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A NOVEL ELECTROPHORETIC FRACTIONATION OF *ESCHERICHIA COLI* ENVELOPES

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SUMMARY

Particulate fractions of *Escherichia coli* have been submitted to electrophoretic fractionation in a buffer stabilized by sucrose gradient. Inner membrane and outer membrane were readily resolved. A combination of electrophoresis, fractional centrifugation and gel filtration can remove remaining contamination by ribosomes and cytoplasm.

The presence of particles containing no phospholipids was detected after differential centrifugation. The nature of this fraction is unknown. The inner membrane exhibited heterogeneity on electrophoresis.

INTRODUCTION

Envelopes of gram-negative bacteria are complex structures [1, 2]. They include an outer layer, called outer membrane since it contains proteins and phospholipids and gives often an electron microscopic image of a triple layer, but also called cell wall or lipopolysaccharide-containing layer [3, 4]. It is separated by a rigid but thin layer, formed by a single covalent murein macromolecule called the sacculus [5–8] from the inner membrane (cell membrane or plasma membrane), which is the most important layer for vital functions. Some proteins are located between the two membrane layers, in a region which is called the periplasmic space [9, 10].

Preparation of pure fractions from the complex envelopes has been attempted in a variety of ways. Penicillin inhibition of the synthesis of murein or its digestion by lysozyme produce spheroplasts [11] whose osmotic lysis in carefully controlled conditions gave fairly reproducible membrane vesicle preparations which proved useful to solve a number of important problems [12–15]. Techniques were also devised to yield the outer layer, based on the solubilisation of the plasma membrane in non-ionic detergents [16, 17], leaving the outer layer intact.

In order to avoid alterations of the native membrane during preincubation, mechanical disruption was also used followed by fractionations yielding two main layers. Fractionations have been usually based on isopycnic centrifugation [18–20] or a combination of a continuous free flow electrophoresis [21, 22] with isopycnic centrifugation. The overall yield of this preparation was around 20 %.

All these methods are relatively time consuming. Preincubations with lysozyme range from 30 to 60 min at room temperature, isopycnic centrifugations are run for 16–40 h, and free flow electrophoresis takes several hours to furnish significant amounts of preparations.

In the present article, we describe the fractionation of mechanically disrupted envelopes of *Escherichia coli* by zonal electrophoresis in a liquid column stabilized by a sucrose gradient which yields in 30–60 min two visibly and clearly separated fractions, one containing the cell wall, the other the cell membrane. The fractionations have been monitored by the measurement of membrane and cell wall markers, and combinations of the electrophoretic separation with differential centrifugation and gel filtration are also described.

MATERIALS AND METHODS

Bacteria. Escherichia coli K12 strain 3300 was grown in 50-l fermentors under aeration in a medium of the following composition: 40 mM K_2HPO_4, 100 mM KH_2PO_4, 30 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 1.8 μM $FeSO_4$, 5 g/l glycerol.

Smaller cultures were grown in 500-ml batches in Fernbach flasks. When phospholipids were labeled with [2-^{3}H]glycerol the main carbon source was lactose.

Preparation of particulate and soluble fractions. Bacteria, harvested by centrifugation were washed with 1 mM Tris · HCl buffer, pH 7.5, with 1 mM magnesium acetate, resuspended in the same buffer at a cell density of approx. 30 mg dry weight per cm^3 and submitted to disruption under a pressure of 30 000 lb/inch^2 (approx. 1600 bars) in a Ribi cell fractionator (Sorvall) at 5 °C. Large debris were eliminated by sedimenting 10 min at 10 000 $\times$ g. From the supernatant fraction of this step (the crude extract), the cell envelope particles were collected either in a single step centrifugation, 90 min at 165 000 $\times$ g which yielded the crude sedimentable fraction or in two steps: the first 20 min at 60 000 $\times$ g sedimenting the heavy particles, and the second, 90 min at 165 000 $\times$ g, furnishing the light particles and the cytoplasmic supernatant fraction. Pellets were washed once with 1 mM Tris · HCl buffer, pH 7.5.

Gel filtration. Columns (2.6 $\times$ 40 cm) were packed with 150 M biogel equilibrated with buffer. 1–5 ml (20–60 mg protein) suspension of sedimentable fraction, were applied to the column and eluted with the same buffer. 5-ml fractions were collected.

Electrophoresis. Electrophoresis was carried out in the vertical cylindrical apparatus represented on Fig. 1a which has a migration chamber of 50 ml. A larger apparatus with a migration chamber of 300 ml was used occasionally. These incorporate the feature of a small diameter central cylinder for the passage of the lower electrode and the escape of the lower electrode gases. This chimney communicates with the main outer cylinder through openings near the bottom. The main cylinder is provided with side tubes for the introduction of the sample and for the harvest of fractions after the run.

The main cylinder, when functioning, includes three sections, the lower electrode vessel, the migration chamber and the upper electrode vessel. The electrode vessels contain high conductance electrolyte while the migration chamber contains

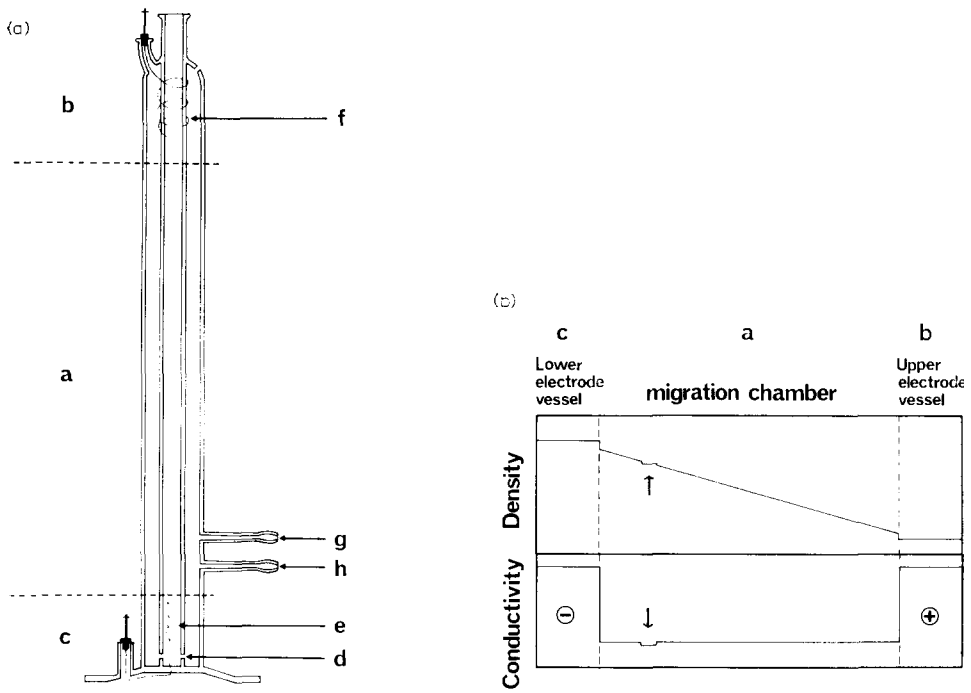

Fig. 1 (a) and (b). See opposite page for legend.

low conductance buffer. The separation of these solutions and the stabilization of the electrolyte in the migration chamber are insured exclusively by the density gradient obtained with sucrose. The field is uniform between the two horizontal planes which separate the content of the migration chamber from the electrode vessels.

Table I gives sets of values for the composition and volume of the three compartments which have been varied experimentally. The total volume, the presence or absence of Mg^{2+}, the range of sucrose concentration have been varied with no substantial change in the results. All results reported here have been obtained with Tris · HCl, pH 7.5.

Operation. Lower electrode fluid is introduced through the central cylinder, followed by the building of the linear gradient in the migration chamber.

Before introducing the sample, a small volume is substracted from the migration chamber through the side tube (g), so that a discontinuity is formed in the linear gradient. The sample containing the particles in buffer is then adjusted with a concentrated solution of sucrose to the same refractive index as the liquid withdrawn from the migration chamber (see Fig. 1b), and carefully injected through the same side tube. The dc power supply can now be switched on. We currently used 800 V, which give a field of 37 V/cm and a current of 5 mA/cm^2. The separation of two fractions becomes visible after 15 min. After the run, fractions are collected through the side arm.

Analytical techniques. Particulate material was monitored by nephelometry at 350 nm in cells of 1 cm light path on a Zeiss PMQ2 spectrophotometer.

Protein was measured by absorbance at 280 nm or by the method of Lowry et

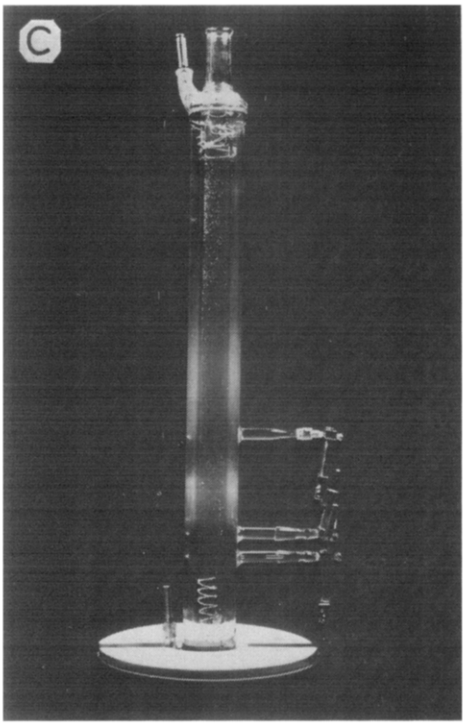

Fig. 1. (a) A schematic representation of the apparatus for zonal electrophoresis. a, migration chamber with upper, b, and lower, c, sections of electrode vessel; d, openings for communication between small central cylinder, and the main outer cylinder, in its lower electrode vessel section; e, lower electrode; f, upper electrode, g, side arm for introduction of sample after withdrawal of a small volume of the migration electrolyte; h, side arm for harvesting. (b) Schematic density and conductivity distribution of the electrolyte along the length of the main cylinder. The arrows indicate the place of the sample. (c) Photograph of the electrophoresis apparatus after 20 min migration of a crude sedimentable fraction of *E. coli*.

TABLE I

	Small cell		Large cell
	Standard conditions	Variants	
Upper chamber	10 ml 100 mM Tris · HCl, pH 7.5	Tris · HCl, pH 8.1 potassium phosphate, pH 6.5, 1 mM Mg^{2+}	50 ml
Migration chamber	50 ml 1 mM Tris · HCl, 30–5 % sucrose	1–2 mM electrolyte as above, 15–5 % sucrose	300 ml
Lower chamber	15 ml 100 mM Tris · HCl, pH 7.5, 45 % sucrose	Electrolyte as in upper vessel, 45 % sucrose	100 ml
Sample	10 mg protein	10 mg protein	80 mg protein

al. [23] with bovine serum albumin as the standard. Nucleic acids were detected by absorbance at 260 nm.

Lipopolysaccharide was measured by its content of ketodeoxyoctulosonate [24] according to Ashwell [25]. 2-ml samples were precipitated with 10 % trichloroacetic acid and washed twice by centrifugation to remove sucrose which interferes with the reaction.

Phospholipids were measured after extraction with chloroform/methanol/water (1 : 2 : 0.7, v/v) according to Bligh and Dyer [26]. After wet ashing 2 h at 180 °C with a 4 : 1 (v/v) mixture of H_2SO_4 and $HClO_4$ containing 2 mM ammonium molybdate, inorganic phosphate was measured by the method of Fiske and SubbaRow [27]. In some experiments, phospholipids were measured by radioactive counts after incorporation of [2-^{3}H]glycerol during culture for three generation times. At least 98 % of ^{3}H radioactivity was extractable with chloroform/methanol.

Phospholipids were analyzed by chromatography on silica-impregnated paper (Whatman SG81) using chloroform/methanol/ammonia/water (100 : 40 : 0.75 : 2.25, v/v) as solvent in an ascending system.

NADH oxidase was measured by the decrease in absorbance at 340 nm. The reaction mixture contained in 1 ml buffer 0.25 mM NADH and 0.2 ml of membrane fraction from electrophoresis or 50–100 μg membrane protein.

Cytochromes were measured by reduced-oxidized difference spectra in an Aminco-Chance double beam spectrophotometer.

β-Galactosidase was measured by the rate of hydrolysis of *o*-nitrophenol β-D-galactoside [28].

RESULTS

Electrophoresis of the crude sedimentable fraction

Fig. 1c shows the visible results of the electrophoretic migration, and Fig. 2

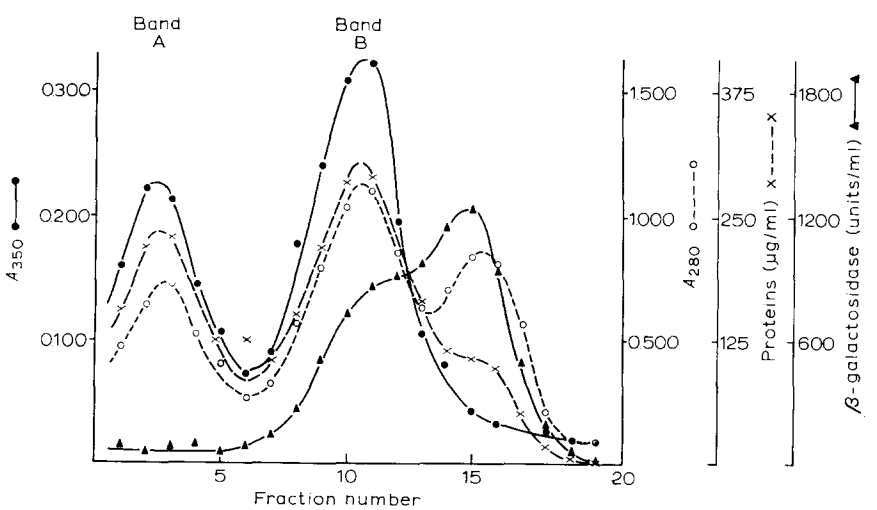

Fig. 2. Profile obtained after electrophoretic migration of a crude sedimentable fraction of *E. coli*. Fractions of 1.5 ml were collected. ●—●, turbidity measured at 350 nm; ○—○, absorbance at 280 nm; ▲—▲, β-galactosidase; ×- - -×, protein.

represents the profile obtained by migration of a crude sedimentable fraction. Turbidimetry at 350 nm in the collected fractions showed two peaks A and B of approximately equal size.

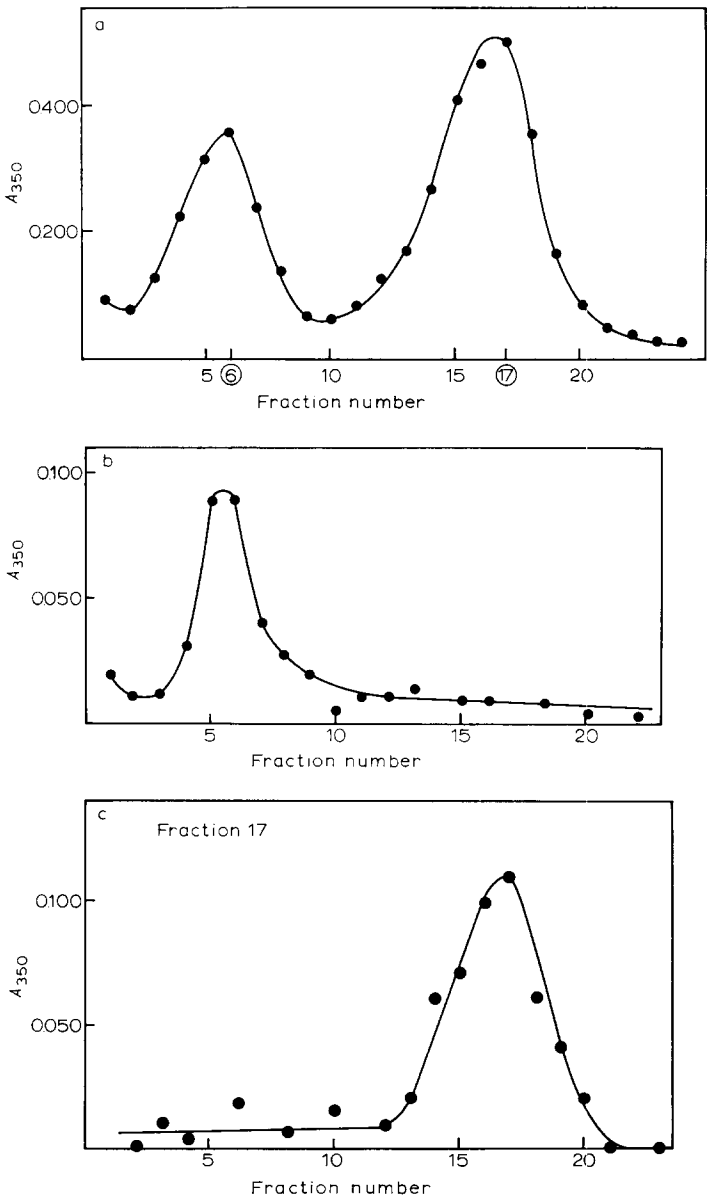

Fig. 3. Reproducibility of the electrophoretic migration. After a first migration of a crude sedimentable fraction as in Fig. 2, yielding the turbidity profile shown in (a) fraction 6 of band A and fraction 17 of band B were centrifuged at 165 000 × *g*. Pellets were resuspended in 10^{-3} M Tris · HCl (pH 7.5) and submitted to electrophoresis. (b) Profile obtained after rerun of fraction 6. (c) Profile obtained after rerun of fraction 17.

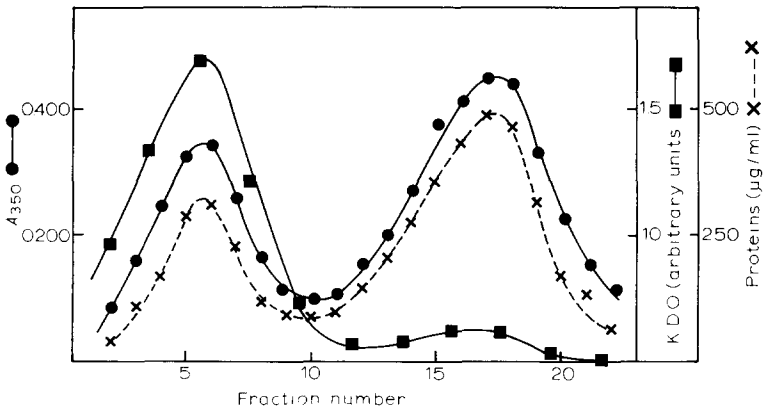

Fig. 4. Distribution of lipopolysaccharide. ● — ●, turbidity measured at 350 nm; ■ — ■, ketodeoxyoctulosonate (KDO); × — ×, protein.

The separation of the two turbid fractions was reproducible. Fig. 3 shows the single peak obtained upon a rerun of peaks A and B, respectively, after harvest, centrifugation and resuspension in the original buffer. Similarly reproducible patterns were found when particles from peak A and B were submitted to sonication before the second run.

Protein showed a similar distribution except that a third peak appeared also as a shoulder. The absorption spectrum of this peak showed that it was rich in nucleic acid, so in all likelihood it contained the ribosomes which had been sedimented together with the envelopes. β-Galactosidase was found to contaminate peak B but two-thirds of this enzyme migrated ahead of this peak (Fig. 2).

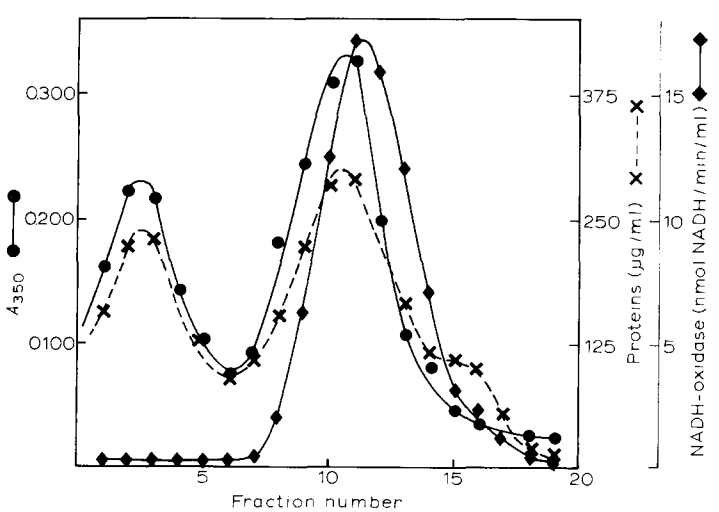

Fig. 5. Distribution of NADH oxidase activity. ● — ●, turbidity measured at 350 nm; × — ×, protein; ◆ — ◆, NADH oxidase activity.

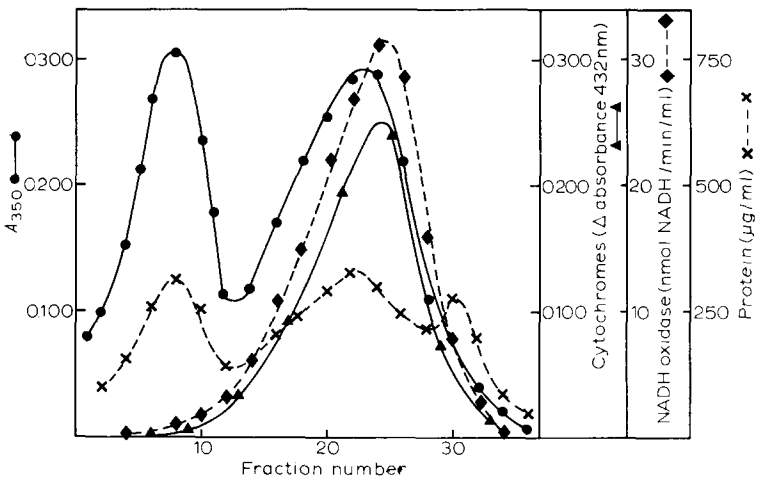

Fig. 6. Distribution of cytochromes. 70 mg of a crude sedimentable fraction were submitted to electrophoresis in a large size apparatus for 1.5 h. Fractions of 3 ml were collected. ● — ●, $A_{350\,nm}$; × — ×, protein; ◆ — ◆, NADH oxidase activity; ▲ — ▲, cytochromes measured by reduced minus oxidized difference spectrum at 432 nm.

Fig. 4 represents the distribution of a typical cell wall marker: ketodeoxyoctulosonate, part of the lipopolysaccharide molecule. This sugar was reproducibly associated with peak A but consistently 5–8 % migrated with peak B. NADH oxidase, a typical marker of inner membrane (Fig. 5), was associated exclusively with peak B.

Peak B was always much broader than peak A. The maximum of NADH oxidase was found regularly one or two fractions more anodic than the maximum of turbidity or of protein. The distribution of cytochromes measured by the difference spectrum in the Soret region exhibited the same shift (Fig. 6) indicating that the heterogeneity could not be due only to a different accessibility to NADH.

Fractional centrifugation

It has been commonly admitted that light particles represent a better membrane preparation than total sedimentable particles. Since sedimentation depends much on the particle size, each technique of disruption had to be associated with the suitable cut in the sedimentation programme.

For particles obtained by extrusion used in the present study, equal aliquots of crude extract were submitted to centrifugation for 20 min at different speeds. Proteins sedimented in two distinct steps. NADH oxidase activity was sedimented with the second wave of protein. Subsequently a centrifugation at 30 000 rev./min for 20 min was adopted to sediment the heavy particles containing about 5 % of the total NADH oxidase with 35 % of the total sedimentable protein.

Gel filtration

As expected from the speed of the ultracentrifugation and as suggested by results shown on Fig. 2, ribosomes were the main contaminants of cell envelopes in the crude sedimentable fraction.

The gel filtration on 150 M agarose was designed to obviate the contamination.

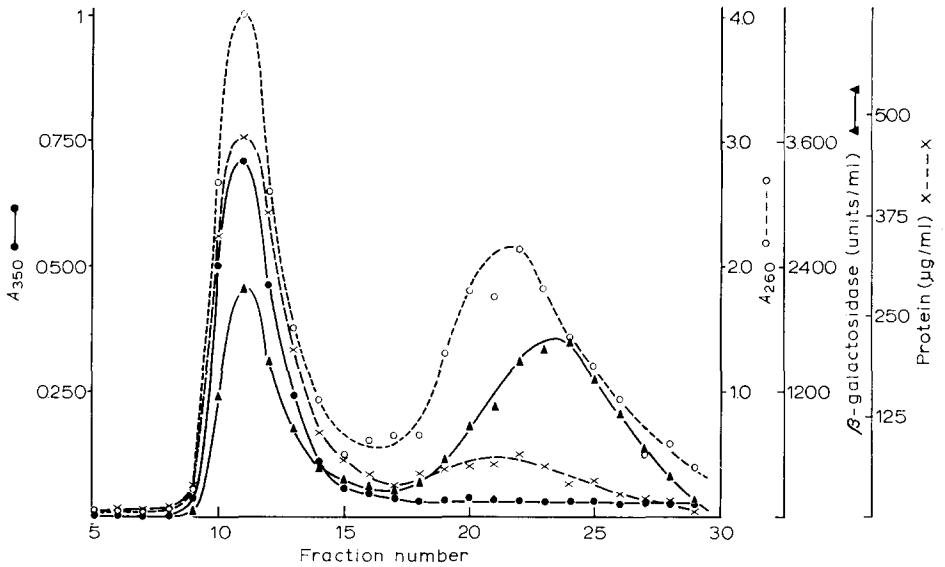

Fig. 7. Gel filtration of the crude sedimentable fraction. ●—●, $A_{350\,nm}$; ○—○, $A_{260\,nm}$; ×---×, protein; ▲—▲, β-galactosidase activity.

As shown on Fig. 7 the peak in the exclusion volume contained most membrane and cell wall particles. When light scattering calculated from $A_{350\,nm}$ by extrapolation to 260 nm was subtracted, only trace amounts of nucleic acid were found associated with the main membrane peak. Ribosomes were notably retarded and rather well separated from the envelope particles. About 3 % of cellular β-galactosidase contaminate the crude sedimentable fraction. Agarose filtration permits the elimination of 70 % of this contamination.

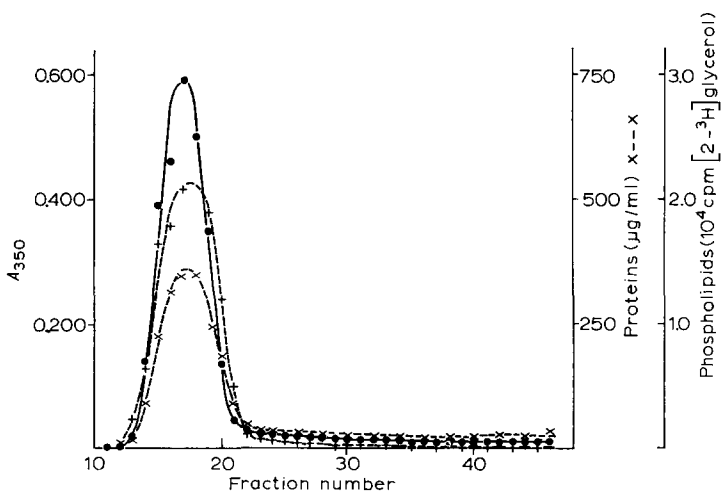

Fig. 8. Gel filtration of heavy particles. ●—●, $A_{350\,nm}$; ×---×, protein; +---+, phospholipids measured by ^{3}H radioactivity incorporated after growth on [2-^{3}H]glycerol.

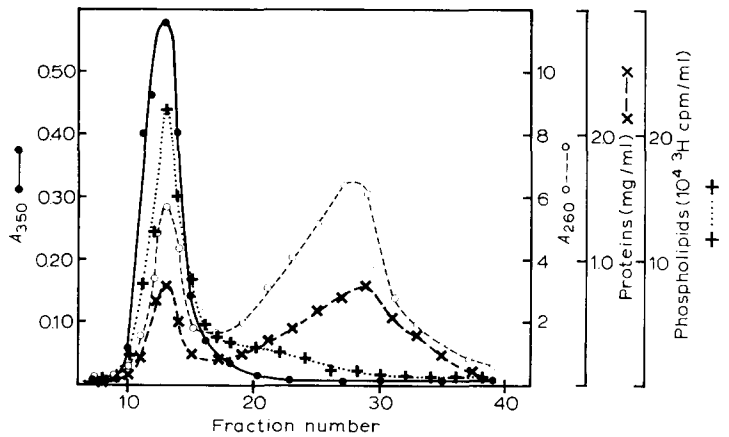

Fig. 9. Gel filtration of light particles. ● — ●, $A_{350\,nm}$; ○ — ○, $A_{260\,nm}$; × --- ×, protein; + --- +, phospholipids.

Combined utilization of gel filtration differential centrifugation and electrophoresis

Figs 8 and 9 show the gel filtration profile of heavy and light particles, respectively. Heavy particles did not contain ribosomal and soluble contaminants. The light particles were separated from these contaminants by the gel filtration, but at the same time, some small size membrane particles, identified by their phospholipid content and also by their NADH oxidase activity were trailing, so as to be eluted together with the ribosomal peak. The NADH oxidase to phospholipid ratio in the retarded fractions was not significantly different from that found in the main peak. Assuming that the small membrane fragments retarded on the gel have the same protein to phospholipid ratio as the main peak, the non-membrane proteins amount to 60 % of the input.

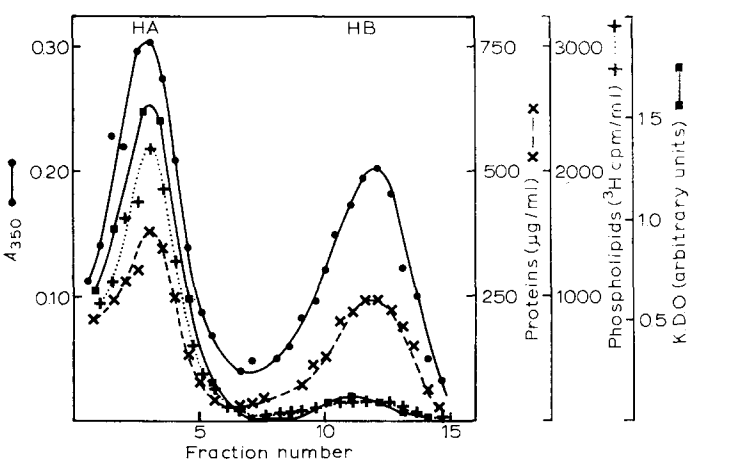

Fig. 10. Electrophoresis profile of heavy particles. Heavy particles from the exclusion volume of the agarose column represented in the figure were collected and submitted to electrophoresis. ● — ●, $A_{350\,nm}$; × --- ×, protein; ■ — ■, ketodeoxyoctulosonate (KDO); + --- +, phospholipids.

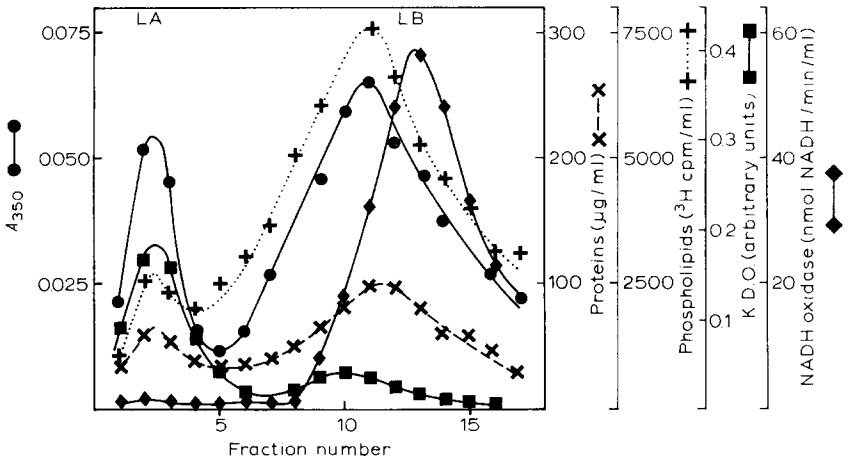

Fig. 11. Electrophoretic profile of light particles. Light particles from the exclusion volume of the agarose column represented in the figure were collected and submitted to electrophoresis. ●—●, $A_{350\,nm}$; ×—×, protein; ■—■, ketodeoxyoctulosonate (KDO); +---+, phospholipids; ◆—◆, NADH oxidase.

Figs 10 and 11 show the electrophoretic profile obtained with the heavy and light particles which had been submitted to the agarose column filtration and collected with the exclusion volume.

Table II shows the distribution of protein, phospholipid, lipopolysaccharide and NADH oxidase between the four peaks obtained in percent of the amounts found in the crude sedimentable fraction. Components of the light membranes as recovered were corrected for the loss of recovery after gel filtration. NADH oxidase was localized almost exclusively in peak B of the light particles. Ketodeoxyoctulosonate distributed in contrast between the light and heavy particles, 91 % being in the two A peaks; however, about 9 % was found associated with the B peaks, even if these were submitted to a second run of electrophoresis. The lipopolysaccharide to protein ratio and the phospholipid to protein ratio were identical in heavy and light A particles.

Peak B of the heavy particles contained only trace amounts of phospholipids

TABLE II

DISTRIBUTION OF MAIN COMPONENTS AND SPECIFIC MARKERS AFTER CENTRIFUGATION, FILTRATION AND ELECTROPHORETIC FRACTIONATION

Fractional centrifugation yielded heavy (H) and light (L) particles which were subfractionated into peaks HA, HB, LA and LB respectively. Losses of membrane material during gel filtration of light particles have been corrected. The results are expressed as percent of crude sedimented fraction.

Fractions	HA	HB	LA	LB
Proteins	20	20	6	18*
Phospholipids	18	2	7	73
Ketodeoxyoctulosonate	67	3	24	6
NADH oxidase	< 1	6	< 1	94

* The missing 36 % is due mainly to ribosomes.

TABLE III

DISTRIBUTION OF CELLULAR PROTEIN AND PHOSPHOLIPID BETWEEN CELL WALL, MEMBRANE AND CYTOPLASM

For definition of fractions, see legend to Table II.

	Proteins	Phospholipids
Cell wall or lipopolysaccharide layer (HA+LA)	12 %	25 %
Plasma membrane (LB+part of HB)	9 %	72 %
Total	21 %	97 %
Unidentified particulate fraction (bulk of HB)	9 %	trace
Cytoplasm	70 %	trace

and of NADH oxidase; thus the bulk of it does not qualify as a membrane layer and will be referred to as the unidentified particulate fraction.

Table III shows the distribution of cellular protein and phospholipid in the two recognizable layers, cell wall and plasma membrane, in the unidentified particulate fraction and in the cytoplasm.

The ratio phospholipid to protein is about 0.3 in the plasma membrane and only 0.1 in the cell wall.

The distribution of main phospholipids was studied in the four peaks by chromatography, after incorporation of [2-^{3}H]glycerol for three generation times. The ratio phosphatidylglycerol to phosphatidylethanolamine was twice as high in the membrane fraction as in the cell wall.

DISCUSSION

The electrophoretic fractionation of bacterial envelopes here described results in a particularly rapid and clearcut separation of the concentric layers, and might be a valuable addition to existing techniques. Two features merit special emphasis. (a) The method is conservative, virtually 100 % of the sedimentable material can be collected with little or no overlap of identifiable fractions, so that a complete inventory and balance sheet are possible. (b) The rapidity of the method is not only a convenience, but also provides optimal conditions for functional integrity of the collected membrane fractions.

Contamination of envelope particles is due essentially to ribosomes and to soluble proteins. Centrifugation and washing are effective in eliminating contamination by the soluble fractions, and the electrophoresis provides a fair selectivity between plasma membrane and ribosomes. Nevertheless, the best remedy for the two contaminants is gel filtration, and this can be rapid if no further selectivity is expected from this step. The contamination which remains is due mainly to inclusion of soluble proteins into closed membrane vesicles. The amount of such contamination depends largely on the concentration of bacteria during disruption. Cell wall material in the membrane fraction resists further attempts of separation. It is possible that its

presence is a direct consequence of the bacterial anatomy: it might be due e.g. to cell wall precursors being synthesized in the membrane layer just before they reach their final location [29, 30].

There is no difficulty with the assignment of markers such as ketodeoxyoctulosonate and NADH oxidase to cell wall and plasma membrane, respectively. The difficulty is raised only by the finding that electrophoresis after previous fractional centrifugation reveals a particulate fraction with a membrane-like electrophoretic mobility but a non-membrane composition. The existence of such a fraction would have escaped attention without the centrifugal step. No interpretation is offered presently about the nature and in vivo localization of this component.

One important point concerns homogeneity, especially that of the inner-membrane fractions. Theoretically, a heterogeneity might be expected on several grounds. Such dualities as cylindrical vs polar parts [31] of the membrane, plasma membrane vs mesosomal particles [32], inside out artifacts vs right side out vesicles [33], growing membranes vs resting membrane [34], could result in subfractions.

Experimentally, the asymmetric distribution of NADH oxidase suggests that a subfractionation actually occurs during electrophoresis. Attempts to improve further fractionation are underway.

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