Heterogeneity of Lipid A: Structural Determination by ^{13}C and ^{31}P NMR of Lipid A Fractions from Lipopolysaccharide of *Escherichia coli* 0111†

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ABSTRACT: Purified lipid A from *Escherichia coli* 0111 was fractionated by thin-layer chromatography, and seven major bands were studied by ^{13}C and ^{31}P NMR. All lipid A fractions except one had fatty acids, 3-hydroxytetradecanoic acid, 3-(acyloxy)tetradecanoic acid, and phosphate groups bonded to the diglucosamine backbone. The remaining fraction was shown to be phosphatidylethanolamine. The number of substituents found showed that in all fractions all sites available for C-acylation (C-3, C-4, and C-3′) and N-acylation (C-2 and C-2′) carried acylic substituents. The number, ranging from four to six, and type of ester-bound carboxylic acid residues as well as the number of phosphate groups differed among the fractions. The three fastest moving bands all had three unsubstituted hydroxy fatty acids and one phosphate group (C-4′), while the slower moving bands had four hydroxy fatty acids and two phosphate groups. Unsubstituted 3-hydroxytetradecanoic acid residues were amide-bound to the disaccharide in all but one of the fractions. In summary, the heterogeneity of *E. coli* 0111 lipid A is found to be a consequence of a variation of the number and composition of carboxylic acid residues and of varying phosphate content.

Lipid A, the lipid moiety of the lipopolysaccharide (LPS)[1] from the cell wall of Gram-negative bacteria, is responsible for most of the endotoxic properties of LPS (Galanos et al., 1977), and its structure is therefore of considerable interest. Lipid A is heterogeneous (Nowotny, 1963; Kasai, 1966; Drewry et al., 1973; Chang & Nowotny, 1975; Hase & Rietschel, 1977; Lehmann & Rupprecht, 1977; Mühlradt et al., 1977; Qureshi et al., 1982; Rosner et al., 1979a,b; Banerji & Alving, 1979; Mattsby-Baltzer et al., 1984a), and several subfractions may be isolated as was recently done with eight major fractions from *Shigella flexneri* lipid A by thin-layer chromatography (TLC; Banerji & Alving, 1979). In this investigation, we present a NMR study of the molecular basis for the heterogeneity of lipid A in seven major fractions from *Escherichia coli* 0111 (Mattsby-Baltzer et al., 1984a).

The lipid A molecule of many Gram-negative bacteria consists of a β-1,6-linked diglucosamine backbone [6-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-amino-2-deoxy-β-D-glucopyranose], long-chain fatty acids (FA), 3-hydroxytetradecanoic acid (HTA), 3-(acyloxy)tetradecanoic acid (ATA), and phosphorylic residues (Wollenweber et al., 1982). On the backbone, positions C-3, C-4, and C-3′ are available for O-acylation and C-2 and C-2′ for N-acylation. Position C-6′ has recently been shown to be the site of KDO linkage to LPS in a heptose-less mutant of *E. coli* K 2 (Strain et al., 1983). The phosporylic substituents are found in positions C-1 and C-4′ (Lehmann & Rupprecht, 1977; Mühlradt et al., 1977). In studies on nonfractionated material, it has been concluded that the amide-bound acid is HTA (Rosner et al., 1979b). On nonfractionated material from *E. coli* (Seydel et al., 1984; Wollenweber et al., 1982, 1984) and on fractionated material from *Salmonella typhimurium* (Takayama et al., 1983), it has been concluded that one of the amide-bound acids is ATA. The number of carboxylic acids as well as their position is believed to constitute one source of heterogeneity. Also, the number and nature of the phosphorylic residues may vary (Hase & Rietschel, 1977; Rosner et al., 1979a).

The aim of this investigation was to study the structure and structural heterogeneity of lipid A in intact, free lipid A molecules from *E. coli* 0111. The NMR method was chosen because it is noninvasive and a powerful tool for structural elucidation.

Materials and Methods

Lipid A was prepared from *E. coli* 0111 LPS (Westphal procedure, purchased from Difco, Detroit, MI) by boiling with 1% acetic acid for 2 h (10 mg/mL). The procedure for purification of lipid A and the separation of the various fractions have been described earlier (Banerji & Alving, 1979; Mattsby-Baltzer et al., 1984a). Approximately 10% of chloroform-soluble material was obtained from the LPS. Seven major fractions (bands 2–8) were isolated from the *E. coli* 0111 lipid A (Figure 1). Amounts of 5–25 mg of each fraction were used for the NMR studies. One of these (band 8) is phosphatidylethanolamine (see Results). The fractions have recently been characterized by TLC and compared with

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[1] Abbreviations: FA, fatty acid; HTA, 3-hydroxytetradecanoic acid; ATA, 3-(acyloxy)tetradecanoic acid; SDS, sodium dodecyl sulfate; TEA, triethylamine; PE, phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid.

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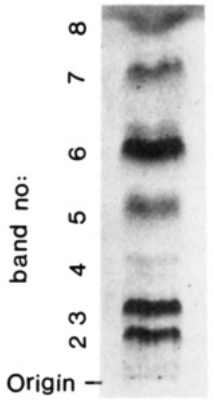

FIGURE 1: Chromatogram of purified lipid A from *E. coli* 0111 (200 nmol of phosphate). Lipid A, prepared by hydrolysis of LPS with 1% acetic acid, was purified by EDTA treatment followed by chloroform extraction. The chromatogram was charred with sulfuric acid.

lipid A fractions from other Gram-negative bacteria. The fractions are numbered and named according to the order they are eluted on the chromatography plate under the conditions described in that paper (Mattsby-Baltzer et al., 1984a). In short, the higher numbered fractions are eluted first; and we are here concerned with fractions 2–7, fraction 1 being available only in negligible amounts (Figure 1).

A small sample of each fraction was hydrazinolyzed according to the standard procedure. Hydrazine (Eastman, 95%), freshly distilled from sodium hydroxide under dry argon, was added (0.3 mL) to 1–2 mg of lipid A in a glass ampule. After being sealed under vacuum at −15 °C, the ampule was kept in a water bath at 60 °C for 30 min. The ampule was cooled and opened, and its contents were transferred to a few milliliters of acetone. After evaportion to dryness, the residue was washed with acetone and used for ^{31}P NMR without further purification.

The following reference compounds were purchased from the listed suppliers and used without further purification: HTA (Tokyo KASEI, Tokyo, Japan), 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (pentaacetylglucosamine) (Aldrich Chemical Co.), 3-deoxyoctulosonic acid (KDO), *O*-phosphoethanolamine, phosphatidylethanolamine, phosphatidylglycerol, and glucosamine 1-phosphate (Sigma Chemical Co.). 3-Tetradecanoyltetradecanoic acid was prepared from equal amounts of HTA and tetradecanoyl chloride (Aldrich) in benzene in the presence of pyridine. The reaction products were chromatographed on a silica gel column (Kiselgel 60, EM Products) with chloroform as the eluent. Sodium dodecyl sulfate (SDS, Polysciences Inc.) and triethylamine (TEA, Eastman Chemical Co.) were used as supplied. Chloroform-*d*, methanol-d_4, and D_2O (Merck Isotopes) were used as supplied for the NMR measurements.

NMR measurements were performed on a NIC 500 Spectrometer equipped with a Nicolet 1280 computer. The frequencies were 125.75 MHz for ^{13}C and 202.45 MHz for ^{31}P.

For carbon, the 90° pulse was 22 μs, and an 80° pulse was used with 16K memory and a sweep width of 20000 Hz. The preacquisition delay was 125 μs, and the pulse repetition time was 500 ms. Chemical shifts were determined relative to internal tetramethylsilane (Me_4Si).

For phosphorus, a 90° pulse was 24 μs, and a 45° pulse was used with 16K memory and 10000 Hz sweep width. Preacquisition delay was 100 μs, and the pulse repetition time was 1.8 s. Chemical shifts are relative to external phosphoric acid (85%). The spectra were run under proton decoupling at 45 °C. The samples were prepared by taking 1–2 mg of lipid A for hydrazinolysis and by using all the acetone-insoluble product in 2 mL of solvent.

Table I: Solvents for ^{13}C NMR of Lipid A Fractions

fraction no.	$CDCl_3$:CD_3OD:D_2O
2	1.2:1.02:0.1
3	1:0.58:0.05
4	2:1:0[a]
5	1:0.58:0.05
6	1:0.58:0.05
7	0.99:0.77

[a] Not optimized.

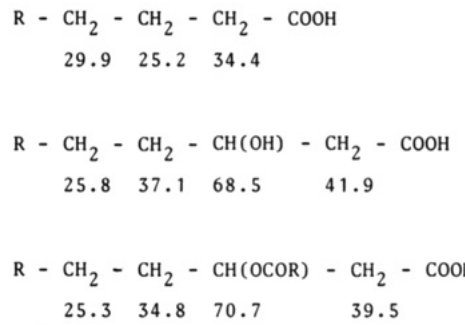

FIGURE 2: ^{13}C chemical shifts of carboxylic acids found in *E. coli* 0111 lipid A.

Depending on the availability of each fraction, the carbon spectra required 20000–150000 transients (5–21 h) and the phosphorus spectra 5000–20000 transients (2.5–10 h).

The extensive aggregation of lipid A is an experimental difficulty. The resonances of carbon atoms and phosphorus atoms close to the diglucosamine moiety as well as those of the diglucosamine itself are broadened due to aggregation. Solvents for ^{13}C NMR therefore had to be chosen that would minimize aggregation. Chloroform is a solvent that dissolves lipid A, but the aggregation is too extensive to permit any meaningful NMR studies. The line widths of the ^{31}P NMR resonances were >100 Hz. Addition of methanol and a small amount of water in slightly different proportions was optimal for the studies conducted here (Table I). The solvent was chosen that would minimize the ^1H NMR line widths of the sugar moiety for each fraction. The resonances of the carbon atoms in the lipophilic end of the carboxylic acids were all sharp in chloroform as well as in chloroform/methanol/water but were also less informative, due to their insensitivity to variation in diglucosamine substitution.

Assignments of peaks in the carbon spectra were made according to the literature (Krasikova et al., 1982; Tullock, 1978; Bundle et al., 1973; Koerner et al., 1979) (Figure 2). 2-Tetradecanoyltetradecanoic acid was synthesized and its spectrum recorded to assign unequivocally the shifts of that compound.

The hydrazinolyzed materials were run in D_2O, with EDTA and TEA added. ^{31}P resonances were assigned according to the literature (Mühlradt et al., 1977), with correction for magnetic susceptibility (Batley & Redmond, 1982), and that of the diglucosamine 1-phosphate was confirmed by comparison with glucosamine 1-phosphate. The ^{31}P NMR spectra of fractions 5 and 6 (see below) revealed the presence of 10–20% of impurities in these fractions. Lyso-PE comigrated with fraction 5 and phosphatidylglycerol comigrated with fraction 6 on TLC. Furthermore, ninhydrin spraying of the TLC peak gave a color reaction with fraction 5 but not with fraction 6, supporting our suggestion about the identity of these impurities. Interpretation of the spectra has been done with

Table II: Composition of Lipid A Fractions from *E. coli* 0111[a]

fraction no.	GlcNHCCH$_2$C(O)(OCR)—	GlcOCCH$_2$C(O)(OCR)—	GlcNHCCH$_2$CH(OH)—	—OCCH$_2$CH(OH)—	—OCCH$_2$R	total no. of acid subst	–OPO$_3$[b]
2	0	1	2	2	1	5	2
3	0	3	2	2	1	5	2
4	*c*	*c*	*c*	*c*	*c*	*c*	2
5	0	1	2	1	2	5	1
6	0	1	2	1	2	5	1
7	1	2	1	2	2	5	1

[a] The presence of the molecular fragments shown in the table has been determined for each fraction with ^{13}C NMR. To determine the number of substituents in the disaccharide molecule, each fragment in the second and third columns is combined with one fragment from the fifth and sixth columns. If the total number of FA and HTA (fifth and sixth columns) exceeds the total number in columns 2 and 3, then there are FA and HTA directly bound to the diglucosamine backbone. The sum of columns 2 and 3, column 4, and the number of HTA and FA bound directly to the backbone is the number of acid substituents for each molecule (fraction). [b] The phosphate groups of fractions 2–4 are linked to C-1 and C-4′ and to C-4′ in fractions 5–7. [c] Not determined.

due consideration given to these molecules.

Results

The ^{13}C and ^{31}P NMR spectra have been recorded for seven major fractions of lipid A from *E. coli* 0111. The objective has been to study the structural heterogeneity of these fractions with respect to substituents such as FA, HTA, ATA, and phosphate esters, as well as the possibility of other substituents. The fractions were purified on preparative TLC, and there were no free acids in the fractions used for NMR. For simplicity, the use of the abbreviations HTA, ATA, etc. refer to the acylic residues of these acids in ester or amide linkage.

The ^{13}C chemical shifts of FA, HTA, and ATA of interest to the structural assignment are given in Figure 2. HTA, ATA, and FA were observed in fractions 2–7 with enough resolution to allow observations of individual substituents. Fraction 4 was, however, available only in very small amounts, and only the presence of FA, HTA, and ATA could be concluded, as well as phosphate groups, without permitting a detailed structural analysis. A summary of the results are given in Table II. Fraction 8 was shown to be phosphatidylethanolamine (PE) by comparison of its ^{13}C NMR spectrum with that of commerically available PE. The ^{13}C NMR resonances of fraction 8 matched those of dimyristoyl-PE within a few tenths of a ppm. In addition, fraction 8 showed resonances at 11.0, 16.0, 16.1, 29.0, and 30.5 ppm relative to Me_4Si, typical of a cyclopropyl structure in fatty acids such as lactobacillic acid and *cis*-9,10-methylenehexadecanoic acid that have been found in *E. coli*. The finding was confirmed by TLC (Mattsby-Baltzer et al., 1984b).

The number of positions for substitution in each molecule is limited. Given the presence of two sites for phosphate linkage, one for KDO attachement, and two for the joining of the two glucosamine moieties leaves five sites for acylation, two for N-linkage and three for O-linkage. Also C-3 of HTA is available for esterification.

The identification of individual substituents allows a summation to be made of all the substituents in the molecule. The fact that the individual substituents are identified permits assignments to various positions on the backbone. For example, the observation of a ^{13}C resonance at 44 ppm downfield from Me_4Si, C-2 of HTA, shows the presence of an N-linked HTA in that molecule. N-Acylation shifts the C-2 resonance of a carboxylic acid 2–3 ppm downfield. The chemical shift of C-2 of the methyl ester of myristic acid in $CDCl_3$ is 34.1 ppm whereas the chemical shift of C-2 of an N-linked fatty acid is 36.9 ppm (Koerner et al., 1979). A hydroxy group on C-3 of the methyl ester of a long-chain fatty acid will shift

Table III: ^{13}C NMR Chemical Shifts of C-1, C-1′, C-2, C-2′, and C-6′ Carbon Atoms of Diglucosamine Disaccharide of Lipid A from *E. coli* 0111[a]

fraction no.	chemical shift (ppm) of C-1	C-1′	C-2	C-2′	C-6′
2	93.5	102.5			60.3
3	93.3	102.7	51.3	52.1	60.0
5	91.7	102.3	53.9	54.4	60.4
6	91.7	102.3	52.4	53.8	60.3
7	91.8	102.4	54.4	54.0	
pentaacetylglucosamine	92.0				62.3
O-deacylated, N-acetylated lipid A from *Y. tuberculosis*				56.23	61.5

[a] For solvents, see Table II.

C-2 to 41.2 ppm, and an acetoxy group on C-3 will shift the resonance to 39.0 ppm (Tulloch, 1978). Since methyl esters of fatty acids are good models for O-acylated fatty acids in terms of chemical shift, we expect O-linked HTA around 41.2 ppm, and since ^{13}C chemical shifts are additive, we expect N-linkage to shift C-2 downfield 2–3 ppm. The uncertainty arises partly from small solvent effects. Also, O-acylated ATA is expected on the basis of this model around 39.0 ppm and N-linked ATA consequently at 41–42 ppm. So from the observation of a single resonance, the presence and functionality of one N-linked acylic substituent can be established. This is not possible for example with ^{1}H NMR. On the other hand, in the narrow range from 25.2 to 26.2 ppm resonances due to C-3 of FA, C-5 of HTA, and C-5 of ATA appear. The resonances from FA and ATA appear between 25.2 and 25.5 ppm and those from HTA around 25.8 ppm. Here then, only the sum of ATA and FA can be found, and one will have to look elsewhere in the spectrum to distinguish between ATA and FA. Fortunately, resonances are available for such assignments. The ^{13}C chemical shifts of fatty acids do not discriminate between O-acylation on C-3 of HTA or on the diglucosamine backbone. In prinicple, both FA and HTA can 3-O-acylate HTA to form two different homologues of ATA.

Information about ring substitution is obtained from the chemical shifts of the diglucosamine carbon atoms (Table III). The chemical shifts of the C-1 carbons in fractions 2 and 3 appear 1.5 and 1.9 ppm downfield of the corresponding carbon of fractions 5, 6, and 7 in agreement with a phosphate substituent in that position. The C-1′ carbon atom of all the fractions are at 102.2–102.8 ppm. The principle that O-acylation at C-3 shifts the resonance at C-2 of N-acetylated glucosamine is well established in carbohydrate research (Jennings & Smith, 1978). The C-2 and C-2′ carbon atoms

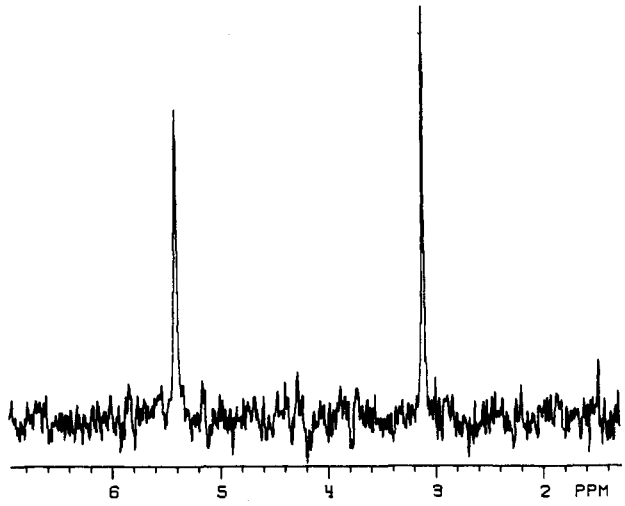

FIGURE 3: ^{31}P NMR spectra of hydrazinolyzed material from fraction 2 at 202.45 MHz. For discussion of spectra, see text.

in all fractions are a few ppm upfield from the corresponding carbon atom in N-acylated, O-deacylated, dephosphorylated lipid A from *Yersinia tuberculosis* (Krasnikova et al., 1982) in agreement with C-3 and C-3′ acylation in all the fractions. This is in agreement with the fact that C-3′ is not the site of KDO linkage but rather C-6′ as has recently been shown by Strain et al. The chemical shifts of C-6′ may be compared with those of the model substance pentaacetylglucosamine. An upfield shift is noted in these fractions compared to the model substance in agreement with the absence of acylation at C-6′. However, C-6′ of de-O-acylated *Yersinia tuberculosis* appears at 61.5 ppm, closer to pentaacetylglucosamine than to C-6′ of lipid A, and some caution should be exercised in interpreting these data until de-O-acylated, N-acylated lipid A with a phosphate group on C-4′ is available as a reference. The remaining carbon atoms could in principle give information too about substitution, but they all appear within a few ppm around 70 ppm, and assignment has not been possible, partly because the C-3 carbon of HTA and ATA also appears in that region, with greater intensity. Unfortunately then, no reference evidence has been obtained concerning the interesting question of acylation at C-4 of the diglucosamine backbone. But, it is clear that the position of KDO and phosphate-linkage is not a variable in the heterogeneity of lipid A.

Having provided this background, the structural determination may be discussed fraction by fraction (Table II). To determine whether the heterogeneity is manifested in the phosphorus content of the lipid A molecules, ^{31}P NMR was employed (Table II). First, a noninvasive approach was tried, and second, a small amount of each fraction was subjected to hydrazinolysis, and spectra were recorded in the presence of EDTA and surfactants.

Under noninvasive conditions, the peaks were very wide, at least 20 Hz or more. Hydrazinolysis, and the addition of surfactant and EDTA in connection with high temperature, reduced the line width to 3–4 Hz. SDS was tried with fraction 6 at pH 8 but did not improve the line width satisfactorily. Instead, triethylamine was used throughout (0.5% v/v). The pH under these conditions was around 11, but this did not affect the de-O-acylated molecules as monitored by NMR.

Fractions 2 (Figure 3) and 3 show two peaks at 5.4 and 3.1 ppm downfield from external 85% phosphoric acid. Fractions 5 and 6 show peaks at 5.5 ppm and much weaker ones at 1.7 ppm with intensity about 10–20% of that of the main peaks. These peaks are assigned to lyso-PE and phosphatidylglycerol (see Materials and Methods). Fraction 7 shows only one resonance at 5.5 ppm. The external reference is measured immediately after the sample is ejected from the magnet, and the chemical shifts are believed to be better than ±0.1 ppm. In the case of fraction 4, too little material was available to allow chemical degradation. However, fraction 4 is similar to fractions 2 and 3 under noninvasive conditions, and it is most probable that fraction 4 also has two phosphate groups.

The ^{13}C NMR spectrum of fraction 2 shows one peak at 44.1 ppm that is twice as intense as peaks at 42.4 and 42.9 ppm, respectively, showing that the intense peak is in fact two coinciding peaks. The two downfield peaks correspond to N-linked HTA and the other two to O-linked HTA. At 39.6 and 35.0 ppm are resonances corresponding to C-2 and C-4 of an ATA residue, and at 34.5 ppm is a single peak corresponding to C-2 of a long-chain FA. It follows that the sum of substituents is five.

Additional evidence is a very intense resonance at 37.5 ppm but without good resolution, corresponding to C-4 of HTA, in agreement with the presence of four HTA. Fraction 3 shows three well-resolved peaks at 37.6, 37.4, and 37.1 ppm, the first one being of double intensity. The same holds for signals at 43.9, 42.6, and 41.4 ppm. There are therefore two *N*-linked HTA and two O-linked HTA in this molecule. A sharp signal at 34.3 ppm together with three at 34.7, 34.8, and 34.9 ppm and together with four peaks between 25.3 and 25.6 ppm shows the presence of three ATA and one FA in this fraction.

Fractions 5–7 all have one phosphate group according to ^{31}P NMR. Fraction 5 shows three well-resolved ^{13}C NMR peaks between 37.4 and 37.8 ppm together with one double-intensity peak at 43.9 ppm and one single-intensity peak at 43.1, indicating two N-linked and one O-linked HTA. One ester function is evident from a resonance at 39.4 ppm and one sharp resonance at 34.8 ppm. The number of FA is two as concluded from the two signals at 34.4 ppm and in the 25.2–25.6 range. Fraction 6 shows very good resolution, and three HTA can be found both in the C-2 and in the C-4 regions where three peaks of equal intensity are observed at 43.9, 43.6, 42.8 ppm and 37.7, 37.6, and 37.3 ppm, respectively (Figure 4). Sharp signals at 39.5 and 34.8 ppm corresponding to C-2 and C-4 of an ATA substituent and two resonances at 34.4 ppm show that two FA are present. Peaks of minor intensity at 25.2 and 34.5 ppm are due to an impurity (see Materials and Methods).

Fraction 7 shows less good resolution, and the region at 37.5 is not well resolved although the intensity is strong. However, at 43.6, 42.8, and 42.4 ppm are three signals of equal intensity from C-2 of three HTA, suggesting the presence of only one N-linked HTA. The resonance at 41.4 is C-2 of ATA shifted downfield 2 ppm because of N-substitution. At 39.5 ppm is a peak of double intensity to that at 41.4 ppm, and this suggests three ATA in this molecule. The five peaks between 34.4 and 35.0 ppm will then have to be assigned as three ATA and two FA.

The spectra of the reference compounds KDO (2-keto-3-deoxyoctulosonic acid) and *O*-phosphoethanolamine were also recorded for comparison with those of the fractions, but no evidence was found for the presence of either of them.

Discussion

In this investigation we have studied the structure of six major lipid A fractions from *E. coli* 0111 with ^{13}C and ^{31}P NMR. NMR allows identification of molecular fragments such as carbon atoms in ester and amide-linked carboxylic acids (Tullock,1978; Bundle et al., 1973; Koerner et al., 1979; Strain et al., 1983) as well as phosphate groups. Furthermore, on the basis of carbon chemical shifts, it is possible to dis-

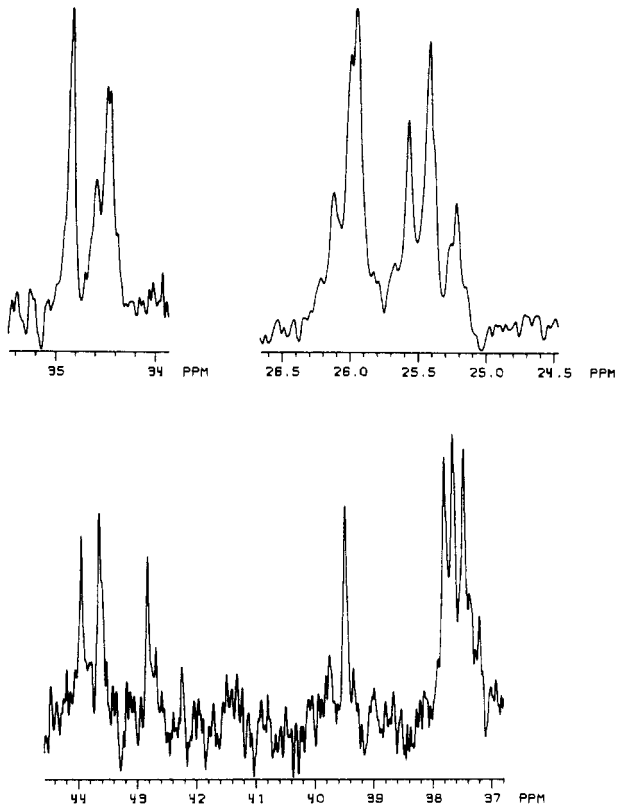

FIGURE 4: ^{13}C NMR spectra of fraction 6 at 125.75 MHz. For discussion of spectra, see text.

criminate between esterification or no esterification of the 3-O position on HTA (Tullock, 1978). The total number of carboxylic acids in the lipid A molecule can therefore be used to determine the number of ring substituents on the diglucosamine backbone.

The total number of substituents in fractions 2, 3, 5, 6, and 7 can be determined from examination of Table II. We find that in all these fractions there are five substituents and that the backbone therefore in all these cases is suggested to be fully substituted. The possiblity of free hydroxylic groups or amino groups on the backbone is therefore not a variable in the heterogeneity of *E. coli* 0111 lipid A.

Although determination of the exact position on the diglucosamine of all the various substituents is not possible, the amide-linked ones may be distinguished. N-Linkage in comparison with O-linkage shifts C-3 and C-3′ downfield (Bundle et al., 1973).

A direct observation of resonances in this region is therefore very strong evidence for N-linked HTA. It has, however, been suggested with *E. coli* and other bacterial strains that only one amide substituent is HTA and that the other is ATA (Takayama et al., 1983; Seydel et al., 1984; Wollenweber et al., 1982, 1984). In this investigation, we find that fractions 2, 3, 5, and 6 have two HTA in the 2- and 2′-positions and that in one of the fractions, fraction 7, one HTA and one ATA are in the amide positions. The studies by Seydel and Wollenweber were performed on nonfractionated material, and it is therefore difficult to ascertain whether a structural component is present in all of the material or only in a certain fraction. The study by Takayama et al. was performed on a different bacterial strain. Thus, these results are not necessarily comparable to or at variance with the present results.

The positions of the phosphate groups at C-1 and C-4′ are in agreement with the generally accepted view. The number of carboxylic acids found in these fractions may be compared with those found in some recent investigations of the chemical structure of lipid A. Wollenweber et al. (1982) find that approximately seven carboxylic acids are linked to the backbone in heterogeneous *Salmonella minnesota* lipid A, with ATA linked to at least one of the amino groups. The number of acids are in agreement with our results, provided that we take the average over fractions 2, 3, 5, 6, and 7. The authors find that, in contrast to the present results, it is likely that one position on the backbone apart from the site of KDO linkage is free from substituents. In the study by Qureshi et al. (1982), LPS from *Salmonella typhimurium* is hydrolyzed in 0.1 M HCl/methanol to give lipid A that is subsequently fractionated by chromatography. The five monophosphorylated fractions are isolated and subjected to FAB mass spectrometry to yield the molecular weights of these fractions. The slower moving zones contain fewer fatty acids per molecule than in this study. Their faster moving fractions have molecular weights that correspond to six acids per molecule, in agreement with fractions 5 and 6 of the present investigation. The authors did not provide evidence concerning the question of complete substitution of the backbone. In a ^1H NMR study of this bacterial strain, Takayama et al. showed that it did lack an acylic substituent in the C-4 position (Takayama et al., 1983). NMR studies have been performed on "*Escherichia coli* lipid A" that suggest the number of fatty acids to be six (Shiba et al., 1984) and that C-4 of the diglucosamine backbone is nonsubstituted. The material used may have been nonfractionated.

The question of substitution at C-4 is probably the most interesting one, and as mentioned above, other groups have reported that C-4 does not carry a substituent. Our result shows that in *E. coli* 0111 C-4 does carry an acylic residue, and the reason for this discrepancy is not understood by us. Different procedures in preparing lipid A must, of course, be taken into consideration.

The question of attachment of KDO to the diglucosamine backbone has received some attention recently. Contrary to the earlier suggestion that C-3′ was the site of linkage, it has been shown that KDO in LPS from heptose-less *E. coli* is bonded to the C-6′ position (Strain et al., 1983). Our results (Table III) are in agreement with, although not evidence for, such an interpretation. The chemical shifts of C-2 and C-2′ in the fractions are compared with those of N-acylated, O-deacylated lipid A from *Yersinia tuberculosis* appear 2–4 ppm to higher field, as would be expected when C-3 and C-3′ are O-acylated. One should, however, note that the shifts of C-6′ in the degraded lipid A did not agree perfectly with the shifts of C-6′ in the fractions. On the basis of the results of Strain, C-6′ is assigned as the position of KDO linkage.

The suggested structure for each fraction is summarized in Figure 5. The heterogeneity observed in free lipid A suggests that the parent LPS is also heterogeneous in its lipid portion. Variation in the preparation conditions such as prolonged hydrolysis (up to 16 h) did not affect the TLC pattern (Mattsby-Baltzer et al., 1984a), indicating that none of the fractions, at least not to a higher extent, degrade into another one. Certain fractions are also consistent in their appearance on TLC even though the lipid A is obtained from different bacteria (Mattsby-Baltzer et al., 1984a). That various fractions of lipid A exert different biological activities has been shown concerning activities such as *Limulus* gelation, mitogenicity, antigenicity, lethality for chick embryo, and tumor regression (Qureshi et al., 1982; Takayama et al., 1984; Banerji & Alving, 1979; Alving et al., 1980; Richardson et al., 1983; Richardson & Alving, 1984; Mattsby-Baltzer & Alving, 1984).

Fraction No	ester - linked (C-3,C-4,C-3')	PO_4
2	$R_2R_2R_3$ or $R_1R_2R_4$	2
3	$R_3R_4R_4$	2
4	—	2
5	$R_1R_2R_3$ or $R_1R_1R_4$	1
6	" "	1
7	$R_1R_3R_4$, $R_1R_4R_4$, $R_2R_3R_3$ or $R_2R_3R_4$	1

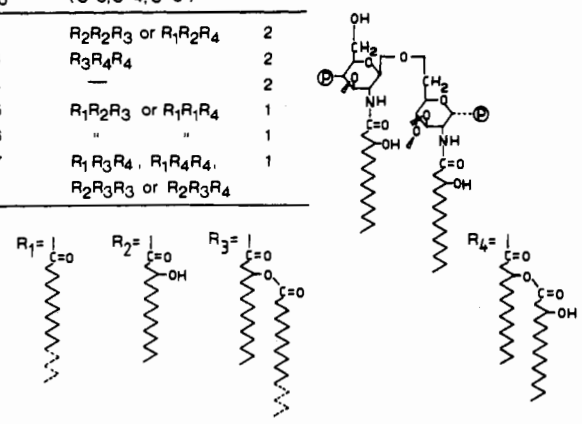

FIGURE 5: Suggested composition of lipid A fractions from *E. coli* 0111. In all fractions, the amino groups are substituted with HTA except in fraction 7, where one HTA and one ATA are N-linked.

The results from these studies may indicate that the regions on the lipid A molecule responsible for the different biological activities vary.

In conclusion, we have shown that the heterogeneity of lipid A from *E. coli* 0111 is manifested in a variation of the number and types of carboxylic acids. Also, the phosphate content varies among the fractions. All sites available for acylation on the diglucosamine backbone are substituted, the two amino groups with HTA except in fraction 7, where one HTA and one ATA are N-linked. Evidence is presented that the positions of phosphate groups are always C-1 and C-4′ or C-4′. We conclude that the heterogeneity with respect to carboxylic content is manifested in varying amounts of HTA, FA, and ATA in positions C-3, C-4, C-3′, and one of the amide positions and that the heterogeneity is also present in the parent LPS. Substituents other than these were not detected.

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