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PROLINE TRANSPORT ACTIVITY IN *ESCHERICHIA COLI* MEMBRANE VESICLES OF DIFFERENT BUOYANT DENSITIES

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Summary

Cytoplasmic membrane vesicles prepared by lysis of *Escherichia coli* W 3110 spheroplasts in a French press at 0°C are heterogeneous with respect to density due to membrane protein aggregation as a result of lateral phase separation of membrane phospholipids and to the presence of more or less outer membrane. These different vesicle classes can be separated on isopycnic density gradients. Assays for various membrane-associated functions show that the membranes differ not only with respect to density and structure but also with respect to function.

The proline transport system (as detected by uptake experiments with the artificial electron donor ascorbate-phenazine methosulfate) shows maximal activities in membrane fractions that have considerably higher densities than the normal cytoplasmic membrane. This is always the case, whether vesicles are isolated from membranes that exhibit a temperature-induced protein aggregation or not. A correlation between high proline transport activity and the presence of vesicles with double membranes (consisting of outer and inner membrane) has been established. The possibility that the outer membrane protects the transport system in the cytoplasmic membrane during the isolation of vesicles is discussed.

Introduction

We have recently shown that the lipid phase separation which occurs in the cytoplasmic membrane of *Escherichia coli* when the temperature is lowered, causes the lateral displacement of membrane proteins, resulting in membrane domains of varying phospholipid and protein content and composition, which

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can be separated and isolated on isopycnic density gradients [1]. Although it might have been expected that different proteins of a given multicomponent membrane system should be displaced together during a phase separation, this was not the case for some of the proteins involved in electron transport. While succinate dehydrogenase and NADH oxidase activities remained associated with protein-rich cytoplasmic membrane fragments or vesicles, D-lactate dehydrogenase activity was clearly separated from the other two activities and remained associated with lipid rich membrane vesicles.

As an extension of these observations we became interested in the behaviour of complex membrane associated systems, such as those responsible for active transport of amino acids; as an example of such processes we have studied the proline transport system. In this paper we show that proline uptake activity is not affected by phase separations, but is found predominantly in a high density membrane fraction, which contains significant amounts of outer membrane.

Materials and Methods

Cell growth

Stationary phase cultures of *E. coli* W 3110 were grown in minimal medium as described previously [2]. Cell densities were determined from their absorbances at 450 nm and were always expressed as mg of cell dry mass per ml [3].

Preparation of membrane fractions

Membranes were prepared as described earlier [1] with the exception that the spheroplasts were stabilized by the addition of 10 mM $MgCl_2$ before they were collected by centrifugation [2], and were resuspended in 10 mM Tris · HCl buffer (pH 8.0), 1 mM EDTA containing 20% (w/w) sucrose.

Uptake experiments

Proline transport was performed essentially according to Konings and Freese [4] with the exception that no water-saturated oxygen was blown over the incubation mixture. The incubation mixture of 100 µl consisted of 50 mM K-phosphate buffer (pH 6.6), 10 mM $MgSO_4$, 50 µg of membrane protein and D-lactate or ascorbate-phenazine methosulfate. The reaction was started by addition of [U-^{14}C]proline (The Radiochemical Centre, Amersham, Buckinghamshire, U.K.), with a specific activity of 290 mCi/mmol (final concentration 4.6 µM). All uptake experiments were performed at 25°C. Initial rates of transport were measured after a 1-min incubation of the membrane vesicles with the electron donor.

Electron microscopy

Membrane fractions were prefixed in 1.5% glutaraldehyde in 0.01 M sodium cacodylate buffer (pH 7.0) for 1 h, washed and postfixed in 1% OsO_4 in the same buffer. After dehydration in acetone the membranes were embedded in Vestopal W. Ultrathin sections were cut on an LKB ultramicrotome with glass knives, picked up on uncoated 400 mesh grids and stained with lead citrate [5] and uranyl acetate [6]. Sections were examined in a Philips EM 200 or EM 201 electron microscope at 80 kV.

Freeze-fracturing of membrane fractions was performed as described [1].

Enzyme assays

D-Lactate dehydrogenase (EC 1.1.1.28) and reduced nicotinamide adenine dinucleotide (NADH) oxidase were measured as described previously [1].

Analytical procedures

Protein was determined according to Lowry et al. [7] using bovine serum albumin as a standard.

Slab gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed in 12% polyacrylamide gels, using the buffer system of Laemmli [8]. About 20—30 μg of protein was applied per slot. Bovine serum albumin, ovalbumin, aldolase, chymotrypsinogen and cytochrome *c*, were used as molecular weight standards. Gels were run for approximately 5—6 h at 100 V per slab and stained and destained as described by Lugtenberg et al. [9].

Results

Transport in vesicles prepared by lysis in a French press

Based on a number of different criteria several investigators have concluded that vesicles prepared by lysis in a French press are inverted [10—12]. As it is generally agreed that active uptake of amino acids and sugars only takes place in vesicles in which the membrane orientation is identical to that of the cytoplasmic membrane in intact cells (see ref. 13 for a review), the finding that French press vesicles do not accumulate proline in an energy-stimulated reaction [11,12] is in good agreement with the "inside-out" orientation of these vesicles.

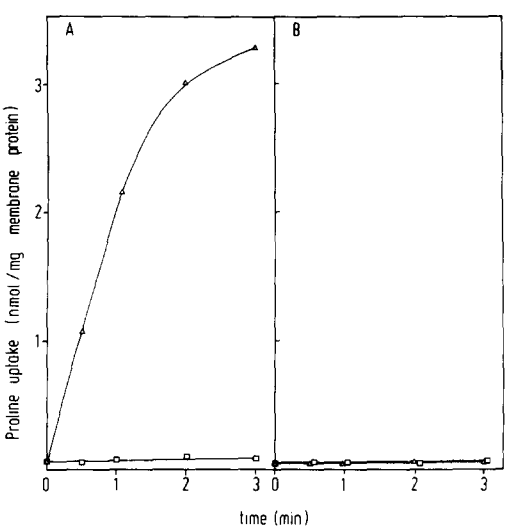

Fig. 1. Uptake of [^{14}C]proline by *E. coli* W 3110 membrane vesicles made by different procedures. A. Vesicles were prepared by lysis of EDTA-lysozyme spheroplasts in a French press. B. Vesicles were prepared by lysis of whole cells in a French press according to Rosen and McClees [12]. △———△, in the presence of ascorbate (10 mM) plus phenazine methosulfate (0.1 mM); □———□, no energy source added.

In our experiments however, French press vesicles are prefectly capable of proline uptake, as shown in Fig. 1A. The activity of these vesicles is as high as that reported for osmotic shock vesicles, for which proline uptake rates varying from 1 to 1.3 nmol/min per mg membrane protein have been found [14—16]. The French press vesicles described here must therefore have, at least partly, a right-side out orientation. The fact that we did observe proline transport in French press vesicles while other investigators did not, may be the result of one of the following differences in the vesicle preparation procedure. First, we lysed EDTA-lysozyme spheroplasts [2] rather than whole cells; when vesicles were obtained by lysis of whole cells, as described by Rosen and McClees [12], there was no stimulation of the proline uptake above the nonenergized level (Fig. 1B). Second, during the lysis step the spheroplasts were suspended in a buffer that contained 20% sucrose, the presence of which improved the proline uptake activity of the resulting vesicles by a factor of 1.5—2.5, compared to vesicles that were lysed in the absence of sucrose (data not shown). Third, Hertzberg and Hinkle [10] and Rosen and McClees [12] used much higher centrifugal forces (10 min at 27 000—39 000 × *g* as opposed to 10 min at 5000 × *g*) for clearing the lysate of unbroken cells. This procedure leaves only very small vesicles, which make up a minor part of the total membranes in the supernatant as illustrated by the very low yield of membranes in this procedure: only about 7.5 mg of membrane protein per g of cells (dry weight), as opposed to approximately 80 mg of membrane protein per g of cells (dry weight) in our procedure. It may be that these small vescles preferentially have an inside-out orientation.

Distribution of proline transport activity in a density gradient

When *E. coli* W 3110 spheroplasts are lysed at 0°C the resulting membranes show a considerable heterogeneity in their buoyant density [1]. This density variability is partly caused by the inhomogeneous distribution of membrane proteins in the plane of the membrane at the time of lysis [1]. This is due to a lipid phase separation which is induced by the low temperature (0°C) (see ref. 1) and high pressure (250—500 atm) * at which lysis occurs. The effect of such a lipid phase separation on the transport activity of proline was investigated. Fig. 2 shows the activity of proline transport as well as the activities of NADH oxidase and D-lactate dehydrogenase, since these two activities are associated with the normal cytoplasmic membrane and the low density membranes respectively [1]. High specific activities for proline transport are found in vesicle fractions that have considerably higher densities (fractions 12—16, with an average density of 1.20 g/ml) than the normal cytoplasmic membranes (fractions 9—11, which have an average density of 1.17 g/ml). The transport rates found for the higher density vesicles are similar to or exceed the rates observed for osmotic shock vesicles (14—16).

The same experiment was repeated using membrane vesicles isolated at 37°C, which temperature is high enough to prevent the occurrence of phase separa-

* High pressure has a substantial influence on the transition temperature of phospholipids. It has been shown [25] that the transition temperature of phospholipids increases 20—22°C per 1000 atm, the exact value being only slightly dependent on chain length and phospholipid headgroup. As we have used pressures of 250—500 atm, an increase in transition temperature of 5—11°C can be expected.

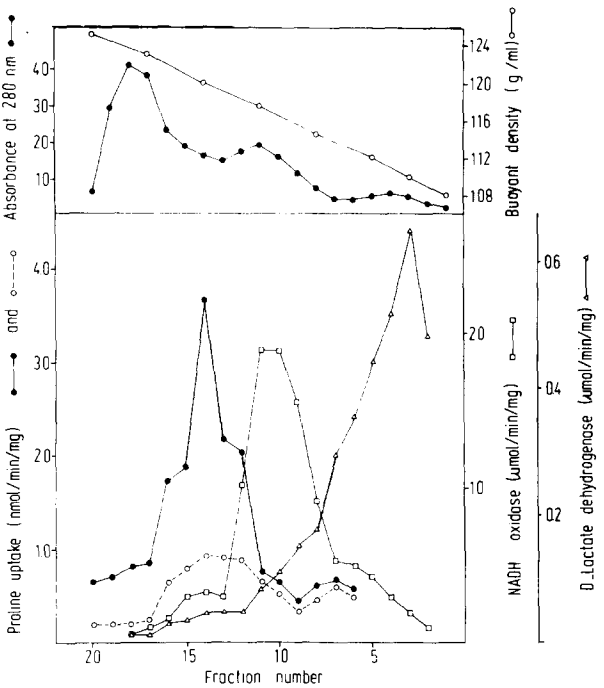

Fig. 2. Sucrose gradient centrifugation of total membranes of *E. coli* W 3110 isolated at 0—4°C and the distribution in the gradient of the specific activities of NADH oxidase, D-lactate dehydrogenase and the uptake of [^{14}C]proline. Total membranes were isolated from 3 g of cells. Isopycnic centrifugation was carried out in the SW 25.2 rotor for 63 h at 25 000 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 0.1 M K-phosphate buffer (pH 6.6) the membranes were collected by centrifugation (2 h, 176 000 × *g*) and resuspended in the same buffer. Determination of protein and assay of enzyme and uptake activities were carried out as described in Materials and Methods. Upper panel: absorbance at 280 nm (●———●) and buoyant density (○———○). Lower panel: NADH oxidase (□———□) and D-lactate dehydrogenase (△———△) expressed as μmol/min per mg protein, [^{14}C]proline uptake expressed as nmol/min per mg protein, energized by ascorbate (10 mM) plus phenazine methosulfate (0.1 mM) (●———●) or by D-lactate (20 mM) (○ - - - - - ○).

tions due to the high lysis pressure (ref. 1, see also footnote 1). Fig. 3 shows that the D-lactate dehydrogenase activity shifted towards the normal cytoplasmic membrane as was to be expected in the absence of a phase separation. Membrane fractions with highest proline uptake are still found in the same density region of the gradient however, indicating that the presence of the proline transport components in relatively dense membranes cannot be due to a phase separation. Instead, the higher density of vesicles with maximal proline uptake activity is probably due to the fact that they consisted of concentric outer- and cytoplasmic membrane, as indicated by the following lines of evidence.

First, sodium dodecyl sulphate polyacrylamide gel electrophoresis shows that these fractions contained outer membrane as well as cytoplasmic membrane proteins. This can be seen in Fig. 5 by comparing fractions 12—16 (which showed the highest proline uptake activities) to fractions 9—11 (cytoplasmic membrane) and fractions 17—20 (outer membrane). Although the data of Fig. 5 were not quantitated, it is clear that outer membrane proteins accounted for

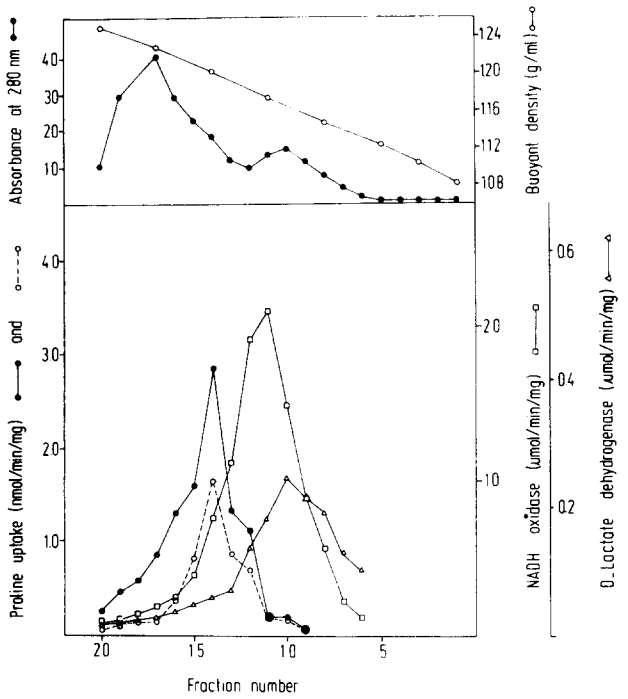

Fig. 3. Sucrose gradient centrifugation of total membranes of *E. coli* W 3110 isolated at 37°C and the distribution in the gradient of NADH oxidase, D-lactate dehydrogenase and the uptake of proline. For further details and explanation of symbols see legend to Fig. 2.

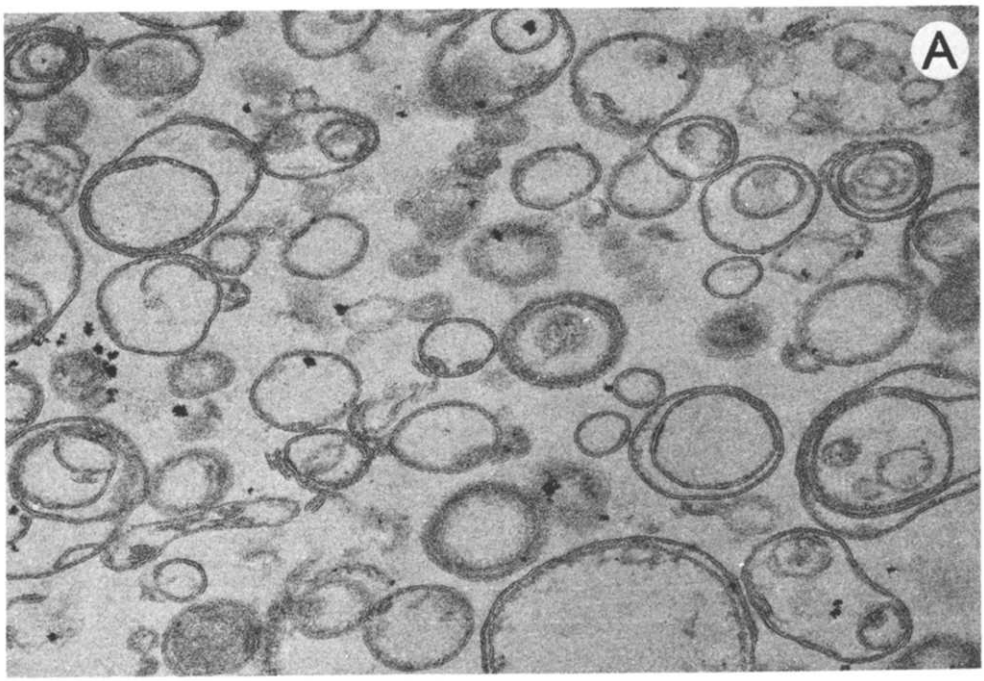

Fig. 4A.

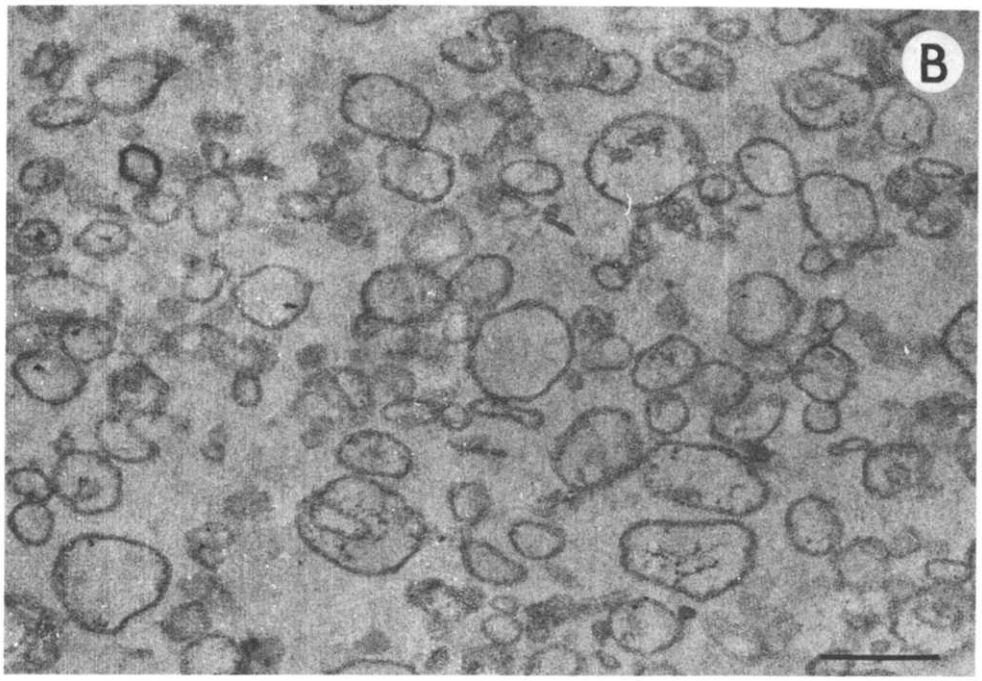

Fig. 4. Thin section electron micrograph of *E. coli* W 3110 membrane fractions of different buoyant densities. A. Subfraction having a density of about 1.20 g/ml (i.e. a fraction analogous to fraction 14 of Fig. 2). B. Subfraction having a density of about 1.15 g/ml (i.e. a fraction analogous to fraction 9 of Fig. 2). The bar represents 0.2 μm.

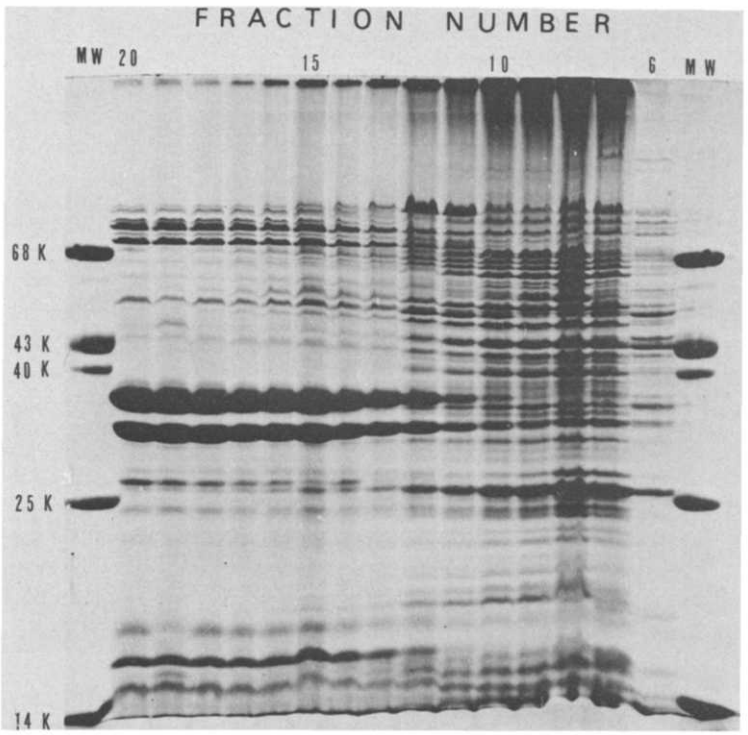

Fig. 5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of *E. coli* W 3110 membrane proteins after sucrose gradient centrifugation of total membranes isolated at 37°C (see legend to Figs. 2 and 3).

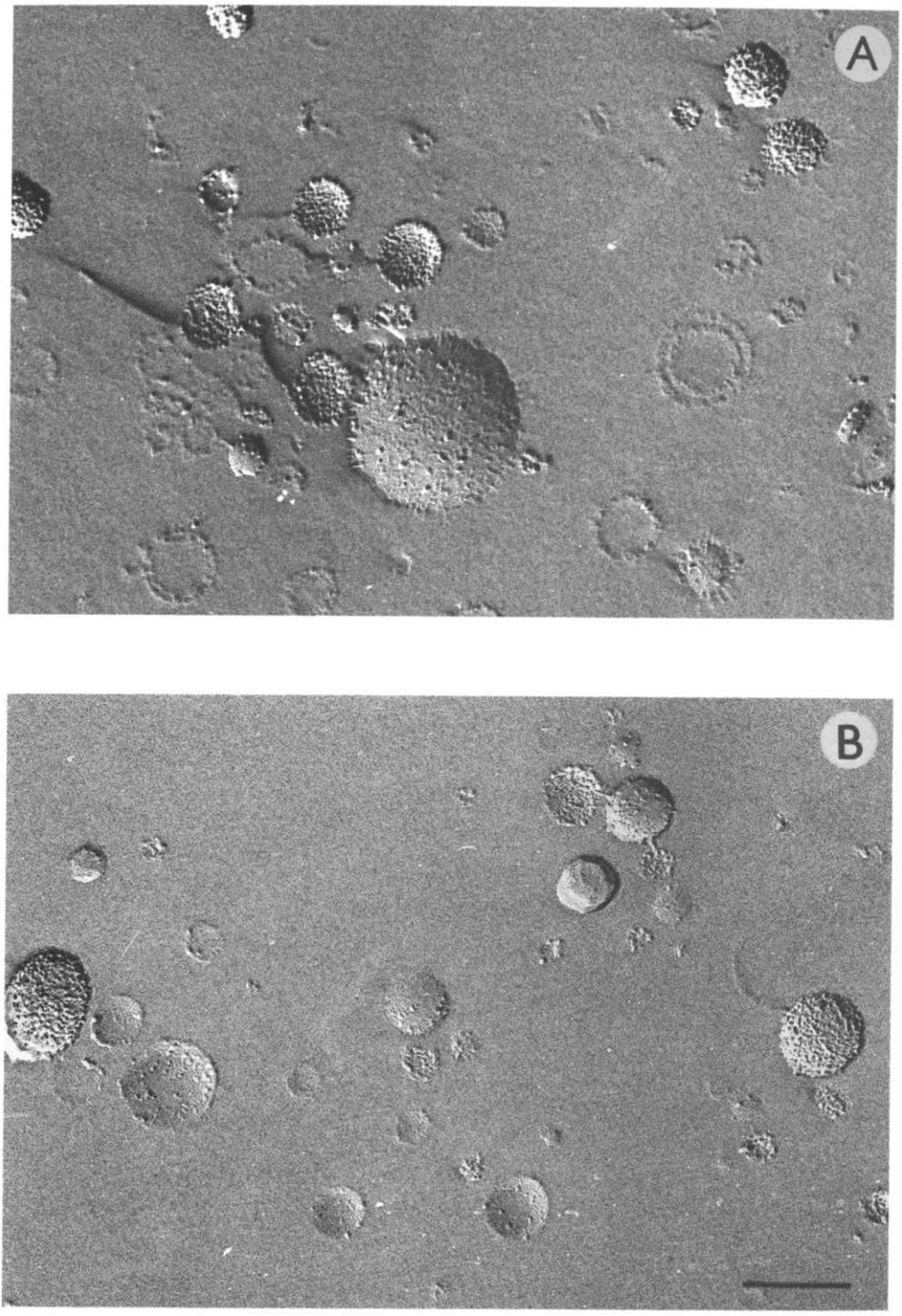

Fig. 6. Freeze-fracture micrographs of *E. coli* W 3110 membrane fractions of different buoyant densities. A. Subfraction with a density of about 1.20 g/ml. B. Subfraction with a density of about 1.15 g/ml. The bar represents 0.2 μm.

at least 50%, and possibly as much as 80% of the total protein in fractions 14—16. Second, it is likely that each individual vesicle of fractions 12—16 contained both outer membrane and cytoplasmic membrane, because the density of these vesicles was intermediate between that of pure outer membrane and pure cytoplasmic membrane. Finally, as illustrated in Fig. 4A, 70—80% of these vesicles (fraction 14) consisted of two or more concentric membranes. In contrast, normal-density cytoplasmic membranes (fraction 9) consisted almost solely of single walled vesicles (Fig. 4B); only a very low percentage of double membranes was counted. Whether the 20—30% single walled vesicles in the high density fraction (Fig. 4A) consist of only outer membrane with a deviating density, or of fused inner and outer membranes, cannot be determined.

The possibility that a significant difference in membrane orientation between the mixed concentric vesicles and the single walled cytoplasmic membrane vesicles could be the reason for the large differences in proline uptake by these two fractions was tested for by freeze fracture electron microscopy (see Fig. 6). This technique has already been used successfully for the determination of vesicle sidedness by Altendorf and Staehelin [17]. Briefly, it makes use of the fact that the inner fracture face (normally convex) of the cytoplasmic membrane is highly particulated, whereas the outer fracture face (normally concave) is only sparsely particulated. Examination of over 400 vesicles of each type indicated that 63% of the vesicles of the mixed membrane fraction and 46% of the cytoplasmic membrane vesicles had a right-side out orientation.

Discussion

Membrane vesicles prepared by French press treatment of *E. coli* spheroplasts appear to be very heterogeneous, according to structure as well as to function.

Heterogeneity of vesicle structure and composition

Vesicles isolated from the cytoplasmic membrane of *E. coli* might be heterogeneous with respect to structure and composition due to several causes, including phospholipid phase separation, the presence of variable amounts of outer membrane fragments and other non-cytoplasmic membrane components, and intrinsic in vivo heterogeneity of the cytoplasmic membrane.

Phase separations, which may occur when membranes are isolated at 0°C and at high pressures *, cause membrane proteins to aggregate because phospholipids with a high transition temperature crystallize into solid patches from which most of the intrinsic membrane proteins are excluded [1,18]. Since in these experiments phase separations no longer occur at 37°C [1], even when pressures of 250—500 atm are used *, membrane vesicles isolated at 37°C should be more homogeneous, as was in fact, found. The low density fraction observed in membranes isolated at 0°C, which represents the bare phospholipid patches in the cytoplasmic membrane after phase separation [1], was absent when membranes were isolated at 37°C. Similarly, D-lactate dehydrogenase, one of the proteins associated with the patches, shifted to the main cytoplasmic membrane fraction when membranes were isolated at 37°C, confirming the view that D-lactate dehydrogenase is concentrated in the low den-

sity fraction because of the phase separation.

Nevertheless, although the separate low density fraction was no longer present when membranes were isolated at 37°C, the cytoplasmic membrane fraction remained heterogeneous with respect to both density and function. This heterogeneity is real and is not the result of gradient overloading or diffusion in the gradient, because isolated subfractions of specific densities have been shown to reequilibrate at their respective original densities upon recentrifugation (de Leij, L. and Witholt, B., to be published). One of the reasons for this heterogeneity is that, in addition to vesicles of outer membrane only (with high buoyant density), vesicles containing outer and inner membrane in different ratios (and thus of intermediate buoyant densities) were also obtained during membrane isolation [19].

In addition, although at physiological temperature the membrane lipids are in the liquid crystalline state [20], all membrane constituents need not necessarily be distributed homogeneously. The cytoplasmic and outer membrane could be intrinsically heterogeneous due to the possible anchoring of the cytoplasmic membrane proteins to outer membrane constituents at the adhesion sites [21], due to the anchoring of cytoplasmic membrane proteins to cytoplasmic factors, or due to local variations in the phospholipid, protein, and (in the case of outer membrane) lipolysaccharide content of different membrane regions [22]. It does not appear however, that there are obvious differences in the detailed protein composition of different subfractions of the same membrane; those differences which were observed (Fig. 5) may be explained by varying amounts of cross-contamination between the outer and the inner membranes.

Heterogeneity of vesicle function

The results of Figs. 2 and 3 showed that the proline transport system which was assayed using ascorbate plus phenazine methosulfate as an artificial energy donor, was associated neither with the D-lactate dehydrogenase-rich fraction, nor with the main cytoplasmic membrane fraction. Instead, proline uptake was maximal in the mixed vesicle region; this was true independently of whether there was a phase separation or not.

As expected, when D-lactate was used as the energy source, proline uptake depended on the abundance of D-lactate dehydrogenase in a particular membrane fraction. Figs. 2 and 3 show that D-lactate dehydrogenase was apparently not limiting in most of the cytoplasmic membrane, so that D-lactate was nearly as effective as ascorbate plus phenazine methosulfate in these fractions. In the mixed vesicles however, the specific activity of D-lactate dehydrogenase was low, and D-lactate was less effective than ascorbate plus phenazine methosulfate in energizing proline uptake.

The fractions in which D-lactate oxidation was rate-limiting for proline transport are particularly interesting. Based on the data of Fig. 2, it can be calculated that the ratio of D-lactate oxidation to proline transport (energized by D-lactate) in fractions 12—16 (Fig. 2) is about 40. If only the vesicles which contribute to proline uptake are considered, this ratio is still lower. About 40% of the vesicles in these fractions are inverted and hence do not transport proline, while these same vesicles oxidize D-lactate more efficiently than do the

right-side-out vesicles [23]. Thus, right-side-out vesicles can oxidize at most 24 nmol of D-lactate for each nmol of proline taken up. It appears therefore that these vesicles are considerably more efficient than osmotic shock vesicles with respect to their energization of proline transport, since osmotic shock vesicles oxidize about 100 nmol of D-lactate per nmol of proline taken up [14].

The effect of the outer membrane on active transport by membrane vesicles obtained by lysis in a French press

Active transport systems of gram-negative bacteria are located in the cytoplasmic membrane [13,24]. The results of this paper show however, that the specific uptake of proline was from 5 to 8 times lower in the cytoplasmic membrane fraction compared to the mixed membrane fraction; expressed in terms of cytoplasmic membrane protein only, the specific uptake of proline in the cytoplasmic membrane fraction was about 10 to 16 times lower than in the mixed membrane fraction, since at least 50% of the protein in the mixed membrane vesicles was outer membrane protein.

This difference cannot be explained by the slight difference in the fraction of inverted vesicles between mixed vesicles and pure cytoplasmic membrane vesicles. A more likely explanation is the following. When spheroplasts are extruded from the French press they explode; this explosion may damage the cytoplasmic membrane in one or more unspecified ways such that it is no longer capable of full uptake activity. Such postulated damage may be lower when adjoining outer and inner membrane fragments remain together as they vesicularize. It is interesting that uptake rates for proline in vesicles prepared by osmotic shock [14—16] are of the same magnitude as the uptake rates in mixed membrane fractions; the uptake in cytoplasmic membrane fractions of French press vesicles is much lower. This may be either because the lysis of spheroplasts by osmotic shock is much less violent than the explosion of spheroplasts in a French press, and therefore less harmful to the uptake activity, or it may be because many of the vesicles obtained after osmotic shock also have two concentric membranes, as is the case for the mixed fraction of the French press vesicles.

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