An Extracellular Lipopolysaccharide-Phospholipid-Protein Complex Produced by *Escherichia coli* Grown under Lysine-Limiting Conditions

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Lysine limitation during growth of the lysine-requiring mutant of *Escherichia coli* 12408 resulted in the excretion of a complex containing 60% of lipopolysaccharide, 26% of extractable phospholipid and 11% of protein. The complex was obtained from the culture filtrate in yields of about 0.5 g./l. by precipitation with chloroform or gel filtration; some purification steps are described. The greater part of the phospholipid consisted of phosphatidylethanolamine, which contained four main fatty acids; two monoenoic acids and a cyclopropane acid were esterified mainly in the β -position, and a saturated acid was located mainly in the γ -position. The protein component was relatively insoluble and contained an excess of acidic over basic amino acids and little cystine. The lipopolysaccharide resembled in composition the intracellular lipopolysaccharides from rough strains of *E. coli*. Both protein and lipopolysaccharide constituents of the complex were serologically active; the complex was less toxic than the purified lipopolysaccharide. In the electron microscope the complex showed a mixture of particles of various sizes and shapes. Rods and hollow spheroids (diameter 12–200 m μ) were most common and resembled the particles previously found surrounding cells actively excreting the complex. The chloroform-precipitated material showed a tubular lamellar structure. Soluble lipopolysaccharide prepared from the complex also consisted of hollow spheres and rods.

Studies on the products excreted by certain lysine-requiring mutants of *Escherichia coli* grown under lysine-limiting conditions have shown that they include one substance related to the cell wall; this has been variously called lipomucoprotein (Meadow, 1958), mucopeptide (Municio, Diaz & Martinez, 1963, 1964) and lipoglycopeptide (Bishop & Work, 1965). Lipoglycopeptide was an insoluble substance that was precipitated from concentrated culture filtrates of *E. coli* A.T.C.C. 12408 after extraction with chloroform. It contained phospholipid, carbohydrate and a limited range of amino acids. Further examination showed that the carbohydrate component was part of a lipopolysaccharide indistinguishable chemically and serologically from lipopolysaccharide extracted from cells (Taylor, Knox & Work, 1966).

The present paper describes the isolation and properties of a water-soluble complex of the extracellular lipopolysaccharide. It can be obtained from the culture filtrate as an aggregate produced by addition of chloroform; on removal of chloroform

from the harvested aggregate the complex re-disperses in water. A similar product can be obtained by gel filtration. The major components of the complex have been separated and analysed. Besides lipopolysaccharide they were phosphatidylethanolamine and a protein containing a more complete range of amino acids than that reported for lipoglycopeptide. This complex of lipopolysaccharide-phospholipid-protein is referred to below as 'lipopolysaccharide complex'. It resembles the O-somatic antigens extracted from Gram-negative bacteria (see review by Davies, 1960) in its biological properties and in having phospholipid and protein associated with the lipopolysaccharide.

The accumulation of extracellular products by this mutant under lysine-limiting conditions is associated with a biphasic type of growth. A study with the electron microscope of the morphology of cells at all stages of growth had shown no cellular lysis, but marked structural changes in the cell wall; globules, now known to consist of the lipopolysaccharide complex, were seen outside the cells (Work, Knox & Veski, 1966; Knox, Veski & Work, 1966). The present paper describes the morphology

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of the extracellular lipopolysaccharide complex, obtained either by gel filtration or by chloroform precipitation. The appearance of the lipopolysaccharide after removal of protein and phospholipid is also described.

MATERIALS AND METHODS

Micro-organisms and method of culture. The lysine-requiring strain of *E. coli*, A.T.C.C. 12408, was that used in previous studies (Bishop & Work, 1965; Taylor *et al.* 1966). The parent wild-type strain, A.T.C.C. 11396, was also grown. Cultures of strain 12408 were grown at 37° in a stirred aerated fermenter (30l.) containing 14–15l. of medium; the medium contained lysine hydrochloride (60 mg./l.), inorganic salts and glycerol (2.0%, w/v) (Bishop & Work, 1965). After 26 hr. growth, the cells were removed by centrifuging and the culture fluid was clarified by passing through a membrane filter (Membranfilter-Entkeimungsschichten no. 1121; Carl Schleicher und Schüll, Dassel/Kr. Einbeck, Germany). The cells were washed with distilled water and freeze-dried if desired.

Preparation of cell walls. Cells were disintegrated with glass beads and the cell-wall fraction was purified by the method of Allsop & Work (1963).

Isolation of lipopolysaccharide complex from culture fluid. A trial portion (1l.) of the culture fluid was shaken vigorously in a separating funnel with 10ml. of chloroform. The separating funnel was allowed to stand for a few minutes and then examined for the presence of a lower chloroform layer. During this period the culture fluid became opalescent. If a chloroform layer did not separate, an additional 5ml. of chloroform was added and the mixture again shaken. The procedure was repeated until a separate chloroform layer formed; with each addition the opalescence of the culture fluid increased. From the result of this trial experiment, chloroform was added to the remainder of the culture fluid in an amount just insufficient to form a separate chloroform layer on standing. Generally the amount required was 20–50 ml./l. of culture fluid. After the final addition of chloroform, the culture fluid was kept at room temperature for at least an hour (usually overnight) and then centrifuged at 14 000g for 20 min. or in a Sharples centrifuge. The deposit was suspended in water and the chloroform was removed *in vacuo* at room temperature in a vacuum rotary evaporator. The resulting aqueous solution is referred to as crude lipopolysaccharide complex. To remove contaminating substances from this solution, several methods of purification were employed; these were dialysis, ultracentrifugation, reprecipitation with chloroform and precipitation with 25 mm-MgCl₂, and are described in the Results section.

The lipopolysaccharide complex remaining in the culture fluid after precipitation with chloroform could be estimated or isolated by gel filtration. This method was also used to recover total complex from untreated culture filtrate. Culture fluid (250 ml.) was concentrated fivefold *in vacuo* at 35°, applied to a column (40 cm. × 2.5 cm.) of Sephadex G-75 and eluted with water. The first carbohydrate-containing fraction, as determined by the phenol-H₂SO₄ method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), contained the lipopolysaccharide complex.

Separation of lipid, protein and lipopolysaccharide components of lipopolysaccharide complex. Lipids were extracted

from the freeze-dried crude lipopolysaccharide complex with methanol and chloroform. Complex (1g.) was suspended in methanol (80 ml.) and ground to a fine slurry in a Potter-Elvehjem homogenizer; the suspension was stirred at 60° for 15 min. The insoluble material, separated by centrifuging, was re-extracted with methanol as before, and then with chloroform (80 ml.) at room temperature. The combined extracts were evaporated to dryness, dissolved in chloroform and filtered. The chloroform-soluble material containing extractable lipids was dried *in vacuo* to constant weight.

The chloroform-insoluble material, containing protein and lipopolysaccharide, was suspended in aq. 45% (w/v) phenol and heated at 65° for 10 min. to separate the components (Westphal, Lüderitz & Bister, 1952). The cooled mixture (4°) was separated into two phases by centrifuging; the lower phenol phase was extracted a second time with an equal volume of water under the same conditions. Protein was recovered as a precipitate from the lower phenol phase either by dialysis against water until the phenol had been removed or by treatment with 2 vol. of methanol. The pooled upper aqueous-phenol phase containing lipopolysaccharide was dialysed free of phenol and centrifuged at 100 000g for 1 hr., yielding a solution containing the bulk of the lipopolysaccharide and a pellet also containing some lipopolysaccharide. Portions of the solution were further fractionated with ethanol and MgCl₂ as described by Taylor *et al.* (1966) or by a subsequent modification of this method, described in the Results section.

Analytical methods. Nitrogen, phosphorus and carbohydrates were estimated by the methods used by Taylor *et al.* (1966). Amino acids were determined on an EEL automatic amino acid analyser. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), the material being dissolved in N-KOH and subsequently neutralized with perchloric acid; an ovalbumin standard was treated similarly. Esterified fatty acids were determined by the method of Stern & Shapiro (1953). Methyl esters of fatty acids were prepared from analytical samples and analysed by gas-liquid chromatography by the methods described by Knivett & Cullen (1965).

Examination of lipid components of lipopolysaccharide complex and cells. Lipids extracted from the lipopolysaccharide complex were dissolved in chloroform and applied to columns of DEAE-cellulose (acetate form) (Whatman Column Chromedia DE11), prepared according to the method of Rouser, Kritchevsky, Heller & Lieber (1963). After elution with chloroform-methanol (9:1, v/v) to remove pigments, phosphatidylethanolamine was eluted with chloroform-methanol (7:3, v/v). The solution was evaporated to dryness, the phosphatidylethanolamine was redissolved in chloroform and precipitated with 4–5 vol. of acetone. After removal of non-lipid components from the DEAE-cellulose with methanol, acidic lipids were eluted from the column with chloroform-methanol (2:1, v/v) saturated with ammonia. The acidic lipids were purified, and stored, as barium salts (Macfarlane, 1961). The lipids were examined qualitatively by thin-layer chromatography with Kieselgel G (E. Merck A.-G., Darmstadt, Germany) and solvent systems chloroform-methanol-acetic acid (65:25:8, by vol.) (Nichols, 1963), chloroform-methanol-water (65:25:4, by vol.) (Wagner, Hörhammer & Wolff, 1961) and the two-dimensional system of Nichols (1964), chloroform-methanol-7 N-ammonia (65:30:4, by vol.) and

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chloroform-methanol-acetic acid-water (170:25:25:6, by vol.). Components were detected by spraying with 0.5% (w/v) ninhydrin in butan-1-ol, the modified Zinzadze reagent (Dittmer & Lester, 1964) or chromic acid (Mangold & Kammereck, 1962).

For the estimation of phosphatidylethanolamine in the complex samples of extractable lipid containing 30–80 µg. of P were chromatographed as 1.5 cm. streaks (20–40 µl. of chloroform solution) on paper loaded with silica gel (Whatman Paper Chromedia SG 81) with chloroform-methanol-water (65:25:4, by vol.) as solvent (Marinetti, 1965). The paper was treated with ammonium molybdate-perchloric acid reagent to detect phospholipids (Burrows, Grylls & Harrison, 1952) and the stained areas corresponding to phosphatidylethanolamine were then removed, cut into pieces and placed in Pyrex centrifuge tubes. The contents of each tube were digested in 72% (w/w) perchloric acid (2 ml.) for 1–2 hr. and then concentrated to approx. 0.6 ml. Water (5 ml.) was added to each tube and the resulting suspension was centrifuged until the silica gel formed a firm pad; the clear supernatant liquor was removed as completely as possible and its phosphorous content was estimated (Allen, 1940). Blank determinations were carried out by comparing the total phosphorous content of an untreated sample of lipid with that of the same amount of lipid, streaked on the paper but not developed by solvent, and then treated as the chromatographed sample. Negligible differences were usually obtained.

The method of Hildebrande & Law (1964), with *Crotalus adamanteus* venom (Sigma (London) Chemical Co. Ltd., London, S.W. 6), was employed for examination of the distribution of fatty acids in purified phosphatidylethanolamine. This venom contains phospholipase A (EC 3.1.1.4), which specifically hydrolyses the β -fatty ester linkage of phospholipids (van Deenen & de Haas, 1963). Hydrolysis products were separated by thin-layer chromatography with either chloroform-methanol-water (65:25:4, by vol.) to obtain the lyso compound, or hexane-diethyl ether-acetic acid (70:30:1, by vol.) (Nichols, 1963) to obtain free fatty acids.

The 'residual fatty acids' (fatty acids released after hydrolysis) were obtained from the material remaining after extraction of lipid from lipopolysaccharide complex, and from a soluble lipopolysaccharide preparation, by refluxing in a tenfold excess of 10% (w/v) KOH in methanol for 3 hr. The fatty acids were extracted from the acidified hydrolysate, after addition of water, by diethyl ether, esterified and examined by gas-liquid chromatography. The methyl ester of 3-hydroxytetradecanoic acid was adsorbed by certain Apiezon columns, even where the column efficiency was still high. Accordingly 'residual fatty acid' samples were chromatographed on Apiezon columns capable of detecting this hydroxy acid methyl ester.

Lipids were extracted from freeze-dried cells and cell walls with methanol and chloroform by the procedure described for the extraction of lipids from freeze-dried lipopolysaccharide complex.

Electron microscopy. For negative staining, a drop of preparation was placed on a grid (covered with a carbon-coated nitrocellulose film) and mixed with an equal volume of aq. 2% (w/v) ammonium molybdate or aq. 1% (w/v) phosphotungstic acid that had been adjusted to pH 7 with N-KOH. After 5–30 sec. the liquid was withdrawn with filter paper, leaving a thin film that was allowed to dry at

room temperature. For the preparation of sections of complex, the material precipitated by chloroform was fixed with 1% (w/v) osmium tetroxide in phosphate buffer, pH 7, and embedded in Epon. Specimens were examined in a Philips EM100 apparatus operated at 60 kv and fitted with a 0.25 µ objective aperture.

Biological tests. Toxicity for chick embryos was estimated by injecting 0.2 ml. doses (sterilized by filtration) into the yolk sacs of 5-day-old embryos, which were incubated at 37° for a further 2 days. Mouse toxicity tests were carried out on groups of ten mice (each weighing 20 g.) that had been sensitized 5 days previously with formalized *Haemophilus influenzae*. In both cases LD₅₀ values were calculated by the method of Reed & Muench (1938). Rabbit-skin sensitivity tests (Shwartzman reaction) were carried out as described by Taylor *et al.* (1966).

RESULTS

Preparation and purification of crude lipopolysaccharide complex

Chloroform (3%, v/v), added to 11 l. of culture fluid as described in the Materials and Methods section, produced a creamy-white precipitate of crude lipopolysaccharide complex, which was recovered by centrifuging and dispersed in 150 ml. of water; on removal of chloroform the mixture changed from a milky suspension to an opalescent solution (fraction 1). A portion (30 ml.) on freeze-drying yielded 620 mg., giving an overall yield of 280 mg./l. of culture fluid.

For the removal of possible contaminating material from the crude lipopolysaccharide complex, several methods of purification were tried on 30 ml. portions of fraction 1. The recovery of freeze-dried material was compared with the amount present in the unfractionated solution (620 mg./30 ml.).

Dialysis. Fraction 1 was dialysed for several days at 2° against daily changes of distilled water. The recovery of indiffusible material was 90% (fraction 3).

Ultracentrifugation. Fraction 1 (30 ml. diluted to 100 ml. with water) was centrifuged at 100 000g for 1 hr.; the gelatinous pellet was dispersed in 100 ml. of water and centrifuged again. The final pellet contained 350 mg. of solid (56% recovery).

Reprecipitation with chloroform. Preliminary experiments had indicated that reprecipitation from water with chloroform gave recoveries of only 40%. A comparison of the effect of addition of the various salts present in the culture medium showed that only magnesium sulphate (concentration in medium 0.2 mM) gave enhanced precipitation. Accordingly, magnesium sulphate was added to fraction 1 to a final concentration of 1 mM, followed by 5% (v/v) chloroform; 60% of the starting material was recovered in the precipitate.

Precipitation with magnesium chloride. Precipi-

tation with 25mM-magnesium chloride has been used for the quantitative recovery of lipopolysaccharide from solutions (Osborn, Rosen, Rothfield & Horecker, 1962; Taylor *et al.* 1966); this concentration also precipitated lipopolysaccharide complex even in the absence of chloroform. Fraction 1 (30ml.) was diluted to 45ml. and 0.25M-magnesium chloride (5ml.) was added with stirring. After standing at 2°, a flocculent precipitate was separated by centrifuging and was dissolved in 20mM-EDTA. Both this solution and the supernatant solution from the magnesium chloride precipitation were dialysed against mM-EDTA, followed by distilled water. The yields of freeze-dried materials were: precipitate (fraction 4a), 554mg., 89% recovery; supernatant (fraction 4b), 22mg.

Properties and analyses of preparations of lipopolysaccharide complex

Of the possible methods of purifying the lipopolysaccharide complex, only dialysis (fraction 3) and precipitation with 25mM-magnesium chloride (fraction 4a) gave satisfactory recoveries. Solutions (5mg./ml.) of preparations purified by these methods were stable, opalescent and viscous. Analyses were carried out for heptose (*L-glycero-D-manno*-heptose) and glucose, both components of the lipopolysaccharide, and also total nitrogen, total phosphorus, protein and extractable lipid (Table 1). The results indicated that the crude chloroform-precipitated complex (fraction 1) contained mainly lipopolysaccharide (heptose content was 8.6%), lipid (26%) and protein (11%). Dialysis

lowered the content of nitrogen and phosphorus (fraction 3) possibly because of removal of ammonium phosphate, a major component of the culture medium (cf. Bishop & Work, 1965). Magnesium chloride-precipitated material (fraction 4a) contained 9% of protein and 22% of extractable lipid. The nitrogen content was less than in fraction 3 because of the removal of a minor nitrogen-rich fraction (4b) and, more particularly, the probable displacement by Mg^{2+} of NH_4^+ ions (0.7% of inorganic N found in fraction 3; Table 1).

The lipopolysaccharide complex remaining in the culture fluid after chloroform precipitation was isolated by column chromatography on Sephadex (fraction 2). It was similar in composition to the precipitated preparation purified by dialysis (fraction 3). Qualitative paper chromatography of hydrolysed fraction 2 showed that it contained a protein component similar to that of fraction 4a (Table 2).

The residues remaining after the removal of extractable lipid from fractions 3 and 4a were also analysed (Table 1).

In other experiments, a second treatment with chloroform enabled nearly 90% of the lipopolysaccharide complex to be recovered from the culture fluid.

Aggregation of lipopolysaccharide complex from culture filtrate by organic compounds. Thirty miscellaneous organic compounds were mixed with 100ml. samples of culture filtrate to find out if aggregation of the lipopolysaccharide complex was specific to chloroform. Evidence for the presence of the complex in freeze-dried preparations from

Table 1. *Composition of preparations of lipopolysaccharide complexes*

Details of the procedures are given in the Materials and Methods section. —, Not analysed.

Fraction	Procedure	Composition (% w/w)					
		Nitrogen	Phosphorus	Heptose	Glucose	Protein	Extractable lipid
1	CHCl ₃ precipitation	4.6	5.5	8.6	7.0	11†	26†
2	Sephadex G-75 chromatography of supernatant from fraction 1	3.0	3.2	11.6	8.0	—	27
3	Dialysis of fraction 1	3.1*	3.3	11.2	6.4	11†	24
4a	Reprecipitation of fraction 1 with MgCl ₂	2.3†	3.2	10.0	7.0	9†	22
4b	Supernatant from fraction 4a	10.5	0.6	2.3	5.4	—	—
5	Methanol- and CHCl ₃ -extracted fraction 3	3.6	3.2	15.1	9.0	—	0
6	Methanol- and CHCl ₃ -extracted fraction 4a	3.3	3.4	15.2	8.5	—	0

* Includes 0.7% of inorganic nitrogen.

† Includes 0.1% of inorganic nitrogen.

‡ Not estimated on the same sample as the other analyses.

Table 2. *Amino acid composition of protein component obtained from purified lipopolysaccharide complex (fraction 4a) by phenol extraction and dialysis*

Amino acid	Amount (moles/10 ⁴ g.)	Amino acid	Amount (moles/10 ⁴ g.)
Ala	4.9	Leu	2.9
Gly	5.6	Ile	1.2
Ser	3.7	Val	1.9
Thr	3.0	Met	0.2
Glu	4.8	Cys	Trace
Asp	7.6	Phe	1.7
Lys	2.8	Tyr	2.0
Arg	1.8	His	0.4

dialysed aggregated materials was obtained by analysing for glucose, heptose, protein, organic phosphorus and extractable lipid phosphorus. Water-soluble preparations consisting mainly of lipopolysaccharide complex were produced by some other halogenated hydrocarbons, such as methylene dichloride, trichloroethylene, trichloroethane and tetrachloroethane; bromoform produced a water-insoluble complex. Various other polar and non-polar compounds produced aggregates; with methanol (1 vol.) and ethanol (2 vol.), the precipitates were insoluble in water after freeze-drying and appeared to consist mainly of lipopolysaccharide complexes low in phospholipids. In other cases (pentan-1-ol, butan-2-one, ethyl acetate, diethylamine) the yields were low (less than 25% of the highest yields). Soluble products containing mainly lipopolysaccharide complex were obtained in fair yields with di-isopropyl ether and ethylene glycol dimethyl ether. With some non-polar compounds such as heptane and cyclohexane, the products were insoluble and either low in protein or lipid or high in glucose (suggesting that other polymers were also precipitated).

Properties of lipopolysaccharide and protein components of the complex. Lipid was extracted from a preparation of dialysed lipopolysaccharide complex and the residue (3.0 g.) was separated into the lipopolysaccharide and protein components by phenol extraction as described in the Materials and Methods section. After centrifuging the lipopolysaccharide solution at 100 000g for 1 hr., only a small amount of material (0.18 g.) was in the pellet; this contained 20.2% of heptose and was considered to be a high-molecular-weight lipopolysaccharide (Lüderitz *et al.* 1965). The supernatant liquid, which was water-clear and slightly viscous, contained 1.71 g. of solid at a concentration of 9 mg./ml. (Found: heptose, 19.4; glucose, 8.9; N, 1.5; P, 4.0%). Further purification by precipitation with ethanol followed by 25 mM-magnesium chloride lowered the nitrogen

and phosphorus contents and gave a product in 60% yield with an analysis similar to that of lipopolysaccharide previously purified in this way from lipoglycopeptide [Found: heptose, 19.8; glucose, 10.4; N, 1.3; P, 3.3%. Published analyses: heptose, 18.9; glucose, 9.6; N, 1.1; P, 3.9% (Taylor *et al.* 1966)]. In an attempt to omit the ethanol precipitation step, it was found that magnesium chloride alone would not precipitate lipopolysaccharide from aqueous solutions unless small amounts (5%, v/v) of ether or ethanol were present.

The phenolic phase, which contained protein, was divided into two portions. One-third (equivalent to 1.0 g. of starting material) was dialysed to give 100 mg. of an insoluble product containing 11.4% of nitrogen and 0.8% of phosphorus. The remainder was treated with 2 vol. of methanol; the precipitate (140 mg.) contained 13.0% of nitrogen and 0.3% of phosphorus; the supernatant liquid yielded 30 mg. of solid containing 2.0% of nitrogen and 2.6% of phosphorus. This indicates that the crude protein component in the phenol phase was contaminated with a phosphorus-containing compound.

The analyses in Table 1 showed that the crude lipopolysaccharide complex (fraction 1) contained a contaminating nitrogen-rich fraction (4b) that was removed by magnesium chloride precipitation; accordingly, before quantitative amino acid analysis of the protein component, the complex was purified by magnesium chloride precipitation. The protein was then obtained from phenol solution by dialysis; amino acid analysis showed that it contained a preponderance of acidic over basic amino acids and only a little cystine (Table 2). The water-insoluble protein was partially soluble in acetic acid or 8 M-urea, and soluble in phenol-acetic acid-water (2:1:1, by vol.).

Analysis of lipid fractions of lipopolysaccharide complex. Material extracted with methanol-chloroform from a number of preparations of lipopolysaccharide complex varied from 24 to 28% of the dry wt. of the complex. Phosphatidylethanolamine was the major component (60–75%, w/w); only small amounts of lysophosphatidylethanolamine were present in most lipid mixtures examined. In a typical experiment, in which 71.5% (w/w) of the extractable lipid was phosphatidylethanolamine, the minor components were: pigments, 9.2%; non-lipid material, 8.3%; acidic lipids, 6.0% (recovery 95%). The yellow-pigment fraction contained no detectable free fatty acids or neutral lipids; the non-lipid components gave positive ninhydrin reactions and were presumably amino acids or peptides; the acidic lipids had the mobilities of phosphatidylglycerol and cardiolipin on thin-layer chromatograms (Nichols, 1964).

The specimen of phosphatidylethanolamine con-

Table 3. *Distribution of fatty acids in phosphatidylethanolamine*

The first two columns give results on six separate preparations: the next three columns relate to a single preparation treated with phospholipase A to release the fatty acids in the β -position and yield lysophosphatidylethanolamine with the fatty acid in the γ -position. The values given are the percentages of the total fatty acid methyl esters in each sample. The fatty acids are designated thus: the number before the colon is the number of carbon atoms; that after the colon is the number of double bonds; Δ represents a cyclopropane ring.

Fatty acid	Total fatty acids (six preparations)		Position of fatty acid (one preparation)		
			Total (phosphatidylethanolamine)	β -Position (free fatty acids)	γ -Position (lysophosphatidylethanolamine)
	Mean	S.D.			
14:0	1.7	0.5	1.0	1.1	1.4
16:1	20.9	1.3	17.5	27.0	2.2
16:0	44.5	2.1	38.9	15.1	69.7
17:0 Δ	6.3	2.1	7.5	9.5	1.0
18:1	25.6	2.3	33.3	43.0	22.1
18:0	1.3	0.5	1.3	2.0	3.5
19:0 Δ	<1.0	—	0.5	2.3	—

Table 4. *Fatty acid composition of various lipids from E. coli 12408*

Lipids of cells and lipopolysaccharide (LPS) complex from five different batches were extracted with hot methanol and chloroform. 'Residual fatty acids' remaining after removal of lipid from one preparation of lipopolysaccharide complex were released by hydrolysis in hot methanolic KOH; 'residual fatty acids' of a soluble preparation of lipopolysaccharide were released in the same way. The 'residual fatty acids' contained significant amounts of two unidentified constituents with carbon numbers 14.6 and about 15 on Apiezon and 15.5 and 16.2 respectively on polyethylene glycol adipate. Fatty acid esters were prepared and analysed as described in the Materials and Methods section. Fatty acids are designated as indicated in Table 3.

Fatty acid	Extractable lipid (five preparations)				'Residual fatty acids'	
	Cells		LPS complex		LPS complex (one preparation)	LPS (one preparation)
	Mean	S.D.	Mean	S.D.		
12:0	0	—	0	—	18.3	36.8
14:0	1.6	—	1.3	—	25.7	27.3
OH-14:0	0	—	0	—	31.0	25.5
16:1	21.7	1.1	20.2	0.6	1.8	0
16:0	44.0	3.6	43.1	4.5	7.0*	4.8*
17:0 Δ	6.2	2.3	5.3	2.8	0.5	0
18:1	24.6	4.6	28.8	4.5	3.5	0

* Not checked for second component (Bishop & Work, 1965).

tained 1.77% of nitrogen, 4.15% of phosphorus and 65% of esterified fatty acids calculated as hexadecanoic acid. The molar ratio of nitrogen to phosphorus was 0.95:1.0, and of fatty acid to phosphorus 1.9:1.0 (theoretical 1:1 and 2:1 respectively). The main fatty acids were hexadecanoic acid (39%) and octadecenoic acid (33%); there was also hexadecenoic acid (17%) and C₁₇ cyclopropane fatty acid (7.5%) (Table 3). There were insignificant differences between the proportions of individual fatty acids in phosphatidylethanolamine extracted from six different batches of lipopoly-

saccharide complex. The distribution of fatty acids in the β - and γ -positions of one sample of phosphatidylethanolamine showed a preponderance of saturated acids esterified in the γ -position and of unsaturated and cyclopropane acids in the β -position.

The fatty acid composition of the total lipid extracted from the lipopolysaccharide complex (Table 4) was similar to that of purified phosphatidylethanolamine. It also resembled that of lipids extracted from whole cells; there was good correlation between the composition, with respect to the

Table 5. *Biological tests on lipopolysaccharide preparation*

Test		Result
		LD ₅₀ (μg./egg)
Complete complex, fraction 3*	Chick-embryo toxicity test	8.5
Lipid-free complex, fraction 5*		9.0
Soluble lipopolysaccharide		7.0
Lipopolysaccharide†		3.3
		LD ₅₀ (μg./20 g. mouse)
Complete complex, fraction 3*	Mouse toxicity test	750
Lipopolysaccharide†		250
		Lowest reacting dose (μg./rabbit)
Complete complex, fraction 3*	Shwartzman skin test	0.25
Lipid-free complex, fraction 5*		0.025
Lipopolysaccharide†		0.025

* See Table 1 and the text.

† Taylor *et al.* (1966).

four main fatty acids, of the lipid extracted from either cells or complex in five successive experiments (Table 4).

The 'residual fatty acids', left after removal of extractable lipid from the lipopolysaccharide complex, contained mainly the two saturated shorter-chain fatty acids (C₁₂ and C₁₄) and hydroxy C₁₄ acid (Table 4). These three fatty acids accounted for 90% of the fatty acid content of soluble lipopolysaccharide prepared from the complex and are known to be constituents of the lipid A moiety (Taylor *et al.* 1966).

Biological properties of lipopolysaccharide complex. Table 5 shows that, weight for weight, the toxicity for mice or for chick embryos of the lipopolysaccharide complex was about one-third that of the purified lipopolysaccharide prepared by phenol extraction, ethanol and magnesium chloride precipitation (Taylor *et al.* 1966). Removal of lipid from the complex had little effect on toxicity.

All preparations were highly active in the Shwartzman skin-sensitizing test, the lowest reacting doses varying from 0.025 to 0.25 μg. in a 3 kg. rabbit.

The lipopolysaccharide complex produced an antiserum reacting with lipopolysaccharide complex, protein and purified lipopolysaccharide. On Ouchterlony plates, there was only one line of precipitation with lipopolysaccharide and two to four with complex. The complex also reacted with antisera prepared against lipopolysaccharide or protein; the protein antiserum gave a single line with the complex and did not react with lipopolysaccharide.

Lipid component of cell walls

Since the lipopolysaccharide of the complex is known to be a constituent of cell walls, it was

decided to determine the origin of the phosphatidylethanolamine. Bishop & Work (1965) were unable to detect this lipid in a cell-wall preparation, though there was an unidentified phosphate-containing product reacting with ninhydrin (now thought to be lysophosphatidylethanolamine) together with free fatty acids. Seven preparations of freeze-dried cell walls were extracted by the method described for extraction of lipid from freeze-dried complex. Only one preparation contained phosphatidylethanolamine as the major phospholipid; the other preparations had mainly lysophosphatidylethanolamine and only traces of phosphatidylethanolamine. The seven freeze-dried wall preparations were from separate batches of cells known to contain phosphatidylethanolamine and included walls from both fresh and freeze-dried cells of *E. coli* 12408 and of the parent wild-type strain (11396). During wall preparation, the use of crystalline trypsin instead of the usual crude trypsin preparation, which possibly contained phospholipase A, had no effect on lipid composition. No reason has been found for the apparent conversion of phosphatidylethanolamine of cells into its lyso compound in wall preparations.

EXPLANATION OF PLATE I

(a) Negatively stained (ammonium molybdate) preparation of aqueous solution (5 mg./ml.) of lipopolysaccharide complex separated from culture filtrate by gel filtration. Magnification × 120 000. (b) Negatively stained (phosphotungstic acid) preparation of lipopolysaccharide complex separated from culture filtrate by precipitation with chloroform and centrifuging; the sediment was resuspended in water. A drop of chloroform was added to the preparation on the grid before adding the stain. Magnification × 80 000. (c) Thin section of lipopolysaccharide complex precipitated from culture filtrate by chloroform. Magnification × 160 000.

Morphology of lipopolysaccharide complex

The appearance of negatively stained preparations of extracellular particles, both in the whole cultures actively excreting lipopolysaccharide complex and in the pellet deposited by centrifuging the culture filtrate at 100000*g* for 1 hr., has already been described (Knox *et al.* 1966). In all cases there were mixtures of spheres, having boundary membranes, globules and stacked leaflets ranging in diameter from 12 to 200 μ . The aqueous solutions of the lipopolysaccharide complex that had been separated from the culture filtrate on Sephadex also showed spheroids ranging in diameter from 12 to 200 μ mixed with large irregularly shaped particles, and rods of varying lengths with diameter 12 μ ; no stacking was observed. The spheroids had boundary membranes that were sometimes double, having the appearance of two light bands separated by a dark line of variable thickness (Plate 1*a*). The ammonium molybdate stain appeared to have penetrated into all the spheroids.

The complex separated by chloroform precipitation was of very variable appearance. When fixed in the presence of chloroform it presented a lamellar appearance both in negatively stained preparation (Plate 1*b*) and in section (Plate 1*c*). The lamellae were derived from pleated structures resembling unit membranes. When the chloroform was removed and the complex was dispersed in water to give a 0.5% (w/v) solution, various forms were apparent, even on the same grid (Plate 2*a* and 2*b*). Closely packed spheroids had their boundaries coalescing or overlapping to produce regions of lamellar structures (Plate 2*a*). Some of the spheroids were very large (up to 2000 μ , not shown here). In certain regions of the grid there were white dense areas round which were clustered numerous spheroids. No rods were visible in the chloroform-precipitated complex, but many small spheres (12 μ) were always present.

After removal of protein and lipid from the complex, the water-soluble lipopolysaccharide presented a mixture of hollow spheroids and rods of the usual size range (Plate 2*c*). A preparation dissolved in buffer at pH 4.6 (Plate 2*d*) appeared to have

aggregated; the rods were often aligned in parallel groups.

DISCUSSION

When *E. coli* A.T.C.C. 12408 is grown under specific lysine-limiting conditions a variety of products are known to accumulate in the culture fluid; one of these, designated lipoglycopeptide (Bishop & Work, 1965), contains a lipopolysaccharide component (Taylor *et al.* 1966). The procedure of Bishop & Work (1965) for isolating lipoglycopeptide included the prior extraction of some phospholipid from the culture filtrate with chloroform; this apparently rendered the remaining lipopolysaccharide complex insoluble in water. The phosphatidylethanolamine detected in the final preparation was then regarded as a contaminant. It has now been shown that the lipopolysaccharide is present in the culture fluid as part of a complex containing approximately 60% of lipopolysaccharide, 26% of lipid (mostly phosphatidylethanolamine) and 11% of protein. Isolation depends on aggregation of the complex by chloroform, or other organic compounds, into micellar-type structures of high specific gravity. As a product of similar composition was obtained by Sephadex chromatography of culture filtrate, it is concluded that the association of the components was not an artifact caused by chloroform.

The three components of the complex are the same as those found in O-somatic antigen preparations obtained by extraction of smooth strains of Gram-negative bacteria with various solvents such as trichloroacetic acid or glycols (for reviews see Davies, 1960; Lüderitz, Staub & Westphal, 1966). *E. coli* A.T.C.C. 12408 is a rough strain; these solvents do not extract the somatic antigen from rough organisms, and therefore it has not been possible to compare our extracellular complex with the complete somatic antigen.

The complex isolated from the culture fluid contains the lipopolysaccharide component already shown to be indistinguishable chemically and serologically from the cellular lipopolysaccharide (a known component of cell walls) and having the biological properties of an endotoxin (Taylor *et al.* 1966). Phosphatidylethanolamine, another major component of the complex, is the main phospholipid of cell walls of *E. coli* (Gray & Wilkinson, 1965); tests for its presence in the cell-wall preparations from both parent and mutant strains used in these Laboratories have given variable results, due apparently to its conversion into the lyso form during the preparation of walls. An active association of lipopolysaccharide with phosphatidylethanolamine, at least during the biosynthesis of lipopolysaccharide, is indicated by the studies of Rothfield & Takeshita (1965) and Rothfield &

EXPLANATION OF PLATE 2

(*a*) and (*b*) Negatively stained (phosphotungstic acid) preparation of lipopolysaccharide complex precipitated from culture filtrate with chloroform, resuspended in water and chloroform removed (fraction 1; see the Results section) (5 mg./ml.). Magnification $\times 80000$. (*c*) Negatively stained (ammonium molybdate) preparation of soluble lipopolysaccharide (9 mg./ml.). Magnification $\times 80000$. (*d*) Negatively stained (phosphotungstic acid) preparation of soluble lipopolysaccharide (8 mg./ml.) in 25 mm.-ammonium acetate buffer, pH 4.6. Magnification $\times 80000$.

Pearlman (1966), who showed that the enzymic transfer of a glycosyl residue to lipopolysaccharide was dependent on the prior formation of a lipopolysaccharide-phosphatidylethanolamine complex. The fatty acids of the extractable lipids and of phosphatidylethanolamine are those commonly found in bacteria; hexadecanoic acid (palmitic acid) was the main saturated fatty acid, and the two monoenoic acids, hexadecenoic acid and octadecenoic acid, constituted the other main acids. There was always less than 10% of the cyclopropane acid methylenehexadecanoic acid, sometimes less than 5%. In phosphatidylethanolamine, hexadecenoic acid and methylenehexadecanoic acid were almost exclusively esterified in the β -position, a result confirmed by van Golde & van Deenen (1967) on another sample. This is in accord with the known distribution of fatty acids in most naturally occurring phospholipids, including those from *E. coli* (Hildebrande & Law, 1964).

Under the cultural conditions used biphasic growth occurred; the serologically active lipopolysaccharide complex was excreted continuously from the end of the first exponential growth phase at 8 hr. through a stationary phase lasting about 10 hr., and into a secondary growth phase, ending at 26 hr. (Knox *et al.* 1966; Work *et al.* 1966). This pattern of excretion differs from that reported by Maurino & Municio (1966); biphasic growth of lysine-requiring *E. coli* 26-26 also caused a rise in serological activity of the culture filtrate, but only in phases of active growth. Our lipopolysaccharide complex represented a cumulative sample of material produced only during the stationary phase and the secondary growth phase. After 26 hr. in the fermenters, the highly significant similarity between the fatty acid content of extractable lipids of whole cells and of the complex suggests that there could have been no major changes in fatty acid composition of the cells after the end of the first exponential growth phase. In *E. coli*, as in other bacteria that synthesize cyclopropane acids, an accumulation of cyclopropane acids can occur in the post-exponential phase under certain cultural conditions (Knivett & Cullen, 1965, 1967; Marr & Ingraham, 1962; Law, Zalkin & Kaneshiro, 1963). In the present case, the low concentrations of cyclopropane acids found both in the lysine-limited cells and in the lipopolysaccharide complex suggests that the cultural conditions were not conducive to cyclopropane acid accumulation, but the concentrations have not been followed over the whole growth cycle. These results resemble those of Kates, Adams & Martin (1964), who found that a crude preparation of extracellular lipopolysaccharide complexes from *Serratia marcescens* contained similar extractable lipids to those in whole cells and that a white strain

of the organism produced only low amounts of cyclopropane fatty acids.

In *E. coli* 12408 there was a marked difference between the main fatty acid components of the extractable lipids of the lipopolysaccharide complex (C_{16} - C_{18} fatty acids) and the covalently bound lipid A of the pure lipopolysaccharide (mainly C_{12} - C_{14} acids, including hydroxy C_{14} acid) (see also Bishop & Work, 1965; Taylor *et al.* 1966). Removal of extractable lipids from the complex left only the saturated shorter-chain acids specific to lipid A in the residue, suggesting that lipid A was the only covalently linked lipid in the complex. The lipid A moiety can only be separated by a hydrolytic step and so would not be evident in lipid extracts made from lipopolysaccharides under milder conditions such as those used by Kates *et al.* (1964).

Protein, the third major component of the lipopolysaccharide complex, is also an integral component of cell walls of Gram-negative bacteria (Salton, 1964). The separated protein component of the lipopolysaccharide complex resembles in its solubility the proteins of membranous structures such as mitochondrial or chloroplast membranes. In composition it resembles that of other bacterial membrane proteins in having an excess of acidic over basic amino acids (Brown, 1964), but the low cystine content is consistent with that found in other bacterial extracellular proteins (Pollock & Richmond, 1962). Homma & Suzuki (1964, 1966) isolated and analysed the proteins both of the lipopolysaccharide complex and of a fraction of the walls of *Pseudomonas aeruginosa*; the two proteins had identical serological properties and amino acid contents. Although these proteins differed from that of the *E. coli* 12408 lipopolysaccharide complex in amino acid ratios, here too there was very little cystine and an excess of acidic over basic amino acids.

Evidence is now accumulating for the localization of cellular lipopolysaccharides in the outer layers of cell walls (Leive, 1965; Homma & Suzuki, 1966; Mergenhagen, Bladen & Hsu, 1966). It has also been suggested (Work *et al.* 1966; Knox *et al.* 1966) that with *E. coli* 12408 the extracellular lipopolysaccharide complex originates from the outer layers of the walls by a process of unbalanced growth.

Studies on O-somatic endotoxin complexes have shown that they were heterogeneous, both physically and morphologically (Beer, Braude & Brinton, 1966; Nowotny *et al.* 1966). Similar results have been obtained with extracellular lipopolysaccharide complex (B. D. Ward & E. Work, unpublished work; Plate 1a and Plate 2a and 2b). The usual picture of all these complexes shows a mixture of irregularly distributed rods and hollow spheres of varying sizes; the spheres were bounded by leaflets and in certain pictures (Plate 2b) were very similar

in size and appearance to those seen in preparations of lipopolysaccharide (Plate 2c; Rothfield & Horne, 1967). When these authors combined lipopolysaccharide with phosphatidylethanolamine they found continuity between the leaflets of the envelope of the lipopolysaccharide spheres and the lamellae of the phospholipid. The marked orientating effect (Plate 1b) of chloroform on the lipopolysaccharide complex suggests that under these conditions the phospholipids in the complex tend to form lamellae; the picture resembles the 'fingerprint' appearance of phosphatidylethanolamine and phosphatidylcholine (Rothfield & Horne, 1966; Bangham & Horne, 1964; Lucy & Glauert, 1964). Under other conditions the phospholipid appears to form a globule round which are clustered numerous spheroids resembling lipopolysaccharides (Plate 2a); these appear to merge into the phospholipid globules as in certain cases noted by Rothfield & Horne (1966). The protein appears to have little influence on the general appearance of the complex.

The fact that the isolated lipopolysaccharide complex resembled the wall blebs and extracellular globules of cells actively excreting complex supports the idea that this complex originates from the outer membranes of the walls (Knox *et al.* 1966).

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