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ORGANIZATION OF PROTEINS IN THE NATIVE AND REFORMED OUTER MEMBRANE OF *ESCHERICHIA COLI*

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SUMMARY

1. The proteins of the outer membrane of *Escherichia coli* were characterized by polyacrylamide gel electrophoresis and their molecular weights determined.

2. A buffer system is described which permits reproducible resolution of three proteins which do not resolve on polyacrylamide gel electrophoresis using conventional phosphate–sodium dodecyl sulfate buffer systems.

3. Pronase digestion of spheroplast membranes, Triton-treated cell envelopes and isolated outer membranes of *E. coli* showed that only two major proteins were accessible to the enzyme in spheroplast membranes, while Triton treatment revealed several others to pronase attack. Several other proteins were not attacked by pronase under the conditions employed. An asymmetric arrangement of proteins within the membrane is suggested.

4. Pronase digestion of reformed outer membrane gave a result similar to that obtained for the original outer membrane preparation. It is suggested that reaggregation of the components of the solubilized outer membrane has occurred in a specific manner with the proteins organized as in the native membrane.

INTRODUCTION

Razin *et al.*[1] demonstrated that the membrane of *Mycoplasma laidlawii* could be solubilized by detergent and that the solubilized material would subsequently reassociate to membrane-like structures on removal of the detergent in the presence of Mg^{2+}. Later work showed that the reformed membranes were derived from protein and lipid which had been separated by the detergent and not from solubilized lipoprotein subunits[2,3]. The reconstituted membrane had a composition and appearance similar to that of the native membrane[3,4], although the exact protein composition of the reformed membranes depended on the concentration of Mg^{2+} present during removal of the detergent[5]. Subsequently, freeze-etching studies revealed that the ultrastructure of the reformed membrane was not exactly the same as that of the native membrane[6]. Membranes from other microorganisms also have been disaggregated, and reassociated to membrane-like structures as observed by electron microscopy[7–10]. However, in none of these examples is there evidence that the proteins in the reformed membranes are organized in the same manner as in the native membrane.

DePamphilis and Adler[11] have recently described a convenient method to prepare the outer membrane of *Escherichia coli*. This membrane has a relatively small number of proteins present in comparison with the inner membrane[12], and it can be disaggregated by detergent and reformed[10]. In the present paper we have studied the organization of proteins in this membrane, before and after reconstitution using the technique of pronase digestion which has been successfully applied to the erythrocyte membrane[13–15]. Since the outer membrane prepared by the technique of DePamphilis and Adler[10], is partially depleted of phospholipid (and possibly of hydrophobic proteins), the intact spheroplast membrane has been used as a control in our experiments. The outer membrane as prepared appears to consist largely of vesicles[11]. Some of these vesicles could be open so that pronase might attack both the inner and outer surface of the membrane. Thus, we have used as an additional control a Triton-treated envelope preparation which, like the outer membrane, is partially depleted of phospholipid (and possibly of hydrophobic proteins) but in which the inner surface of the outer membrane is protected by the murein layer. The inner membrane of this preparation has been removed by Triton[16].

MATERIALS AND METHODS

Reagents

Hog pepsin, trypsin, horse heart myoglobin, and bovine pancreatic α-chymotrypsin were purchased from Mann Research Laboratories. Bovine serum albumin, human γ-globulin, pronase, Triton X-100 and phenylmethanesulfonyl fluoride were obtained from Calbiochem.

Organism and growth conditions

E. coli (strain 482 of the culture collection of the National Research Council of Canada) was grown with vigorous aeration at 37 °C on minimal salts medium as described previously[17].

Preparation of outer membrane

Spheroplast membranes were prepared as described by Davies and Bragg[17]. Outer membranes were obtained from them by treatment with Triton X-100 in the presence of Mg^{2+} (ref. 11), and purified on a gradient of CsCl as described by DePamphilis and Adler[11].

Disaggregation and reformation

The outer membrane (7.8 mg protein) derived from the spheroplast membrane (31.5 mg protein) was purified on a CsCl gradient as above. The membrane band from the gradient was diluted to 10 ml with 15 mM Tris–HCl buffer (pH 8.0) containing 6.5 mM EDTA. The solution was incubated with 0.5 ml 10% (w/v) sodium dodecyl sulfate at 37 °C for 40 min and then centrifuged at 100000 × *g* for 2 h at 4 °C. The white, crystalline pellet of sedimented detergent was discarded. The supernatant solution was dialysed against 20 mM Tris–HCl (pH 7.4) containing 20 mM $MgCl_2$ and 0.1 mM dithiothreitol. The buffer (1 l) was changed after 4 and 16 h dialysis at room temperature. The dialysis was then continued at 4 °C for 48 h with the buffer being changed at 24 h. The dialysed membrane solution was diluted to 9.9 ml with

the same buffer and 4.46 g CsCl added. The resulting solution was centrifuged at 43000 rev./min for 28 h in two tubes of a Beckman SW 50L rotor. The reconstituted outer membrane was obtained as a band with a buoyant density of 1.33 g/cm^3. The band (3.4 mg protein) was collected and dialysed against 10 mM Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$ until salt-free.

Preparation of Triton-treated cell envelope fraction

Cells were grown and harvested as in the preparation of spheroplast membranes. The cells (0.9 g) were suspended to a volume of 10 ml in 10 mM Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$. The suspension was disrupted in a French pressure cell (American Instrument Co.) at 12500 lb/inch^2. Unbroken cells were removed from the cell extract by centrifuging it at 5000 × *g* for 5 min. The cell envelope fraction was then sedimented from the supernatant solution by centrifuging it at 37000 × *g* for 20 min. The envelope fraction (11.2 mg protein) suspended in 13.5 ml 0.1 M Tris–HCl (pH 8.0) containing 35 mM $MgCl_2$ was stirred at room temperature for 30 min with 2.36 ml 10% (w/v) Triton X-100. The suspension was then centrifuged at 100000 × *g* for 30 min. The resultant pellet was suspended in 6 ml buffer and resedimented as above to give the "Triton-treated cell envelope fraction" (6.1 mg protein).

Digestion of membranes with pronase

The sample (0.45 mg protein/ml) in 0.01 M Tris–HCl (pH 7.5) containing 0.01 M $MgCl_2$ was treated at 37 °C for 2 h with pronase (0.1 mg/ml). The reaction was stopped by adding 0.05 ml phenylmethanesulfonyl fluoride (7 mg/ml ethanol) per ml[13]. After at least 30 min at 4 °C, the solution was centrifuged at 100000 × *g* for 2 h. In the case of the outer membrane and Triton-extracted envelope preparations the pellet so obtained was lyophilized and then submitted to gel electrophoresis. In the case of the spheroplast membrane preparation the outer membrane was prepared from this pellet, and purified on a CsCl gradient prior to gel electrophoresis. Control preparations were submitted to identical treatments except for the omission of pronase, and comparable amounts of material were examined by gel electrophoresis.

Sequential extraction of cell envelope fraction

The Triton-extracted envelope fraction from 10 g cells was prepared as previously described[18]. This fraction was then extracted in sequence with 38.5 ml 0.5% (w/v) sodium dodecyl sulfate (adjusted to pH 9.0) at 37 °C for 1 h, 20 ml 4 M urea containing 0.05% dithiothreitol and 1% sodium dodecyl sulfate at room temperature for 16 h, and finally with 9 ml 2.5% sodium dodecyl sulfate at 100 °C for 4 min. After each treatment the extracted envelopes were sedimented by centrifuging at 120000 × *g* for 30 min. In this way three extracts ("first, second and third extracts") were obtained. This is a modification of the method previously described[18].

Gel electrophoresis

Electrophoresis was performed on 10% polyacrylamide gels prepared in 0.1% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer (pH 7.2). In System 1 both buffer compartments contained the buffer used in the preparation of the gels. In System 2 the cathodic buffer compartment contained 0.1 M Na_2HPO_4–0.1% sodium dodecyl sulfate. adjusted to pH 11.4 with NaOH, while the anodic buffer compartment

contained 0.1 M NaH_2PO_4–0.1 % sodium dodecyl sulfate (pH 4.1). Samples (2–3 mg/ml) were dissolved in 1 % (w/v) sodium dodecyl sulfate–4 M urea–1 % (v/v) β-mercaptoethanol and incubated at 37 °C for 1 h. Then, unless noted otherwise, the solution was heated at 100 °C for 3 min prior to application to the gel. The gels were stained with Coomassie Blue (and destained electrolytically), or with periodic acid–Schiff stain[19].

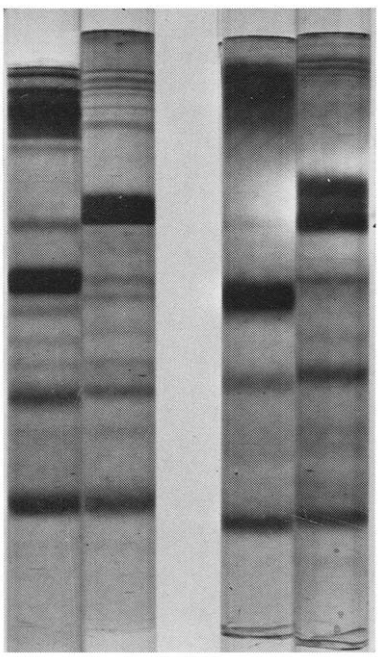
For molecular weight determinations, electrophoresis was performed on 10 % polyacrylamide gels in System 1 using the split gel technique[20]. Marker proteins used were bovine serum albumin (monomer and dimer), γ-globulin (heavy and light chains), pepsin, trypsin, myoglobin, and chymotrypsin (B and C chains).

Analytical methods

Protein was determined by the method of Lowry *et al.*[21]. Heptose and 2-keto-3-deoxyoctonate were assayed as described by Osborn[22] using the extinction coefficients determined by her. Total phosphate, total carbohydrate and RNA were determined by the methods of Ames[23], Montgomery[24], and Schneider[25], respectively.

Electron microscopy

Dialysed samples were negatively stained with 2 % potassium phosphotungstate



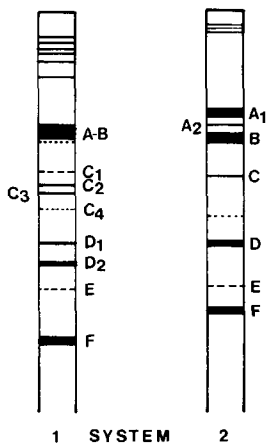


Fig. 1. Electrophoresis on 10 % polyacrylamide gels of proteins of the *E. coli* outer membrane. The gels from left to right are (a) membrane dissolved in dodecyl sulfate–urea–β-mercaptoethanol at 37 °C; (b) solution subsequently heated at 100 °C; (c) same as (a); (d) same as (b). Electrophoresis was carried out with System 1 for gels (a) and (b), and with System 2 for gels (c) and (d).

Fig. 2. Schematic diagram of separation of outer membrane proteins of *E. coli* on 10 % polyacrylamide gels using Systems 1 and 2. The samples in dodecyl sulfate–urea–β-mercaptoethanol had been heated at 100 °C.

pH 6.8, containing 0.01% bovine serum albumin, and examined in a Cambridge Instrument Co. AEI-801A electron microscope operated at 60 kV.

RESULTS

Proteins of outer membrane

When the outer membrane of *E. coli* was dissolved in dodecyl sulfate–urea–β-mercaptoethanol at 37 °C and submitted to gel electrophoresis using system 1, the left-hand gel of Fig. 1 was obtained. (The gels were overloaded with sample to show minor bands). If the solution was heated at 100 °C prior to electrophoresis (Fig. 1; second gel from left), the major protein ("heat-modifiable protein"; apparent molecular weight, approx. 28500) was found to migrate more slowly (apparent molecular weight, approx. 33400). This behaviour has been previously observed by Smart *et al.*[26]. Further disaggregation of higher molecular weight proteins also occurred on heating. For purposes of further discussion the main protein bands seen on gel electrophoresis are named as shown in Fig. 2.

The band A–B was generally found as a single band even when low amounts of protein were applied to the gel. Our previous work[18] had shown that this band actually contained three proteins (A_1, A_2, B). Reproducible resolution of this band into its components on gel electrophoresis was obtained with System 2 (Fig. 1; right-hand pair of gels).

Previous data[18] indicated that proteins A_1, A_2 and B had molecular weights of 44000, 38100 and 33400, respectively. Our previous values for proteins C and D are

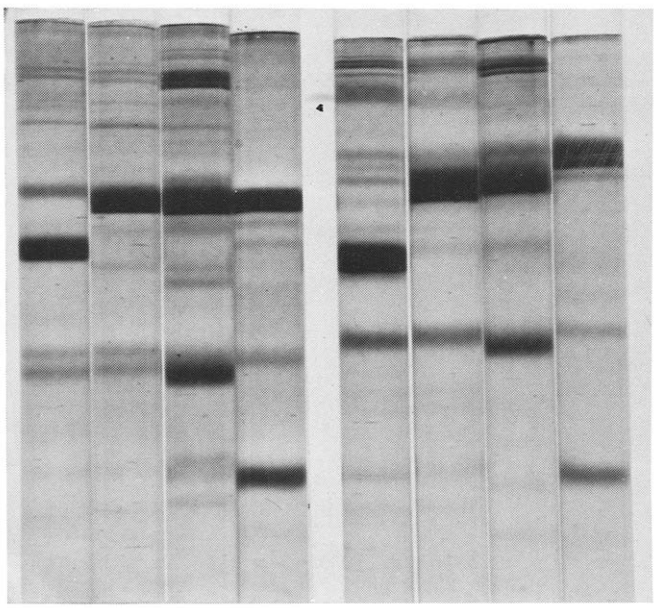


Fig. 3. Electrophoresis on 10% polyacrylamide gels of proteins sequentially extracted from Triton-extracted envelopes of *E. coli* as described in Materials and Methods. The left-hand four gels were run with System 1 and the right-hand four gels with System 2. The gels from left to right in each group of four are (a) first extract (not heated at 100 °C); (b) first extract (heated); (c) second extract (heated); (d) third extract (heated).

erroneous due to incomplete resolution of the component proteins of these bands on the gels. Redetermination of the molecular weights gave the following values (mean of three determinations): C_2(26300), C_3(23600), D_1(18900), D_2(17900), E(14460) and F(10500).

The heat-modifiable protein after heating migrates on gel electrophoresis in the region occupied by proteins A_1, A_2 and B. The following experiment showed that protein B originated from the heat-modifiable protein. Envelopes which had been stripped of the inner membrane[18] were sequentially extracted as described in Materials and Methods. Gel electrophoresis of the extracts gave the results shown in Fig. 3. The first extract contained mainly the heat-modifiable protein in its non-modified form. The second extract was enriched in proteins B and D_2, whereas the third extract contained mainly proteins A_1, A_2 and F. When the first extract was heated at 100 °C

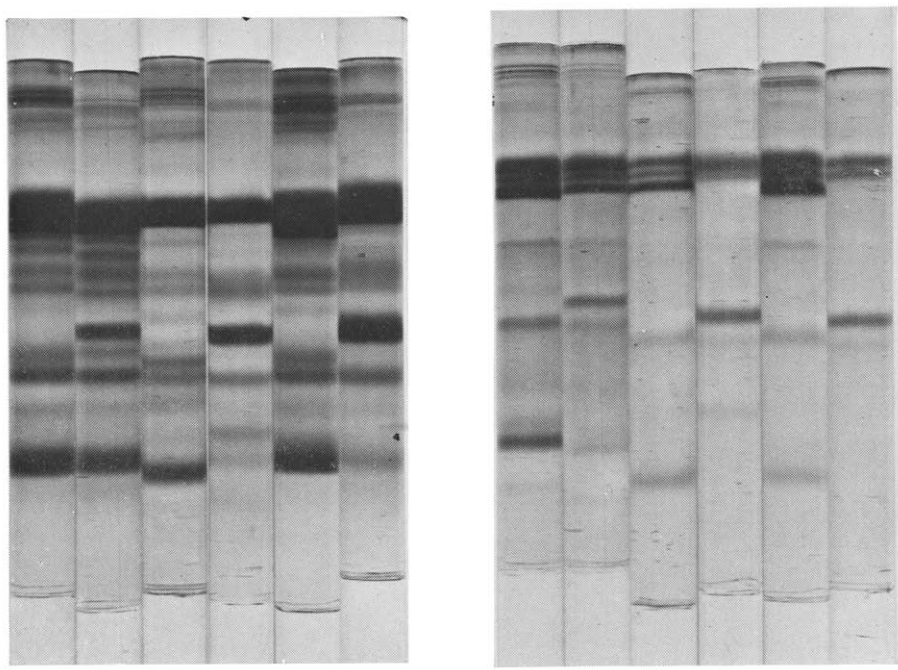


Fig. 4. Electrophoresis on 10% polyacrylamide gels of outer membrane proteins of *E. coli* obtained after treatment of spheroplast membrane, Triton-treated envelope, and outer membrane fractions with pronase as described in Materials and Methods. The gels were run with System 1 and from left to right are (a) outer membrane prepared from spheroplast membrane control; (b) outer membrane prepared from pronase-treated spheroplast membrane; (c) Triton-treated envelope control; (d) pronase-treated Triton-treated envelope; (e) outer membrane control; (f) pronase-treated outer membrane. Each control sample was worked up in parallel with the pronase-treated preparation. All fractions had been heated in dodecyl sulfate–urea–β-mercaptoethanol at 100 °C.

Fig. 5. Electrophoresis on 10% polyacrylamide gels of outer membrane proteins of *E. coli* obtained after treatment of spheroplast membrane, Triton-treated envelope, and outer membrane fractions with pronase as described in Materials and Methods. The gels were run with System 2 and from left to right are (a) outer membrane prepared from spheroplast membrane control; (b) outer membrane prepared from pronase-treated spheroplast membrane; (c) Triton-treated envelope control; (d) pronase-treated Triton-treated envelope; (e) outer membrane control; (f) pronase-treated outer membrane. Each control sample was worked up in parallel with the pronase-treated preparation. All fractions had been heated in dodecyl sulfate–urea–β-mercaptoethanol at 100 °C.

prior to electrophoresis the heat-modifiable protein now migrated as protein B. Thus, proteins A_1 and A_2, which are found in outer membrane extracts only after heating at 100 °C, must originate by further disaggregation of the material of higher molecular weight seen on the gels of unheated extracts (Fig. 1). Auborn *et al.*[27] have also observed that heating markedly increases the dissociation of membrane components by sodium dodecyl sulfate.

Effect of pronase on outer membrane proteins

Spheroplast membranes, Triton-treated envelopes and isolated outer membranes were digested with pronase as described in Materials and Methods. The outer membrane was subsequently prepared from the treated spheroplast membranes so that the effect of pronase on the outer membrane only of the spheroplast membranes would be considered. Solution of the control and treated membranes in dodecyl sulfate–urea–β-mercaptoethanol at 100 °C, and subsequent gel electrophoresis in Systems 1 and 2 gave the results shown in Figs 4 and 5. Pronase digestion of spheroplast membranes resulted in loss of some of protein B, and possibly of protein F. A new protein band of molecular weight about 20000 appeared in the pronase-digested samples. That this new protein was derived from protein B is more clearly seen in the gels showing the effect of pronase digestion on the Triton-treated envelopes. Here, complete loss of protein B on pronase digestion was correlated with the appearance of the new band in about the same amount. Pronase digestion of the Triton-treated envelopes also completely destroyed proteins D_1, E and F, and some of the proteins migrating more slowly than A_1 on electrophoresis. Proteins A_1, A_2, C_2, C_3 and D_2 were

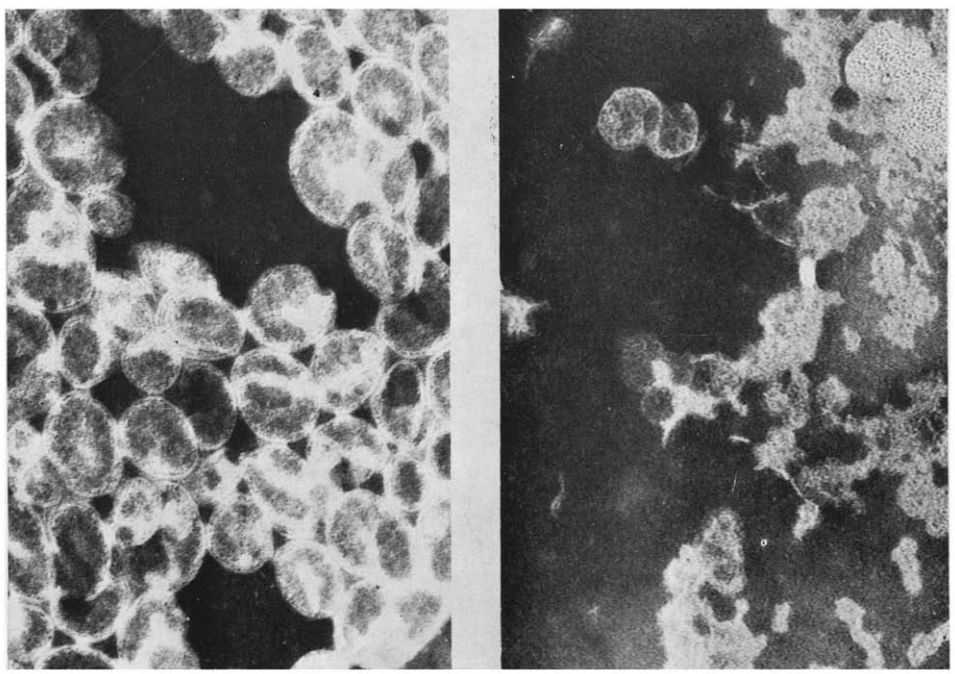


Fig. 6. Electron micrographs of negatively stained outer membrane (left) and reformed outer membrane (right) of *E. coli*. Magnification, 90000 ×.

not attacked significantly. Digestion of the outer membrane gave results similar to those obtained with the Triton-treated envelopes.

Disaggregation and reformation of outer membrane

DePamphilis[10] solubilized the outer membrane of *E. coli* with Triton X-100 in the presence of EDTA. Reformation of membrane-like structures as observed by electron microscopy was obtained by dialysis of the solubilized material against buffer containing magnesium ions. A modification of this procedure using sodium dodecyl sulfate as the disaggregating agent is described in Materials and Methods. The reaggregated material banded in CsCl with a buoyant density of 1.33 g/cm^3 compared to 1.34 g/cm^3 for the original outer membrane. The material was membranous as seen by electron microscopy (Fig. 6) of negatively stained preparations. Gel electrophoresis of the original and reformed outer membrane showed closely similar band patterns (Figs 7 and 8) indicating that all the proteins previously present in the outer membrane had been reincorporated into the reaggregated material, and in approximately the same ratio. Analysis of the reaggregated material (values for original outer membrane in parentheses) for protein, total carbohydrate, total phosphate, RNA, heptose and 2-keto-3-deoxyoctonate gave (% of dry weight) 35.3 % (42.7 %), 16.0 % (16.2 %), 4.6 % (3.8 %), 0.74 % (0.98 %), 4.3 % (3.6 %) and 2.3 % (2.0 %), respectively. These values indicate that the lipopolysaccharide also was reincorporated into the reaggregated material.

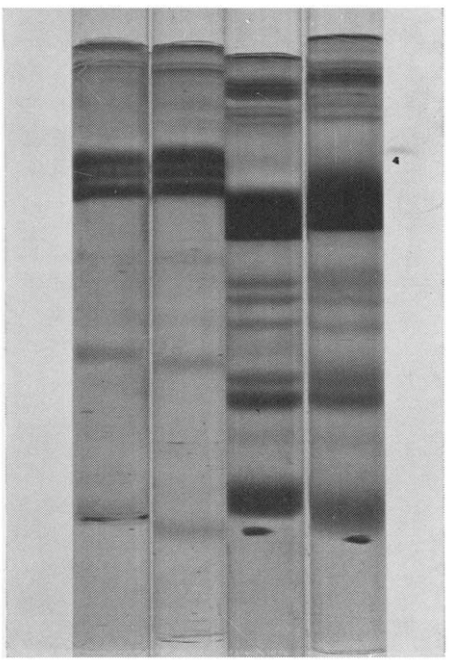


Fig. 7. Electrophoresis on 10 % polyacrylamide gels of proteins of outer membrane and reformed outer membrane of *E. coli*. The left-hand pair of gels were run with System 2 and the right-hand pair were run with System 1. The left-hand gel of each pair is the outer membrane and the right-hand gel is the reformed outer membrane. The samples were heated at 100 °C in dodecyl sulfate–urea–β-mercaptoethanol prior to electrophoresis.

Effect of pronase on reformed outer membrane

The effect of pronase digestion on the original and reaggregated outer membrane is shown in Fig. 8. Pronase had a similar effect on both membranes, the only marked difference being the more extensive digestion of proteins C_2 and C_3 in the reaggregated preparation. Of particular interest is the fact that proteins A_1, A_2 and D_2 were reincorporated into the reformed membrane such that they were not accessible to pronase, and that protein B was orientated so that it gave the same digestion fragment as that obtained from the control preparations.

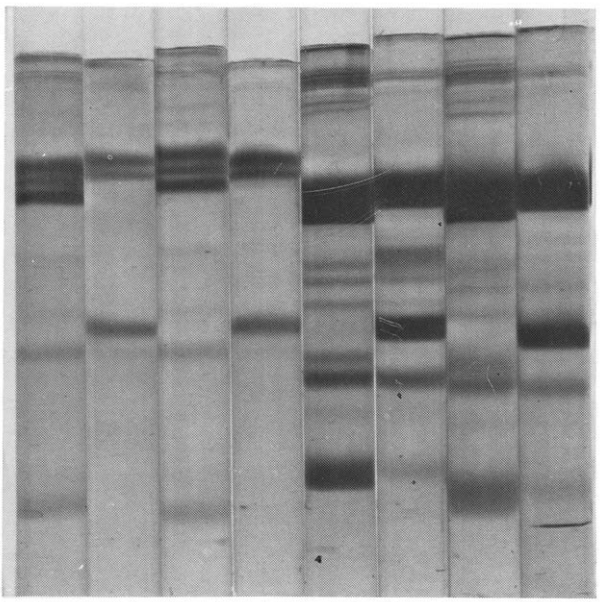


Fig. 8. Electrophoresis on 10% polyacrylamide gels of outer membrane proteins of *E. coli* after the outer membrane and reformed outer membrane had been treated with pronase as described in Materials and Methods. The left-hand four gels were run with System 2 and the right-hand four gels with System 1. The gels from left to right in each set of four are (a) outer membrane control; (b) pronase-treated outer membrane; (c) reformed outer membrane; (d) pronase-treated reformed outer membrane. Each control was worked up in parallel with the pronase-treated preparation. All fractions had been heated in dodecyl sulfate–urea-β-mercaptoethanol at 100 °C.

DISCUSSION

Current ideas of membrane structure envisage areas of phospholipid bilayer into which individual proteins, or groups of proteins, penetrate[28]. The results of the present work are in accord with this hypothesis. At least one of the outer membrane proteins (*e.g.* protein B) must project from the membrane surface and still be part of the membrane. This is shown by the fact that pronase will attack protein B in the spheroplast membrane but still leave a signficant portion of the protein B molecule undigested. (Pronase digestion of isolated proteins A_1, A_2 and B yields small peptides, showing that these proteins have no intrinsic resistance to pronase, although it is possible that more limited digestion might occur in the presence of phospholipid).

Other proteins (*e.g.* proteins D_1, E and F) must be adjacent to phospholipid bilayer regions (or regions of Triton-extractable proteins) since these proteins are attacked only in the phospholipid-depleted preparations. Still other proteins (*e.g.* proteins A_1, A_2, C_2, C_3 and D_2) must either be in interior regions of the protein part of the membrane or be shielded by molecules of lipopolysaccharides. An asymmetric arrangement of proteins within the membrane is suggested by these results.

This asymmetric arrangement of proteins appears to be reformed during reconstitution of the outer membrane. Reassembly of the membrane must involve specific interactions between the individual components presumably determined by their three-dimensional structures. The formation of the UDP-galactase–lipopolysaccharide-α-3-galactosyl transferase system of *Salmonella typhimurium*[29] by sequential association of lipopolysaccharide with phospholipid, and then with enzyme, could represent a model for processes occurring during reassembly of the outer membrane of *E. coli*.

Two proteins from the outer membrane of *E. coli* have been well characterized. Phospholipase A1 has been shown to have a molecular weight of 29000 ± 2000 (ref. 30) and so might be one of the proteins detected on our gels. However, we have not investigated the enzyme activity of any of our protein fractions. A lipoprotein (minimum molecular weight, 8000) isolated by Braun and Wolff[31] is unlikely to be similar to protein F since former was released from the murein layer only after treatment with trypsin.

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