Amino Sugars in the Cell Walls of *Pseudomonas* Species

By S. G. WILKINSON AND K. A. CARBY

Department of Chemistry, University of Hull, Hull, HU6 7RX

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SUMMARY

Fucosamine has been identified as a component of the walls of *Pseudomonas denitrificans* and *P. fragi*, and quinovosamine as a component of the walls of *P. fluorescens*, *P. mucidolens*, *P. putida*, *P. stutzeri*, *P. syncyanea* and *P. synxantha*. A third amino sugar, present in the walls of *P. synxantha* and possibly in those of *P. fluorescens*, was apparently 3-amino-3,6-dideoxyglucose.

INTRODUCTION

Bacterial walls contain a wide variety of unusual amino sugars, including 2-amino-2,6-dideoxyhexoses and 3-amino-3,6-dideoxyhexoses. In Gram-negative bacteria such compounds occur in the lipopolysaccharide of the wall (Lüderitz, Jann & Wheat, 1968). Although lipopolysaccharides from members of the Enterobacteriaceae have been the most widely studied, the finding of fucosamine (2-amino-2,6-dideoxygalactose) in lipopolysaccharides from strains of *Pseudomonas aeruginosa* (Fensom & Gray, 1969; Suzuki, 1969) suggested that such compounds might be widely distributed. During a study of the walls of *Pseudomonas* species sensitive to ethylenediaminetetraacetic acid (Wilkinson, 1970), several unidentified compounds thought to be amino sugars were detected. This paper reports the identification of two of these compounds (previously designated Unknowns IV and VI) as quinovosamine (2-amino-2,6-dideoxyglucose) and fucosamine, respectively. A third compound (Unknown V) is apparently 3-amino-3,6-dideoxyglucose. Since the completion of this work, the isolation of D-quinovosamine from a strain of *P. aeruginosa* has been reported (Suzuki, Suzuki & Fukasawa, 1970).

METHODS

Organisms and preparation of cell walls. Walls were prepared from *Pseudomonas denitrificans* (NCIB 8376), *P. fluorescens* (NCTC 10038), *P. fragi* (NCIB 8542), *P. mucidolens* (NCTC 8068), *P. putida* (NCIB 9034), *P. stutzeri* (NCIB 9040), *P. syncyanea* (NCTC 9943) and *P. synxantha* (NCIB 8178) as described previously (Wilkinson, 1970).

Isolation of amino sugars. The following procedure was used to isolate samples of Unknown IV (from *Pseudomonas putida*), Unknown VI (from *P. fragi*), a mixture of Unknowns IV and V (from *P. synxantha*), and fucosamine (from the specific polysaccharide of *Pneumococcus* type XII; Cifonelli, Rebers, Perry & Jones, 1966). The walls and pneumococcal polysaccharide were hydrolysed for 4 h. at 105° with 6·1 N-HCl. After neutralization with Dowex 2 resin in the bicarbonate form, the hydrolysates were filtered and dried. The residues were subjected to preparative electrophoresis on Whatman no. 3 paper for 2 h. at about 20 V/cm., using aqueous pyridine + acetic acid buffer (pH 5·3; Lüderitz *et al.* 1968). Substances having a mobility equal to or slightly greater than that of glucosamine were detected by spraying guide strips with ninhydrin and were eluted. Amino sugars present

in the eluates were separated from each other (except for Unknowns IV and V from *P. synxantha*) and from basic amino acids by preparative paper chromatography on water-washed Whatman no. 1 paper, using solvent system A descending for 12 to 14 h. Amino compounds were again located by spraying guide strips with ninhydrin, and were eluted using deionized water.

Paper chromatography. Analytical chromatography was done on Whatman no. 1 paper using the following solvent systems: A, ethyl acetate + pyridine + water + acetic acid (5 + 5 + 3 + 1, by vol.); B, *n*-butanol + acetic acid + water (5 + 1 + 2, by vol.); C, *n*-butanol + pyridine + water (6 + 4 + 3, by vol.); D, *n*-butanol + ethanol + water (13 + 8 + 4, by vol.); E, *sec*-butanol + 88 % formic acid + water (15 + 3 + 2, by vol.); F, phenol + water + aq. ammonia sp.gr. 0.88 (80 + 20 + 1, by wt); G, the upper phase of ethyl acetate + pyridine + water (5 + 2 + 5, by vol.); H, the upper phase of *n*-butanol + ethanol + water + aq. ammonia sp.gr. 0.88 (40 + 10 + 49 + 1, by vol.). Spots were detected using ninhydrin, alkaline AgNO₃ (Trevelyan, Procter & Harrison, 1950), aniline phosphate, the Elson-Morgan reagents (Partridge, 1948) and the reagents of Edward & Waldron (1952).

N-acetylation of amino sugars. This was done as described by Strominger, Park & Thompson (1959). Solutions containing the *N*-acetyl derivatives were deionized by passage down columns containing Dowex 50 resin (hydrogen form) overlying Dowex 2 resin (bicarbonate form).

Reduction of amino sugars. Reduction using NaBH₄ was done by the method of Jann & Jann (1968). The amino sugar alcohols formed were subjected to high voltage electrophoresis in molybdate buffer (pH 5.0) as described by Mayer & Westphal (1968). Spots were detected using alkaline AgNO₃.

Degradation of amino sugars by ninhydrin. This was done by the method of Spiro (1966). Neutral sugars formed were detected after paper chromatography using solvent systems G and H.

Oxidation of amino sugars by periodate. To a sample (about 0.25 μ mole) of amino sugar dissolved in cooled, deionized water (100 μ l.) was added 17 mm-NaIO₄ (AnalaR, 150 μ l.), and oxidation was allowed to proceed in the dark at 4°. Samples (10 μ l.) were taken periodically for the estimation of residual periodate by the method of Avigad (1969). Further samples (50 μ l.) were eventually taken for the estimation of acetaldehyde by the method of Kabat & Mayer (1961): the formation of acetaldehyde was confirmed by using a modification of the method (Simmons, 1969) which incorporates a micro-distillation.

Analytical methods. Amino sugars were estimated by reaction with ninhydrin using a Technicon AutoAnalyzer. The method of Park & Johnson (1949) was used for the estimation of reducing sugars and the method of Rondle & Morgan (1955) for the estimation of 2-amino-2-deoxyhexoses, using glucosamine as a standard in both cases. The reaction with cysteine-H₂SO₄ (Dische, 1962) was used for the detection of 6-deoxyhexoses, and the method of Ashwell, Brown & Volk (1965) as a test for 3-acetamido-3-deoxyhexoses.

RESULTS

The unidentified, ninhydrin-positive compounds Unknowns IV, V and VI were considered to be amino sugars because of their behaviour during ion-exchange chromatography using the AutoAnalyzer (Wilkinson, 1970). Unknown IV apparently occurred in *Pseudomonas fluorescens*, *P. mucidolens*, *P. putida*, *P. stutzeri*, *P. synxantha* and *P. synxantha*, Unknown V in *P. fluorescens* and *P. synxantha*, and Unknown VI in *P. denitrificans* and *P. fragi*. For the isolation and detailed study of these compounds, previously analysed

batches of walls (Wilkinson, 1970) from the following bacteria were chosen: *P. putida* (because it contained the largest amount of Unknown IV), *P. synxantha* (because the alternative, *P. fluorescens*, contained galactosamine in addition to Unknown V and the other amino sugars present in *P. synxantha*), and *P. fragi* (because it contained rather more Unknown VI and less galactosamine than *P. denitrificans*). Hydrolysates of walls from the other species were used for making chromatographic comparisons of amino sugars.

Crude hexosamine-containing fractions were isolated from hydrolysates of walls from *Pseudomonas putida*, *P. synxantha* and *P. fragi* by high voltage electrophoresis, and were examined by descending paper chromatography using solvent system A. In addition to glucosamine, galactosamine and some basic amino acids, the fraction from *P. putida* had a ninhydrin-positive component with a mobility similar to that of mannose. The fraction from *P. fragi* contained glucosamine, galactosamine, basic amino acids and a ninhydrin-positive component with a mobility similar to that of galactose. The two unidentified components were isolated and shown, by using the AutoAnalyzer, to correspond to Unknowns IV and VI. Except for the absence of galactosamine, the hexosamine-containing fraction from *P. synxantha* resembled that from *P. putida* on paper chromatography. However, the material isolated from the region of a chromatogram corresponding to Unknown IV was shown, by using the AutoAnalyzer, to contain both Unknowns IV and V. Chromatographic and electrophoretic data for the three compounds are given in Table 1.

Table 1. *Chromatographic and electrophoretic properties of amino sugars*

Analytical method	$R_{\text{glucosamine}}$		
	Unknown IV	Unknown V	Unknown VI
Paper chromatography*			
Solvent A	1.40 ± 0.05	c. 1.35	1.21 ± 0.04
Solvent B	1.80 ± 0.05	c. 1.70	1.49 ± 0.03
Paper electrophoresis at pH 5.3	1.06 ± 0.02	c. 1.06	1.13 ± 0.02
Autoanalysis (21 h. elution programme)	1.23 ± 0.02	1.27 ± 0.02	1.34 ± 0.02

* A tendency to streak or form double spots was observed when solvents C, D and G were used.

Although purple spots were eventually formed for all three Unknowns after treating chromatograms with ninhydrin, the colours produced initially were: Unknown IV, grey-pink; Unknowns V and VI, brown. All three compounds reduced alkaline AgNO_3 (Unknown V more strongly than Unknown IV) but gave no detectable reaction with aniline phosphate. Unknowns IV and VI gave a positive reaction with the Elson-Morgan reagents for 2-amino-2-deoxy sugars; Unknown V seemed to give little or no colour, but a firm conclusion was not possible because of the poor separation from Unknown IV on chromatograms. The relatively high mobilities of the compounds on paper chromatograms suggested that they might be 6-deoxyhexosamines, and this was confirmed for Unknowns IV and VI. Both compounds gave positive reactions with reagents A (but not B or C) of Edward & Waldron (1952), gave products with weak extinction maxima (relative to that for rhamnose) at about 400 nm. in the cysteine- H_2SO_4 reaction, and gave acetaldehyde on oxidation with periodate. By assuming that the colour yields on reaction of Unknowns with ninhydrin were equal to that for glucosamine in order to calculate the amounts of amino sugars oxidized, the yields of acetaldehyde after 48 h. were 71 % (Unknown IV) and 79 % (Unknown VI). By using the same assumption, the colour yields for Unknowns IV and VI, respectively, relative to those for glucosamine in an estimation of reducing sugars (Park & Johnson,

1949) were 90 % and 85 %, and in an estimation of 2-amino-2-deoxyhexoses (Rondle & Morgan, 1955) were 94 % and 92 %. In the latter estimation, the extinction maximum (530 nm.) of the products from the Unknowns was the same as that for glucosamine.

A comparison of $R_{\text{glucosamine}}$ values (Table 1) for Unknowns IV and VI with those reported for 2-amino-2,6-dideoxyhexoses (Lüderitz *et al.* 1968) was insufficient to identify the compounds. Comparisons of the Unknowns with authentic quinovosamine and fucosamine were therefore made by the following methods: (a) descending, one-dimensional paper chromatography using solvent systems A, B, C, D and F, and ascending, two-dimensional paper chromatography using solvent systems E followed by F; (b) high voltage paper electrophoresis at pH 5.3; (c) ion-exchange chromatography using the AutoAnalyzer; (d) descending, one-dimensional paper chromatography of the *N*-acetyl derivatives using solvent systems A, B and C; (e) descending, one-dimensional paper chromatography of the products of ninhydrin degradation using solvent systems G and H; (f) high voltage paper electrophoresis in molybdate buffer of the amino sugar alcohols. In all tests Unknown IV had the properties of quinovosamine and Unknown VI those of fucosamine. The combination of methods used differentiates these amino sugars from other known 2-amino-2,6-dideoxyhexoses (Jann & Jann, 1968; Lüderitz *et al.* 1968; Suzuki, 1969). The amounts of Unknowns available were too small for the determination of optical rotation or for confirmation of their identities by the ion-exchange technique of Crumpton (1959). However, by the latter technique using a column of Dowex 50 resin packed without the application of pressure, Unknown IV from the lipopolysaccharide of *Pseudomonas stutzeri* had $R_{\text{glucosamine}}$ 1.38 (S. G. Wilkinson and G. A. Lightfoot, unpublished results) as expected for quinovosamine (Wheat, 1966). Although Unknowns IV and VI were not isolated from the walls of other species in which they occurred, limited studies on hydrolysates indicated that these components were the same as for *P. putida* and *P. fragi*. For example, ninhydrin degradation of hydrolysates of all walls containing Unknown IV gave rise to a new neutral sugar which reduced alkaline AgNO_3 , reacted with aniline phosphate to give a brown spot which had a strong blue-white fluorescence under ultraviolet light, and had the chromatographic mobility of 5-deoxyarabinose.

Although Unknowns IV and V were clearly separated using the AutoAnalyzer, a separation of the amino sugars (or their *N*-acetyl derivatives) adequate for preparative purposes was not achieved. Consequently, studies on Unknown V have largely been confined to the mixture isolated from *Pseudomonas synxantha*. Like Unknowns IV and VI, Unknown V was reasonably stable to acid: it had first been detected in hydrolysates prepared using 6*N*-HCl for 16 h. at 105°. This fact suggested that it was more likely to be a 2- or 3-amino sugar rather than a 4-amino sugar (Jann & Jann, 1967). After ninhydrin degradation of the mixed Unknowns IV and V only 5-deoxyarabinose was detected. The possibility that Unknown V was rhamnosamine, which, like quinovosamine, would give 5-deoxyarabinose on degradation, was eliminated by electrophoresis of the amino sugar alcohols in molybdate buffer. Initially only 2-amino-2,6-dideoxyglucitol (from quinovosamine) was detected, but there was ionic interference with the detection of possible cationic spots. After removal of inorganic ions by prior electrophoresis in pyridine + acetic acid buffer (pH 5.3), a second component was detected after electrophoresis in molybdate buffer. The mobility of this component ($R_{\text{glucosaminol}}$ 0.29) suggested that it might be 3-amino-3,6-dideoxyglucitol ($R_{\text{glucosaminol}}$ 0.31; Mayer & Westphal, 1968). The component was eluted and shown to yield acetaldehyde on oxidation by periodate. Further evidence that Unknown V was a 3-amino hexose was obtained by the method of Ashwell *et al.* (1965): the reactivity of the mixed *N*-acetyl derivatives of Unknowns IV and V with the Morgan-Elson reagents was

significantly greater after periodate oxidation than before. The *N*-acetyl derivative of quinovosamine would only react before oxidation, whereas the derivative of a 3-amino hexose would only react after oxidation. Unknown V could not be distinguished from authentic 3-amino-3,6-dideoxyglucose by the methods (a) to (f) used in the identification of Unknowns IV and VI.

DISCUSSION

Quinovosamine has previously been found in *Achromobacter georgiopolitanum* (Smith, 1964; Colwell, Smith & Chapman, 1968), strains of *Salmonella*, Arizona and *Proteus vulgaris* (Lüderitz *et al.* 1968), and *Pseudomonas aeruginosa* (Suzuki *et al.* 1970). In most cases the amino sugar has been shown to occur in lipopolysaccharides. Similarly, fucosamine has been identified as a component of lipopolysaccharides from *Chromobacterium violaceum* (Crumpton & Davies, 1958), *Escherichia coli* (Ørskov *et al.* 1967) and *P. aeruginosa* (Fensom & Gray, 1969; Suzuki, 1969) and of other bacterial polysaccharides. The location of these amino sugars within the cell wall was not studied during the present work, but further studies (S. G. Wilkinson, L. Galbraith and G. A. Lightfoot, unpublished results) have shown that quinovosamine occurs in the lipopolysaccharide fractions from *P. stutzeri* and *P. synxantha*. Thus the present results and those of Suzuki (1969) indicate that 2-amino-2,6-dideoxyhexoses may occur commonly in members of the family Pseudomonadaceae as well as in the Enterobacteriaceae. The occurrence of 3-amino-3,6-dideoxyglucose in *P. synxantha* and possibly in *P. fluorescens* is also of interest as 3-amino-3,6-dideoxyhexoses have been found in *Xanthomonas campestris*, a member of Pseudomonadaceae (Ashwell & Volk, 1965), as well as in Enterobacteriaceae (Raff & Wheat, 1966, 1967; Jann, Jann & Müller-Seitz, 1967; Lüderitz *et al.* 1967). The close similarity in composition between the walls of *P. synxantha* and *P. fluorescens* (both included in *P. fluorescens* as defined by Stanier, Palleroni & Doudoroff, 1966) has been noted previously (Wilkinson, 1970). The present results underline the basic similarity in composition between lipopolysaccharides from pseudomonads and from members of the Enterobacteriaceae.

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