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IMMUNOLOGICAL PROPERTIES OF MEMBRANE FRACTIONS FROM WILD TYPE AND *dna*A MUTANTS OF *ESCHERICHIA COLI*

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SUMMARY

Membrane vesicles from *Escherichia coli* wild type and an otherwise isogenic *dna*A mutant were used to immunize rabbits. In addition, a membrane protein fraction, containing the material found deficient in *dna*A mutants, was purified by preparative polyacrylamide gel electrophoresis in sodium dodecylsulfate, and used for immunization. The antisera produced were analyzed by immunoelectrophoresis and immunofluorescence microscopy. The antisera obtained by immunization with membrane vesicles from either wild type or *dna*A mutant membrane preparations were qualitatively similar in the precipitin bands seen after immunoelectrophoresis. The antisera obtained by immunization with the purified protein fraction contained a subset of the antibodies seen when whole vesicles were used for immunization. In a semiquantitative precipitin assay, the antisera prepared against whole membrane vesicles or the isolated protein fraction both caused the precipitation of more protein from sodium dodecylsulfate-solubilized membranes of wild type than of *dna*A mutants. No difference was seen by immunoelectrophoresis between the protein composition of wild type or *dna*A membrane preparations. Thus, the *dna*A mutant appears to differ from the wild type in the quantitative composition of its membrane proteins, whereas no qualitative differences were detected.

Fluorescein-conjugated antiserum preparations were employed to assess the reactivity of intact cells, spheroplasts and membrane vesicles with the antisera studied above. Wild type cells of *E. coli* have a barrier to reaction with the antisera; this barrier is removed when the cells are converted to spheroplasts or to membrane vesicles. Similarly, a highly permeable mutant of *E. coli* permits reaction of the antisera with unaltered cells. Antisera to both whole membrane vesicles and to the isolated protein fraction react identically with the cellular and subcellular preparations. Thus, antisera prepared from membrane proteins isolated after sodium dodecylsulfate–polyacrylamide gel electrophoresis can still recognize some antigens present in membrane vesicle preparations.

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INTRODUCTION

Previous investigation of temperature-sensitive DNA replication mutants of *Escherichia coli* [1–5] has shown that two classes of such mutants have alterations in membrane protein composition. One class (*dna*A mutants), affected in the initiation of chromosomal replication, is deficient in two protein fractions, as determined by sodium dodecylsulfate gel electrophoresis, and has an excess of a third component, when compared with isogenic wild type strains [2, 4, 5]. The fractions which are deficient have molecular weights of approximately 60 000 (Fraction MP60)* and 40 000 (Fraction MP40)*. In previous studies we examined the localization of these altered components in the inner and outer membranes [5], and also the effect of phenotypic suppression of the DNA synthesis mutations [3, 5] on the membrane protein composition. We concluded that the alteration in Fraction MP60 is a direct consequence of the DNA synthesis mutations, but is not necessarily directly related to DNA replication per se; the alteration at Fraction MP40 seems to be directly related to DNA replication, although it might have been a secondary response [5].

The present studies were initiated to define more clearly the nature of the alteration in membrane protein composition seen with *dna*A mutants, and additionally to evaluate any physiologic significance of such membrane changes. The altered membrane components serve as markers which are associated, however indirectly, with DNA replication, and might show whether membrane structure and function is involved in this process. To this end, we have prepared antibodies against membrane proteins and used these antibodies as reagents for probing the structure of the *E. coli* surface. Several other recent studies have been directed toward the use of membrane proteins as antigens, in an attempt to evaluate membrane structure. Such immunological studies of *Mycoplasma* [6, 7] and *Micrococcus* [8] membrane preparations have shown that membrane proteins exist as unique antigenic determinants which may be used to follow membrane disaggregation and reaggregation. A recent examination of purification methods for *E. coli* membrane proteins has also shown that membrane protein fractions are distinct antigenic components [9].

This study analyzes antibodies produced against whole membrane vesicles as contrasted with those directed against an isolated subfraction of membrane proteins. The subfraction examined contains the Fraction MP60 and Fraction MP40 components and was isolated by preparative sodium dodecylsulfate–polyacrylamide gel electrophoresis. These fractions, which are deficient in *dna*A strains [2, 4, 5], have been defined by their migration on sodium dodecylsulfate–polyacrylamide gels, so the protein subfraction used for immunization is a partially purified preparation of these proteins. We have been able to use such sodium dodecylsulfate-denatured material as an antigen, and have found that the population of antibodies elicited by it is a subset of that seen when whole membrane vesicles are used in the immunization. By examining the reaction of different antisera with membrane protein components, we have obtained evidence that the proteins previously found to be deficient in *dna*A

* Fraction MP60 is used to refer to a membrane protein fraction which has a molecular weight approximately 60 000. In this operational definition, nothing is implied about the number of molecular components which comprise Fraction MP60, nor even that it represents a single complex species. The terminology is used merely to facilitate reference to areas of the electrophoretograms being discussed.

mutants [2, 4, 5] reflect a quantitative, rather than a qualitative, alteration in membrane protein composition.

MATERIALS AND METHODS

Bacterial strains

The following strains of *E. coli* K-12 were employed: CRT 4614, *thi thy leu str*^{r} *lac*$_Y$ *dna*A; CRT 4615, a temperature-resistant derivative otherwise isogenic with CRT 4614; $P_{4\times8}$, *leu ts*$_{84}$, a temperature-sensitive filament-forming strain containing the previously described [10] PAT-84 mutation; MAD-1, a mutant with increased permeability and an alteration in cellular division and morphology [11].

Media and supplies

The medium used in labeling experiments was a minimal salts medium as described previously [2] supplemented with 4 mg/ml of glucose and 20 μg/ml leucine. Non-labeled cells were grown in a bactotryptone–yeast extract medium [2]. Radioisotopes were uniformly labeled L-[^{14}C]leucine, L-[4,5-^{3}H]leucine, and sodium dodecyl-[^{35}S]sulfate. All were obtained from New England Nuclear. Fluorescein isothiocyanate was purchased from Miles. All other chemicals were of the highest commercial purity.

Growth of radioactively labeled bacteria and preparation of membrane

Logarithmically growing bacterial cultures were used in all experiments as previously described [2–5]. In all experiments where the immunological properties of mutant membranes were tested, wild type and mutant strains were grown at 40 °C for 3 h to permit expression of the *dna*A mutation. For isotope incorporation experiments, cultures were grown to an $A_{600\ nm} = 0.6$–0.8, and growth was stopped by centrifugation and washing with 0.03 M Tris–HCl, pH 8.1. Membrane preparation steps were exactly as in our previous papers, which contain a full description of the state of purity of the membrane vesicles obtained [2–5].

The filament-forming strain ($P_{4\times8}$ *ts*$_{84}$) was grown in a tryptone–yeast extract medium. Late log phase overnight cultures grown at 30 °C were diluted into fresh medium at an $A_{600\ nm} = 0.07$ and grown aerobically at 30 °C until $A_{600\ nm} = 0.1$. The exponentially growing cultures were transferred to 41 °C and incubated with shaking for 2 h (at that temperature). At this time the presence of long filaments was confirmed by phase-contrast microscopy. Strain MAD-1 was grown in glucose minimal salts medium as previously described [11], under which conditions the morphologic and permeability abnormalities are expressed. Membrane vesicles were prepared from the filament-forming strain exactly as was done with wild type strains [2] except that the extensive homogenization and wash procedure were omitted. All protein determinations were by the Lowry method [12].

Separation of inner and outer membrane fractions

Cytoplasmic membrane enriched and wall enriched fractions of the *E. coli* cell surface were prepared by the method of Schnaitman [13]. This procedure involves disruption of the cells with a French pressure cell and separation of cell surface fractions by layering on sucrose gradients. The gradients were fractionated in an

ISCO gradient fraction collector and monitored for absorbance at 280 nm. The resulting cytoplasmic membrane-enriched and wall-enriched fractions were washed in 0.1 M sodium phosphate buffer, pH 6.6, containing 1 mM EDTA, and were stored in the same buffer. The cytoplasmic membrane fraction was found to have a specific activity of NADH oxidase some 8–10 times greater than the wall enriched fraction, a purification which is similar to that reported by Schnaitman [13] for other cytoplasmic membrane markers.

Membrane disaggregation, polyacrylamide gel electrophoresis, and fractionation of gels

Conditions for gel electrophoresis were as previously described [2–5] with electrophoresis being performed in 5 % acrylamide gels containing 0.1 % sodium dodecylsulfate in 0.1 M sodium phosphate buffer (pH 7.1). Membranes were first disaggregated in 1 % sodium dodecylsulfate at 40 °C (analytical electrophoresis) or 2 % sodium dodecylsulfate at 40 °C (preparative electrophoresis). Electrophoresis was at 8 mA per tube and continued until the tracking dye (bromophenol blue) had travelled 7 cm in 10-cm gel columns. Gels were fractionated with the aid of an Autogel divider (Savant), the gel being eluted with 0.1 % sodium dodecylsulfate directly into scintillation vials, after which 10 ml of Instagel (Packard) emulsifier–scintillant was added to each vial. When gels were stained, they were fixed in methanol–acetic acid–water (5 : 1 : 5, by vol.) and stained in the same solvent containing 0.25 % Coomassie Blue stain and then decolorized in 5 % methanol–7 % acetic acid.

Preparative gel electrophoresis was carried out with a Shandon apparatus using the same 5 % polyacrylamide–0.1 % sodium dodecylsulfate gels employed in the analytic gel system. A 5-cm gel was run at 75 mA for 9 h while being cooled with tap water. The elution buffer was 5 mM Tris–HCl, 0.1 % sodium dodecylsulfate, pH 8.1. Fractions were monitored for absorbance, at 280 nm by the use of an ISCO Model UA-4 absorbance monitor. Radioactivity of the fractions was assessed in a Beckman LS-230 liquid scintillation spectrometer using Instagel (Packard) scintillant.

Pooled fractions from the preparative electrophoreses were dialyzed against a buffer containing 5 mM Tris–HCl, pH 8.1, and 1 mM dithiothreitol in order to remove as much of the sodium dodecylsulfate as possible. With dialysis it was possible to remove over 99 % of the sodium dodecylsulfate as monitored by the inclusion of sodium dodecyl-[^{35}S]sulfate in the samples. Samples were then concentrated at room temperature by ultrafiltration through a Diaflo PM-10 filter (Amicon).

Preparation of antiserum

Female New Zealand white rabbits were used as the source of antiserum in all the experiments. After obtaining non-immune serum by a control bleeding, rabbits were immunized with membrane vesicle preparation (1.5 mg of membrane protein in Freund's incomplete adjuvant in a total of 3.0 ml) by subcutaneous injection at several sites. All animals received six injections of antigen at 1-week intervals and were bled 3–5 days after the sixth injection. Rabbits were given booster injections several weeks after the initial immunization and were bled 3–5 days later. Serum was separated from coagulated blood, and stored at −20 °C. Immunization was also performed with an isolated fraction from the preparative gel electrophoresis. In this case 0.5 mg of membrane protein in 0.01 M phosphate, 1 mM EDTA, pH 6.6

buffer (containing 0.5–1 % sodium dodecylsulfate) was used in place of the membrane vesicles, also in Freund's adjuvant.

Antigenic analysis

Double diffusion assays were performed on immunodiffusion discs (Miles) in a moisture chamber at room temperature for 1–3 days. In addition, a technique was developed for utilizing sodium dodecylsulfate gel electrophoresis in conjunction with immunodiffusion. For these experiments sodium dodecylsulfate gel electrophoresis was performed according to our routine procedures. The gel was removed from the tube and cut in half along its long axis. One-half of the gel was stained and the other half placed in a petri dish. Agarose, 1 %, in 0.05 M barbital buffer, pH 8.4, was pipetted into the dish and allowed to solidify. Trenches were cut in the agar and filled with the appropriate antiserum. The plates were then incubated in a moisture chamber at room temperature for 5–7 days. Precipitin bands were detected after washing with 1 % NaCl and staining the petri dishes with 1 % amido schwarz stain in 7 % acetic acid.

Other immunological techniques

Antisera were absorbed with lipopolysaccharide or membrane protein fractions by addition of the antigen of interest to an antibody preparation. The quantitative precipitin assays were performed by incubating [^{3}H]leucine-labeled membranes, solubilized as for electrophoresis, with antisera, at 37 °C for 0.5 h, followed by filtering the complexes through Whatman GF/C glass fiber filters and washing with 0.01 M phosphate, 1 mM EDTA, pH 6.6. The filters were dried, put into 6 ml of Liquifluor (New England Nuclear), and counted in a liquid scintillation counter. To obtain reproducible results with this technique it was necessary to wash all the samples with identical amounts of the salt solution.

Fluorescein labeling of antiserum

Following the procedure outlined by Clausen [14], rabbit antiserum was purified by precipitation with 2 M $(NH_4)_2SO_4$ (pH 6.8). The $(NH_4)_2SO_4$–antiserum mixture sat at room temperature for 18 h and the precipitate was recovered by centrifugation for 15 min at $5000 \times g$. The precipitate was washed twice with 2 M $(NH_4)_2SO_4$, dissolved in 0.1 M $NaHCO_3$, and then dialyzed against 0.1 M $NaHCO_3$ for 18 h. The protein concentration was determined by the method of Lowry et al. [12].

The immunoglobulin fraction (100–150 mg of protein in 3–5 ml) was mixed with 10.5 ml of 0.9 % NaCl and 3.0 ml of a CO_3^{2-}–HCO_3^- buffer at pH 9.2. The buffered immunoglobulin fraction was then mixed with 7.5 mg of fluorescein isothiocyanate dissolved in 1.0 ml dioxane and 0.5 ml acetone, and the mixture was stirred for 18 h (0 °C). Most of the unreacted fluorescein isothiocyanate was then removed by dialysis of the mixture against 8 l of 0.9 % NaCl. The contents of the dialysis sacs were adjusted to pH 7.0 and the solution was extracted with 2 vol. of ethyl acetate. The aqueous phase was concentrated to dryness; the conjugated immunoglobulin fraction was then redissolved in 1–3 ml of 0.1 M sodium bicarbonate. This material was stored at −20 °C.

Reaction of bacteria, spheroplasts, and membrane vesicles with fluorescein isothiocyanate-labeled antiserum

Cells were grown to mid-log phase and were harvested by centrifugation. They were washed once with 0.03 M Tris–HCl, pH 8.1, and resuspended in the same buffer to $A_{600\ nm} = 0.1$–0.2. Cells (1.0 ml) were incubated for 0.5 h at 37 °C with 0.5 ml of fluorescein isothiocyanate-labeled antiserum diluted 10-fold with 0.1 M $NaHCO_3$. In some experiments the washed cells were suspended in 0.03 M Tris– HCl, 10 mM EDTA, 20 % sucrose, pH 8.1 for reaction with the fluorescein isothiocyanate-labeled antibody, which had been similarly diluted in sucrose containing buffer. In both cases after the incubation with labeled antibody the cells were washed twice and then resuspended in 1.0 ml of the buffer used for the incubation.

Spheroplasts were prepared by incubation in the sucrose–EDTA-Tris buffer described above, containing lysozyme (0.5 mg/ml). After 30 min of incubation with lysozyme, 1.0-ml aliquots of the spheroplast preparations were incubated directly with fluorescein isothiocyanate-labeled antibody preparation diluted 10-fold in the Tris–EDTA–sucrose buffer. After 30 min at 37 °C, the spheroplasts were washed twice with the Tris–EDTA–sucrose buffer and then examined by microscopy.

Membrane vesicles, prepared as described above, were washed twice in 0.1 M $NaHCO_3$ or another buffer for incubation with labeled antibodies. The protein concentration of the membrane vesicles during incubation was 0.5 mg/ml. The reaction with fluorescein isothiocyanate-labeled antiserum was as described for cells and spheroplasts. After a 30 min incubation at 37 °C, the membrane vesicles were washed twice and resuspended in the appropriate buffer before microscopy.

Fluorescence microscopy

Samples of cell, spheroplast, or membrane vesicle suspensions were placed on clean glass slides and covered with a glass cover slip. The preparations were examined by dark field fluorescence microscopy using a Zeiss microscope system. The light source was a high pressure mercury lamp (HBO-200). The excitation filter employed was a cesium interference filter (Zeiss fluorescein isothiocyanate-excitation filter), designed for the fluorescein label we have employed. Zeiss barrier filters 50 and 47 were used. Observations were made using dark-field optics at 40× magnification; the magnification factor at the camera back was 160×. Black and white photographs were on Kodak TriX film, exposed for 5–10 min. A 35 mm camera (Zeiss) was employed, and enlargements were made from the 35 mm film.

RESULTS

Immunochemical analysis of membranes from a dnaA mutant and an isogenic wild type strain

Fig. 1 shows the results of a combination of sodium dodecylsulfate–polyacrylamide gel electrophoresis of membrane preparations and subsequent immunodiffusion. The antiserum in this experiment was obtained by immunizing rabbits with isolated membrane vesicles which contained inner and outer membrane components from wild type strain CRT 4615. Gel electrophoreses were performed with membrane vesicles from strain CRT 4615 and an isogenic strain carrying, in addition the *dna*A mutation *ts* 46 (strain CRT 4614). Three classes of precipitin

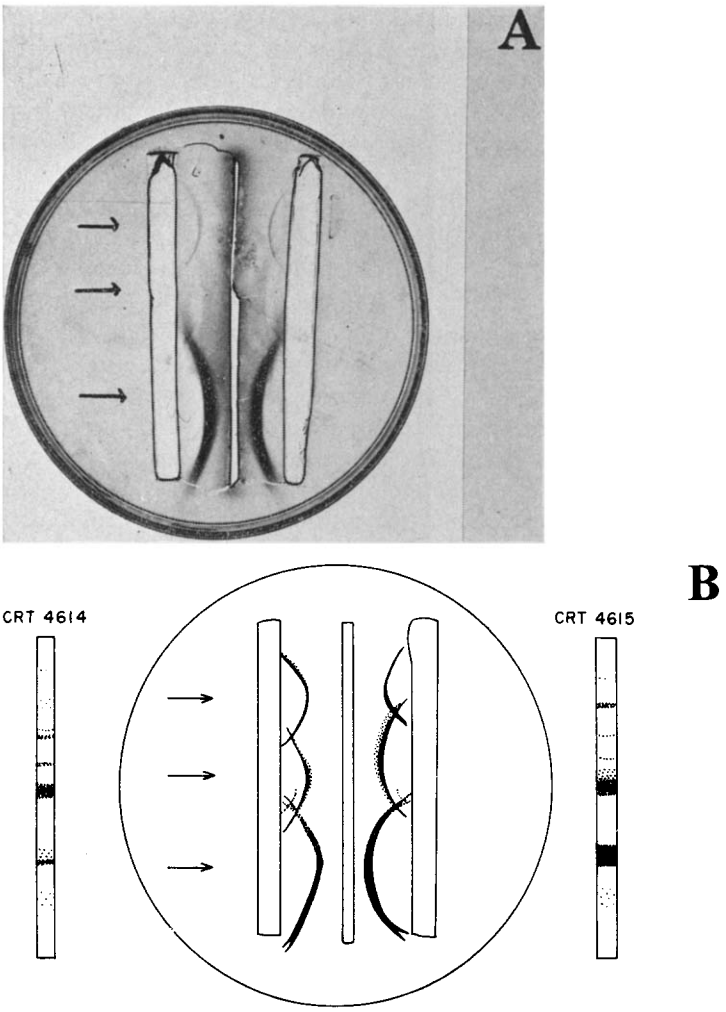


Fig. 1. A. Polyacrylamide gel immunoelectrophoresis of sodium dodecylsulfate-soluble membrane proteins of *E. coli* strains CRT 4615 (right) and CRT 4614 (left). The center well was filled with antiserum prepared against membrane vesicles from the wild type bacteria (CRT 4615). The arrows indicate the three major antibody classes: high molecular weight antigen, intermediate molecular weight antigen, and low molecular weight antigen. B. Drawing of plate shown in A to illustrate all of the bands we have seen in such an electrophoretic separation. Drawings of the sodium dodecylsulfate–acrylamide separation of proteins, stained with Coomassie blue, are shown at the sides of the immunodiffusion experiment. Electrophoresis was from top to bottom in all such immunoelectrophoretic experiments.

bands are formed, corresponding to high, intermediate and low molecular weight antigen, respectively (Fig. 1). The intermediate molecular weight antigens migrate in the area of the membrane protein components we had previously termed Fractions MP40 and MP60. Although it is not clear from the photograph in Fig. 1A, there were three components of the low molecular weight material, two components of the intermediate molecular weight material, and one antigenic component in the high

molecular weight area (Fig. 1B). A similar pattern was seen with antiserum preparations from two different rabbits, although the relative intensities of the precipitin bands varied from one preparation of antiserum to another. There was no reaction with control antiserum. Antiserum prepared by immunizing with membranes from the *dna*A mutant (CRT 4614) gave qualitatively similar results (data not shown).

The chemical nature of the membrane antigens

In order to examine the effect of lipid components on the antigenic reactivity of solubilized membrane, we extracted membrane vesicles with a mixture of chloroform-methanol [15] and solubilized the residue in 2 % sodium dodecylsulfate. The proteins were then separated by analytical sodium dodecylsulfate gels as described above, and gave a protein distribution essentially identical to membrane proteins not extracted with organic solvents. As shown in Fig. 2, this organic solvent extraction did not alter the antigenic properties of the membrane components.

Since lipopolysaccharide is a major surface antigen of *E. coli* and isolated membrane preparations contain varying amounts of lipopolysaccharide [16], the lipopolysaccharide might have contributed to the precipitin bands seen in Fig. 1. When sodium dodecylsulfate gels of *E. coli* membrane proteins were stained by the HIO_4–Schiff reaction for lipopolysaccharide we found positive reaction exclusively in the lower molecular weight area. To see whether the lower molecular weight antigen we saw was lipopolysaccharide, antiserum directed against strain CRT 4615 was absorbed with purified lipopolysaccharide; some precipitate was noted in the mixture. After removal of this precipitate, the absorbed antiserum was reacted against sodium dodecylsulfate gels of membranes from strain CRT 4615 and no differences were seen between absorbed and unabsorbed sera (data not shown), suggesting that the low molecular weight antigen we seen is not lipopolysaccharide. In addition, when purified lipopolysaccharide is dissolved in sodium dodecylsulfate and subject to the immuno-

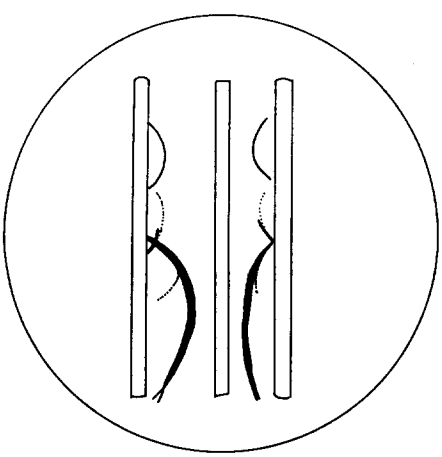


Fig. 2. Polyacrylamide gel immunodiffusion of sodium dodecylsulfate-soluble membrane proteins of CRT 4615 (left) and of chloroform–methanol (2 : 1, v/v) extracted membrane proteins of CRT 4615 (right) dissolved in 2 % sodium dodecylsulfate. The center well contained antiserum directed against unfractionated membrane vesicles.

electrophoretic analysis, no precipitin bands were seen (data not shown), whereas lipopolysaccharide could be detected in the gels by HIO_4–Schiff staining. It is possible that upon diffusing from the gel the lipopolysaccharide aggregates when it reaches a lower sodium dodecylsulfate concentration, and thus is not seen at all in our immunoelectrophoretic assay. Thus, neither lipid nor lipopolysaccharide seem to be complicating our assay system.

Isolation of intermediate molecular weight antigen

Since the intermediate molecular weight antigenic material was that which corresponded to the MP60 and MP40 fractions found to be deficient in *dnaA* mutants,

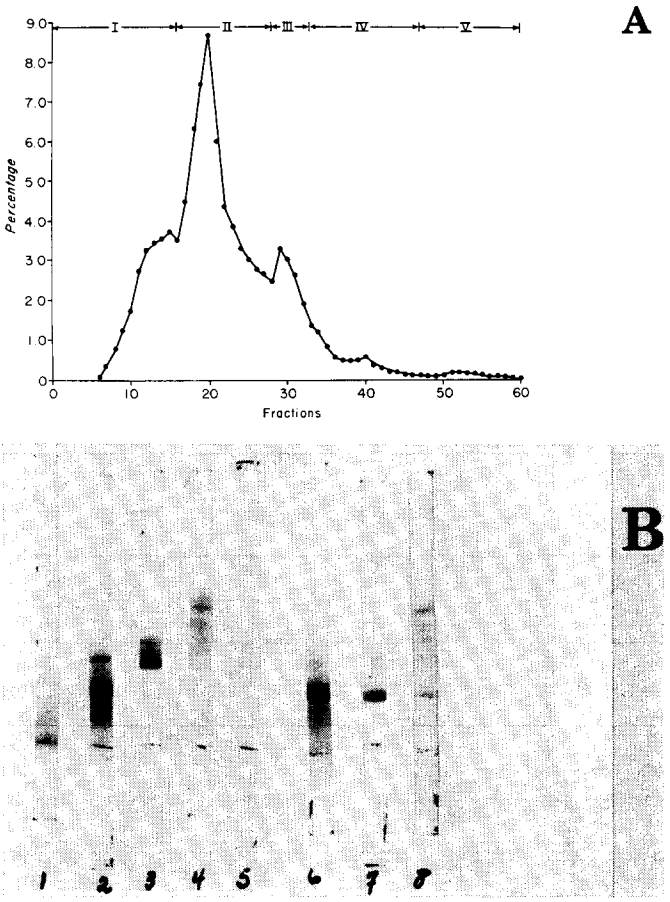


Fig. 3. A. Profile of ^3H radioactivity eluted from an sodium dodecylsulfate–5 % polyacrylamide preparative gel column. 2 % sodium dodecylsulfate solubilized [^3H]leucine-labeled CRT 4615 membranes were applied to the column and run at 76 mA for 12 h. The graph represents the percentage contribution of each fraction to the total radioactivity recovered from the column. B. Electrophoretic patterns of pooled fractions from preparative gel column, shown in Fig. 7A. The fractions were disaggregated at either 40 °C (Fractions I–V for gels 1–5) or 100 °C (Fraction II = gel 6, Fraction III = gel 7, and Fraction IV = gel 8). Fraction III is referred to as the intermediate molecular weight antigen.

we preferred to have antiserum directed specifically against this fraction. An antibody against the intermediate molecular weight antigens would both serve as a reagent to identify this class of membrane proteins and might be useful in attempts to ascertain the role of the MP40 and MP60 fractions, for these membrane proteins have been identified only in terms of their electrophoretic characteristics.

The distribution of labeled membrane protein eluting from the preparative gel was similar to that seen upon fractionating an analytical gel [2–5]. Fig. 3A shows the elution profile of a sample of [^{3}H]leucine-labeled membranes. Fractions were pooled as indicated in Fig. 3A and termed Fractions I, II, III, IV and V. When these fractions were examined by analytical polyacrylamide gel electrophoresis (Fig. 3B) it was seen that a rather good separation of bands was achieved by preparative electrophoresis. Fraction III was selected as the antigen to be used to immunize rabbits, since it both corresponds to the Fraction MP60 area of our analytical gels and also shows the shift in migration typical of Fraction MP60 [3, 4] when it is disaggregated at 100 °C. In the experiment shown, we recovered 95 % of the counts applied to the preparative electrophoresis, with the IMA fraction accounting for 10 % of these. Thus, about a 10-fold purification of the labeled membrane protein was achieved.

Immunological analysis of antibodies directed against intermediate molecular weight antigens

We used pooled preparations of Fraction III isolated from several preparative gel separations, to immunize rabbits. As seen in Fig. 4A, the antiserum obtained by

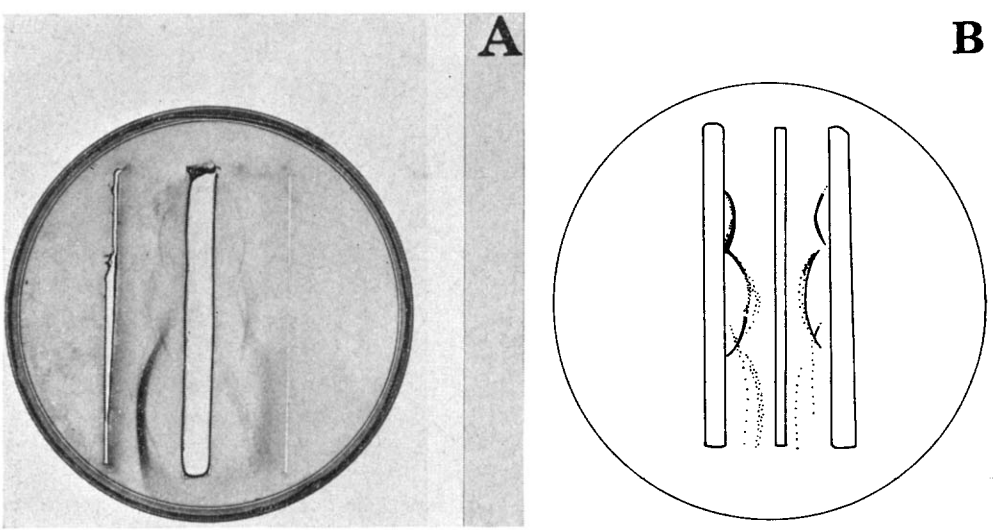


Fig. 4. A. Polyacrylamide gel immunoelectrophoresis of sodium dodecylsulfate-soluble membrane proteins of CRT 4615 diffused against anti-unfractionated membrane antiserum (left well) and anti-intermediate molecular weight antigen antiserum (right well). B. Polyacrylamide gel immunoelectrophoresis of sodium dodecylsulfate-soluble membrane proteins of CRT 4615 (right) and CRT 4614 (left). The center well was filled with antiserum prepared against the intermediate molecular weight antigen fraction of CRT 4615.

such an immunization procedure yielded a somewhat different immunoprecipitation pattern than that directed against unfractionated membrane vesicles. The class of antibodies which was most decreased in antiserum directed against intermediate molecular weight antigen was that which reacted with lower molecular weight membrane material (Fig. 4A). However, the antiserum obtained by immunization of rabbits with sodium dodecylsulfate-solubilized intermediate molecular weight protein behaved in a similar fashion toward intermediate molecular weight antigen and higher molecular weight antigens as did that obtained by immunizing rabbits with unfractionated membrane vesicles. Thus, the antisera directed against a membrane protein fraction (intermediate molecular weight antigen) solubilized in 1 % sodium dodecylsulfate gave precipitin patterns similar to those seen with antisera directed against unfractionated membrane vesicles, with respect to the intermediate and higher molecular weight membrane antigens. The antiserum has a low level of antibodies directed against material other than isolated intermediate molecular weight antigen, probably because trace components in the Fraction III preparations served as stimulators of the immune response.

Fig. 4B shows the reaction of the anti-intermediate molecular weight antigen antiserum with membrane proteins from the *dna*A mutant and its isogenic wild type. As with antibody directed against whole membrane vesicles (Fig. 1) there is no qualitative difference between the mutant and wild type. To test further for qualitative antigenic differences, the antisera directed against membrane preparations from the wild type strain were absorbed with membrane proteins from the otherwise isogenic *dna*A mutant. This absorption did not reveal any components which might have been masked in the unfractionated antisera. At all levels of absorption, all that was detected was a gradual diminution of the bands originally seen. No one band persisted after the absorption.

Quantitative assay of membrane protein precipitation by antiserum

As shown in Figs 1 and 4, there is no qualitative immunological difference between membrane preparations from *dna*A mutant and wild type strains, even though differences in their membrane protein composition [2, 5] had previously been detected. Two possibilities may account for the inability to detect a qualitative difference in membrane protein immunological activity. First, the detergent-solubilized membrane preparations we employ may select for a subpopulation of antigens, that is, those which can react with antiserum after being treated with 1 % sodium dodecylsulfate at 40 °C. This subpopulation might be common to both mutant and wild type membranes and thus qualitative differences between the preparations might be obscured. On the other hand, the difference in membrane protein composition may be a quantitative rather than a qualitative one. In order to see whether we could detect a difference between mutant and wild type membrane preparations by immunologic techniques in the presence of sodium dodecylsulfate we employed a semiquantitative precipitin assay, which is illustrated in Fig. 5.

When [^{3}H]leucine-labeled membranes from strain CRT 4614 (*dna*A) and CRT 4615 (wild type) are solubilized in 1 % sodium dodecylsulfate and then reacted with antisera, precipitates occur which may then be resolved from the soluble membrane proteins by filtration through Whatman GF/C filter discs. When the membrane proteins are examined at the same protein concentration and reacted with antibody

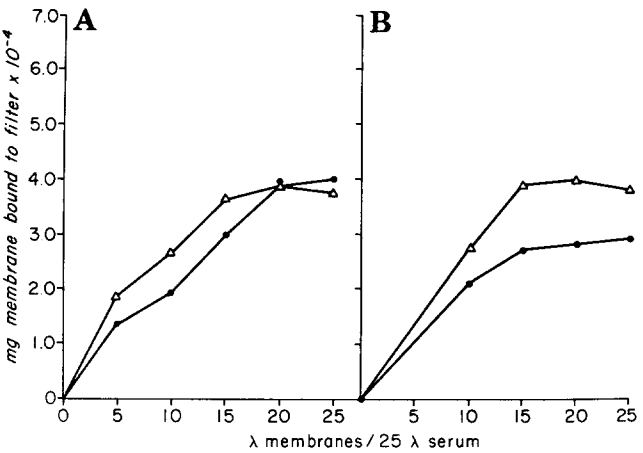


Fig. 5. A. Effect of increasing membrane concentration on precipitate formation with antiserum directed against whole membrane vesicles. Sodium dodecylsulfate-soluble CRT 4615 (△) or CRT 4614 (●) membrane proteins at a concentration of 0.23 mg/ml were used. The amount of membrane protein bound per filter is plotted against increasing membrane/serum ratio. B. Effect of increasing membrane concentration on precipitate formation with antiserum directed against intermediate molecular weight antigen. Sodium dodecylsulfate-soluble CRT 4615 (△) or CRT 4614 (●) membrane proteins at a concentration of 0.23 mg/ml were used. The amount of membrane protein bound per filter is plotted against increasing membrane/serum ratio. In each case a maximum of 8–10 % of the membrane protein was bound to the filter.

directed against whole membrane vesicles, labeled material precipitated at several different ratios of antigen to antibody (Fig. 5A). There is slightly more wild type than mutant membrane material precipitated by the antiserum directed against whole membrane vesicles (Fig. 5A). The more specific antiserum, directed against intermediate molecular weight antigen, caused more precipitation of wild type membrane protein than mutant membrane protein at each ratio of antigen to antibody (Fig. 5B). This precipitation assay, although somewhat crude, suggests that the antibody preparation we have can indeed discriminate between membrane preparations from the wild type strain and an otherwise isogenic *dnaA* mutant, which differs some 5 % in membrane protein composition [2, 5].

Location of the antigens in the E. coli cell envelope

We have previously shown [5] that the Fraction MP40 component is enriched in the cytoplasmic membrane whereas the Fraction MP60 component seems to be distributed in both cytoplasmic and wall fractions. To analyze distribution of the membrane protein antigens examined above, we have isolated cytoplasmic membrane enriched and wall enriched preparations by the method of Schnaitman [13] (described in Methods).

An analysis of the sodium dodecylsulfate gel electrophoresis and subsequent immunodiffusion patterns of the separated inner and outer membrane preparation is shown in Fig. 6. Fig. 6A shows the results obtained when gels of the cytoplasmic membrane fraction (left well) or outer membrane fraction (right well) are diffused against an antiserum directed against the intermediate molecular weight antigen.

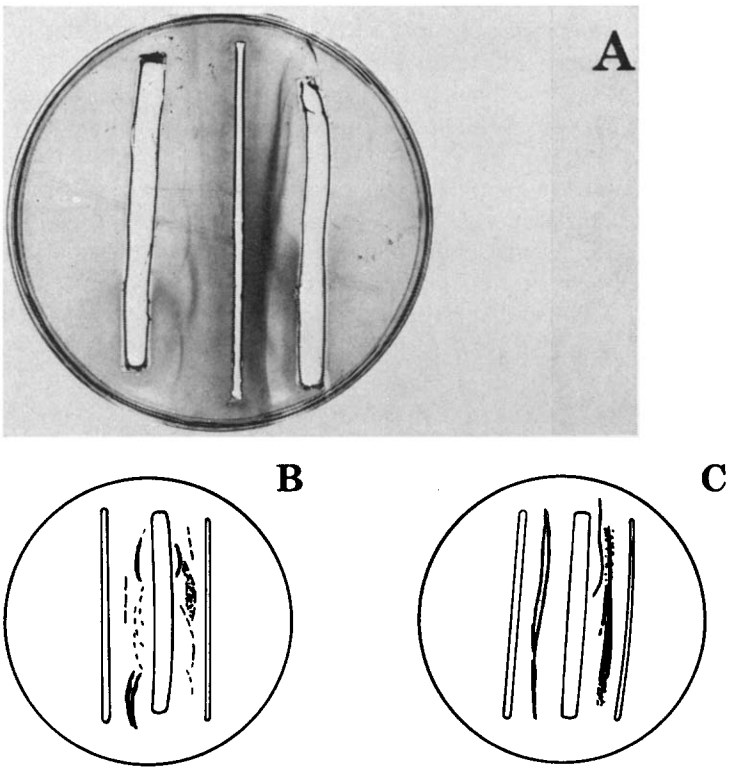


Fig. 6. Analysis of cytoplasmic membrane enriched and cell wall enriched preparations by sodium dodecylsulfate gel electrophoresis and subsequent immunodiffusion. The cytoplasmic membrane enriched and cell wall enriched fractions were prepared by the method of Schnaitman [13]. In A the center well was filled with antiserum prepared against intermediate molecular weight antigen; the left well contained a gel in which cytoplasmic membrane enriched fractions were separated by sodium dodecylsulfate–acrylamide electrophoresis; the right well contained gel in which the cell wall enriched fraction was separated in a similar system. As in the other experiments the direction of migration of electrophoresis was from the top to bottom. All other conditions were as described in Methods. In B the cytoplasmic membrane-enriched fraction was separated by electrophoresis and placed in the center with subsequent immunodiffusion against another preparation of anti-unfractionated membrane antiserum (left well) and anti-intermediate molecular weight antigen antiserum (right well). In C a polyacrylamide gel containing separated cell wall components was diffused against anti-unfractionated membrane antiserum (right well) or anti-intermediate molecular weight antigen antiserum (left well).

The cell wall-enriched preparation (right well) exhibits broad diffuse precipitin bands with few discrete components. On the other hand, the cytoplasmic membrane-enriched fraction (left well) exhibits a population of discrete antigenic components reminiscent of those seen with whole membrane preparations. The heavy precipitin bands seen in the cell wall enriched fraction was not nearly so apparent in our previous experiments (Figs 1, 2 and 4), in which the membrane preparations were from lysozyme-induced spheroplasts. We have not pursued the significance of these differences which appear to be related to the membrane preparation technique employed. The differences between the cytoplasmic membrane fractions or cell wall-enriched

membrane fractions are further illustrated in Figs 6B and 6C. In Fig. 6B the cytoplasmic membrane fraction was separated by sodium dodecylsulfate gel electrophoresis and diffused against either anti-unfractionated membrane antiserum (left well) or anti-intermediate molecular weight antigen antiserum (right well). Several precipitin lines are seen which again look much like those found in the experiments on unfractionated bacterial membrane preparations (Figs 1, 2 and 4). When the cell wall enriched fraction is examined in a similar fashion using the same antisera (Fig. 6C) diffuse precipitin lines again occur with little evidence of discrete bands. Thus, although it is difficult to argue for identity from the kinds of experiments introduced here, the cytoplasmic membrane preparation appears to have an antigenic composition much more like that of unfractionated membrane vesicles than does the isolated cell wall-enriched fraction.

The nature of antiserum binding to membrane vesicles

We have shown (above) that antisera directed against an isolated membrane protein subfraction (intermediate molecular weight antigen) contained a class of antibodies which are a subset of those seen with antisera directed against whole membrane vesicles. This conclusion was obtained from experiments in which membrane proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and then immunodiffused against different antisera. We wanted to assess whether the two types of antisera would react with membrane proteins as they exist in relatively native conformation, that is, within isolated membrane vesicles from *E. coli*. Toward this end we have prepared fluorescent-labeled derivatives of each antiserum. The labeling technique is described in Methods.

As seen in Fig. 7 antiserum directed against unfractionated membrane vesicles (Fig. 7A) yields brilliantly fluorescent particles when reacting with membrane vesicles. Fluorescein-conjugated control antiserum prepared from a rabbit before immunization gave essentially no fluorescence when reacted in a similar fashion (data not shown). Thus, the fluorescence obtained is dependent upon the presence of antibodies directed against bacterial membrane components. All of our conclusions from the types of experiments to be presented below are based upon examination of single particles or small aggregates, since large aggregates fluoresce greatly even with the fluorescein labeled control serum. This spurious fluorescence was probably caused by difficulty in efficiently washing aggregated clumps of particles.

Membrane vesicles in *E. coli* are 0.1–1.5 μm in diameter, a size which is at the limit of resolution for the fluorescent microscopy technique. However, by employing a filament forming mutant of *E. coli*, one may obtain huge membrane vesicles, some 10 times the diameter of those from wild type cells. This mutant strain, which contains the PAT-84 mutation, produce filaments at 41 °C. Large vesicles may be obtained from these filaments, as long as the homogenization used in washing the vesicles is not too vigorous. Such large membrane vesicles react with fluorescein labeled antisera in a uniform fashion with occasional bright spots seen upon the surface (Fig. 7B). The results obtained in Fig. 7 were the same whether the conjugated antiserum preparation was one directed against whole membrane vesicles or against the intermediate molecular weight antigen fraction.

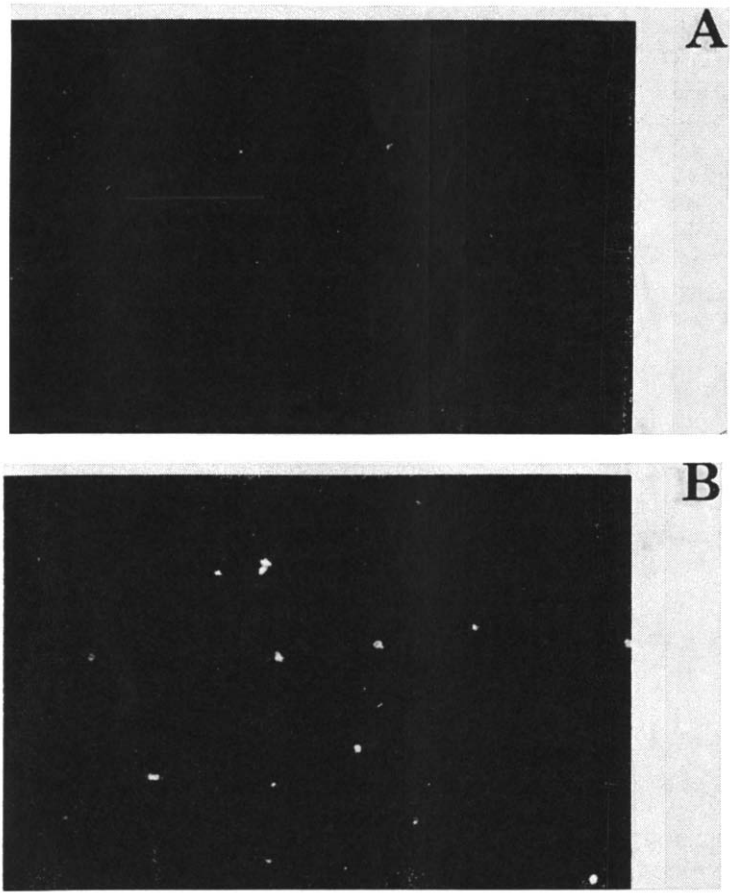
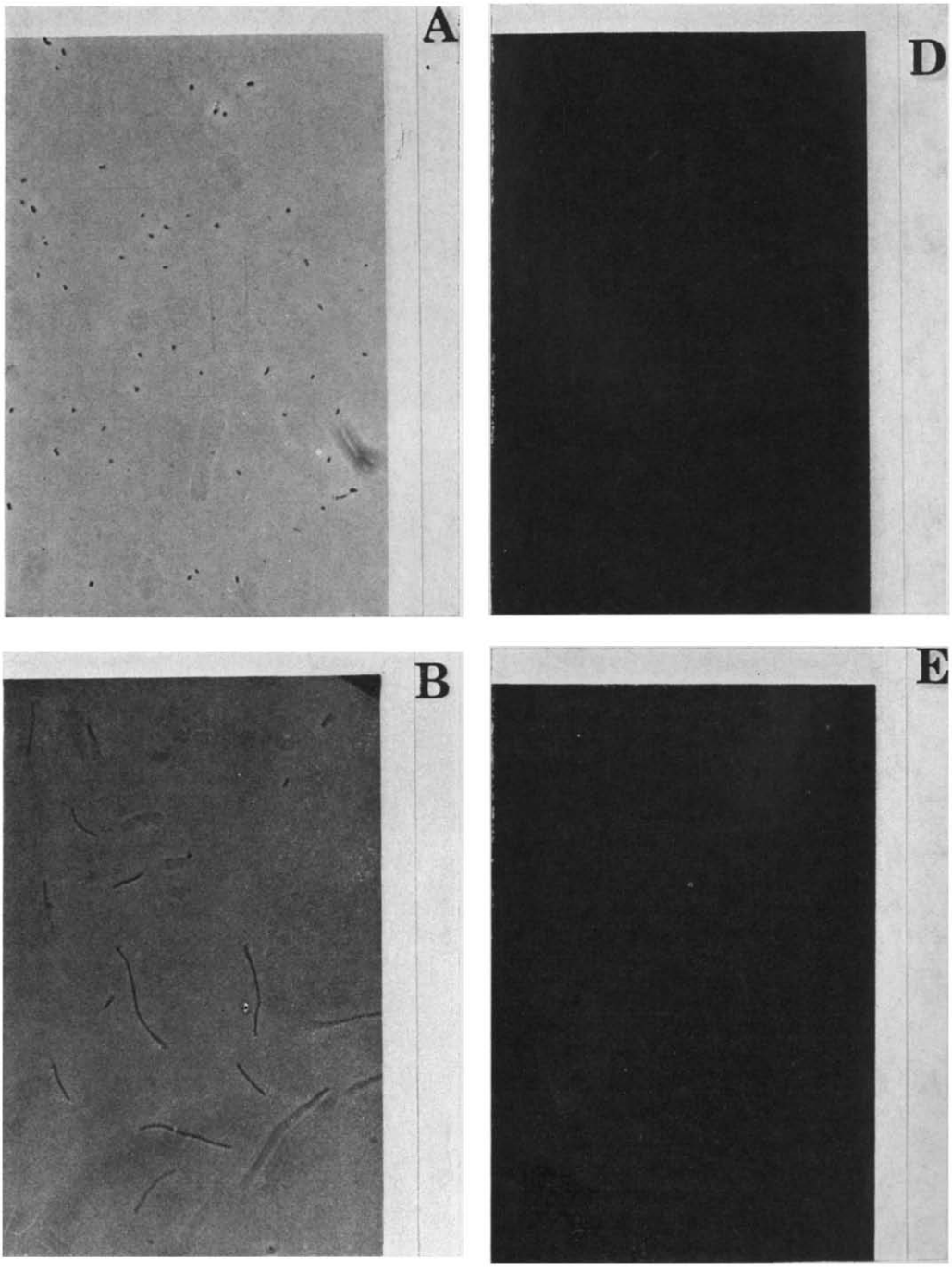


Fig. 7. Immunofluorescence of *E. coli* membrane vesicles after reaction with fluorescein labeled antiserum. Membrane vesicles were reacted with fluorescein isothiocyanate-conjugated antisera and examined as described in Methods. The conjugated antiserum preparation employed was that directed against whole membrane vesicles. (a) Membrane vesicles obtained from strain CRT 4615; (b) Membrane vesicles obtained from strain $P_{4\times8}$, containing the PAT-84 mutation which leads to filament formation at 41 °C.

A barrier to reaction with antiserum exists in intact cells

Membrane vesicles become brilliantly fluorescent when reacted with fluorescein isothiocyanate-conjugated antiserum, as shown above (Fig. 7). However, intact cells from wild type *E. coli* are not fluorescent under similar conditions (Fig. 8). In Fig. 8A and 8D are shown phase-contrast microscopy and dark field microscopy of the same field of a suspension of *E. coli* CR34. Although there are many cells in the field, none of these is fluorescent. If these cells are treated with EDTA and lysozyme, however, to make spheroplasts, they become brilliantly fluorescent when reacted with the same antiserum (data not shown). When membrane vesicles are purified from such cells they are likewise brilliantly fluorescent. The minimal reactivity with antiserum exhibited by whole cells is also demonstrated with a filament forming strain, PAT 84. Filaments, seen in phase in Fig. 8B, are only minimally fluorescent,


A
D
B
E

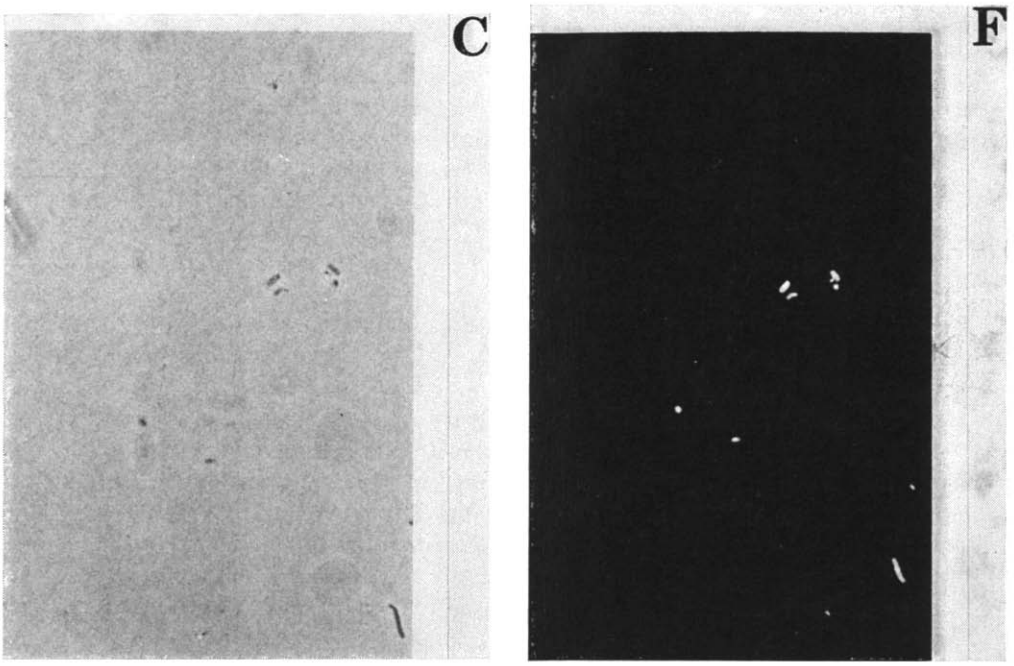


Fig. 8. Fluorescence of *E. coli* cells reacted with fluorescein conjugated antiserum. The same preparation of cells was examined by either phase-contrast microscopy (A, B, C) or dark-field fluorescence microscopy (D, E, F). The fluorescein conjugated antiserum preparation was that directed against isolated membrane protein subfraction. The strains examined were: CR34 (A, B); $P_{4\times8}$ *ts*$_{84}$ (C, D); the MAD-1 derivative of CR34 (E, F). The same results were seen when the antiserum preparation was that directed against whole vesicles.

as shown by the dark field fluorescence microscopy employed in 8E. Both spheroplasts and membrane preparations from this strain are highly fluorescent when reacted with fluorescein isothiocyanate labeled antiserum.

A surface altered mutant of *E. coli* has been described by Lazdunski and Shapiro [11]. The strain, the MAD mutant, has an increased permeability and an alteration in its morphology and cell division properties. Unlike the two strains just described, the MAD mutant does fluoresce brightly when reacted with fluorescein isothiocyanate-labeled antisera (Fig. 8F).

We have studied the reactivity of antiserum with whole cells, spheroplasts, and membrane vesicles, by employing ferritin-conjugated antiserum preparations and electron microscopy. The results found with this technique (data not shown) were identical to the ones seen with fluorescence microscopy. Membrane vesicles and spheroplasts from all the strains examined had many ferritin particles localized over them. The extent of ferritin binding was the same, whether the conjugated antiserum employed was that directed against unfractionated membrane or against the intermediate molecular weight antigen. Of the intact cells examined by this technique, the MAD mutant was the only cell type with ferritin molecules distributed over its surface. There was no localization of ferritin molecules seen by this technique either in membrane vesicles or in the MAD mutant.

DISCUSSION

A striking conclusion from the above data is that membrane proteins isolated by polyacrylamide gel electrophoresis in 1 % sodium dodecylsulfate may be used to immunize rabbits; they then lead to the production of antibodies which are subset of those seen when whole membrane vesicles are used in the immunization. Such a preliminary isolation of proteins by sodium dodecylsulfate gel electrophoresis may be a general useful technique for obtaining antisera to specific membrane components which are otherwise difficult to fractionate. Furthermore, as demonstrated in the fluorescence microscopy experiments (Figs 7 and 8). when antisera are prepared against isolated membrane protein fractions, they are reactive with intact membrane vesicles. Thus, such antisera might be used to assess the functional significance of membrane protein components.

There appears to be some specificity in these antiserum preparations. As shown in Fig. 5, the antisera can discriminate between membranes from wild type cells and those from *dna*A mutants. Less protein is precipitated from solubilized preparations of membranes from *dna*A mutants than from similarly treated membrane preparations from wild type. Since the *dna*A mutant is missing several membrane protein components [2, 5] and the precipitation difference is more pronounced with anti-intermediate molecular weight antigen antiserum, directed against such components (Fig. 5B), the data suggest that the antisera can assay for the amounts of the protein com ponent altered in *dna*A strains. It is then interesting that we find no qualitative difference between the *dna*A strain and wild type (Figs 1 and 4). Thus the *dna*A mutant differs from the isogenic wild type in its quantitative membrane protein composition. A qualitative difference might exist and not be detected by our techniques; however, since we do see a change in the amount of precipitable material, we suggest that the antiserum does identify the protein components altered in *dna*A mutants. We have attempted to use the antimembrane protein antibodies to inhibit several in vitro DNA replication systems without obtaining any specific inhibition (Giza and Shapiro, unpublished). Thus, the question of whether the membrane alterations in *dna*A mutants are significant for the physiology of the *dna*A mutation remains unclear. As we have emphasized in our previous papers, while these changes are clearly concomitants of the *dna*A mutation, they may not be linked to the physiology of DNA replication.

The antibodies which we have produced when either whole membrane vesicles or isolated membrane proteins were used as immunizing antigens are restricted from reacting with intact *E. coli* cells. However, in a highly permeable MAD mutant of *E. coli* which has an as yet undefined alteration on its cell surface, the antisera react with intact cells. Likewise, the antisera react with both spheroplasts and with isolated membrane vesicles. Thus, the antigenic sites are cryptic in intact cells, but are exposed either in a surface-altered mutant or when the cells are treated in a fashion to alter the cell surface. This suggests that the antigens are either localized on the inner membrane or are buried within the outer membrane. When we examined fractionated membrane preparations (Fig. 6) we found that the inner membrane preparation gave a precipitin pattern most like that seen with whole membrane vesicles. This data likewise supports the idea that the antigens examined are localized to the inner membrane, but in all cases, diffuse precipitates were seen with outer membrane

preparations (Fig. 6). Thus, an unequivocal assignment of the antigens to the inner or outer membranes cannot be made.

In this study, we have disaggregated *E. coli* membrane preparations at 40 °C and separated the proteins by preparative gel electrophoresis. We have previously shown that the migration of proteins from the *E. coli* envelope differs depending upon whether disaggregation is at 40 or 100 °C; in addition, the protein components altered in *dna*A mutants are among those whose migration rate differs [3, 4]. After the membrane proteins are disaggregated at 100 °C, none of the antigens may be detected by the immunoelectrophoretic techniques described above (Smith, R. P. P., unpublished). Thus, by employing milder conditions of disaggregation, even in 1 % sodium dodecylsulfate, it is possible to perform immunological experiments. The use of such mild disaggregation conditions, followed by separation of specific protein fractions by gel electrophoresis, might prove generally useful in immunological studies of membrane protein function.

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