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CHARACTERIZATION OF A LOW DENSITY CYTOPLASMIC MEMBRANE SUBFRACTION ISOLATED FROM *ESCHERICHIA COLI*

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SUMMARY

We have used freeze fracture electron microscopy to study the distribution of membrane proteins in the cytoplasmic membrane of *Escherichia coli* W 3110. While these proteins were distributed randomly at the growth temperature (37 °C), there was extensive protein lipid segregation when the temperature was lowered, resulting in bare patches containing no visible particles (protein), and areas of tightly packed or aggregated particles. To understand the segregation process, we have separated the bare patches from the particle rich membrane areas. Lysis of spheroplasts at 0 °C leads to cytoplasmic membrane fragments with different amounts of membrane particles per unit area; such fragments have been separated on isopycnic sucrose gradients. The bare patches occurred as low density membranes which were completely devoid of particles. They were compared to normal density cytoplasmic membranes with respect to fatty acid composition, protein distribution as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and their content of several cytoplasmic membrane marker enzymes.

The phospholipid to protein ratio of low density membranes was five times greater than that of normal membranes; unsaturated fatty acids were more abundant in the low density membranes. Most proteins had disappeared from the low density membranes. One protein, which had an apparent molecular weight of 26000 on sodium dodecyl sulfate gels appeared to be concentrated in the low density membranes; it accounted for about 50% of the total protein found in this membrane fraction.

Of the cytoplasmic membrane markers tested, NADH oxidase and succinate dehydrogenase were excluded, while D-lactate dehydrogenase remained, and even appeared to be concentrated in the low density membranes.

These results indicate that while most membrane proteins are associated with the fluid portion of the bilayer, some proteins evidently associate preferentially with phospholipids in the gel or frozen state.

INTRODUCTION

Freeze fracture electron microscopy can give ultrastructural information on the hydrophobic interior of membranes. Except for myelin [1] fracture faces of membranes have been found covered with particles. It has been generally accepted that these particles represent or contain proteins and glycoproteins which are intercalated into and may traverse the bilayer [2, 3]. The distribution of these particles in the plane of the membrane depends on temperature in a number of cases; cooling of membranes results in such cases in aggregation of the particles while large parts of the membrane become devoid of particles [4–7]. The present paper describes the separation, isolation and characterization of the membrane domains devoid of the particles mentioned above.

MATERIALS AND METHODS

Bacteria and media

Escherichia coli W 3110 was grown to the stationary phase in a minimal medium containing 0.5 % dextrose and E salts [8] as follows. Frozen cultures of *E. coli* W 3110 were used over a period of several months to inoculate small amounts of medium. The resulting late log phase cultures were diluted into fresh medium to a cell density of 0.01 mg/ml, and shaken at 37 °C for 15 h to produce stationary phase cells. Such cultures stopped growing after about 8 h, and achieved final cell densities of approximately 0.8–1.0 mg cells/ml. Cell densities were determined as described previously and were always expressed as mg of cell dry mass per ml [9].

Preparation and isolation of membrane fractions

Cells were harvested by centrifugation for 10 min at $5000 \times g$ or with a Sharples centrifuge (Sharples Centrifuges Ltd, Camberley, England). Spheroplasts were made at 0–4 °C as follows. The cells were suspended in 200 mM Tris · HCl (pH 8.0) to a cell density of 20 mg/ml. At 0 min EDTA was added to a final concentration of 1 mM. At 1 min the cell suspension was diluted twofold with 200 mM Tris · HCl, 1 M sucrose (pH 8.0). At 2.5 min egg white lysozyme (EC 3.2.1.17, C. F. Boehringer & Soehne, GmbH Mannheim, Germany) was added to a final concentration of 60 μg/ml. At 3 min the cells were exposed to a mild osmotic shock by the addition of an equal volume of water, which triggered murein degradation by lysozyme (Witholt, B., Van Heerikhuizen, H. and de Leij, L., to be published). While the cells remained insensitive to a severe osmotic shock (11-fold dilution in water) at 0 °C, they became progressively more sensitive to a similar osmotic shock at 20–22 °C, indicating that the murein layer was being degraded by lysozyme.

At 8–10 min the resulting spheroplasts were collected by centrifugation (0–4 °C, 10 min, $5000 \times g$) and resuspended in ice cold 10 mM Tris · HCl, 1 mM EDTA (pH 8.0) at 25 mg/ml. The spheroplasts were disrupted in a Ribi RM-cellfractionator equipped with a DC-1 dry cooler unit (Sorvall Inc., Norwalk, Conn., U.S.A.), at an operating pressure of 5000–15000 lbs/inch^2, while the temperature was maintained between 0 and 4 °C. After removal of whole cells and large fragments by low speed centrifugation (10 min, $5000 \times g$), the total membrane fractions were isolated by high speed centrifugation (2 h, $176000 \times g$), resuspended in 20 % (w/w) sucrose, 10 mM

Tris · HCl, 1 mM EDTA (pH 8.0) and layered on top of a discontinuous 25–55 % (w/w) sucrose gradient containing 10 mM Tris and 1 mM EDTA (pH 8.0). The gradients were centrifuged for 12–16 h at 38 000 rev./min in a SW 41 rotor (Beckman Instruments Inc., Fullerton, Calif., U.S.A.), or in the case of large scale preparations for 40–60 h at 25 000 rev./min in a SW 25-2 rotor. Gradients were fractionated by pumping in 65 % (w/w) sucrose at the bottom of the tube. Fractions were diluted at least four times with 10 mM Tris, 1 mM EDTA (pH 8.0) and the membranes were collected by centrifugation for 2 h at 176 000 $\times g$ and resuspended in 10 mM Tris (pH 8.0)

Freeze-fracturing

Whole cells were resuspended in minimal medium containing 30 % glycerol (to permit cooling to temperatures below 0 °C) and spray frozen according to Bachmann [10] from a thermostated spray-gun after temperature equilibration for 30 min in the spray gun.

Membrane fractions were frozen conventionally by dipping in liquid Freon 22. The fracture process was carried out at −150 °C for the spray frozen preparation and at −100 °C for the conventionally frozen samples on a Balzers BAF 301 instrument (Balzers, Fürstentum Liechtenstein). Replicas were cleaned in commercial bleach and examined with a Philips EM 200 or EM 300.

Analytical procedures

Protein was determined according to Lowry et al. [11]. Phosphate was assayed for as described by Chen et al. [12]. Phospholipid contents were calculated assuming an average molecular weight of 700.

Electrophoresis of about 30 μg of protein, solubilized by heating in 1 % sodium dodecyl sulfate for 5 min at 100 °C, was carried out on 7.5 % acrylamide gels containing 1 % sodium dodecyl sulfate as described by Lee and Inouye [13]. Bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c* were used as molecular weight standards. Gels were scanned at 630 nm with a Gilford linear transport apparatus attached to a Zeiss PM QII spectrophotometer. Phospholipids were extracted according to Bligh and Dyer [14] and transesterified with methanol-sulfuric acid. The resulting methylesters were analysed on a Hewlett-Packard F & M 5750 gas chromatograph using a 1/8 inch × 12 ft column of 10 % diethyleneglycol-succinate on 80–100 mesh Chromosorb WAW. The column was run at 180 °C. The relative amounts of the different fatty acids were determined according to the peak height × retention time method of Carrol [15].

Enzyme assays

Succinate dehydrogenase (EC 1.3.99.1), D-lactate dehydrogenase (EC 1.1.1.28) and reduced nicotinamide adenine dinucleotide (NADH) oxidase (EC 1.6.3.1) were measured according to Osborn et al. [16].

RESULTS

Effect of temperature on particle aggregation

In agreement with similar results obtained for *Tetrahymena pyriformis* [5],

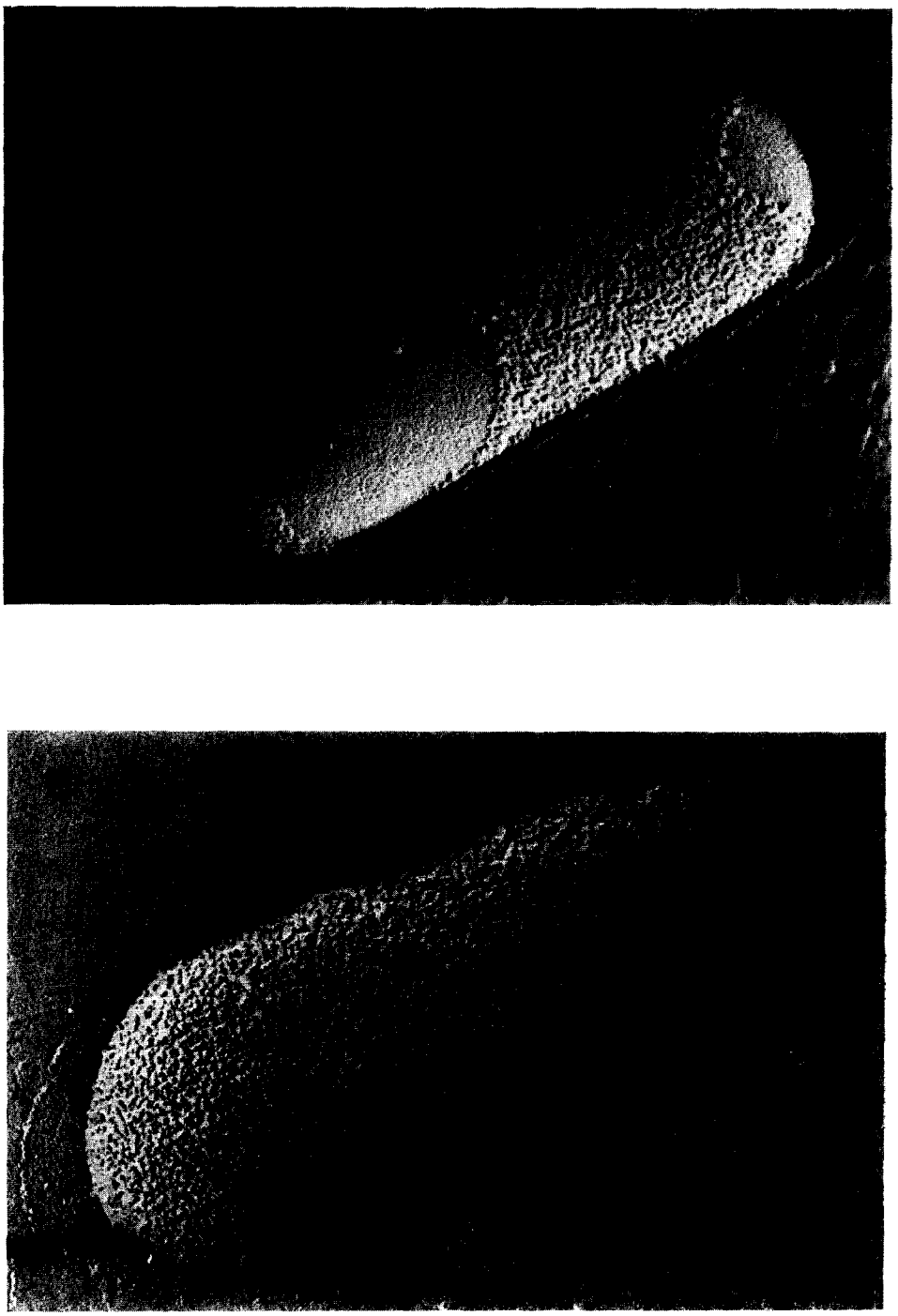

Fig. 1. Freeze fracture micrographs of the cytoplasmic membrane of *E. coli* W 3110 incubated at 0 °C (A) and 37 °C (B) before being frozen. The bar represents 0.5 μm.

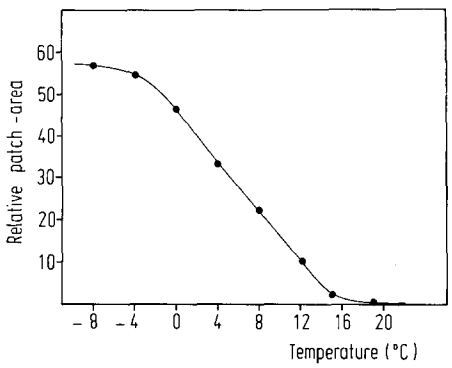

Fig. 2. The effect of temperature on the extent of particle aggregation in the cytoplasmic membrane of *E. coli* W 3110 cells. Cells were equilibrated at the desired temperature for 30 min and spray frozen as described in Materials and Methods. The particle-free area relative to the total fracture area (%) is an average determined from freeze fracture micrographs of 50 cells at each temperature.

Streptococcus faecalis [17] and fatty acid auxotrophs of *E. coli* [7, 18] we find for both exponentially growing and stationary phase *E. coli* W 3110 that freezing from low temperatures results in aggregation of cytoplasmic membrane particles (Fig. 1A), while freezing from growth temperature shows a random dispersion (Fig. 1B). The netlike distribution of particles found by several authors [4, 6] after freezing from growth temperature is an artifact created by cooling rates which are too slow, as has been previously suggested [6, 18]. We have found similar netlike particle distributions in conventionally frozen preparations (cooled by dipping in fluid Freon 22), while the much faster freezing process of spray freezing produces a fully random particle distribution.

The effect of temperature on the cytoplasmic membrane particle distribution is shown in Fig. 2. Although it is difficult to interpret this curve absolutely quantitatively due to possible non random fracturing, it is clear that above 20 °C the particle distribution in the cytoplasmic membrane is fully random. At 16–20 °C particle aggregation starts, increasing gradually as the temperature prior to spray freezing is lowered and reaching a maximum at about −4 °C.

Particle densities of cytoplasmic membrane vesicles obtained by lysis of EDTA-lysozyme spheroplasts at 0 °C and at room temperature

Rupturing of EDTA-lysozyme spheroplasts, either by osmotic shock or by passage through a Ribi pressure cell, leads to fragmentation of the cell envelope into smaller membrane vesicles [16, 19]. If the cytoplasmic membrane contains particle rich areas and patches devoid of particles, spheroplast lysis may be expected to lead to the formation of vesicles with various particle densities. This was in fact observed, as can be seen in Fig. 3. These vesicles show considerable differences in particle density, while lysis of the same spheroplasts at room temperature only showed vesicles with a uniform particle density (not shown).

Particle aggregation due to a lowering of the temperature from room temperature to 0 °C was reversible in vesicles as well as in the cells from which they originated.

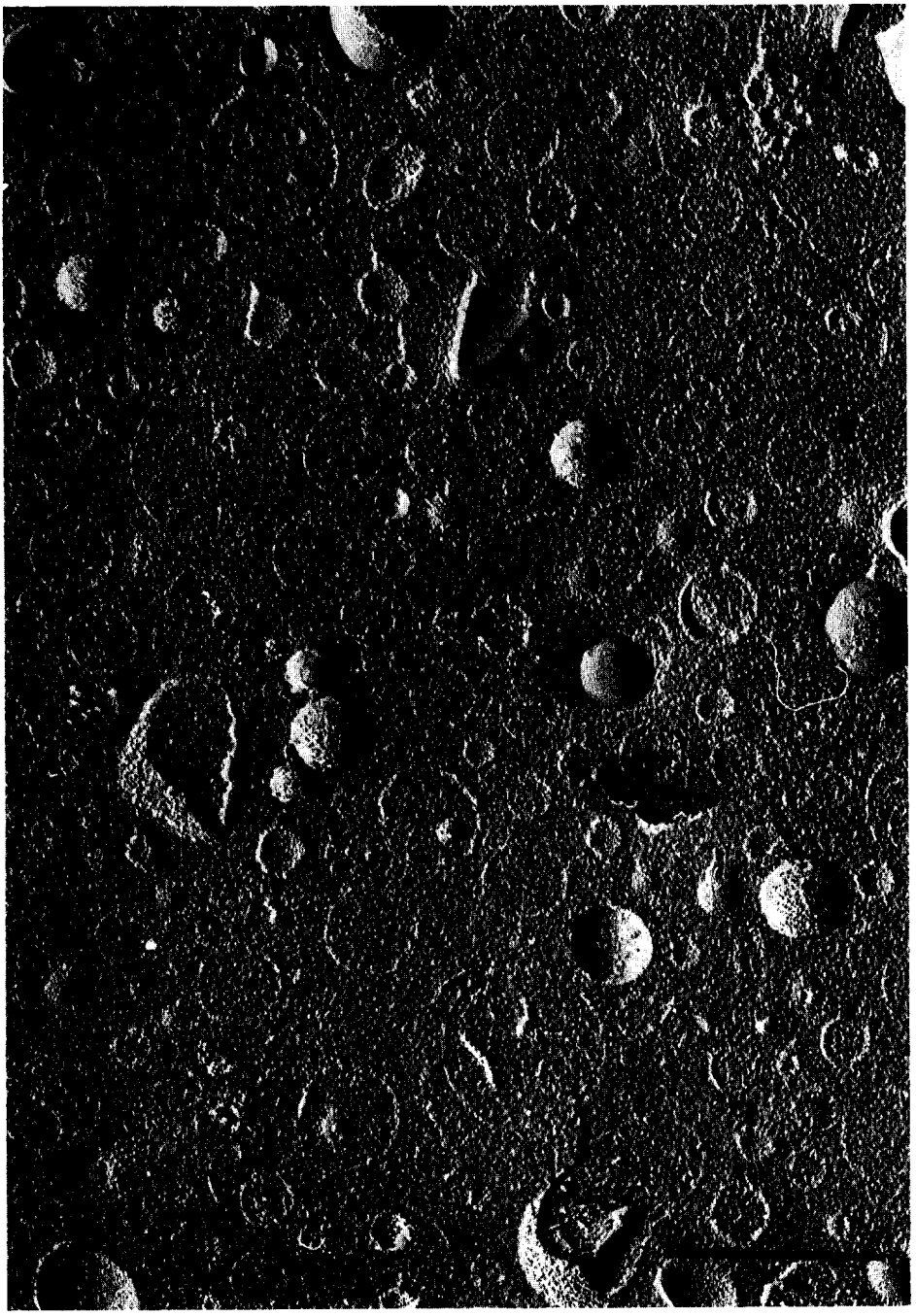

Fig. 3. See opposite page for legend.

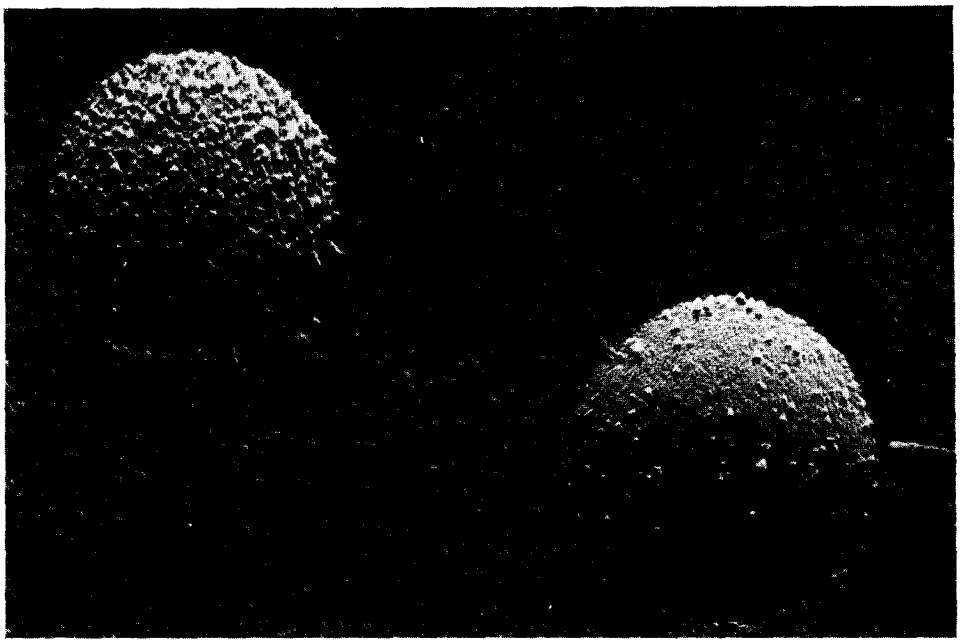

Fig. 3. Freeze fracture micrographs of *E. coli* W 3110 cytoplasmic membrane vesicles obtained by breakage of EDTA-lysozyme spheroplasts at 0–4 °C in a Ribi press at 15000 lbs/inch^2. The sample was equilibrated at and frozen from 25 °C to insure a random distribution of particles in all vesicles. A: Typical lysate at low magnification (bar represents 0.5 μm). B: Detail of vesicles with low and high particle density (bar represents 0.2 μm).

Subfractionation of the cytoplasmic membrane vesicles of a lysate obtained at 0 °C

When *E. coli* spheroplasts were lysed at 0 °C by osmotic shock or in a Ribi press the resulting lysates contained a variety of different membrane fragments and vesicles. In addition to the characteristic outer membrane, cytoplasmic membrane and mixed fragments observed by other authors [16, 20], we found considerable heterogeneity within the class of cytoplasmic membrane fragments as might be expected, in view of the variations in particle density illustrated in Fig. 3. Because the fragments without any particles were expected to have a buoyant density lower than the 1.14–1.16 g/cc found by several authors [16, 20] for the cytoplasmic membrane band, we used density gradients containing 20–55 % (w/w) sucrose (ρ = 1.08-1.26 g/cc) to separate all of these membrane fragments.

When spheroplast lysates prepared at 0 °C were fractionated on such gradients we found a small broad membrane band at a low buoyant density (ρ = 1.10 g/cc) in addition to the typical cytoplasmic and outer membrane bands found by other workers [20, 21], as shown in Fig. 4A. Investigation of this low density membrane band by freeze fracture electron microscopy revealed a membrane preparation with very few or no membrane particles per unit area (Fig. 5).

Fatty acid composition

Membrane fractions showing no particles in freeze fracture electron micrographs (fractions 1 to 4) were pooled. Their fatty acid composition was determined

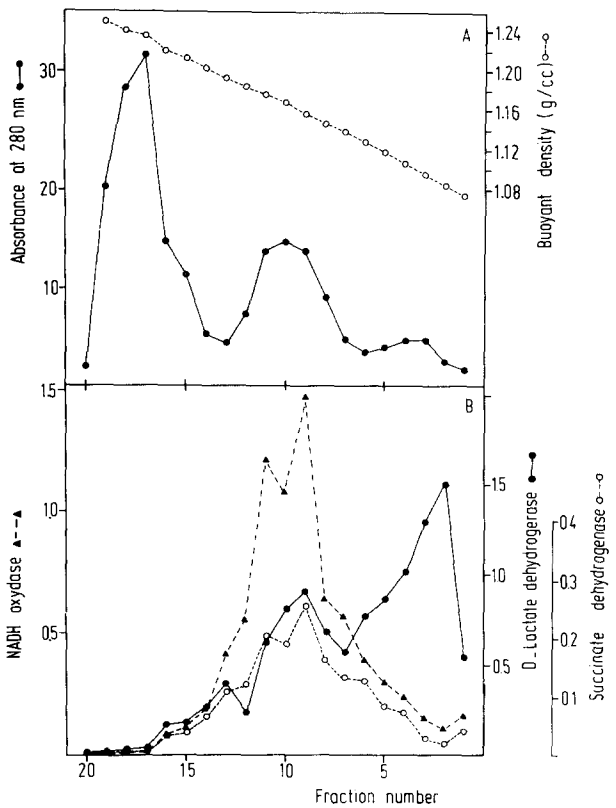

Fig. 4. Sucrose gradient centrifugation of total membranes of *E. coli* W 3110 and the distribution in the gradient of the specific activities of NADH oxidase, succinate dehydrogenase and D lactate dehydrogenase. Total membranes were isolated from 2 g of cells. Isopycnic sucrose gradient centrifugation was carried out in the SW 25.2 rotor for 60 h at 25000 rev./min and 4 °C. Fractions of 50 drops (2.9 ml) were collected. Refractive indices and absorbances at 280 nm were measured. After dilution with 10 mM Tris (pH 8.0) the membranes were collected by centrifugation (2 h, 176000 × *g*) and resuspended in the same buffer. Determination of protein and assay of enzyme activities were carried out as described in Methods and Materials. A. Absorbance at 280 nm (●—●) and buoyant density (○ – – – ○) B. Enzyme specific activities expressed as μmol/min/mg protein. NADH oxidase (▲ – – – ▲), D-lactate dehydrogenase (●—●) and succinate dehydrogenase (○ – – – ○).

and compared with that of pooled cytoplasmic membrane fractions banding at normal density (fractions 9 to 11). The results are shown in Table I; similar results have also been obtained for another preparation. The pooled low density fractions, which originate from the bare patches in the cooled membrane, possess about 20 to 25 % more saturated fatty acids than the pooled cytoplasmic membrane fractions of normal buoyant density, resulting in less fluid behaviour of the phospholipids.

Protein composition

The protein composition of normal cytoplasmic membranes was compared to that of the low density membranes by solubilization of membrane proteins in the

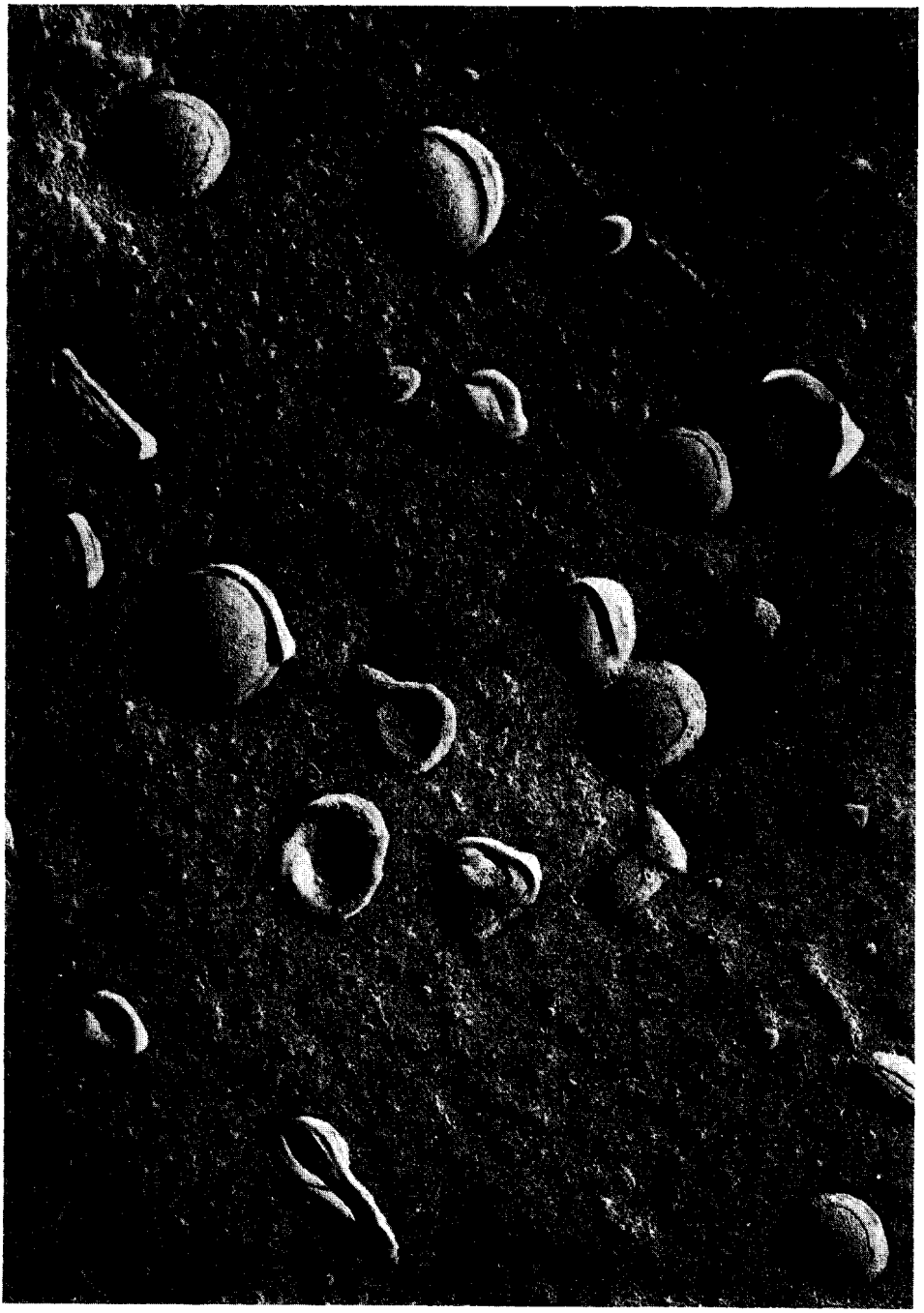

Fig. 5. Freeze fracture micrograph of a low density cytoplasmic membrane subfraction. The subfraction was isolated as described in the legend to Fig. 4 and consists of the fractions 1 to 4. The samples were equilibrated at and frozen from 25 °C. The bar represents 0.2 μm.

TABLE I

PHOSPHOLIPID TO PROTEIN RATIO AND FATTY ACID COMPOSITION OF NORMAL CYTOPLASMIC MEMBRANES AND A LOW DENSITY SUBFRACTION

Phospholipid/protein (mg/mg):	Normal cytoplasmic membranes 0.37	Low density subfraction 1.74
Fatty acid*		
14 : 0	2.9	6.1
16 : 0	43.2	52.8
16 : 1	5.7	5.2
17 : cy	33.9	21.1
18 : 1	6.2	5.9
19 : cy	5.1	3.4
Unidentified	3.0	5.6
Saturated	46.1	58.9
Unsaturated + cyclopropane	50.9	35.6

* Results of fatty acid composition are expressed as percent total fatty acid.

presence of 1 % sodium dodecyl sulfate and subsequent electrophoresis of the resulting polypeptides. Fig. 6 shows spectrophotometric scans of the electrophoretic patterns obtained. A single band at a molecular weight position of about 26 000 dominated the low density membrane fraction (Fig. 6B). This band was consistently found in a number of different preparations. The normal cytoplasmic membrane fraction (Fig. 6A) showed the typical heterogeneous protein distribution observed by other authors [16, 20, 22]; no clear major protein band was present. The material at a molecular weight position of 26 000 was in most cases poorly resolved and consisted of only a small fraction of the total protein.

A moderate band at an estimated molecular weight of 67 000 (arrow in Fig.

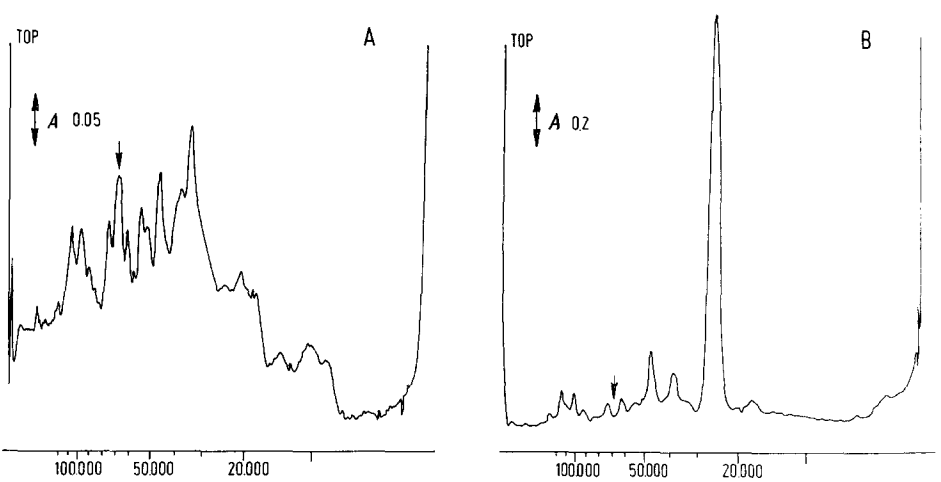

Fig. 6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of membrane proteins of the normal cytoplasmic membrane fraction (A) and the low density cytoplasmic membrane subfraction (B) of *E. coli* W 3110.

6A), shown by Spencer and Guest [22] to be succinate dehydrogenase, was clearly absent from the low density membranes (arrow in Fig. 6B).

Distribution of cytoplasmic membrane marker enzymes

As was shown by Osborn et al. [16] electron transfer enzymes are almost solely localized in the cytoplasmic membrane and can thus be used as marker enzymes for the cytoplasmic membrane. The distribution of NADH oxidase, succinate dehydrogenase and D-lactate dehydrogenase in the sucrose gradient is illustrated in Fig. 4B.

NADH oxidase and succinate dehydrogenase behave quite similary; high specific activities are associated with the normal cytoplasmic membrane which bands at $\rho = 1.17$ g/cc. Low density cytoplasmic membrane vesicles, which contain few or no visible particles after freeze fracturing, have very low specific activities of these two enzymes.

Strikingly different from the behaviour of these two enzymes is that of D-lactate dehydrogenase. The low density membranes contain specific D-lactate dehydrogenase activities which are considerably higher than those found in the normal cytoplasmic membranes. The distribution of enzyme specific activities as given in Fig. 4B were consistently found in different preparations. The ratio of the specific activity of D-lactate dehydrogenase in the low density membranes relative to its specific activity in the normal cytoplasmic membranes, which was about 2 in the experiment shown in Fig. 4B, varied between 1 and 4 in other preparations. Succinate dehydrogenase and NADH oxidase were essentially absent in the low density membranes in these same preparations.

DISCUSSION

We have shown that fragmenting of *E. coli* W3110 spheroplasts at a temperature where the cytoplasmic membrane shows extensive particle aggregation leads to the formation of cytoplasmic membrane vesicles which differ considerably in the number of particles per unit area. Recently Altendorf and Staehelin [23] produced vesicles of *E. coli* ML 308-225 by osmotic shock and French press treatment, but they did not report any heterogeneity in particle density in their preparations. The difference in their findings, compared to those reported here is probably due to the fact that the temperature at which Altendorf and Staehelin [23] produced vesicles exceeded the transition temperature of the membrane phospholipids.

It proved possible to isolate a cytoplasmic membrane subfraction with a very low buoyant density banding at around $\rho = 1.10$ g/cc, by means of isopycnic sucrose density gradient centrifugation. This subfraction had very few or no membrane particles per unit area as was shown by freeze fracture electron microscopy (Fig. 5). Total separation of cytoplasmic membrane vesicles according to their particle densities by means of sucrose density centrifugation appears to be impossible. Fractions banding at higher densities than the low density subfraction still contain a number of vesicles with low particle density. The reason for this probably lies in the variable amount of contamination of the cytoplasmic membrane vesicles with outer membrane, an explanation already suggested by Osborn et al. [16] for the existence of the two different cytoplasmic membrane bands L_1 and L_2 found in *Salmonella typhimurium*. Furthermore cytoplasmic membrane vesicles with very high particle densities

will band in the outer membrane region of the gradient (this was confirmed by freeze fracture electron microscopy) and thus cannot be separated from outer membrane on isopycnic density gradients. Consequently the only subfraction that can be isolated in pure form consists of cytoplasmic membrane vesicles with very low particle density.

When stationary phase *E. coli* W3110 cells were frozen from temperatures below 20 °C the cytoplasmic membrane particles were found to be aggregated. The extent of aggregation increased as the cells were cooled from 20 °C to about −4 °C and then remained unchanged.

The explanation for such temperature dependent formation of bare membrane patches is that, as already suggested by Verkleij et al. [4], the membrane phospholipids crystallize into solid patches which exclude the membrane particles and force them into aggregates. In addition, it is possible that some membrane particles disappear from the fracture face altogether, as suggested by Speth et al. [5, 24].

The crystallization behaviour of complex lipid mixtures such as those found in natural membranes is not known, but recently the freezing behaviour of some binary lipid mixtures has been studied [25, 26]. It was found that cooling causes a lateral phase separation of lipids into liquid crystalline phase and gel phase regions; the lipids in the gel phase are enriched in the higher melting phospholipid. We may expect a similar segregation of the different lipid species present in natural membranes upon phase separation; the extent of this segregation will depend on the structural differences between the lipid species. Thus the membrane patches may be expected to contain higher melting phospholipids than the bulk phospholipids. Our finding that the light subfraction originating from the bare membrane patches contains 20 to 25 % more saturated fatty acids compared to the normal cytoplasmic membranes, is in accord with this expectation.

The effect of the lipid phase separation on the membrane proteins was quite interesting. While the bulk of the proteins was excluded from the patches, it is clear that the phase separation did not affect all proteins equally. Instead, Fig. 6 shows that the distribution of the various proteins present in a patch differs drastically from that found for normal cytoplasmic membranes. There is a striking increase in the relative intensity of one band at a molecular weight position of about 26 000, comprising about 50 % of the protein present. In principle, this enrichment could be due to the disappearance of other proteins from the membrane patches. However, the observed enrichment of 10–20 fold (Fig. 6) is considerably greater than that expected based on the phospholipid to protein ratios of Table I.

If it is assumed that phospholipid and protein with densities of 1.05 and 1.35 g/cc respectively determine the total membrane mass, and that the thickness of the bilayer is 40 Å, while on the average proteins protrude 5 Å from either side of the membrane, a 2.5-fold enrichment may be expected. Deviations from the above assumptions alter the enrichment only slightly.

It appears therefore that the localization of this protein must be ascribed to its preferential segregation towards growing patches of high melting phospholipids during phase separation.

Although this protein dominates the profile of the low density membrane fraction, a number of other membrane proteins can still be found to varying extents. Thus, D-lactate dehydrogenase was present in both low density and normal cytoplasmic membranes (Fig. 4B). Futai [27] has shown that D-lactate dehydrogenase has a

molecular weight of 72 000 on sodium dodecyl sulfate gel electrophoresis; the presence of such a band in both low density as well as normal cytoplasmic membranes (immediately to the left of the arrows in Fig. 6) supports the data of Fig. 4B.

Some proteins were excluded essentially completely from the low density membrane fraction. This was the case for succinate dehydrogenase and NADH oxidase as shown in Fig. 4B. The exclusion of succinate dehydrogenase from patches is corroborated by the sodium dodecyl sulfate gel data of Fig. 6: the arrows indicate a protein band at a molecular weight position of about 67 000 which has been shown to be succinate dehydrogenase by Spencer and Guest [22]. This band, which is seen in the normal membranes, is absent from the low density subfraction, in good agreement with our enzymatic data.

The correlation between the specific activities of succinate dehydrogenase and NADH oxidase and the presence of membrane particles as seen in freeze fracture electron micrographs strongly suggests a close connection between the particles and the enzymes. These two enzymes may well be true intrinsic membrane proteins.

In contrast, the localization of D-lactate dehydrogenase in the membrane does not seem to be affected by the gross particle aggregation observed at low temperatures. Thus, although D-lactate dehydrogenase is undoubtedly closely associated with the cytoplasmic membrane [27, 28], it seems not to have any connection with the membrane particles seen in freeze fracture electron micrographs, which may be explained in several ways. First, D-lactate dehydrogenase may be localized at the periphery of the membrane, having little or no interaction with the fatty acid chains of the membrane phospholipids; this might be the case if its association with the membrane is

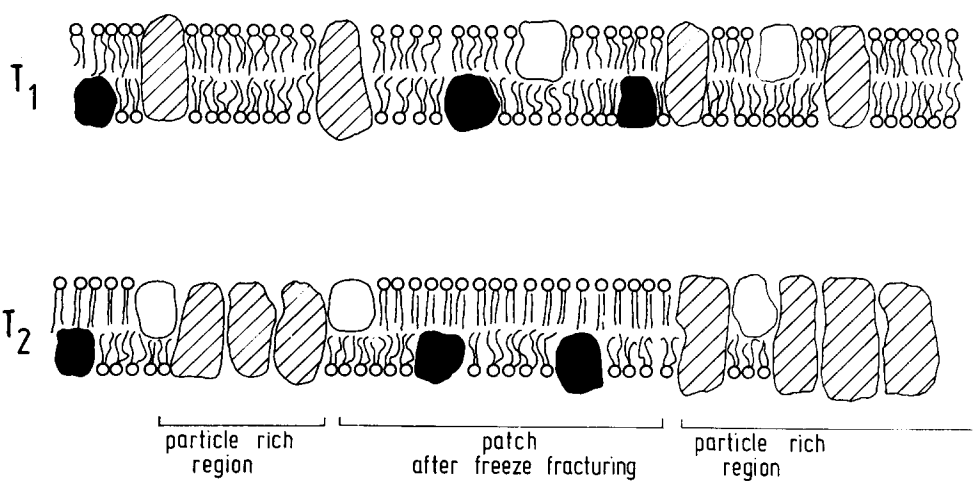

Fig. 7. Possible model for the specific aggregation of membrane proteins during a phase separation. Some proteins (hatched oblongs) penetrate through the entire bilayer, while others occur mainly in only one of the two membrane monolayers (open and solid shapes). At temperature T_1 (upper membrane) essentially all phospholipids are in the fluid state, as indicated by ♀. If, upon lowering the temperature to T_2, a phase separation occurs in only one monolayer, (i.e. the phospholipids of this monolayer enter the gel state, indicated by ♀ in the lower membrane), there is aggregation of those proteins which penetrate that monolayer (hatched and open shapes). Proteins which penetrate only through the other monolayer (solid shapes) remain randomly distributed in this monolayer.

caused primarily by ionic interactions with the phospholipid headgroups. Second, D-lactate dehydrogenase may penetrate only one monolayer and may interact equally well with lipids in a fluid or in a gel state; it would then fail to be excluded from the patches, and it would also not be seen upon freeze fracturing. Third, our findings can also be explained by assuming an asymmetric phospholipid distribution in the lipid bilayer of the cytoplasmic membrane, such as has been found in erythrocytes by Zwaal et al. [29, 30]. This explanation is illustrated schematically in Fig. 7. Upon cooling ($T_1 \rightarrow T_2$) one monolayer starts to gel, excluding the proteins embedded in this monolayer only, as well as the proteins which penetrate through the whole bilayer. NADH oxidase and succinate dehydrogenase may be representatives of one of these classes of proteins. Proteins embedded in the other monolayer only, which at T_2 is still in the fluid state, remain randomly dispersed. D-lactate dehydrogenase may be a representative of this class of membrane proteins.

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