An Extracellular Glycolipid Produced by *Escherichia coli* Grown under Lysine-Limiting Conditions

BY D. G. BISHOP* AND ELIZABETH WORK
Twyford Laboratories, Twyford Abbey Road, London, N.W. 10

(*Received* 31 *December* 1964)

1. Some of the products excreted by cultures of lysine-requiring *Escherichia coli* A.T.C.C. 12408 grown under lysine-limiting conditions have been studied. 2. A glycolipid designated 'extracellular lipoglycopeptide' was prepared from culture filtrates of such organisms. It contained 35% of lipid, 19% of carbohydrate, 3·4% of P and 3·7% of N. 3. Comparison of the lipids, fatty acids, carbohydrates and amino acids of this lipoglycopeptide with those of whole cells, cell walls and cellular lipopolysaccharides shows that it has few features (except its residual lipids) in common with any of these fractions. 4. The lipoglycopeptide was antigenically related to both walls and lipopolysaccharide.

Certain lysine-requiring mutants of *Escherichia coli* that lack *meso*-2,6-diaminopimelate carboxy-lyase are known to excrete a variety of products when grown under lysine-limiting conditions. The excreted compounds include amino acids, especially diaminopimelic acid (Davis, 1952; Work & Denman, 1953; Cassida, 1956; Angulo, Diaz, Hererra, Municio & Rivero, 1960*a*), nucleotides and flavines (Lilly, Clarke & Meadow, 1963) and lipid-containing products designated as lipomucoprotein (Meadow, 1958) or mucopeptide (Municio, Diaz & Martinez, 1963, 1964). These lipid-containing substances were thought to be related to cell walls, on the basis of their chemical composition or immunological properties. An intracellular lipid-containing material possibly originating in cell walls is the lipopolysaccharide, known also as endotoxin, extracted by hot aqueous phenol from walls or whole cells of various Gram-negative bacteria (Westphal, Lüderitz & Bister, 1952; Westphal, 1960).

The present paper describes the preparation of a high-molecular-weight material (designated extracellular lipoglycopeptide) from the culture filtrate of the lysine-requiring mutant of *E. coli*, A.T.C.C. 12408, and compares its composition and immunological properties with those of whole cells, cell walls and intracellular lipopolysaccharide.

METHODS

Organisms and growth conditions. Escherichia coli A.T.C.C. 12408 was mainly used; this is a lysine-requiring strain selected by Cassida (1956) for its ability to excrete large amounts of diaminopimelic acid during growth. Another lysine-requiring mutant, *E. coli* 26–26, originated from Professor B. Davis (1952). Cells were grown at 37°. Stock cultures were maintained on nutrient–agar slopes stored at 4°. The basal medium selected for production of lipoglycopeptide contained (g./l.): $(NH_4)_2HPO_4$, 15; KH_2PO_4, 2; $MgSO_4,7H_2O$, 0·05; $Na_2SO_4,10H_2O$, 0·1; glycerol, 16; L-lysine monohydrochloride, 0·06; the pH was adjusted to 7·0. Experiments with the same inorganic salt medium with variations in energy source and lysine concentrations were first made to establish conditions for maximum production of extracellular products. These experiments were carried out in L-shaped tubes containing 40ml. of culture shaken horizontally to provide good aeration; one end of each tube was fused to a narrower optically homogeneous tube designed to fit into the EEL photoelectric colorimeter, so that the extinction of cultures could be measured during growth; a vertical arm plugged with cotton wool provided means of entrance to the growth compartment. Subsequently, the cultures were grown on a rotary shaker in 2l. Erlenmeyer flasks containing 80ml. of medium; the inocula were either one-sixth of the saline washings from an overnight culture on nutrient–agar, or 0·1ml. of an overnight culture grown in basal medium. Larger batches (10l.) were grown in a stirred fermenter (30l. capacity) with an aeration rate of 5l. of air/min.; this was inoculated with the overnight growth from six shake flasks each containing 80ml. of culture grown in basal medium. Antifoam [70% (v/v) silicone MS antifoam A in silicone fluid MS 200/10CS (Pirt & Callow, 1958)] was added automatically when foaming occurred. After 26hr., most of the cells were removed from the culture by centrifugation in a Sharples centrifuge and the supernatant solution was freed from all bacteria by membrane filtration.

Preparation of fractions. Harvested cells were washed twice with 0·85% NaCl and freeze-dried. Cell walls were prepared from wet washed cells as described by Allsop & Work (1963) and freeze-dried. Cellular lipopolysaccharide was prepared by aqueous phenol extraction of freeze-dried whole cells (Westphal *et al.* 1952).

* Present address: Department of Chemistry, Harvard University, Cambridge, Mass., U.S.A.

The cell-free culture filtrate was, if necessary, concentrated fivefold, by vacuum-distillation, and extracted four times with an equal volume of chloroform. The aqueous layer was briefly warmed to 35° under reduced pressure to remove all traces of chloroform and stored at 4° for 72hr. The precipitate that had formed was collected by centrifugation, suspended in water and dialysed against 300vol. of water at 4° for 24hr. The non-diffusible material was collected and freeze-dried; it is referred to henceforth as 'extracellular lipoglycopeptide'. Extracellular extractable lipids were obtained for analysis from the chloroform extracts, which were dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was taken up in a small amount of chloroform, centrifuged to remove insoluble material and evaporated to dryness under reduced pressure.

Lipid analysis. 'Free' and 'bound' lipids were successively extracted from freeze-dried material with ethanol–ether (3:1, v/v) and ethanol–ether–12N-HCl (20:20:1, by vol.) respectively (Bishop & Still, 1963). The extracts and the residue remaining after the extraction of 'bound' lipid were saponified in a tenfold excess of N-KOH in methanol for 6hr. After removal of non-saponifiable material, the fatty acids were extracted with ether and methylated with diazomethane, after which the reagents were removed by evaporation at room temperature under N_2. 'Free' and 'bound' lipids (100μg. samples dissolved in chloroform) were examined by thin-layer chromatography on plates prepared by standard techniques with Kieselgel G (E. Merck A.G., Darmstadt, Germany). The solvent systems employed were hexane–ether–acetic acid (70:30:1, by vol.) to separate non-polar components, and chloroform–methanol–acetic acid (65:25:8, by vol.) for polar components (Nichols, 1963). Spots were detected by spraying with either chromic acid (Mangold & Kammereck, 1962), 0·5% ninhydrin in butanol, or the modified Zinzadze reagent for phospholipids (Dittmer & Lester, 1964). Methyl esters of fatty acids were analysed by gas–liquid chromatography in a Pye Panchromatograph, with an Apiezon column at 196°, and a flame ionization detector. Further confirmation of the identity of certain components was obtained with a polyethylene glycol adipate column at 180°.

Determination of other materials. Total carbohydrate was estimated by the phenol–H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers & Smith (1956); glucose was used as a standard and results are expressed as glucose equivalents. Nitrogen was determined by the micro-Kjeldahl method, and total phosphorus by the method of Allen (1940). Diaminopimelic acid was measured in the culture filtrate (0·05ml.) by the acid-ninhydrin method (Work, 1957) and protein with modified Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951), with crystalline bovine serum albumin as a standard. Flavine was estimated by measuring the extinction of a sample of the culture filtrate in phosphate buffer, pH7·0, by using the molar extinction coefficient $E_{450m\mu}$ $11{\cdot}3\times10^6$cm.^{2}/mole given for oxidized FAD by Beinert & Page (1957). Glycerol was measured as described by Hartman (1953).

Amino acids of fractions were examined after hydrolysis in 6N-HCl for 18hr. at 105°, HCl being removed by several distillations *in vacuo*. Quantitative analysis was carried out by the method of Moore, Spackman & Stein (1958) on an EEL Automatic Analyser. Two-dimensional chromatography of the hydrolysate on Whatman no. 4 paper was carried out in aqueous phenol (ammonia atmosphere) and butan-1-ol–acetic acid–water (67:10:23, by vol.) respectively. Spots were detected with 1·0% (w/v) ninhydrin in acetone. For chromatography of sugars, samples were hydrolysed for 2hr. in 2N-HCl at 105° and HCl was removed *in vacuo*. The solvent was the organic phase from ethyl acetate–pyridine–water (5:2:7, by vol.); spots were detected with $AgNO_3$ (Trevelyan, Procter & Harrison, 1950). Hexosamines were separated with butan-1-ol–pyridine–water (6:4:3, by vol.) and detected with Ehrlich's reagent (Partridge, 1948).

Measurements of opacity of cultures were made in the EEL portable colorimeter with filter OB10, 5·76 units being equivalent to 1mg. dry wt. of cells/ml. Above 4 units there was no direct proportionality between opacity and dry weight. Other colorimetric measurements employed a Unicam SP.600 photoelectric colorimeter.

Immunological methods. Antisera were prepared by intraperitoneal injections three times weekly into female New Zealand White rabbits weighing about 2·5kg. Suspensions or solutions containing 10mg. of material/ml. of saline (0·85% NaCl) were injected. Cell walls and whole cells were prepared from cultures grown on synthetic media containing adequate lysine. The first two injections were 0·5ml. and eight injections of 1·0ml. followed, a total of 90mg. of material being administered. One week after the last injection, the rabbits were bled from the heart and the resulting antiserum was treated with 0·01% merthiolate and stored at −10°. Antisera were concentrated where necessary by freeze-drying, and subsequently the requisite amount of water was added.

Agglutination and precipitation reactions were carried out in Perspex haemagglutination plates (M.R.C. pattern) containing 0·25ml. of saline in each well. Fivefold-diluted antiserum (0·25ml.) was added to the contents of the first well (making first dilution of 1:10), and thereafter it was diluted serially from this well by a factor of 2 in each succeeding well across the plate. Material under test (0·05mg. in 0·25ml. of saline) was added to each well except the control, to which only saline was added. The trays were covered and rocked gently at 37° for 2hr., after which they stood for several hours at room temperature before the results were recorded.

Gel diffusion analysis was carried out on glass microscope slides with agar as described by Mansi (1957). Fivefold-concentrated antisera were used; the best concentration of antigen was determined in each case.

RESULTS

Effect of culture conditions on excretion of products by cells. The conditions for optimum production of diaminopimelic acid by *E. coli* 12408 were first studied in shaken L-tubes. Table 1 shows the results of a single experiment in which various tubes contained media of slightly differing composition. Within a certain concentration range of lysine hydrochloride (10–100mg./l.), the concentration of lysine in a suitable medium determined the amount of diaminopimelic acid excreted (tubes 3, 4 and 5). With 2–2·5% glycerol, the optimum lysine concentration was about 58·5mg./l.; at this or lower

Table 1. *Effect of variation of organic constituents of the basal medium on the production of diaminopimelic acid in shaken* L*-tubes by* E. coli 12408

Tube no.	Energy source	L-Lysine hydrochloride in medium (mg./l.)	Type of growth	Final extinction	Peak concn. of diaminopimelic acid (mg./ml.)
1	Glucose (1%)	54	Diphasic	7·9	1·22
2	Glucose (1%)	72	Diphasic	8·2	0·8
3	Glycerol (2·5%)	54	Diphasic	11·0	2·09
4	Glycerol (2·5%)	40	Diphasic	10·5	1·8
5	Glycerol (2·5%)	72	Normal	6·8	0·7
6	Glycerol (1·25%)	54	Normal	4·0	0·3

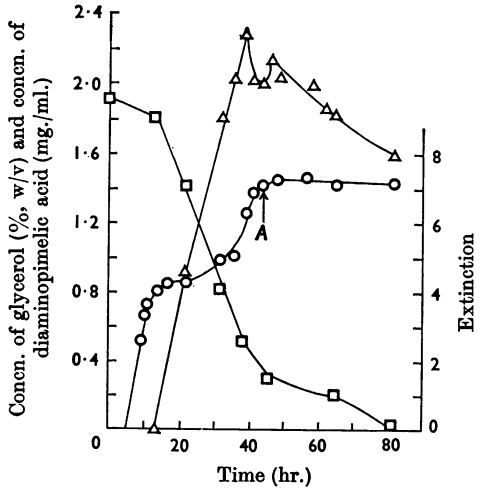

Fig. 1. Time-course of growth and excretion of diaminopimelic acid when *E. coli* 12408 was grown in shaken L-tubes in basal medium containing 58·5 mg. of lysine hydrochloride/l. and 1·9% of glycerol. At point *A* 0·8 mg. of lysine hydrochloride was added to the contents of one tube. △, Diaminopimelic acid in medium; ○, growth of organism (extinction); □, glycerol in medium.

concentrations, diphasic growth (Fig. 1) occurred, and the diaminopimelic acid excretion varied directly with lysine concentration. When the medium contained a little more lysine (e.g. 72 mg./l., tube 5) growth was normal and the final cell density and diaminopimelic acid excretion were very low. Diphasic growth has also been noted by Lilly *et al.* (1963) with *E. coli* 26–26, and by Angulo, Diaz, Municio & Rivero (1960*b*) with *E. coli* 12408. Soon after the end of exponential growth (about 12–15 hr. after inoculation), measurable amounts of diaminopimelic acid appeared in the culture filtrate; at this stage lysine is exhausted from the medium (Lilly *et al.* 1963). A second phase of growth soon started, and production of diaminopimelic acid then continued at a steady rate until there was no further increase in opacity of the culture. At this point there was often an abrupt small fall in diaminopimelic acid concentration followed by a temporary increase and either a slow fall or no further change. Addition of lysine after all growth had ceased (point *A* in Fig. 1) had no effect on either growth or diaminopimelic acid concentration. Diphasic growth was a necessary condition for high production of diaminopimelate, but no correlation was found between final cell density and diaminopimelate concentration. In the experiment shown in Fig. 1, where the peak concentration of diaminopimelate (2·3 mg./ml.) was higher than that obtained in Table 1 (tube 3), the final opacity was very much less than in tube 3.

The nature and amount of carbohydrate in the medium also influenced diaminopimelate production. In confirmation of the findings of Angulo *et al.* (1960*a*), at suitable lysine concentration, more diaminopimelic acid was produced when glycerol (2·5%) was the source of energy than when glucose was used (Table 1, tubes 1 and 3); lowering the glycerol concentration to 1·25% resulted in a normal type of growth and little production of diaminopimelic acid (tube 6). Other experiments showed that a mixture of glycerol (1·5%) and sucrose (1·5%) led to greater excretion of diaminopimelic acid than when either compound was used alone. Fig. 1 shows that practically all the glycerol originally present (1·9%) was used during the 80 hr. experiment; the highest rate of utilization occurred during the period of rapid diaminopimelate production.

It has been suggested (Lilly *et al.* 1963; Angulo *et al.* 1960*b*) that the secondary growth which occurs after virtual exhaustion of lysine from the medium is due to reversion of the culture to lysine-independence. This was not invariably the case in our experiments; the cells had not always changed, with regard to either their requirement for lysine or their content of diaminopimelate carboxy-lyase, when examined at various periods of growth. In

the experiment illustrated in Fig. 1, the cells had apparently reverted, as they grew when subcultured into minimal medium without lysine after 24 or 80hr.; in the experiment shown in Table 1 there was no reversion after 30hr.

Angulo, Diaz & Municio (1961) reported a high requirement for oxygen for maximal diaminopimelic acid production; this was confirmed by using shake-cultures in 2l. conical flasks, where the best results were obtained when the volume of medium was reduced to 80ml. In the tube cultures, high flavine and lipoglycopeptide or protein contents had been observed in all cases where maximal diaminopimelic acid excretion occurred. Experiments in shake flasks and the fermenter confirmed these results, although under both these conditions the maximum concentrations of diaminopimelic acid were reached after only 26hr. and very little decline was noted thereafter. The flask cultures grown for 26hr. contained 1·3mg. dry wt. of bacterial cells/ml., and were intensely yellow owing to their content of flavines (12·5 μmoles/ml.); they also contained Folin-positive materials equivalent to 0·55mg. of protein N/ml.; some of this was diffusible. Comparative studies with *E. coli* 26–26 gave essentially similar results, but with lower concentrations of all extracellular products. The medium finally chosen for use in the fermenter with *E. coli* 12408 contained 60mg. of L-lysine hydrochloride/l. and (for ease of sterilization) had only glycerol as energy source.

Composition of products. The analytical data presented in Tables 2–5 were of materials obtained from one experiment on 10l. of basal medium in the fermenter. Samples of cells harvested after 26hr. growth were taken for preparation of cell walls and cellular lipopolysaccharide, and the extracellular lipoglycopeptide and chloroform-extractable lipid were prepared from the culture filtrate. The lipoglycopeptide was a white powder that gave a highly opalescent solution in water. The average yield was about 0·15g./l. of culture medium.

The overall compositions of whole cells, cell walls, extracellular lipoglycopeptide and cellular lipopolysaccharide are shown in Table 2; a commercial sample (Difco) of *E. coli* O111:B4 lipopolysaccharide is included for comparison. The nitrogen content of these cell walls (6·7%) is lower than that (8·2%) found in walls of the organism grown in the same medium containing an excess of lysine (200mg./l.). The extracellular lipoglycopeptide contained less carbohydrate, nitrogen and phosphorus but more lipid than the cellular lipopolysaccharide; its carbohydrate content was higher than that of walls but its total lipid content was about the same. The major carbohydrate components of all fractions were glucose, galactose and glucosamine (Table 3). Muramic acid was detected only in the cell walls. Ribose, found only in lipopolysaccharide, may have originated in nucleic acid-containing contaminants. The quantitative amino acid analysis of cell walls is given in Table 3; as in all walls of Gram-negative bacteria (Work, 1961) all amino acids of proteins and also diaminopimelic acid were present. The content of lysine was slightly lower than that reported for another strain of *E. coli* (Salton, 1964). The analysis of walls of *E. coli* 12408 grown in the presence of an excess of lysine was very similar to that from walls of lysine-limited cells and did not account for the higher N content of these walls. Extracellular lipoglycopeptide had proportionately fewer amino acids compared with walls and very little diaminopimelic acid; cellular lipopolysaccharide contained only glutamic acid, glycine, serine and alanine. Ethanolamine was a major ninhydrin-positive component of lipoglycopeptide and lipopolysaccharide, even after extraction of 'free' and 'bound' lipids.

Analysis of lipids. All four products contained lipids, referred to as 'residual', that were not extracted in either the 'free' or the 'bound' fractions. The fatty acids of the 'residual' lipids were liberated by saponification for analysis. The products showed marked differences in the distribu-

Table 2. *Composition* (%, *w/w*) *of fractions of* E. coli 12408 (*lysine-limited*)

	Whole cells	Cell walls	Extracellular lipoglycopeptide	Cellular lipopoly-saccharide	*E. coli* O111:B4 cellular lipopolysaccharide
Carbohydrate (as glucose)	11·6	7·0	19·0	25·5	30·2
Nitrogen	11·6	6·7	3·7	4·6	6·7
Phosphorus	2·4	1·9	3·4	4·5	3·3
'Free' lipid	9·4	14·9	5·8	0·8	—
'Bound' lipid	2·6	7·1	21·4	0·8	—
Fatty acid from 'free' lipid	6·1	11·6	3·6	0·4	—
Fatty acid from 'bound' lipid	1·4	3·6	7·3	0·5	—
Residual fatty acid	5·1	4·2	8·1	5·1	—
Total fatty acid	12·6	19·4	19·0	6·0	—

Table 3. *Sugars and amino acids found in hydrolysates of fractions from* E. coli 12408 *grown under lysine-limiting conditions*

Amino acids and amino sugars were estimated on the automatic amino acid analyser. In other cases, results were obtained from visual observation of paper chromatograms. A blank space indicates uncertainty about presence or absence; tr. indicates a just-detectable spot.

	Cell walls (moles/10^4g.)	Extracellular lipoglycopeptide	Cellular lipopolysaccharide
Glucose	+++	+++	+++
Galactose	+++	+++	+++
Ribose	–	–	++
Glucosamine	1·9	++	+
Muramic acid	1·0	–	–
Alanine	5·3	++	+
Arginine	0·8	+	–
Aspartic acid	4·7	++	tr.
Cystine	+		
Diaminopimelic acid	1·4	tr.	tr.
Glutamic acid	3·4	++	++
Glycine	3·0	+++	++
Histidine	0·07	–	–
Leucine	1·9	+	–
Isoleucine	0·8		–
Lysine	1·4	+	–
Methionine	0·4		
Phenylalanine	2·3	–	–
Proline	0·6	–	–
Serine	1·6	+++	+
Threonine	1·5	+	–
Tyrosine	1·2	tr.	–
Valine	1·2	+	–
Ethanolamine	–	++++	++

tion of lipids in these various fractions (Table 2). Cell walls had a higher content of both 'free' and 'bound' lipids than whole cells, whereas intracellular lipopolysaccharide contained only very small amounts of both types of lipids. Walls prepared from cells grown in an excess of lysine were found to contain similar amounts of 'free' and 'bound' lipid to these walls from lysine-limited cells. The content of total fatty acid (19%, w/v) of extracellular lipoglycopeptide was about the same as that of walls, but the proportional distribution in the three types of lipid fractions differed from that of walls. About 35% of the lipoglycopeptide was composed of lipid, most of which occurred in the 'bound' fraction, whereas 'free' lipids represented the major wall fraction. The content of 'free' lipid of lipoglycopeptide varied in different preparations from about 1 to 8%, but the amount of 'bound' lipid was constant.

Thin-layer chromatograms showed that the only major component of both 'free' and 'bound' lipid of whole cells and extracellular lipoglycopeptide had a mobility similar to that of authentic phosphatidylethanolamine (Table 4). The presence of this extractable phospholipid in the 'bound' lipid can be attributed to the fact that phospholipids are not completely extracted with the ethanol–ether mixture used here to remove 'free' lipids (Insull & Ahrens, 1959). Phosphatidylethanolamine was also a major component of the chloroform-soluble lipid extracted from the culture filtrate. As already stated, the 'free' lipid content of lipoglycopeptide varied between preparations, and, as this fraction consists almost entirely of phosphatidylethanolamine, it can be presumed that the variation is due to incomplete extraction of the phosphatidylethanolamine from the medium before precipitation of the lipoglycopeptide. The small amounts of 'free' and 'bound' lipid found in cellular lipopolysaccharide appear to be composed entirely of phosphatidylethanolamine.

No phosphatidylethanolamine was detectable in any of the lipids of cell walls, whether prepared from cells grown with limiting or with an excess of lysine. Thin-layer chromatography with a non-polar solvent showed that the 'free' lipid of cell walls contained about 30% of a component having the same R_F as free fatty acids; the remainder of the lipid stayed at the origin and was only detected with the non-specific chromic acid spray. 'Free' lipids

Table 4. *Examination of lipid fractions of* E. coli 12408 (*lysine-limited*) *by thin-layer chromatography on Kieselgel G*

Solvent 1, chloroform–methanol–acetic acid (65:25:8, by vol.); solvent 2, hexane–ether–acetic acid (70:30:1, by vol.). F, 'Free' lipid; B, 'bound' lipid.

Solvent	Spot	R_F	Whole cells		Cell walls		Chloroform-extractable lipid	Extracellular lipoglycopeptide		Cellular lipopolysaccharide	
			F	B	F	B	F	F	B	F	B
1	Phosphatidylethanolamine	0·66	+++	+	−	−	++++	++++	+++	+	+
1	Ninhydrin-positive, phosphate-positive	0·14	−	+	+	++	−	−	+++	−	−
1	Ninhydrin-positive, phosphate-negative	0·08	−	−	−	++	+	−	++	−	−
1	Ninhydrin-positive, phosphate-positive	0	−	++	−	+	+	−	+	−	−
2	Free fatty acids	0·53	+	−	++	−	+	+	+	−	−
2	Organic material*	0	+++	+++	+++	+++	+++	+++	+++	+++	+++

* Detectable by non-specific chromic acid spray.

from other fractions also contained small amounts of free fatty acids. In addition to phosphatidylethanolamine, the 'bound' lipids of whole cells, walls and lipoglycopeptide were found to contain appreciable amounts of other unidentified compounds giving positive reactions for amino and phosphate groups. A spot reacting only with ninhydrin was also detected on chromatograms from extracts of cell walls and lipoglycopeptide.

The analyses of fatty acid methyl esters are shown in Table 5. The major components of the fatty acids of whole cells were palmitic acid, palmitoleic acid and *cis*-vaccenic acid. They were not equally distributed between the three lipid fractions: palmitoleic acid and *cis*-vaccenic acid were predominant in the 'free' lipid, accounting for 45% of this fraction, whereas they only made up 17% of the 'bound' lipid. An unidentified peak with a retention volume (R_v) 4·32 times that of methyl myristate was found only in the 'bound' lipid fraction. The composition of the 'residual' fatty acids of whole cells differed greatly from that of the 'free' and 'bound' fatty acids. Here, the major component was β-hydroxymyristic acid; there were also substantial amounts of lauric acid and myristic acid and two other unidentified substances with R_v values relative to myristic acid ester of 1·32 and 1·51. These 'residual' fatty acid esters of whole cells showed a large peak corresponding to the ester of palmitic acid on the Apiezon column, but on a polyethylene glycol adipate column about 80% of the material making up this peak emerged well after all the other fatty acid esters (R_v relative to myristic acid ester of 10·70). Only traces of unsaturated acids were detected in the 'residual' fatty acids of whole cells.

The relative distribution of fatty acids in the cell-wall lipid fractions followed a similar pattern to that found in whole cells, the differences in the content of unsaturated acids in the 'free' and 'bound' lipids being even more marked. Hydroxymyristic acid, myristic acid and lauric acid were again the major components of the 'residual' fatty acids, but palmitic acid accounted for over 70% of the mixed '16:0' Apiezon peak. The lipids of extracellular lipoglycopeptide differed from those of whole cells and walls in having similar fatty acids in the 'free' and 'bound' lipids, with unsaturated acids (palmitoleic acid and *cis*-vaccenic acid) amounting to 64% of the total fatty acids in both cases. The 'residual' fatty acids were again characterized by a lack of unsaturated fatty acids; the major components resembled those of 'residues' from whole cells and walls, being mainly β-hydroxymyristic acid, palmitic acid, lauric acid and myristic acid. The 'free' and 'bound' lipids of cellular lipopolysaccharide contained only small amounts of unsaturated acids; the major component

Table 5. *Fatty acid composition (%, w/w) of lipids from fractions of* E. coli 12408 *grown under lysine-limiting conditions*

Results of the gas–liquid chromatography of fatty acid methyl esters with an Apiezon column at 196° are given. The percentage composition was estimated by the method of triangulation. F, 'Free' lipid; B, 'bound' lipid; R, 'residual' fatty acids. + indicates that the acid ester was detected and identified on the basis of retention data but that it occurred to an extent of less than 0·2%.

	Cells			Walls			Extracellular lipoglycopeptide			Cellular lipopolysaccharide			Extracellular extractable
Fatty acid*	F	B	R	F	B	R	F	B	R	F	B	R	lipid
OH, 10:0	0·8	3·1	0·2	0·5	2·3	—	0·7	0·7	—	7·8	1·7	—	1·5
12:0	0·6	1·1	9·6	0·2	1·4	12·3	0·9	0·7	16·2	0·7	2·3	15·1	5·4
14:1	0·6	+	−	+	−	−	0·3	0·2	−	−	−	−	+
14:0	2·3	3·2	7·1	2·4	3·6	14·2	2·2	1·9	21·0	2·1	4·6	18·3	6·5
Unknown (R_v 1·32†)	−	0·2	4·4	0·2	0·4	4·6	0·2	+	7·0	0·4	1·5	6·6	2·2
Unknown (R_v 1·51†)	0·4	0·6	10·0	0·2	1·2	10·7	0·8	0·7	13·0	0·7	2·3	11·8	2·9
OH, 14:0	+	3·4	33·2	0·5	4·5	36·7	1·5	0·8	34·0	1·8	6·9	27·4	5·8
16:1	21·9	6·5	+	24·0	2·2	+	20·8	17·0	+	5·4	+	2·1	16·9
16:0	42·5	56·8	34·0‡	28·5	61·3	20·1‡	33·5	35·4	7·3‡	68·2	64·5	13·6‡	28·2
17:0△	3·0	2·6	+	3·5	1·8	−	3·9	4·2	−	0·7	+	1·5	2·6
17:0	+	0·6	−	+	0·7	−	0·7	0·6	−	−	−	−	+
Unknown (R_v 4·32†)	−	6·3	−	−	2·9	−	−	2·8	−	−	−	−	−
18:1	23·6	10·6	+	35·3	6·8	+	31·0	28·4	+	8·6	+	1·4	20·8
18:0	4·0	4·2	−	3·6	5·1	−	2·9	2·3	−	1·8	−	−	0·9
19:0△	−	−	−	+	−	−	0·3	−	−	1·1	−	−	−

* The number before the colon gives the number of carbon atoms; that after the colon is the number of double bonds; △, cyclopropane ring.

† Retention volume relative to that of myristic acid ester.

‡ This value for 16:0 includes some other acid (see the text).

Table 6. *Tests with antisera prepared against whole cells, cell walls and extracellular lipoglycopeptide of* E. coli 12408

Dilution 1 represented a tenfold dilution of the antiserum; all subsequent dilutions were twofold. Numbers represent strength of agglutination reaction, judged by eye. Reactions with lipoglycopeptide and lipopolysaccharide were not assessed quantitatively.

Antiserum	Antigen	Test	Antisera dilutions ...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Whole cells	Whole cells	Agglutination		3	2	2	2	2	1	1	1	1	1	½					
	Cell walls	Agglutination		2	2	1	1	1	1	1	1	½	½	½	½				
	Lipoglycopeptide	Precipitation		+	+	+	+	+											
	Lipopolysaccharide*	Agglutination		+	+	+	+	+											
Cell walls	Whole cells	Agglutination		3	3	3	3	3	3	2	2	2	2	2	2	2	1	½	½
	Cell walls	Agglutination		3	3	3	3	3	3	3	3	3	2	2	2	2	1	1	
	Lipoglycopeptide	Precipitation		+	+	+	+	+	+	+	+	+	±	±					
	Lipopolysaccharide*	Agglutination		+	+	+													
Lipoglycopeptide	Whole cells	Agglutination		2	2	2	2	1	1	1	1	1	½						
	Cell walls	Agglutination		3	3	2	2	2	2	2	2	1	1	1	1	1			
	Lipoglycopeptide	Precipitation		+	+	+	+	+	+	+	+	+	+	+	+	+			
	Lipopolysaccharide*	Agglutination		+	+	+	+	+	+	+	+	+	+	+	+	+	+		

* From *E. coli* 12408 or O111:B4.

was palmitic acid, which accounted for over 64% of both fractions. The 'residual' fatty acids resembled those of lipoglycopeptide and the other cell fractions.

Immunological properties. Table 6 shows the results of agglutination and precipitation tests carried out on the antisera prepared against whole cells, cell walls and extracellular lipoglycopeptide. Antiserum against extracellular lipoglycopeptide reacted with both lipoglycopeptide and cellular lipopolysaccharide at dilutions of 1:20480, but cell-wall antiserum, though precipitating lipoglycopeptide strongly, only reacted with lipopolysaccharide at 1:40 dilution. Antisera against cell walls and lipoglycopeptide both agglutinated walls at high dilutions (1:163840 and 1:40960 respectively). The dilutions of all three antisera that reacted with commercial *E. coli* O111:B4 lipopolysaccharide were the same as those agglutinating our cellular lipopolysaccharide from *E. coli* 12408. Gel diffusion tests with lipoglycopeptide (1–5mg./ml. of 0·85% sodium chloride) produced at least two lines with fivefold-concentrated antisera against whole cells, cell walls and lipoglycopeptide.

DISCUSSION

The peculiar behaviour of lysine-requiring mutants of *E. coli* grown under specified conditions cannot yet be explained. The excretion of a variety of products, such as lipids, diaminopimelic acid, flavines and nucleotides, seems to be a characteristic of this particular type of mutant and is not confined to one strain of *E. coli*. The mechanisms of diphasic growth and of production of these substances, excreted in amounts that are often far in excess of simple leakage or lysis, have yet to be elucidated. It is certain, however, from work done in these and other Laboratories (Lilly *et al.* 1963; Angulo *et al.* 1960*b*), that all these phenomena result from limitation of the amount of lysine supplied to the growing culture. The second phase of growth is sometimes considered to be a consequence of reversion of the mutant, since *meso*-diaminopimelate carboxy-lyase, absent from the culture originally, was often detectable in cells examined during this growth phase (V. A. Knivett, personal communication; Lilly *et al.* 1963). This does not, however, explain the continued production of extracellular materials during this time.

The composition of the extracellular lipoglycopeptide resulting from lysine limitation of *E. coli* 12408 was such that this substance cannot yet be said to originate in any one cellular fraction. The characteristic ingredients of cell-wall mucopeptide, muramic acid and diaminopimelic acid (Work, 1961), were not major constituents of the lipoglyco-

peptide, making it unlikely that it originated from or was destined for the mucopeptide wall component. Neither did it resemble in all aspects the cellular lipopolysaccharide, another component of walls of Gram-negative bacteria. However, the 'residual' lipids of lipoglycopeptide resemble those of both lipopolysaccharide and cell walls in containing mainly the four fatty acids, β-hydroxymyristic acid, myristic acid, palmitic acid and lauric acid. This combination of fatty acids has already been found in the lipid component, known sometimes as lipid A (Westphal & Lüderitz, 1954), of cellular lipopolysaccharides from *E. coli* (Burton & Carter, 1964; Ikawa, Koepfli, Mudd & Niemann, 1953). Our methods of extracting 'free' and 'bound' lipids would not have extracted lipid A, and so it is possible that extracellular lipoglycopeptide may also be a complex of other molecules with the lipid A moiety. Unlike cellular lipopolysaccharide, which had very little extractable lipids, lipoglycopeptide contained considerable amounts of 'free' and 'bound' lipids in which phosphatidylethanolamine was found. As phosphatidylethanolamine occurred also in the extracellular chloroform-extractable lipids, it is possible that the lipoglycopeptide was contaminated by phosphatidylethanolamine from this source. The small amounts of extractable lipids in cellular lipopolysaccharide also consisted of phosphatidylethanolamine. A 'kephalin-like' lipid (lipid B), probably phosphatidylethanolamine, has been reported as a minor component of lipopolysaccharides from salmonellae (Westphal & Lüderitz, 1954; Westphal, 1960).

The 'bound' lipids of extracellular lipoglycopeptide differ in several respects from those of cell walls. They have a high content of unsaturated fatty acids, and they also contain phosphatidylethanolamine. Neither phosphatidylethanolamine, phosphatidylserine, nor phosphatidic acids were detected in lipid fractions of cell walls of *E. coli* 12408, irrespective of the lysine content of the growth medium. It has been suggested that, in certain Gram-positive bacteria, phospholipids are contained exclusively in the cytoplasmic membranes (Kolb, Weidner & Toennies, 1963). In Gram-negative organisms, such as *E. coli*, there is a complicated and so far unknown relationship between cell wall and cytoplasmic membrane, and so it is not possible to say whether our wall preparations were free from cytoplasmic membranes. The electron micrographs of these walls indicated that they contained more than one structure, as is usual with this type of organism (H. Griffiths, unpublished work).

All fractions of *E. coli* examined were immunologically active; the results suggest that extracellular lipoglycopeptide is related antigenically to cell walls and to lipopolysaccharide, but it contains a cell-wall antigen that is not present in lipopolysaccharide. The results of gel diffusion analysis also suggest that lipoglycopeptide contains more than one antigen. A material called 'mucopeptide' has been prepared from the culture filtrate of lysine-limited *E. coli* 12408 by using a slightly different method of isolation (Municio *et al.* 1963). It had very similar immunological properties to our extracellular lipoglycopeptide. Overall analyses, quoted briefly on more purified material (Municio *et al.* 1964), also correspond, with the exception of lower lipid content, to those on extracellular lipoglycopeptide reported in the present paper.

We are grateful to Dr V. A. Knivett and Miss J. Cullen for helpful advice and discussion. The technical assistance of Mr J. Murdoch, Miss M. Leyland, Miss J. Allsop and Mr V. Okoro is gratefully acknowledged. We also thank Dr H. Griffiths for electron micrographs, Dr O. Lüderitz for a sample of β-hydroxymyristic acid and Mr M. Hatton for amino acid analysis.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Allsop, J. & Work, E. (1963). *Biochem. J.* **87**, 512.
Angulo, J., Diaz, T., Herrera, J., Municio, A. M. & Rivero, W. (1960*a*). *An. R. Soc. esp. Fis. Quim.* **56**, 311.
Angulo, J., Diaz, T. & Municio, A. M. (1961). *An. R. Soc. esp. Fis. Quim.* **57**, 211.
Angulo, J., Diaz, T., Municio, A. M. & Rivero, W. (1960*b*). *An. R. Soc. esp. Fis. Quim.* **56**, 413.
Beinert, H. & Page, E. (1957). *J. biol. Chem.* **225**, 479.
Bishop, D. G. & Still, J. L. (1963). *J. Lipid Res.* **4**, 81.
Burton, A. J. & Carter, H. E. (1964). *Biochemistry*, **3**, 411.
Cassida, L. E. (1956). *U.S. Patent* 2771396.
Davis, B. D. (1952). *Nature, Lond.*, **169**, 533.
Dittmer, J. C. & Lester, R. L. (1964). *J. Lipid Res.* **5**, 126.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Hartman, L. (1953). *J. appl. Chem.* **3**, 308.
Ikawa, M., Koepfli, J. B., Mudd, S. G. & Niemann, C. (1953). *J. Amer. chem. Soc.* **75**, 1035.
Insull, W. & Ahrens, E. H. (1959). *Biochem. J.* **72**, 27.
Kolb, J. J., Weidner, M. A. & Toennies, G. (1963). *Analyt. Biochem.* **5**, 78.
Lilly, M. D., Clarke, P. H. & Meadow, P. M. (1963). *J. gen. Microbiol.* **32**, 103.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mangold, H. K. & Kammereck, R. (1962). *J. Amer. Oil Chem. Soc.* **39**, 201.
Mansi, W. (1957). *J. comp. Path.* **67**, 297.
Meadow, P. M. (1958). *J. gen. Microbiol.* **18**, iii.
Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
Municio, A. M., Diaz, T. & Martinez, A. (1963). *Biochem. biophys. Res. Commun.* **11**, 195.

Municio, A. M., Diaz, T. & Martinez, A. (1964). *Abstr. 6th int. Congr. Biochem., New York*, p. 519.
Nichols, B. W. (1963). *Biochim. biophys. Acta*, **70**, 417.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Pirt, S. J. & Callow, D. S. (1958). *J. appl. Bact.* **21**, 211.
Salton, M. R. J. (1964). *The Bacterial Cell Wall*, p. 255. Amsterdam: Elsevier Publishing Co.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Westphal, O. (1960). *Ann. Inst. Pasteur*, **98**, 789.
Westphal, O. & Lüderitz, O. (1954). *Angew. Chem.* **66**, 407.
Westphal, O., Lüderitz, O. & Bister, F. (1952). *Z. Naturf.* **7B**, 148.
Work, E. (1957). *Biochem. J.* **67**, 416.
Work, E. (1961). *J. gen. Microbiol.* **25**, 167.
Work, E. & Denman, R. F. (1953). *Biochim. biophys. Acta*, **10**, 183.