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Antigenic and enzymatic architecture of *Micrococcus lysodeikticus* membranes established by crossed immunoelectrophoresis

(crossed affino-immunoelectrophoresis/zymogram techniques)

PETER OWEN AND MILTON R. J. SALTON

Department of Microbiology, New York University School of Medicine, New York, N.Y. 10016

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ABSTRACT **By crossed immunoelectrophoresis with membrane antiserum, 17 antigens have been detected in fractions from plasma membranes of *M. lysodeikticus* solubilized with Triton X-100. Absorption tests with protoplasts have demonstrated that eight of the antigens are expressed on the surface. Of these antigens the major one has been identified as a succinylated mannan. Five of the principal immunoprecipitates unaffected by absorption with protoplasts were shown by zymograms to possess the following enzymic activities: succinate dehydrogenase (EC 1.3.99.1), ATPase (EC 3.6.1.3), NADH dehyrogenase (EC 1.6.99.3)(two separate components), and malate dehydrogenase (EC 1.1.1.37). These enzymes or enzyme-complexes are, therefore, not expressed on the outer surface of the protoplast membrane.**

Bacterial membranes perform many of the functions found in separate membranous organelles in the eukaryotic cell. Thus functions of electron transport, of active transport, and the biosynthesis of phospholipids and cell walls all occur in the bacterial membrane (1). In membranes of Gram-positive bacteria two principal regions, the plasma or cytoplasmic membrane and the mesosome, are recognizable (2) and these two entities have been isolated as homogeneous fractions from several species (3). Our investigations have been directed towards the elucidation of the structure–function relationships of the membranes of *Micrococcus lysodeikticus* by a multidisciplinary approach combining biochemical, immunochemical, and electron microscopic techniques. With conventional immunochemical methods, earlier studies in this laboratory showed the presence of three principal immunoprecipitates when membrane fractions of *M. lysodeikticus* reacted with membrane antiserum (4). These results did not reflect the anticipated complexity of the membrane, considering the multiplicity of the functions it performs and the number (about 30) of individual polypeptides found by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels (5, 6).

The potentials of crossed immunoelectrophoresis (CIE) and related techniques of tandem CIE, together with the use of intermediate gels containing monospecific antisera or lectins acting as specific affinity absorbents (crossed affino-immunoelectrophoresis) have seldom been exploited in the study of the molecular architecture of microbial membranes and their antigens (see, however, ref. 7). In order to gain further understanding of the biochemical architecture of the membranes of *M. lysodeikticus* we have accordingly undertaken such a study.

MATERIALS AND METHODS

Preparation of Protoplasts and Membrane Fractions. Cells of *Micrococcus lysodeikticus* (NCTC 2665) were cultured and harvested as described (5). Washed cells were resuspended in Buffer I (0.8 M sucrose, 10 mM Mg^{2+}, 70 mM NaCl in 50 mM Tris·HCl, pH 7.5), lysozyme was added to 200 μg/ml, and the cell suspension (OD_{625nm}^{1cm} = 16) was held at 22° for 45 min. Protoplasts were sedimented (9000) × *g*, 30 min, 22°), washed once, and resuspended in Buffer I (OD_{625nm}^{1cm} = 10).

Methods for the preparation of "total" membranes (i.e., plasma and mesosomal membranes), plasma membranes, and purified mannan from *M. lysodeikticus* are detailed elsewhere (5, 8). Triton X-100 extracts of plasma membranes were obtained by treatment with detergent (final concentrations of 4%, w/v) for 1 hr at 22°. Membrane residues (about 20%) sedimentable at 17,500 × *g* for 45 min were removed and the supernatant fractions (19 mg of protein per ml) were retained for analysis.

Preparation of Antisera. Rabbits were immunized with "total" membranes (2 mg) given initially into both footpads and intradermally with Freund's complete adjuvant. Further injections of antigen without adjuvant were given weekly by subcutaneous injection at multiple sites. Subsequently, sera from four consecutive bleedings were pooled, and immunoglobulins were partially purified by precipitation with ammonium sulfate and dialysis against acetate buffer, pH 5.0 (9), and then concentrated by ultrafiltration to one-tenth of the original serum volume.

Absorption of Anti-Membrane Serum with Protoplasts. Concentrated immunoglobulins (2.0 ml) were dialyzed against Buffer II (0.8 M sucrose, 10 mM Mg^{2+}, 70 mM NaCl, 50 mM Tris·HCl, pH 8.6) and the final volume was adjusted to 6.0 ml. Aliquots (1.0 ml) were incubated for 1 hr at 22° with 0–3.0 ml protoplast suspension and sufficient Buffer II to give a final volume of 6.0 ml. Protoplasts were removed by centrifugation, the supernatant fraction was dialyzed against 0.1 M NaCl, and precipitated (9) immunoglobulins were made up to a final volume of 3.5 ml.

Crossed Immunoelectrophoresis. The method for performing CIE was based upon a modification (10) of Laurell's technique (11). Barbital–HCl buffer (ionic strength μ = 0.02, pH 8.6) containing 1% (v/v) Triton X-100 was used throughout and incorporated into all gels. 1% agarose gels were cast on glass plates (50 mm × 50 mm × 0.6 mm) to give a volume to surface area ratio of 0.132 ml/cm^2. Samples, applied to wells 1.5 mm in diameter, were subjected to electrophoresis at 5 V/cm for 65 min in a Behring Diagnostic water-cooled immunoelectrophoresis cell. An agarose strip (10 mm × 50 mm) containing antigen was retained on the plate after removal of the rest of the gel which was replaced with an adjacent gel (40 mm × 50 mm) containing antibody. Electrophoresis in the second direction was then performed at 2 V/cm for 12–18 hr.

Crossed Affino-Immunoelectrophoresis. The method employed was a modification of that of Bøg-Hansen (12).

Abbreviation: CIE, crossed immunoelectrophoresis.

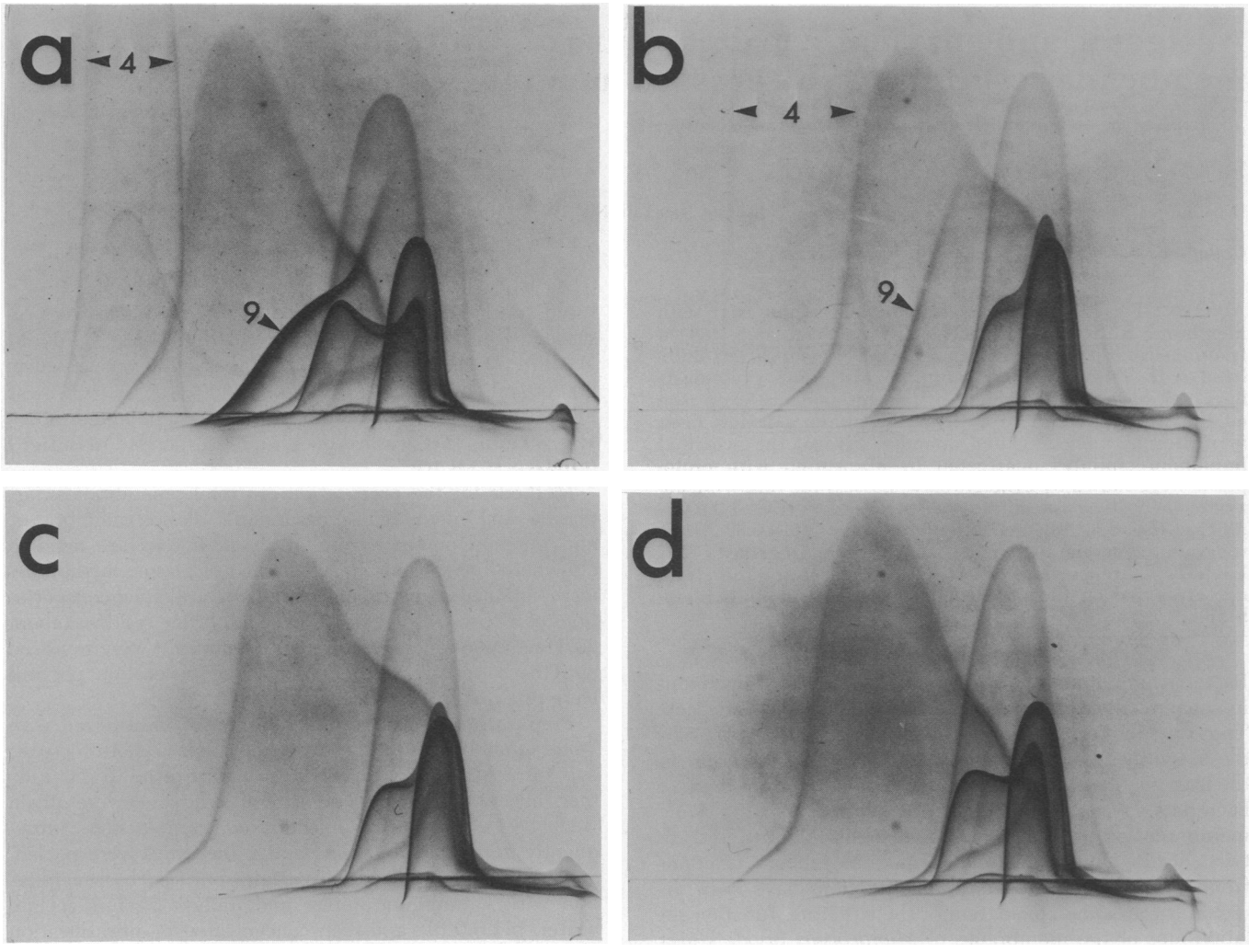

FIG. 1. Effect of absorption of anti-membrane serum with protoplasts of *M. lysodeikticus* upon the pattern obtained by CIE of a Triton X-100 extract of plasma membranes. Anti-membrane serum was absorbed with 0 ml (a), 0.5 ml (b), 1.0 ml (c), and 3.0 ml (d) of washed protoplast suspension and the immunoglobulin fraction was incorporated into agarose gels (145 μl/ml). Membrane protein (57 μg) was analyzed by CIE in all instances. Areas under immunoprecipitates 4 and 9 increased upon absorption (compare a with b) and were totally absent from patterns (c) and (d). Similar phenomena were observed for precipitates 1–3, 5, 7, and 16 indicated in Fig. 2. Precipitates 6, 8, 10–15, and 17 (see Fig. 2) were unaffected by absorption.

Agarose gels were cast on glass plates (102 mm × 82 mm × 0.8 mm), antigen was applied to wells 2.5 mm in diameter, and electrophoresis was performed at 6 V/cm for 100 min. The upper portion of the gel was discarded, leaving an agarose strip (14 mm × 82 mm) containing antigen. Agarose gels were cast and cut to give a final plate consisting of four parallel gels (width 82 mm) 14, 10, 5, and 73 mm in length, containing membrane antigen, concanavalin A, agarose alone, and antibody, respectively. In control runs, the concanavalin A gel and its spacer gel were replaced with a single spacer gel (15 mm × 82 mm). Electrophoresis in the second dimension was performed at 2.5 V/cm for 12–18 hr.

Techniques of Staining and Preparation of Zymograms. After electrophoresis, gels were pressed, washed twice in 0.1 M NaCl, air-dried, and stained with Coomassie brilliant blue (13). Immunoprecipitates containing specific enzymes were revealed by immersing pressed gels in appropriate incubation mixtures (20 ml) for the following activities: (*a*) succinate dehydrogenase (EC 1.3.99.1): tetranitrobluetetrazolium, 6 mg; 1.0 M disodium succinate (pH 7.0), 2.0 ml; 0.1 M KCN, 1.0 ml; 50 mM Tris·HCl (pH 7.5), 17.0 ml; (*b*) malate dehydrogenase (EC 1.1.1.37): tetranitrobluetetrazolium, 6 mg; phenazine methosulphate, 0.5 mg, NAD, 10 mg; 1.0 M sodium DL-malate (pH 7.0), 2.0 ml; 0.1 M KCN, 1.0 ml; 50 mM Tris·HCl (pH 7.5), 17.0 ml; (*c*) NADH dehydrogenase (EC 1.6.99.3): tetranitrobluetetrazolium, 6 mg; NADH, 7 mg; 0.1 M Tris·HCl (pH 7.5), 20.0 ml; (*d*) ATPase (EC 3.6.1.3): 0.02 M ATP (pH 7.0), 8.0 ml; 2% lead acetate, 1.2 ml; 0.1 M $MgSO_4$, 2.0 ml; 0.1 M Tris·HCl (pH 7.5), 8.0 ml; distilled water, 0.8 ml. Gels stained for ATPase were developed with 0.1% Na_2S.

Chemicals. Concanavalin A and agarose were obtained from Miles Labs and Triton X-100 from Baker Chemical Co.

RESULTS

The pattern obtained following CIE of a Triton X-100 extract of *M. lysodeikticus* membranes against unabsorbed rabbit anti-membrane serum is shown in Fig. 1a. At least seventeen discrete immunoprecipitates, some showing heterogeneity, were observed. A schematic diagram of this pattern with the numbers assigned is given in Fig. 2. Few, if any of the antigens in this complex pattern showed characteristics compatible with the suggestion that they could be

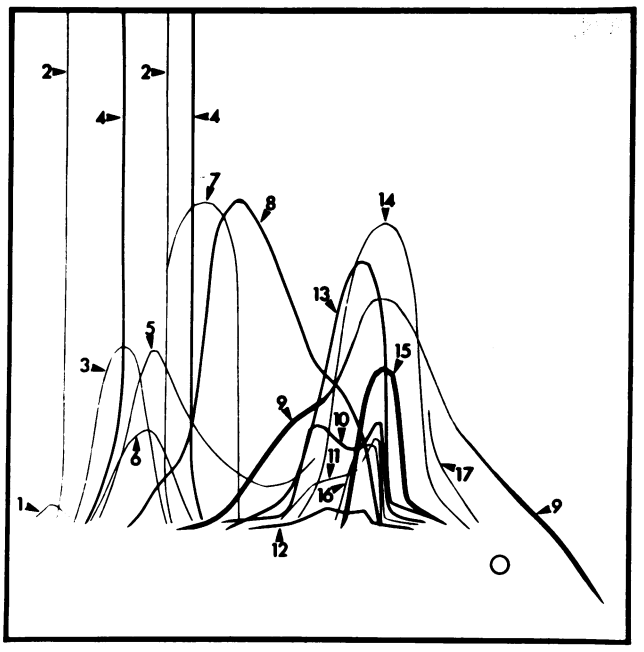

FIG. 2. Schematic representation of the CIE pattern shown in Fig. 1a. Width of lines is intended to indicate the observed intensity of immunoprecipitates. Many of the weaker precipitates shown here are lost upon photographic reproduction. The upper part of precipitate 9 was often very faint (as in Fig. 1a) or lost completely (as in Figs. 4a, 5a, and 5b). Similarly, precipitates 5 and 17 were often difficult to follow.

multiple products of proteolytic digestion caused by plasmin (14). Differences were occasionally noted in the quantities of some of the minor immunogens and also in the degree of heterogeneity between different membrane extracts (compare Fig. 1a with Fig. 4a). It is possible that these differences reflect changes in the efficiency of extraction and alterations in the degree of association of antigen complexes.

Absorption of anti-membrane serum with increasing volumes of stable protoplasts would be expected to reduce antibodies directed against immunogens expressed on the surface. Since the area under an immunoprecipitate in the CIE system is proportional to the antigen/antibody ratio (13), it follows that, for a given amount of electrophoresed antigen, absorption of antibody will be reflected by an increase in the area under the immunoprecipitate, since the antigen must travel further in order to form a stable precipitate. Conversely, antibodies directed against membrane immunogens which are not expressed on the surface should be unaffected by absorption, resulting in immunoprecipitates of constant area. Fig. 1a–d demonstrates that antigens 6, 8, 10–15, and 17 are unaffected by absorption, even by quantities of protoplasts three times those required to remove antibodies to antigens 1–5, 7, 9, and 16. It follows that the former group of antigens does not have determinants accessible on the protoplast surface. Furthermore, antigen 9 appears to be the major surface immunogen.

Five of the major immunoprecipitates unaffected by absorption were shown by the use of zymograms to contain the following enzymes: no. 8—succinate dehydrogenase (Fig. 3a); no. 10—ATPase (Fig. 3b); nos. 12 and 13—NADH dehydrogenase (Fig. 3c); no. 14—malate dehydrogenase (Fig. 3d).

Of the surface antigens, three (nos. 7, 9, and 16) could be preferentially removed by crossed affino-immunoelectrophoresis with an intermediate gel containing the lectin concanavalin A (compare Fig. 4a and b). This procedure would be expected to absorb only molecules containing sugar residues in α-D-mannopyranoside or α-D-glucopyranoside configurations (15), although some nonspecific interactions have been reported (16). A succinylated mannan is known to be the major carbohydrate component of washed membrane fractions isolated from *M. lysodeikticus* (8, 17), and antigen 9 was identified as this polymer by coelectrophoresis of the Triton X-100 membrane extract with the purified polysaccharide (compare Fig. 5a and b).

DISCUSSION

The multiplicity of immunoprecipitates obtained by CIE of Triton X-100 extracts of plasma membranes of *M. lysodeikticus* dramatically establishes the antigenic complexity of this membrane structure and demonstrates the enhanced resolution of the technique over conventional immunochemical methods (4). Other unique antigens may also be present in the Triton-insoluble membrane residues which have not yet been examined by dissolution in stronger surfactants.

Whether the individual immunoprecipitates represent single membrane components or multienzyme complexes is uncertain at present. Blomberg and Raftell (18) found evidence of multienzyme complexes in detergent-solubilized preparations from rat liver microsomes and plasma membranes. Furthermore, by using different solubilization procedures these authors showed that the complexes were not artifacts caused by interaction of enzyme molecules with detergent micelles (19). The observation that few, if any, of the immunoprecipitates detected in our study show lines of partial identity would argue against complexes arising from a random association of components following solubilization

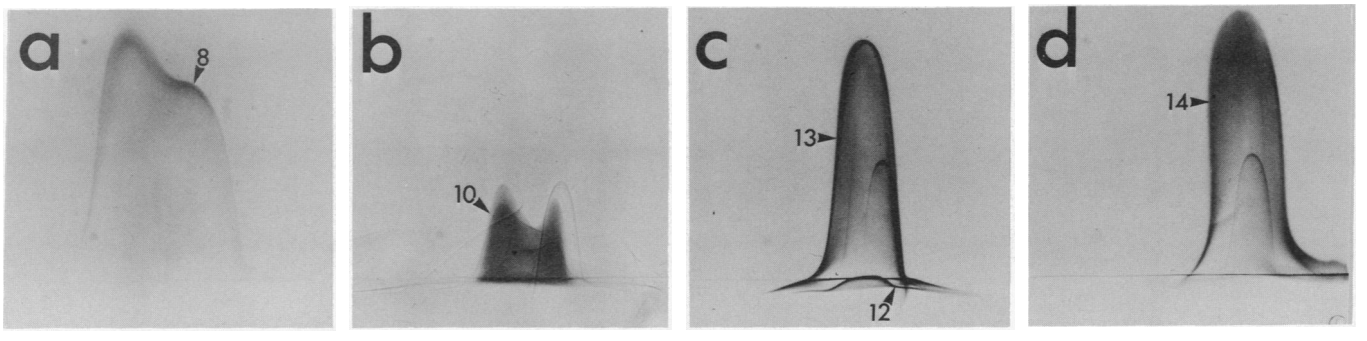

FIG. 3. Characterization of immunoprecipitates by zymograms. (a–d) represent gels similar to the one illustrated in Fig. 1a, stained for succinate dehydrogenase, ATPase, NADH dehydrogenase, and malate dehydrogenase, respectively. Enzymatically stained gels could be subsequently stained with Coomassie brilliant blue to confirm the identification as indicated. Parts of overlapping precipitates were frequently reinforced within the zymogram staining areas (b, c, d), phenomena which appear to be due to nonspecific absorption of stain.

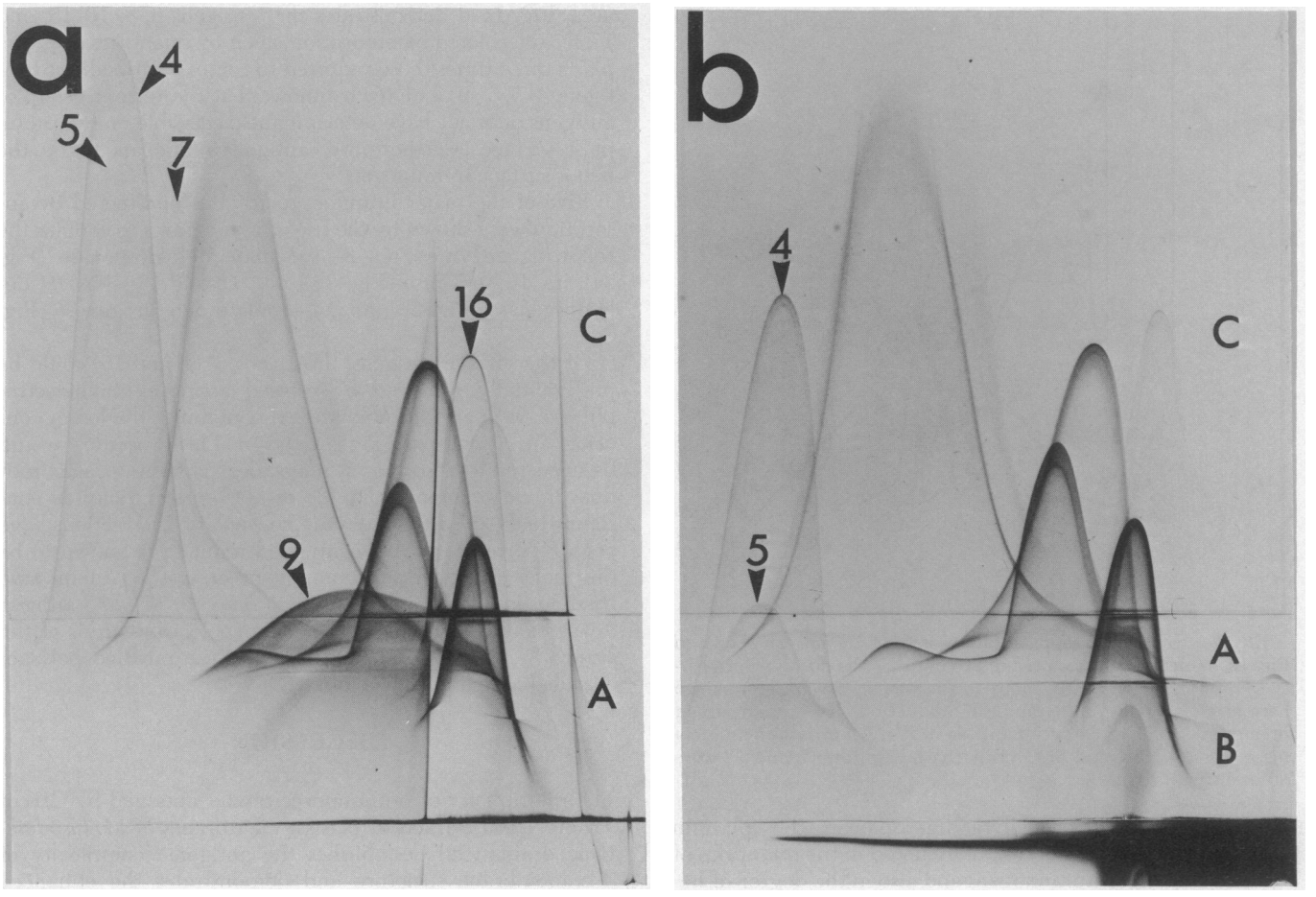

FIG. 4. Crossed affino-immunoelectrophoresis of a Triton X-100 extract of *M. lysodeikticus* membranes. Gel regions marked A, B, and C contain agarose alone, concanavalin A (3 mg/ml), and concentrated anti-membrane serum (10 μl/ml), respectively. Similar amounts (85 μg of protein) of a Triton X-100 membrane extract were then subjected to CIE. Antigens 7, 9, and 16 present in (a) are absent in (b), and 4 and 5 appear to have been reduced in area owing to absorption.

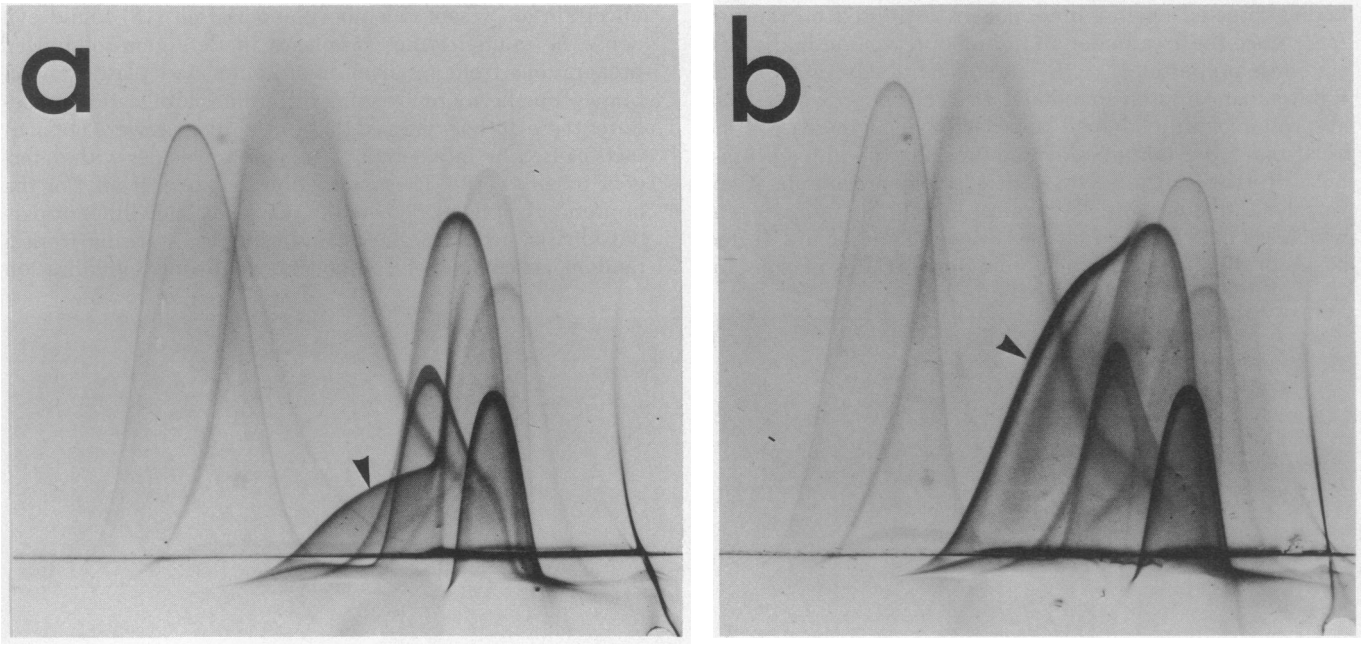

FIG. 5. Characterization of the major surface antigen of stable protoplasts. A Triton X-100 extract of *M. lysodeikticus* membranes (38 μg of membrane protein) was co-electrophoresed in the CIE system with (b) and without (a) purified mannan (480 ng). Electrophoresis in the second dimension was conducted into gels containing concentrated anti-membrane immunoglobulins (11 μl/ml). Only the area under precipitate 9 (arrowed) increased, indicating the identity of the major surface antigen of stable protoplasts (see Fig. 1).

by detergent. Although some of the immunoprecipitates exhibited consistent polydispersity (e.g., nos. 8 and 10), possibly reflecting heterogeneity in size and charge of complexes, we have so far been unable to detect more than one enzyme activity in any one immunoprecipitate. However, we have recently shown by CIE and tandem CIE that ATPase complex extracted by Triton X-100 (antigen 10, Fig. 2) from *M. lysodeikticus* membranes has approximately one-half the relative migration of partially purified, free ATPase isolated by the shock-wash method (6). Thus the Triton X-100 extractable "ATPase antigen" may represent a native enzyme complex coupled with other components of the membrane energizing system. Analysis of individual immunoprecipitates recovered from CIE plates would help to resolve these possibilities.

The absorption experiments clearly demonstrate that only antigens 1–5, 7, 9, and 16 have determinants exposed on the protoplast surface. Further experiments would be needed to determine whether they are also present on the inner face of the membrane. The preparation of homogeneous inside-out and right-side-out membrane vesicle fractions for absorption experiments should enable us to establish the asymmetry of distribution of the various antigens. Our results do show, however, that the membrane enzymes ATPase, succinate dehydrogenase, NADH dehydrogenase, and malate dehydrogenase, and unidentified antigens 6, 11, and 17 are not expressed as protoplast surface antigens. By labeling with ferritin, ATPase has been localized only on the inner face of the membrane (20) and it may be anticipated for functional reasons that the dehydrogenases we have identified would also share such asymmetry of localization.

An unusual feature of the membranes of *M. lysodeikticus* is the presence of a mannan polymer (17, 21) which we have recently shown to be succinylated (8). The results presented in this study indicate that the mannan is the major surface antigen of intact protoplasts. At least two, and possibly as many as four, other minor surface antigens also appear to contain sugar residues in conformations permitting interaction with concanavalin A.

The ability to distinguish between antigens exposed on the outer surface of the bacterial protoplast and those on the inner face of the membrane should provide a more complete picture of the molecular organization of the *M. lysodeikticus* plasma and mesosomal membrane system. Moreover, the identification of immunoprecipitates as specific enzymatic entities should lead to the resolution of some of the complex functional associations existing in the cell membrane.

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