The Isolation of D-glycero-D-galactoHeptose and other Sugar Components from the Specific Polysaccharide of *Chromobacterium violaceum* (BN)

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Although ketoheptose sugars are known to occur in Nature both free (La Forge, 1916-17; La Forge & Hudson, 1917) and in phosphorylated form (Robison, Macfarlane & Tazelaar, 1938; Benson, Bassham & Calvin, 1951; Horecker & Smyrniotis, 1952; Buchanan, 1953), it is only in very recent years that aldoheptose sugars have been detected in natural products. The aldoheptoses have been found, up to the present time, only as constituents of polysaccharides extracted from Gram-negative bacteria.

From a strain of *Shigella sonnei*, Jesaitis & Goebel (1952) isolated a polysaccharide which they showed to contain glucose, galactose, glucosamine and an aldoheptose. This heptose was not identified but its behaviour on paper chromatograms was similar to that of D-gala-L-mannoheptose (D-glycero-L-mannoheptose). The specific polysaccharide of a strain of *Shigella flexneri* was described by Slein & Schnell (1953); this contained glucose, rhamnose, glucosamine and phosphorylated sugars, one of which was an aldoheptose. Derivatives of this heptose had very similar properties to those of D-gala-L-mannoheptose but the melting points of mercaptals and hexa-acetyl-mercaptals were lowered in the presence of the corresponding products of authentic D-gala-L-mannoheptose. It was considered that the sugar was probably the optical enantiomorph, L-gala-D-mannoheptose (L-glycero-D-mannoheptose). Chromatographic evidence for the occurrence of aldoheptose sugars in *Escherichia coli* polysaccharides has been presented by Weidel, Koch & Bobosch (1954) and Fromme, Lüderitz & Westphal (1954). The somatic antigen of *Shigella dysenteriae* has been reported to contain a similar sugar (Davies, Morgan & Record, 1955). From *E. coli* cell walls, Weidel (1955) has isolated and unequivocally identified L-glycero-D-mannoheptose.

In addition to their occurrence in the Enterobacteriaceae aldoheptoses have been found in organisms of the genera *Pasteurella* and *Bordetella*. The 'rough' somatic polysaccharide of *Pasteurella pestis* is composed largely of an aldoheptose sugar; glucose and glucosamine are present in smaller amounts (Davies, 1956). All serological groups of *Pasteurella pseudotuberculosis* (Thal, 1954) are

characterized by somatic antigens which contain the same heptose as that found in *P. pestis* (Davies, 1957a) and strains of *Bordetella bronchiseptica* also possess a common specific polysaccharide containing heptose (MacLennan, 1957).

In the course of examining the composition of polysaccharides obtained from a range of Gram-negative bacteria, aldoheptoses were detected in three strains of *Chromobacterium violaceum* (Davies, 1955). In two of these strains the sugar seemed likely to be D-glycero-D-galactoheptose, but only chromatographic evidence was presented.

With the exception of L-glycero-D-mannoheptose, isolated and identified by Weidel (1955), the identity of the various bacterial aldoheptoses remains uncertain. The heptose sugar component of the somatic antigen of *C. violaceum* (BN) was therefore chosen for isolation and the results are described in this paper. A preliminary report has already appeared (MacLennan & Davies, 1956).

MATERIALS AND METHODS

Bacteria. *Chromobacterium violaceum* (BN) was isolated from a fatal human infection by this organism in Malaya (Sneath, Whelan, Singh & Edwards, 1953; Sneath, 1956).

Materials for analysis. Samples for analysis were thoroughly dialysed against distilled water at 0-2° and dried from the frozen state. They were further dried to constant weight *in vacuo* at 78°.

Total nitrogen. The Kjeldahl method was used 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 µg. of P by a modification of the method of Fiske & Subbarow (1925).

Sugars. Estimations were carried out by the following methods: hexosamine, Elson & Morgan (1933); N-acetylhexosamine, Morgan & Elson (1934); methyl pentose, Dische & Shettles (1948); heptose, Dische (1953); total reducing sugars, Somogyi (1937).

EXPERIMENTAL AND RESULTS

Growth and recovery of organisms

C. violaceum (BN) was grown on peptone-agar in enamelled-steel trays each containing 500 ml. of medium. After growth at 37° for 22 hr. the bacteria were harvested by

suction, filtered through glass wool and washed once with saline at 0–2°. The centrifuged cells were resuspended in water and killed by pouring the suspension into 10 vol. of acetone at –15°. After checking for sterility and purity the cells were washed several times with dry acetone at –15°, collected by centrifuging and dried *in vacuo* over H₂SO₄. The yield of cells from 200 trays was 40 g. (dry wt.).

Preparation of the somatic antigen

Acetone-dried bacteria (40 g.) were suspended in water (800 ml.) at 0–2° and an equal volume of cold *N*-trichloroacetic acid was added (Boivin, Mesrobianu & Mesrobianu, 1933). After stirring the mixture for 3 hr., in the cold, the cells were removed by centrifuging at 18000 *g*. The supernatant fluid was dialysed against distilled water until largely free of acid, and concentrated at reduced pressure (16°) to about 1% (w/v) of total solid material. The extract was then fractionated with ethanol at 0–2°. The addition of 2.5 vol. of ethanol brought down a precipitate, which was recovered by centrifuging, potassium acetate being added to assist flocculation. From the supernatant solution a small amount of material was obtained by the addition of a further 7.5 vol. of ethanol; this was largely polyglucose, probably from inside the bacterial cells. The precipitate with 2.5 vol. of ethanol was redissolved in water and reprecipitated twice at the same ethanol concentration, redissolved in water, dialysed and freeze-dried. The product weighed 2.58 g. and contained 5.8% of N and 0.2% of P.

Degradation of the antigenic complex

The somatic antigenic complex, which consisted of polysaccharide–lipid–protein (2.3 g.), was dissolved in 1% (v/v) acetic acid (200 ml.) and heated at 100° for 3 hr. in an atmosphere of N₂. The insoluble lipoprotein thus liberated (Morgan & Partridge, 1940; Davies & Morgan, 1953) was recovered by filtration, dissolved in 0.1*N*-NaOH, reprecipitated by the addition of dilute HCl to pH 4 and the precipitate washed twice with cold water and dried from the frozen state. This fraction weighed 630 mg. and contained 8.8% of N and 0.5% of P.

The acetic acid supernatant solution was concentrated under reduced pressure (16°) to about 60 ml. and poured into 600 ml. of cold ethanol containing a little potassium acetate. The flocculent precipitate thus obtained was redissolved in and thoroughly dialysed against distilled water, and the aqueous solution centrifuged at 105 000 *g* for 2 hr.; the small amount of material sedimented was discarded. The material recovered from the supernatant fluid by freeze-drying did not give a clear solution on redissolving in water, but appeared to retain some lipoprotein which had now become insoluble as a result of freeze-drying. Centrifuging for a further 2 hr. period at 105 000 *g* after adjustment to pH 4 with dilute HCl gave a small amount of sediment, which was discarded. The supernatant solution was poured into 10 vol. of cold ethanol and the precipitated polysaccharide redissolved in water, dialysed and freeze-dried. This degraded polysaccharide weighed 980 mg. and contained 3.7% of N and less than 0.1% of P.

Composition of the degraded polysaccharide

Course of acid hydrolysis. Samples of polysaccharide (2 mg.) were hydrolysed at 100° in sealed ampoules with 0.5*N*-HCl in a volume of 1 ml.;

pairs of ampoules were removed from the water bath at various times for the estimation of hexosamine and of total reducing sugars released. The hydrolysis curves constructed from these data are illustrated in Fig. 1. The total reduction, measured as glucose, reached a value of about 50% after 12 hr. Estimations of hexosamine showed this to represent about 12% of the weight of the polysaccharide. It is clear that the hexosamine did not account for all of the N present in the preparation, but it was thought that small amounts of other nitrogenous materials, such as peptides, would not interfere with the isolation of the neutral sugars.

Chromatographic examination of acid hydrolysates. Polysaccharide samples (10 mg.) were hydrolysed with 0.5*N*-H₂SO₄ for 16 hr. at 100° in sealed ampoules and the hydrolysates neutralized with Ba(OH)₂. After removal of BaSO₄, the salt-free solutions were evaporated to dryness under reduced pressure and redissolved at approx. 5% (w/v) in water for application to paper chromatograms. Whatman no. 1 paper was used with the following solvent systems: butanol–pyridine–water (6:4:3, by vol.), butanol–acetic acid (4:1, v/v) and phenol–aq. NH₃ soln. With anisidine hydrochloride (Hough, Jones & Wadman, 1950) or ammoniacal AgNO₃ as spray reagent, only three major sugar components

Fig. 1. Hydrolysis curves of *C. violaceum* (BN) degraded polysaccharide (0.5*N*-HCl at 100°). O, Glucosamine released (duplicate points, not distinguishable); Δ, free reducing sugars, estimated as glucose.

were found for each solvent system. These seemed likely to be glucosamine, rhamnose and an aldohexose. The last-named component had a smaller *R* value than any aldohexose sugar and gave a grey-green colour reaction with the anisidine spray reagent, which is characteristic of aldohexose sugars. The reducing sugar in the position for glucosamine was shown on separate papers to react both with ninhydrin and with Ehrlich's reagent (Partridge, 1948). No evidence for the presence of ketoheptose could be obtained by spraying chromatograms with the orcinol reagent of Bevenue & Williams (1951).

As it was intended to separate the aldohexose from rhamnose by using a column of powdered cellulose, further solvent systems were examined with paper chromatograms in order to obtain the greatest possible separation of these two sugars. The best results were found with 95% (v/v) aq. acetone, a solvent previously used successfully by Hough, Jones & Wadman (1949) for the separation of some mixtures of sugars.

Estimation of aldohexose and rhamnose. In addition to 12% of glucosamine (Fig. 1) the degraded polysaccharide was found to contain 15% of rhamnose (Dische & Shettles, 1948) and 26% of aldohexose (Dische, 1953), measured against standards of authentic L-rhamnose and D-glycero-D-galactohexose respectively.

Hydrolysis of the degraded polysaccharide

In view of the results shown in Fig. 1, the polysaccharide (920 mg.) was hydrolysed for 16 hr. at 100° with 0.5N-H₂SO₄ (50 ml.) in an atmosphere of N₂. After neutralization and removal of salt as already described the hydrolysate was concentrated *in vacuo* (16°) to approx. 15 ml. The examination of a sample of this hydrolysate by paper chromatography confirmed the presence of the three components previously detected; traces of glucose and galactose were also found and peptides were revealed by the ninhydrin spray reagent.

Separation of amino sugar and peptides from neutral sugars

A glass column, 1.2 cm. internal diam., was packed with 40 g. of damp Zeo-Karb 225 (wet wt. to dry wt. ratio, 1.9; The Permutit Co. Ltd.). The resin was packed in the H⁺ form after two cycles through the Na⁺ form. After thorough washing of the column in water, the concentrated hydrolysate (approx. 15 ml.) was placed on the resin and the column eluted with water. Elution was stopped when approx. 10 μ l. samples of the eluate, 'spotted' on to filter paper and sprayed with anisidine hydrochloride, no longer gave a positive reaction for reducing sugars. The sugar solution eluted was neutralized and concentrated to a syrup; chromato-

graphy revealed two major sugar components but there remained a faint reaction with ninhydrin. The syrup was therefore diluted to approx. 10 ml. and passed through a second column of Zeo-Karb 225 exactly as previously described. The syrup obtained by concentrating the neutralized eluate of the second column contained only rhamnose and aldohexose, with traces of glucose and galactose; this was preserved at 0–2° in the presence of CHCl₃.

Isolation and identification of D-glucosamine hydrochloride

The first resin column containing the adsorbed amino sugar was eluted with 0.3N-HCl; only those fractions of the eluate were retained which, when spotted on filter paper, reacted with the ninhydrin spray reagent. The hexosamine solution thus obtained was evaporated almost to dryness *in vacuo*. Crystals formed on standing in the cold; these were redissolved in water and the material was recrystallized by the addition of 10 vol. of acetone and leaving overnight at 0–2°. The crystals were then washed with acetone and with ether and dried over P₂O₅. The dry crystals weighed 35 mg., $[\alpha]_D^{20} + 73^\circ$ in water (c, 2); authentic D-glucosamine hydrochloride has $[\alpha]_D^{20} + 72.5^\circ$.

Within the limits of accuracy of the methods used the amino sugar gave the same amount of colour (measured in the Hilger Spekker absorptiometer) as that given by a sample of authentic D-glucosamine hydrochloride in the Elson & Morgan (1933) test. The same amount of colour as that produced in the Morgan & Elson (1934) test by N-acetyl-D-glucosamine was obtained when a sample of the isolated sugar was tested after acetylation by the method of Smithies (1953). After treatment with ninhydrin according to the method of Stoffyn & Jeanloz (1954) arabinose could be detected chromatographically. The amino sugar was therefore considered to be D-glucosamine.

Separation of neutral sugars

The neutral sugars present in the water eluate of the resin columns were separated on a column of cellulose powder. A glass tube, 1.8 cm. internal diam. and 66 cm. long, was packed with Whatman no. 1 analytical-grade cellulose powder (100 g.). For elution 95% (v/v) aq. acetone was used. This solvent had given good results in filter-paper separations and it offered the additional advantage of being easily removed from the column effluent by distillation. The column was packed dry and thoroughly washed through with the solvent. Preliminary experiments showed that mixtures of galactose and rhamnose could readily be separated and the sugars recovered. Hydrolysates of the specific degraded polysaccharide of *Shigella dysenteriae* (Davies *et al.* 1955) were also separable into

their component neutral sugars after removal of glucosamine on a Zeo-Karb 225 column. These experiments also revealed that it was unnecessary to remove traces of salt from neutralized resin-column eluates before application to the cellulose column.

The mixture of aldohexose and rhamnose, from which glucosamine and peptides had been removed, was diluted to 3 ml., placed on the cellulose column and eluted at 20°, the flow rate being approx. 50 ml./hr. The effluent was collected in 5 ml. fractions and the presence of reducing sugars detected by spotting on filter paper as already described. With anisidine hydrochloride as a spray reagent the colour given by rhamnose (yellow) could readily be distinguished from that given by the aldohexose (grey-green).

The first anisidine-reacting material to emerge from the column gave a bright-yellow spot without heating and appeared to be a substance derived from the resin column. This fraction was recovered as a non-hygroscopic reddish powder (7.6 mg.) and was not examined further. Rhamnose then appeared and all fractions containing this sugar were pooled and evaporated to a syrup. No reducing sugar appeared in the next 2 l. of column eluate; the elution of heptose was therefore hastened by running water down the column. This was unfortunate since the column had not been washed with water and as a result the heptose was later found to be contaminated with a water-soluble impurity from the cellulose.

Identification of L-rhamnose

The rhamnose syrup was crystallized from acetone which contained a trace of water and a yield of 59 mg. was obtained ($[\alpha]_D^{20} + 9^\circ$ in water (c, 2)). After recrystallization from the same solvent the product gave an m.p. of 91°, and a mixed m.p. with authentic L-rhamnose gave the same figure. The m.p. and mixed m.p. of the phenylhydrazone, prepared as described by Butler & Cretcher (1931), were in the range 146–153°. The absorption curve given by the isolated sugar in the H_2SO_4 -cysteine reaction (Dische & Shettles, 1948) was quantitatively identical with that given by authentic L-rhamnose.

Isolation and identification of heptose

The eluted heptose solution was evaporated to a syrup which, when dried over P_2O_5 in *vacuo*, weighed 270 mg. On paper chromatograms sprayed with anisidine a sample of this syrup gave only one spot which had the characteristic aldohexose colour. Its position corresponded to that of D-glycero-D-galactohexose in all solvent systems used, and differed in position in one or more solvent systems from the other aldohexoses available for comparison (D-glycero-D-glucose, D-glycero-L-glucose, D-glycero-D-mannose, D-glycero-L-mannose, D-glycero-

L-galactose, D-glycero-D-allose, D-glycero-D-gulose, D-glycero-D-idose, D-glycero-D-talose, D-glycero-L-talose and D-glycero-D-altrose-heptoses).

The optical rotations of those aldohexose sugars described in the literature are all rather small, with the exception of D-glycero-D-galactohexose ($[\alpha]_D + 68^\circ$). The specific rotation of a sample of the syrup after drying by grinding under several changes of dry acetone was $[\alpha]_D^{20} + 52^\circ$ in water (c, 2). This figure supported a tentative identification as D-glycero-D-galactohexose by ruling out the possibility of it being the optical enantiomorph L-glycero-L-galactohexose; these two sugars would not be distinguishable either chromatographically or by their infrared-absorption spectra.

Fig. 2. Infrared-absorption spectra of aldohexose sugars, samples incorporated in KCl disks. (A) Specimen isolated from *C. violaceum* (BN); (B) and (C), D-glycero-D-galactose; (D), D-glycero-L-galactose; (E), D-glycero-L-glucose; (F), D-glycero-L-mannose; (G), D-glycero-D-allose; (H), D-glycero-D-gulose; (J), D-glycero-L-talose; (K), D-glycero-D-idose. (B) is a sample of (C) diluted to match (A).

Attempts to crystallize the sugar from various water-organic solvent mixtures were unsuccessful and resulted in considerable loss of material. A white amorphous precipitate was obtained, however, when acetone or ethanol was added to aqueous solutions of the sugar. This was recovered and dried; unlike the sugar syrup, which was extremely hygroscopic, the dried powder did not absorb moisture from the atmosphere. This material seemed likely to be a polysaccharide and was therefore hydrolysed for 16 hr. at 100° with 0.5N-H₂SO₄, and the hydrolysate examined chromatographically. A number of components were thus revealed: xylose and glucose were present, two slow-moving components which may have been sugar acids and traces of some substances which reacted with ninhydrin. It was subsequently found possible to recover a polysaccharide having this same composition from the aqueous washings of powdered cellulose. The material closely resembles that described by Huffman, Rebers, Spriestersbach & Smith (1955), which was obtained from cellulose in the same way. A further quantity of the contaminating polysaccharide was removed from the heptose sugar solution, to which acetone had been added to 75 % (v/v), by sedimentation at 105 000 g for 2 hr. As a result of this treatment the optical rotation of a dried sample of the heptose syrup rose to $[\alpha]_D^{20} + 60^\circ$ in water (c, 2).

From a 90 % (v/v) ethanol solution of sugar, 1.5 mg. of microcrystalline material was finally obtained. In the hope of obtaining an unequivocal identification with this small sample, the infrared-absorption spectra of eight of the available synthetic aldoheptoses were obtained, together with that of the unknown heptose. These are shown in Fig. 2. It can be seen that the spectra of the eight known heptoses all show considerable differences from each other, but that the spectrum of the *C. violaceum* heptose cannot be distinguished from that of D-glycero-D-galactoheptose.

DISCUSSION

Recent techniques have made the detection of aldoheptose sugars a relatively simple matter. On chromatograms the anisidine spray reagent of Hough *et al.* (1950) gives a characteristic colour when papers are heated at 130° for a few minutes; when this is obscured by failure of the heptose to separate from other sugars in a mixture, the sensitive H₂SO₄-cysteine reaction of Dische (1953) can be used.

Information about the movement of a few aldoheptoses on paper chromatograms has been given by Isherwood & Jermyn (1951) and Kowkabany (1954); additional data on all heptoses at present available are about to appear (Davies, 1957*b*). Since

all of the aldoheptoses have not yet been synthesized, however, a definite identification cannot be made by paper chromatography alone; in addition this method does not distinguish between pairs of optical enantiomorphs. Apart from D-glycero-D-galactoheptose, which has now been identified, the only other aldoheptose sugar isolated from a natural product is in fact an L-sugar, namely L-glycero-D-mannoheptose (Slein & Schnell, 1953; Weidel, 1955).

Up to the time of writing, the specific polysaccharides of Gram-negative bacteria are the only known natural sources of aldoheptoses. Future publications from this Establishment will show that at least twenty-five different polysaccharides prepared from organisms of this group contain heptose sugars, which are thus of much more frequent occurrence than had previously been realized.

The recognition of glucosamine by chromatography alone should not be relied upon because chromatographic data for other 2-amino sugars is limited; several new sugars of this class have recently been reported to occur in natural products (Strange & Dark, 1956; Strange, 1956; Crumpton & Davies, 1956; Tamelen, Dyer, Carter, Pierce & Daniels, 1956).

SUMMARY

1. A preparation of the somatic antigen of *Chromobacterium violaceum* (BN) was obtained by extraction of acetone-dried cells with trichloroacetic acid.
2. By hydrolysis with 1 % (v/v) acetic acid at 100°, the somatic antigen was separated into degraded polysaccharide and lipoprotein components.
3. The degraded polysaccharide was hydrolysed to release its component sugars and the hydrolysate passed through an ion-exchange resin.
4. D-Glucosamine hydrochloride was isolated from an acidic eluate of the resin column.
5. The neutral sugars, obtained by eluting the resin column with water before the acid elution, were separated on a column of powdered cellulose with 95 % (v/v) aqueous acetone as the developing solvent.
6. From the earlier fractions of the cellulose-column effluent L-rhamnose was isolated.
7. From the later fractions of the cellulose-column effluent D-glycero-D-galactoheptose was isolated, a sugar not previously known to occur in Nature.

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prepare solutions of the free sugars. Our thanks are due to Mr L. C. Thomas, Chemical Defence Experimental Establishment, Porton, for producing the infrared-absorption spectra and to Mr D. C. Hawkins for technical assistance.

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Uridine Diphosphoglucose Dehydrogenase of Pea Seedlings

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The discovery of uridine 5'-diphosphoglucuronic acid (Dutton & Storey, 1954; Storey & Dutton, 1955) as the intermediate in the formation of 'ether-type' glucuronides by cell-free liver preparations has led to renewed interest in and a new approach to many problems of uronic acid metabolism in both plants and animals, e.g. the forma-

tion of other types of glucuronides (Isselbacher & Axelrod, 1955; Dutton, 1955, 1956), the synthesis of polysaccharides containing uronic acid (Glaser & Brown, 1955), and the biosynthesis of ascorbic acid (Isherwood, Chen & Mapson, 1954). In the liver of several animals, the synthesis of uridine diphosphoglucuronic acid is carried out by a diphospho-