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Analytical studies of lipopolysaccharide and its derivatives from *Salmonella minnesota* R595. II. Proton and carbon magnetic resonance spectra

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^{1}H and ^{13}C nuclear magnetic resonance spectroscopy are of limited value for study of native lipopolysaccharide from *Salmonella minnesota* R595. Methylation and acetylation of derived lipid A gave preparations with better solubility properties in a number of solvents, but sharpest spectra were obtained with lipopolysaccharide which had been treated with hydrazine to remove ester-linked fatty acids. ^{13}C-NMR of lipid A indicates that hydroxy fatty acid is present as 3-hydroxyacyl amide, 3-acyloxy amide and 3-acyloxy ester. All normal fatty acids appear to be present as substituents on the hydroxyacyl residues. These conclusions are consistent with kinetics of release of fatty acids and component estimates using improved procedures.

Introduction

Lipopolysaccharide occupies such a large part of the surface area of Gram-negative bacteria that the details of its molecular structure are of great importance for understanding the bacterial cell wall. Possible functions for the various parts of the molecule have been discussed in a comprehensive review by Nikaido and Nakae [1].

The polysaccharide may have an evolutionary advantage for the organism, enabling it to create new surfaces and evade antibodies or phages. It will also increase the hydrophilicity of the cell surface, thereby protecting the cell from phagocytosis [2].

The role of the lipidic part of the molecule is largely structural. Lipopolysaccharide completely blocks the passage of small hydrophobic molecules through the bilayer, unlike phospholipid, which can provide a hydrophobic pathway for diffusion [3]. Absence of the hydrophobic pathway is particularly advantageous to enteric bacteria, which need protection against penetration of bile salts. Nikaido and Nakae [1] point out that the hydrophobic pathway is present in some Gram-negative species, but not in enterobacteria.

The reasons for the effectiveness of lipopolysaccharide as a barrier to hydrophobic molecules remain undefined, but a number of types intermolecular interaction have been proposed. There is considerable evidence that ionic interactions stabilize the membrane [1]. Divalent ions certainly bind to [4,5] and cause aggregation of lipopolysaccharide [6]. Cross-linking of the core oligosaccharide [7] and pyrophosphoryl bridges between lipid A subunits have been ruled out by the experiments of Mühlradt and coworkers [8,9]. It is now believed that the molecules are held together by ions or hydrogen bonds [1]. The latter interaction has been proposed as the reason for strong association between phospholipids and other galactolipids [10]. If the finding, reported in an accompanying paper [11], that lipopolysaccharide from

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

Salmonella minnesota R595 has only about four fatty acid residues per molecule is true for other species, specific interactions of this type will be required to explain the special properties of lipopolysaccharide. At the commencement of this work a number of questions concerning the structure of lipopolysaccharide remained unanswered. The aim of the present work was to explore the extent to which proton and carbon NMR spectroscopy could answer these questions. A similar study of the spectra of lipopolysaccharide from *Escherichia coli* has recently been reported [12].

Materials and Methods

Bacteria and lipopolysaccharide. The samples were prepared, purified and analysed as described [11,13,14].

Modified lipopolysaccharide. Lipopolysaccharide was treated with 10 M hydrochloric acid as described [15] and the precipitate was washed repeatedly with ice-cold water, dispersed in water and freeze-dried. The resultant lipid A was dispersed in chloroform/methanol (2 : 1) and treated with an excess of diazomethane in chloroform for 1 h at 0°C. The mixture was allowed to warm to room temperature, the precipitate of insoluble polymethylene was removed by centrifugation and the supernatant evaporated. The residue was dispersed in water with sonication, dialysed and the dispersion was freeze-dried.

Acetylation of lipopolysaccharide was carried out as described [16].

Nuclear magnetic resonance. Spectra were recorded on a Varian XL-200 spectrometer operating at 50.3 MHz for carbon and 200 MHz for protons. The field was locked to the deuterium signal from the ^2H_2O solvent. For quantitative measurements with ^{13}C, the spectra were obtained using gated decoupling.

Results

Proton spectra

The proton spectra of lipopolysaccharide dispersed in water were very broad. At 25°C the linewidths of the fatty acid peaks were greater than 1 kHz, indicating the formation of large aggregates. The lines were broader than those that have been observed for the large multilamellar liposomes formed by unsonicated phospholipids [17]. Other solvents for lipopolysaccharide were tried, but none gave spectra with resolved spin-spin splitting. The observed linewidths, summarized in Table I, indicate that aggregation occurred in all the solvents. Estimation of the linewidth expected for monomeric lipopolysaccharide is difficult because, unlike ^{13}C relaxation, proton relaxation is not caused by a single dominant interaction. The lines are, however, broader than those observed for other lipidic molecules, such as cerebrosides in dimethyl sulphoxide [18] and lysophosphatidylcholine in ^2H_2O [19].

Acetylation of lipopolysaccharide and methylation of the lipid A were tried as methods for improving resolution. Both materials were very soluble in chloroform, but the proton spectra were still broad and impurities were difficult to remove. The greatest improvement was obtained by partial de-acylation with hydrazine, followed by electrodialysis. Before dialysis, the linewidth depended on the amount of divalent ions and a progressive sharpening was produced by added EDTA.

Identification of some proton resonances in the spectrum of electrodialysed, hydrazine-treated lipopolysaccharide was possible. (i) Anomeric protons were observed with chemical shifts of 5.38 ppm and 5.88 ppm, which are the same, within experimental error, as those found by selective proton decoupling of the ^{31}P resonances of the 1- and 4′-phosphates [14]. (ii) Peaks corresponding to the 2- and 4-methylene groups of 3-hydroxytetradecanoic acid, were seen at 3.08 ppm and 1.79 ppm. (iii) Peaks from H-3 and H-3′ of KDO were observed at 2.06 and 2.44 ppm.

Carbon spectra

The limited solubility of lipopolysaccharide in phenol and dimethyl sulphoxide made these solvents unsuitable for natural-abundance ^{13}C-NMR spectroscopy. Chloroform and ^2H_2O solutions gave spectra with reasonable signal-to-noise ratios, but linewidths of 30 Hz for all carbons except those near the ends of the aliphatic chains. Broad lines were expected for the aqueous solution, in which aggregates are known to exist [6], but in chloroform, Re lipopolysaccharide is reported to form small oligomers [20]. The spectra of

TABLE I

LINEWIDTHS FOR ^1H SPECTRA OF LIPOPOLYSACCHARIDE AND ITS DERIVATIVES

LPS, lipopolysaccharide; DMSO, dimethyl sulphoxide.

Sample	α-CH_2 [a]	β-CH_2	$(CH_2)_n$	CH_3	C-1
Electrodialysed LPS in ^2H_2O	–	–	≈1000	–	–
LPS in phenol	50	–	24	16	40
LPS in DMSO-d_6	–	24	3	2	20
LPS in DMSO-d_6 + 5% ^2H_2O	10	–	10	4	20
LPS in C^2HCl_3	32	–	12	≈10	–
Acetylated LPS in C^2HCl_3	24	30	6	2	–
Diazomethane-treated lipid A in C^2HCl_3	–	22	6	4	–
Hydrazine-treated LPS in ^2H_2O	20	–	18	16	–
Hydrazine-treated LPS electrodialysed in ^2H_2O	–	16	– [b]	2	1.5

[a] This peak represents α-CH_2 protons in normal fatty acids, but γ-CH_2 protons in 3-hydroxytetradecanoic acid.
[b] The peak was obscured by triethylammonium ion.

acetylated lipopolysaccharide were equally broad. These observations are consistent with the linewidths observed in the proton spectra. Spectra similar to those reported recently [12] were obtained when SDS was used to disperse the lipopolysaccharide in ^2H_2O. A high ionic strength, provided in our case by 80 mM triethylammonium chloride, was essential for preventing precipitation and consequent loss of resolution.

Lipid A, on the other hand, is quite soluble in chloroform and gives interpretable spectra, as shown in Fig. 1. Aliphatic resonances were about 20 Hz wide, the carbonyl and fatty acid C-3 peaks were 30 Hz wide, and the sugar resonances were completely unresolved. Addition of about 100 μmol of triethylamine to a solution containing 30 μmol of lipid A sharpened the spectrum only slightly.

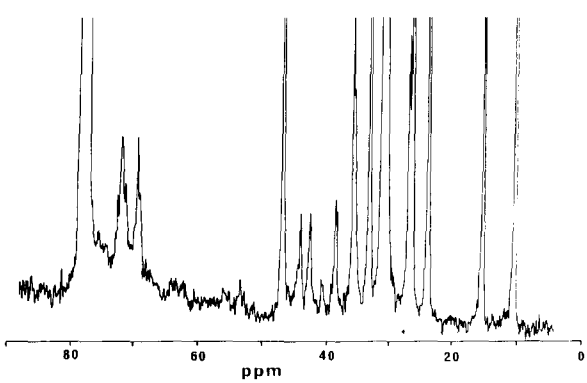

Fig. 1. ^{13}C-NMR spectrum of 25 μmol of lipid A plus 40 μmol of triethylamine in C^2HCl_3.

Methylation of lipid A greatly improved the resolution, so that individual sugar resonances could be seen, but a number of small impurity peaks were also introduced. They were very sharp, had no counterpart in the spectrum of lipid A, and could be reduced in intensity (but not eliminated) by purification. The measured chemical shifts are reported in Table II. Because of the possibility of buried impurity peaks, the spectra of methylated lipid A were not used for quantitative measurements.

Reduction of the molecular size by partial deacylation resulted in a further improvement in resolution. The spectrum of hydrazine-treated lipopolysaccharide is shown in Fig. 2.

The above spectra were assigned by comparison with the reported spectra of fatty acyl esters and glucosyl ceramides [21,22]. Such comparisons are reliable to better than 1 ppm [23]. Lipopolysaccharide may contain six types of fatty acid: normal fatty esters, normal fatty amides, 3-hydroxy fatty esters, 3-acyloxy fatty esters, 3-hydroxy fatty amides and 3-acyloxy amides. If present, 2-hydroxytetradecanoic acid would be hard to distinguish from 3-hydroxy fatty acid, but no significant amounts of it were detected by gas chromatography [11].

The chemical shifts of the carboxylic carbons

TABLE II

^{13}C CHEMICAL SHIFTS AND ASSIGNMENTS

Chemical shifts were measured relative to C^2HCl_3 at 77.00 ppm or glycerol at 62.69 ppm. Both secondary standards were calibrated using TMS in a 25% solution in acetone in a 5 mm tube placed concentrically in the 10 mm sample tube. LPS, lipopolysaccharide.

Assignment	LPS in ^2H_2O	LPS in C^2HCl_3	Lipid A in C^2HCl_3	Methylated lipid A in C^2HCl_3	Hydrazine-treated LPS in ^2H_2O
Lipid CH_3	13.76	13.95	13.9	14.04	14.05
Lipid ($n-1$)	22.61	22.55	23.1	22.64	22.78
BHM [a] C-5					
Acyl BHM C-5	–	25.03	25.0	25.02	25.65
n-Acyl C-3		25.8	25.6	25.64	25.95
Lipid ($n-3$)	29.57	29.30	29.3	–	28.98 29.62
Lipid $(CH_2)_n$	29.96	29.64	29.7	29.67	29.99
Lipid ($n-2$)	32.01	31.82	31.9	31.89	32.11
KDO [b] C-3?	34.1				33.37
Acyl BHM C-4	34.6	33.94	34.2	33.87	–
n-Acyl C-2		34.42	34.5	34.47	
KDO C-3?	36.2				34.76
BHM C-4	38.1	37.6 (br)	37.2	37.23	36.76 37.34
Acyl BHM ester C-2	39.1 39.3	– [b]	39.3	39.1	
BHM ester C-2	40.2	– [b]	41.4	41.18	
Acyl BHM amide C-2	40.7 41.9				
BHM amide C-2	43.1	– [b]	42.9	42.5 43.3	4.47 44.20
Glucosamine C-2′	– [c]	52.3	52.4 (br)	52.55	54.40 (br)
Glucosamine C-2	53.5	54.8	54.7 (br)	– [c]	55.3 (br)
Methyl phosphate				54.68	
?	59.6			58.93	
?	62.5		61.3		
Glucosamine C-6′	63.2		62.8	62.8	63.23
KDO C-8					63.58
Unassigned sugar carbons	64.2				63.85 ?
64.74					
					64.38
					64.74
	66.2			65.83	66.13
				66.35	66.4
	67.8			67.92	68.56

TABLE II (continued)

Assignment	LPS in ^2H_2O	LPS in C^2HCl_3	Lipid A in C^2HCl_3	Methylated lipid A in C^2HCl_3	Hydrazin-treated LPS in ^2H_2O
BHM C-3		68.1	68.7	68.42	69.2
Unassigned sugar carbons	69.7			69.75 70.3	69.5
Acyl BHM C-3	70.6	70.7	71.0	70.85	70.17
Unassigned sugar carbons	71.3				71.1 71.55
	72.5			73.0 73.28 74.5	72.82 73.5 74.5 76.3
Glucosamine C-1		93.5 [d]	91.8	91.3	93.5
?	95.5		96.2	95.9	–
KDO C-2?	99.1 99.9	98.9 [d]			99.2 100.05
Glucosamine C-1′	102.1	102.6 [d]	101.8 [d]	100.8	102.3
Acyl BHM carbonyl		170.1	170.7	170.25 [e]	
n-Acyl carbon		173.1	173.5	173.38 [f]	173.6 174.9
KDO carboxyl		175.8			176.0

[a] BHM = 3-hydroxytetradecanoic acid.
[b] Peaks were too weak to be identified reliably.
[c] The signal may be buried under nearby solvent peak.
[d] A series of broad, ill-defined peaks.
[f] Refers to the maximum of an assembly of many sharper peaks.

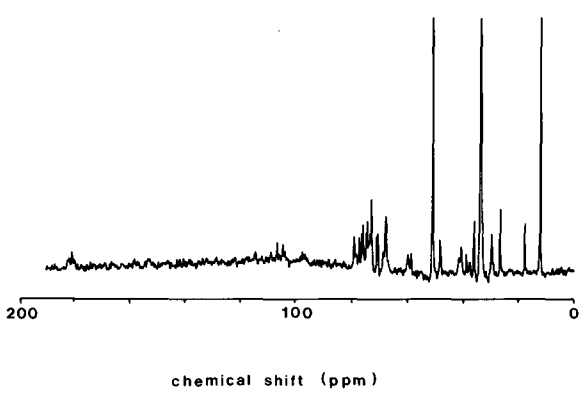

Fig. 2. ^{13}C-NMR spectrum of electrodialysed, hydrazine-treated lipopolysaccharide in ^2H_2O, pH 9.1, 30°C.

can distinguish between only some of these possibilities. Model compounds [21] lead to the prediction that the carboxyl carbons of 3-acyloxy fatty acids will be found 2.6 ppm upfield from the corresponding hydroxy acid. The differences between ester and amide [22], and between normal and 3-hydroxy fatty acids [21], are smaller. The predicted chemical shifts for ester-linked normal fatty acids, amide-linked and ester-linked hydroxytetradecanoic acids are 174.0, 174.2 and 173.2 ppm, respectively. 3-Acyloxy fatty esters should appear at 170.6 ppm, and the amides at 171.6 ppm. These predictions are in excellent agreement with the observed spectra. Lipid A shows a peak at

170.7 ppm, corresponding to acyloxy esters and amides, and another at 173.5 ppm, due to all the other species. (The spectrum of methylated lipid A contained too many peaks from uncharacterized impurities, as discussed above, to permit reliable interpretation of this part of the spectrum.) The spectrum of lipopolysaccharide in C^2HCl_3 had similar peaks at 170.1 and 173.1 ppm, plus an additional signal at 175.8 ppm, which is therefore assigned to the carboxyls of KDO. Removal of all ester-linked fatty acids by hydrazinolysis caused the high-field peak to vanish and left resonances at 173.5, 174.8 and 175.9 ppm, corresponding to carbonyls in the 3-hydroxytetradecanoic amides and KDO. Acetylation of lipopolysaccharide increased the intensity of the peak at 170.3 ppm, which is the result expected for esterification of the 3-hydroxy groups. The acetyl groups, themselves, will also contribute to the intensity of this peak.

The chemical shift for the KDO carboxyls cannot at present be used to determine the anomeric configuration because the chemical shift differences are small [24] and close proximity of KDO groups is known to produce unusual chemical shift values [25,26]. The nearby 4′-phosphate group may also affect the KDO carboxyl resonances. Similar comments apply to the C-2 signals of KDO.

Sugar peaks in the 60–80 ppm region are unresolved in the spectra of lipopolysaccharide and lipid A, apart from two prominent peaks which we assign to the acyl C-3 carbons of the hydroxy fatty acids, because the lipid chains are expected to be more mobile than the sugar rings. The peak positions are close to those for model compounds, which show that acylation at C-3 produces a 2.5 ppm downfield shift [21]. The reported spectra of ceramides suggest that amides should appear 1.2 ppm downfield from the corresponding ester [22]. No splitting of the peak was observed, so the amide shift in lipid A appears to be less than expected. The peak width at half height was, however, 1.2 ppm, so the expected splitting would have been barely observable. Corresponding peaks in the spectra of methylated lipid A and hydrazinolysed lipopolysaccharide were not easily distinguishable from the surrounding sugar resonances.

The C-2 and C-4 carbons of 3-hydroxytetradecanoic acid should resonate between 30 and 40 ppm. The C-2 carbon is sensitive to the nature of the acyl linkage, with amides 2.6 ppm downfield from esters [21,22], while acylation of 3-hydroxytetradecanoic acid moves the C-2 signals 2.2 ppm upfield. The C-2 carbons of acylated amide and non-acylated ester should behave similarly and, instead for four peaks, only three are expected for the four types of hydroxyacid. Non-acylated amide should appear at 43.8 ppm, acylated ester at 39.0 ppm and the other two species between 41 and 42 ppm. As shown in Table II, the spectrum of lipid A contained peaks close to these positions, but the amide shift is slightly less than predicted. Hydrazine-treated material, which contains only amide-linked, non-acylated hydroxy fatty acid, confirmed the assignment of the 44 ppm peak, and because of the improved resolution a 0.4 ppm splitting of the C-2 peak was revealed. The spectrum of unmodified lipopolysaccharide was too broad to permit observation of the C-2 resonances.

Complete assignment of the sugar resonances was not attempted because of the large number of overlapping peaks in the region between 60 and 80 ppm. The one spectrum with sufficient resolution, that for methylated lipid A, was complicated by the presence of unremovable impurities. Despite the congestion, some of the thirty carbons should have characteristic chemical shifts. For example, glucosamine C-2 peaks should appear in a region free of other peaks. The reducing terminal glucosamine should behave like 2-acetamido-2-dexoy-α-D-glucose 1-phosphate or its 3-*O*-acetyl derivative, in which the C-2 carbons have chemical shifts of 55.3 and 53.4 ppm, respectively [27]. The non-reducing glucosamine will be similar to 2-acetamido-2-deoxy-β-D-glucoside, which has a C-2 peak at 56.9 ppm when 1,4-linked to another sugar [28]. Acetylation of the 3 position leads to an upfield shift of 1.8 ppm in the free sugar. The predicted chemical shift for C-2′ is therefore 56.9 ppm in hydrazinolysed lipopolysaccharide and 55.0 ppm in lipid A, if the 3′ position is acylated. The latter prediction does not allow for any solvent shift.

Both glucosamine signals were observed. Among the very few sugar peaks in the ^{13}C spectrum of lipopolysaccharide in chloroform are two at 52.3 and 54.8 ppm, which are assigned to C-2 and C-2′,

respectively. Peaks at 51.4 ppm and 53.6 ppm were seen when the lipopolysaccharide was dispersed in ^2H_2O with the help of SDS. Similar peaks were observed in the spectrum of lipid A, but they were broad.

Anomeric resonances were weak and rather broad in most spectra. Two glucosamine peaks were expected at 93.9 and 100.7 ppm, based on the reported values for 2-acetamido-2-deoxy-α-D-glucose 1-phosphate and 1,4-linked 2-acetamido-2-deoxy-β-D-glucose [28]. In the latter case a −2.0 ppm shift was included to take account of the 1,6-glycosyl linkage in lipopolysaccharide [29]. The position of the C-2 resonance in aminoarabinose is difficult to predict, especially as the ring adopts a conformation unlike that for α-D-glucose 1-phosphate [14]. The part of the molecule near C-1 is expected to be most like α-D-glucose 1-phosphodiester or α-D-galactose 1-phosphodiester, which show C-1 resonances at 93.9 ppm [30]. Formation of the diester produces a shift of 1.3–2.3 ppm [27]. One or two peaks for the C-2 carbons in KDO are predicted near 103 ppm if pyranose rings are present [24], and near 107 ppm if they are in the furanose form [31].

The spectrum of unmodified lipopolysaccharide was too broad in the anomeric region for interpretation, but that of lipid A contained peaks at 91.8, 96.2 and 101.8 ppm. The first is likely to be the C-1 resonance of the diglucosamine subunit, but it is at lower field than expected, possibly as the result of a solvent effect. The peak at 96 ppm could be from C-1 in aminoarabinose, as the intensity was slightly less than that for the other peaks, but the r.m.s. signal-to-noise ratio in all spectra was only 3 : 1, making such conclusions tentative. In aqueous solution, hydrazine-treated lipopolysaccharide showed peaks at 94.4 and 102.6 ppm, but no separate peak attributable to aminoarabinose. It may contribute part of the intensity of the 94.4 ppm peak. A pair of closely spaced signals at 99.7 and 100.1 ppm had no counterpart in the spectrum of lipid A, and differ from the peak at 95.7 ppm produced by hydrolysis of the KDO. This leads to their assignment as the C-2 resonances of KDO in the pyranose ring form. An attempt was made to find the KDO signals by comparing the spectra of hydrazine-treated samples before and after mild acid hydrolysis, but precipitation occurred when KDO was removed.

Other distinctive resonances are those for methylene groups, which can be distinguished from other carbons by use of the INEPT pulse sequence [32]. In this way, CH_2 signals were found at 62.8 ppm in the spectrum of methylated lipid A, and at 62.6 and 63.4 ppm in the spectrum of hydrazine-treated lipopolysaccharide. The signal common to both samples was therefore assigned to unsubstituted C-6′, and the lower-field, more intense peak to the C-8 carbons of KDO. The model compound, 2-acetamido-2-deoxy-β-D-glucose, has a chemical shift of 62.0 ppm for C-6 [27], and C-8 in methyl 3-deoxy-β-D-*manno*-octulosidonic acid occurs at 65.2 ppm [24].

The substituted methylene peak for C-6 is harder to distinguish. In an INEPT spectrum in which the methylene peaks were selectively inverted, there was, in addition to the inverted peaks, a region at 73 ppm where the signal height was reduced, but not negative. It is believed that this is due to incomplete resolution of neighbouring CH and CH_2 peaks. This may be C-6, although the corresponding carbon in 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-2-deoxy-D-galactopyranose appears at 69.0 ppm [30].

One further point is worthy of comment. No peaks were found between 75 and 90 ppm in the spectra of either methylated lipid A or hydrazine-treated lipopolysaccharide. In the former case the INEPT sequence was used to suppress the signal from the solvent, deuterochloroform. The two signals expected in this region are those for C-5′ on glucosamine and C-7 on KDO if there is a 2,7 linkage [26]. The absence of the KDO signal is consistent with a 2,4 or 2,5 link between the two KDOs, as reported for those rough strains of *E. coli* and *Salmonella* that have only two KDO groups [12,35].

The absence of the C-5′ signal is more surprising. Disaccharides containing 2-acetamido-2-deoxy-β-D-glucose show peaks from C-5 at 76.5 to 77.0 ppm [30]. The 4′-phosphate may produce an upfield shift of about 0.5 ppm, but this is insufficient to explain the spectrum of methylated lipid A. We are forced to assume that geometric constraints on the molecule lead to distortions of the glucosamine ring that are not encountered in the model compounds. No specific model is proposed.

Discussion

Fatty acid substitution pattern

Our revised estimate for the glucosamine content of lipopolysaccharide [11] has consequences for the fatty acid substitution pattern. The proportion of hydroxylated fatty acid remains unchanged, but a greater fraction of the 3-hydroxytetradecanoic acid is believed to be amide-linked. This, in turn, means that more of the amide-linked fatty acids must have unsubstituted 3-hydroxy groups than was previously thought. The ^{13}C-NMR spectra support these conclusions and the suggestion that only hydroxy acids are attached to the diglucosamine backbone [33].

Quantitative measurements accurate to $\pm 5\%$ were made on the spectrum of lipid A in chloroform, using gated decoupling. Progressive saturation was used to verify that the spin-lattice relaxation time for the signals was less than 0.5 s, so that the 3.2 s recovery time between pulses was adequate. The area of the peaks between 25.0 and 25.6 ppm was taken as a measure of the total fatty acid content. This area was compared with the areas of the peaks for the n, $n-1$ and $n-2$ carbons of the lipid chains and all four values agreed within $\pm 5\%$.

The total 3-hydroxytetradecanoic acid content determined from the NMR spectrum is in reasonable agreement with our analytical results, as shown in Table III. The estimate can be made in a number of independent ways. The sum of the areas of the C-2 peaks in hydroxy acids indicate that 55% of the total fatty acid is 3-hydroxy acid, while the C-3 peaks yielded a value of 60%. The latter resonances coincide with unresolved sugar peaks and are therefore harder to integrate reliably. Nevertheless, the NMR results are consistent with chemical analysis in this and previous studies [11,33,34].

The NMR spectra also support the suggestion of Rietschel and coworkers concerning the location of normal fatty acids in lipid A [33]. The amount of 3-acyloxytetradecanoic acid is equal to the proportion of normal acyl groups, suggesting that they are attached exclusively to the hydroxytetradecanoic chains. Again, the estimates can be made in a number of ways. The C-3 peaks give a direct ratio of hydroxy to acyloxy species, and indicate that 37% of the total fatty acid is acyloxytetradecanoic acid. The areas of the quaternary C-1 carbons lead to an estimate of 30% for the same ratio, which is in reasonable agreement with the previous figure, since quaternary carbons are those most likely to give inaccurate intensities as a result of long relaxation times. A figure of 32% based on the area of the C-2 peaks was obtained by a less direct argument. The peak at 39.3 ppm is assigned to acylated ester, and at 41.4 ppm corresponds to both acylated amide and

TABLE III

PROPORTIONS OF 3-HYDROXYTETRADECANOIC ACID SPECIES PRESENT IN LIPID A, EXPRESSED AS A PERCENTAGE OF THE TOTAL FATTY ACID CONTENT

Species	Carbon peak used	Proportion (%)		
		NMR	analysis [a]	previous [b] work
Total 3-hydroxy and 3-acyloxy	C-2,C-3	55–60	58	61
3-Acyloxy ester and amide	C-3,C-2,C-1	30–37	39 [c]	38 [c]
3-Hydroxy and 3-acyloxy amide	C-3,C-2	48	46	28
3-Hydroxy and 3-acyloxy ester	C-3,C-2	8	15	32
3-Acyloxy ester	C-2	8		10
3-Hydroxy ester	C-2,C-3	0		22
3-Acyloxy amide	C-2	24	1	
3-Hydroxy amide	C-2,C-3	24		27

[a] Results reported in an accompanying paper [11].

[b] Ref. 33.

[c] These estimates were obtained by assuming that all the normal fatty acid residues are ester-linked to 3-hydroxytetradecanoic residues.

non-acylated ester. Since the sum of the areas of these two peaks is equivalent to 32% of the total fatty acid, we conclude that the amount of non-acylated ester is small.

The main difference between our analytical results and those reported previously, apart from the total number of fatty acids per molecule, is the proportion of 3-hydroxytetradecanoic acid present as the amide. The rates of release of fatty acids show that, per mol lipid A, 2 mol of hydroxytetradecanoic acid are amide-linked and only 0.6 mol is ester-linked. The NMR results in Table III clearly support this proposition and not the former view that half the hydroxy acids are esters [33].

Our lipid A preparation contained no detectable free fatty acid ($< 1\%$) and the ratios of the fatty acids were the same within experimental error as those reported in an accompanying paper [11] for the original lipopolysaccharide. We are, therefore, confident that the carbon spectra of lipid A properly reflect the structure of lipopolysaccharide.

Conclusion

The state of aggregation of lipopolysaccharide and its derivatives severely limits ^{1}H- and ^{13}C-NMR studies. ^{13}C magnetic resonance results indicate that hydroxy fatty acid is present as 3-hydroxyamide, 3-acyloxyamide and 3-acyloxyester. No significant amounts of 3-hydroxyester are present. These conclusions are consistent with results from ^{31}P-NMR spectroscopy [13], kinetics of fatty acid release and component estimates [11].

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