Structure of the Terminal Reducing Heptasaccharide of Polysaccharide 1 Isolated from the *Bordetella pertussis* Endotoxin

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The tetrasaccharide β-D-glucopyranosyl-(1,3)-β-D-glucopyranuronyl-(1,2)-L-*glycero*-α-D-*manno*-heptopyranosyl-(1,5)-3-deoxy-D-*manno*-2-octulosonic acid was isolated after treatment of polysaccharide 1 of *Bordetella pertussis* endotoxin with nitrous acid. Taking into account previously identified di- and trisaccharide fragments and analytical data obtained for the intact polysaccharide 1, we present the structure of a heptasaccharide that is thought to represent the region immediately adjacent to the hydrophobic (lipid A) moiety of lipopolysaccharide 1 of the *B. pertussis* endotoxin. This heptasaccharide represents 50 to 60% of the complete polysaccharide structure.

Polysaccharides isolated from endotoxins of smooth-type, gram-negative bacteria usually contain two regions, one composed of repeating oligosaccharide units and the other composed of nonrepeating monosaccharides. In the case of endotoxins isolated from enterobacteria, the first region is referred to as the O-specific chain and the second as the core. While exact structures are known for many repeating units present in endotoxins (17), core regions have been less frequently investigated. For the latter studies, R forms of enteric bacteria have been used primarily (5, 19).

In previous studies we have established that the endotoxin isolated from vaccinal, phase I strains of *Bordetella pertussis* is made up of two major lipopolysaccharides, lipopolysaccharides 1 and 2 (which account for almost all of the mass of the endotoxin), and that the isolated polysaccharide chains (polysaccharides 1 [PS-1] and 2) of these are, in fact, oligosaccharides; their estimated molecular masses of about 3,000 daltons indicate that they contain approximately 12 to 16 monosaccharide units. As their ^{13}C nuclear magnetic resonance (NMR) spectra do not suggest the presence of repeating units, they could represent core-type oligosaccharides. In agreement with this, the rate of migration of *B. pertussis* endotoxin was found to be similar to that of endotoxins isolated from R mutants of enteric bacteria (22, 25). It has also been shown that at least one of the isolated oligosaccharide chains has unexpected immunological properties, such as being mitogenic for mouse lymphocytes (7) and being a polyclonal activator for rabbit splenocytes (8). Furthermore, it has been demonstrated that the binding of the *B. pertussis* endotoxin to rabbit peritoneal macrophages is mediated by the polysaccharide region (9) and that in human monocytes, secretion of interleukin 1 is stimulated by the isolated PS-1 of this endotoxin (7a). Knowledge of the exact structure of this polysaccharide is therefore clearly of interest. In the present work the structure of the tetrasaccharide that contains the terminal reducing 3-deoxy-D-*manno*-2-octulosonic acid (KDO) unit of PS-1 is described.

MATERIALS AND METHODS

Endotoxin. *B. pertussis* 1414 (phase I, vaccinal; Institut Mérieux, Lyon, France) was grown in the liquid medium of Cohen and Wheeler, and the endotoxin was isolated by the phenol-water method as previously described (13).

General methods. For descending chromatography on Whatman no. 1 paper, the solvents butan-2-one–methanol–formic acid–water (8:4:4:1, vol/vol) (S1) and butan-1-ol–pyridine–water (6:4:3, vol/vol) (S2) were used. High-voltage paper electrophoresis was carried out on Whatman 3MM paper in a flat-plate electrophoresis apparatus with a pyridine-acetic acid-water (16:11:973, vol/vol; pH 5) buffer. Compounds containing vicinal diols were detected by treatment with periodate-alkaline silver nitrate (24). On paper chromatograms and electropherograms radioactive material was located with a Berthold LB 280 scanner; in solutions it was measured by scintillation with 2,5-di-(5-*tert*-butyl-2-benzoxazolyl)thiophene (4 g/liter in toluene), using an Intertechnique SL 30 counter.

Sugars and polyols were peracetylated with sodium acetate-acetic anhydride at 100°C for 1 h, solvents were removed, the products were extracted from the residue with chloroform, which was then evaporated, and the peracetates, dissolved in ethyl acetate, were used for analysis. For combined gas-liquid chromatography and mass spectrometry (GLC-MS), a stainless steel column (2,000 by 3.2 mm, charged with 3% SE-30 on GasChrom Q, 100-120 mesh) was used with a temperature gradient of 2°C/min from 120 to 200°C.

The analytical methods used to estimate hexoses, heptoses, hexosamines, hexuronic acids, and nitrogen were those employed previously (3, 20). A modification (2) of the periodate-thiobarbiturate reaction (27) was used to estimate KDO (absorption maximum at 550 nm) and to detect 3-deoxyoctitol (absorption maximum at 532 nm). D-Glucose was estimated with the hexokinase–glucose-6-phosphate dehydrogenase reaction (Boehringer, Mannheim, Federal Republic of Germany).

^{1}**H NMR.** Fourier transform spectra (40 scans) of β-D-glucopyranosyl-(1,3)-β-D-glucopyranuronyl-(1,2)-L-*glycero*-α-D-*manno*-heptopyranosyl-(1,5)-3-deoxy-D-*manno*-2-octulosonic acid (tetrasaccharide K) (16 mg/ml in ^{2}H$_2$O) were

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TABLE 1. Analytical data for PS-1 and compound K[a]

Compound	Amt (μmol/mg)					
	Hexoses	Heptoses	Hexosamines	Hexuronic acids[b]	KDO[c]	Nitrogen
PS-1	0.5	1.1	1.1	0.5	0.5	2.8
K	0.6	1.1	0	1.0	0.8	0

[a] Isolated tetrasaccharide: β-D-glucopyranosyl-(1,3)-β-D-glucopyranuronyl-(1,2)-L-*glycero*-α-D-*manno*-heptopyranosyl-(1,5)-3-deoxy-D-*manno*-2-octulosonic acid.

[b] Values are corrected for interference from heptose.

[c] Estimated by the semicarbazide test (18).

obtained on a 400-MHz instrument (Institut d'Electronique Fondamentale, Université de Paris Sud, Orsay, France). The resonance line (2,225 ppm) of added acetone (0.5%, vol/vol) was used as an internal standard, and chemical shifts are given in parts per million on the δ scale. The HDO resonance was saturated by two successive pulses at 180 and 90°, separated by an optimized delay time.

Chemicals and enzymes. 3-Deoxy-5-*O*-(β-D-glucopyrano-syl)-2-octulosonic acid (a mixture of the D-*manno* and D-*gluco* isomers) was prepared (23) by S. R. Sarfati (Châtenay Malabry, France). Yeast α-D-glucosidase, sweet almond β-D-glucosidase, and β-D-glucuronidase from *Helix pomatia* were purchased from Boehringer. NaB[3]H$_4$ was from the Commissariat à l'Energie Atomique (Saclay, France).

RESULTS

Preparation of PS-1 of *B. pertussis* endotoxin. PS-1 was prepared as described previously (12). Briefly, endotoxin of *B. pertussis* 1414, phase I (5.4 g), obtained by the phenol-water procedure (28), was treated with trifluoroacetic acid (pH 3, 50°C) until the thiobarbiturate reaction (27) of the samples reached a constant value (100 to 150 h). After removal of the precipitate by centrifugation (16,000 × *g*, 30 min), the supernatant was purified by chromatography on a Bio-Gel P10 column (100-120 mesh, 95 by 2.5 cm, with water as the eluant). Material appearing in the void volume was discarded, and the main fraction (purified PS-1, 850 mg) was recovered by lyophilization. Analytical data, obtained by colorimetric methods, are given in Table 1.

Isolation of the KDO-containing fragment. A lyophilized sample of PS-1 (20 mg) was dissolved in 1 M NaNO$_2$ (1 ml), and acetic acid (0.1 M, 1.2 ml) was added in three portions at 30-min intervals at room temperature. Solvents were removed, and the residue was desalted by electrophoresis on a glass fiber sheet (Whatman GF1, pH 5, 26 V/cm, 60 min). Material located between the base line and picric acid, used as a marker, was eluted with water, recovered by lyophilization, and subjected to column chromatography (Bio-Gel P2, 200-400 mesh, 48 by 2 cm, with water as the eluant); the elution profile obtained is shown in Fig. 1. The material of each peak was analyzed for KDO by the thiobarbiturate reaction (λ_{max} = 550 nm) applied either directly to samples of the eluate or to revealing electropherograms (pH 5, 54 V/cm, 60 min) of the material recovered by lyophilization. It was found that most of the material giving a λ_{max} at 550 nm in the thiobarbiturate test was located in peak 3 (Fig. 1). In the other peaks the λ_{max} of the thiobarbiturate-positive material was at 532 nm, suggesting that products formed from 2-amino sugars during treatment with nitrous acid were present; indeed, it was observed that treatment of glucosamine with nitrous acid gave a chromogen absorbing at 532 nm in the thiobarbiturate reaction. It was also established that the main product formed upon nitrous deamination of glucosam-

ine, namely, 2,5-anhydromannose, gave a positive reaction in this test, the λ_{max} being at 532 nm.

For preparative purposes, the procedure described above was repeated with 150 mg of PS-1. The material from peak 3 (Fig. 1) was recovered by lyophilization and further purified by paper electrophoresis (pH 5, 54 V/cm, 60 min, $R_{picric\ acid}$ = 0.77 to 0.79), yielding 8 mg of a compound, designated compound K, which gave a single spot upon chromatography in solvent S1 when visualized by the periodate-alkaline silver nitrate method. Its composition, as established by colorimetric methods, is given in Table 1.

Identification of the components of compound K by GLC-MS. Compound K (1 mg) was subjected to methanolysis (2 M HCl in methanol, 1 ml, 100°C, 24 h), and after removal of the solvent and residual acid by coevaporation with toluene, the residue was acetylated and analyzed by GLC-MS. Three peaks were detected and identified on the basis of their retention times and mass spectra as per-O-acetylated derivatives of the methyl esters of methyl glucuronide, methyl glucoside, and methyl heptoside, appearing in that order. Derivatives of KDO were not detected, although the presence of this component in compound K was indicated by the thiobarbiturate reaction; it has been established that, when subjected to methanolysis under the conditions indicated, the ammonium salt of KDO gives a number of small peaks.

To two other samples (1 mg each), aqueous sodium borohydride solution (1 ml, 1 mg/ml, 0°C) was added to
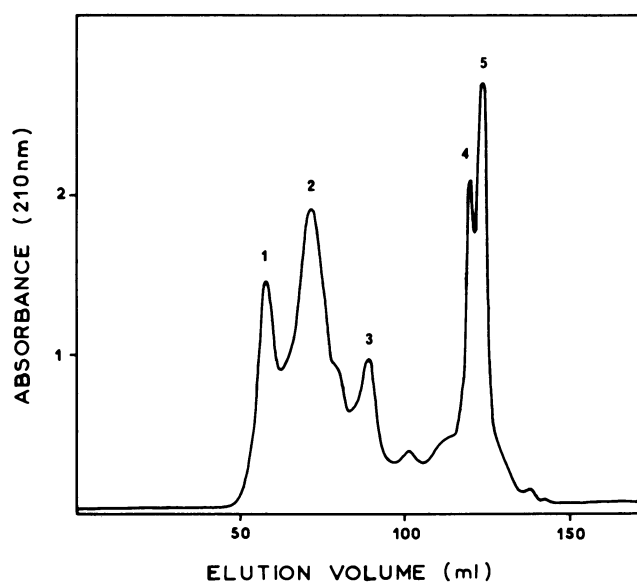

FIG. 1. Elution pattern of fragments formed upon treatment of PS-1 with nitrous acid (Bio-Gel P2 column, 48 by 2 cm).

TABLE 2. Methylation analysis of compound K

Derivative[a]	Main mass fragments[b]
A	45 (36.4), 75 (50.0), 88 (100), 101 (27.3), 111 (0.5), 127 (0.9), 149 (7.3), 176 (0.9), 187 (3.4), 219 (2.3)
B	75 (100), 88 (56.8), 101 (77.3), 129 (11.4), 141 (11.4), 159 (14.8), 185 (27.3), 233 (31.8), 245 (5.2), 277 (6.6)
C	43 (100), 101 (88.0), 113 (16.0), 117 (48.0), 129 (48.0), 145 (44.0), 161 (72.0), 173 (2.0), 205 (26.0), 217 (1.2)
D	43 (100), 89 (59.1), 129 (36.6), 143 (18.9), 173 (16.5), 189 (30.5), 205 (47.6), 291 (8.5), 335 (3.1), 349 (5.5)

[a] A, Methyl-2,3,4,6-tetra-*O*-methylglucopyranoside; B, methyl-2-*O*-acetyl-3,4,6,7-tetra-*O*-methylheptoside; C, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol; D, 1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methylheptitol.

[b] Expressed as z/e. Numbers in parentheses are relative intensities, expressed as a percentage of the base peak.

reduce the terminal KDO unit; 30 min later the solvent was evaporated and boric acid was removed by repeated addition and evaporation of acidified methanol. The residual material (K-1) of both samples was dried and methylated according to the method of Hakomori (10).

The first sample was then submitted to methanolysis (2 M HCl in methanol, 1 ml, 100°C, 24 h); the resulting mixture of partially methylated methyl glycosides was acetylated as described above and analyzed by GLC-MS. Two main peaks of comparable surface area were detected; the first (peak A) had a retention time and mass spectrum which indicated that it was methyl 2,3,4,6-tetra-*O*-methylglucopyranoside, while the second (peak B) (for which no standard was available to check the retention time) gave a mass spectrum which defined it as methyl-2-*O*-acetyl-3,4,6,7-tetra-*O*-methylheptoside.

The second methylated sample was hydrolyzed (2 M HCl, 1 ml, 100°C, 2 h) and, after removal of the solvent and residual acid with toluene, reduced with sodium borohydride and then acetylated as described above. Analysis by GLC-MS of the methylated alditol acetates thus formed gave two peaks: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (peak C) and 1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methylheptitol (peak D). The main fragments of the mass spectra of peaks A, B, C, and D are listed in Table 2. Methylated derivatives of the uronic acid and the epimeric 3-deoxyoctonic acids (formed by the reduction of the terminal KDO unit) did not elute from the column, as their carboxyl groups had not been esterified.

Recognition of compound K as a tetrasaccharide with a terminal reducing KDO unit. The data given above strongly suggested that compound K was a tetrasaccharide composed of a terminal reducing KDO, a terminal nonreducing glucose, a 2-*O*-substituted heptose, and a hexuronic acid. The terminal reducing position of the KDO unit was readily proved; after sodium borohydride treatment of compound K, the resulting material (K-1) no longer gave a positive reaction in the thiobarbiturate test. A similar reduction with NaB³H₄ was used to label compound K-1 ([³H]K-1). The paper chromatographic mobility (R_f = 0.21 in solvent S1, descending) of [³H]K-1 relative to that of an unseparated mixture of synthetic 2-deoxy-5-*O*-(β-D-glucopyranosyl)-D-*gluco*- and -D-*manno*-2-octulosonic acids (23) indicated that [³H]K-1 was larger than a disaccharide.

Upon paper electrophoresis at pH 5, the mobility of [³H]K-1 relative to the same standard as above was 1.21. As compound K did not contain basic groups, it could be deduced from the mobility of [³H]K-1 that in compound K the ratio of neutral sugars to acidic sugars was 1. This and the ¹H NMR spectrum of compound K provided proof that compound K was a tetrasaccharide. Indeed, in the low-field region (δ < 4.0 ppm) of the spectrum (Fig. 2), only three signals corresponding to anomeric protons were found, their ratio being 1:1:1. It was thus clear that, in addition to KDO

(which has no anomeric proton), only three other sugars were present in compound K.

Establishment of the substitution position of the KDO unit in tetrasaccharide K. A sample of [³H]K-1 (5 × 10⁵ cpm) was treated with aqueous NaIO₄ (7 × 10⁻² M, 20 μl, 20°C), followed, 70 h later at 0°C, by aqueous sodium borohydride solution (2 mg/ml), 150 μl, 1 h). The resulting material was then hydrolyzed with HCl (0.25 M, 1 ml, 100°C, 45 min), and after evaporation of the solvent and removal of residual acid by coevaporation with toluene, the residue was treated with dilute NaOH (pH 9.5) to hydrolyze any lactones present. The radioactive material ([³H]K-2 [Fig. 3]) was then examined by paper electrophoresis (pH 5) and descending chromatography in solvent S2. The material comigrated in both instances with a mixture of epimeric 3-deoxy-[2-³H]hexonic acids and was easily distinguished from a similar mixture of 3-deoxy-[2-³H]heptonic acids (Fig. 4). It follows that the terminal KDO unit present in tetrasaccharide K carried no substituent in positions 6 and 7 but was substituted at least in position 5.

Identification of the sequence 2-*O*-(β-D-glucopyranuronyl)-*glycero-manno*-heptose as part of tetrasaccharide K. Tetrasaccharide K (600 μg) was treated with aqueous sodium borohydride (0.5 ml, 1 mg/ml) to reduce the carbonyl group of the KDO unit. After removal of boric acid by repeated evaporation of acidified methanol, the product was treated briefly (25 min) with sodium periodate solution (25 μl, 7 × 10⁻² M, 20°C), and then, at 0°C, a large excess of NaB³H₄ (25 mCi, 2 mg, 1 h) was added to transform the heptose constituent into the corresponding 6-³H-labeled hexose. This was expected to occur, as it has been shown that analogous treatment of the intact *B. pertussis* endotoxin leads to the destruction of heptose units with the concomitant appearance of mannose (3) and that the heptose present in tetrasaccharide K was unsubstituted in positions 6 and 7 (see above). Upon paper electrophoresis (pH 5, 54 V/cm, 60 min) a negatively
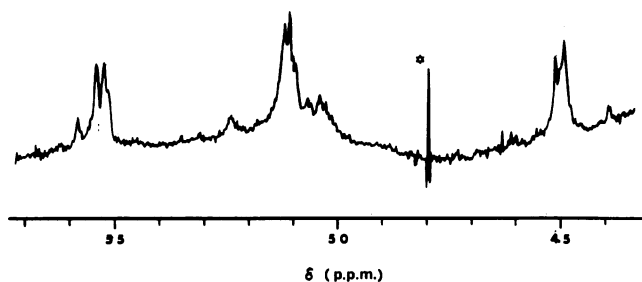

FIG. 2. Low-field region of the Fourier transform 400-MHz proton magnetic resonance spectrum of a 2.2% solution of compound K in deuterium oxide. Asterisk indicates HDO residual resonance.
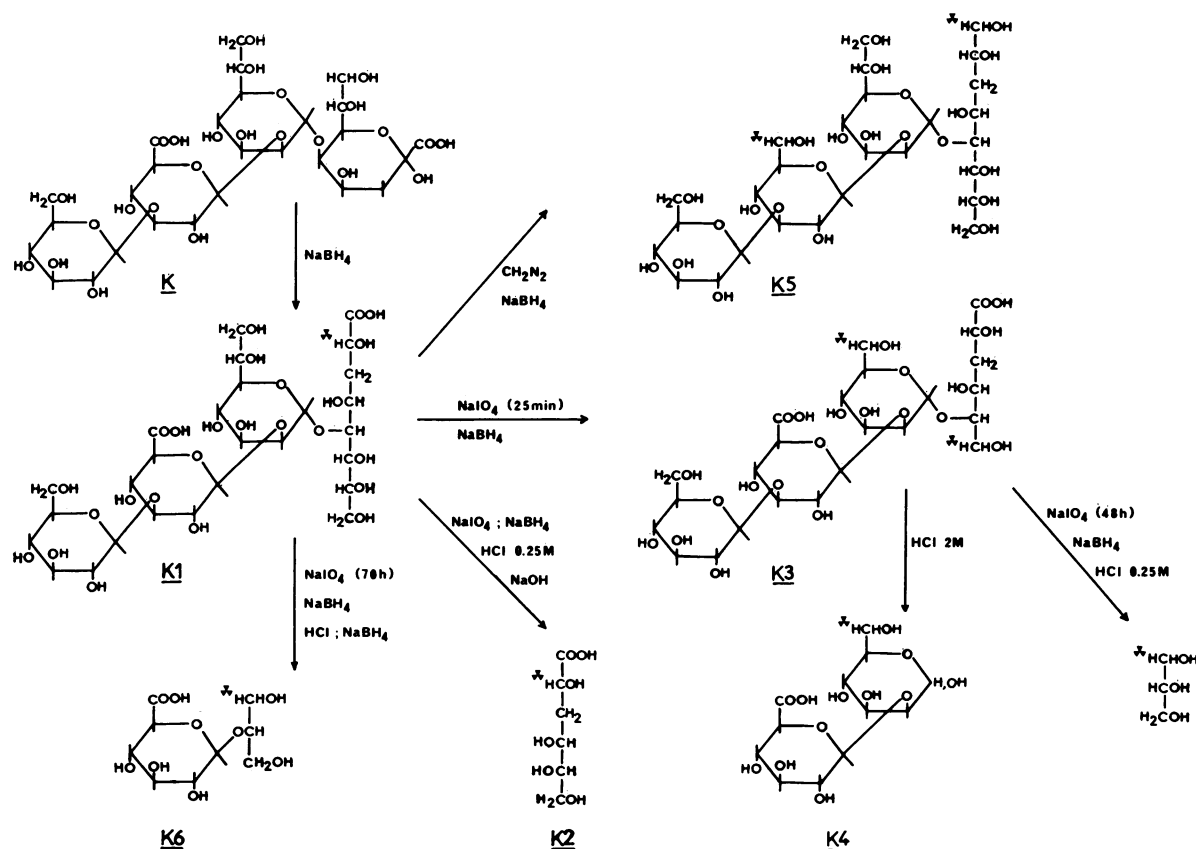
FIG. 3. Fragmentation scheme of tetrasaccharide K. Asterisk indicates position of label when NaB^3H_4 was used instead of $NaBH_4$.

charged, radioactive compound ($[^3H]$K-3 [Fig. 3]) having an $R_{picric\ acid}$ of 0.83 was detected and recovered by elution with water from a preparative run. A sample of this compound was hydrolyzed with acid (2 M HCl, 100°C, 1 h), and the hydrolysate, freed of acid, was subjected to paper electrophoresis (pH 5). One uncharged and two negatively charged radioactive substances ($R_{picric\ acid}$ = 0.79 and 1.1) were detected and recovered by elution with water.

One of the radioactive substances ($R_{picric\ acid}$ = 0.79) ($[^3H]$K-4 [Fig. 3]) was dissolved in acetate buffer (0.1 M, pH 4.5, 100 μl) and incubated (24 h, 37°C) with β-D-glucuronidase; paper chromatography (solvent S2, 30 h, descending) revealed that the main radioactive product now migrated like D-mannose (Fig. 5). It was concluded that the heptose unit (from which the radioactive mannose was formed) of tetrasaccharide K was substituted by a β-D-glucopyranuronyl residue. The other radioactive substance ($R_{picric\ acid}$ = 1.1) was not examined. The neutral material isolated in the hydrolysate of $[^3H]$K-3, when analyzed by paper chromatography (solvent S2, 26 h, descending), appeared to consist of a major radioactive compound having the same mobility as D-mannose and of a minor product ($R_{mannose}$ = 0.28) which was not identified.

To determine the substitution position of the heptose unit, $[^3H]$K-3 (5 × 10^5 cpm) was subjected to a Smith degradation (oxidation: 7 × 10^{-2} M $NaIO_4$, 50 μl, 48 h; reduction: 0.5 mg of $NaBH_4$ in 0.5 ml of water, 0°C, 1 h; hydrolysis: 0.25 M HCl, 1 ml, 100°C, 30 min), and the material obtained after removal of the acid was analyzed for radioactive compounds. Upon paper electrophoresis (pH 5, 54 V/cm, 1 h)

only neutral radioactive material was found. It was recovered by elution and subjected to paper chromatography (solvent S2, 18 h, descending), and a single radioactive compound that had the mobility of glycerol was detected. It was concluded that the glucuronic acid unit was attached to
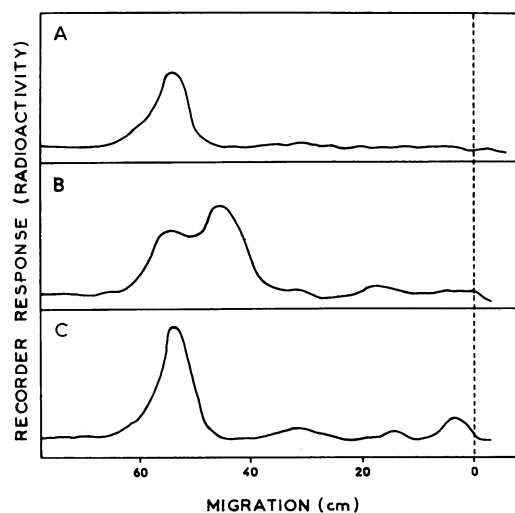

FIG. 4. Electropherograms (pH 5) of $[^3H]$K-2 alone (A), with added 3-deoxy-$[2-^3H]$heptonic acid (B), and with added 3-deoxy-$[2-^3H]$hexonic acid (C).
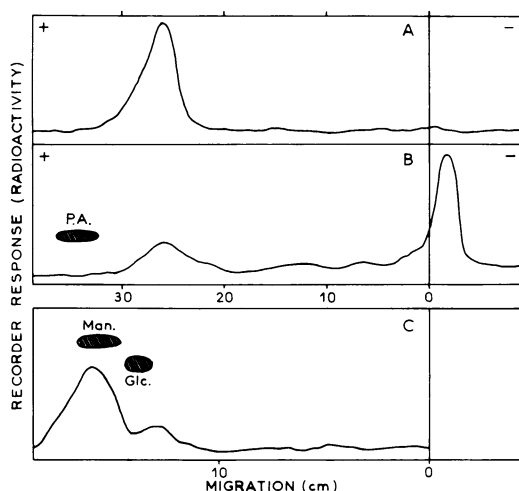
FIG. 5. Electropherogram (pH 5) of [³H]K-4 before treatment with β-D-glucuronidase (A) and electropherogram (pH 5) (B) and chromatogram (solvent S2, 30 h) (C) of [³H]K-4 after treatment with β-D-glucuronidase.

position 2 of the heptose; this is consistent with results obtained by methylation analysis (see above).

Substitution pattern of the glucuronic acid unit present in tetrasaccharide K. A sample (100 μg) of the tetrasaccharide was subjected to a Smith degradation (oxidation: 7 × 10⁻² M NaIO₄, 100 μl, 70 h; reduction: NaB³H₄, 25 mCi, 2 mg in 1 ml of water, 0°C, 1 h; hydrolysis: 0.5 M HCl, 1 ml, 100°C, 30 min) with a long period for the oxidation step to ensure complete oxidation of all vicinal diol groups present. After removal of HCl by coevaporation with toluene, the material was reduced (NaBH₄, 1 mg in 1 ml of water, 0°C, 1 h) and submitted to paper electrophoresis at pH 5. Scanning of the electropherogram revealed one neutral and two anionic substances. One of the latter ($R_{picric\ acid}$ = 0.9) ([³H]K-6 [Fig. 3]), when recovered and compared with synthetic 2-O-(β-D-glucopyranuronyl)glycerol by both paper electrophoresis (pH 5) and paper chromatography (solvent S1, 18 h, descending; $R_{maltose}$ = 1.5), had mobilities identical with those of the reference compound. The same substance (4 × 10⁵ cpm) was then incubated with β-D-glucuronidase (5 μl, 5.2 U/ml) in an acetate buffer (pH 4.5, 0.1 M, 100 μl, 37°C, 24 h); upon analysis of the mixture by paper electrophoresis (pH 5), a neutral, radioactive compound was found. Upon paper chromatography (solvent S2, 18 h, descending), the mobility of this substance, recovered from the electropherogram, was identical to that of glycerol and different from those of ethylene glycol ($R_{glycerol}$ = 1.2) and threitol ($R_{glycerol}$ = 0.8). It was concluded that the substance having an $R_{picric\ acid}$ of 0.9 was 2-O-(β-D-glucopyranuronyl)glycerol ([³H]K-6) and that, as the β-D-glycopyranuronyl unit was not destroyed during the exhaustive periodate oxidation, it was substituted in position 3 by the terminal nonreducing glucopyranose unit present in tetrasaccharide K.

The reference compound, 2-O-(β-D-glucopyranuronyl)glycerol, was prepared by NaBH₄ reduction of 2-O-(β-D-glucopyranuronyl)-D-arabinose (23) to the corresponding arabinitol derivative, which after oxidation with 2.5 molar equivalents of NaIO₄, followed by reduction with a large excess of NaBH₄, gave the required glucuronide.

Enantiomeric and anomeric configuration of the terminal nonreducing glycopyranose unit in tetrasaccharide K. The

identification of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol as one of the products formed upon methylation, acid hydrolysis, reduction, and acetylation of tetrasaccharide K established that the glucose constituent was an unsubstituted glucopyranose unit, but revealed neither the enantiomeric nor the anomeric configuration of the sugar. It was expected, however, that both of these features could be established by use of enzymes. As it has been observed (15) that the presence of acidic sugars may inhibit the action of glycosidases, both carboxyl groups of tetrasaccharide K-1 were first reduced by treating tetrasaccharide K-1 (800 μg), dispersed in diethyl ether (1.5 ml) containing some diazomethane, in an ultrasonic bath at 0°C for 5 min. After removal of the solvent, an aqueous solution of NaBH₄ (25 mCi, 4 mg, 0°C, 1 h) was added, and the radioactive material (presumed to be K-5 [Fig. 3]) present in the residue obtained after removal of water was purified by chromatography on a Bio-Gel P2 column. The efficiency of the reduction was evaluated by repeating the experiment, but using [³H]K-1; more than 80% of the originally anionic material was found to be neutral by paper electrophoresis (pH 5). In addition, when a sample of K-5 was hydrolyzed with acid (1 M HCl, 100°C, 1 h) and the hydrolysate was assayed by the thiobarbiturate reaction, the absorption maximum was found to be at 532 nm, indicating that the terminal 3-deoxyaldonic acid had been transformed into 3-deoxyoctitol.

Four samples of compound K-5, each containing 72 μg of glucose released upon hydrolysis with acid (0.5 ml of HCl, 100°C, 1 h), were prepared, and the following additions were made. To the first sample, α-glucosidase (10 μl, 100 U/ml) and acetate buffer (pH 6.5, 0.1 M, 50 μl) were added; to the second sample, β-glucosidase (5 μl, 200 U/ml) and acetate buffer (pH 5, 0.1 M, 50 μl) were added; and to the third and fourth samples, identical amounts of buffers and denatured (100°C, 10 min) enzymes, respectively, were added. All samples were incubated at 37°C overnight, whereafter the D-glucose content of each sample was estimated by the hexokinase–glucose-6-phosphate dehydrogenase reaction. It was found that the sample treated with active β-glucosidase released 11% of its glucose content, whereas no glucose was released from the other samples. The conclusion, corroborated by the ¹H NMR study reported below, was reached that the nonreducing terminal unit of tetrasaccharide K was a β-D-glucopyranose unit.

Anomeric configuration of the heptose constituent. In an aqueous solution of tetrasaccharide K only three signals appeared in the region of the 400-MHz ¹H NMR spectrum where anomeric protons usually are found (Fig. 2). Resonances at 5.53 ppm ($J_{1,2}$ = 7.5 Hz), 5.11 ppm ($J_{1,2}$ = 3 Hz), and 4.50 ppm ($J_{1,2}$ = 7.5 Hz) relative to tetramethylsilane were observed. It has been established that the heptose present in tetrasaccharide K is a homomorph of mannose (3); it followed that only the signal at 5.11 ppm could be that of the anomeric proton of the heptose. Indeed, in both anomers of aldopyranosides homomorphic with mannose the dihedral angle between H-1 and H-2 is about 60°C, and as a corollary $J_{1,2}$ is smaller than 5 Hz. The chemical shift of this signal, 5.11 ppm, is within the range (5.1 to 5.38 ppm) where anomeric protons of 2-O-substituted α-D-mannopyranosides resonate (4, 6, 14, 26); it is different from the range (4.8 to 4.9 ppm) where the anomeric protons of β-mannopyranosides are observed. It was therefore concluded that the heptose present in tetrasaccharide K, known to be L-*glycero*-D-*manno*-heptose, had the α-D-*manno* configuration. Incidentally, the relatively large coupling constants (7.5 Hz) measured for the signals at 5.53 and 4.5 ppm, due to the anomeric

protons of the glucopyranuronyl and glucopyranosyl units, corroborated the β-anomeric configuration assigned to these constituents on the basis of their cleavage by the appropriate enzymes.

DISCUSSION

It has been observed during previous studies that only very small amounts of KDO-containing fragments are released upon acid hydrolysis of PS-1 of the *B. pertussis* endotoxin, with most of the KDO being set free as a monosaccharide. It was thus mandatory to use other methods of fragmentation for the structural analysis of the region adjacent to this constituent. As it has also been noticed that free amino groups are present in PS-1, and as treatment of 2-amino sugars with nitrous acid is known to lead, concomitantly with deamination, to cleavage of the glycosidic bonds of the deaminated sugar (16), this method was used to produce a fragment containing the KDO unit. Unexpectedly, this fragment (tetrasaccharide K), which was detected in and recovered from the mixture formed upon deamination of PS-1 because of its λ_{max} at 550 nm in the thiobarbiturate reaction (27), was formed in a very low yield, about 5% instead of the calculated 30 to 35%. The reason for this could, perhaps, be the preponderance of usually secondary reactions occurring during the deamination process, some of which are known not to be accompanied by cleavage of the glycosidic bond of the deaminated sugar (16). It is also possible, however, that due to the treatment with nitrous acid or to the acid hydrolysis employed to release PS-1, the terminal KDO unit of PS-1 is present in a form (e.g., lactone) that does not react in the thiobarbiturate test in the expected manner and thus escaped recognition.

The presence of free amino groups in the polysaccharide chain of an endotoxin is not exceptional; sugars bearing such groups have been reported to occur in core oligosaccharides of endotoxins of *Pseudomonas maltophilia* (21), *Shigella flexneri* (11), and *Escherichia coli* (5).

Although not demonstrated by experiments ad hoc, the presence of a KDO unit in compound K was obvious because the tetrasaccharide gave a positive reaction in the thiobarbiturate test, with a λ_{max} at 550 nm (27); because of the previous demonstration (12) that the starting material, PS-1, contained a single, 5-*O*-substituted KDO molecule; and because upon Smith degradation the reduced tetrasaccharide, K-1, released a 3-deoxyaldohexonic acid, expected to be produced from a 5-*O*-substituted KDO unit. Because of the very small amount of material available, methylation analysis of the tetrasaccharide after reduction of its carbonyl and carboxyl groups was not attempted. The proof of substitution in position 5 is unequivocal; its unicity follows from the linearity of the tetrasaccharide.

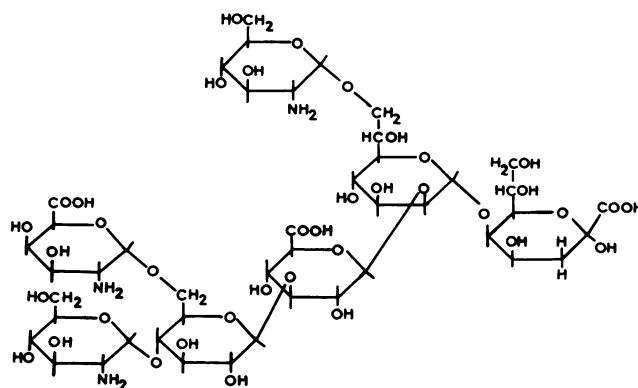Difficulties were encountered when the determination of

FIG. 6. Proposed structure of the heptasaccharide present at the reducing end of PS-1 isolated from *B. pertussis* endotoxin.

the anomeric configuration of the glucose unit was attempted by use of enzymes; unexpectedly, and despite a number of experiments done, only about 10% of the glucose present in the tetrasaccharide was released by β-glucosidase. An unambiguous assignment could be made, however, on the basis of the ^{1}H NMR spectrum for both the glucose and the heptose constituents. The configuration of the latter sugar as L-*glycero*-D-*manno*-heptose can be inferred from previous work (3) in which the trisaccharide 7-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-*O*-(β-D-glucopyranuronyl)-L-*glycero*-D-*manno*-heptose (1) was shown to be part of PS-1. The absence of the amino sugar in tetrasaccharide K, which was released by deamination with nitrous acid, and the presence of a heptose with a free primary alcohol and substituted in position 2 by β-D-glucuronic acid would both be expected if these constituents of the above-mentioned trisaccharide and those of tetrasaccharide K were one and the same unit of PS-1. It follows that the heptose present in tetrasaccharide K has the L-*glycero*-D-*manno* configuration.
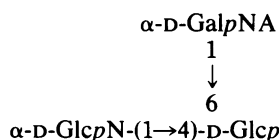
With the analytical data obtained for the intact PS-1 and the overlap of partial structures so far identified, it appears possible to reconstruct that part of the carbohydrate chain which, in PS-1, is adjacent to the hydrophobic (lipid A) region of lipopolysaccharide 1, one of the two major constituents of *B. pertussis* endotoxin. Indeed, colorimetric estimations of the hexose and hexuronic acid contents indicated (Table 1) that only single units of these constituents were present in PS-1, which also contains only a single unit of KDO. The sequence β-D-GlcpA-(1→2)-L-*glycero*-D-*manno*-Hep is common is tetrasaccharide K and the previously identified trisaccharide:

$$\text{α-D-GlcpN}$$
$$1$$
$$\downarrow$$
$$7$$
$$\text{β-D-GlcpA-(1→2)-L-}\textit{glycero}\text{-D-}\textit{manno}\text{-Hep}$$
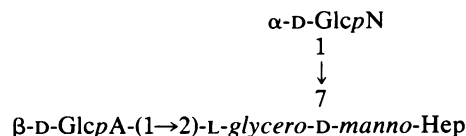
Thus, tetrasaccharide K may be enlarged to the branched pentasaccharide:

$$\text{α-D-GlcpN}$$
$$1$$
$$\downarrow$$
$$7$$
$$\text{β-D-Glcp-(1→3)-β-D-GlcpA-(1→2)-L-}\textit{glycero}\text{-D-}\textit{manno}\text{-Hep-(1→5)-KDO}$$
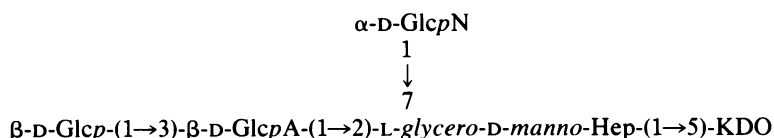
Furthermore, as the trisaccharide (20)

α-D-GalpNA

1

↓

6

α-D-GlcpN-(1→4)-D-Glcp

and the pentasaccharide share the single glucuronic acid residue of PS-1, the structure may be completed to the heptasaccharide displayed in Fig. 6. As the molecular weight of PS-1, calculated from its colorimetrically estimated KDO content, appears to be 2,100 to 2,300, this heptasaccharide should represent about 50% of the entire structure of PS-1.

The exactness of the proposed structure of the heptasaccharide, as reconstructed by the overlap of isolated oligosaccharides, depends on the accuracy of the colorimetric estimation of hexose and hexuronic acid in the isolated PS-1; it should, therefore, be considered tentative until confirmed by direct methods.

ACKNOWLEDGMENTS

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