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Chemical, Physical, and Biological Properties of a Lipopolysaccharide from *Escherichia coli* K-235*

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ABSTRACT: Studies on a lipopolysaccharide (LPS) from the cells of *Escherichia coli* K-235 were focused upon the relationship of toxicity, pyrogenicity, and antibody neutralization to the state of aggregation, molecular charge, and lipid content. Disaggregation to a unit of 400,000 mol wt was achieved by three different procedures: (1) removal of esterified fatty acids by alkaline hydroxylaminolysis, (2) the introduction of approximately 200 succinyl groups/molecule, and (3) dissolution with an equal weight of sodium dodecyl sulfate (SDS). Succinylation markedly increased the anionic character of LPS. Both succinylation and the removal of lipid gave high yields of completely water-soluble products which showed surprisingly little evidence of heterogeneity. A high degree of molecular asymmetry is indicated by very low $s_{20,w}$ values in relation to light-scattering figures for molecular weights. Disaggregation by SDS did not decrease pyrogenicity in the rabbit. Compared to LPS dissolved with SDS, the succinyl derivative showed no great loss of pyrogenicity and toxicity, but there was a marked loss of ability to neutralize antibody to LPS. The removal of lipid resulted in a very great loss of pyrogenicity and toxicity, but only slight loss of antibody-neutralizing ability.

Lipopolysaccharides[1] from Gram-negative bacteria are usually obtained as aggregates with particle weights of millions when prepared by the phenol–water extraction (Westphal and Jann, 1965; Schramm *et al.*, 1952) which allows a minimum of opportunity for the cleavage of covalent bonds. The high particle weight of these preparations and their low solubility in water have made homogeneity studies difficult (Nowotny *et al.*, 1966; Beer *et al.*, 1966), and thus there is a question as to whether the many interesting biological properties belong to one or several different molecules in the same preparation.

In most investigations in the past a reduction in particle size of LPS preparations has been achieved only by procedures which would break covalent bonds (Neter *et al.*, 1956; Nowotny, 1963; Tripody and Nowotny, 1966; Ribi *et al.*, 1962; Haskins *et al.*, 1961; Johnson and Nowotny, 1964). In these studies one cannot tell whether changes in physical state and biological properties resulted from disaggregation (intermolecular) or degradation (intramolecular). Recently evidence has been presented for the reversible dissociation and inactivation of LPS preparations by the use of SDS (Oroszlan and Mora, 1963) and NaD (Rudbach *et al.*, 1966). While the dissociation with either SDS or NaD indicates the importance of hydrophobic bonding in the aggregation of LPS molecules, there is no suggestion as to the involvement of other types of intermolecular forces. The molecular size has not been defined; usually only sedimentation coefficients have been reported without other data necessary for meaningful calculations, and light scattering has been employed either inadequately or not at all.

Our investigations of the relationships among the physical, chemical, and biological properties of an *Escherichia coli* LPS are presented here, with emphasis on homogeneity, the factors responsible for molecular aggregation, and the relationship of aggregation to toxicity.

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[1] Abbreviations used: LPS, lipopolysaccharide; PS, polysaccharide obtained after removal of lipid from LPS; SDS, sodium dodecyl sulfate; NaD, sodium deoxycholate; KDO, 2-keto-3-deoxyoctonic acid; MPD-3, minimal pyrogenic dose, 3 hr.

Materials and Methods

Preparation of the Lipopolysaccharide. E. coli K-235

L+OC+[2] (Barry *et al.*, 1962) was grown on a medium which contained salts, cerelose, Casamino Acids, and Difco yeast extract, in a 1000-l. tank with aeration. Cells were collected in a Sharples centrifuge and washed. The following procedure was carried out at 4° unless otherwise specified. To each 5 kg of cell paste 5 l. of 88% phenol (Mallinckrodt AR) and 10 l. of freshly distilled water were added. After 36 hr of stirring, the suspension was allowed to settle for 7–9 days. The cloudy supernatant (usually 10 or 11 l.) was decanted, concentrated *in vacuo* at 35° to approximately 2 l., and allowed to stand overnight. The aqueous (upper) layer was dialyzed at constant volume against several changes of distilled water for 4 days. The dialyzed solution was stirred and heated at 65° for 15 min with an equal volume of 88% phenol. After cooling overnight, the aqueous phase was separated and dialyzed for 4 or 5 days against several changes of water. This solution was made 0.15 M with NaCl and the LPS was removed by sedimentation for 4–6 hr at 29,000*g*, 5°. The sediment was resuspended in pyrogen-free saline and centrifuged again. If the absorption spectrum suggested the presence of nucleic acid, it was removed by resuspending the gel in pyrogen-free saline and centrifuging. The LPS gel was suspended in a minimum of pyrogen-free water, dialyzed, and either stored frozen or lyophylized.

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl at 110° for 24 hr in an evacuated sealed tube. After removal of the acid *in vacuo*, samples were analyzed in a Spinco Model 120B amino acid analyzer by the accelerated modification (Benson and Patterson, 1965) of the Spackman *et al.* (1958) system. The instrument was standardized to determine glucosamine, galactosamine, and ethanolamine along with the amino acids.

Hydroxylaminolysis. The conditions of the analytical method (Verheyden and Nys, 1962) were used for preparative hydroxylaminolysis as follows. Four volumes of 2.5% sodium hydroxide (Baker's AR) and three volumes of 2.5% hydroxylamine hydrochloride (Baker's AR), both in 95% ethanol, were mixed and the sodium chloride was removed by centrifugation. Water (2 ml), LPS (200 mg), and the alkaline hydroxylamine solution (40 ml) were mixed well in a glass tissue homogenizer, and the suspension was stirred under nitrogen for 1 hr at room temperature. The insoluble material was removed by centrifugation and washed twice by resuspending and centrifuging in 95% ethanol, once in 0.01 N acetic acid in 95% ethanol, and again in alcohol. The vacuum-dried polysaccharide was 80% of the weight of the LPS. Nondialyzable material was 60–65% of the LPS.

Succinylation. LPS (1 g) was suspended in 50 ml of 1.7 M sodium succinate and 4.8 g of succinic anhydride was added in small aliquots over a 90-min period at room temperature. The pH of the reaction was maintained at 8.5 ± 0.5 by the addition of 0.5 N NaOH. The product was dialyzed against distilled water at 4°, concentrated to 30 mg/ml by ultrafiltration, then sterile filtered and stored at 4°. An aliquot was lyophilized for chromatographic studies and solids determination.

Nitrogen. The micro-Kjeldahl method (Ma and Zuazaga, 1942) was used, with fuming sulfuric acid substituted for concentrated sulfuric acid and sodium sulfate.

Phosphorus. We used a modification (Bartlett, 1959) of the colorimetric method of Fiske and Subbarow (1925).

Paper Chromatography of Carbohydrates. Following hydrolysis of the macromolecules at 100°, 22 hr, in 2 N HCl, chromatography was carried out on Whatman No. 1 paper, with a solvent system of 1-butanol–pyridine–water (3:2:1.5) and with silver nitrate stain. A mixture of known sugars was used for reference.

Heptose. The orcinol and cysteine reactions were used (Dische, 1953) with D-glycero-L-mannoheptose[3] as a reference.

2-Keto-3-deoxyoctonic Acid (KDO).[4] The colorimetric reaction with thiobarbituric acid (Weissbach and Hurwitz, 1959; Osborn, 1963) was employed.

Gas–Liquid Partition Chromatography of Fatty Acid Methyl Esters. Nonvolatile methyl esters were prepared by transesterification in absolute methanol–BF_3 reagent (Applied Science Laboratories, State College, Pa.) at 63°, in a glass-stoppered test tube. The methanol solution was cooled and extracted with four volumes of petroleum ether (bp 50–57°). Aliquots of the petroleum ether extract were evaporated to dryness and the residues were dissolved in carbon disulfide for analysis. Two microliters was injected into a Wilkins Aerograph Hy-Fi Model 550 B flame-ionization unit equipped with a 5 ft × 1/8 in. glass column packed with 20% diethylene glycol succinate on Gas Pack W 60–80. The column was maintained at 180° and the injector port at 200°. Nitrogen inlet pressure was held at 18 psi.

Light Scattering. Molecular weights were determined on a Brice-Phoenix light-scattering photometer at 4358 A. At this wavelength the refractive index increment (dn/dc) on a Phoenix differential refractometer was 0.151 ml/g.

Sedimentation. Data were obtained with a Spinco Model E analytical ultracentrifuge. All runs were at 59,780 rpm, at or near room temperature. Calculations of sedimentation coefficients and their subsequent correction to standard conditions were made as described by Schachman (1957).

Diffusion. The diffusion coefficient was determined in the Spinco Model H electrophoresis diffusion apparatus at 4°. Calculations were made from the

[2] A culture of this organism was obtained from Dr. Stephen B. Binkley, University of Illinois, Chicago, who obtained it from Dr. G. T. Barry.

[3] A sample was generously provided by Dr. N. K. Richtmyer, National Institutes of Health.

[4] A sample of pentaacetyl-3-deoxyoctulosonate was generously provided by Dr. E. C. Heath, Johns Hopkins University.

Rayleigh fringe patterns and corrected to standard conditions. The zero-time correction was 20 sec.

Electrophoresis. Measurements were made in the Spinco Model H electrophoresis diffusion apparatus. All experiments were performed in the 2-ml microcell. The samples were dialyzed overnight against PO_4–NaCl buffer (pH 7.4), ionic strength 0.05 and 0.15, respectively.

Column Chromatography. LPS and its derivatives were chromatographed by gel permeation on 4% Agarose beads in a column 1.3 × 100 cm. For PS and succinyl-LPS the eluent was 0.15 M NaCl in 0.05 M PO_4 buffer (pH 7.0); for LPS 0.5% SDS was added to the eluent.

Pyrogen Assays. The U.S.P. XVII conditions were followed and temperatures were recorded every 30 min for 6 hr after injection of the samples. The same colony of New Zealand White rabbits was used for one assay every 2 weeks over a period of several months. For pyrogen and toxicity studies the native LPS was dissolved with the assistance of an equal weight of SDS (95% sodium dodecyl sulfate, Matheson Colman and Bell, Norwood, Ohio) which was pyrogen free.

Toxicity. The toxicity of LPS and derivatives was studied in female CFW mice weighing 20 ± 2 g. Mouse sensitivity to endotoxin was enhanced by the administration of pertussis vaccine (Parfentjev, 1954). Each animal received approximately 6,000,000 cells in a volume of 0.1 ml intraperitoneally, and 5 days later the endotoxin samples were administered intraperitoneally in a volume of 0.25 ml. Survival was recorded at 24 and 72 hr.

The toxicities of LPS and PS were also compared in dogs. Mixed breed dogs with a mean body weight of 7.1 kg were administered either LPS at 1 mg/kg or PS at 100 mg/kg intravenously. Rectal temperature and clinical symptoms were recorded.

Immunologic Studies. LPS and its derivatives were examined by quantitative precipitin, agar gel diffusion, passive hemagglutination, and immunoelectrophoretic methods. The quantitative precipitin analyses were performed by a microprecipitin technique using absorbancy at 280 mμ for determination of protein (Kabat and Mayer, 1961). Agar diffusion studies were performed by the technique of Ouchterlony (1948) in 1% gels of Difco Agar Noble. Hemagglutination studies were performed with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) with 0.025-ml loops and a 1% erythrocyte suspension at room temperature. Rabbit erythrocytes were treated with pyruvic aldehyde and formaldehyde prior to coating with LPS (A. Hirata, personal communication). Immunoelectrophoresis was carried out in a 1% agar or Agarose gel containing barbital–acetate buffer at pH 8.6 and ionic strength 0.012. A potential difference of 8 v/cm was applied for 45 min prior to diffusion against antibody.

The antibody used in these studies was prepared in New Zealand rabbits immunized with acetone-dried *E. coli* K-235 cells suspended in saline (Landy *et al.*, 1955). The rabbits received nine injections of 0.5 mg of dried cells each during an 8-week period. Low precipitin titers were obtained. Ten weeks later, the animals were given two additional doses of 0.5 mg of cells, 1 week apart, and their sera were harvested 2 days later. A globulin concentrate was prepared from some of the sera by ammonium sulfate precipitation, and a 3% solution of the precipitated protein was prepared in borate-buffered saline (pH 7.6) (Campbell *et al.*, 1963).

Results

General Properties and Composition. Our lyophylized LPS preparations are colorless amorphous solids which hydrate slowly but do not dissolve in water or 0.15 M NaCl. Although we have not attempted to identify all constituent small molecules, by paper chromatography

TABLE I: Analytical Values on LPS and PS.

	LPS (%)	PS[a] (%)
C	43.0	40.4
H	7.6	6.9
N	2.4	2.0
P	2.5	2.2
Ash (Na)	2.7	3.3
	Moles/400,000 Mol Wt	Moles/350,000 Mol Wt[e]
Free amino groups[b,c]	120.0	40.0
Glucosamine	345.0	336.0
Galactosamine	44.0	32.0
Ethanolamine	137.0	25.6
Glycine	35.0	0.9
Glutamic acid	2.3	<0.4
Histidine	2.0	0.7
Lysine	3.3	1.6
Methionine sulfoxide	27.0	12.0
All other amino acids	<0.5 each	<0.5 each
Lauric acid[d]	50.0	2.6
Myristic acid	50.0	1.7
β-Hydroxymyristic acid	155.0	68.0

[a] Nondialyzable product of hydroxylaminolysis. [b] Calculated from ninhydrin determination with glucosamine as reference. [c] Because of no correction for loss on hydrolysis, the values for amino compounds are probably minimal. These analyses were obtained on one large batch of LPS. In another large batch the free amino groups were only 64/molecule of 400,000. [d] To compensate for loss of fatty acids during the transesterification procedure, a mixture of known quantities of the acids was carried through the whole procedure as a recovery standard. [e] A figure of 350,000 is used for PS because the loss of fatty acids and other known groups would reduce the molecular weight of LPS by approximately 50,000.

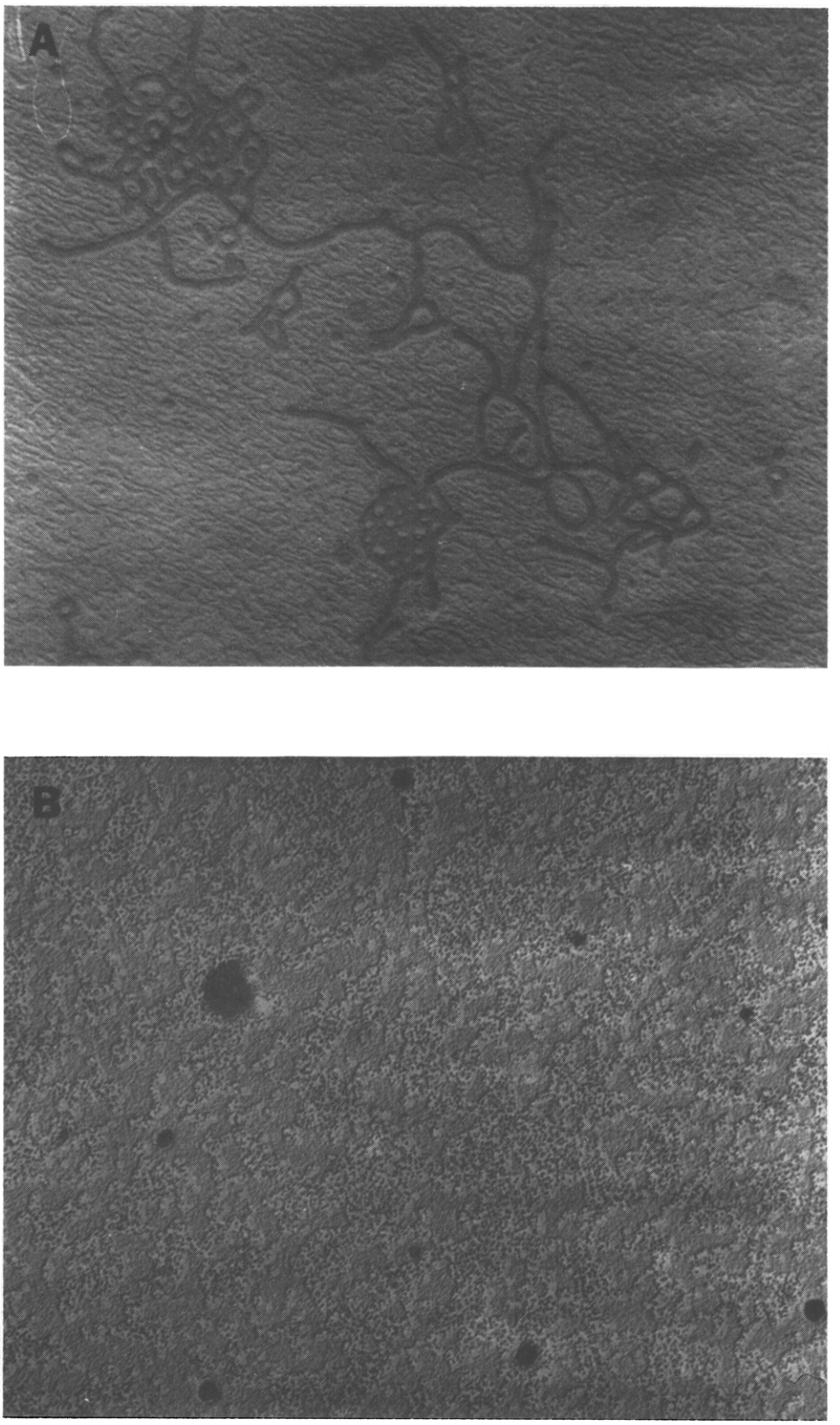

FIGURE 1: Electron micrographs of (A) suspension of LPS in sodium phosphate–chloride buffer and (B) LPS plus an equal weight of sodium dodecyl sulfate in sodium phosphate–chloride buffer. Shadow cast preparations with chromium and carbon (magnification, 65,000×).

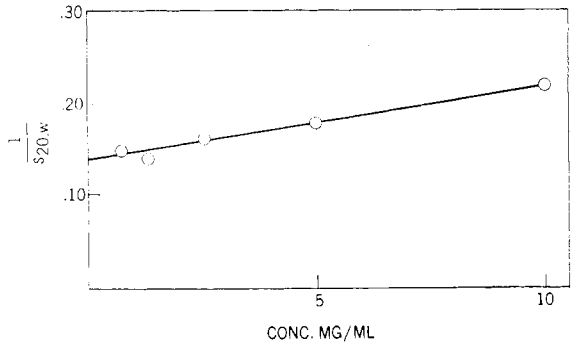

FIGURE 2: Sedimentation coefficient of PS as a function of concentration. $s^0_{20,w}$ = 7.3 S.

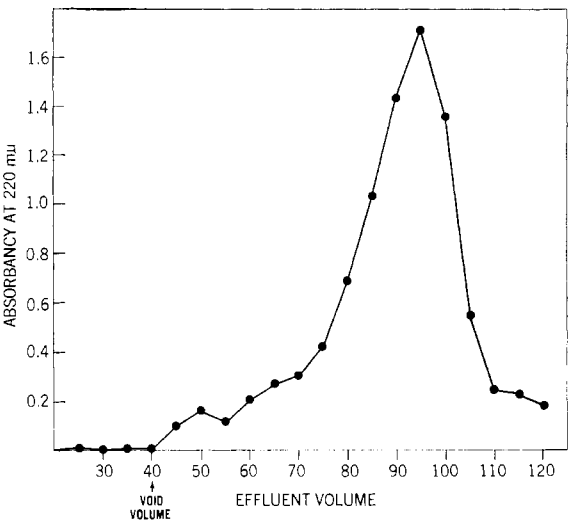

FIGURE 3: Chromatography of succinylated LPS on 4% Agarose.

we have demonstrated the presence of glucosamine, galactosamine, glucose, galactose, rhamnose, and probably *N*-acetylglucosamine. The orcinol and cysteine colorimetric methods indicated the presence of heptose. Although the data can have no real quantitative significance without absolute identification of the heptose, the amount present might be as high as 5% of the LPS. The thiobarbituric acid reaction indicated that KDO could account for as much as 6%.

Table I shows the quantitative composition of LPS and PS in terms of elements, amino compounds, and long-chain fatty acids. The amino acids present account for less than 10% of the total nitrogen, and their distribution indicates that they do not represent an ordinary protein contamination. The absorption spectrum indicated that LPS contained neither aromatic amino acids nor nucleic acid.

Free amino groups account for as much as 16% of the total nitrogen. Although no attempt has been made to identify the constituents which carry the free amino groups, the data would allow ethanolamine to be mainly responsible. Because our autoanalyzer was operated on a fast schedule, the analyses for amino compounds may not be any better than ±10%, and we made no attempt to allow for the loss during hydrolysis. Inasmuch as the identified amino compounds account for 90% of the total nitrogen under these circumstances, probably no significant source of nitrogen remains to be identified.

In the determination of fatty acids the transesterification of all lauric and myristic and approximately one-half of the β-hydroxymyristic was complete in 18 hr. The remainder of the β-hydroxymyristic required an additional 30 hr; possibly this portion of the acid is amide linked.

Molecular Size. When an equal weight of SDS is added to a turbid 0.1% suspension of LPS with gentle agitation, within a few minutes at room temperature the LPS particles dissociate to form a perfectly clear solution. More sophisticated evidence of the disaggregation is presented in the electron micrographs of Figure 1. That the disaggregation is reversible has been demonstrated by electron micrographs and sedimentation studies after removal of SDS by either dialysis or low-temperature precipitation.

The sedimentation pattern for LPS in the presence of SDS showed a single symmetrical peak with an $s_{20,w}$ of 4.7 S.

Calculated from light-scattering data, the disaggregated LPS had a molecular weight of 410,000, which is much higher than ordinarily would be consistent with the observed sedimentation coefficient.

The PS from hydroxylaminolysis of LPS dissolved easily in water. In sedimentation experiments it always gave a hypersharp boundary, and, as shown in Figure 2, the sedimentation coefficient had only a low degree of dependence upon the concentration of PS. (For comparison, DNA also shows a hypersharp boundary, but with the sedimentation coefficient more dependent upon concentration.) Determination of the diffusion coefficient of PS gave a $D_{20,w}$ value of 0.96×10^{-7} cm²/sec; the partial specific volume measured densitometrically was 0.57 ml/g. These values, along with the $s^0_{20,w}$ of 7.3 S, in the Svedberg equation gave a molecular weight of 330,000. Calculated from light-scattering measurements the molecular weight was 431,000.

Disaggregation of the native LPS also was achieved by acetylation and more effectively by succinylation. The sodium salt of the succinyl derivative dissolved quickly in water to give a completely clear solution. A preparation, in which more than 90% of the free amino groups and approximately an equal number of hydroxyls were succinylated (a total of approximately 200 succinyl groups/molecular weight of 400,000), gave a sedimentation pattern with a single peak which had a slight shoulder on the trailing edge. The main component had an $s_{20,w}$ of 9.1 S; from light-scattering measurements the molecular weight was 485,000. Lyophylization of salt-free succinyl-LPS

TABLE II: Toxicity in Pertussis Vaccine Treated Mice and Pyrogenicity in Rabbits of *E. coli* K-235 Lipopolysaccharide and Derivatives.

Material	Mouse Toxicity			Rabbit Pyrogenicity
		Survival		
	μg/Mouse	24 hr	72 hr	MPD-3[b] (g/kg body wt)
LPS	1	$^4/_{10}$	$^4/_{10}$	1.0×10^{-9}
LPS	5	$^5/_{12}$	$^5/_{12}$	
LPS	10	$^0/_{10}$	$^0/_{10}$	
PS	3000	$^{10}/_{10}$	$^{10}/_{10}$	$<1.0 \times 10^{-3}$
PS	6000	$^{10}/_{10}$	$^8/_{10}$	
Succinyl-LPS	1	$^3/_5$	$^3/_5$	3.0×10^{-9}
Succinyl-LPS	20	$^1/_5$	$^0/_5$	
SDS[a]	20	$^5/_5$	$^5/_5$	

[a] Sodium dodecyl sulfate control. [b] MPD-3: minimal pyrogenic dose, 3 hr (Watson and Kim, 1963).

caused a partial reaggregation which could be demonstrated by analytical ultracentrifugation or by gel permeation chromatography. The reaggregation could be reversed by SDS but not by boiling in water.

Gel permeation chromatography on calibrated columns showed that LPS, PS, and succinyl-LPS all had approximately the same molecular weight consistent with 400,000.

Molecular Charge. In moving-boundary electrophoresis PS gave a single sharp symmetrical peak with a mobility of 0.7×10^{-5} cm²/sec v. Succinyl-LPS gave a single broad asymmetric peak with a mobility of 6.9×10^{-5} cm²/sec v. LPS could not be examined satisfactorily in moving-boundary electrophoresis because of the SDS required to keep it disaggregated.

Homogeneity. Although the electrophoresis and sedimentation patterns gave no evidence for heterogeneity of LPS and PS additional criteria were desired. In gel permeation chromatography LPS and PS each gave a single symmetrical peak, but succinyl-LPS appeared somewhat heterogeneous as indicated in Figure 3. Sampling of the main succinyl-LPS peak at several points indicated constant ratios among anthrone color, absorption spectra, and pyrogenicity. Compared to the main peak the small amount of material in the shoulders was less than 1% as pyrogenic and had lower anthrone analyses and greater absorbancy at 260 mμ.

Pyrogenicity and Toxocity. The acute toxicity of LPS in mice or pyrogenicity in rabbits was relatively unchanged by succinylation; however, a dramatic change in toxicity was effected by hydroxylamine treatment (Table II). The toxicity of PS in mice was reduced by a factor of at least 10^3 and pyrogenicity in rabbits by 10^6.

Dogs receiving LPS at 1 mg/kg intravenously displayed a biphasic febrile response with an average rectal temperature elevation of 2.1° 4–5 hr after injection. In addition these animals exhibited mydriasis, retching, and vomiting, and defecated watery stools. There was general lethargy and the animals declined food on the following morning.

Dogs receiving the PS material at 100 mg/kg showed only mild and transient symptoms. One out of four dogs showed an elevation of rectal temperature at 1 hr which promptly returned to base line. There was mydriasis and defecation of normal stool, but all animals ate their food the next day and appeared quite normal.

Immunology. The ability of LPS derivatives to react with antibody to LPS was examined by absorption studies (Table III). The concentration of globulin prepared from the antisera was adjusted to give a hemagglutination titer of $^1/_{6400}$ with LPS-coated rabbit erythrocytes. The antibody solution was absorbed with increasing amounts of erythrocytes coated with LPS, PS, or succinyl-LPS and then tested with each type of antigen-coated cells.

Absorption of antibody by LPS-coated cells eliminated the hemagglutination response with each type of antigen-coated cell. Absorption of antibody with an excess of cells coated with either PS or succinyl-LPS eliminated agglutination of the same type of cells but only reduced the titer with LPS-coated cells. The succinyl-LPS-coated cells were the least effective in absorbing antibody. Absorption with PS-coated cells prevented subsequent agglutination of succinyl-LPS-coated erythrocytes, but the reverse was not observed.

Analogous results were obtained with the precipitin reaction. Optimum amounts of antigen were determined from quantitative precipitin curves, and these concentrations were used for the single precipitation of antibody from the sera. The resulting supernatant solutions were then tested by agar double-diffusion precipitin analyses, using a wide range of antigen concentrations. Precipitation of antibody by LPS prevented subsequent precipitation with LPS or PS in agar. Prior precipitation of antibody with PS prevented a subsequent precipita-

tion in agar with PS but only reduced the precipitation with LPS. The quantitative precipitin curves for LPS and succinyl-LPS were similar in shape, but the succinyl-LPS precipitated only one-half as much antibody as the LPS (Figure 4).

Immunoelectrophoresis demonstrated the increased anionic behavior of succinyl-LPS (Figure 5). In these experiments the precipitin bands formed with succinyl-LPS were markedly reduced in intensity after the slides were soaked in pH 7.5 buffered saline to remove the excess protein prior to staining.

Agar immunodiffusion analyses of LPS, PS, and succinyl-LPS showed a reduction in precipitin band intensity for PS and succinyl-LPS, and the formation of multiple diffuse bands with PS (Figure 6). At equal concentrations the rate of diffusion for PS was considerably greater than for LPS or the succinyl-LPS. In order to examine the confluence of precipitin bands, we placed the trough containing antibody at an angle to give increasing distances for migration of antigen and antibody. The LPS precipitin band was confluent with succinyl-LPS which in turn was confluent with the middle band of the PS precipitin pattern. The LPS precipitin band was also confluent with the slower migrating band in the PS pattern. The disparity in band intensity and migration characteristics between LPS and the third band of the PS pattern prevented the demonstration of confluence under a variety of conditions of varying concentration, distance, and time of diffusion.

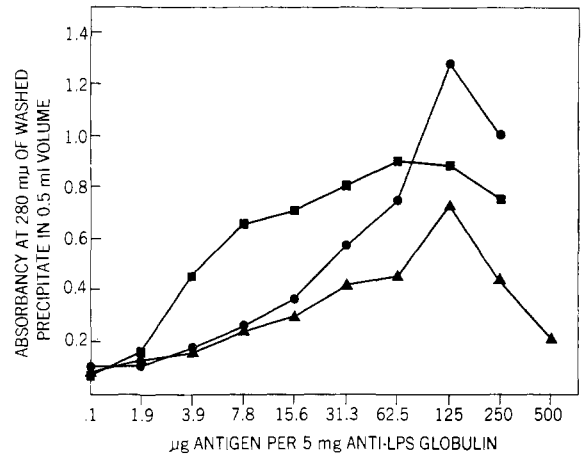

FIGURE 4: Microquantitative precipitation analysis of LPS and derivatives. (●——●) LPS dispersed with SDS, (■——■) PS, and (▲——▲) succinylated LPS.

Discussion

The LPS from *E. coli* K-235 appears to be a molecule with a weight near 400,000 which reversibly forms large aggregates that are held together by hydrophobic and possible electrostatic forces. The purified LPS aggregates, free of protein and nucleic acid, appear to be made up of a population of molecules with very little, if any, evidence of heterogeneity, and therefore the biological properties belong either to only one kind of molecule, or to molecules too similar to be separated by the means employed. The strength of these conclusions is based upon good agreement among studies in which LPS was disaggregated by three different procedures.

In the forces which hold the aggregates together, the importance of hydrophobic bonds is demonstrated by (a) the disaggregation in the presence of SDS, (b) the lack of any evidence of aggregation even in lyophylized PS which is essentially free of esterified fatty acids, and (c) the partial reaggregation of lyophylized succinyl-LPS, in contrast with PS. That electrostatic interactions among molecules may be involved is suggested by the partial disaggregation which accompanies acetylation of the amino groups. Data not presented indicate a partial disaggregation when only the amino groups are succinylated. Complete disaggregation requires additional succinylation, presumably on hydroxyl groups.

The dearth of evidence for heterogeneity in LPS is indeed surprising. With such a complex molecule one must be cautious about speaking of a truly homogeneous preparation; there is the possibility of subtle differences among the molecules which could not be detected by the available separation methods and which may or may not affect the biological properties. The suggestion of heterogeneity by the immunological data of Figures 5 and 6 is tenuous because of the probable complexity of the antibody preparation. Some degree of heterogeneity in PS, a product of partial alkaline degradation, and in succinyl-LPS, a partially succinylated derivative, should be expected even if LPS were truly homogeneous.

TABLE III: Absorption of Antibody by Rabbit Erythrocytes Coated with *E. coli* K-235 Lipopolysaccharide and Derivatives.

Antibody Absorbed by Cells Coated with		Hemagglutination Titer of Absorbed Globulin Cells Coated with		
Material	Packed Cell Vol. (ml)	LPS	PS	Succinyl-LPS
LPS	0.1	1/50	1/50	1/50
	0.2	1/50	1/50	1/50
	0.3	1/50	1/50	1/50
	0.4	1/50	1/50	1/50
PS	0.1	1/50	1/50	1/50
	0.2	1/100	1/50	1/50
	0.3	1/100	1/50	1/50
	0.4	1/200	1/50	1/50
Succinyl-LPS	0.1	1/6400	1/1600	1/100
	0.2	1/3200	1/800	1/50
	0.3	1/1600	1/800	1/50
	0.4	1/1600	1/800	1/50
Control	None	1/6400	1/3200	1/800

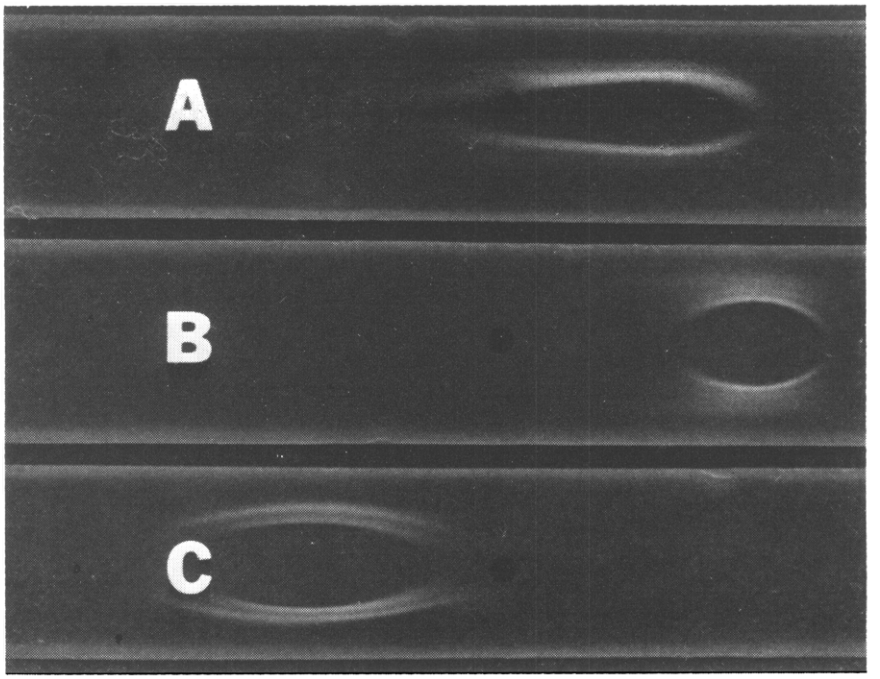

FIGURE 5: Immunoelectrophoresis of *E. coli* K-235 lipopolysaccharide and derivatives: (A) 10 μg of LPS, (B) 5 μg of PS, and (C) 10 μg of succinyl-LPS. The anode is to the left.

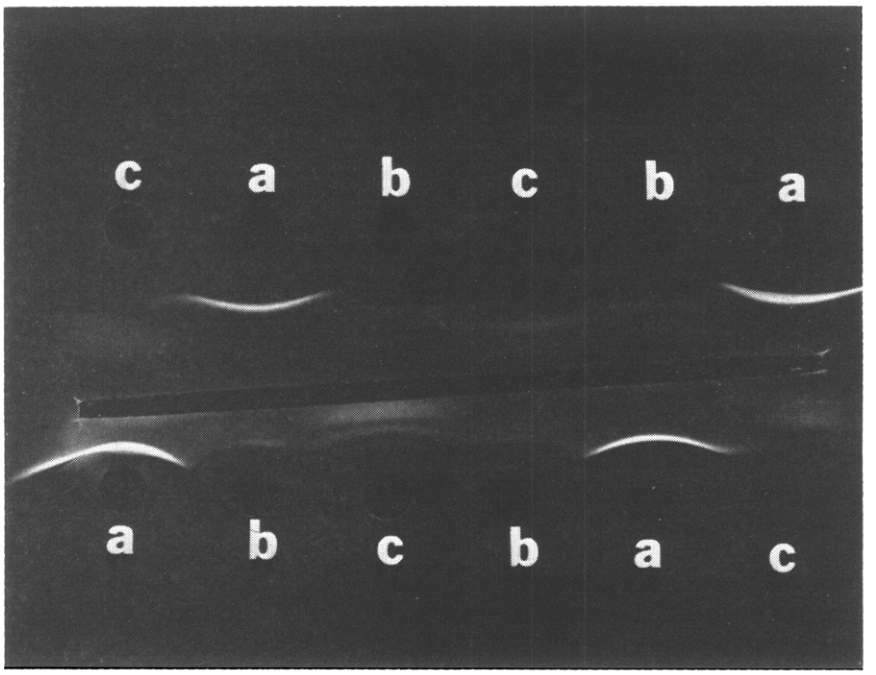

FIGURE 6: Agar immunodiffusion analyses. Antigen concentration = 2.5 mg/ml; (a) LPS, (b) succinyl-LPS, and (c) PS. Rabbit antibody to *E. coli* K-235 was diffused from the trough.

The absence of pyrogenicity in PS and its very low toxicity for both the mouse and the dog are associated with a loss of more than 90% of the lauryl and myristyl and one-half of the β-hydroxymyristyl groups. These results confirm and extend the observation of Tauber *et al.* (1961) on the effect of fatty acid ester removal by hydroxylaminolysis and support Westphal's view of the importance of lipid for the toxicity of lipopolysaccha-

rides from Gram-negative bacteria (Westphal, 1956). However, from the available evidence one cannot tell whether the loss of toxicity is a result of a change in conformation or the loss of lipid irrespective of the change in conformation. The hypersharp sedimentation boundary of PS without a pronounced concentration dependence and the very low diffusion rate suggest that removal of the fatty acids has allowed the molecule to become very extended. A change in dissymmetry of an endotoxin following treatment with aqueous sodium hydroxide was reported by Tripodi and Nowotny (1966) who suggested that a change in conformation is related to detoxification.

One of the most important points upon which our data have a cogent bearing is the relationship between the molecular or aggregate size and the toxicity or pyrogenicity of lipopolysaccharides from Gram-negative bacteria. Based upon sedimentation coefficients and pyrogen assays of preparations disaggregated by the presence of 0.25% NaD, Rudbach *et al.* (1966) have postulated that a lipopolysaccharide is composed of subunits with a molecular weight somewhere between 10,000 and 80,000 which may be reversibly disaggregated by agents such as NaD, that the monomers are biologically inactive, and that the smallest aggregate which has full toxicity or pyrogenicity is in the 500,000 weight range. With our own LPS from *E. coli* K-235 we have confirmed the observation of Rudbach *et al.* that dilution of LPS in 0.25% NaD does indeed block the pyrogen response to as much as 100 MPD-3 of LPS, but, for the following reasons, we cannot agree with their explanation of the phenomenon. (1) The molecular weight estimates of Rudbach *et al.* are not realistic because they were based only on sedimentation coefficients (apparently not corrected to $s_{20,w}$) without consideration of partial specific volume and diffusion coefficient. Our experience has shown that, taken alone, the $s_{20,w}$ of LPS disaggregated by SDS is very misleading; the $s_{20,w}$ was 4.7 S and yet the molecular weight by light scattering was 410,000. (Rudbach *et al.* obtained a sedimentation coefficient of 4.0 in 0.25% NaD.) (2) Our LPS was fully pyrogenic when diluted in the same concentration of SDS that was used for disaggregation of LPS in the sedimentation experiments. This means that maximum disaggregation by SDS does not interfere with pyrogenicity. (3) Our nonpyrogenic derivative, PS, had a molecular weight in the 400,000 range, calculated from light scattering, $s_{20,w}$, partial specific volume, and diffusion coefficient. The fact that two full toxic and pyrogenic preparations, our succinyl-LPS and our LPS disaggregated in SDS, also showed light-scattering molecular weights near 400,000 probably is more than a coincidence, and we therefore favor a molecule of this size as fully active biologically and the ultimte in disaggregation (without the cleavage of covalent, intramolecular bonds). We believe then that the reversible inhibition of pyrogenicity by NaD does not depend upon the reversible disaggregation of a toxic polymer. Perhaps this interesting phenomenon indicates the formation of nontoxic molecular complexes between NaD and LPS, complexes which are easily dissociated and which require a certain concentration of NaD and a very high ratio of this molecule over LPS. The plausibility of this explanation was suggested when we observed the formation of a gel structure in solutions which contained 0.25% NaD and 0.1% or less LPS.

The observation by Oroszlan and Mora (1963) of the reversible inactivation of an LPS by SDS is at variance with our experience, but their experiments differed from ours in both the biological assay and the LPS employed. Their molecular weight conclusions were based on sedimentation coefficients alone and therefore suffer limitations already mentioned.

The immunologic studies indicate a loss or modification of determinant groups when LPS is either succinylated or detoxified by removal of fatty acids in ester linkage. A comparison of LPS, succinyl-LPS, and PS, with regard to their toxicity and their ability to react with precipitating and hemagglutinating antibodies, suggests that most of the determinants which react with these antibodies are not related to the pyrogenicity and toxicity of LPS. The succinyl-LPS may be useful for investigations of the extent to which pyrogenicity and toxicity are the result of antigen–antibody interactions and of the involvement of protective antibodies in the development of immunity to pyrogenicity (Kim and Watson, 1966). Preliminary results indicate that rabbits treated with LPS become equally immune to the pyrogenic effects of LPS and succinyl-LPS, while rabbits treated with succinyl-LPS become immune to it but much less immune to LPS.

Molecular charge, within the limits possessed by LPS and succinyl-LPS, apparently has a minor effect upon pyrogenicity and toxicity. While the succinylation of LPS has markedly altered its reaction with precipitating and hemagglutinating antibodies, we cannot at present suggest the degree to which this may be attributed to a change of charge, independent of steric and other effects of acylation on or near immunologically determinant sites.

Acknowledgments

Assistance from colleagues at Abbott Laboratories is gratefully acknowledged as follows: pyrogen assays, R. Cantrell, R. J. Miller and R. T. Olsen; production of bacterial cells, D. D. Dibble; large-batch preparation of LPS, Dr. G. G. Post; C, H, and N analyses, V. Rauschel; amino acid analyses, R. L. Gardisky and T. F. Tanner; synthesis of β-hydroxymyristic acid reference, F. E. Fischer and E. V. Schuber; gas chromatography, D. Robinson; dog toxicity, Dr. E. J. Fedor; electron microscopy, Dr. W. R. Richter and E. J. Rdzok; and electrophoresis, L. Coen.

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