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# STRUCTURAL HETEROGENEITY OF THE CYTOPLASMIC AND OUTER MEMBRANES OF *ESCHERICHIA COLI*

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## Summary

The cytoplasmic and outer membranes of gram-negative bacteria can be isolated from spheroplasts, and separated on sucrose density gradients. Lysis of spheroplasts causes extensive membrane fragmentation and since the characteristics of the fragments obtained by different lysis procedures need not be identical, the influence of the disruption method on membrane composition has been examined. Spheroplasts of *Escherichia coli* J5 were lysed by osmotic shock, which did not significantly separate the cytoplasmic and outer membranes, but resulted in mixed membrane vesicles. Lysis in the French press and by sonication caused extensive membrane fragmentation and separation. Sonication, however, also caused some fusion between fragments of the outer and the cytoplasmic membranes; this intermembrane fusion increased with sonication time.

When the cytoplasmic and outer membranes were well separated and intermembrane fusion was minimal or absent, the cytoplasmic and outer membrane fragments were heterogeneous with respect to density and overall phospholipid, protein and lipopolysaccharide composition. In addition, cytoplasmic, but not outer, membrane fragments were also heterogeneous with respect to protein composition.

It is concluded, therefore, that membrane fragments obtained from the cytoplasmic and outer membranes are heterogeneous independently of the lysis procedures used to obtain these fragments. Possible reasons for this heterogeneity are discussed.

## Introduction

The cell envelope of *Escherichia coli* and other gram-negative bacteria consists of a cytoplasmic membrane, a peptidoglycan layer and an outer membrane [1—3]. The major approach used to separate and purify the cytoplasmic and

outer membranes of gram-negative bacteria [4—7] has been to disrupt spheroplasts by osmotic shock or sonication, followed by isopycnic sucrose gradient centrifugation, which has usually yielded an outer membrane fraction (H fraction), one or more cytoplasmic membrane fractions ($L_1$ and $L_2$ fraction) and an intermediate fraction (M fraction) [6]. The existence of more than the expected two fractions has been ascribed to contamination of cytoplasmic membrane vesicles by outer membrane material and by the existence of completely unseparated or reaggregated envelope fragments [6].

The possibility remains, however, that isolated cytoplasmic and outer membrane vesicle populations are heterogeneous even when inter-membrane contamination does not occur, for several reasons. First, there is differentiation within a single membrane (membrane growth areas [8], cell division site [9], deoxyribonucleic acid attachment sites [10], sites of specific membrane associated enzyme complexes, polar versus cylindrical parts [11]), which could cause intrinsic density differences within both cytoplasmic and outer membranes. Second, density differences within a membrane population could arise artificially during the isolation procedure. It is known, for instance, that membrane components segregate in the plane of the membrane at the low temperatures at which spheroplasts are normally lysed [12,13]; this process can introduce density differences within a membrane [12]. It is likely therefore that the procedures used to separate and purify membranes significantly affect the structural characteristics of the resulting membranes.

Accordingly, we have examined the effect of various lysis conditions on the separation of bacterial membranes. In this paper we show that even under optimal lysis and separation conditions, the cytoplasmic and outer membranes are still heterogeneous; the outer membrane vesicles vary in density, while the cytoplasmic membrane vesicles vary not only in density but also in protein composition.

## Materials and Methods

### *Organism and growth conditions*

Strain J5, a mutant of *E. coli* 0111 : K58 (B4) which lacks uridine diphosphate galactose-4-epimerase, was grown in minimal medium as described previously [14]; the doubling time was 83 min under these conditions. Exponential phase cells were harvested when the cell density had reached 0.17 mg cell dry mass/ml [15].

### *Preparation of membranes*

Cells were harvested by centrifugation (Sharples Centrifuge Ltd., Chamberley, U.K.) at room temperature and converted to spheroplasts at 0°C [14,16]. Spheroplasts were lysed by three methods.

*Osmotic shock.* The spheroplasts suspension was directly diluted 10-fold into $H_2O$. As a result, the spheroplasts lysed immediately.

*Sonication and French press.* The spheroplast suspension was first concentrated: $MgCl_2$ was added to a final concentration of 10 mM, after which the spheroplasts were collected by centrifugation (10 min, 5000 × *g*) and resuspended to 25 mg/ml in 10 mM Tris · HCl (pH 8.0), containing 0.01 mg/ml

DNAase (EC 3.1.4.5, Sigma, St. Louis, U.S.A.) and 0.01 mg/ml RNAase (EC 3.1.4.22, Miles-Seravac Ltd., Berkshire, U.K.). Subsequently, EDTA was added to a concentration of 2.5 mM and the spheroplasts were either disrupted by a single passage at 0°C through a French pressure cell (Aminco, Silver Spring, Md., U.S.A.), or by sonication (Branson no B12, Branson Sonic Power Co., Danburry, Conn., U.S.A.) at 150 W in a 50 ml vessel. During sonication the temperature of the spheroplast suspension was always kept below 5°C.

After removal of cell debris and unlysed spheroplasts from the lysate by low speed centrifugation (10 min, 5000 × *g*), total membranes were isolated by high speed centrifugation (2 h, 360 000 × *g*). After resuspending in buffer containing 1 mM EDTA and 10 mM Tris · HCl (pH 8.0), followed by a similar high speed centrifugation, the membrane pellet was resuspended in 25% (w/w) sucrose, 10 mM Tris · HCl, 1 mM EDTA (pH 8.0) and layered on top of a discontinuous sucrose gradient containing 10 mM Tris · HCl and 1 mM EDTA (pH 8.0), made according to Osborn et al. [6], unless otherwise stated. The gradients were centrifuged at 40000 rev./min in an SW 41 rotor (Beckman Instruments Inc., Fullerton, Calif., U.S.A.) at 0°C for 62 h, unless otherwise stated, and fractionated as described previously [14].

*Chemical analyses*

Total protein was determined according to the method of Lowry et al. [17], as modified by Yocum et al. [18], using bovine serum albumin as standard.

The phospholipids were extracted according to the procedure of Bligh and Dyer [19]. The lipid extract was concentrated to dryness for determination of phosphate according to the method of Ames and Dubin [20]. Phospholipid contents were calculated assuming an average molecular weight of 700.

Lipopolysaccharide content was estimated after determination of 2-keto-3-deoxyoctonate as described by Osborn [21], based on the assumption that the lipopolysaccharide of *E. coli* J5 grown in the absence of galactose contains 7.4% (w/w) 2-keto-3-deoxyoctonate [22].

Protein analysis was carried out by gel electrophoresis of about 20 μg protein, solubilized by heating in 2% sodium dodecyl sulphate (SDS) for 5 min at 100°C, on 12.5% polyacrylamide gels containing 0.2% SDS as described by Laemmli [23]. Gels were stained and photographed, and the negatives were scanned with a flying spot densitometer, after which computer-generated protein profiles were obtained as described previously [24].

*Electron microscopy*

Electron microscopy was carried out as described previously [12,25].

## Results and Discussion

*Effect of the spheroplasting procedure and the centrifugation time on the separation of membranes on continuous and discontinuous gradients*

Adequate membrane separation was possible only when cells had been fully converted to spheroplasts, in agreement with the finding of Osborn et al. [6]; this was achieved with an effective lysozyme procedure, which has been described previously [14,16]. In addition, it was noticed in early experiments

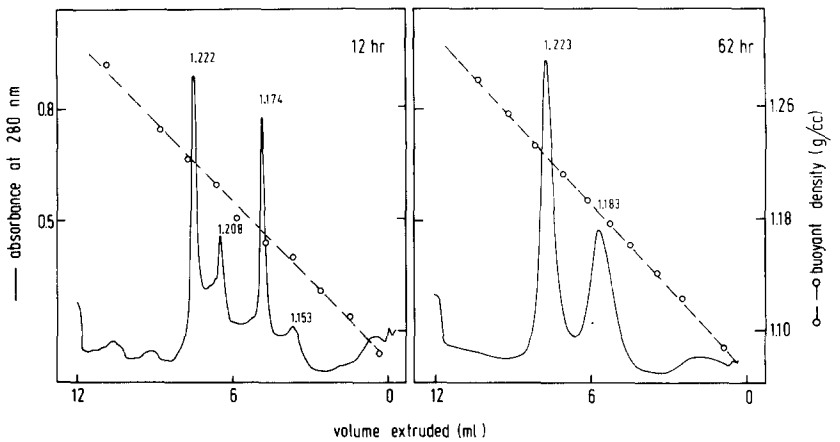



**Fig. 1. Effect of centrifugation time on membrane separation. Spheroplasts were disrupted in the French press at 800 atm. Membranes were suspended in 20% (w/w) sucrose and loaded on top of a discontinuous gradient, ranging from 25 to 60% (w/w) sucrose. Centrifugation times and apparent mean densities are indicated.**

that the centrifugation conditions also affected the separation of membranes obtained from lysed spheroplasts; dependent on the lysis conditions, the different membrane fractions appeared not always to attain density equilibrium within the centrifugation times described previously [6,26].

To investigate this effect, membrane preparations were centrifuged for 12, 15, 20 or 62 h on discontinuous and continuous gradients. Although in all cases a continuous sucrose gradient appeared to be established, membrane equilibrium was not reached for the shorter centrifugation times. The apparent mean density of the cytoplasmic membrane fraction on continuous gradients shifted from 1.173 g/cm$^3$ after centrifugation for 12 h to 1.183 g/cm$^3$ after centrifugation for 61 h. Furthermore, shorter centrifugation times resulted in peak broadening and tailing for the continuous gradients, while in the case of discontinuous gradients they resulted in the formation of artificial peaks and shoulders (Fig. 1). Spurious peaks arose because vesicles accumulated at the density interfaces of discontinuous gradients; when the interfaces were moved (from 35/40% to 37/42% sucrose, for instance), the corresponding spurious peaks moved accordingly (data not shown). Thus, as a discontinuous gradient becomes continuous, vesicles float to equilibrium, but this takes longer than the time required for the sucrose gradient to reach equilibrium. Based on these results, all subsequent density gradients were centrifuged for 62 h.

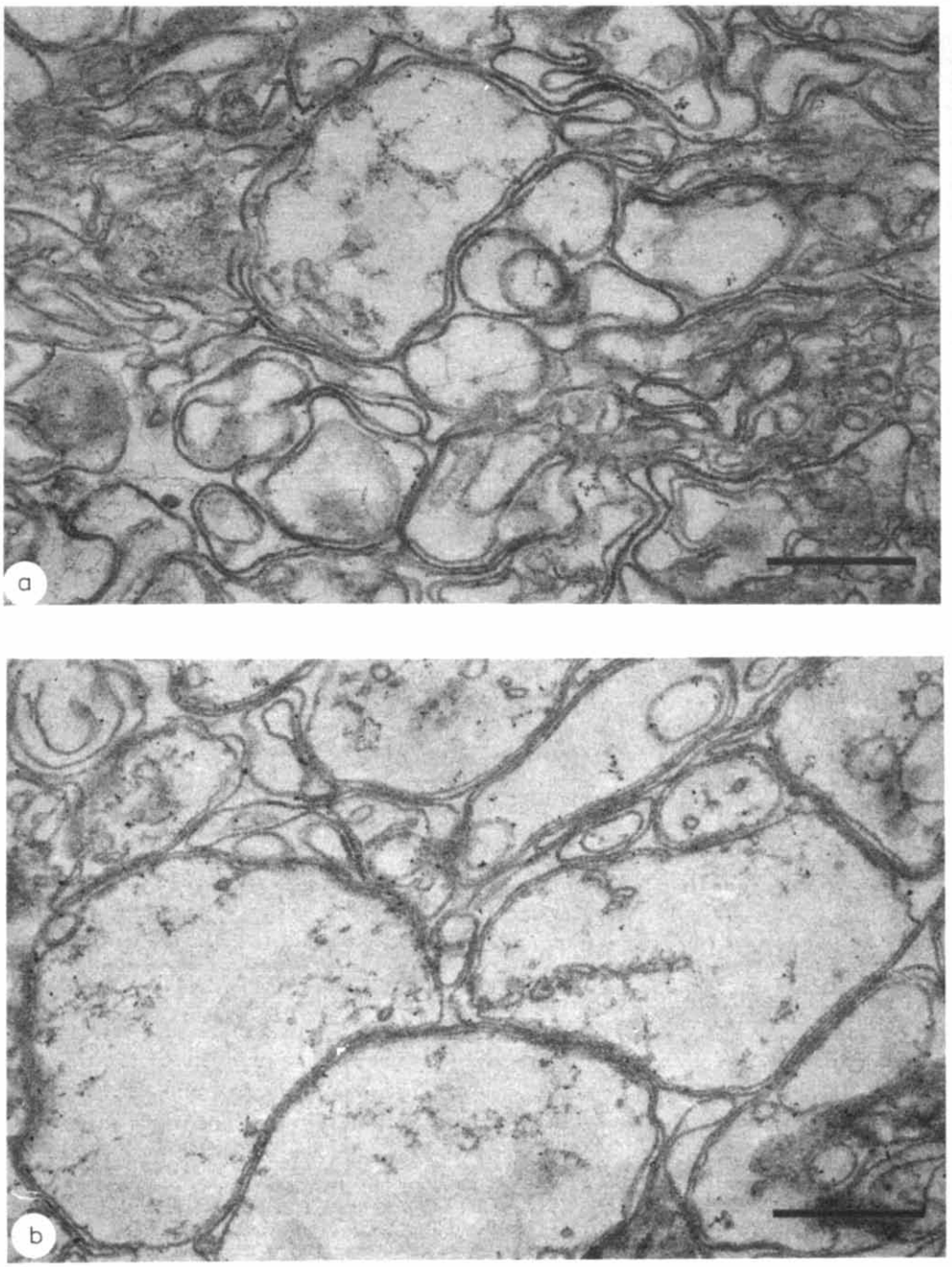
*Spheroplast disruption*

The effects of applying various lysis methods to spheroplasts are examined below.

*Osmotic shock.* In our hands, lysis of spheroplasts by osmotic shock proved to be too mild for obtaining separate outer and cytoplasmic membranes *.

* A 10-fold dilution of spheroplasts in $H_2O$ produces an osmotic pressure differential of about 9 atm. Since increasing the dilution volume further only increases the osmotic pressure differential to a maximum of about 10 atm, it is assumed that dilutions greater than that used in this study will not produce significantly better results.

After isopycnic centrifugation only one membrane fraction with an intermediate density of 1.20 g/cm$^3$ could be observed. Thin sectioning of this material showed large vesicles, often consisting of several concentric membranes (Fig. 2a), while the protein profile of this fraction on SDS-polyacrylamide gel electrophoresis revealed all the protein bands characteristic of the outer as well as the cytoplasmic membrane (Fig. 3).



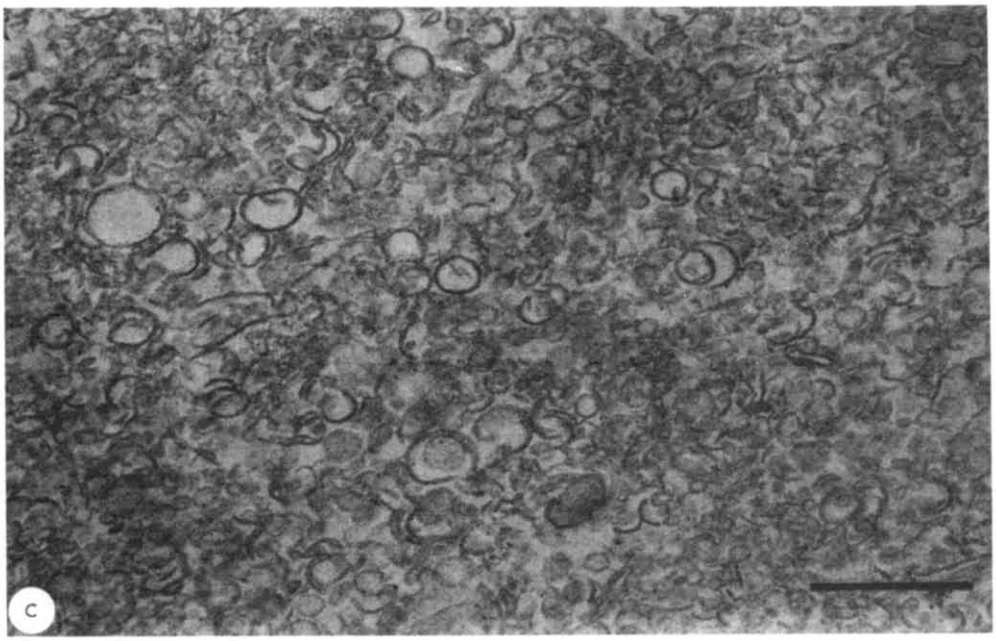



Fig. 2. Thin section electron micrographs of M fractions isolated after isopycnic centrifugation for 62 h. Vesicles were concentrated and freed from sucrose by centrifugation at 360000 × *g* for 2 h. a, M fraction isolated after spheroplast lysis by a 10-fold dilution in water; b, M fraction isolated after spheroplast lysis by sonication for 15 s; c. M fraction isolated after spheroplast lysis by sonication for 450 s. The bar represents 0.5 μm.

*Sonication.* In agreement with the results of others [6,26], we found that sonication could adequately separate the outer and cytoplasmic membranes. Fig. 4 shows that there was a definite optimum sonication period however, which could vary depending on the concentration and state of the spheroplasts. Sonication for shorter, as well as longer, periods produced M fraction material (Figs. 4a, 4b, 4e and 4f). The protein profile on SDS-polyacrylamide gels was similar for both types of M fraction to that of the M fraction isolated after osmotic shock (Fig. 3). Thin sectioning of the M fraction isolated after the

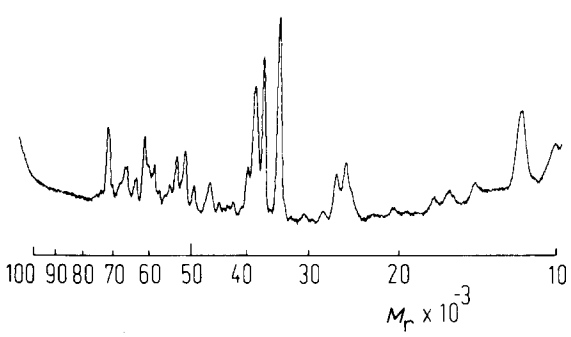



Fig. 3. SDS-polyacrylamide gel electrophoresis of the M fraction isolated after osmotic shock.

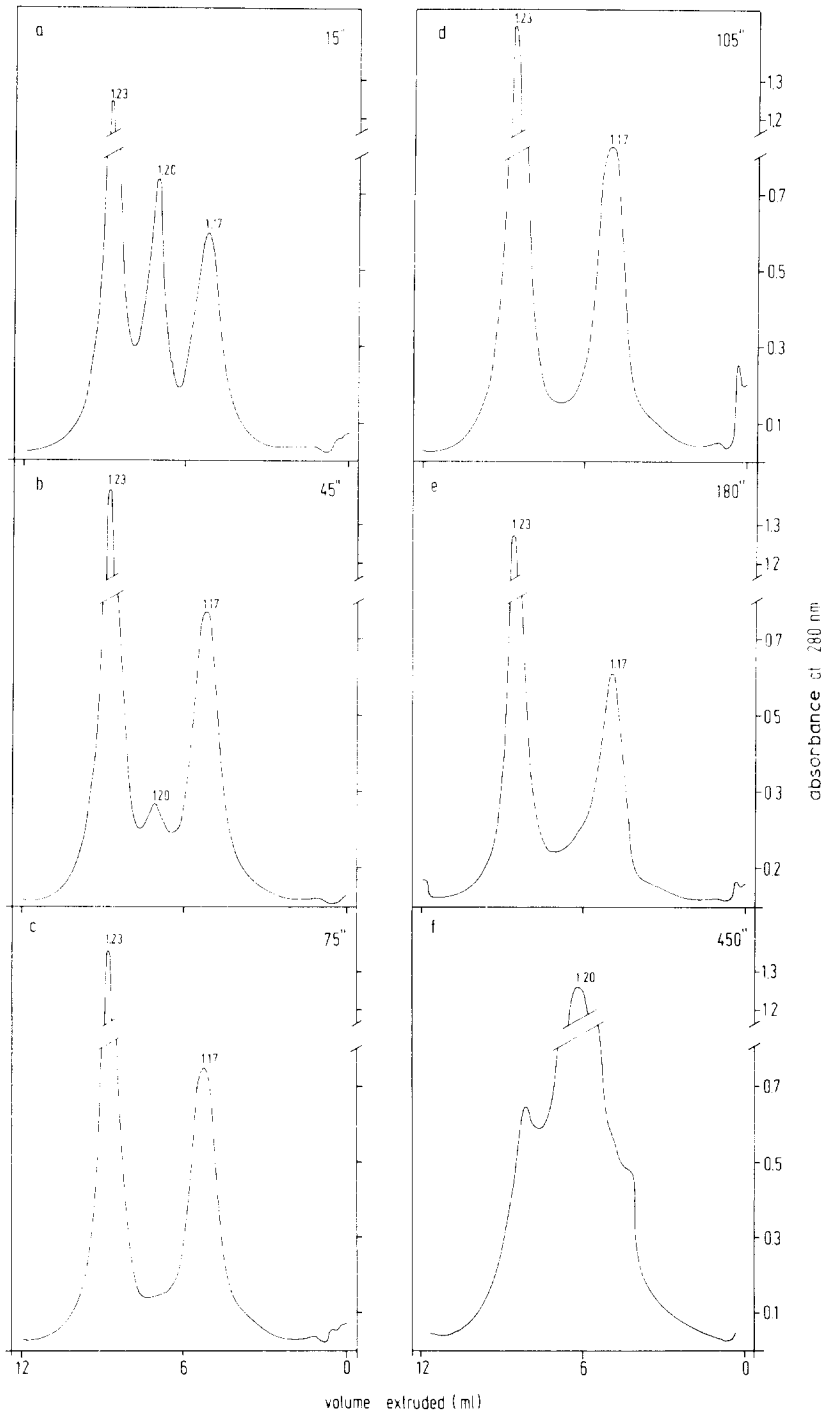



**Fig. 4. Isopycnic sucrose gradient centrifugation of total membranes isolated from spheroplasts lysed by sonication for different times. In this experiment spheroplast lysis was complete after a sonication time of 75 s. Sonication times and apparent mean densities of membrane fractions are indicated.**

shorter sonication times showed very large vesicles with concentric outer and cytoplasmic membranes (Fig. 2b); in contrast, small vesicles, bordered by a single membrane were observed for the longer sonication times (Fig. 2c).

These results indicate that very short sonication times did not separate all membranes, while longer sonication times caused fusion between previously separated membranes. Since fusion is likely to be a continuous process, it must also occur to some degree at the optimum sonication time and, it can be concluded therefore, that a certain amount of mixing of membrane components is inherent to this isolation procedure. As a result, the structure and composition of membrane vesicles obtained by sonication of spheroplasts might be different from that of membranes in situ.

*French press.* The mixing of membrane components, which occurs during sonication, might be avoided in the French press, where the spheroplasts are ruptured only once during the abrupt transition from high to low pressure. Accordingly, spheroplasts were ruptured by a single passage through a French press with pressures ranging from 400 to 1200 atm (see for instance, for a pressure of 400 atm, Fig. 6a and, for a pressure of 800 atm, Fig. 1). In each case the cytoplasmic and outer membranes were well separated. Freeze-fractured cytoplasmic membrane vesicles (Fig. 5a) showed the characteristic fracture faces normally seen in this membrane in freeze-fractured cells. In agreement with others [27,28] we found that the orientation of the membranes in these vesicles was largely inside-out. In the preparation of Fig. 5a, fewer than 5% of the vesicles was right-side-out. Outer membrane fragments (Fig. 5b) generally showed cross-fracture rather than fracture faces, which is typical of the outer membrane in freeze-fractured cells.

In spite of the fact that lysis of spheroplasts in the French press resulted in separate membrane fractions, the mean density of the cytoplasmic membrane fraction increased from 1.17 to 1.18 to 1.19 g/cm$^3$ as the lysis pressure increased from 400 to 800 to 1200 atm respectively (not shown). This was due to increasing contamination of the cytoplasmic membrane fraction by outer membrane material, as was shown by SDS-polyacrylamide gel electrophoresis (data not shown). In contrast, the protein profile of the different outer membrane fractions was not influenced by the lysis pressure (data not shown).

Thus, it is probable that neither lysis in a French press nor lysis by sonication yields truly physiological membrane preparations. Nevertheless, lysis in a French press at least generates vesicles which are likely to resemble the cell membranes immediately prior to lysis and by operating at low lysis pressures, contamination of the cytoplasmic membrane fraction by outer membrane material can be minimized.

*Heterogeneity of the cytoplasmic and outer membranes*

The results of Fig. 1 indicate that the time required to reach equilibrium on a sucrose density gradient was not equal for all vesicles, which could be due to the fact that the cytoplasmic as well as the outer membrane vesicles were heterogeneous with respect to size, as shown in Fig. 5. To determine whether these vesicles were heterogeneous with respect to density as well, a single batch of spheroplasts was disrupted either by sonication during 105 s or by passage through a French press at 400 atm. The resulting outer membrane vesicles

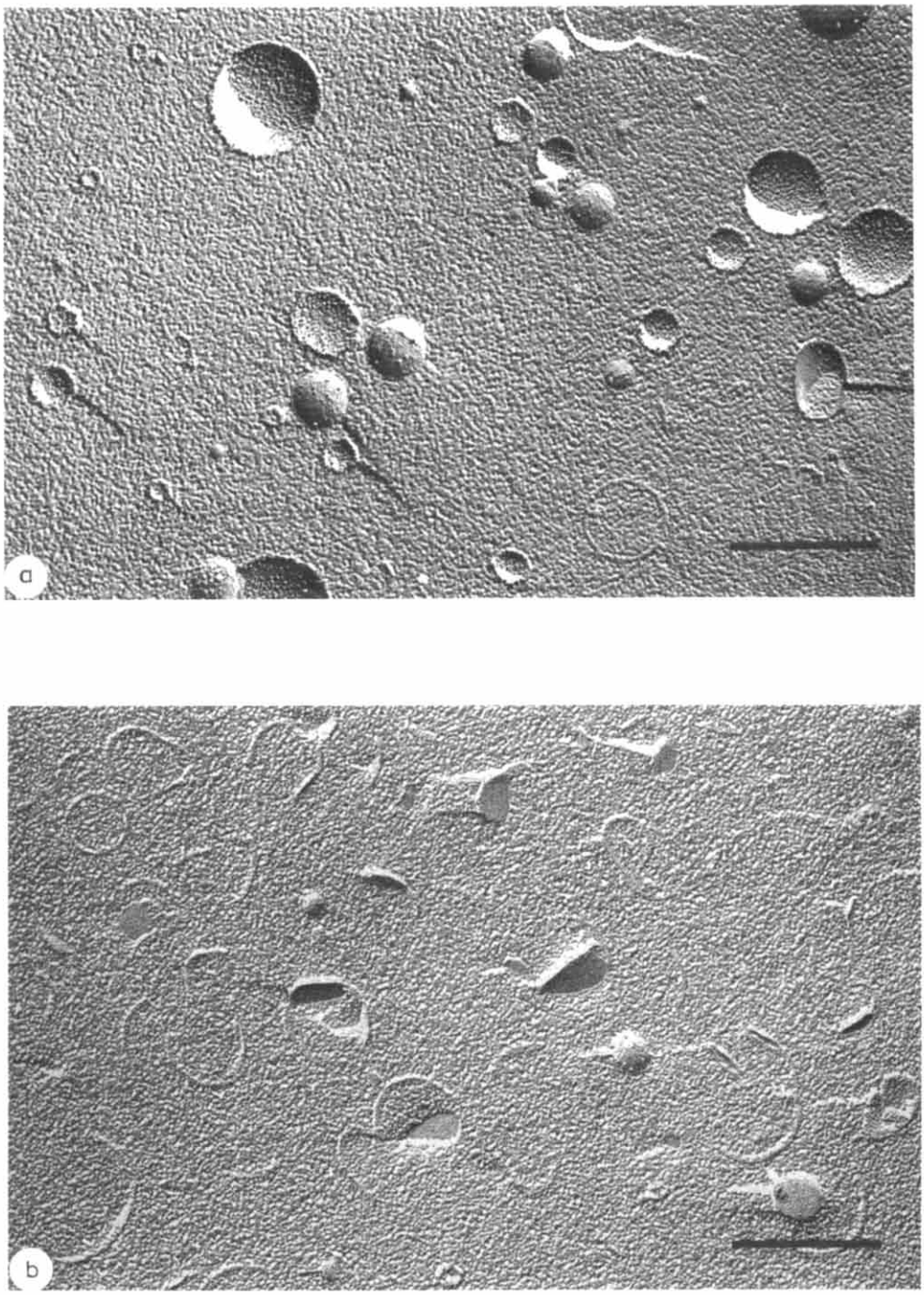



Fig. 5. Freeze-fracture electron micrograph of the cytoplasmic and outer membrane fraction, isolated from spheroplasts lysed by passage through the French press at an operating pressure of 400 atm. a, Cytoplasmic membrane vesicles; b, outer membrane fragments. The bar represents 0.5 $\mu$m.

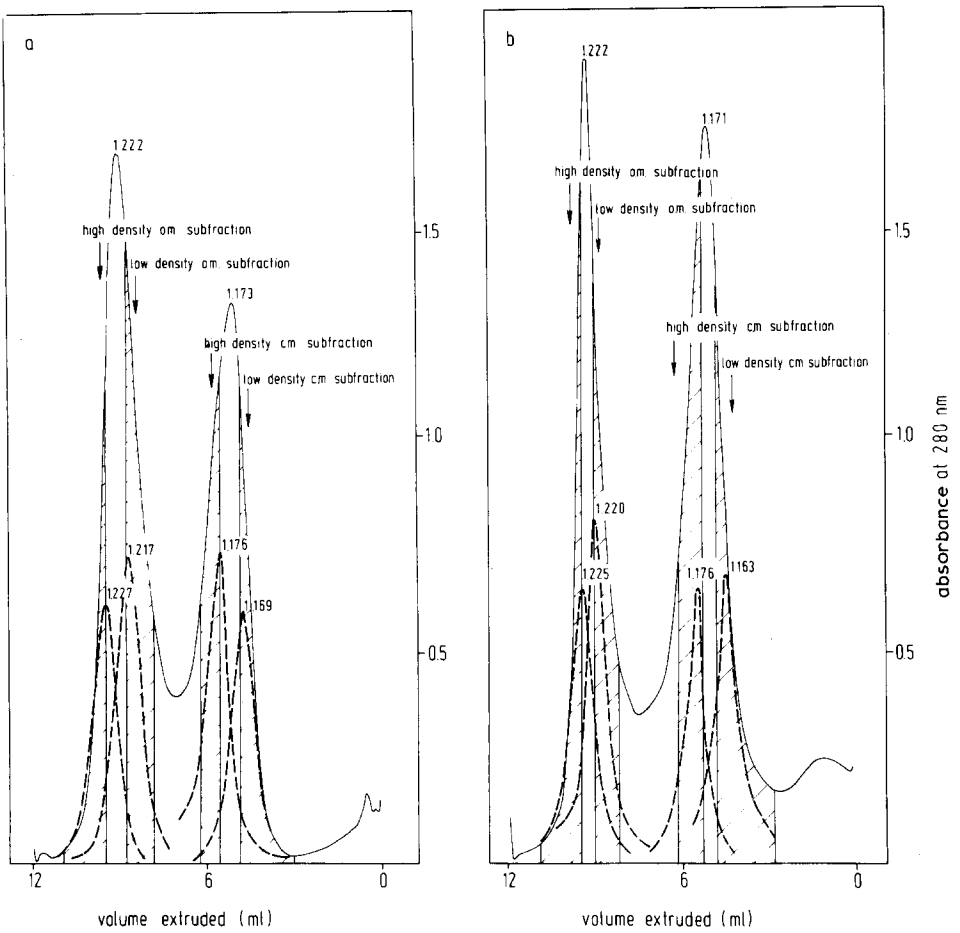



**Fig. 6. Isopycnic sucrose gradient centrifugation of total membranes isolated from spheroplasts lysed by passage through the French press at an operating pressure of 400 atm (panel a) and by sonication for 105 s (panel b). Subfractions were taken as indicated by hatched areas. The absorbance profiles of the recentrifuged subfractions are shown as broken lines. Apparent mean densities are indicated. c.m., Cytoplasmic membrane; o.m., outer membrane.**

TABLE I

MEAN DENSITY AND CHEMICAL COMPOSITION OF ISOLATED SUBFRACTIONS

| | Lysis by sonication | | Lysis by French press | |
|---|---|---|---|---|
| | Light subfraction | Heavy subfraction | Light subfraction | Heavy subfraction |
| Cytoplasmic membrane | | | | |
| Mean density (g/cm$^3$) | 1.163 | 1.176 | 1.169 | 1.176 |
| Phospholipid/protein (g/g) | 0.55 | 0.42 | 0.46 | 0.37 |
| Lipopolysaccharide/protein (g/g) | 0.02 | 0.06 | 0.01 | 0.04 |
| Outer membrane | | | | |
| Mean density (g/cm$^3$) | 1.220 | 1.225 | 1.217 | 1.227 |
| Phospholipid/protein (g/g) | 0.26 | 0.17 | 0.23 | 0.21 |
| Lipopolysaccharide/protein (g/g) | 0.21 | 0.17 | 0.16 | 0.18 |

banded from 1.20 to 1.24 g/cm$^3$, while the cytoplasmic membrane vesicles sedimented from 1.14 to 1.20 g/cm$^3$ (Fig. 6). The density distribution within one band did not result from diffusion alone, but reflected a real density heterogeneity as was shown by recentrifugation of subfractions of the different membrane fractions (Fig. 6, broken lines).

Table I shows that the density heterogeneity of the membrane fractions was related to variations in the phospholipid/protein and lipopolysaccharide/protein ratios of their subfractions. The lipopolysaccharide/protein ratio was higher and the phospholipid/protein ratio was lower in the denser subfraction in every case, except when the outer membrane was obtained after sonication. In that case, the lipopolysaccharide/protein ratio was higher in the light

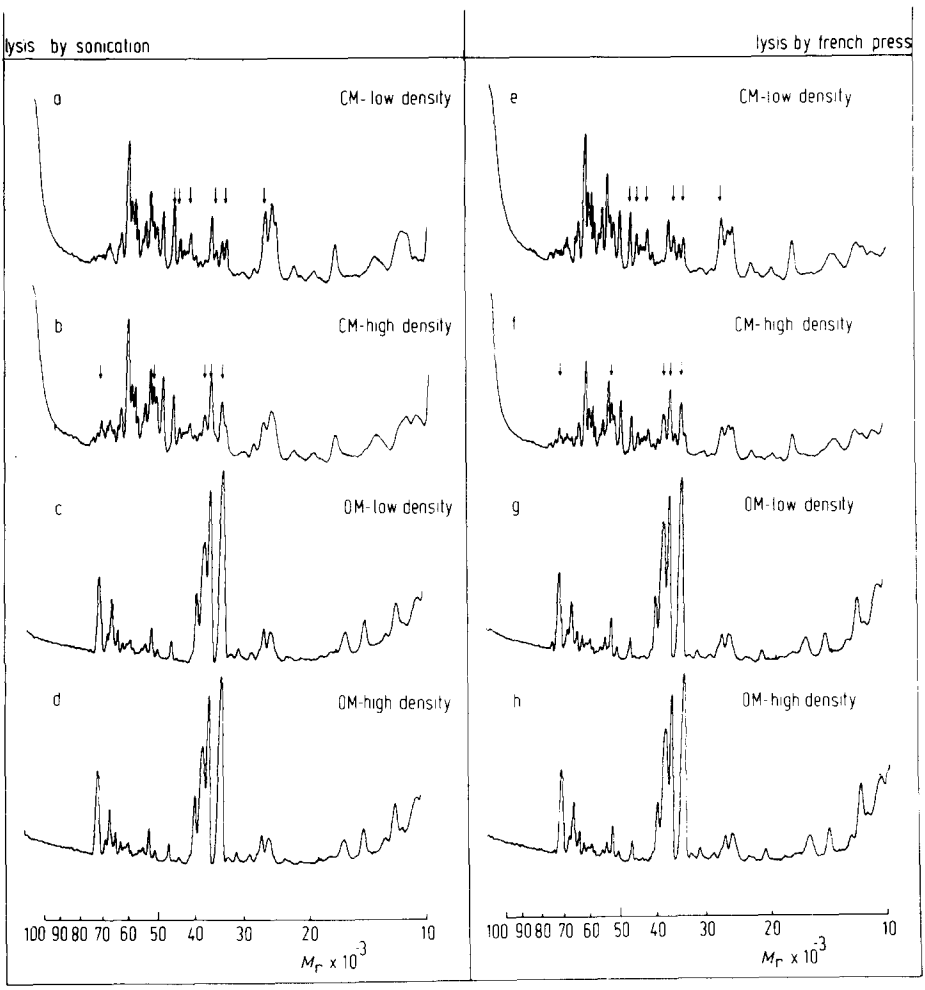



**Fig. 7. SDS-polyacrylamide gel electrophoresis of subfractions from the cytoplasmic and outer membrane isolated from spheroplasts lysed by sonication for 105 s and passage through the French press at 400 atm. Low (a, e) and high (b, f) density subfractions of the cytoplasmic membrane. Low (c, g) and high (d, h) density subfractions of the outer membrane. Peaks which are enriched in the low density membrane subfractions relative to the corresponding high density subfractions, and vice-versa, are indicated by arrows. CM, cytoplasmic membrane; OM, outer membrane.**

subfraction, but the effect of the increased lipopolysaccharide/protein ratio on the density was apparently offset by the relatively high phospholipid/protein ratio in this subfraction.

The protein distribution of the membrane subfractions was determined by SDS-polyacrylamide gel electrophoresis (Fig. 7). Differences in the protein composition of the subfractions of the cytoplasmic membrane fractions (indicated by arrows in Fig. 7) were small but reproducible. The low-density subfraction was enriched with proteins with apparent molecular weights of 26000, 34000, 37000, 43000, 46000 and 48000, while the high density subfraction was enriched with proteins with apparent molecular weights of 35000, 38000, 38 500, 53 000 and 72 000. In contrast, the protein compositions of the different subfractions of the outer membrane were identical.

The heterogeneity of the cytoplasmic membrane fraction was not simply due to a variable contamination with outer membrane material, because not all outer membrane proteins were enriched equally in the heavy cytoplasmic membrane subfraction (for instance those with apparent molecular weights of 16 000, 40 000, and 67 000 were almost absent from both cytoplasmic membrane subfractions).

The density heterogeneity of the outer membrane must also be due to intrinsic variations in the lipopolysaccharide, phsopholipid and protein content of different subfractions. It cannot be ascribed to a variable contamination with cytoplasmic membrane fragments since the protein profiles of the different outer membrane subfractions were identical: if contamination had occurred, the light outer membrane subfractions could be expected to be more contaminated and, therefore, to be different from the heavy outer membrane subfractions.

Thus, the heterogeneity of the outer and cytoplasmic membranes must be due to a degree of variability in the composition of individual membrane vesicles. This variability could be partly artifactual, because of the segregation of proteins and lipopolysaccharide in the plane of the membrane as a result of lipid phase separations [12,13]. The occurrence of phase separations depends on the transition temperature of the membrane phospholipids, on the lysis temperature and on the lysis pressure, since the transition temperature of membrane phospholipids increases 2°C/100 atm independently of the detailed phospholipid structure [32]. Since phase separations may occur in the outer membrane [12,13], it is clear that depending on the strain and conditions used for cell growth (which are likely to influence the composition and transition temperature of the membrane phospholipids) and the temperature and pressure at which spheroplasts are lysed in the French press, membrane vesicle populations are obtained which are heterogeneous to various extents with respect to their phospholipid, lipopolysaccharide and protein content (Table I). Since not all proteins necessarily behave identically during a phase separation, it is possible that differences in density correlate with differences in protein composition; we have shown previously that a protein with a molecular weight of 26000 is greatly enriched in a low density subfraction of the cytoplasmic membrane [12].

Heterogeneity within a membrane fraction could also be due to intrinsic variations in the local composition of different areas of a single membrane. Thus, the outer membrane of minicells, which are believed to be enriched in

polar area membranes, is denser than the outer membrane of the parent cells [31], while the cytoplasmic membrane of minicells has been found to be enriched in a protein with a molecular weight of 48000 [11].

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