ISOLATION OF A NEW AMINO SUGAR FROM
CHROMOBACTERIUM VIOLACEUM

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ABSTRACT


Crumpton and Davies (1958) isolated and characterized D-fucosamine (2-aminoo-2,6-di-deoxy-D-galactose) from the polysaccharide moiety of lipopolysaccharide prepared from Chromobacterium violaceum NCTC 7917. During an investigation of the intracellular nucleotides of this organism, a routine check of the lipopolysaccharide for the presence of fucosamine revealed fucosamine and an additional cationic, reducing compound (Smith, 1960; Smith and Wheat, 1962; Wheat and Rollins, 1962) which did not react in the Rondle-Morgan (1955) or Levey-McAllan (1959) assays for 2-amino sugars (Kent and Whitehouse, 1955; Foster and Horton, 1959; Crumpton, 1959; Sharon and Jeanloz, 1960). This report describes these observations.

MATERIALS AND METHODS

A strain of C. violaceum NCTC 7917, maintained on nutrient agar slants, was originally obtained from D. A. L. Davies. The organism was grown aerobically on a medium containing 0.3% Basamin-yeast extract (Anheuser-Busch, Inc.), 0.25% (NH4)2SO4, 0.67% KH2PO4, 0.17% K2HPO4, 0.005% MgSO4-7H2O, and 10% tap water for trace metals. Glucose (1%) was autoclaved and added separately.

Preparation of lipopolysaccharide. After harvesting at 5 to 12 C in a DeLaval-gyrottest centrifuge, the cells were extracted with ice-cold 10% trichloroacetic acid. The cell residues were removed by centrifugation, and the supernatant solution was extracted with diethyl ether to remove the trichloroacetic acid. The extract, neutralized to pH 7.0 with ammonium hydroxide, was percolated through Dowex-1-formate to adsorb nucleotides and nucleic acids. Pooled water effluents were concentrated in vacuo, dialyzed overnight against distilled water in the cold, and precipitated by addition to 10 volumes of 95% ethanol. The precipitate was collected by centrifugation, washed with acetone and ether, and dried in vacuo. The yield of dried powder obtained from approximately 580 g wet wt (about 90 g dry wt) of C. violaceum was 2.34 g. The presence of nucleic acid or protein appeared unlikely, since this material did not exhibit absorption in the 260- or 280-mu regions.

"Degraded" polysaccharide. Lipopolysaccharide was subjected to mild acid hydrolysis (Crumpton and Davies, 1958) to remove the "lipid A" moiety; 1 g was hydrolyzed at 100 C in 35 ml of 1% acetic acid for 4 hr under nitrogen. Insoluble humin and debris were removed by centrifugation at 25,000 x g for 30 min, and the supernatant was precipitated with 10 volumes of 95% ethanol, yielding 0.5 g of "degraded" polysaccharide.

Isolation of amino sugars. Samples (100 mg) of lipopolysaccharides and "degaded" polysaccharides were hydrolyzed for 2 hr in 5 ml of 6 N HCl. The hydrolysates were taken to dryness under reduced pressure and redissolved in approximately 2 ml of water. The solutions were adsorbed onto columns (1 by 40 cm) of Dowex 50-H* and eluted with 0.33 N HCl (Crumpton and Davies, 1958). Fractions (5 ml) were collected: 1-ml

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samples were taken for amino sugar determination, and 0.1-ml samples were used for reducing-sugar analysis. Fractions from peaks were pooled, dried in vacuo, and redissolved in a minimum of water, for further analyses.

**Paper chromatography.** The following solvents were used: (1) n-butanol-glacial acetic acid-water (5:1:2); (2) n-butanol-pyridine-water (6:4:3); (3) 75% aqueous phenol; and (4) 90% acetone. Ninhydrin was used to detect amino sugar and amino acids; alkaline silver nitrate was used for reducing compounds; and p-anisidine hydrochloride was used to detect heptoses, hexoses, pentoses, and other reducing sugars.

**Chemical analyses.** Methyl pentose was determined by the cysteine-sulfuric acid method (Dische and Shettles, 1948), with L-fucose as standard. Neutralized solutions were assayed for amino sugars by the procedure of Elson and Morgan as modified by Rondle and Morgan (1955), and N-acetylamino sugars were assayed by the Morgan-Elson procedure as modified by Levvy and McAllan (1959). Glucose was determined with glucostat (i.e., combined glucose oxidase, peroxidase, and p-anisidine reagent; Worthington Biochemical Corp., Freehold, N.J.). Reducing sugar was determined by the procedure of Park and Johnson (1949). Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

**RESULTS**

Results shown in Fig. 1 are from data obtained with “degraded” polysaccharide. Roughly similar results were obtained with lipopolysaccharide. The initial reducing-sugar peak, which is not numbered, contained some glucose, as determined with glucose oxidase. The finding of smaller amounts of reducing sugar in peak 1 with longer hydrolysis indicated that this component was probably an oligosaccharide, and no attempt was made to characterize it further. Peaks 2 and 3 coincided with elution positions for known glucosamine and fucosamine (elution volume, 1.67 × glucosamine, or elution rate relative to glucosamine of 1 + 1.67 = R$_{glucosamine}$ = 0.6; Crumpton and Davies, 1958), and also migrated as glucosamine and fucosamine when chromatographed on paper according to Crumpton (1959), using known markers of glucosamine and a sample of crystalline D-fucosamine-HCl which was generously furnished by D. A. L. Davies.

The reducing compound in peak X ($R_{glucosamine}$ of 0.7) which did not react in hexosamine or acetylatedhexosamine assays for 2-amino sugars had not been previously reported. It did not react in the Molisch test but did react with ninhydrin and alkaline silver nitrate, and gave a characteristic orange-brown color with p-anisidine. It migrated with mobility relative to glucosamine ($R_{glucosamine}$) of 1.45, 1.47, and 1.3 with solvent systems 1, 2, and 3, respectively. Fucosamine-HCl yielded mobilities in solvents 1 and 2 of R$_{glucosamine}$ = 2.0 and 1.32. Mobilities compared with fucosamine on Dowex 50-H$^+$ and paper chromatograms suggested a methyl-group constituent. Acetaldehyde was obtained by periodate oxidation, using the assay procedure described by Crumpton and Davies (1958); a positive methyl pentose test was also obtained (Dische and Shettles, 1948). These results indicated the compound to be similar to fucosamine in that it contains a terminal-CH$_3$ group next to a pair of periodate-reactive groups. The possibility that the new compound might be produced from fucosamine as an artifact of hydrolysis was partially ruled out when no increase of the new compound was observed after adding excess fucosamine to cells and polysaccharide preparations, which were then hydrolyzed and assayed by Dowex 50-H$^+$ chromatography as above.

Preliminary attempts to crystallize large-scale preparations of the compound from crude lipopolysaccharide (Crumpton and Davies, 1958)
were unsuccessful; small amounts of impurities, including amino acids, were shown to be present by paper chromatography. However, elemental analyses on such amorphous material (C, 38.04%; H, 7.16%; N, 6.17%; Cl, 17.37%) were in the range expected for a di- or possibly tri-deoxy sugar monomine. Therefore, the following experiment was performed to purify the compound and to obtain evidence to support the assumption that it was an amino sugar monomer and not a di- or oligosaccharide, nor a sugar-amino acid compound. An aqueous solution of the impure material was N-acetylated by treatment with acetic anhydride in acetone in the presence of borate (Levvy and McAllan, 1959) or 0.1 M sodium bicarbonate (Roseman and Daffner, 1956). After 15 min at room temperature, the solution was deionized with mixed-bed resin without heating, to destroy excess acetic anhydride, and the solution was concentrated in vacuo at reduced temperature. A single spot, which did not react with ninhydrin, was seen on chromatograms treated with alkaline silver nitrate. The \( R_f \) in 90% acetone was \(<0.4\), compared with \(<0.5\) for N-acetyl-fucosamine. The N-acetylated material was hydrolyzed in 1 N HCl for 2 hr, dried in vacuo to remove HCl, and chromatographed on paper. Only two spots were seen: one which corresponded to the N-acetylated material and which reacted with ammoniacal silver nitrate only, and a second spot which also reacted faintly with ninhydrin and which migrated as the nonacetylated compound. This would indicate that, although hydrolysis was incomplete, only the original material was recovered after hydrolysis. Since other products were not found, this observation may be interpreted to indicate that the original free amino compound is a monomer (Stacey, 1958), and that the N-acetylation, deionization, and hydrolysis procedure may also serve for purification of amino sugars present in amino acid hydrolysates.

**Discu$$

It may be tentatively concluded from the data presented that the new amino sugar is a deoxyhexosamine with a terminal methyl group. The position of the amino group and configuration remains to be determined, although lack of reaction in the Elson-Morgan assay for 2-amino-2-decoxy sugars (Kent and Whitehouse, 1955; Crumpton, 1959; Foster and Horton, 1959) indicates that the compound is not 6-deoxytalosamine, the C-2 epimer of \( \beta \)-fucosamine (Crumpton and Davies, 1958). It is pertinent to note that C-3, C-4, C-5, and C-6 amino sugars are known not to react in the Elson-Morgan reaction (Whitehouse, Kent, and Pasternak, 1954; Kent and Whitehouse, 1955; Foster and Horton, 1959). A C-6 amino sugar is presumably ruled out because of the presence of a terminal methyl group. [The compound has been recently characterized as a 4-amino-4,6-dideoxylactohexose (Wheat, Rollins, and Leatherwood, unpublished data).]

This is apparently the first amino sugar of this type to be isolated from a naturally occurring polysaccharide. Possibly similar N-acetyl-deoxyamino sugars, obtained from *Escherichia coli* as thymidine diphosphate compounds, have been reported (Okazaki et al., 1962).

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**Literature Cited**


