The Isolation of D-Fucosamine from the Specific Polysaccharide of *Chromobacterium violaceum* (NCTC 7917)

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In many groups of Gram-negative bacteria the dominant serological character is due to an 'O' somatic antigen whose specificity resides in a polysaccharide component. Material carrying the 'O' somatic specificity can be conveniently extracted as a lipopolysaccharide (polysaccharide + lipid 'A') in 45% phenol (Westphal, Lüderitz & Bister, 1952). For a large number of bacterial polysaccharides extracted by this and other methods the major sugar constituents are known, those most frequently found being galactose, glucose, mannose, rhamnose, fucose, glucosamine and galactosamine. Recent studies have, however, revealed the presence in many instances of additional sugars which were not known to occur in Nature before the advent of paper chromatography. Some of these have been isolated and identified: for example, the 3:6-dideoxyhexoses tyvelose and abequose (Westphal & Lüderitz, 1953, 1954), and the aldoheptoses L-glycero-D-mannoheptose (Slein & Schnell, 1953; Weidel, 1955) and D-glycero-D-galactoheptose (MacLennan & Davies, 1957).

The compositions of polysaccharides obtained from some strains of *Chromobacterium violaceum* have already been described (Davies, 1955). In one of these (*C. violaceum* NCTC 7917) an unidentified aldoheptose and an unspecified hexosamine were recorded, in addition to other sugars. Further examination of the polysaccharide revealed that the aldoheptose was most probably D-glycero-D-mannoheptose (Davies, 1957) and that two amino sugars were present, one of which differed from all the known synthetic and naturally occurring amino sugars.

The only 2-amino-2-deoxyaldose sugars that have been described as constituents of bacterial polysaccharides are D-glucosamine, D-galactosamine, 'muramic acid' (3-O- α -carboxyethyl-D-glucosamine) (Strange, 1956; Kent, 1957), an N-acetylaminohexuronic acid (Clark, McLaughlin & Webster, 1958) and one unidentified amino sugar (Goebel & Jesaitis, 1952). D-Gulosamine has been isolated from Streptothricin and Streptolin B by van Tamelen, Dyer, Carter, Pierce & Daniels (1956), D-mannosamine from neuraminic acid by

Comb & Roseman (1958) and talosamine has been reported to occur naturally (Muir, 1957). Talosamine has also been separated from a preparation of N-acetylgalactosamine although in this case it could not be assumed that the sugar was of natural occurrence (Crumpton, 1957).

The new amino sugar component of the *C. violaceum* (NCTC 7917) specific polysaccharide has now been isolated and identified. The results, together with some properties of the polysaccharide, are presented in this paper, a preliminary account having already appeared (Crumpton & Davies, 1956).

METHODS AND MATERIALS

Materials for analysis. Samples of extraction products for analysis were thoroughly dialysed against distilled water at 2° and dried from the frozen state. They were further dried to constant weight *in vacuo* at 78°.

Nitrogen. Total N was determined by the Kjeldahl method with the distillation apparatus of Markham (1942), and the mixed bromocresol green-methyl red indicator of Ma & Zuazaga (1942).

Phosphorus. Determinations were made on samples containing 5–20 μ g. of P by a modification of the method of Fiske & Subbarow (1925).

Sugars. Estimations were carried out by the following methods: amino sugars, by the method of Rondle & Morgan (1955a), after hydrolysis for 16 hr. at 100° with 0.5N-HCl unless otherwise stated, amounts of amino sugar being expressed in terms of glucosamine base; N-acetyl amino sugars, by the method of Aminoff, Morgan & Watkins (1952), amounts being given in terms of N-acetylglucosamine; aldoheptoses by the method of Dische (1953), amounts being expressed in terms of D-glycero-L-mannoheptose.

Nucleic acid. Nucleic acid was estimated by measuring the absorption at 260 m μ of material dissolved in 0.05N-NaOH. A purified preparation of ribonucleic acid was used as a standard for reference.

Chromatography. Samples were hydrolysed in sealed tubes at 100° for 16 hr. with 0.5N-HCl except where otherwise stated. Solutions were evaporated to dryness *in vacuo* in the presence of NaOH pellets and redissolved at suitable concentrations for application to paper chromatograms. Whatman no. 1 paper was used with the following solvent systems: phenol-aq. NH₃ soln.; butanol-acetic acid-water, 4:1:5 (by vol.); butanol-pyridine-water, 6:4:3 (by vol.). Papers were sprayed with either alkaline

acetylacetone followed by Ehrlich's reagent (Partridge, 1948) to reveal amino sugars and their *N*-acetyl derivatives, or anisidine hydrochloride (Hough, Jones & Wadman, 1950), which distinguishes by colour the different classes of sugars.

Separation and identification of amino sugars. Hydrolysates of the polysaccharide were analysed for amino sugars by chromatography on columns of 200-mesh Zeo-Karb 225 resin (The Permutit Co. Ltd., London, W. 4) in the H^+ form eluted with 0.33*N*-HCl, by a modification (Rondle & Morgan, 1955*b*) of the method of Gardell (1953). Recoveries of approx. 90% of the amino sugars added to the column were usually obtained. Glucosamine standards at concentrations of 20–50 μ g. were employed for the estimation of the eluted sugars.

Periodate oxidation. Amounts (moles/mole of amino sugar) of periodate consumed and of formic acid produced by oxidation of amino sugar with periodate were estimated by a modification of the method of Jeanloz & Forchielli (1951). The oxidation was carried out in the dark at 4°, at pH 4.5 for 18 hr. Glucosamine was included as a control in each test; about 95% of the theoretical periodate consumption and 90% of the theoretical amount of formic acid were estimated. Acetaldehyde was estimated in Conway units, as described by Aminoff, Morgan & Watkins (1950) for the determination of fucose. The method was modified by carrying out the oxidation in a 0.4*M*-phosphate buffer, pH 7.5. Under these conditions 95–100% of the theoretical amount of acetaldehyde was recovered from known amounts of fucose which were included as controls in each test.

Growth and recovery of organisms. *Chromobacterium violaceum* (lividum) NCTC 7917 (Sneath, 1956) was grown on peptone–agar in enamelled-steel trays. After 4 days at 20° the bacteria were harvested, resuspended in cold saline, washed and poured into 10 vol. of acetone at –10°. The acetone was changed several times and the cells were dried *in vacuo* over H_2SO_4 . The yield was 90 g. (dry wt.) from 200 trays.

Specific lipopolysaccharide. Lipopolysaccharide was extracted from dried organisms (90 g.) with warm 45% (w/v) phenol (Westphal *et al.* 1952) and separated from nucleic acid by ultracentrifuging. The product (6.3 g.) contained N, 3.4%; P, 0.8%; amino sugar, 16%; aldoheptose, 21% and not more than 0.2% of nucleic acid.

Degraded polysaccharide. The degraded polysaccharide was prepared from the lipopolysaccharide (5.6 g.) by hydrolysis with 1% (v/v) of acetic acid for 4 hr. at 100° and separated from insoluble phospholipid according to Davies (1956). The phospholipid fraction (970 mg.) contained N, 2.3%; P (organic), 1.5%; amino sugar, 12% and aldoheptose, 5%. The degraded polysaccharide (3.6 g.) contained N, 3.3%; P (organic), 0.3%; amino sugar, 18% and aldoheptose 23%.

RESULTS

Composition of the specific polysaccharide

Chromatographic analysis. Samples of polysaccharide hydrolysates examined by paper chromatography with various solvent systems showed five major spots. The colour and positions of two of these spots were identical with those of glucose and

galactose run at the same time; a third spot gave the colour characteristic of aldoheptoses and most probably represents *D*-glycero-*D*-mannoheptose (Davies, 1957). When chromatograms of the hydrolysed lipopolysaccharide with each solvent system were sprayed with the amino sugar reagents, two spots were distinguished. The fainter spot occupied a position identical with that of glucosamine run at the same time. The other could not, however, be identified with any of the amino sugars available for comparison. The R_{glucose} values (± 0.04) for this component in butanol–pyridine–water, phenol–aq. NH_3 soln. and butanol–acetic acid–water were 1.00, 2.03 and 1.32 respectively. Lipopolysaccharide which had been hydrolysed with 6*N*-HCl for 16 hr. at 100° gave an identical result. Unlike the lipopolysaccharide, 0.5*N*- and 6*N*-HCl hydrolysates of the degraded polysaccharide gave only one amino sugar spot; this was in the position of the unidentified sugar.

Polysaccharide which had been hydrolysed with *N*-acetic acid for 64 hr. at 100° was examined by paper chromatography with butanol–pyridine–water as solvent. When sprayed with ethanolic alkali followed by Ehrlich's reagent to reveal *N*-acetyl amino sugars, a reddish purple spot appeared (R_{glucose} value 1.60 ± 0.04) which could not be identified with any of the *N*-acetyl amino sugars available for comparison. This spot most probably represented the *N*-acetyl derivative of the new amino sugar.

Separation of amino sugars. Samples of the hydrolysed polysaccharides containing 200–400 μ g. of amino sugar were analysed on columns of Zeo-Karb 225 resin eluted with 0.33*N*-HCl. The columns used had been standardized with a mixture of authentic *D*-glucosamine (200 μ g.) and *D*-galactosamine (200 μ g.); these emerged in peak positions (in terms of ml. of effluent) which were characteristic for the column provided that HCl of the same normality was used as eluent. Lipopolysaccharide hydrolysed with 0.5*N*-HCl for 16 hr. at 100° gave two peaks; the peak position (125 ml. of effluent) of one of these was identical with that for glucosamine, but the peak position (242 ml.) of the other component differed from that of any of the amino sugars available for comparison (Fig. 1*A*). The ratio of the amount of new amino sugar to that of glucosamine was 6.4:1. When glucosamine and galactosamine were added to the hydrolysate three components separated completely from one another, emerging in the order glucosamine (126 ml.), galactosamine (153 ml.) and new amino sugar (245 ml.) (Fig. 1*B*). The amount of glucosamine recovered was equal to the amount of authentic sugar added plus that previously calculated to be present in the lipopolysaccharide. Strong acid hydrolysates (6*N*-HCl, 100°, 3 hr.) of

the lipopolysaccharide gave glucosamine and new amino sugar in the same respective positions as those shown in Fig. 1A; the sugars were present in a similar ratio but recoveries of only about 70% were obtained. Analysis of 0.5N- and 6N-HCl hydrolysates of the degraded polysaccharide revealed the presence of only one component which emerged in the position of the new amino sugar. On the other hand, the phospholipid, after hydrolysis (0.5N-HCl, 16 hr., 100°), contained both sugars; the ratio of glucosamine to new amino sugar had, however, increased to 1:1. Thus the glucosamine present in the lipopolysaccharide seemed likely to be a component only of the phospholipid moiety. For this reason the degraded polysaccharide was chosen as starting material for the isolation of the new amino sugar.

As it was intended to separate the amino sugar from the neutral sugars and traces of glucosamine by elution with HCl from a column of Zeo-Karb 225 resin, the effect of HCl normality on the positions in which glucosamine and the new amino sugar emerged was investigated. A complete separation of the amino sugar in the shortest possible time was found to occur on eluting with 0.8N-HCl.

Detection of N-acetyl amino sugar. Unhydrolysed polysaccharides that contain a 2-acetamido-2-

deoxy sugar with an unsubstituted hydroxyl group attached to C₄, and either linked through C₁ by an alkali-labile bond or with a free reducing group, produce, when treated with dilute alkali, a chromogen which forms a coloured complex with the *p*-dimethylaminobenzaldehyde-HCl reagent (Aminoff *et al.* 1952; Kuhn, Gauhe & Baer, 1954). The maximum colour intensity given by known weights of materials can be expressed in terms of the maximum colour intensity developed under defined conditions by *N*-acetylglucosamine. It is, however, necessary to determine for each material the optimum time of heating with alkali (0.05N-Na₂CO₃, 100°) which subsequently gives the maximum colour intensity on the addition of Ehrlich's reagent. Under the conditions employed the optimum heating period for *N*-acetylglucosamine was 4 min.

The influence of the time of heating with alkali on the intensity of colour produced by 1 mg. samples of the lipopolysaccharide and degraded polysaccharide is shown in Fig. 2; maximum colour intensities equivalent to 15 and 45 µg. of *N*-acetylglucosamine coincided with 18–20 min. and 10 min. heating periods respectively. In each case the absorption spectrum of the coloured complex

Fig. 1. Separation of amino sugars on a column of Zeo-Karb 225 resin eluted with 0.33N-HCl. A, *C. violaceum* (NCTC 7917) lipopolysaccharide hydrolysed with 0.5N-HCl for 16 hr. at 100°; B, a sample of the same hydrolysate with the addition of glucosamine and galactosamine. Amino sugars were eluted in the order: glucosamine, galactosamine, the new amino sugar.

Fig. 2. Influence of time of heating in 0.05N-Na₂CO₃ on the intensity of colour produced by: ○, lipopolysaccharide; △, degraded polysaccharide and □, lipopolysaccharide hydrolysed with *n*-acetic acid for 12 hr. at 100°.

was identical with that of *N*-acetylglucosamine, with maxima at 550 and 590 $m\mu$, and there was no preformed chromogenic structure. Examination of lipopolysaccharide which had been hydrolysed in sealed ampoules at 100° with *N*-acetic acid for different periods revealed that the optimum time of heating with alkali and the maximum colour intensity reached were dependent on the period of hydrolysis. After hydrolysis for 12–18 hr. a maximum colour equivalent to about 18% of *N*-acetylglucosamine was produced after heating for 4 min. (Fig. 2). Hydrolysis periods greater than 24 hr. resulted in a decrease in the maximum colour intensity; this was probably due to deacetylation of the *N*-acetyl amino sugar that is released from the lipopolysaccharide on hydrolysis with mild acid.

Isolation of the new amino sugar

Hydrolysis of the degraded polysaccharide. To determine the conditions of acid hydrolysis necessary to release the maximum amount of amino sugar from the degraded polysaccharide, rates of liberation with 0.5*N*- and 6*N*-HCl were measured. Maximum liberation with 0.5*N*-HCl occurred at approx. 12 hr. and was equivalent to 18%, estimated as glucosamine, whereas hydrolysis with 6*N*-HCl apparently released a greater amount of amino sugar (20%) in a much shorter time (2 hr.), although extensive decomposition occurred with the formation of a precipitate of humin.

In view of these results and since only 70% of the amino sugar present in 6*N*-HCl hydrolysates of the lipopolysaccharide was recovered from columns of Zeo-Karb 225 resin, the polysaccharide (3.26 g.) was hydrolysed for 16 hr. at 100° with 0.5*N*-HCl (336 ml.) in sealed ampoules. The hydrolysate was filtered, evaporated to dryness *in vacuo* in the presence of NaOH pellets and redissolved in approx. 25 ml. of 0.8*N*-HCl. Examination of a sample of this hydrolysate by chromatography on paper, and on columns of Zeo-Karb 225 resin eluted with 0.33*N*-HCl, confirmed the presence of the new amino sugar together with glucose, galactose and *D*-glycero-*D*-mannoheptose; glucosamine could not be detected.

Separation of amino sugar from neutral sugars. A glass column, 5.1 cm. internal diam. and 53 cm. long, was packed with Zeo-Karb 225 resin in the H^+ form after two cycles through the Na^+ form. After thorough washing of the column with 0.8*N*-HCl the concentrated hydrolysate containing about 420 mg. of amino sugar was placed on the resin and the column eluted with 0.8*N*-HCl at a rate of 10 ml./hr. The effluent was collected in fractions of 50 ml. Samples (0.5 ml.) of these fractions were examined for the presence of neutral reducing sugars with the Molisch test since this does not detect amino sugar

and could be used without further treatment of the acid eluates. Positive fractions were pooled, neutralized and concentrated *in vacuo* to a syrup which was found to contain only glucose, galactose and *D*-glycero-*D*-mannoheptose. Amino sugar was detected and estimated in suitable dilutions of the fractions, positive fractions being pooled and evaporated *in vacuo* almost to dryness. Pale-yellow crystals formed on allowing the product to stand in the cold; these were dissolved in water and the sugar was recrystallized by adding 10 vol. of acetone and leaving overnight at 2°. The product was recrystallized three times from aqueous acetone, after which its specific rotation was not altered by further recrystallization. The colourless crystals obtained were washed with acetone and with ether and dried over P_2O_5 (Fig. 3). The dry crystals weighed 260 mg., giving a yield of about 60%.

Properties and identification of the amino sugar

Physical. The specific rotation, the mean value obtained by examination of several separate solutions of the $\alpha\beta$ -equilibrium mixture, is $[\alpha]_D^{20} + 91 \pm 2^\circ$ (c, 1.0; H_2O), the molecular rotation being $+181.5$. On heating, charring occurred at about 155° followed by decomposition at 170–175°. No decrease in weight was observed on heating at 110° *in vacuo* over P_2O_5 , suggesting that the crystals were unlikely to contain water or solvent of crystallization. This was confirmed by examination of the infrared-absorption spectrum. Comparison of this spectrum with those of six of the aldohexoses and seven of the 6-deoxyhexoses revealed that, after allowing for the $-NH_2$ bands, it resembled most closely that of L -fucose.

Chemical. Elementary analysis showed C, H, N and Cl [Found: C, 36.5; H, 6.7; Cl, 18.0; N (Dumas),

Fig. 3. The new amino sugar (*D*-fucosamine hydrochloride) crystallized from aqueous acetone ($\times 80$).

7.2; N(Kjeldahl), 6.8. Calc. for $C_6H_{14}O_4NCl$: C, 36.2; H, 7.0; Cl, 17.8; N, 7.0%].

On paper chromatograms sprayed either with anisidine hydrochloride or the amino sugar reagents the sugar gave only one spot, the colour of which, in each case, was characteristic of amino sugars. Its position corresponded in each solvent system to that previously described for acid hydrolysates of the polysaccharide. The sugar was eluted with 0.33N-HCl from a column of Zeo-Karb 225 resin in a position identical with that obtained with a polysaccharide hydrolysate; approx. 95 % of the total amount added to the column was recovered in a single symmetrical peak.

In the Rondle & Morgan (1955a) test for hexosamines the sugar gave 75 % of the colour given by an equal weight of glucosamine base; the absorption spectrum of the chromophore was identical with that for glucosamine with a maximum at 530 $m\mu$. On the assumption that the sugar might be a methylpentosamine hydrochloride, the amount of base used in the above test gave 92 % of the colour given by an equal weight of glucosamine base.

At this stage an indication of the identity of the sugar was obtained by the spectrophotometric examination of the chromophore formed on treatment with nitrous acid followed by H_2SO_4 -cysteine. The sugar (0.5 ml.; 0.02 %) was deaminated by treatment with equal volumes of 5 % (w/v) $NaNO_2$ and 33 % (v/v) acetic acid at 20° for 10 min. Excess of nitrous acid was destroyed by the addition of 0.5 ml. of 12.5 % (w/v) ammonium sulphamate and repeated shaking for 30 min. The mixture was diluted with water to 4 ml. and the anhydro sugar formed (1 ml. sample) was identified by the H_2SO_4 -cysteine reaction of Dische & Shettles (1948). A strong absorption with a maximum at 400 $m\mu$ was found, which is characteristic of methylpentoses. Glucosamine, which was included in the above test as a control, gave the maximum absorption at 415 $m\mu$ that is characteristic of hexoses.

Periodate oxidation. Formaldehyde was not detected by the specific colorimetric method of O'Dea & Gibbons (1953) in the products formed by oxidation of the sugar with periodate for 3 hr. in the dark at 20°. Glucosamine, which was included as a control, gave under the same conditions about 98 % of the theoretical yield of formaldehyde. On oxidation with periodate 3.8 moles were consumed and 2.6 moles of formic acid were produced for each mole of amino sugar. Glucosamine, which was included as a control, consumed 4.8 moles of periodate during the oxidation of 1 mole and gave 3.6 moles of formic acid. Thus 1 mole of the amino sugar consumed 1 mole less periodate and produced 1 mole less formic acid than did 1 mole of

glucosamine. These results suggest that the sugar is a methylpentosamine since in this case $C_{(5)}$ and $C_{(6)}$ atoms would be liberated as acetaldehyde. The production of acetaldehyde on periodate oxidation was tested for in the following way. Oxidation was carried out in Conway units; a sample (0.5 ml.) was removed from the centre well and examined by the method described by Barker & Summerson (1941). A purple colour was obtained with a maximum absorption at 560 $m\mu$ which is characteristic of acetaldehyde. Glucosamine and fucose which had been treated in the same way were included as negative and positive controls. The amount of acetaldehyde formed by each mole of the amino sugar was 0.96 mole. Fucose was included as a control and gave 95 % of the theoretical amount of acetaldehyde. Thus 4 moles of periodate are consumed in the oxidation of 1 mole of the amino sugar, with the production of 3 moles of formic acid and 1 mole of acetaldehyde.

Characterization of the phenylosazone. The phenylosazone was chosen as a suitable derivative because the same osazone would be obtained from the 2-amino sugar as from the parent 2-hydroxy sugar. The very insoluble phenylglucosazone, which can be isolated in almost theoretical yield from glucose, was obtained in only about 30 % yield, however, when D-glucosamine was used as starting material. Preliminary experiments showed that whereas phenyl-L-rhamnosazone readily crystallized in good yield, phenyl-L-fucosazone was more difficult to crystallize.

A sample of the *C. violaceum* amino sugar hydrochloride (60 mg.) was dissolved in water (1 ml.) and acetic acid (0.1 ml.), phenylhydrazine (120 mg.) and sodium acetate (180 mg.) were added. The reaction mixture was heated for 90 min. at 100°, and the insoluble material was filtered off, washed and dried. This was redissolved in hot 70 % (v/v) ethanol, cooled and, after it had stood at -10° for some weeks, 25 mg. of the product was recovered. Rather than lose material in attempts to purify this by recrystallization, the product was dissolved in 0.5 ml. of ethanol and diluted to 3 ml. with chloroform; this solution was placed on a column of $CaCO_3$ (length 45 cm., internal diam. 3.2 cm.) and eluted with chloroform containing 3 % (v/v) of ethanol (Jorgensen, 1950). Three bands separated in the order brown, pink, yellow; the last was recognized as the osazone from preliminary runs with various osazones. This fraction was collected, evaporated to small volume, run again through the column to ensure complete separation from the other components and finally recovered (10.2 mg.).

The infrared-absorption spectrum of this phenylosazone (Fig. 4A) is closely similar to that of phenylfucosazone (Fig. 4B), the only difference

Fig. 4. Infrared-absorption spectra of osazones (Perkin-Elmer Model 21 spectrophotometer, NaCl prism, samples incorporated in KCl disks). A, Phenyl-L-fucosazone of the amino sugar isolated from *C. violaceum* (NCTC 7917) polysaccharide; B, phenyl-L-rhamnosazone; C, phenyl-L-rhamnosazone.

being a band at 7.2μ which appears to be due to a small amount of impurity in the phenylfucosazone standard. These spectra differ markedly from that of phenyl-L-rhamnosazone (Fig. 4C).

The only figure found in the literature for the optical rotation of phenyl-L-fucosazone was $[\alpha]_D = -70^\circ$ (pyridine 2, ethanol 3) (Freudenberg & Raschig, 1929). Initially this could not be confirmed, but later, a figure $[\alpha]_D^{20} = -66 \pm 5^\circ$ (c, 1.0; pyridine 2, ethanol 3) was obtained with a freshly made solution. After the solution had stood for 24 hr. the figure dropped to about 60% of the initial value, i.e. $[\alpha]_D^{20} = -40 \pm 5^\circ$. The *C. violaceum* product, however, gave the approximately equal but opposite rotation, $[\alpha]_D^{20} = +38 \pm 5^\circ$ (c, 1.0; pyridine 2, ethanol 3; at 24 hr.). A similar figure of $[\alpha]_D^{20} = +42 \pm 5^\circ$ was subsequently found under the same conditions for a sample of phenyl-D-fucosazone prepared from D-fucose.

DISCUSSION

The specific lipopolysaccharide of *Chromobacterium violaceum* (NCTC 7917) is unusual in that it contains two sugars, D-glycero-D-mannoheptose (Davies, 1957) and an amino sugar, which have not previously been found in Nature. Evidence has also

been obtained for the presence of glucose, galactose and glucosamine.

Hydrolysis of the lipopolysaccharide with 1% acetic acid resulted in the separation of a soluble degraded polysaccharide from an insoluble material which contained a chloroform-soluble, ether-insoluble phospholipid. Glucosamine was not detected in hydrolysates of the degraded polysaccharide when these were analysed for amino sugars by chromatography on columns of Zeo-Karb 225 resin. The phospholipid fraction was, however, shown by the same method to contain a much greater proportion of glucosamine than did the lipopolysaccharide. It would thus appear that the phospholipid part of the lipopolysaccharide contains all the glucosamine. Ikawa, Koepfli, Mudd & Niemann (1953) have previously demonstrated the presence of glucosamine in the lipid part of a lipopolysaccharide isolated from a strain of *Escherichia coli*.

Analysis of the lipopolysaccharide and degraded polysaccharide for the presence of N-acetyl amino sugar by the method of Aminoff *et al.* (1952) gave a positive result. Lipopolysaccharide that had been hydrolysed with N-acetic acid for 12–18 hr. gave, after heating for 4 min. with alkali, a maximum colour intensity equivalent to 18% of N-acetylglucosamine. This material contained 16% of amino sugar estimated as glucosamine, which corresponds to 20% of N-acetylglucosamine. Thus unless the N-acetyl derivative of the new amino sugar gives, weight for weight, a much greater colour than N-acetylglucosamine, it would appear that the amino sugar occurs naturally as its N-acetyl derivative and in a form which is readily released by mild acid hydrolysis.

The amino sugar was separated from the neutral sugars present in the hydrolysed degraded polysaccharide by elution from a column of Zeo-Karb 225 resin. It was isolated as a dextrorotatory crystalline hydrochloride with an analytical composition which indicated that it was probably a methylpentosamine. After deamination with nitrous acid the sugar reacted with sulphuric acid-cysteine (Dische & Shettles, 1948) to give a chromophore with an absorption spectrum identical with that of a methylpentose. Although hexosamines do not give chromophores with sulphuric acid-cysteine, treatment with nitrous acid produces a 2:5-anhydro sugar which is probably the first product of the reaction between hexoses and sulphuric acid. Oxidation of 1 mole of the sugar required 4 moles of periodate; 3 moles of formic acid and 1 mole of acetaldehyde were formed, but no formaldehyde appeared. These results, together with the fact that the sugar gave an absorption spectrum in the Rondle & Morgan (1955a) test which was identical with that of glucosamine,

indicate that the sugar is a 2-amino-2:6-dideoxy-hexose. The infrared-absorption spectrum of the phenylosazone formed by the sugar was not appreciably different from that of phenylfucosazone, and the rotation indicates that it is the D-form. The sugar is thus either 2-amino-2:6-dideoxy-D-galactose or 2-amino-2:6-dideoxy-D-talose, since both of these would give the same osazone. The infrared-absorption spectrum of the amino sugar resembled that of fucose but not that of 6-deoxytalose. Moreover, the molecular rotation is consistent with the D-galactose configuration (D-galactose, +144; D-fucose, +124.6; D-galactosamine, +224) but it is not consistent with the D-talose configuration (D-talose, +36; 6-deoxy-D-talose, +32.6; D-talosamine, -12.3). It is thus concluded that the sugar is 2-amino-2:6-dideoxy-D-galactose (D-fucosamine). Whereas L-fucose occurs frequently in Nature, the D-form has not been reported as a constituent of bacterial polysaccharides. D-Fucosamine is closely related structurally, however, to D-galactosamine, which is of frequent occurrence. Another D-fucose derivative has recently been recognized as a component of some bacterial polysaccharides. This is abequose (Westphal & Lüderitz, 1953, 1954; Fromme, Himmelsbach, Lüderitz & Westphal, 1957), which has now been shown to be 3:6-dideoxy-D-galactose, i.e. 3-deoxy-D-fucose (Fouquey *et al.* 1958).

Fucosamine can be distinguished from glucosamine, galactosamine and muramic acid, which are the only other 2-amino sugars known to occur naturally in bacteria, by its chromatographic behaviour on paper and columns of Zeo-Karb 225 resin. The peak volume (ml. of effluent) at which an amino sugar is eluted from a column of Zeo-Karb 225 may be expressed in relation to that of glucosamine. The resultant ratio ('the $R_{\text{glucosamine}}$ value') is characteristic of the sugar and is independent of column length, amount of resin and the hydrochloric acid normality used for elution, at least within the range 0.25–0.50N; the $R_{\text{glucosamine}}$ values for muramic acid, galactosamine and fucosamine are 1.10, 1.21 and 1.94 respectively (Crumpton, 1958).

SUMMARY

1. The specific lipopolysaccharide of *Chromobacterium violaceum* (NCTC 7917) was extracted from acetone-dried cells with warm aqueous phenol. Mild acid hydrolysis of the lipopolysaccharide resulted in the separation of a specific degraded polysaccharide from a chloroform-soluble phospholipid.

2. The lipopolysaccharide contains a new 2-amino-2-deoxyaldose sugar which occurs naturally as its N-acetyl derivative; glucosamine, D-glycero-D-mannoheptose, glucose and galactose are also

present. Glucosamine is not present in the degraded polysaccharide.

3. The acid-hydrolysate of the degraded polysaccharide was separated into neutral and amino sugar fractions by passage through Zeo-Karb 225 resin.

4. The crystalline hydrochloride of the new amino sugar was isolated, and, by elementary analysis, deamination, periodate oxidation and examination of its phenylosazone, it was shown to be 2-amino-2:6-dideoxy-D-galactose hydrochloride (D-fucosamine hydrochloride).

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