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LOCALIZATION OF PROTEINS IN THE INNER AND OUTER MEMBRANES OF *CAULOBACTER CRESCENTUS*

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Cytoplasmic and outer membranes of *Caulobacter crescentus* were separated by isopycnic sucrose gradient centrifugation into two peaks with buoyant densities 1.22 and 1.14 g/cm^3. These peaks were identified as outer and cytoplasmic membranes by the enrichment of malate dehydrogenase and NADH oxidase in the lower density peak and the presence of flagellin, a cell surface protein, in the heavier peak. The identity of the heavier peak as outer membrane was confirmed by labeling of cells with diazotized [^{35}S]sulfanilic acid, a reagent that does not penetrate intact cells. Under these conditions only outer membrane proteins were substituted by the sulfanilic acid. The distribution of proteins between the cytoplasmic and outer membranes were examined by the analysis of [^{35}S]methionine-labeled membranes by SDS-polyacrylamide and two-dimensional gel electrophoresis. These results showed that the inner and outer membranes contain approximately equal numbers of proteins, and that the distribution of these proteins between the two layers is highly asymmetric. Although many of the proteins could be assigned to one or the other membrane fraction, a number of the outer membrane proteins in the 32000–100000 molecular weight range frequently contaminate the inner membrane fractions. The implications of these results for membrane isolation and separation in *C. crescentus* are discussed.

Introduction

The cell envelope interacts directly with the external medium and it is responsible for maintaining the internal environment within the cell. In *Caulobacter crescentus*, the envelope is also the site of structural changes that occur periodically throughout the cell cycle (for review, see Refs. 1 and 2): examples of these changes are formation of the stalk, pili, and flagellum. All of these structures are assembled at only one of the cell poles, and this structural asymmetry leads to the production of two different cell types at division, the flagellated swarmer cell and the nonmotile stalked cell. Each cell then follows a characteristic program of chromosome replication [3] and protein synthesis [4]. Since the cell envelope is directly involved in stalk formation and events required for spatial localization of other surface structures in *C. crescentus*, the membrane composition of these cells is of particular interest.

Separation of the inner and outer membrane has been reported for *C. crescentus* [5–7], and one of these reports [5] suggested that many of the major membrane proteins were present in both membrane fractions. Because of the limited resolution of one-dimensional gel analysis used in these earlier studies, we have examined the distribution of proteins between inner and outer membranes in *C. crescentus* using a two-dimensional system which separates proteins on the basis of both size and isoelectric point. We also report the use of di-

azosulfanilic acid as a relatively specific reagent to label outer membrane proteins of intact cells. These results show a high degree of asymmetry in the localization of membrane proteins in *C. crescentus,* with the majority of these proteins localized in either the inner or the outer membrane.

Materials and Methods

Strains and culture conditions. Caulobacter crescentus strain CB15 (ATCC 19089) was routinely grown in minimal salts medium (M3; Ref. 1) containing 0.2% glucose as a carbon source. Exponentially growing cells were used for all experiments.

Labeling. Exponentially-growing cultures (usually $3 \cdot 10^8$ cells/ml) were labeled by the addition of 10–50 μCi/ml of [^{35}S]methionine (40–50 Ci/mmol); when necessary, carrier methionine (0.1 μg/ml) was added to insure linear incorporation of the precursor during the labeling period.

Membrane preparation. Inner and outer membranes were prepared by the method of Osborn et al. [8] except that the concentrations of lysozyme and EDTA required for satisfactory lysis of the cells were higher than those used for *Salmonella.* Cells at a final concentration of $3 \cdot 10^{10}$ cells/ml were suspended in a freshly prepared lysis buffer that contained lysozyme (250 μg/ml), 0.75 M sucrose and 10 mM Tris, pH 7.8. The suspension was incubated at 4°C for 2 min and then slowly diluted over a period of about 10 min in 2 vol. of 10 mM EDTA, pH 7.5. Formation of spheroplasts and cell disruption were monitored by phase contrast microscopy. Under the conditions used (see above; Ref. 8) spheroplasts were formed from all cells. Spheroplasts were lysed by sonic disruption for 15 s which resulted in approximately 95% lysis of the spheroplasts. Adequate lysis was not obtained by dilution of the spheroplasts in distilled water, and this procedure also resulted in very poor membrane separation. We have observed that the quality of distilled water used for preparing reagent solutions was critical for spheroplasting and lysis to occur properly; only doubly quartz distilled water gave satisfactory results. Membranes were collected by centrifugation and then centrifuged to equilibrium in sucrose gradients as described by Osborn et al. [8].

Membrane recovery. Total membranes recovered represented about 9–10% of the [^{35}S]methionine incorporated into cellular protein. Recovery of membranes after equilibrium sucrose centrifugation was calculated to estimate the overall efficiency of membrane separation. $8.7 \cdot 10^6$ cpm were applied to one gradient (Fig. 1) and $8.1 \cdot 10^6$ cpm, or approx. 93%, was recovered in the high and low density fractions. Thus, membranes from swarmer, stalked and dividing cells apparently separate into inner and outer membrane.

Sulfanilic acid labeling. Diazotized sulfanilic acid, a surface labeling reagent, was prepared immediately before use from [^{35}S]sulfanilic acid (Amersham) by a modification of the method of Berg [9]. [^{35}S]Sulfanilic acid (0.5 mCi) was dried under nitrogen and 5 μl of 1 M HCl and 5 μl of 1 M $NaNO_2$ were added at 4°C. After 20 min the diazotized sulfanilic acid was neutralized by the addition of 5 μl of 1 M NaOH and the volume was brought to 0.25 ml with 0.1 M phosphate buffer, pH 7.5.

Procedure A was used when the proteins from total membranes were to be examined by gel electrophoresis. Exponential cells were suspended in 0.1 M sodium phosphate buffer, pH 7.5 at 10^9 cells/ml and 10–100 μCi of diazo[^{35}S]sulfanilic acid reagent was added. The reaction was allowed to proceed for 10–20 min at 4°C and stopped by the addition of an equal volume of 10% trichloroacetic acid plus 50 μg/ml sodium deoxycholate. After 1–2 h, precipitates were collected by centrifugation at $10000 \times g$, for 10 min and were washed four times with 5% trichloroacetic acid and once with water. The extent of incorporation was dependent on cell concentration and was linear with time when either procedure was used.

Procedure B was used when inner and outer membranes were to be isolated. Growing cells were labeled in M3 medium, pH 7.5. Diazo-[^{35}S]sulfanilic acid (50–250 μCi) was added to 2 ml of cells ($3 \cdot 10^8$ cells/ml) and incubated for 20 min. The cells were collected by centrifugation at $10000 \times g$ for 10 min and the membrane isolated as described above. Predominantly outer membrane proteins are labeled under these conditions (see Results). It was also shown that soluble proteins of *C. crescentus* are labeled by the ^{35}S reagent only when the cells were broken by sonic disruption before carrying out the reaction (data not

shown). Thus, the cells are not freely permeable to diazotized sulfanilic acid.

Enzyme assays. NADH oxidase (EC 1.6.99.2) was measured by decrease in absorbance at 340 nm. Incubation mixtures contained 0.1 mM NADH, 0.2 mM dithiothreitol in 50 mM Tris, pH 7.5 [8]. The reaction was started by the addition of 50–500 μg of membrane protein.

Malate dehydrogenase (EC 1.1.1.37) was assayed by measuring decrease of absorbance at 340 nm using NADH and oxaloacetate as substrates [10]. 0.1 M sodium phosphate buffer (pH 7.4) was used. Inner and outer membranes were assayed for activity immediately after collection of the gradient fractions.

Radioimmune precipitation. The radioimmune precipitation assay used was described by Sheffery and Newton [11]. Flagellin antibody was the gift of Michael Sheffery.

Gel electrophoresis. Electrophoresis on polyacrylamide gels was performed as described by Laemmli [12] and two-dimensional gel electrophoresis was carried out by the method of O'Farrell [13]. Gels were fixed in 10% acetic acid–50% methanol followed by incubation in 10% acetic acid–5% methanol. They were then prepared for fluorography by incubation in two washes of dimethyl sulfoxide (DMSO) followed by incubation in 20% 2,5-diphenyloxazole (PPO) in DMSO for 3 h. The gels were finally washed in water for several hours before drying onto Dhalnaur filter paper, dried and exposed to Kodak X-OMAT film that had been prefogged [14]. Individual proteins analyzed by one- and two-dimensional electrophoresis are referred to by their molecular weights and position in the electrofocusing dimension.

Results

Separation and characterization of inner and outer membranes

Membranes were isolated from exponentially growing cells of *C. crescentus* that had been labeled for 10 minutes with [^{35}S]-methionine and the inner and outer membranes were separated by equilibrium centrifugation on a discontinuous sucrose gradient (Fig. 1). The two major peaks correspond to buoyant densities of 1.22 g/cc and 1.14 g/cc. Densities of 1.22 and 1.19 g/cc have been reported previously for the inner and outer membranes of *C. crescentus* strain CB13 [6]. The higher density for the inner membrane could result from some contamination by outer membrane (see Discussion) or a difference between strain CB13 and the strain CB15 used in the present studies.

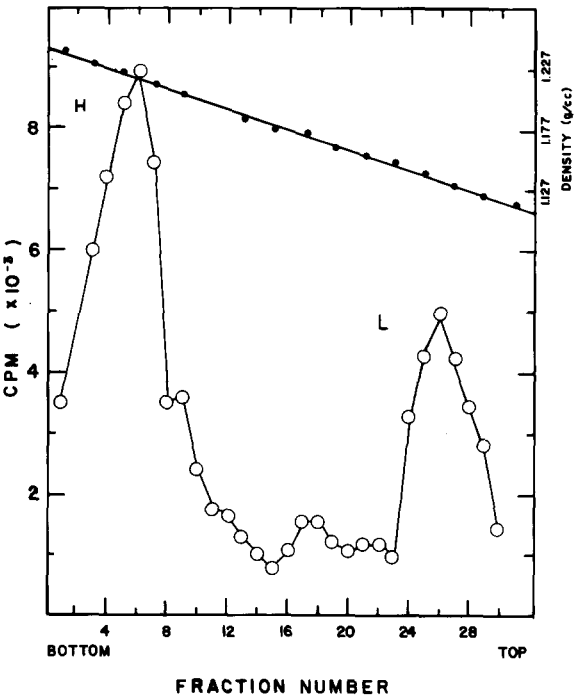

Fig. 1. Separation of inner and outer membranes of *C. crescentus*. Exponentially growing cells (5-ml culture, $A_{650} = 0.3$) were labeled with 250 μCi of carrier free [^{35}S]methionine (40–80 Ci/mmol) for 10 min, chilled and immediately harvested by centrifugation. Inner and outer membranes were prepared from these preparations by centrifugation on a 30% to 50% sucrose gradient as described (Ref. 8; Materials and Methods). Radioactivity in samples from gradient fractions was determined by counting in liquid scintillation fluid (○) and buoyant densities in fractions were determined by the refractive index (●). 'H' refers to the heavy peak (outer membrane) and 'L' to the light (inner membrane) peak.

The material banding in the heavier (H) peak was identified as outer membrane and the material in the lighter (L) peak was identified as inner membrane from the distribution of marker enzymes and surface proteins in the two peaks (Table 1 and Fig. 2). NADH oxidase, an enzyme characteristic of the inner membrane, was enriched in the lighter member fractions; a significant but

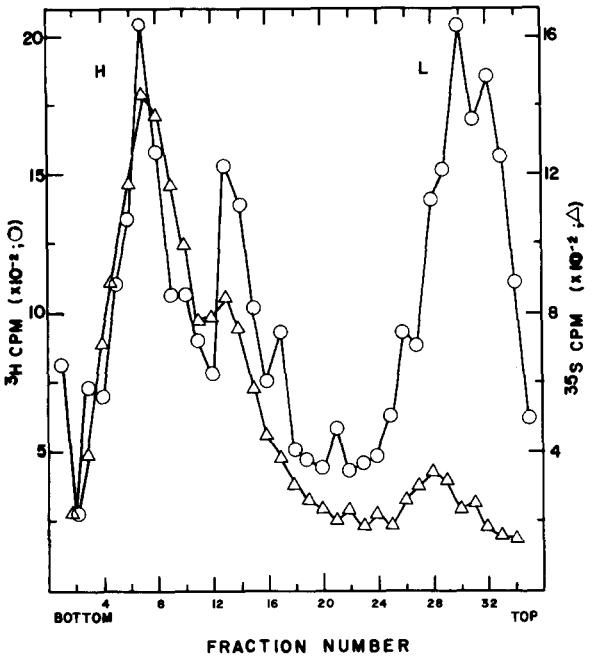

Fig. 2. Substitution of whole cells and outer membranes with diazo[^{35}S]sulfanilic acid. Exponentially growing *C. crescentus* cells (A_{650} = 0.250) were labeled with diazotized [^{35}S]sulfanilic acid by Procedure B (Materials and Methods). The ^{35}S-labeled cells were then harvested and the inner and outer membranes were immediately prepared as described in Fig. 1 (△). Inner and outer membranes were also prepared from a duplicate 5-ml culture of cells that had been labeled with [^{3}H]leucine (50 μCi) and sedimented to equilibrium on a parallel sucrose gradient (○).

much smaller fraction of the activity was associated with the outer membrane (Table I). We also examined the distribution of malic dehydrogenase in *C. crescentus* cells. Although only about 10% of the total activity was bound to membranes after extensive washing (data not shown), the bulk of this activity sedimented with the inner membrane. The observed specific activities were generally lower than those in *Salmonella typhimurium* [8], and consequently we were unable to use lactate dehydrogenase as a marker enzyme in these studies.

The identification of the L and H peaks as inner and outer membranes was confirmed by labeling the whole cells with diazo[^{35}S]sulfanilic acid [9]. This reagent, used previously to label the surface proteins of red blood cells, does not penetrate the envelope of *C. crescentus* cells (see Materials and Methods), and we have used it as a probe to examine the exposed surface proteins of the outer membrane. Exponentially growing cells of strain CB15 were labeled with either [^{3}H]leucine or diazo[^{35}S]sulfanilic acid. Membranes were prepared from both samples and centrifuged through sucrose density gradients as described in Fig. 1. The ^{3}H-labeled membrane separated into a characteristic low density and high density peak plus a minor peak (fractions 12–16) of intermediate density that may represent unresolved inner and outer membrane (Fig. 2). The ^{35}S-label from the membranes of intact cells substituted with the sulfanilic acid reagent sedimented almost exclusively with the higher density fractions, suggesting that these fractions do indeed represent separated outer membrane.

The lack of label at the density corresponding to inner membrane was not due to the absence of reactive sites on inner membrane proteins. Membranes were separated by sucrose density centrifugation, collected into H and L fractions and then reacted with diazo[^{35}S]sulfanilic acid in vitro.

TABLE I

DISTRIBUTION OF ENZYMES AND FLAGELLIN BETWEEN INNER AND OUTER MEMBRANE FRACTIONS

Enzyme or protein	Inner membrane		Outer membrane	
	% of total activity or protein	Spec. act.	% of total activity or protein	Spec. act.
NADH oxidase [a]	80	36	20	9.2
Malate dehydrogenase [a]	97	580	3	4.6
Flagellin [b]	13		87	

[a] Unlabeled inner and outer membrane were prepared for enzyme assays from approximately $3 \cdot 10^{10}$ cells as described in Fig. 1 by pooling fractions and centrifuging at 150000 × *g* for 6 h. Inner and outer membrane peaks on the sucrose gradient were located by measuring A_{280}.

[b] Inner and outer membrane fractions from the experiment shown in Fig. 1 were diluted directly into an equal volume of 50 mM Tris, pH 7.6, 2 M KCl and 2% Triton and X-100 and assayed by radioimmunoassay as described previously [21].

[c] Specific activity is defined as nmol NADH hydrolyzed per min per mg protein.

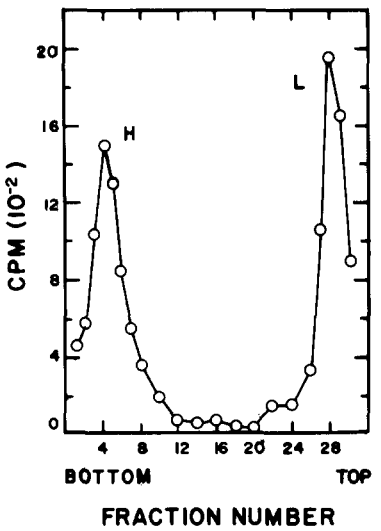

Fig. 3. Substitution of isolated inner and outer membranes with diazo[^{35}S]sulfanilic acid. Unlabeled inner and outer membranes were prepared from 20 ml of exponentially-growing CB15 ($A_{650}=0.445$) and separated by isopycnic sucrose gradient centrifugation. The outer and inner membrane peaks located by A_{280} were pooled, repelleted by centrifugation, and suspended in 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.5) for reaction with 50 μCi of freshly prepared diazo[^{35}S]sulfanilic acid. Equal numbers of acid precipitable counts from the inner and outer membrane fractions were loaded onto a discontinuous sucrose gradient and sedimented to equilibrium. Radioactivity in acid precipitable counts was determined after extensive washing in 5% trichloroacetic acid and ethanol.

When the membranes were sedimented to equilibrium on sucrose gradients (Fig. 3) the ^{35}S-label was associated with membranes in both the H and L peaks. Thus inner membrane and outer membrane proteins will react with diazo[^{35}S]sulfanilic acid when they are exposed to the reagent (Fig. 3 and data not shown).

Membrane fractions were also examined for the presence of flagellins A and B. Radioimmunoassay showed that the proteins were present almost exclusively in the outer membrane fractions (Table I); these proteins were also present in autoradiograms of outer membrane proteins separated by two-dimensional gel electrophoresis (see below, Fig. 5). Although flagellins A and B should not be considered intrinsic outer membrane proteins, their presence in the heavy membrane fractions is consistent with the surface localization of the flagellar filament in these cells.

One-dimensional analysis of inner and outer membrane proteins

Exponentially growing cells of *C. crescentus* were labeled for 10 min with [^{35}S]methionine and the inner and outer membranes isolated as in Fig. 1. Fractions from the two peaks were pooled, concentrated by centrifugation at $105000 \times g$ for 6 h and then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Fig. 4 compares the protein patterns in total membrane (lanes G–I), inner membrane (lanes A–C) and outer membrane (lanes D–F). While the exact number of protein bands observed varied somewhat according to the resolution of the particular gel and the degree of exposure, approximately 30 bands could be routinely detected in the outer membrane and approximately 27 in the inner membrane profiles. The distribution of proteins between the two membrane layers was quite asymmetric; major protein bands observed at 121, 110, 55, 48, 36 and 33 kDa in the inner membrane preparation were absent or present in extremely low amounts in the outer membrane preparation (Fig. 4 D–F). Prominent inner membrane proteins were also observed at 63, 48, and 23 kDa, although bands at these positions were also present in the outer membrane in lesser amounts.

Major outer membrane proteins are detected at 93, 86, 74, 50, 32, and 17 kDa (Fig. 4, D–F). These same bands also appeared at much lower levels in the inner membrane lanes; in other inner membrane preparations, e.g., lanes L and M, these proteins are also present in reduced amounts in the L peak fractions. These results and cell surface labeling experiments discussed below suggest that the indicated bands (Fig. 4, D–F) represent outer membrane proteins and that these proteins contaminate the inner membrane to a variable degree, depending on the conditions of membrane preparation and separation. Little contamination of the outer membrane by most of the inner membrane proteins has been observed (see also next section).

We have also examined the membrane proteins of intact cells of *C. crescentus* that had been labeled with diazo[^{35}S]sulfanilic acid. The only heavily substituted bands corresponded to the major outer membrane proteins, i.e., 93, 86, 74, 50, and 17 kDa (Fig. 4, J–K). Although not all outer membrane

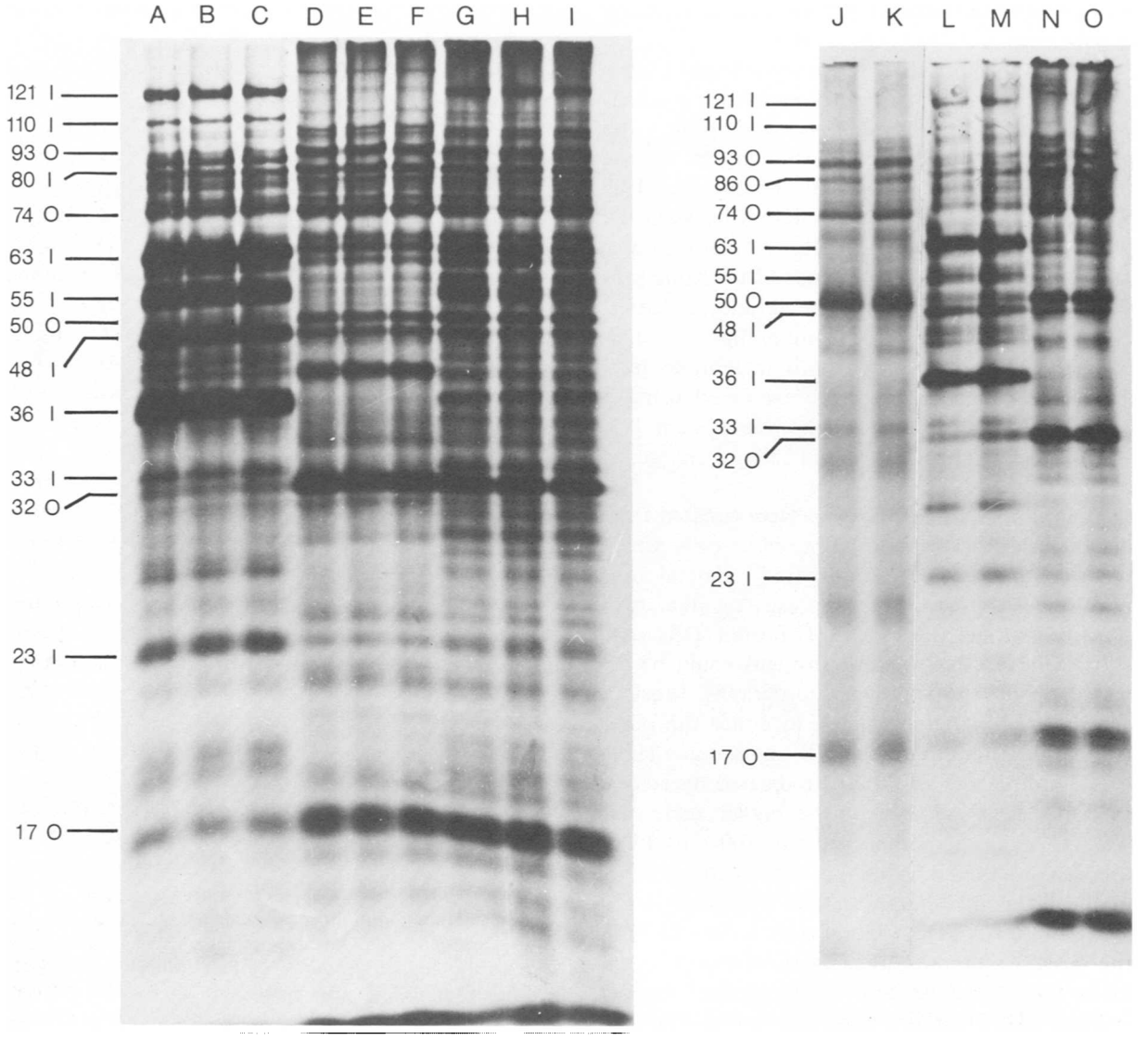

Fig. 4. Protein patterns of inner and outer membranes and sulfanated cell surfaces of *C. crescentus*. Inner (A–C) and outer (D–F) membranes from *C. crescentus* cells labeled with [^{35}S]methionine were separated as described in Fig. 1 and electrophoresed on a 12.5% polyacrylamide SDS gel [12]. Inner membranes (fractions Nos. 23–29; Fig. 1) and outer membranes (Nos. 2–10, Fig. 1) were diluted 5-fold in 10 mM Tris/10 mM EDTA buffer, pH 7.5, and collected by centrifugation at 105000×*g* for 6 h. These preparations were stored frozen or analyzed directly by electrophoresis. Unfractionated membranes (G–I) were analyzed in the same way. In a different experiment the surface proteins of *C. crescentus* were substituted by Procedure A (Materials and Methods) using 50 μCi of diazo-[^{35}S]sulfanilic acid and then fractionated on a 12.5% polyacrylamide gel (J and K). Inner (L and M) and outer (N and O) membrane preparations labeled in vivo with [^{35}S]methionine were run in the same gel for comparison. Approx. 75000 cpm were applied in samples to the gels; autoradiograms were prepared as described above (Materials and Methods). The molecular weights of major protein bands are given in kDa with designations indicating the assignment to the inner (I) or outer (O) membranes.

proteins were labeled, none of the major inner membrane proteins reacted with the reagent to a significant degree. Other experiments (see Materials and Methods; Fig. 3) show that the inner membrane proteins do react with the diazotized reagent after the cells are first disrupted.

Two-dimensional analysis of inner and outer membrane proteins

Despite the apparently good separation of inner and outer membrane by sucrose density gradient centrifugation, some protein bands appear to be present in both the heavy and light membrane fractions (see also Refs. 5–7 and Discussion). This could be due to cross-contamination between the two membranes, as proposed above for some outer membrane proteins, the presence of the same protein in both inner and outer membranes, or different proteins with the same mobilities on SDS-polyacrylamide gels in the two membrane fractions. These possibilities were examined using a two-dimensional gel electrophoresis system [13] which separates proteins on the basis of charge, as well as size.

Inner and outer membranes were isolated from exponentially growing *C. Crescentus* cells which had been labeled for 15 min with [^{35}S]methionine and analyzed by electrophoresis on the two-dimensional gel system of O'Farrell [13]. Approximately 140 membrane proteins could be detected on heavily exposed fluorographs. Most of the proteins can be assigned to either the outer (Fig. 5A) or the inner (Fig. 5B) membrane. However, as discussed above and indicated by arrows in panel B, a number of the higher molecular weight, outer membrane proteins (60–110 kDa) are also observed in lower, but significant amounts in the inner membrane fraction. Flagellins A (26 kDa) and B (28 kDa) were also detected mainly in the outer membrane preparation where they separate into three or four characteristic charged species [11]. Their positions (arrows in Fig. 5A) were determined by the mobility of purified flagellin in the same gel system.

The major inner membrane proteins were present at extremely low levels, if at all, in the outer membrane preparations. The relative positions of these proteins are indicated by circles on the outer membrane pattern (panel A). It is also apparent from Fig. 5 that many of the major bands observed by SDS-polyacrylamide gel electrophoresis actually represent multiple protein species with similar molecular weights. For example, only eight bands could be detected in the molecular weight range of 74000 to 32000 in the inner membrane by SDS gel analysis (Fig. 4, A–C); over 40 spots

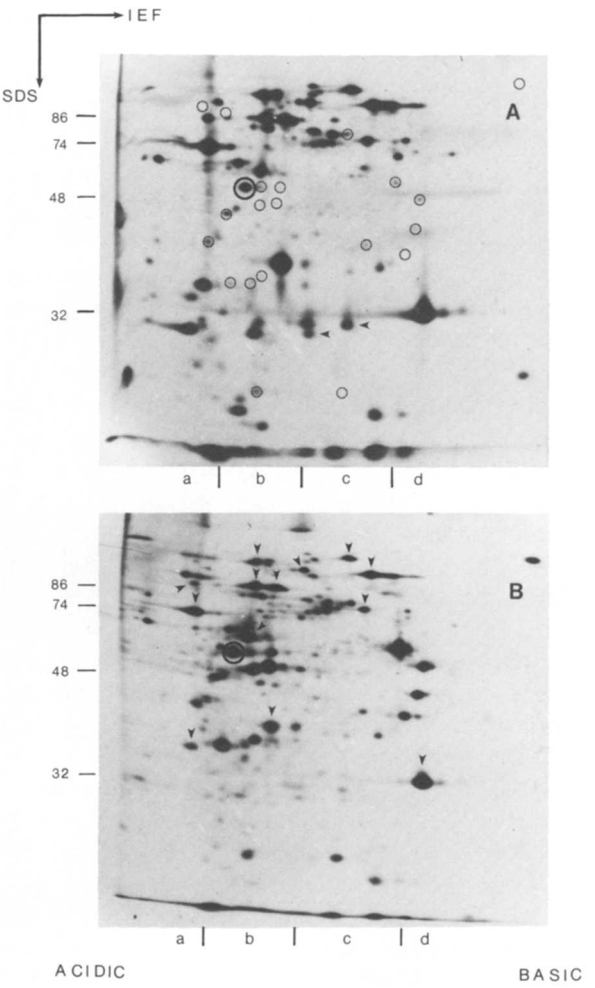

Fig. 5. Distribution of inner and outer membrane proteins on two-dimensional gels. Outer (A) and inner (B) membranes were prepared from *C. crescentus* cells labeled with [^{35}S]methionine as described in Fig. 4. The pellets were suspended in 0.15 ml lysis buffer by freezing and thawing several times and then vortexing. Inner and outer membranes ($1.5 \cdot 10^5$ cpm) were applied to the first (isoelectric focusing) dimension which was run for 14 h at 400 V. The second dimension was 10% SDS polyacrylamide gel electrophoresis [11]; the gels were equilibrated 2 h at room temperature in SDS buffer O prior to running. Basic and acidic ends are oriented as shown. The second dimension was 12.5% polyacrylamide and autoradiograms were prepared as described (Materials and Methods). Molecular weights indicated on the left side were calibrated using the mobilities of major membrane proteins (see Fig. 4). The positions of flagellin A (26 kDa) and of flagellin B (28 kDa) proteins are marked in panel A with arrows. Protein 55 kDa (see text) is marked with a heavy circle in panels A and B. Outer membrane proteins that tend to contaminate the inner membrane are indicated by arrows in panel B. The positions of the major inner membrane proteins are indicated by small circles on the outer membrane pattern (panel A).

could be detected in this range by two-dimensional analysis.

The protein at 55 kDa which is heavily circled in Fig. 5A and B is one exception to the generally asymmetric distribution of most major proteins. This protein has been observed at about the same level in inner and outer membranes in all preparations, regardless of the amount of contamination by the outer membrane proteins. With the exception of the protein at 55 kDa, then, our general conclusion is that the coincidence of protein bands from inner and outer membrane fractions by SDS-polyacrylamide gel electrophoresis is due to proteins of similar molecular weights in the two membrane layers of *C. crescentus* and to some contamination of the inner membrane by outer membrane proteins.

Discussion

The experiments reported here are the first detailed analysis of proteins from the cytoplasmic and outer membranes of *C. crescentus* by both SDS-polyacrylamide and two-dimensional gel electrophoresis. The results, which are based on the distribution of proteins labeled in vivo with [^{35}S]methionine and on the cell surface with diazo[^{35}S]sulfanilic acid, show a generally asymmetric localization of major proteins between the inner and outer membrane layers. They also suggest that contamination of inner membrane fractions with outer membrane proteins may be a significant problem in membrane separation and isolation in *C. crescentus*.

Conclusions about membrane protein localization in Gram-negative bacteria depend critically on the effectiveness of separating the two membrane layers. In *C. crescentus* a significant fraction of the NADH oxidase activity contaminates the high density or outer membrane fractions (Table I), and small amounts of several higher molecular weight outer membrane proteins are present in the low density or inner membrane fraction (Fig. 5A). These results are consistent with a variable, but limited cross-contamination between the membrane fractions, rather than membrane subfractionation as a result of either membrane heterogeneity or lipid phase separation, as reported in *Escherichia coli* [16]. Incomplete spheroplasting or disruption under high pressure can result in extensive cross-contamination between membrane fractions [16,18] and this may partially explain an earlier report that inner and outer membrane proteins are very similar in *C. crescentus* [5]. Several results indicate that the bulk of the membrane in the H and L fractions described in this paper (Fig. 1) represents outer and inner membrane, and not artifactually generated membrane vesicles: (a) cells were lysed under conditions in which membrane fusion does not occur to a significant extent (cf., Materials and Methods; Ref. 16), (b) continuous density gradients were established during membrane separation (Fig. 1; Refs. 8, 16) to give densities for the H and L peaks similar to those reported for membranes of *Salmonella* [8], *E. coli* [17] and other Gram-negative species [18–20], and (c) the inner membrane fractions, which contain the most of the NADH oxidase (Table I), represent approx. 35–40% of the membrane protein, while artifactually generated, protein-depleted vesicles with the same density would contain only a small fraction of the membrane protein [21]. The results of in vivo and surface labeling experiments discussed below are also consistent with the conclusion that inner and outer membranes are effectively separated into the H and L density fractions.

The major membrane proteins observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis at 121, 110, 93, 86, 74, 50, and 17 kDa (Fig. 4) apparently correspond to membrane proteins at 127, 113, 95, 88, 74, 47 and 17.5 kDa reported by Agabian et al. [7]. In agreement with this latter study, we assign the 121, 110 and 48 kDa proteins to the inner membrane; in addition, we can also assign the 63, 55, 36 and 23 kDa proteins to this membrane layer (Fig. 4). The two-dimensional cell analysis (Fig. 5) shows that these inner membrane proteins are found at extremely low levels in the outer membrane.

The outer membrane contains proteins at 93, 86, 50, 32 and 17 kDa. These designations are supported by the distribution of these proteins between the H and L membrane fractions (Figs. 4 and 5) and the surface labeling experiments. With the exception of the 32 kDa protein, all of the presumptive outer membrane proteins were substituted when intact cells were reacted with

[^{35}S]sulfanilic acid (Fig. 4, J and K). Some of the outer membrane proteins appeared to contaminate the inner membrane fractions to a limited extent while others did not (Fig. 5). This observation could reflect a differential organization of outer membrane proteins in which some proteins are more closely associated with, or have a higher affinity for, the inner membrane than others.

The outer membrane protein pattern of *C. crescentus* appears complex by one-dimensional analysis compared to that of other Gram-negative species [18–20]. About 30 bands are routinely seen on our gels of the outer membrane and seven to eight of the proteins account for approx. 70% of the outer membrane protein. Three of the major proteins have molecular weights over 70000. In *E. coli,* by contrast, only five proteins with molecular weights from 7000 to 50000 account for more than 90% of the outer membrane protein [22]. The short exposure times required for resolution of these major proteins in *E. coli* may then produce a relatively low estimate of the number of outer membrane proteins. In fact, approx. 50 total proteins in the *E. coli* outer membrane were detected by two-dimensional gel analysis [22], which is similar to the 60 to 70 proteins estimated for the *C. crescentus* outer membrane by the same techniques (Fig. 5).

The inner membrane protein pattern, on the other hand, is relatively simple in *C. crescentus*, with about 70 proteins detected by two-dimensional gel analysis. This number compares with 120 proteins in the corresponding *E. coli* membrane fraction [22]. This difference may also be more apparent than real since the presence of 8 to 10 major proteins in the inner membranes of *C. crescentus* (Fig. 5) necessitates relatively shorter exposures, even on two-dimensional gels (see above discussion and outer membrane proteins of *E. coli*). In comparing the present results with those published previously, it should be noted that the pulse labeling procedure described in these studies allows detection only of methionine-containing proteins that are rapidly incorporated into the membrane.

Surface labeling of intact cells with diazotised sulfanilic acid confirmed the identification of density fractions containing inner and outer membrane, and it also showed that there is no extensive mixing of proteins in the two membrane layers during the isolation procedure. Not all of the outer membrane proteins were substituted by the reagent, however. This may result from the limited availability of the active residues to the surface reagent, i.e., some proteins are 'buried', or the presence of few or no histidine and tyrosine residues in some of the proteins. One low molecular weight, outer membrane protein of 7 kDa is not substituted. This protein, which may correspond to the lipoprotein described by Braun [23] in *E. coli*, does not contain histidine (Weir, P. and Newton, A., unpublished data), which along with tyrosine reacts with diazosulfanilic acid. We have also failed to detect flagellin [24,25] and pilin [26] in sulfanilic acid substituted membranes. These surface proteins contain few histidine and tyrosine residues [24,26], and we have observed that purified flagellin reacts poorly with diazotized sulfanilic acid (unpublished data).

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