HCl at 110 C for hexosamines.

Other quantitative estimations. Quantitation of uronic acid was made on unhydrolyzed samples of slime by the carbazole method of Bitter and Muir (9) against standards of D-glucuronic acid (K & K Laboratories). Determinations for RNA and DNA were made by the method of Schneider (26). Analyses were also performed for *N*-acetyl (8), *O*-acetyl (18), ketose (14), and sialic acid (29).

Serology. Rabbit immune sera were produced by intravenous inoculation of heat-inactivated (100 C, 1.5 hr) suspensions of strain BI, or by intramuscular injection of slime at a concentration of 20 mg/ml (0.5 ml at each of four sites) suspended in Freund's complete adjuvant (Difco). The antisera produced in this manner reacted with concentrations of slime ranging from 10 to 15 μg to 16 mg/ml. Ring test precipitation in capillary tubes was performed with dilutions of antigen in saline-layered immune serum, and agar-gel immunodiffusion was performed by using the immuno-plate, pattern B (Hyland Laboratories Los Angeles, Calif.; reference 18).

Passive-protection tests were performed in 18-g Swiss mice that were injected intraperitoneally with 0.5 ml of various dilutions of rabbit immune sera 4 hr before challenge with *P. aeruginosa* strain BI. In these experiments infection was accomplished by intraperitoneal injection of 4×10^9 viable organisms.

Enzyme and slime substrate interaction. Assays were based on the release of hexosamines (7) that were determined by the method of Rondle and Morgan (25) as modified by Boas (10). At concentrations of 20 mg/ml in 0.1 M sodium phosphate buffer (*p*H 7.5), purified slime polysaccharide was mixed with an equal volume of polysaccharide depolymerase PDB_2 (6) and incubated at 37 C. A standard of D-glucosamine hydrochloride (Eastman Organic Chemicals, Rochester, N.Y.) was included in each hexosamine determination.

RESULTS

Crude extracts of slime gave a positive anthrone test and a positive Lowry reaction, and the ratio of carbohydrate to protein was about 1:6. This ratio was increased to 1:4 after precipitation with ethanol, deproteinization with trichloroacetic acid, and reprecipitation with ethanol. The antigenic nature of the slime preparation was demonstrated by the production of precipitating antibodies after the immunization of rabbits. When the crude preparations of slime were tested against homologous rabbit antisera by the ring test, a sharp disc of precipitate was observed, even at high dilutions. When examined by immunodiffusion, by using antisera prepared against the crude slime, four bands were readily discernible. As previously reported (6), the extracted slime was found to be an excellent substrate for the *Pseudomonas* phage 2 depolymerase PDB_2, and its interaction with this enzyme was demonstrated by measuring the release of hexosamines.

A 3-ml sample (containing 9 mg of carbohydrate) of the ethanol-precipitated, trichloroacetic acid-treated slime was then introduced into a Sephadex G-100 column and eluted with 0.05 M Tris buffer, *p*H 7.2. The elution profile is illustrated in Fig. 1. Most of the carbohydrate was eluted as a single peak in fractions 7 to 11 immediately following the void volume, and, although protein was distributed among 6 peaks, the major protein peak appeared in fractions 7 to 11. The effluents containing the carbohydrate peak were then pooled, concentrated, and applied to a column composed of DEAE cellulose as described. The gradient elution profile obtained with potassium chloride is shown in Fig. 2. A minor carbohydrate peak appeared in fraction 5, overlapping a protein peak that was contained in fractions 5, 6, and 7. These effluents failed to give a positive ring precipitin reaction when tested with antisera prepared against crude slime. The nature of this material remains to be determined. However, it obviously does not represent the intact, antigenic slime molecule.

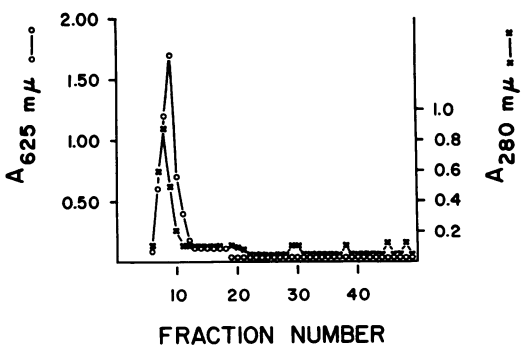

FIG. 1. *Elution profile from Sephadex G-100 column chromatography. Carbohydrates were determined with anthrone and measured by absorbance at 625 nm. Proteins were measured at 280 nm.*

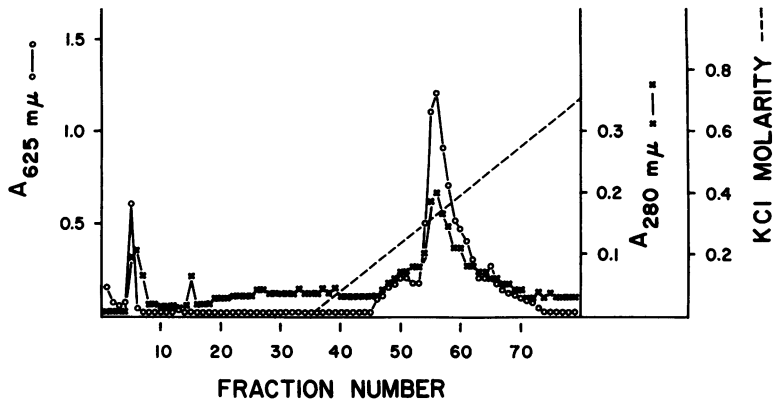

FIG. 2. *Elution profile from anion DEAE cellulose column with increasing molarities of potassium chloride. Carbohydrates were determined with anthrone and measured by absorbance at 625 nm. Proteins were measured at 280 nm.*

TABLE 1. *Passive-protection test in mice challenged with P. aeruginosa strain BI after injection of rabbit antiserum prepared against purified slime*

Dilution of antiserum (0.5 ml, ip[b])	Mortality of mice in days[a]					
	1	2	3	4	5	6
Undiluted	0/10	0/10	0/10	0/10	0/10	0/10
1/2	0/10	0/10	0/10	0/10	0/10	0/10
1/4	0/10	0/10	0/10	0/10	0/10	0/10
1/8	0/10	1/10	1/10	1/10	1/10	1/10
1/16	3/10	5/10	5/10	5/10	5/10	5/10
1/32	2/10	4/10	4/10	5/10	5/10	5/10
Normal serum, undiluted	6/10	10/10				

[a] Numerator indicates the cumulative number of mice that died and denominator indicates the number of mice used.

[b] Intraperitoneal.

A more pronounced carbohydrate peak was observed in fractions 54 to 61, being eluted at a potassium chloride molarity of 0.30 to 0.40. It should be noted that these same fractions contained a significant amount of protein. A strongly positive precipitin test was observed with fractions 54 to 61, but all other fractions were serologically negative, failing to react with antisera prepared against crude slime. When further tested by immunodiffusion, a pooled and concentrated specimen of fractions 54 to 61 showed only a single band when reacted with antisera prepared against crude slime, thus indicating the absence of other antigenic impurities that were present in the crude preparations of slime.

The immunization of rabbits with the purified slime resulted in the production of precipitating antibodies which, when reacted with crude slime

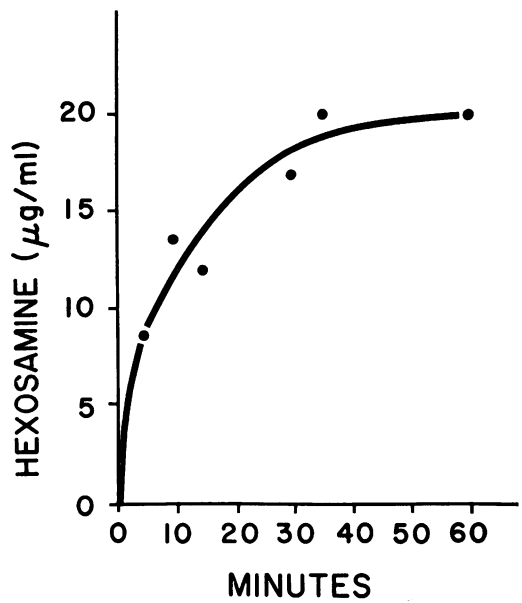

FIG. 3. *Release of hexosamines from purified slime in the presence of pseudomonas phage 2 depolymerase PDB₂.*

in immunodiffusion plates, produced a single band. These results suggest that the purified slime, as a homogeneous antigen, stimulated the production of a single species of antibody. It was also demonstrated that mice were protected against infection by *P. aeruginosa* strain BI after injection of rabbit antiserum prepared against the purified slime. The results of a typical experiment are shown in Table 1.

It was also of interest to reaffirm the substrate characteristic of slime by using purified prepara-

tions. Figure 3 depicts the release of hexosamines from the purified slime in the presence of the *Pseudomonas* phage 2 depolymerase PDB_2, confirming the suitability of the purified material as substrate for the enzyme.

Table 2 lists the chemical components found in acid hydrolysates of purified slime, and the results of the quantitative analyses as well. Glucuronic acid was identified as the only uronic acid present; accordingly, it represents all of the 13% value shown in the table for uronic acid. Since the total per cent of the two amino sugars present, glucosamine and galactosamine, equals 12%, the slime contains approximately equal amounts of uronic acid and hexosamine. In sharp contrast to other studies (11), only trace amounts of DNA or RNA could be detected in the purified slime. A considerable amount of protein was found to be associated with the carbohydrate moiety throughout the purification procedure. Hydrolysis of the protein revealed the presence of most of the known amino acids, with alanine being present in the highest concentration. Proline and cystine were absent. Two unknown ninhydrin-positive peaks appeared between histidine and arginine. The neutral sugars present in hydrolysates of purified slime were glucose, rhamnose, and small amounts of mannose. Rhamnose was present in the highest concentration, whereas only small amounts of mannose could be detected. The observed R_{glc} values of these sugars are presented in Table 3.

TABLE 2. *Components found in hydrolyzed preparations of purified slime derived from P. aeruginosa strain BI*

Component	Analysis
	% dry wt
Glucose	2
Rhamnose	10
Mannose	<1
Glucuronic acid	13
Glucosamine	8
Galactosamine	4
Total % sugar	37
Protein	18
N-acetyl	13
O-acetyl	<1
Ash	10
DNA	Trace
RNA	Trace

TABLE 3. *Observed R_{glc} values of neutral sugars found in purified slime of P. aeruginosa strain BI*

Sugar	R_{glc}	
	Solvent no. 1	Solvent no. 2
Glucose, authentic	1.00	1.00
Glucose, isolated	1.00	1.01
Rhamnose, authentic	2.07	2.84
Rhamnose, isolated	2.07	2.82
Mannose, authentic	1.26	1.27
Mannose, isolated	1.25	1.27

DISCUSSION

The results of this study indicate that the slime of *P. aeruginosa* strain BI consists mainly of polysaccharide and contains a significant amount of protein. In other studies the slime polysaccharide was principally obtained and purified by ethanol (11), ethanol-benzene (15), and cetylpyridinium (13) precipitation procedures. In the present study, the purification procedure additionally included gel filtration and ion-exchange chromatographic procedures to obtain more homogeneous preparations of slime for chemical analysis. This procedure resulted in the elution of the purified slime as a single peak at a potassium chloride molarity of 0.30 to 0.40. Immunodiffusion tests, which were employed throughout the procedure, demonstrated the elimination of antigenically contaminating substances. Only a single band was observed when the purified slime was reacted with antisera prepared against the crude slime. The purified slime appeared to be a homogeneous antigen, stimulating the production of antibody that produced only a single band when reacted with crude slime preparations. The antigenic nature of the slime polysaccharide was further demonstrated in mouse passive-protection tests using rabbit antisera. In addition, the purified slime remained consistent in regard to its character as substrate for the *Pseudomonas* phage 2 depolymerase PDB_2.

Hydrolysates of slime that were purified in this manner contain two hexosamines, glucosamine and galactosamine, as well as glucuronic acid. Of the neutral sugars, rhamnose was present in highest concentrations, with lesser amounts of glucose and mannose. Protein was a significant component, but only trace amounts of nucleic acid could be detected. These results fail to corroborate the findings of other investigators (11, 13, 15, 16, 21) and suggest that the observed differences in composition may, in part, reflect variations that exist from one strain to another. This was first suggested by the studies of Liu et al. (22) who observed protection of mice only when they were challenged with a homologous strain, but not a heterologous strain. Another line of evidence supporting this possible

heterogeneity in the slime polysaccharides was derived from enzymatic studies (6, 7) by using the phage depolymerases PDB_2 and PDA_8. This heterogeneity of slime polysaccharides was further indicated by studies (V. M. Young, R. Moody, D. M. Kenton, and G. Vermeulen, Bacteriol. Proc., p. 87, 1969) which established seven stable serotypes among *P. aeruginosa* strains isolated from human clinical conditions, with use of antisera prepared against extracted slimes. Serotyping was accomplished by passive-protection tests in mice.

The relationship between the slime layer of *P. aeruginosa* and infecting phage remains to be clarified. However, certain observations have been made that relate to the virus life cycle (6) and suggest the possibility that the slime layer may play a role in the adsorptive process. Experiments designed to establish the relationship between the slime layer and receptor sites of the host cell are in progress.

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LITERATURE CITED

1. Alexander, J. W., W. Brown, H. Walker, A. D. Mason, and J. A. Moncrief. 1966. Studies on the isolation of an infection-protective antigen from *Pseudomonas aeruginosa*. Surg. Gynecol. Obstet. **123**:965–977.
2. Alms, T. H., and J. A. Bass. 1965. Mouse protective antigen from *Pseudomonas aeruginosa*. Tex. Rep. Biol. Med. **23**:140.
3. Alms, T. H., and J. A. Bass. 1967. Immunization against *Pseudomonas aeruginosa*. I. Induction of protection by an alcohol-precipitated fraction from the slime layer. J. Infec. Dis. **117**:249–256.
4. Alms, T. H., and J. A. Bass. 1967. Immunization against *Pseudomonas aeruginosa*. II. Purification and characterization of the protective factor from the alcohol-precipitated fraction. J. Infec. Dis. **117**:257–264.
5. Bartell, P. F., and T. E. Orr. 1969. Origin of polysaccharide depolymerase associated with bacteriophage infection. J. Virol. **3**:290–296.
6. Bartell, P. F., and T. E. Orr. 1969. Distinct slime polysaccharide depolymerases of phage-infected *Pseudomonas aeruginosa*: evidence of close association with the structured phage particle. J. Virol. **4**:580–584.
7. Bartell, P. F., T. E. Orr, and G. K. H. Lam. 1966. Polysaccharide depolymerase associated with bacteriophage infection. J. Bacteriol. **92**:56–62.
8. Belcher, R., and A. L. Godbert. 1954. Semi-micro quantitative organic analysis, p. 42–44 and p. 123–125. Longmans Green and Co., London.
9. Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. **4**:330–334.
10. Boas, N. F. 1953. Method for the determination of hexosamines in tissues. J. Biol. Chem. **204**:553–563.
11. Brown, M. R. W., J. H. S. Foster, and J. R. Clamp. 1969. Composition of *Pseudomonas aeruginosa* slime. Biochem. J. **112**:521–525.
12. Brown, M. R. W., and R. M. E. Richards. 1964. Effect of polysorbate (tween) 80 on the resistance of *Pseudomonas aeruginosa* to chemical inactivation. J. Pharm. Pharmacol. London **16**: Supplement, p. 51T–55T.
13. Carlson, D. M., and L. W. Matthews. 1966. Polyuronic acids produced by *Pseudomonas aeruginosa*. Biochemistry **5**:2817–2822.
14. Dische, Z., and E. Borenfreund. 1951. A new spectrophotometric method for the detection and determination of keto sugars and trioses. J. Biol. Chem. **192**:583–587.
15. Doggett, R. G., G. M. Harrison, and E. S. Wallis. 1964. Comparison of some properties of *Pseudomonas aeruginosa* isolated from infections in persons with and without cystic fibrosis. J. Bacteriol. **87**:427–431.
16. Eagon, R. G. 1962. Composition of an extracellular slime produced by *Pseudomonas aeruginosa*. Can. J. Microbiol. **8**: 585–586.
17. Haynes, W. C. 1954. *Pseudomonas aeruginosa*—its characterization and identification. J. Gen. Microbiol. **5**:939–950.
18. Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochemistry, p. 22–96 and p. 493–495. Charles C Thomas, Publisher, Springfield, Ill.
19. Krause, R. M. 1963. Symposium on relationship of structure of microorganisms to their immunological properties. IV. Antigenic and biochemical composition of hemolytic streptococcal cell walls. Bacteriol. Rev. **27**:369–380.
20. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins, p. 447–454. *In* S. P. Colowick and N. D. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
21. Linker, A., and R. S. Jones. 1964. A polysaccharide resembling alginic acid from a *Pseudomonas* micro-organism. Nature **204**:187–188.
22. Liu, P. V., Y. Abe, and J. L. Bates. 1961. The role of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. J. Infec. Dis. **108**:218–228.
23. 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.
24. Piez, K. A., and L. Morris. 1960. A modified procedure for the automatic analysis of amino acids. Anal. Biochem. **1**: 187–201.
25. Rondle, C. J. M., and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. Biochem. J. **61**:586–589.
26. Schneider, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis, p. 680–684. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
27. Spiro, R. G. 1966. Analysis of sugars found in glycoproteins, p. 3–26. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
28. Sutherland, I. W., and J. F. Wilkinson. 1968. The exopolysaccharide of Klebsiella aerogenes $A_3(S1)$ (type 54): the isolation of *O*-acetylated octasaccharide, tetrasaccharide and trisaccharide. Biochem. J. **110**:749–754.
29. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta **24**:605–611.
30. Trevelyan, W. E., D. P. Procter, and J. S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature **166**: 444–445.