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Analytical studies of lipopolysaccharide and its derivatives from *Salmonella minnesota* R595. III. Reappraisal of established methods

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Established methods for analysis of components of lipopolysaccharides were assessed. Optimal release of glucosamine from lipopolysaccharide occurs after hydrolysis in 6 M hydrochloric acid at 100°C for 4 h and fatty acids are best released by treatment with boron trifluoride/methanol at 100°C for 6 h. The semicarbazide assay for 3-deoxy-D-*manno*-octulosonic acid was modified to give results comparable to those from the periodate/thiobarbituric acid method. It was concluded that each molecule of lipopolysaccharide from *Salmonella minnesota* R595 contains two octulosonic acid residues and only four fatty acids, on average. There are two amide-linked hydroxyacids, together with, on average, 0.5 residues of ester hydroxyacid and a total of 1.5 residues of ester-linked normal fatty acids. This conclusion differs from the accepted view of *Salmonella* lipid A, but is supported by NMR results.

Introduction

Accompanying reports [1,2] describe the use of NMR spectroscopy for structural analysis of lipopolysaccharide from *Salmonella minnesota* R595. The good resolution for ^{31}P spectra of hydrazine-treated lipopolysaccharide has allowed us to identify all the different phosphorylated species and, for the first time, to make a spectroscopic estimate of the overall phosphorus-glucosamine ratio. This ratio was not in agreement with analytical estimates of phosphorus and glucosamine using published methods [3,4] and necessitated a critical reappraisal of the reliability of classical approaches to analysis of lipopolysaccharides.

Our results highlight the central role of glucosamine analysis in lipopolysaccharide structural studies and lead us to propose a new and simpler model for the structure of lipopolysaccharide from *S. minnesota* R595.

Materials and Methods

General. Boron trifluoride/methanol (14%) was obtained from Sigma Chemical Co. (St. Louis, MO). Anhydrous hydrazine was prepared from the monohydrate [5] and stored in sealed glass ampoules. 3-Deoxy-D-*manno*-octulosonic acid was prepared as described [6].

Lipopolysaccharide was extracted from acetone-dried *S. minnesota* R595 cells and treated with hydrazine as described in an accompanying paper [2]. Since the moisture content of lipopolysaccharide varies by at least $\pm 5\%$ when exposed to the laboratory atmosphere, it was routinely stored over concentrated sulphuric acid in an unevacuated desiccator. All analyses were carried out on aliquots of the same stock solution (10.00 mg/ml) prepared from this lipopolysaccharide.

Abbreviations: NMR, nuclear magnetic resonance; KDO, 3-deoxy-D-*manno*-octulosonic acid.

Glucosamine estimations. Aliquots of lipopolysaccharide stock solution, equivalent to approx. 100 nmol glucosamine, were added to fusion tubes and carefully evaporated in vacuo. After addition of 0.4 ml of hydrochloric acid (4 or 6 M), the tubes were flame-sealed and heated at 100°C for various times. The tubes were then cooled, mixed and centrifuged briefly before opening. Triplicate 0.050 ml samples were evaporated in vacuo and analyzed by the method of Strominger [7].

Fatty acid estimations. Samples (0.050 ml) of lipopolysaccharide stock solution were thoroughly dried in a desiccator, 50 μg of heptadecanoic acid in toluene were added as an internal standard and the solvent was carefully evaporated in a nitrogen stream. Boron trifluoride in methanol (14%, 1 ml) was then added and the tubes were immediately flame-sealed and heated at 100°C for 6 h. The cooled tubes were opened and 2 ml each of saturated sodium chloride solution and pentane were added. The mixtures were thoroughly agitated on a vortex mixer and centrifuged to give clear separation of the phases. The organic layers were removed and concentrated very carefully in a nitrogen stream.

Hydrolysis of lipopolysaccharide was achieved with 4 M sodium hydroxide [3,8,9], 4 M hydrochloric acid [3,9,10], and acid followed by alkali [11,12] according to published methods. Isolated fatty acids were esterified using diazomethane before gas chromatography.

Separation of fatty acid methyl esters was carried out using a 2 m glass column packed with 5% SP2340 on Chromosorb W/AW (Supelco Inc., Bellefonte, PA), a column temperature of 165°C and a nitrogen flow rate of 60 ml/min. The total time for analysis was 12 min. Based on active carbon contents, the Ackman-Sipos correction factors [13] for detection of methyl esters were: methyl dodecanoate, 1.07; methyl tetradecanoate, 1.03; methyl hexadecanoate, 1.01; methyl heptadecanoate, 1.00; methyl 3-hydroxytetradecanoate, 1.10.

Release of 3-deoxy-D-manno-octulosonic acid. Hydrolysis was carried out by dilution of aliquots of stock solution of either lipopolysaccharide or KDO, equivalent to between 10 and 100 μg KDO, with an equal volume of either 0.25 M sulphuric acid or 50 mM sodium acetate buffer (pH 4.5). The mixtures were heated at 100°C for 8 min (sulphuric acid) and 30, 60 and 90 min (acetate buffer).

Analysis of 3-deoxy-D-manno-octulosonic acid. KDO was estimated by the thiobarbituric acid method as described [14]. Alternatively, a solution of 0.600 mg (2.35 μmol) KDO ammonium salt in 3.00 ml water was treated with 3.00 ml of reagent consisting of 1% semicarbazide hydrochloride and 1.5% sodium acetate trihydrate in water [15]. After incubation at 30°C or 60°C, 1.00 ml aliquots were removed, diluted with 1.50 ml water, and read at 250 nm against a reagent blank.

Portions (1.00 ml) of sulphuric acid hydrolysate, and authentic KDO which had been identically treated, were neutralized with 0.100 ml 2.5 M sodium hydroxide and 1.00 ml of semicarbazide reagent was added. The mixtures were heated at 60°C for 30 min, cooled and diluted with 3.00 ml of water. After addition of 0.50 ml chloroform, the samples were mixed thoroughly and centrifuged to remove lipid. The absorbance of the aqueous phase was read at 250 nm. When lipopolysaccharide had been hydrolyzed using acetate buffer, the sodium hydroxide was omitted.

Total phosphorus estimation. The total phosphorus content of *S. minnesota* R595 lipopolysaccharide was determined by the method of Ames [16].

NMR spectroscopy. Total fatty acid content was determined from the ^1H spectrum by integration of methyl and methylene peaks relative to internal sodium benzoate. This, in turn, was checked by comparison with a known concentration of sodium stearate. Total phosphate was determined by integration relative to an external sample of methylene diphosphonate. This external reference was standardized against a known concentration of inorganic phosphate in deuterium oxide. Gated decoupling and a pulse repetition time of 5-times T_1 were used and care was taken to ensure similar sample geometries, even though the sample active volume was more than filled. Other conditions are specified in accompanying papers.

Results

Glucosamine estimations

Lipopolysaccharide must be subjected to acidic

hydrolysis before determination of its glucosamine content. Most commonly, 4 M hydrochloric acid has been used for this purpose, for times varying from 4 to 20 h [3,4,8,10,17–20], and the estimates for lipopolysaccharide from *S. minnesota* R595 have been 0.70–0.75 μmol/mg [3,4,17–20]. Consistent with this, we obtained a limiting value of 0.73 ± 0.01 μmol/mg after hydrolysis of lipopolysaccharide in 4 M acid for 6–20 h (Fig. 1). When 6 M acid was used for 6 h, however, a higher peak value of 0.87 ± 0.01 μmol/mg was obtained. Longer heating gave lower values as a result of destruction, and extrapolation to zero time gave a value of 0.92 ± 0.02 μmol/mg (Fig. 1).

As detailed in the discussion section, the results using 4 M acid must be underestimates because of the known presence of phosphorylated glucosamine in the hydrolysates [8,18]. The limiting value obtained over a range of hydrolysis times is probably due to fortuitous balancing of phosphate hydrolysis and glucosamine destruction.

Fatty acid estimations

Many different hydrolysis conditions have been used to release fatty acids from lipopolysaccharide. Hydrolysis using mineral acid has been most common [3,9,10], but alkali has sometimes given higher values [8,9]. Because of the formation

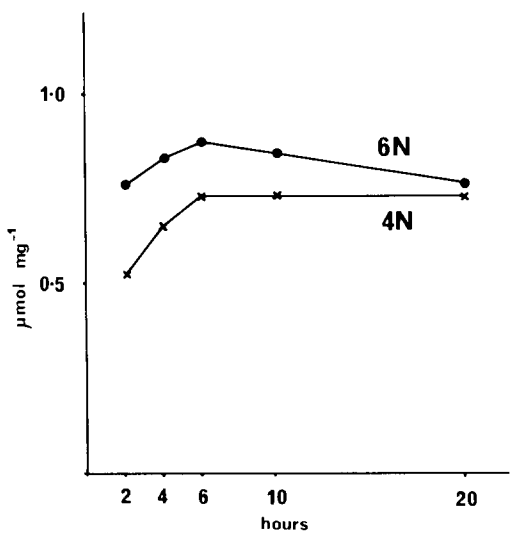

Fig. 1. Release of glucosamine from *S. minnesota* lipopolysaccharide R595 (Batch B2) by hydrochloric acid at 100°C, as assessed by colorimetric analysis [7].

of acyloxy fatty acids under acidic conditions [11], a subsequent treatment with alkali has been recommended [11,12]. Accurate assessment of the 3-hydroxytetradecanoic acid content has been especially difficult because of the instability of this acid under the conditions necessary to hydrolyze amide linkages [3]. As it has not been normal practice to report results on a weight basis, it is difficult to compare results from different groups. We have therefore reinvestigated hydrolysis under some of the more commonly reported conditions (Table I).

The results of alkaline hydrolysis (methods A and B) permit the simplest interpretation. Regardless of whether the standard is added before or after heating, the estimates of normal fatty acids are the same, indicating lack of destruction during hydrolysis (Table I). Comparison of the corresponding acidic hydrolyses (methods C and D) shows 30–32% loss of the normal fatty acids during heating. This loss is largely due to formation of acyloxytetradecanoic acids, and a subsequent treatment with alkali gives higher values (methods E and F). The effect of adding standard after heating is then much less, but some loss of the normal fatty acids still occurs, presumably through destruction. Provided the standard is included before hydrolysis, all hydrolytic methods give consistent results for normal fatty acids. The greatest variability occurs for dodecanoic acid, because of the possibility of loss of the volatile methyl ester during handling.

Analysis of 3-hydroxytetradecanoic acid is less satisfactory. As observed by others [8,9], alkaline hydrolysis (methods A and B) gives somewhat higher values than acid (methods C and D). The losses of hydroxyacid on heating in acid are largely the result of acyloxytetradecanoic acid formation, and higher estimates are obtained by the two-stage procedure (methods E and F). We have, however, found the method rather erratic for hydroxytetradecanoic acid analysis. Possibly saponification of the acyloxy acids is accompanied by a variable amount of destruction of the hydroxyacid.

Transacylation of fatty acids in lipopolysaccharide using boron trifluoride/methanol (method G) gives values of normal fatty acids which agree well with those from hydrolyses (Table I). The estimate of hydroxyacid, however, is much higher

TABLE I

FATTY ACID CONTENT OF *SALMONELLA MINNESOTA* R595 LIPOPOLYSACCHARIDE (BATCH B2) ESTIMATED BY DIFFERENT METHODS

Method	Treatment	Fatty acid (μmol/mg)			
		12:0	14:0	16:0	3-OH-14:0 [a]
A	4 M NaOH,	0.35	0.31	0.06	0.67
B [b]	100°C, 4 h	0.35	0.31	0.06	0.67
C	4 M HCl,	0.36	0.31	0.06	0.61
D [b]	100°C, 4 h	0.25	0.22	0.05	0.36
E	4 M HCl, 100°C,	0.33	0.31	0.06	0.86–1.11
F [b]	4 h; then 1 M NaOH, 100°C, 1 h	0.30	0.26	0.05	0.58–0.68
G	BF/MeOH, 100°C, 6 h	0.33	0.31	0.05	1.18

[a] Estimate includes artifacts.
[b] 17:0 standard added after hydrolysis.

than those obtained by hydrolysis. Some hydroxyacid is converted by the reagent to methyl *trans*-tetradec-2-enoate and methyl 3-methoxytetradecanoate [4]. These products are stable and well-separated from the other methyl esters and are included in the estimate of the total hydroxyacid (Table I).

Despite the formation of small amounts of artifacts, transacylation of fatty acids using boron trifluoride/methanol for 6 h at 100°C appears to be the method of choice. This method has been used by others, but without detailed study of conditions [4,21]. Traces of fluoroborate in the crude ester product lead to deterioration of WCOT capillary and EGSS-X packed columns, but we have used SP2340 nitrile phase in packed columns over extended periods with no change in chromatographic performance. The only complication associated with the transacylation method is variability in quality of the reagent. Batches which have been stored at room temperature over several months are less reactive and require heating for 24 h or more to effect complete cleavage of amide fatty acids. Storage at −18°C is satisfactory for extended periods.

The fatty acid estimates which we have obtained by transacylation give a ratio of normal acids to hydroxyacid which is very similar to those reported for a wide range of lipopolysaccharides [22] without the necessity for correction for destruction of hydroxyacid. Using hydrolysis, however, our hydroxyacid values would require correction factors of 2 or greater to make them conform to this ratio. Our estimate of 1.87 ± 0.02 μmol/mg of total fatty acids corresponds to 44% of the total lipopolysaccharide weight. The only literature values are 42% [20] and approx. 50% [3].

Lipopolysaccharide is soluble in the boron trifluoride reagent and it is possible to carry out a kinetic study of deacylation. The transacylation of lipopolysaccharide fatty acids as a function of time is shown in Table II. Graphical treatment (Fig. 2) indicates that release of hydroxytetradecanoic acid from lipopolysaccharide is the sum of two pseudo-first-order processes. The initial rapid stage corresponds to transacylation of ester-bound acids and the subsequent phase represents the relatively slow release of amide-linked residues. Hydrazine-treated lipopolysaccharide, which contains only amide-bound fatty acids [3,12], was included as a control to confirm the rate of release of amide fatty acids. Extrapolation of the linear part of the plot for lipopolysaccharide shows 0.89 ± 0.02 μmol/mg of amide-linked hydroxyacid, equivalent to 1.99 ± 0.07 residues per diglucosamine unit. This corresponds to 75% of the total hydroxyacid content. The remainder (0.29 ± 0.04 μmol/mg or 0.65 ± 0.10 residues per molecule) is

TABLE II

FATTY ACID RELEASE FROM *SALMONELLA MINNESOTA* R595 LIPOPOLYSACCHARIDE (BATCH B2) USING BORON TRIFLUORIDE/METHANOL

Time (h)	Fatty acid (μmol/mg)					
	12:0	14:0	16:0	14:1 artifact	3-OMe-14:0 artifact	3-OH-14:0
0.17	0.23	0.22	0.05	0	0	0.29
0.5	0.28	0.28	0.06	0.01	0	0.47
1	0.30	0.29	0.05	0.02	0	0.63
2	0.30	0.30	0.05	0.03	0.01	0.81
6	0.33	0.31	0.05	0.06	0.05	1.07
24	0.32	0.30	0.05	0.08	0.06	1.04

ester-linked. This conclusion is quite inconsistent with published views [12,23].

A general check on possible systematic errors in our chromatographic estimates of fatty acid contents was carried out using ^{1}H-NMR spectroscopy as described above. For this experiment, we chose hydrazine-treated lipopolysaccharide, rather than native material, as it is much better dispersed in water without the use of a surfactant. Estimated by NMR, the sample contained 0.92 ± 0.05 μmol/mg of 3-hydroxytetradecanoic acid, compared with a value of 0.94 ± 0.02 μmol/mg obtained by gas chromatography. We are therefore confident that our chromatographic results do not understate the acyl content of lipopolysaccharide.

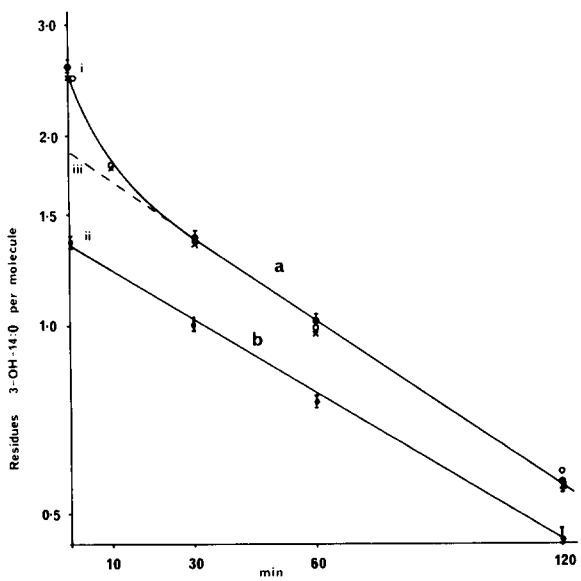

Fig. 2. Kinetics of release of 3-hydroxytetradecanoic acid from (a) lipopolysaccharide and (b) hydrazine-treated lipopolysaccharide from *S. minnesota* R595 (Batch B2) using boron trifluoride/methanol at 100°C. The intercepts are (i) total hydroxyacid residues in lipopolysaccharide, (ii) total hydroxyacid residues in hydrazine-treated lipopolysaccharide and (iii) amide-linked hydroxyacids in lipopolysaccharide.

In an accompanying paper [1] we have described determination of the types of hydroxyacids present in lipid A using ^{13}C-NMR spectroscopy. This approach gives an even lower estimate for the amount of ester hydroxyacid than our kinetic studies, but clearly supports the conclusion that the majority of hydroxyacid is amide-linked. It also indicates that all of the ester and half of the amide hydroxyacid is substituted by acyl groups (Table III), although the errors in intensity measurements are such that the presence of 5% unacylated ester could not be ruled out.

Finally, our methods were checked using a sample of lipopolysaccharide prepared in another laboratory (kindly provided by Dr. Otto Lüderitz, Freiburg). The same level and distribution of normal and hydroxyacids and of ester- and amide-linked hydroxyacids were found.

KDO estimations

A chromatographic method has been proposed for analysis of KDO [24], but determination of its level in lipopolysaccharide is usually carried out by means of either the periodate/thiobarbituric acid [25,26] or the semicarbazide test [15,25]. In using the former procedure, precise control of

TABLE III

TYPES OF 3-HYDROXYTETRADECANOIC ACID RESIDUES IN LIPID A FROM *SALMONELLA MINNESOTA* R595

Residue type	Percent of total fatty acid residues		
	NMR analysis[a]	gas chromatography	
		this work	previous work[b]
Acylated ester	8 (C-2)		11
Unacylated ester	0 (C-2, C-3)		20
Acylated amide	24 (C-2, C-3)		27
Unacylated amido	24 (C-2)		1
Total amide	48	47	29
Total ester	8	16	32
Total acylated residues	30–37	37[c]	39
Total hydroxyacid	55–60	63	61

[a] The ^{13}C resonances used in the estimate are given in parentheses. The areas of these peaks were compared with the area of the group of peaks between 25 and 26 ppm.

[b] Calculated from results of total analysis and selective hydrolysis [12].

[c] Assuming the amount of 3-acyloxytetradecanoic acid is equal to the amount of non-hydroxy acids [12].

conditions at the periodate oxidation stage is important in order to avoid overoxidation [27], but the major source of difficulty with the method has been the instability of the chromophore after extraction into organic solvents. This problem is completely avoided by the use of dimethyl sulphoxide as cosolvent [14,27].

The simpler semicarbazide method [25] has been used less frequently. We obtained variable results in preliminary experiments, and therefore investigated the kinetics of semicarbazone formation from authentic KDO. A limiting value is obtained after 30 min at 60°C, but reaction at 30°C, the temperature normally employed [15], is slow and incomplete (Fig. 3). This procedure is less sensitive than the periodate/thiobarbituric acid method, but is much simpler and provides consistent results at 60°C.

^{1}H-NMR experiments indicate that KDO is converted to artifacts on heating at pH 3.4 and 100°C for 30 min. Resonances characteristic of KDO disappear completely during this treatment. This is consistent with earlier reports [28–30] and shows that artifacts, and not true KDO, are obtained by hydrolysis of lipopolysaccharide. These artifacts respond to both the periodate/thiobarbituric acid and semicarbazide assays, albeit with varying sensitivity (Table IV), and it is therefore essential that all measurements be made relative to authentic KDO which has been subjected to the same acidic treatment. The increasing estimates by the semicarbazide method after extended heating in acetate buffer suggest that such conditions are not optimal for lipopolysaccharide hydrolysis.

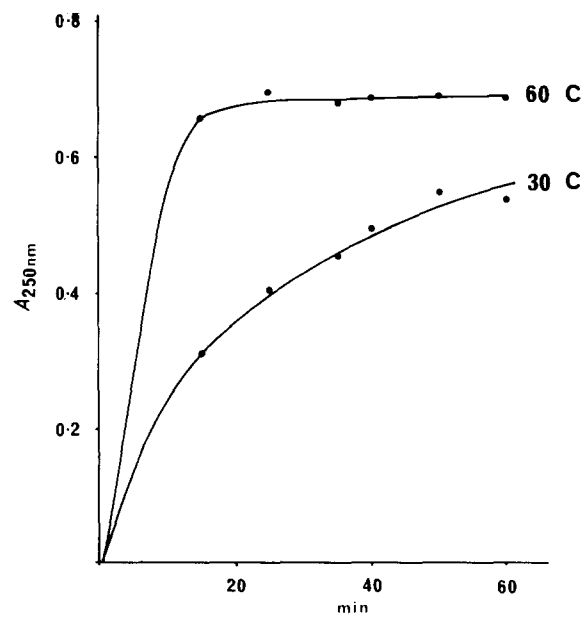

Fig. 3. Influence of derivatization conditions on estimates of 3-deoxy-D-*manno*-octulosonic acid by the semicarbazide method.

TABLE IV

INFLUENCE OF ACID TREATMENTS ON DETERMINATION OF STANDARD 3-DEOXY-D-*MANNO*-OCTULOSONIC ACID (KDO)

Unheated KDO = 100.

Acid treatment	Time (min at 100–C)	Thiobarbituric acid method	Semicarbazide method
0.25 M sulphuric acid	8	69	76
Acetate buffer (pH 4.5)	30	94	95
	60	91	87
	90	80	93
	120	75	97

Lipopolysaccharide and authentic KDO were subjected in parallel to acid treatment and assay. The results in Table V indicate that optimal release of KDO from lipopolysaccharide occurs after 8 min in mineral acid or 30 min in acetate buffer. Our estimate of 1.72–2.15 KDO residues per diglucosamine unit contrasts with the traditional view [23,25,30], but is supported by recent studies [32,41].

Phosphorus estimations

S. minnesota R595 lipopolysaccharide contains 0.92 ± 0.01 μmol/mg total phosphorus, determined by the Ames dry ashing procedure [16], compared with 0.85 ± 0.04 μmol by ^{31}P-NMR spectroscopy. A check was carried out using the older Lowry method [33] to demonstrate the absence of systematic errors. Few absolute values for the phosphate content of *S. minnesota* R595 lipopolysaccharide are available, but our result appears to be distinctly higher than those reported by others [4,17] and corresponds to 2.00 ± 0.06 phosphorus residues per diglucosamine unit. It seems that others have not achieved complete ashing of the organic phosphate.

High-resolution ^{31}P-NMR in the presence of surfactant permits the determination of each phosphorylated species present in lipopolysaccharide and it is possible to make an estimate of the overall phosphorus-glucosamine ratio in a sample that is completely independent of the estimates of each component reported above. Within the precision of the NMR method ($\pm 5\%$), agreement with our analytical ratios is excellent (Table VI). The one assumption is that the 4′ position is completely substituted. As discussed in an accompanying paper, this assumption is supported strongly by the ^{31}P spectra of hydrazine-treated lipopolysaccharides.

Effects of hydrazine on lipopolysaccharide

^{31}P-NMR spectra of hydrazine-treated lipopolysaccharides (Fig. 4) provide some insight into the effect of the reagent on both the phosphates and the amide-linked fatty acids. With the exception of peak e, the resonances have been characterized in an accompanying paper [1]. Pyrophosphate resonances are completely absent from both spectra because of rapid conversion to monophosphate. Comparison of spectra A and B shows that peak d (assigned to 1-monophosphate in a mole-

TABLE V

ESTIMATES OF 3-DEOXY-D-*MANNO*-OCTULOSONIC ACID (KDO) CONTENT OF *SALMONELLA MINNESOTA* R595 LIPOPOLYSACCHARIDE (BATCH B2)

Acid treatment	Time (min at 100°C)	Semicarbazide method		Thiobarbituric acid method	
		μmol/mg	residues per molecule	μmol/mg	residues per molecule
0.25 M sulphuric acid	8	0.93	2.02	0.86	1.87
Acetate buffer	30	0.87	1.89	0.81	1.76
	60	0.85	1.85	0.80	1.74
	90	0.96	2.09	0.79	1.72
	120	0.99	2.15	0.75	1.63

TABLE VI

COMPARISON OF ESTIMATES OF PHOSPHATE/GLUCOSAMINE RATIOS IN *SALMONELLA MINNESOTA* LIPOPOLYSACCHARIDE

Batch	Chemical analysis (±0.03)	NMR analysis (±0.05)
G4	1.00	1.07
G9	1.02	1.03
G11	1.14	1.07
B1	1.07	1.06

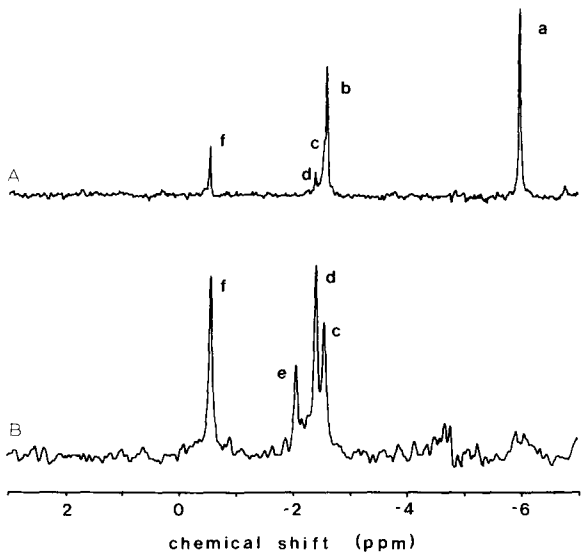

Fig. 4. ^{31}P-NMR spectra of lipopolysaccharide from *S. minnesota* R595 lipopolysaccharide after treatment with anhydrous hydrazine for (A) 30 min at 80°C, (B) 180 min at 100°C. Spectra were measured at 45°C in $^{2}H_2O$ at pH 7.7 with 3% sodium taurodeoxycholate and 5 mM ethylenediamine tetraacetate. Peak assignments are (a) 4′-diphosphate; (b) 1-monophosphate on molecules with 4′-diphosphate; (c) 1-monophosphate on molecules with 4′-monophosphate; (d) 1-monophosphate on molecules with no 4′ substituent; (e) 1-monophosphate with no 2-acyl group (tentative assignment); (f) 4′-monophosphate. All molecules have two KDO residues.

cule lacking any 4′-monophosphate) and peak f (4′-monophosphate) result equally from extended hydrazinolysis of the 4′-diester. Nucleophilic cleavage occurs equally on either side of the diester linkage. Peak e, not previously characterized, is tentatively assigned to 1-monophosphate in molecules lacking the adjacent 2-fatty acid. The peak occurs downfield from the other 1-monophosphates, indicating a decreased pK_a. This is the same type of change as that observed when hydroxyl groups adjacent to the 4′-monophosphate become available for intramolecular hydrogen bonding.

By comparison of the area of peak e with those of peaks b and c in spectrum B, we calculate that per mol lipopolysaccharide there is a deficit of 0.23 mol of hydroxyacid from the 2 position after treatment with hydrazine for 3 h. Chromatographic results show, however, that only 1.02 residues of hydroxyacid are present per molecule (Table VII). Therefore a much greater proportion of the acid (0.75 mol) must have been lost from the 2′ position. We emphasize that these values are approximate, but they leave little doubt that amide fatty acid is lost less readily from the 2 position, probably as a result of repulsion between the 1-phosphate and the incoming nucleophile.

Discussion

The accepted structure of lipid A from *Salmonella* and many other Gram-negative bacteria is based on a β-1,6-linked-D-glucosamine disaccharide which is substituted with seven fatty acids, two phosphates and three KDO residues [18,23,31]. Until the introduction of spectroscopic techniques, our understanding of the structural details of lipo-

TABLE VII

ANALYSES OF LIPOPOLYSACCHARIDES FROM *SALMONELLA MINNESOTA* R595 AFTER TREATMENT WITH HYDRAZINE

Hydrazine treatment		μmol/mg (mol ratio)		
time (min)	temp. (°C)	glucosamine	phosphate	hydroxytetradecanoic acid
30	80	1.20 (2.00)	1.03 (1.71)	0.78 (1.30)
30	100	1.38 (2.00)	1.22 (1.77)	0.94 (1.36)
180	100	1.29 (2.00)	0.85 (0.69)	0.69 (1.05)

polysaccharides was based on selective chemical modifications and determination of ratios of individual components. The quantitative studies reported here, and in accompanying papers, lead us to propose a simpler structure for the lipid A molecule, whereby only four fatty acids and two KDO residues are present in each molecule (Table VIII).

Glucosamine

The glucosamine content of hydrolysates of lipopolysaccharides is normally estimated by the Morgan-Elson procedure [3,4,8,10,17–20]. Early studies of the method demonstrated that substitution of glucosamine in the 6 position has no effect on the color yield, that substitution in the 3 position enhances the yield and that a substituent in the 4 position blocks color production [34].

Analysis of glucosamine in lipopolysaccharide is complicated by the presence of residual phosphate in the 4 position. Using an amino acid analyzer, Hase and Rietschel showed that 36% of the non-reducing terminal glucosamine of deacylated lipopolysaccharide is still phosphorylated after hydrolysis with 4 M acid for 8 h [18]. Furthermore, *S. typhimurium* lipid A precursor gives significant amounts of phosphorylated glucosamine after 14 h [8]. It is then likely that the problem of hydrolytic removal of phosphate from glucosamine is a general one. Since, however, the phosphate appears to migrate from the 4′ position to the 6′ position under acid conditions [18], the glucosamine estimates have been considered reliable.

More recently, glucosamine 6-phosphate was shown to give only 70% of the color yield of glucosamine in the Morgan-Elson test [35]. Even if phosphate migration to the 6′ position is complete, it must be concluded that estimates of glucosamine after hydrolysis of lipopolysaccharide in 4 M hydrochloric acid are low. Our value of 0.92 ± 0.01 μmol/mg, based on hydrolysis in 6 M acid, is 26% higher than that obtained with 4 M acid (0.73 ± 0.01 μmol/mg), presumably because of more effective removal of phosphate. Interference from 4-amino-4-deoxy-L-arabinose is very unlikely as its phosphate linkage is extremely acid-labile and the released sugar is unstable under the hydrolysis conditions [36].

Our conclusions demonstrate the importance of obtaining a satisfactory estimate for the glucosamine content of a lipopolysaccharide. A glucosamine disaccharide is the basic backbone of lipid A from a wide range of lipopolysaccharides [18], but the numbers of substituents on this backbone (fatty acids, KDO and phosphate) are variable to some degree. The ^{31}P-NMR spectra of hydrazine-treated lipopolysaccharides are sensitive to such small changes in molecular structure that it is extremely unlikely that molecules with a backbone other than the disaccharide could be present. Estimates of the average numbers of substituents on the backbone are therefore totally dependent on a correct estimate of the glucosamine content.

Fatty acids

Two ester- and two amide-linked 3-hydroxytetradecanoic acid residues have been reported to be substituted on each lipid A backbone [12]. Only hydroxyacid is present in lipopolysaccharide which has been treated with hydrazine, dilute alkali or alkaline hydroxylamine [3,12], indicating that normal fatty acids are ester-linked. A recent study, using selective cleavage of amide-linked fatty acids, demonstrated that three normal fatty acids are present as secondary substituents on the hydroxyacids [12].

Using our revised value for glucosamine and fatty acid values from transacylation, we estimate 0.69 ± 0.02 μmol/mg (1.50 ± 0.04 residues per molecule) of normal fatty acids and 1.18 ± 0.01 μmol/mg (2.57 ± 0.05 residues per molecule) of total hydroxyacid in lipopolysaccharide. Moreover, the kinetics of release of hydroxyacid in boron trifluoride/methanol (Fig. 2) demonstrate

TABLE VIII

ANALYTICAL SUMMARY FOR *SALMONELLA MINNESOTA* R595 LIPOPOLYSACCHARIDE

Component	Content	Literature range (μmol/mg)
	μmol/mg ratio	
Glucosamine	0.92 2.00	0.60–0.75
KDO	0.81–0.93 1.76–2.02	0.58–1.26
3-OH-14:0 acid	1.18 2.56	–
12:0 acid	0.33 0.72	–
14:0 acid	0.31 0.67	–
16:0 acid	0.05 0.11	–

directly that the ratio of ester- and amide-bound hydroxyacids is 25 : 75. This shows that 0.89 ± 0.02 μmol/mg of hydroxyacid are amide-linked, equivalent to 1.92 ± 0.07 residues per molecule. Since there cannot be more than two amide fatty acids per molecule, this result provides independent support for our higher value for the glucosamine content of the lipopolysaccharide. Furthermore, the ratio of amide- to ester-bound fatty acids cannot be affected by systematic errors in the total fatty acid content. ^{13}C-NMR spectra confirmed the low proportion of ester-linked hydroxyacid (Table III). This conclusion is surprising in view of the demonstrated role of bis(2-hydroxytetradecanoyl)-α-D-glucosamine 1-phosphate in the biosynthesis of lipid A in *E. coli* [37] and indicates that some removal of ester-linked hydroxyacid takes place at about the stage of incorporation of the normal fatty acids.

Elegant studies have recently been carried out on chromatographic fractions of lipid A obtained by acidic hydrolysis of deep rough lipopolysaccharide [10,38,39]. These fractions contain as many as seven fatty acids per molecule. These, and similar synthetic lipid A preparations [40], have significant endotoxic activity, but it is difficult to judge how relevant these results are to the present conclusions concerning the average composition of heterogeneous native lipopolysaccharide. Certainly some free fatty acids are produced during the lipid A preparation [10] and it is possible that transacylation of acids occurs, similar to that observed under strongly acidic conditions [11], to give some species with a higher degree of acylation than the starting lipopolysaccharide.

Previous estimates of the proportions of ester- and amide-linked hydroxyacid have been consistent with the degree of fatty acid loss under mildly nucleophilic conditions [3,12]. We have found that treatment of lipopolysaccharide with hydrazine for 30 min at 100°C gives a product with a hydroxyacid-glucosamine ratio which is approximately half that in the starting lipopolysaccharide. The product contains, however, only 1.38 ± 0.04 residues of 3-hydroxytetradecanoic acid per diglucosamine unit. Mild alkaline treatment gives a similar product [12], which therefore must also contain less than two amide-linked acid residues. No kinetic studies have been reported, but evidently loss of some of the amide-linked fatty acids occurs under quite mild conditions.

The precise extent of removal of fatty acids by hydrazine appears difficult to control. On one occasion, greater deamidation was obtained by heating at 80°C than at 100°C for the same time (Table VII). This might be due to variations in the physical state of the lipopolysaccharide or the quality of the anhydrous hydrazine used. Moreover, recovery of backbone glucosamine is only 46–60% after microfiltration. Even poorer yields of deacylated lipopolysaccharide have been obtained after solvent precipitation [12]. Quantitative arguments should not therefore be based on the content of hydroxyacid in the fractions of material isolated after hydrazine treatment.

3-Deoxy-D-manno-octulosonic acid

The combination of instability and unsatisfactory analytical tests has made difficult the accurate assessment of KDO content of lipopolysaccharides [25,27]. Based on semiquantitative observations of the release of the subunit under mildly acidic conditions and its susceptibility to periodate oxidation and methylation in the intact lipopolysaccharide, it has been proposed that a branched KDO trisaccharide provides the link between lipid A and the core oligosaccharide in *Salmonella* and *E. coli* [25,41]. Very recently, a reinvestigation has introduced some doubt as to the generality of the trisaccharide link [39,41] in *Salmonella* and an NMR study has indicated the presence of a KDO disaccharide in a deep rough *E. coli* mutant [32].

The fate of KDO on heating in acid is not clearly understood [28–30]. The decrease in response to the periodate/thiobarbituric acid test has been used as a criterion for destruction in acid [27], but our observation by ^{1}H-NMR of the rapid conversion to artifacts indicates that the slower changes in response to the color tests are the result of subsequent modification of the primary artifacts. Nevertheless, because this modification is slower than hydrolysis and formation of primary artifacts, parallel treatment of authentic KDO provides a fairly satisfactory control and the conclusion that there are only two KDO residues per lipopolysaccharide molecule (Table V) is well based.

Conclusion

From the results of this and accompanying papers [1,2], we conclude that there are two KDO residues and only four fatty acid substituents per molecule of lipopolysaccharide from *S. minnesota* R595. One amide-linked hydroxyacid and one amide-linked acyloxyacid, together with (on average) 0.5 residues of acyloxy ester, at least part of which is in the 3′ position. These conclusions are based on improved analyses, kinetics of fatty acid release and ^{31}P-, ^{1}H- and ^{13}C-NMR spectroscopy.

Much of the present work has been repetition of experiments carried out by others. This was necessary because the existing literature cannot properly be assessed in view of the practice of reporting ratios of components without their absolute values. Our conclusion concerning the structure of the lipopolysaccharide of *S. minnesota* R595 (Table VIII) is based on a set of closely interdependent analytical estimates. An error in any one of our determinations would make all the other analytical values untenable. As an example, if the true value of glucosamine were lower than our estimate, we would have to conclude that (i) there are more than two amide-linked hydroxyacids per molecule, (ii) that the phosphorus-glucosamine ratios which we have obtained analytically, and by NMR, are wrong and (iii) that the types of hydroxyacids, assessed by NMR, are wrong.

S. minnesota R595 may well produce an atypical lipid A subunit and the generality of our conclusions has yet to be demonstrated, but there is good evidence that the proportions of normal and hydroxyacids are conserved through a wide range of organisms [22]. Our results indicate that lipid A is much smaller than previously believed [10,23,32] and this has important implications for the role of lipopolysaccharide in the outer membrane. Furthermore, the low biological activity of some synthetic lipid A analogues may be the result of incorporation of excessive amounts of fatty acids [40,43].

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