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Immunochemical Analysis of Inner and Outer Membranes of *Escherichia coli* by Crossed Immunoelectrophoresis

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Isolated membrane fractions of *Escherichia coli* K-12 yielded complex immunoprecipitate patterns when Triton X-100 and sodium dodecyl sulfate extracts were examined by crossed immunoelectrophoresis with antienvelope immunoglobulins. Twelve of the 46 antigens in the immunoprecipitate patterns of inner (plasma) membranes were identified by zymograms and/or by the use of specific antisera. The following enzyme activities were detected in immunoprecipitates: 6-phosphogluconate dehydrogenase (EC 1.1.1.43); adenosine triphosphatase (EC 3.6.1.3); glutamate dehydrogenase (EC 1.4.1.4), two separate components; malate dehydrogenase (EC 1.1.1.37); dihydroorotate dehydrogenase (EC 1.3.3.1); succinate dehydrogenase (EC 1.3.99.1); lactate dehydrogenase (EC 1.1.1.27); reduced nicotinamide adenine dinucleotide dehydrogenase (EC 1.6.99.3); protease (EC 3.4.21.1); and glycerol 3-phosphate dehydrogenase (EC 1.1.99.5). The corresponding immunoprecipitate pattern for isolated outer membranes consisted of at least 25 discrete antigens and differed strikingly from that obtained with inner membranes. Two major immunogens were identified as lipopolysaccharide and Braun lipoprotein. A protease-active immunoprecipitate was also detected in this fraction, but attempts to identify the Rosenbusch matrix protein in the crossed immunoelectrophoretic profile were unsuccessful.

The development of procedures for the successful separation of inner and outer membranes from gram-negative bacteria (33, 44, 54) has proved to be of immense value in furthering our understanding of the structure and distribution of functions within the bacterial cell envelope (8, 41, 43). Significant differences in the morphology and ultrastructure of the two membrane fractions have been established by a variety of electron microscopic techniques (43), and the characteristic distribution of enzymes and of other chemically defined components has been demonstrated (8, 41, 43). In addition, the polypeptide profiles of inner and outer membranes have been compared by numerous investigators, using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (e.g., 44, 45). Although these latter studies have revealed characteristic differences in the polypeptide band patterns, the denaturing effect of SDS generally limits the ability to identify the biological activities or functions of the individual components (16). In contrast, cell components analyzed by immunochemical techniques often retain sufficient biological activity to permit functional characterization (2, 37, 52). However, gram-negative cell envelopes have rarely been analyzed in this way (18, 49), largely because of the poor resolution of these complex systems achieved by classical immunochemical methods (e.g., immunodiffusion and immunoelectrophoresis).

Significant technical advances have been made recently which permit superior resolution of immunoprecipitate patterns (2, 37) by the crossed (two-dimensional) immunoelectrophoresis system originally developed by Laurell (26). Crossed immunoelectrophoresis has been used successfully in studies of mammalian (2, 37) and mycoplasma membrane proteins (20, 21), and, in our studies, it has been of exceptional value in establishing the architecture of plasma and mesosomal membranes of *Micrococcus lysodeikticus* (36, 43) and in the analysis of cell envelope antigens of *Neisseria gonorrhoeae* (50; C. J. Smyth and M. R. J. Salton, *in* R. B. Roberts [ed.], *The Gonococcus,* in press). In the latter investigations, methodological problems associated with analysis of detergent extracts of gram-negative cell envelopes were also evaluated (Smyth and Salton, in press).

The analysis and characterization of the im-

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munoprecipitate patterns obtained by crossed immunoelectrophoresis of detergent extracts of both inner and outer membranes of *Escherichia coli* K-12 are described in this report. The novel, high-resolution reference systems characterized here should facilitate detailed analyses of the surface exposure and distribution of envelope antigens on cells, spheroplasts, and membrane vesicles of gram-negative bacteria.

(This investigation was presented in part at the 8th Meeting of the North West European Microbiology Group, 16 to 18 June 1976, Helsinki, Finland [abstract, p. 156], and at the 77th Annual Meeting of the American Society for Microbiology, 8 to 13 May 1977, New Orleans, La. [abstract K97, p. 202].)

MATERIALS AND METHODS

Bacterial strain and growth conditions. *E. coli* K-12 F⁻ strain 44, a β-lactamase-negative mutant, was derived from *E. coli* K-12 F⁻ strain Pa601 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (32). Cells were grown in LB broth (1) on a shaker (200 rpm) at 37°C. Overnight cultures were diluted 1:10 with fresh LB broth and, after a further 5-h growth period (late logarithmic phase of growth), the cells were harvested by centrifugation (5,000 × *g* for 10 min at 4°C). The wet weight yield of cells was about 3.5 to 4.0 g/liter.

Preparation of envelope fractions. Cells (20 to 30 g, wet weight) were washed once, suspended in buffered sucrose [30 mM tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (pH 8), 20% (wt/vol) sucrose], and treated with lysozyme-ethylenediaminetetraacetic acid (EDTA), and the resulting spheroplasts were harvested by centrifugation by the procedure of Yamato et al. (54). Spheroplasts were then disrupted in an Aminco French pressure cell at 6,000 lb/in², and envelopes were harvested by centrifugation at 40,000 × *g* for 30 min at 4°C and washed by a modification of the method of Yamato et al. (54). The crude envelope fraction was suspended with a Dounce homogenizer, washed three times with buffered sucrose (100 ml/6 g [wet-weight equivalent] of bacteria), suspended in 50 ml of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 2 mM $MgCl_2$ and deoxyribonuclease (DNase, 10 μg/ml), and incubated at 37°C for 15 min. DNase treatment proved to be essential to obtain good separation of inner and outer membranes by sucrose density centrifugation. DNase-treated envelopes were then washed twice with buffered sucrose and dialyzed overnight against 3 mM EDTA (pH 7.2) at 4°C (54).

Isolation of inner and outer membranes. Inner and outer membranes were separated by two-stage discontinuous sucrose density gradient centrifugation (78,000 × *g* at 4°C for 12 to 18 h) in an angle rotor 54) and washed once with 50 mM Tris-hydrochloride buffer (pH 7.5). Membranes were stored as portions of concentrated suspensions (20 mg of protein per ml) in 50 mM Tris-hydrochloride buffer (pH 7.5) at −70°C before detergent extraction. The whole process, from growth of bacteria to isolation of inner and outer membranes, was performed as a continuous operation.

Preparation of detergent extracts of membrane fractions. Membranes were extracted with Triton X-100 or Triton X-100–EDTA essentially by the method of Schnaitman (45, 46). Inner membranes were treated with 4% (vol/vol) Triton X-100 in 50 mM Tris-hydrochloride buffer (pH 8.6) at 23°C for 2 h. Isolated outer membranes were extracted in a similar manner with 4% (vol/vol) Triton X-100 in Tris-hydrochloride buffer (pH 8.6) containing 5 mM EDTA.

Extractions were performed with final membrane protein concentrations of 7 to 18 mg/ml. Supernatant fractions obtained after centrifugation of extracts at 17,500 × *g* for 30 min at 4°C (36) were designated as IM1 (inner membranes) and OM1 (outer membranes). Pelleted materials were extracted again in their respective original volumes of Triton X-100 or Triton X-100–EDTA under identical conditions. Supernatant fractions obtained after centrifugation at 39,000 × *g* for 30 min at 4°C were designated as IM2 and OM2, respectively. (To obtain firm pellets with these and subsequent outer membrane extracts, it was necessary to alter the centrifugation conditions.) Triton X-100-insoluble residues from inner membranes and Triton X-100–EDTA-insoluble residues from outer membranes were washed with 50 mM Tris-hydrochloride buffer (pH 8.6) and 50 mM Tris-hydrochloride buffer (pH 8.6) containing 5 mM EDTA, respectively. The volumes used were equivalent to those of the initial extractions. Suspensions were then centrifuged at 39,000 × *g* for 30 min at 4°C to give two supernatant fractions, designated IM3 and OM3, respectively. The washed Triton X-100-insoluble and Triton X-100–EDTA-insoluble residues were then suspended to their original volumes with 0.1% (wt/vol) SDS in 50 mM Tris-hydrochloride buffer (pH 8.6) and heated at 100°C for 1 min. The SDS extracts were then centrifuged at 39,000 × *g* for 30 min at 4°C, and the supernatant fractions were designated IM4 and OM4. There were no visible residues after SDS extraction.

Portions of IM1 and OM1 were centrifuged at 165,000 × *g* for 2 h at 4°C, and the high-speed supernatant fractions were designated IM-U and OM-U, respectively. Total envelope (outer and inner membrane) fractions were extracted in a manner identical to that for outer membranes, and the extracts were designated as TM1 through TM4.

All detergent extracts were stored, as 50- or 100-μl portions, at −70°C immediately after preparation and thawed only once, either for chemical analysis or for crossed immunoelectrophoresis.

Isolation of Rosenbusch matrix protein. Matrix protein (synonym: protein 1 [14]) was isolated from envelopes of *E. coli* K-12 strain 44 essentially as described by Rosenbusch (40). Envelopes were prepared by disruption of the bacteria in a Mickle apparatus at 4°C and were isolated as previously described (42). Envelope pellets were washed four times in 50 mM Tris-hydrochloride buffer (pH 7.5) after treatment with DNase (10 μg/ml) at 37°C for 15 min. The matrix protein was then extracted and purified from envelopes (8.3 g [wet weight] of cells) by the method of Rosenbusch (40) with only minor modifications (e.g., heating in the extraction buffer at 100°C for 10 min instead of for 5 min; threefold concentration by ultra-

filtration on an Amicon PM-10 filter before chromatography). The preparation showed a major band (apparent molecular weight, 36,000) and several minor ones when analyzed by SDS-polyacrylamide gel electrophoresis.

Isolation of lipopolysaccharide. Lipopolysaccharide was prepared by the method of Galanos et al. (13) from washed bacterial cells. Approximately 300 mg of purified lipopolysaccharide was obtained from 16.5 g (dry weight) of cells. The lipopolysaccharide had the following composition: 18.5% keto-3-deoxyoctonate, 12.5% total hexose, 1.5% protein. The neutral sugars galactose and glucose (molar ratio, 1.0:0.89), together with an unidentified component, were detected by gas chromatographic analysis.

Preparation of envelope antisera and purification of immunoglobulins. Rabbits (New Zealand white; either sex) were immunized with washed Mickle envelopes from *E. coli* (42) according to the schedule described earlier for gonococcal envelopes (50), were bled approximately every 14 days (40 to 50 ml of blood), and were given booster doses of envelopes, with or without Freund incomplete adjuvant at monthly intervals. A serum pool (230 ml) obtained from two rabbits over a period of 3 months was used for our studies. Immunoglobulins were purified by ammonium sulfate precipitation, acetate dialysis, and diethylaminoethyl-cellulose chromatography (15). After dialysis against 0.1 M NaCl containing 15 mM sodium azide as a preservative, purified immunoglobulins were concentrated by ultrafiltration on an Amicon PM-10 membrane to one-fifth the original serum volume and stored at 4°C.

Preparation of antiserum to lipopolysaccharide. Rabbits were given intravenous doses of purified lipopolysaccharide in isotonic saline according to the following schedule: day 1, 5 μg; day 3, 10 μg; day 5, 15 μg; increasing by 5 μg per injection to day 15. On day 25 the schedule was continued as before up to a final dose of 500 μg. Immunization had to be continued for 4 months before immunoprecipitates were detected by immunodiffusion tests. Immunoglobulins were purified as described above.

Specific antisera. The antiserum preparations to specific *E. coli* membrane antigens were obtained as follows: anti-adenosine triphosphatase (anti-ATPase; EC 3.6.1.3) from L. Heppel, Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, N.Y.; anti-lipoprotein III from V. Braun, Lehrstuhl Mikrobiologie II, University of Tübingen, Tübingen, Germany; anti-D-lactate dehydrogenase (EC 1.1.1.27) from H. R. Kaback, Roche Institute of Molecular Biology, Nutley, N. J.; anti-reduced nicotinamide adenine dinucleotide (anti-NADH) dehydrogenase (EC 1.6.99.3) from B. M. Shapiro, Department of Biochemistry, University of Washington, Seattle, Wash. Immunoglobulins either precipitated with ammonium sulfate or purified as described above were used.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed by a modification (36) of the Laurell technique (26). Barbital-HCl buffer (ionic strength, 0.02; pH 8.6) containing 1% (vol/vol) Triton X-100 was used throughout and incorporated into all gels. Unless otherwise stated, 1% (wt/vol) agarose gels were prepared from Seakem HGT or HGT(P) agarose (Marine Colloids, Inc., Rockland, Me.). The gels were cast on glass plates (50 by 50 by 0.6 mm) to give a volume-to-surface area ratio of 0.132 ml/cm^2. Samples, applied to wells of minimal diameter, were subjected to electrophoresis at 5.4 V/cm for 70 min in a Behring Diagnostic water-cooled immunoelectrophoresis cell. An agarose strip (10 by 50 mm) containing the antigens that had been subjected to electrophoresis was retained on the plate after removal of the rest of the gel, which was replaced with an adjacent gel (40 by 50 mm) containing antienvelope immunoglobulins. Electrophoresis in the second direction was then performed at 2 V/cm for 18 to 24 h. Gels were finally pressed, washed twice in 0.1 M NaCl, air dried, and stained with Coomassie brilliant blue. The relative concentrations of discrete antigens in membrane extracts were estimated by weighing, in triplicate, photocopies of the areas subtended by corresponding immunoprecipitate arcs.

Crossed immunoelectrophoresis with intermediate gel. The initial step in crossed immunoelectrophoresis with an intermediate gel was identical to that described above. However, after electrophoresis in the first direction, a larger agarose strip (20 by 50 mm) was retained on the plate, the rest of the gel being replaced by an agarose gel (30 by 50 mm) containing antienvelope immunoglobulins. An intermediate strip of agarose (10 by 50 mm) immediately below this antibody-containing gel was then cut and removed. A third gel, containing another antibody, was then cast in its place. In control plates this latter step was omitted, leaving a spacer gel of dimensions (10 by 50 mm) identical to those of the intermediary gel but containing no antibodies. Electrophoresis in the second dimension was then performed at 2 V/cm for 18 to 24 h.

Crossed immunoaffinoelectrophoresis. Crossed immunoaffinoelectrophoresis (4) was performed in a manner almost identical to that described for crossed immunoelectrophoresis with an intermediate gel. However, after electrophoresis in the first direction, the upper portion of the gel was discarded, leaving an agarose strip (10 by 50 mm) containing antigen. Agarose gels were then cast and cut to give a final plate consisting of four parallel gels (width, 50 mm), 10, 8, 2, and 30 mm in length, containing membrane antigens that had been subjected to electrophoresis, lectin, agarose alone, and antienvelope immunoglobulins, respectively. In control runs, the affinity gel containing the lectin and its 2-mm spacer were replaced with a single spacer gel (10 by 50 mm). Concanavalin A was incorporated into the affinity gels at a concentration of 3 mg/ml, and wheat germ agglutinin was incorporated at a concentration of either 100 μg/ml or 3 mg/ml. Electrophoresis in the second direction was performed at 2 V/cm for 18 to 24 h.

Identification of immunoprecipitates by enzyme-staining (zymogram) techniques. The methods described by Owen and Salton (36) were used to detect immunoprecipitates possessing the following enzyme activities: malate dehydrogenase (EC 1.1.1.37), succinate dehydrogenase (EC 1.3.99.1), NADH dehydrogenase (EC 1.6.99.3), and ATPase (EC 3.6.1.3). Immunoprecipitates containing glucose 6-phosphate

dehydrogenase (EC 1.1.1.49), glutamate dehydrogenase (EC 1.4.1.4), and chymotrypsin-like protease (EC 3.4.21.1) were identified by the methods of Uriel (52), and those possessing D-lactate dehydrogenase (EC 1.1.1.27) activity were identified by the method described by Smyth et al. (50). Other enzyme activities were detected by incubating pressed immunoplates individually in the following incubation mixtures (final volumes, 20 ml each): (i) 6-phosphogluconate dehydrogenase (EC 1.1.1.43)—tetranitroblue tetrazolium (10 mg), 6-phosphogluconate (40 mg), nicotinamide adenine dinucleotide phosphate (10 mg), phenazine methosulfate (0.5 mg), 0.1 M Tris-hydrochloride buffer (pH 7.0; 20 ml); (ii) glycerol 3-phosphate dehydrogenase (EC 1.1.99.5)—tetranitroblue tetrazolium (10 mg), 1.0 M DL-α-glycerophosphate (disodium salt; pH 7.0; 2.0 ml), phenazine methosulfate (0.5 mg), 0.1 M Tris-hydrochloride buffer (pH 7.0; 18 ml); and (iii) dihydroorotate dehydrogenase (EC 1.3.3.1)—tetranitroblue tetrazolium (6 mg), 1.0 M dihydroorotate (pH 7.5; 2.0 ml), nicotinamide adenine dinucleotide (10 mg), phenazine methosulfate (0.5 mg), 0.1 M Tris-hydrochloride buffer (pH 7.5; 18 ml).

Analytical procedures. Protein was determined by the method of Lowry et al. (28), with bovine serum albumin as the standard. Controls were included to correct for the interfering effects of Tris, EDTA, detergents, and reducing agents. Total hexose was determined by the anthrone method of Morris (30), with glucose as the standard; keto-3-deoxyoctonate was determined by the procedure of Droge et al. (12). Neutral sugars were analyzed as their trimethyl silyl ether derivatives by gas chromatography after acid hydrolysis in 1 M H_2SO_4 and neutralization with Dowex AG-X8 (HCO_3^-) resin (35).

Chemicals. Agarose [Seakem HGT and HGT(P)] was obtained from Marine Colloid, Inc., Rockland, Me.; Triton X-100 (scintillation grade) was from Research Products International, Elk Grove Village, Ill.; sucrose (ultrapure) was from Fisher Chemicals, Pittsburgh, Pa.; DNase (EC 3.1.4.5; 2,000 U/mg) and keto-3-deoxyoctonate were from Sigma Chemical Co., St. Louis, Mo.; and concanavalin A was from Miles Labs, Inc., Elkart, Ind. Wheat germ agglutinin was a gift from Joel D. Oppenheim, Department of Microbiology, New York University School of Medicine, New York. All other chemicals and reagents were of analytical grade. L-(+)-, and D-(−)-lactic acids were the lithium salts.

RESULTS

Electron microscopy of inner and outer membranes. Electron micrographs of negatively stained outer and inner membranes, isolated by the procedure outlined in Materials and Methods, are shown in Fig. 1A and B. Their appearance is in accord with the known morphological characteristics of these two types of membrane (39, 54): the inner membranes (Fig. 1B) consisting of large flat or folded sheets of membrane possessing a fine granular appearance, and the outer membranes (Fig. 1A) consisting of spherical or concentric structures of smoother texture than that of the inner membranes. There was very little evidence of cross-contamination of one fraction with the other, and pili, which contaminated plasma membrane fractions prepared from this organism by other methods (39), were rarely seen in our preparations. It is possible that these structures were removed by entrapment in the viscous sucrose-DNA suspension after dispersion in a Dounce homogenizer.

Detergent extraction of membrane fractions. The nonionic detergent Triton X-100 has proved to be extremely useful in the extraction of bacterial membrane antigens and in their subsequent analysis by crossed immunoelectrophoresis (36, 50). However, optimal conditions for membrane protein extraction often require the use of fairly dilute membrane suspensions (45). Since the maximum volume of detergent extract that can be conveniently analyzed by crossed immunoelectrophoresis in our system is about 10 μl and since it is necessary to analyze about 50 μg of membrane protein for optimum resolution, it is apparent that detergent extracts of complex antigen mixtures should possess a protein concentration in excess of 5 mg/ml. Consequently, membrane fractions often have to be at an initial protein concentration as high as 20 mg/ml (36, 50) and must be treated repeatedly with detergent if full extraction is desired.

Membrane fractions (7 to 20 mg of protein per ml) isolated from *E. coli* were thus extracted twice with 4% Triton X-100, washed once with buffer to remove excess Triton X-100, and finally extracted with 0.1% SDS. This procedure was adopted in the hope that it would permit the analysis of as many major membrane antigens as possible. EDTA (5 mM) was added to detergent solutions and buffers during extraction of outer membranes and envelopes to achieve maximal dispersion of wall components (46). The protein contents of the extracts are summarized in Table 1. Unlike inner membranes, both outer membranes and envelopes had to be treated twice with Triton X-100–EDTA to extract over 85% of the total protein. The Triton X-100-insoluble and Triton X-100–EDTA-insoluble membrane residues could be fully solubilized, as anticipated, by treatment at 100°C with 0.1% SDS after the buffer wash. It should be noted that about 47, 45, and 19% of the protein present in the first detergent extracts of inner membranes, envelopes, and outer membranes, respectively, could be removed after ultracentrifugation at 165,000 × *g* for 2 h.

Crossed immunoelectrophoresis of detergent extracts. Preliminary experiments demonstrated that optimum resolution of immunoprecipitate patterns for Triton X-100 extracts of

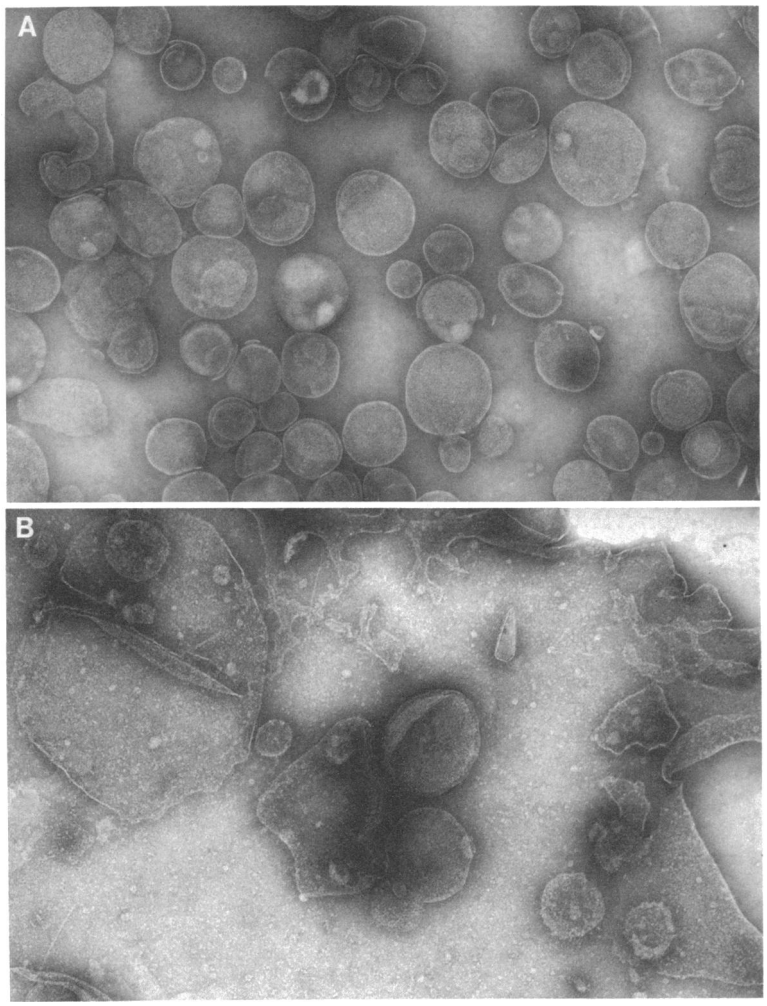

FIG. 1. *Electron micrographs of E. coli K-12 strain 44 membrane fractions isolated by the procedures described in the text. (A) Outer membranes; magnification, ×36,000. (B) Inner (plasma) membranes, magnification, ×47,200. Preparations were negatively stained with 2% ammonium molybdate.*

TABLE 1. *Detergent extraction of membrane fractions from E. coli K-12*

Membrane fraction	Inner membrane			Envelope			Outer membrane		
	Extract	Protein (mg/ml)	% Protein extracted	Extract	Protein (mg/ml)	% Protein extracted	Extract	Protein (mg/ml)	% Protein extracted
Isolated membranes	IM	9.6		TM	7.1		OM	18.1	
1st Triton X-100–(EDTA) extract	IM1	8.2	85.7	TM1	3.1	43.2	OM1	11.8	64.8
2nd Triton X-100–(EDTA) extract	IM2	0.2	1.6	TM2	3.0	42.5	OM2	4.8	26.4
Buffer wash	IM3	0.1	1.2	TM3	0.4	5.9	OM3	0.6	3.5
SDS extract	IM4	0.4	4.0	TM4	0.3	4.5	OM4	1.8	9.2
1st Triton extract after ultracentrifugation	IM-U	4.4	(45.7)[a]	TM-U	1.7	(23.8)[a]	OM-U	9.5	(52.6)[a]
Recovery (%)			92.5			96.1			103.9

[a] Figures not included in percent recovery.

both inner and outer membranes of *E. coli* was only achieved with agarose of low electroendosmotic flow. Agaroses from Bio-Rad Laboratories, Sigma Chemical Co., Behring Diagnostic, and Miles Laboratories all gave poor resolution and were deemed to be unsatisfactory. Seakem HGT and HGT(P) agaroses had previously proved to be the most suitable for analysis of gonococcal envelope extracts (50; Smyth and Salton, in press), and in the studies with *E. coli* membranes they too gave excellent results. From a study of over 300 immunoplates run under conditions of different antigen and antibody concentrations, at least 42 discrete immunoprecipitates stained with Coomassie brilliant blue were detected in the Triton X-100 extracts of inner membranes of *E. coli* (IM1). Many of the immunoprecipitates in this complex pattern are visible in Fig. 2A. Some immunoprecipitates showed considerable heterogeneity, a phenomenon that has been observed during crossed immunoelectrophoresis of other bacterial membrane extracts (36, 50). However, very few of the immunoprecipitates in this complex pattern showed characteristics typical of multiple products originating from proteolytic digestion caused by plasmin (2).

Crossed immunoelectrophoresis is not only a qualitative method of antigen analysis, but it also gives quantitative information where standard conditions of electrophoresis and antibody concentrations are used (2, 37). Thus, the area of individual immunoprecipitates can be shown to be proportional to the amount of each antigen present in the sample (2, 37). Since equivalent volumes of all extracts of *E. coli* inner membranes were analyzed, Fig. 2A through E give not only a qualitative, but also a quantitative comparison of the antigens present in these extracts. The extractibility profiles presented in Fig. 2A and C through E compare very favorably with the known solubility properties of inner membranes of *E. coli* (45) and also with the analytical data shown in Table 1.

Several antigens present in IM1 could be completely removed by ultracentrifugation at 165,000 × *g* (cf. Fig. 2A with B), whereas others, e.g., antigen 8 (identified subsequently as lactate dehydrogenase, see Fig. 3G), remained virtually unaffected. Many others, identifiable by a partial decrease in the areas of their corresponding immunoprecipitates after ultracentrifugation, appeared to separate to varying extents into the pellet and supernatant fraction (cf. Fig. 2A and B).

The immunoprecipitate pattern obtained by crossed immunoelectrophoresis of Triton X-100–EDTA extracts of outer membranes (OM1) was dramatically different from that found for IM1 (cf. Fig. 2A and K) and consisted principally of a group of about seven slow-moving antigens. Several other minor antigens are also evident in Fig. 2K. Indeed, as many as 25 Coomassie brilliant blue-stained immunoprecipitates were detected for this extract by manipulating the concentrations of antigen and/or antibody. Additional protein was extracted from outer membranes during the second Triton X-100–EDTA extraction (Table 1) in contrast to the results obtained for inner membranes. Crossed immunoelectrophoretic analysis of this second extract (OM2) revealed the presence of the same major group of antigens detectable in the first extract, OM1 (Fig. 2K and M). Based on the similarity of electrophoretic profiles and staining intensities of the immunoprecipitates, the presumed identity of the major components in these extracts was confirmed in tandem crossed immunoelectrophoresis experiments with OM1 and OM2 (immunoplates not shown).

Residual membrane protein resistant to two extractions with Triton X-100–EDTA was amenable to analysis after a buffer wash (Fig. 2N) and subsequent extraction with 0.1% SDS (Fig. 2O). The conditions employed for sample treatment, which were designed to remove residual SDS by formation of mixed micelles with excess Triton X-100 (16), appeared to successfully normalize the electrophoretic profile (Fig. 2O). The two major immunoprecipitates present in this pattern seemed to correspond to immunoprecipitates of similar electrophoretic mobilities and staining intensities present in OM1 and OM2. The 19% decrease in the protein content of OM1 noted upon ultracentrifugation was reflected by the total or partial loss of some of the minor antigens and by only a marginal decrease in the concentration of major antigens (cf. Fig. 2K and L).

The immunoprecipitate patterns obtained by crossed immunoelectrophoresis of sequential detergent extracts of isolated cell envelopes (total membranes) are shown in Fig. 2F through J. The immunoprecipitate patterns for the envelope extracts (TM1, TM-U) illustrated in Fig. 2F and G are in remarkably good qualitative agreement with the summation of the patterns seen individually for inner (IM1, IM-U) and outer (OM1, OM-U) membranes in Fig. 2A and B and K and L, respectively. A close examination of Fig. 2A, F, and K revealed that some antigens appeared to partition between isolated inner and outer membranes. Two such antigens are indicated by arrows in Fig. 2B, G, and L and are readily recognizable on the original immunoplates by the shapes and staining intensities of

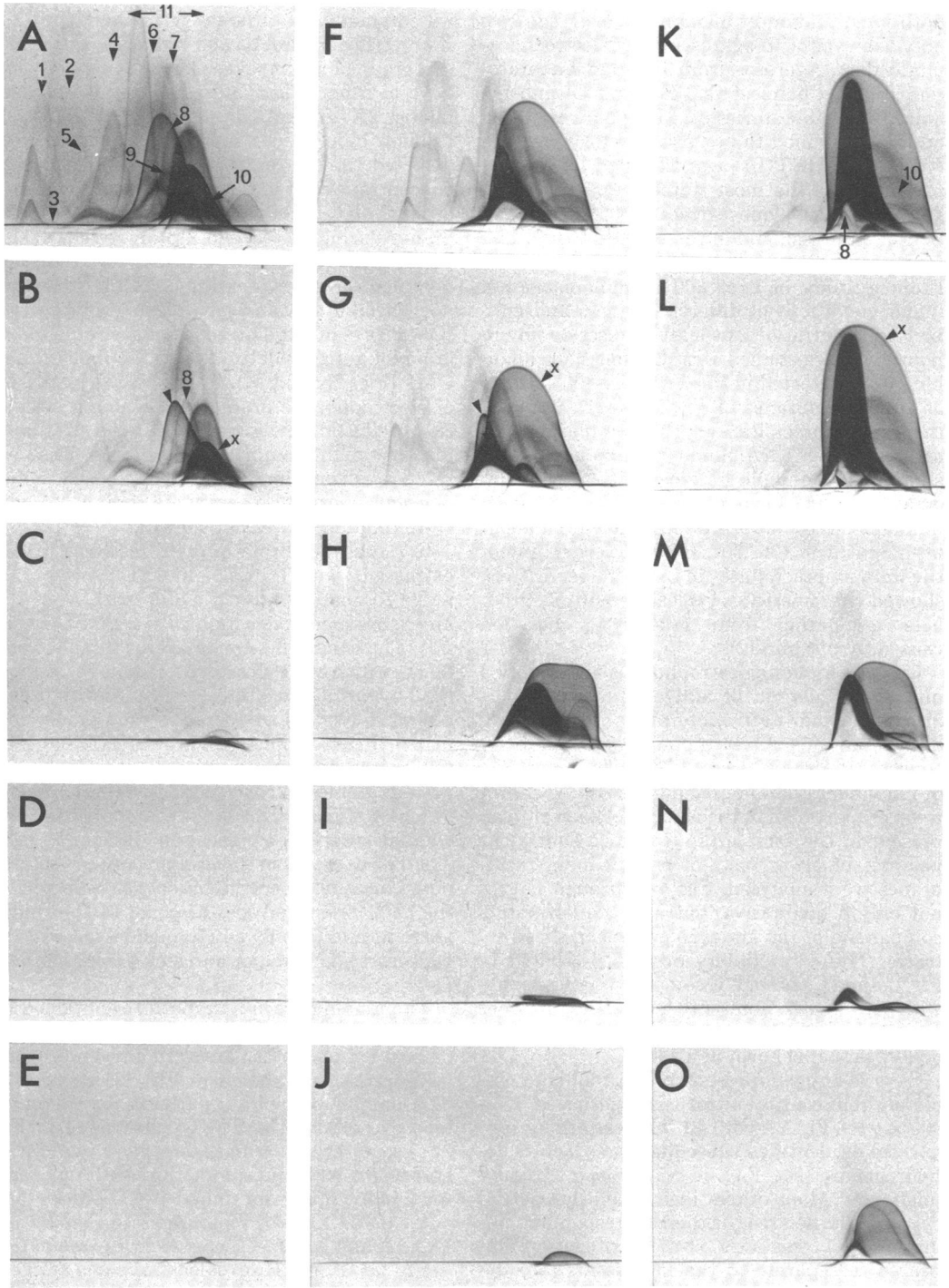

FIG. 2. *Crossed immunoelectrophoretic analysis of detergent extracts of membrane fractions from E. coli K-12. Isolated inner membranes (A to E), envelopes (F to J), and outer membranes (K to O) were subjected to repeated detergent extraction as described in the text. (A, F, and K) First Triton X-100–(EDTA) extracts, i.e., IM1, TM1, and OM1, respectively. (B, G, and L) First Triton X-100–(EDTA) extracts after high-speed centrifugation (165,000 × g), i.e., fractions designated IM-U, TM-U, and OM-U, respectively. (C, H, and M)*

their immunoprecipitates. As anticipated from their probable identity in both inner and outer membrane fractions, only single immunoprecipitates of each of these two antigens were seen in the pattern for the cell envelopes (see precipitates marked by arrows in Fig. 2G). In most instances, such antigens appear to segregate predominantly in one of the isolated membrane fractions. For example, calculations based on measurements of peak area showed that the antigen (Fig. 2B, G, and L) identified as Braun lipoprotein (see Fig. 4A and B) partitioned on a protein basis between inner and outer membranes in the concentration ratio 1:9.3. The other antigen, in contrast, fractionated largely with inner membrane (concentration ratio inner/outer membranes, 6.6:1).

Characterization of immunoprecipitates in the inner membrane reference pattern. Many antigens associated with electron transport and metabolic processes in gram-negative bacteria have been shown to be associated with the inner membrane (41). The detection of such enzyme activities in immunoprecipitates by use of zymograms represents a useful approach to the characterization of complex immunoprecipitate patterns (37, 52). In this way, immunoprecipitates 1 through 11 (Fig. 2A) have been shown to contain the following enzymes: no. 1, 6-phosphogluconate dehydrogenase (Fig. 3A); no. 2, ATPase (Fig. 3B); no. 3 and 4, glutamate dehydrogenase (Fig. 3C); no. 5, malate dehydrogenase (Fig. 3D); no. 6, dihydroorotate dehydrogenase (Fig. 3E); no. 7, succinate dehydrogenase (Fig. 3F); no. 8, lactate dehydrogenase (Fig. 3G); no. 9, NADH dehydrogenase (Fig. 3H); no. 10, protease (Fig. 3I); no. 11, glycerol 3-phosphate dehydrogenase (Fig. 3J). In all cases the enzyme-staining reaction was specific, and none of the identifiable immunoprecipitates stained for more than one enzyme activity.

The specificity of the enzyme staining reaction was further demonstrated for lactate dehydrogenase, ATPase, and NADH dehydrogenase by using crossed immunoelectrophoresis with intermediate gels containing antienvelope immunoglobulins in the reference gel and antiserum to the purified or partially purified enzyme (11, 47, 48) in the intermediate gel. Immunoprecipitates staining for lactate dehydrogenase (no. 8, Fig. 2A), ATPase (no. 2, Fig. 2A) and NADH dehydrogenase (no. 9, Fig. 2A) were depressed from the reference pattern into the intermediate gel containing antiserum for its respective enzyme (immunoplates not shown). The intermediate gel technique used to confirm the identity of the three enzymes was also used for the identification of Braun lipoprotein (see Fig. 4A and B). This technique was of special importance in confirming the identity of precipitates 2 and 9, since no. 2 stained only weakly for ATPase (Fig. 3B) and no. 9 gave a diffuse and poorly defined zymogram (Fig. 3H; see also reference 11).

Immunoprecipitate 8 stained very faintly when L-(+)-lactic acid was used as the substrate, compared with the intense staining reaction given with D-(−)-lactic acid (Fig. 3G). This is in complete agreement with the known substrate specificity of *E. coli* lactate dehydrogenase (24). Immunoprecipitates corresponding to antigens 3, 4, 6, and 11 were not detected when immunoplates were stained with Coomassie brilliant blue. These enzymes must therefore be considered as only very minor antigenic components of the inner membrane. Immunoprecipitates possessing the following enzyme activities were not detected by zymogram techniques: isocitrate dehydrogenase (EC 1.1.1.42), glucose 6-phosphate dehydrogenase (EC 1.1.2.49), polynucleotide phosphorylase (EC 2.3.7.8), 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), alkaline phosphodiesterase (EC 3.1.4.1), and trypsin-like protease (EC 3.4.21.4).

Second Triton X-100-(EDTA) extracts, i.e., IM2, TM2, and OM2, respectively. (D, I, and N) Supernatants after a buffer wash, fractions IM3, TM3, and OM3, respectively. (E, J, and O) Final extraction with 0.1% SDS, i.e., IM4, TM4, and OM4, respectively. Before analysis, samples representing buffer washes and SDS extracts were made 4% (vol/vol) with respect to Triton X-100 by the addition of 1/4 volume of 20% (vol/vol) Triton X-100. This was performed to normalize the Triton concentration of all samples and also to reduce the denaturing and charge effect of SDS (16). Equivalent volumes of consecutive extracts were analyzd in all instances. Volumes and protein concentrations of individual samples were as follows: IM1, 5 μl, 41 μg of protein; IM-U, 5 μl, 22 μg of protein; IM2, 5 μl, 1 μg of protein; IM3, 6.25 μl, 0.5 μg of protein; IM4, 6.25 μl, 2 μg of protein; TM1, 8 μl, 25 μg of protein; TM-U, 8 μl, 13.6 μg of protein; TM2, 8 μl, 24 μg of protein; TM3, 10 μl, 3.2 μg of protein; TM4, 10 μl, 2.4 μg of protein; OM1, 5 μl, 59 μg of protein; OM-U, 5 μl, 48 μg of protein; OM2, 5 μl, 24 μg of protein; OM3, 6.25 μl, 3 μg of protein; OM4, 6.25 μl, 9 μg of protein. Crossed immunoelectrophoresis was performed by using Seakem HGT(P) agarose, and antibody gels contained 94 μl of concentrated antienvelope immunoglobulins per ml. Numbered arrows in (A) denote the positions of the enzyme-active immunoprecipitates identified in Fig. 3. Immunoprecipitates possessing D-lactate dehydrogenase (no. 8) and protease (no. 10) activity are also indicated in (K). Arrowed immunoprecipitates in (B), (G), and (L) indicate two major antigens that fractionate to differing extents in the three membrane fractions. "X" indicates Braun lipoprotein. Anode to left and top of all gels.

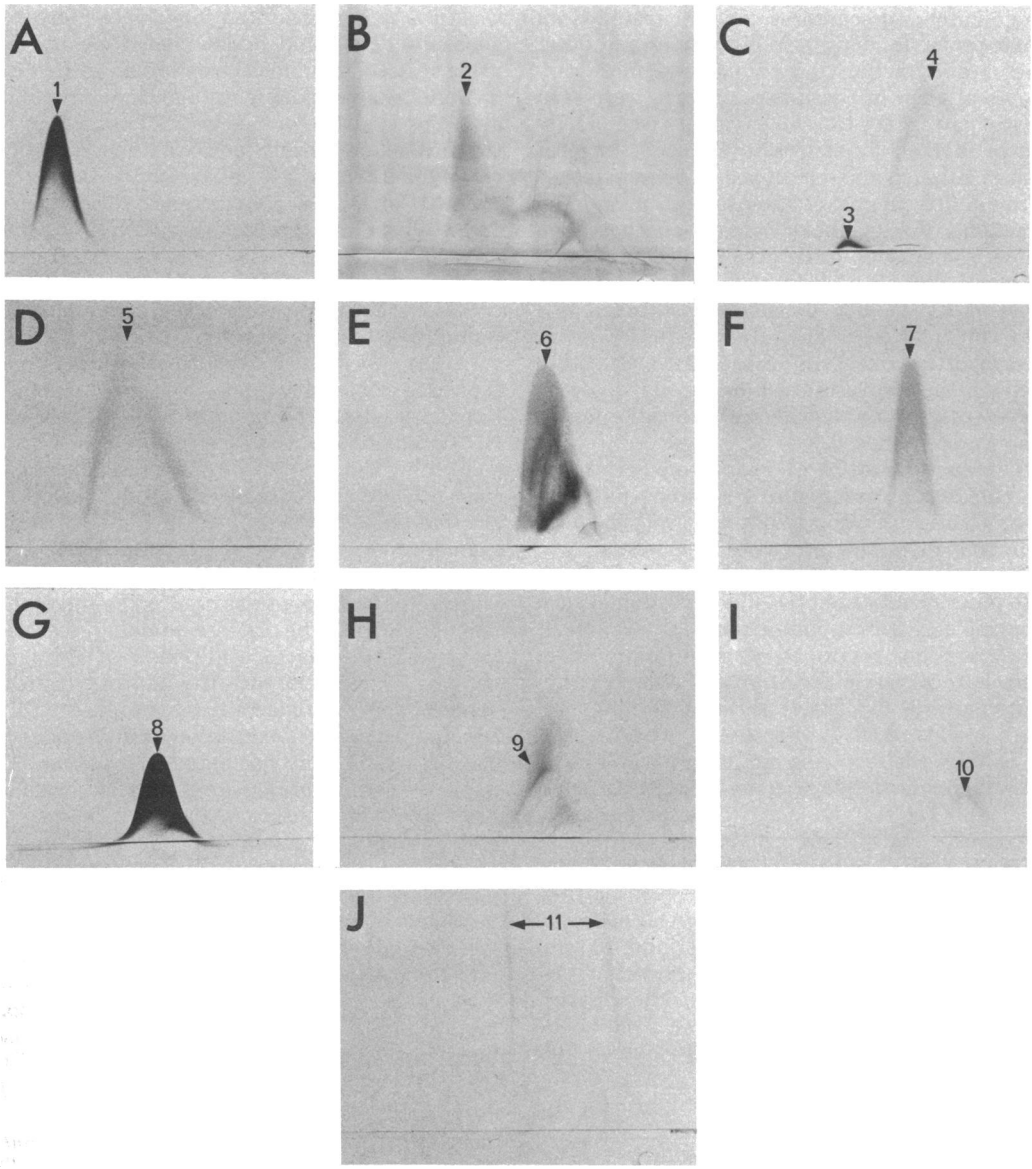

FIG. 3. *Characterization by zymograms of immunoprecipitates in the crossed immunoelectrophoretic reference pattern for isolated inner membranes of E. coli. (A to J) Gels, similar to the one illustrated in Fig. 2A, stained for (A) 6-phosphogluconate dehydrogenase, (B) ATPase, (C) glutamate dehydrogenase, (D) malate dehydrogenase, (E) dihydroorotate dehydrogenase, (F) succinate dehydrogenase, (G) D-lactate dehydrogenase, (H) NADH dehydrogenase, (I) protease (chymotrypsin-like), (J) glycerol 3-phosphate dehydrogenase. Enzymatically stained gels could be subsequently stained with Coomassie brilliant blue to confirm the identification as indicated in Fig. 2A, except for the four immunoprecipitates only detectable by zymogram staining (antigens 3, 4, 6, and 11). Parts of overlapping precipitates were frequently reinforced within the zymogram staining areas (e.g., E), phenomena that appear to be due to nonspecific adsorption of stain or nonspecific entrapment of enzyme. Anode to left and top of all gels.*

One other immunoprecipitate in the reference pattern of inner membranes (indicated by X in Fig. 2B) was shown by intermediate gel techniques, similar to those illustrated for outer membranes in Fig. 4A through C, to be identifiable as Braun lipoprotein (5). Thus, 12 of the 46 discrete immunoprecipitates detected by protein or by zymogram staining of detergent ex-

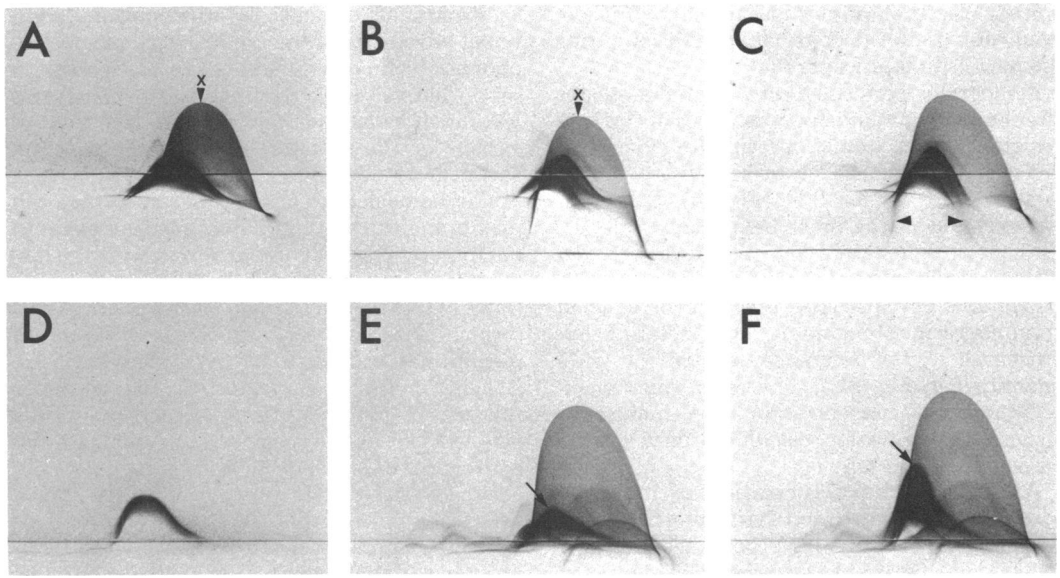

FIG. 4. *Characterization of the major antigens present in Triton X-100–EDTA extracts of outer membranes of E. coli K-12 by crossed immunoelectrophoresis with intermediate gel (A to C) and by co-crossed immunoelectrophoresis (D to F). (A to C) Similar amounts (2 μl, 23 μg of protein) of OM1 were analyzed in all instances. (A) Control plate with no immunoglobulins in the intermediate gel. (B) Test plate with 500 μl of anti-lipoprotein III (5) immunoglobulins per ml of intermediate gel. (C) Test plate with 500 μl of anti-lipopolysaccharide immunoglobulins per ml of intermediate gel. (A to C) All reference gels contained 113 μl of concentrated antienvelope immunoglobulins per ml. The main immunoprecipitate (X) in (A) was specifically depressed into the intermediate gel in (B), thus indicating its identity as Braun lipoprotein. (C) Reactions of "inward feet" (arrowed) tentatively identify the lipopolysaccharide antigen. In co-crossed immunoelectrophoretic experiments, (D) purified lipopolysaccharide (40 μg in 10 μl of 1.6% Triton X-100), (E) OM1 (23 μg of protein in 10 μl of 1.6% Triton X-100), and (F) a mixture of lipopolysaccharide (40 μg) and OM1 (23 μg of protein in 10 μl of 1.6% Triton X-100) were subjected to crossed immunoelectrophoresis against antienvelope immunoglobulins (151 μl of concentrated immunoglobulins per ml of gel). Only the area under the arrowed precipitate in (E) increased in (F), thus indicating its identity as lipopolysaccharide. Anode to left and top of all gels.*

tracts of isolated inner membranes have been identified.

Characterization of immunoprecipitates in the outer membrane reference pattern. Two of the antigens in the outer membrane immunoprecipitate reference system were conclusively identified by two variations of the crossed immunoelectrophoresis technique, the intermediate gel procedure and coelectrophoresis. Braun lipoprotein (5) was identified by using antiserum to this component in the intermediate gel (cf. Fig.4A with B). The immunoprecipitate of this component was formed in the intermediate gel region only in the presence of its specific antibody (Fig. 4B). Similar experiments were performed with antiserum to purified lipopolysaccharide included in the intermediate gel. The reactions obtained under these conditions only permitted tentative identification of this antigen (Fig. 4C). Such a result may have been due to the low titer of antibodies formed in response to immunization with highly purified endotoxin (29). Confirmation of the identity of this component was achieved when the purified lipopolysaccharide was subjected to coelectrophoresis with the Triton X-100–EDTA extract of the outer membranes (cf. Fig. 4D, E, and F).

Another major, well-characterized component of the outer membrane of *E. coli* is the "matrix protein" described by Rosenbusch (40). This protein is identical to membrane protein 1 of Garten et al. (14) and appears to be closely, but not covalently, associated with the peptidoglycan layer (14, 40). The polypeptide was purified from envelopes of *E. coli* K-12 by the differential SDS-extraction procedure of Rosenbusch (40). However, attempts to identify it in the crossed immunoelectrophoresis reference patterns were unsuccessful. The purified protein failed to give an immunoprecipitate against antienvelope immunoglobulins when tested by either crossed

immunoelectrophoresis or immunodiffusion, even after removal of residual SDS as during the purification procedure (40).

Zymograms performed on wet immunoplates after crossed immunoelectrophoresis of OM1 revealed that one immunoprecipitate (identified in Fig. 2K) possessed protease activity similar to that observed for IM1 (Fig. 3I). Other enzymes found in the inner membranes (Fig. 3A through J) either were not present or were detected in extremely low quantities. For example, zymograms revealed the presence of a small immunoprecipitate staining for D-lactate dehydrogenase in the reference pattern for OM1 (identified in Fig. 2K). The ratio inner membrane enzyme concentration/outer membrane enzyme concentration calculated on a protein basis was 12.5:1.

Attempted characterization of immunoprecipitates by crossed immunoaffinoelectrophoresis. Lectins have been used successfully as specific affinity absorbents to characterize membrane glycoproteins by crossed immunoaffinoelectrophoresis (34, 36) by placing the lectin in an intermediate gel between the antigen subjected to electrophoresis and the antibody reference gel. Any antigenic components reacting with the lectin to form affinity precipitates on electrophoresis in the second dimension are normally recognized by the absence of their corresponding immunoprecipitates from the reference antibody gel. Affinity experiments of this type were performed with the lectins, concanavalin A showing primary sugar specificities for α-D-mannopyranoside and α-D-glucopyranoside residues (51), and wheat germ agglutinin showing a primary sugar specificity for *N*-acetyl-D-glucosaminyl residues (7). Neither lectin had any observable effect on the reference immunoprecipitate patterns of IM1, OM1, or TM1. Wheat germ agglutinin often requires the presence of relatively high saline concentrations for optimum precipitation with glycoproteins (34), but addition of saline (25 mM) to wheat germ agglutinin gel did not alter any of the immunoprecipitate patterns.

DISCUSSION

These investigations provide a model system for establishing the complexity of the antigenic architecture of the gram-negative, bacterial cell envelope. The immunoprecipitate patterns of Triton X-100-solubilized antigens of isolated inner and outer membranes of *E. coli* provide the first detailed immunochemical analysis with a resolution of heterogeneity approaching that of SDS-polyacrylamide gel electrophoresis (14, 44, 45). Moreover, the ability to identify antigens as membrane enzymes is an important additional advantage of the crossed immunoelectrophoresis technique. Such high-resolution analyses of "native" antigens still possessing biochemical functions adds a new dimension to investigations of the structure-function relationship within gram-negative cell envelopes.

The homogeneity of the inner and outer cell membrane fractions as determined by electron microscopy (Fig. 1) correlates well with the distribution of enzymatically active immunoprecipitates in the crossed immunoelectrophoresis patterns of detergent extracts of the two types of membranes examined by zymogram staining. Enzyme markers characteristic of the inner (plasma) membranes of *E. coli*, such as ATPase (48), NADH dehydrogenase (10), D-lactate dehydrogenase (24), and succinate and malate dehydrogenases (33, 41) were also detected almost exclusively in the inner membrane preparations by crossed immunoelectrophoresis. Glycerol 3-phosphate dehydrogenase, an inducible enzyme, is also considered to be membrane associated (53), and its detection in inner membranes of *E. coli* may reflect either the presence of low basal levels of the enzyme or the presence of low concentrations of inducer in the medium. The significance of detectable levels of glutamate, dihydroorotate and 6-phosphogluconate dehydrogenases in the plasma membrane is less clear, since data on their intracellular distribution are lacking. The absence of immunoprecipitates staining for glucose 6-phosphate dehydrogenase, polynucleotide phosphorylase, and acid and alkaline phosphatases provides strong evidence against gross contamination of membrane fractions with soluble cytoplasmic or periplasmic enzymes (17).

The relationship between the protease activity detected by zymograms of inner and outer membranes of *E. coli* and an endopeptidase purified from this organism (protease I; 38) is unclear. Both enzymes have similar substrate specificities in that they can hydrolyze acetylphenylalanine naphthyl esters. However, protease I is generally considered to be a periplasmic enzyme (25, 38), although activity has been detected in isolated envelope fractions (25). There is evidence to suggest that an enzyme possessing aminoendopeptidase activity is expressed on the cell surface of *E. coli* and is released less readily by osmotic shock than the periplasmic enzyme marker, alkaline phosphatase (27). It thus seems probable that the endopeptidase detected in the present study segregates between the periplasmic and membranous compartments of the cell envelope.

Lipopolysaccharide and Braun lipoprotein are

well characterized marker molecules for the outer membrane of *E. coli* (5, 33), and these components have been localized predominantly in outer membrane fractions by crossed immunoelectrophoresis. Because crossed immunoelectrophoresis is a quantitative technique, it offers a convenient and rapid method for monitoring the levels of cellular antigens, especially those of the outer membrane, which appear to lack catalytic activity and cannot be estimated by enzyme assay. Unlike direct enzyme assay, crossed immunoelectrophoresis generally indicates the total amount of antigen present. It will thus give an estimate of enzyme concentration independent of factors, such as the presence of inhibitors or orientation of membrane vesicles, which might affect the expression of catalytic activity (37).

The inability to detect an immunoprecipitate accounting for the Rosenbusch matrix protein (40) was disappointing, especially in view of its abundance in the outer membrane and the potential use of antimatrix protein in resolving some of the controversy surrounding the nature of the passive diffusion channels in the outer membrane (19, 31). It is possible that this protein does not regain its native antigenicity after removal of SDS even though considerable refolding of the molecule is known to occur under these conditions (40).

Crossed immunoaffinoelectrophoresis has been used successfully in this laboratory to characterize several of the membrane antigens of *M. lysodeikticus* (36). Attempts to detect *E. coli* envelope antigens possessing sugar residues interacting with the lectins concanavalin A and wheat germ agglutinin were unsuccessful. Although the negative results obtained with concanavalin A can be rationalized from a consideration of the sugar composition of *E. coli* envelopes, the reasons why lipopolysaccharide or other envelope components containing glucosamine (22, 23) were not detected by this technique are less apparent. The recent demonstration that wheat germ agglutinin would not coprecipitate with lipopolysaccharide isolated from these organisms in gels containing concentrations of Triton X-100 in excess of 0.1% (vol/vol) (D. Fitzgerald and P. Owen, unpublished data) could explain the absence of any affinity reactions due to the high concentration of 1% (vol/vol) Triton X-100 normally used in immunoplates together with the high salt requirements for this lectin (34).

The observation that some membrane antigens can be selectively removed from Triton X-100 membrane extracts by ultracentrifugation may be of particular significance for studies of the possible existence of antigen complexes. It has been suggested that some immunoprecipitates obtained after crossed immunoelectrophoresis of detergent-"solubilized" membranes may represent antigen complexes rather than discrete antigens (3). It is therefore possible that some of the immunoprecipitates we have detected represent close associations of several membrane antigens. These complexes may reflect an in vivo association of membrane components due to cohesive interactions in membrane microenvironments. Much of the compelling evidence in favor of this proposal has recently been critically reviewed by Owen and Smyth (37). The sedimentation properties of such complexes would be expected to differ from those of their component antigens. In contrast, immunoprecipitates that are not diminished in area after ultracentrifugation may reflect the presence of discrete antigens. These and related problems might be resolved by analysis of the interaction of radiolabeled membrane components with antisera raised to excised immunoprecipitates (9).

In summary, high-resolution crossed immunoelectrophoretic reference patterns for isolated inner and outer membranes of *E. coli* K-12 have been established and partly characterized. These results should provide a basis for future absorption studies designed to resolve some of the intriguing problems concerning the structural asymmetry and functional architecture of the gram-negative cell envelope (43).

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