Chemical and Biological Properties of an Extracellular Lipopolysaccharide from *Escherichia coli* Grown under Lysine-Limiting Conditions

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Lipopolysaccharide was prepared from the extracellular lipoglycopeptide produced by the lysine-requiring mutant *Escherichia coli* A.T.C.C. 12408 grown under lysine-limiting conditions. The lipid moiety, containing glucosamine phosphate and four fatty acids (lauric acid, myristic acid, β-hydroxymyristic acid and palmitic acid) corresponded in composition to lipid A of known bacterial lipopolysaccharides. The components of the polysaccharide moiety were D-glucose, D-galactose, L-*glycero*-D-*manno*-heptose, 3-deoxy-2-oxo-octonic acid, ethanolamine and phosphate. These are the constituents of the polysaccharide of the cell-wall antigens from rough strains of *E. coli*. Lipopolysaccharides were also prepared from whole cells of *E. coli* 12408 grown with excess or limited amounts of lysine; they were identical in carbohydrate composition with the extracellular lipopolysaccharide. The biological properties of this material also resembled those of known lipopolysaccharides; it was antigenic, pyrogenic, toxic and had adjuvant activity.

The lysine-dependent mutant A.T.C.C. 12408 of *Escherichia coli* produces an extracellular high-molecular-weight lipid, designated lipoglycopeptide, when grown under lysine-limiting conditions (Bishop & Work, 1965). This substance, which contained lipids, sugars and amino acids, proved to be related antigenically to certain fractions, including the lipopolysaccharide, from cell walls; the fatty acid composition of one of its lipid fractions suggested that lipoglycopeptide was a complex of other molecules with lipid A, the lipid moiety of lipopolysaccharides from Gram-negative bacteria (Westphal & Lüderitz, 1954). It seemed reasonable to assume therefore that another part of the complex was the polysaccharide based on a poly-heptose phosphate which is a characteristic component of lipopolysaccharides.

The present work describes the identification of the constituents of lipopolysaccharides prepared both from the extracellular lipoglycopeptide of *E. coli* 12408 and from the cells of this organism. The extracellular lipopolysaccharide was also examined for the physical and biological properties known to be associated with endotoxins.

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MATERIALS AND METHODS

Micro-organism and method of culture. The strain of *E. coli* A.T.C.C. 12408 was that used by Bishop & Work (1965). Dr J. Taylor, Central Public Health Laboratory, Colindale, London, N.W. 9, has kindly identified the organism as a rough strain of *E. coli* (personal communication). Cultures were grown for 26 hr. in defined medium (101.) in a fermenter under the conditions described by Bishop & Work (1965). 'Lysine-deficient' cells were grown in the defined medium containing glycerol and 60 mg. of lysine hydrochloride/l.; the medium for control experiments on 'normal' cells contained 200 mg. of lysine hydrochloride/l. After growing the bacteria in either medium for 26 hr. at 37°, culture filtrate was separated from the cells by centrifugation and filtration; the cells were washed with distilled water and dried from the frozen state.

Cellular and extracellular lipopolysaccharides from E. coli. Lipoglycopeptide was prepared from the culture filtrate of 'lysine-deficient' cells as described by Bishop & Work (1965): concentrated culture filtrate was extracted with chloroform and the aqueous phase left for several days at 4°; the precipitate (lipoglycopeptide) that formed was collected, dialysed and dried from the frozen state. Purification involved as a first step the procedure of Westphal, Lüderitz & Bister (1952) for extracting lipopolysaccharide from bacterial cells; in this case, undialysed lipoglycopeptide was extracted since only inorganic phosphates (constituents of medium) and amino acids were removed by dialysis. Analyses were carried out on dialysed material. Lipo-glycopeptide (undialysed, 10 g.) was suspended in 500 ml. of water at 65° and 500 ml. of aq. 90% (w/v) phenol at 65°

was added. The mixture was stirred at 65–68° for 20 min. and cooled to 4°; the resulting two layers were separated by centrifuging at 4° and the lower phenol layer was extracted a second time with 500 ml. of water under the same conditions. The pooled aqueous layers were extracted twice with 300 ml. of diethyl ether, concentrated to 500 ml. *in vacuo* at 35° and treated with ethanol (10 vol.) together with a few milligrams of sodium acetate. After standing overnight at 2°, the resulting precipitate was removed by centrifugation, dissolved in 0·05 M-tris–HCl buffer, pH 7·2 (1750 ml.), and reprecipitated by the addition of $MgCl_2$ (final concn. 0·025 M) (Osborn, Rosen, Rothfield & Horecker, 1962). The precipitate was dissolved in 0·02 M-EDTA, pH 7·0, and dialysed against 0·001 M-EDTA (daily changes for 3 days) followed by glass-distilled water (daily changes for 2 days). The preparation, designated 'extracellular lipopolysaccharide', was dried from the frozen state.

Cellular lipopolysaccharides were extracted from 'normal' and 'lysine-deficient' cells by the method of Westphal *et al.* (1952) as modified by Lüderitz *et al.* (1965); in this procedure lipopolysaccharide extracted with hot aqueous phenol is purified by repeated ultracentrifugation at 100000 g. In order that a comparison of these preparations with extracellular lipopolysaccharide should be valid, lipopolysaccharide extracted from another preparation of lipoglycopeptide was also isolated from the aqueous phase by ultracentrifugation instead of by precipitation. Studies on this preparation were confined to quantitative analyses for carbohydrates, nitrogen and phosphorus.

A commercial preparation of *E. coli* O111:B4 lipopolysaccharide, prepared by the method of Westphal *et al.* (1952), was obtained from Difco Laboratories, Detroit, Mich., U.S.A.

Carbohydrates. Samples of L-*glycero*-D-*manno*-heptose and colitose were kindly provided by Dr H. Nikaido, Harvard Medical School, Boston, Mass., U.S.A., and of 3-deoxy-2-oxo-octonic acid by Dr E. C. Heath, Johns Hopkins University School of Medicine, Baltimore, Md., U.S.A. Crystalline 3-deoxy-2-oxogluconic acid, synthesized by Dr G. B. Paerels (Paerels, 1961), was kindly made available by Dr J. N. Walop, N.V. Philips–Duphar, Weesp, The Netherlands.

Enzymes. Glucose-oxidase reagent (Glucostat) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; galactose oxidase (grade X3) was purchased from Hughes and Hughes (Enzymes) Ltd., London, W. 1.

Paper electrophoresis and chromatography. The conditions of hydrolysis differed for the compounds under investigation and are described in the Results section. Electrophoresis was carried out on Whatman no. 3 paper at 7–10 v/cm. in either 0·1 M-pyridine–acetic acid buffer, pH 5·3, or in 0·025 M-sodium tetraborate. For chromatography the following solvents were used: *A*, saturated aq. phenol, NH_3 atmosphere; *B*, butan-1-ol–pyridine–water (6:4:3, by vol.); *C*, butan-1-ol–acetic acid–water (4:1:5, by vol.); *D*, acetone–water (17:3, v/v); *E*, acetone–water (19:1, v/v); *F*, butan-2-one–acetic acid–water (8:1:1, by vol.); *G*, ethyl acetate–pyridine–water (2:1:2, by vol.); *H*, ethyl acetate–acetic acid–water (3:1:3, by vol.); *I*, saturated aq. phenol, acetic acid atmosphere. Components were revealed by the following reagents: reducing sugars with alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950); 3-deoxy-2-oxo-octonic acid with thiobarbituric acid (Warren, 1960) or semicarbazide (MacGee & Doudoroff,

1954); hexoses and heptoses with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950); amino compounds with ninhydrin (0·1%, w/v) in acetone; organic phosphorus with molybdate–perchlorate (Bandurski & Axelrod, 1951).

Analytical methods. Total nitrogen was determined by the Kjeldahl procedure and total phosphorus as described by Allen (1940). Hexosamines were determined by the method of Rondle & Morgan (1955), after hydrolysis for 4 hr. at 100° in 4 N-HCl. D-Glucose and D-galactose were estimated enzymically, after hydrolysis for 4 hr. at 100° in N-HCl, with glucose oxidase (Huggett & Nixon, 1957) and galactose oxidase (Roth, Segal & Bertoli, 1965) respectively. Heptose was determined by a modification (Osborn, 1963) of the method of Dische (1953). 3-Deoxy-2-oxo-octonic acid was determined by the thiobarbituric acid method of Weissbach & Hurwitz (1959) as modified by Osborn (1963), with 3-deoxy-2-oxogluconic acid as a standard. Free amino groups were estimated with 1-fluoro-2,4-dinitrobenzene (Ghuysen & Strominger, 1963).

Lipid components. Fatty acids were identified and estimated by gas–liquid chromatography of their methyl esters (Metcalfe & Schmitz, 1961) in the Pye Chromatograph with an Apiezon L column at 197°. A sample of β-hydroxymyristic acid was kindly supplied by Dr O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, Germany. Acetyl groups were estimated by the method of Ludowieg & Dorfman (1960).

Physical properties. Ultracentrifugal studies were performed in the Spinco analytical centrifuge (model E) at 31410 rev./min. Lipopolysaccharide was dissolved (4 mg./ml.) in each of the following buffers: 0·05 M-tris buffer, pH 8·6, containing 5% (v/v) of 0·02 M-EDTA; pyridine–acetic acid buffer, pH 6·1 (50 ml. of pyridine plus 4 ml. of acetic acid, diluted to 1250 ml. with water). Free electrophoresis in the Perkin–Elmer apparatus was carried out on solutions (5 mg./ml.) of lipopolysaccharide in each of the above buffers; at pH 8·6, the voltage applied was 185 v (27 min.), and at pH 6·1, 120 v (100 min.). Viscosities were measured at 25° in a standard 2 ml. Ostwald viscometer.

Biological methods

Extracellular lipopolysaccharide was dissolved (5 mg./ml.) by standing overnight in aq. 1% (w/v) phenol, and diluted to appropriate concentrations with 0·9% NaCl.

Antigenicity of preparations. Antisera to cells of *E. coli* 12408 were prepared by injecting New Zealand White rabbits (2·5–3 kg.) with two doses (intraperitoneal) of 5 mg. (dry wt.) of a washed cell suspension followed by eight doses of 10 mg. on alternate days. Antisera to extracellular lipopolysaccharide were prepared by injecting rabbits with 20 daily doses (intraperitoneal) of lipopolysaccharide or lipoglycopeptide; animals were bled 12 days after the last injection (Westphal *et al.* 1952).

The effectiveness of the antisera was tested by agglutination of a suspension of *E. coli* cells (Bishop & Work, 1965), and also in some cases by reaction with extracellular lipopolysaccharide, as determined by the quantitative precipitin method with 0·5 ml. of serum and increasing amounts of lipopolysaccharide up to 30 µg.; antibody protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Pyrogenicity test. Dutch rabbits (approx. 1·5–2·5 kg. body wt.) were used; they had been previously tested in a

trial with pyrogen-free saline (British Pharmacopoeia). Their mean body temperature was established over a period of 2 hr. on the test day. Each rabbit was injected intravenously (1 ml./kg. body wt.) with the test solution appropriately diluted; each substance was tested at two dose levels, three rabbits being used for each level. The body temperature was recorded for the next 5 hr. at intervals of 20 min.

Skin tests on rabbits. The primary inflammatory response of rabbits to endotoxin, giving rise to oedema or erythema or both, was examined (Westphal & Lüderitz, 1954; Larson, Ribi, Milner & Lieberman, 1960). Single doses of 0·2–8 μg. of lipoglycopeptide, extracellular lipopolysaccharide or *E. coli* O111:B4 lipopolysaccharide in 0·1 ml. of 0·9% NaCl were injected into the depilated skin of New Zealand White rabbits. Two rabbits were used for each substance. At 24 hr. after injection a provoking dose of 200 μg. of the corresponding substance/kg. body wt. was given intravenously. Readings of the reaction at the site of injection were taken after 2, 4, 6 and 24 hr., the skin being wiped with ethanol to make lesions visible (Larson *et al.* 1960).

Enhancement of primary antibody response. New Zealand White rabbits (2·5–3 kg.) were divided into five groups of five animals and all injected with 2 mg. of twice-recrystallized ovalbumin (Koch–Light Laboratories Ltd., Colnbrook, Bucks.). One group served as a control, while animals in each of the other groups received, simultaneously to the ovalbumin, either 2 μg. or 10 μg. of lipoglycopeptide or lipopolysaccharide. The rabbits were bled after 11 days and the anti-ovalbumin antibody content of the serum was determined by mixing serum (0·5 ml. or 1·0 ml.) with suitable amounts (5–50 μg.) of ovalbumin in a final volume of 1·5 ml.; five different concentrations of ovalbumin were used for each serum. After standing for 7 days at 2–4° the protein content of the precipitate was determined by the method of Lowry *et al.* (1951).

Toxicity. Mice (CFW strain; 15–20 g.) were sensitized by intraperitoneal injections of 0·5 ml. of a phenol-killed suspension of *Haemophilus influenzae* containing $1·5 \times 10^9$ organisms/dose. After 6 days, 0·2 ml. of the test solution was injected intraperitoneally and deaths were recorded over a period of 2 days. Five mice were used in each group.

RESULTS

Properties of the extracellular lipopolysaccharide. A yield of 3·0 g. of lipopolysaccharide was obtained from 10·0 g. of undialysed lipoglycopeptide (equivalent to 7 g. of dialysed lipoglycopeptide). Free amino acids were absent from the purified lipopolysaccharide, although detectable at stages before the final dialysis. Protein was not detectable by the method of Lowry *et al.* (1951). A solution (1 mg./ml.) of lipopolysaccharide in 0·05 m-tris–hydrochloric acid buffer, pH 7·2, was examined for light-absorption in the ultraviolet; nucleic acid was not detectable. During the preparation of lipopolysaccharide it was noticed that Mg^{2+}-precipitated material in the process of being dissolved in 0·02 m-EDTA was stringy and sticky, reminiscent of strong solutions of DNA. On examination by electron microscopy, after negative staining with phosphotungstic acid, long threads were seen, forming bunches of parallel filaments (Fig. 1).

The lipopolysaccharides gave clear aqueous solutions only when organic compounds were present, and such solutions (5 mg./ml.) became turbid when inorganic salts were added. For this reason solutions of extracellular lipopolysaccharides
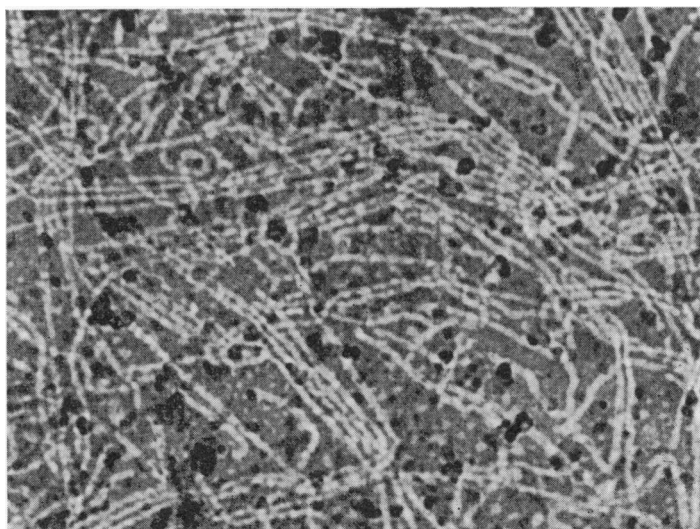

Fig. 1. Electron microphotograph of Mg^{2+}-precipitated lipopolysaccharide redissolving in 0·02 m-EDTA, pH 7·0. Negative staining with phosphotungstic acid was used. Magnification × 84000.

in tris–EDTA buffer, pH 8·6 (0·05 M), and pyridine-acetic acid buffer, pH 6·0 (0·1 M), were used for the examination of some physical properties. Free electrophoresis showed single peaks moving towards the anode; the peak in tris–EDTA buffer was abnormally sharp. Examination of the lipopolysaccharide in the analytical ultracentrifuge gave values for the sedimentation coefficient that depended on the buffer employed: S values were 8 s in pyridine–acetic acid buffer, pH 6·1, and 58 s (for the major component) in tris–EDTA buffer, pH 8·6. These differences are attributed to varying degrees of aggregation in the different buffers; this effect has frequently been described for other lipopolysaccharides. Concentrated solutions were markedly viscous; a concentration of 5 mg./ml. in 0·02 M-tris–0·01 M-EDTA buffer, pH 7·0, gave a reduced viscosity of 242 ml./g.

Identification of water-soluble components of acid-hydrolysed extracellular lipopolysaccharide. Lipopolysaccharide was hydrolysed for 30 min. at 100° in N-sulphuric acid; the insoluble lipid was removed and the soluble oligosaccharides were hydrolysed for a further 15 hr. at 100° to liberate heptose (Kauffman, Lüderitz, Stierlin & Westphal, 1960a). After cooling, the solution was neutralized with barium hydroxide, barium sulphate was removed by centrifugation and the supernatant solution evaporated *in vacuo*. For detection of carbohydrates a chromatogram on Whatman no. 1 paper was run in solvent A and sprayed with p-anisidine hydrochloride. The components detectable were indistinguishable from glucose, galactose (both giving a greenish-brown spot) and L-*glycero*-D-*manno*-heptose (purple spot). Paper electrophoresis in 0·025 M-sodium tetraborate (Ribi *et al.* 1960) for 8·5 hr. at 7 v/cm. confirmed this result. Further evidence for the presence of D-glucose, D-galactose and heptose has been provided by the results of quantitative analyses (see below). Hydrolysis of lipopolysaccharide for a shorter interval (2 hr. at 100° in N-sulphuric acid) followed by chromatography in solvents A, B, C and E failed to reveal the presence of colitose, pentoses or 6-deoxyhexoses.

To test for the presence of 3-deoxy-2-oxo-octonic acid, lipopolysaccharide was hydrolysed in 0·25 N-sulphuric acid at 100° for 25 min. A component, indistinguishable from authentic 3-deoxy-2-oxo-octonic acid on chromatography in solvent B, was eluted from a chromatogram run in this solvent, and examined further by chromatography in solvents D, F, G and H, and by electrophoresis at pH 5·3 for 3 hr. at 9 v/cm. In each instance the eluted compound had the same mobility as the authentic compound. Further, the eluate gave a reaction in the thiobarbituric acid test characteristic of a 3-deoxy-2-oxoaldonic acid ($\lambda_{max.}$ 550 mμ).

For investigating amino compounds, lipopoly-saccharide was hydrolysed with 5 N-hydrochloric acid for 16 hr. at 100°, and hydrochloric acid was then removed by repeated evaporation *in vacuo* over potassium hydroxide. The hydrolysate was examined by electrophoresis in pyridine–acetic acid buffer, pH 5·3. Three ninhydrin-positive components were detected. A minor fast-moving electropositive component was identical in mobility with ethanolamine; its identity as ethanolamine was confirmed by chromatography in solvent I. A slower-moving electropositive component had the same mobility as glucosamine and was indistinguishable from glucosamine on chromatography in solvent B; identification of glucosamine was supported by its conversion with ninhydrin into arabinose (Stoffyn & Jeanloz, 1954). The third component obtained on paper electrophoresis remained at the origin; it reacted with alkaline silver nitrate and gave a positive reaction for organic phosphate. An eluate containing this component, on hydrolysis for a further 16 hr. at 100° in 5 N-hydrochloric acid, produced additional glucosamine and phosphate; the compound is thus presumed to be glucosamine phosphate. No amino acids were detectable in hydrolysates of lipopolysaccharide applied to chromatograms in amounts equivalent to 0·4 mg. of original material.

Analysis of the lipid component of extracellular lipopolysaccharide. Bound lipid was released from lipopolysaccharide (200 mg.) by refluxing a suspension in 8 ml. of N-sulphuric acid for 30 min. The precipitated lipid was thoroughly washed with water and dried from the frozen state (yield, 55 mg.).

Before further analysis, free fatty acids were selectively extracted from the dried lipid with freshly redistilled ethyl formate (11 ml.) (Wander-Forschungsinstitut, 1960) by shaking vigorously for 1 hr. at 18°, followed by 5 min. at 54°. The insoluble residue (lipid A) was removed by centrifugation and dried. The supernatant solution, containing free fatty acids, was evaporated to dryness *in vacuo*, redissolved in a small volume of chloroform and dried (yield, 7 mg.). The results of the analysis of fatty acid components by gas–liquid chromatography are shown in Table 1; β-hydroxymyristic acid, lauric acid and myristic acid were the main components.

A portion (28 mg.) of the lipid A was hydrolysed for 16 hr. at 100° in 5 N-hydrochloric acid, and the hydrolysate extracted three times with 3 ml. portions of diethyl ether and twice with 2 ml. portions of chloroform. The combined ether–chloroform extracts were washed with an equal volume of water and the aqueous phase was retained for subsequent examination (see below). The organic phase was dried over sodium sulphate and evaporated to dryness *in vacuo*. The yield of fatty acids thus obtained was 17·5 mg., representing

62% of the weight of lipid hydrolysed. Gas–liquid chromatography of methyl esters indicated that β-hydroxymyristic acid was the main component, with myristic acid and lauric acid as the other major components of this fraction (Table 1).

The aqueous phase remaining after extraction of fatty acids from the hydrolysate of lipid was examined for the presence of amino compounds by the methods used above. Glucosamine, glucosamine phosphate and a trace of ethanolamine were detected.

Quantitative analysis of lipid A showed the presence of 20·9% of hexosamine and 2·75% of organic phosphorus (8·7% of phosphate). Fatty acids, glucosamine and phosphate thus accounted for 92% of the lipid fraction (without correction for water regain during hydrolysis).

Quantitative analysis of cellular and extracellular lipopolysaccharides. Analyses for carbohydrate components, nitrogen and phosphorus were carried out on lipoglycopeptide, and the lipopolysaccharide obtained from lipoglycopeptide by phenol extraction followed by precipitation with ethanol and Mg^{2+}. These results are compared in Table 2 with those for the lipopolysaccharide fractions obtained by ultracentrifugation from lipoglycopeptide and from 'normal' and 'lysine-deficient' cells.

The carbohydrate composition of extracellular lipopolysaccharide was similar to that of both cellular lipopolysaccharides, the range of values being 9–12% for glucose and galactose, 18–19% for heptose and 5–6% for glucosamine. The results also suggest that lipopolysaccharide accounts for approx. 90% of the lipoglycopeptide. Lipoglycopeptide and cellular lipopolysaccharides contain more nitrogen than extracellular lipopolysaccharides; this can be attributed to their amino acid content.

Chromatographic studies of extracellular lipopolysaccharide showed the presence of ethanolamine, but no amino acids; quantitative analysis gave 0·23% of amino N, indicating that ethanolamine did not exceed 1%. Extracellular lipopolysaccharide also contained 0·1 μmole of bound acetic acid/mg.

The polysaccharide component of extracellular lipopolysaccharide (200 mg.) was obtained with minimal degradation by heating at 100° for 15 min. in 10 ml. of 0·01 N-acetic acid (Osborn, 1963). (On more prolonged hydrolysis some galactose was liberated.) The suspension was then cooled, centrifuged at 100 000 g for 2 hr. and the supernatant liquid dialysed and dried from the frozen state (yield, 60 mg.). The results of analysis of the polysaccharide (Table 2) indicated that the major carbohydrate components are heptose, glucose and galactose; glucosamine was present only in trace amounts. The relative rates of release of galactose

Table 1. *Fatty acid composition of ethyl formate extract and residual lipid (lipid A) from acid-hydrolysed lipopolysaccharide*

The results of gas–liquid chromatography of fatty acid methyl esters on Apiezon L at 197° are given.

Fatty acid	Composition (%, w/w, of total methyl esters)	
	Ethyl formate extract	Lipid A
Lauric acid	24·0	26·8
Myristic acid	32·7	16·5
β-Hydroxymyristic acid	29·9	42·8
Palmitic acid	10·4	3·1
Hexadecenoic acid	1·3	1·6
Unknown*	1·6	8·3

* Carbon number 14·6.

Table 2. *Comparison of composition of cellular and extracellular lipopolysaccharides*

Results are expressed as percentages of the dry weight of the fraction. LPS, Lipopolysaccharide.

Fraction	Heptose*	D-Glucose	D-Galactose	Glucosamine	3-Deoxy-2-oxo-octonic acid	Nitrogen	Phosphorus
Lipoglycopeptide	16·4	8·7	9·1	3·5	1·4	1·8	3·4
Extracellular LPS (Mg^{2+}-precipitated)	18·9	9·6	10·1	6·2	1·7	1·1	3·9
Extracellular LPS (ultracentrifuged)	18·7	10·5	9·2	5·4	1·9	1·0	3·8
LPS from normal cells	17·8	11·9	10·7	5·2	1·8	1·7	4·1
LPS from lysine-deficient cells	19·2	11·8	11·7	6·3	1·8	1·7	3·7
Polysaccharide from extracellular LPS	30·0	18·5	14·4	1·0	0·9	0·4	3·8

* Estimated as L-*glycero*-D-*manno*-heptose.

Table 3. *Agglutination of cells of* E. coli 12408 *by antisera to whole cells and to extracellular products*

A fresh suspension of cells grown on nutrient agar was mixed with serial dilutions of antisera in Perspex haemagglutination plates. Plates were rocked gently for 2 hr. at 37° and the greatest dilution giving agglutination of cells was recorded after 20 hr. (agglutination titre before injection, 1:20–1:80). Antisera were prepared as described in the Materials and Methods section.

Substance injected	No. of doses and amount injected	Agglutination titre for each antiserum
Whole cells	2 × 5 mg., 8 × 10 mg.	1:2560
Extracellular lipopolysaccharide	20 × 1 μg.	1:160 1:320
Extracellular lipopolysaccharide	20 × 10 μg.	1:1280 1:2560
Extracellular lipopolysaccharide	20 × 100 μg.	1:2560 1:10240
Lipoglycopeptide	2 × 5 μg., 8 × 10 μg.	1:1280 1:1280
Lipoglycopeptide	2 × 50 μg., 2 × 100 μg.	1:2560 1:2560

and glucose from polysaccharide on hydrolysis in 0·1 N-hydrochloric acid at 100° were such that after 30 min. all of the galactose was present as the monosaccharide, whereas only 10% of the glucose had been released.

Biological properties of the extracellular lipopolysaccharide

The biological activity of extracellular lipopolysaccharide was established by tests for antigenicity, pyrogenicity, toxicity, skin reaction in rabbits and ability to act as an adjuvant. Its activity was compared with that of the original lipoglycopeptide from which it was obtained.

Antigenicity. Extracellular lipopolysaccharide and lipoglycopeptide were antigenic, repeated injections of 10 μg. amounts giving antisera with moderate antibody titres (Table 3). A higher titre was obtained in one animal receiving 100 μg. injections of lipopolysaccharide. Antiserum to extracellular lipopolysaccharide, with an agglutination titre of 1:2560, was shown (quantitative precipitin) to contain 120 μg. of antibody protein/ml. The reaction between extracellular lipopolysaccharide and antiserum to whole cells was followed (Fig. 2); the antiserum in this experiment contained 270 μg. of antibody protein/ml.

The conclusion of Bishop & Work (1965) that lipopolysaccharide from *E. coli* O111:B4 cross-reacted with antisera to *E. coli* 12408 cells, cell wall and lipoglycopeptide depended on what now appears to be a non-specific agglutination, as it could not be confirmed by the qualitative precipitin (ring) test; nor did extracellular lipopolysaccharide cross-react with antiserum to *E. coli* O111:B4.
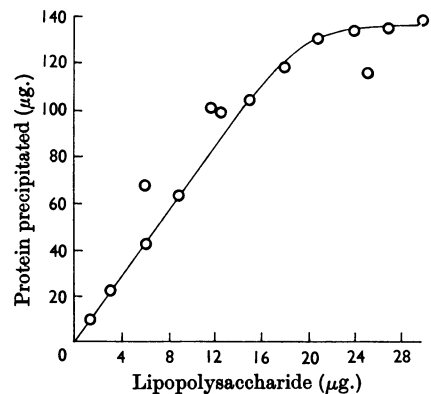

Fig. 2. Precipitin reaction between extracellular lipopolysaccharide (in 1·5 ml. of 0·9% NaCl) and antiserum to whole cells (0·5 ml.). The amount of antibody in the precipitate was determined by the method of Lowry *et al.* (1951).

Colitose, the specific determinant of *E. coli* O111:B4 lipopolysaccharide (Kauffman, Braun, Lüderitz, Stierlin & Westphal, 1960b), was not detected in the extracellular lipopolysaccharide.

Pyrogenicity. Extracellular lipopolysaccharide was highly pyrogenic, a dose of $2·5 \times 10^{-5}$ μg./kg. body wt. producing temperature rises of 0·55°, 0·85° and 1·2° in each of three rabbits tested; doses of 25×10^{-5} μg./kg. gave rises of 1·25°, 1·45° and 1·50°. With extracellular lipoglycopeptide and *E. coli* O111:B4 lipopolysaccharide, doses of 250×10^{-5} μg./kg. were required to give temperature rises comparable with those obtained with 25×10^{-5} μg. of extracellular lipopolysaccharide/kg. In all cases,

Table 4. *Enhancement of antibody response to ovalbumin by simultaneous administration of lipoglycopeptide or extracellular lipopolysaccharide*

The antibody content of sera was determined by the quantitative precipitin method. Results, expressed as means $\pm$ S.E.M., were examined by Student's t test and values for P are given.

Material injected with ovalbumin	No. of rabbits	Antibody protein (μg./ml. of serum)	P
None	5	71 ± 23	—
Lipoglycopeptide ($2\,\mu$g.)	5	120 ± 26	> 0.05
Lipoglycopeptide ($10\,\mu$g.)	5	280 ± 55	< 0.01
Lipopolysaccharide ($2\,\mu$g.)	5	263 ± 39	< 0.01
Lipopolysaccharide ($10\,\mu$g.)	4*	411 ± 34	< 0.001

* The fifth rabbit died within 1 day of injection.

the rise and fall of temperature characteristic of pyrogenic responses were observed.

Skin tests on rabbits. Lipoglycopeptide, extracellular lipopolysaccharide and *E. coli* O111:B4 lipopolysaccharide had comparable activities in producing local inflammation in the skin of sensitized rabbits, doses of 0.5–$1\,\mu$g. of each substance giving a positive reaction in 4 hr.; with the extracellular products erythema was more pronounced, whereas with *E. coli* O111:B4 lipopolysaccharide the response was more oedematous.

Enhancement of primary antibody response. Extracellular lipopolysaccharide was an effective adjuvant, the injection of $2\,\mu$g. simultaneously with ovalbumin causing a marked increase in the anti-ovalbumin antibody content of serum (Table 4). Lipoglycopeptide was less effective, $10\,\mu$g. being required to give an increase comparable with that given by $2\,\mu$g. of lipopolysaccharide.

Toxicity. The LD$_{50}$ values for lipoglycopeptide and extracellular lipopolysaccharide were $900\,\mu$g. and $300\,\mu$g. respectively.

DISCUSSION

The cell-wall lipopolysaccharides that determine the O-somatic antigen specificity of the Enterobacteriaceae are known to consist of a complex polysaccharide joined to a glucosamine-containing lipid (Westphal & Lüderitz, 1954). The composition of the polysaccharide shows wide variability between strains, but glucose, galactose and L-*glycero*-D-*manno*-heptose are characteristic components (Kauffman *et al.* 1960*a,b*). The heptose is phosphorylated (Slein & Schnell, 1953), and studies on the structure and biosynthesis of lipopolysaccharide have shown the presence of a polyheptose phosphate 'backbone' to which the other sugars are attached (Kauffman, Krüger, Lüderitz & Westphal, 1961; Osborn, Rosen, Rothfield, Zeleznick & Horecker, 1964).

Ethanolamine (Grollman & Osborn, 1964) and

3-deoxy-2-oxo-octonic acid (Heath & Ghalambor, 1963) have also been identified as components of lipopolysaccharide, and there is evidence that the latter compound is involved in the linkage of the polysaccharide to the lipid moiety (Osborn, 1963). The lipid, designated lipid A by Westphal & Lüderitz (1954), contains mainly glucosamine, phosphate and four fatty acids (lauric acid, myristic acid, palmitic acid and β-hydroxymyristic acid).

The extracellular lipoglycopeptide that had been prepared by Bishop & Work (1965) from *E. coli* grown under conditions of lysine-limitation had been examined, without further purification, for carbohydrate and lipid components. Glucose, galactose and glucosamine were identified chromatographically, but heptose was not detected in the solvent system employed. The residual unextractable lipid was, however, found to resemble lipid A, as it contained the four characteristic fatty acids. It is now obvious that extracellular lipopolysaccharide was present in a complex with a lipoprotein that can be dissociated by aqueous phenol: a few more steps have produced a pure material identical with the cellular lipopolysaccharide present in the cell wall. The lipid component of the pure extracellular lipopolysaccharide is very similar to that reported by Burton & Carter (1964) for purified lipid A from *E. coli* O111:B4 lipopolysaccharide. Other preparations of lipid A obtained from *E. coli* by Ikawa, Koepfli, Mudd & Niemann (1953) and Wander Forschungsinstitut (1960) have contained amino acids as additional components, but, as Burton & Carter (1964) showed, these are not components of purified lipid A.

The studies of Kauffman *et al.* (1960*b*), and more recently Jann (1965), on strains of *E. coli* belonging to 100 different serological types showed that they could be divided into 20 different groups, or chemotypes, depending on the carbohydrate components of the lipopolysaccharide O-antigens. The carbohydrate components of both the cellular and extracellular lipopolysaccharide of *E. coli* 12408 are

typical of organisms of chemotype I; they are present in lipopolysaccharides of strains of *E. coli* showing 13 different O-specificities and also in those of the rough types of organism. The relative molar proportions of these components in the extracellular lipopolysaccharide prepared by precipitation with Mg^{2+} are: glucose, 1·0; galactose, 1·1; heptose, 1·7; glucosamine, 0·6; 3-deoxy-2-oxo-octonic acid, 0·1; and also phosphorus, 2·2. Studies on the structure and biosynthesis of lipopolysaccharides from *E. coli* and salmonellae (Edstrom & Heath, 1964; Staub & Westphal, 1964) have shown that glucosamine is present in the polysaccharide moiety of lipopolysaccharides from smooth strains, whereas it is absent from some rough strains, e.g. the R I mutants of salmonellae (Lüderitz *et al.* 1965). The presence of only a trace of glucosamine in the polysaccharide fraction obtained from the extracellular lipopolysaccharide therefore supports the identification of *E. coli* 12408 as a rough strain. Our analytical results are consistent with the results of other studies (Edstrom & Heath, 1964; Staub & Westphal, 1964; Lüderitz *et al.* 1965) that such 'rough' polysaccharides consist of a polyheptose phosphate 'backbone' to which are attached short side chains containing glucose and galactose. The preferential release of galactose from extracellular lipopolysaccharide under very mild conditions of acid hydrolysis indicates that most of the galactose residues are terminal, and possibly joined to glucose by a (1→3)-linkage (Rosen, Osborn & Horecker, 1964). However, as emphasized by the investigation of Sutherland, Lüderitz & Westphal (1965) on, among others, a strain of *Salmonella typhimurium* (TV 166) with a similar carbohydrate composition, further deductions on the nature of the side chains are premature.

Lipopolysaccharides isolated from bacterial cells by phenol extraction possess a number of physical and biological properties that are also shared by the extracellular lipopolysaccharide. They are of high molecular weight. They aggregate easily and their physical properties depend on the degree of aggregation (Westphal & Lüderitz, 1954; Oroszlan & Mora, 1963). More specifically they are toxic, pyrogenic (Westphal & Lüderitz, 1954), effective adjuvants (Johnson, Gaines & Landy, 1956) and produce inflammatory lesions in the skin of rabbits (Larson *et al.* 1960).

Extracellular complexes containing O-antigenic lipopolysaccharides have also been obtained from colicinogenic strains of *E. coli* (Goebel, 1962). In this case, colicin activity was associated with O-antigen, but a variant that elaborated no colicine still produced an extracellular O-antigen (Rüde & Goebel, 1962).

The similarity between the compositions of extracellular lipopolysaccharide of *E. coli* 12408 and

the cell-wall lipopolysaccharide extracted from 'normal' and 'lysine-deficient' cells of the same organism indicates that the extracellular component is derived from the cell wall. Lipoglycopeptide appears in the culture fluid only when the organism is grown under lysine-limiting conditions, and is probably related to the 'lipomucoprotein' (Meadow, 1958) and 'mucopeptide' (Municio, Diaz & Martinez, 1963, 1964) obtained previously from the cultures of another lysine-requiring mutant, *E. coli* 26–26, grown under similar conditions. Other compounds are also excreted by both mutant strains under lysine-limiting growth conditions; these include diaminopimelic acid (Work & Denman, 1953), nucleotides and flavines (Lilly, Clarke & Meadow, 1963). However, as discussed by Bishop & Work (1965), the mechanism of production of these extracellular components has not been elucidated.

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