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Ciliostatic, Hemagglutinating, and Proteolytic Activities in a Cell Extract of *Mycoplasma pneumoniae*

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An extract of *Mycoplasma pneumoniae*, prepared from glass-grown organisms by extraction with 2 M NaCl, followed by freeze-thaw, ultracentrifugation, dialysis, and lyophilization, yielded approximately 20% of the total mycoplasmal protein. The extract contained at least 20 protein bands on sodium dodecyl sulfate-polyacrylamide gels and 2 to 5% carbohydrate and inhibited 70 to 100% of the ciliary activity of hamster tracheal organ cultures (ciliostasis). The extent of ciliostasis was dependent on the concentration of the extract. The extract also produced hemagglutination of human O-positive erythrocytes and showed proteolytic activity with a synthetic tetrapeptide substrate, S-2222. These in vitro tissue-damaging activities may be associated with the virulence of the mycoplasmas and with the pathogenesis of *M. pneumoniae* disease.

Infections with *Mycoplasma pneumoniae*, the cause of human primary atypical pneumonia (6), are endemic, with epidemics occurring at approximately 5-year intervals (15, 16). Epidemiological studies indicate that clinically symptomatic pneumonia represents only a fraction of the total *M. pneumoniae* infections and that *M. pneumoniae* infections are responsible for a significant portion of respiratory illness among school-age children (15, 16) and young adults (16).

Information regarding the pathogenesis of *M. pneumoniae* disease has been obtained from hamster tracheal organ cultures. These studies have shown that infection is initiated by attachment of the organism to ciliated cells followed by ciliostasis, alteration and loss of the cilia, and destruction of the mucosal epithelial cells (5, 9). The ability of the organism to inhibit normal ciliary function is considered an important virulence factor. That is, ciliostasis can be produced with virulent strains of *M. pneumoniae* (5, 10, 11, 13, 19), but not with avirulent strains (5, 11) or with heat-killed organisms (10). In addition, heavily ciliated areas of the hamster trachea do not permit attachment as well as areas with less ciliation (17). This effect was attributed to the physiological function of vigorously beating cilia, which prevented the *M. pneumoniae* from adhering to the cell surfaces (17). Ciliostasis may thus enhance attachment and colonization, contributing to the mechanisms by which the parasite produces and maintains the infectious process. Ciliostasis has also been observed with membrane preparations of *M. pneumoniae* (18). Hence, studies were initiated to examine component(s) of the mycoplasma cell responsible for these pathological activities.

This report describes the preparation of a cell-free extract of *M. pneumoniae* which possesses ciliostatic, hemagglutinating, and proteolytic activities. Thus, the extract contains solubilized, biologically active components of the mycoplasma cell which are potentially related to virulence and represents starting material for the purification and characterization of these factors.

MATERIALS AND METHODS

Mycoplasma cultures. Cultures of *M. pneumoniae* M129 (kindly provided by Albert Collier, Chapel Hill, N.C.) were used during passages 12 to 25 and grown attached to glass in 32-oz (ca. 0.9-liter) prescription bottles containing 65 ml of mycoplasma complete broth (BBL Microbiology Systems, Cockeysville, Md.) with 10% yeast extract (Microbiological Associates, Inc., Bethesda, Md.), 10% heat-inactivated, filtered horse serum (Microbiological Associates), 0.5% glucose, and 1,000 U of penicillin G per ml (1).

Preparation of extract. Cultures of *M. pneumoniae* M129 were grown at 37°C to confluency (usually 5 to 7 days) in 32-oz (ca. 0.9-liter) prescription bottles and harvested before significant metabolic products accumulated (pH 6.0 to 7.0). Attached colonies were washed two or three times with phosphate-buffered saline (pH 7.2), 5 ml of 2 M NaCl was added to each bottle, and the cultures were incubated at 36°C for 30 min. The colonies were scraped from the glass with a rubber policeman, and the cell suspension was frozen in a dry ice-ethanol bath and then thawed slowly at room temperature. The freeze-thaw procedure was performed five times. The resultant cell suspension was centrifuged at 100,000 × *g* for 60 min, and the supernatant was dialyzed against 40 volumes of deion-

ized water at 4°C with four to six changes. The dialyzed cell-free extract was placed in vials, freeze-dried, and stored desiccated at 4°C until used. With most lots, 10 to 15 culture bottles were processed at one time and yielded approximately 200 μg of extract protein per bottle of culture. With some lots, dialysis against deionized water caused precipitation of the extract. This precipitation was associated with lots made from cultures which produced a pH near 6.0. The precipitate was sedimented by centrifugation at 10,000 × *g* for 15 min and designated fraction DP. The supernatant was removed, lyophilized as described above, and designated fraction DS. For ciliostasis, hemagglutination, and protease assays, the fractions were suspended in Hanks balanced salt solution (HBSS).

Hamster tracheal ring organ cultures. The procedure used was described by Collier and co-workers (10, 13). Tracheas were removed by sterile procedures and cut into rings, which were placed on 5-mm cover slips (Bellco, Vineland, N.J.) in 35-mm petri dishes (Costar, Cambridge, Mass.) containing 0.5 ml of Eagle minimal essential medium supplemented with 10% heat-inactivated fetal calf serum (Reheis Chemical Co., Phoenix, Ariz.), 2 mM glutamine, and 1,000 U of penicillin G per ml. The tracheal rings were incubated in 5% CO_2–95% air at 36°C until used, usually 3 to 5 days. The medium was changed after 24 h of culture and twice per week thereafter. Normal ciliary activity could be maintained for at least 2 weeks under these conditions.

Ciliostatic assay. The assay was performed in flat-bottomed, 96-well microtiter plates (Falcon no. 3040, Oxnard, Calif.). Tracheal rings were transferred to wells containing either 100 μl of HBSS, which served as a negative control, or given concentrations of the mycoplasma extract suspended in HBSS, and incubated at 36°C in 5% CO_2–95% air overnight (18 h). The ciliary activity was observed with a Zeiss inverted light microscope at ×300 magnification, and the ciliary activity score given was the product of "ciliary vigor" (from 0 for no detectable activity [complete ciliostasis] to 3 for normal ciliary activity) times the percent of the tracheal ring retaining ciliary activity (18). Scores ranged from 0, no ciliary activity on any portion of the ring, to a maximum of 300 for normal vigorous activity over the entire ring (i.e., 3 times 100%). Two or more tracheal rings were used for each preparation tested. A unit of ciliostatic activity was defined as the minimal amount of extract that reduced ciliary activity to a score of 20 or less (i.e., inhibition of approximately 90% or more of normal activity) for rings incubated in the standard assay. The designation of a unit as a 90% reduction instead of the conventional 50% reduction of the normal activity was chosen because it gave more consistent and reproducible results. In some experiments ciliostatic activity was determined for nonmycoplasmal protein such as bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo.), myoglobulin (Sigma), and normal horse serum (Microbiological Associates).

Hemagglutination procedure. Blood from O-positive donors was obtained at the National Institutes of Health Blood Bank Center from a number of subjects. The blood was stored at 4°C and used within 10 days. Approximately 1 ml of whole blood was added to 10 ml of buffer A [0.1 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane-chloride, 1 mM $CaCl_2$, pH 7.2]. The cell suspension was mixed gently, sedimented by centrifugation at 500 × *g* for 3 to 5 min, washed twice, and suspended in buffer A or HBSS to a final concentration (vol/vol) of 2.5%. For the test, 40 μl of the mycoplasma extract was added to round-bottomed wells of a microtiter plate followed by 10 μl of the 2.5% erythrocyte suspension. Negative controls using either buffer A or HBSS alone were included in each test. The plates were incubated on an orbital shaker at 36°C, and hemagglutination was observed after 30 to 90 min.

Protease assays. Protease activity was measured by release of *p*-nitroaniline from the chromogenic synthetic tetrapeptide S-2222 (*N*-benzoyl-isoleucyl-glutamyl-glycyl-arginyl-*para*-nitroanilide-hydrochloride; Ortho Diagnostics, Raritan, N.J.). The assay mixture contained 0.2 ml of the test extract material, 0.2 mM substrate S-2222, 10 mM $CaCl_2$, and 50 mM tris(hydroxymethyl)aminomethane (pH 8.2) in a final volume of 1 ml. An assay (with water substituted for the extract material) was performed to measure any endogenous hydrolysis of the substrate. Absorbance was monitored at 410 nm, and nanomoles of *p*-nitroaniline released per minute at 37°C was calculated by using a molar extinction coefficient of 8,800 (3). The rate of any endogenous hydrolysis of S-2222 was subtracted from the rate obtained with the test material before calculating the rate of product formation. Trypsin (Armour Chemical Co., Chicago, Ill.) served as the standard.

Analytical procedures. Protein was determined by the Lowry procedure (21) with bovine serum albumin (fraction V; Sigma) as a standard. Carbohydrate analyses were performed by R. Boykins, Bureau of Biologics, using an automated analyzer which employed reverse-phase partition chromatography after hydrolysis with 0.16 N methane sulfonic acid (4). Polyacrylamide gel electrophoresis was performed with PAA 4/30 gradient gels (Pharmacia, Inc., Piscataway, N.J.) in the presence of sodium dodecyl sulfate (SDS) with electrophoresis buffer containing 0.04 M tris(hydroxymethyl)aminomethane (pH 7.4), 0.02 M sodium acetate, 2 mM ethylenediaminetetraacetic acid, and 0.2% SDS for 2 h at 140 V (Pharmacia, Inc. Electrophoresis Manual, p. 11). Samples were digested in 10 mM tris(hydroxymethyl)aminomethane-chloride (pH 8.0), 1 mM ethylenediaminetetraacetic acid, 1% SDS, and 5% β-mercaptoethanol by boiling for 5 min. Gels were stained overnight with 0.1% Coomassie brilliant blue R-250 (BioRad Laboratories, Richmond, Calif.) in 40% methanol–10% acetic acid and destained in 7% acetic acid–10% methanol. Protein standards used were low-molecular-weight calibration proteins obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

RESULTS

Properties of the *M. pneumoniae* extract. Cultures of glass-grown *M. pneumoniae* produced confluent colony growth within 5 to 7 days of incubation. Each culture bottle con-

tained 10^8 to 10^9 colony-forming units and about 1 mg of protein. A precise enumeration of viable cells was difficult to determine because mycoplasmas tend to aggregate during colony growth. Because mycoplasmas normally are grown in an enriched medium containing 20% horse serum, the extract was subject to contamination by medium components. Contamination was minimized by growing mycoplasmas attached to glass in medium containing only 10% horse serum which had been heat inactivated and filtered (0.2 μm). Glass-grown cultures can be vigorously and repeatedly washed and harvested by scraping with a rubber policeman. This method eliminates concentration and washing of organisms by centrifugation, which is a major source of medium contamination in broth-grown cultures (2, 26).

Approximately 20% of the total mycoplasmal protein was recovered by the extraction procedure. Using the SDS-polyacrylamide gel electrophoresis procedure, at least 20 protein-containing bands were detected in the extract with the Coomassie blue stain (Fig. 1). Carbohydrate analysis of three different lots indicated that the extract contained 2 to 5% neutral sugars. Glucose (0.8 to 2.2%) and galactose (0.3 to 1.0%) were present in a ratio of about 2:1 to 3:1, and ribose was present at 0.2 to 2.1%. No deoxyribose was found, but a trace amount of glucosamine was detected. Thus, the cell-free extract contained a mixture of proteins with a small amount of carbohydrate, possibly as glycoprotein.

Ciliostatic activity of the extract. The effect of the *M. pneumoniae* extract on the ciliary activity of hamster tracheal organ cultures is shown in Tables 1 and 2. The ciliary activity was completely inhibited by mycoplasmal protein extract (140 μg) after overnight incubation, and the activity was markedly inhibited within 5.5 h. Six different lots of extract were examined in eight separate experiments, and the mean score of ciliary activity ± standard error was 170.4 ± 9.6 (n = 33) for control tracheal rings but only 24.0 ± 6.3 for extract-treated rings (n = 21) (60 to 140 μg of protein, 36°C, 16 to 20 h). Bovine serum albumin, myoglobulin, and normal horse serum (which served as nonmycoplasmal control proteins, 100 to 200 μg) did not inhibit the ciliary activity.

The ciliostatic effect was dependent on the concentration of the extract used (Table 2). Similar dose responses were observed in repeated experiments and indicated that the assay can be used for quantitative determination of the ciliostatic activity.

Hemagglutination activity. Adherence of *M. pneumoniae* to ciliated epithelial cells initiates infection of tracheal organ cultures (9). The interaction of *M. pneumoniae* with mammalian cells has also been determined by hemadsorption (2, 20). Hence, the ability of the extract to interact with mammalian cells was tested by hemagglutination. Table 3 shows that the extract exhibited hemagglutinating activity with human O-positive erythrocytes. The minimum amount of extract that produced hemagglutination was 6 to 20 μg of protein for the three lots tested.

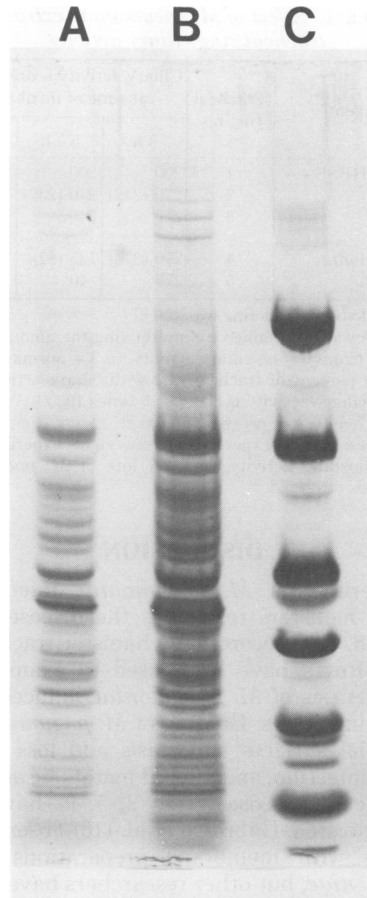

FIG. 1. *SDS-polyacrylamide gel electrophoresis on PAA 4/30 gradient gel of (a) 5 μg of extract; (b) 20 μg of extract; (c) protein standards (molecular weight): phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic analydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). Stained with Coomassie brilliant blue R-250.*

Proteolytic activity. The proteolytic activity of the extract was examined using substrate S-2222, a synthetic chromogenic tetrapeptide (Table 4). Proteolytic activity with substrate S-2222 was present in each of the lots tested.

TABLE 1. *Effect of M. pneumoniae extract on tracheal ring ciliary activity*

Reagent[a]	Tracheal ring no.	Ciliary activity score[b] (mean) at time of incubation		
		0 h	5.5 h	18 h
Control (HBSS alone)	1	200)	200	150
	2	270 (242)	270 (237)	175 (148)
	3	255	240	120
M. pneumoniae extract[c]	4	270 (270)	125 (82)	0 (0)[d]
	5	279	40	0

[a] Total volume per ring was 100 μl.

[b] Score was determined by multiplying the amount of ciliary vigor (from 0 = no ciliary activity, to 3 = normal activity) times the percent of tracheal ring with ciliary activity (e.g., maximal ciliary activity is 300; i.e., 3 times 100%).

[c] Lot 17, 140 μg of protein per 100 μl.

[d] Representative experiment from eight experiments in which ciliostatic activity with six lots of *M. pneumoniae* extract was observed.

DISCUSSION

Experimental *M. pneumoniae* infection in Syrian hamsters resembles the disease in humans (8, 12). Accordingly, hamster tracheal organ cultures have been used to examine the pathogenesis of *M. pneumoniae*-induced respiratory infections. Binding of *M. pneumoniae* to epithelial surfaces, ciliostasis and loss of cilia due to infection, and altered metabolic activities of infected mucosal cells (2, 5, 9) have been demonstrated. Gabridge et al. (18) observed ciliostasis with membrane preparations of *M. pneumoniae*, but other researchers have not reproduced this observation. This report documents that a cell-free extract of *M. pneumoniae* can produce ciliostasis as well as hemagglutination and proteolysis. The cell-free extract is important because these biological factors have been solubilized in an active form. This solubilized preparation can be used as a starting material for the purification of these factors, necessary for their identification and characterization. The assays for the ciliostatic, hemagglutinating, and proteolytic factors can also be used to examine the relative efficiency of other procedures such as sonication or detergent extraction, using the NaCl extract as reference material.

The 2 M NaCl treatment was selected because it is a mild extraction procedure. In addition, high ionic strength has been used to solubilize erythrocyte membrane proteins (7, 25) and bacterial membranes (T.-Y. Liu, personal communication). Although the freeze-thaw process aids in solubilization, it may also release intercellular components. SDS-polyacrylamide gel electrophoresis showed that the cell-free extract contained approximately 20 protein bands, primarily with molecular weights ranging from 20,000 to 70,000. The extract also contained small amounts of carbohydrate (approximately 2 to 5%).

The ciliostatic activity of the extract appears to be similar to ciliostasis observed with virulent *M. pneumoniae* infection (10, 11, 13) or with *M. pneumoniae* membranes (18); thus, it is likely that an active component(s) of the mycoplasmal cell responsible for ciliostatic activity has been solubilized and recovered by the extraction procedure used in our studies. The heat stability of the ciliostatic factor (resisted boiling or 80°C for 10 min) argues against a protein factor, but heat stability has been observed with certain microbial proteins such as fragment A of diphtheria toxin, which retains its adenosine 5'-diphosphate-ribosylating activity after temperatures of 100°C for short periods of time at extreme pH ranges (14). However, the possibility that the ciliostatic activity is due to some other component such as glycolipid cannot be excluded. Whereas heat did not affect the ciliostatic properties, treatment at 80°C for 10 min partially inactivated (40 to 60%) the proteolytic activity of the extract. The specific activity of the protease was low in comparison with purified trypsin and may reflect the concentration of the protease in a mixed population of proteins or

TABLE 2. *Dose response of M. pneumoniae extract in the ciliostasis assay*

Reagent[a]	Concentration of extract (μg/100 μl)	Ciliary activity score[b]	Ciliostatic units per mg[c]
Control (HBSS alone)	None	223	—
Fraction DS (lot 19)	12	105	40
	25	5	
	50	0	
Fraction DP (lot 19)	32	120	7.7
	65	65	
	130	12	

[a] Total volume per ring was 100 μl: fraction DP was the sediment that occurred during dialysis of the extract against water and was collected by centrifugation; fraction DS was the lyophilized supernatant from the centrifugation of fraction DP.

[b] Ciliary activity score was determined as described in Table 1 after 18 h of incubation. Score represents the mean from duplicate or triplicate rings. Dose response is representative of three experiments.

[c] Number of units: a unit is defined as the minimal amount of mycoplasma extract that reduces the ciliary activity score (200 to 300 at zero time) to 20 or less after overnight incubation, i.e., inhibition of normal ciliary activity by approximately 90%.

differences in substrate specificity. However, the effect of the protease activity on the stability of other protein components in the extract must be considered. The possible pathogenic role of the protease is presently under examination because of the specific immunoglobulin A protease activities associated with other mucosal pathogens (22-24).

The ability to hemagglutinate suggests that the extract contains component(s) of the mycoplasma cell capable of interacting with and attaching to human erythrocytes, resulting in agglutination. Virulent *M. pneumoniae* cells agglutinate human erythrocytes, which suggests that the mycoplasma hemagglutinin is located on the outer surface of the cell. The presence of hemagglutinating activity in the extract argues that the salt extraction procedure effectively solubilizes outer surface components of the mycoplasma cell. These component(s) appear to have lectin-like properties and may be similar to the binding moiety of the mycoplasma that mediates adherence of the organism to the host target cell.

In summary, we have described an extraction procedure to solubilize active factors from *M. pneumoniae* cultures which may be related to the virulence of this organism. These procedures and assays will be used to isolate and characterize the mycoplasma cell components possessing specific biological activities so that their role in the initiation, maintenance, and progression of mycoplasma infections can be determined. The effect of the extract as an immunogen in preventing mycoplasma disease in hamsters challenged with virulent *M. pneumoniae* is also under investigation.

TABLE 3. *Hemagglutination of human erythrocytes by M. pneumoniae extract*

M. pneumoniae extract	Minimal concn of extract producing hemagglutination (μg per 50 μl)[a]
Lot 17	13
Lot 19[b]	
Fraction DP	≤12
Fraction DS	20
Lot 25	
Expt 1	10
Expt 2	6

[a] A 10-μl sample of a 2.5% suspension of human 0+ erythrocytes added to 40 μl of mycoplasma extract at appropriate dilutions (0.5 to 100 μg) and incubated for 30 to 90 min at 36°C. Controls with HBSS showed no hemagglutination. Hemagglutination with each lot of extract represents a separate experiment with different donor O+ erythrocytes. Hemagglutination was also observed with three other lots of extract in which the minimal hemagglutinating concentration was not determined.

[b] Fraction DP was the sediment that occurred during dialysis of the extract against water and was collected by centrifugation; fraction DS was the lyophilized supernatant from the centrifugation of fraction DP.

TABLE 4. *Proteolytic activity of M. pneumoniae extract with substrate S-2222*

Reagent	Reagent concn (μg/ml)	Proteolytic activity[a]	
		nmol of pNA per min	nmol of pNA per min per mg of protein
M. pneumoniae extract[b]			
Lot 14	159	3.18	20.0
Lot 17	77	1.07	13.9
Lot 19	195	1.86	9.6
Trypsin	0.01-0.05	7.17-24.80	7.2×10^{5}[c]

[a] By measuring the release of *p*-nitroaniline (pNA) from the chromogenic tetrapeptide S-2222.

[b] Each lot assayed represents a separate experiment.

[c] Mean value obtained from six determinations of trypsin activity with 0.01 to 0.05 μg/ml.

LITERATURE CITED

1. **Barile, M. F.** 1974. General principles of isolation and detection of mycoplasmas. Colloq. Inst. Natl. Santé Rech. Med. **33**:135-142.
2. **Barile, M. F.** 1979. Mycoplasma-tissue cell interactions, p. 425-474. *In* J. G. Tully and R. F. Whitcomb (ed.), The mycoplasmas, vol. 2: human and animal mycoplasmas. Academic Press, Inc., New York.
3. **Bergstrom, K., and M. Blomback.** 1974. Determination of plasma prothrombin with a reaction rate analyzer using a synthetic substrate. Thromb. Res. **4**:719-729.
4. **Boykins, R. A., and T.-Y. Liu.** 1980. Automatic analysis of neutral sugar components in glycoproteins and complex carbohydrates. J. Biochem. Biophys. Methods **2**: 71-78.
5. **Carson, J. L., A. M. Collier, and W. A. Clyde, Jr.** 1979. Ciliary membrane alterations occurring in experimental *Mycoplasma pneumoniae* infection. Science **206**:349-351.
6. **Chanock, R. M., L. Hayflick, and M. F. Barile.** 1962. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natl. Acad. Sci. U.S.A. **48**:41-9.
7. **Chavin, S. I.** 1971. Isolation and study of functional membrane proteins. FEBS Lett. **14**:269-282.
8. **Clyde, W. A., Jr.** 1971. Immunopathology of experimental *Mycoplasma pneumoniae* disease. Infect. Immun. **4**: 757-763.
9. **Collier, A. M.** 1979. Mycoplasmas in organ culture, p. 475-493. *In* J. G. Tully and R. F. Whitcomb (ed.), The mycoplasmas, vol. 2: human and animal mycoplasmas. Academic Press, Inc., New York.
10. **Collier, A. M., and J. B. Baseman.** 1973. Organ culture techniques with mycoplasmas. Ann. N.Y. Acad. Sci. **225**:277-289.

11. **Collier, A. M., and W. A. Clyde, Jr.** 1971. Relationships between *Mycoplasma pneumoniae* and human respiratory epithelium. Infect. Immun. **3**:694-701.
12. **Collier, A. M., and W. A. Clyde, Jr.** 1974. Appearance of *Mycoplasma pneumoniae* in lungs of experimentally infected hamsters and sputum from patients with natural disease. Am. Rev. Respir. Dis. **110**:765-773.
13. **Collier, A. M., W. A. Clyde, Jr., and F. W. Denny.** 1969. Biologic effects of *Mycoplasma pneumoniae* and other mycoplasmas from man on hamster tracheal organ culture. Proc. Soc. Exp. Biol. Med. **132**:1153-1158.
14. **Collier, R. J.** 1977. Inhibition of protein synthesis by exotoxins from *Corynebacterium diphtheriae* and *Pseudomonas aeruginosa*, p. 69-98. *In* P. Cuatrecasas (ed.), The specificity and action of animal, bacterial, and plant toxins. John Wiley and Sons, New York.
15. **Fernald, G. W., A. M. Collier, and W. A. Clyde, Jr.** 1975. Respiratory infections due to *Mycoplasma pneumoniae* in infants and children. Pediatrics **55**:327.
16. **Foy, H. M., G. E. Kenny, M. K. Cooney, and I. D. Allan.** 1979. Long-term epidemiology of infections with *Mycoplasma pneumoniae*. J. Infect. Dis. **139**:681-687.
17. **Gabridge, M. G., C. C. Agee, and A. M. Cameron.** 1977. Differential distribution of ciliated epithelial cells in the trachea of hamsters: implications for studies of pathogenesis. J. Infect. Dis. **135**:9-19.
18. **Gabridge, M. G., C. K. Johnson, and A. M. Cameron.** 1974. Cytotoxicity of *Mycoplasma pneumoniae* membranes. Infect. Immun. **10**:1127-1134.
19. **Gabridge, M. G., and R. B. Polisky.** 1977. Intracellular levels of adenosine triphosphate in hamster trachea organ cultures exposed to *Mycoplasma pneumoniae* cells or membranes. In Vitro **13**:510-516.
20. **Hansen, E. J., R. M. Wilson, and J. B. Baseman.** 1979. Isolation of mutants of *Mycoplasma pneumoniae* defective in hemadsorption. Infect. Immun. **23**:903-906.
21. **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.
22. **Male, C. J.** 1979. Immunoglobulin A1 protease production by *Haemophilus influenzae* and *Streptococcus pneumoniae*. Infect. Immun. **26**:254-261.
23. **Mulks, M. H., and A. G. Plaut.** 1978. IgA protease as a characteristic distinguishing pathogenic from harmless *Neisseriaceae*. N. Engl. J. Med. **299**:973-976.
24. **Plaut, A. G., J. V. Gilbert, and A. H. Rule.** 1978. Isolation and properties of the immunoglobulin A protease of *Neisseria gonorrhoeae* and *Streptococcus sanguis*, p. 279-284. *In* G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), Immunobiology of *Neisseria gonorrheae*. American Society for Microbiology, Washington, D.C.
25. **Rosenberg, S. A., and G. Guidotti.** 1969. Fractionation of the protein components of human erythrocyte membranes. J. Biol. Chem. **244**:5118-5124.
26. **Somerson, N. L., W. D. James, B. E. Walls, and R. M. Chanock.** 1967. Growth of *Mycoplasma pneumoniae* on a glass surface. Ann. N. Y. Acad. Sci. **143**:384-389.