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RELEASE OF OUTER MEMBRANE FRAGMENTS FROM NORMALLY GROWING *ESCHERICHIA COLI*

DICK HOEKSTRA*, JAN WILLEM VAN DER LAAN, LOE DE LEIJ and BERNARD WITHOLT**

Biochemisch Laboratorium, Rijksuniversiteit Groningen, Groningen, (The Netherlands)

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SUMMARY

A complex containing lipopolysaccharides, phospholipids and proteins is released into the culture medium by *Escherichia coli* during normal growth. It can be separated from the medium by gelfiltration on Sephadex G-200 or by centrifugation. Electron microscopy revealed that this material is released as vesicles and membrane fragments. To determine the origin of these fragments, they were compared to outer and cytoplasmic membranes with respect to keto-deoxyoctulosonic acid, phospholipid, and protein content, phospholipid composition, fatty acid composition, protein distribution on sodium dodecyl sulfate-polyacrylamide gels, buoyant density, and content of several membrane marker enzymes. The results of this comparison indicate that the membrane fragments found in the culture supernatant of normally growing *Escherichia coli* consist of practically unmodified outer membrane. Possible mechanisms as to the cause of the release of outer membrane fragments, and its relationship to cell-division, are discussed.

INTRODUCTION

The cell envelope of gram-negative bacteria, which has recently been reviewed [1], consists of a cytoplasmic membrane, a peptidoglycan (or murein) layer and an outer membrane. The outer membrane contains protein, phospholipid and most or all of the lipopolysaccharide of the cell envelope [2–4]. Under appropriate experimental conditions, for instance when growing cells are limited for a required amino acid [5–7] or exposed to phage [8] or when cells are suspended in saline or Tris-EDTA buffers [9–11], substantial amounts of these components have been shown to be released from gram-negative cells in the form of complexes; normally growing bacteria also excrete such complexes to some extent [7, 8]. The composition of these

* Present address: Department of Physiological Chemistry, University of Groningen, Bloemsingel 10, Groningen, The Netherlands.

** Correspondence to: B. Witholt, Biochemisch Laboratorium, Zernikelaan, Groningen, The Netherlands.

complexes is variable; they generally contain 60–90 % lipopolysaccharide and 5–26 % phospholipid by weight, while their phospholipid/protein ratio varies from 1 to 2.5.

Although it has been assumed that these complexes are related to the outer membrane [6–8], this has not been demonstrated directly since the complexes have not yet been compared to the corresponding outer membranes. In fact, the hitherto published data concerning the overall composition of these complexes differ markedly from data available nowadays about the outer membrane composition [2–4], especially with respect to protein content.

Thus, it remains to be shown where and how these complexes originate. They may be aggregates of membrane components released in the medium nonspecifically due to cell lysis, or specifically due to excretion of membrane components; they may be membrane fragments which are released as such from the outer and possibly the inner membrane; or they may be fragments originating specifically from certain areas of a heterogeneous outer membrane. To determine which of these possibilities is correct, we have examined in detail the membrane fragments excreted by normally growing *Escherichia coli*, and compared them to the isolated outer and cytoplasmic membranes from the same cells.

In this paper we show that the membranes found in the culture supernatant of normally growing cells consist solely of outer membranes, which appear to be released from the cells as intact vesicles and membrane fragments. Although these vesicles closely resemble the outer membrane, some low molecular weight proteins (including Braun's lipoprotein and protein G) occur in reduced amounts. Moreover, in contrast to isolated outer membrane, these vesicles show a cleavage plane during freeze fracturing. The relationship between these findings, which suggest that the excreted vesicles may originate from specific outer membrane regions, and the phenomena which occur during cell division, are discussed.

MATERIALS AND METHODS

Organisms and cultivation. E. coli JC 411 (*leu*$^-$ *his*$^-$ *arg*$^-$ *met*$^-$ *lac*$^-$ *mal*$^-$ *xyl*$^-$ *mtl*$^-$ str^r) and *E. coli* W 3110 were grown to the stationary phase in minimal medium as described previously [12]. Cell density (mg/ml dry mass) was determined as described elsewhere [13].

Isolation of membrane vesicles and fragments from the culture medium (medium vesicles). Stationary phase cultures of 1 l (final cell density 1.5 mg/ml) were harvested at 0–4 °C by centrifugation at 5000 × *g* for 10 min. The supernatant was collected and concentrated by lyophilization. The lyophilized fraction was suspended in 10 ml 0.1 M NH_4 acetate (pH 8.0, 0–4 °C), applied to a Sephadex G-200 column (2.1 × 50 cm), and eluted with the same buffer at 0–4 °C. The void volume fraction (25–30 ml) was lyophilized and resuspended in 12 ml 10 mM $MgCl_2$ and 200 mM Tris · HCl (pH 8.0).

For isolation of vesicles on a large scale, the supernatant was dialyzed and concentrated with an artificial kidney (C-DAK, model 4, Cordis Corp., Miami, Florida 33137, U.S.A.). The artificial kidney, which is normally used as the filtration unit in artificial kidney machines, appears to be similar to hollow fiber miniplants such as those produced by Bio-Rad (for instance, Bio-Fiber 50). The Cordis artificial kidney was preferred, however, because it is 20–30 times cheaper than a hollow fiber

miniplant. The procedure, performed at 15–18 °C, was as follows. Typically, 6 l supernatant was pumped continuously through the blood compartment of two kidneys (connected in series) with a flow rate of 10 l/min. A partial vacuum was applied to the dialysate compartment to generate a total pressure differential of 0.9 atm. Water (and small molecules) was removed at a rate of 1.4 l/h under these conditions. After concentrating the supernatant 2- to 4-fold, 0.1 M NH_4 acetate (pH 8.0) was added to the circulating supernatant to replace the small molecules originally present in the supernatant with NH_4 acetate. This process was continued for 6 h, after which the supply of NH_4 acetate was stopped and the supernatant was concentrated to a final volume of 200–300 ml. This concentrate was subsequently centrifuged at 40 000 × *g* for 2 h at 0–4 °C. The resulting pellet was resuspended in a buffer containing 10 mM $MgCl_2$ and 200 mM Tris · HCl (pH 8.0).

Preparation and isolation of membrane fractions. Outer and inner membrane fractions were prepared from stationary phase cells as described previously [12].

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Electrophoresis of about 50 μg of protein, solubilized by heating in 2 % sodium dodecyl sulfate for 5 min at 100 °C, was carried out on 11 % polyacrylamide slab gels as described by Lugtenberg et al. [14]. Stained gels [14] were photographed with a view-camera equipped with a red-filter on Agfapan 25 Professional film (9 × 12 cm) rated at 25 ASA. To obtain uniform lighting the gels were transilluminated with light from 4 incandescent 100 W bulbs reflected by an opaque white surface. Films were developed in Kodak HRP developer for 3 min at 20 °C. The negative was transferred to magnetic tape with a flying spot densitometer (Photoscan System P-1000, Optronics International, Inc., Chelmsford, Mass., U.S.A.) using a 50 × 50 μm raster. Since on the negative each sample occupies a column of about 80 × 5 mm, each sample was scanned 100 times along its entire length. A Fortran IV Extended program averaged the central 10 or 20 scans for each sample column, resulting in an absorbance profile (about 1600 points along the length of each sample column) for the central 0.5–1.0 mm of each sample column. All absorbance data in each sample slot were expressed as a percentage of the total absorbance. Generated absorbance profiles for different slots could therefore be compared directly and manipulated in various ways; for instance, differential profiles were generated to determine the precise location of peaks and shoulders, and integrated profiles were tabulated to determine the contribution of each peak to the profile. In this paper, sodium dodecyl sulfate-polyacrylamide gel data are presented only as computer plots of the absorbance profiles.

Phospholipid extraction and analysis. Phospholipids were extracted by the method of Bligh and Dyer [15]. The subclasses were separated by thin-layer chromatography on plates of kieselgel H (Merck), and visualized with iodine vapor. Fatty acids were obtained by saponification of the phospholipids in methanol/sulfuric acid at 70 °C for 2 h. The resulting methylesters were analysed as described previously [16].

Chemical analyses. Total protein was determined by the method of Lowry et al. [17], using bovine serum albumin as the standard. Total phosphorus was assayed by the procedure of Chen et al. [18]. Phospholipid contents were calculated assuming an average molecular weight of 700. 2-Keto-3-deoxyoctulosonic acid was estimated according to the method of Osborn et al. [3].

Enzyme assays. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed at 37 °C in a mixture containing 0.45 mM NADP, 1.1 mM glucose-6-phosphate,

11 mM $MgCl_2$, and 55 mM Tris · HCl (pH 7.5). Enzyme activity was calculated from the initial rate at which $A_{340\ nm}$ increased after the addition of enzyme. Succinate dehydrogenase (EC 1.3.99.1), D-lactate dehydrogenase (EC 1.1.1.28) and reduced nicotinamide adenine dinucleotide (NADH) oxidase (EC 1.6.3.1.) were measured according to Osborn et al. [3].

Electron microscopy. Vesicle preparations were pelleted (40 000 × *g* for 2 h) and resuspended in several drops of the remaining buffer; these concentrated preparations (about 10 mg vesicle protein/ml) were freeze-fractured and examined under the electron microscope as described previously [16].

RESULTS

Appearance of protein containing vesicles in the culture medium of stationary phase cells

When *E. coli* JC 411 was grown to the stationary phase in minimal medium, protein was found to be released into the medium. To study the nature of this protein, the cell-free supernatant was concentrated and applied on a Sephadex G-200 column. After elution with 0.1 M NH_4 acetate (pH 8.0), two fractions were obtained (Fig. 1). No glucose-6-phosphate dehydrogenase, a cytoplasmic marker enzyme [19], was detected in either fraction or in the unfractionated supernatant, indicating that the protein accumulated in the medium did not result from cell lysis.

The low molecular weight fraction eluted with the salt peak from Sephadex G-200. Although it contained protein according to the Lowry determination, no

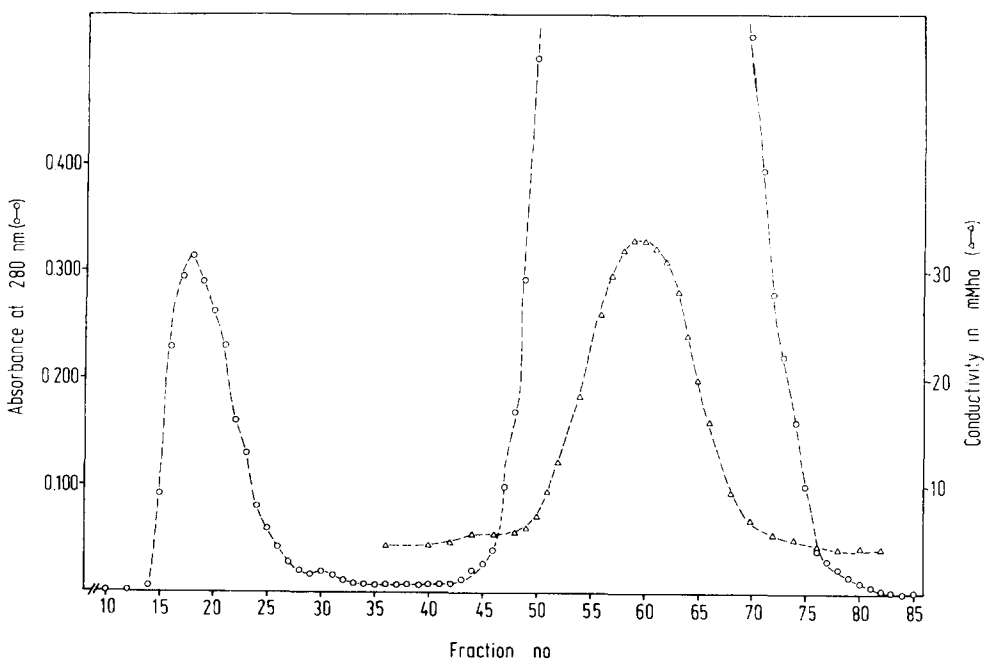

Fig. 1. Gelfiltration of a concentrated supernatant fraction. The supernatant was applied to a column (2.1 × 50 cm) of Sephadex G-200 and eluted with 0.1 M NH_4 acetate (pH 8.0) at 0–3 °C.

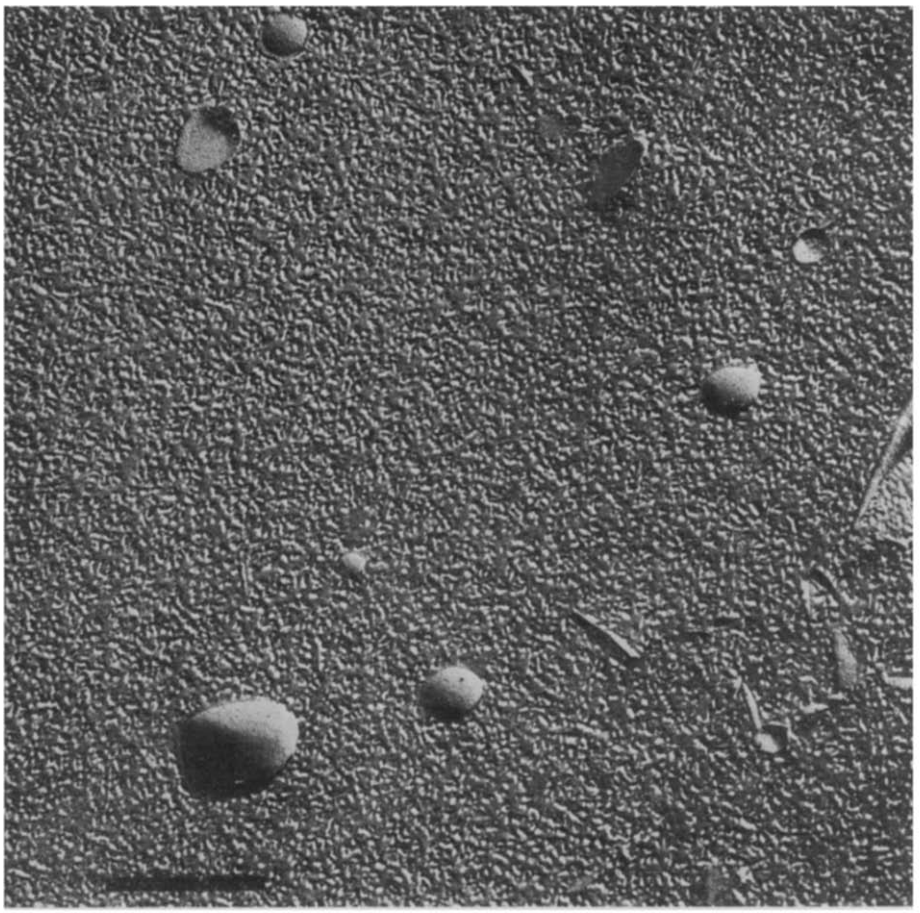

Fig. 2. Freeze-fracture electron micrograph of supernatant vesicles. Bar equals 300 nm.

protein was detected on sodium dodecyl sulfate-polyacrylamide gels. Chromatography of this material on Sephadex G-25 resolved it into two broad bands. The first of these preceded and overlapped the salt peak and probably consisted of small peptides [8], while the second peak trailed behind the salt peak and probably consisted of ultraviolet absorbing material such as nucleotides and tryptophane, which are retarded anomalously upon gel filtration on Sephadex. Further study of the low molecular weight material was deemed unnecessary.

The high molecular weight fraction appeared in the void volume (determined with Blue Dextran), suggesting that the proteins were present in a complex form and possibly aggregated with other cell components. Electron microscopy of this fraction showed that it did in fact consist of vesicular material (Fig. 2).

Similar results were obtained when *E. coli* W 3110 was grown to the stationary phase. The protein found in medium vesicles amounted to 0.3 and 0.5 % of the total cell protein of *E. coli* W 3110 and *E. coli* JC 411, respectively.

TABLE I

COMPARISON OF THE MEDIUM VESICLES TO THE CYTOPLASMIC AND OUTER MEMBRANES OF *E. COLI* JC 411

Analysis	Cytoplasmic membrane	Vesicles	Outer membrane
Phospholipid/protein (mg/mg)	0.56	0.20	0.22
Keto-deoxyoctulosonic acid (μmol/mg protein)	0.014–0.030	0.068–0.125	0.100–0.135
Buoyant density (g/cm^3)	1.17	1.24–1.28	1.234

TABLE II

COMPARISON OF THE PHOSPHOLIPID COMPOSITION OF THE CELLULAR MEMBRANES AND THE VESICLES RELEASED INTO THE MEDIUM BY *E. COLI* JC 411

Results are expressed as percent total phosphorus present.

Phospholipid	Cytoplasmic membrane	Vesicles	Outer membrane
Phosphatidylglycerol	8.1	13.6	14.0
Lysophosphatidylethanolamine	1.9	10.9	10.9
Phosphatidylethanolamine	78.9	65.1	61.1
Cardiolipin	11.0	10.3	14.1

TABLE III

FATTY ACID COMPOSITION OF CELLULAR MEMBRANES AND VESICLES RELEASED INTO THE MEDIUM BY *E. COLI* JC 411

Results of fatty acid composition are expressed as percentage of total fatty acids.

Fatty acid	Cytoplasmic membrane		Vesicles		Outer membrane	
12 : 0	0.2		1.1		0.3	
14 : 0	2.0		5.1		3.2	
16 : 0	47.5		50.7		50.0	
16 : 1	3.5	35.8	16.6	29.2	3.3	33.9
17 : cy	32.3		12.6		30.6	
18 : 1	5.0	14.2	9.8	14.1	4.1	12.5
19 : cy	9.2		4.3		8.4	
Saturated	49.7		56.9		53.5	
Unsaturated + cyclopropane	50.0		43.3		46.4	

Comparison of the medium vesicles to cytoplasmic and outer membrane

Membranes were obtained from stationary phase cells and fractionated on 20–60 % (w/w) sucrose gradients as described previously [12, 16]. The buoyant densities of the resulting outer ($\rho = 1.23$ g/cm^3) and cytoplasmic ($\rho = 1.17$ g/cm^3) membrane preparations are in good agreement with values published previously [3–4, 16]. Medium vesicles, prepared as described above, were heterogeneous with respect to buoyant density, and were found in a relatively broad band with $\rho = 1.24$ g/cm^3 and a heavier fraction which sedimented through the gradient ($\rho \geqslant 1.28$ g/cm^3).

Table I shows that in terms of the overall chemical composition, the medium vesicles resembled the outer membrane. More detailed analyses are described below.

Phospholipid and fatty acid composition

The relative phospholipid content of the cytoplasmic membrane fraction was appreciably higher than that of the outer membrane (Table I), in agreement with previous results [2–4]. The two membranes also differed significantly in phospholipid composition (Table II). As can be seen in Tables I and II, the medium vesicles were very similar to the outer membrane with respect to phospholipid content and composition. The high concentration of lysophosphatidylethanolamine in the outer membrane and the medium vesicles may be due to the activity of phospholipase A, which is known to be present in the outer membrane [3, 20].

Table III shows that the fatty acid compositions of the outer and cytoplasmic membrane differed only to a slight extent. The medium vesicles contained similar amounts of the saturated fatty acids, more unsaturated fatty acids and fewer cyclopropane fatty acids than were found in the cell membranes. Nevertheless, the total amounts of palmitoleic acid+cis-9 10-methylene hexadecanoic acid ($C_{16:1}+C_{17:cy}$) and cis-vaccenic acid+lactobacillic acid ($C_{18:1}+C_{19:cy}$), respectively were similar in the medium vesicles and in outer and cytoplasmic membranes.

Membrane enzymes and sodium dodecyl sulfate-polyacrylamide protein profiles

Table IV shows the activities of NADH oxidase, succinate dehydrogenase and D-lactate dehydrogenase in the membrane fractions and medium vesicles. As expected [2–4, 16], hardly any activity is present in the outer membrane fractions. The activity of these specific enzymes is as low in the medium vesicle fraction.

Fig. 3 shows protein profiles obtained after sodium dodecyl sulfate-polyacrylamide electrophoresis of medium vesicles and isolated cytoplasmic and outer

TABLE IV

ACTIVITY OF SEVERAL ENZYMES IN THE CELLULAR MEMBRANES AND THE VESICLES RELEASED BY *E. COLI* JC 411

Specific activity expressed as μmol of substrate converted per minute per mg of protein.

Enzyme	Cytoplasmic membrane	Vesicles	Outer membrane
NADH oxidase	1.540	0.007	0.015
D-Lactate dehydrogenase	0.165	0.006	0.005
Succinic dehydrogenase	0.033	0.003	0.002

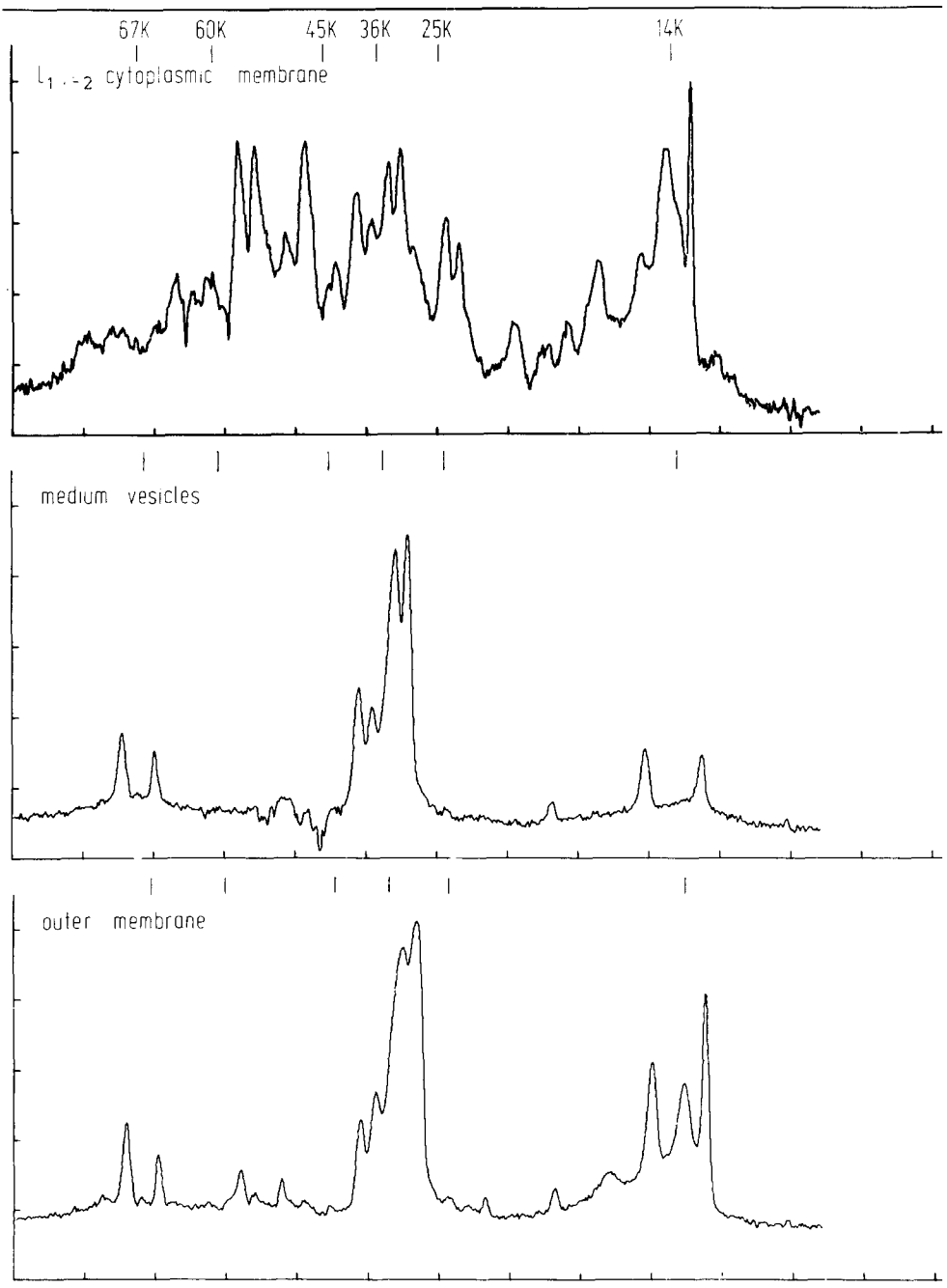

Fig. 3. Electrophoresis of membrane and vesicle proteins in sodium dodecyl sulfate-polyacrylamide slab gels. Vertical hatchmarks show position of reference proteins [14].

membranes. The vesicle protein profile changes when *E. coli* JC 411 is limited for its required amino acids (Kuipers, M and Witholt, B., unpublished results); the effect of changes in the growth conditions on the resulting medium vesicles are being investigated. The vesicle protein profile shown in Fig. 3 is representative for vesicles obtained from stationary phase cells which were limited for oxygen.

DISCUSSION

The release of complexes from gram-negative bacteria has been described by several authors. The conditions under which such complexes can be released are rather variable. Knox et al. [5, 6] demonstrated the excretion of a lipopolysaccharide-phospholipid-protein complex from a lysine auxotroph of *E. coli*, grown under lysine-limiting conditions. Rothfield and Pearlman-Kothencz extended these observations and showed that chloramphenicol as well as amino acid starvation of *E. coli* led to increased release of complex [7]. Infection of *E. coli* with phage T4 also caused the release of a lipopolysaccharide-phospholipid-protein complex [8]. Release of complex into the medium appears to be a general phenomenon; even under normal growth conditions small amounts are released [7, 8].

Although it has previously been suggested that these complexes are derived from the outer membrane [5–9], this has not been demonstrated explicitly by a comparison of their chemical composition to that of isolated outer membrane. There are in fact several other possibilities as to how these complexes might be formed. They might result from the lysis of a small percentage of cells, in which case both cytoplasmic and outer membrane fragments should be found in the medium. Alternatively, they might be formed because specific membrane components are excreted and aggregate in the medium following excretion. Variations in the growth conditions could alter the extent and the nature of the excretion, leading to variable amounts and compositions of the complexes. Cytoplasmic membrane components might be excreted as well as outer membrane components, resulting in mixed aggregates of doubtful origin*. Given the variable composition of the complexes isolated so far, and the fact that the 40 % protein content (w/w) found for the outer membrane [2–4] is considerably higher than the 10–15 % reported for the complexes, we have specifically compared complexes to the isolated membranes from which they might have been derived.

The results of this paper show that the composition of the medium membrane fragments closely resembles that of the outer membrane. Cytoplasmic membrane marker enzymes, which were absent from the outer membrane, were also absent from the medium membrane vesicles. The keto-deoxyoctulosonic acid content and the buoyant density of the supernatant vesicles were similar to those of the outer membrane. The clearest indication however, that these vesicles were derived from the outer membrane, was the striking resemblance of their protein and phospholipid compositions.

Thus, the supernatant material released by *E. coli* JC 411 under normal growth

* As an example, *Bifidobacterium bifidum* excretes substantial amounts of lipids into its medium under certain conditions. *B. bifidum* is gram-positive; the lipids must therefore originate in the cytoplasmic membrane. Moreover, no macromolecules (protein, nucleic acids) are released; the lipid excretion must therefore be specific [27].

conditions consists of outer membrane vesicles. Similar results have been obtained for *E. coli* W 3110, a K-12 strain [12], and two *E. coli* strains isolated from porcine feces at the Rijks Instituut voor de Volksgezondheid, Bilthoven, the Netherlands, and grown in rich medium (Gankema, H., Wensink, J. and Witholt, B., unpublished observations).

The fatty acid composition of the vesicles differed from that of the outer membrane with respect to the unsaturated and cyclopropane fatty acids. Although the sum of the unsaturated and cyclopropane fatty acids was approximately equal in the supernatant vesicles and the membranes, the vesicles contained more unsaturated fatty acids, while the membranes contained more cyclopropane derivatives of the corresponding fatty acids. Since unsaturated fatty acids are substituted by cyclopropane derivatives when cells enter the stationary phase [21], these data suggest that the vesicles accumulated in the supernatant in part during the exponential growth phase, in agreement with the results of Loeb [8] and Rothfield and Pearlman-Kothencz [7].

The outer membrane of *E. coli* contains a lipoprotein which occurs in one of two states. Per cell there are about 250 000 lipoprotein molecules which are bound covalently to the murein layer [22], while about 500 000 lipoprotein molecules occur in the free form [23]. The surface of a cell is about 4 μm^2 [24], while the area of the outer membrane vesicles described in this paper ranges from 10^4–10^5 nm^2. The vesicles must therefore be derived from outer membrane segments which could contain as many as 10^3–10^4 bound lipoprotein molecules, if the lipoprotein molecules are distributed homogeneously over the entire outer membrane surface. The release of outer membrane vesicles therefore requires either that 10^3–10^4 covalent murein-lipoprotein linkages be severed, or else that bound lipoprotein molecules are not distributed homogeneously over the entire cell surface. Severing of the covalent linkage between murein and outer membrane lipoprotein does occur to some extent, since it has been shown that the bound and free forms of the lipoprotein are interconvertible [23]. Heterogeneity of the surface distribution of bound lipoprotein is also possible, however. It could arise if in certain regions fewer of the newly inserted free lipoprotein molecules are linked covalently to the murein layer, or if the absolute number of lipoproteins introduced into these regions is low. This last possibility is supported by the fact that the outer membrane vesicles we found in the culture supernatants contain less lipoprotein than the outer membrane of the corresponding cells. Moreover, freeze-fracture preparations of *E. coli* normally do not show cleavage planes in the outer membrane which might be, at least in part, because the penetration of the lipoprotein through the outer membrane prevents the formation of such cleavage planes. The supernatant vesicles do show cleavage planes however, which correlates with their decreased lipoprotein content.

Thus, vesicles are probably formed in areas where there are few outer membrane-murein connections. Such areas could arise due to the cleavage of existing lipoprotein-murein linkages as discussed above, or they could be formed due to the preferential insertion of lipopolysaccharides, phospholipids, and proteins other than the lipoprotein into specific outer membrane zones. Such zones might occur at the site of cell division. Weigand and Rothfield [25] have recently described *Salmonella* mutants which form large outer membrane blebs at the septum, while Burdett and Murray, who have investigated septum formation during cell division of *E. coli* [26], found that the outer membrane appears to form groups of vesicles at the site of divi-

sion; these vesicles are probably identical to those described in this paper. We are now performing experiments to test whether the release of outer membrane fragments does result from cell division.

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