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THE OUTER MEMBRANE OF *PROTEUS MIRABILIS*

IV. SOLUBILIZATION AND FRACTIONATION OF THE OUTER AND CYTOPLASMIC MEMBRANE COMPONENTS

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SUMMARY

1. The solubilization and fractionation of the outer and cytoplasmic membrane components of *Proteus mirabilis* were studied as a first step towards the reconstitution of the membranes from their solubilized components.

2. Membrane phospholipids were specifically labeled by growing the organisms with radioactive oleic acid. The lipopolysaccharide component of the outer membrane was effectively labeled by adding radioactive galactose to the medium. Less than 5 % of the label derived from galactose was found in the outer membrane phospholipids and none in the protein.

3. Sodium dodecyl sulfate effectively solubilized all three components of the outer membrane while sodium deoxycholate and Triton X-100 solubilized almost all the phospholipid, but only about half of the protein and lipopolysaccharide. The cytoplasmic membranes of *P. mirabilis* were much more susceptible to solubilization by the three detergents tested. Treatment of the outer membrane with aqueous butan-1-ol separated the phospholipid into the butanol phase, while most of the protein and lipopolysaccharide was found in the aqueous phase.

4. Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate separated the lipopolysaccharide into two bands. Both bands were stained with the periodic acid-Schiff reagent, but only the slower migrating one stained with Coomassie blue. Filtration of the solubilized outer membranes through a Sephadex G-200 column containing sodium dodecyl sulfate separated the two lipopolysaccharide types. Our results support and supplement those of Gmeiner (Eur. J. Biochem. (1975) 58, 621–626) in showing that *P. mirabilis* produces two lipopolysaccharide types, one having long 0-antigenic side chains, rich in lysine and amino sugars, while the other one is poorer in 0-antigenic chain components and more hydrophobic in character.

INTRODUCTION

Elucidation of the molecular organization of the proteins, lipopolysaccharide and phospholipids in the outer membrane of Gram-negative bacteria has been the

major goal of our studies on *Proteus mirabilis* [1–3]. One approach to this problem, based on the specific labeling and enzymic degradation of the outer membrane components in intact cells and isolated membranes, has been tested and the results are reported in the accompanying communication [3]. Another approach to the elucidation of the molecular organization of biomembranes is based on the solubilization of the membrane to its building blocks, followed by the biophysical and biochemical characterization of the solubilization products and their reassembly to a membrane identical to the native membrane in structure and function [4]. The present communication deals primarily with the solubilization and the fractionation of the outer membrane components by detergents and provides data on the specific labeling of these components, in particular on the lipopolysaccharide of *P. mirabilis*.

MATERIALS AND METHODS

Organism and membrane fractions. P. mirabilis strain 19, kindly provided by Professor H. H. Martin (Technische Hochschule, Darmstadt, Germany), was grown as described previously [1]. To label membrane phospholipids, 15 μCi of [9, 10-$^3H_2$]oleic acid (3 Ci/mmol, The Radiochemical Centre, Amersham, England) were added to each liter of the growth medium. To label lipopolysaccharides, each liter of the growth medium was supplemented with 15 μCi of D-[1-^{3}H]galactose (13 Ci/mmol), or 5 μCi of D-[1-^{14}C]galactose (30 Ci/mol). For simultaneous labeling of phospholipids and lipopolysaccharides, each liter of the growth medium was supplemented with 25 μCi of D-[1-^{3}H]galactose and 4.5 μCi of [1-^{14}C]oleic acid (60 Ci/mol). The outer and cytoplasmic membrane fractions were isolated by sucrose density gradient centrifugation of the crude cell envelope fraction obtained by sonication of the cells in the presence of lysozyme [1].

Solubilization of membrane components. Washed membrane suspensions (containing 1 mg membrane protein/ml) in NaCl/Tris/β-mercaptoethanol buffer (β-buffer [5]) diluted 1 : 20 in deionized water (dilute β-buffer) were treated for 15 min at 37 °C with different concentrations of sodium dodecyl sulfate, sodium deoxycholate or Triton X-100. The solubilized membrane material was separated from the nonsoluble residue by centrifugation at 100 000 $\times g$ for 1 h. Solubilization of membrane components by butan-1-ol was carried out by a modification of Maddy's procedure [6] as described in detail by Rottem et al. [7].

Gel filtration. A Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (90 $\times$ 2.5 cm) was equilibrated with dilute β-buffer containing 1 % sodium dodecyl sulfate. The void volume of the column, as determined by Blue Dextran 2000, was found to be about 120 ml. Washed membranes (containing about 20 mg protein) isolated from cells grown with [^{3}H]galactose and [^{14}C]oleic acid were solubilized in 4 % sodium dodecyl sulfate (8 mg detergent/mg membrane protein) in dilute β-buffer as described above. About 4 ml of the solubilized membrane material were applied to the column. Filtration was carried out at 37 °C and 4-ml fractions were collected. Protein in the fractions was determined according to Lowry et al. [8] and the phospholipid and lipopolysaccharide content was assessed by radioactivity measurements in a Packard Tri-Carb liquid scintillation spectrometer using a toluene/Triton X-100 (2 : 1, v/v) scintillation liquor [11].

Lipid extraction. Lipids were extracted from sedimented membranes labeled with [^{3}H]oleic acid or [^{14}C]galactose by two successive extractions with chloroform/methanol (2 : 1, v/v), the first at 45 °C for 2 h and the second at room temperature overnight. The extracts were combined and dried under a stream of N_2. Thin-layer chromatography of the extracted lipids was carried out as described before [2].

Lipopolysaccharide extraction. Lipopolysaccharide was extracted from whole cells or membrane preparations with phenol/water according to Westphal et al. [9]. The water phase was dialyzed for 24 h against deionized water and then freeze dried. This preparation consisted of the crude, unpurified lipopolysaccharide heavily contaminated with nucleic acids. Purified lipopolysaccharide was obtained by extensive treatment of the crude lipopolysaccharide preparation with ribonuclease, deoxyribonuclease and pronase [1] followed by electrodialysis [10].

Gel electrophoresis. The sodium dodecyl sulfate gel electrophoresis system of Fairbanks et al. [12] was used with the modifications outlined in detail by Amar et al. [13]. To detect the proteins, the gels were fixed overnight at 37 °C in 25 % isopropanol containing 10 % acetic acid and stained by Coomassie blue. To detect the lipopolysaccharide, the gels were fixed overnight at 37 °C in 40 % ethanol containing 5 % acetic acid, and stained with the periodic acid-Schiff reagent as described by Segrest and Jackson [14]. Densitometer tracings of the stained gels were made in a Kipp and Zonen densitometer model DD2.

Localization of the lipopolysaccharide in the gels was also made by determination of radioactivity derived from galactose in gel sections. For this purpose, after electrophoresis the gels were frozen and sliced laterally into 2-mm sections which were transferred to scintillation vials containing 0.5 ml of Soluene 350 (Packard). The slices were solubilized by warming the solution to 65 °C for 2 h, and counted after the addition of the dioxane/toluene scintillation liquor [1].

RESULTS

Selective labeling of the phospholipid and lipopolysaccharide membrane components

To facilitate the assessment of the selective solubilization of the outer membrane components, simple and rapid means for the quantitation of phospholipid and lipopolysaccharide were sought. The amount of phospholipid was effectively assessed by determining the radioactivity derived from labeled oleic acid added to the growth medium. Over 90 % of the radioactivity derived from oleic acid was found in the lipid fraction extracted from the membranes with chloroform/methanol.

Estimation of the lipopolysaccharide component posed greater difficulties. The conventional method based on the determination of 2-keto-3-deoxyoctonoic acid is laborious and requires large quantities of material [1]. An attempt to label lipopolysaccharide with D-galactose was therefore made.

When radioactive D-galactose was added to the medium, over 90 % of the label was found in the outer membrane fraction, and less than 10 % in the cytoplasmic membrane fraction. Extraction of the labeled outer membrane with chloroform/methanol (2 : 1, v/v) removed less than 5 % of the radioactivity into the extractable lipid fraction consisting of phospholipids [2]. However, similar treatment of labeled cytoplasmic membranes showed over 50 % of the radioactivity to be associated with the major phospholipids: phosphatidylethanolamine and diphosphatidylglycerol. To

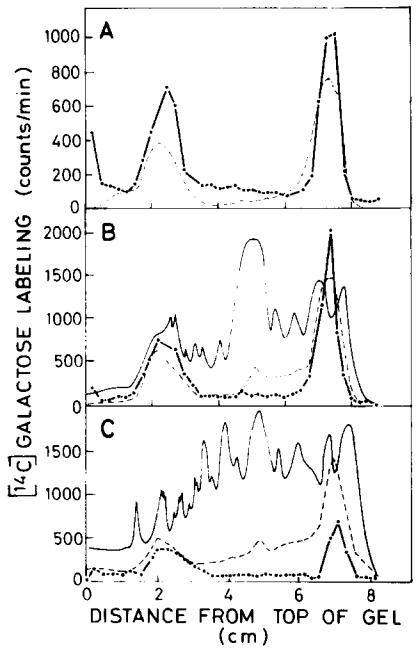


Fig. 1. Electrophoretic analysis of purified *P. mirabilis* lipopolysaccharide (A), outer membranes (B) and cytoplasmic membranes (C) in polyacrylamide gels containing sodium dodecyl sulfate. ———, densitometer tracing of bands stained with Coomassie blue; – – –, densitometer tracing of bands stained with periodic acid-Schiff reagent; ●–●, radioactivity derived from [^{14}C]galactose.

test membrane proteins for the presence or absence of radioactivity derived from D-galactose, labeled outer and cytoplasmic membranes were subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Fig. 1 shows that the radioactivity from both membrane types was not associated with the protein bands but was localized in two distinct regions in the gels, both of which gave a positive staining reaction (red) with the periodic acid-Schiff reagent. The upper band (designated PAS_1) was also stained by Coomassie blue, exhibiting a pink-violet color as opposed to the blue color characterizing the protein bands (Fig. 2). A very similar picture was given by the purified lipopolysaccharide itself (Figs 1 and 2). Careful inspection of Figs 1 and 2 also reveals a band weakly stained by the periodic acid-Schiff reagent in the region of the major protein band of the outer membrane. This band (designated PAS_3) could not be detected in the electrophoretogram of the purified lipopolysaccharide and was not associated with any radioactivity peak.

Solubilization of the outer and cytoplasmic membrane components

For membrane solubilization three detergents were selected: sodium dodecyl sulfate, sodium deoxycholate and Triton X-100. Fig. 3 shows that sodium dodecyl sulfate was the most effective, solubilizing more than 90 % of each of the three outer membrane components. Deoxycholate and Triton X-100, though quite effective in the solubilization of the cytoplasmic membranes, were much more selective with respect to the outer membrane components. These detergents solubilized the phospholipid

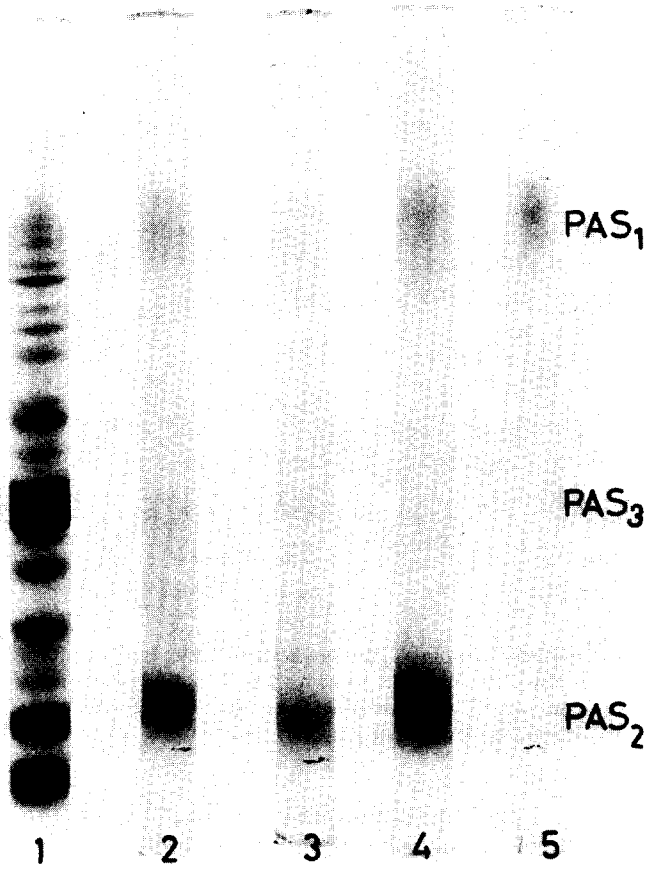


Fig. 2. Electrophoretic separation of *P. mirabilis* lipopolysaccharides in polyacrylamide gels containing sodium dodecyl sulfate and stained with Coomassie blue (gels 1 and 5) or by the periodic acid-Schiff reagent (gels 2–4). Gels 1 and 2, outer membranes; gel 3, cytoplasmic membranes; gels 4 and 5, extracted lipopolysaccharide.

component of the outer membrane quite effectively, but solibilized less than half of the outer membrane protein and lipopolysaccharide (Fig. 3). The degree of solubilization of the cytoplasmic membrane components labeled by radioactive galactose corresponded rather closely with that of the phospholipids, contrary to the findings with the outer membrane.

Organic solvents, owing to their ability to separate lipid from protein, have been utilized for the solubilization of biomembranes [6, 7, 15]. Treatment of the outer and cytoplasmic membranes of *P. mirabilis* with cold butanol, followed by centrifugation, resulted in the separation of four phases, resembling those obtained by Nachbar et al. [15] with *Micrococcus lysodeikticus* membranes (Table I). Almost all the outer membrane phospholipid was separated into the upper butan-1-ol phase, while most of the protein and lipopolysaccharide components were found in the aqueous phase. The results with the cytoplasmic membrane differed in that only about 40 % of the

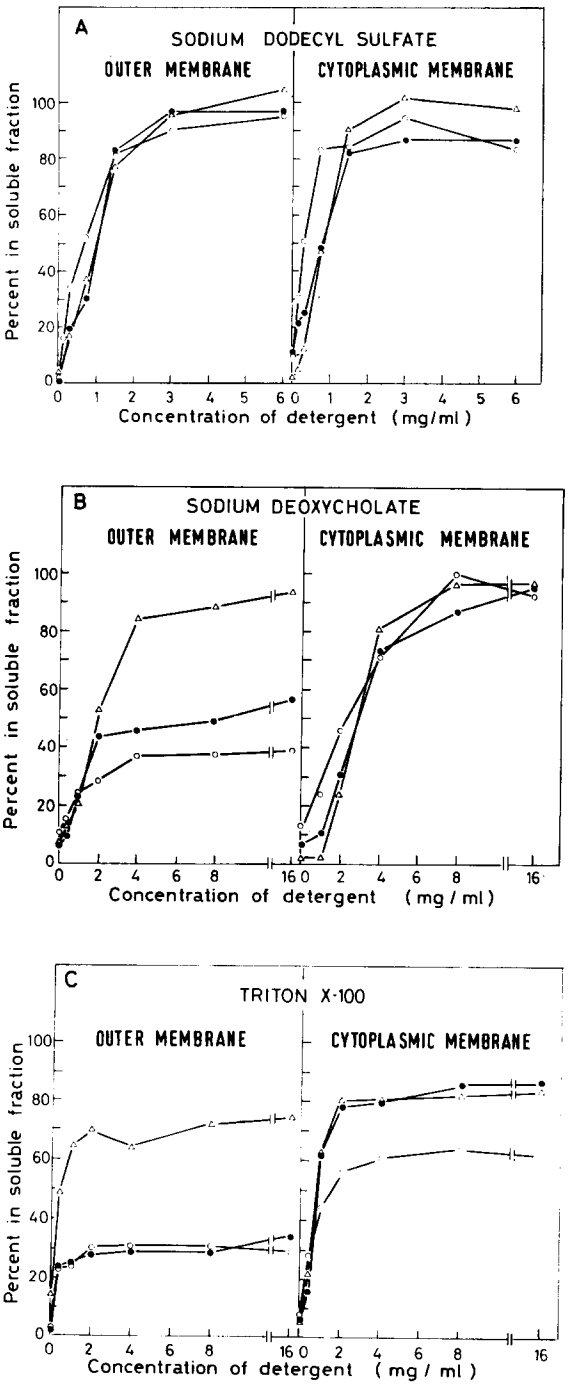


Fig. 3. Solubilization of the outer and cytoplasmic membrane components by various detergents. (A) Sodium dodecyl sulfate; (B) Sodium deoxycholate; (C) Triton X-100; (○) membrane protein; (△) [^{3}H]oleate-labeled material; (●) [^{3}H]galactose-labeled material.

TABLE I

SOLUBILIZATION OF THE OUTER AND CYTOPLASMIC MEMBRANE COMPONENTS BY BUTAN-1-OL

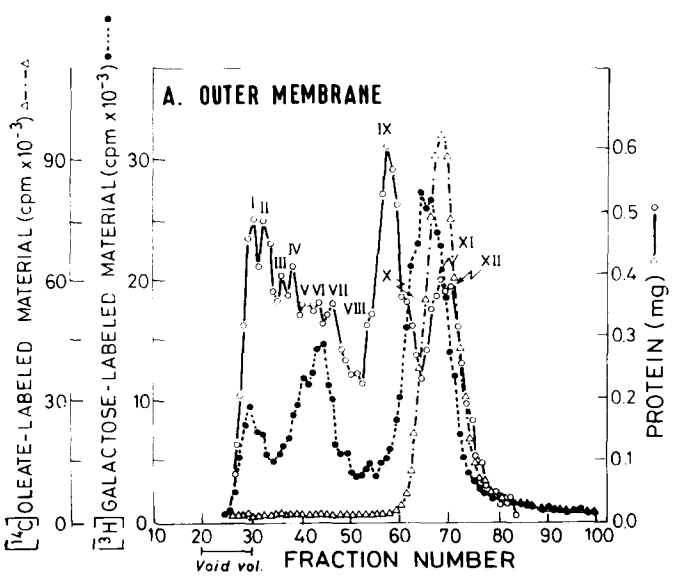
Phase	Percent of initial quantity in membrane				
	Outer membrane			Cytoplasmic membrane	
	Protein	Phospho-lipid*	Lipopoly-saccharide**	Protein	Phospho-lipid*
Butan-1-ol saturated with water	2.6	88.5	4.5	1.6	102.8
Interphase	3.7	2.0	3.4	n.d.	0.1
Water saturated with butan-1-ol	75.7	4.1	66.7	38.2	1.7
Sediment	7.9	0.3	8.2	9.5	0.5

* As determined by radioactivity derived from [^{3}H]oleic acid.
** As determined by radioactivity derived from [^{3}H]galactose.
n.d., not determined because of difficulties in resuspension of this phase.

protein were found in the aqueous phase, the remainder apparently located in the interphase which was sticky and resisted dispersion.

Fractionation of the solubilized membrane components by gel filtration

Fig. 4A shows that filtration of outer membranes solubilized by sodium dodecyl sulfate through a Sephadex G-200 column equilibrated with this detergent resulted in the elution of the phospholipid component in a single peak near the end of the elution volume. The lipopolysaccharide component was eluted in three peaks, the



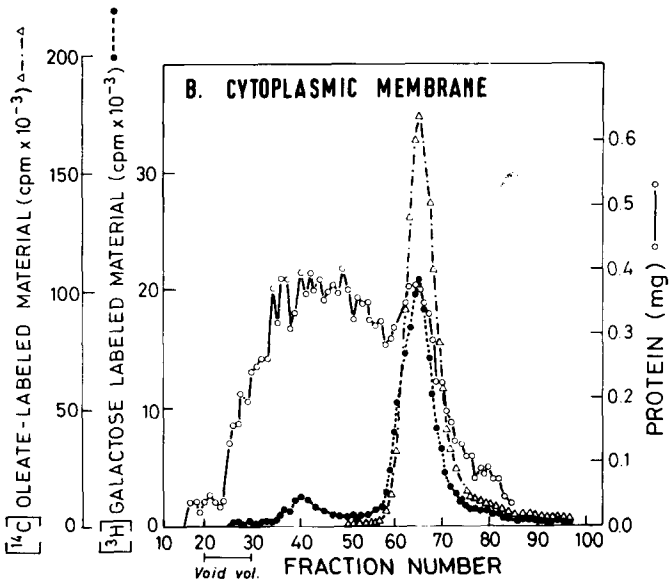


Fig. 4. Filtration of outer membranes (A) and cytoplasmic membranes (B) solubilized by sodium dodecyl sulfate through a Sephadex G-200 column equilibrated with the detergent.

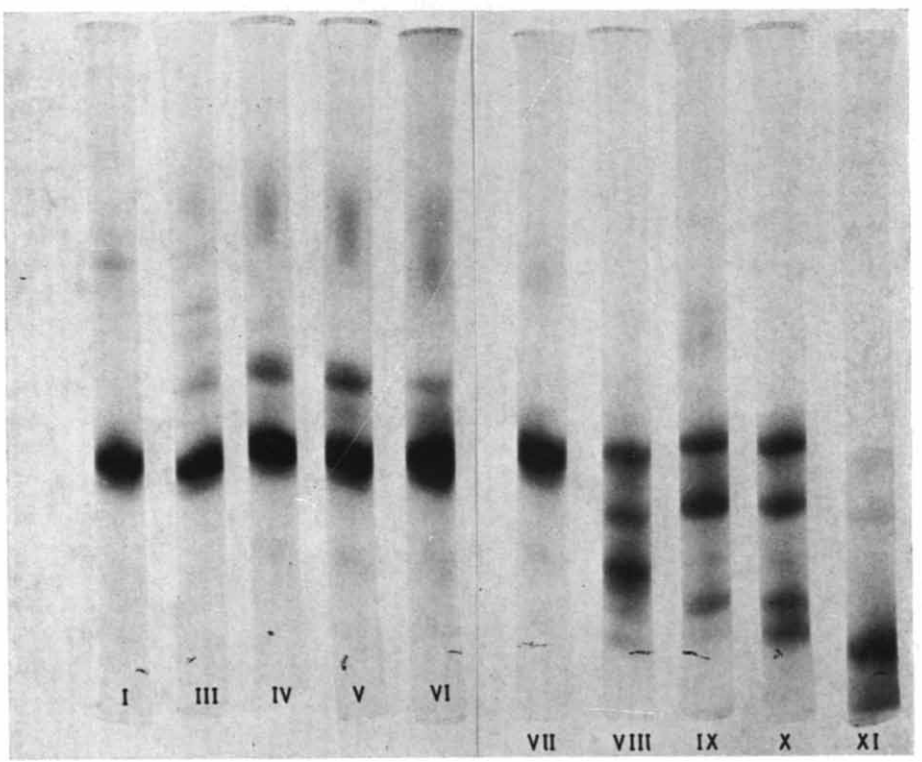


Fig. 5. Electrophoretic patterns of outer membrane fractions eluted from Sephadex G-200 column containing sodium dodecyl sulfate (see Fig. 4A). Electrophoresis was carried out in polyacrylamide gels containing 1 % sodium dodecyl sulfate and staining was done with Coomassie blue. Roman numerals on the gels are equivalent to the designation of the protein peaks in the fractions eluted from the column, as given in Fig. 4A.

first one in the void volume, while the last one slightly preceded the phospholipid peak but for the most part overlapped it. The protein was distributed throughout the elution volume forming about 12 peaks. Electrophoretic analysis of the fractions (Fig. 5) showed that the proteins were generally eluted according to their molecular weight, the high molecular weight proteins being eluted first. The major protein band, which apparently consists of several closely migrating proteins [1, 16], appeared in almost all the electrophoresed fractions.

Electrophoretic analysis of the outer membrane fractions containing the [^{3}H]-galactose-labeled material showed (Figs 6 and 7) that the first peak, which was eluted in the void volume, consisted of the two lipopolysaccharide types, PAS_1 and PAS_2. The second elution peak consisted almost entirely of PAS_1, while the third elution peak essentially contained only PAS_2.

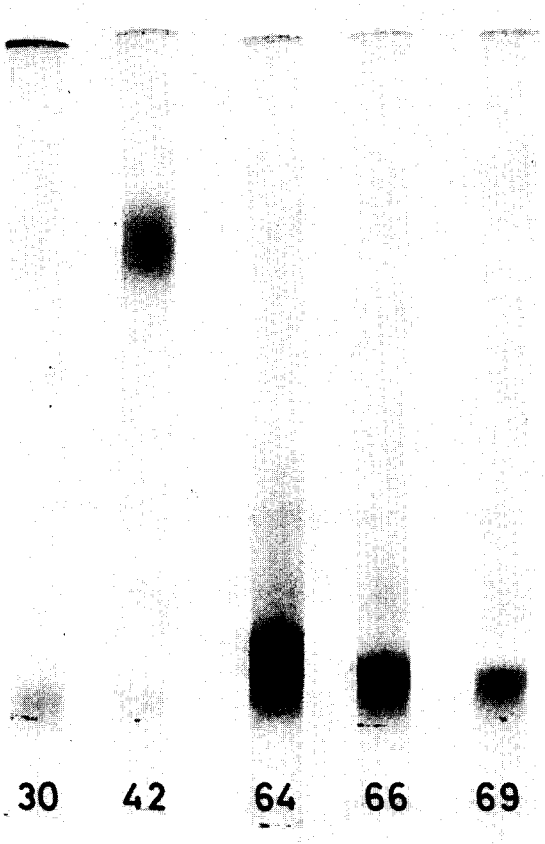


Fig. 6. Electrophoretic patterns of outer membrane fractions eluted from Sephadex G-200 column containing sodium dodecyl sulfate (see Fig. 4A). Electrophoresis was carried out in polyacrylamide gels containing 1 % sodium dodecyl sulfate and staining was done with the periodic acid-Schiff reagent. Numbers on gels denote the number of the fraction eluted from the column, as given in Fig. 4A.

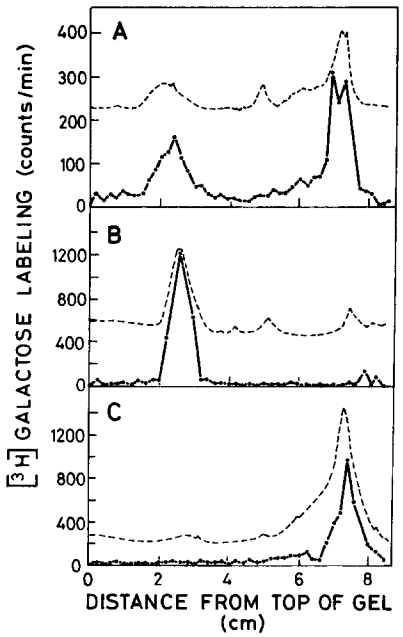


Fig. 7. Electrophoretic analysis of outer membranes (A) and outer membrane fractions 42 (B) and 66 (C) eluted from the Sephadex G-200 column shown in Fig. 4A. ---, densitometer tracing of bands stained with the periodic acid-Schiff reagent; ●–●, radioactivity derived from [^{3}H]galactose.

Fig. 4B shows that the filtration of the solubilized cytoplasmic membranes through the Sephadex G-200 column gave results resembling those obtained with the outer membrane in showing the elution of the phospholipid component in a single peak and the distribution of the proteins almost throughout the elution volume. The two membranes differed, however, with respect to the elution of the [^{3}H]-galactose-labeled material. In the cytoplasmic membrane most of this material was eluted in a peak which coincided with the phospholipid peak.

DISCUSSION

Specific labeling of *Salmonella* and *Escherichia coli* lipopolysaccharides has been achieved by growing mutants lacking the enzyme UDPgalactose-4-epimerase with [^{14}C]galactose [17]. A mutant of this kind is not yet available for *P. mirabilis* strain 19. However, our data show that labeling limited almost exclusively to the lipopolysaccharide component of the cell envelope can be achieved by growing the wild type of *P. mirabilis* strain 19 with radioactive galactose.

Electrophoretic analysis and Sephadex gel filtration of the labeled lipopolysaccharide of *P. mirabilis* showed it to consist of two different molecular types, differing in size and in stainability by Coomassie blue. Two lipopolysaccharide fractions have been described in the outer membrane of *Salmonella typhimurium* [18] and *E. coli* [19, 20] and as suggested by Schnaitman [19] one of the fractions may represent unsubstituted "cores" and the other, complete lipopolysaccharide molecules.

Hence, the finding of two lipopolysaccharide types is not a unique property of *P. mirabilis*. Yet, the staining of the high molecular weight type lipopolysaccharide of *P. mirabilis* with Coomassie blue has not been reported before and may be due to the high lysine and amino sugar content of *P. mirabilis* lipopolysaccharide [10, 21].

In parallel to our studies, Gmeiner [10] isolated from several *P. mirabilis*. strains (including strain 19) two types of lipopolysaccharide by ultracentrifugation of the crude lipopolysaccharide fraction extracted from the cells with phenol/water. Comparison of our data with Gmeiner's [10] indicates that the lipopolysaccharide type which migrates slower in the gels, stains with Coomassie blue and elutes from the Sephadex column first (our PAS_1) corresponds to the complete hydrophilic lipopolysaccharide molecules, with the long lysine-containing 0-antigenic side chain, designated by Gmeiner [10] as lipopolysaccharide II. The second lipopolysaccharide type, which migrates faster in the gels, elutes last from the column, and is not stained by Coomassie blue (our PAS_2) apparently corresponds to the lipopolysaccharide type I that was found to be deficient in the 0-antigenic chain components, smaller in size and more hydrophobic [10].

Apart from the two lipopolysaccharide bands, which were intensely stained by the periodic acid-Schiff reagent, the third, weakly stained band (PAS_3) was not labeled by [^{14}C]galactose and apparently does not represent lipopolysaccharide. It could represent a glycoprotein. The presence of glycoproteins in the Gram-negative cell envelope has been claimed before [22, 23]. However, more work is needed before any definite conclusion as to the identity of PAS_3 can be drawn.

Susceptibility of the cytoplasmic membrane of *P. mirabilis* to solubilization by detergents was very similar to that of other microbial plasma membranes [4]. Sodium dodecyl sulfate and sodium deoxycholate effectively solubilized both the phospholipid and protein components of this membrane, whereas Triton X-100 was less effective in the solubilization of the protein, in agreement with the findings with mycoplasmas [24] and erythrocyte membranes [25]. The outer membrane of *P. mirabilis* was less susceptible to solubilization by deoxycholate and Triton X-100 than the cytoplasmic membrane. These detergents solubilized less than 50 % of the protein and lipopolysaccharide components, though they solubilized almost all the outer membrane phospholipids.

The finding that the percentage of the outer membrane protein solubilized by deoxycholate, Triton X-100, and butan-1-ol (Table I) paralleled that of the solubilized lipopolysaccharide rather than that of the phospholipids can be interpreted as the lipopolysaccharides being more tightly bound to the outer membrane proteins than the phospholipids. Wu and Heath [26] go as far as to claim that all the lipopolysaccharide in the outer membrane of *E. coli* is covalently bound to a protein, forming a complex which resists dissociation by 1 % sodium dodecyl sulfate. Our gel electrophoresis and gel filtration data indicate that almost all of the outer membrane protein of *P. mirabilis* is separable from the two lipopolysaccharide types, in the presence of sodium dodecyl sulfate. Nevertheless, this does not rule out the possibility that a substantial part of the protein and lipopolysaccharide in the outer membrane exist as complexes resisting dissociation by the weaker detergents, Triton X-100 and deoxycholate. Most of the phospholipids, on the other hand, appear to be only weakly associated with the two other membrane components, possibly forming "islets" of phospholipids readily extractable with detergents or organic solvents. The total

removal of the phospholipids from the outer membrane with organic solvents did not destroy membrane structure nor its characteristic trilaminar shape in thin sections [2].

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