Membrane Asymmetry and Expression of Cell Surface Antigens of *Micrococcus lysodeikticus* Established by Crossed Immunoelectrophoresis

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Crossed immunoelectrophoresis of Triton X-100-solubilized plasma membranes of *Micrococcus lysodeikticus* established the presence of 27 discrete antigens. Individual antigens were identified as membrane components possessing enzyme activity by zymogram staining procedures and by reactivity of certain antigens with a selection of four lectins in the crossed-immunoelectrophoresis (immunofinoelectrophoresis) system. Absorption experiments with intact, stable protoplasts and isolated membranes established the asymmetric nature of the *M. lysodeikticus* plasma membranes. Of the 14 antigens with determinants accessible solely on the cytoplasmic face of the membrane, four possessed individual dehydrogenase activities, and a fifth was identifiable as a component possessing adenosine triphosphatase (EC 3.6.1.3) activity. Evidence from absorption studies with isolated membranes suggested that antigens such as the adenosine triphosphatase complex were more readily accessible to reaction with antibodies than was succinate dehydrogenase (EC 1.3.99.1), for example. Twelve antigens were located on the protoplast surface as determined by antibody absorption, and the succinylated lipomannan was identified as a major antigen. At least five other antigens possessed sugar residues that interacted with concanavalin A. With the antiserum generated to isolated membranes, there was no evidence suggesting that any of these antigens was not detectable on either surface of the plasma membrane. From absorption experiments with washed, whole cells of *M. lysodeikticus*, it was concluded that the immunogens on the protoplast surface were also detectable on the surface of the intact cell. However, some of the components such as the succinylated lipomannan appeared to be exposed to a greater extent than others. The cytoplasmic fraction from *M. lysodeikticus* was used as an antigen source to generate antibodies, and 97 immunoprecipitates were resolvable by crossed immunoelectrophoresis. In the cytoplasm-anticytoplasm reference immunoelectrophoresis pattern of precipitates, three of the immunoprecipitates unique to the cytoplasmic fraction were identifiable by zymogram staining procedures as catalase (EC 1.11.1.6), isocitrate dehydrogenase (EC 1.1.1.42), and polynucleotide phosphorylase (EC 2.7.7.8). The identification of membrane and cytoplasmic antigens (including the above-mentioned enzymes) provides a sensitive analytical system for monitoring cross-contamination and antigen distribution in cellular fractions.

Crossed or two-dimensional immunoelectrophoresis (CIE) is a high-resolution procedure that is particularly suitable for the immunochromical analysis of complex mixtures of antigens such as solubilized membrane fractions and cell cytoplasmic components. The basic feature of the CIE procedure (2, 11, 17) involves electrophoresis of antigen fractions in agarose gels (molecular exclusion limit of approximately 10⁶ daltons) in the first direction; the separated antigens are then electrophoresed at right angles (second dimension) into the agarose containing the antibodies under conditions whereby the immunoglobulins are virtually immobile (2, 11, 17). As the antigens are electrophoresed into the antibodies, the antigen-antibody complexes...
formed ultimately coalesce and produce immobile immunoprecipitates, giving a two-dimensional display of rockets or peaks of immunoprecipitates (2, 11, 17). This procedure possesses several advantages over the classical analytical immunoelectrochemical methods of immunodiffusion and immunoelectrophoresis (2, 11, 17). First, the technique is essentially quantitative, the peak areas of individual immunoprecipitates being proportional to the antigen/antibody ratios (2). Second, the resolution achieved by CIE is far superior to that obtained by the other methods. Indeed, as many as 70 discrete immunoprecipitates can be readily resolved on one CIE test plate with a high degree of reproducibility (1, 34).

Although CIE has been used for some time in the analysis of serum proteins (2, 11, 17), its potential in the study of cell surface antigens and biological membranes has only recently been exploited (5, 14, 28). Both one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedures have been widely used with high resolution of solubilized membrane proteins, but these techniques suffer from the disadvantage that any intrinsic biological activity associated with individual polypeptides is usually destroyed in the process by the denaturing effects of sodium dodecyl sulfate. However, immunoprecipitates formed after CIE of detergent-solubilized membrane fractions often exhibit sufficient enzyme activity to allow specific characterization of their component antigens (10, 28). Furthermore, comparative CIE studies of antiserum serum absorbed with whole cells or with isolated membranes permit conclusions to be made as to the surface location of various membrane immunogens (14, 28). In this way the molecular architecture of the membrane can be successfully investigated (6, 14, 25).

Studies in our laboratory have been directed towards an understanding of the structure-function relationships existing in the membrane system of the gram-positive organism Micrococcus lysodeikticus (30, 32). The enzyme adenosine triphosphatase has been located on the inner, cytoplasmic face of the plasma membrane by both 125I-labeling with lactoperoxidase or 125IICl and by the ferritin-labeled antibody method of immunoelectron microscopy (23, 30). Moreover, our preliminary CIE study has revealed that 8 of the 17 discrete antigens observed for plasma membranes of this organism could be shown by absorption experiments to be exposed on the protoplast surface (i.e., outer surface of the membrane). Five of the antigens that were not detectable on the protoplast membrane surface were shown by zymogram staining to possess enzyme activity (25).

The results presented in this communication provide the first detailed investigation of the CIE resolution of antigens of solubilized plasma membranes of a bacterial species (M. lysodeikticus), together with the asymmetry of distribution of individual membrane antigens, their reactivity towards lectins as determined by immunoflorescentelectrophoresis absorption experiments (8), and the detection of membrane antigens on the surface of intact cells. In addition, a CIE reference system for the cytoplastic fraction of M. lysodeikticus has been established, and its potential for determining antigen distribution in cellular fractions and for monitoring membrane isolation is indicated.

MATERIALS AND METHODS

Preparation of cell fractions. Cells of M. lysodeikticus (NCTC 2665) were grown on peptone water-yeast extract medium and harvested in the stationary phase (18 to 24 h) as previously described (24, 27). For the preparation of plasma membranes, M. lysodeikticus cells washed and suspended in 50 mM Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) to give a suspension containing approximately 50 mg (dry weight) per ml were treated with lysozyme (100 μg/ml) for 45 min at 30°C to remove the cell walls. Deoxyribonuclease (10 μg/ml) was added to the total cell lysate to reduce the viscosity, and the plasma membranes were then sedimented by centrifugation at 38,000 × g for 50 min at 0°C. “Standard” plasma membrane fractions were prepared by six consecutive washes of the membrane deposits with 50 mM Tris-hydrochloride buffer (pH 7.5) by centrifugation and resuspension (24, 27). Methods for the preparation of washed-cell suspensions and protoplast suspensions have been described in detail elsewhere (24, 27). A soluble cytoplasmic fraction was obtained from washed protoplasts of M. lysodeikticus (25) by osmotic lysis (24) and centrifugation at 39,000 × g for 30 min. The supernatant fraction was then centrifuged at 150,000 × g for 1 h to remove residual membrane fragments and concentrated to 22.6 mg of protein per ml by ultrafiltration in an Amicon cell with a PM-10 filter. The concentrated cytoplasmic fraction was then dispensed into 50-μl portions and stored at −70°C until required for further use.

Preparation of Triton X-100 extracts of plasma membranes. Plasma membranes were adjusted to either 40 or 20 mg (dry weight) per ml and extracted with 4% (wt/vol) Triton X-100 as previously described (25). The 17,500 × g supernatant extract was distributed into 50-μl portions and stored at −70°C until use.

Preparation of antisera. Antiserum to “total” membranes (i.e., plasma and mesosomal membranes) was raised in a rabbit as described by Owen and Salton (25), and antiserum to the soluble cytoplasmic fraction was raised similarly. Sera from at least five consecutive bleedings were pooled, and immunoglobulins were partially purified by precipitation with (NH4)2SO4 and dialysis against acetate buffer, pH 5.0 (13). After subsequent dialysis against 0.1 M NaCl containing 15 mM NaN3, the antibody preparations were concentrated by ultrafiltration to about one-tenth of the original serum volume.
Absorption of antimembrane sera with whole cells. Concentrated immunoglobulins (6.5 ml) were dialyzed against two changes of 50 mM Tris-hydrochloride (pH 7.5) containing 0.1 M NaCl, and the final volume was adjusted to 9.0 ml. Portions (1.7 ml) were incubated for 1 h at 22°C with 0 to 3.0 ml of a cell suspension (E₅₄₀ = 10) and sufficient buffer to give a final volume of 6.0 ml. Cells were then removed by centrifugation at 12,000 × g for 15 min, and immunoglobulins in the supernatant fluids were precipitated by the addition of solid (NH₄)₂SO₄ to 50% saturation. Final volumes were adjusted to 4.0 ml after dialysis of the precipitated immunoglobulins against 0.1 M NaCl containing 15 mM NaN₃.

Absorption of antimembrane sera with proteoplasts. The method for obtaining antimembrane serum that had been progressively absorbed with increasing amounts of stable protoplasts has been described in detail elsewhere (25).

Absorption of antimembrane sera with isolated membranes. Concentrated immunoglobulins (2.0 ml) were dialyzed against two changes of 50 mM Tris-hydrochloride (pH 7.5), and the final volume was adjusted to 6.2 ml. Portions (1.0 ml) were incubated for 1 h at 22°C with 0 to 1.0 ml of plasma membrane suspension (22.7 mg [dry weight] per ml) and sufficient buffer to give a final volume of 2.0 ml. Membranes were removed by centrifugation at 12,000 × g for 30 min, and supernatant fractions were dialyzed against 0.1 M NaCl containing 15 mM NaN₃. Because of the small volumes of antisera in this absorption experiment, representation and dialysis of immunoglobulins were omitted and the resultant antisera was used directly without further fractionation.

Preparation of lectins. Wheat germ agglutinin was purified from raw wheat germ as described by Kahane et al. (16), soybean agglutinin was purified from raw soybeans by the method of Nachbar and Oppenheim (20), ricin agglutinin was purified from raw castor beans by the method of Nicolson and Blaustein (22), and Ulex agglutinin I was purified from gorse seeds as described by Matsumoto and Osaka (19). Concanavalin A (3× crystallized and lyophilized) was purchased from Miles-Yeda Laboratory and used without further purification.

Quantitative immunoelectrophoretic techniques. The methods for performing CIE, CIE with intermediate gel, and crossed immunoaffinelectrophoresis in the presence of Triton X-100 have been described in detail elsewhere (2, 25, 34). In all crossed immunoaffinelectrophoresis experiments, lectins were included in the affinity gel at a final concentration of 1 mg/ml. During tests of absorbed antisera, the progress of absorption was monitored by measuring peak heights of the individual antigens on millimeter-ruled graph paper. Under normalized conditions of electrophoresis and antigen loading, the peak height of immunoprecipitates should be proportional to the area subtended by those immunoprecipitates and thus be a reflection of the antibody concentration.

Techniques of staining and preparation of zymograms. After completion of electrophoresis, gels were pressed, washed twice in 0.1 M NaCl, air dried, and stained with Coomassie brilliant blue R-250 by the method of Axelsen et al. (2). Immunoprecipitates containing the enzymes adenosine triphosphatase (EC 3.6.1.3), reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase (EC 1.6.99.3), succinate dehydrogenase (EC 1.3.99.1), and malate dehydrogenase (EC 1.1.1.37) were revealed as previously described (25). Other enzyme-active immunoprecipitates were located by incubating pressed immunoplates individually in the following incubation mixtures (20 ml): (i) isocitrate dehydrogenase (EC 1.1.1.42)—tetrani troblue tetrazolium, 6 mg; 0.1 M sodium isocitrate (pH 7.5), 2.0 ml; 0.1 M KCN, 1.0 ml; nicotinamide adenine dinucleotide phosphate, 4 mg; MnCl₂, H₂O, 4 mg; phenazine methosulfate, 0.5 mg; and 0.1 M Tris-hydrochloride (pH 7.0), 17.0 ml (incubation was continued at 22°C for 10 to 15 min); (ii) catalase (EC 1.11.1.6)—3% H₂O₂, 1.0 ml; 0.6 M Na₂SO₄, 5H₂O, 1.4 ml; 0.1 M Tris-hydrochloride (pH 7.0), 2.0 ml; and water, 15.6 ml (incubation was continued at 22°C for 15 min, followed by immersion of gels in 20 ml of 45 mM KI until the background was light blue [about 1 min]); (iii) polynucleotide phosphorylase (EC 2.7.7.8)—adenosine 5′-diphosphate, 125 mg; 0.1 M MgSO₄, 2.5 ml; 2% (wt/vol) Pb(NO₃)₂, 1.5 ml; and 0.1 M Tris-hydrochloride (pH 7.5), 17.0 ml (incubation was continued at 37°C for 3 h, and gels were pressed, washed, and finally developed with 0.1% [wt/vol] Na₂S).

The methods outlined by Uriel (35) were followed in attempts to detect precipitates having the following enzyme activities: glucose 6-phosphate dehydrogenase (EC 1.1.2.49), alanine dehydrogenase (EC 1.4.1.1), glutamate dehydrogenase (EC 1.4.1.2), lipase (EC 3.1.1.3), alkaline phosphatase (EC 3.1.3.1), acid phosphatase (EC 3.1.3.2), chymotrypsin (EC 3.4.21.1), and trypsin (EC 3.4.21.4). The method described by Smyth et al. (34) was used in an attempt to detect immunoprecipitates possessing lactate dehydrogenase activities (EC 1.1.1.27), and those described by Uriel (35) or by Brogren and Bog-Hansen (10) were used to detect esterase-active immunoprecipitates. Location of immunoprecipitates possessing muralytic enzyme activity was attempted by overlaying pressed immunoplates with an agarose gel (about 2 mm thick) made by mixing equal volumes of 2% (wt/vol) agarose and a sonically dispersed suspension of isolated M. lysodeikticus cell walls in 50 mM Tris-hydrochloride (pH 7.5) at a concentration of 10 mg (dry weight) of wall per ml. Incubation was continued in a humid atmosphere at 37°C for several weeks.

After zymogram development, gels were routinely rinsed with distilled water, pressed, and washed twice with 0.1 M NaCl before final pressing and air drying. Gels stained for catalase had to be rapidly air dried after background color development to retain a sharply defined, negatively stained image.

Chemical procedures. Protein was determined by the method of Lowry et al. (18), with bovine serum albumin as the standard.

Chemicals. All chemicals were obtained from standard commercial sources and were used without further purification.

RESULTS

Reference CIE pattern for plasma membranes. For a detailed analysis of a complex
mixture of antigens such as those present in detergent-solubilized membranes of *M. lysodeikticus*, it was necessary to perform CIE over a wide range of antigen and antibody concentrations. This analysis has been performed with four different plasma membrane preparations, and the number of resolvable immunoprecipitates totaled 27 discretely separated antigens. A schematic diagram showing the relative positions and intensities of all 27 immunoprecipitates numbered in order of decreasing electrophoretic mobility of their peak maxima is shown in Fig. 1. The most common immunoprecipitates found in the preliminary study (25) and the present investigation appear unchanged in relative height and staining intensity. Differences observed in the intensities and peak heights of some, notably no. 4 and 21, appear to reflect an increased antibody titer in later serum pools, since their peak heights in the four different membrane preparations were fairly similar when tested against any one serum pool (cf. Fig. 3A and 5A). However, peak heights of other antigens, such as no. 1 and 10, varied considerably between different membrane preparations. This phenomenon may either reflect differences in the efficiency of extractions or be due to minor variations in the methods of membrane preparation.

Most of the additional 10 precipitates detected in the present study stained weakly and could only be readily observed either at relatively high antigen loadings (e.g., no. 12, 15, 22, and 27) or at relatively low antigen loadings (e.g., no. 9, 23, and 24; see Fig. 2A). However, two additional immunoprecipitates (no. 19 and 26) stained more intensely. Immunoprecipitate 19 followed the profile of no. 20 very closely and only became resolved in later pools of antimembrane serum, presumably because of changing antibody titers. Precipitate 26 was observed infrequently and usually only at lower loadings of antigen (Fig. 2A).

The majority of immunoprecipitates were observed consistently in all four plasma membrane preparations tested, but some, notably no. 6, 12, and 22, were not. This may reflect their weak intensity and/or positioning close to heavier-staining precipitates or the loose association of

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**Fig. 1.** Schematic representation of the CIE pattern obtained for plasma membranes of *M. lysodeikticus*, revised to accommodate the 10 additional precipitates detected in the present study. The width of lines is intended to indicate the observed intensities of immunoprecipitates. Many of the weaker immunoprecipitates shown here are lost upon photographic reproduction, and the outline of some precipitates, e.g., precipitate 22, was often difficult to follow. Anode to left and top.
these antigens with the membrane. Many of the resolved immunoprecipitates showed consistent and marked heterogeneities (e.g., no. 5, 8, 10, 11, 16, and 18; see Fig. 1, 2A, 3A, and 5A). However, only one pair of antigens (no. 4 and 17) showed reactions of partial identity when membrane extracts were subjected to electrophoresis for a short time in the first dimension (Fig. 2B). A curious feature of antigen 8 was its occasional tendency to appear resolved as more than one immunoprecipitate (Fig. 2A). This multiplicity, when evident, was more noticeable towards the cathodal half of the precipitate, all ramifications of the basic immunoprecipitate fusing at or before the extreme anodal foot. This phenomenon is reminiscent of the multiplicity of precipitates induced by plasmin (7) and consistently observed for spectrin during CIE of solubilized erythrocyte membranes (4, 6, 9). However, the multiplicity observed for precipitate 8 in the present study was unaffected by inclusion into the antibody gel of the protease inhibitor Trasylol at a final concentration of 1,000 U/ml (7).

Absorption of antimembrane serum with stable protoplasts. Absorption of antimembrane serum with increasing volumes of washed protoplasts of *M. lysodeikticus* has established that four antigens (no. 12, 15, 23, and 24) of the additional 10 immunoprecipitates resolved in this study have determinants exposed on the protoplast surface, together with the eight (no. 1 to 5, 7, 17, and 18) previously found (and reconfirmed). The surface location of antigen 26 was not conclusively determined due to its unpredictable behavior. Absorption of antimembrane serum with membranes. Absorption of antimembrane serum with increasing volumes of washed membranes would be expected to reduce antibodies directed against immunogens exposed on either the outer protoplast surface or the inner, cytoplasmic face of the membrane. Absorption of antibody directed against any membrane surface component will be reflected by an increase in the area under that immunoprecipitate, since the area is proportional to the antigen-antibody ratio (2) for a constant amount of antigen subjected to electrophoresis. Conversely, antibodies directed against membrane immunogens that are not present on either face of the membrane should be unaffected by absorption, resulting in immunoprecipitates of constant area. Figure 3 (A to E) demonstrates that all detectable antigens were affected by absorption and that membrane immunogens (apart from the uncertainty of no. 26) have their determinants accessible on either or both membrane surfaces.

One of the most interesting features of the absorption experiments was that antibodies to the various antigens showed distinctly different rates of absorption and removal (Fig. 3A to E and 4). Thus, for example, antibodies to the succinylated lipomannan (antigen 18, Fig. 3A) were readily absorbed, and absorption was almost complete, as shown in Fig. 3B in the initial absorption. Immunoglobulins to antigens 4, 5, and 11 (adenosine triphosphatase) were also readily absorbed, whereas those to antigens 10 (NADH dehydrogenase) and 20 were removed much less readily (Fig. 3A to E and 4).
whole cells. To determine whether any of the membrane immunogens were detectable on the bacterial cell surface, antimembrane serum was absorbed with increasing quantities of washed cells of *M. lysodeikticus*, and the resultant sera were tested against Triton X-100 extracts of plasma membranes as described above. Antigens 6, 8 to 11, 13, 14, 16, 19 to 22, 25, and 27 were unaffected by cell quantities several times those required to remove antibodies to antigens 1, 2, and 18 (among others) (Fig. 5A to D), thus indicating that the determinants of these antigens were not exposed on the cell surface. Antibodies to all 12 antigens detected on the proto-

**FIG. 3.** Effect of absorption of antimembrane serum with membranes of *M. lysodeikticus* upon the pattern obtained by CIE of a Triton X-100 extract of plasma membranes. Antimembrane serum was absorbed with (A) 0 ml, (B) 0.05 ml, (C) 0.10 ml, (D) 0.25 ml, and (E) 1.00 ml of washed plasma membranes as described in the text, and the immunoglobulin fractions were incorporated into agarose gels (95 µl/ml). Membrane protein (76 µg) was analyzed by CIE in all instances. Areas under immunoprecipitates 18 and 11 increased rapidly upon absorption, whereas those under no. 10 and 20 increased more slowly. No immunoprecipitates were detected in (E). Anode to left and top.
plast surface were also removed by absorption with the intact whole cells. From a close examination of Fig. 5A to D and from Fig. 6 it is evident that antibodies to the membrane immunogens that were detectable on the cell surface were absorbed at greatly differing rates. For example, antibodies to antigens 2 and 18 (succinylated lipomannan) were absorbed some six times more readily than antibodies to antigens 5 and 17 (Fig. 6).

Characterization of immunoprecipitates in the plasma membrane reference pattern. The identities of the five enzyme-active immunoprecipitates (no. 8 [succinate dehydrogenase], 10 and 13 [NADH dehydrogenase], 11 [adenosine triphosphatase], 21 [malate dehydrogenase]) and antigen 18, the succinylated lipomannan (26, 29) in the plasma membrane reference pattern (25), were confirmed in all four plasma membrane preparations. Attempts to identify other immunoprecipitates as enzymes by the following zymogram techniques failed: viz., lactate dehydrogenase, isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase, glutamate dehydrogenase, alanine dehydrogenase, acid and alkaline phosphatases, polynucleotide phosphorylase, esterase, lipase, trypsin, chymotrypsin, catalase, and cell wall muraisin.

By using the lectin concanavalin A as an affinity absorbent in crossed immunoaffinoelectrophoresis experiments, we had previously shown that antigens 7, 17, and 18 (and also possibly antigens 4 and 5) have sugar residues in conformations allowing interaction with this lectin (25). Of the additional antigens detected in the present study, two (no. 15 and 23) were also absorbed in crossed immunoaffinoelectrophoresis experiments with concanavalin A. Furthermore, the affinity experiments have been extended to include the lectins, wheat germ agglutinin, soybean, ricin and Ulex agglutinins, which have primary sugar specificities for N-acetyl-d-glucosaminyl, N-acetyl-d-galactosaminyl, d-galactosyl, and l-fucosyl residues, respectively (19, 22, 33). Both ricin and soybean agglutinins appeared to absorb antigen 14, whereas neither wheat germ nor Ulex agglutinin had any observable effects on any of the antigens in the plasma membrane reference pattern. Many of the weaker immunoprecipitates (such as no. 12, 22, 24, and 27) were rather difficult to detect except under optimized conditions, and, due to the limited availability of both lectins and antisera, it has been impossible to document completely all of the reactions in these affinity experiments.

Reference CIE precipitin pattern for the cytoplasmic fraction. CIE of the cytoplasmic antigen fraction under conditions of varying antigen and anticytoplasm fraction concentrations has resolved at least 97 distinct immunoprecipitates. The complexity of the system is illustrated in Fig. 7A. Two discrete immunoprecipitates can be shown by zymograms to possess NADH dehydrogenase activities (Fig. 8A), one immunoprecipitate possessing isocitrate dehydrogenase activity (Fig. 8B) and two others possessing polynucleotide phosphorylase activity (Fig. 8C) and catalase activity (Fig. 8D). Zymogram tests designed to detect other enzyme activities (see Materials and Methods) were negative.

CIE analysis with intermediate gels. CIE with an intermediate gel (2) provides a simple and sensitive technique for comparing complex antibody populations in two different antisera reacting against antigens in a single immunoplate. Thus, the cellular origins of immunogens (e.g., cytoplasmic versus membrane) in heterogeneous antigen fractions can be determined by the intermediate-gel variation of CIE. In our specific studies, the distribution of cellular antigens in cytoplasmic and membrane fractions of M. lysodeikticus was determined. When an intermediate gel contained antimembranous serum below cytoplasmic antisera in the analysis of cytoplasmic antigens, several immunoprecipitates were depressed into the intermediate gel (Fig. 7B). One of these stained for NADH de-
hydrogenase, and another appeared to have no counterpart in the reference gel (Fig. 7A). Electrophoretic profiles and zymogram staining suggest that they correspond to antigens 10 and 18, respectively, in the plasma membrane reference pattern presented in Fig. 1.

When an intermediate gel containing anticytoplasm fraction was introduced below the antimmembrane gel during the analysis of plasma membrane extracts, only 3 (no. 2, 9, and 10) of the 27 plasma membrane immunoprecipitates were found in the intermediate gels (gels not shown). NADH dehydrogenase antigen 10 was included in the intermediate gel, in accord with its partitioning between both membrane and cytoplasm fractions. Several other antigens (no.

**Fig. 5.** Effect of absorption of antimmembrane serum with whole cells of *M. lysodeikticus* upon the pattern obtained by CIE of a Triton X-100 extract of plasma membranes. Antimmembrane serum was absorbed with (A) 0 ml, (B) 0.25 ml, (C) 0.5 ml, (D) 1.5 ml, and (E) 3.0 ml of washed cells as described in the text, and the immunoglobulin fractions were incorporated into agarose gels (95 µl/ml). Membrane protein (47 µg) was analyzed by CIE in all instances. Areas under immunoprecipitates 4 and 18 (among others) increased at different rates upon absorption. Precipitates 6, 8 to 11, 13, 14, 16, 19 to 22, 25, and 27 appear to be unaffected by absorption (see Fig. 1). Anode to left and top.
Progress of Absorption of Antimembrane Immunoglobulins during Absorption with Increasing Amounts of Whole M. lysodeikticus Cells. One unit on the abscissa represents the absorption of antimembrane immunoglobulins by 0.25 ml of cell suspension as detailed in the text. The absorption of immunoglobulins was monitored by measuring the relative increase in peak area for the particular antigen in question. The symbols *, □, △, ○, □, and Δ represent antibodies to antigens 18, 2, 1, 6, 17, and 4, respectively.

**DISCUSSION**

Identification of the complex variety of membrane antigens, including enzymes, has been hampered in the past by the poor resolving power of conventional immunodiffusion and immunoelectrophoresis. Analysis of cell membranes by CIE has, however, provided a reproducible system for a high degree of resolution of *M. lysodeikticus* membrane antigens. Our preliminary studies have shown that only certain membrane antigens were found on the outer membrane surface of protoplasts (25). The comprehensive analysis presented in this paper has clearly established the asymmetrical distribution of membrane antigens by absorption experiments with intact protoplasts and isolated plasma membrane preparations in which both outer and cytoplasmic faces of the membranes are freely accessible for reaction with antibodies. Twelve antigens are exposed on the outer surface of the membrane and, of these, six or pos-

![Graph showing the progress of absorption](image-url)

**Fig. 6.** Progress of the removal of antimembrane immunoglobulins during absorption with increasing amounts of whole *M. lysodeikticus* cells. One unit on the abscissa represents the absorption of antimembrane immunoglobulins by 0.25 ml of cell suspension as detailed in the text. The absorption of immunoglobulins was monitored by measuring the relative increase in peak area for the particular antigen in question. The symbols *, □, △, ○, □, and Δ represent antibodies to antigens 18, 2, 1, 6, 17, and 4, respectively.

![CIE with intermediate gel with the cytoplasm anticytoplasm reference system](image-url)

**Fig. 7.** CIE with intermediate gel with the cytoplasm anticytoplasm reference system. Gel regions marked (a), (b), and (c) contain anticytoplasm serum (9.5 μl/ml), concentrated antimembrane serum (13.2 μl/ml), and agarose alone, respectively. Similar amounts (118 μg of protein) of concentrated cytoplasmic antigen were subjected to CIE in both instances. In (A), NADH dehydrogenase (i and ii), isocitrate dehydrogenase (iii), polynucleotide phosphorylase (iv), and catalase (v) are indicated (see Fig. 8 also). In (B), the probable identities of two major immunoprecipitates fully included in the antimembrane intermediate gel are indicated. Anode to left and top.
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Fig. 8. Characterization of immunoprecipitates in the cytoplasm-anticytoplasm reference system by zymogram techniques. Similar amounts (45 μg) of concentrated cytoplasmic antigen were subjected to CIE (34) in all instances. Antibody gels contained anticytoplasm immunoglobulins (9.5 μl/ml). Gels were subsequently stained for (A) NADH dehydrogenase, (B) isocitrate dehydrogenase, (C) polynucleotide phosphorylase, and (D) catalase. Enzymatically stained gels could be subsequently counterstained with Coomassie brilliant blue to confirm their identity in the reference pattern (Fig. 7A). Parts of an overlapping precipitate within the heavily staining NADH dehydrogenase peak appear stained due to nonspecific absorption of the enzyme stain or nonspecific entrapment of enzyme (unnumbered arrow in A). Anode to left and top.

Possibly seven appear to possess sugar residues interacting with concanavalin A, thus suggesting a protoplast surface rich in antigens possessing carbohydrate residues, a feature shared with most mammalian cells (33). The asymmetry of this membrane structure was further emphasized by the detection of a membrane antigen (no. 14) that was detectable only on the cytoplasmic face of the membrane and was reactive with both ricin and soybean lectins. The presence of such a glycoprotein antigen (apparently possessing galactosyl and/or N-acetyl-D-galactosaminy1 residues) on the inner face of the membrane is intriguing and worthy of further investigation. None of the five membrane antigens identifiable as a specific enzyme by zymogram staining was detectable on the surface of stable protoplasts, but absorption experiments with isolated membranes exposing outer and cytoplasmic faces established that antibodies to all five enzymes were removed (Fig. 3A to E).

Thus, the absorption experiments have clearly shown that all detectable immunogens have determinants accessible on one or both sides of the membrane and that the five enzyme antigens are present only on the cytoplasmic face of the membrane (Fig. 3A to E).

These investigations do not permit the identification of antigens (transmembrane) with determinants exposed on both faces of the membrane. Further detailed investigations would be needed to specifically identify such immunogens by labeling techniques, proteolytic cleavage of surface exposed antigens, or absorption experiments with homogeneous populations of inside-out membrane vesicles (a goal not yet achieved for any membrane system; 32). Moreover, the present studies have not explored the possible existence of immunogens totally buried within the membrane. However, it should be noted that absorption with isolated membranes may remove antibodies to antigens exposed along mem-
brane fracture surfaces should these develop during membrane isolation (14). All of these possibilities would require further exploration.

An interesting feature of the absorption experiments with whole membranes was that antibodies to some immunogens were absorbed at different rates. This is contrary to the findings of Johansson and Hjerten in a study of Achopleasma laidlawii membrane antigens (14). It could be argued that our findings reflect differing concentrations and antigenicities of the various membrane immunogens in our system. However, it is difficult to accept this explanation since antibodies to antigen 8 were absorbed some six times less readily than those to antigen 11, and both antigens appeared to be equally immunogenic. It seems probable that differential rates of antibody absorption could in part reflect the relative accessibilities of the antigens in the membrane. It is significant that antigen 11, antibodies to which are readily absorbed, has been characterized as adenosine triphosphatase, an enzyme peripherally located on the membrane of *M. lysodeikticus* and releasable by osmotic shock treatment (23, 30). In contrast, antibodies to antigen 8 were absorbed relatively slowly, and this antigen was identified as succinate dehydrogenase, an enzyme firmly associated with membrane fragments after extraction with sodium deoxycholate (31). Furthermore, in a CIE study of mouse liver plasma membranes, Gard et al. (12) found evidence that certain membrane antigens became more fully “expressed” after removal of other membrane components.

Absorption experiments with whole cells revealed a differential removal of antibodies to some membrane immunogens. This may also reflect their relative accessibilities on the cell surface, a possibility supported by the observations that the succinylated lipomannan (antigen 18), antibodies to which are absorbed relatively rapidly (Fig. 6), appears to be secreted into the medium during the late logarithmic phase of growth (27) in a manner analogous to that of lipoteichoic acid of other bacterial species (15). The other major antigens of the protoplast surface (no. 4 and 17) do not appear to share the same degree of exposure and may be located closer to the membrane surface.

The cytoplasmic-anticytoplasmic reference system revealed a complex pattern of immunoprecipitates markedly different from that observed for plasma membranes, a reflection of the variety and distribution of functions between these two cellular compartments. The fact that the enzymes catalase, polynucleotide phosphorylase, and isocitrate dehydrogenase can be shown by zymogram techniques to be localized in the cytoplasmic fraction attests both to the specificity of the zymogram reactions and to the cell fractionation procedures. All three enzymes have previously been shown to be localized largely in the cytoplasm of *M. lysodeikticus* (21).

From CIE experiments with intermediate gels, some components, notably no. 10 (NADH dehydrogenase) and no. 18 (succinylated lipomannan), partition between both cytoplasm and plasma membrane fractions. NADH dehydrogenase activity has been detected previously in both fractions by direct enzyme assay (21). Of the three NADH dehydrogenase enzymes detected for *M. lysodeikticus*, one (antigen i in Fig. 8A) appears to be largely membrane associated, one (antigen j in Fig. 8A) appears to be largely cytoplasmic, and one (no. 10) distributes between both fractions (Fig. 7). One form of the membrane-associated mannan can also be readily removed from the membrane (27) and may be solubilized to some extent during protoplast rupture used in separating cytoplasmic fractions. This low-molecular-weight polysaccharide (27) proved to be a very poor immunogen in the purified state, only displaying immunogenicity in the membrane-bound form (28); this could explain why antigen 18 was only observed in the cytoplasmic antigen fraction when antimembrane serum was used in the intermediate gel. The cytoplasmic-anticytoplasmic reference system thus provides a valuable adjunct to membrane antigen studies in which distributions in cell fractions can be studied with the intermediate gels, and cross-contamination and monitoring of membrane isolation can be followed.

The studies presented in this article and elsewhere have led to a fuller understanding of the asymmetrical distribution of membrane proteins (23, 30) and phospholipids (3) of this bacterial membrane system and will ultimately broaden our knowledge of membrane structure-function relationships in procaryotic cells.

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**LITERATURE CITED**


