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Characterization of Lipid A and Polysaccharide Moieties of the Lipopolysaccharides from *Vibrio cholerae*

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Lipid A and polysaccharide moieties obtained by mild acid hydrolysis of the lipopolysaccharides from *Vibrio cholerae* 569 B (Inaba) and *Vibrio el-tor* (Inaba) were characterized. Heterogeneity of lipid A fractions was indicated by t.l.c. and by gel filtration of the de-*O*-acylated products from mild alkaline methanolysis of the lipids. Presumably lipid A contains a glucosamine backbone, and the fatty acids are probably bound to the hydroxyl and amino groups of glucosamine residues. Approximately equal amounts of fatty acids $C_{16:0}$, $C_{18:1}$ and 3-hydroxylauric acid were involved in ester linkages, but 3-hydroxymyristic acid was the only amide-linked fatty acid. Sephadex chromatography of the polysaccharide moiety showed the presence of a high-molecular-weight heptose-free fraction and a low-molecular-weight heptose-containing fraction. Haemagglutination-inhibition assays of these fractions showed the heptose-free fraction to be an *O*-specific side-chain polysaccharide, whereas the heptose-containing fraction was the core polysaccharide region of the lipopolysaccharides. Identical results were obtained for both organisms.

The lipopolysaccharides (endotoxins) of Gram-negative bacteria are composed of two distinct regions of contrasting character, a hydrophilic polar region, the polysaccharide, consisting of the *O*-specific chain and the basal core, and a hydrophobic lipid portion, lipid A (Luderitz *et al.*, 1966; Gmeiner *et al.*, 1969; Luderitz *et al.*, 1971). Lipid A is covalently bound within the lipopolysaccharides, from which it can be released by gentle acid hydrolysis, which simultaneously liberates polysaccharide. Lipid A is the endotoxic component of the lipopolysaccharides (Gmeiner *et al.*, 1971; Galanos *et al.*, 1972; Luderitz *et al.*, 1973), whereas the polysaccharide moiety is responsible for serological specificity (Luderitz *et al.*, 1968).

Although the lipopolysaccharides of a large number of micro-organisms have been studied in greater detail, knowledge of the exact chemical composition of lipid and polysaccharide moieties of the lipopolysaccharides is limited to certain representatives of *Enterobacteriaceae* and *Pseudomonas* families (Gmeiner *et al.*, 1969; Luderitz *et al.*, 1971; Hewett *et al.*, 1971; Weckesser *et al.*, 1972; Rooney *et al.*, 1972; Drewry *et al.*, 1973; Wilkinson *et al.*, 1973; Lomax *et al.*, 1974; Wilkinson & Galbraith, 1975). Previously, lipopolysaccharides isolated from both whole cells and cell walls of different stains of *Vibrio cholerae* have been analysed (Jann *et al.*, 1973; Jackson & Redmond, 1974; Armstrong & Redmond, 1974; Redmond, 1975; Raziuddin & Kawasaki, 1976; Raziuddin & Ambegaokar, 1976). The present paper describes the further characterization of the lipid and polysaccharide moieties of the lipopolysaccharides from *Vibrio cholerae* 569 B (Inaba) and *Vibrio el-tor* (Inaba).

Materials and Methods

Growth of bacteria and isolation of lipopolysaccharides

Vibrio cholerae 569 B (Inaba) and *Vibrio el-tor* (Inaba) were grown in a synthetic medium and lipopolysaccharides were isolated from the defatted cell walls by treatment with aq. 45% (w/v) phenol at 72°C by the method of Westphal & Jann (1965), as described earlier (Raziuddin & Kawasaki, 1976).

Isolation of lipid A and polysaccharide moieties

Lipid A and polysaccharide moieties of the lipopolysaccharide were cleaved by the method of Wilkinson *et al.* (1973). To cleave lipopolysaccharide into lipid A and polysaccharide, samples (50mg) were hydrolysed with 1% acetic acid (5ml) at 105°C for 2.5h. Each hydrolysate was separated into chloroform-soluble, water-soluble and interfacial materials by thorough mixing with an equal volume of chloroform, followed by low-speed centrifugation at 18000*g* for 20min. The chloroform layer was isolated, washed twice with water and dried to give lipid A. Similarly, the aqueous layer was isolated, washed twice with chloroform and dried *in vacuo* over P_2O_5 to give polysaccharide. They were purified as described previously (Raziuddin & Kawasaki, 1976).

Fractionation of lipid A and further analysis

Fatty acid residues (ester- and amide-linked) were removed from lipid A by hydrazinolysis with anhydrous hydrazine at 100°C for 10h as described by Gmeiner *et al.* (1969), and lipid A was de-*O*-acylated by mild alkaline methanolysis as follows. Lipid A (15mg) was dissolved in chloroform (6ml), 0.2M-KOH (6ml) was added and the mixture was incubated at 37°C for 2.5h. Then chloroform (6ml) and ethyl formate (40μl) were added, and incubation was continued for a further 10min. Water-soluble products were isolated by mixing the solution with deionized water (5ml), separating the two phases by centrifugation at 3000rev./min in an MSE centrifuge and collecting the upper phase. The lower phase was washed three times with fresh upper-phase solvent, and the washings were combined with the original upper phase. It was then freeze-dried. Mild alkaline methanolysis of the lipid was carried out by the method of Rietschel *et al.* (1972).

The water-soluble products from lipid A were fractionated by column chromatography as follows. Samples (15mg) were applied to a column (1.5cm × 70cm) of Sephadex G-25. Elution was carried out with 20% (v/v) methanol at a flow rate of 10ml/h. Fractions (2ml) were analysed for phosphorus and were then pooled according to the respective peaks and analysed.

Liberation of fatty acids from lipid A with 2.0M-HCl or 10% (w/v) KOH in 50% (v/v) methanol and extraction from the hydrolysate was carried out as described by Rooney & Goldfine (1972). Methyl esters of the free fatty acids were prepared by reaction with diazomethane by the method of Schlenk & Gellerman (1960). The relative proportions of *O*- and *N*-substitution with fatty acid were determined as described by Fensom & Gray (1969). For identification of hydroxy fatty acids, the mixture of fatty acid methyl esters was trimethylsilylated as described previously (Raziuddin & Kawasaki, 1976). Methylation of the hydroxyl group of hydroxy fatty acids was carried out as described by Hakomori (1964).

Fractionation of polysaccharide moiety and further analysis

The polysaccharide moiety obtained as described above was fractionated on a Sephadex G-50 column (1.5cm × 200cm) with pyridine/acetic acid/water (10:4:1, by vol.), pH5.4, as eluent. Fractions (1ml) were collected at a flow rate of 15ml/h, analysed for phosphorus and carbohydrate content and were pooled according to the respective peaks and analysed.

Dephosphorylation of polysaccharide

Polysaccharides and fractions were dephosphorylated by the method of Baddiley *et al.* (1957). Samples (10mg) were dissolved in 0.2M-ammonium acetate buffer, pH3.8 (1ml), and heated for 42h at 100°C. The dephosphorylated polysaccharide was separated from buffer and low-molecular-weight products by chromatography on Sephadex G-15 with pyridine/acetic acid/water (10:4:1, by vol.), pH5.4, as eluent and analysed (Lomax *et al.*, 1974).

Periodate oxidation

Polysaccharides were oxidized with 50mM-$NaIO_4$ in the dark at 4°C and the consumption of $NaIO_4$ was determined by the method of Avigad (1969). After the completion of oxidation, residual $NaIO_4$ was destroyed by ethylene glycol and the oxidation product was further treated for 12h with 2% (w/w) $NaBH_4$. The excess of $NaBH_4$ was destroyed with dilute acetic acid, and after drying, boric acid was removed by repeated distillation with methanol.

Methylation

Methylation of polysaccharide was carried out by the modified method of Lomax *et al.* (1974). Polysaccharide (5mg) was dissolved in dry dimethyl sulphoxide (1ml) by sonication for 5min at 1.2A in an MSE ultrasonic disintegrator, and kept overnight. A solution (1.5ml) containing the dimsyl ion (dimethylsulphinyl carbanion) in dimethyl sulphoxide was added dropwise under N_2, and the mixture was sonicated for 45min as described above and then kept at room temperature (27°C) for 10h. Methyl iodide (0.25ml) was added with cooling, and the mixture was sonicated for 30min. When a single methylation was inadequate, further dimsyl reagent (1.5ml) and methyl iodide (1ml) were used in a second treatment. The final solution was poured into deionized water (50ml) and dialysed against distilled water for 24h to remove salts and reagents. The non-diffusible residue was dried by rotary evaporation and then subjected to formolysis with 80% (w/w) formic acid at 105°C for 6h as described by Percival (1971). It was then diluted 4-fold with deionized water and heated for 2.5h, and the hydrolysate was dried by rotary evaporation. Methylated sugars were converted into alditol acetates (Sarwardekar *et al.*, 1965).

Chromatography

Paper chromatography was carried out on sheets of Whatman no. 1 filter paper. The following solvents were used: a, butanol/pyridine/water (6:4:3, by vol.); b, ethyl acetate/acetic acid/88% (v/v) formic acid/water (18:3:1:4, by vol.); c, ethyl acetate/pyridine/water (10:4:3, by vol.); d, butanol/pyridine/0.1M-HCl (5:3:2, by vol.); e, methanol/water/pyridine/10M-HCl (32:7:4:1, by vol.); f, acetone/

water (19:1, v/v); g, chloroform/methanol/water (65:25:4, by vol.); h, propanol/water (7:1, v/v).

T.l.c. of lipid A was carried out on 20cm×20cm glass plates coated (0.35mm thick) with Stahl silica gel G (E. Merck A.-G., Darmstadt, Germany) with the solvent systems chloroform/methanol/water (65:25:4, by vol.) (g) and chloroform/methanol/7M-ammonia (12:7:1, by vol.).

Electrophoresis

High-voltage electrophoresis was carried out on Whatman no. 1 and 3 MM filter papers, at about 30V/cm for 1h on no. 1 or at about 12V/cm for 3h on 3 MM filter paper, in pyridine/acetic acid/water (5:2:43, by vol.).

Components were detected by alkaline $AgNO_3$ reagent, Ehrlich's reagent, ninhydrin (0.2%) in acetone, periodate/Schiff's reagent and aniline phosphate reagent as described earlier (Raziuddin & Kawasaki, 1976).

Gas–liquid chromatography

The detailed procedures for methylation of fatty acids and g.l.c. were described previously (Raziuddin & Kawasaki, 1976; Raziuddin, 1976; Raziuddin & Ambegaokar, 1976). The fatty acid methyl esters were analysed on a Shandon FB4 gas chromatograph by using 15% ethylene glycol succinate polyester on Chromosorb W (80–100 mesh). Monosaccharides were reduced to alditols with $NaBH_4$ and converted into alditol acetates or *O*-trimethylsilyl derivatives. Alditol acetates were characterized on columns containing 3% (w/w) ECNSS-M as stationary phase, and *O*-trimethylsilyl derivatives on columns containing 3% (w/w) OV-225, by the methods of Bjorndal *et al.* (1967) and Lonngren & Pilotti (1971) respectively. Mass spectra were recorded on an LKB 9000 spectrometer.

Haemagglutination test

The test was carried out on fractions of the polysaccharide moiety in large haemagglutination-titre plates (well diameter 15mm, capacity 1ml) by using veronal buffer (pH7.8) as eluent. Human erythrocytes were used. Serial dilution by a factor of 2 (20μl) with the inhibitor substance (fractions P1 and P2, obtained by gel filtration of polysaccharide by Sephadex G-50, Fig. 2) at concentrations between 400 and 0.40μg/ml was done with veronal buffer (pH7.8), and an appropriate serum dilution of 20μl of a guinea-pig serum was added. After 1h of incubation at 37°C, 50μl of the lipopolysaccharide-sensitized cells (human erythrocytes were sensitized with alkali-treated lipopolysaccharides at 37°C for 30min) was added, and the plates were again incubated at 37°C for 1h. Total inhibition of haemagglutination was checked after 24h.

Analytical methods

D-Perosamine (4-amino-4,6-dideoxy-D-mannose) was determined as described by Redmond (1975). Methods used for determination of carbohydrates, glucose, rhamnose, heptose, mannose, fructose, glucosamine and phosphorus have been described previously (Raziuddin & Kawasaki, 1976).

Results

Lipopolysaccharides isolated from the defatted cell walls of *V. cholerae* 569 B (Inaba) and *V. el-tor* (Inaba) were purified by ultracentrifugation (Raziuddin & Kawasaki, 1976), freeze-dried and were further hydrolysed to separate lipid A and polysaccharide moieties.

The lipid-A fractions were obtained as white waxy solids soluble in chloroform but insoluble in water. Analysis of lipid A showed the presence of glucosamine as the only amino sugar. After hydrolysis in 1M-HCl at 105°C for 4h, traces of glucose, heptose and also fructose were detected by paper chromatography. These sugars might be present in lipid A because of slight contamination with the polysaccharide moiety during fractionation. T.l.c. of lipid-A preparations showed a considerable

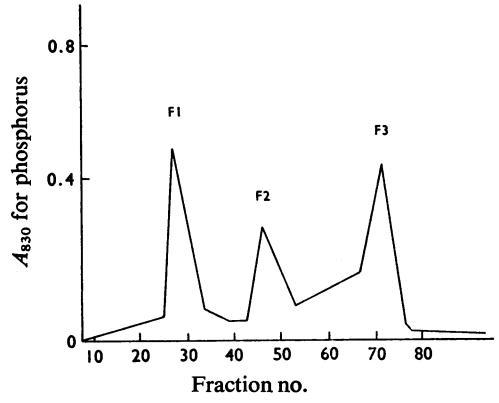

Fig. 1. *Fractionation of de-O-acylated lipid A from V. cholerae*

The water-soluble products (de-*O*-acylated lipid) from lipid A (15mg) were fractionated on a column of Sephadex G-25 (1.5cm×70cm). Elution was carried out with 20% (v/v) methanol at a flow rate of 10ml/h. Fractions (2ml) were collected and screened for phosphorus content and were then pooled and analysed as described in Table 1. Similar results were obtained for *V. cholerae* 569 B (Inaba) and *V. el-tor* (Inaba).

heterogeneity (Raziuddin & Kawasaki, 1976), which could partly be due to the existence of extended structures in which acylated disaccharide units are linked together by phosphodiester or pyrophosphate bridges. The structural studies of the backbone of lipid A can be simplified by the prior removal of fatty acid residues. Hydrazinolysis was therefore used to remove the fatty acids, both ester- and amide-linked, from the lipid-A preparations of *V. cholerae* and *V. el-tor*. The de-*O*-acylated lipid A (water-soluble products of lipid A), obtained as a result of mild alkaline methanolysis, was proved to be a mixture of phosphorylated oligosaccharides. De-*O*-acylated lipid A was further fractionated by column chromatography on Sephadex G-25, giving the results shown in Fig. 1 for *V. cholerae* and *V. el-tor*. Three major fractions designated F1, F2 and F3 were resolved by fractionation of the de-*O*-acylated lipid A of these bacteria. The distributions of components between these three fractions are given in Table 1. Glucosamine and phosphate were present at different concentrations in F1, F2 and F3 fractions. No definite conclusions can be drawn from these results, except that de-*O*-acylated lipid A of *V. cholerae* and *V. el-tor* can be resolved into three distinct fractions.

Table 1. *Analysis of fractions of de-O-acylated lipid A from V. cholerae and V. el-tor*
Water-soluble products of lipid A (de-*O*-acylated lipid A) were fractionated on a column of Sephadex G-25 (1.5cm×70cm) into fractions F1, F2 and F3, and analysed.

	V. cholerae		*V. el-tor*	
Fractions	Glucosamine (μg)	Total phosphorus (μg)	Glucosamine (μg)	Total phosphorus (μg)
F1	250	12.5	280	28
F2	190	20.0	65	18
F3	405	60.0	510	48

The results of the g.l.c. analysis of the fatty acids liberated by acidic (2.0M-HCl, 2.5h, 105°C) and alkaline (10% KOH in 50% methanol; 2.5h refluxing) hydrolysis of lipid A from *V. cholerae* and *V. el-tor* are given in Table 2. The fatty acids were identified by comparison of their retention times with those of authentic standards. Further, the hydroxylauric acid (OH-$C_{12:0}$) and hydroxymyristic acid (OH-$C_{14:0}$) were compared as the acetylated compounds with methyl acetoxylaurate and methyl acetoxymyristate as described previously (Raziuddin & Kawasaki, 1976). Both of the hydrolytic procedures used gave an identical quantitative fatty acid pattern, with $C_{14:0}$, $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ fatty acids, 3-hydroxy-$C_{12:0}$ and 3-hydroxy-$C_{14:0}$ as the major fatty acids, and differing only in $C_{16:1}$ fatty acid content. The alkaline hydrolysis of lipid A yielded a smaller amount of $C_{16:1}$ fatty acid. 3-Hydroxy-$C_{12:0}$ was the main fatty acid component of lipid A from *V. cholerae* and *V. el-tor*. Moreover, hydroxy fatty acids were absent from free lipids and phospholipids of these bacteria (Raziuddin, 1976; Raziuddin & Kawasaki, 1976).

Mild alkaline methanolysis was used to distinguish between the ester- and amide-linked fatty acids from lipid A of *V. cholerae* and *V. el-tor*. Methanolysis transesterifies *O*-acyl residues into methyl esters, but in contrast, amide-linked fatty acids are not liberated by this treatment and are still present in the de-*O*-acylated lipid A, obtained after the centrifugation of the methanolysates as a sediment. Strong alkaline hydrolysis (4M-NaOH, 6h, 105°C) was therefore used to release amide-linked fatty acids from these products. In the de-esterified sediments of lipid A from *V. cholerae* and *V. el-tor*, 3-hydroxymyristic acid was the only fatty acid component identified by g.l.c. Therefore these

Table 2. *Fatty acid composition determined by acid and alkaline hydrolysis of lipid A from V. cholerae and V. el-tor*
After hydrolysis with either acid (2.0M-HCl at 105°C) or alkali (10% KOH in 50% methanol, 2.5h refluxing) and methylation with diazomethane, the fatty acid methyl esters were analysed by g.l.c. on a 15% (w/v) ethylene glycol succinate polyester column.

	V. cholerae		*V. el-tor*	
Fatty acids	Acid hydrolysis (%, w/w)	Alkaline hydrolysis (%, w/w)	Acid hydrolysis (%, w/w)	Alkaline hydrolysis (%, w/w)
$C_{14:0}$	12.8	10.2	13.4	10.8
$C_{15:0}$	1.0	0.6	1.6	0.8
$C_{16:0}$	9.2	11.4	13.6	12.2
$C_{16:1}$	10.8	4.2	12.8	3.8
$C_{18:0}$	2.8	Trace	3.0	Trace
$C_{18:1}$	12.8	11.6	11.8	10.6
3-OH-$C_{10:0}$	2.5	1.6	1.8	0.6
3-OH-$C_{12:0}$	28.6	26.4	22.4	25.4
3-OH-$C_{14:0}$	14.2	13.4	15.6	14.2

Table 3. *Quantification of fatty acids in methanolysates of lipid A and relative proportions of O- and N-linked esters of fatty acids from V. cholerae and V. el-tor*

Total fatty acids were determined in a separate experiment from the mild alkaline methanolysates carried out by treatment of lipid A (5mg) with methanolic 0.25M-sodium methoxide at 37°C for 12h. Fatty acids released as methyl esters were measured by g.l.c. on 15% ethylene glycol succinate polyester columns.

	V. cholerae			*V. el-tor*		
Fatty acids	Total (%)	*O*-Acyl (%)	*N*-Acyl (%)	Total (%)	*O*-Acyl (%)	*N*-Acyl (%)
$C_{14:0}$	6.8	6.8	—	5.4	3.2	0.5
$C_{15:0}$	Trace	3.0	6.8	1.2	7.1	4.2
$C_{16:0}$	18.6	16.4	12.5	16.0	14.4	10.2
$C_{16:1}$	11.8	—	14.8	10.2	—	18.2
$C_{18:0}$	8.0	11.4	—	8.8	10.2	2.4
$C_{18:1}$	22.2	12.8	14.5	20.8	10.8	—
3-OH-$C_{10:0}$	1.4	1.2	—	3.2	—	9.8
3-OH-$C_{12:0}$	32.2	32.6	26.8	34.0	30.4	34.0
3-OH-$C_{14:0}$	—	18.0	24.4	—	24.0	16.2

results indicate that 3-hydroxymyristic acid is an amide-linked fatty acid in lipid A of *V. cholerae* and *V. el-tor*. On the other hand, ester-linked fatty acids were identified in the supernatants, obtained after the centrifugation of the methanolysates, in the form of methyl esters. The ester-bound major fatty acids from lipid A of *V. cholerae* and *V. el-tor* were $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ fatty acids and 3-hydroxylauric acid. Of these ester-bound fatty acids, 3-hydroxylauric acid was the main component and 3-hydroxymyristic acid was not detected (Table 3). Thus these results clearly indicate that 3-hydroxymyristic acid is an amide-linked fatty acid, and $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ fatty acids and 3-hydroxylauric acid are ester-linked fatty acids in lipid A of *V. cholerae* and *V. el-tor*.

The relative proportion of *O*- and *N*-substitutions in fatty acids of lipid A from *V. cholerae* and *V. el-tor* are also given in Table 3. Hydroxylaminolysis of lipid A showed that $C_{14:0}$, $C_{18:0}$ and 3-hydroxy-$C_{10:0}$ fatty acids were *O*-linked in *V. cholerae*, whereas the $C_{18:1}$ fatty acids was *O*-linked in *V. el-tor*. Similarly, *N*-linked fatty acids were $C_{16:1}$ from *V. cholerae* and $C_{16:1}$ and 3-hydroxy-$C_{10:0}$ from *V. el-tor*. Approximately equal amounts of *O*-linked $C_{16:0}$ and $C_{18:1}$ fatty acids and 3-hydroxylauric acid were found to be involved in ester linkages. Except for these above-mentioned differences, all other fatty acids were present as both the *O*-acyl and *N*-acyl esters. These results cannot readily be interpreted.

The polysaccharide moiety obtained by acetic acid hydrolysis of lipopolysaccharides from *V. cholerae* and *V. el-tor* was soluble in water but insoluble in chloroform, and scarcely soluble in pyridine. The substance after gel filtration on Sephadex G-50 and elution with pyridine/acetic acid/water (10:4:1, by vol.), pH 5.4, was resolved into two major fractions (P1 and P2, Fig. 2). These fractions were then separated, freeze-dried, dephosphorylated and analysed for different components (Table 4). Glucose, mannose, rhamnose, fructose and D-perosamine made up both of these fractions, although there were large variations in individual quantities of these components in the two fractions. The P2 fraction was the major fraction, and it contained all the constituents of the lipopolysaccharides of these bacteria, except glucosamine, fatty acids (Redmond, 1975; Raziuddin & Kawasaki, 1976) and D-

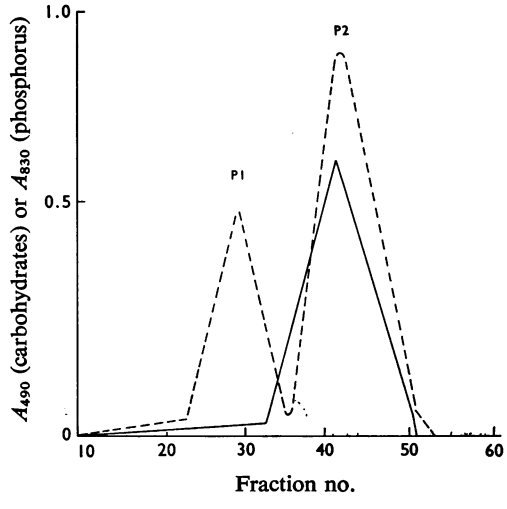

Fig. 2. *Fractionation of the polysaccharide from V. cholerae*

Polysaccharide was fractionated on a column of Sephadex G-50 (1.5cm×200cm). Elution was carried out with pyridine/acetic acid/water (10:4:1, by vol.) pH 5.4, at a flow rate of 15ml/h. Fractions (1ml) were analysed for total phosphorus (——) and for total carbohydrates (----). Almost similar results were obtained for *V. cholerae* 569 B (Inaba) and *V. el-tor* (Inaba).

Table 4. *Analysis of dephosphorylated polysaccharides from V. cholerae and V. el-tor*
P1 and P2 are the fractions after gel chromatography of total dephosphorylated polysaccharides (de-Phos). Polysaccharides were fractionated on a column of Sephadex G-50 (1.5cm×200cm) with pyridine/acetic acid/water (10:4:1, by vol.), pH5.4, as eluent. Fractions (P1 and P2) obtained from the corresponding peaks (shown in Fig. 2) were analysed.

	Content (%, w/w)					
	V. cholerae			*V. el-tor*		
Components	P1	P2	Total de-Phos.	P1	P2	Total de-Phos.
Phosphorus	—	0.3	0.4	—	0.2	0.3
Heptose	—	14.2	14.2	—	13.8	14.0
Glucose	4.8	13.8	18.4	4.0	14.6	19.8
Fructose	2.4	4.2	6.8	2.2	3.2	5.2
Mannose	3.0	Trace	3.2	2.8	0.6	3.0
Rhamnose	3.2	Trace	3.2	2.8	Trace	2.8
Glucosamine	3.2	—	3.4	3.4	—	3.4
D-Perosamine	2.4	Trace	2.6	2.6	Trace	2.8

Table 5. *Methylation analysis of the phosphorylated and dephosphorylated polysaccharides from V. cholerae and V. el-tor*
Polysaccharides were methylated by using dimethyl sulphoxide, and the methylated sugars released by acid hydrolysis were analysed as alditol acetates by g.l.c.–mass spectrometry at 165°C with ECNSS-M as column stationary phase, as described in the Materials and Methods section. Five peaks, A, B, C, D and E were obtained by g.l.c., and characterized. Abbreviations: Phos., phosphorylated polysaccharide; de-Phos., de-phosphorylated polysaccharide. Retention time is expressed relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucose.

		Retention time			
		V. cholerae		*V. el-tor*	
Components	Peak	Phos.	de-Phos.	Phos.	de-Phos.
2,3,4,6-Tetra-*O*-methylglucose	A	0.90	0.80	0.80	0.75
2,3,4-Tri-*O*-methylfructose	B	1.0	1.20	1.0	1.26
2,3,4-Tri-*O*-methylrhamnose	C	1.60	1.50	1.70	1.65
2,3,4-Tri-*O*-methylmannose	D	1.98	2.12	2.0	2.20
2,3,4,6-Tetra-*O*-methylheptose	E	2.82	5.84	2.92	6.20

quinovosamine (2-amino-2,6-dideoxy-D-glucose), which was previously identified as a component of the lipopolysaccharides isolated from the whole cells of *V. cholerae* 569 B (Inaba) and Ogawa NIH 41 (Jann *et al.*, 1973). The most characteristic features of fraction P2 were the presence of heptose and minute quantities of phosphorus, which were not detected in P1 fraction. Most of the phosphorus was lost from the P2 fraction owing to dephosphorylation by selective hydrolysis of phosphomonoester bonds at pH3.8, as phosphorus analysis of the phosphorylated polysaccharide P2 fraction showed it to contain significant quantities of phosphate (results not shown). These fractions (P1 and P2) of the polysaccharide moiety were tested for serological specificities. The results of the haemagglutination-inhibition test of fractions P1 and P2 from both *V. cholerae* and *V. el-tor* showed that serological specificity of the polysaccharide moiety was restricted to fraction P1, whereas fraction P2 was the serologically inactive component. These results suggested that in these bacteria, fraction P2, which contains heptose and phosphate, represents the core-polysaccharide region of the lipopolysaccharides. The high-molecular-weight fraction P1, which is the smaller fraction, was found to contain glucosamine and represents the *O*-specific side-chain polysaccharide.

Methylation analysis of the polysaccharides (phosphorylated or dephosphorylated) gave identical results, except those for heptose content, as shown in Table 5. The g.l.c. pattern of methylated polysaccharide and dephosphorylated polysaccharide gave five peaks (A, B, C, D and E) (results not shown) corresponding to the five components. The retention times of these components were the same as those of the alditol acetates of the reference compounds 2,3,4,6-tetra-*O*-methylglucose, 2,3,4-tri-*O*-methylfructose, 2,3,4-tri-*O*-methylrhamnose, 2,3,4-tri-*O*-methylmannose and 2,3,4,6-tetra-*O*-methylheptose respectively. The identities of these components were

also confirmed by using paper chromatography, electrophoresis and chemical reactions as well as by combined g.l.c.–mass spectrometry (Bjorndal *et al.*, 1970).

Discussion

From the work described in this paper and that reported earlier (Raziuddin & Kawasaki, 1976), the chemical compositions of lipopolysaccharides isolated from the cell walls of *V. cholerae* 569 B (Inaba) and *V. el-tor* (Inaba), and of acid-hydrolysis products of lipopolysaccharides, lipid A and polysaccharide moieties, have been determined.

The de-*O*-acylated lipid A after fractionation by gel filtration was resolved into three major distinct fractions containing glucosamine and phosphorus (Fig. 1). The fact that similar molar ratios of glucosamine/phosphorus were obtained for these fractions of the solubilized lipids supports the supposition that the fractionation was indeed based on molecular size. In a similar study, however, the fractionation of de-*O*-acylated lipid A from *Pseudomonas aeruginosa* and *Pseudomomas alcaligenes* (Drewry *et al.*, 1973) did not yield clear fractions like those obtained from de-*O*-acylated lipid A of *V. cholerae* and *V. el-tor*.

There were intriguing questions about the structure of lipid A in Gram-negative bacteria. Westphal & Luderitz (1954) have described lipid A as a single molecular entity constituting an integral part of the endotoxic complex. However, the fractionation of lipid A released by mild acid hydrolysis of lipopolysaccharides disclosed a variety of compounds such as free fatty acids and glucosamine on the one hand and unaltered phosphorylated acylpolyglucosamine of different chain lengths on the other hand (Nowotny, 1963; Kasai & Nowotny, 1967; Adams & Singh, 1970*a*; Wober & Alaupovic, 1971*a*,*b*; Drewry *et al.*, 1973). Nevertheless, it is difficult to visualize lipid A as a simple linear structure consisting of a phosphorylated polyglucosamine chain with ester- and amide-linked fatty acids. More likely, lipid A possesses a branched structure consisting of several polyglucosamine chains of different lengths linked together through phosphodiester linkages, as demonstrated by fractionation of the de-*O*-acylated lipid A (Fig. 1) from *V. cholerae* and *V. el-tor*.

The main component of the total fatty acids of lipid A from both the organisms was 3-hydroxylauric acid. However, 3-hydroxymyristic acid, a major component of lipid A in other *Vibrio* species (*Vibrio metchnikovii* and *Vibrio parahemolyticus*) and various other Gram-negative bacteria (Rietschel *et al.*, 1973; Rusa & Lorkiewicz, 1974), which was exclusively involved in amide linkages, is an amide-linked second major fatty acid of lipid A from *V. cholerae* and *V. el-tor*. Similarly, in lipopolysaccharides from *Salmonella*, *Escherichia*, *Klebsiella*, *Serratia*, *Proteus* and *Citrobacter* the major fatty acids, lauric and myristic acid and palmitic acid are ester-linked, whereas 3-hydroxymyristic acid is an amide-linked component of lipid A (Gmeiner *et al.*, 1969; Adams & Singh, 1970*a*,*b*). The absence of 3-hydroxymyristic acid, a characteristic component of lipid A, from a number of Gram-negative bacteria, was particularly striking in *Pseudomonas syncyanea*, *Ps. alcaligenes* and *Ps. aeruginosa* (Hancock *et al.*, 1970; Wilkinson *et al.*, 1973; Drewry *et al.*, 1973). However, an amide-linked 3-hydroxy acid has been found in all the lipopolysaccharides of Gram-negative bacteria investigated so far, except for *Brucella* (Lacave *et al.*, 1969; Berger *et al.*, 1969).

Phenol used for the extraction of lipopolysaccharides removes some ester- and amide-linked fatty acids and dissociates the protein moiety by splitting a particular (phosphodiester?) linkage within lipid A. Acetic acid, however, seems to break specifically the linkages between lipid A and the polysaccharide moiety, as well as the linkages between the side chain and the core portions of the polysaccharide. Therefore the polysaccharide moiety obtained by acetic acid hydrolysis of the lipopolysaccharides from *V. cholerae* and *V. el-tor* gave two major fractions (P1 and P2, Fig. 2) on Sephadex column chromatography, one of which was of low molecular weight and the other of high molecular weight. On the basis of the molecular structure and separation by gel filtration, fraction P2, which is inactive in the haemagglutination-inhibition test, represents the core polysaccharide. However, the sugars of the serologically active fraction P1 can be ascribed to the determinant group, *O*-specific side chain of the lipopolysaccharides. The association of serological specificity with this fraction (*O*-specific side chain) is potentially of greater importance in *V. cholerae* and *V. el-tor*. Such fractions of core polysaccharide and *O*-specific side chain have been characterized in relatively few Gram-negative bacteria, particularly with several strains of *Pseudomonas* and smooth strains of Enterobacteriaceae (Schmidt *et al.*, 1969; Fensom & Meadow, 1970; Drewry *et al.*, 1972; Chester *et al.*, 1973; Wilkinson & Galbraith, 1975). Further, it is noteworthy that in *V. cholerae* and *V. el-tor*, the serologically inactive core-polysaccharide fraction containing heptose and phosphate did not contain glucosamine, the main amino sugar of lipid A, whereas glucosamine was exclusively detected in the *O*-specific side-chain polysaccharide. These differences are further important in biological properties of lipid A and polysaccharide moieties in these bacteria.

It is suggested that lipopolysaccharides of *V. cholerae* and *V. el-tor* have a core structure similar to that of other Gram-negative bacteria, where heptose is the basal sugar of the core (Luderitz *et al.*, 1966; Hammerling *et al.*, 1971, 1973; Lomax *et al.*, 1974),

but differ in that they lack 3-deoxy-2-octulonic acid (2-keto-3-deoxyoctonoate; KDO), which provides the bridge between the polysaccharide and lipid moieties of the enterobacterial lipopolysaccharides (Osborn, 1963; Rooney *et al.*, 1972).

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