Structural Heterogeneity in the Lipopolysaccharide of *Aerobacter aerogenes* NCTC 243*

D. E. Koeltzow† and H. E. Conrad‡

ABSTRACT: The lipopolysaccharide (LPS) of *Aerobacter aerogenes* NCTC 243 can be separated into two distinct fractions which are free of RNA and the capsular polysaccharide produced by this organism. One of these (LPS_{III}) appears to be primarily a rough type LPS composed of lipid A and an oligosaccharide core containing 2-keto-3-deoxyoctulosonate, heptose, glucose, galactose, and glucosamine. The other (LPS_{II}) is composed of lipid A and three major polysaccharide (PS) components, all of which contain the galactan described earlier (Koeltzow, D. E., Epley, J. D., and Conrad, H. E. (1968) *Biochemistry 7*, 2920). The latter apparently is the O-antigen side chain of the complete LPS and is linked to the core in two of the PS fractions from LPS_{II}. The third PS fraction is a mixture of at least two PS types and is devoid of heptose. The major component in the mixture is the galactan. Both LPS fractions contain labile phosphate residues which are released by mild acid hydrolysis as orthophosphate at rates similar to the release of some of the PS fractions from lipid A. The amount of labile phosphate is equivalent to the amount of ester phosphate which precipitates with lipid A. The polysaccharide fractions also contain ester phosphate. The lipid A fractions obtained from the two LPS's are identical and contain equimolar amounts of glucosamine, ester phosphate, and total fatty acid. β-Hydroxymyristic acid is found exclusively in amide linkage to the nitrogen of glucosamine.

The lipopolysaccharide of *Aerobacter aerogenes* strain NCTC 243 on mild acid hydrolysis releases a polysaccharide fraction which is a complex mixture of fragments varying both in monosaccharide content and molecular weight (Koeltzow *et al.*, 1968). The major portion of the polysaccharide fraction is a branched galactan which appears to occupy a position in the LPS[1] analogous to that of the O-antigen side chain in *Salmonella* LPS's (for a review of the literature in this area, see Lüderitz *et al.*, 1966; Nikaido, 1969; and Osborn, 1969). The earlier observations on the NCTC 243 LPS are extended in this report, which shows that the crude LPS is an extremely complex mixture of lipid-linked carbohydrate polymers and that even when the LPS is chromatographically separated into several discrete fractions, each, on cleavage of the carbohydrate–lipid linkage, yields several polysaccharide components. The monosaccharide ratios of the individual polysaccharide fractions differ from each other, from those in the crude LPS, and from those in the purified LPS from which they are derived. Determination of the degree of polymerization and the reducing terminal residue for each of the polysaccharide fragments gives data consistent with the interpretation that only one or two of the polysaccharide fractions represent completed LPS (*i.e.*, core plus O-antigen side chain) and that the others represent metabolic intermediates in lipopolysaccharide biosynthesis.

Methods

Total carbohydrate was determined on unhydrolyzed samples by the phenol–sulfuric acid method (Dubois *et al.*, 1956) and expressed in terms of glucose equivalents. Monosaccharide content of fractions was determined by radiochromatographic analysis (Koeltzow *et al.*, 1968) of samples that had been hydrolyzed in 1 N sulfuric acid at 100° for 6 hr. In samples containing monosaccharide-linked ester phosphate in which the phosphate residues were not completely removed under these hydrolysis conditions, hydrolysates were brought to pH 8.5–9 with an equal volume of 1 M sodium carbonate and treated for 30 min at room temperature with one-half volume of a solution containing 1 mg/ml of alkaline phosphatase (calf mucosa, Sigma, Type II) prior to radiochromatography. To determine glucosamine in samples containing stable glucosaminyl linkages, hydrolysates were treated with sodium nitrite prior to radiochromatography and glucosamine was determined as the chitose peak on the radiochromatograms (Shively and Conrad, 1970). D-Glucose and D-galactose were determined in hydrolysates using the Glucostat and Galactostat reagents (Worthington Biochemical Corp., Freehold, N. J.). Total phosphate was determined by a modification (Shin, 1962) of the method of Bartlett (1959). Free and labile phosphate was estimated by the method of Fiske and Subbarow (1925) as described by Leloir and Cardini (1957). Labile phosphate is defined as that bound phosphate which is converted to orthophosphate by hydrolysis in 1 N hydrochloric acid at 100° for 7 min.

KDO was determined without prior hydrolysis using the TBA assay described by Osborn (1963). Values obtained in this assay are only approximate since a number of experimental parameters which affect the color yield could not be uniformly controlled. The main variations in conditions

* From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received August 10, 1970.* Supported by a grant from the U. S. Public Health Service, National Institute of Allergy and Infectious Diseases (AI 05696).

† Holder of a National Institutes of Health predoctoral traineeship on U. S. Public Health Service Training Grant GM-321. Present address: Department of Medical Microbiology, Stanford University School of Medicine, Stanford, Calif. 94305.

‡ To whom to address correspondence.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: LPS, lipopolysaccharide; PS, polysaccharide: KDO, 2-keto-3-deoxyoctulosonate; TBA, thiobarbituric acid; LPS_{II} and LPS_{III}, purified fractions of LPS (see Figure 1); PS_{II} and PS_{III}, the total PS fractions from LPS_{II} and LPS_{III}, respectively; PS_{III}-A, PS_{III}-B, PS_{III}-C, etc., purified PS fractions from PS_{III} (same designations for PS_{II} fractions).

were in the buffers from which KDO was sampled; the latter were chosen according to the conditions required for hydrolysis and/or column chromatography. For example, when the ammonium salt of KDO, prepared as described by Hersberger *et al.* (1968), is assayed at intervals after heating at 100° in 0.1 M Tris-HCl buffer (pH 8.5), its color yield *increased* to 150% of the original intensity in 5–10 min. On the other hand, when heated in 1% acetic acid (pH 3.5) at 100° its color yield is *decreased* to 70% of the original in 15–30 min. If the latter solution is cooled to room temperature and brought to pH 8.35 by adding solid Tris, the color yield increases immediately to 85% of the original. These changes are presumed to be due to shifts in equilibria among the various ring forms (lactone and hemiacetal) in which KDO can exist, which in turn can affect the course of the periodate oxidation. A further complication in the TBA assay is observed in solutions of KDO containing Tris-HCl buffers at concentrations greater than 0.1 M. In this situation, a precipitate of salt begins to form approximately 10 min after removing the assay tube from the 100° heating bath. Therefore, the color intensity must be measured before the precipitating salt begins to interfere. However, the color intensity decreases continually during the cooling period until, at the time the salt begins to precipitate, the sample has about 80% of the intensity observed for the hot solution.

Complications in quantitation of KDO arise in this work because, as described below, part of the examination of LPS fractions involves hydrolysis of purified LPS in 1% acetic acid for varying periods of time, followed by chromatography of the resulting polysaccharide solutions on DEAE-cellulose in a 0–0.2 M Tris-HCl gradient. Consequently, the assay conditions for the sample applied to the column cannot be reproduced in analysis of the column fractions. Analysis of the later fractions removed from the column is further complicated by the salt precipitation problem.

Reducing Terminal and Degree of Polymerization Determination. Aliquots (20 μl) of polysaccharide solutions containing 10–60 mg/ml of water were mixed with 5 μl of a solution of [^{14}C]glucose (50 mCi/ml, 200 mCi/mmole), 5 μl of 2 N sodium carbonate, and 5 μl of 0.05 N [^{3}H]sodium borohydride (200 mCi/mmole) in 0.1 N sodium hydroxide. This mixture was heated at 50° for 40 min. After cooling, 10 μl of 2 N sulfuric acid was added to destroy excess borohydride and the resulting solution was used for the degree of polymerization and reducing terminal analysis.

For determination of the degree of polymerization, an aliquot of the solution of reduced polysaccharide was spotted on a 1-in. wide strip of Whatman No. 1 paper and chromatographed for 12–15 hr using 1-propanol–ethanol–water (17:43:40, v/v; see Huber *et al.*, 1968). The strip was scanned for radioactivity in a strip scanner to locate the [^{14}C]glucitol, which is the only radioactive component which migrates from the origin during the short development period. The part of the strip containing the [^{14}C]glucitol was cut off of the strip and the amount of ^{14}C present was measured by scintillation counting and used to calculate the amount of the polysaccharide solution actually spotted on the chromatogram. A fresh strip of paper, equal in length to the one removed, was sewed to the bottom end of the original chromatogram which was then developed in the same solvent for an additional 36 hr before drying and counting the strip for ^{3}H by the normal procedure used for radiochromatography. The total ^{3}H was used to calculate the number of moles of oligo- or polysaccharide on the strip. The total moles of bound monosaccharide in the same polysaccharide solution was determined by hydrolysis and radiochromatography as described above. The degree of polymerization is the ratio of total moles of monosaccharides to total moles of oligo- or polysaccharide. Polymers with degrees of polymerization greater than 30 did not migrate from the origin of the chromatogram. In these cases, the polysaccharide was assumed to be pure on the basis of its DEAE-cellulose elution profile and the total ^{3}H counts per minute at the origin were used to calculate the moles of polysaccharide on the chromatogram. In this solvent system, background counts at the origin are negligible.

The residue at the reducing terminal of the polymer was determined by hydrolyzing the [^{3}H]borohydride-reduced polysaccharide in 1 N sulfuric acid at 100° for 6 hr to release the ^{3}H-labeled reducing residue as a glycitol. The latter was identified by radiochromatography in ethyl acetate–acetic acid–formic acid–water (18:3:1:4, v/v) using the [^{14}C]glucitol to characterize the [^{3}H]monosaccharide on the basis of its $R_{glucitol}$ value. When a [^{3}H]borohydride-reduced KDO standard was treated according to the hydrolysis conditions used here, it was converted to a mixture of ^{3}H-labeled degradation products which gave a characteristic multi-peak radiochromatogram. Several of the purified polysaccharide fractions analyzed here gave the KDO-type radiochromatogram in the reducing terminal analysis.

Fatty Acid Content of Lipid A Fractions. The lipid A fractions which precipitated from aqueous solution during the acetic acid hydrolysis of LPS_{II} and LPS_{III} were removed by centrifugation and dissolved in chloroform–methanol (2:1, v/v). The small amount of insoluble material was centrifuged out and the soluble fraction was concentrated to dryness and extracted with ethyl ether to remove neutral lipids. The ether-insoluble phospholipid fraction was taken to dryness and treated with 1 N methanolic sodium hydroxide at room temperature for 30 min to saponify the *O*-acyl fatty acids. The hydrolysates were acidified with 1 N hydrochloric acid and the free fatty acids were extracted with ether. The ether extracts were concentrated to dryness and esterified with diazomethane for vapor-phase chromatographic analysis.

The remaining aqueous phase was concentrated to dryness under nitrogen and taken up in 1 N hydrochloric acid in 60% methanol. This solution was heated at 80° for 16 hr to remove the *N*-acyl fatty acids which were then extracted with ethyl ether and esterified for analysis as above. The remaining water-soluble material was analyzed by radiochromatography.

For vapor-phase chromatography the methyl esters of the fatty acids were dissolved in hexane and chromatographed on 6-ft columns of 5% DEGS on Chromosorb W (60–80 mesh) at 170° or 3.8% SE-30 on Diatoport S (80–100 mesh) at 145° using an F & M Model 400 gas chromatograph. Peaks were identified and quantitated by comparison of retention times and peak heights with those of standards.

To purify sufficient β-hydroxymyristic acid for infrared and mass spectrographic analysis, the esterified *N*-acyl fraction from above was applied in petroleum ether containing 5% ethyl ether to a 1 × 20 cm column of silicic acid packed in petroleum ether (bp 30–60°). The column was eluted successively with 100 ml each of petroleum ether–ethyl ether mixtures having the following ratios: 100:0, 90:10, 85:15, 80:20, 70:30, and 60:40. The bulk of the lipid eluted with the 85:15 and 80:20 mixtures. Both thin-layer and vapor-phase chromatography showed that a single fatty acid ester was present in greater than 99% purity. This material was crystallized from petroleum ether at 5° and analyzed.

Purification of LPS and PS Fractions. A. aerogenes NCTC

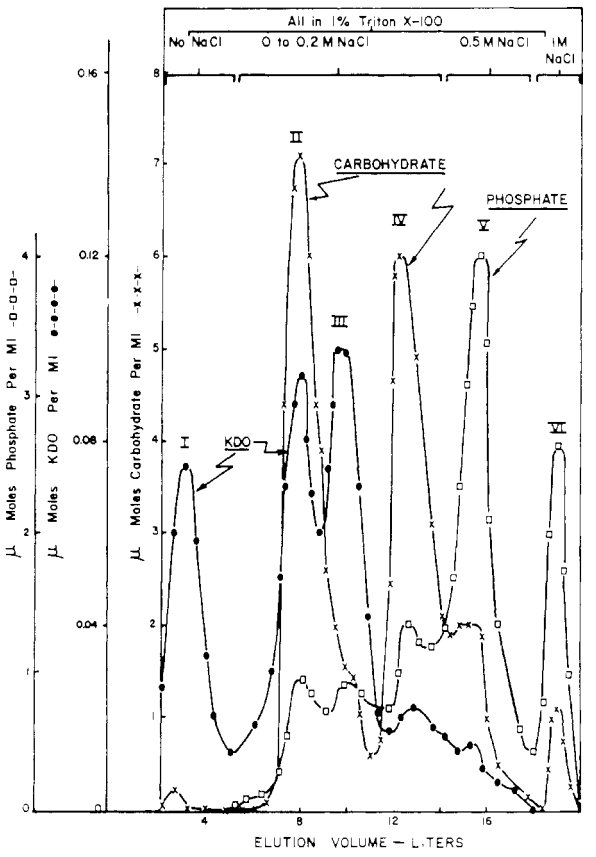

FIGURE 1: DEAE-cellulose chromatography of crude LPS. A solution containing 15 g of crude LPS in 375 ml of 1% (v/v) Triton X-100 was loaded onto a 7.9 × 75 cm column of DEAE-cellulose packed in 1% Triton X-100. The column was eluted first with 3 l. of 1% Triton X-100, then with a linear salt gradient formed from 4.5 l. each of 1% Triton X-100 and 0.2 M sodium chloride in 1% Triton X-100, followed by 4.5 l. of 0.5 M sodium chloride, and finally 4.5 l. of 1.0 M sodium chloride, both in 1% Triton X-100. Elution curves plotted are KDO (●-●-●), total carbohydrate (×-×-×), and total phosphate (□—□--□).

243 cells were grown and decapsulated as described previously (Koeltzow *et al.*, 1968). Cell paste (500 g) was treated with 2.5 l. of 45% phenol at 70° for 15 min and, after cooling to 0°, the water phase was removed and the precipitate at the interface and the phenol phase were reextracted at 70° with 1.25 l. of water. The combined aqueous layers were dialyzed exhaustively against deionized water and concentrated to 350 ml. The crude LPS was precipitated by addition of four volumes of acetone, washed with acetone and ethyl ether, and dried *in vacuo* at 60°; yield 8.3 g/500 g of cell paste.

Crude LPS (15 g) was dissolved in 375 ml of a 1% (v/v) solution of Triton X-100 and fractionated on a DEAE-cellulose column (7.9 × 75 cm) packed in 1% Triton X-100. The column was eluted first with 3 l. of 1% Triton X-100, then with a linear gradient generated from 4.5 l. each of 1% Triton X-100 and 0.2 M sodium chloride in 1% Triton X-100. The column was finally eluted with 4.5 l. each of 0.5 M sodium chloride and 1 M sodium chloride, both in 1% Triton X-100. Fractions (50 ml) were collected at a flow rate of 2 ml/min and analyzed for total carbohydrate, KDO, phosphate, and monosaccharide components.

The fractions in the six peaks recovered from the large column were combined and the Triton X-100 was removed by four successive extractions with four volumes of ethyl ether–ethanol (4:1, v/v). The aqueous solutions were concentrated to one-tenth their original volumes and, with the exception of peak I, were dialyzed exhaustively to remove the sodium chloride. Peak I was deionized on Sephadex G-25 since it was found to contain low molecular weight LPS components. Each of these fractions was then concentrated to a thick solution. The carbohydrates were precipitated by addition of several volumes of acetone, washed with acetone and ether, and dried *in vacuo* at 60°.

For preparation of PS components, 2% solutions of the LPS fractions were hydrolyzed in 1% acetic acid at 100° for an appropriate period of time (1 hr for LPS_{II} and 3 hr for LPS_{III}). The precipitated lipid A's were removed by centrifugation and saved for analysis, and each of the supernatants, containing mixtures of polysaccharides, was applied to a 3.3 × 115 cm column of DEAE-cellulose packed in 0.01 M Tris-HCl buffer (pH 8.35). Each column was eluted with a linear gradient generated from 3 l. each of 0.1 M and 0.5 M Tris-HCl buffer (pH 8.35). Fractions (15 ml) were collected at a flow rate of 1 ml/min and analyzed for total carbohydrate and KDO. Each of the carbohydrate peaks was concentrated *in vacuo* to a volume of 10–30 ml and deionized by gel filtration on a column of Sephadex G-25. The deionized peaks were concentrated to small volumes, precipitated, washed with acetone and ethyl ether, and dried *in vacuo* as above.

Results

Purification of Crude LPS. Crude LPS contains, in addition to its typical LPS constituents, RNA (15%), capsular polysaccharide (15%), protein (20% based on % N in excess of RNA and GlcN), and ash (15%). Figure 1 shows the chromatographic separation of crude LPS on DEAE-cellulose into six uniquely defined peaks. Analyses of the combined peak fractions after deionization are given in Table I. As described below, peaks I, II, and III can be identified as LPS components, peak IV as capsular polysaccharide (Gahan *et al.*, 1967), and peaks V and VI as RNA. Most of the protein and ash present in the crude LPS are not recovered in the combined peak fractions. The separation shown in Figure 1 is accomplished using a 0–0.2 M sodium chloride gradient in 1% Triton X-100, followed by stepwise elution first with 0.5 M sodium chloride, and then with 1 M sodium chloride, both in 1% Triton X-100. In the absence of detergent, only capsular polysaccharide and RNA can be eluted from DEAE-cellulose since the LPS forms micelles which become entrapped at the top of the column. Summation of the contents of all column fractions shows recoveries of 95% of the applied carbohydrate, 164% of the KDO (see Methods), and 95% of the phosphate.

To emphasize several features of the data, different coordinates are used to show the total carbohydrate, KDO, and phosphate elution profiles. It is seen that KDO is found mainly in peaks I, II, and III but trails into peaks IV and V. Phosphate is found in all fractions but is present in largest amounts in the two RNA peaks. The KDO and phosphate in peak I are both free, *i.e.*, uncombined (in spite of the fact that the crude LPS had been dialyzed exhaustively before application to the column), and are removed from the small amounts of polymeric carbohydrate in peak I by gel filtration prior to the analysis shown in Table I. The bound phosphate in peaks II and III (and IV) is in part ester phosphate and in part labile phosphate (see below). Glucosamine and heptose

TABLE I: Analysis of Combined and Deionized Fractions from DEAE-Cellulose Chromatography of Crude LPS.[a]

Component[b]	Crude LPS	Peak I	Peak II	Peak III	Peak IV	Peak V	Peak VI	Total
Glucosamine	6.2 (1.0)	0.2 (1.0)	3.0 (1.0)	3.7 (1.0)	0.2	0.1	0.0	7.2
Aldobiouronic acid[c]	15.0	0	0	0	4.8	1.8	0.2	6.8
Heptose	4.3 (0.7)	0 (0)	1.1 (0.4)	2.3 (0.6)	0.7	0.2	0.03	4.3
Hexose	125.4 (20.2)	1.5 (7.5)	59.4 (19.8)	18.0 (4.9)	30.3	2.7	0.3	112.2
KDO	2.6 (0.4)	0.3 (1.5)	0.5 (0.2)	0.8 (0.2)	0.2	0.2	0	1.9
Ribose	26.0 (4.2)	0 (0)	0 (0)	trace	4.0	5.4	3.0	12.4
PO_4	117.5 (18.9)	0.3 (0.2)	2.3 (0.8)	3.9 (1.1)	4.3	14.7	9.2	34.7
Total CHO (%)	100.0	0.7	30.4	16.1	34.7	11.5	2.4	95.8
Weight (mg)	100.0	4.2	15.2	9.7	11.1	9.5	4.0	53.7

[a] Values in parentheses are molar ratios in each fraction. [b] All values are per 100 mg of crude LPS. Phosphate and monosaccharide values are in micromoles. [c] From the capsular polysaccharide produced by this organism. See text.

(not plotted in Figure 1) parallel the KDO profiles in peaks II and III and trail into peak IV.

Of the carbohydrate components quantitated in Table I, glucosamine, heptose, and KDO are the ones which are typical components of enteric lipopolysaccharides. These are recovered from the column quantitatively and are concentrated in peaks II (LPS_{II}) and III (LPS_{III}). These two fractions are devoid of both RNA (see ribose values) and the aldobiouronic acid which characterizes the capsular polysaccharide (see Gahan *et al.*, 1967). Together, they represent about 50% of the carbohydrate and 25% of the weight of the crude LPS.

Peak IV is identified as capsular polysaccharide. When it is hydrolyzed in 1 N sulfuric acid at 100° for only 30 min, it gives a paper chromatographic fingerprint identical with that obtained when the capsular polysaccharide is treated in the same way (Gahan *et al.*, 1967). As shown in the earlier studies, an aldobiouronic acid, 3-*O*-α-D-glucopyranosyluronic acid D-mannose, makes up 50% of the carbohydrate in the capsule. This disaccharide is very resistant to acid hydrolysis, and, under the hydrolysis conditions used for the analyses in Table I, more than 80% of this disaccharide remains intact. Thus, the amount of aldobiouronic acid present in a hydrolysate can be used to calculate the amount of capsular polysaccharide originally present. It may be noted that there is some aldobiouronic acid found in fractions V and VI, indicating a strong absorption of the capsule to the column. This adsorption results in a loss of more than half of the capsular polysaccharide applied to the column (based on total aldobiouronic acid recovery). Identical behavior has been observed with preparations of the capsular polysaccharide isolated without disruption of the cells. Based on its glucosamine, heptose, and KDO content, peak IV obviously contains a significant amount of LPS, but this small percentage of the LPS was not purified and examined further.

The data presented below show a detailed examination of LPS_{II} and LPS_{III}. Before further study, LPS_{III} was treated exhaustively with ribonuclease and dialyzed to remove the trace amount of RNA present.

Time Course of Hydrolysis of Lipopolysaccharide Fractions. Upon initial examination of the cleavage of LPS_{II} and LPS_{III} into polysaccharide and lipid A in 1% acetic acid, several important observations were made. Both LPS fractions were found to give several polysaccharide fractions separable on DEAE-cellulose columns using a linear 0–0.2 M Tris-HCl (pH 8.35) gradient. Some of these polysaccharides were found in both fractions while others were unique to one of the fractions.

The rates of release of the different polysaccharides from a given LPS differed quite markedly from each other and from that of free KDO release. The effect of hydrolysis time on the PS and KDO elution profile is shown in Figure 2 for LPS_{II} and LPS_{III}. In these experiments the LPS's were hydrolyzed in 1% acetic acid at 100° and at intervals aliquots were removed for column chromatographic analysis. Peaks containing significant levels of carbohydrate are given letter designations and are plotted in the closed circles; the TBA-reactive fractions appear primarily in the free KDO peak and are indicated by the open circles. For the carbohydrate peaks the values given in parentheses are the percentages of applied carbohydrate recovered in that peak. Similarly the percentage of the zero time TBA-reactive material recovered in the free KDO peak is given in parentheses. Because of some variability in the total recoveries from one column to the next the progress of hydrolysis is most appropriately presented in the individual profiles rather than in hydrolysis progress curves. Prior to hydrolysis none of the carbohydrate or the KDO in either LPS fraction can be eluted from the columns under the conditions used in these elutions (*i.e.*, in the absence of Triton X-100).

Examination of the elution profiles shows that for LPS_{II} there is a progressive increase in PS_{II}-A until it reaches a maximum at approximately 3 hr. On the other hand, PS_{II}-C reaches its maximum at 60 min and then is slowly destroyed during the further hydrolysis. At the earliest sampling PS_{II}-D has reached its maximum and it then shows a slow decline. The KDO recoveries are somewhat variable (see Methods), but most of the recovered KDO appears as a separate peak of free KDO. In only one of the carbohydrate peaks, PS_{II}-C, is there found a significant amount of TBA-reactive material. After hydrolysis for 360 min, the KDO appears to be largely destroyed.

The hydrolysis of LPS_{III} proceeds in a similar manner. In this case, however, PS_{III}-C does not show a decline after peaking early, as found for PS_{II}-C. A unique fraction, PS_{III}-E, appears in the LPS_{III} hydrolysis mixture, reaching its maximum at about 3 hr. Again, KDO appears primarily as free KDO.

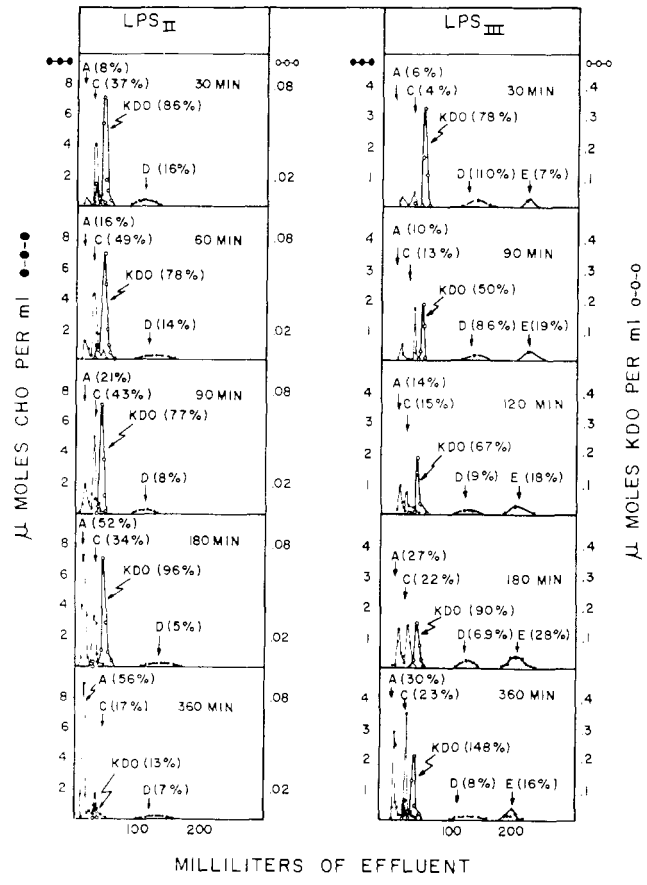

FIGURE 2: The progress of PS release during acetic acid hydrolysis of LPS. A 1% solution of each LPS in 1% acetic acid was heated at 100°. At each indicated time an aliquot was taken and the precipitated lipid A was removed by centrifugation. Supernatant (1.5 ml) was applied to an 0.9 × 30 cm column of DEAE-cellulose and eluted with a linear salt gradient formed from 300 ml each of 0.01 and 0.2 M Tris-HCl, both at pH 8.35. Fractions were analyzed for total carbohydrate (●—●—●) and TBA-reactive KDO (○—○—○). The numbers in parentheses above each peak are the percentages of the applied material recovered in the peaks. Before hydrolysis, none of the carbohydrate can be eluted from the column under these conditions.

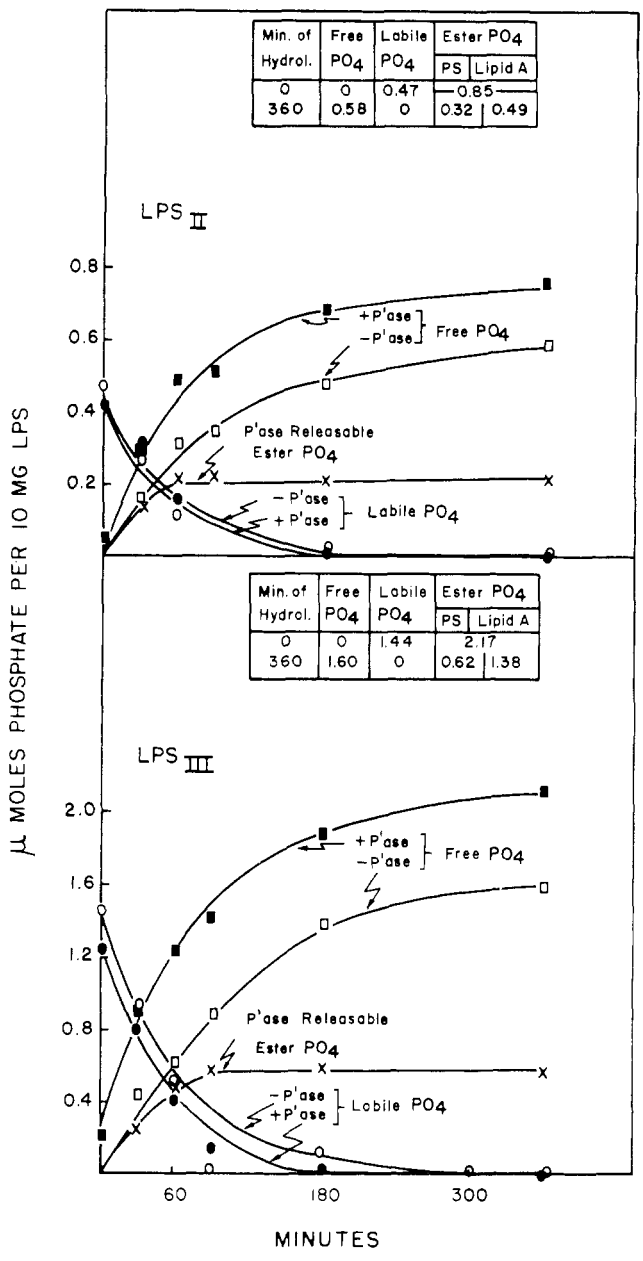

FIGURE 3: Phosphate release during acetic acid hydrolysis of LPS. Solutions containing 10 mg of LPS/ml of 1% acetic acid were heated at 100° and aliquots were taken at appropriate intervals. The lipid A was removed by low-speed centrifugation and the supernatants were made slightly alkaline by addition of 80 μl of 2 N sodium carbonate/ml of supernatant. Aliquots of the neutralized solutions were taken for total phosphate analysis. To determine free and labile phosphate, 300 μl of the neutralized supernatant was mixed with 25 μl of 16% (v/v) Triton X-100 and 75 μl of a solution containing 10 mg of alkaline phosphatase/ml of water. Free and labile phosphate were determined immediately (−P'ase) and after a 60-min incubation at room temperature [+P'ase) as described in Methods. The Triton X-100 was necessary to prevent formation of a precipitate in the samples taken before precipitation of lipid A (0-, 30-, and 60-min samples). The P'ase-releasable ester phosphate is the amount of free phosphate released by phosphatase. Since it remains the same after precipitation of lipid A between 60 and 90 min, it arises from ester phosphate in the supernatant fraction.

The Release of Phosphate and Precipitation of Lipid A During LPS Hydrolysis. Prior to hydrolysis, the LPS fractions give clear (LPS_{II}) to slightly turbid (LPS_{III}) solutions which become progressively more cloudy during hydrolysis until lipid A finally precipitates. The time at which lipid A finally flocculates is a function of the purity and concentration of the LPS. In crude LPS solutions, addition of acetic acid to begin the hydrolysis causes immediate precipitation of RNA and this seems to form a surface on which lipid A aggregates as it is released. In the purified LPS hydrolysates, lipid A could not be centrifuged out of solution until after heating at 100° for 60–90 min.

The bound phosphate in the LPS fractions is in part labile phosphate and in part ester phosphate. As hydrolysis proceeds, the labile phosphate is converted completely to orthophosphate, while the ester phosphate is recovered in both the lipid A and the PS fractions. The progress of phosphate release is shown in Figure 3. During hydrolysis aliquots were withdrawn at intervals and centrifuged to remove the flocculated lipid A. The supernatants were analyzed for orthophosphate, labile phosphate, and total phosphate. The supernatants were then treated exhaustively with alkaline phosphatase and the phosphate analyses were repeated. Values for ester phosphate in the lipid A fraction were calculated by subtracting the total phosphate found in the

TABLE II: First-Order Rate Constants for Hydrolysis of LPS Fractions and Model Compounds in 1% Acetic Acid at 100°.

Reaction	k (sec^{-1}) × 10^4	Reaction	k (sec^{-1}) × 10^4
$LPS_{II} \rightarrow PS_{II}$-A	1.1	$LPS_{II} \rightarrow P_i$[a]	3.4
$LPS_{III} \rightarrow PS_{III}$-A	1.1	$LPS_{III} \rightarrow P_i$[a]	2.5
$LPS_{II} \rightarrow PS_{II}$-C	6.2		
$LPS_{III} \rightarrow PS_{III}$-C	1.4	UDPG $\rightarrow P_i$[a]	1.8
$LPS_{II} \rightarrow PS_{II}$-D	>25.0	ADP $\rightarrow P_i$[a]	2.3
$LPS_{III} \rightarrow PS_{III}$-D	>25.0	G-1-P $\rightarrow P_i$	1.2
$LPS_{III} \rightarrow PS_{III}$-E	2.5	G-6-P $\rightarrow P_i$	0.04
ADPG → Glc	24.8	$LPS_{II} \rightarrow$ KDO[b]	>25.0
Maltose → 2Glc	0.02	$LPS_{III} \rightarrow$ KDO[b]	>25.0
Sucrose → Glc + Fru	5.1		

[a] Labile phosphate only. [b] Free KDO.

TABLE III: Molar Ratios in Polysaccharide Fractions from LPS_{II}.

Component	Polysaccharide			
	Total[a]	A	C	D
Glucosamine	1.0	1.0	1.0	1.0
Heptose	0.6	0.0	0.6	0.7
Total hexose[b]	21.7	61.4	24.8	21.2
Galactose[b]	20.4 (25.3)	61.1 (56.1)	23.9 (18.8)	20.4 (19.1)
Glucose	1.3	0.3	0.9	0.8
KDO	0.2	0.02	0.03	0.03
PO_4	0.7	0.0	0.05	0.04
Minimum DP[c]		62	26	23
Experimental DP		66	54	132
Reducing terminal		Gal (79%), GlcN (21%)	KDO	KDO

[a] The unfractionated PS obtained by 1% acetic acid hydrolysis of LPS_{II}. [b] Glucose and galactose are the only hexoses in these polysaccharides. Total hexose is the sum of these sugars, determined by radiochromatography, while glucose and galactose values are determined using the glucostat and galactostat reagents. Since the total hexose and the glucostat values are most reliable, galactose is recorded as the difference between these values. The galactostat determination is given in parentheses. [c] Minimum degree of polymerization (DP) is the sum of the molar ratios of glucosamine, heptose, hexose, and KDO. The experimental degree of polymerization and reducing terminal values are obtained as described in Methods.

supernatant from the total phosphate in the zero time sample. Ester phosphate in the supernatant was obtained by subtracting both free and labile phosphate from the total supernatant phosphate.

The hydrolysis patterns are very similar for LPS_{II} and LPS_{III}. The labile phosphate is converted completely into orthophosphate in 180 min, but the ester phosphate is cleaved at a very slow rate. Alkaline phosphatase has very little effect on the labile phosphate, but the supernatant ester phosphate becomes accessible to the enzyme as the hydrolysis proceeds. It is not clear what implications concerning the nature of the supernatant ester phosphate may be drawn from the phosphatase data. In control experiments the phosphatase preparation was found to release all of the phosphate residues (anhydride, ester, and glycosyl) from UTP, ADP, AMP, UDP-glucose, or glucose-1-P. The supernatant ester phosphate has not been studied further, but it is of note that it is not found in any of the major polysaccharide fractions described below.

In the inserts on the graph, the nature and amounts of phosphate present initially and after 6-hr hydrolysis are shown. In both fractions the amount of ester phosphate that precipitates with lipid A is equal to the original amount of labile phosphate. A significantly smaller amount of ester phosphate appears in the supernatant.

Relative Rates of Release of Carbohydrate, KDO, and Phosphate. The data in Figures 2 and 3 can be used to calculate first order rate constants for hydrolytic conversion of LPS to its various fragments. The semiquantitative nature of the experimental manipulations required to obtain the polysaccharide release data (Figure 2) dictate that the rate constants for appearance of the various polysaccharide fragments can only indicate *relative* labilities of the bonds linking the polysaccharide to lipid. The rate constants for phosphate release are more precise numbers. The results of this treatment of the data are shown in Table II. Values obtained with pertinent standard compounds are also given. The rate constants for conversion of labile phosphate in these LPS's into orthophosphate are somewhat greater than those found for UDP-glucose, ADP, or glucose-1-P and significantly greater than the glucose-6-P value. The carbohydrate hydrolytic release constants present a more complex picture. Only PS_{II}-D and PS_{III}-D are released at rates comparable to the rate of appearance of free KDO. The hydrolysis of glucose from ADP-glucose occurs at a comparable rate. All of the other PS fragments are released at rates much slower than either the free KDO or the glucose from ADP-glucose, but their rate constants are still much greater than that for the glycosidic bond of maltose. These PS's could be formed by hydrolysis of a very labile glycosidic bond such as that in sucrose. It is clear that further information is required to explain these kinetics. The rate constants do suggest, however, that in LPS_{II} and LPS_{III}, the PS-A's are identically linked, as are the PS-D's. For reasons not yet clear, PS_{II}-C and PS_{III}-C are released at different rates, in spite of their strong chemical similarities shown below.

Characterization of Polysaccharide Fractions. To obtain sufficient amounts of the PS fractions for analysis, the preparations shown in Figure 2 were scaled up. To maximize the yield of each PS, LPS_{II} was hydrolyzed for 60 min and LPS_{III} for 180 min prior to DEAE-cellulose chromatography of the soluble part of each hydrolysate. The separated PS fractions were deionized on Sephadex G-25 prior to analysis. After concentration all PS fractions were found to contain significant amounts of free KDO, most of which was removed readily during the deionization process. In addition to the main carbohydrate fractions described here, several very minor peaks (less than 1% of the total carbohydrate) were recovered from the DEAE-cellulose columns, but recoveries were too low to permit further analysis. Polysaccharide fractions from LPS_{II} and LPS_{III} which behaved identically on both DEAE-cellulose and on Sephadex are given the

TABLE IV: Molar Ratios in Polysaccharide Fractions from LPS_{III}.[a]

Component	Polysaccharide					
	Total	A	B[b]	C	D	E
Glucosamine	1.0	1.0	1.0	1.0	1.0	1.0
Heptose	1.0	0.0	0.4	0.5	1.0	1.8
Total Hexose	6.4	50.8	17.3	33.2	30.4	5.5
Galactose	4.5 (5.0)	50.4 (40.0)	15.6 (14.8)	31.4 (34.1)	28.5 (18.3)	3.2 (1.3)
Glucose	1.9	0.4	1.7	1.8	1.9	2.3
KDO	0.3	0.1	1.3	0.0	0.1	0.1
PO_4	0.9	0.0	0.0	0.0	0.3	0.0
Minimum DP		52	20	35	32	8
Experimental DP		77	16	63	126	12
Reducing terminal	Gal (87%)	GlcN (13%)	KDO	KDO	KDO	KDO

[a] See footnotes to Table III. [b] PS_{III}-B is a minor fraction (1% of the carbohydrate) which chromatographed with PS_{III}-C on small columns (Figure 2) but which separated in the shallower gradient used in the large-scale preparation.

same letter designation, and either II or III is used as a subscript to indicate the LPS from which the PS is derived. The monosaccharide ratios of the PS's, relative to glucosamine, are given in Tables III and IV, along with the ratios in LPS_{II} and LPS_{III} and their corresponding whole polysaccharide fractions.

The important features of the data may be summarized as follows. Only trace amounts of both KDO (TBA positive) and phosphate are present in these PS fractions after gel filtration. With the exception of the A polysaccharides, the reducing terminal of all polysaccharides appears to be KDO (borohydride-reduced KDO is converted to several fragments under the conditions used here to hydrolyze the [^3H]BH_4-reduced polysaccharide, but a KDO standard gives a characteristic multipeak radiochromatogram identical with those obtained from the PS fractions). The KDO in these polysaccharides is not TBA reactive. Both PS_{II}-A and PS_{III}-A show two reducing terminals—galactose (80%) and glucosamine (20%)—and are, therefore, mixtures. PS fractions from LPS_{II} and LPS_{III} which are given the same letter designation because of their similar chromatographic behavior have very similar molar ratios. The degrees of polymerization of the C fractions are approximately 50, those of the D fractions are approximately 100, and the *average* degrees of polymerization of the A fraction mixtures are intermediate between these two extremes. There is no heptose in either PS_{II}-A or PS_{III}-A. PS_{II}-C, PS_{II}-D, PS_{III}-B, PS_{III}-C, and PS_{III}-D all have very similar molar ratios with the main variation found in the amount of galactose. The molar ratio of glucose to glucosamine is approximately 2 in the PS_{III} fractions and 1 in the PS_{II} fractions. PS_{III}-E is unique both in degree of polymerization and in molar ratios, and is very reminiscent of the *Salmonella* core. It may be noted that KDO is apparently the only anionic residue in those fractions which are retarded on the DEAE-cellulose columns. Amino acid analyses show that only trace amounts of peptide or protein are present in these polysaccharides.

Relative Abundance of Each Type of Polysaccharide in Crude LPS and in LPS_{II} and LPS_{III}. The data presented in Tables III and IV give no indication of the relative contributions of each type of PS to the total make-up of the LPS. Because of the kinetics of appearance and disappearance of the PS fractions during hydrolysis and because of the uncertainty as to how the PS's are related to each other, it is not possible to state in precise terms how much of each is originally present in the LPS. Table V shows the relative amounts of total carbohydrate in each polysaccharide in LPS_{II} and LPS_{III} at 180- and 360-min hydrolysis. The overall picture is very nearly the same at both times. In progressing from PS-A to PS-E, two points are evident: (1) the per cent of total carbohydrate recovered in each fraction becomes progressively smaller, and (2) the per cent of each type of PS recovered from LPS_{II} decreases while that recovered from LPS_{III} increases. Since LPS_{II} and LPS_{III} represent 46% of the crude LPS and since together they may be considered as the *total* purified LPS from this organism, PS-A represents about 40% of the total carbohydrate in the purified LPS, PS-C about 20%, and PS-D and PS-E 5 to 10% each. The table also shows the number of micromoles of each type of PS which are recovered in LPS_{II} and LPS_{III}. These values are calculated using the experimentally determined degrees of polymerization and represent only approximate values. However, they serve to illustrate a much sharper distinction between LPS_{II} and LPS_{III} than the total carbohydrate data. Thus, the A and C type polysaccharides are very markedly concentrated in LPS_{II} while LPS_{III} is characterized by the predominance of PS_{III}-E.

Characterization of the Lipid A Fractions from LPS_{II} and LPS_{III}. Lipid A was isolated as the chloroform–methanol-(2:1, v/v) soluble, ether-insoluble material from the precipitates formed during the acetic acid hydrolysis of LPS_{II} and LPS_{III}. Table VI shows the analytical characterization of these fractions. The lipid A is 6% of the weight of LPS_{II} and 15% of LPS_{III}. This is consistent with the observations that, on a weight basis, LPS_{II} is richer in total carbohydrate (Table I) and that LPS_{III} is composed largely of a low molecular weight carbohydrate fraction. Thus, the conclusion that LPS_{II} is a relatively smooth LPS while LPS_{III} is relatively rough is reflected in its content of both carbohydrate and lipid A.

The fatty acid content of the two lipid A fractions is essentially identical when compared on the basis of percentage of total fatty acids represented by each individual fatty acid. The major fatty acids present are lauric, myristic, β-hydroxymyristic, palmitic, a C-17 cyclopropane acid, and an unidentified acid which plots as a C-15 monounsaturated acid (but which may be a C-15 cyclopropane acid). The total number of moles of fatty acids is found to be equivalent to the number

TABLE V: Amount of Each Type of PS Obtainable from LPS_{II} and LPS_{III}.

Type of PS	Hydrolysis Time (min)	% of CHO in Crude LPS[a]			μmoles/100 mg of Crude LPS[b]	
		LPS_{II}	LPS_{III}	Total	In LPS_{II}	In LPS_{III}
A	180	15.8	4.3	20.1		
	360	17.0	4.9	21.9	0.54	0.10
C	60				0.58	
	180	10.3	3.5	13.8		
	360	5.2	3.8	9.0	0.20	0.09
D	30				0.08	0.02
	180	1.5	1.1	2.6		
	360	2.0	1.3	3.3	0.03	0.02
E	180	0	4.4	4.4	0	0.57
	360	0	2.5	2.5	0	0.33

[a] These values are the product of the per cent of the carbohydrate (phenol–sulfuric acid) from the crude LPS recovered in the LPS fraction (Table I) and the per cent of carbohydrate from the LPS fraction recovered in the PS fraction (Figure 2). [b] The values are approximations calculated as follows: the total μmoles of all monosaccharides recovered from 100 mg of crude LPS is 64 for LPS_{II} and 24.8 for LPS_{III} (see Table I). At different intervals in the hydrolysis, these monosaccharides are distributed in the various PS fractions in the proportions shown in Figure 2. Thus, an approximate value for the total monosaccharides in any given PS is given by the product of the total monosaccharides in the LPS fraction and the fraction of the LPS fraction recovered in the PS. Division of this value by the DP of the PS fraction (Tables III and IV) gives the μmoles of the PS/100 mg of crude LPS. Data are given for 360 min and for the time when the PS is at its maximum level.

TABLE VI: Composition of Lipid A Fractions from LPS_{II} and LPS_{III}.

Analysis[a]	Lipid A_{II}	Lipid A_{III}
Weight (mg)	6.1	14.7
Fatty acids (μmoles)[b]		
12:0	0.48 (7.9)	1.87 (11.3)
12:1	0.04 (0.7)	0.19 (1.2)
14:0	1.73 (28.4)	4.94 (30.0)
14:1	0.07 (1.2)	0.00
β-HO-14:0[c]	0.53 (8.7)	1.53 (9.3)
15:1[d]	1.24 (20.4)	3.22 (19.6)
16:0	1.54 (25.4)	4.01 (24.4)
17:cyclopropane	0.33 (5.4)	0.68 (4.1)
18:0	Trace	Trace
18:1	0.12 (2.0)	Trace
Total	6.08	16.44
Glucosamine (μmoles)	6.13	13.81
Ester phosphate (μmoles)	5.65	13.92

[a] All values are per 100 mg of the LPS fraction in question. [b] Values in parentheses give the per cent of the total moles of fatty acid represented by each fatty acid. [c] The β-hydroxymyristic acid is found exclusively in amide linkage to the nitrogen of glucosamine. See Figure 5 and text. [d] Plots as a C_{15} monounsaturated acid, but may be a C_{15} cyclopropane acid.

of moles of glucosamine, which in turn is equal to the moles of ester phosphate. The β-hydroxymyristic acid was recovered almost quantitatively in the *N*-acyl fatty acid fraction and is identified as the only fatty acid present in amide linkage to the glucosamine nitrogen. Thus, it is found in the *A. aerogenes* lipid A in the same combination as noted in lipid A preparations from *Salmonella* (Fromme and O. Lüderitz, unpublished results quoted in Lüderitz *et al.*, 1968) and *E. coli* (Heath *et al.*, 1966) strains. Its identity is established by its position of migration in vapor-phase chromatography, its infrared spectrum, and its mass spectrum shown in Figure 4. The latter, which was kindly run for us by Professor L. Glaser, shows a molecular ion peak at *m/e* 258 and a peak at *m/e* 103 which is the characteristic ($HOCH\text{-}CH_2COOCH_3$) fragment obtained from β-hydroxy fatty acid methyl esters (Ryhage and Stenphages, 1960). The data show that only 10% of the glucosamine residues are substituted with β-hydroxylmyristyl residues. Whether the other amino groups are substituted with acetyl or other volatile acyl residues was not determined.

Discussion

The lipopolysaccharide fraction from *A. aerogenes* is a complex mixture of components containing covalently linked lipid and carbohydrate constituents. In this work, the LPS has been completely separated from capsular polysaccharide and RNA and resolved into two distinct fractions containing more than 80% of those bound monosaccharide components which characterize lipopolysaccharides from enteric organisms. The relatively high carbohydrate and low lipid A content of LPS_{II} when compared to LPS_{III} suggests, by analogy with the *Salmonella* system, that LPS_{II} is a "smooth" LPS while LPS_{III} is a "rough" LPS. Consistent with this interpretation is the observation that LPS_{II} on hydrolysis yields primarily high molecular weight (DP >50) polysaccharides and no significant amounts of oligosaccharide fragments, while the major carbohydrate component of LPS_{III} is a low molecular weight polysaccharide. The high content of polymeric galactose in all PS fragments from LPS_{II} indicates that the galactan characterized previously (Koeltzow *et al.*, 1968) is analogous to the O-antigenic side chains found

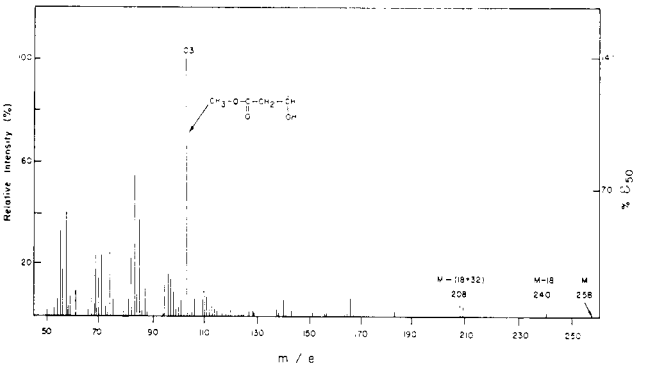

FIGURE 4: The mass spectrum of methyl β-hydroxymyristate.

TABLE VII: Relative Amounts of the Components in Lipid A and Polysaccharide Fractions of LPS.

Component[a]	LPS_{II}	LPS_{III}
Lipid A fraction[b]		
Glucosamine	6.1 (1.0)	13.8 (1.0)
Ester phosphate	5.7 (0.9)	13.9 (1.0)
β-Hydroxymyristate	0.5 (0.1)	1.5 (0.1)
O-Acyl fatty acids	5.6 (0.9)	14.9 (1.1)
Polysaccharide fraction[c]		
Glucosamine	14.0 (2.3)	25.8 (1.9)
KDO	3.0 (0.5)	7.6 (0.6)
Heptose	8.0 (1.3)	26.0 (1.9)
Glucose	18.8 (3.1)	49.3 (3.6)
Labile phosphate	4.7 (0.8)	14.4 (1.0)
Ester phosphate	3.6 (0.6)	7.9 (0.6)
Galactose	285.2 (46.8)	114.7 (8.3)
PS-A	3.3 (0.5)	1.3 (0.1)
PS-C	4.0 (0.7)	1.3 (0.1)
PS-D	0.5 (0.1)	0.2 (0.02)
PS-E	0.0 (0.0)	7.9 (0.6)

[a] All values are in μmoles/100 mg of LPS fraction. Numbers in parentheses are molar ratios based on lipid A glucosamine as 1.0. [b] The lipid A fraction is the $CHCl_3$–MeOH- (2:1, v/v) soluble material which precipitates during acetic acid hydrolysis of LPS. [c] Analyses are for the total PS after precipitation of lipid A.

in *Salmonella* smooth lipopolysaccharides (Lüderitz *et al.*, 1966).

The lipid A fractions obtained from LPS_{II} and LPS_{III} are identical in all respects and contain equimolar amounts of glucosamine, ester phosphate, and total fatty acids. Since monomeric glucosamine was not observed after complete removal of fatty acids, the glucosamine must exist in lipid A in an oligomeric or polymeric form just as found in lipid A fractions from *E. coli* (Burton and Carter, 1964) and *Salmonella minnesota* (Gmeiner *et al.*, 1969). The glucosaminyl linkages are quite stable and only 20% of the glucosamine is released as free glucosamine by hydrolysis in 1 N sulfuric acid at 100° for 6 hr. Treatment of such hydrolysates with alkaline phosphatase yields an additional 10% of the residues as free glucosamine, indicating that some (and perhaps all) of the ester phosphate residues are linked to glucosamine. Cleavage of *all* of the glucosaminidic linkages requires nitrous acid treatment of the acid hydrolysate. This treatment converts all of the glucosamine to "chitose" (Shively and Conrad, 1970)

Since the lipid A represents the "anchor" for the PS fractions, and since it is identical in LPS_{II} and LPS_{III}, it is of interest to compare the molar ratios of the monosaccharides in each total polysaccharide fraction with that of glucosamine in lipid A to see if LPS_{II} and LPS_{III} have any common ratios relative to lipid A. A comparison of the *total* PS fraction to the total lipid A seems more appropriate than comparison of ratios of separated PS fractions to lipid A since it is possible that some of the PS fractions are fragments resulting from hydrolysis of labile glycosidic bonds in others. This treatment of the data is shown in Table VII. As indicated in Results, both LPS fractions contain identical amounts of labile phosphate and lipid A ester phosphate. In addition, it is seen in Table VII that there are 2 moles of PS glucosamine and 0.5 mole of KDO/mole of lipid A glucosamine, suggesting that these ratios are a basic feature of the LPS complex in *A. aerogenes*. The heptose and glucose ratios to lipid A glucosamine are similar but the differences noted seem to be significant since they have shown up consistently in analyses throughout this work. Certainly, these ratios of core-type monosaccharides are much less variable than the galactose ratios. The ester phosphate ratios in the two PS fractions are also identical and therefore presumably represent an additional basic feature of the LPS structure. The nature of this phosphate has not yet been determined, but it is not found in the major PS components and it may, in fact, be a part of a low molecular weight, noncarbohydrate (or low carbohydrate) component since the previous work (Koeltzow *et al.*, 1968) showed that more than 75% of the PS phosphate is lost on dialysis. It is also seen that, per mole of lipid A glucosamine, there are a total of 1.3 moles of PS in LPS_{II} but only 0.8 mole of PS in LPS_{III}. Thus, the lipid A in LPS_{II} appears to be more highly substituted with PS than that in LPS_{III}. An alternate interpretation of this observation, namely, that one or more of the polysaccharides in LPS_{II} is not attached to lipid A, is suggested below.

From the data obtained here, one can only speculate about how the several polysaccharides produced during acetic acid hydrolysis may be structurally and biologically related to each other. The formulated concepts of the structure and biosynthesis of the *Salmonella* LPS, however, may be used as a model which leads to several interesting assignments of structure and biological role for the carbohydrate fragments observed here. PS_{III}-E shows strong similarities to the complete *Salmonella* core PS. It apparently cannot be a hydrolytic fragment produced by cleavage of a labile glycosidic bond in a completed LPS structure because it is obtained only from LPS_{III} while those structures which more nearly resemble the complete LPS (*i.e.*, core plus O-antigenic side chain) are concentrated in LPS_{II}. Therefore, it seems reasonable to suggest that LPS_{III} is primarily a metabolic intermediate in the biosynthesis of the complete *Aerobacter* LPS; *i.e.*, it is a completed core structure which in the normal biosynthetic sequence serves as acceptor for the galactan side chain in the final step in LPS synthesis. It may be noted that the low molecular weight of PS_{III}-E renders it dialyzable; consequently, if PS fractions are dialyzed before analysis, as has been reported in some of the earlier LPS literature, such fragments would not be observed. PS_{III}-E is the main fragment which accounts for the loss of core monosaccharides on dialysis which we noted in our previous report.

PS-A is unique in that it lacks heptose. The A fractions derived from LPS_{II} and LPS_{III} are essentially identical. They contain glucosamine and are devoid of phosphate and relatively low in glucose. According to the reducing terminal analyses, they are mixtures in which more than 80% of the polysaccharide has galactose at the reducing terminal while the remainder has glucosamine. It is tempting to speculate that the major part of the PS-A fraction is pure galactan which exists in the original LPS, not as a galactan linked to lipid A, but as a biosynthetic intermediate in which the activated galactan is linked through a phosphate or pyrophosphate bridge to a polyisoprenoid carrier lipid as found in *Salmonella* O-antigen biosynthesis (Wright *et al.*, 1967) and in *Micrococcus lysodeikticus* mannan biosynthesis (Scher *et al.*, 1968). Such a carrier lipidwould not have been detected in the procedures used here in recovery and analysis

of lipid A. Accordingly, one might hope to separate LPS_{II} into one fraction which contains only the A polysaccharide and another which contains the C and D polysaccharides. Although the kinetics of hydrolysis of LPS_{II} suggest that PS_{II}-A is formed from PS_{II}-C, this is not reflected in the analyses of these fractions. Cleavage of PS_{II}-C between the galactan and the heptose containing portion should yield a fragment which has a very high ratio of heptose to glucosamine. No such fragment has been observed here. Further work is required to clarify this point.

PS_{II}-C and PS_{II}-D (and their counterparts from LPS_{III}) appear to be complete polysaccharides containing both galactan and core and differing only in DP. PS_{II}-D appears to be a complete dimer of PS_{II}-C (as opposed to a polymer containing the same amount of core but twice as much galactan). It is interesting to note that if the galactan of PS_{II}-A or PS_{III}-A were transferred to the PS_{III}-E core, as proposed above, the resulting polysaccharides would approximate PS_{II}-D or PS_{III}-D both in molar ratios of monosaccharides and in DP. Whether these postulated relationships among the PS fractions are real is, of course, not known, but the suggestion that LPS is a mixture of biosynthetic intermediates and final product serves as a working hypothesis for continuation of this work.

The final question which is raised here concerning the structure of the *Aerobacter* LPS is that of the linkages between the PS fractions and lipid A. Clearly, if LPS is the type of mixture suggested, more than one type of linkage of PS to lipid may be found and the lipid fraction which precipitates when LPS is hydrolyzed may be a mixture of carrier lipid and lipid A. The striking observation here is that hydrolysis of both LPS fractions yields free phosphate in an amount equal to the ester phosphate which precipitates with the lipid but in significant excess over the amount of ester phosphate in the PS fractions. This 1:1 ratio of labile phosphate and lipid A ester phosphate suggests the possibility that in the intact LPS these two phosphate residues occur as a pyrophosphate moiety which may be a basic feature of the LPS structure. If the phosphate data are considered in terms of the pyrophosphate hypothesis, two interpretations are possible: (1) there is a glycosyl pyrophosphoryl linkage from some of the PS fractions to the lipid fraction, or (2) there is a pyrophosphate residue linked to the lipid fraction. Other possible forms of bound phosphate appear to be ruled out. For example, a pyrophosphate linked to the PS would yield one labile phosphate but would leave an equal amount of ester phosphate in the PS fraction; the same would be true if several of the polysaccharide chains were linked together by glycosyl pyrophosphoryl linkages; ethanolamine in pyrophosphate linkage to the PS, as found in *Salmonella typhimurium* LPS (M. J. Osborn and Slomka, unpublished results quoted in Osborn, 1969), would not yield a labile phosphate; a glycosyl phosphate at the reducing end of a polysaccharide would yield a labile phosphate provided the phosphate was not linked also to the lipid, but this type of structure seems to be ruled out by the failure of any of the carbohydrate from the LPS to elute from DEAE-cellulose columns unless detergent is present. Thus, when these considerations are taken together with the rate constant data in Table II, it is a reasonable, but not a necessary, conclusion that there is a glycosyl pyrophosphoryl linkage between the PS and the lipid fractions. This could not be accounted for if such a linkage existed solely in the PS-A fraction because in LPS_{III} the molar amount of labile phosphate is much greater than that of PS_{III}-A.

The above hypothesis is complicated by the fact that, except for PS-A, all PS fractions appear to have KDO as their reducing terminals. This KDO is TBA insensitive. A PS → KDO → pyrophosphate → lipid A structure presumably would be much more labile to acid than those observed in LPS_{II} and LPS_{III}. The TBA-positive KDO in the *Aerobacter* LPS seems to occur as nonreducing residues linked either to the polysaccharide or to lipid A. This tentative conclusion is based on the fact that in short term hydrolysis, prior to complete release of all free KDO, no oligosaccharides of KDO are detected on paper chromatograms either by spraying with TBA reagent or by the sensitive radiochromatographic methods used here.

Finally, there are two features of the physical behavior of these materials that seem quite anomalous to us. First, the DEAE-cellulose chromatographic separation of the PS fractions initially was presumed to depend upon the ion-exchange interactions between the polymers being separated and the column material. However, analysis of the eluted polysaccharides shows that they are devoid of all anionic groups except for one KDO residue for 50–100 neutral monosaccharides. This single negative charge would hardly seem to serve as an explanation for the absolute and reproducible separations observed in Figure 2 where, *e.g.*, PS_{II}-C has a DP:KDO ratio of 54:1 and PS_{II}-D a ratio of 132:1. Furthermore, on these columns, fractions D and E are eluted at approximately 0.1 and 0.2 M Tris-HCl (pH 8.3), respectively. In the earlier study, quantitative recovery of all the PS carbohydrate from a DEAE-cellulose column was obtained by eluting only with 0.05 M potassium phosphate buffer (pH 7.0). We have also found that in a gradient of pyridinium acetate (pH 5.3) all of the carbohydrate of PS_{III} is completely eluted when the pyridinium acetate concentration reaches 0.075 M. Since in all of these buffers the KDO carboxyl would be completely ionized, it is difficult to explain why an ion-exchange process would account for these differences in ionic strengths at which the PS's are eluted. It is our tentative conclusion, therefore, that the chromatographic separations are more reasonably accounted for in terms of different degrees of adsorption of the PS's to DEAE-cellulose than in terms of differences in their ionic properties.

The second type of anomalous behavior is the apparent noncovalent binding of KDO to these PS fractions. Throughout this work it has been found that varying amounts of TBA-reactive KDO appear in all of the polysaccharide fractions; yet, when the combined peak fractions are concentrated and deionized on Sephadex G-25 columns, the TBA-reactive KDO is recovered with the salt peak and is found to migrate on paper chromatograms as free KDO. As stated above, the reducing terminal KDO in the polymeric carbohydrate is not TBA reactive. Thus, in our experience, what appears in the initial chromatography to be bound TBA-reactive KDO is, in fact, free KDO which cochromatographs with each of the PS fractions. The amount of free KDO recovered in the PS fractions varies with the nature of the eluting buffer.

Acknowledgments

The authors express their thanks to Dr. Luis Glaser for the mass spectrographic analysis reported here and to Drs. George Schroepfer and Alemka Kisic for much helpful advice in the analyses of lipid A fractions. The excellent technical assistance of Mr. Charles Slife and Mr. Jerry Henderson is also acknowledged.

References

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.
Burton, A. J., and Carter, H. E. (1964), *Biochemistry 3*, 411.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.
Gahan, L. C., Sandford, P. A., and Conrad, H. E. (1967), *Biochemistry 6*, 2755.
Gmeiner, J., Lüderitz, O., and Westphal, O. (1969), *Eur. J. Biochem. 7*, 370.
Heath, E. C., Mayer, R. M., Edstrom, R. D., and Beaudreau, C. A. (1966), *Ann. N. Y. Acad. Sci. 133*, 315.
Hersberger, C., David, M., and Binkley, S. B. (1968), *J. Biol. Chem. 243*, 1585.
Huber, C. N., Scobell, H. D., Tai, H., and Fisher, E. E. (1968), *Anal. Chem. 40*, 207.
Koeltzow, D. E., Epley, J. D., and Conrad, H. E. (1968), *Biochemistry 7*, 2920.
Leloir, L. F., and Cardini, C. E. (1957), *Methods Enzymol. 3*, 840.
Lüderitz, O., Jann, K., and Wheat, R. (1968), *Comp. Biochem. 26A*, 105.
Lüderitz, O., Staub, A. M., and Westphal, O. (1966), *Bacteriol. Rev. 30*, 192.
Nikaido, H. (1969), *Advan. Enzymol. 31*, 77.
Osborn, M. J. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 499.
Osborn, M. J. (1969), *Annu. Rev. Biochem. 38*, 501.
Ryhage, R., and Stenphages, E. (1960), *Ark. Kemi 15*, 551.
Scher, M., Lennarz, W. J., and Sweeley, C. C. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 1313.
Shin, J. M. (1962), *Anal. Chem. 34*, 1164.
Shively, J. E., and Conrad, H. E. (1970), *Biochemistry 9*, 33.
Wright, A., Dankert, M., Fennessey, P., and Robbins, P. W. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1798.

Amino Acid Sequence around 3-Methylhistidine in Rabbit Skeletal Muscle Actin*

Marshall Elzinga

ABSTRACT: Actin contains 1 mole of the unusual amino acid 3-methylhistidine [2-amino-3-(1-methyl-4-imidazolyl)propanoic acid] per mole of protein. All of the 3-methylhistidine is found in only 1 of the 17 cyanogen bromide peptides of actin; this peptide contains a total of 35 amino acid residues, and the determination of its primary structure is reported in this paper. The sequence is: Gly-Gln-Lys-Asp-Ser-Tyr-Val-Gly-Asp-Glu-Ala-Gln-Ser-Lys-Arg-Gly-Ile-Leu-Thr-Leu-Lys-Tyr-Pro-Ile-Glu-3-methylhistidine-Trp-Gly-Ile-Ile-Thr-Asn-Asp-Asp-Hse.

The unusual amino acid, 2-amino-3-(1-methyl-4-imidazolyl)propanoic acid (3-methylhistidine), has been identified as a natural constituent of the myofibrillar proteins actin and myosin (Asatoor and Armstrong, 1967; Johnson *et al.*, 1967). There is one residue of 3-methylhistidine in the single polypeptide chain of rabbit muscle actin while in myosin, which is composed of two heavy chains and two (or three) light chains, there is an average of one residue of 3-methylhistidine in each of the two heavy subunits in white skeletal muscle myosin.

Studies on peptides from actin have suggested that the 3-methylhistidine is present as a single fully methylated residue; all the 3-methylhistidine in actin is found in only 1 of the 17 peptides that are released when actin is cleaved with cyanogen bromide (Elzinga, 1970; Adelstein and Kuehl, 1970). Previously Johnson *et al.* (1967) found that when the soluble portion of a tryptic digest of *S*-β-carboxymethylactin was chromatographed on a column of Dowex 1, 3-methylhistidine was localized in one of the effluent peaks.

The presence of derivatives of both lysine and histidine has been shown in a variety of proteins besides actin. Among these are histones, which contain ε-*N*-acetyllysine, ε-*N*-monomethyllysine, and ε-*N*-dimethyllysine (Ogawa *et al.*, 1969; DeLange *et al.*, 1969), and possibly 3-methylhistidine (Gershey *et al.*, 1969); myosin, with 3-methylhistidine, ε-*N*-monomethyllysine, and ε-*N*-trimethyllysine (Trayer *et al.*, 1968; Kuehl and Adelstein, 1969; Hardy and Perry, 1969; Huszar and Elzinga, 1969); and wheat germ cytochrome *c*, with ε-*N*-trimethyllysine (DeLange *et al.*, 1970).

In the case of actin and myosin there is evidence that the methylated derivatives of both histidine and lysine are not directly incorporated into the polypeptide chains, but rather the methyl groups are added enzymatically at specific positions, with *S*-adenosylmethionine serving as the methyl donor (Asatoor and Armstrong, 1967; Trayer *et al.*, 1968).

As part of a study of the complete amino acid sequence of rabbit skeletal muscle actin, the sequence of the cyanogen bromide peptide that contains 3-methylhistidine has been determined. It is anticipated that this basic structural informa-

* From the Department of Muscle Research, Boston Biomedical Research Institute and the Department of Neurology, Harvard Medical School, Boston, Massachusetts 02114. *Received August 24, 1970.* This work was carried out while the Department of Muscle Research was a part of the Retina Foundation. The author is an Established Investigator of the American Heart Association, Inc. The work was supported by research grants from the Medical Foundation of Boston, the National Institutes of Health (H-5949 and 1-S01-FR-05527), and the National Science Foundation. A preliminary report of this work was presented at the 3rd International Biophysics Congress, Cambridge, Mass., August 1969.