

Physicochemical Studies on a Lipopolysaccharide from the Cell Wall of *Azotobacter vinelandii**

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SUMMARY

Lipopolysaccharide prepared from the cell wall of *Azotobacter vinelandii* was found to consist of two polydisperse components: $s_{20} = 8$ to 16 S and $s_{20} = 70$ to 100 S. Treatment of this material with ethylenediaminetetraacetate resulted in dissociation into a smaller homogeneous unit with a molecular weight of 134,000 daltons. Subsequent dialysis in the presence of CaCl_2 produced a reassociated polymer with a molecular weight of 873,000 daltons. Dissociation of the lipopolysaccharide was also effected by dodecyl sulfate. The smallest subunit, obtained by the combined action of dodecyl sulfate and ethylenediaminetetraacetate, had a molecular weight of 65,600 daltons when corrected for binding of dodecyl sulfate. Equilibrium and velocity sedimentation and viscosity measurements characterize both the dissociated and reassociated species as relatively homogeneous, highly asymmetric particles. The hydrogen ion titration curve has clearly identifiable segments due to the titration of carboxyl and amino groups. The latter appear to have a lower intrinsic pK than expected from the partially known structure. The lipopolysaccharide was shown to contain phosphate, glucose, and a 2-keto-3-deoxy sugar. Ribose, rhamnose, and hexosamine were identified tentatively.

The lipopolysaccharides of gram-negative bacteria have been studied extensively with respect to their endotoxic, antigenic, and chemical properties (1). Significant heterogeneity and high molecular weights (1 to 24×10^6 daltons) of isolated lipopolysaccharide were revealed in early physical chemical studies (2-5). These results could not distinguish between a continuous molec-

ular network and a subunit structure of lipopolysaccharide. More recently, endotoxin has been dissociated by SDS¹ (6) and sodium deoxycholate (7). In the latter investigation, size and shape of subunits were determined. Another indication of subunit structure is the solubilization of lipopolysaccharide after treatment of gram-negative cells with EDTA (8, 9).

The dissociation of isolated lipopolysaccharide from *Azotobacter vinelandii* by EDTA and by SDS is described in this paper. We have studied some physical properties of the molecular subunits and of the calcium-reassociated products. In addition we present some characterization of the carbohydrate components of the lipopolysaccharide.

EXPERIMENTAL PROCEDURE

Materials and Methods—Sugars were obtained from commercial sources, except for 2-keto-3-deoxyoctonate, which was a gift of Dr. Mary Jane Osborn. SDS was twice crystallized from hot 95% ethanol. Crystalline *Escherichia coli* alkaline phosphatase was a gift of Dr. Michael H. Malamy.

Analytical ultracentrifugation and apparent specific volume determinations were carried out at 20° as previously described (10). The partial specific volume, \bar{v} , was determined from the apparent volume at three concentrations. Molecular weights were calculated from short column equilibrium centrifugation by the method of La Bar and Baldwin (11). Viscosities were measured in a Ubbelohde viscometer. The buoyant density in Cs_2SO_4 was calculated by the method of Ludlum and Warner (12).

Acid hydrolysis of the lipopolysaccharide was carried out in sealed tubes. At the end of the hydrolysis the tubes were immediately cooled to 0° and the solution was neutralized with NaOH. Samples to be used for chromatography were dialyzed against glass-distilled water before hydrolysis in HCl. Hydrochloric acid was removed with AG11A8 resin (Bio-Rad Laboratories).

Inorganic phosphate and total phosphate were determined by the method of Dryer, Tammes, and Routh (13). The total phosphate determination has been used as the basis for relating all analyses to dry weight. The determination of total phosphate in samples of lipopolysaccharide dried over P_2O_5 were in good agreement with additional phosphate analyses performed by Schwarzkopf Microanalytical Laboratory. The method of Berenblum and Chain (14), as modified by Ennor and Stocken (15), was used to determine inorganic phosphate released by alkaline

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¹ The abbreviation used is: SDS, sodium dodecyl sulfate.

phosphatase. Reducing sugar was determined by the method of Nelson (16), with ribose as a standard. The 2-keto-3-deoxy sugar acid was assayed with thiobarbituric acid by the method of Weissbach and Hurwitz (17), as modified by Osborn (18). The cystein- H_2SO_4 reaction for analysis of carbohydrates was carried out according to Dische (19). Glucose was determined enzymatically by the use of Glucostat (Worthington). Hexosamine was determined by the Elson-Morgan method as modified by Boas (20). Hydrolysis in a sealed tube with 1 M HCl was carried out for 16 hours at 100°. The orcinol reaction for pentose was performed according to Dische (21).

The reduction of ribose sugars with sodium borohydride was carried out according to the following procedure. The sugar was incubated at room temperature for 4 hours with a 50-fold excess of NaBH_4 . The reaction was stopped by the addition of HCl until the evolution of hydrogen had ceased. The solution which resulted was evaporated to dryness. Excess borate was removed by repeated additions of methanol and evaporation of trimethyl borate. Salts were removed with Amberlite MB-3.

The spectrum of lipopolysaccharide was determined on a Perkin-Elmer model 237B infrared spectrophotometer. A solution which contained 2 mg of polysaccharide was dialyzed against distilled water, mixed with 180 mg of KBr, lyophilized, and pressed into a pellet.

Paper chromatograms were developed in ethyl acetate-acetic acid-water, 3:1:3, and butanol-pyridine-water, 6:4:3. Thin layer chromatograms, made of silica gel, were developed in butanol-pyridine-water, 6:4:3. The silver nitrate-sodium hydroxide stain for the detection of reducing sugars was used according to Trevelyan, Proctor, and Harrison (22), except that 5% Na_2SO_3 was used to remove excess silver oxide. Ninhydrin spray was used to show the position of amino groups on chromatograms.

Columns of Chelex 100, analytical grade chelating resin, 100 to 200 mesh (Bio-Rad Laboratories) were used for the removal of divalent cations from the lipopolysaccharide. The column was monitored by spot tests on filter paper which were stained with periodate and silver nitrate (23).

The hydrogen ion titration of the lipopolysaccharide was carried out with a Beckman GS pH meter at 20°. The electrodes were enclosed in a Faraday cage of copper mesh. The National Bureau of Standards reagents and pH scale specifications were used for buffer standards. Continuous titrations of lipopolysaccharide and its dialysate (0.1 M KCl) were performed with a 0.5-ml micrometer buret in a nitrogen atmosphere.

Preparation of Lipopolysaccharide—The lipopolysaccharide was originally isolated as a contaminant of RNA preparations. Its presence was recognized by the high amount of orcinol-reactive material in proportion to ultraviolet absorbance. Until the substance was identified as a cell wall lipopolysaccharide, it was extracted from whole cells with cold phenol. Preparations were made from *A. vinelandii* (American Type Collection 9104) grown as described by Newton, Wilson, and Burris (24). The cells were separated from the growth medium by centrifugation and stored frozen. Thawed cells were mixed with an equal volume of 0.05 M sodium phosphate or 0.05 M Tris buffer, pH 7 to 8. After 2½ min of sonic oscillation in a Raytheon model DF101 sonic oscillator, the broken cells were centrifuged for 1 hour at 4,000 × *g*. The precipitate was discarded and the supernatant solution was centrifuged for 1 hour at 20,000 × *g*. The precipitated cell wall fraction was suspended on 0.005 M Tris buffer, pH 8.0. Distilled phenol was added to make a 45% phenol

solution. This mixture was shaken at 68–72° for 45 min and cooled in ice. Centrifugation of this material in the cold allows separation of several phases. The upper aqueous phase was carefully removed with a pipette. Early preparations involved cold phenol extraction of whole cells. However, they did not differ in the subsequent steps of the procedure. All of the physical studies and some of the chemical studies were done on material in which the cell wall fraction was extracted with hot phenol.

The supernatant solution from the aqueous phenol treatment was acidified at room temperature with 0.1 M acetic acid to pH 5.0 and with 0.1 M HCl to pH 2.2. The precipitate, chiefly nucleic acid, was removed from the solution by centrifugation and the solution was neutralized with NaOH. Five volumes of 95% ethanol were added slowly at 0° with stirring and the solution was refrigerated for several hours, or preferably overnight. Centrifugation yielded a clear solution which was discarded and a precipitate which was dissolved in a minimal amount of 0.05 M Tris buffer at pH 8.0. This viscous solution was kept as a stock in the freezer. Aliquots were subsequently diluted with 0.05 M Tris buffer and incubated with 1 µg of RNase per ml at 37° for 1 hour with shaking. This preparation was cooled and dialyzed at 4° for a minimum period of 2 days with at least four changes of buffer. Visking tubing was treated with dilute EDTA and rinsed before use. Tris buffers were 0.05 M and had a pH between 8.0 and 8.2. These buffers, after addition of 0.005 M EDTA, 0.001 M SDS, 0.01 M CaCl_2 or no additions, are referred to in the text as Tris-EDTA, Tris-SDS, Tris-Ca, and Tris buffer, respectively.

The amount of nucleic acid which contaminated various preparations was estimated by assuming that the absorbance at 260 mµ was all due to nucleic acid. For this calculation a specific extinction coefficient of 26.5 liters $\text{g}^{-1} \text{cm}^{-1}$ was used. The average amount found was 0.0036 g of nucleic acid per g of lipopolysaccharide.

RESULTS

Identification of Sugars—A sugar which had the same mobility as glucose on paper and thin layer chromatograms was observed in acid hydrolysates of lipopolysaccharide. When this sugar was eluted from a paper chromatogram, it gave the same spectrum as glucose in the cysteine- H_2SO_4 reaction and was a substrate for glucose oxidase.

The presence of a 2-keto-3-deoxy sugar was indicated by the spectrum of the product in the thiobarbituric acid assay (17, 18). It showed a sharp maximum at 549 mµ, as did a known sample of 2-keto-3-deoxyoctonate. Preliminary data indicate a minimum of 1.33 mmoles of sugar per g of lipopolysaccharide.

A positive orcinol reaction was given by all preparations. A sugar which had the same mobility as ribose was observed on paper and thin layer chromatograms of lipopolysaccharide hydrolysates. After elution, this sugar could be reduced with NaBH_4 to give a product with the same electrophoretic mobility in 0.05 M borate buffer, pH 9.2, as similarly treated ribose and untreated ribitol. No attempt was made to determine the ribose quantitatively because of the contribution by other sugars to the orcinol color.

The presence of rhamnose was indicated by paper and thin layer chromatography of hydrolysates. The sugar eluted from paper chromatograms had the same mobility as rhamnose and a

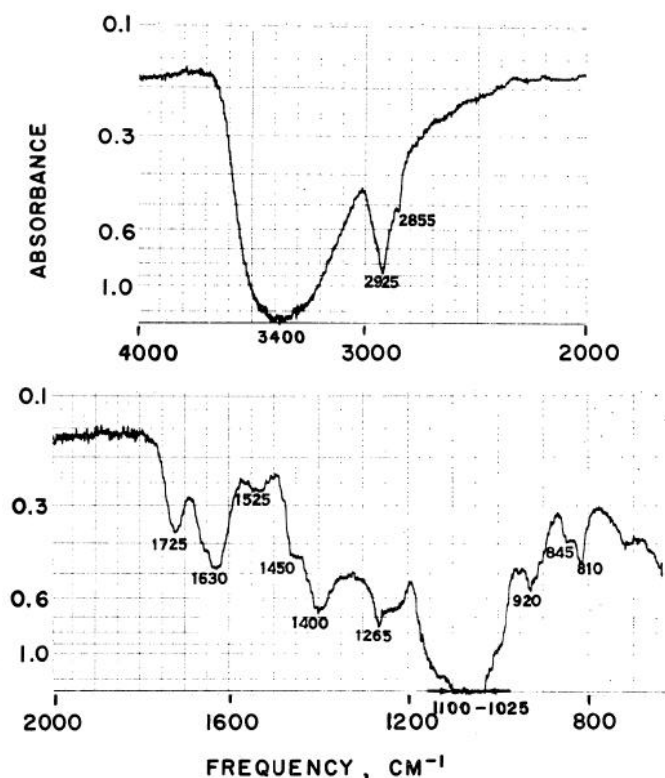


FIG. 1. The infrared spectrum of a sample of lipopolysaccharide in a KBr pellet.

TABLE I
Quantitative elemental analysis

Analysis of lipopolysaccharide for certain constituents. The buffer against which the sample was first dialyzed is shown in the first column (see the text). Analyses were done by the Schwarzkopf Microanalytical Laboratories, Woodside, New York

Buffer	Calcium	Nitrogen	Sulfur	Phosphate	Phosphate
	<i>mmoles/mole P</i>				<i>mmoles/g</i>
Tris-EDTA.....	0.147			1.00	0.661
Tris.....	0.218			1.00	0.651
Tris-EDTA followed by Tris-Ca.....	0.623	0.97	0.070	1.00	0.677
Tris-SDS.....	0.192		0.12	1.00	0.625

spectrum similar to that of known rhamnose in the cysteine- H_2SO_4 reaction.

The Elson-Morgan test for hexosamine, as modified by Boas (20), showed the presence of 0.21 mmole of hexosamine per g of lipopolysaccharide, calculated as glucosamine. It should be noted that the assay based on glucosamine is subject to error if sugar amines with groups in positions other than C-2 or diamino sugars are present (25).

Alkaline Phosphatase—No phosphomonoester could be detected when lipopolysaccharide was treated with alkaline phosphatase. Less than 1% of the total phosphate was released after 90 min of incubation with enzyme. After 24 hours of incubation, a maximum of 3% of the total phosphate was released. A simultaneous control with fructose-1,6-diphosphate as substrate showed release of 63% of the phosphate after 90 min and 97% of the phosphate after 24 hours.

Acid Hydrolysis—Half of the total phosphate was released as orthophosphate by hydrolysis in 0.45 M HCl at 100° in about 1 hour with very little increase after 6 hours. Under the same conditions, a maximum of 12 moles of reducing sugar (as ribose) per mole of total phosphate was released in 6 hours. Thiobarbituric acid-positive material reached its maximum value after 30 min at 100° in 0.025 M HCl. The lipid moiety of the lipopolysaccharide was released as an insoluble precipitate during these hydrolyses.

It is probable that the phosphate that remained esterified after 1 hour at 100° in 0.45 M HCl can be attributed to that present as *O*-phosphorylethanolamine. This substance was isolated by Grollman and Osborn (26) from a lipopolysaccharide obtained from *Salmonella*. It accounted for half of the phosphate and all of the nitrogen of a polysaccharide fraction obtained from the lipopolysaccharide. The rapidly liberated orthophosphate may be in a diester linkage between two sugars similar to that presumed to be present in the backbone of other bacterial lipopolysaccharides (1).

Infrared Spectrum—The infrared spectrum of lipopolysaccharide in a KBr pellet is shown in Fig. 1. The assignment of absorption bands to functional groups is, of necessity, tentative. The broad band of 3500 to 3200 cm^{-1} is probably due to hydroxyl and amine groups. At 1725 cm^{-1} is a band attributable to a carbonyl group due to an uncharged carboxylic acid, an ester, or a lactone. The presence of a carboxylate ion is indicated by the absorption bands at 1630 cm^{-1} and 1400 cm^{-1} . However, another assignment for these two bands may be an amine and a carboxyl group, respectively. The band at 1525 cm^{-1} can be assigned to an amine. The absorption band at 1265 cm^{-1} is probable due to an organic phosphate.

Elemental Analysis—A lyophilized sample of lipopolysaccharide was analyzed for C, H, N, and P by the Schwarzkopf Microanalytical Laboratory. The sample had been dialyzed against Tris buffer followed by glass distilled water. This sample contained 40.87% C, 7.09% H, 1.40% N, and 2.22% P. An emission spectrum revealed the presence of faint traces of aluminum, copper and manganese; trace amounts of silicon, magnesium and iron; and a measurable quantity of calcium. Some analytical data on four samples prepared in different ways are shown in Table I. Each sample was dialyzed against the buffer listed in the table, characterized with respect to its sedimentation coefficient, dialyzed against glass distilled water, and lyophilized. These samples were dried over magnesium perchlorate anhydride at room temperature to constant weight and analyzed. After determination of the phosphorus content on a dry weight basis, the concentration of lipopolysaccharide for chemical and physical measurements was calculated from total phosphate determinations.

Acid-Base Equilibrium—Continuous titration in the acid and the alkaline range was carried out on a sample of lipopolysaccharide dialyzed against Tris-EDTA buffer and then dialyzed against a 0.1 M KCl solution. Effective activity coefficients were determined by titrating the 0.1 M KCl dialysate with the standard acid and the standard base solutions. The activity coefficients for H^+ and OH^- of 0.71 and 0.66, respectively, were obtained. With the use of these values and a molecular weight of 134,000 daltons, the titration of lipopolysaccharide can be represented by the data in Fig. 2.

The theoretical interpretation of the electrostatic effect in the titration of polyelectrolytes is on an unsatisfactory basis. For

an approximate treatment we have used the equation

$$\text{pH} - \log \frac{\alpha}{1 - \alpha} = \text{pK}_{\text{int}} - 0.868wZ$$

with the designation of w as an empirical electrostatic interaction factor which may be expected to be a function of Z (27, 28). Application of this equation has been based on the following considerations. The analytical data and the molecular weight determination indicated 89 phosphate groups and 89 nitrogen atoms per molecule of EDTA-treated lipopolysaccharide. The presence of a minimum of 178 potential carboxyl groups was shown by the amount of 2-keto-3-deoxy sugar detected. The net negative charge on the lipopolysaccharide expected from these figures has been confirmed by paper electrophoresis at pH 3.5, 6.0, and 10.0. It was assumed that the phosphate groups are in diester linkage. For the purpose of calculating overlap with the ionization of carboxyl groups, it was assumed that the pK of the phosphodiester is 1.3, the same as in glycerol phosphate (28). Because of this overlap it is not possible to obtain a direct estimate of the number of carboxyl groups by titration. Values in the range of 178 groups per molecule were tried in further calculations. The calculated curve in Fig. 2 is based on 130 groups per molecule, but this value is highly dependent on the assumptions made about ionization of the primary phosphate groups. The indication is that most or all of the carboxyl groups of the 2-keto-3-deoxy sugar are titratable.

The inflection point in the titration curve near pH 6, together with the plateau at high pH, defines an alkaline segment of the curve that is in agreement with the number of total nitrogen atoms found by analysis. The nature of the nitrogen-containing groups is not clear. Half of them may be due to ethanolamine, as suggested above. The hexosamine determination accounts for about 31% of the total nitrogen, but if these are acetylated they would not contribute to the titration curve. A quantitative discrepancy thus exists, unless the sugar amino groups are assumed to be free rather than acetylated. There is little precedent for nonacetylated amino sugars, although galactosamine in this form has been identified in a polymer from *Aspergillus parasiticus* (29).

Preliminary calculations on the basis of these and other assumptions showed that w must decrease with Z both for the amino and the carboxyl segments of the curve. This is a consequence of the large spread of each segment of the pH axis and cannot be eliminated even by assigning more than one pK value to each kind of group. A similar behavior of w has been found for other polyelectrolytes. It may be ascribed in part to cation binding, which would make the effective Z less than that determined by proton titration (27). Another contributing factor may be extension of a somewhat flexible molecule as Z assumes large negative values. Attempts to fit the curve by assuming that w must be a continuous function of Z in the region of overlap of amino and carboxyl titrations greatly restrict the choice of pK_{int} for the two types of groups. No greater difference than about 3 pH units between them will allow continuity of w . If w is not allowed to assume negative values, the pK_{int} for the carboxyl region cannot be above 2.5. This places the pK_{int} for the amino groups at an unreasonably low value. Amino sugars have been reported to have pK values ranging from 7.3 to 7.8 (30), and the assumption of a lower value than 6.5 does not seem warranted. If the amino groups are those of ethanolamine, a still higher pK of perhaps 9.4 should be assumed. However, a

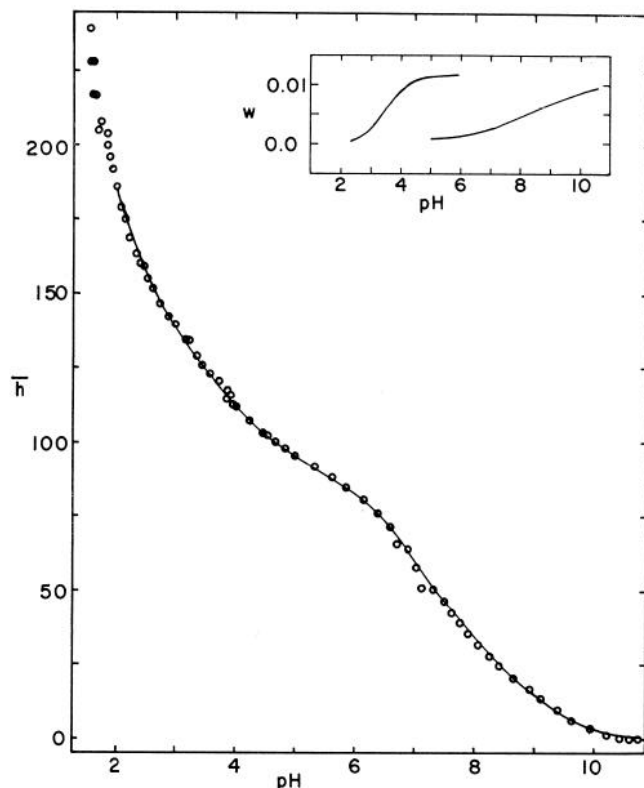


FIG. 2. Hydrogen ion titration of lipopolysaccharide after treatment with EDTA. The points show the number of protons bound per 134,000 daltons, \bar{h} . —, calculated titration curve based on the values of w shown in the inset, pK_{int} = 2.5 for 130 carboxyl groups and pK_{int} = 6.5 for 89 amino groups. A small allowance for the titration of 89 primary phosphate groups, pK_{int} = 1.3, has been made at the acid end of the curve by assuming $w = 0$ for this ionization.

value higher than 6.5 results in a negative w at low values of α for amino titration.

Because of the uncertainty in the number and kind of groups on chemical grounds, a calculated curve is presented in Fig. 2 based on the simplest possible assumptions derived from the curve itself. The pK values and numbers of groups are summarized in the legend and the values of w are shown in the inset. The assumed lack of continuity in w on going from the carboxyl to the amino ionization might result from specific local arrangements of these groups with respect to each other in the structure of the lipopolysaccharide. Both are probably part of the backbone structure as distinguished from the antigenic sidechains and may be so spaced as to produce unusual electrostatic effects in the ionization of all or a portion of the groups. The low pK_{int} for the carboxyl groups could also be a result of such a specific configuration. The possibility of effects of this nature has been considered by Tanford (28) and by Tanford and Kirkwood (31) for the case of globular molecules with a fixed geometry.

Macromolecular Structure—The lipopolysaccharide prepared by the standard method had a variable sedimentation behavior. The pattern was usually similar to that in Fig. 3A with a fast, polydisperse peak followed by a slower, less diffuse boundary. The pattern shifted, on addition of a divalent cation, to one in which the fast component was increased in amount. This is shown in Table II. It is evident that a variable amount of divalent ion was removed in the preparative procedure. Sub-

sequent sedimentation experiments were done after removing most of the divalent ion with EDTA or restoring it to a known concentration by dialysis. The effect of dialysis against Tris-EDTA buffer is shown in Fig 3B. A single, symmetric peak with $s_{20,w}^0 = 5.66$ S was observed. The molecular weight of this material by equilibrium sedimentation (average of four experiments) was 134,000 daltons. Its homogeneity is indicated by an estimate of M_z from the same data of 155,000. The concentration dependence of the sedimentation coefficient and the intrinsic viscosity determination of this and other preparations are shown Figs. 4 and 5, respectively. A summary of the values is given in Table III. Treatment in Tris buffer with Chelex 100 yielded a similar product with an uncorrected sedimentation coefficient of 5 S.

Restoration of the calcium by dialysis against Tris-Ca buffer resulted in a somewhat homogeneous aggregate with $s_{20,w}^0 = 16.7$ S (Fig. 3C). The molecular weight of 873,000 (Table III) indicates an association of 6 or 7 of the subunits obtained in EDTA. When this material was frozen and thawed, a further

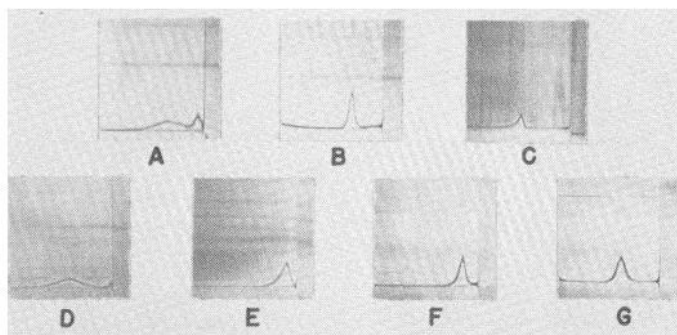


FIG. 3. Sedimentation velocity patterns of lipopolysaccharide. Direction of sedimentation is from right to left. A, Tris buffer with no additions made after ethanol precipitation, 16 min at 29,500 rpm. B, Tris-EDTA buffer, 50 min at 56,100 rpm. C, dissociated in Tris-EDTA buffer and reassociated in Tris-Ca buffer, 46 min at 39,460 rpm. D, treated as in C, then frozen and thawed, 8 min at 39,460 rpm. E, Tris-SDS buffer after one ethanol precipitation, 32 min at 39,460 rpm. F, Tris-SDS buffer after repeated ethanol precipitation, each followed by dialysis against Tris-SDS buffer, 26 min at 56,100 rpm. G, Tris-SDS buffer, sample was previously dialyzed against Tris-EDTA buffer, 86 min at 56,100 rpm.

TABLE II

Sedimentation properties of lipopolysaccharide

Sedimentation velocity in Tris buffer of preparations precipitated under various conditions (see the text). The additions to 5 volumes of 95% ethanol used in the precipitation are given in the first column.

Additions	s_{20}	
	Fast peak	Slow peak
	svedbergs	
None ^a	45	9
0.05 M K acetate.....	39	8 ^b
0.001 M MgCl ₂ ; 0.05 M K acetate.....	52 to 61 ^b	8 to 9
0.001 M CaCl ₂ ; 0.05 M K acetate.....	49 ^b	Very small

^a Pattern shown in Fig. 3A.

^b Peak having the larger area.

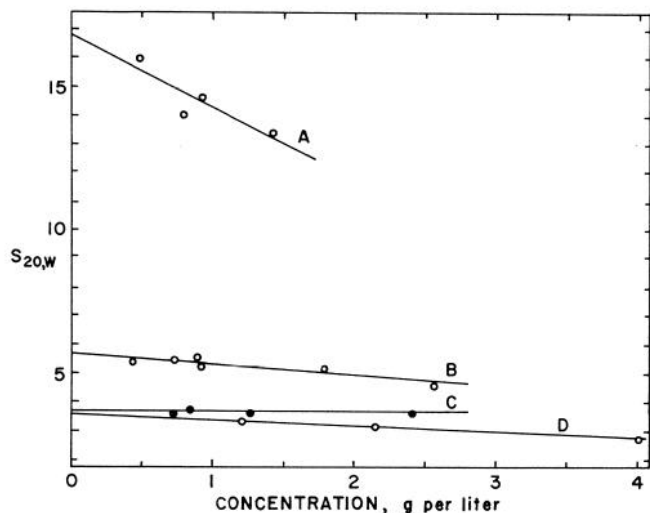


FIG. 4. Concentration dependence of the sedimentation coefficients of lipopolysaccharide. A, Tris-Ca buffer. B, Tris-EDTA buffer. C, Tris-SDS buffer. D, Tris-SDS after dialysis against Tris-EDTA buffer. The straight lines have been fitted to the points by the least squares method.

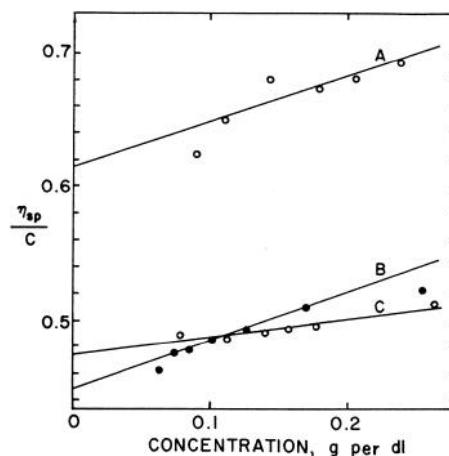


FIG. 5. Determination of the intrinsic viscosity of lipopolysaccharide. A, Tris-SDS buffer. B, Tris-EDTA buffer. C, Tris-Ca buffer. The straight lines have been fitted to the points by the least squares method. Concentration of lipopolysaccharide, C , is given in grams per dl.

increase in sedimentation coefficient to 99 S and a large increase in polydispersity occurred (Fig. 3D).

The lipopolysaccharide was also dissociated in the presence of SDS. Equilibrium was attained only after prolonged dialysis. A more rapidly formed and reproducible subunit was obtained by the combined use of EDTA and SDS by dialyzing against Tris-SDS buffer after dialysis against Tris-EDTA buffer. The preparation which resulted sedimented as a symmetric peak (Fig. 3G) with $s_{20,w}^0 = 3.59$ S.

Regardless of the method of calculation, equilibrium sedimentation yields a value for $M(1-\bar{v}\rho)$ for the sedimenting species. In this case the species includes bound SDS. Correction for this was made by the method of Hersh and Schachman (33). The extent of binding, expressed as grams of dodecyl sulfate per g of lipopolysaccharide, x , was determined from the synthetic boundary cell run of the solution used for equilibrium sedimentation against its equilibrium dialysate. This determination gives

TABLE III
Molecular weight and hydrodynamic properties of lipopolysaccharide

Data and calculations are given for four different kinds of preparations (see the text). In the last two columns, M_c , \bar{v}_c , and the calculated hydrodynamic parameters refer to the complex including bound SDS. The quantity in Line *f* is the ratio of interference fringes due to lipopolysaccharide to total fringes in a centrifuge experiment with the use of a synthetic boundary cell. Lines *i* and *j* were calculated from M and s only, assuming the particle volume $V_0 = M\bar{v}/N_0$. Lines *k* and *l* were calculated from the viscosity by the same assumption. In Line *m*, β , as defined by Scheraga and Mandelkern (32), is given, followed in Lines *n*, *o*, *p*, and *q* by quantities derived from β . No allowance has been made for hydration in any of the calculated values.

Line	Quantity	Dissociated: Tris-EDTA buffer	Reassociated: Tris-Ca buffer	Dissociated: Tris-SDS buffer	Dissociated: Tris-EDTA plus Tris-SDS buffer
a	M (daltons)	134,000	873,000	96,200	65,600
b	M_c (daltons)			162,500	114,900
c	s_{20w}^0 (svedbergs)	5.66	16.7	3.67	3.59
d	$[\eta]$ (dl/g)	0.451	0.475	0.611	
e	\bar{v} (ml/g)	0.667	0.667		
f	$\Delta N_{LPS}/\Delta N_T$			0.63	0.61
g	x (gSDS/gLPS)			0.690	0.752
h	\bar{v}_c			0.756	0.760
i	f/f_0	2.10	2.48	2.59	2.06
j	a/b	22.8	33.9	37.5	21.8
k	ν	67.6	71.2	80.8	
l	a/b	28.3	29.3	31.5	
m	$\beta \times 10^{-6}$	3.01	2.60	2.60	
n	f/t_h	3.14	1.91	1.91	
o	ν	225	32.5	32.5	
p	a/b	57.7	17.9	17.9	
q	V_c/V_0	0.203	2.19	2.49	

a fringe number, f_0 , proportional to refractive index contribution of the complex. The fraction of these fringes due to the lipopolysaccharide was calculated from its known concentration, its specific refractive increment, and the optical constants of the centrifuge. This fraction is proportional to the contribution of the polysaccharide to the refractive index increment, ΔN_{LPS} . From this, x was obtained by the relation

$$x = \frac{\Delta N_{SDS}}{\Delta N_{LPS}} \cdot \frac{k_{LPS}}{k_{SDS}}$$

where the specific refractive increment of lipopolysaccharide, $k_{LPS} = 1.445 \times 10^{-4}$ ml mg^{-1} , was obtained from the fringe count in a separate synthetic boundary run with lipopolysaccharide solution dialyzed against Tris-EDTA buffer. The value of $k_{SDS} = 1.23 \times 10^{-4}$ ml mg^{-1} was obtained by direct measurement with a differential refractometer and is in good agreement with a tentative value quoted by Hersh and Schachman (33). The optical constants of the centrifuge were checked with solutions of known refractive index by a fringe count as well as with a calibration cell furnished by the manufacturer.² With the use of this value of x and $\bar{v}_{SDS} = 0.885$ (34), the molecular weight of the polysaccharide in the complex can be obtained from

$$M_{LPS} = \frac{M(1 - \bar{v}_p)_{\text{exptl}}}{(1 - \bar{v}_{LPS}\rho) + x(1 - \bar{v}_{SDS}\rho)}$$

where the numerator is the experimental value (33). In the calculation, additivity of refractive index and molar volumes are assumed. This point has been discussed by Carusi and Sinsheimer (35) in connection with a similar calculation. Under the conditions of SDS concentration, 0.001 M, and centrifuge

speed 10,589 rpm or lower, the small contribution of detergent micelles to the measured fringe displacements was negligible. This was verified by examination of fringe pattern after centrifugation of 0.001 M SDS at the same concentration in both sectors and against water.

The same calculation was applied to lipopolysaccharide precipitated by ethanol and dialyzed against Tris-SDS. A low sedimentation coefficient was obtained, but the peak was asymmetric (Fig. 3E). In order to obtain a homogenous preparation (Fig. 3F), the ethanol precipitation and the Tris-SDS dialysis had to be repeated several times. If SDS was simply added in Tris buffer, several peaks with intermediate sedimentation coefficients were seen. Therefore, all samples were extensively dialyzed against Tris-SDS buffer. The effect of concentration on sedimentation coefficient and viscosity are shown in Figs. 4 and 5. The data and molecular weight calculations are summarized in Table III.

Removal of the SDS by precipitation with ethanol and dialysis of the precipitate against various buffers resulted in aggregated preparations with sedimentation coefficients in the range of 21 to 74. Reaggregation occurred regardless of whether Ca^{2+} or Mg^{2+} was present in the buffer, presumably because the divalent cation was not removed by the original SDS treatment.

The buoyant density of a sample of lipopolysaccharide which had been dialyzed against Tris buffer was 1.608 g per ml in a Cs_2SO_4 gradient.

DISCUSSION

Lipopolysaccharides similar to that from *A. vinelandii* have been isolated from the cell walls of a number of gram-negative bacteria (1). Interest has focused on the properties of these substances as structural elements of the cell wall, as endotoxins, or as antigens. Characteristic components were identified that

² Spinco Division, Beckman Instruments, Palo Alto, California.

classify the *Azotobacter* lipopolysaccharide as belonging to this group of substances. These include a lipid released by mild acid hydrolysis, glucose, rhamnose, hexosamine, a thiobarbituric acid-positive sugar, and phosphate. There appears to be considerable variation in the relative amount of these and other components in lipopolysaccharides from various sources. The reader should therefore not infer that the lipopolysaccharide which we have studied is closely related in character to those that have been obtained from *Salmonella* and *E. coli*. This cannot be decided until a much more extensive chemical characterization of the preparation from *Azotobacter* is available.

The lipopolysaccharide was isolated as a large heterodisperse polymer that could be dissociated into subunits by treatment with EDTA or SDS. The EDTA subunit reassociated in the presence of Ca^{2+} . The data on these forms, summarized in Table III, indicate an association of 6 or 7 molecules of the EDTA subunit to form the aggregate. Since the more homogeneous subunit has come from a still larger species resulting from the isolation procedure and this, in turn, from some network in the fabric of the cell wall, it may be presumed that the polymerization is sequential and multivalent and that the aggregated species examined here represents a size distribution characteristic of an arbitrary procedure for the reconstitution.

In spite of the larger size of the reassociated particle, its intrinsic viscosity is only slightly greater than that of the subunit. The possible conclusions about the size and shape of the particles are shown by the calculations summarized in Table III. Lines *i* and *j* give f/f_0 calculated in the usual manner from M and s . The value of a/b is that obtained by application of the Perrin equation for a prolate ellipsoid (36). In Lines *k* and *l*, the viscosity increment, ν , and the corresponding a/b for a prolate ellipsoid from Simha's theory (37, 38) are given. It is clear from the values of f/f_0 and $[\eta]$ that the particles are highly asymmetric and that there is not much change in the asymmetry on association. When the data are interpreted by the classical treatment on the assumption that the molecule is a prolate ellipsoid of revolution, an axial ratio of near 30 is obtained for both particles from either s or $[\eta]$. A description of the particles in terms of an equivalent ellipsoid is the only one that can be insisted on. The partially known structure of other lipopolysaccharides suggests an irregular, somewhat rigid particle. Rigidity can also be deduced from the fact that the viscosity does not change much on association and is reflected in the similarity of the axial ratios for the two particles deduced from the two methods of measurement. The dimensions of the equivalent, unhydrated ellipsoid would be about 22×600 Å for the EDTA particle and 39×1200 Å for the reassociated particle. If the equivalent ellipsoid approximates the real particle, this suggests end-to-end as well as lateral aggregation or perhaps more likely some side-by-side overlapping pattern.

The limitations on the conclusions that can be drawn about size and shape from data of this kind have been critically discussed by Yang (39). Some of these problems are shown in a particularly acute manner by our data. The Scheraga and Mandelkern (32) function, β , given in Table III, although characteristically insensitive to variations in shape, is high for both particles. Interpretation of β in terms of an axial ratio implies the derivation of an effective volume, V_e , which together with the axial ratio characterizes the equivalent ellipsoid. This requirement, coupled with the insensitivity of β to shape, produces the results for a/b and V_e/V_0 given in Lines *p* and *q* of Table

III. The difference in a/b in Line *p* from those in Lines *j* and *l* is a result of the implicit requirement that values for a/b and V_e be found that will simultaneously satisfy the Perrin and Simha theories. The difficulty of relating the physical particle to this effective model is evident in the case of V_e/V_0 , which is required to increase 15-fold in the aggregation process even though a comparison is being made of two particles of the same chemical composition. The V_e/V_0 values can also be expressed as the effective specific volume of the equivalent ellipsoid to yield 0.135 ml per g for the EDTA subunit and 2.07 ml per g for the aggregate.

Treatment of the lipopolysaccharide with SDS also resulted in dissociation. The dialysis procedure used did not remove all of the calcium and the product reassociated on removing the SDS. The SDS product was not as homogeneous as that produced by EDTA and the somewhat lower molecular weight (Table III) is probably an average of a heterogeneous distribution. The sequential action of EDTA and SDS produced a subunit of about half the size of the EDTA subunit. Both SDS products were studied in the presence of SDS. It is evident from Table III that the binding of SDS is extensive, and the contribution to the molecular weight of the lipopolysaccharide can be obtained only by correcting for the bound SDS and for the effect of the bound SDS on the partial specific volume. Neglect of these factors raises the apparent molecular weight by about 25%. These corrections were not made in a study of lipopolysaccharides produced by deoxycholate from several gram-negative bacteria (7).

The bound SDS also affects the hydrodynamic parameters, s and $[\eta]$, so that these refer to the complex. Interpretation of the results is uncertain, but some calculations are included in Table III based on s and for the product obtained by Tris-SDS treatment on $[\eta]$ also. The bound detergent slightly increased the asymmetry of the particle, as indicated by the increase in the viscosity and decrease in the sedimentation coefficient.

The effect of EDTA in dissociating the lipopolysaccharide implicates calcium ion in cross-linking between the subunits. Analyses (Table I) showed that most, but not all, of this cation was removed by EDTA, which thus confirms its chelating action under our experimental conditions. More of the calcium remained bound after SDS treatment. In view of the additional dissociation resulting from addition of SDS after dialysis against EDTA, it is probable that the detergent caused dissociation by some other mechanism than EDTA did. We also found that Chelex, a chelating resin, could dissociate the lipopolysaccharide, thereby excluding the necessity that the agent must bind to the lipopolysaccharide to produce dissociation. A high negative charge on lipopolysaccharide subunits was found by titration and electrophoresis. Both phosphate and carboxylate anions, which are responsible for the negative charge, provide possible sites for calcium binding.

The binding of SDS is in marked contrast to its behavior with purely anionic macromolecules such as nucleic acids and may be related to the presence of cationic sites plus adjacent hydrophobic regions, as has been presumed in the case of proteins (40, 41). The hydrophobic binding sites could be located in the fatty acid side chains of the lipid (42). These considerations suggest that a combination of calcium ion binding and hydrophobic interactions is responsible for linking the subunits.

The titration curve reflects certain features of the structural organization of the polymer. Although not much significance

can be attached to a precise fitting of the curve, several qualitative conclusions can be drawn. The alkaline branch of the curve is consistent with titratable amino groups equal to the total nitrogen. A discrepancy exists with the hexosamine determination unless these amino groups are not acetylated. It is not possible to distinguish sets of groups having different pK values within the alkaline branch of the curve. The carboxyl groups can be accounted for by the amount of 2-keto-3-deoxyoctonate detected colorimetrically. No secondary phosphate ionization is evident, in confirmation of the failure of phosphomonoesterase to liberate orthophosphate. A consistent treatment of the electrostatic interaction effect even on an empirical basis was not possible. Complications that cannot be allowed for may result from some flexibility of the molecule, cation binding, positional effects due to location of carboxyl groups close to amino groups, and possibly from further dissociation of the molecule at extreme pH values.

The present demonstration that isolated lipopolysaccharide can be dissociated by EDTA and SDS may explain some of the observations of whole gram-negative bacterial cells treated with the same reagents. In 1956 Repaske (43) showed that gram-negative cells can be made lysozyme sensitive by EDTA treatment. More recently, Leive (8) and Gray and Wilkinson (9) showed that lipopolysaccharide is removed by EDTA. Furthermore, Asbell and Eagon (44) have reported evidence that multivalent cations reassociated the EDTA-removed material (presumably lipopolysaccharide) onto the surface of bacteria. Removal of lipopolysaccharide from *Salmonella* cells, by SDS, has also been shown (45). It seems likely, therefore, that the lipopolysaccharide is sufficiently close to the surface of the cell to permit penetration of EDTA and SDS, dissociation, and release of subunits.

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