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LOCALIZATION AND DISTRIBUTION OF *MICROCOCCUS LYSODEIKTICUS* MEMBRANE ATPase DETERMINED BY FERRITIN LABELING

JOEL D. OPPENHEIM and MILTON R. J. SALTON

Department of Microbiology, New York University School of Medicine, New York, N.Y. 10016 (U.S.A.)

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SUMMARY

Antiserum to Ca^{2+}-activated ATP phosphohydrolase (EC 3.6.1.3) isolated and purified from membranes of *Micrococcus lysodeikticus* was prepared in rabbits and guinea pigs. The γ-globulin fractions of these antisera reacted with and inhibited ATPase activity in isolated membranes but failed to absorb to intact protoplasts or purified mesosome fractions. ATPase activity was not detectable in the purified mesosomal preparations and trypsin treatment and sonication failed to release any activity. Ferritin conjugated to the γ-globulin fractions of the antiserum reacted with the ATPase particles on the membrane as visualized in negatively stained preparations examined in the electron microscope. Labeled membranes showed a distribution of ferritin very similar to the patterns observed for ATPase particles on untreated membranes. No significant labeling occurred when the ferritin conjugate was reacted with intact protoplasts or mesosome fractions. Thin sections of ferritin-labeled membranes established the asymmetric disposition of the ATPase, with the conjugate visible on only one side of the membrane. The results indicate that the ATPase protein occurs on the inner face of the membrane. All labeling experiments were verified immunologically. When ferritin-labeled membranes were subjected to the selective release procedure used in releasing the ATPase-like particles from the membranes, a complex of ferritin-conjugate associated with the ATPase particles was released. The selective release of ferritin–antibody–enzyme complexes from the membrane opens up a new way of studying the molecular architecture of cell membranes.

INTRODUCTION

In order to understand the molecular architecture of cell membranes of bacteria numerous attempts have been made to identify specific functional proteins on the membranes. Direct observation by electron microscopy of isolated bacterial membranes using negative staining techniques[1–3] has been of some value in the elucidation process, but little can be deduced about the specific molecular architecture from the sole use of this technique. Cytochemical staining combined with electron microscopy has also been widely employed in the localization of specific enzymes in a variety of cells, cellular organelles and in the surface structures of bacteria[4–7]. Although these methods are highly specific for a particular enzymatic activity the resolution is rarely

good enough to indicate the precise location of the protein. The cytochemical staining has, however, been successful in demonstrating the location of ATPase on the inner face of the erythrocyte membrane[8].

In this paper we have investigated the localization and distribution of a specific protein, the membrane-bound form of ATP phosphohydrolase (ATPase), by the highly selective method utilizing ferritin-labeled antibody specific for this enzyme. Through the use of this technique we have been able to specifically localize the enzyme with a high degree of resolution, establish its position on the membrane and isolate the ferritin–antibody–enzyme complex after its removal from the membrane.

MATERIALS AND METHODS

Growth and harvest

Micrococcus lysodeikticus (NCTC 2665) was grown and harvested as previously described[9–11].

Preparation of purified ATPase

Membranes were prepared from 20 l of cells by the standard method[9,12] from lysozyme lysates, except that the buffer strength was reduced from 0.1 M to 0.03 M Tris–HCl, pH 7.5. Membranes were washed five times with the 0.03 M Tris–HCl buffer, and sedimented after each wash by centrifugation at $30000 \times g$ for 30 min at 4 °C. The membrane pellets from the last wash were then suspended in 0.03 M Tris–HCl buffer containing 0.005 M EDTA and left overnight at 0–4 °C to release the bulk of the NADH dehydrogenase[13]. The membranes were centrifuged ($30000 \times g$ for 30 min, 4 °C) and supernatants were discarded. Ca^{2+}-activated ATPase was then obtained by a modification of the selective release, shock-wash method[11] from the membranes. The membranes were washed twice in 0.003 M Tris–HCl buffer containing 0.0005 M $CaCl_2$. After each of these treatments membranes were deposited by centrifugation ($30000 \times g$ for 30 min, 4 °C). The two supernatant washes were pooled and after an additional centrifugation ($30000 \times g$ for 30 min, 4 °C) the supernatant fractions were concentrated 10-fold in an Amicon Ultrafiltration unit (Amicon Corp., Lexington, Mass.) using an XM-50 membrane. The filtrate was then centrifuged at low speed as above, the residual membrane pellet discarded, and the supernatant fluids then subjected to two consecutive high-speed centrifugations ($147000 \times g$ for 60 min, 4 °C) in a Spinco L-2 ultracentrifuge. The resulting supernatants were pooled and concentrated 5-fold in the Amicon unit, yielding a slightly yellowish solution with a marked increase in specific activity (ATPase units/mg protein of 88.0 compared to the initial shock wash of 27.0).

The concentrated protein solution was then fractionated on a Sephadex G-200 as previously described[14] using a 4 cm × 70 cm column. Fractions containing "soluble ATPase III" activity[14] were pooled, concentrated and refractionated on the same G-200 column. The fractions exhibiting "soluble ATPase III" activity were again pooled and concentrated. The ATPase which was fractionated twice on Sephadex G-200 was finally chromatographed on a 2.5 cm × 120 cm Agarose A 1.5 (Bio-Rad) column eluted with 0.03 M Tris–HCl. Enzyme fractions containing a constant specific activity were pooled, concentrated to 1.5 mg protein/ml and stored at −70 °C. From 20 l of stationary phase culture yielding approximately 150 g wet weight of cells, 25 mg of highly purified ATPase were obtained.

Preparation and purification of antisera

Rabbit and guinea pig antisera to the purified ATPase described above were prepared essentially according to the procedure of Whiteside and Salton[15]. Relatively pure preparations of the γ-globulin fraction were prepared from whole sera by salt precipitation followed by chromatography on DEAE-cellulose as described by Campbell *et al.*[16,17]. The antibody titer of the purified γ-globulin fraction from rabbit anti-ATPase was determined by the precipitin ring test as described in Campbell[18]. All sera were stored in the absence of preservative at -20 °C.

Rabbit antisera to whole and sonicated membranes, prepared as outlined by Salton[12], were obtained as described by Whiteside and Salton[15].

Rabbit anti-horse ferritin antisera, sheep anti-rabbit γ-globulin and sheep anti-guinea pig γ-globulin were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio).

Immunodiffusion and immunoelectrophoresis

Double diffusion tests were performed in agar coated microscope slides by the method in Campbell *et al.*[19]. Immunoelectrophoresis was performed on agar coated microscope slides according to Campbell *et al.*[20]. Electrophoresis was carried out for 90–120 min at 4 °C with a current of 5 mA/slide.

Both immunodiffusion and immunoelectrophoresis slides were developed in a moisture chamber at room temperature (22–24 °C) for 18–24 h. Precipitin patterns were photographed in a Cordis immunodiffusion camera.

Preparation of ferritin conjugates

Horse-spleen ferritin (6× recrystallized, Cd-free, Miles-Pentex, Inc., Kankakee, Ill.) was further purified by crystallization and ultracentrifugation as described by Andres *et al.*[21]. The purified ferritin was conjugated to the γ-globulin fractions of rabbit anti-ATPase, guinea pig anti-ATPase and rabbit non-immune sera using toluene 2,4-diisocyanate (Polysciences, Inc., Rydal, Pa.) essentially as described by Singer and Schick[22]. Conjugates were separated from unconjugated protein by three successive centrifugations at $100000 \times g$ for 3 h. All preparations were tested by immunodiffusion analysis for the presence of the conjugate and to determine whether or not the conjugated antibody was still reactive with its specific antigen.

Preparation of membranes for labeling and labeling procedure

Membranes were prepared from 300-ml cultures, from protoplasts as described by Muñoz *et al.*[10] with the exception that 0.005 M $MgCl_2$ was added to the protoplast lysis buffer. Membranes so obtained were washed three times with 0.03 M Tris–HCl buffer, pH 7.5, containing 0.005 M $MgCl_2$ and then twice in the Tris buffer containing 0.005 M $MgCl_2$ and 0.15 M NaCl (hereafter referred to as Tris–$MgCl_2$–NaCl buffer). Membranes were sedimented after each wash by centrifugation at $30000 \times g$ for 30 min at 4 °C. The membrane pellet from the last wash was suspended in Tris–$MgCl_2$–NaCl buffer so as to obtain a suspension containing 2.0–2.5 mg protein/ml. Membrane preparations were tested for ATPase activity and examined under the electron microscope before proceeding.

2 ml of the membrane suspension were mixed with 2 ml of 0.05 M Tris–$MgCl_2$–NaCl buffer and then 2 ml of the purified ferritin-conjugated antibody were added.

The mixture was incubated for 30 min at 23 °C. After incubation membranes were washed three times by centrifugation at 20000 × *g* for 10 min at 4 °C and resuspension in 10 ml of Tris–$MgCl_2$–NaCl buffer. The supernatants from each wash were analyzed for the presence of free conjugate and γ-globulin by immunodiffusion testing.

In order to simulate the selective release method using the labeled membranes, membrane suspensions were dialyzed overnight against 0.003 M Tris–HCl buffer. Membranes were pelleted by centrifugation, and then washed twice with 0.003 M Tris–HCl, all supernatants being saved. The final pellets were resuspended in 4 ml of 0.003 M Tris–HCl.

Preparation of mesosomes for labeling and labeling procedure

Mesosomes were prepared from 1 l cultures. After harvesting, the cells were washed twice in 0.1 M Tris–HCl buffer, pH 7.5, containing 0.04 M $MgCl_2$. Cells were sedimented after each wash by centrifugation at 6000 × *g* for 10 min at 23 °C. The cell pellet from the last wash was suspended in 50 ml of 0.1 M Tris–HCl, pH 7.5, containing 2.0 M sucrose and 0.04 M $MgCl_2$ and allowed to incubate for 30 min at 23 °C. The cell suspension was then diluted with 0.1 M Tris–HCl containing 0.04 M $MgCl_2$, to give a 0.8 M sucrose concentration. This suspension was allowed to stand for an additional 30 min at 23 °C. At the end of this second period, lysozyme was added to give a final concentration of 150 μg/ml. The cell-lysozyme suspension was then incubated for 30–40 min in a 37 °C water bath. Protoplasts so formed were then pelleted by centrifugation at 12000 × *g* for 40 min at 23 °C. The protoplast pellets were then resuspended in 100 ml of 0.8 M sucrose in 0.1 M Tris–HCl containing 0.01 M $MgCl_2$ and allowed to stand 10 min at 23 °C. The suspension was then centrifuged at 12000 × *g* for 40 min at 23 °C. The supernatant, which contained whole and fragmented mesosomes[23], was decanted off and recentrifuged at 30000 × *g* for 1 h at 4 °C to remove any membrane fragments. The low-speed supernatant containing the mesosomes was then subjected to a high speed centrifugation (147000 × *g* for 90 min, 4 °C) in a Spinco L-2 ultracentrifuge. The supernatant was discarded while the pellets were resuspended in a minimal volume of 0.1 M Tris–HCl buffer containing 0.005 M $MgCl_2$ to give a suspension containing 1.0–1.5 mg protein/ml. The resuspended mesosome preparation was then centrifuged at 12000 × *g* for 30 min at 4 °C to remove any large aggregates. The mesosome preparations were tested for ATPase activity and examined under the electron microscope before proceeding.

2 ml of the mesosome suspension were mixed with 2 ml of 0.1 M Tris–$MgCl_2$–NaCl buffer and then 0.5 ml of the purified ferritin-conjugated anti-ATPase was added. The mixture was incubated for 30 min at 23 °C with gentle stirring. After incubation anti-ferritin $F(ab')_2$ fraction was slowly added in an amount calculated to give a slight antibody excess in relation to the ferritin present. The mixture was allowed to incubate for an additional 30 min at 23 °C. A flocculant precipitate gradually formed and settled out. The mixture was then centrifuged at 3000 × *g* for 10 min to firmly pack the precipitate. The supernatant was carefully decanted off and saved. The precipitate was dissociated by suspension in 0.2 M glycine–HCl–saline buffer, pH 2.5.

Preparation of protoplasts for labeling and labeling procedure

Protoplasts of *M. lysodeikticus* were prepared by washing the cells in 0.8 M

sucrose in 0.05 M Tris–HCl buffer, pH 7.5, containing 0.025 M $MgCl_2$. Washed cells were resuspended in the same buffer and treated with lysozyme (200 μg/ml final concentration) and protoplasts allowed to form by incubation for 30–40 min at 23 °C. Wall digestion was considered complete when a diluted suspension of protoplasts lysed rapidly. Protoplasts so formed were centrifuged and washed with the same sucrose–Tris buffer at $12000 \times g$ for 40 min at 23 °C. The washed protoplasts were resuspended in the sucrose–Tris–Mg^{2+} buffer.

An attempt to ferritin label protoplasts was carried out by using a modification of the technique described by Fukui *et al.*[24] for the absorption of membrane antiserum with protoplasts. 2-ml portions of a protoplast suspension were layered over discontinuous sucrose gradients in tubes for the SW-25 rotor. The gradients consisted of a bottom layer of 5 ml of 70% sucrose, 5 ml of 50% sucrose, 5 ml of 40% sucrose, 10 ml of 34% sucrose and a top layer of 3 ml of the ferritin-conjugated antibody which had been dialyzed against saline containing 0.8 M sucrose. All of the sucrose solutions for the gradient were prepared in 0.05 M Tris–HCl buffer, pH 7.5, containing 0.025 M $MgCl_2$. The gradient tubes were then centrifuged at 23 °C at $5000 \times g$ for 30 min to sediment the protoplasts into the layer containing the labeled antibody. Possible absorption of labeled antibody by protoplasts was allowed to proceed by holding the gradients at 23 °C for 1 h. The protoplasts were then separated from the labeled antibody layer by centrifugation further into the gradient ($20000 \times g$, 1 h at 23 °C). After centrifugation the protoplasts, which banded at the interface between the 50% and 70% sucrose layers, were syphoned off and washed twice in 0.05 M Tris–HCl, pH 7.5, containing 0.025 M $MgCl_2$, by centrifugation at $12000 \times g$ for 30 min at 23 °C.

Inhibition of membrane-bound ATPase

The ability of the purified ATPase antisera to block the enzymatic activity of the membrane-bound ATPase, as detected by trypsin stimulation[11] was tested with 1 ml samples of a five times washed membrane preparation in 0.03 M Tris–$MgCl_2$–NaCl buffer (2.0 mg protein/ml). Samples were incubated for 30 min at room temperature (22–24 °C) with varying amounts of purified anti-ATPase γ-globulin (0–0.8 ml of a 15.0 mg/ml preparation in 0.03 M Tris–$MgCl_2$–NaCl buffer). After incubation, treated membranes were washed three times by centrifugation at $20000 \times g$ for 20 min at 4 °C and resuspended in 10 ml of the Tris–$MgCl_2$–NaCl buffer. The final pellets were resuspended in 1.0 ml of the buffer and ATPase and trypsin-activated ATPase assays were performed on each sample.

Electron microscopy

Membranes, protoplasts, mesosome fractions, and ATPase preparations were examined after negative staining with ammonium molybdate essentially as described by Muñoz *et al.*[10]. For thin sectioning specimens were fixed in 1% osmium tetroxide in Kellenberger's veronal–acetate buffer[25] at 4 °C for 16 h. The fixed pellets were washed twice in the veronal–acetate buffer and thereafter embedded in 1% Noble agar in the veronal buffer. The agar blocks (0.5 mm^3) were stained with 0.5% magnesium uranylacetate in veronal buffer and then dehydrated in a graded series of ethanol and finally embedded in Epon 812 according to Luft[26]. Thin sections of cured blocks were cut on a Reichert $Om4_2$ automatic ultramicrotome with a Dupont

diamond knife and were mounted on Formvar, carbon-coated grids. Sections were doubly stained according to the method of Frasca and Parks[27]. Sections and negatively stained samples were examined in a Siemens Elmiskop I.

Other procedures

ATPase activity was measured by the liberation of inorganic phosphorus (P_i) from ATP and specific activities determined as described by Muñoz *et al.*[10,11]. Protein was determined by the method of Lowry *et al.*[28].

Disc gel electrophoresis was performed under the standard alkaline conditions as described by Salton[12]. Proteins were stained with Coomassie blue[29] while the lead acetate method described by Weinbaum and Markman[30] was used to visualize ATPase activity in gels.

RESULTS

Preparation of purified ATPase

Since only a portion of the γ-globulin fraction of antisera is conjugated in the Singer and Schick[22] toluene 2,4-diisocyanate–ferritin labeling procedure, specific antisera of high titer are essential. For this reason we embarked on the preparation of highly purified ATPase for use in animal inoculation. Through the use of techniques

Fig. 1. Polyacrylamide gel electrophoresis of highly purified ATPase. (A) Gel of ATPase stained with Coomassie blue. (B) Gel stained for ATPase activity with lead acetate, as described by Weinbaum and Markman[30].

already developed in this laboratory for the purification of this enzyme[10,11,14] together with additional techniques developed specifically for this task (*e.g.* the use of multiple columns, series of high-speed centrifugations, etc.) we were able to prepare a relatively large quantity (25 mg) of highly purified enzyme. This preparation had a specific activity of more than twice that previously reported from this laboratory[14]. The preparation was judged to be homogeneous on the basis polyacrylamide gel electrophoresis (Fig. 1), immunoelectrophoresis developed against antisera to whole membranes and anti-ATPase previously prepared in this laboratory[15] (Fig. 2) and by electron microscopy.

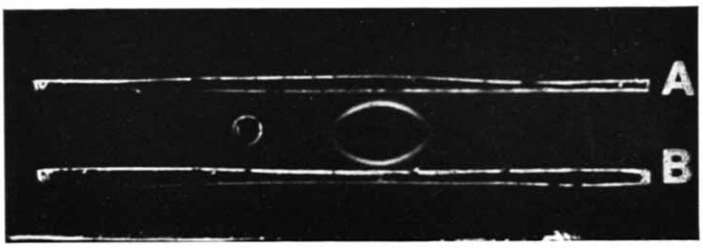

Fig. 2. Immunoelectrophoresis slide of the purified ATPase preparation used for animal injections. 4 μg of the ATPase was placed in the center well and electrophoresed for 90 min at 5 mA at pH 8.2. Troughs A and B were filled with anti-ATPase and whole membrane antiserum, respectively, and incubated overnight.

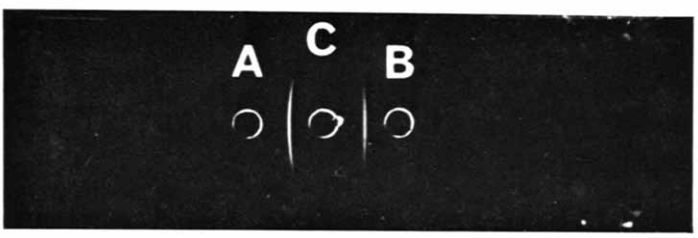

Fig. 3. Double diffusion slide showing the precipitin reactions between purified rabbit anti-ATPase γ-globulin (Well A), purified guinea pig anti-ATPase γ-globulin (Well B) and the purified illiciting antigen ATPase (Well C).

It has been previously shown that the soluble form of the Ca^{2+}-dependent ATPase from *M. lysodeikticus* is antigenic in rabbits[15] and it appears to exhibit the same antigenicity in guinea pigs. The solubilized enzyme was antigenically homogeneous and both the rabbit and guinea pig antibodies were specific for the purified ATPase, as shown by the characteristic single precipitin lines observed for each in a double diffusion slide (Fig. 3).

Inhibition of membrane-bound enzymatic activity by antibody

An essential requirement for the localization of the ATPase on the membrane by ferritin labeling is that the antibody must react with the ATPase (antigen) when it is in the membrane-bound state. It was therefore necessary to determine whether or not the antisera would react with the membrane-bound ATPase. Accordingly, inhibition of the activity by the purified γ-globulin fraction of the rabbit anti-ATPase

was determined and the results are shown in Fig. 4. An almost complete inhibition of the membrane-bound ATPase, as determined by trypsin activation assays, was obtained with the use of suitable concentrations of anti-ATPase γ-globulin. Similar inhibition has been shown using the soluble form of ATPase[15]. These results thus clearly establish the reactivity of the antiserum to the ATPase antigen when it is bound to the membrane.

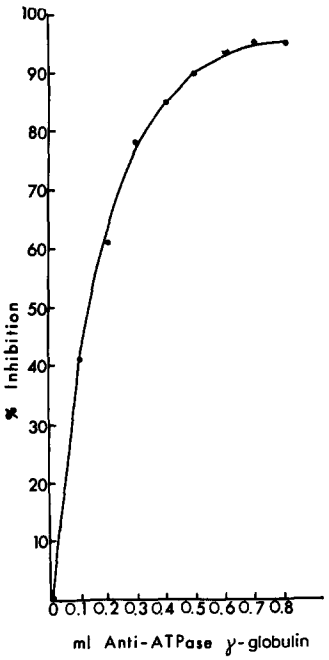

Fig. 4. Inhibition of membrane-bound ATPase by specific antisera. Each tube contained 1 ml of a four times washed membrane preparation (2.0 mg protein/ml) in Tris–$MgCl_2$–NaCl buffer and anti-ATPase γ-globulin as indicated. Procedures as described in Materials and Methods. Non-immune rabbit γ-globulin was used as a control.

General membrane and protoplast architecture

A negatively-stained preparation of five times washed membranes of *M. lysodeikticus* is illustrated in Fig. 5. The distribution of particles believed to possess the ATPase activity, on the membrane surface and along the peripheries of membrane fragments is evident. Fig. 6 shows a negatively-stained membrane preparation which had been previously subjected to a shock wash, causing the release of the stalk-like particles associated with ATPase activity[10]. The distribution of ATPase-like particles on an intact membrane of a lysed protoplast, obtained by a modification of the Nicolson and Singer technique[31], is exhibited in Fig. 7. The particles appear to be distributed evenly on the membranes. There are no ATPase particles visible on the vesicular mesosomal material isolated from intact protoplasts by the procedure of Ellar and Freer[23] (Fig. 8).

Ferritin labeling

Five times washed membranes were reacted with ferritin-conjugated anti-

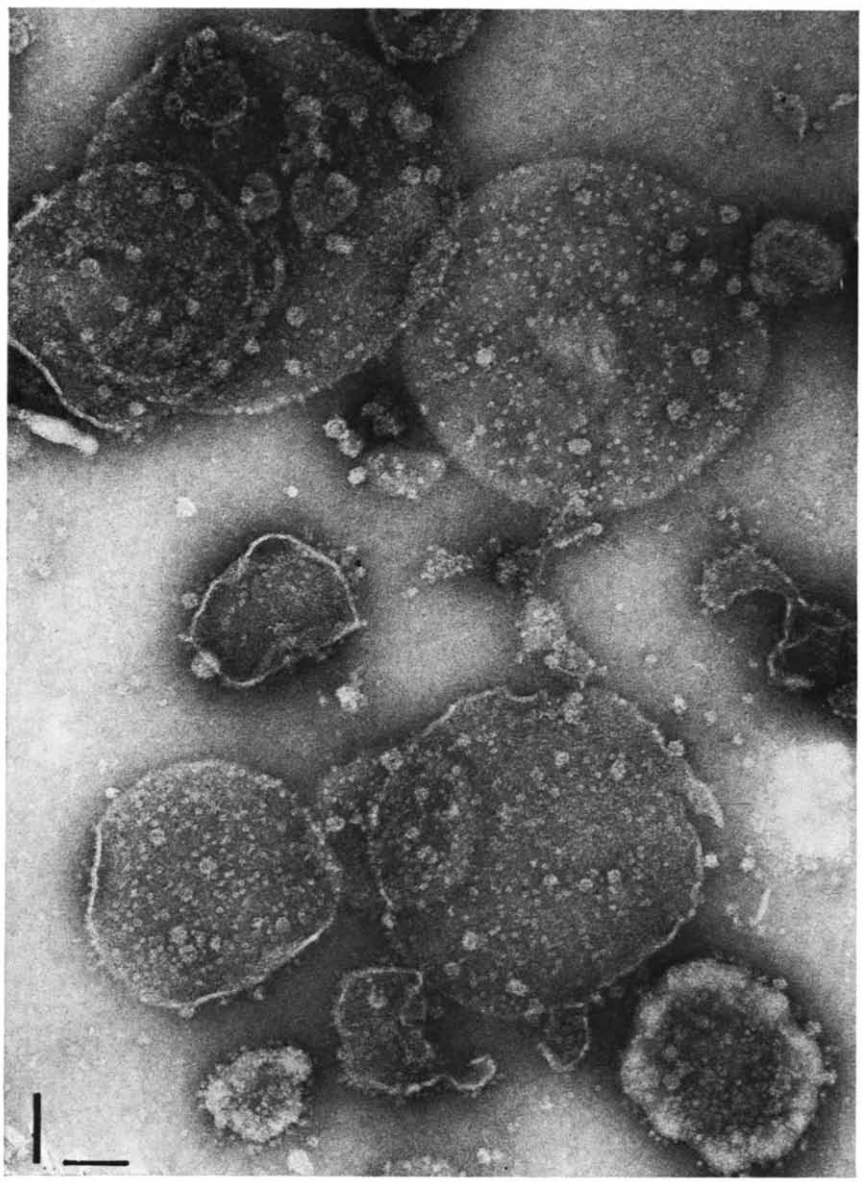

Fig. 5. Electron micrograph of five times washed untreated membranes of *M. lysodeikticus* negatively stained with ammonium molybdate. ×90000. Bar represents 0.1 μm.

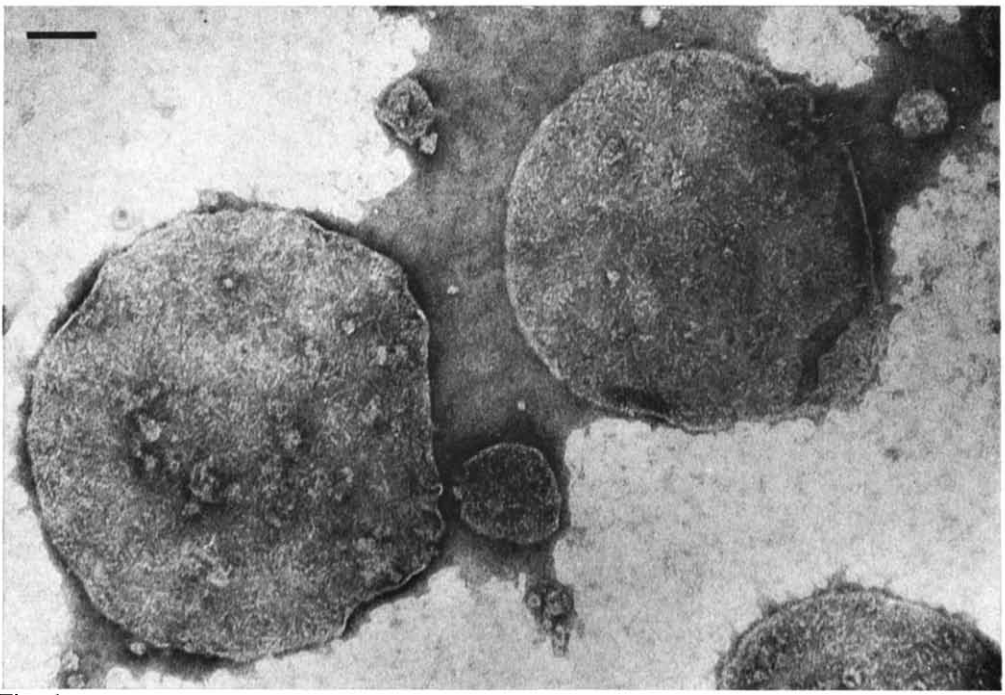

Fig. 6

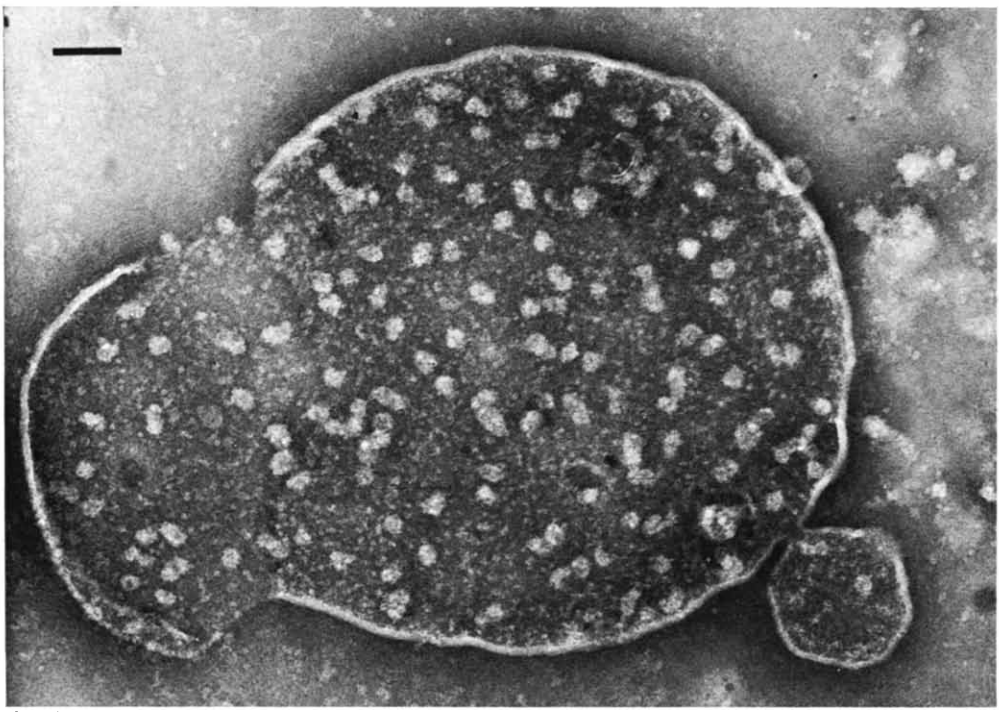

Fig. 7

Fig. 6. Electron micrograph of a shock-washed membrane preparation of *M. lysodeikticus* negatively stained. ×90000. Bar represents 0.1 μm.

Fig. 7. Electron micrograph of a lysed whole protoplast of *M. lysodeikticus*, prepared using a modification of the Nicolson and Singer technique[31], negatively stained with ammonium molybdate. ×90000. Bar represents 0.1 μm.

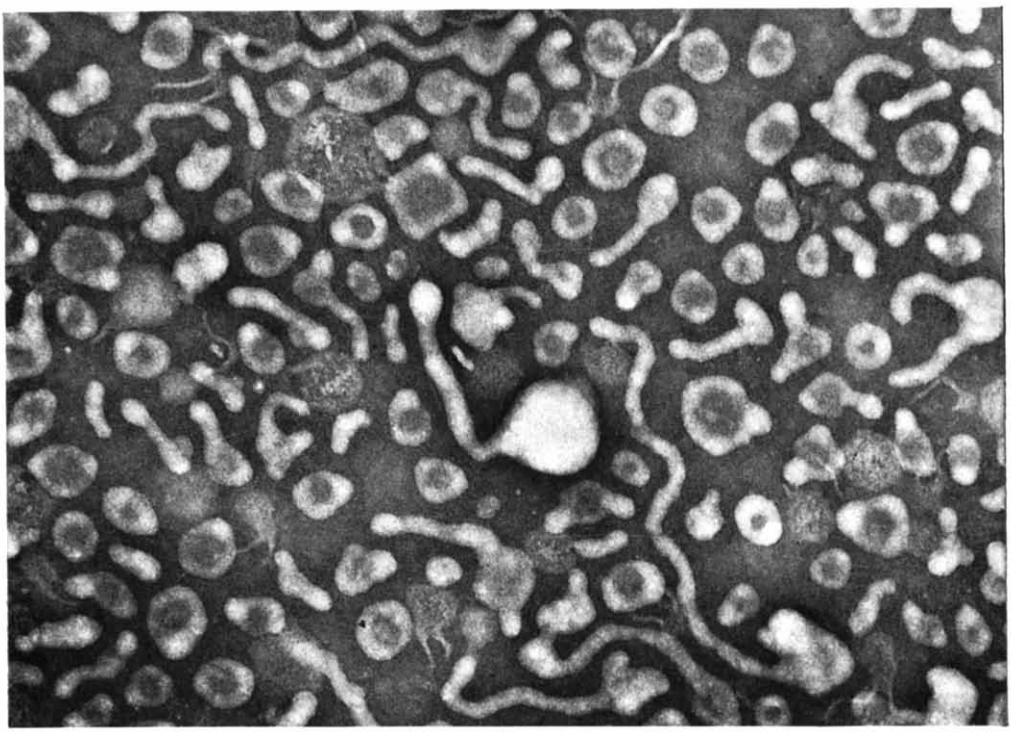

Fig. 8. Electron micrograph of mesosomes of *M. lysodeikticus* prepared by the procedure of Ellar and Freer[23], negatively stained. ×90000.

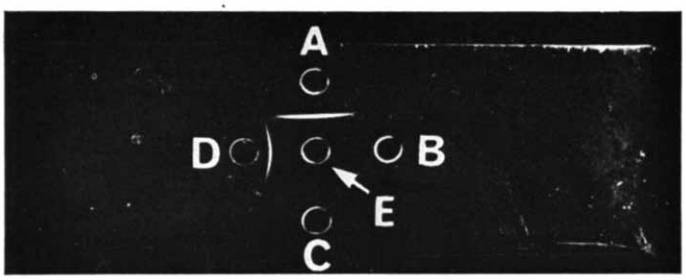

Fig. 9. Immunodiffusion slide showing the distribution of ferritin in three successive washes (Wells A, B and C) and in the residual membrane preparation (Well D) after ferritin labeling as detected by anti-ferritin antisera (Well E).

ATPase and after labeling the membranes were washed three times in buffer. Each wash and the final membrane suspensions were monitored biochemically for ATPase activity (Table I) and immunologically for unbound ferritin (Fig. 9).

As indicated in Table I the ferritin labeling procedure used does not cause the release of ATPase activity from the membrane. No enzymatic activity was observed in any of the washes while almost all of the activity on the original membranes was still associated with control membranes which were subjected to the same manipulations as for the labeled membranes. While detectable ATPase activity dropped at least 80% after labeling, some residual enzymatic activity remained on the washed membranes. We believe that such activity may be in part due to sequestered ATPase which was not exposed (*e.g.* in closed vesicles) during the labeling procedure but which became exposed during the washings and/or steric hindrance of some enzyme sites to reaction with antibody in the membrane-bound state. Such phenomena could explain why we were unable to obtain 100% enzyme blockage in the inhibition experiment performed on membranes (Fig. 4).

The presence of a strong precipitin band (Fig. 9) between anti-ferritin antisera (Well E) and wash I (Well A) is not unexpected since this wash is a dilution of the original labeling reaction mixture. Wash I, thus contains unconjugated ferritin, ferritin conjugated to nonspecific γ-globulin and any unreacted specific conjugate. Washes II and III (Wells B and C) show little or no reactivity against the anti-ferritin antisera, while the washed resuspended membranes (Well D) show a moderate reaction. These results suggest that all unreacted ferritin conjugate has been successfully removed from the treated membranes and that remaining label must be the result of specific antigen–antibody interaction.

In Fig. 10A, a negatively stained membrane preparation, the regular distribution of ferritin (conjugated to rabbit anti-ATPase γ-globulin) is apparent. In many instances ferritin molecules can be seen in direct contact with "ATPase-like" structures along the membrane periphery. This distribution becomes more readily discernable

TABLE I

DISTRIBUTION OF ATPase AND FERRITIN IN MEMBRANE FRACTIONS AND WASHES OF FERRITIN-LABELED MEMBRANES

Sample	*ATPase units*	*Ferritin***
Membrane suspension	+++*	−
Wash I	0	++++
Wash II	0	±
Wash III	0	−
Labeled membrane	±*	++
Control membrane	+++*	−

* Indicates (+ → +++) relative amount present or (−) absence of trypsin activated ATPase: owing to complexity of kinetics on trypsin activation exact units not given.

** Indicates (+ → ++++) relative amount present or (−) absence of ferritin as detected in immunodiffusion assay (see Fig. 9).

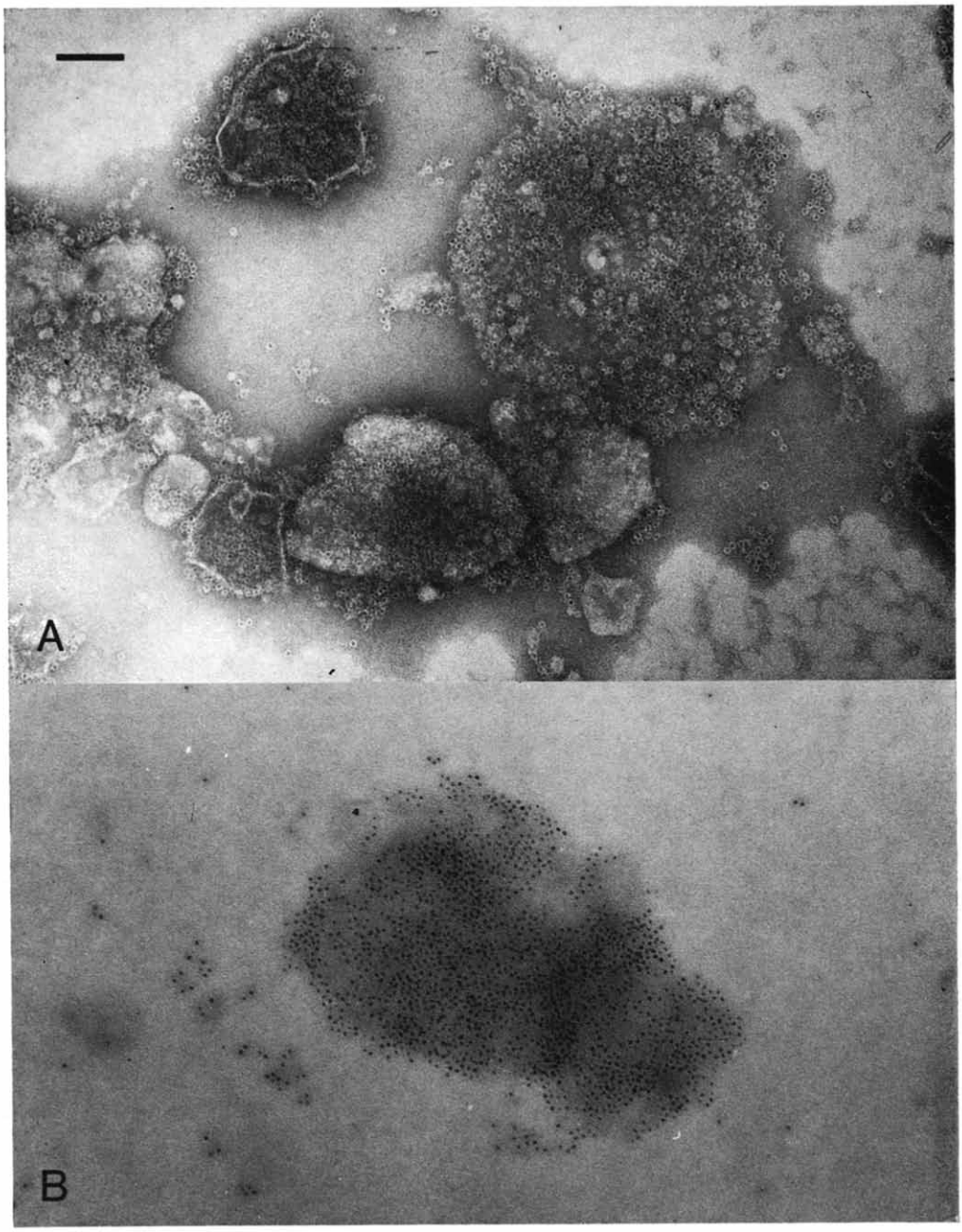

Fig. 10. Electron micrographs of five times washed membranes of *M. lysodeikticus* treated with anti-ATPase γ-globulin conjugated to ferritin. Preparation A was negatively stained with ammonium molybdate while Preparation B was unstained. In both micrographs the even distribution of ferritin on the membrane is discernable. $\times 90000$. Bar represents 0.1 μm.

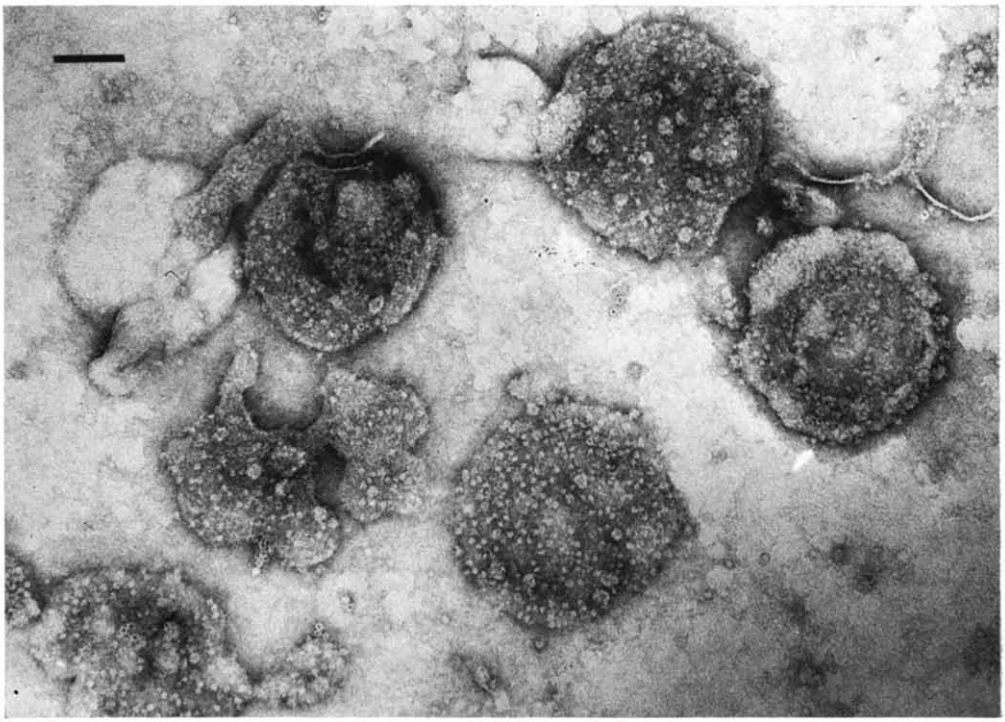

Fig. 11. Electron micrograph of five times washed membranes treated with ferritin conjugate preabsorbed with soluble ATPase. ×90000. Bar represents 0.1 μm.

in an unstained preparation (Fig. 10B). Similar results were obtained using ferritin conjugated to guinea pig anti-ATPase γ-globulin. The labeling reaction was blocked by either the absorption of the membrane with excess unconjugated antibody or the absorption of the conjugated antisera with excess antigen prior to labeling (Fig. 11). Membranes treated with either free ferritin or ferritin-conjugated non-immune rabbit globulin similarly failed to react. The shock-wash procedure has been shown to be effective in removing most of the detectable ATPase from the membrane[11] and when membranes were subjected to this treatment prior to labeling, they also failed to react with the conjugate or showed only sparse labeling as seen in Fig. 12.

From the examination of thin sections of unlabeled and labeled membranes (Figs 13A and 13B) it is apparent that the ferritin conjugate reacts only with one side of the membrane surface. It is well known that cellular membranes exhibit a marked propensity for the formation of closed vesicular-like structures and in this respect the isolated membranes of *M. lysodeikticus* are no exception since such vesicles were observed in earlier studies[12]. The labeling of some of these vesicles (Fig. 13B) in contrast to the absence of significant labeling of the outer membrane of intact protoplasts does show labeled ATPase particles on the "outer" surface of the membrane vesicles and thus suggests that some appear to have turned inside out with respect to surface orientation. The vesicles which do not exhibit ferritin labeling would thus either lack the ATPase or the antigen may be inaccessible for reaction in vesicles which are completely closed prior to labeling. That the isolated membrane suspensions con-

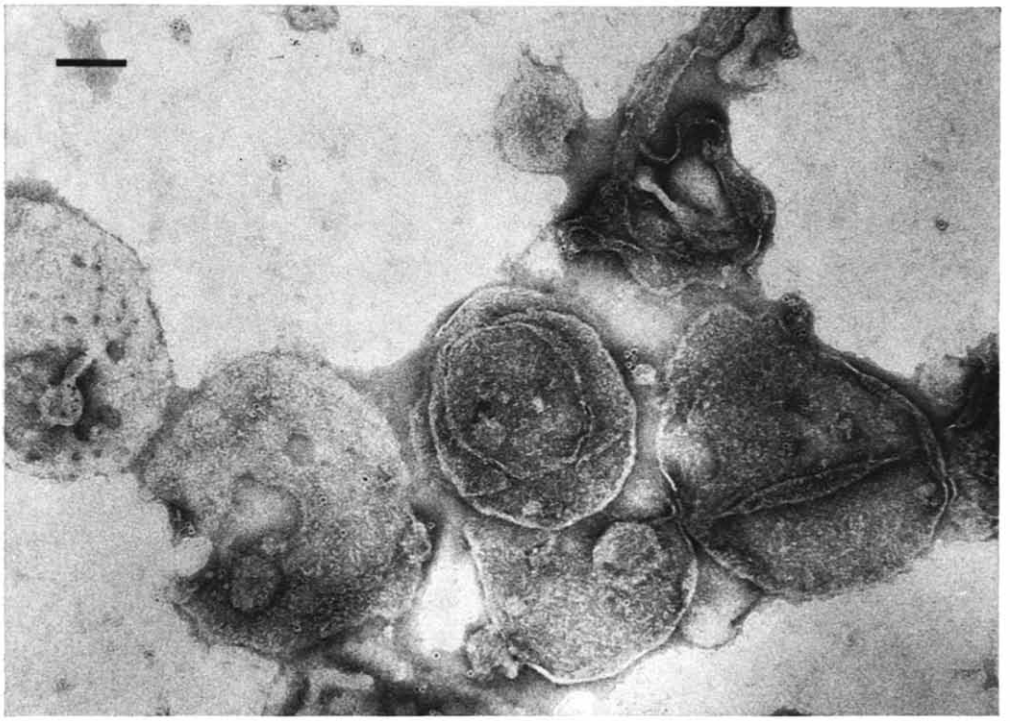

Fig. 12. Electron micrograph of shock-washed membranes treated with anti-ATPase ferritin conjugate. ×90000. Bar represents 0.1 μm.

tained a mixture of inside-out and right-side-out (*i.e.* the same orientation as in intact protoplasts) vesicles was further supported by the partial precipitation of such preparations with the ATPase antiserum.

All the preparations which were examined for ferritin labeling as described above were also subjected to immunodiffusion analysis. The results of these reactions can be seen in Fig. 14. A strong reaction between anti-ferritin and labeled membranes was always observed as shown in Figs 14A and 14E. A much weaker reaction can also be seen between the sheep anti-rabbit globulin or the rabbit anti-guinea pig globulin and the labeled membranes (Figs 14A and 14E). No reactions were observed between the membranes in other preparations, Figs 14B, 14C, 14D or 14F, reacted against the anti-ferritin or anti-globulin fractions, thus indicating that little if any labeling has occurred. In all slides ATPase antisera showed a strong reaction against their corresponding anti-globulin antisera. These positive reactions are expected from this system of testing. It is interesting to note that not even with untreated control membrane suspensions, containing substantial ATPase enzymatic activity (Table I), did the anti-ATPase give a line of precipitation in the agar diffusion tests. While this results is not unexpected in membrane samples which were successfully labeled, since the ATPase on these membranes was blocked, we were surprised to observe this with untreated membranes. A possible explanation may be found in the steric conformation of the ATPase molecules on the membrane. Since anti-ATPase was prepared from solubilized ATPase, a number of the antigenic determinant sites

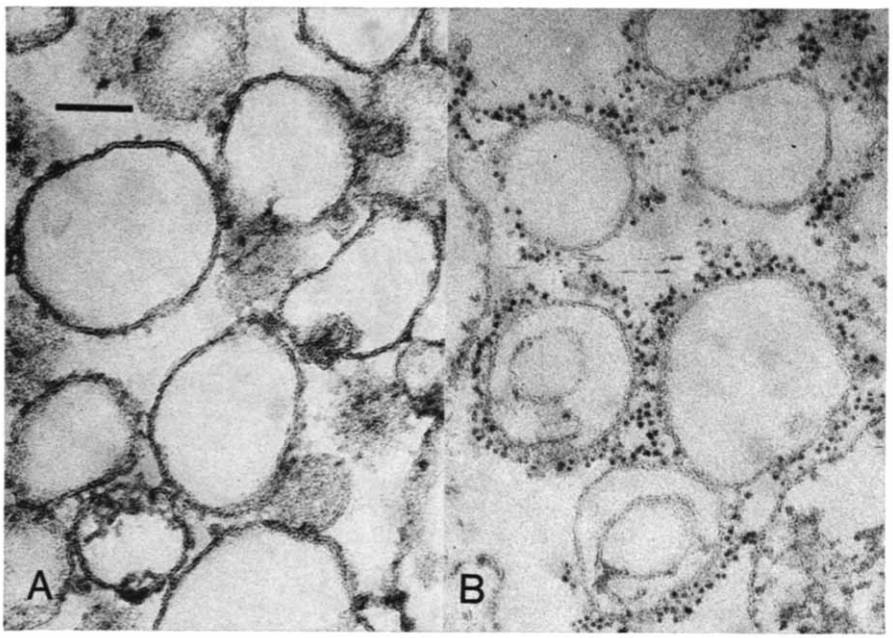

Fig. 13. Electron micrographs of sectioned membranes. Preparation A was unlabeled, whereas B was ferritin labeled. Sections were stained with magnesium uranyl acetate and lead citrate. Ferritin is present only on one side of the membrane in the labeled preparation. ×100000. Bar represents 0.1 μm.

located on the molecule may no longer be available to react immunologically when the enzyme is positioned on the membrane in its bound form, thereby minimizing the chances of precipitate formation. This may also explain the surprisingly large amount of antisera in relation to membrane protein which was required for enzyme inhibition of membrane-bound ATPase (Fig. 4). When membranes were subjected to sonic oscillation at pH 9.0 or sonication at pH 7.5 with subsequent incubation with trypsin (100 μg/ml) for 1 h at 37 °C, the treatments broke up the membranes into small diffusible pieces and caused the release of some ATPase molecules[24]. Such preparations were then able to give precipitin reactions against the anti-ATPase (Fig. 15).

Examination of whole protoplasts incubated with ferritin conjugated rabbit anti-ATPase, washed and then negatively stained, indicated that there were relatively few sites reactive for antibody on the outer membrane surface (Fig. 16). A few ferritin molecules can be seen adhering to the outer membrane surface, while numerous ATPase-like particles can be readily identified, thus indicating that these particles are localized on the internal membrane surface.

Mesosomes isolated from intact protoplasts were reacted with ferritin-conjugated anti-ATPase. Separation of mesosome material from ferritin was achieved by precipitating out the ferritin by the addition of the $F(ab')_2$ fraction of purified anti-ferritin γ-globulin. After centrifugation the supernatant was decanted off and saved while the pelleted precipitate was dissociated by suspension in 0.2 M glycine–

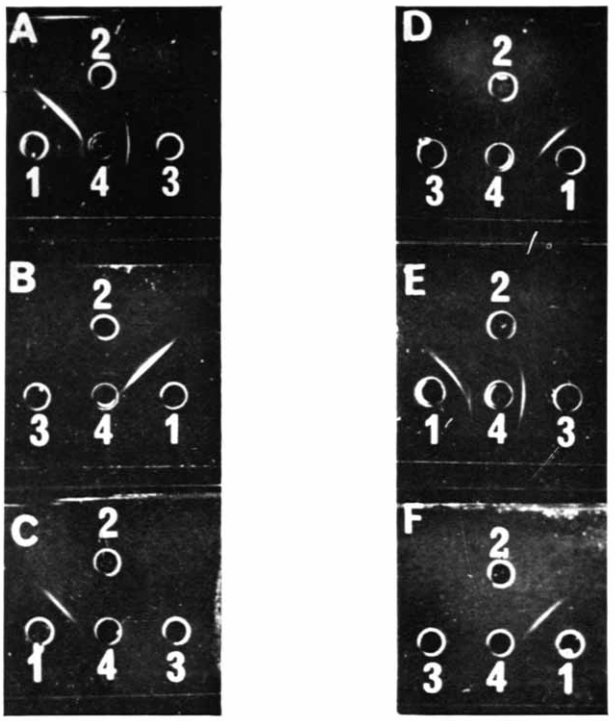

Fig. 14. Reactions of ferritin-labeled membranes, unlabeled membranes and membranes subjected to non-specific ferritin labeling procedures against antisera in agar diffusion tests. Wells 1, 2 and 3 on each slide, except Slide E contained sheep anti-rabbit γ-globulin, rabbit anti-ATPase γ-globulin fraction and sheep anti-horse ferritin antisera, respectively. Wells 1, 2 and 3 on Slide E contained rabbit anti-guinea pig γ-globulin, guinea pig anti-ATPase γ-globulin fraction and sheep anti-horse ferritin antisera. Ferritin-labeled membranes were placed in Well 4 of Slide A; membranes treated with free, unconjugated ferritin in Well 4 of Slide B; membranes treated with ferritin conjugated to non-immune rabbit γ-globulin in Well 4 of Slide C; membranes treated with ferritin conjugated anti-ATPase which was preabsorbed with an excess of ATPase in Well 4 of Slide D; membranes treated with ferritin conjugated to guinea pig anti-ATPase γ-globulin in Well 4 of Slide E; and untreated membranes in Well 4 of slide F.

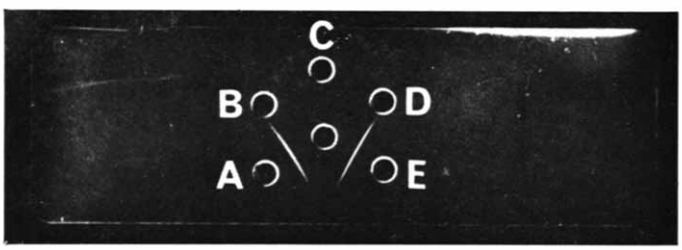

Fig. 15. Immunodiffusion slide showing the reactions of untreated membranes (Well B), untreated mesosomal material (Well D), membranes (Well A) and mesosomes (Well C) subjected to sonication at pH 7.5 with subsequent incubation with trypsin (100 μg/ml) for 1 h at 37 °C, and purified ATPase (Well E) *versus* rabbit anti-ATPase γ-globulin fraction (center well).

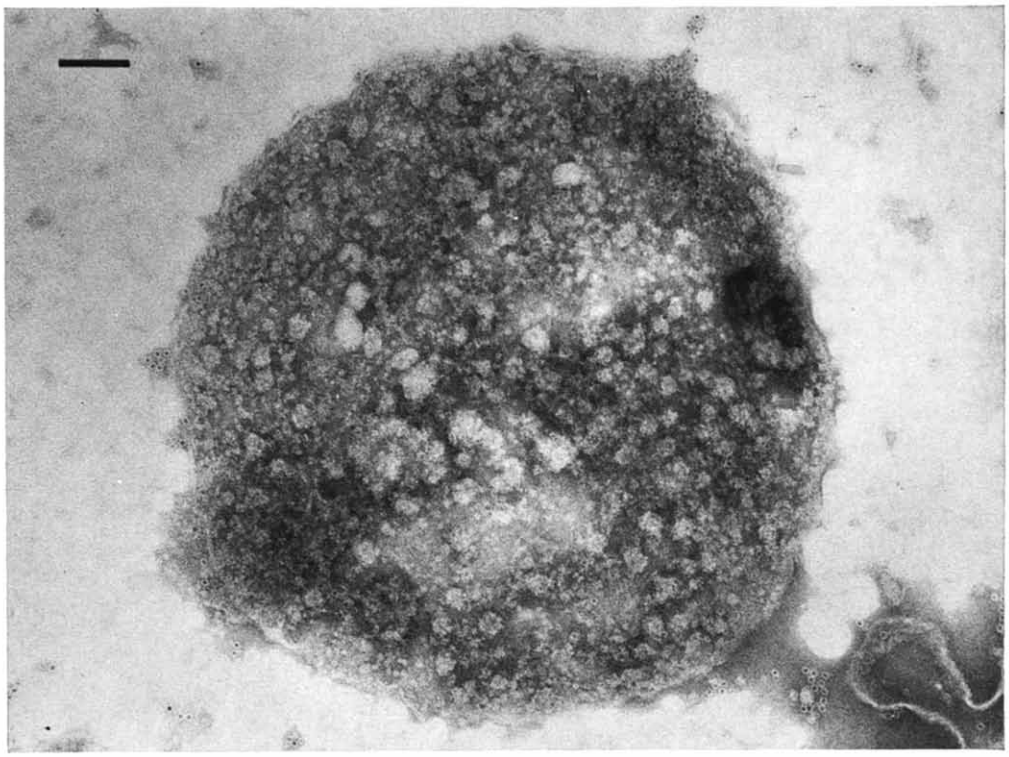

Fig. 16. Electron micrograph of a stabilized intact protoplasts of *M. lysodeikticus* treated with ferritin conjugated to rabbit anti-ATPase γ-globulin fraction, negatively stained with ammonium molybdate. $\times 90000$. Bar represents 0.1 μm.

HCl saline. The supernatant and dissociated precipitate material were examined under the electron microscope. Negatively stained supernatant material appeared identical to untreated mesosomes as seen in Fig. 8 with the exception of a few ferritin molecules which were randomly located in any given field. The dissociated precipitate consisted almost entirely of ferritin molecules and some small pieces of membrane material and contained no discernable mesosomes. The inability to label mesosomes with ferritin conjugated to anti-ATPase is not surprising in that ATPase was undetectable by either enzymatic assay (direct or trypsin activation) or immunologically (Fig. 15) using purified mesosomes or sonically treated mesosome material.

Isolation of ferritin–antibody–enzyme complex

Since Ca^{2+}-activated ATPase can be selectively released from *M. lysodeikticus* membranes by lowering the ionic strength of the buffer, an attempt was made to isolate the ferritin-labeled ATPase complex using a modification of this procedure. Fig. 17A shows a typical ATPase preparation obtained after the treatment of five times washed membranes by this selective release method. The preparation consisted primarily of 10 nm diameter spherical particles associated with the Ca^{2+}-activated ATPase activity of the membrane and some small membrane fragments. When ferritin-labeled membranes were subjected to similar treatment ATPase particles

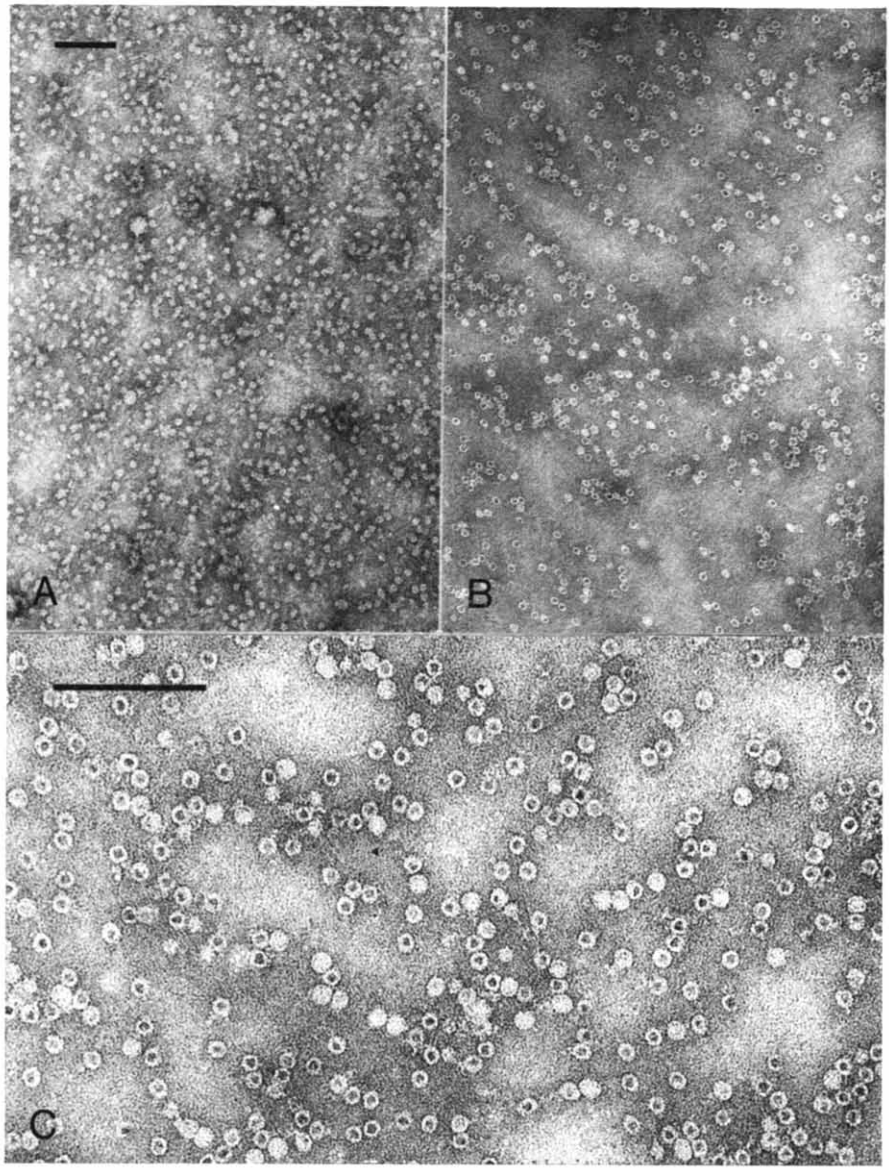

Fig. 17. Electron micrographs of (a), shock-wash from five times washed membranes, (b) and (c), shock-wash I from ferritin-labeled membranes, negatively stained with ammonium molybdate. (A and B), ×100000; (C) ×300000. Bars represent 0.1 μm.

associated with ferritin molecule(s) were released (Figs 17B and 17C). One or two ferritin molecules per ATPase particle were commonly seen. The shocked ferritin-labeled membranes (Fig. 18) look strikingly similar to normal shocked membranes (Fig. 6) and shown the attachment of very few residual ferritin or ATPase molecules.

Table II summarizes the distribution of protein, ATPase and ferritin in the

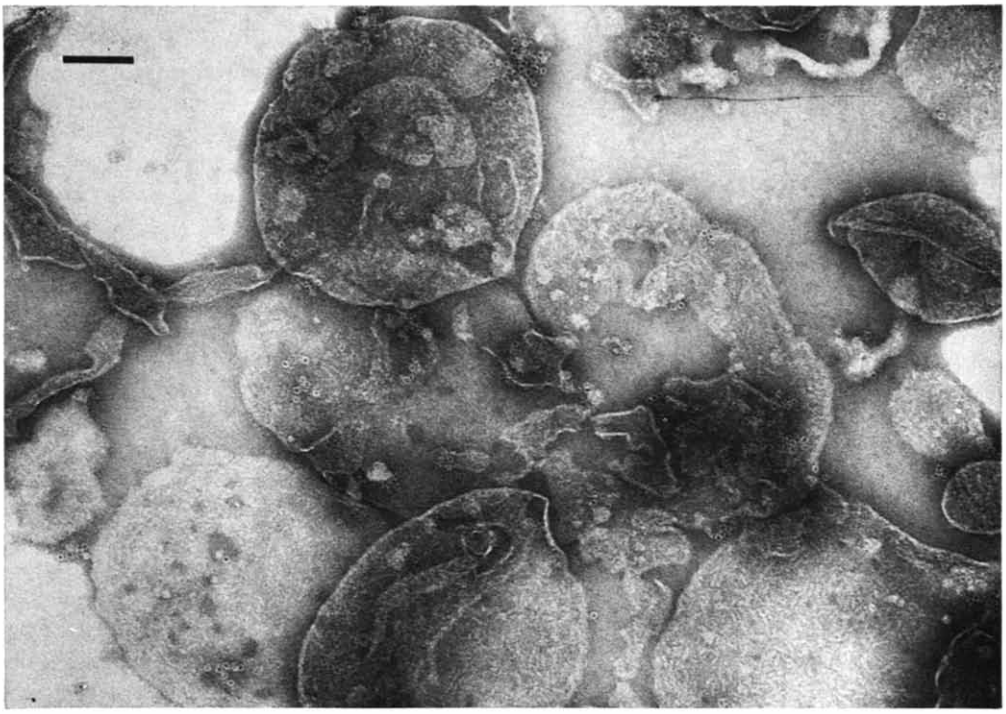

Fig. 18. Electron micrograph of ferritin-labeled membrane subjected to selective release treatment, negatively stained. ×90000. Bar represents 0.1 μm.

various fractions in a shock-wash treatment experiment of unlabeled and ferritin-labeled membranes. As previously observed (Table I) ATPase activity dropped drastically after ferritin labeling. It is interesting to note, however, that corresponding to the decrease in enzymatic activity there is a subsequent increase in the amount of protein associated with the labeled membranes. Such an increase in protein can readily be accounted for by the addition of the ferritin-conjugated antibody to these membranes. After the labeled membranes were exposed to low ionic strength buffer which caused the release of ATPase from untreated membranes, most of the additional protein was observed in the shock-wash fractions. Ferritin which was previously detectable on the membranes was also found to reside almost exclusively in these fractions. Neither the labeled nor the unlabeled membranes exhibited any residual ATPase activity nor were they able to react with anti-ferritin antisera after shock-wash treatment. Dialysis of labeled or unlabeled membranes in normal ionic strength buffer in which the membranes were initially prepared failed to cause the release of any significant amounts of ATPase, protein or ferritin.

Fig. 19 displays immunodiffusion slide assay performed on some of the fractions described in Table II. Figs 19A and 19B, show control reactions of labeled and unlabeled membranes against various antisera (results similar to Figs 14A and 14E discussed earlier). The reactions of the shock-wash fractions from the corresponding membranes can be seen in Figs 19C and 19D. Strong reactions between the labeled membrane shock-wash and anti-globulin, anti-ATPase and anti-ferritin

TABLE II

DISTRIBUTION OF PROTEIN, ATPase AND FERRITIN IN FRACTIONS OBTAINED IN A "SHOCK-WASH" TREATMENT OF UNLABELED AND FERRITIN-LABELED MEMBRANES

See Materials and Methods for procedures used.

Sample	*Protein (mg)*	*ATPase units*	*Ferritin***
Membrane	3.6	+++*	−
Ferritin-labeled membranes	5.1	±*	+++
Shock-wash of membranes I	0.6	47.0	−
Shock-wash of membranes II	0.4	8.8	−
Shock-wash of ferritin-labeled membranes I	1.7	6.6	+++
Shock-wash of ferritin-labeled membranes II	0.65	0.4	±
Shock-washed membranes	2.25	−*	−
Shock-washed ferritin-labeled membranes	2.65	−*	−

* Indicates (+ → + + +) relative amount present or (−) absence of trypsin activated ATPase; owing to complexity of kinetics on trypsin activation exact units not given.

** Indicates (+ → + + + +) relative amount present or (−) absence of ferritin as detected in immunodiffusion assay (see Fig. 19).

antisera are apparent (Fig. 19C). The shock-wash of unlabeled membranes also showed a strong reaction against anti-ATPase (Fig. 19D), a reaction which was undetectable when the ATPase was in the bound membrane state (Fig. 19B). These results suggest that in the unbound, solubilized state, ATPase and certain components of the ATPase-ferritin complex are much more accessible and thus susceptible to immunological detection. Both residual membrane preparations failed to react with any of the antisera (Figs 19E and 19F). The reaction of rabbit anti-ATPase against sheep anti-rabbit γ-globulin observed in all slides were a normal result of the testing system.

Immunoelectrophoresis analysis of some of the fractions described in Table II are seen in Fig. 20. Fig. 20A shows the electrophoretic pattern of the shock-wash fraction from unlabeled membranes when developed against rabbit anti-ATPase (Trough 2) and sheep anti-horse ferritin (Trough 1). A single band migrating towards the anode was observed in reaction against anti-ATPase. There was no visible reaction against the anti-ferritin, indicating no cross-reactivity of this antisera with any component of the shock-wash. Fig. 20B depicts the electrophoretic pattern of the shock-wash fraction from the ferritin-labeled membranes. Single bands, showing little diffusion capabilities, exhibiting very slight anodal migration, were observed in reactions against either anti-ferritin (Trough 1) or anti-ATPase (Trough 2). There was no detectable band in the position observed in Fig. 20A against anti-ATPase, thus indicating a new region of material which reacts with anti-ATPase. Samples of purified ferritin (Fig. 20C, Well 1) and shock-wash from labeled membranes (Well 2) were co-electrophoresed and developed against anti-ferritin antisera, the results can be seen in Fig. 20C. The purified ferritin showed a slight anodal migration and gave a large arching precipitin band, whereas, the shock-wash showed a similar reaction

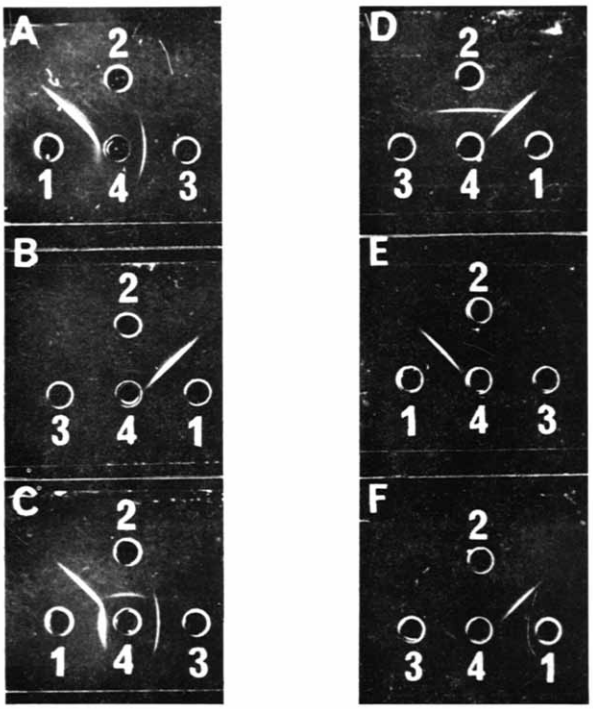

Fig. 19. Immunodiffusion slides of fractions obtained by shock-wash treatment of unlabeled and ferritin-labeled membranes reacted against various antisera. Wells 1, 2 and 3 on each slide contained sheep anti-rabbit γ-globulin, rabbit anti-ATPase γ-globulin, and sheep anti-horse ferritin antisera, respectively. Ferritin-labeled membranes were placed in Well 4 of Slide A; untreated membranes in Well 4 of Slide B; the first shock-wash from ferritin treated membranes in Well 4 of Slide C; the first shock-wash unlabeled membranes in Well 4 of Slide D; ferritin-labeled membranes after shock-wash in Well 4 of Slide E; and unlabeled membranes after shock-wash in Well 4 of Slide F.

to that observed in Fig. 20B. To eliminate the possibility that free ferritin might be reacting with the ATPase in the test system and forming a non-specific complex, purified ferritin and shock-wash from unlabeled membranes were mixed in a single well, electrophoresed, and the gel slide developed against anti-ATPase (Trough 1) antisera and anti-ferritin (Fig. 20D, Trough 2). From the precipitin pattern developed in Fig. 20D it is apparent that both components migrated to their normal positions in the gel without showing evidence of interaction.

DISCUSSION

The identification of specific proteins and glycoproteins in cell membranes is a prerequisite for an understanding of the molecular architecture of these structures. One of the most powerful and specific techniques available for the molecular mapping of membranes is that of ferritin labeling (by conjugation to specific antibody or to sugar-specific lectins such as concanavalin A) and visualization of the reactive sites in the electron microscope. This method has been used for the detection of a variety

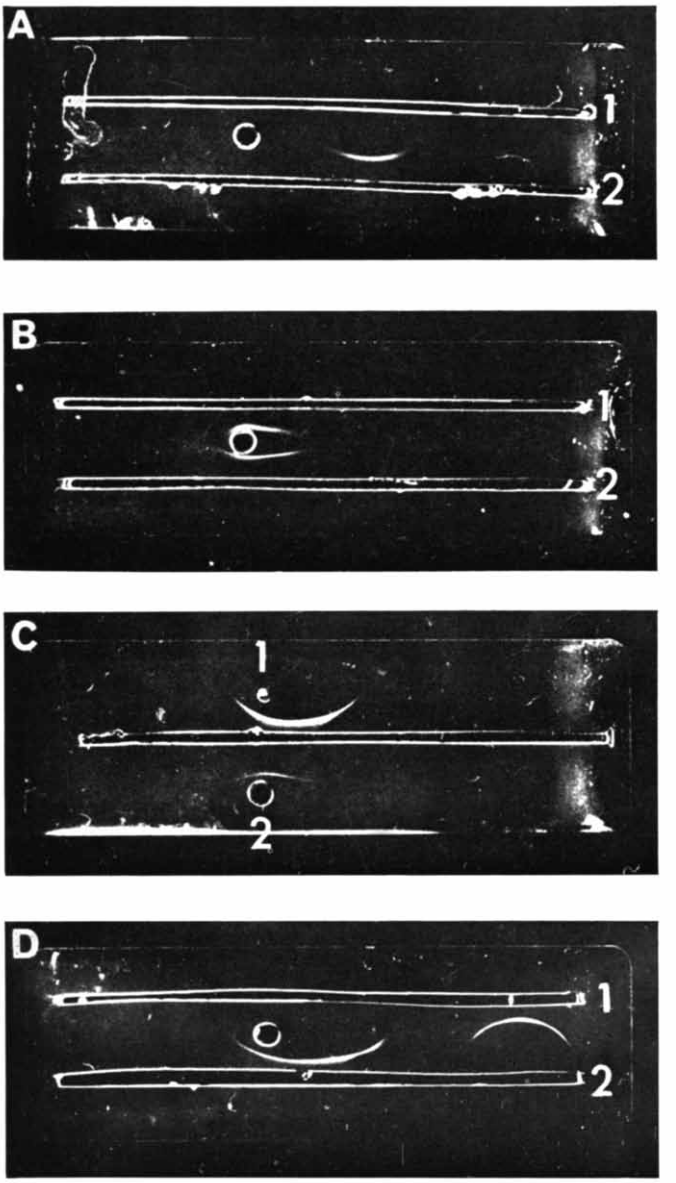

Fig. 20. Immunoelectrophoresis slide tests on fractions obtained by shock-wash treatment of unlabeled and ferritin-labeled membranes reacted against various antisera. (A), Shock-wash I from untreated membranes (well) electrophoresed and reacted against sheep anti-horse antisera ferritin (Trough 1) and rabbit anti-ATPase (Trough 2). (B), Shock-wash I of ferritin-labeled membranes (well) electrophoresed and then reacted against anti-ferritin (Trough 1) and anti-ATPase (Trough 2). (C), Purified, unconjugated ferritin (well 1) and shock-wash I of ferritin-labeled membranes (Well 2), electrophoresed and then reacted against anti-ferritin (trough). (D), Mixture of purified ferritin and shock-wash I from untreated membranes (center well) electrophoresed and then reacted against anti-ATPase (Trough 1) and anti-ferritin (Trough 2). Slides A, B and C were electrophoresed for 90 min while Slide D was run for 120 min.

of protein antigens and surface glycoproteins[21,22,31]. The resolution and specificity of the ferritin–antibody and ferritin–lectin techniques are of a high order and have generally been superior to cytochemical staining methods available for enzyme localization in cells and cell surfaces. Although the asymmetric disposition of the enzyme ATPase in erythrocyte membranes was established by Marchesi and Palade[8] by cytochemical staining with Pb, the method does not reveal the density of specific sites on the inner membrane face.

Our investigations have been concerned with the structure–function relationships of *M. lysodeikticus* membranes and one of the prominent enzymatic activities and proteins in the membrane is a Ca^{2+}-activated ATPase[14]. We have accordingly been interested in establishing the asymmetric disposition of this enzyme and "mapping" its sites on the membrane by means of ferritin-labeled antibody. Previous work from this laboratory has suggested that the ATPase activity is associated with a 100 Å diameter particle composed of six spherical subunits[3] and the current investigations have provided a unique opportunity of confirming that these particles are indeed the site of the membrane ATPase. The distribution of these "ATPase-like" particles on the *M. lysodeikticus* membrane system is apparent from negatively-stained preparations of plasma (Fig. 5) and mesosomal (Fig. 8) membranes and whole protoplasts (Fig. 7) observed under the electron microscope. These particles appear to be evenly distributed on the plasma membrane in the whole protoplast and on the membrane fragments, but are not visible on mesosomal membrane. ATPase activity was found on plasma membrane fragments, but no activity was detectable in the purified mesosome preparations. Moreover, antisera to highly purified ATPase reacted with and inhibited most of the ATPase activity on the membrane fragments, but there was little absorption of such antisera (as ferritin conjugate) to whole protoplasts or mesosome material.

In order to specifically localize the enzyme and to establish that the particles which stud the membrane are in fact ATPase, membrane preparations were treated with ferritin conjugated to the γ-globulin fraction of anti-ATPase (produced either in rabbit or guinea pig). Labeling was deemed successful when after repeated washes of the treated membranes, the ferritin was not released into the supernatant but was still detectable immunologically (Fig. 9) and when ATPase activity was reduced by at least 75% in the reacted membrane suspension as compared to control membranes (Table I). Examination of such labeled membranes under the electron microscope showed a distribution of ferritin particles very similar to the patterns observed for ATPase particles on untreated membranes (Figs 10A and 10B). In some instances ferritin molecules could be seen in direct contact with the "ATPase-like" structures along the membrane periphery. Membranes treated with free ferritin, ferritin conjugated to non-immune rabbit or guinea pig γ-globulin or ferritin conjugated with the specific antisera, but blocked with excess antigen prior to labeling, failed to show significant attachment of ferritin or loss of enzymatic activity after treatment. When membranes were treated with excess specific antisera prior to exposure to conjugated ferritin, labeling was also blocked and little enzymatic activity could be detected on these membranes (Fig. 11). Shock-washed membranes similarly failed to react with conjugated ferritin (Fig. 12).

Earlier studies based on the failure of intact protoplasts to absorb out ATPase antibodies from membrane antisera suggested an internal location of the ATPase[24]

and this result is verified by the ferritin-labeling procedure. From thin section studies it is apparent that the specific ferritin conjugate reacts with only one side of the membrane (Fig. 13B) thus establishing the asymmetric disposition of this enzyme. Protoplasts possessing intact outer membrane surfaces were not significantly labeled (Fig. 16) whereas specific labeling of the ATPase was readily observed upon lysis and washing (Figs 10A and 10B). Our results with the thin sections of ferritin-labeled isolated membrane vesicle fractions (Fig. 13B) are very similar to those reported for cytochemical localization of red cell ghost ATPase by Marchesi and Palade[8] in that the labeling occurs on the "external" surface of some of the closed vesicles, which incidently these authors refer to as the "inner surface". Purified mesosome and sonicated mesosome fractions also failed to react with the specific ferritin conjugate.

In addition to the above evidence attempts to isolate the ferritin–antibody–enzyme complex from labeled membranes have also been successful. When ferritin-labeled membranes were subjected to the same selective release procedure which is used in releasing "ATPase-like" particles and activity from the membranes, ferritin conjugate associated with ATPase molecules were released. As expected from reaction with antibody such complexes were enzymatically inactive (Table II) but gave immunological reactions against each of the components of the complex (*i.e.* ferritin, γ-globulin and ATPase). That ATPase was in fact an integral part of the complex was thus established by immunoelectrophoresis studies (Figs 20A–20D) and electron microscopy (Figs 17B and 17C). All of these results add conclusive support to the identification of these membrane particles as the sites of ATPase activity.

The ability to specifically label a membrane component, determine its position and then isolated it after removal from that membrane therefore offers a valuable new tool in the study of membrane architecture. Such a technique clearly establishes a structure–function relationship and should be applicable to any of a number of membrane proteins which are readily obtainable. Work is continuing in this laboratory to determine the distribution of other specific proteins (*e.g.* succinate dehydrogenase, NADH dehydrogenase, other electron transport components and phospholipid synthesizing enzymes) in the plasma and mesosomal membranes, *in situ* in whole cells, in protoplasts and in isolated membranes and mesosome structures. In this way it is hoped that we shall be able to elucidate and understand the structural organization of this multifunctional membrane system at the molecular level.

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