JOURNAL OF BACTERIOLOGY, Dec. 1979, p. 881-887
0021-9193/79/12-0881/07$02.00/0

Vol. 140, No. 3

Immunochemical Analysis of Triton X-100-Insoluble Residues from *Micrococcus lysodeikticus* Membranes

PETER OWEN* AND HELEN DOHERTY

Department of Microbiology, Trinity College, Dublin, Ireland

Received for publication 25 September 1979

Triton X-100-insoluble residues from *Micrococcus lysodeikticus* membranes were analyzed by crossed immunoelectrophoresis after dispersal of the residues in sodium dodecyl sulfate (SDS). Conditions which produce no obvious distortion of the immunoprecipitate profile and which allow qualitative and quantitative analyses of the antigens present in the extracts are described. Two main antigens were detected; these were identified as succinate dehydrogenase (EC 1.3.99.1) and adenosine triphosphatase (EC 3.6.1.3). As determined by peak area estimations, the maximal release of succinate dehydrogenase and of adenosine triphosphatase from Triton X-100-insoluble membrane residues occurred at protein/SDS ratios of about 4.3:1 (0.2% SDS) and 6.8:1 (0.13% SDS), respectively. A comparison of enzyme activities of SDS extracts with those of untreated, control Triton X-100-insoluble membrane residues indicated that both the succinate dehydrogenase and the adenosine triphosphatase antigens were released with a full (or enhanced) catalytic potential at or below concentrations of SDS required to effect maximal solubilization of the enzyme in question. Evidence is also presented to suggest that the more acidic of the two components detected by crossed immunoelectrophoresis for the heterogeneous adenosine triphosphatase antigen is more sensitive to SDS than is the other. Both succinate dehydrogenase and adenosine triphosphatase lost catalytic activity and were denatured at protein/SDS ratios lower than 3.4:1.

Crossed immunoelectrophoresis (CIE) has proved to be an extremely useful technique for the analysis of bacterial membranes (1, 2, 9, 12, 16–20, 24, 25). By this method, membrane immunogens are resolved as clear two-dimensional arrays of immunoprecipitates. The identity of individual antigens can be established in a variety of ways (21); one of the most useful in the study of membrane-bound enzymes is that of zymogram staining (26). This method relies on the fact that enzyme antigens usually express some of their intrinsic catalytic activity after immunoprecipitation in the CIE system. Furthermore, the quantitative aspects of CIE allow the method to be combined with adsorption experiments designed to probe the molecular architecture of the membrane (9, 16, 18–20).

One of the current limitations of CIE is that analysis is usually restricted to those antigens which are soluble in nonionic detergents (normally Triton X-100). This is because the Triton X-100-insoluble membrane residues are particulate in nature and fail to enter agarose gels. Consequently, a significant percentage of the total quantity of membrane protein is often refractory to direct CIE analysis (19, 20). Clearly, the use of more rigorous solubilization procedures is necessary if a complete picture of the antigenic character of the membrane is to be achieved. However, for CIE analysis to be effective, it is equally obvious that full solubilization of membrane antigens should not be accompanied by a loss of intrinsic biological activity or denaturation. In the present paper, the feasibility of using controlled amounts of the ionic detergent sodium dodecyl sulfate (SDS) is studied in relation to effecting maximal solubilization of Triton X-100-insoluble membrane antigens with a concomitant retention of biological and immunological specificities. SDS was chosen in preference to other detergents since it is capable of fully solubilizing most bacterial membranes (8), and the membranes of the gram-positive organism *Micrococcus lysodeikticus* were used since the CIE profile for Triton X-100-soluble membrane antigens from this organism has been characterized in detail (19, 20).

MATERIALS AND METHODS

Preparation of plasma membranes. Cells of *M. lysodeikticus* NCTC 2665 were grown on peptone-water-yeast extract medium and harvested in early

stationary phase (18 h) as previously described (15). Plasma membranes were collected by centrifugation at 38,000 × *g* for 40 min at 4°C after treatment of washed cells with lysozyme in hypotonic buffer (20). Membrane preparations were routinely washed six times with 50 mM Tris-hydrochloride buffer (pH 7.5) and stored as a thick suspension (about 20 mg of protein per ml) at −70°C before use.

Preparation of detergent extracts of plasma membranes. Plasma membranes were adjusted to a concentration of between 17 and 20 mg of protein per ml, and Triton X-100 (20%, vol/vol) was added to a final concentration of 4% (vol/vol). Incubation was continued with occasional shaking for 1 h at 20°C, and the extract was then centrifuged at 48,000 × *g* for 45 min at 4°C. The supernatant fraction was carefully removed, and the pellet was suspended in an equal volume of 4% (vol/vol) Triton X-100 in 50 mM Tris-hydrochloride buffer (pH 7.5). Incubation was continued for 1 h more at 20°C, and the extract was then centrifuged as before. This process was repeated a third time, and the Triton X-100-insoluble membrane residue was finally washed in 50 mM Tris-hydrochloride buffer (pH 7.5) to remove excess Triton X-100.

Before extraction with SDS, Triton X-100-insoluble membrane residues were suspended to a uniform consistency with 50 mM Tris-hydrochloride buffer (pH 7.5). The protein concentration of this suspension was routinely about 10 to 20 mg/ml, and the concentration of residual free Triton X-100 was estimated to be less than 0.02% based upon volume considerations. Volumes (1 ml each) of 50 mM Tris-hydrochloride buffer (pH 7.5) containing SDS at various concentrations were then added to 1-ml volumes of the Triton X-100-insoluble membrane residue. The solutions were incubated at 20°C with occasional shaking for 30 min, and extracts were then centrifuged at 48,000 × *g* for 45 min (at 15°C, to prevent crystallization of the SDS). Supernatant fractions were carefully removed and retained for analysis.

All detergent extracts were stored, as 50 to 100-μl volumes, at −70°C immediately after preparation and thawed only once, either for biochemical analysis or for CIE.

Immunochemical analysis. Methods for raising antiserum to plasma membranes and for performing CIE in the presence of Triton X-100 have been described in detail elsewhere (19, 20, 25). Immunoprecipitates containing the enzymes adenosine triphosphatase (ATPase; EC 3.6.1.3) and succinate dehydrogenase (SDH; EC 1.3.99.1) were revealed by zymograms as previously described (19). Peak areas subtended by individual immunoprecipitates were estimated by photocopying dried immunoplates and subsequently excising and weighing 10 copies of each peak (16). Photocopy paper routinely weighed 7.89 mg/cm^2.

Analytical procedures. ATPase activity was measured by the liberation of inorganic phosphorus from ATP as previously described (13, 14). In view of the latency described for this enzyme, assays were routinely performed in the presence of trypsin (14). Color development was initiated by the addition of 1% (wt/vol) amidol in 3% (wt/vol) $Na_2S_2O_5$ (6) and was complete after incubation at 37°C for 10 min. SDH activity was estimated spectrophotometrically as described by Owen and Freer (15).

Protein determinations were carried out with a modification (5) of the method of Lowry et al. (11) that eliminates interference by Triton X-100. Bovine serum albumin was used as a standard.

Chemicals. Agarose was obtained from Miles Laboratories, Inc., Slough, England, and Triton X-100 was from Research Products International, Elk Grove Village, Ill. Bovine pancreatic trypsin (type III) was the product of Sigma Chemical Co., St. Louis, Mo. All other chemicals and reagents were of analytical grade.

RESULTS

Analysis of Triton X-100 extracts of *M. lysodeikticus* membranes. Under the experimental conditions adopted, about 50% of the total protein was routinely extracted from *M. lysodeikticus* membranes by Triton X-100. Most of the Triton X-100-extractable protein was detected in the first Triton X-100 extract (about 37% of the total membrane protein). Greatly decreased levels of protein were observed in the other Triton X-100 extracts and buffer washes (about 7, 3 and 2% of the total membrane protein in the second Triton X-100 extract, third Triton X-100 extract, and buffer wash, respectively). Analysis of the first Triton X-100 extract by CIE revealed a complex immunoprecipitate pattern (Fig. 1A), which resembled in many details that described previously for a similar preparation (19, 20). The reader is thus referred to earlier publications for a detailed analysis of this CIE profile and for the system adopted to number immunoprecipitates (19, 20). Suffice to say that the identities of two antigens which are relevant to the present study and which are shown in Fig. 1A were confirmed as SDH (antigen no. 8) and ATPase (antigen no. 11) by zymograms. Analysis by CIE of the other Triton X-100 extracts and buffer wash did not reveal any additional immunoprecipitate bands. Indeed, the CIE profiles were qualitatively very similar to that of the first Triton X-100 extract. However, from a comparison of peak areas, it was obvious that the concentrations of most antigens in these extracts were much lower than those in the first Triton X-100 extract (data not shown). Thus, the CIE profile for the first Triton X-100 extract (Fig. 1A) gives a realistic impression of the spectrum of membrane antigens amenable to extraction with this nonionic detergent.

Analysis of Triton X-100-insoluble membrane residues. About 50% of the total membrane protein from *M. lysodeikticus* resisted extraction with Triton X-100 and, consequently, could not be analyzed directly by CIE. Further study indicated that the anionic detergent SDS

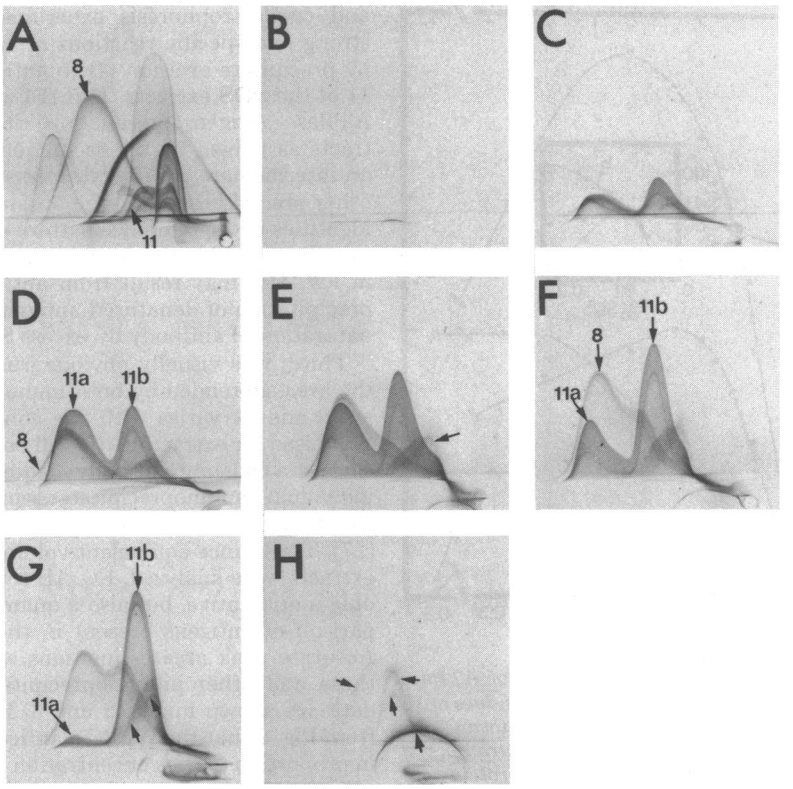

FIG. 1. *CIE of detergent extracts of M. lysodeikticus membranes. Plasma membranes of M. lysodeikticus were first extracted with Triton X-100 and subsequently with various concentrations of SDS as detailed in the text. Detergent extracts were then subjected to CIE against anti-membrane serum (35 μl of concentrated immunoglobulins per ml of gel). (A) represents the immunoprecipitate profile obtained after CIE of the first Triton X-100 extract (88 μg of protein). (B) through (H) represent the corresponding profiles obtained by CIE of the soluble fractions obtained after treatment of Triton X-100-insoluble membrane residues (final concentration, 8.5 mg of protein per ml) with SDS at final concentrations of 0.01% (B), 0.05% (C), 0.083% (D), 0.125% (E), 0.167% (F), 0.25% (G), and 0.5% (H). Similar volumes (4 μl each) of SDS extracts were analyzed in all instances. Immunoprecipitates possessing ATPase (no. 11) and SDH (no. 8) activities have been indicated in several of the panels. Note that the ATPase antigen is heterogeneous and gives two immunologically related precipitin peaks denoted no. 11a and 11b. Several other faint immunoprecipitates observed in (B) through (H) are also indicated with arrows. The anode is to the left and top of all gels.*

could be used to disperse the Triton X-100-insoluble membrane residues into a form which was amenable to CIE analysis. However, it became apparent that the concentration of SDS (or, perhaps more importantly, the protein/SDS ratio) had to be carefully controlled to obtain both maximal solubilization and retention of full catalytic function and antigenic specificity. These points are shown in Fig. 1 through 3, which depict the results of a typical series of experiments in which the properties of SDS extracts are monitored as a function of the concentration of detergent used. Several features are noteworthy.

First, there is an almost linear relationship between the amount of protein released from the Triton X-100-insoluble residue and the concentration of SDS used to effect extraction (Fig. 2 and 3). Maximal solubilization (about 97%) was achieved at a concentration of SDS of 0.25% (wt/vol), which, under the experimental conditions adopted, corresponds to a protein/SDS ratio of about 3.4:1.

Second, SDS extracts which were obtained with protein/SDS ratios of 3.4:1 or above (i.e., at or below 0.25% SDS) gave well-defined, recognizable, undistorted, and simple immunoprecipitate profiles when analyzed by CIE (Fig. 1B to G). Most of these extracts contained two main antigenic components. One was identical with the SDH antigen (antigen no. 8; Fig. 1A) and the other (specifically no. 11b; Fig. 1C to G) was

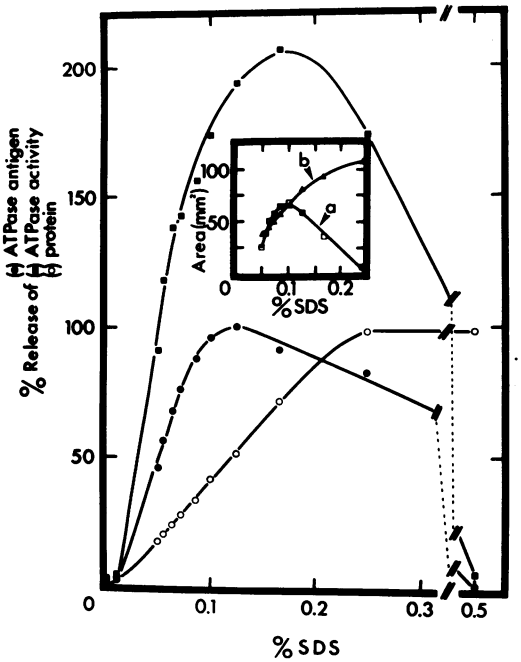

FIG. 2. *Effect of SDS on the release of ATPase from Triton X-100-insoluble membrane residues of M. lysodeikticus. Triton X-100-insoluble membrane residues were treated with different concentrations of SDS as described in the text. The final protein concentration in all cases was 8.5 mg/ml. Supernatant fractions obtained after centrifugation at 48,000 × g were analyzed colorimetrically for protein* (○) *and for ATPase activity* (■) *and by CIE to quantitate the amount of ATPase antigen* (●) *present in each extract. Protein and ATPase activity are expressed as a percentage of the amounts observed in untreated Triton X-100-insoluble membrane residues. Similar calculations are not feasible for the estimation of ATPase antigen, since the Triton X-100-insoluble residues are not amenable to direct CIE analysis. Consequently, values in this instance are quoted as a percentage of the maximal amount of antigen released (i.e., by SDS at a final concentration of 0.125%). The specific activity of the ATPase in the Triton X-100-insoluble membrane residue was 1.61 μmol of ATP hydrolyzed per 10 min per mg of protein. The inset shows the differential effect of SDS on the peak areas of the two components of the ATPase immunoprecipitate (i.e., no. 11a and 11b; see Fig. 1).*

similar to the ATPase antigen (antigen no. 11; Fig. 1A) of the first Triton X-100 extract as determined by the following multiple and independent criteria: (i) similarities in electrophoretic mobilities and intensities of immunoprecipitation (compare Fig. 1A and F); (ii) identical enzyme staining potential (zymograms are not shown since they are very similar to those presented in an earlier publication [19]); (iii) reactions of complete identity in tandem CIE (10) and co-electrophoresis experiments; and (iv) strong and specific reactions of antisera raised by precipitate excision (4) to antigen no. 8 and 11 of the SDS extracts (Fig. 1F) with SDH and ATPase antigens present in Triton X-100 extracts as judged by the technique of CIE with an intermediate gel (3; data not shown). A few other precipitates were also observed, but their identities in the reference pattern remain uncertain. It is possible that some (e.g., the major one in Fig. 1H) may result from antibody-induced precipitation of denatured antigens or from denaturation of antibody by excess SDS.

Third, it is visually obvious from Fig. 1 that the area subtended by both immunoprecipitate no. 8 and 11 varies with the concentration of SDS used for extraction. (It will be remembered that, at a constant antibody loading, the area of individual immunoprecipitates is proportional to the amount of antigen present in the sample [27]. Thus, since equivalent volumes of all SDS extracts were analyzed, Fig. 1B to H shows not only a qualitative, but also a quantitative, comparison of antigens present in these extracts.) Accurate peak area estimations were made on these and other immunoprecipitates, and the data are shown in Fig. 2 and 3. It is apparent from Fig. 2 that the ATPase antigen is released in proportion to the concentration of SDS in the extract and that the maximal release is achieved at a concentration of about 0.13% SDS. This corresponds to a protein/SDS ratio of 6.8:1 and to a point in the extraction process at which

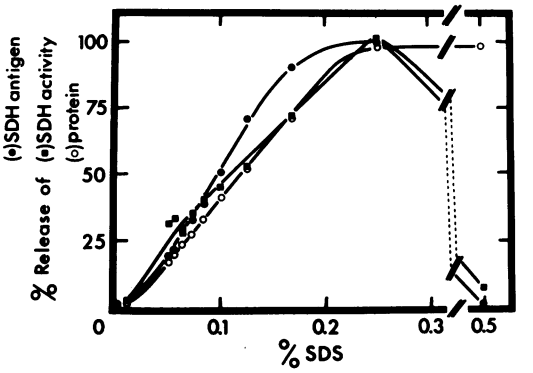

FIG. 3. *Effect of SDS on the release of SDH from Triton X-100-insoluble membrane residues of M. lysodeikticus. Experimentation was carried out in a manner identical to that described in the legend to Fig. 2. SDS extracts were analyzed colorimetrically for protein* (○), *spectrophotometrically for SDH activity* (■), *and by CIE to quantitate the SDH antigen* (●). *The data are presented in a manner similar to those of Fig. 2. The specific activity of SDH in the Triton X-100-insoluble membrane residue was 0.11 μmol of substrate oxidized per min per mg of protein.*

about 50% of the total protein of the Triton X-100-insoluble residue has been solubilized (Fig. 2). It should be recognized that these values pertain to an ATPase antigen which is clearly heterogeneous, displaying two precipitate arcs when analyzed by CIE (i.e., antigen no. 11a and 11b in Fig. 1D). These two components clearly differ in their individual responses to SDS (Fig. 1 B to H and Fig. 2, inset). The more acidic species (antigen no. 11a) is released maximally by 0.1% SDS and is observed to decrease at higher concentrations of SDS. In contrast, antigen no. 11b is progressively released with increasing concentrations of SDS up to a final concentration of SDS of at least 0.25%. Notably, both immunoprecipitate no. 11a and 11b possess ATPase activity as judged by zymogram staining (data not shown). The SDH antigen is also progressively released from Triton X-100-insoluble residues by increasing concentrations of SDS (Fig. 3). However, in this instance the release of antigen parallels more closely the release of total protein and is maximal at about 0.2% SDS (or a protein/SDS ratio of about 4.3:1). It is also important to note that both the ATPase and the SDH antigens appear to be denatured at concentrations of SDS exceeding 0.25% (Fig. 1H, 2, and 3), since no recognizable immunoprecipitates can be detected for either antigen at these concentrations of detergent.

The experiments detailed above have clearly shown that controlled levels of SDS can effect the release of the two main (enzyme) antigens present in Triton X-100-insoluble membrane residues of *M. lysodeikticus*. However, it is impossible to compute from these data what proportion of the total ATPase and SDH antigens available for extraction is released by SDS in an undenatured form. It is also important to know whether the enzymes in question retain full catalytic potential (as opposed to the retention of immunological specificities) after release by SDS. In an attempt to answer these questions, the ATPase and the SDH activities of the various SDS extracts were monitored and compared with the corresponding activities expressed by untreated Triton X-100-insoluble membrane residues. These results are also shown in Fig. 2 and 3. For both enzymes the release of catalytic activity by SDS followed very closely the release of the corresponding enzyme antigen. Thus, conditions which effect the maximal release of SDH antigen also effect the full quantitative release (102%) of the SDH activity present in the Triton X-100-insoluble residue (Fig. 3). The results for ATPase (Fig. 2) are complicated slightly by the fact that the enzyme is apparently activated by SDS. Thus, the maximal ATPase activity observed in SDS extracts is over 200% of that detected in untreated Triton X-100-insoluble residues. If this activation is taken into account, then the similarity between the release of ATPase activity and the release of ATPase antigen (antigen no. 11a plus antigen no. 11b) becomes striking (Fig. 2).

DISCUSSION

The main results of the present study may be summarized as follows. First, the Triton X-100-insoluble membrane residue from *M. lysodeikticus* can be dispersed by SDS into a form which is amenable to CIE analysis. Second, when so analyzed, the Triton X-100-insoluble membrane residue from this organism appears to possess only two main antigens, viz., SDH and ATPase. Third, the maximal release of these enzymes is achieved at different concentrations of SDS, viz., about 0.2% for SDH and 0.13% for ATPase. Furthermore, the release of SDH and ATPase at optimal or suboptimal concentrations of SDS is accompanied by the retention of both immunological specificity and catalytic potential. Finally, both enzymes lose immunological specificity and catalytic potential and appear to be denatured at concentrations of SDS above 0.25% (i.e., below a protein/SDS ratio of 3.4:1).

The practical implications of these novel observations are obvious. It should now be feasible to analyze and quantitate by CIE the full complement of antigens in a preparation of bacterial membranes. However, care should be taken to choose a concentration of SDS appropriate to the antigen under investigation. The optimal concentration of detergent could readily be determined by pilot experiments of the type shown in Fig. 1B to H. On the basis of the results obtained in this study for ATPase and SDH, it seems reasonable to conclude that the optimal concentration of SDS will vary from antigen to antigen. This may reflect in part the strength and extent of the protein-protein interactions involved in the integration of the particular antigen into the membrane residue (8). In view of these and other considerations, such as charge shift effects (2, 17), we cannot recommend SDS as a detergent of choice to solubilize isolated membranes, since, among other things, this is unlikely to generate both a quantitatively and a qualitatively complete spectrum of membrane-associated antigens. Rather, the use of SDS should be restricted to those instances in which it is necessary to analyze fully a particular antigen which partitions to some extent in the Triton X-100-insoluble membrane residues.

It is difficult to draw firm conclusions from the absolute concentrations of SDS necessary to give the optimal release of the enzymes in the present study since it is probable that some

Triton X-100 remains associated with the Triton X-100-insoluble residue even after washing. Suffice to note that the optimal release of SDH and ATPase occurs near or below the critical micelle concentration of SDS (0.23%; see reference 8) and at SDS/protein ratios below that required to effect saturating binding of the detergent (i.e., at an SDS/protein ratio of 1.4:1; see reference 8).

It could be argued that peak area determinations cannot be applied reliably to the quantitation of two antigens (such as no. 11a and 11b) which have different electrophoretic mobilities, since the more acidic species might tend to form an immunoprecipitate whose area is greater than that produced by the same antigen concentration of the less acidic species. However, whereas this phenomenon could very well apply to two antigenically distinct isoenzymes of widely differing electrophoretic mobility, it is unlikely to occur to a significant extent for ATPase antigen no. 11a and 11b, whose electrophoretic mobilities differ by a factor of 1.7 and which precipitate with immunoglobulins of the same specificity. Furthermore, if such an effect were occurring, one would expect to observe noticeable differences in the intensities of immunoprecipitate no. 11a and 11b. This is clearly not the case (Fig. 1C to G). Moreover, the fact that there is a close correlation between the amount of enzyme antigen and the amount of enzyme activity released over the whole course of solubilization and the fact that there is a total (or enhanced) recovery of catalytic potential after the optimal release of the enzymes strongly suggest that peak area determinations and enzyme assay both reflect fairly accurately the amount of active enzyme present in the various detergent extracts. The possibility that quantitation by both techniques is biased in the same direction but for different reasons seems unlikely. However, this cannot be ruled out completely at present.

The simplicity of the CIE profile for the Triton X-100-insoluble residue after dispersal in SDS is surprising, especially since the residue accounts for about half of the total protein. There are several possible explanations for this phenomenon. First, other membrane antigens could be present in the Triton X-100-insoluble fraction, but these are readily denatured by SDS. It is difficult to rule out this possibility, but it seems unlikely in view of the wide range of SDS concentrations examined and in view of the stabilities of the ATPase and SDH antigens in the test system. An additional possibility is that many of the proteins in the Triton X-100-insoluble membrane residue are very poor immunogens. Alternatively, it could be argued that the ATPase and SDH antigens represent not simply the basic catalytic molecules but complexes containing additional components with which the enzymes might be associated for functional reasons. There are precedents in the literature to support either of the latter two possibilities (17, 21, 22), and the situation seems unlikely to be resolved until a comprehensive analysis of the components present in each immunoprecipitate is undertaken.

An intriguing and possibly related phenomenon is the pronounced heterogeneity of the ATPase antigen. In a previous communication it was reported that partially purified F_1·ATPase from *M. lysodeikticus* had approximately twice the electrophoretic mobility of the ATPase antigen detected in Triton X-100-solubilized membranes from this organism (19). This latter antigen is thought to represent the F_0F_1·ATPase (23). Moreover, the electrophoretic mobility of F_1·ATPase has been shown to depend to a certain extent on whether the enzyme is in a "latent" or fully activated form (21). It is significant that antigen no. 11b has an electrophoretic mobility similar to that of the ATPase antigen observed in the first Triton X-100 extract, whereas antigen no. 11a displays an electrophoretic mobility nearer that of partially purified F_1·ATPase. It thus seems reasonable to suggest that antigen no. 11b represents a more integrated form of ATPase (e.g., F_1F_0·ATPase) and that antigen no. 11a represents a degenerate form of this complex which has lost some of the components (or subunits) which are themselves not involved in the primary catalytic event (i.e., the hydrolysis of ATP; see reference 7). It is notable that antigen no. 11a appears to be more readily denatured by SDS than does antigen no. 11b (Fig. 2, inset). This is not incompatible with the above interpretation and could imply a greater stability for the membrane-associated form of the enzyme. It could be argued that the more acidic component (antigen no. 11a) is not denatured by SDS at concentrations in excess of 0.1% but instead is converted to antigen no. 11b. It is difficult to accept this interpretation since one would expect it to result in a point of inflection in the plot of the peak area of antigen no. 11b against the SDS concentration. This was not observed (Fig. 2, inset). Additionally, it could be argued that antigen no. 11a and 11b do not reflect ATPase heterogeneity but result from differences in the binding of SDS. Although this possibility cannot be eliminated in the present instance, it should be noted that an immunoprecipitate displaying all of the properties of no. 11a is observed not infrequently in Triton X-100 extracts of *M. lysodeikticus* membranes (unpublished data).

It is interesting that the two major antigens

detected for Triton X-100-insoluble membrane residues are also present in the Triton X-100 extracts. Indeed, calculations based upon estimations either of peak area or of enzyme activity indicate that 34% of the SDH partitions into the Triton X-100-insoluble membrane fraction. In contrast, the Triton X-100-insoluble residue contains about 71% of the total ATPase antigen, as estimated by measurements of peak areas. (Similar calculations based on ATPase activity are not possible since Triton X-100 interferes with the estimation of enzyme activity in the various Triton X-100 extracts.) Although the precise reasons for these phenomena are not understood, it is relevant to note that previous conclusions regarding membrane architecture and based upon the analysis of Triton X-100-soluble antigens from *M. lysodeikticus* (19, 20) were fully justified, since they obviously relied upon a qualitatively (rather than quantitatively) competent spectrum of membrane immunogens. On the other hand, should quantitative data be required regarding, for example, the distribution of membrane antigens in various fractions, then it is equally apparent that immunological analysis cannot be restricted arbitrarily to those components which are "soluble" in Triton X-100. Account must be taken of that proportion of the individual antigens which partitions into the insoluble residues.

LITERATURE CITED

1. **Alexander, A. G., and G. E. Kenny.** 1977. Characterization of membrane and cytoplasmic antigens of *Mycoplasma arginini* by two-dimensional (crossed) immunoelectrophoresis. Infect. Immun. **15**:313-321.
2. **Alexander, A. G., and G. E. Kenny.** 1978. Application of charge shift electrophoresis to antigenic analysis of mycoplasmic membranes by two-dimensional (crossed) immunoelectrophoresis. Infect. Immun. **20**:861-863.
3. **Axelson, N. H.** 1973. Intermediate gel in crossed and in fused rocket immunoelectrophoresis. Scand. J. Immunol. **2**(Suppl. 1):71-77.
4. **Crowle, A. J., G. J. Revis, and K. Jarret.** 1972. Preparatory electroimmunodiffusion for making precipitins to selected native antigens. Immunol. Commun. **1**:325-336.
5. **Dulley, J. R., and P. A. Grieve.** 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal. Biochem. **64**: 136-141.
6. **Fiske, C. H., and Y. Sabbarow.** 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375-400.
7. **Haddock, B. A., and C. W. Jones.** 1977. Bacterial respiration. Bacteriol. Rev. **41**:47-99.
8. **Helenius, A., and K. Simons.** 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta **415**: 29-79.
9. **Johansson, K.-E., and S. Hjertén.** 1974. Localization of the Tween 20-soluble membrane proteins of *Acholeplasma laidlawii* by crossed immunoelectrophoresis. J. Mol. Biol. **86**:341-348.
10. **Krøll, J.** 1973. Tandem-crossed immunoelectrophoresis. Scand. J. Immunol. **2**(Suppl. 1):57-59.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
12. **McLaughlin, J., and E. Meerovitch.** 1975. Immunochemical studies of the surface and cytoplasmic membranes of *Entamoeba invadens* (Rodhain 1934). Can. J. Microbiol. **21**:1635-1646.
13. **Muñoz, E., M. S. Nachbar, M. T. Schor, and M. R. J. Salton.** 1968. Adenosinetriphosphatase of *Micrococcus lysodeikticus*: selective release and relationship to membrane structure. Biochem. Biophys. Res. Commun. **32**:539-546.
14. **Muñoz, E., M. R. J. Salton, M. H. Ng., and M. T. Schor.** 1969. Membrane adenosine triphosphatase of *Micrococcus lysodeikticus*. Eur. J. Biochem. **7**:490-501.
15. **Owen, P., and J. H. Freer.** 1972. Isolation and properties of mesosomal membrane fractions from *Micrococcus lysodeikticus*. Biochem. J. **129**:907-917.
16. **Owen, P., and H. R. Kaback.** 1978. Molecular structure of membrane vesicles from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **75**:3148-3152.
17. **Owen, P., and H. R. Kaback.** 1979. Immunochemical analysis of membrane vesicles from *Escherichia coli*. Biochemistry **18**:1413-1422.
18. **Owen, P., and H. R. Kaback.** 1979. Antigenic architecture of membrane vesicles from *Escherichia coli*. Biochemistry **18**:1422-1426.
19. **Owen, P., and M. R. J. Salton.** 1975. Antigenic and enzymatic architecture of *Micrococcus lysodeikticus* membranes established by crossed immunoelectrophoresis. Proc. Natl. Acad. Sci. U.S.A. **72**:3711-3715.
20. **Owen, P., and M. R. J. Salton.** 1977. Membrane asymmetry and expression of cell surface antigens of *Micrococcus lysodeikticus* established by crossed immunoelectrophoresis. J. Bacteriol. **132**:974-985.
21. **Owen, P., and C. J. Smyth.** 1977. Enzyme analysis by quantitative immunoelectrophoresis. p. 147-202. *In* M. R. J. Salton (ed.), Immunochemistry of enzymes and their antibodies. John Wiley & Sons, Inc., New York.
22. **Rutberg, B., L. Hederstedt, E. Holmgren, and L. Rutberg.** 1978. Characterization of succinic dehydrogenase mutants of *Bacillus subtilis* by crossed immunoelectrophoresis. J. Bacteriol. **136**:304-311.
23. **Schmitt, M., K. Rittinghaus, P. Scheurich, U. Schwulera, and K. Dose.** 1978. Immunological properties of membrane-bound adenosine triphosphatase. Immunological identification of rutamycin-sensitive F_0-F_1 ATPase from *Micrococcus luteus* ATCC 4698 established by crossed immunoelectrophoresis. Biochim. Biophys. Acta **509**:410-418.
24. **Smyth, C. J., A. E. Friedman-Kien, and M. R. J. Salton.** 1976. Antigenic analysis of *Neisseria gonorrhoeae* by crossed immunoelectrophoresis. Infect. Immun. **13**:1273-1288.
25. **Smyth, C. J., J. Siegel, M. R. J. Salton, and P. Owen.** 1978. Immunological analysis of inner and outer membranes of *Escherichia coli* by crossed immunoelectrophoresis. J. Bacteriol. **133**:306-319.
26. **Uriel, J.** 1971. Characterization of precipitates in gels. I. Color reactions for identification of antigen-antibody precipitates in gels. Methods Immunol. Immunochem. **3**:294-321.
27. **Weeke, B.** 1973. Crossed immunoelectrophoresis. Scand. J. Immunol. **2**(Suppl. 1):47-56.