STRUCTURAL INVESTIGATION OF THE LIPOPOLYSACCHARIDE OF *Yersinia pseudotuberculosis* OF TYPE IB

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The lipopolysaccharides (LPSs) of *Yersinia pseudotuberculosis* have recently been investigated chemically and immunochemically [1, 2] and a general scheme of the structure of the O-specific side chains for the LPSs from the microorganisms of the serological subtypes IIA and IIB has been suggested (I). We have shown previously [2] that the LPSs from *Y. pseudotuberculosis* of type IB contain the following monosaccharide residues: paratose (3,6-dideoxy-D-ribohexose), L-fucose, D-mannose, D-glucose, D-galactose, D-glucosamine, 2-oxo-3-deoxyoctonic acid (ODO), L-glycero-D-mannoheptose, and D-glycero-D-mannoheptose.

The present paper gives the results of a study of the same LPSs with the use of methylation.

The lipopolysaccharide was isolated from bacteria of the *Y. pseudotuberculosis* IB type (strain 12) by Westphal's phenyl–water method in agreement with the procedure described [2]. The acid hydrolysis of the LPS and the analysis of the monosaccharides in the form of acetates of the aldononitriles gave paratose, fucose, mannose, galactose, and the sum of the heptoses in the relative molar percentages of 20 : 14 : 25 : 15 : traces : 25, respectively. The results of the identification of the monosaccharides were confirmed by the mass spectra of the acetates of the aldononitriles and the acetates of the methyl glycosides. These monosaccharides were also partially characterized in preceding investigations [2, 3]. The acetates of the aldononitriles of the two heptoses had the same retention time on GLC as the acetates of the aldononitriles of L-glycero-D-mannoheptose (16%) and D-glycero-D-mannoheptose (9%).

The lipopolysaccharide was methylated in dimethyl sulfoxide by treatment with methyl iodide in the presence of dimethylsulfinylsodium by Hakomori's method [4]. In methylation, the usual mechanical stirring was used, or the reaction mixture was subjected to ultrasonic treatment [5]. In the second case the methylation of the initial LPC took place faster. The completely methylated LPS was hydrolyzed and the resulting mixture of methylated sugars was analyzed in the form of the corresponding acetates of methyl glycosides or acetates of aldononitriles [6] by chromato-mass spectrometry (GLC–MS). For separation we used a column containing QF-1 (Fig. 1) and neopentyl glycol succinate (NPGS). The methyl glycosides and aldononitriles of the partially methylated sugars were separated considerably better on the QF-1.

The methyl ethers of fucose and the hexoses were identified by the T values and mass spectra as described elsewhere [7]. The mass spectra of 2,5-di-O-methylparatose in the form of the corresponding acetate of the aldononitrile and the acetate of the methyl glycoside are given in Tables 1 and 2. It follows from this that the paratose is present in the LPS in the furanose form. In the mass spectrum of 2,5-di-O-methylparatitol (I) the main ions are those with m/e 43, 57, 59, 69, 71, 75, 85, 97, 101, 117, 129, 170, and 189 (the ions with the maximum intensities are underlined). Of these, the ions m/e 59, 117, and 189 are primary ions. If position 4, and not 5, were methylated, there should be an ion with m/e 131 (as in the case of 2,4-di-O-methylabequose [5] and 2,3,4-trimethylfucitol [8]).

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TABLE 1. The Mass Spectra of the Acetates of the Methyl Glycosides of the Partially Methylated Heptoses and Paratose

2,5-Di-O-methylparatose			2,3,4,6,7-Penta-O-methylheptose			2,3,4,6-Tetra-O-methylheptose			2,4,6-Tri-O-methylheptose			2,6,7-Tri-O-methylheptose		
type of ion*	m/e	rel. intensity, %	type of ion*	m/e	rel. intensity, %	type of ion*	m/e	rel. intensity, %	type of ion*	m/e	rel. intensity, %	type of ion*	m/e	rel. intensity, %
A_1	159	2,8	A_1	263	0,3	A_1	291	2,6	A_1	319	4	A_1	319	7
E_1	131	26	M—32	262	0,35	M—59	263	0,4	M—59	291	0,5	E_1	305	15,5
A_1-32	127	4,5	E_1'	249	1,1	A_1—32	259	0,8	M—60	290	0,4	M—59	291	0,9
	103	14	A_1—32	231	1	E_1'	249	0,5	C_2	259	0,6	E_1	261	8
	100	3,3		217	1,5	A_1—60	231	0,8		245	0,6	A_1—60	259	1,3
E_1-32	99	6,4	E_1	205	1,8		227	1,7	E_1	233	2,9	E_1'—60	245	17,5
	89	10,5		199	3,2	D_1	221	15		231	2,6	C_2	231	13,5
	88	5,5	D_1	193	12,5		217	1,4		217	1,5		213	58
	75	100		185	0,9	E_1	205	5		201	0,4		203	8,5
	72	12	B_1	176	0,7		199	1,6		199	3,5	E_1—60	201	13,5
G_1	71	50	E_1—32	173	4,1		185	1,2	E_1—60	173	12,3		199	10
S	59	67	D_1—32	161	2,9	B_1	176	1,1		157	6		185	64
	55	11		145	10		173	10		143	6		171	44
	45	61		144	1,4		161	3,7		129	5		159	21
	43	55		131	5,2		145	8	S	117	54		143	64
				116	2,9	S	117	16		116	25	G_1	129	60
			G_1	101	90	G_1	101	96	G_1	101	100	H_1	116	35
			S	89	32	H_1	88	100	H_1	88	43		111	49
			J_1	75	87	J_1	75	92		85	7,4		97	104
				73	15		73	8	J_1	75	58	S	89	73
			S'	45	36		45	18		74	49		88	86
			H_1	88	100		43	29		73	11	J_1	75	80
										45	19	H_1—42	74	100
										43	84		59	32
												S'	45	82
													43	†

*The symbols for the ions are given in accordance with those used previously [9].

TABLE 2. Mass Spectra of the Acetates of the Aldononitriles of the Partially Methylated Heptoses and of Paratose

2,5-Di-O-methylparatose			2,3,4,6-Tetra-O-methylheptose			2,4,6-Tri-O-methylheptose			2,6,7-Tri-O-methylheptose		
type of ion	m/e	rel. intensity, %	type of ion	m/e	rel. intensity, %	type of ion	m/e	rel. intensity, %	type of ion	m/e	rel. intensity, %
M—31	184	12,5	M—59	288	0,45	M—59	316	0,4	A_6	330	100
M—32	183	3,75	B_5	277	3,6	B_5	305	0,4	M—59	316	2,4
	173	15	A_6	274	2,8	A_6	302	3,8	B_5	305	5
M—60	155	11		242	2,3	A_5	258	0,7	A_5	286	2
A_4	116	67	B_4	233	23		242	1,4		270	6,5
	145	10		214	1,2	B_4	233	22	B_4	233	2
	141	42		201	4,8		203	1,8		228	15,5
	131	37	B_3	189	0,9		201	1,2	A_4	214	4
	129	42		173	3,7	B_3	189	1,4		203	34
	124	35	A_4	158	9	A_4	186	12,6	B_3	161	3,7
	114	26		131	4,7		173	17		154	†
	113	55		127	22		159	42		129	69
	100	66	B_2	117	100		154	40		99	24
	99	41		85	19	A_3	142	3,2	B_2	89	238
	97	100	B_1	73	12		131	9,6		87	99
	96	37,5	A_2	70	5	B_2	117	100	A_2	70	20
	72	135		45	45		85	9,6		45	90
	70	145		43	†		70	9		43	†
B_2	59	†					45	48			
							43	†			

A represents an ion including the carbon atoms of the molecule beginning from the first, and B those beginning from the last. The subscript figure relates to the number of C atoms in the ion.

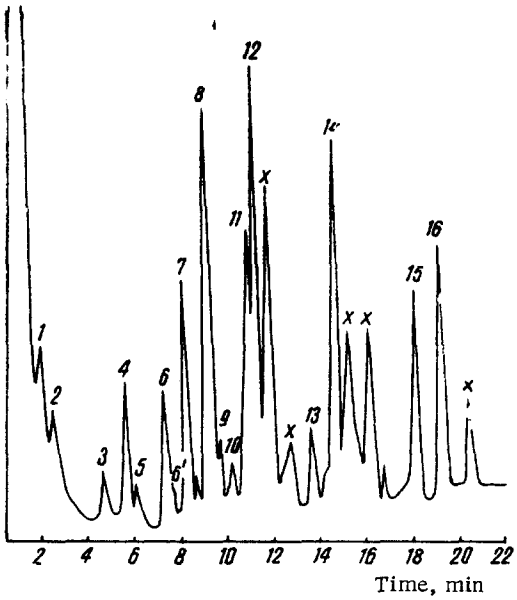
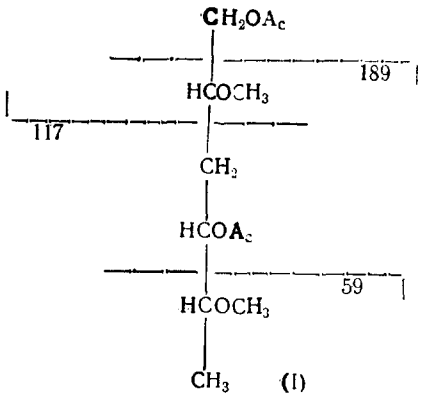
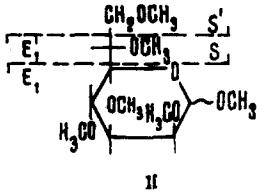


Fig. 1. GLC of the acetates of the methyl glycosides from a hydrolyzate of the methylated glycopolysaccharide of *Y. pseudotuberculosis* on QF-1 (the designation of the peaks are given in Table 1, X representing unidentified peaks).

This peak was absent in our case.



The methyl ethers of the heptoses were identified by the mass sepctra of the corresponding acetates of the methyl glycosides and aldononitriles (see Tables 1 and 2). The pattern of decomposition of the methyl esters of the methyl heptosides under electron impact is similar to that of the decomposition of the corresponding methyl esters of methyl hexosides [9]. The presence of a side chain with two carbon atoms leads to the formation of ions of types E' and S' (II)



II

The results of the analysis of the methylated sugars are summarized in Tables 3 and 4.

It can be seen from the facts given that all the monosaccharide residues with the exception of the paratose are present in the carbohydrate chain of the LPS in the pyranose form. Fucose residues are included in the carbohydrate chain only by a 1,3-bond. All the paratose and galactose residues are present at nonreducing ends of the carbohydrate chain. Furthermore, the terminal position is occupied by some of the heptose, glucose, and possibly mannose residues. The few glucose residues are included in a linear chain by 1,2- and 1,4-bonds. The linear sections of the chain also include the mannose and heptose residues, and in a number of cases the residues of the same monosaccharides are points of branching of the LPS carbohydrate chain.

The partial hydrolysis of the LPS with 1% acetic acid led to the precipitation of lipid A. In addition to this, a polysaccharide hapten was formed. The complete acid hydrolysis of the latter gave paratose, fucose, mannose, glucose, galactose, L-glycero-D-mannoheptose and D-glycero-D-mannoheptose in relative molar percentages of 15 : 17 : 27 : 15.5 : traces : 17 : 7, respectively. The compound obtained was methylated, hydrolyzed, and analyzed in the form of acetates of the methyl glycosides of the partially methylated sugars by GLC-MS (see Table 3).

The almost complete disappearance of the 2,4,6-tri-O-methylmannose and the considerable decrease in the amount of 4,6-di-O-methylmannose show that the paratose residue is attached to the mannose in position 3 (this is indicated by the increase in the amount of 3,4,6-tri-O-methylmannose in the hapten in comparison with the LPS, since some of the paratose residues are split off on hydrolysis).

TABLE 3. Acetates of Methyl Glycosides of Partially Methylated Sugars from a Hydrolyzate of Methylated LPS (columnn A) and from a Hydrolyzate of the Methylated Hapten (column B).

Methylated sugars	QF-1				NPGS
	No. of peak	T†	A	B	T†
2,5-Di-O-methyl-β-Par *	1	−1,3	+	+	−1
2,5-Di-O-methyl-α-Par	2	1,3	+	+	+1
2,3,4,6-Tetra-O-methyl-β-D-Glc	3	12,6	+	Tr.	12,7
2,3,4,6-Tetra-O-methyl-α-D-Glc‡ (α-D-Man)	4	16,3	++	+	18,4
2,3,4,6-Tetra-O-methyl-α(β)-Gal**	5	19,1	+	Tr.	20
2,3,4,6,7-Penta-O-methyl-Hep	6	24,8	++	+	27,8
3,4,6-Tri-O-methyl-α-D-Man	6′	26	Tr.	+	22,5
2,4-Di-O-methyl-β-L-Fuc	7	28,5	++	+	22,0
2,4-Di-O-methyl-α-L-Fuc	8	33	++++	++	27
3,4,6-Tri-O-methyl-α-D-Glc	9	36	+	—	30,6
2,3,6-Tri-O-methyl-β-D-Glc	10	39	+	—	33
2,4,6-Tri-O-methyl-α-D-Man	11	42	++	—	36,5
2,3,6-Tri-O-methyl-α-D-Man	12	43	++++	+++	40
—,—,6,7-Tetra-O-methyl-Hep	13	54	+	—	52,5
4,6,Di-O-methyl-α-D-Man	14	58	+++	++	46,4
2,3,4,6-Tetra-O-methyl-Hep	14	58			56,4
2,6,7-Tri-O-methyl-Hep	15	75,7	++	+	67
2,4,6-Tri-O-methyl-Hep	16	80,6	++	+	71,4

*Par) paratose

†The relative retention times were determined on a scale in which T_{sp} for 2,3,4-tri-O-methyl-β-xyloside was taken as zero and T_s of the peracetate of the aldononitrile of galactose as 100.

‡T_{sp} of 2,3,4,6-tetra-O-methyl-α-D-glucose and of 2,3,4,6-tetra-O-methyl-α-D-mannose coincided on QF-1, NPGS, and OV-225.

**T_{sp} of 2,3,4,6-tetra-O-methyl-α- and -β-D-galactosides coincided on QF-1, NPGS, and OV-225.

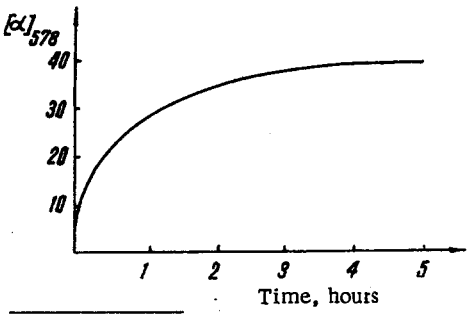


Fig. 2. Curve of the change in optical density on the acid hydrolysis of the hapten with 0.5 N H_2SO_4 at 80°C.

If it is assumed that the O-specific side chains consist of oligosaccharide repeating units, the simplest unit for this LPS must probably consist of one residue each of paratose and fucose and two mannose residues. The other monosaccharides are possibly obtained from the skeletal polysaccharide; 2,4,6-tri-O-methylmannose is formed from the terminal repeating unit of the O-specific chains.

The course of the hydrolysis of the hapten was investigated polarimetrically. The curve of the change in optical rotation (increase in rotation) shows that the most readily hydrolyzed bonds of the paratose have the β configuration (Fig. 2).

EXPERIMENTAL

For growth we used a local strain (No. 12) of the microbe *Yersinia pseudotuberculosis* type IB. The technology of the production of the microbial mass has been described elsewhere [2]. The LPS was isolated by phenol–water extraction and was purified by ultracentrifugation at 105,000 g.

Hydrolysis of the LPS. The LPS (10 mg) was hydrolyzed in 1 ml of 1 N H_2SO_4 in a sealed tube at 100°C for 4 h. The acid was neutralized with Dowex-1 (HCO_3), and the solution was evaporated in vacuum at 45°C. This gave the acetates of the aldononitriles [10], and these were analyzed by the GLC–MS method.

TABLE 4. Acetates of Aldononitriles of Partially Methylated Monosaccharides from a Hydrolyzate of Methylated LPS

Methylated sugars	QF-1	
	No. of peak	T*
2,5-Di-O-methyl-Par*	1	28,5
2,3,4,6-Tetra-O-methyl-Glc (Man)	2	50
2,4-Di-O-methyl-Fuc	3	53
2,4,6-Tri-O-methyl-Man	4	62,5
2,3,6-Tri-O-methyl-Man	5	64,2
2,3,4,6,7-Penta-O-methyl-Hep	5	64,2
2,3,6-Tri-O-methyl-Glc	6	69,5
3,4,6-Tri-O-methyl-Glc	7	71,5
4,6-Di-O-methyl-Man	8	76
Tetra-O-	8	76
2,6,7-Tri-O-methyl-Hep	9	81,7
2,3,4,6-Tetra-O-methyl-Hep	10	86
2,4,6-Tri-O-methyl-Hep	11	95

* and †: see Table 3.

Preparation of the Hapten (the polysaccharide part of the LPS). The LPS (50 mg) was heated in 1% acetic acid (6 ml) at 100°C in a sealed tube for 2 h, and the mixture was then cooled and concentrated to 2 ml. Ethanol (8 ml) was added and the mixture was extracted with hexane (10 ml). The polysaccharide (hapten) was isolated from the ethanolic aqueous phase after dialysis by lyophilization (20 mg). Part of the substance was hydrolyzed with 1 N H_2SO_4 at 100°C for 4 h and was neutralized (Dowex-1, HCO_3^-) and the monosaccharides were converted into the acetates of the aldononitriles and were analyzed by GLC. Another part was methylated.

The methylation of the LPS was performed by Hakomori's method [4] in two ways: with mechanical stirring in the usual way, and under the action of ultrasound [5].

In the second case, the LPS (10 mg, dried in vacuum over P_2O_5 at 78°C) was dissolved in dry dimethyl sulfoxide (DMSO) (1 ml) in an atmosphere of argon in a 5-ml test tube. Then 1 ml of a 1.8 N solution of dimethylsulfinylsodium was added and the mixture was kept for 30 min in an ultrasonic bath and for another 8 h at room bemperature with magnetic stirring. Then, with external ice cooling, CH_3I (1 ml) was added in drops and the resulting solution was kept in the ultrasonic bath until a clear yellowish solution had been obtained (30 min). This solution was poured into ice water (20 ml), dialyzed, and evaporated to dryness. The completeness of methylation was determined by IR spectroscopy from the absence of an absorption band at 3400-3600 cm^{-1} 26.9% of OCH_3 was found.

The hydrolysis of the methylated LPS (and hapten) was performed by two ways:

A. Half the methylated product was dissolved in 90% formic acid (2 ml), and the solution was heated at 100°C for 2 h and was evaporated to dryness. The residue was dissolved in 0.13 M H_2SO_4 (2 ml) and hydrolyzed at 100°C for 12 h.

The hydrolyzate was neutralized with $BaCO_3$ or Dowex-1 (HCO_3^-) and evaporated to dryness. The mixture of methylated sugars was converted into the acetates of the aldononitriles and analyzed by GLC—MS.

B. Another part of the methylated LPS (or hapten) was heated in a methanolysis mixture (3 ml of $HClO_4$—methanol, 1:10 v/v) at 100°C for 3 h. After cooling, it was neutralized with Dowex-1 (HCO_3^-), evaporated to dryness, and acetylated. The mixture of acetates of the partially methylated methyl glycosides was analyzed by GLC—MS. All the solutions were concentrated under reduced pressure at 30-35°C.

The GLC analysis of the hydrolyzate of the initial LPS and of the products of its methylation was performed on a Pye Unicam-104 gas chromatograph with a flame-ionization detector using steel columns (200 cm × 4 mm) filled with 3% (v/v) of QF-1 on Gas-Chrom Q (100-200 mesh) and 3% of NPGS (v/v) on Aeropak-30 (60-80 mesh) washed with acid and silanized, with programming of the temperature from 110 to 230°C on the QF-1 and from 125 to 225°C on the NPGS (5°C/min) for the acetates of the methyl glycosides and for the aldononitriles of the partially methylated sugars. The temperature conditions of working for the acetates of the aldononitriles of the monosaccharides were from 175 to 225°C (5°C/min).

The rate of flow of the carrier gas (argon) and of the hydrogen was 60 ml/min. The GLC—MS analysis of the mixture of methylated products was performed on an LKB-9000 apparatus with glass columns (300 cm × 4 mm) filled with the phases described above. The mass spectra were recorded at an ionization potential of 70 eV.

Hydrolysis of the Hapten at 80°C. A sample of the hapten (3.75 mg) was dissolved in 0.5 N H_2SO_4 (1.5 ml), transferred to a polarimetric cell (10 cm) and heated at 80°C. The optical rotation was measured after predetermined intervals of time on a Perkin—Elmer polarimeter.

SUMMARY

Using the chromato–mass spectrometry of the methylated sugars formed in the hydrolysis of the fully methylated lipopolysaccharide from *Yersinia pseudotuberculosis* of type IB the nature of the glycosidic bonds between the monosaccharide residues has been established. The hapten formed in the partial hydrolysis of the lipopolysaccharide has also been studied by methylation. The results obtained have confirmed the results of an investigation of the methylated lipopolysaccharide; they have enabled an idea to be put forward of the composition of the repeating unit of the O-specific side chains of the lipopolysaccharide.

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