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Composition of *Pseudomonas aeruginosa* Slime

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1. The slime produced by eight strains of *Pseudomonas aeruginosa* on a number of different media was demonstrated to be qualitatively the same. Small quantitative differences may be occasioned by differences in the extraction procedure, the growth medium or the strain of organism used. 2. The slime was shown to be predominantly polysaccharide with some nucleic acid material and a small amount of protein. 3. The hydrolysed polysaccharide fraction consists mainly of glucose with smaller amounts of mannose. This accounts for some 50–60% of the total slime. In addition, there is some 5% of hyaluronic acid. The nucleic acid material represents approx. 20% of the total weight, and is composed of both RNA and DNA. 4. Minor components are protein, rhamnose and glucosamine, the protein being less than 5% of the total. 5. Hyaluronic acid is produced in greater quantities from nutrient broth than from chemically defined media, and is more firmly attached to the cells than the other components.

A characteristic of liquid cultures of *Pseudomonas aeruginosa* is that they become viscous after a few days. This property is the result of extracellular slime. Haynes (1951) found that all strains of *P. aeruginosa* he tested produced slime in a gluconate–tryptone broth, but not in glucose media. The gluconate was oxidized to 2-oxogluconate; slime production and formation of 2-oxogluconate from gluconate were regarded as reliable taxonomic characters of the species.

The literature on the composition of the slime is contradictory. Eagon & Randles (1954) reported that *Pseudomonas* species produced large amounts of polysaccharide slime, which was shown to consist largely of mannan. Subsequently Eagon (1956) showed slime composed entirely of mannose residues was produced in a mineral salts medium from a variety of low-molecular-weight carbon sources, but not from carbohydrates. He did not detect uronic acids or amino sugars. Later, Eagon (1962) showed that, in addition to the mannose, which he estimated to account for about 50% of the material, the slime contained appreciable amounts of nucleic acid (mostly DNA) and small amounts of protein.

Warren & Gray (1954, 1955) demonstrated that *P. aeruginosa* produced a polysaccharide that could be degraded by hyaluronidase. Bonde, Carlsen & Jensen (1957) found that *P. aeruginosa* produced a polysaccharide that they concluded to be hyaluronic acid.

In addition to these workers, Halleck, Durkin & Guschlbauer (1961) stated that the slime produced by two strains of *Pseudomonas* species was highly polymerized DNA. Carlson & Matthews (1966) have reported that *P. aeruginosa* slime is a polymer composed of uronic acids. Klyhn & Gorrill (1967) reported that the slime produced by several strains of *P. aeruginosa* contained several sugars: glucose, mannose, fucose, galactose, ribose and rhamnose, together with two hexosamines that they identified as glucosamine and galactosamine.

The slime of *P. aeruginosa* has been implicated in resistance to chemotherapy (Brown & Richards, 1964) and in the toxicity and pathogenicity of this organism (Liu, Abe & Bates, 1961). The present paper attempts to elucidate the conflicting reports on the composition of the slime by using eight strains of *P. aeruginosa* and various cultural conditions.

MATERIALS AND METHODS

Materials and organisms. Eight strains of *Pseudomonas aeruginosa* were used in this study. Strains N.C.T.C. 7244, 6750, 8203 and 1999 were obtained as freeze-dried desiccated samples from the National Collection of Type Cultures, London N.W.9; strain O.S.U. (Ohio State University) 64 was obtained from Professor R. G. Eagon, Department of Bacteriology, University of Georgia, Athens, Ga., U.S.A.; strains CST 651, 652 and 653 were clinical isolates obtained from the Bristol Public Health Laboratory.

The organisms were grown in 100ml. of either Oxoid Nutrient Broth no. 2 (C.M. 4) (Oxoid Ltd., London S.E.1) with 1% (w/v) glucose added or a chemically defined medium containing $(NH_4)_2HPO_4$ (0·6g.), NaCl (1g.),

KH_2PO_4 (0·4g.), $MgSO_4,H_2O$ (0·4g.) and water to 1l. in 1l. flasks for 5 days at 37° without shaking. Various carbon sources were added to the chemically defined media as required at a concentration of 4%.

All reagents were AnalaR grade (British Drug Houses Ltd., Poole, Dorset) unless otherwise stated. The potassium gluconate used as carbon source was B.P.C. grade and obtained from Pfizer Chemicals Ltd., Sandwich, Kent.

Extraction of slime. Five-day-old static cultures of *P. aeruginosa* in liquid media were treated in the following manner. The slime was removed from the bacteria either by treatment for 30sec. in an Ato-Mix homogenizer (Measuring and Scientific Equipment Ltd., London S.W.1) or by shaking for 30min. at 37° with 10% (v/v) ethylene glycol. The cells were removed by centrifuging at 2000*g* for 2hr. at 20°. Slime was precipitated from the supernatant by the addition of an equal volume of ethanol. After 24hr. at 4° the supernatant was decanted and the slime redissolved in the minimum volume of hot (60°) 0·1% KOH. When cold (18°) the slime was reprecipitated with an equal volume of ethanol. The precipitate was kept overnight at 4° and the supernatant decanted, the slime washed with 90% (v/v) ethanol, ethanol and finally ether. After drying over P_2O_5 and KOH *in vacuo*, the yield of slime was determined by weighing. The slime was stored in a desiccator over P_2O_5.

Identification of amino sugar. Hydrolysates (6M-HCl for 16hr. at 100°) were examined by descending paper chromatography with the solvent butan-1-ol–ethanol–water (4:1:2, by vol.) containing 1% (v/v) of aq. NH_3 (sp. gr. 0·88) on Whatman 3MM paper for 18hr. Spots were located by means of the Elson–Morgan reagent.

Identification of sugars. Samples of slime were hydrolysed by means of Dowex 50 (X4: H^+ form) ion-exchange resin of 200–400 mesh (Clamp & Putnam, 1964). The hydrolysate was examined by descending paper chromatography with the following solvents: butan-1-ol–ethanol–water (4:1:2, by vol.) containing 1% (v/v) of aq. NH_3 (sp.gr. 0·88) and ethyl acetate–pyridine–water (12:5:4, by vol.) on Whatman 3MM paper for 18hr. The sugars were located by means of a *p*-anisidine–HCl reagent (Hough, Jones & Wadman, 1950).

Mucopolysaccharides. The mucopolysaccharides were precipitated by adding cetylpyridinium chloride to a final concentration of 0·2% to a solution (100mg. in 100ml.) of the slime. The precipitate was washed with ethanol saturated with potassium acetate after settling for 18hr. at 4° (Di Ferranti, 1967). The precipitate was dried as described above. The mucopolysaccharides in the precipitate were identified by means of zone electrophoresis, paper chromatography and their i.r. spectra.

Zone electrophoresis. The precipitate from the cetylpyridinium chloride precipitation was dissolved in 2ml. of water and 10μl. applied 3cm. from the anode end of cellulose acetate electrophoresis strips (2·5cm.×20cm.) (Oxoid Ltd., London S.E.1). The strips were placed on special carriers in an EEL electrophoresis tank. Electrophoresis was carried out with sodium barbitone buffer, pH8·6 and I 0·25. A current of 1mA/strip at 200v was applied for 3hr. Hyaluronic acid and chondroitin sulphate [Sigma (London) Chemical Co., London S.W.6] were used as markers. The strips were removed and dried between glass plates at 100° for 30min. The strips were stained in 1% Alcian Blue in 90% (v/v) ethanol containing 5% (v/v) of acetic acid for 10min., and the background was destained with 5% (v/v) acetic acid until almost white. A further sample (5mg.) was digested with 3000 units of hyaluronidase (Rondase; Evans Medical Ltd., Speke, Liverpool) in 5ml. of McIlvaine (1921) phosphate buffer, pH7·4, for 18hr. at 37° before electrophoretic examination.

Chromatography. The solution prepared above was examined by ascending paper chromatography in a solvent composed of 37% (v/v) propan-1-ol in 67mM-potassium phosphate buffer, pH6·5, on Whatman no. 1 paper for 48hr. (Kerby, 1954). Markers of hyaluronic acid and chondroitin sulphate were used. After chromatography the chromatograms were air-dried for 24hr. and stained by the toluidine blue technique of Leitner & Kerby (1954).

Infrared spectra. The i.r. spectra of the cetylpyridinium chloride precipitates were obtained by using a Unicam SP.200 spectrophotometer. The samples were dispersed in anhydrous KBr and the mixture was compressed into a disk. Pure hyaluronic acid from human umbilical cords was used as a reference sample.

Quantitative examination of the slime preparations. The protein content of the slime was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951). The sugar content was assayed as total reducing sugar in terms of glucose by the Somogyi (1945) colorimetric method with the Nelson (1944) arsenomolybdate colour reagent after the resin hydrolysate was deproteinized with trichloroacetic acid. Additionally glucose was assayed in the deproteinized hydrolysate by the colorimetric glucose oxidase method of Huggett & Nixon (1957). The hyaluronic acid content was assayed in terms of uronic acid (Bitter & Muir, 1962) and also of amino sugar (Gatt & Berman, 1966) with the cetylpyridinium chloride precipitate. DNA was estimated by the diphenylamine reaction (Burton, 1956) after fractionating the slime by the method of Schneider (1957). RNA was estimated by the orcinol reaction (Schneider, 1957). Ash values were also determined.

RESULTS

Yields. All strains grown on glucose broth produced slime in approximately equal amounts (1·2–1·3g./l. of culture).

All strains grown on chemically defined media produced slime in approximately equal amounts when the carbon source was gluconate, acetate, citrate, glycerol or other low-molecular-weight organic compounds but slime was not produced when glucose or other sugar was carbon source. When the glucose was supplemented with Oxoid yeast extract (1%), potassium nitrate (1%) and sodium nitrate (1%) the slime was produced. The yield for all strains from the chemically defined media was constant at 1·1–1·2g./l. of culture, whatever the carbon source.

Composition of the slime. All of the slime samples produced by the eight different strains of *P. aeruginosa* on the different culture media had qualitatively the same composition. All gave a strongly positive Molisch test and a positive biuret reaction. The only amino sugar identified was

glucosamine. In addition glucose, mannose, glucuronic acid, deoxyribose, ribose and traces of rhamnose were identified in the hydrolysates. Table 1 gives the R_{Glc} values observed for these sugars. Gas – liquid - chromatographic analysis (Clamp, Dawson & Hough, 1967) of representative samples of the slime showed glucose to be present on all occasions and mannose on all but one.

Examination of the cetylpyridinium chloride precipitate

Zone electrophoresis. All of the samples showed a single distinct band that migrated 5cm. towards the anode. This corresponded to the hyaluronic acid marker, but had a mobility less than that of the chondroitin sulphate. This disappeared after digestion with hyaluronidase.

Paper chromatography. The only component identified by this method was hyaluronic acid, which showed as a clear blue spot against the light-blue background.

Infrared spectra. These were obtained from the samples and from the reference sample of hyaluronic acid. This method has been used to identify mucopolysaccharides from various sources (Orr, 1954; Clausen & Hansen, 1963; Clausen & Anderson, 1963), and the results obtained here indicated that this method gave useful information. The samples here were clearly identical with hyaluronic acid, showing distinct and characteristic peaks at 3400, 2900, 1640, 1420, 1160 and 1040cm.$^{-1}$, with a noticeable absence of any strong absorption in the 'fingerprint' area, below 1000cm.$^{-1}$.

Table 1. *Observed R_{Glc} values of sugars found in* P. aeruginosa *slime*

Values were determined by descending chromatography on Whatman 3MM paper for 18hr. in two solvents. Location reagent: *p*-anisidine–HCl.

	R_{Glc}	
Sugar	Butan-1-ol–ethanol–water (4:1:2, by vol.) containing 1% (v/v) of aq. NH_3 (sp.gr. 0·88)	Ethyl acetate–pyridine–water (12:5:4, by vol.)
Glucuronic acid	15	20
Glucose	100	100
Mannose	130	125
Ribose	200	158
Rhamnose	280	193
Deoxyribose	300	220

Quantitative analysis

Table 2 shows the results of the quantitative analysis of the slime samples. These results show that qualitatively all of the slime samples produced by the different strains of *P. aeruginosa* from the different media are of similar composition. Differences of media and extraction procedure caused more differences in quantitative composition than did strain differences.

The glucose broth gave a higher protein content than did the chemically defined media with gluconate as carbon source (3–4% as against 1–2%).

The hyaluronic acid content was higher from glucose broth than from chemically defined media.

Table 2. *Quantitative analysis of* P. aeruginosa *slime*

Ranges of results with eight strains, grown on two different media and extracted by treatment with ethylene glycol or by homogenization, are shown.

		Analysis (% of dry wt. of slime)			
		Nutrient broth with glucose		Gluconate–mineral salts medium	
Component	Coefficient of variation* (%) Extraction method ...	Ethylene glycol	Homogenization	Ethylene glycol	Homogenization
Reducing sugar (as glucose)	±1·7	52–54	52–54	49–51	49–51
Glucose†	±5·4	39–42	39–42	44–46	44–46
Protein	±4·15	3–4	3–4	1–2	1–2
Hyaluronic acid‡	±6·03	5–6	2–3	3–4	1–2
Hyaluronic acid§	±7·6	6–7	3–4	4–5	2–3
DNA	±5·6	11–12	11–12	10–11	10–11
RNA	±5·2	8–9	8–9	7–8	7–8
Ash	—	13–15	13–15	12–14	12–14

* Coefficient of variation calculated from six replicate determinations.
† By glucose oxidase method.
‡ Calculated from uronic acid assay.
§ Calculated from glucosamine assay.

Here the method of extraction influenced the results considerably. About twice as much hyaluronic acid was obtained when the slime was removed from the cells by shaking with ethylene glycol as when it was removed by homogenization. In addition, the hyaluronic acid values were all higher when calculated from the amino sugar (glucosamine) values than from the uronic acid (glucuronic acid) values. This suggested that some free glucosamine was present in the slime, over and above that which could be accounted for by the hyaluronic acid.

The DNA content was constant at 11–12% for the glucose broth and 10–11% for the chemically defined media. The RNA content was 8–9% for the glucose broth and 7–8% for the chemically defined media. Ash values were 13–15% for glucose broth and 12–14% for chemically defined media.

The reducing sugar, assayed in terms of glucose, was similarly constant at 52–54% for the glucose broth and 49–51% for the chemically defined media. The only reducing sugars identified in the hydrolysate were glucose and mannose. This value therefore represented the sum of the two components.

The glucose alone assayed by the glucose oxidase method was shown to be 39–42% for the glucose broth and 44–46% for the chemically defined media. This represents some 70–75% of the reducing sugar for the slime produced in glucose broth, and approximately 90% of the reducing sugar for the slime produced in the chemically defined media.

The glucose/mannose ratio determined by gas–liquid chromatography (Clamp *et al.* 1967) was 3–4 in the slime from glucose broth and about 10 in the slime from the chemically defined media. In one sample of this latter group mannose could not be detected.

DISCUSSION

The present results show clearly that the slime produced by *P. aeruginosa* is predominantly polysaccharide, with smaller amounts of nucleic acids and protein.

The slime samples from eight strains of *P. aeruginosa* grown in a variety of culture media were all qualitatively similar, but there were small quantitative differences. The differences may be accounted for by small errors in the analytical procedures, and were not significantly greater than the coefficient of variation for each assay (Table 2).

The presence of hyaluronic acid was demonstrated. It was the only fraction to be influenced by the extraction procedure, being present in twice the amount in slime samples prepared by extraction with ethylene glycol. This is a more vigorous extraction procedure, and will therefore extract material that is more closely bound to the cell surface, compared with the vigorous agitation in the homogenizer (Wilkinson, 1958). This being so, it is possible that the hyaluronic acid fraction is more closely bound to the cell than the other polysaccharide and nucleic acid fractions. Indeed, since hyaluronic acid has been shown to be a component of the capsule of Gram-positive organisms (Lancefield, 1943), it may be that this component is more capsule-like, possibly corresponding to a micro-capsule.

There is an apparent conflict between our results and those of Eagon (1956, 1962), who specifically excluded uronic acid and demonstrated mannose as the only sugar present in the hydrolysed slime. He thus concluded that the slime is largely composed of a polysaccharide comprising mannose residues.

The explanation probably lies in the method used to hydrolyse the slime, since the uronic acid can easily be degraded by treatment with sulphuric acid under the conditions used by Eagon (1956, 1962). A similar degradation of uronic acid by sulphuric acid has been observed by Carlson & Matthews (1966). Likewise the methods used by Eagon (1956, 1962) to identify the mannose and to estimate sugar [osazone; determination by the Somogyi method (Nelson, 1944)] are applicable equally to glucose and mannose.

It is difficult to explain the difference between our paper-chromatographic results and those of Eagon (1956, 1962) and Carson & Eagon (1964). However, our results, showing both glucose and mannose, are confirmed by both the glucose oxidase assay for the glucose and the glucose/mannose ratios by gas–liquid chromatography. In addition our results were confirmed with a sample of slime kindly supplied by Professor Eagon, which showed no significant differences from our preparations.

The main polysaccharide fraction clearly contains both glucose and mannose. The gas–liquid-chromatographic analysis of representative slime samples showed that the ratio of the one to the other was variable. It is impossible from the evidence presented here to determine whether the glucose and mannose units are present as a copolymer, or if the polysaccharide is a mixture of a glucan and mannan. There was more glucosamine than could be accounted for on the basis of the hyaluronic acid (calculated from the glucuronic acid assay). This may be a component of the polysaccharide, as indicated by Klyhn & Gorrill (1967), or it may arise from the cell walls, being released by cell lysis. The same explanation may apply to the traces of rhamnose identified in the slime hydrolysates, since these two sugars have been identified in cell-wall fractions of *P. aeruginosa* (Salton, 1964; Clarke, Gray & Reaveley, 1967). In addition, aging cultures of *P. aeruginosa* produce a rhamnose-containing glycolipid (Jarvis & Johnson, 1949;

Edwards & Hayashi, 1965) and this may have been produced in small amounts by the cultures from which the slime was extracted, contributing to the rhamnose identified in the hydrolysates.

The ribose and deoxyribose in the hydrolysates can be explained satisfactorily on the basis of the nucleic acid component of the slime, but possibly may have arisen from GDP derivatives occurring as intermediates in the synthesis of the polysaccharide from the monosaccharides.

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