JOURNAL OF BACTERIOLOGY, Sept., 1966
Vol. 92, No. 3

Adenosine Triphosphatase Activity of Mycoplasma Membranes[1]

SHLOMO ROTTEM AND SHMUEL RAZIN
Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

Received for publication 18 April 1966

ABSTRACT

ROTTEM, SHLOMO (Hebrew University, Jerusalem, Israel), AND SHMUEL RAZIN. Adenosine triphosphatase activity of mycoplasma membranes. J. Bacteriol. **92**:714–722. 1966.—Adenosine triphosphatase activity of *Mycoplasma laidlawii*, *M. gallisepticum*, and *Mycoplasma* sp. strain 14 was confined to the cell membrane. The enzymatic activity was dependent on magnesium, but was not activated by sodium and potassium. Ouabain did not inhibit the adenosine triphosphatase activity of the mycoplasmas, and did not interfere with the active accumulation of potassium by *M. laidlawii* cells. Sulfhydryl-blocking reagents and fluoride inhibited the enzymatic activity, whereas 2,4-dinitrophenol was without any effect. Membranes of *M. laidlawii* hydrolyzed other nucleotide triphosphates and adenosine diphosphate (ADP), but at a lower rate than adenosine triphosphate (ATP). Nucleoside-2′-(3′)-phosphates, ribose-5-phosphate, glucose-6-phosphate, and pyrophosphate were not hydrolyzed by the membrane preparations. It seems that the enzyme(s) involved in ATP hydrolysis by *M. laidlawii* membranes is strongly bound to the membrane subunits, which would account for the failure to purify the enzyme by protein fractionation techniques. The adenosine triphosphatase activity of mycoplasma membranes resembles in its properties that of similar enzymes studied in bacteria. The mycoplasma enzyme(s) seems to differ from the adenosine triphosphatase associated with ion transport in mammalian cell membranes and from mitochondrial adenosine triphosphatase.

Adenosine triphosphatase activity has been detected in all *Mycoplasma* strains tested so far (13). Cell fractionation procedures (14) and cytochemical techniques (H. Maniloff, H. J. Morowitz, R. J. Barrnett, and M. Munkres, Abstr., 9th Annual Meeting Biophys. Soc., San Francisco, 1965) have shown this enzymatic activity to be confined to the cell membrane of mycoplasmas. Enzymes hydrolyzing adenosine triphosphate (ATP) are ubiquitous, and are usually associated with biological membranes. These enzymes have been implicated in muscular and membrane contraction, active transport of ions, oxidative phosphorylation, and photosynthesis (3, 9, 10, 16). The transport of sodium and potassium through animal cell membranes is mediated by a $(Na^+ + K^+)$-activated adenosine triphosphatase enzyme system located in the membrane (23). This enzyme system has hitherto not been described in bacteria. Mycoplasmas differ from bacteria in lacking a rigid cell wall. The presence of cholesterol in mycoplasma membranes relates them to animal cell membranes (18). It was therefore of interest to compare the properties of the adenosine triphosphatase of mycoplasmas with those of animal cells and bacteria.

[1] Taken in part from a dissertation to be submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree from the Hebrew University, Jerusalem, Israel.

MATERIALS AND METHODS

Organisms. M. laidlawii (oral strain) was isolated in our laboratory from the human oral cavity. This strain was serologically related to *M. laidlawii* strain A (M. Argaman, *unpublished data*). *M. gallisepticum* strain A5969 was obtained from M. E. Tourtellotte (Department of Animal Diseases, University of Connecticut, Storrs). *Mycoplasma* sp. strain 14 (goat strain), was obtained from H. E. Adler (School of Veterinary Medicine, University of California, Davis). Most of the work to be described was done with *M. laidlawii*.

Media and growth conditions. Cultures were grown statically in 6-liter Erlenmeyer flasks containing 3 liters of a modified Edward medium (19). The or-

ganisms were harvested after 18 to 24 hr of incubation at 37 C by centrifugation at 13,000 × *g* for 10 min. The sedimented cells were washed twice in β buffer containing 0.15 M NaCl, 0.05 M tris(hydroxymethyl)-aminomethane (Tris), and 0.01 M β-mercaptoethanol, in deionized water adjusted to *p*H 7.4 with HCl (13).

Preparation of cellular fractions. Cells were disrupted by osmotic lysis (13, 19), or by ultrasonic treatment. For osmotic lysis, the washed sedimented cells (about 0.5 to 1.5 g, wet weight) were resuspended in 30 to 50 ml of β buffer diluted 1:20 in deionized water, and were incubated at 37 C for 15 min. The treated suspensions were centrifuged at 8,000 × *g* for 5 min to remove clumps of unbroken cells. The supernatant fraction was carefully decanted and centrifuged for 30 min at 34,000 × *g* to collect membranes. The membranes were washed twice and resuspended in 1:20 β buffer. Disruption of cells by ultrasonic treatment was carried out in an MSE ultrasonic disintegrator (60 w). A 7-ml amount of a heavy suspension of the washed organisms in 0.25 M NaCl was treated at 1.5 amp for 3 min. The suspension was then centrifuged at 34,000 × *g* for 30 min, and the supernatant fluid was separated from the sediment. The sediment was washed twice in β buffer. For testing cation requirements of adenosine triphosphatase activity, the membrane preparation was dialyzed against 6 liters of deionized water. Dialysis was carried out at 4 C for 24 hr, with constant stirring and two medium replacements. The amount of protein in cell fractions was determined according to Lowry et al. (11) with crystalline bovine albumin as standard. All cell fractions were kept at −20 C until used.

Enzymatic digestion of membranes. Membranes (3 mg, dry weight) suspended in 0.05 M Tris-maleate buffer (*p*H 7.6) were treated for 30 min at 37 C with 0.1 mg of pancreatic lipase, papain, trypsin, or chymotrypsin. Cysteine (2×10^{-3} M) and ethylenediaminetetraacetic acid (10^{-2} M) were in the reaction mixture containing papain. The reaction mixtures were centrifuged at 34,000 × *g* for 30 min, and adenosine triphosphatase activity was determined in the sediment and the supernatant fluid.

Disaggregation of cell membranes. Membranes of *M. laidlawii* were disaggregated by detergents or by ultrasonic treatment. For disaggregation by detergents, the methods described previously (21) were followed. Increasing amounts of sodium lauryl sulfate or sodium deoxycholate were added to membrane suspensions. The degree of membrane disaggregation was estimated by measuring the decrease in optical density of the membrane suspension at 400 mμ. The disaggregated membrane preparation was then centrifuged at 34,000 × *g* for 30 min, and the supernatant fluid was separated from the sediment. Adenosine triphosphatase activity was determined in the disaggregated membrane preparation before centrifugation, and in the supernatant fluid and sediment obtained after centrifugation. For disaggregation of membranes by ultrasound, 7 ml of membrane suspension, containing about 35 mg of membrane protein, was treated at 1.5 amp for 10 min. The treated material was then centrifuged at 100,000 × *g* for 45 min, and the supernatant fluid was separated from the sediment. This fluid was centrifuged at 59,780 rev/min in a Spinco model E analytical ultracentrifuge at 11 C. Sedimentation peaks were observed by use of Schlieren optics. Sedimentation constants were calculated without correcting for medium viscosity or density.

Reaggregation of membrane material was carried out according to Razin, Morowitz, and Terry (21). The supernatant fluid obtained after centrifugation at 100,000 × *g* of membranes disaggregated by ultrasonic treatment was dialyzed in the cold for 2 to 3 days against 1:20 β buffer containing 0.01 M $MgCl_2$. The reaggregated membrane material was collected by centrifugation at 34,000 × *g* for 60 min and resuspended in 1:20 β buffer.

Determination of adenosine triphosphatase activity. Activity was measured by the release of inorganic phosphorus in reaction mixtures incubated at 37 C for 15 min and 30 min. The reaction mixtures (1.0 ml) contained 0.3 to 0.5 mg of cell protein, and the following (in micromoles): $MgCl_2$, 2; Tris-HCl (*p*H 7.6), 50; ATP, 2. The reaction was stopped by addition of 1.0 ml of cold 10% trichloroacetic acid to each test tube. The test tubes were immediately centrifuged at 5,000 × *g*, and the amount of inorganic phosphorus in the deproteinized supernatant fluid was determined according to Fiske and SubbaRow (5). Results were expressed as micromoles of inorganic phosphorus released per milligram of cell protein in 30 min. The rate of ATP hydrolysis by the membranes was found to be constant during the first 30 min of incubation.

Degradation products of ATP were identified by paper electrophoresis (22). Samples of 10 μliters were applied to Whatman no. 1 paper strips (34 by 3 cm). Electrophoresis was carried out in 0.05 M ammonium acetate-acetic acid buffer (*p*H 3.5) with the use of a potential gradient of 20 v/cm for 3 hr, or 5 v/cm for 16 hr. The paper strips were then air-dried, and examined under ultraviolet light. The nucleotide spots were identified by comparison with known samples.

Accumulation of potassium by mycoplasma cells. M. laidlawii was grown in Edward medium from which K_2HPO_4 was omitted. The organisms were harvested after 18 to 20 hr of incubation at 37 C, washed twice, and resuspended in the partially defined medium of Razin and Cohen (20) modified by the omission of KCl. To deplete the cells of potassium, the suspension was incubated for 6 hr at 37 C in a water-bath shaker. At 2-hr intervals the cells were sedimented by centrifugation, and the medium was replaced. After this preincubation period, potassium was added, and incubation continued for another 3 hr. Samples (5 ml) were withdrawn at various time intervals and centrifuged immediately at 9,000 × *g* for 15 min in the cold. The supernatant fluids were separated, and the cell pellets were washed in the cold with 10 ml of the modified partially defined medium. The resulting pellets were then extracted by boiling with 5 ml of 0.1 N HCl for 30 min. The cell residue was removed by centrifugation, and the sodium and potassium content of the supernatant fluid was estimated with a flame photometer (model KY Baird-Atomic Inc., Cambridge, Mass.). Potassium accumulation by human erythrocytes was tested according to Post et al.

(15). The erythrocytes were depleted of potassium by preincubation in a potassium-free medium at 4 C. Potassium accumulation was tested as described above for *Mycoplasma*.

RESULTS

The three *Mycoplasma* species tested showed adenosine triphosphatase activity localized in the cell membrane, confirming previous results (13). Adenosine triphosphatase activity of *M. laidlawii* membranes was highest when the cells were harvested at the early logarithmic phase of growth (Fig. 1). Optimal temperature for activity was 37 C. No sharp *p*H optimum was noted; activity was more or less constant at *p*H values ranging from 6.7 to 8.4 in 0.05 M Tris-maleate buffer, declining steeply at *p*H values below 6.7 and above 8.4.

Cation requirements. Magnesium was found to be essential for adenosine triphosphatase activity of membranes of the mycoplasma studied. This requirement could be partially met by manganese or cobalt; calcium was much less effective as a magnesium substitute (Table 1). Addition to magnesium-containing reaction mixtures of equivalent amounts of several divalent cations decreased or inhibited adenosine triphosphatase activity. Ba^{++}, Ca^{++}, or Cu^{++} decreased the activity by about 50%, and Zn^{++} inhibited the activity almost completely. The concentration of magnesium required for optimal activity depended on the ATP concentration; highest activity was demonstrated at an ATP-Mg^{++} molar ratio of 1.

TABLE 1. *Activation of adenosine triphosphatase of Mycoplasma laidlawii membranes by divalent cations*

Cation (0.02 M)	Specific activity[a]	Per cent activity[b]
Mg^{++}	4.50	100
Mn^{++}	3.35	75
Co^{++}	2.82	63
Fe^{++}	2.08	46
Ca^{++}	1.25	28
Ba^{++}	0.80	17
Zn^{++}	0.55	12
Sn^{++}	0.34	7
Cu^{++}	0.25	6
Pb^{++}	0.20	4
None	0.26	6

[a] Expressed as micromoles of inorganic phosphorus released per milligram of protein per 30 min.

[b] As compared to activity with Mg^{++}.

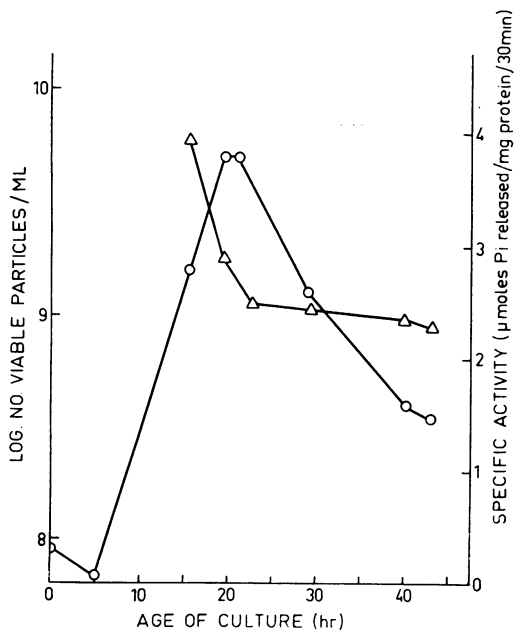

FIG. 1. *Adenosine triphosphatase activity of Mycoplasma laidlawii membranes harvested at various growth phases. Symbols: logarithm of number of viable particles per milliliter of medium, ○; adenosine triphosphatase activity, △.*

The monovalent cations sodium, potassium, and ammonium had no stimulating effect on the adenosine triphosphatase activity of the three *Mycoplasma* strains when tested alone, or in various combinations, at concentrations ranging from 5 to 100 μmoles/ml. Sodium decreased the enzymatic activity at a concentration of 100 μmoles/ml.

Hydrolysis of nucleotides other than ATP. Membranes of *M. laidlawii* hydrolyzed several nucleoside triphosphates at a slower rate than ATP. Adenosine diphosphate (ADP) was hydrolyzed at close to 30% the rate of ATP. The membrane preparation hydrolyzed adenosine-5′-monophosphate (5-AMP) at a very slow rate, and none of the nucleoside-2′-(3′)-phosphates tested was attacked (Table 2). Paper electrophoresis of the products accumulating in the reaction mixture after hydrolysis of ATP by *M. laidlawii* membranes showed the presence of ADP, 5-AMP, and small amounts of adenosine. Pyrophosphate, ribose-5-phosphate, and glucose-6-phosphate were not hydrolyzed by the membrane preparation.

Addition of the hydrolyzable nucleotides to a reaction mixture containing an excess of ATP did not increase the amount of inorganic phosphorus released, indicating that the same enzyme hydrolyzes all the different nucleotides. Lineweaver-Burk plots for ATP hydroysis gave K_m

TABLE 2. *Hydrolysis of various nucleotides by membranes of Mycoplasma laidlawii*

Nucleotide (2 μmoles)	Specific activity[a]	Per cent activity[b]
Adenosine triphosphate	5.62	100
Adenosine diphosphate	1.69	30
Adenosine-5'-monophosphate	0.89	16
Adenosine-2'(3')-monophosphate	0	0
Guanosine triphosphate	3.14	55
Guanosine-2'(3')-monophosphate	0	0
Cytidine triphosphate	2.54	45
Cytidine-2'(3')-monophosphate	0	0
Uridine triphosphate	2.43	43
Uridine-2'(3')-monophosphate	0	0

[a] Expressed as micromoles of inorganic phosphorous released per milligram of protein per 30 min.

[b] As compared to activity with adenosine triphosphate.

values of 2×10^{-2} M and of V of 15 μmoles of inorganic phosphorus released per mg of membrane protein per 30 min.

Physical factors affecting enzymatic activity. Heating of *M. laidlawii* membranes at 47 C for 10 min did not reduce their adenosine triphosphatase activity; heating at 65 C for 10 min completely abolished all activity. Enzymatic activity was, however, not affected by 10 cycles of alternate freezing and thawing, or by freeze-drying of the membranes. Storage of the membranes suspended in deionized water at −20 C for 7 days reduced their adenosine triphosphatase activity by about 40%; when suspended in β buffer under the same conditions, they lost only 20% of their initial activity. Inactivation of the enzymatic activity was more rapid on storage of the membranes at 4 or 37 C.

As shown in Fig. 2, the adenosine triphosphatase activity of *M. laidlawii* membranes was relatively stable to ultrasonic treatment. Treatment of the membranes for 25 min decreased the enzymatic activity by 50%. Some protection against enzyme inactivation was afforded by the incorporation of 10^{-2} M glutathione in the medium in which the membranes were disrupted (Fig. 2).

Inhibitors of the adenosine triphosphatase activity. Sulfhydryl-blocking reagents inhibited the adenosine triphosphatase activity of *M. laidlawii* membranes (Table 3). A similar degree of inhibition was demonstrated with membranes of *M. gallisepticum* and the goat mycoplasma. *p*-Chloromercuribenzoate at a concentration of 4×10^{-3} M inhibited hydrolysis of ATP, guanosine triphosphate (GTP), cytidine triphosphate (CTP), ADP, and 5-AMP to about the same degree (60 to 75% inhibition). The adenosine triphosphatase activity of the three mycoplasmas was equally inhibited by sodium fluoride. 2,4-Dinitrophenol, known to stimulate mitochondrial adenosine triphosphatase (16), had no significant effect on the adenosine triphosphatase activity of the three *Mycoplasma* strains, in concentrations up to 5×10^{-3} M. Potassium cyanide (10^{-3} to 10^{-1} M), sodium azide (10^{-2} M), sodium nitrite (10^{-3} to 10^{-1} M), sodium deoxycholate (2×10^{-3} M), sodium taurocholate (2×10^{-3} M), thallium acetate (5×10^{-3} M), and diisopropyl fluorophosphate (10^{-3} M) did not affect the adenosine triphosphatase activity of the mycoplasmas.

The inhibitory effect of *N*-ethylmaleimide (NEM) and 2,4-dinitrofluorobenzene (DNFB) was markedly diminished when ATP was simultaneously added to the reaction mixture. No such effect was noticed with *p*-chloromercuribenzoate (PCMB). Addition of β mercaptoethanol to the reaction mixture almost completely abolished the inhibitory effect of PCMB, but only partially counteracted the inhibition caused by NEM and DNFB (Table 4).

Ouabain (*g*-strophanthin) did not inhibit adenosine triphosphatase activity of *M. laidlawii* membranes (Fig. 3). An experiment run simultaneously with ghosts of human erythrocytes

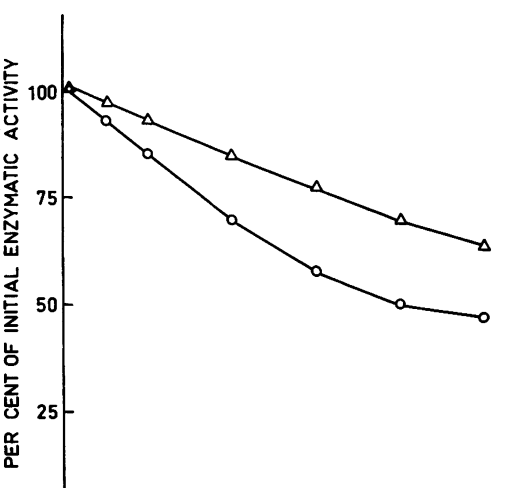

FIG. 2. *Inactivation of adenosine triphosphatase activity of Mycoplasma laidlawii membranes by ultrasonic treatment. Symbols: membranes suspended in 0.25 M NaCl, ○; membranes suspended in 0.25 M NaCl containing 10^{-2} M glutathione, △.*

demonstrated that ouabain inhibited that part of the adenosine triphosphatase activity which is activated by Na^+ and K^+, whereas, as in mycoplasma membranes, the magnesium-dependent adenosine triphosphatase activity of the erythrocytes was not affected.

Association of adenosine triphosphatase activity with membrane subunits. Several attempts were made to release the adenosine triphosphatase activity of *M. laidlawii* membranes in a soluble form. The membranes were treated with several proteolytic and lipolytic enzymes. Trypsin and chymotrypsin inactivated the enzymatic activity, whereas papain or pancreatic lipase had no such effect. However, papain or pancreatic lipase did not release any of the adenosine triphosphatase activity into the "soluble" fraction.

Detergents were found to disaggregate *M. laidlawii* membranes to lipid-protein subunits (21). Partial disaggregation of *M. laidlawii* membranes by sodium deoxycholate increased the specific adenosine triphosphatase activity of the still sedimentable membrane material, but no significant enzymatic activity could be detected in the soluble supernatant fraction. Further increase in the concentration of the detergent caused a decrease in the specific enzymatic activity of the sedimentable membrane material (Fig. 4). Other experiments with various sodium deoxycholate concentrations (0.001 to 100 μmoles/ml), at incubation periods of 10 min to 24 hr at 4 C and at room temperature, failed to release the membrane bound adenosine triphosphatase in a soluble form. Sodium lauryl sulfate caused rapid inactivation of the adenosine triphosphatase activity at concentrations insufficient for membrane disaggregation.

TABLE 3. *Effect of inhibitors on hydrolysis of adenosine triphosphate by Mycoplasma laidlawii membranes*

Inhibitor[a]	Concn of inhibitor	Per cent inhibition[b]
	M	
p-Chloromercuribenzoate	1×10^{-3}	60
	1×10^{-4}	65
Mercurous chloride	5×10^{-2}	65
	5×10^{-3}	40
Potassium iodoacetate	1×10^{-1}	100
	1×10^{-2}	60
	1×10^{-3}	0
N-ethylmaleimide	1×10^{-2}	65
	1×10^{-3}	35
2,4-Dinitrofluorobenzene	1×10^{-2}	36
	1×10^{-3}	10
Potassium ferricyanide	1×10^{-1}	100
	1×10^{-2}	25
	1×10^{-3}	0
Sodium fluoride	5×10^{-3}	42
	1×10^{-3}	12
Potassium thiocyanate	1×10^{-1}	50
	1×10^{-2}	22
	1×10^{-3}	0

[a] Membranes were suspended in deionized water instead of β buffer. The inhibitors were added to the reaction mixture 10 min before addition of the substrate.

[b] As compared to activity with no inhibitor.

TABLE 4. *Protective effect of adenosine triphosphate and β-mercaptoethanol against inhibition of the adenosine triphosphatase activity of Mycoplasma laidlawii membranes by several inhibitors*

Inhibitor	Concn of inhibitor	β-Mercaptoethanol	Per cent inhibition	
			Without preincubation[a]	With preincubation[b]
	M	*M*		
p-Chloromercuribenzoate	5×10^{-3}	0	58	66
		10^{-2}	ND[c]	8
N-ethylmaleimide	1×10^{-2}	0	15	48
		10^{-2}	ND	23
2,4-Dinitrofluorobenzene	1×10^{-2}	0	17	40
		10^{-2}	ND	30

[a] Substrate (4 μmoles of ATP) added simultaneously with inhibitor.

[b] Substrate and β-mercaptoethanol were added after 10 min of incubation of the membranes with inhibitor.

[c] Not done.

The finding that adenosine triphosphatase activity of *M. laidlawii* membranes is relatively stable to prolonged periods of ultrasonic treatment prompted its application for the release of this enzyme from the membranes. More than 50% of membrane material became nonsedimentable by centrifugation at 100,000 × *g* for 45 min after 10 min of ultrasonic treatment (Table 5). Ultracentrifugal analysis of the soluble membrane material showed a single peak having an uncorrected sedimentation coefficient ($S_{20,w}$) of 6.2 Svedberg units. However, the peak was not symmetrical, indicating some heterogeneity of particles. All the protein in this soluble membrane material could be adsorbed on diethylaminoethyl (DEAE) cellulose, but not on carboxymethyl cellulose. However, the membrane protein adsorbed on DEAE cellulose could not be eluted with NaCl. Ammonium sulfate at 30 to 50% saturation precipitated almost all soluble membrane protein, resulting in no significant

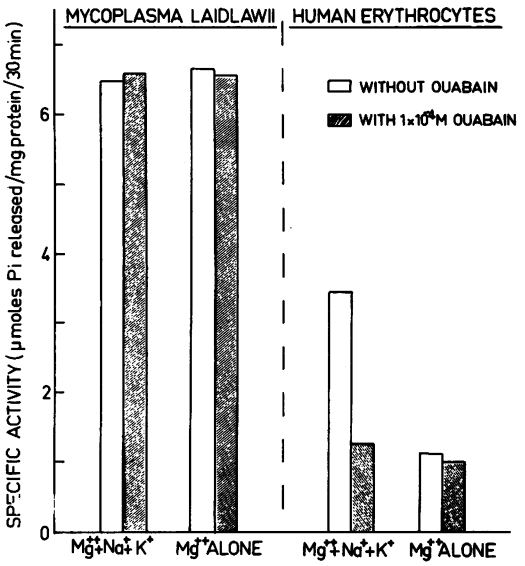

FIG. 3. *Effect of cations and ouabain on the adenosine triphosphatase activity of Mycoplasma laidlawii membranes and human erythrocyte ghosts. Human erythrocytes were dialyzed against deionized water for 2 hr. The ghosts obtained were sedimented by centrifugation at 17,000 × g for 15 min and washed five times with deionized water. The washed ghosts were then dialyzed for 24 hr against deionized water, sedimented by centrifugation, and resuspended in 0.05 M Tris buffer (pH 7.6). Reaction mixtures contained 2 μmoles of Mg^{++} alone or in combination with 100 μmoles of Na^+ and 20 μmoles of K^+. Ouabain was incubated with the mixtures for 10 min at 37 C before addition of ATP.*

increase in specific adenosine triphosphatase activity.

The soluble membrane material obtained by ultrasonic treatment was dialyzed against 1:20 β buffer containing 0.01 M $MgCl_2$. Turbidity appeared in the dialysis bag after 2 to 3 days of incubation at 4 C. Centrifugation of the contents of the dialysis bag at 34,000 × *g* for 60 min yielded a yellow translucent sediment. Almost all of the adenosine triphosphatase activity of the soluble fraction was found in this sediment (Table 5). About 30% of the membrane protein was not sedimented and remained in the supernatant fluid. The reaggregated membrane material hydrolyzed ADP, CTP, GTP, uridine triphosphate (UTP), and 5-AMP. The rate of hydrolysis of these nucleotides, relative to that of ATP, remained the same as in Table 2.

Accumulation of potassium by M. laidlawii. Accumulation of potassium by various mammalian cells has been shown to be associated with a (Na^+ + K^+)-activated adenosine triphosphatase system located in the cell membrane. Ouabain inhibits this enzymatic activity, as well as potassium accumulation by the cells (23). It was, therefore, of interest to test the ability of *Mycoplasma* cells to accumulate potassium, and to determine whether this accumulation is sensitive to ouabain. Potassium could be shown to be actively accumulated by *M. laidlawii* cells. The presence of a utilizable energy source, such as glucose, was essential for the accumulation. Iodoacetate inhibited potassium accumulation apparently by inhibiting glucose utilization. Figure 5 shows the accumulation of potassium by *M. laidlawii* cells. Accumulation was almost complete within 15 min of incubation at 37 C to a level of 7 mg of potassium per g of cell protein. Ouabain at a concentration of 5 × 10^{-5} M did not inhibit potassium uptake by *M. laidlawii*, but in a simultaneous experiment was found to inhibit completely the potassium uptake by human erythrocytes (Fig. 5). Variations in the

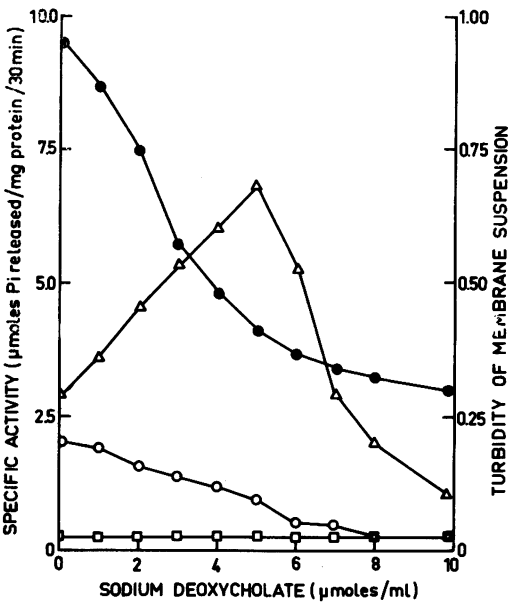

FIG. 4. *Effect of disaggregation of Mycoplasma laidlawii membranes by sodium deoxycholate on their adenosine triphosphatase activity. Membrane suspensions (containing 0.5 mg of protein per ml) were treated with various amounts of sodium deoxycholate for 10 min at room temperature, and their turbidity was measured at 400 mμ. Adenosine triphosphatase activity was determined in the treated suspensions and in the supernatant fluids and sediments obtained after centrifugation of the suspensions at 34,000 × g for 30 min. Symbols: enzymatic activity in treated suspension before centrifugation, ○; enzymatic activity in sediment, △; enzymatic activity in supernatant fluid, □; turbidity of treated suspension, ●.*

TABLE 5. *Adenosine triphosphatase activity of disaggregated Mycoplasma laidlawii membranes and reaggregated membrane material*[a]

Step	Prepn	Total membrane protein	Specific activity[b]	Per cent of initial activity
		mg		
A	Ultrasonic treatment			
	Untreated membranes	42.5	4.10	100
	Treated membranes	42.5	3.85	95
B	Treated membranes from A centrifuged at 100,000 × g for 45 min			
	Sediment	15.6	3.85	37
	Supernatant fluid	19.7	3.90	44
C	Reaggregation of membrane material from supernatant obtained in B			
	Reaggregated material	13.5	5.90	45
	Supernatant fluid	7.1	0.51	2

[a] Membrane suspensions (containing 4 mg of protein per ml) in 1:20 β buffer were treated for 10 min in an ultrasonic disintegrator. The treated suspensions were fractionated, reaggregated, and assayed for adenosine triphosphatase activity as described in Materials and Methods.

[b] Expressed as micromoles of inorganic phosphorus released per milligram of protein per 30 min.

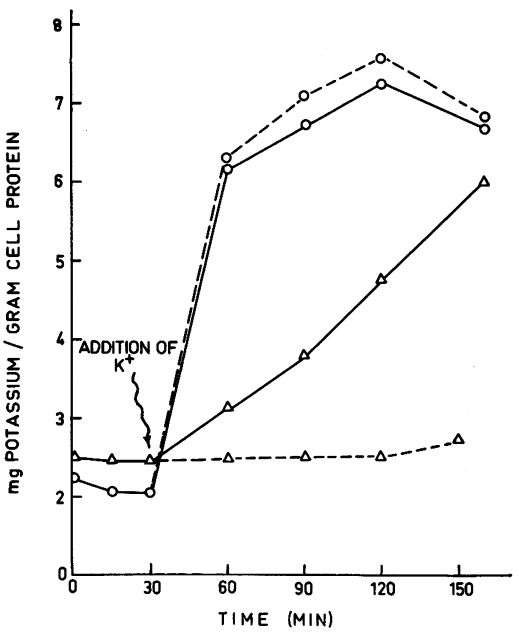

FIG. 5. *Effect of ouabain on potassium accumulation by Mycoplasma laidlawii cells and by human erythrocytes. Accumulation was tested as described in Materials and Methods. Potassium was added to a final concentration of 100 μg/ml. Symbols: Mycoplasma laidlawii, ○; human erythrocytes, △; solid lines, without ouabain; broken lines, with 5 × 10⁻⁵ M ouabain.*

sodium content of the suspending medium (from 5 to 2,000 μg/ml) did not affect potassium accumulation by *M. laidlawii* cells. Potassium uptake by *M. laidlawii* did not seem to be associated with changes in the intracellular sodium content.

DISCUSSION

The close association of enzymes hydrolyzing ATP with membranes of various cells and organelles very frequently requires the use of drastic procedures to "solubilize" the enzymes. These procedures include the use of detergents or mechanical means for disaggregation of the membranes (8, 26). Our attempts to liberate the adenosine triphosphatase activity from mycoplasma membranes by detergents failed, owing to the high sensitivity of the mycoplasma enzyme(s) to inactivation by detergents. Disaggregation of the membranes by ultrasound was more successful, since the enzymatic activity proved to be relatively stable even to prolonged treatment, especially in the presence of a reducing agent such as glutathione. The reducing agent apparently acts by neutralizing the oxidizing radicals generated by ultrasound energy (7). Part of the disaggregation products of *M. laidlawii* membranes obtained by ultrasonic treatment resemble the subunits obtained by disaggregation of the membranes by detergents (21). However, the uncorrected sedimentation coefficient of the slowest moving peak obtained by ultrasonic treatment was about 6.2, compared with an *S* value of 3.3 observed for subunits obtained with sodium lauryl sulfate, indicating that the particles are not identical. Our results show that the adenosine triphosphatase activity of *M. laidlawii* is part of, or is tightly bound to, the membrane subunits obtained by ultrasonic treatment, since the entire enzymatic activity could be recovered in the reaggregated membrane subunits. Thus, the mycoplasma enzyme(s) is not

truly soluble, but is part of a complex containing other proteins and phospholipids (21; Razin and Rottem, *unpublished data*). This explains our failure to purify the enzyme by the conventional protein fractionation methods.

The adenosine triphosphatase activity of the three mycoplasma strains tested in the present investigation largely corresponds in its properties to that described for other bacteria, mainly with respect to activation by cations. The mycoplasma enzyme(s) depended on magnesium for activity. This cation could be replaced by Mn^{++} or Co^{++}, but not by Ca^{++}, as in the adenosine triphosphatase activity of *Streptococcus pyogenes* (25), *Vibrio parahemolyticus* (6), *Streptococcus faecalis* (1), and *Bacillus stearothermophilus* (12). Unlike the adenosine triphosphatase of mammalian cell membranes, the adenosine triphosphatase of the mycoplasmas was not activated by sodium and potassium. A literature survey revealed that a combination of Na^+ and K^+ is not required for the activation of the adenosine triphosphatases of bacteria. Ouabain and oligomycin, which are potent inhibitors of the ($Na^+ + K^+$)-activated adenosine triphosphatase systems of animal cells, were without effect on the adenosine triphosphatase activity of bacteria (2, 6; G. Drapeau and R. A. MacLeod, Bacteriol. Proc., p. 122, 1962), as of mycoplasmas. The adenosine triphosphatase of the halophilic bacterium *V. parahemolyticus* was enhanced by sodium or potassium ions, but, unlike the mammalian enzyme, the enzymatic activity of the bacterium was not inhibited by ouabain and did not require both ions for optimal activity (6).

One of the arguments for the coupling of the ($Na^+ + K^+$)-activated adenosine triphosphatase with the transport of Na^+ and K^+ through the mammalian cell membrane is complete repression of this ion transport by ouabain (23). We were able to show that *M. laidlawii* accumulated potassium by an active transport mechanism which was not affected by ouabain. Accumulation of potassium by mammalian cells is usually linked with the extrusion of sodium from the cells. Our data showed that sodium concentration outside and inside the mycoplasma cells had no effect on potassium accumulation. According to Epstein and Schultz (4), the accumulation of K^+ by *Escherichia coli* cells is accompanied by the extrusion of H^+ rather than Na^+ from the cells. However, in a more recent communication (10th Annual Meeting Biophy. Soc., Boston, 1966), these authors found some evidence for the extrusion of Na^+ from *E. coli* cells during accumulation of potassium.

M. laidlawii membranes are able to hydrolyze a variety of nucleotides in addition to ATP, as is the case with the several other bacteria examined so far (1, 2, 6, 25). Our results do not allow any conclusion to be drawn as to this hydrolytic activity being brought about by one enzyme or more. Some indications in favor of one enzyme active in the hydrolysis of all nucleotides were that: (i) hydrolysis of all nucleotides was inhibited to about the same degree by *p*-chloromercuribenzoate; (ii) there was no cumulative phosphorus release on addition of various nucleotides to the reaction mixture containing ATP in excess; (iii) the reaggregated membrane material hydrolyzed the various nucleotides at the same rate as untreated *M. laidlawii* membranes. Although the rate of hydrolysis of 5-AMP by *M. laidlawii* membranes was very slow, it appeared consistently in each of the membrane preparations examined. The ability of *M. laidlawii* to hydrolyze 5-AMP as against its inability to hydrolyze the 2′-(3′)-mononucleotides may explain previous nutritional findings. The nutritional requirement of *M. laidlawii* for nucleosides could be fulfilled by 5′-mononucleotides, but not by the 2′-(3′)-mononucleotides (17).

Like adenosine triphosphatases of microbial and animal cells, the mycoplasma enzymes were also inhibited by sulfhydryl-blocking reagents and by fluoride. Inhibition of the mycoplasma enzyme by PCMB could be reversed almost completely by β-mercaptoethanol. However, β-mercaptoethanol could only partially reverse the inhibitory activity of NEM and DNFB. These inhibitors are known to react with free amino groups, as well as with -SH groups. The protection afforded by ATP against inhibition by NEM or DNFB may be due to the barrier erected by this substrate when combining with the active site of the enzyme, against changes in steric configurations of the enzyme molecule brought about by the inhibitors (24).

In light of the present findings, it may be concluded that, with respect to adenosine triphosphatase activity, mycoplasma membranes more closely resemble membranes of bacteria than of animal cells, mainly because of the independence of the mycoplasma enzymes of Na^+ and K^+ for activity, and their insensitivity to ouabain. In these properties, the mycoplasma adenosine triphosphatase activity resembles that of mitochondrial membranes. However, the mitochondrial enzyme differs from that of mycoplasmas in that it is stimulated by 2,4-dinitrophenol and sensitive to inactivation in the cold (16).

ACKNOWLEDGMENT

This research was supported by grant PG-Is-174 made by the U.S. Department of Agriculture under P.L. 480.

LITERATURE CITED

1. ABRAMS, A. 1965. The release of bound adenosine triphosphatase from isolated bacterial membranes and the properties of the solubilized enzyme. J. Biol. Chem. **240**:3675–3681.
2. COLE, H. A., AND D. E. HUGHES. 1965. The enzymic activity of the outer shell of *Lactobacillus arabinosus*. J. Gen. Microbiol. **40**:81–95.
3. CSÁKY, T. Z. 1965. Transport through biological membranes. Ann. Rev. Physiol. **27**:415–450.
4. EPSTEIN, W., AND S. G. SCHULTZ. 1965. Cation transport in *Escherichia coli*. V. Regulation of cation content. J. Gen. Physiol. **49**:221–234.
5. FISKE, C. H., AND Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**:375–400.
6. HAYASHI, M., AND R. UCHIDA. 1965. A cation activated adenosine triphosphatase in cell membranes of halophilic *Vibrio parahemolyticus*. Biochim. Biophys. Acta **110**:207–209.
7. HUGHES, D. E. 1961. The disintegration of bacteria and other microorganisms by the M.S.E.-Mullard ultrasonic disintegrator. J. Biochem. Microbiol. Technol. Eng. **3**:405–433.
8. ISHIKAWA, S., AND A. L. LEHNINGER. 1961. Reconstitution of oxidative phosphorylation in preparations from *Micrococcus lysodeikticus*. J. Biol. Chem. **237**:2401–2408.
9. KIELLEY, W. W. 1961. Myosin adenosine triphosphatase, p. 159–168. *In* P. D. Boyer, H. Lardy, and K. Myrbäck [ed.], The enzymes, vol. 5. Academic Press, Inc., New York.
10. LEHNINGER, A. L. 1962. Water uptake and extrusion by mitochondria in relation to oxidative phosphorylation. Physiol. Rev. **42**:467–516.
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. MILITZER, W., AND L. C. TUTTLE. 1952. Thermal enzymes. IV. Partial separation of an adenosine triphosphatase from an apyrase fraction. Arch. Biochem. Biophys. **39**:379–386.
13. POLLACK, J. D., S. RAZIN, AND R. C. CLEVERDON. 1965. Localization of enzymes in *Mycoplasma*. J. Bacteriol. **90**:617–622.
14. POLLACK, J. D., S. RAZIN, M. E. POLLACK, AND R. C. CLEVERDON. 1965. Fractionation of *Mycoplasma* cells for enzyme localization. Life Sci. **4**:973–977.
15. POST, R. L., C. R. MERRIT, C. R. KINSOLVING, AND C. D. ALBRIGHT. 1960. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. J. Biol. Chem. **235**:1796–1802.
16. PULLMAN, M. E., H. S. PENEFSKY, A. DATA, AND E. RACKER. 1960. Partial resolution of the enzymes catalysing oxidative phosphorylation. I. Purification and properties of soluble, dinitrophenol-stimulated adenosine triphosphatase. J. Biol. Chem. **235**:3322–3329.
17. RAZIN, S. 1962. Nucleic acid precursor requirements of *Mycoplasma laidlawii*. J. Gen. Microbiol. **28**:243–250.
18. RAZIN, S. 1963. Structure, composition and properties of the PPLO cell envelope, p. 526–534. *In* N. E. Gibbons [ed.], Recent progress in microbiology, vol. 8. Univ. Toronto Press, Toronto.
19. RAZIN, S. 1963. Osmotic lysis of *Mycoplasma*. J. Gen. Microbiol. **33**:471–475.
20. RAZIN, S., AND A. COHEN. 1963. Nutritional requirements and metabolism of *Mycoplasma laidlawii*. J. Gen. Microbiol. **30**:141–154.
21. RAZIN, S., H. J. MOROWITZ, AND T. M. TERRY. 1965. Membrane subunits of *Mycoplasma laidlawii* and their assembly to membranelike structures. Proc. Natl. Acad. Sci. U.S. **54**:219–225.
22. RIBEIRO, L. P., E. MILIDIERI, AND O. R. AFFONSO. 1961. Paper electrophoresis, p. 303–313. Elsevier Publishing Co., Amsterdam.
23. SKOU, J. C., 1965. Enzymatic basis for active transport of Na^+ and K^+ across cell membranes. Physiol. Rev. **45**:596–616.
24. SKOU, J. C., AND C. HILBERG. 1965. The effect of sulphydryl-blocking reagents and of urea on the $(Na^+ + K^+)$-activated enzyme system. Biochim. Biophys. Acta **110**:359–369.
25. SOKAWA, Y. 1965. Liberation of inorganic phosphate from ATP and GTP by a protoplast membrane fraction of *Streptococcus pyogenes*. J. Biochem. (Tokyo) **57**:707–708.
26. TANAKA, R., AND K. P. STRICKLAND. 1965. Role of phospholipid in the activation of Na^+, K^+-activated adenosine triphosphatase of beef brain. Arch. Biochem. Biophys. **111**:583–592.