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# Antibody to Adenosine Triphosphatase from Membranes of *Micrococcus lysodeikticus**

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ABSTRACT: Antisera to $Ca^{2+}$-activated adenosine triphosphatase, isolated and purified from membranes of *Micrococcus lysodeikticus*, were prepared in rabbits and tested by the quantitative precipitin reaction. The antibody was specific for adenosine triphosphatase: it gave a single band against pure or crude enzyme and sonicated membranes; electrophoresis of the enzyme, followed by development with the antiserum gave a single band which had migrated toward the anode. The pooled antiserum contained approximately 0.4 mg of antienzyme/ml of serum. Complete inhibition of adenosine triphosphatase activity, noncompetitive with respect to the substrate, was obtained. Radial immunodiffusion was employed as a quantitative assay for adenosine triphosphatase.

The antibody to the *M. lysodeikticus* enzyme cross-reacted with and partially inhibited the activity of adenosine triphosphatases from membranes of *Sarcina flava*, *Sarcina lutea*, and *Micrococcus varians*. Treatment of the enzyme with 1% sodium dodecyl sulfate or 2.6 M guanidine hydrochloride yielded "subunits" which gave a precipitin line with antisera to membranes, but not with antiserum to adenosine triphosphatase.

Adenosine triphosphatase is a ubiquitous and important component of biological membranes of diverse origins including those of red blood cells (Askari and Rao, 1969), inner mitochondrial membranes (Racker, 1967), and the plasma membranes of bacterial cells (Abrams, 1965; Abrams and Baron, 1967, 1968; Evans, 1969). The enzyme has been identified as a particulate entity of the mitochondrial membrane (Racker and Horstman, 1967) and as an integral part of the red cell membrane (Marchesi and Palade, 1967). However, the identity of the "stalked" particles seen in negatively stained preparations of bacterial membranes (Abrams, 1965; Biryuzova *et al.*, 1964) has not yet been conclusively established, although there is clear-cut evidence that the ATPase[1] activity is associated with a well-defined particle possessing a central unit surrounded by six peripheral subunits (Muñoz *et al.*, 1968a). Furthermore, lead-staining techniques used in localization studies (Voelz and Ortigoza, 1968) yielded little information as to the precise mode of attachment or distribution of the enzyme on the bacterial membrane.

Muñoz *et al.* (1968b, 1969) purified a $Ca^{2+}$-dependent ATPase from the membranes of *Micrococcus lysodeikticus*, and it was identified as a major antigen of the membrane. As the enzyme could be readily purified, it became possible to prepare an antiserum specific for the membrane ATPase. It was our feeling that such an antiserum would provide a valuable reagent for structural studies as well as an aide in defining the properties of the enzyme itself. Moreover, such an antienzyme serum would be useful in recognizing structural and antigenic differences and similarities among ATPase proteins from different bacterial membranes. The present report deals with the preparation and characterization of a specific anti-ATPase serum.

## Experimental Procedures

*Purification of ATPase.* $Ca^{2+}$-activated ATPase was obtained by a "selective release" method (Muñoz *et al.*, 1968b) from membranes of *M. lysodeikticus* (NCTC 2665) and purified by gel filtration on Sephadex G-200 as described by Muñoz

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[1] Abbreviations used in this work are: ATPase, adenosine triphosphatase or ATP phosphohydrolase (EC 3.6.1.3); Gd·HCl, guanidine hydrochloride.

*et al.* (1969). In some experiments ATPase was obtained by a modification of the butanol extraction method of Maddy (1966), developed for the isolated membranes of *M. lysodeikticus* in this laboratory (M. R. J. Salton and M. T. Schor, unpublished data), and purified by a single filtration on Sephadex. ATPases from membranes of *Sarcina flava*, *Sarcina lutea*, *Micrococcus varians* (NCTC 7281), *Sporosarcina ureae* (CCM 981), and *Bacillus subtilis* (strains 42 and 67 kindly provided by Dr. Leonard Mindich) were partially purified by the method of Muñoz *et al.* (1969).

*Preparation of Antisera.* Purified ATPase from membranes of *M. lysodeikticus* was judged homogeneous on the basis of polyacrylamide gel electrophoresis, electron microscopic examination, and agar gel diffusion against antisera to the membrane. The enzyme was incorporated in Freund's incomplete adjuvant (Difco) and injected subcutaneously into young Angora rabbits. Three injections, each containing 0.5 mg of ATPase protein, were given to every rabbit at weekly intervals. Two weeks after the third injection, the rabbits were given a subcutaneous booster shot of 250 $\mu$g of ATPase. The animals were bled by heart puncture 5 days later. After an additional 2 days, they were sacrificed and total bleeding was performed. Sera were separated by standard techniques (Campbell *et al.*, 1964), tested for antibody titer by the precipitin ring test, pooled, and stored in the absence of preservative at $-20°$.

Rabbit antisera to whole and sonicated membranes, prepared as described by Salton (1967), and to sodium deoxycholate insoluble residue (Salton *et al.*, 1968) of *M. lysodeikticus* were obtained by multiple subcutaneous injections of the fractions (5 mg/ml) mixed with equal volumes of Freund's incomplete adjuvant, given biweekly for 4 weeks followed by a subcutaneous booster shot after a week's rest. Blood was collected by heart puncture and sera from weekly bleedings were tested for antibody content, pooled, and stored with 0.01% merthiolate at $-20°$.

*Precipitin Test.* Varying amounts (1–60 $\mu$g) of purified ATPase in 0.03 M Tris-HCl buffer (pH 7.5) were added to a constant volume (50 $\mu$l) of rabbit anti-ATPase. The reaction mixtures were adjusted to a final volume of 0.5 ml with 0.9% sodium chloride solution and incubated at room temperature for 2 hr and then in the cold (4°) for 2 days. After centrifugation, the supernatants were carefully removed and assayed for ATPase activity. The precipitates were washed three times with 0.9% sodium chloride solution and assayed for total nitrogen by the microKjeldahl procedure (Kabat and Mayer, 1967).

*Immunoelectrophoresis* was performed on agar-coated microscope slides according to Campbell *et al.* (1964). Barbital buffer (pH 8.2, ionic strength 0.05) was used. Electrophoresis was carried out for 45 min at 4° with a current of 5 mA/slide. After addition of antiserum, slides were incubated in a moisture chamber at room temperature for 18–24 hr.

*Radial Immunodiffusion Assay.* The method of Mancini *et al.* (1964) was used to develop a quantitative assay for ATPase. Noble agar (1.5%) in borate–saline buffer at pH 8.2 was cooled to about 50° and 3 ml was mixed with 0.5 ml of ATPase antiserum warmed to the same temperature. The mixture was poured onto a clean microscope slide, allowed to harden and, after the addition of antigen to the wells, the slide was developed overnight in a moisture chamber at room temperature. Diameters of the precipitate rings were estimated in a Leitz microscope equipped with an ocular micrometer.

*Double Diffusion in Agar.* Double-diffusion studies were carried out on microscope slides in a moisture chamber at room temperature according to Campbell *et al.* (1964). After overnight incubation, precipitin patterns were photographed in a Cordis immunodiffusion camera.

*Inhibition Experiments.* The ability of the anti-ATPase serum to inhibit liberation of $P_i$ from ATP by ATPase was demonstrated by preincubation of the enzyme for 30 min at 25° with varying amounts of anti-ATPase $\gamma$-globulin (0.1–1.0 mg as protein) prepared by precipitation with ammonium sulfate (Campbell *et al.*, 1964). The effect of substrate on the inhibition reaction was also determined.

To estimate the maximum levels of inhibition of ATPases from different bacterial species by *M. lysodeikticus* anti-ATPase, excess of antibody was used. However, in all cases preliminary titration curves were run to determine the smallest amount of antibody giving maximal inhibition.

*Treatment with Sodium Dodecyl Sulfate and Gd·HCl.* Sodium dodecyl sulfate (Fisher Scientific Co.) or Gd·HCl (Mann Research Laboratories) solutions in 0.03 M Tris-HCl buffer (pH 7.5) were added to the enzyme solution (1 mg of protein/ml) at final concentrations of 1% sodium dodecyl sulfate and 1.3 or 2.6 M Gd·HCl. After 2 hr at room temperature, samples were taken for disc gel electrophoresis, for gel diffusion, and for determinations of activity; the remainder was dialyzed against several changes of the Tris-HCl buffer at 25°.

*Disc Gel Electrophoresis.* Electrophoresis was performed under the standard alkaline conditions (stacking gel at pH 8.9 and separating gel at pH 9.5) described by Salton (1967). Proteins were stained with Amido Black or coomasie blue (Chrambach *et al.*, 1967). The lead acetate method described by Weinbaum and Markman (1966) was used to visualize ATPase activity in gels. Incubation for 5–10 min at 37° usually sufficed, and faintly stained ATPase bands could be cut out from the gels and set in double-diffusion agar plates against the anti-ATPase serum.

*Other Procedures.* ATPase activity was measured as described by Muñoz *et al.* (1968a,b). Protein was determined by the method of Lowry *et al.* (1951).

## Results

The antigenicity of a $Ca^{2+}$-dependent, purified ATPase, isolated by selective release from membranes of *M. lysodeikticus*, was demonstrated by the successful production of antisera in rabbits. The purity of the ATPase used for the inoculations was checked by disc gel electrophoresis (Muñoz *et al.*, 1969; see also Figure 8a), electron microscopy (Figure 1), and agar gel double diffusion against antisera to membranes of *M. lysodeikticus* (Figure 2). The characteristic single precipitin line observed in Ouchterlony plates between the ATPase and the antimembrane serum was also formed when serum absorbed with whole stabilized protoplasts (prepared by Dr. Yoshio Fukui) was used (Figure 2). Since antibodies against surface antigens are expected to be absent from such absorbed serum, the formation of a precipitin line indicates that the ATPase antigen is not accessible to the surface.

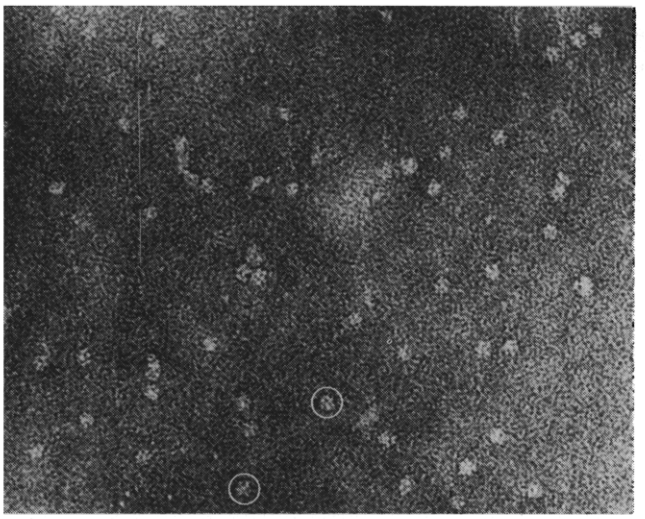

FIGURE 1: Purified ATPase preparation negatively stained with ammonium molybdate as seen in the electron microscope. The enzymatic activity is associated with the "rosettes" which are about 100 Å in diameter and are composed of one central subunit surrounded by six others. ×250,000.

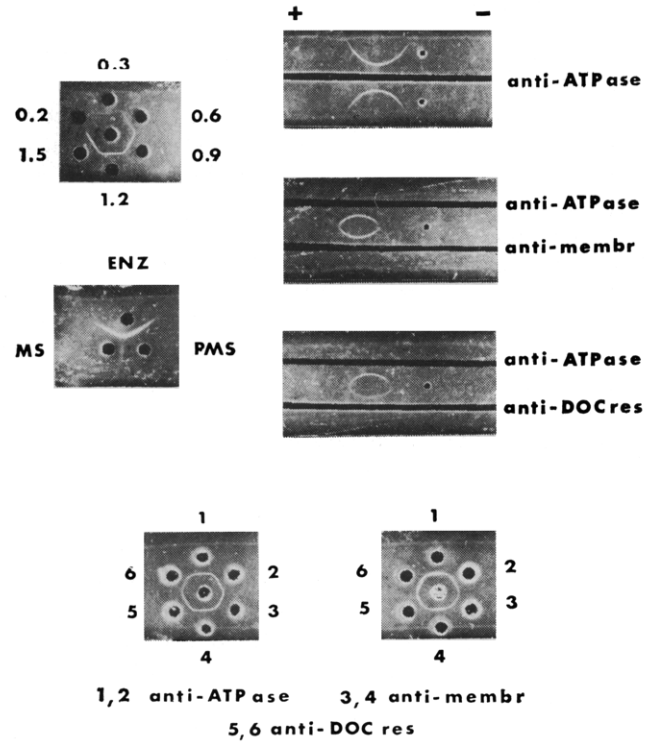


FIGURE 2: Agar double diffusion and immunoelectrophoresis of ATPase. Upper left: increasing amounts (0.3–1.5 μg) of the purified ATPase were placed in the outer wells. The center well contained the anti-ATPase. Lower left: reaction of the ATPase (ENZ) with the anti-membrane serum (MS) and the antimembrane serum absorbed with protoplasts (PMS). Bottom: the right center well contained the upper component of a double ATPase band (see Figure 8a) cut out from a polyacrylamide gel. The left center well contained ATPase eluted from a Sephadex G-200 column. Right: immunoelectrophoresis of the purified ATPase. Each well contained about 2 μg of the ATPase. After electrophoresis at pH 8.2, as described in the text, troughs were filled with antisera and incubated overnight.

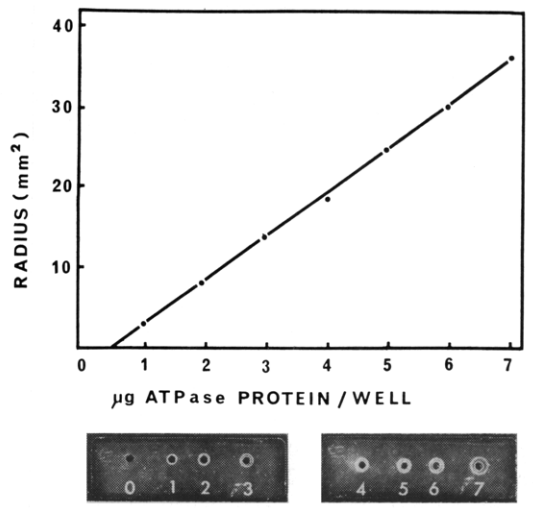


FIGURE 3: Radial immunodiffusion of the purified ATPase. Varying amounts of the ATPase ranging from 0 (control) to 7 μg of protein were placed in the wells. The slides were incubated overnight and the radii of the precipitin rings were measured. The upper part of the figure is a plot of squares of the radii of the precipitin rings against the amount of the enzyme in each well.

*Precipitin Analyses of Enzyme–Antibody Complexes.* Figure 2 demonstrates some of the precipitin reactions of the enzyme with the anti-ATPase, antimembrane, and anti-sodium deoxycholate residue sera. The sodium deoxycholate insoluble residue used for the preparation of anti-sodium deoxycholate residue serum is a lipid-depleted fraction of protein nature containing cytochromes and exhibiting succinic dehydrogenase activity (Salton *et al.*, 1968) and, in some preparations, residual ATPase activity. The ATPase antiserum gave a single precipitation line when tested against the purified or crude enzyme preparation or sonicated membranes. Electrophoresis of the enzyme also resulted in a single band migrating toward the anode upon development with the anti-ATPase, anti-membrane, or anti-sodium deoxycholate residue sera (Figure 2).

The purified ATPase often, but not always, gives a double band after electrophoresis in polyacrylamide gels under standard alkaline conditions (see Figure 8a). The double band may be cut out from a polyacrylamide gel, as described in Experimental Procedures, and reacted with anti-ATPase and/or antimembrane sera in Ouchterlony plates. A single precipitin line was always observed even after prolonged incubation, indicating that the purified ATPase is antigenically homogeneous. Furthermore, when the upper and the lower components of this double band were cut out and separately set against the antisera (as in Figure 2, bottom), they both produced identical precipitin patterns.

Precipitin analysis in agar proved to be a useful quantitative method for assaying ATPase as an antigen. Figure 3 shows the radial precipitin patterns obtained when increasing amounts of the enzyme were allowed to diffuse into the agar containing the anti-ATPase serum. The areas of the precipitin rings were proportional to the amounts of the enzyme placed in the wells, and a plot of the radius$^2$ *vs.* micrograms of the enzyme protein per well resulted in a linear relationship.

Figure 4 shows a characteristic precipitin curve obtained when increasing amounts of the purified ATPase were added

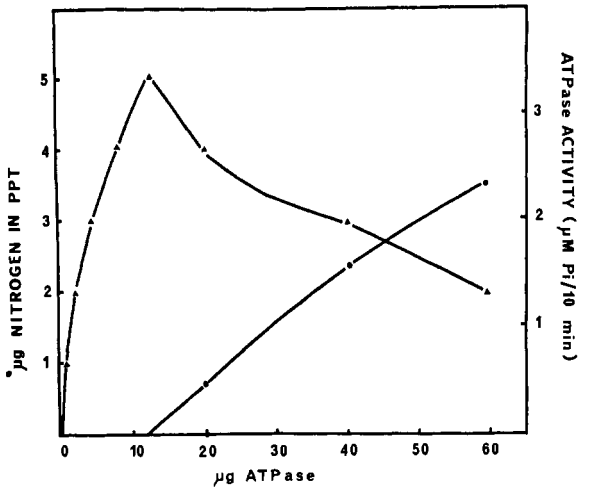


FIGURE 4: Titration of the anti-ATPase γ-globulin with the purified enzyme. Each tube contained 50 µl of the antiserum, increasing amounts of antigen (ATPase), and NaCl to the total volume of 0.5 ml. The reaction mixtures were incubated as described in the text. Total nitrogen determinations were performed on the washed precipitates (triangles). Enzymatic activities were determined on the supernatants (circles).

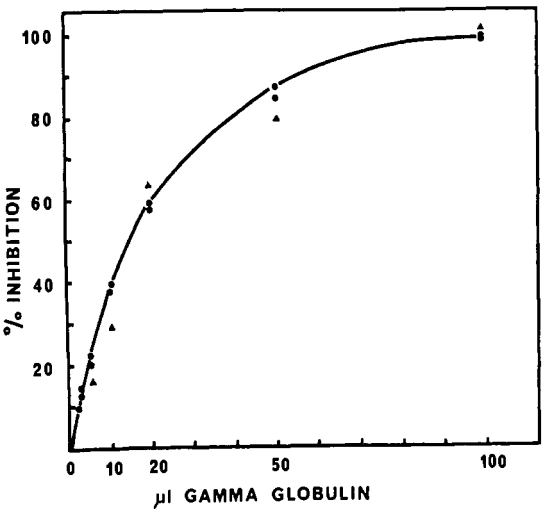


FIGURE 5: Inhibition of ATPase activity by the specific antiserum. Each tube contained 20 µg of the purified enzyme, the anti-ATPase γ-globulin as indicated, and 0.1 M Tris-HCl buffer (pH 7.5) to the total volume of 0.3 ml. The mixtures were preincubated at room temperature for 30 min, and 0.1-ml samples were then taken for the ATPase assay. Normal rabbit γ-globulin was used as a control. In one set of experiments precipitates formed during preincubation were centrifuged, and the activity present in the supernatants was determined. The triangles represent levels of inhibition measured in the supernatants.

to a constant amount of anti-ATPase γ-globulin. The equivalence point is 240 µg of antigen/ml of antibody. Supernatants at the equivalence point showed no ATPase activity, indicating that the added enzyme had been precipitated. If all added antigen nitrogen was also precipitated, the antibody content would be 385 µg of anti-ATPase/ml of antiserum. The supernatants from all the tubes were tested for ATPase activity, and only those on the antigen-excess side of the curve were active.

*Inhibition of Enzymatic Activity by Antibody.* Inhibition of ATPase activity by the specific antiserum is shown in Figure 5. A complete inhibition of the enzymatic activity can be obtained with suitably high concentrations of anti-ATPase γ-globulin. Thus, to inactivate 20 µg of pure ATPase, about 80 µl of the γ-globulin is needed. Time-course experiments indicate that at a given level of enzyme and antibody, the inhibition is complete within 20 min. Figure 5 shows that the levels of inhibition measured in the supernatants after centrifugation of the precipitates were similar to those in a complete reaction mixture. Small differences were due to trapping of enzyme in the precipitate during centrifugation.

To determine whether the inhibition of ATPase by the antibody was competitive or noncompetitive with respect to the substrate, suitable volumes of enzyme solution and antibody were preincubated for 30 min to give about 50% inhibition of activity. Samples were then added to increasing concentrations of the substrate to measure ATPase activity. As seen in Figure 6A, in the preincubation experiment the anti-ATPase acted as a noncompetitive inhibitor as measured in the first 10 min of the reaction with the substrate. To make sure that this was not caused by the slowness of the displacement of the inhibitor (the antibody) by the substrate, a second experiment was set up in which the substrate, antibody, and enzyme were added to the reaction mixture at the same time. Figure 6B demonstrates that again the curves were noncompetitive. The anti-ATPase appeared to be a less effective inhibitor when combining with ATPase in the presence of the substrate. Such substrate protection has been demonstrated for a number of enzyme–antienzyme systems (Cinader, 1963).

*Effect of the Antibody on Other Bacterial ATPases.* The bacterial ATPases isolated from different species were tested for cross-reactions with the antiserum against *M. lysodeikticus* ATPase. A selective release method described by Muñoz *et al.* (1968b) was used for their isolation. Figure 7 demonstrates that purified ATPases of *S. lutea*, *S. flava*, and *M. varians* did indeed cross-react with the antiserum to *M. lysodeikticus* ATPase, while those of *Sporosarcina ureae* and *B. subtilis* did not cross-react.

Different degrees of cross-reaction were observed among the bacterial ATPases. Table I shows that the activities of the cross-reacting ATPases were inhibited by the antiserum, although there was a wide variation in the degree of inhibition by excess of the antibody. Purified and crude enzyme preparations gave essentially the same results. The ATPases from *S. flava* and *S. lutea* gave a line of complete identity, without spur development, even after a prolonged incubation (Figure 7). The ATPase from *M. varians* showed a very weak precipitin reaction in agar gel, with a distinctive spur after 18 hr. When the enzymes from all three species were purified, electrophoresed in agar gel, and reacted with the antiserum to *M. lysodeikticus* ATPase, all gave a characteristic single band after 24-hr incubation and their mobilities were similar to that of *M. lysodeikticus* ATPase, except that *M. varians* enzyme had a slightly lower mobility.

*Effect of Dissociation on Reaction with Antisera.* Muñoz *et al.* (1969) reported that dissociation of the ATPase molecule into 3.5S subunits occurred upon treatment with 1% sodium dodecyl sulfate. Sodium dodecyl sulfate dissociated ATPase is not only inactive enzymatically but is also incapable of

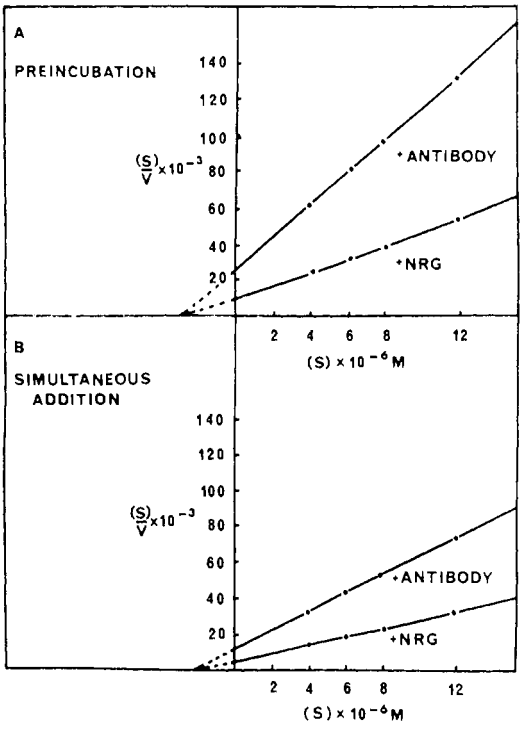


FIGURE 6: Inhibition of the ATPase activity by the specific antiserum in the presence of the substrate. In experiment A (preincubation with the antibody), 0.1 ml of the enzyme solution was incubated with 0.1 ml of the anti-ATPase $\gamma$-globulin for 30 min at room temperature. The concentrations of the enzyme and the antiserum were so adjusted as to give a final inhibition of about 50%. After preincubation, ATP and $Ca^{2+}$ (in a 1:1 ratio) were added to each tube, and ATPase activity was measured. The concentration of the substrate varied from 4 to 12 $\mu$M. In experiment B, the enzyme, the antibody, and the substrate were added to each tube at the same time. After the addition of the required cation ($Ca^{2+}$), the tubes were incubated for 10 min at 37° and assayed for ATPase. Normal rabbit $\gamma$-globulin (NRG) was used as control for both experiments.

TABLE I: Inhibition of ATPase from Different Bacteria by Antiserum to *M. lysodeikticus* ATPase.[a]

| Source of Enzyme | Inhibn by Excess Antibody (%) |
|---|---|
| *M. lysodeikticus* | 100 |
| *S. flava* | 87 |
| *S. lutea* | 75 |
| *M. varians* | 25 |
| *S. ureae* | 0 |
| *B. subtilis* | 0 |

[a] A constant volume (50 $\mu$l) of undiluted antiserum (globulin fraction) of *M. lysodeikticus* ATPase was added to a constant volume (100 $\mu$l) of the enzyme tested. The mixture was incubated for 30 min at 25°, and then immediately after addition of the substrate and $CaCl_2$, the ATPase activity was assayed as described in Experimental Procedures. To measure normal enzyme activity, samples of each enzyme solution were incubated with a diluent instead of an antibody. As a control, samples of each enzyme solution were also incubated with normal rabbit globulin at concentrations equivalent to those of the antibody.

reacting with anti-ATPase or with antiserum to whole membranes in agar gel even after prolonged incubation. Neither enzymatic activity nor ability to react with the anti-ATPase was restored by removal of sodium dodecyl sulfate by dialysis.

Dissociation of the enzyme with Gd·HCl at concentrations higher than 1.3 M yielded products similar to those obtained with sodium dodecyl sulfate, with respect to migration in acrylamide gel, response to antisera, and loss of activity. No loss of antigenicity and only 60% reduction in total enzymatic activity was observed when the enzyme was treated with 1.3 M Gd·HCl for 2 hr at room temperature. On examination by polyacrylamide gel electrophoresis a major faster moving component was found (Figure 8) but no such band was detectable when the supernatant solution from ATPase reacted with antiserum was treated with Gd·HCl (2 hr at room temperature). This suggests that the dissociation product is indeed derived from ATPase. This dissociation was reversible because dialysis restored both the enzymatic activity and the electrophoretic mobility typical for the untreated enzyme (Figure 8).

## Discussion

Antigenic analysis of individual protein components of a biological membrane is difficult as it involves their solubilization, preferably without denaturation or conformational changes. In such studies it is obviously a great advantage to be able to deal with a protein which is not only a major membrane component (and also a major antigen) but is also a "functional" protein so that its release from a membrane and its purification can be monitored in terms of its enzymatic activity. $Ca^{2+}$-dependent ATPase obtained in soluble form from membranes of *M. lysodeikticus* was antigenic in rabbits. The solubilized enzyme was antigenically homogeneous, and the rabbit antibody was specific for ATPase. In contrast, purified mitochondrial ATPase from baker's yeast was not immunologically homogeneous when reacted with antiserum prepared in rabbits by Schatz *et al.* (1967). Homogeneity and specificity are absolute essentials for an antiserum that is to be used in ferritin labeling of enzymes for their localization on cell membranes. We prepared our antiserum to ATPase with this end in mind.

Radial immunodiffusion, initially developed to assay single serum proteins (Mancini *et al.*, 1964) without using long and laborious purification procedures, proved to be a sensitive and reliable test for ATPase. Such a method does not depend on substrate utilization or product formation, but requires an antiserum specific for the enzyme being studied. Since our antiserum was of this character, it provided us with a simple and convenient method of detecting very small amounts of the enzyme in membrane washes, column eluates, and solutions of purified enzyme.

A survey of the literature shows that some enzymes, notably alkaline phosphatase (Schlamowitz, 1954), are not inhibited by their antibodies. Others, like acetylcholinesterase from the electric organs of eels (Williams, 1969), are inhibited partially only, while those acting on substrates with high molecular weights, such as ribonucleases or lysozymes (Cinader, 1967) are completely inhibited. Antiserum to ATPase from membranes of *M. lysodeikticus* caused a 100% inhibition of enzymatic activity, an observation not unex-

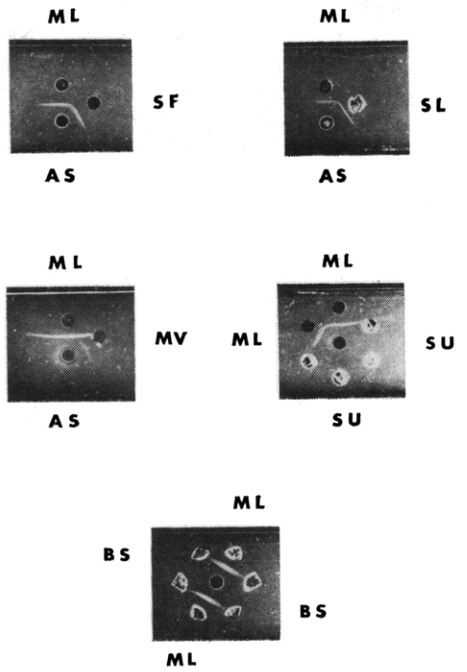


FIGURE 7: Reaction of the ATPases from various bacterial membranes with the *M. lysodeikticus* antiserum. The anti-ATPase serum ("AS" or in center wells) was reacted in double-diffusion agar plates against the ATPases obtained from membranes of *S. flava* (SF), *S. lutea* (SL), *M. varians* (MV), *S. ureae* (SU), *B. subtilis* (BS), and, with the ATPase from *M. lysodeikticus* (ML) for the homologous system.

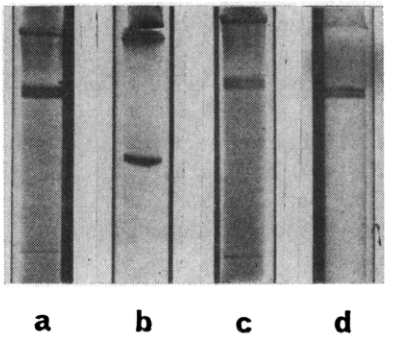


FIGURE 8: Disc gel electrophoresis of the ATPase before and after treatment with 1.3 M Gd·HCl. Electrophoresis was performed under the standard conditions as described in the text. (a) Control enzyme, (b) treated ATPase, (c) treated ATPase after dialysis against Tris-HCl buffer, and (d) enzymatic stain of c.

pected, as ATPases from other sources (beef heart and yeast mitochondria, spinach chloroplasts) were also inhibited by their homologous antisera (Schatz *et al.*, 1967). The discovery that the membrane ATPases from several different bacterial species were inhibited by the antiserum to ATPase from *M. lysodeikticus* is of interest.

Cross-reaction and inhibition of ATPase from different bacterial membranes by an antiserum specific to ATPase from *M. lysodeikticus* provide us with a remarkably sensitive means of studying the relationships between the enzymes and the different species. The continuity of the precipitin bands for ATPases from *M. lysodeikticus*, *S. flava*, and *S. lutea* (all closely "related" species), and their apparently identical electrophoretic mobilities indicated that the sizes and surface charges of these enzymes were very similar, if not the same. In contrast, the ATPase of *M. varians* had a slightly different electrophoretic mobility and gave only partial cross-reaction with the antiserum, while ATPases from unrelated species (*Sporosarcina ureae* and *Bacillus subtilis*) did not cross-react at all. Cross-reactivity among enzymes of similar biochemical specificity from different organisms is not frequently observed. Pollock (1963) found no cross-reactions between *B. subtilis* and *B. cereus* penicillinases and antisera to enzymes of the other species. In higher organisms, Williams (1969) reported that antiserum to eel acetylcholinesterase did not cross-react or inhibit the activity of horse serum or bovine erythrocyte acetylcholinesterase. On the other hand, the antibody to pure rabbit liver fructose 1,6-diphosphatase inhibited both the liver and the kidney enzyme, but not the muscle enzyme (Enser *et al.*, 1969).

The inhibition of an enzyme by the antiserum specific to it could involve either the catalytic site or the sites adjacent to it. Alternatively, the antibody might cause a conformational change at the active site, or inhibition might be due to antibody–antigen complexes blocking access to this site (Cinader, 1963, 1967). We have attempted to find out which of these mechanisms may be responsible for the inhibition of ATPase by its antiserum. The kinetics of the inhibition indicated that it was of a noncompetitive type, and the degree of inhibition caused by a given amount of the antibody was independent of the substrate concentration. It appeared, then, that the antiserum was not affecting the active center but was either blocking access to it or acting on an antigenic site or sites adjacent to it.

Dissociating agents such as sodium dodecyl sulfate or Gd·HCl have been used in studies of subunit structure of purified enzymes. In many cases, however, dissociation into subunits may result in a loss of enzymatic activity (Rottem and Razin, 1966) or antigenicity, or both. Our experiments on the dissociation of the ATPase with 1.3 M Gd·HCl have shown that it is reversible. On the other hand, dissociation with sodium dodecyl sulfate or with 2.6 M Gd·HCl results in enzymatically inactive "subunits" which do not react with the anti-ATPase serum but which do give a precipitin line with the antiserum to sonicated membranes. It is conceivable that a "masked" subunit exists in the ATPase and that this can be uncovered by sonication or dissociation with sodium dodecyl sulfate or Gd·HCl. Further work is under way in this laboratory to determine the nature of the subunit structure or structures of this enzyme.

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# Change in Conformation of the Rabbit γG-Immunoglobulin Molecule with Various Chemical Treatments*

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ABSTRACT: Changes in the three-dimensional structure of rabbit γG-immunoglobulin and rabbit anti-lactoside antibody have been studied using the technique of differential sedimentation. The molecules were subjected to reduction, reduction and alkylation, and pH change. The sedimentation coefficient of the anti-lactoside antibody was about 0.3 S smaller than that of the nonspecific γG-immunoglobulin. However, both molecules showed the same behavior when subjected to reduction, and to reduction and alkylation. Reduction caused a *decrease* in sedimentation coefficient, corresponding to an increase in frictional coefficient, whereas reduction and alkylation caused an *increase* in sedimentation coefficient, corresponding to a decrease in frictional coefficient. Change of pH, away from neutrality, caused a decrease in the sedimentation coefficient of γG-immunoglobulin. A model for γG-immunoglobulin structure, based on a flexible Y, was proposed to explain the observed changes in frictional coefficient. It was found that the frictional coefficient of the γG-immunoglobulin molecule increases as the angle between the arms of the Y increases.

The three-dimensional structure of the γG-immunoglobulin molecule is a problem which still eludes biochemists. It has been suggested (Noelken *et al.*, 1965) that the molecule is Y shaped, consisting of three compact globularr egions (the two Fab pieces and the Fc piece) connected by somewhat flexible regions. This model is supported by the hydrodynamic data which give a frictional coefficient ratio of 1.47 for the entire IgG molecule, but 1.24 for the Fab piece and 1.21 for the Fc piece (Noelken *et al.*, 1965). Also, the intrinsic viscosity is 6–8 cc/g (Jirgensons, 1962) which is higher than one would predict for a typical globular protein. This model is also supported by various fluorescence depolarization studies (Chowdhury and Johnson, 1961; Steiner and Edelhock, 1962) which show that the rotational relaxation time of a fluorescent dye attached to the IgG molecule is smaller than that predicted for a compact sphere, thus indicating that the

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