Immunochemical Characterization of Major Outer Membrane Components from *Salmonella typhimurium*

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We used crossed immunoelectrophoresis to study detergent-solubilized components of the outer membrane of *Salmonella typhimurium* under nondenaturing conditions. The antisera used were raised against nondenatured outer membrane preparations. Lipopolysaccharide and lipoprotein were identified easily as discrete precipitates when they were solubilized with Triton X-100. However, solubilization of the porins with Triton X-100 resulted in a complex precipitate pattern, indicating incomplete dissociation of protein-protein interactions. A clear-cut pattern was obtained when the porins were first solubilized and denatured with hot sodium dodecyl sulfate, followed by removal of the sodium dodecyl sulfate and renaturation in the presence of Triton X-100. Our findings suggested that crossed immunoelectrophoresis can be used to study the antigenicity of nondenatured porins and the antibody responses to them.

The main components of the outer membranes of gram-negative bacteria are lipopolysaccharide (LPS) and the major proteins, including lipoprotein (LP), *ompA* protein, and the porins (7). The porins, which form diffusion pores for small hydrophilic molecules (23), span the lipid bilayer of the membrane and are exposed on the membrane surface (6, 15, 16). Recent studies have suggested that porins could be used as vaccines against infections caused by gram-negative bacteria (19, 29; J. Dankert, H. Hofstra, and T. S. Veninga, Abstr. FEMS Symp. Microbiol. Envelopes, abstr. no. 51, 1980). This has increased the need for immunochemical characterization of the components of outer membranes.

Crossed immunoelectrophoresis (CRIE) is a rapid and sensitive method for immunochemical characterization of antigens and has the special advantage that it permits identification of individual components in crude antigen preparations. This technique was first developed to study soluble proteins (4, 12), but later it was adapted for analyses of membrane proteins solubilized with nonionic detergents (2, 26). However, the use of this technique for the study of outer membrane proteins presents problems. Most of these proteins are not properly solubilized by nonionic detergents (1). Discrete precipitates of outer membrane proteins have been obtained by CRIE if the first electrophoresis takes place in a polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) (30). However, this treatment also denatures proteins,

which is an obvious disadvantage, especially for studying outer membrane proteins as potential vaccines. In this paper we describe a CRIE system that overcomes some of these problems and can be used to study the antigenic properties of nondenatured outer membrane components.

MATERIALS AND METHODS

Bacterial strains. The porin preparations were made from strain SL1909, a rough (Rb$_2$) derivative of subline SD 14 of *Salmonella typhimurium* line LT-2 (28). This strain lacks the 33,000-dalton (33K) *ompA* protein. *Escherichia coli* strain JE 5506 (13) was used to isolate LP.

LPS. The LPS of the Rb$_2$ chemotype (22) was purified by the phenol-chloroform-petroleum ether method (8) from strain SH 5014 (25).

LP. LP was extracted by using 4% (wt/vol) SDS at 100°C, as described by Braun and Rehn (5). However, instead of using lysozyme, we broke the cells by sonication at 0°C. After trypsin treatment and ultracentrifugation, the supernatant containing free LP was used in the experiments. SDS-polyacrylamide gel electrophoresis (PAGE) of this preparation showed no contaminating protein (see Fig. 5A, lane 1), and there was no cross-reaction with purified LPS in CRIE (data not shown).

Porin preparations. Three kinds of porin preparations of different degrees of purification were prepared from Triton X-100-lysozyme-ethylenediaminetetraacetate (EDTA)-treated envelopes (24).

(i) Preparation A. Preparation A was a crude porin preparation obtained as described by Nurminen (24). Lysozyme-EDTA-treated envelopes (25) were extracted twice with 2% (wt/vol) Triton X-100 in the presence of 0.01 M MgCl$_2$; 0.1 g (wet weight) of the sediment from centrifugation at 3,000 × *g* (designated

Triton X-100–lysozyme–EDTA-treated envelopes) was suspended in 1 ml of 0.01 M EDTA containing 0.2% (wt/vol) Triton X-100 and incubated overnight at 37°C with trypsin (0.5 mg/ml). Then, 100 ml of the resulting digested mixture was centrifuged at 20,000 × g, the supernatant was collected, and the sediment was digested once more with trypsin and centrifuged. The supernatants were transferred to Diaflo apparatus (Amicon Corp., Lexington, Mass.) equipped with an XM 50 filter and subjected to ultrafiltration. The retained material was washed with 2 liters of distilled water by further ultrafiltration. This precipitated the porin fraction. The precipitate was suspended in 100 ml of distilled water and centrifuged at 20,000 × g for 20 min. The resulting sediment was washed once with distilled water and twice with 0.05 M EDTA and then solubilized in 0.02% (wt/vol) Triton X-100. This sediment was designated preparation A.

(ii) **Preparation B.** Preparation B was prepared by a method which will be described fully elsewhere (Nurminen, manuscript in preparation). Triton X-100–lysozyme–EDTA-treated envelope preparations (see above) were electrodialyzed against distilled water in approximately 20 ml of 0.2% (wt/vol) Triton X-100 in dialysis bags in a custom-made electrophoresis apparatus that was cooled with circulating ice-cold water. The temperature in the bag did not rise above 37°C. After dialysis, the pH was raised to 8.0, the remaining sediment was removed by centrifugation, and the supernatant was treated with trypsin (0.5 mg/ml) overnight at 37°C and cleared by centrifugation. The resulting supernatant was designated preparation B.

(iii) **Preparation C.** Preparation C was a purified preparation obtained by precipitating preparation B in 0.3 M NaCl at pH 6.0. The precipitate collected by centrifugation was dissolved in 0.01 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 8.6) containing 0.2% (wt/vol) Triton X-100. This procedure was repeated two more times (Nurminen, manuscript in preparation). SDS-PAGE by the method of Laemmli (20) showed that most of the protein was in the position of porins (36K, 34K, and a little 35K, typical of this bacterial line) (25) (see Fig. 3).

(iv) **[125]I-labeled preparation C.** Preparation C (10 μg of protein in 60 μl of 0.5 M sodium phosphate buffer, pH 7.0) was iodinated with 0.5 mCi of ^{125}I by using the conventional chloramine-t method (9); 0.1% (wt/vol) Triton X-100 was present throughout the procedure. After bovine serum albumin was added to a final concentration of 0.04% (wt/vol), excess iodine was removed by exhaustive dialysis against 0.05% (wt/vol) Triton X-100 at room temperature. This final preparation contained 200,000 cpm/μl.

(v) **36K porin.** The 36K porin was purified by preparative SDS-PAGE. Samples containing 3 × 10^7 cpm of ^{125}I-labeled preparation C were placed into 10 adjacent 1-cm slots (thickness of gel, 1.2 mm). After electrophoresis the porin bands were located by autoradiography of the wet gel. The 36K porin band was cut out, and the slice was eluted by overnight incubation at 37°C in 3 ml of a solution containing 0.1% (wt/vol) SDS and 0.02% (wt/vol) bovine serum albumin and saturated with phenylmethylsulfonyl fluoride. The eluted 36K protein was concentrated by ultrafil-

tration in collodion bags (type SM 132,000; Sartorius GmbH, Göttingen, West Germany).

Antisera. White New Zealand rabbits were immunized by subcutaneous injections of various crude porin preparations. Preparation A was used to prepare antiserum 1, a crude preparation obtained by treating Triton X-100–lysozyme–EDTA-treated envelopes (see above) with SDS at 50°C. Trypsin (19) was used to produce antiserum 2, and a crude Triton X-100–EDTA extract of Triton X-100–lysozyme–EDTA-treated envelopes (19) was used to prepare antiserum 3. Injections (500 μg of protein per injection) were given at zero time and on days 14, 28, and 42. Antisera were collected 10 days after the last injection. Immunoglobulins were precipitated with ammonium sulfate (50% saturation) and were concentrated after dialysis to one-half of the original volume (18). These immunoglobulins gave 22 separate precipitates in CRIE with crude outer membrane preparations, as described previously (18).

In absorption studies the immunoglobulin solutions were incubated with either LPS (400 μg/ml) or LP (625 μg/ml) for 2 h at 37°C.

Immunoelectrophoresis. CRIE and tandem CRIE were performed as described previously (17, 18) by using the tris(hydroxymethyl)aminomethane-glysin-barbital buffer described by Bjerrum and Lundahl (3); the buffer which we used also contained 0.2% (wt/vol) Triton X-100.

Elution of the CRIE precipitates. Each gel was autoradiographed without drying at 4°C, the radioactive precipitates were cut off separately, and the proteins were eluted from the pieces of gel by incubating them overnight at 37°C in 400 μl of a solution containing 1% (wt/vol) SDS, 0.075% (vol/vol) mercaptoethanol, and 0.02% (wt/vol) bovine serum albumin. The remnants of the agarose gel were removed by centrifugation, and the supernatant was lyophilized and dissolved in 100 μl of SDS-containing sample buffer for SDS-PAGE (20).

Other methods. For autoradiography, the gels were exposed to Kodak X-Omatic XR1 film with an intensifying fluorescent screen. Densitometric scanning of the film was performed with an autoscanner (Helena Laboratories, Beaumont, Tex.); 36K/34K ratios were calculated from the areas of the scanning curves. Proteins were determined by the method of Lowry et al. (21).

RESULTS

Detection of LPS. A complex pattern of precipitates was produced when crude porin preparation A was immunoelectrophoresed across antisera raised against similar preparations (Fig. 1A and C). The porin was solubilized with and electrophoresed in Triton X-100. At least six precipitates were resolved, and the pattern varied according to the antiserum used (Fig. 1A and C).

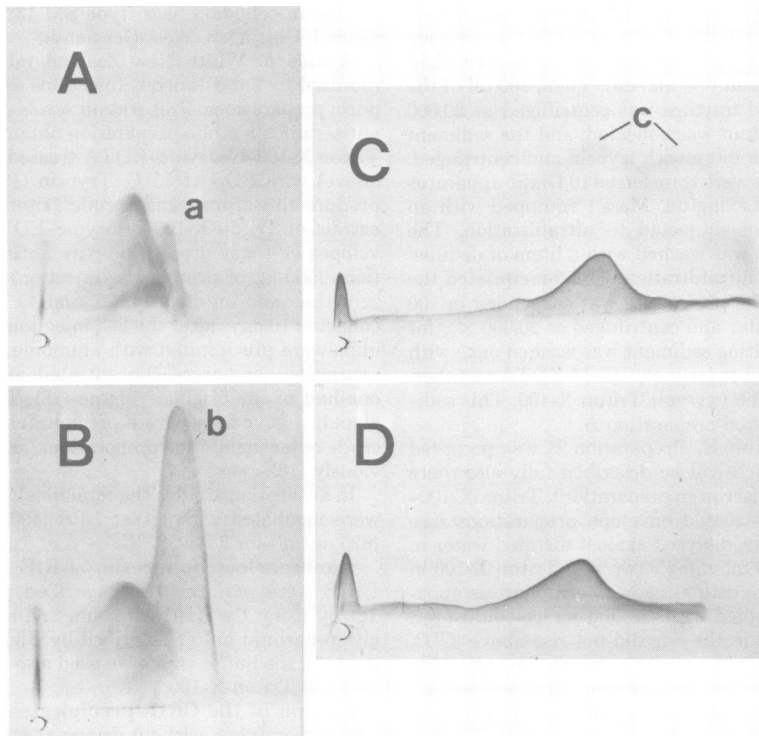We expected that a major component of this crude porin preparation would be LPS. Figure

FIG. 1. *Identification of LPS by CRIE. (A) Crude porin preparation A (40 μg of protein) subjected to CRIE against antiserum 1. The first electrophoresis was for 2 h. (B) Tandem CRIE of porin preparation A (40 μg of protein in the first well) and purified LPS (12 μg in the second well). Other conditions as in (A). (C) CRIE of preparation A (40 μg of protein) against antiserum 2. The first electrophoresis was for 4 h. (D) CRIE of preparation A against antiserum absorbed with LPS (400 μg/ml). Other conditions as in (C).*

1B shows a tandem CRIE comparison of preparation A and highly purified LPS. The LPS precipitate (Fig. 1B, precipitate b) fused with one of the major precipitates of preparation A (Fig. 1A, precipitate a), indicating the presence of LPS in this precipitate.

In another set of experiments, the LPS precipitate was identified by comparing the precipitation patterns obtained with untreated sera and with sera preabsorbed with purified LPS. Figure 1C shows that a prominent fast-moving, weakly stained precipitate (Fig. 1C, precipitate c) and a weak precipitate at the origin were observed with the nonabsorbed serum, but not after absorption of the serum with LPS (Fig. 1D). Absorption with LPS had no effect on the other precipitates.

The putative LPS precipitates were heterogeneous and characteristically weak and hazy. They were always fast moving. We regularly obtained this same pattern with several antisera irrespective of whether the membrane preparations were treated with SDS before the run. This characteristic appearance permitted rapid pre-liminary identification of LPS under standard conditions.

Detection of LP. SDS-PAGE indicated that the semipurified porin preparation (preparation B) consisted of only three major proteins, the LP and the complex containing the 34K and 36K porins. The precipitation patterns of preparation B in CRIE (Fig. 2A) were also simpler than the patterns of crude porin preparations. Two major precipitates were obtained when the antigen was solubilized with Triton X-100. Neither of these precipitates contained LPS when analyzed by tandem CRIE (data not shown).

One of the precipitates (Fig. 2A, precipitate d) was found at the same position as the precipitate obtained with purified LP against the same antiserum (Fig. 2B, precipitate e). Precipitate d fused with the LP precipitate in tandem CRIE (Fig. 2C). After partial preabsorption of the antiserum with purified LP, the precipitate d peak became very weak (Fig. 2D), whereas there was no change in the other major precipitate (precipitate f) or in any of the minor precipitates. No precipitate d was observed when the serum was
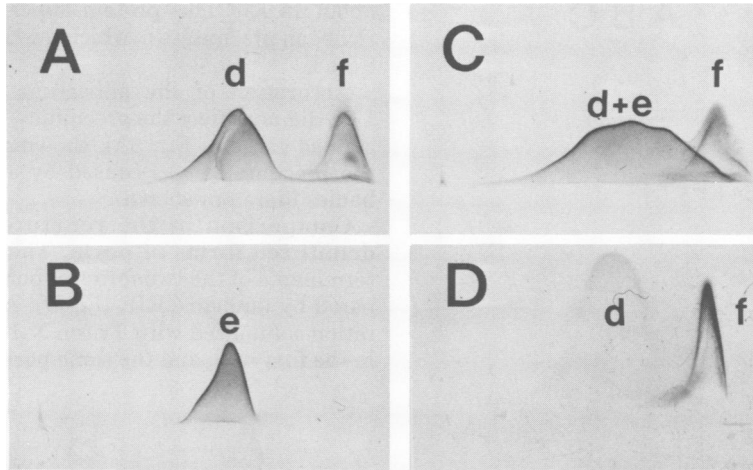
FIG. 2. *Identification of LP by CRIE. (A) CRIE of semipurified porin preparation B (40 μg of protein) against antiserum 2. (B) CRIE of LP (20 μg of protein) against antiserum 2. (C) Tandem CRIE of preparation B (40 μg of protein in the first well) and LP (20 μg in the second well). (D) Tandem CRIE as in (C) against antiserum 2 absorbed with LP (625 μg/ml). In each case the first electrophoresis was for 4 h.*

preabsorbed with larger amounts of LP. Thus, there was little doubt that precipitate d was caused by LP. Furthermore, although LP was solubilized only with Triton X-100, it did not seem to be significantly associated with the other outer membrane components, which presumably cause the other, faster-moving major precipitate.

Detection of porins. A problem with immunological analyses of porins is the inadequate solubilization of these molecules with nondenaturing detergents. We tried to overcome this by the following two-step treatment. The porins were first solubilized (and denatured) with SDS at 100°C. Then SDS was exchanged for the weak nondenaturing detergent Triton X-100. We expected that this detergent replacement would result in at least partial renaturation of the original antigenic determinants.

An extensively purified porin preparation (preparation C) was labeled with ^{125}I. When this preparation was subjected to SDS-PAGE, both Coomassie blue staining and autoradiography (Fig. 3, lanes A and B) showed two major bands at the positions of the 36K and 34K porins. Densitometric scanning of the autoradiograms indicated that the porins comprised more than 90% of the labeled proteins in the preparation. This preparation was then subjected to CRIE after solubilization with SDS at 100°C and renaturation with Triton X-100. The details of this procedure are given in the legend to Fig. 4. Both staining (Fig. 4A) and autoradiography (Fig. 4B) showed only two distinct precipitates, which mi-

grated almost at the same rate in the first run (precipitates g and h).

The identification of nondenatured porins by conventional tandem CRIE comparisons and CRIE absorption procedures was not possible because of a lack of appropriate purified porin preparations. Therefore, we studied the presence of porins in the CRIE precipitates by eluting individual precipitates from the agarose layer and analyzing them by SDS-PAGE. Precipitate h of Fig. 4 contained only 36K and 34K porins (Fig. 5, lane 4). Precipitate g (Fig. 5, lane 3) contained both LP and porins. Densitometric scanning indicated a high enrichment of LP in this precipitate; in fact, the amount of porins was so small that they could have been derived from overlapping precipitate h.

The presence of porins in precipitate h was also shown by the following experiment. The 36K porin was purified by preparative SDS-PAGE (Fig. 3C), solubilized with SDS at 100°C, renatured with Triton X-100, and subjected to CRIE with unlabeled porin (preparation C) as carrier. Only one radioactive precipitate was found (Fig. 4D), at approximately the position of porin precipitate h (Fig. 4A and B). SDS-PAGE confirmed the presence of radioactive 36K porin in the precipitate.

CRIE of Triton X-100-solubilized porins. Radioactive porin preparation C was applied to CRIE gels without prior SDS treatment. In agreement with the weaker solubilizing power of nonionic detergents on membrane proteins, a much more complex pattern of precipitates was
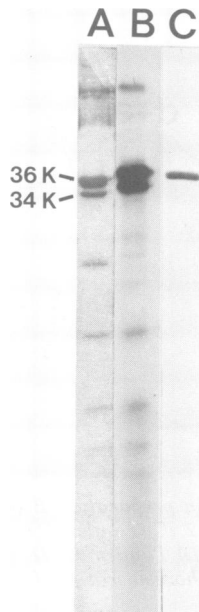
FIG. 3. SDS-PAGE of ^{125}I-labeled preparation C and ^{125}I-labeled purified 36K porin. A total of 5 μg of non-iodinated porin preparation C (lane A), 2 × 10⁵ cpm of ^{125}I-labeled preparation C (lane B), or 10⁵ cpm of purified ^{125}I-labeled 36K porin (lane C) was applied to a 14% gel; 36K and 34K indicate the positions of the 36K and 34K porins, respectively. Lane A was stained with Coomassie blue; lanes B and C were autoradiographed for 24 and 48 h, respectively.

obtained (Fig. 6). Autoradiography revealed seven major, distinct precipitates (Fig. 6B), most of which were also observed in a stained gel (Fig. 6A).

The tops of the major precipitates were cut out from the gel in the pattern shown schematically in Fig. 6C, and the contents of these gel sections were analyzed by SDS-PAGE (Fig. 5, lanes 5 through 10). Because of extensive overlapping of the precipitates, most of the eluates obviously were derived from more than one precipitate. All eluates contained significant amounts of porins. The most clear-cut conclusions could be reached from the tops of precipitates i and j, both of which contained the 36K and 34K porins in roughly the same proportions as the preparation C applied to the CRIE gel. These precipitates also clearly fused to each other. In contrast, the 36K porin was strongly enriched in the eluate of precipitate k; in this respect this precipitate was unique. LP was found in two precipitates (precipitates l and m), both of which also contained porins. However, both of these precipitates were overlapped significantly by porin precipitates i, j, and k. A prominent contaminant of preparation C was a protein having an apparent molecular weight of

about 15,000. This protein appeared to be enriched in precipitate n, which overlapped precipitate l.

Absorption of the antiserum with purified LPS did not affect the precipitate pattern of the stained gel (e.g., Fig. 6A), showing that none of the precipitates was caused by anti-LPS antibodies (data not shown).

Comparison of the renatured and nondenatured forms of porin. The antigenic determinants of the two forms of porins were compared by tandem CRIE (Fig. 7). A porin preparation solubilized with Triton X-100 was placed in the first well, and the same porin preparation
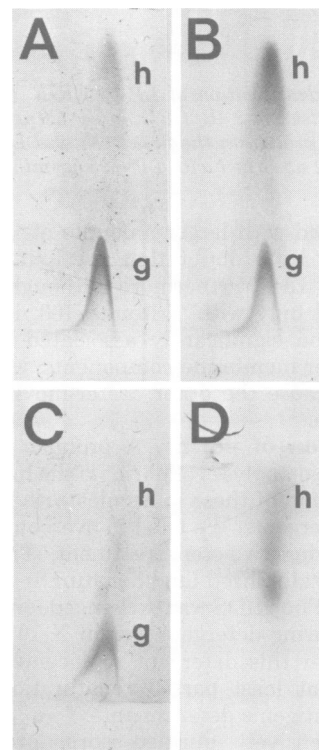

FIG. 4. CIRE of ^{125}I-labeled porins solubilized with SDS and renatured in the presence of Triton X-100. (A and B) ^{125}I-labeled porin. A total of 6 × 10⁵ cpm of ^{125}I-labeled porin and 10 μg of porin preparation C were made to contain 1% SDS and incubated at 100°C for 5 min. After the preparation was cooled to 20°C, Triton X-100 was added to a final concentration of 2% (wt/vol), and the two samples were mixed and subjected to CRIE against antiserum 3. (A) Amido black staining. (B) Autoradiography (exposure time, 24 h) of the same gel. (C and D) Purified ^{125}I-labeled 36K porin. A total of 0.3 × 10⁵ cpm of 36K porin purified by preparative SDS-PAGE and 10 μg of unlabeled porin preparation C were subjected to SDS treatment and CRIE as described above. (C) Amido black staining. (D) Autoradiography (exposure time, 14 days) of the same gel.
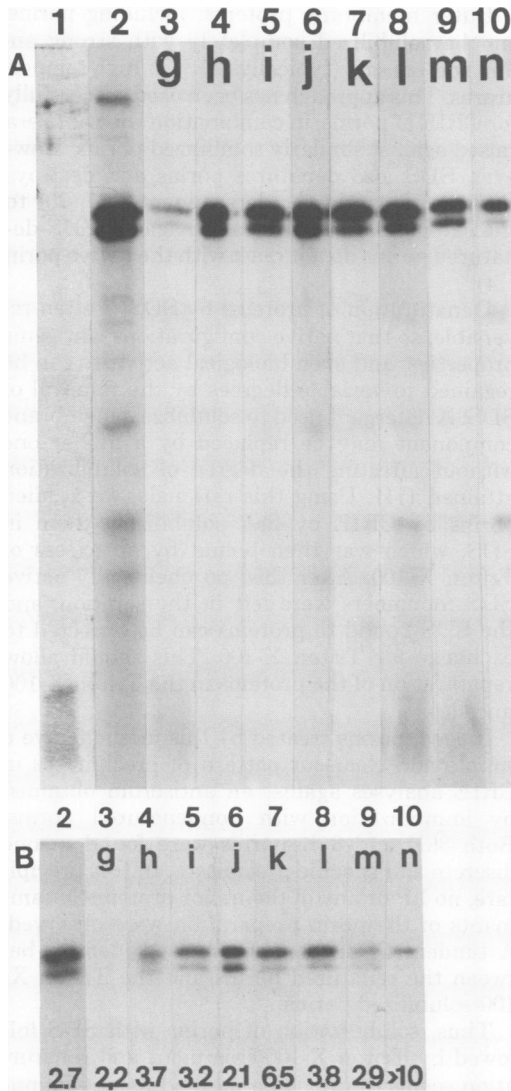
FIG. 5. SDS-PAGE of the proteins eluted from the radioactive precipitates shown in Fig. 4 and 6. Radioactive proteins eluted from the CRIE precipitates as described in the text were applied to a 14% acrylamide gel. The letter symbols on lanes 3 through 10 indicate the precipitates which the lanes contained, as shown in Fig. 4 and 6. Lane 1 contained 10 µg of unlabeled Coomassie blue-stained LP, and lane 2 contained preparation C labeled with ^{125}I. Exposures were for 7 days (A) or 24 h (B). The numbers at the bottoms of the lanes in (B) indicate the ratio of the amount of 36K protein to the amount of 34K protein, as determined by densitometric scanning of the autoradiograms.

first denatured with SDS and then renatured with Triton X-100, was placed in the second well (nearer the anode). Because SDS treatment increased the rate of migration of porins, it was

necessary to make the first electrophoretic run very short (30 min). Precipitates h and g represented the SDS-treated preparation in a pattern very similar to the pattern in Fig. 4A. Precipitates j and l were derived from the nondenatured preparation and were obviously analogous to precipitates j and l in Fig. 6. The LP precipitate of the SDS-treated sample (precipitate g) clearly fused with precipitate l, which represented the LP precipitate of the nondenatured preparation, as described above. The major precipitate j of the nondenatured porin preparation likewise fused with the main precipitate of the SDS-treated preparation (analogous to precipitate h in Fig. 4). This was in good agreement with the finding that both precipitates contained only 36K and 34K porins as determined by SDS-PAGE. It also strongly suggested that the major antigenic determinants recognized by this antiserum were identical in the nondenatured and renatured porin preparations.

DISCUSSION

Of the major components of outer membranes, LPS and LP can be recognized readily in CRIE analyses (27). This was also true in this study, in
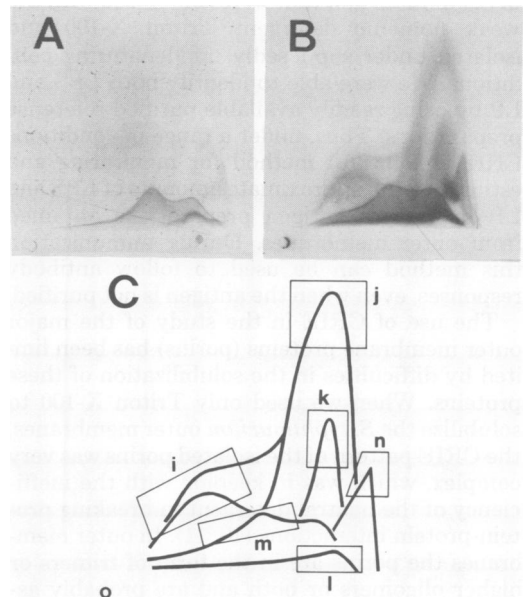

FIG. 6. CRIE of a Triton X-100-solubilized preparation labeled with ^{125}I. A total of 6×10^5 cpm of ^{125}I-labeled preparation C and 10 µg of unlabeled preparation C were mixed and subjected to CRIE against antiserum 3. The first electrophoresis was for 4 h. (A) Stained with amido black. (B) Autoradiography (exposure time, 24 h). (C) Schematic diagram of the precipitates and the portions which were cut out for elution.
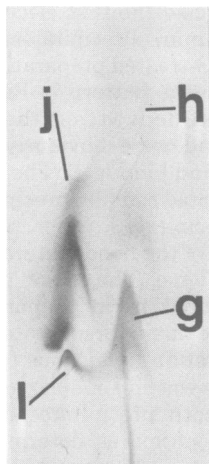
FIG. 7. *Tandem CRIE of the SDS-treated and nondenatured porins. The first well contained 40 µg of porin preparation C, and the second well contained 10 µg of porin preparation C which had been treated with SDS and renatured with Triton X-100 as described in the legend to Fig. 4. The first electrophoresis was for 30 min. The antiserum used was antiserum 3.*

which we analyzed outer membrane components of *S. typhimurium* that were solubilized with the weak nonionic detergent Triton X-100 and isolated under supposedly nondenaturing conditions. We were able to identify both LPS and LP by using readily available purified reference preparations. Thus, under a range of conditions CRIE is a useful method for monitoring and estimating the approximate amounts of LPS and LP in various antigen preparations obtained from outer membranes. During immunization this method can be used to follow antibody responses, even when the antigen is not purified.

The use of CRIE in the study of the major outer membrane proteins (porins) has been limited by difficulties in the solubilization of these proteins. When we used only Triton X-100 to solubilize the *S. typhimurium* outer membranes, the CRIE pattern of the isolated porins was very complex, which was in keeping with the inefficiency of the neutral detergent in breaking protein-protein interactions (10, 11). In outer membranes the porins are in the form of trimers or higher oligomers or both and are probably associated with other proteins and membrane components. Some reassociation of solubilized proteins could also take place during purification. In agreement with this, we found porins in several discrete precipitates. The relative amounts of the 36K and 34K porins varied, and many precipitates contained a mixture of porins and other proteins.

Outer membrane proteins, including porins, can be solubilized completely with strong anionic detergents (typically SDS) at high temperatures. This approach has been used successfully for CRIE of porins, in combination with antisera raised against similarly solubilized porins. However, SDS also denatures porins and destroys their native antigenic determinants (27, 30) to the extent that antisera raised against SDS-denatured porins do not react with the native porin (14).

Denaturation of proteins by SDS is often reversible, so that native configurations, antigenic properties, and even biological activities can be regained to variable degrees by the removal of SDS. A detergent used to solubilize a membrane component may be replaced by a milder one without affecting the degree of solubilization attained (11). Using this rationale, we studied porins by CRIE by first solubilizing them in SDS, which was then bound by an excess of Triton X-100. After this, no chemically active SDS monomers were left in the solution, and the SDS bound to proteins can be expected to exchange for Triton X-100. This should allow renaturation of the proteins in the Triton X-100 micelles.

Indeed, porins treated by this method gave a simple and clear-cut pattern of precipitates in CRIE analyses against an antiserum obtained by immunization with nondenatured porins. Both 36K and 34K porins were found in one discrete and specific precipitate. In this precipitate, no LP or any of the minor protein contaminants of the porin preparation were observed. A tandem CRIE analysis showed identity between the renatured porins and the Triton X-100-solubilized porins.

Thus, solubilization of porins with SDS followed by Triton X-100 treatment and renaturation seems to provide a method for the immunological assay of native antigenic determinants of porins with CRIE. This may be especially useful for studying antibodies or antibody responses to native porins. The lack of reasonably pure nondenatured porin preparations makes CRIE more applicable than any other immunological method for this purpose. However, it remains to be seen whether all important antigenic determinants of native porins are present in the renatured porin preparation. Furthermore, we have not studied the reliability of the method for detecting or assaying porins from a complex mixture of proteins.

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