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Immunological Properties of *Micrococcus lysodeikticus* Membranes

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Membranes of *Micrococcus lysodeikticus* possess antigens which are distinct from other cellular components such as cytoplasm, ribosomes, and cell walls. Only a few (two to three) components are found when dissociated membranes are examined by immunodiffusion and immunoelectrophoresis techniques. Membranes treated with 0.3% sodium dodecyl sulfate, 0.3% Triton X-100, trypsin, phospholipase A or C, or by sonic oscillation at *p*H 9.0, all showed the same pattern (three major bands) when examined against membrane antisera by immunoelectrophoresis. Immunological analysis of fractions isolated by sucrose gradient centrifugation or by polyacrylamide gel electrophoresis suggests that individual components cross-react. Antibodies to adenosine triphosphatase (EC 3.6.1.3) and fast-moving component are not removed by absorption with protoplasts. Removal of antibody to one of the membrane antigens by protoplast absorption indicated a surface location. Glutaraldehyde fixation of protoplasts resulted in the loss of membrane antigens detectable by immunodiffusion.

The use of specific markers is an essential approach in fully understanding the molecular architecture and organization of cell membranes as well as being valuable in tracing the fate of membrane components during isolation and fractionation. Such markers may be functional proteins (enzymes, electron transport components, transport carriers) or they may be identifiable as antigenic components (e.g., blood group substances, virus receptors, etc.). Salton (6) indicated the usefulness of membrane antigens for following the isolation and characterization of membranes of *Micrococcus lysodeikticus*. The existence of membrane-specific antigens would thus be of great value in elucidating the structure of a multifunctional organelle such as the bacterial membrane. For this reason we have undertaken an investigation of the immunological properties of the membranes of *M. lysodeikticus*.

Although the immunochemical analysis of surface components of the bacterial cell, including lipopolysaccharides, teichoic acids, and capsular polysaccharides, has been investigated intensively, there is comparatively little information on the immunological properties of bacterial membranes. An earlier study by Vennes and Gerhardt (11) showed that antisera for flagella, walls, and membranes of *Bacillus megaterium* were specific for each structure. In studying the immunological properties of group A streptococcal membranes, Freimer (2) reported the release of a specific antigen by treatment with trypsin. More recently, Kahane and Razin (3) have investigated the immunogenicity of mycoplasma membranes and suggested that the antigenic determinants are proteins.

In the present investigation, an attempt has been made to clarify the presence of membrane-specific antigens and to define some of the immunological properties of the membranes of *M. lysodeikticus*. It will be recalled that one of the major membrane antigens detected in this organism (6) was later identified as a Ca^{2+}-dependent adenosine triphosphatase (ATPase, EC 3.6.1.3; reference 4).

MATERIALS AND METHODS

Preparation of membranes, cell walls, ribosomes, and cytoplasm. Membranes were isolated from *M. lysodeikticus* (NCTC 2665) by the procedures previously described (6, 7). Cell walls were prepared by disintegrating the cells with glass beads in the Braun shaker and isolating the fractions by differential centrifugation by the method of Salton and Horne (8). Ribosomes were isolated from protoplast lysates by centrifugation of the cytoplasmic fraction on a 5 to 32% sucrose gradient (linear) formed above a 70% sucrose cushion. The ribosome fraction was collected after centrifugation for 3 hr at 25,000 rev/min in a SW 25 rotor. Further purification of the 70*S* ribosome fraction was achieved by centrifugation on a linear gradient of 5 to 30% sucrose. All

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sucrose solutions were prepared in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.5) containing 10 mM $MgCl_2$ and 60 mM KCl. The 280 nm/260 nm ratio for the ribosome fraction so obtained was 0.49. Cytoplasmic fractions were prepared from lysates by centrifugation at 105,000 × *g* for 2 hr in a Beckman-Spinco ultracentrifuge (model L2); pellets were discarded, and the supernatant fluid which was carefully removed constituted the cytoplasmic fraction.

Preparation of antisera and gamma globulin fractions. Rabbits were immunized with membrane preparations (2 mg of antigen in Freund's incomplete adjuvant in a total of 4 ml) by intramuscular injection at several sites. Each animal received three injections of the antigen (each injection at intervals of 2 weeks), and all were bled out 10 days after the third injection.

Antiserum against purified ATPase was kindly provided by Theresa L. Whiteside in our laboratory.

Isolation of the gamma globulin fraction from antisera to membranes was performed by repeated precipitation with ammonium sulfate at a final concentration of one-third saturation, by the methods of Campbell et al. (1).

Antigenic analysis. Immunoelectrophoresis was carried out on microscope slides covered with a layer of 1% (w/v) agar in borate-phosphate buffer (*p*H 8.3; ionic strength, 0.01). Electrophoresis was performed in the Durrum apparatus (Beckman, Palo Alto, Calif.) at an applied potential of 5 mamp/slide for 90 min in the cold, by the method of Campbell et al. (1). Ouchterlony plates were prepared on microscope slides by using 1% (w/v) agar in buffered saline, and immunodiffusion was allowed to take place in a moist chamber at room temperature (approximately 21 to 24 C) for 1 to 3 days. Slides were photographed with the Cordis Immunodiffusion Camera (Cordis Corp., Miami, Fla.).

Treatment of membranes. The immunochemical properties of membranes were examined after treatment with trypsin, phospholipases, and surface-active agents. Trypsin and other enzymes at a final concentration of 100 μg/ml were added to sonically treated membranes (2 mg of protein/ml), suspended in 0.05 M Tris-hydrochloride buffer (*p*H 7.5), and incubated at 37 C for 1 hr. Sonic oscillation treatments were all performed in a MSE (Measuring and Scientific Equipment, Ltd., London, England) sonic disintegrator as previously described (9). Membranes (3 mg of protein/ml) were treated with Triton X-100 or sodium dodecyl sulfate (SDS) at final concentrations of 0.3%.

Trypsin (crystalline, salt-free, A grade) and phospholipase A (from *Crotalus terr*; B grade) were obtained from Calbiochem, Los Angeles, Calif.; phospholipase C purified from *Clostridium welchii* was a gift from Alan W. Bernheimer.

Absorption of membrane antiserum with protoplasts. Protoplasts of *M. lysodeikticus* were prepared at about 21 to 24 C by adding lysozyme (200 μg/ml, final concentration) to washed cells suspended in 0.8 M sucrose in 0.05 M Tris-hydrochloride buffer (*p*H 7.5). The density of cell suspensions was adjusted to give an optical density of 10 at 600 nm in a Bausch & Lomb Spectronic 20 spectrophotometer. Four-milliliter portions of the suspensions were immediately layered over discontinuous sucrose gradients in tubes for the SW 25 rotor. The gradients consisted of a bottom layer of 5 ml of 50% sucrose, 5 ml of 41% sucrose, 10 ml of 34% sucrose, and a top layer of 5 ml of the gamma globulin fraction from membrane antiserum which had been dialyzed against saline containing 0.8 M sucrose. All of the sucrose solutions for the gradients were prepared with 0.05 M Tris-hydrochloride buffer (*p*H 7.5) containing 5 mM $CaCl_2$. After layering on the gradients, the tubes were held at room temperature for 30 min for complete conversion of the cells to protoplasts. The tubes were then centrifuged at room temperature at 5,000 × *g* for 30 min to sediment the protoplasts into the layer containing the gamma globulin fraction. Absorption of antibody by protoplasts was allowed to proceed by holding at room temperature for 1 hr. The protoplasts were then separated from the gamma globulin layer by centrifugation further into the gradient (10,000 × *g*, 1 hr, at about 21 to 24 C). The upper portion of the gradient containing the absorbed gamma globulin fraction was collected; the gamma globulin was precipitated with 50% saturation of ammonium sulfate and dialyzed against buffered saline.

Sucrose density gradient centrifugation of sonically treated membrane. One-milliliter portions of membrane suspensions (5 mg of protein/ml) which had been sonically oscillated at *p*H 9.0 were layered over a linear gradient of 5 to 20% (w/v) sucrose in 0.03 M Tris-hydrochloride buffer (*p*H 7.5) and centrifuged in a SW 25 rotor at 25,000 rev/min for 16 hr.

Fixation of protoplasts with glutaraldehyde. Protoplasts were prepared with lysozyme in 0.8 M sucrose in 0.05 M Tris-hydrochloride buffer (*p*H 7.5) containing 5 mM $CaCl_2$ as described above. After 30 min at approximately 21 to 24 C, glutaraldehyde was added to the protoplast suspensions to give final concentrations of 0.05, 0.5, and 1.0% and immediately centrifuged at 30,000 × *g* for 30 min. The pellets were washed twice with saline, and membrane fractions prepared by sonic disruption of the fixed protoplasts for 2 to 3 min at 0 C and collecting the membrane fragments by centrifugation at 30,000 × *g* for 30 min. The membrane deposits were washed four times with 0.05 M Tris-hydrochloride (*p*H 7.5). Before immunodiffusion tests, these membrane fractions were dissociated by sonic oscillation at *p*H 9.0 or by treatment with trypsin as described above.

RESULTS

Analysis of membrane and cellular fractions by agar diffusion method. For the analysis of the antigenic specificity of the membranes, their reactivity was compared to that of other cellular fractions, including cytoplasm, ribosomes, and purified cell wall preparations. By double-diffusion precipitin reactions in agar, the membrane preparations sonically oscillated at *p*H 9.0 gave three major lines of precipitation against membrane antiserum (Fig. 1a). One of the lines fuses with a precipitation band formed with cytoplasm and exhibits spur formation. Ribosome and cell wall fractions dispersed by sonic oscillation before addition to the wells did not give rise to any precipitation lines against the membrane antiserum. Antisera prepared individually to mem-

brane, cytoplasmic, and ribosome fractions showed many precipitation bands which fused when mixed and reacted against crude, unwashed membrane fractions (Fig. 1b). This result confirmed earlier results from this laboratory (6) and indicated that unwashed membrane fractions are contaminated with cytoplasmic and possibly ribosomal antigens. Except for one antigen which appears to be common to cytoplasm and membrane, the results indicate that adequately washed membranes do not appear to contain other cellular components detectable by antigen-antibody precipitin reactions in agar and that the antigens appear to be membrane specific under these test conditions.

Immunoelectrophoresis of membranes treated with detergents and enzymes. Immunoelectrophoresis of membranes which had been dissociated by sonic oscillation at *p*H 9.0, by treatment with 0.3% SDS, 0.3% Triton X-100, or digested with trypsin, phospholipase A, or phospholipase C showed the presence of three major precipitation arcs on reaction against membrane antiserum. Some typical results are illustrated in Fig. 2a which compares the treated membranes with those dispersed by sonic oscillation in 0.05 M Tris-hydrochloride buffer at *p*H 7.0. The results indicate that the three major antigenic entities of the membranes are released or "solubilized," irrespective of the method used to disrupt the membrane. It should be noted that the immunoelectrophoretic patterns obtained from membranes sonically treated at *p*H 7.0 (Fig. 2a) differed from those observed when sonic disruption was performed at *p*H 9.0 (*compare* with Fig. 4b). One possible explanation of this difference is that more complete dissociation of membrane complexes occurs at the higher *p*H.

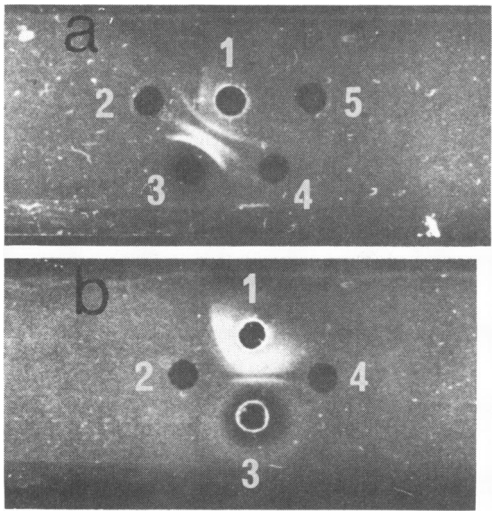

FIG. 1. *Tests with cellular components. (a) Immunodiffusion test of cellular components against membrane antiserum (well 1). Fractions placed in the peripheral wells were: cytoplasm (well 2), washed membranes sonically treated at pH 9.0 (well 3), sonically treated ribosome fraction (well 4), sonically treated cell walls (well 5). (b) Reaction of cellular fractions against a mixture of antiserum to each of the membrane, cytoplasmic and ribosomal fractions (well 1), cytoplasm in well 2, unwashed membranes sonically treated at pH 9.0 in well 3; and sonically oscillated ribosomes in well 4.*

Membrane which had been dissociated with 0.3% Triton X-100 gave two precipitation lines against antiserum specific for ATPase (Fig. 2b). It is possible that the component of low electrophoretic mobility reacting with the anti-ATPase could be due to the presence of unreleased ATPase on a larger membrane aggregate or due to a cross-reacting determinant.

Behavior of membrane antigens on sucrose gradient centrifugation of sonically oscillated membranes. Attempts were made to resolve the membrane antigens further by sucrose gradient centrifugation of membranes sonically disrupted at *p*H 9.0, as previously described. The 280 nm absorbance profile of the gradient (20 fractions) indicated two broad peaks of protein (fractions 1 to 5 and 6 to 11) and a sharper peak (fractions 12 to 15). The fractions were concentrated to a small volume (0.2 to 0.3 ml) by pervaporation, equilibrated against 0.03 M Tris-hydrochloride buffer (*p*H 7.5), and examined in agar diffusion plates against membrane antiserum. The results are illustrated in Fig. 3. Some separation of the antigens is indicated by comparison of the lines fusing with those from the membrane for fractions 1, 3, 5, and 7 with fusion and spur formation shown for fractions 11 to 17 in Fig. 3.

Antigenic analysis of membrane fractions isolated by polyacrylamide gel electrophoresis. Further resolution of the membrane antigens was attempted by dissociating the membranes with 0.3% SDS and separation by polyacrylamide gel electrophoresis under conditions previously used in this laboratory (6, 10). Membrane components were separated into many individual bands; after electrophoresis, unstained gels were sliced into discs of 2-mm thickness, embedded into the wells of agar diffusion slides, and reacted against membrane antiserum. Fractions separated by polyacrylamide gel electrophoresis possessed some antigenic relationship, as judged by fusion of lines, and may therefore have common antigenic determinants.

Membrane antigens from glutaraldehyde-fixed protoplasts. Membranes prepared from protoplasts fixed with different concentrations of glu-

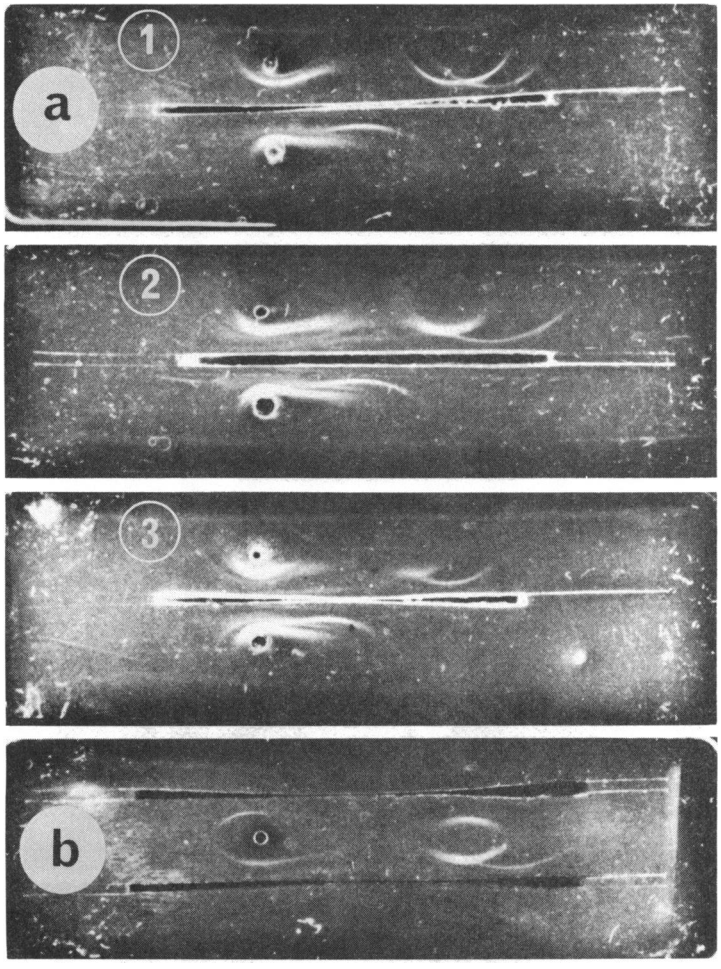

FIG. 2. *Tests with membranes. (a) Immunoelectrophoretic comparison of the reaction of treated membranes (upper wells) and membranes sonically oscillated at pH 7.0 (lower wells) against membrane antiserum. Slide 1, trypsin-treated membranes; slide 2, membranes treated with 0.3% Triton X-100; slide 3, phospholipase A-treated membranes. (b) Immunoelectrophoresis of membranes treated with 0.3% Triton X-100 tested against antiserum to purified ATPase (upper trough) and membrane antiserum (lower trough).*

taraldehyde were treated with trypsin or sonically treated at *p*H 9.0 and reacted against membrane antiserum. It was thought that fixation may possibly change any of the sensitive antigens of the outer surface of the protoplast membrane and leave the "deeper" antigens intact. The untreated membranes gave two lines of precipitation, one of which is absent from the preparation fixed with 0.5% glutaraldehyde (Fig. 4a). Spur formation was observed with the main precipitation band with the 0.05% glutaraldehyde fixed preparation. Immunoelectrophoresis of membrane antigens gives three components (*see* also Fig. 2a); however, after fixation with 0.05% glutaraldehyde, one of the precipitation arcs disappeared (Fig. 4b). Thus one of the membrane antigens could not be released by treatment with trypsin or sonic oscillation after fixation with 0.5% glutaraldehyde. By increasing the glutaraldehyde concentration to 1%, the membrane antigens could no longer be released. The apparent disappearance of the antigens upon glutaraldehyde fixation could be due either to their destruction by the fixative or to cross-linking of the proteins into a less soluble, trypsin-resistant form, or both.

Absorption of membrane antiserum with protoplasts. Absorption of the gamma globulin fraction of membrane antiserum by protoplasts was performed as previously described. One of the two major precipitation bands given against membrane antiserum disappeared upon absorption with protoplasts (Fig. 5a). It should be noted

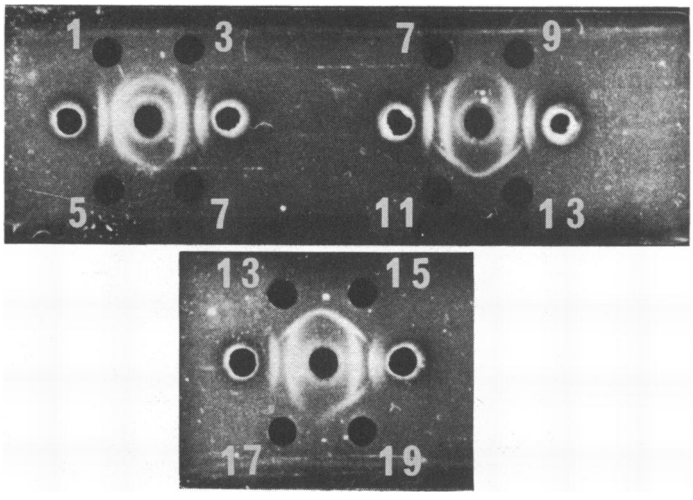

FIG. 3. *Immunological properties of fractions from sucrose density gradient centrifugation of membranes sonically oscillated at pH 9.0. Membrane antiserum was placed in the center wells, and membrane sonically treated at pH 7.0 was placed in the left- and right-hand wells of each set. Fractions from the gradient (top → bottom) were placed in sequence as indicated. Note complete fusion in some fractions and spur formation in others, indicating some separation of antigens on gradient.*

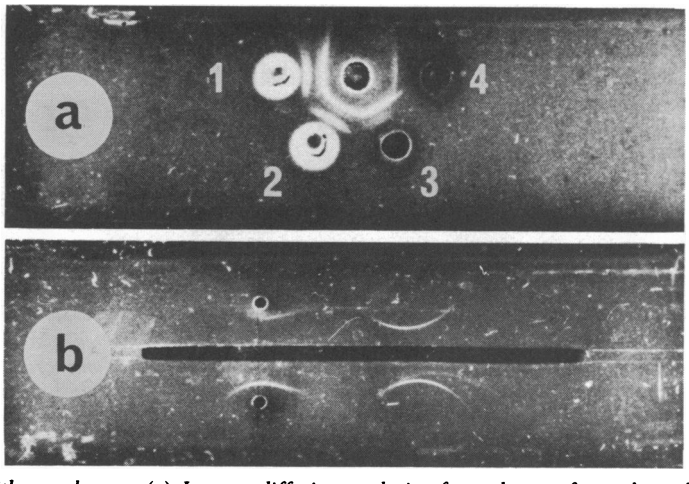

FIG. 4. *Tests with membranes. (a) Immunodiffusion analysis of membranes from glutaraldehyde-fixed protoplasts. Center well contained membrane antiserum; wells 1 and 2, membrane sonically treated at pH 9.0; wells 3 and 4, membrane preparations from 0.05 and 0.5% glutaraldehyde-fixed protoplasts, respectively. (The latter fractions were sonically oscillated at pH 9.0 before being placed in the wells.) (b) Comparison of immunoelectrophoresis pattern of membranes sonically treated at pH 9.0 (lower well) with preparation from 0.05% glutaraldehyde fixation (upper well), as in a, reacted against membrane antiserum.*

that the one which disappears upon absorption appears to be identical to the one lost upon glutaraldehyde fixation (*see* Fig. 4b), thus suggesting a surface location for this antigenic component of the membrane. This observation was confirmed more unequivocally by comparing the absorbed and unabsorbed antiserum against membrane antigens by immunoelectrophoresis (Fig. 5b). The antigenic component involved was further defined by comparing the reactivity with purified ATPase, a fast-moving component (FMC) detected by polyacrylamide gel electrophoresis and SDS-treated ATPase. In Fig. 5c it can be seen that antibodies to ATPase and FMC are not removed by absorption with protoplasts, whereas the antigenic specificity demonstrable with SDS-treated ATPase (a masked antigen?; *see* reference 12) disappeared upon absorption with protoplasts. These results strongly suggest that the antigenic determinants exhibited by purified ATPase and

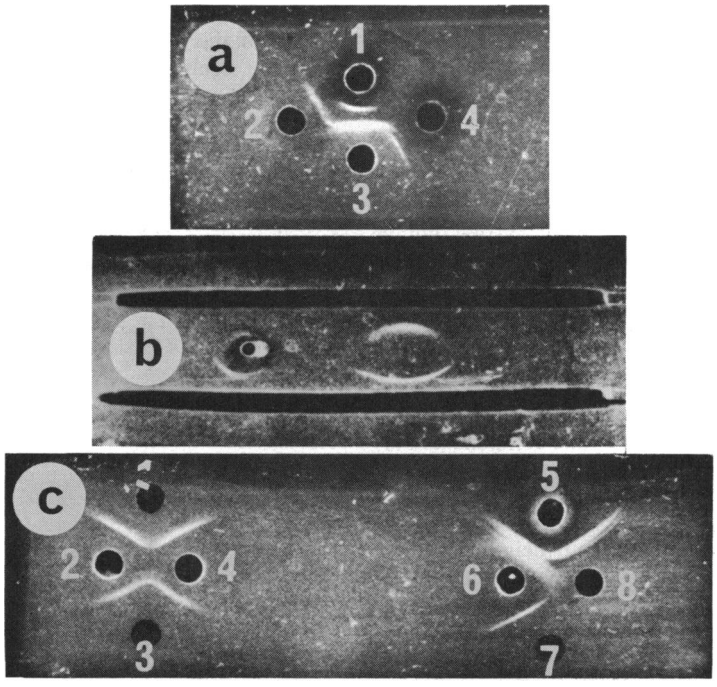

FIG. 5. *Tests with membranes. (a) Immunodiffusion test of membrane antiserum absorbed with protoplasts (well 2) against membranes sonically treated at pH 9.0 (well 1), compared with unabsorbed membrane antiserum (well 3) and membrane preparation (well 4) from 0.5% glutaraldehyde fixation experiment (as for Fig. 4a). (b) Immunoelectrophoresis of sonically treated membrane (pH 9.0) tested against membrane antiserum absorbed with protoplasts (upper trough) and unabsorbed membrane antiserum (lower trough). (c) Immunodiffusion tests of membrane antiserum (wells 2 and 6) and antiserum absorbed with protoplasts (wells 4 and 8) against purified ATPase (well 3), purified FMC (well 1), aqueous-phase protein from n-butanol extracted membranes (well 5), and SDS-treated ATPase (well 7).*

FMC are inaccessible or that they are not located on the outer surface of the membrane. By contrast, the antigenic specificity of the SDS-treated ATPase is detectable by the absorption technique.

DISCUSSION

Earlier studies in this laboratory have shown that it was necessary to wash the membrane preparations four or five times to achieve relative freedom from intracellular proteins (6). The antigenic analysis presented in this paper has shown that apart from one antigen detectable in the cytoplasmic fraction, antisera to washed membranes did not react with other cellular fractions. The antigen in the cytoplasm which reacts with membrane antiserum could originate from the membrane during the process of isolation. The origins of this antigen require further investigation. Apart from this, the antiserum can be considered specific for membrane components.

Perhaps one of the most surprising features of this study was the discovery of the relatively small number of antigenic components detectable by the double-diffusion agar method (two or three) and by immunoelectrophoresis (three). Moreover, the patterns of membrane antigens obtained on immunoelectrophoresis were the same for preparations dissociated by sonic oscillation at *p*H 9.0, by treatment with 0.3% SDS or Triton X-100, or by digestion with trypsin, phospholipase A, or phospholipase C. Identical results were obtained for different batches of membranes and different batches of antiserum. The detection of a relatively small number of membrane antigens in solubilized preparations of *M. lysodeikticus* membranes is similar to the recent observations of Kahane and Razin (3) with *M. laidlawii* membranes. The precipitin patterns obtained with SDS-solubilized membranes of the latter organism showed three bands, and a fourth band was observed fusing with an antigen in the soluble cell fraction (3). It will also be recalled that, in general, similar results were obtained with group A streptococcal membranes (2). All of these studies with bacterial membranes stress the highly specific nature of the membrane antisera. Thus, antisera to *M. lysodeikticus* membranes showed no cross-reaction with *B. megaterium* membranes

in our study, and no cross-reaction was observed between streptococcal and staphylococcal membranes (2). Indeed, no serological cross-reaction could be detected between hydrophobic proteins from *M. laidlawii* and *M. gallisepticum* membranes (3).

The relative simplicity of the immunochemical analysis of the membranes contrasts with the complexity of the bands seen in polyacrylamide gel electrophoresis (5, 10) and with the expectancy that a large number of specific proteins would be found in a multifunctional structure such as a bacterial membrane. Kahane and Razin (3) have suggested that hydrophobic membrane proteins are poorly immunogenic. Apart from this explanation, our results raise the possibility that many individual membrane components may possess a common antigenic determinant or perhaps a common peptide unit. Other explanations for our results and those of other investigators exist; e.g., many proteins and their determinant groups are buried within the membrane and the difficulty of dissociating membrane proteins and keeping them in solution during immunodiffusion testing. Further precise immunochemical analysis will be needed to clarify these various possibilities and to test the significance of the possible existence of a common antigen.

Our results indicate the potential value of the immunological properties of membranes in studying the molecular architecture of these structures. By performing absorption studies with protoplasts, we have shown that the antigenic determinants of the ATPase and FMC do not appear to be localized on the outer membrane surface. At least for functional purposes, it would be reasonable to expect that the ATPase would be located on the inner face of the membrane. The fact that the antigenic specificity exhibited after treatment of the isolated ATPase with SDS is detectable on the outside of the protoplast surface is an intriguing observation and could be related to the allotopic character of this membrane enzyme. Further investigations will be needed to establish the nature of this antigenic specificity and to determine the significance of its presence on the outer protoplast membrane surface. Extension of such studies would undoubtedly lead to a more precise understanding of the asymmetry of the membrane structure in terms of specific proteins.

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