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# The Lipopolysaccharides of *Aerobacter aerogenes* Strains A3(S1) and NCTC 243*

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ABSTRACT: The lipopolysaccharides from *Aerobacter aerogenes* strains A3(S1) and NCTC 243 on mild hydrolysis each yield a polysaccharide fraction which is a complex mixture of fragments varying both in monosaccharide content and molecular weight but having a relatively constant ratio of glucose to heptose. This ratio is approximately 0.5 for the A3(S1) polysaccharide and 2 for that from NCTC 243. The polysaccharide fractions derived from both lipopolysaccharides contain a branched galactan which has 1→3-linked main chains and 1→4-linked branches; these polymers appear to differ structurally only in their degree of branching.

Isotopic methods are described (1) for quantitation of reducing saccharides present in hydrolysates at concentrations down to 0.1 μmole/ml and (2) for methylation analysis of milligram amounts of polysaccharides of unknown structure.

Cell wall lipopolysaccharides (LPS)[1] from *Enterobacteriaceae* are composed of a polysaccharide (PS) fraction and a lipid fraction (lipid A) which may be readily dissociated by mild acid hydrolysis. The polysaccharide portions consist of polymeric oligosaccharides (O-antigenic side chains) joined to a heptose phosphate backbone through a core oligosaccharide containing the sequence, GlcNAc→Glc→Gal→(Gal→)-Glc→. Gross structural features of these complex polysaccharides have been deduced from biosynthetic, immunochemical, and chemical analyses of *Salmonella* and *Escherichia coli* LPS's in a number of laboratories and are reviewed by Lüderitz *et al.* (1966b).

The LPS's from several strains of *Aerobacter* and *Klebsiella*, further members of the enteric group of organisms, have been analyzed by Sutherland and Wilkinson (1966), who found no uniform pattern of chemotypes similar to that in *Salmonella* species. In each of the organisms examined at least one of the core monosaccharides (glucose, glucosamine, or galactose) was present at a level much too low to account for a complete core structure. The analyses were, in fact, reminiscent of those found in the chemotypes, $R_a$, $R_b$, $R_c$, and $R_d$ of the rough mutants of *Salmonella* (Lüderitz *et al.*, 1965, 1966a,b; Sutherland *et al.*, 1965; Osborn, 1966). In several of the strains, however, there was a fraction of the polysaccharide which might be considered analogous to the *Salmonella* O-antigenic side chain which is found covalently linked to the core and which is deleted from the LPS in strains not able to synthesize the complete core sequence. These authors suggested that the LPS's of *Klebsiella* and *Aerobacter* strains may contain polysaccharides directly linked to the heptose phosphate core.

This manuscript describes our initial examination of this suggestion. The data show that the polysaccharide fractions released from the lipopolysaccharides of *Aerobacter aerogenes* strains A3(S1) and NCTC 243 are mixtures of fragments of varying molecular size and monosaccharide content. The LPS's of both strains are shown to contain galactans having basically

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: LPS, lipopolysaccharide; PS, polysaccharide; KDO, 2-keto-3-deoxyoctonate; TBA, thiobarbituric acid.

identical structures which differ only in their degree of branching. In addition, the LPS's contain distinct minor polysaccharide fractions composed of glucose, heptose, and glucosamine in a ratio which is characteristic for each strain. These fragments appear to be oligosaccharides from an incomplete core.

## Methods

Chromatograms were run on Whatman No. 1 paper in the following solvents: (1) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (2) methyl ethyl ketone, saturated with water, and (3) the reversed-phase ethyl ether–dimethyl sulfoxide solvents described by Wickberg (1958). Reducing sugars were detected with aniline acid phthalate reagent (Partridge, 1949), amino sugars with ninhydrin, and KDO with the thiobarbituric acid spray reagent of Warren (1960).

Total carbohydrate was determined on unhydrolyzed samples by the phenol–sulfuric acid method (Dubois *et al.*, 1956) and expressed in terms of glucose equivalents. Phosphorus was assayed by the method of Bartlett (1959). Heptose and KDO were determined without prior hydrolysis as described by Osborn (1963). D-Glucose and D-galactose were assayed in hydrolyzed samples using the Glucostat and Galactostat reagents (Worthington Biochemical Corp., Freehold, N. J.). For hydrolysis of dried samples, 2% solutions in 1 N sulfuric acid were used; for samples already in solution, such as column fractions, 0.5 ml of sample was mixed with 0.1 ml of 6 N sulfuric acid. Hydrolyses were carried out in sealed, 1-ml ampoules at 100° for 6 hr.

*Radiochromatography.* To supplement and confirm the above assays heptose, total hexose, and glucosamine were determined in hydrolyzed samples using the radiochromatographic method described earlier (Gahan *et al.*, 1967) with the following micromodification to increase sensitivity and reduce reagent cost. In this modification microliter aliquots of mixtures are reduced with [$^3$H]sodium borohydride and spotted directly onto paper strips for chromatography. The reaction vessel is prepared by drawing a piece of 6-mm Pyrex tubing sharply to a capillary tip which is then sealed at the end. It is 1.5 in. in over-all length with the 6-mm diameter at the open end extending 0.5 in. before the constriction to the capillary begins. The reaction mixture collects at the constriction in the tube and mixing is assured by the slow bubbling of the borohydride–bicarbonate mixture (see below) during the reaction period. Owing to the difficulty in obtaining quantitative transfer of such small aliquots from the reaction tube to the chromatography strip, it has been necessary to carry out a double isotope dilution assay wherein a 50-$\mu$l aliquot of the hydrolyzed sample is first mixed with 10 $\mu$l of a solution of high specific activity [$^{14}$C]-glucose (New England Nuclear Corp., 200 mCi/mmole, 50 $\mu$Ci/ml) and 50 $\mu$l of 1 M sodium carbonate. A 5-$\mu$l aliquot of this mixture is then transferred to the reaction vessel and 5 $\mu$l of a 0.5 M solution of [$^3$H]sodium borohydride (New England Nuclear Corp., 140 mCi/mmole) in 0.1 N sodium hydroxide is added. The borohydride solution has a half-life of 30 days at 5° but is completely stable when frozen. All transfers of microliter amounts are made conveniently and accurately with Eppendorf pipets (Brinkmann Instruments). The tube containing the reaction mixture is then corked and heated in a sand bath at 50° for 40 min. Following the reaction excess borohydride is destroyed by addition of 10 $\mu$l of 0.75 N sulfuric acid, the tip of the capillary is broken off, and the reduced sample is transferred through the capillary opening to the starting line of a 1-in. wide strip of Whatman No. 1 paper. Chromatograms are developed in solvent 1 and cut into 0.5-in. segments for counting under optimal dual-label-counting conditions as described previously.

The per cent of the original [$^{14}$C]glucose counts recovered on the chromatogram indicates the fraction of the original sample that was actually spotted on the strip. In a separate experiment total $^{14}$C counts to be expected in the aliquot of [$^{14}$C]glucose transferred to the reaction vessel ($^5/_{11}$ $\mu$l) are determined for the [$^{14}$C]-glucose standard. The percentage of the expected $^{14}$C counts actually found on the analytical chromatogram is then used to convert the $^3$H counts for the reduced saccharides on the chromatogram, corrected for a low level of base-line counts (80–350 cpm, depending upon the specific activity of the [$^3$H]borohydride used), into molar equivalents in the original sample using the relationship $\mu$moles/50 $\mu$l of hydrolysate = 22 $\times$ ($^3$H cpm for unknown/$^{14}$C cpm on chromatogram) $\times$ ($^{14}$C cpm/$^5/_{11}$ $\mu$l)/($^3$H cpm/$\mu$mole of reducing sugar). For determination of the hexitols (reduced mannose, glucose, and galactose) which migrate together on the chromatograms, it is necessary to subtract from the total hexitol counts in the $^3$H channel those counts ($^3$H and $^{14}$C) due to the [$^{14}$C]glucose standard. The correction is made using the ratio of counts in the $^3$H and $^{14}$C counting channels for the reduced [$^{14}$C]glucose standard which is determined in a separate experiment. Individual hexoses must be determined by independent means and their sums checked against the radiochromatography value.

The most important requirement for this modification is that accurately measured aliquots of hydrolysates and [$^{14}$C]glucose standard are intimately mixed. Once the ratio of $^{14}$C standard to unknowns is fixed, quantitative precision in the subsequent transfers is not required since all reducing substances, including the [$^{14}$C]glucose standard, are converted stoichiometrically into the [$^3$H]-glycitols under the reduction conditions (see Conrad *et al.* (1966) and below).

Using the reagents with the specific activity described above, this procedure permits quantitative analysis of molar equivalents present in hydrolysates at concentrations as low 0.1 $\mu$mole/ml to $\pm$5% in microliter aliquots of hydrolysates. The specific activity of the [$^3$H]borohydride used can be varied depending upon the sensitivity desired. Results obtained with a standard mixture of typical components found in bacterial LPS's, reduced with [$^3$H]borohydride having a specific activity of 15 mCi/mmole, are shown in Figure 1. Numbers in parentheses represent per cent recovery based on the weight (per cent of TBA assay value, in the case of KDO) of each component in the aliquot applied to the strip.

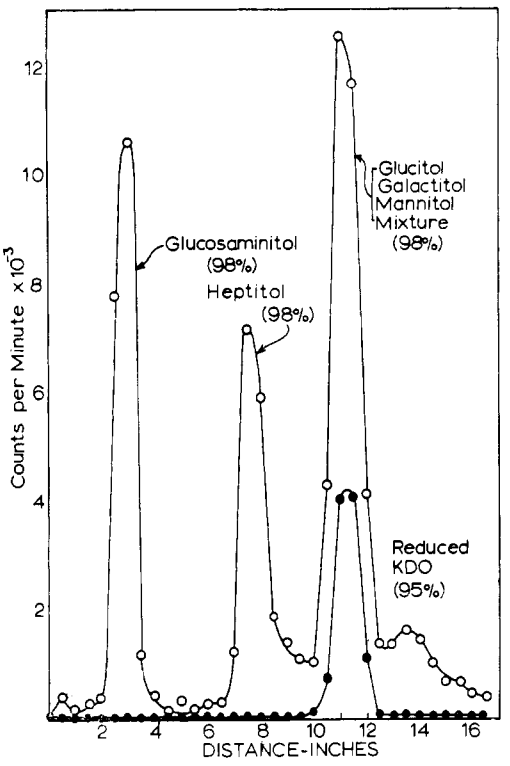


FIGURE 1: Radiochromatographic analysis of a standard mixture of typical LPS monosaccharides. $^3H$, counts per minute (○); $^{14}C$, counts per minute (●). The standard mixture, dissolved in 1 N sulfuric acid, was neutralized, reduced with [$^3H$]sodium borohydride (15 mCi/mmole), chromatographed in solvent 1, and counted as described in Methods. The 5-μl aliquot analyzed here contained 53 nmoles of D-glucosamine, 52 nmoles of D-glycero-D-galactoheptose, 20 nmoles each of D-glucose, D-galactose, and D-mannose, 22 nmoles of KDO, and 0.023 μCi of [$^{14}C$]glucose (210 mCi/mmole). Numbers in parentheses are per cent recoveries in each peak.

There are 20–60 nmoles of each component on the chromatogram shown. For this study samples of several heptoses and of penta-*o*-acetyl–KDO methyl ester were generously supplied by Drs. Nelson Richtmyer and Edward Heath, respectively. The latter sample was converted into KDO by saponification (Ghalambor *et al.*, 1966) and assayed as above. The KDO concentration determined by radiochromatography is essentially identical with that determined by the thiobarbituric acid method using the extinction coefficient given by Osborn (1963). Since reduction of KDO gives two isomers the resulting peak on the chromatogram is rather broad, but can be separated from hexoses in acetic acid chromatography solvents.

Heptoses are reduced quantitatively very early in the reaction period under the conditions employed. Heptose values obtained by radiochromatography were only 35–50% of the values determined by the cysteine–sulfuric acid method using the extinction coefficient of L-glycero-D-mannoheptose given by Osborn, suggesting that the heptose in *A. aerogenes* LPS's may be a different one from that found in *Salmonella*.

The [$^{14}C$]glucose internal standard offers several advantages in addition to its indication of the amount of sample chromatographed. It can be used as an internal chromatographic standard for identification of components of the mixture on the basis of $R_{glucitol}$ values. Also, it can be used to indicate the completion of reduction of the sample with [$^3H$]borohydride. If reduction is incomplete the $^{14}C$ appears in two positions on the chromatogram corresponding to glucose and glucitol. Complete reduction gives only glucitol. The only instances we have observed in which reduction is not complete are those in which the hydrolysates are not completely neutralized prior to addition of [$^3H$]borohydride.

*Preparation of Polysaccharides.* Polysaccharides were isolated from cells of *A. aerogenes* strains A3(S1) (ATCC 12658) and NCTC 243 grown on the glucose–mineral salts medium described by Wilkinson *et al.* (1955). Carboys containing 14 l. of medium were inoculated with 600 ml of an 8-hr shake culture and the cells were grown at 30° with aeration (30 l./min) and vigorous mechanical stirring. Cells were harvested at the top of the logarithmic growth phase in a Sharples supercentrifuge.

Extracellular polysaccharide was measured as follows. The cell paste was homogenized in a Waring Blendor in five volumes of cold 0.01 M potassium phosphate buffer (pH 7.0) for 45 sec to remove residual surface polysaccharide and the wash was added to the spent medium. The volume of the spent medium plus wash was measured and an aliquot was dialyzed overnight against running water and analyzed for total carbohydrate. Capsular and slime polysaccharide was recovered from the dialyzed aliquot by precipitation with ethanol (Sandford and Conrad, 1966).

Glycogen was precipitated from a trichloroacetic acid supernatant of the sonicated cells and purified by passage through a column of DEAE-cellulose as described earlier (Kindt and Conrad, 1967; Strange *et al.*, 1961).

LPS was isolated by phenol extraction of the trichloroacetic acid precipitate. The trichloroacetic acid precipitate was washed twice with water to remove the trichloroacetic acid and extracted with 500 ml of 45% aqueous phenol/100 g of trichloroacetic acid precipitate at 70° for 10 min as described by Westphal *et al.* (1952). The layer recovered from the phenol–water interface was reextracted with water and the combined aqueous phases were dialyzed and concentrated *in vacuo* to 80 ml. The LPS was precipitated from the concentrate with four volumes of acetone, washed with ethanol and ether, and dried *in vacuo* at 60°.

LPS from cells grown in the chemostat under phosphate-limiting conditions (Sandford and Conrad, 1966; Gahan *et al.*, 1967) was prepared by direct extraction of the cell paste with the aqueous phenol and work-up as above.

The polysaccharide (PS) fractions were isolated from acetic acid hydrolysates of LPS. The LPS (500 mg) was suspended in 25 ml of 1% aqueous acetic acid and heated at 100° for 90 min. The precipitated lipid was removed by centrifugation and the supernatant was concentrated to 5 ml and applied to a DEAE-cellulose column (3.5 × 30 cm) prepared in 0.05 M potassium phosphate buffer (pH 7.0). The column was developed

in the same buffer and 5-ml fractions were collected and analyzed.

*Methylation Analysis of the Galactans.* For structural analyses the galactans (major peaks in DEAE-cellulose chromatography of polysaccharides, see Figure 2) were isolated from the phosphorus limited cells of A3(S1) and NCTC 243 described earlier (Sandford and Conrad, 1966; Gahan *et al.*, 1967). Methylation analyses were performed on 25-mg samples of polysaccharide as described for glycogen analysis (Kindt and Conrad, 1967) using relatively high specific activity [$^{14}$C]methyl iodide (44 $\mu$Ci/mmole). These polysaccharides were fully methylated in a single reaction. Ratios of methyl sugars were calculated from $^{14}$C counts on chromatograms of the [$^{14}$C]methyl polysaccharide hydrolysates or from $^{3}$H counts on chromatograms of [$^{3}$H]borohydride-reduced hydrolysates. From the latter chromatograms the number of methoxyls per mole for each methyl sugar, suggested by the $R_F$ in solvent 2, was confirmed by calculations using the $^{14}$C/$^{3}$H ratio, taking the ratio in the trimethylgalactitol (the major component in each hydrolysate) as 3.0.

Identification of the methylgalactoses was complicated by the fact that galactose derivatives substituted in different positions by equal numbers of methoxyl groups have similar physical properties. Consequently, the following modification of the procedure described by Lemieux and Bauer (1953) was used to determine the positions of substitution. Hydrolysates were reduced with [$^{3}$H]sodium borohydride and paper chromatographed on 1-in. wide strips in solvent 2. Positions of migration were determined by scanning for radioactivity using a strip scanner. The separated components were cut out of the chromatogram, a wick and drip tip were sewed on to each segment, and the methyl sugars were eluted with water and collected quantitatively in the first 3–4 drops in the depressions of a Disposo tray (Linbro Chemical Co., Inc.). Eluents were reduced in volume to about 10 $\mu$l in a stream of air and 10 $\mu$l each of 1 N sulfuric acid and of 0.5 M periodic acid was added. After 60 min at room temperature, 10 $\mu$l of 2.5 M ethylene glycol was added to destroy excess periodate. After 10 min the oxidized mixture was transferred to the starting line of a 1-in. wide strip of Whatman No. 1 paper, chromatographed in solvent 2 or 3 at 3°, and cut into 1-in. segments for counting under optimum conditions for dual-label counting.

The $R_F$ values and the $^{14}$C/$^{3}$H ratios of the original methyl sugars and the periodate fragments uniquely characterize each of the methylhexitols. Initial distinction may be made on the basis of substitution at C-2. For derivatives with a free hydroxyl group at C-2 all of the $^{3}$H in C-1 will appear in formaldehyde which is completely volatilized during work-up and chromatography. Complete loss of $^{3}$H therefore identifies those derivatives lacking a methoxyl at C-2. The 2,4,6-tri-*O*-methylhexitol is readily distinguished since it is the only trimethyl derivative which is not oxidized by periodate. The remaining methyl sugars yield as characteristic oxidation products three-, four-, or five-carbon sugars substituted with one, two, or three methoxyl groups. The monomethyltriose and dimethyltetrose derivatives are somewhat volatile and migrate at the solvent front in solvent 2. By running the chromatography at 3° in the reversed-phase system (solvent 3) these compounds are resolved with characteristic $R_F$ values and isotope ratios and are recovered quantitatively.

This approach has been tested with [1-$^{3}$H]glucitol and with the methyl sugars derived from the capsular polysaccharides of A3(S1) and NCTC 243 (Sandford and Conrad, 1966; Gahan *et al.*, 1967). The trimethylhexitols are converted quantitatively into the anticipated products within a few minutes. The dimethylhexitols, however, offer two sites for periodate cleavage and are only slowly oxidized following the initial cleavage. Thus, a dimethylhexitol is oxidized to its most characteristic fragment in only approximately 50% yield. This phenomenon, discussed by Lemieux and Bauer (1953), is not completely understood but cannot be due to slow hydrolysis of formate esters under the acidic oxidation conditions used in this work. Making the oxidation mixture alkaline after destruction of excess periodate did not alter the chromatographic behavior of the fragments. Further study of the reaction is in progress.

With the exception of the incomplete oxidation of the dimethylhexitols, the method has given the anticipated results for each methyl sugar tested. Thus, it appears to be a general method which can be applied using relatively few methyl sugars as standards and which will identify all trimethylhexitols, dimethylpentitols, and C-2-substituted dimethylhexitols even without $^{14}$C-labeled methoxyl groups, provided the ring size of the parent sugar is known.

## Results

The yields and compositions of the polymeric carbohydrates isolated from cultures of A3(S1) and NCTC 243 are compared in Table I. It can be seen that, in terms of yield, capsule is the major polysaccharide formed by both organisms, with one-third to one-fourth as much LPS and quite low levels of glycogen. The 243 strain produces a greater amount of each type of polysaccharide than does strain A3(S1).

Glycogen recoveries are extremely low compared with the levels observed earlier for the same strains harvested after 2 hr in the stationary growth phase (approximately 2000 $\mu$moles of anhydroglucose/100 g of cell paste, see Kindt and Conrad, 1967). The identity of this fraction was checked by testing it as a primer for glycogen synthesis (Kindt and Conrad, 1967). The glycogen fraction from NCTC 243 showed good primer activity. However, the A3(S1) glycogen fraction was devoid of primer activity, even when tested at 200 times the anhydroglucose concentration required to obtain glycogen synthesis. This level was not inhibitory when an active glycogen primer was added to the assay (L. C. Gahan, unpublished data). It is concluded, therefore, that late-log cells of strain A3(S1) contain less glycogen than can be detected by this assay (less than 7 $\mu$g/100 g of cell paste) in spite of the fact that they contain an active glycogen synthetase and are able to store large amounts of glycogen during the stationary growth phase.

TABLE I: Polysaccharide Content of *A. Aerogenes* Strains A3(S1) and NCTC 243.[a,b]

| Strain | Polysaccharide[b,c] | Total CHO | Galactose | Glucose | Total Hexose | Heptose | Glucosamine | KDO | P |
|---|---|---|---|---|---|---|---|---|---|
| A3(S1) | Capsule (5.8 g) | 9,160 | | | | | | | |
| | Glycogen | 15 | | 9 | | | | | |
| | LPS (0.98 g) | 3,420 | 3,300 | 58 | 3,260 | 172 | 163 | 0 | 119 |
| | PS | 3,220 | 3,250 | 53 | 2,790 | 145 | 92 | 36 | 45 |
| | Dialyzed PS[d] | 2,740 | 3,240 | 16 | 2,780 | 65 | 84 | <3 | 5 |
| NCTC 243 | Capsule (8.4 g) | 21,400 | | | | | | | |
| | Glycogen | 86 | | 62 | | | | | |
| | LPS (1.29 g) | 4,300 | 4,040 | 400 | 4,260 | 219 | 298 | 0 | 280 |
| | PS | 4,170 | 3,890 | 331 | 3,900 | 193 | 175 | 54 | 111 |
| | Dialyzed PS[d] | 3,840 | 3,860 | 238 | 3,550 | 167 | 122 | <6 | 27 |

[a] All values are reported in $\mu$moles/100 g of cell paste. [b] Total carbohydrate was determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956) and is expressed as glucose equivalents. Galactose and glucose were determined with the Galactostat and Glucostat reagents (Worthington Biochemical Corp.), respectively. KDO was assayed using the periodate–thiobarbituric acid method (Weissbach and Hurwitz, 1959; Osborn, 1963). Phosphorus was measured by the method of Bartlett (1959). Total hexose, heptose, and glucosamine were determined by radiochromatography as described in Methods. Prior to analyses samples were hydrolyzed for 6 hr in 1 N $H_2SO_4$ at 100°, except for the total carbohydrate and KDO analyses, which were performed on unhydrolyzed samples. [c] Numbers in parentheses are dry weights recovered from 100 g of cell paste. [d] Dialyzed *vs.* 400 volumes of deionized water for 20 hr.

TABLE II: DEAE-cellulose Chromatography of Polysaccharide Fractions of *A. Aerogenes* LPS's.[a]

| Organism | Total CHO | Galactose | Glucose | Total Hexose | Heptose | Glucosamine | KDO |
|---|---|---|---|---|---|---|---|
| A3(S1) | | | | | | | |
| Applied | 1611 | 1660 | 27 | 1425 | 74 | 47 | 18 |
| Recovered | 1077 | 846 | 46 | 1005 | 63 | 27 | 11 |
| NCTC 243 | | | | | | | |
| Applied | 1496 | 1540 | 128 | 1547 | 75 | 68 | 21 |
| Recovered | 1381 | 1273 | 110 | 1509 | 49 | 38 | 17 |

[a] In each case the fraction applied to the column was from 500 mg of LPS. All values are given in total micromoles. For analytical procedures, see footnote *b* of Table I.

The LPS's from both strains are isolated in similar yields. They are not precipitable by $Mg^{2+}$ and contain relatively little RNA as evidenced by levels of ribose too low to detect in hydrolysates. Following acetic acid hydrolysis half of the glucosamine, half of the phosphate, and all of the other monosaccharides are recovered in the soluble polysaccharide fractions. D-Galactose, the major monosaccharide, has been isolated and identified both by melting point and optical rotation and by its reactivity with galactose oxidase. The other typical sugars found in gram-negative LPS's (glucose, heptose, glucosamine, and KDO) make up a relatively small part of the polysaccharide fraction. The identity of these components has been established by paper chromatographic comparison with standards and by characteristic reactions with enzymes and colorimetric reagents. Dialysis of the PS's removes large fractions of the combined glucose, heptose, glucosamine, KDO, and phosphate but none of the galactose, suggesting that the polysaccharide fractions are mixtures of LPS fragments which differ both in composition and molecular weight.

This is confirmed by DEAE-cellulose chromatography of the undialyzed polysaccharides as shown in Figure 2. The elution profiles are quite complex, but there are several significant points. In each case the galactan fraction emerges in the breakthrough volume of the column and dominates the profile as shown in the upper lots, while minor peaks emerge following the galactan. The composition of the minor components in the fractions is shown in the lower plots. The main KDO peak from each column migrates as free KDO

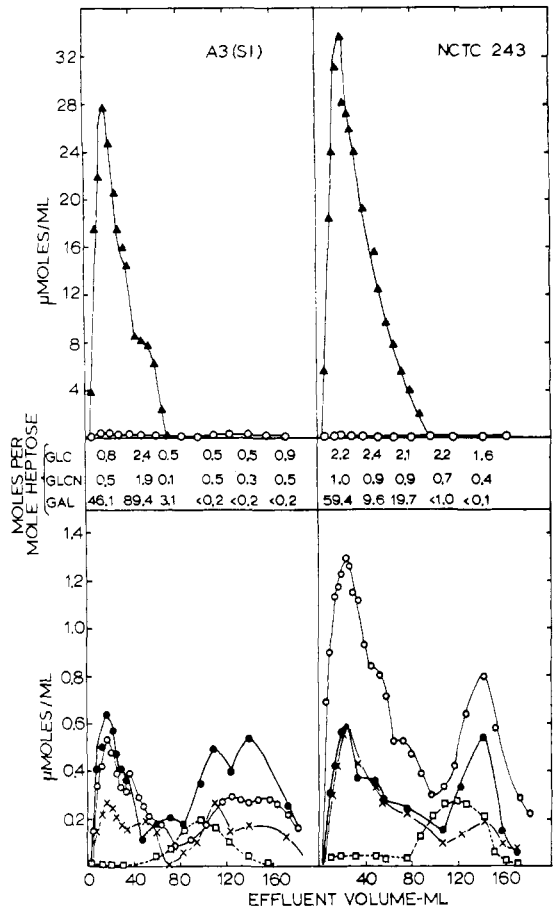


FIGURE 2: DEAE-cellulose chromatography of the PS fractions from the A3(S1) and the NCTC 243 LPS's. The data are plotted on two different scales to emphasize the preponderance of galactose in these fractions. Symbols used are: D-galactose, ▲; D- glucose, ○; heptose, ●; glucosamine, X; and KDO, □. The monosaccharide ratios are for fractions along the elution profiles. The decimal point in each ratio is aligned with the fraction on the profile for which the ratio is given.

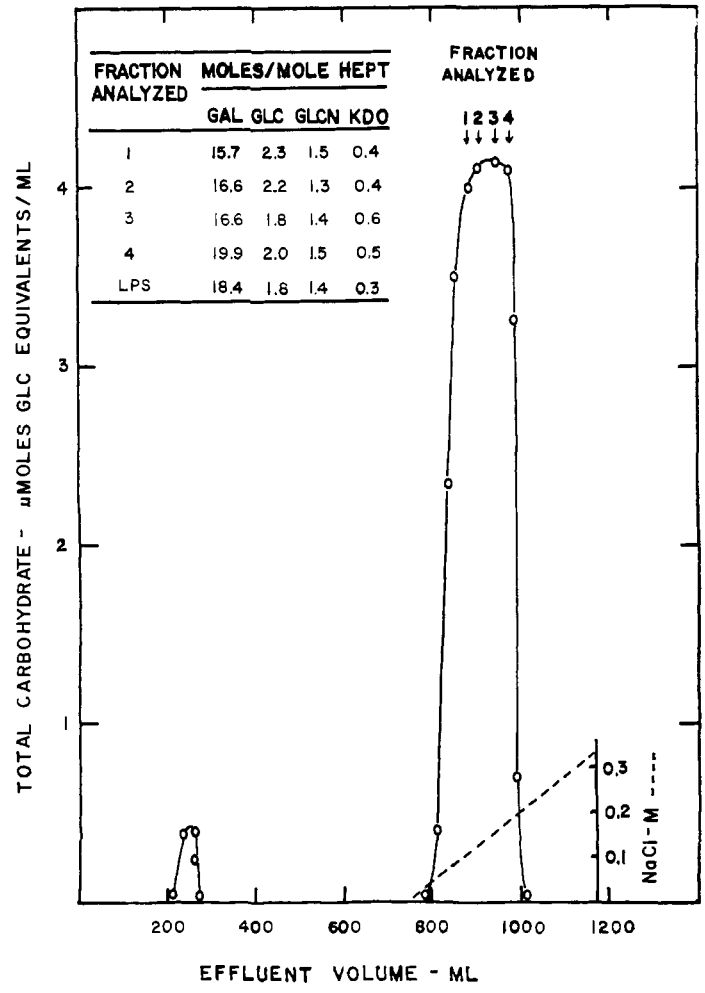


FIGURE 3: DEAE-cellulose chromatography of NCTC 243 LPS. The column (2.8-cm diameter packed to a height of 28 cm) was loaded with 0.23 g of LPS and washed with 700 ml of 0.05 M $KPO_4$ buffer (pH 7.0) containing 0.15% (v/v) Triton X-100, before beginning a linear NaCl gradient with 500 ml each of the 0.05 M $KPO_4$–Triton X-100 buffer and 1 M NaCl in the same buffer. Of the total carbohydrate applied to the column, 3% was recovered in the early peak and 93% in the major peak eluted with the gradient.

on paper chromatograms but there appear to be small amounts of bound KDO associated with most of the fractions. In the NCTC 243 profile the glucose, glucosamine, and heptose molar ratios are quite constant throughout the major peak, while similar ratios of heptose and glucose are recorded for the minor peak, but the glucosamine values drop to about one-half and the galactose values are too low to measure. The A3(S1) polysaccharide presents an even more complex picture with glucose:heptose ratios somewhat variable throughout most of the elution curve. Once again the galactose drops too low for measurement in the minor peak. It is seen that inverse glucose:heptose ratios are found in the NCTC 243 and the A3(S1) polysaccharides.

Similar elution profiles have been observed with the polysaccharide fractions of LPS's isolated from cells of these strains grown in the chemostat under phosphorus-limiting conditions (Sandford and Conrad, 1966; Gahan *et al.*, 1967). The latter LPS's are only 2–3% cell dry weight. They contain amounts of the minor fraction similar to those noted above but levels of galactan are much reduced. The glucose:heptose:glucosamine ratios are identical with those given here and therefore appear to be strain-specific characteristics.

Recoveries of each monosaccharide component from the DEAE-cellulose columns are given in Table II. It was not possible to measure phosphate recoveries since the columns were developed with phosphate buffer. The losses appear mainly in the galactose component. This may be due to failure to obtain quantitative release of the galactan in the acetic acid hydrolysis. The rate of galactan release from LPS with acetic acid has not been determined. When the LPS fractions are chromatographed on DEAE-cellulose columns without prior acetic acid hydrolysis they adhere tightly to the column in the 0.05 M phosphate buffer used in the chromatography experiments in Figure 2 and are only partially recovered upon elution with 1 M sodium chloride or 1 M sodium hydroxide.

The capsular polysaccharides produced by these

TABLE III: Methylation Analysis of A3(S1) and NCTC 243 Galactans.

| Sample | Molar Ratio in Galactan[a] | Characterization: Solvent 2, Before $IO_4$ $R_{Gal}$[b] | Solvent 2, Before $IO_4$ $OCH_3/{}^3H$[c] | Solvent 2, After $IO_4$ $R_{Gal}$[b] | Solvent 2, After $IO_4$ $OCH_3/{}^3H$[c] | Solvent 3, Before $IO_4$ $R_{Gal}$[b] | Solvent 3, Before $IO_4$ $OCH_3/{}^3H$[c] | Solvent 3, After $IO_4$ $R_{Gal}$[b] | Solvent 3, After $IO_4$ $OCH_3/{}^3H$[c] | Positions of $OCH_3$ Substitution |
|---|---|---|---|---|---|---|---|---|---|---|
| A3(S1) | | | | | | | | | | |
| $Me_2$-galactitol | 0.9 | 0.2 | 1.8 | 1.3[d] | 1.2[d] | 0.0 | 2.0 | 1.8[d] | 1.1[d] | 2,6 |
| $Me_3$-galactitol | 3.1 | 0.7 | 3.0 | 0.7 | 3.5 | 0.3 | 3.0 | 0.3 | 2.9 | 2,4,6 |
| $Me_4$-galactitol | 1.0 | 1.0 | 4.2 | 1.0 | 3.7 | 1.0 | 4.0 | 1.0 | 4.1 | 2,3,4,6 |
| NCTC 243 | | | | | | | | | | |
| $Me_2$-galactitol | 1.0 | 0.2 | 2.2 | 1.3[d] | | 0.0 | 2.4 | 1.8[d] | 1.5[d] | 2,6 |
| $Me_3$-galactitol | 23.6 | 0.7 | 3.0 | 0.7 | 3.1 | 0.3 | 3.0 | 0.3 | 3.0 | 2,4,6 |
| $Me_4$-galactitol | 1.0 | 1.0 | 3.9 | 1.0 | 4.1 | 1.0 | 3.8 | 1.1 | 4.1 | 2,3,4,6 |
| Controls | | | | | | | | | | |
| 2,3,6-$Me_3$-D-glucitol | | 0.6 | | 1.4 | | 0.3 | | 3.1 | | |
| 2,6-$Me_2$-D-mannitol | | 0.2 | | 1.3 | | 0.0 | | 1.8 | | |

[a] Based on $^3H$ counting rates in the three components after radiochromatography of hydrolysates of the fully methylated galactans. $Me_4$-galactitol is taken as 1.0. [b] Distance of migration relative to $Me_4$-galactitol. Values are given to only one decimal since compounds were only localized to 1-in. segments of chromatograms. [c] Based on the ratio of $^{14}C$ to $^3H$ counts in each of the components on the chromatograms. Values are normalized to $Me_3$-galactitol in which the $^{14}C/^3H$ ratio is taken as three methoxyls per $^3H$. [d] The $Me_2$-hexitols yield several components on periodate oxidation. The $R_{Gal}$ and $OCH_3/^3H$ values given are for the smallest $^3H$-containing fragment which is characterized as the fragment which migrates the greatest distance in each of the solvents.

organisms (Conrad *et al.*, 1966; Gahan *et al.*, 1967) similarly are bound to the column in 0.05 M phosphate, but are eluted quantitatively midway through a 0–1 M sodium chloride gradient. Acetic acid hydrolysis (1% acetic acid, 100°, 1 hr) of the capsules does not alter this chromatographic behavior. Glycogen emerges from such columns in the hold-up volume (Kindt and Conrad, 1967) and would appear only in the early part of the galactan peak in Figure 2. It is concluded therefore that, in spite of the apparent heterogeneity of the polysaccharides obtained from the LPS's, these materials are distinct from the previously described polysaccharides produced by these organisms.

Direct evidence that the galactan and the minor PS components of the NCTC 243 LPS are all contained in the original LPS fraction of the cell is given in Figure 3, which shows the chromatographic behavior of the LPS when Triton X-100 is a component of the eluting solvent. In this case the LPS adheres tightly to the column during an exhaustive wash with 0.05 M phosphate buffer containing 0.15% (v/v) Triton X-100, but is eluted in a single peak when a linear gradient of sodium chloride in the phosphate–Triton X-100 solvent is passed through the column. Analysis of the monosaccharide components in hydrolysates of peak fractions shows constant molar ratios across the peak which are identical with those found for the original LPS (Table I). Thus, the several PS components of the NCTC 243 LPS are derived from similar, if not identical, LPS molecules.

When the NCTC 243 galactan peak fractions from Figure 2 were combined, hydrolyzed with 1% acetic acid for an additional 90 min, and rechromatographed on DEAE-cellulose, no further amounts of minor peak were found. Thus, the minor peak does not arise by hydrolytic cleavage of the major peak. Hydrofluoric acid hydrolysis (Glaser and Burger, 1964) of the minor fraction had no effect on its chromatographic behavior, but similar experiments have not yet been performed on either of the galactan fractions. Present indications are that the minor fraction is the low molecular weight, dialyzable material noted above. Gel filtration of the NCTC 343 minor fraction on Bio-Gel P2 yields one main peak having an approximate molecular weight of 1500 and a heptose:glucose:glucosamine ratio of 1.0:1.4:0.5, in close agreement with the ratio obtained for the DEAE-cellulose minor fraction.

*Structure of the Galactans.* The methylation analyses were performed on the major DEAE-cellulose peaks similar to those described above, but isolated from the phosphorus-limited chemostat cells described earlier (Sandford and Conrad, 1966; Gahan *et al.*, 1967). In both the A3(S1) and NCTC 243 galactan fractions, galactose made up more than 90% of the total weight. Results in Table III show that the galactans from both organisms yield 2,6-di-*O*-methyl-D-galactose, 2,4,6-

tri-*O*-methyl-D-galactose, and 2,3,4,6-tetra-*O*-methyl-D-galactose. However, the molar ratios in which the three methyl sugars are recovered differ markedly, the A3(S1) galactan being highly branched while that from NCTC 243 is almost linear. The positions of methoxyl substitution are established by the number of methoxyls per mole and the positions of paper chromatographic migration of the $^3H$-containing components before and after periodate oxidation of the [$^3H$]borohydride-reduced methylgalactoses. Neither of the tetramethyl- or the trimethylgalactitols were attacked by periodate. Both dimethylgalactitols yield a monomethyltriose chromatographically identical with that formed from the 2,6-di-*O*-methyl-D-mannitol control and distinct from the fragment from 2,3-di-*O*-methyl-D-glucitol. The effect of periodate on the chromatographic behavior of the standards and of the unknown dimethylgalactitol from the A3(S1) galactan is illustrated in Figure 4. The 2,3-di-*O*-methyl-D-glucitol yields several $^3H$-labeled fragments while 2,3,6-tri-*O*-methyl-D-glucitol is quantitatively converted into a single $^3H$-containing fragment identical with the fastest moving component from 2,3-di-*O*-methyl-D-glucitol in both solvent systems. This is presumed to be 2,3-di-*O*-methyl-L-[4-$^3H$]threose. The 2,6-di-*O*-methyl-D-mannitol also gives several fragments, the fastest of which is presumed to be 2-*O*-methyl-L-[3-$^3H$]glyceraldehyde. This compound is difficult to distinguish from 2,3-di-*O*-methyl-L-threose in the methyl ethyl ketone solvent but is well resolved in ether–dimethyl sulfoxide. The dimethylgalactitols from both galactans yield the monomethyl glyceraldehyde obtainable from a 2,6-di-*O*-methylhexitol as judged both by chromatographic behavior and by the $OCH_3/^3H$ ratios. In all cases described here $^3H$ was recovered quantitatively after periodate oxidation when the chromatograms were run at 3°. Since [$^3H$]formaldehyde from C-1, if formed in the oxidation, would be lost under these conditions, it is clear that standards and unknowns all are substituted at C-2. Of the dimethyl sugars used in this study only the dimethylgalactitols had $^{14}C$-labeled methoxyls. From these only 50% of the $^{14}C$ was recovered after the oxidation, indicating that methylglyoxal formed from carbons 5 and 6 is volatilized under the experimental conditions.

These data show that in both galactans the main chains are linked 1→3 while the branching residues are linked through both C-3 and C-4. Consistent with the failure to find 1→6 linkages is our observation that on acid hydrolysis of the LPS, all of the galactose in the polymer can be assayed by the galactostat reagent at an early stage in hydrolysis when very little free galactose is present. The capacity of galactose oxidase to oxidize polymeric galactose having free hydroxymethyl groups at C-6 has been documented (Avigad *et al.*, 1962). The rate of galactose release during hydrolysis is consistent with its existence in the pyranose ring form as assumed above for assignment of the tetramethylgalactose structures.

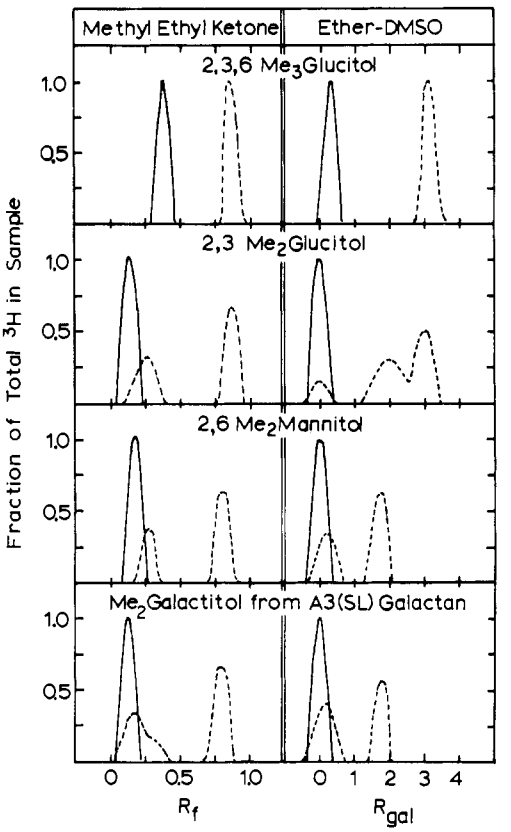


FIGURE 4: Paper chromatographic behavior of [$^3H$]borohydride-reduced methyl sugars before (——) and after (- - - -) periodate oxidation. The oxidations and radiochromatography were performed as described in Methods.

## Discussion

Examination of these results in the light of the general picture that has evolved for *Salmonella* LPS structures (Lüderitz *et al.*, 1966b) indicates several differences in the *Aerobacter* preparations. In the smooth forms of *Salmonella* a characteristic core sequence, GlcNAc →Glc→Gal→(Gal→)→Glc→hept→hept P→KDO→, links the O-antigenic side chain at the nonreducing GlcNAc terminal to lipid A at the reducing KDO terminal. A metabolic deficiency which prevents the specific incorporation of any of the monosaccharides into this core sequence results in the formation of a rough strain. The resulting LPS is characterized by loss from the O-antigen not only of the deficient monosaccharide but of all subsequent monosaccharides distal to the heptose, including the O-antigenic side chain. The latter may appear in cell extracts as a soluble hapten unattached to lipid A or core (Beckmann *et al.*, 1964). The LPS's of such rough mutants are characterized by ratios of heptose, glucose, galactose, and glucosamine which define the point of mutation and which are consistent with the portion of the core sequence which remains.

By analogy the *A. aerogenes* LPS's would appear to be mixtures of rough and smooth O-antigens. In the major fractions one might consider the galactans to be the O-antigenic side chains attached to lipid A through a core which could contain some of the galactose in the fractions. However, this would require a different core sequence than found in *Salmonella* since

in neither *Aerobacter* galactan is the monosaccharide ratio consistent with the *Salmonella* core structure, even if a portion of the galactose is considered to reside in the core sequence. The same nonconformity with *Salmonella* is observed in the minor (rough) fractions, both of which (1) contain glucosamine in the absence of galactose and (2) have unique heptose:glucose ratios. Thus, as concluded by Sutherland and Wilkinson (1966), these LPS's must have core structures different from those found in *Salmonella* strains. These ratios of core monosaccharides remain constant for each of the *A. aerogenes* strains described here under all growth conditions examined to date.

The present data do not rule out the possibility that these LPS preparations are obtained from mixed cultures containing both S and R forms. However, the cells used in this work were grown from single colonies selected at the time of inoculation. It may be noted that Lüderitz *et al.* (1966a) have reported that such mixtures of O-antigenic structures can occur together on the same LPS complex.

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