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Immunochemical Analysis of Membrane Vesicles from *Escherichia coli*†

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With the Technical Assistance of Helen Doherty

ABSTRACT: Membrane vesicles isolated from *Escherichia coli* ML 308-225 have been analyzed by crossed immunoelectrophoresis, and immunoprecipitates corresponding to the following cellular components have been identified: ATPase (EC 3.6.1.3), two or three NADH dehydrogenases (EC 1.6.99.3), D-lactate dehydrogenase (EC 1.1.1.27), glutamate dehydrogenase (EC 1.4.1.4), dihydro-orotate dehydrogenase (EC 1.3.3.1), 6-phosphogluconate dehydrogenase (EC 1.1.1.43), polynucleotide phosphorylase (EC 2.3.7.8), β-galactosidase (EC 3.2.1.23), lipopolysaccharide, and Braun's lipoprotein. The cellular origin of many of the vesicle immunogens is determined, and Braun's lipoprotein is used as a marker to quantitate the extent of outer membrane contamination (less than 3%). Membrane antigens are also characterized with regard to their amphiphilic or hydrophilic properties by charge-shift crossed immunoelectrophoresis. Furthermore, the following immunogens cross-react with components in membrane vesicles prepared from *Salmonella typhimurium*: one of the three NADH dehydrogenases, ATPase, polynucleotide phosphorylase, 6-phosphogluconate dehydrogenase, Braun's lipoprotein, and three unidentified antigens. In the accompanying paper [Owen, P., & Kaback, H. R. (1979) *Biochemistry 18* (following paper in this issue)] quantitative immunoadsorption is utilized to establish the topology of the vesicles with respect to the distribution of antigens on the inner and outer faces of the membrane.

Bacterial membrane vesicles have provided an increasingly important model system for the study of active transport (Kaback, 1972, 1974, 1976), and it is now apparent that they catalyze the accumulation of many solutes by a respiration-dependent mechanism in which chemiosmotic phenomena (Mitchell, 1961, 1966a,b, 1968, 1973) play a central, obligatory role (Schuldiner & Kaback, 1975; Kaback, 1976; Ramos et al., 1976; Ramos & Kaback, 1977a–c; Tokuda & Kaback, 1977). Although various lines of evidence indicate strongly that vesicles prepared by osmotic lysis (Kaback, 1971; Short et al., 1975) consist of topologically sealed plasma membranes with the same polarity as the intact cell (see Stroobant & Kaback, 1975, for a review), doubts have been expressed about the chemical nature and orientation of the preparations (Harold, 1972; Mitchell, 1973; Van Thienen & Postma, 1973; Hare et al., 1974; Futai, 1974; Weiner, 1974; Futai & Tanaka, 1975; Wickner, 1976; Adler & Rosen, 1977; Yamoto et al., 1978). Obviously, resolution of this controversy is of fundamental importance to the interpretation of results obtained from transport experiments conducted with this model system.

Recent studies demonstrate that crossed immunoelectrophoresis (CIE)[1] is an extremely powerful tool for the analysis of membrane immunogens (Johansson & Hjertén, 1974; Owen & Salton, 1975, 1977; McLaughlin & Meerovitch, 1975; Smyth et al., 1976, 1978; Alexander & Kenny, 1977, 1978). Not only does this two-dimensional immunoelectrophoretic technique resolve a spectrum of membrane components to a level approximating that of sodium dodecyl sulfate–polyacrylamide electrophoresis (Owen & Salton, 1975; Smyth et al., 1978) but membrane antigens analyzed by this method often retain sufficient biological activity to permit functional characterization (Owen & Smyth, 1977). Moreover, the quantitative aspects of the method (Weeke, 1973) allow the removal of antibodies during adsorption to be monitored with ease. Thus, expression of cell surface antigens as well as their distribution across the membrane can be established following adsorption of antiserum with whole cells, protoplasts, or isolated membranes (Johansson & Hjertén, 1974; Owen & Salton, 1975; Salton & Owen, 1976; Owen & Kaback, 1978).

Accordingly, we have undertaken a comprehensive immunochemical study of membrane vesicles prepared from *Escherichia coli* ML 308-225, and a preliminary report on some of the results has been presented (Owen & Kaback, 1978). In this paper, many of the antigens comprising the CIE reference pattern for solubilized ML 308-225 vesicles are characterized in detail with respect to their chemical nature, catalytic activity, amphiphilic or hydrophilic properties, and probable cellular origin. In addition, the cross-reactivity of certain ML 308-225 membrane immunogens with components in *Salmonella typhimurium* membrane vesicles is demonstrated.

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[1] Abbreviations used: CIE, crossed immunoelectrophoresis; $NaDodSO_4$ gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CTAB, cetyltrimethylammonium bromide; DOC, sodium deoxycholate; EDTA, ethylenediaminetetraacetic acid.

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Experimental Procedures

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) and ML 30 ($i^+z^+y^+a^+$) were grown on minimal medium A (Davis & Mingioli, 1950) containing 1.0% disodium succinate (hexahydrate) and membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975). Vesicles were suspended in 0.1 M potassium phosphate (pH 6.6), frozen in liquid nitrogen, and maintained at or below −70 °C.

Before use, suspensions of membrane vesicles were thawed rapidly at 45 °C and centrifuged at 30000*g* for 30 min at 4 °C. Vesicles prepared from *E. coli* ML 308-225 and ML 30 were then carefully resuspended in 0.1 M potassium phosphate (pH 6.6) to membrane protein concentrations of about 20 and 10 mg/mL, respectively.

Preparation of Other Cell Fractions. Cell envelopes were isolated following breakage of *E. coli* ML 308-225 in a French pressure cell. Washed cells were suspended in 10 mM sodium phosphate buffer (pH 7.0) containing DNase (40 μg/mL) to a final cell concentration of about 0.3 g wet weight per mL, and 98% disruption was achieved by a single passage through an Aminco French pressure cell at 6000 psi. Whole cells were removed by centrifugation (2600*g* for 15 min) and cell envelopes harvested at 30000*g* for 1 h at 4 °C. Envelopes were subsequently washed twice in 10 mM sodium phosphate (pH 7.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA).

Murein–lipoprotein was isolated from envelopes of *E. coli* ML 308-225 by virtue of its insolubility in boiling 4% sodium dodecyl sulfate (Braun & Sieglin, 1970).

The soluble products of cell lysis, which were recovered in the 30000*g* supernatant fraction obtained following centrifugation of French-pressed cells of *E. coli* ML 308-225, were recentrifuged (30000*g* for 1 h at 4 °C) to remove most of the residual membrane fragments. The supernatant fraction, representing primarily the cytoplasmic and periplasmic components of the cell, was retained.

Preparation of Purified Outer Membrane Components. Lipopolysaccharide was isolated from lyophilized envelopes by phenol–water extraction (Adams, 1972); 118 mg of purified lipopolysaccharide was obtained from 1.6 g of envelopes, and the protein content was less than 0.4%.

Lipoprotein was prepared from murein–lipoprotein by lysozyme digestion of the peptidoglycan followed by acetate precipitation (Braun & Wolff, 1970); 22 mg of lipoprotein was obtained from 411 mg of murein–lipoprotein. Lipoprotein prepared in this manner gave a single immunoprecipitin band when tested against anti-vesicle immunoglobulin by immunodiffusion, by immunoelectrophoresis, and by CIE. Analysis of purified lipoprotein by $NaDodSO_4$ gel electrophoresis combined with diffusion against anti-vesicle serum also revealed a single precipitin band corresponding to a component of apparent molecular weight about 15000. This component reacted specifically with antiserum to lipoprotein III (Braun et al., 1976). Lipopolysaccharide analyzed under similar conditions gave a single precipitin band against anti-vesicle immunoglobulin and had an apparent molecular weight of about 65000.

Rosenbusch matrix protein (Rosenbusch, 1974), also called protein I (Garten et al., 1975), was isolated from washed envelopes by a method that avoids treatment with boiling $NaDodSO_4$ (Nakamura & Mizushima, 1976). Crude matrix protein was further purified by chromatography on Sephadex G-200 (Nakamura & Mizushima, 1976). The final product showed a major band with several minor components when analyzed by $NaDodSO_4$ gel electrophoresis. The apparent molecular weight of the major component was dependent on the temperature to which the sample was heated prior to electrophoresis in a manner identical with that described by Nakamura & Mizushima (1976).

Sonic Disruption. Sonic disruption of membrane vesicles was performed as described by Salton & Netschey (1965), by use of five 1-min pulses from an MSE 100 W ultrasonic disintegrator fitted with a $^3/_8$-in. probe.

Preparation of Detergent Extracts of Membrane Vesicles. For analysis by CIE, membrane vesicles were sedimented by centrifugation (48000*g* for 1 h at 4 °C) and resuspended in 50 mM Tris-HCl (pH 8.6) containing 4% (v/v) Triton X-100 and 5 mM EDTA. The final protein concentration was between 8 and 16 mg/mL. Incubation was continued for 1 h at 25 °C and the extract was then centrifuged at 17000*g* for 45 min at 25 °C. Remaining sediment was extracted again in an identical manner. Insoluble material resisting two such extractions was washed once in twice the original volume of 50 mM Tris-HCl (pH 7.5) in order to remove excess Triton X-100 and extracted at 100 °C with 1 volume of 0.1% $NaDodSO_4$ (pH 7.5).

All detergent extracts were stored in 50-μL aliquots at −70 °C. The samples were frozen immediately after preparation and thawed only once, for either chemical analysis or CIE.

Preparation of ^{125}I-Labeled Detergent Extracts. ML 308-225 vesicles were centrifuged and the pellet was washed and resuspended in 0.1 M sodium borate (pH 8.5) to a concentration of 12–14 mg of protein per mL. Triton X-100 or $NaDodSO_4$ was added to final concentrations of 4% and 0.3%, respectively, and samples containing a total of 4 mg of protein were reacted with 0.5 mCi of ^{125}I-labeled Bolton–Hunter reagent (3,5-diiodo-4-hydroxyphenylpropionic acid *N*-hydroxysuccinimide ester) at a specific activity of 2 Ci/mmol as described (Bolton & Hunter, 1973). The samples were then dialyzed in microdialysis chambers overnight against a large volume of 0.1 M sodium phosphate (pH 6.6) containing the appropriate detergent.

Preparation of Anti-Vesicle Immunoglobulins. Antiserum to membrane vesicles prepared from *E. coli* ML 308-225 was raised in rabbits as described (Owen & Kaback, 1978). Fractionated immunoglobulins from two successive 750-mL serum pools were concentrated ten times with respect to serum volume (final protein concentrations 205 and 150 mg/mL, respectively) and stored in 0.1 M NaCl containing 15 mM NaN_3 at 4 °C. A sample of preimmune serum from the same rabbits was processed in a similar manner. Prior to use in adsorption experiments, concentrated immunoglobulins were extensively dialyzed against the relevant buffer system. The first serum pool (205 mg of protein per mL) was used in all experiments except those presented in Figures 5, 7, and 9, where the second serum pool was utilized.

Adsorption of Anti-Vesicle Immunoglobulins with Membrane Vesicles from E. coli ML 30. Aliquots of concentrated immunoglobulins (0.5 mL containing 89 mg of protein) were incubated with frequent shaking for 1 h at 20 °C with 0–2.0 mL of sonicated vesicles prepared from uninduced *E. coli* ML 30. Sufficient buffer (0.1 M potassium phosphate, pH 6.6) was added to give a final volume of 2.5 mL. Vesicles were then removed by centrifugation (30000*g* for 30 min at 4 °C) and the supernatant fractions were dialyzed against 0.1 M NaCl containing 15 mM NaN_3 and used for CIE.

Crossed Immunoelectrophoresis. Methods for performing CIE in the presence of Triton X-100 and for modifications

of CIE involving the use of intermediate gels containing different antibody preparations (CIE with intermediate gel) or lectins (crossed immunoaffinoelectrophoresis) are described in detail elsewhere (Owen & Salton, 1977; Smyth et al., 1978). Charge-shift crossed immunoelectrophoresis (Helenius & Simons, 1977; Bhakdi et al., 1977) was performed in a manner similar to CIE, with the exception that buffers and gels used during electrophoresis in the first dimension were supplemented with either cetyltrimethylammonium bromide (CTAB) or sodium deoxycholate (DOC) at final concentrations of 0.05% and 0.25%, respectively.

Electrophoresis in the first dimension was routinely performed in a Holm-Nielsen water-cooled immunoelectrophoresis chamber at 5.4 V/cm for 75 min (except where noted). Electrophoresis in the second dimension was performed at 2 V/cm for 18–24 h. To produce permanent records of the immunoprecipitin patterns, immunoplates were washed at least twice in 0.1 M NaCl, air-dried and stained with Coomassie brilliant blue. Peak areas were estimated using a compensating polar planimeter (Model 620005, Keuffel and Esser Co.) following magnification of the image (×6.6) in a standard photographic enlarger. Samples were electrophoresed at least three times and individual peaks areas are the mean of three determinations.

Enzyme Staining Techniques (Zymograms). The method detailed by Owen & Salton (1977) and by Smyth et al. (1978) were used to detect immunoprecipitates possessing the following enzyme activities: D-lactate dehydrogenase (EC 1.1.1.27), 6-phosphogluconate dehydrogenase (EC 1.1.1.43), dihydro-orotate dehydrogenase (EC 1.3.3.1), glutamate dehydrogenase (EC 1.4.1.4), NADH dehydrogenase (EC 1.6.99.3), polynucleotide phosphorylase (EC 2.3.7.8), and ATPase (EC 3.6.1.3). Published procedures (Baptist et al., 1969; Uriel, 1971; Owen & Salton, 1977; Smyth et al., 1978) were also used to attempt detection of the following enzyme activities: malate dehydrogenase (EC 1.1.1.37), isocitrate dehydrogenase (EC 1.1.1.42), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), α-glycerol-3-phosphate dehydrogenase (EC 1.1.99.5), succinate dehydrogenase (EC 1.3.99.1), catalase (EC 1.11.1.6), phosphoglucomutase (EC 2.7.5.1), esterase (EC 3.1.1.—), alkaline phosphatase (EC 3.1.3.1), acid phosphatase (EC 3.1.3.2), phosphodiesterase (EC 3.1.4.1), β-galactosidase (EC 3.2.1.23), aminopeptidases (EC 3.4.11.—), carboxypeptidases (EC 3.4.2.1/2), chymotrypsins (EC 3.4.21.1), and trypsins (EC 3.4.21.4). Detection of other enzyme activities was attempted by incubating pressed immunoplates individually in the following incubation mixtures (final volumes, 20 mL each): (i) L-alanine dehydrogenase (EC 1.4.1.1) —tetranitroblue tetrazolium (8 mg), L-alanine (178 mg), nicotinamide adenine dinucleotide (4 mg), phenazine methosulfate (1 mg), 50 mM Tris-HCl (pH 7.2); and (ii) thiogalactoside transacetylase—*either* (a) acetyl coenzyme A (12.5 mg), isopropyl β-D-thiogalactoside (60 mg), 5,5′-dithiobis(2-nitrobenzoic acid) (6 mg), 50 mM Tris-HCl (pH 7.9) containing 10 mM EDTA *or* (b) acetyl coenzyme A (17 mg), isopropyl β-D-galactoside (48 mg), tetranitroblue tetrazolium (10 mg), phenazine methosulfate (1 mg), 50 mM Tris-HCl (pH 7.5).

Protein Determination. Protein determinations were carried out using a modification (Dulley & Grieve, 1975) of the method of Lowry et al. (1951) that eliminates interference by Triton X-100. Bovine serum albumin was used as standard.

Materials

Agarose and concanavalin A were obtained from Miles Laboratories, and Triton X-100 was from Research Products International. β-Galactosidase was purchased from Sigma Chemical Co. and ^{125}I-labeled Bolton–Hunter reagent from New England Nuclear. Wheat germ agglutinin, soybean agglutinin, and peanut agglutinin were the generous gifts of Dr. Joel Oppenheim of New York University Medical Center, and membrane vesicles from *S. typhimurium*, together with purified components of the phosphoenolpyruvate:sugar phosphotransferase system, were kindly donated by Dr. Saul Roseman of Johns Hopkins University. The authors are also indebted to Dr. William Dowhan of The University of Texas Medical School at Houston for supplying a sample of purified phosphatidylserine synthetase and to Drs. Leon Heppel of Cornell University, Volkmaur Braun of The University of Tubingen, and Bennett Shapiro of The University of Washington for their generous gifts of antisera to ATPase, lipoprotein III, and NADH dehydrogenase, respectively.

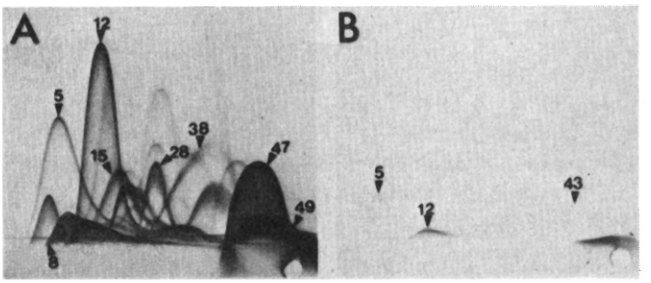

FIGURE 1: CIE of Triton X-100–EDTA extracts of membrane vesicles prepared from *E. coli* ML 308-225. Similar volumes (6 μL) of the first Triton X-100–EDTA extract (67 μg of protein; panel A) and of the second Triton X-100–EDTA extract (5.4 μg of protein; panel B) of membrane vesicles were analyzed by CIE against anti-vesicle immunoglobulins (2.3 mg of protein per mL of gel). Some of the salient precipitates in panel A are identified numerically in order to allow comparison with the complete reference profile (cf. Figure 2). The apparent identities of the few antigens detected for the second Triton X-100–EDTA extract are indicated also (panel B). The anode is to left and top of the gels.

Results

Crossed Immunoelectrophoresis Reference Pattern. The typical pattern of immunoprecipitates obtained following CIE of a Triton X-100–EDTA extract of membrane vesicles isolated from *E. coli* ML 308-225 is shown in Figure 1A. Conditions used for membrane solubilization are based on earlier results obtained with isolated inner and outer membranes from *E. coli* K 12 (Smyth et al., 1978), and in the present study over 80% of the detergent-soluble membrane protein is extracted by a single treatment with Triton X-100–EDTA. An additional 6–7% of the membrane protein can be recovered in a second Triton X-100–EDTA extract, but analysis reveals no additional precipitin bands in this fraction (Figure 1B). Residual membrane protein resisting two extractions with Triton X-100–EDTA is solubilized with 0.1% $NaDodSO_4$, but this material fails to give precipitin bands when analyzed by CIE or immunodiffusion. It is clear, therefore, that studies of the vesicle antigens can be restricted to an analysis of the initial Triton X-100–EDTA extract.

When membrane vesicles from *E. coli* ML 308-225 are analyzed by CIE under optimal conditions of antigen and antibody loading, about 25–30 discrete immunoprecipitates are readily detected, but additional immunoprecipitates can be visualized using more extreme concentrations of antigen or antibody. Indeed, from an analysis of over 1100 immunoplates run at various antigen (1–200 μg of protein) and immunoglobulin (1–16 mg of protein per mL of gel) concentrations, at least 52 discrete precipitates are detected by Coomassie blue staining. For reference, a schematic diagram

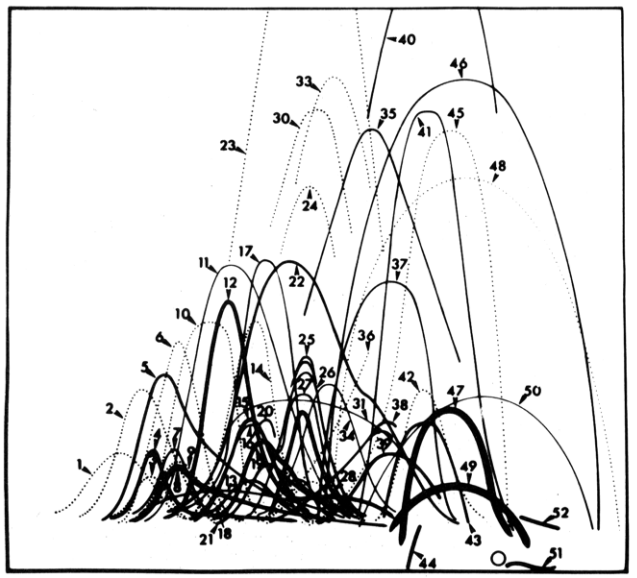

FIGURE 2: Schematic representation of the complete CIE pattern for the first Triton X-100–EDTA extract of plasma membrane vesicles derived from *E. coli* ML 308-225. The anode is to the left and top, and immunoprecipitates are numbered in order of decreasing electrophoretic mobility of the peak maxima. The line width is intended to indicate the intensities observed for the immunoprecipitates, dotted lines (···) denoting precipitates that are detected only at relatively high concentrations of immunoglobulin (over 5.4 mg of protein per mL of gel). The lower portions of immunoprecipitates no. 20, 25, and 35 and the upper parts of immunoprecipitates no. 36 and 38 were often difficult to visualize and have been omitted. Some numbers (i.e., 29 and 32) and the bottoms of some immunoprecipitates (i.e., no. 23, 24, 33, and 40) are omitted for clarity. Many of the weaker immunoprecipitates shown are not visualized on photographic reproduction (cf. Figure 1A). The identities of antigen no. 4, 5, 7, 8, 12, 15, 19, 27, 35, 41, 45, 49, and 51 have been established (cf., Figures 3–6). This figure is taken from a recent article by Owen & Kaback (1978; reprinted with permission) and is presented here for reference.

of this pattern with assigned numbers is presented in Figure 2, and examples of individual immunoplates run at different antigen/antibody ratios are presented in Figures 1A, 3E, and 5B. Although many of the immunoprecipitates are fairly symmetrical, some show consistent heterogeneity (e.g., no. 8 and 22). This phenomenon has been observed in CIE studies of other bacterial membrane extracts (Owen & Salton, 1975; Smyth et al., 1976) and the precise reasons are not completely understood. However, in view of the high exclusion of 1% agarose, it seems reasonable to suggest that the asymmetry may reflect, in part, charge heterogeneity (Owen & Smyth, 1977). Also, antigen no. 27 appears to show lines of partial identity with antigen no. 19, and, in addition, antigen no. 31 is unique in that it appears to fuse with the anodal and cathodal feet of antigen no. 22. It is interesting that a similar immunoprecipitate in the CIE reference pattern for plasma membranes of *Micrococcus lysodeikticus* shows analogous behavior that cannot be attributed to the proteolytic action of plasmin present in the antisera preparations (Owen & Salton, 1977).

Characterization of Immunoprecipitates. Many enzymes have been shown to retain sufficient catalytic activity following CIE to allow detection and localization in complex immunoprecipitin patterns by standard histochemical staining techniques (reviewed by Owen & Smyth, 1977). Application of zymogram staining methods to the identification of antigens detected in the present study reveals that immunoprecipitates no. 5, 7, 12, 35, 41, and 45 possess the following enzyme activities, respectively: polynucleotide phosphorylase, 6-phosphogluconate dehydrogenase, ATPase, glutamate dehydrogenase, dihydro-orotate dehydrogenase, and D-lactate dehydrogenase (Figure 3). Moreover, the vesicles possess at least two immunologically unrelated NADH dehydrogenases, antigens no. 15 and 19/27 (Figure 3D). Antigens no. 19 and 27 appear to show lines of partial identity only, suggesting the presence of perhaps three discrete NADH dehydrogenases. In contrast, the apparent staining of precipitate no. 28 for NADH dehydrogenase activity (Figure 3D) is an artifact as shown by the use of intermediate gels containing antiserum to partially purified NADH dehydrogenase (Dancey & Shapiro, 1976; Dancey et al., 1976; data not shown). The artifact is probably caused by entrapment of small amounts of antigen no. 27 in precipitate no. 28 during electrophoresis in the second dimension (see Brogren & Bøg-Hansen, 1975, for a discussion of this problem). Attempts to detect other

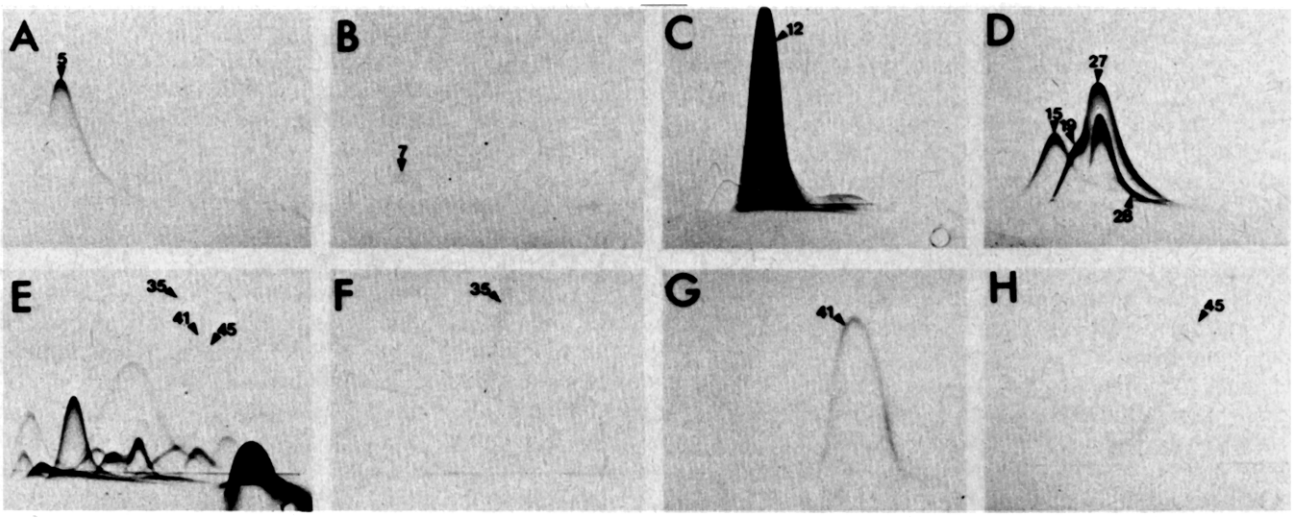

FIGURE 3: Characterization of immunoprecipitates in the vesicle–anti-vesicle reference system by zymogram techniques. Panels A–D represent gels similar to that shown in Figure 1A and stained for polynucleotide phosphorylase, 6-phosphogluconate dehydrogenase, ATPase and NADH dehydrogenase activities, respectively. Panel E shows the CIE profile obtained following electrophoresis of reduced quantities of antigen extract (22 μg of protein) against anti-vesicle immunoglobulins (2.3 mg of protein per mL of gel), and panels F–H represent identical gels stained for glutamate dehydrogenase, dihydro-orotate dehydrogenase, and D-lactate dehydrogenase activities, respectively. Activity stains were performed as described in Methods, and the gels were counterstained with Coomassie brilliant blue to confirm the identification indicated. The discrete nature of antigen no. 35, 41, and 45 was also established by staining individual immunoplates for all three enzyme activities. The anode is to the left and top of all gels.

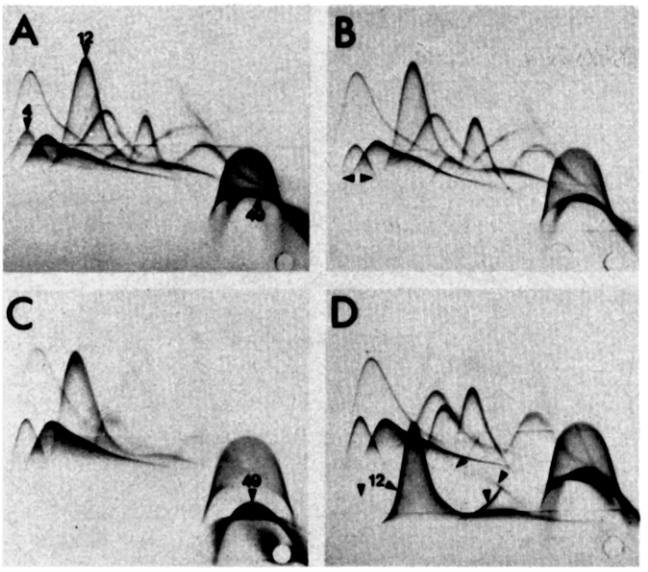

FIGURE 4: Identification of antigen no. 4, 12, and 49 by CIE with intermediate gel. Similar amounts (4.5 μL, 50 μg of protein) of a Triton X-100–EDTA extract of *E. coli* ML 308-225 membrane vesicles were analyzed in all instances. Panel A represents the control immunoplate with no immunoglobulins in the intermediate gel. Test immunoplates are shown in panels B–D where intermediate gels contained preimmune globulin (2.4 mg of protein per mL, panel B), anti-lipoprotein III immunoglobulins (5.5 mg of protein per mL, panel C), and anti-ATPase immunoglobulins (0.39 mg of protein per mL, panel D). All reference gels contained anti-vesicle immunoglobulins (2.3 mg of protein per mL). Antigen no. 4 is the only immunogen affected by preimmune globulin and this is manifested by reactions of "inward feet" (arrows in panel B). Similar reactions are also evident in panels C and D. The only other immunoprecipitate visibly depressed by anti-lipoprotein immunoglobulins is no. 49 (panel C). Similarly, antigen no. 12 is the major vesicle immunogen reacting with anti-ATPase serum (panel D). However, several additional vesicle antigens also appear to react with this serum (unnumbered arrows in panel D). The anode is to the left and top of all gels.

enzymes by the zymogram techniques listed in Methods were unsuccessful.

A powerful variant of CIE is the modification known as CIE with intermediate gel. In this technique, a gel containing a different (and usually monospecific) antiserum is interposed between antigen electrophoresed in the first dimension and the reference antibody gel. Antigens reacting with both antisera are recognized by partial or complete depression of the corresponding immunoprecipitates from the reference gel into the intermediate gel. In this manner antigen no. 4 can be shown to be the only vesicle component that reacts with preimmune serum (Figures 4A and 4B), and it may thus correspond to the "common protein antigen" responsible for cross-reactions between a broad range of Gram-negative organisms (Hoiby, 1975; Smyth et al., 1976). The importance of identifying antigens capable of reacting with preimmune serum is aptly demonstrated in Figure 4C where both antigens no. 4 and 49 appear to react with antiserum directed against lipoprotein III from *E. coli* (Braun et al., 1976). Bearing the discussion in mind, it is clear that antigen no. 49 corresponds to the major outer membrane protein in question. This interpretation is confirmed by purification of the lipoprotein from isolated envelopes of *E. coli* ML 308-225, followed by coelectrophoresis with Triton X-100–EDTA solubilized vesicles (Figures 5D–F). The identification of immunoprecipitates no. 12 (ATPase), 15, 19, and 27 (NADH dehydrogenase), and 45 (D-lactate dehydrogenase) based on zymogram techniques has also been confirmed through use of antisera to the purified or partially purified enzymes in intermediate gels (for example, see Figure 4D).

Identification of antigen no. 49 as lipoprotein suggests the presence of outer membrane in the vesicle preparations. Consequently, it is probable that other precipitates in the CIE reference pattern may correspond to additional outer membrane immunogens. Indeed, antigen no. 51 corresponds to lipopolysaccharide as shown by coelectrophoresis with the purified antigen (Figures 5A–C). This major outer membrane component is one of the few antigens to show cathodal migration at pH 8.6 and thus gives inverse rockets following CIE.

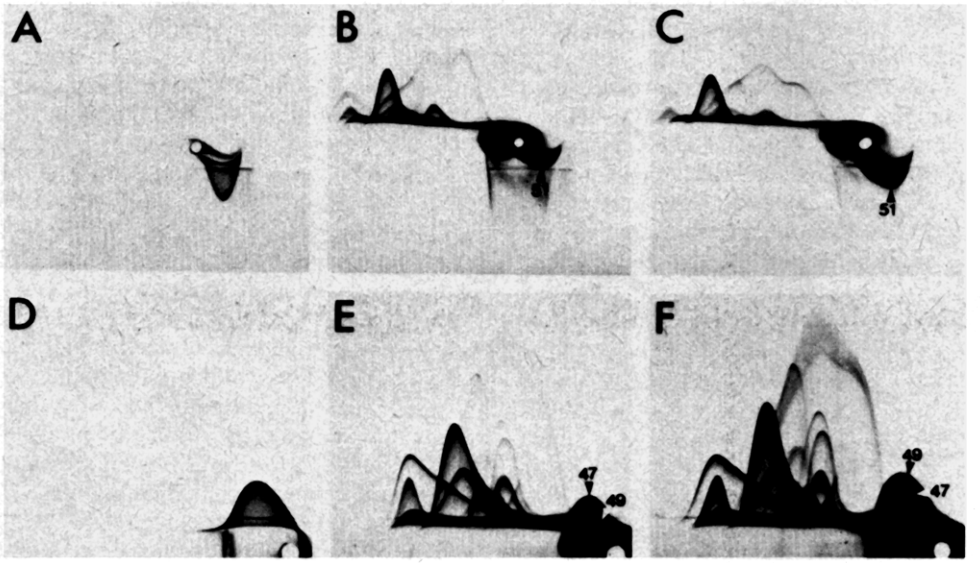

FIGURE 5: Identification of immunoprecipitates corresponding to lipopolysaccharide (panels A–C) and to lipoprotein (panels D–F) by co-CIE. For the identification of lipopolysaccharide, a Triton X-100–EDTA extract of *E. coli* ML 308-225 membrane vesicles (15 μg of protein) was coelectrophoresed in the CIE system with (panel C) and without (panel B) purified lipopolysaccharide (2 μg). Lipopolysaccharide alone (2 μg) was electrophoresed for comparison (panel A). In all cases electrophoresis in the first dimension was for 60 min from a centrally positioned well. For the identification of lipoprotein, a similar Triton X-100 extract of membrane vesicles (66 μg of protein) was coelectrophoresed in the CIE system with (panel F) and without (panel E) purified lipoprotein (5 μg). Lipoprotein alone (5 μg) was electrophoresed for comparison (panel D). For all immunoplates shown, electrophoresis in the second dimension was conducted into gels containing anti-vesicle immunoglobulins at a concentration of 4.0 mg of protein per mL. Only the area subtended by immunoprecipitate no. 51 increased following co-CIE with lipopolysaccharide (panels B and C). Similarly, the area of immunoprecipitate no. 49 only increased following co-CIE with lipoprotein (panels E and F). The anode is to the left and top of all gels.

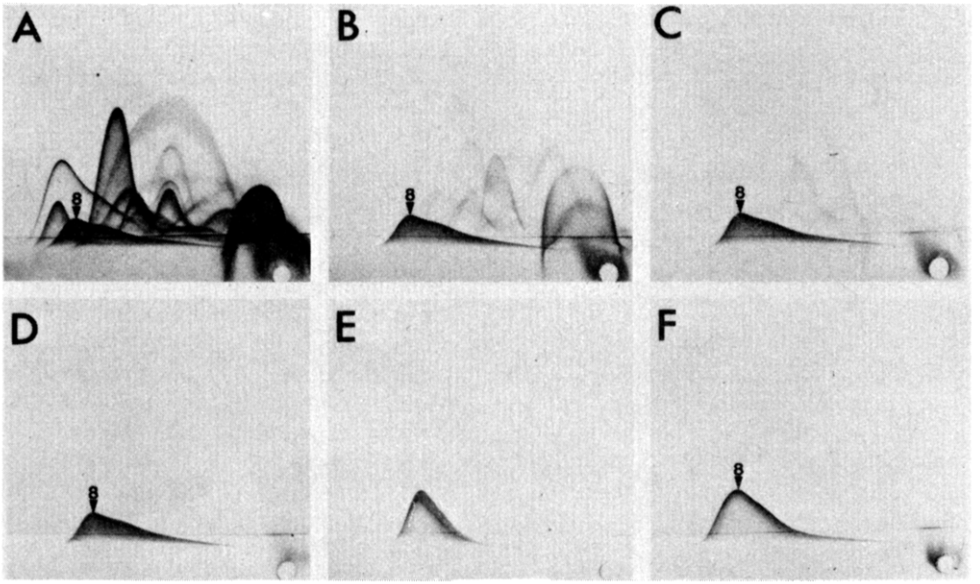

FIGURE 6: Effect of absorbing immunoglobulins against *E. coli* ML 308-225 membrane vesicles with vesicles derived from uninduced *E. coli* ML 30. Antiserum to membrane vesicles from *E. coli* ML 308-225 was adsorbed as described in Methods with 0 (panel A), 5.2 mg (panel B), 10.4 mg (panel C), and 20.8 mg (panels D–F) of sonicated ML 30 membrane vesicle protein. The resultant immunoglobulin fractions were incorporated into agarose gels (94 μL per mL of gel) and tested in the CIE system against a Triton X-100–EDTA extract of membrane vesicles from *E. coli* ML 308-225 (56 μg of protein; panels A-D), commercial β-galactosidase (2 μg of protein; panel E), or a mixture of the two samples (56 μg of membrane protein plus 2 μg of β-galactosidase; panel F). Note that the areas subtended by all immunoprecipitates, except no. 8, increase upon adsorption (compare panels A through D), and that commercial β-galactosidase (panel E) and antigen no. 8 (panel D) are immunologically identical as shown by co-CIE against the same antiserum (panel F). The anode is to the left and top of all gels.

Another well-characterized outer membrane protein, the Rosenbusch "matrix" protein (Rosenbusch, 1974), has escaped immunological characterization by CIE (Smyth et al., 1978). In the present study, preparations of this protein isolated from *E. coli* ML 308-225 by procedures known to retain the intrinsic β structure of the polypeptide (Nakamura & Mizushima, 1976) failed to give a recognizable immunoprecipitate when tested against anti-vesicle immunoglobulin by CIE, immunoelectrophoresis, immunodiffusion, and $NaDodSO_4$ gel electrophoresis followed by diffusion against antiserum. A purified preparation of phosphatidylserine synthetase from *E. coli* ribosomes (Larson & Dowhan, 1976) also fails to react with anti-vesicle immunoglobulins.

Antigens containing carbohydrate are particularly amenable to analysis using a modification of CIE termed crossed immunoaffinoelectrophoresis (Bøg-Hansen, 1973; Owen et al., 1977). In this procedure lectins are incorporated into intermediate gels so as to adsorb interacting glycoproteins or polysaccharides before they enter the reference antibody gel. Affinity experiments of this nature have been performed with lectins bearing primary sugar specificities for α-D-mannopyranoside and α-D-glucopyranoside residues (concanavalin A; So & Goldstein, 1967), D-galactosyl residues (peanut agglutinin; Lotan et al., 1975), *N*-acetyl-D-galactosaminyl residues (soybean agglutinin; Sela et al., 1970), and *N*-acetylglucosaminyl residues (wheat germ agglutinin; Burger & Goldberg, 1967), but none appears to interact with any of the antigens in the reference pattern (data not shown). The observations indicate that glycoproteins are not a major component of the *E. coli* membrane.

Identification of Antigenic Gene Products of the lac Operon. Although the β-galactoside transport system in *E. coli* has been studied intensively, identification and purification of the *lac* carrier protein (the *lac y* gene product) under nondenaturing conditions has been unsuccessful. We have therefore undertaken a series of CIE experiments designed to detect the gene products of the *lac* operon in *E. coli* ML 308-225 membrane vesicles.

In initial experiments, immunoglobulins against *E. coli* ML 308-225 vesicles were adsorbed with increasing quantities of sonicated vesicles prepared from uninduced *E. coli* ML 30. Theoretically, this operation should progressively remove antibodies to all vesicle immunogens except those generated by the products of the *lac* operon. Since the peak area of a precipitate in the CIE system is proportional to the antigen/antibody ratio (Weeke, 1973), analysis of adsorbed immunoglobulins by CIE using fixed amounts of Triton X-100–EDTA antigen extract should yield a series of immunoplates where the only immunoprecipitates that remain constant are those corresponding to products of the *lac* operon. Figure 6 (A–D) reveals that the only immunoprecipitate to behave in this manner is no. 8, and that this component is enzymatically inactive β-galactosidase (*E. coli* ML 308-225 has a point mutation in the *lac z* gene), as evidenced by its coelectrophoresis with the purified enzyme (Figure 6, D–F). Attempts to increase the sensitivity of the procedure with ^{125}I-labeled detergent extracts of ML 308-225 vesicles and radioautography failed to reveal additional immunoprecipitates that are unaffected by adsorption, and comparative CIE analyses of ML 308-225 and ML 30 vesicles confirm the apparent sole absence of antigen no. 8 (data not shown). Finally, sensitive zymogram techniques (cf. Methods) failed to detect an immunoprecipitate with thiogalactoside transacetylase activity (the *lac a* gene product).

Characterization of Vesicle Antigens by Charge-Shift Crossed Immunoelectrophoresis. Charge-shift electrophoresis has been introduced recently by Helenius & Simons (1977) as a simple and effective means of distinguishing between amphiphilic and hydrophilic proteins. The method relies on the fact that amphiphilic proteins bind relatively large amounts of detergent in comparison to soluble hydrophilic proteins. Consequently, the electrophoretic mobility of amphiphilic proteins is altered when electrophoresis is carried out in the presence of cationic and anionic detergents. In order to characterize the important amphiphilic antigens present in membrane vesicles derived from *E. coli* ML 308-225, a

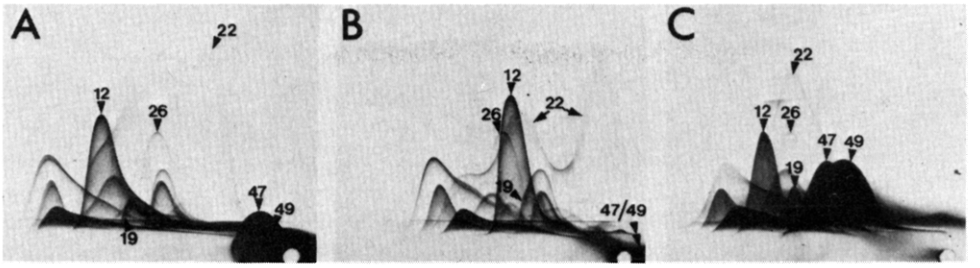

FIGURE 7: Characterization of vesicle antigens by charge-shift CIE. A Triton X-100–EDTA extract of membrane vesicles derived from *E. coli* ML 308-225 (59 μg of protein) was electrophoresed in the first dimension into agarose gels containing Triton X-100 alone (panel A), Triton X-100 supplemented with 0.05% CTAB (panel B), or Triton X-100 supplemented with 0.25% DOC (panel C). Electrophoresis in the second dimension was conducted into gels containing anti-vesicle immunoglobulins (4.0 mg of protein per mL) and Triton X-100 as the sole detergent. Note that antigen no. 26, 47, and 49 show obvious bidirectional charge shifts in the presence of the ionic detergents, while the electrophoretic mobilities of antigen no. 12 and 19 (for example) are affected by CTAB only. Pronounced heterogeneity of immunoprecipitate no. 22 in the presence of CTAB is also evident (panel B). The diffuse staining reaction at the interface between the first dimension gel and the antibody gel in panel C is caused by DOC-induced precipitation of serum proteins and impairs the resolution of some of the antigens in that region of the gel. The anode is to the left and top of all gels.

modification of this technique that allows resolution of the antigens in a CIE system was adapted (charge-shift crossed immunoelectrophoresis; Bhakdi et al., 1977). Figure 7 depicts a typical charge-shift CIE experiment in which Triton X-100–EDTA solubilized vesicles were electrophoresed in the first dimension in the presence of Triton X-100 alone (Figure 7A), Triton X-100 supplemented with CTAB (Figure 7B), and Triton X-100 supplemented with DOC (Figure 7C). It is visually obvious that lipoprotein (antigen no. 49), antigen no. 47 and, to a lesser extent, antigen no. 26 show pronounced bidirectional alterations in electrophoretic mobility in the presence of ionic detergents. Glutamate dehydrogenase (antigen no. 35), dihydro-orotate dehydrogenase (antigen no. 41), and D-lactate dehydrogenase (antigen no. 45) also display charge shifts of similar magnitude to that observed for antigen no. 47 when analyzed by appropriate zymogram techniques (immunoplates not shown). Several other minor immunogens appear to behave similarily, but it is impossible to establish their identity unambiguously. Of the remaining antigens amenable to characterization by this method (i.e., no. 4, 5, 7–9, 12, 13, 15, 17–19, 21, 22, 27, 28, and 34), each (with the exception of no. 22) exhibits less than a 10% alteration in electrophoretic mobility upon electrophoresis in DOC, indicating little binding of this detergent. The observation that many of these antigens show pronounced charge shifts in the presence of CTAB [e.g., ATPase (antigen no. 12) and NADH dehydrogenase (antigen no. 19)] suggests that their ability to bind this detergent results from electrostatic rather than hydrophobic interactions. The characteristic heterogeneity of antigen no. 22 (Figure 7A) is further emphasized when electrophoresis is conducted in the presence of CTAB (Figure 7B). This behavior appears to be largely a consequence of the dramatic charge shift incurred by the less acidic species present in this obviously heterogeneous antigen/antigen complex.

Cross-Reacting Antigens in Membrane Vesicles of Salmonella typhimurium. Membrane vesicles prepared from the related member of the Enterobacteriaceae, *S. typhimurium*, have also been used for transport studies (Konings et al., 1971; Postma & Roseman, 1976; Tokuda & Kaback, 1977). Therefore, it is of interest to characterize the cross-reacting membrane antigens in *E. coli* and *S. typhimurium*. Figure 8 reveals that eight major *S. typhimurium* vesicle antigens and several minor ones are resolved following CIE against anti-*E. coli* vesicle immunoglobulin. Six of these antigens are electrophoretically similar to and cross-react fully with the following membrane immunogens of *E. coli* ML 308-225 as shown by tandem CIE (Krøll, 1973) and co-CIE: polynucleotide phosphorylase (antigen no. 5), ATPase (antigen no.

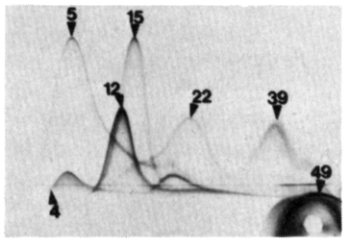

FIGURE 8: Reactivity of *S. typhimurium* vesicle components with immunoglobulins against *E. coli* ML 308-225 vesicles. A Triton X-100–EDTA extract of membrane vesicles from *S. typhimurium* (30 μg of protein) was analyzed by CIE against anti-ML 308-225 vesicle immunoglobulins (2.3 mg of protein per mL of gel) as described in Methods. Numbers indicate the immunogens shown on the reference pattern in Figure 2 with which the components given cross-react in tandem and co-CIE experiments (not shown).

12), NADH dehydrogenase (antigen no. 15), lipoprotein (antigen no. 49), and antigen no. 4 and 22. Another *Salmonella* vesicle component shows lines of partial identity with antigen no. 39 (tandem CIE and co-CIE immunoplates not shown; cf. Figure 8). Furthermore, zymogram staining of co-CIE immunoplates reveals that a component from *S. typhimurium* vesicles with 6-phosphogluconate dehydrogenase activity gives a line of full identity with the corresponding enzyme from *E. coli* (antigen no. 7) but is less acidic. None of the other cross-reacting antigens detected for *S. typhimurium* and *E. coli* vesicles corresponds to components of the phosphoenolpyruvate:sugar phosphotransferase transport system since the following purified proteins from *S. typhimurium* fail to react with the anti-vesicle immunoglobulin: HPr, enzyme I, enzyme IIB (glucose), and factor III (glucose).

Extent of Outer Membrane and Cytoplasmic Contamination. Braun's lipoprotein (antigen no. 49) is an accepted marker for outer membrane, and its presence in vesicles from *E. coli* ML 308-225 can be used to estimate the degree to which the vesicles are contaminated with outer membrane. Results based upon measurements of peak areas under immunoprecipitate no. 49 for solubilized vesicles (two different preparations) and for various concentrations of purified lipoprotein indicate that the vesicles contain about 6.1–6.4 μg of lipoprotein per mg of vesicle protein. Assuming that lipoprotein constitutes about 27% of the total envelope protein (Halegoua et al., 1974), outer membrane contamination of the ML 308-225 vesicle preparations does not exceed about 3%.

Using various intracellular constituents as a measure of cytoplasmic contamination, the vesicle preparations contain less than 3% of the DNA and RNA and less than 2% of the

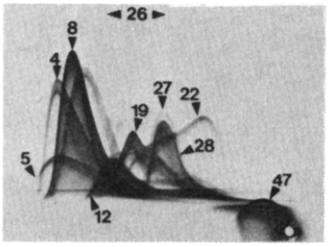

FIGURE 9: Reactivity of *E. coli* ML 308-225 cytoplasmic constituents with immunoglobulins ML 308-225 vesicles. The cytoplasmic fraction from French-pressed *E. coli* ML 308-225 (62 μg of protein) was analyzed by CIE against anti-ML 308-225 vesicle immunoglobulins (4.0 mg of protein per mL of gel) as described in Methods. Numbers indicate the immunogens shown on the reference pattern in Figure 2 with which the components given cross-react in tandem and co-CIE experiments (not shown).

activities of a number of cytoplasmic enzymes relative to the cells from which they were derived (Kaback, 1971). Moreover, visual comparison of the areas subtended by the CIE immunoprecipitates for membrane vesicles and cell cytoplasm (compare Figures 1A and 9) confirms the point that most of the major antigens detected in the vesicles [(e.g., antigen no. 12 (ATPase) and antigen no. 15 (NADH dehydrogenase)] are clearly derived from the plasma membrane. Some antigens [(e.g., no. 5 (polynucleotide phosphorylase) and no. 19/27 (NADH dehydrogenase)] appear to partition between the membrane and soluble fractions to similar extents, and a few antigens [(e.g., no. 4, no. 8 (β-galactosidase) and no. 26] are of cytoplasmic origin.

Table I: Characterization of Antigens Detected for Membrane Vesicles of *E. coli* ML 308-225[a]

antigen no.	identity	amphiphilic nature	reaction with *S. typhimurium* antigens
4	common antigen?		+
5	polynucleotide phosphorylase		+
7	6-phosphogluconate dehydrogenase		+
8	β-galactosidase		
12	ATPase		+
15	NADH dehydrogenase		+
19	NADH dehydrogenase		
22	unknown	+	+
26	unknown	+	
27	NADH dehydrogenase		
35	glutamate dehydrogenase	+	
39	unknown		±
41	dihydro-orotate dehydrogenase	+	
45	D-lactate dehydrogenase	+	
47	unknown	+	
49	Braun's lipoprotein	+	+
51	lipopolysaccharide		

[a] Antigen no. refers to the immunoprecipitates shown in Figure 2. Amphiphilic nature denoted (+) indicates that the antigen in question exhibits bidirectional alteration in electrophoretic mobility during charge-shift CIE experiments. The ability of the antigens to cross-react either fully (+) or partially (±) with membrane antigens from *S. typhimurium* is also indicated.

Discussion

The complexity of the immunoprecipitin reference pattern for membrane vesicles of *E. coli* ML 308-225 dramatically illustrates the resolving power of the two-dimensional CIE technique. Furthermore, the positive identification of 13 of 52 antigens resolved, together with the partial characterization of many others in terms of their amphiphilic or hydrophilic properties, apparent cellular origin and cross-reactivity with membrane immunogens from *S. typhimurium*, emphasizes the potential use of this quantitative technique for the study of cell membranes. Of particular importance is the identification of respiratory enzymes (e.g., D-lactate dehydrogenase and NADH dehydrogenase) and ATPase which are intimately involved in the energetics of the bacterial membrane. Moreover, if the molecular architecture of membrane vesicles is to be fully understood, it is imperative to establish the identity of molecules whose location in the intact cell is known. This has been achieved with the identification of markers for the outer membrane (e.g., Braun's lipoprotein and lipopolysaccharide), the plasma membrane (e.g., D-lactate dehydrogenase, NADH dehydrogenase, and ATPase), and the cytoplasm (e.g., β-galactosidase). A summary of the salient features of the CIE reference pattern is presented in Table I. Several points are worthy of discussion.

It is clear from the identification of marker enzymes for the plasma membrane, from quantitation of markers for the outer membrane and cytoplasm, and from CIE analysis of the cytoplasm that membrane vesicles prepared from *E. coli* ML 308-225 by osmotic lysis (Kaback, 1971; Short et al., 1975) are composed largely of plasma membrane. Only small quantities of outer membrane are detected (estimated at less than 3%), in accordance with results obtained by electron microscopy and by other means (Kaback, 1971). Contamination by cytoplasmic components is also minimal and due probably to the topological constraints imposed by the formation of sealed vesicles during osmotic lysis. On the other hand, although the significance of the observation is uncertain, it is interesting that certain presumably cytoplasmic enzymes such as polynucleotide phosphorylase partition equally between the membrane and the cytoplasm. Finally, the absence of periplasmic constituents has been documented previously (Kaback, 1971; Kerwar et al., 1972) and is confirmed by the lack of immunoprecipitates staining for alkaline phosphatase, acid phosphatase, and phosphodiesterase.

The presence of multiple immunoprecipitates exhibiting NADH dehydrogenase activity is interesting. One enzyme (antigen no. 15) is localized exclusively in the vesicles and can be shown by CIE analysis of ^{59}Fe-labeled membranes to contain nonheme iron (Owen, Kaczorowski, & Kaback, unpublished information). The remaining NADH dehydrogenase(s) (antigens no. 19/27) appears to partition between the membrane and soluble fractions and does not contain bound iron. The latter enzyme(s) probably corresponds to the NADH dehydrogenase described recently by Dancey & Shapiro (1976) and Dancey et al. (1976) since antibody directed against the partially purified enzyme reacts, albeit weakly, with antigens no. 19/27. Moreover, it is noteworthy that antigen no. 39 reacts very strongly with the same antiserum but does not stain for NADH dehydrogenase (data not shown) and is expressed on the outer surface of the vesicles (Owen & Kaback, 1979). Thus, the possibility cannot be excluded that the NADH dehydrogenase isolated by Shapiro and co-workers and estimated to have a molecular weight of 38 000 is contaminated with an membrane protein in the 35 000–40 000 molecular weight range (Garten et al., 1975).

Failure to detect an immunoprecipitate corresponding to the *lac* carrier protein is particularly frustrating in view of the potential use of such an immunoprecipitate for the production of immunoglobulin specific for the *lac y* gene product (cf. Crowle et al., 1972). Clearly the techniques utilized appear to be sufficiently sensitive to allow detection of the *lac* carrier

were it present in the CIE profile. This point is best illustrated by the adsorption experiment shown in Figure 6 where the major product of the *lac* operon (i.e., β-galactosidase) is dramatically defined following adsorption of anti-vesicle immunoglobulins with vesicles from uninduced *E. coli* ML 30. The possibility cannot be ignored that the *lac* carrier protein is a poor antigen perhaps because it is extensively buried in the hydrophobic core of the membrane. This reasoning may also explain, in part, why some of the components of the phosphoenolpyruvate:sugar phosphotransferase system fail to give detectable immunoprecipitates when reacted with anti-vesicle immunóglobulin. Attempts are presently being made to resolve this problem by immunizing with detergent-solubilized membrane vesicles and by analysis of membranes that have been reacted with probes such as aryl azides (Bercovici & Gitler, 1978).

In the following paper (Owen & Kaback, 1979), the topological distribution of antigens between the outer and inner surfaces of the vesicle membrane is described.

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Antigenic Architecture of Membrane Vesicles from *Escherichia coli*†

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With the Technical Assistance of Helen Doherty

ABSTRACT: The antigenic architecture of membrane vesicles prepared from *Escherichia coli* ML 308-225 has been studied using crossed immunoelectrophoresis. Progressive immunoadsorption experiments conducted with control vesicles and with physically disrupted vesicles were used to monitor and quantitate the expression of 14 different immunogens. Eleven immunogens, including NADH dehydrogenase (EC 1.6.99.3), D-lactate dehydrogenase (EC 1.1.1.27), dihydro-orotate dehydrogenase (EC 1.3.3.1), 6-phosphogluconate dehydrogenase (EC 1.1.1.43), polynucleotide phosphorylase (EC 2.3.7.8), and β-galactosidase (EC 3.2.1.23), exhibit minimal expression (10% or less) unless the vesicles are disrupted. Three unidentified antigens are expressed to a similar extent in untreated and disrupted vesicles. Consideration of these and other results [Owen, P., & Kaback, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3148] in terms of membrane polarity, dislocation of antigens, and possible transmembrane orientation of some immunogens reveals that over 95% of the membrane in the vesicle preparations is in the form of sealed sacculi with the same orientation as the intact cell. Furthermore, antigens are distributed across the membrane in a highly asymmetric manner, indicating that dislocation of components from the inner to the outer surface of the membrane during vesicle preparation does not occur to an extent exceeding 10%.

In previous communications (Owen & Kaback, 1978, 1979), a complex crossed immunoelectrophoresis (CIE)[1] reference pattern was established for membrane vesicles prepared from *Escherichia coli* ML 308-225, and many of the 52 component immunogens were characterized. In addition, results of progressive immunoadsorption experiments with certain vesicle immunogens were presented and the relationship

$$\frac{1}{A_v} = \frac{1}{A_0} - Kxv \qquad (1)$$

was derived and shown to apply to the data (Owen & Kaback, 1978) where A_0 and A_v are the peak areas subtended by an immunoprecipitate i following adsorption with 0 and v mL of a vesicle suspension, respectively. K is a constant under the experimental conditions adopted, and x is the degree to which antigen i is expressed in the membrane suspension used for adsorption. Comparison of the values of x for antigen i following adsorption with intact and disrupted vesicles reveals the extent to which i becomes expressed following disruption or, stated conversely, the extent to which i is not expressed in intact vesicles. By using this approach quantitatively, the distribution of i on the inner and outer surface of the membrane can be deduced (Owen & Kaback, 1978).

In this paper, the results of an extensive series of quantitative immunoadsorption experiments of the type outlined above are presented, and the distribution of 14 antigens across the vesicle membrane is established. It is apparent from these and other data (Owen & Kaback, 1978) that the vesicle membrane of *E. coli* ML 308-225 is markedly asymmetric with respect to its antigenic architecture and that it retains the topology of the parent cell. Moreover, dislocation of membrane components from the inner to the outer surface of the membrane occurs to a minimal extent for all of the antigens tested.

Experimental Procedures

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown on minimal medium with 1.0% disodium succinate (hexahydrate) and membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975). Vesicles were suspended in 0.1 M potassium phosphate (pH 6.6), frozen in liquid nitrogen, and maintained at or below −70 °C. Prior to use in adsorption experiments, suspensions were thawed rapidly at 45 °C. When appropriate, the vesicles were washed by centrifugation at 30000*g* for 30 min at 4 °C followed by careful resuspension to their original volume in 0.1 M potassium phosphate (pH 6.6). Concentration was achieved by centrifugation in a similar manner and resuspension of the vesicles in a reduced volume of 0.1 M potassium phosphate, pH 6.6.

Disruption of Membrane Vesicles. Sonic disruption of membrane vesicles was performed at 0 °C using 30-s or 1-min

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[1] Abbreviations used: CIE, crossed immunoelectrophoresis; $NaDodSO_4$, sodium dodecyl sulfate.

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