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# The Role of Pasteurella Pneumotropica and Mycoplasma Pulmonis in the Etiology and Pathogenesis of Murine Pneumonia

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# THE ROLE OF *PASTEURELLA PNEUMOTROPICA* AND *MYCOPLASMA PULMONIS* IN THE ETIOLOGY AND PATHOGENESIS OF MURINE PNEUMONIA*

by

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# LIFE

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From 1954 to January, 1956 she was employed by G. D. Searle, Inc., Skokie, Illinois as a technician.

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

Until recently, many investigators were unconcerned about the health status of their experimental animals, except when disease broke out and the experimenter was faced with the loss of a long term experiment. This complacency has been likened by Nelson (103) to a chemist being unconcerned about the purity of his reagents. Fortunately, such attitudes are changing, and the biomedical researcher now requires the animals he uses to be of such quality and maintained in such a way that his research has a high probability of successful completion. Contemporary investigators often measure more subtle endpoints than were measured in the past. As a result, distortion of these endpoints by extraneous factors becomes critical in evaluating experimental data. Pneumonia in laboratory mice and rats frequently distorts or interferes with results obtained in experiments using these animals. Although standards of care and management have improved vastly in the last 5-10 years, respiratory diseases are still prevalent. In one year, 1966, 261 clinical cases of pneumonia were observed in experimental rodents at Argonne National Laboratory (Brennan, unpublished results). Although respiratory disease in rats and mice does not necessarily preclude their use as experimental animals, Cruickshank (21) and Innes, McAdams, and Yevich (58) have suggested that conventional animals be excluded from research involving toxic inhalants. This might well be extended to studies involving pathogens of the respiratory tract, aging and irradiation. Consequently, studies of the pathogenesis and etiology of murine pneumonia are important to those who use experimental rodents, to those who raise them commercially and to those responsible for the control of their diseases.

Besides the intrinsic value of research on murine pneumonia, it is often possible to extrapolate from diseases of animals to diseases of man. Edward (34) and Klieneberger-Nobel (73) suggested that the study of the pathogenesis of a disease caused by one species of PPLO can provide useful information on the pathogenesis of a disease caused by another species of PPLO. One such disease is primary atypical pneumonia of man caused by Mycoplasma pneumoniae. The extensive literature on M. pneumoniae infection has been reviewed in recent years (16, 32, 42, 80, 144) and will not be reexamined here. Several facts concerning the disease are of particular interest, however, and should be emphasized. M. pneumoniae infection produces no clear-cut pathognomonic syndrome. Although the disease seems to be more severe and of longer duration than viral pneumonias, this does not serve as a distinguishing feature (115). The incubation period is generally longer than pneumonias of different etiology (42) and the disease is generally not fatal. The organism was recovered in tissue culture three times at necropsy (31, 81) but this was before it was grown on artificial culture media. Since improved culture techniques have become available, it has not been recovered from necropsy specimens (42). Without such specimens, the difficulty of determining the pathogenesis of M. pneumoniae pneumonia is obvious. To circumvent these difficulties, Lutsky and Organick (82) used murine pneumonia in axenic mice as an experimental model.

The etiology of murine pneumonia has been obscured by the variety of names given to the disease. For example, Klieneberger-Nobel calls the disease bronchiectasis or bronchopneumonia (72). When she uses these terms, she means the pneumonia associated with M. pulmonis. Nelson, on the other hand, (99, 100, 104) uses enzootic bronchiectasis or the interchangeable term, endemic

pneumonia, to mean a disease which he believes is caused by a virus and is distinguishable from M. pulmonis infection. He calls the disease caused by M. pulmonis "infectious catarrh" (94, 95, 96, 97, 100) and believes that the "chronic respiratory disease" syndrome of rodents is the result of combined infection by M. pulmonis and a virus (104). Other names which have been applied to murine pneumonia are "grey lung disease" (4) and chronic murine pneumonia (108). Joshi, Blackwood and Dale (63) in a review of chronic murine pneumonia considered each named disease separately, thereby giving the impression that a separate etiologic agent was responsible for each syndrome. Their approach unfortunately served only to further confuse an already confused literature.

In the present investigation, the term "murine pneumonia" will be used to denote any pneumonia of mice or rats regardless of etiologic agent. When pathologic lesions are described, the supposed etiologic agent will be named. This will avoid the use of numerous terms for essentially the same syndrome and will avoid implying a particular etiologic agent in the name of a disease. This is essential since this investigation is concerned with the relative contribution of two microorganisms to the pathogenesis of murine pneumonia. Although all previous studies have considered the disease as a simple infection with one etiologic agent, be it viral or bacterial, they have a direct bearing on my investigation and will be reviewed here.

Possible viral agents. In the past 25 years, pneumonia of rats and mice has been attributed to several viruses. The viral nature of three, "grey lung virus", the enzootic bronchiectasis agent, and the mouse pneumonitis agent is doubtful.

Grey lung virus. Andrewes and Glover (4) reported the isolation of "grey lung virus" from mice. They reported that the agent and the lesions produced by it persist indefinitely in infected animals but that death is only a rare occurrence. The histopathology associated with "grey lung virus" infection in mice was reported by Niven (111). She found early enlargement of interalveolar septal cells, congestion and small areas of consolidation 48 hours after intranasal instillation of a lung homogenate. After 96 hours there was an increased number of septal cells and a profusion of polymorphonuclear cells in the more extensively consolidated areas. For 6-8 days there was an increase in the number of neutrophils in the alveoli but they were largely replaced after two weeks by large mononuclear cells. Perivascular and peribronchial cuffing with lymphocytes was apparent by the third week and persisted for as long as ten months. The "virus" did not produce inclusion bodies. This differentiates it from the pneumonitis group. Lesions attributed to grey lung virus were also described in cotton rats by Andrewes and Niven (6). No attempt was made by these workers to grow the agent in a cell free medium. However, they reported that the agent was sensitive in vivo to the tetracyclines (5). The disease could be cured by treating infected animals with chlortetracycline or oxytetracycline. Sulfonamides and penicillin were without effect. Andrewes (3) stated that "grey lung virus" is, in all probability, a mycoplasma in view of its antibiotic sensitivity and general behavior.

Enzootic bronchiectasis virus or endemic pneumonia virus. Little is known regarding the properties of Nelson's enzootic bronchiectasis "virus" or endemic pneumonia "virus". Nelson and Gowan (106) believed they had eliminated chronic respiratory diseases from a colony of rats by

selective breeding. These rats and their progeny subsequently developed pneumonic lesions without middle ear involvement, and on this basis these workers justified the separation of this disease from pneumonia caused by PPLO. Nelson (99) identified the agent as a virus on the basis of negative cultural results for mycoplasma and differences in staining properties. The agent was not readily filterable. Joshi, Blackwood and Dale (64) reported the isolation of the endemic pneumonia virus in tissue culture. They produced lesions in axenic rats with a filtrate of a homogenate of the lungs of conventional rats with pneumonia. The differences between the enzootic bronchiectasis virus or endemic pneumonia virus and mycoplasma may be misleading. Andrewes (3) stated that the agent is probably a mycoplasma.

The lesions associated with the endemic pneumonia virus were transmitted to rats and mice by the nasal instillation of infected lung suspensions or nasal exudate (98, 99). The lesions in these rats were partial or complete consolidation of one or more lobes with localized purulent abscesses (98). In mice infected with the same agent, abscess formation was not evident and the lesions consisted of gray areas of consolidation. In both species the most striking microscopic lesion was an intense peribronchial lymphocytic infiltration. Polymorphonuclear infiltration was observed only in the early stages of the disease (98, 99, 101, 64).

Mouse pneumonitis agent. Nigg (109) and Nigg and Eaton (110) isolated a pneumotropic agent from mice which formed inclusion bodies. Eaton, Beck and Pearson (30) described a similar agent from cases of atypical pneumonia of man, and demonstrated its relation to the psittacosis-lymphogranuloma group. The agent from mice is now called the mouse pneumonitis agent and is placed in the

psittacosis group of bacteria by Moulder (91).

Latent respiratory viruses of mice. Three latent respiratory viruses are known: pneumonia virus of mice (PVM); Sendai virus; and the Kilham K virus.

PVM. The first description of the pneumonia associated with PVM is that of Horsfall and Hahn (56). While attempting to adapt agents causing respiratory diseases in man to mice, they employed serial mouse passage. Lung consolidation developed after a few passages in almost every series. Spontaneous pulmonary consolidation occurred in 1-2% of the animals obtained from eight different suppliers. The serial passage of lung tissue from apparently normal uninoculated mice also resulted in pneumonia after several passes. Filtration of the lung suspension through membranes indicated that infectivity was lost below an average pore diameter of 300 mμ and the virus was estimated to be 150 mμ in diameter. More recent evidence (137) indicates it is 40 mμ in diameter.

PVM causes pulmonary consolidation of 1/2 to 3/4 of the lung (56). The consolidation is more hilar in distribution in the early stages and later radiates along the bronchi. The consolidated areas are dark red in color. Microscopically, the bronchi and blood vessels are surrounded by mononuclear cells. Consolidation is also produced in germfree mice inoculated with the virus (Ward, personal communication).

Horsfall and Hahn (56) recovered PPLO from mice with pneumonia, but the organisms were also recovered from mice without clinical signs of pneumonia. Intranasal instillation of pure cultures of PPLO failed to produce pulmonary consolidation, therefore Horsfall and Hahn concluded that these organisms had no etiologic relationship to the lesions they observed. Tennant, Parker and

Ward (137) in more recent studies with PVM indicated that the virus produced an acute focal enzootic infection which was rarely fatal. They concluded that chronic latent infection did not exist and that the virus was of low infectivity. These workers also found that germfree mice were susceptible to infection and that high antibody titers to the virus could be obtained after intranasal instillation (138). They reported the $LD_{50}$ for PVM to be less than a $10^{-1}$ dilution of virus grown in primary hamster kidney cells. In a survey of germfree mice for several murine viruses, Parker et al. (117) found no evidence of infection with PVM.

PVM is presently unclassified. It can be propagated in primary hamster kidney cells. Infection is demonstrated by hemagglutination inhibition antibody, complement fixing antibody and isolation in cell culture (139).

Sendai virus. Sendai virus was shown to be indigenous to mice in Japan by Fukumi, Nishihawa and Kitayama (45). In Japan the virus produced epizootics of pneumonitis in mouse stocks lasting 3 to 4 months. The virus was easily recovered from apparently healthy mice during the epizootic (46). Tennant, Parker and Ward (139) reported that the onset of infection was acute and rapid. The experimental infection was clinically inapparent and when the mice responded adequately with antibody, the virus disappeared. Sendai virus is classified as a myxovirus, parainfluenza type 1. The diameter has been estimated as 133-250 mμ (3). The virus can be isolated in monkey kidney cells from the lungs, saliva and kidneys of infected mice during the early stages of infection. Diagnosis of infection is by hemagglutination-inhibition or isolation of the virus. No evidence of infection has been found in germfree mice (117) but 35% of conventional colonies were found to be infected (139).

K virus. The K virus was originally isolated from C3H mice (66). It is classified as a papovavirus because its nucleic acid is DNA, it is 50 mμ in diameter, heat stable and has icosahedral symmetry (3). The virus produces a fatal pneumonitis in suckling mice, but not in adult mice (66). Infection is detected by the hemagglutination-inhibition or complement-fixation test. As yet, no cell line has been reported satisfactory for propagation of the virus (139).

Mycoplasma. The generic name Mycoplasma (36) is the generally accepted name for the former designation PPLO (67). However, because the older literature refers to the organisms as PPLO, I have used the two terms synonymously. The Subcommittee on the Taxonomy of the Mycoplasmatales (38) has accepted the name M. pulmonis (129) for the organism associated with pneumonia in rodents. The Mycoplasmataceae have been reviewed extensively over the years. Some of the earlier reviews are those of Sabin (129), Edward (35), Klieneberger-Nobel (73), Freundt (44) and Kohler (75). More recent reviews are those of Klieneberger-Nobel (74), Eaton (32), Adler (1), and Chanock, Mufson and Johnson (17). In 1960 a series of papers on the biology of the pleuropneumonia-like-organisms was published in the Annals of the New York Academy of Sciences. Mycoplasma are the smallest organisms known to multiply in cell free media (17). They are 125-250 mμ in diameter, lack a rigid cell wall, require cholesterol which is incorporated into the limiting cell membrane, exhibit a characteristic form of growth on agar, and are not inhibited by penicillin or thallium acetate.

Klieneberger-Nobel and Steabben (71, 72) isolated PPLO from rats with pneumonia. The organisms were frequently recovered from the lung lesions of

rats with bronchopneumonia. They were not found at that time in young rats with apparently normal lungs but Klieneberger-Nobel and Cheng (70) later found that young rats were also infected. The lesions associated with PPLO are characterized by a severe peribronchial lymphocytic infiltration. The bronchi become distended with an accumulation of mucus, polymorphonuclear cells and monocytes. These form projecting papillae which appear macroscopically as gray translucent nodules. In the final stage, large abscesses filled with inspissated pus are present. Essentially the same pathology has been described by other investigators (58, 108).

In 1937 Nelson (94, 95) described "infectious catarrh" of mice. The first sign of disease was chattering and rapid shallow respiration. The disease progressed slowly over a period of months and eventually 90% of the animals died. Nelson (95) serially produced the same clinical signs by the nasal instillation of exudate from the upper air passages of infected mice. Nelson (95) isolated coccobacilliform bodies in tissue culture from these mice. He also regularly isolated a small nonmotile gram negative bacillus but it was not identified. The lesion associated with the coccobacilliform bodies was lung consolidation. Histologically the early lesions were characterized by occlusion of the bronchi with a polymorphonuclear exudate. Later, hyperplasia of the peribronchial lymphoid tissue was marked. These lesions are similar to the pneumonic lesions already described and to those described by Morse and Smith (89) and Ventura and Domaradzki (145). Nelson (95) reproduced pneumonia in mice by the intranasal instillation of tissue culture fluid containing the coccobacilliform bodies.

Nelson (100) later isolated PPLO from the lungs and middle ears of rats and mice. The organisms were isolated from mice on the 14th passage of apparently normal lung and middle ear suspensions. The organisms were isolated from the 16th passage in rats. Nelson (100) believed that these rats had no respiratory diseases other than endemic pneumonia which he believed was caused by a virus. At the time that Nelson isolated PPLO from mice and rats he thought that they caused pleuropneumonia of rodents and that this disease was distinct from infectious catarrh caused by the coccobacilliform bodies. He regarded the cultural and morphological characteristics of PPLO and coccobacilliform bodies to be distinct enough to warrant their separation. Later Nelson (102, 103, 104, 105) reversed this position and concluded that coccobacilliform bodies were types of PPLO. He considered that the most appropriate designation was M. pulmonis (105). Sullivan and Dienes (135) and Edward (33, 34) also demonstrated that PPLO occurred in normal mice and that they were capable of producing pneumonia.

Formerly, strains of mycoplasma isolated from mice were considered to be different from those isolated from rats. Tully (142), in a study of murine mycoplasma, classified them into three main species. M. neurolyticum is associated with rolling disease in mice; M. arthritidis is associated with polyarthritis of rats and mice and is identical to M. hominis type 2 (37). M. pulmonis is associated with pneumonia of mice and rats. On the basis of Tully's work and that of others (35, 72, 77, 78, 79), there is little valid reason for distinguishing between strains of M. pulmonis isolated from mice and rats. All of these strains are serologically indistinguishable from the

prototype. Of interest is the finding by Leach and Butler (76) that the Negroni agent and strain 880 isolated from human leukemia are related to M. pulmonis.

Lutsky and Organick (82) and Organick, Siegesmund and Lutsky (114) studied M. pulmonis, M. pneumoniae and M. salivarium in conventional and germfree mice. Pneumonia was observed in conventional mice after intranasal instillation of any of the three mycoplasma species. When each species was similarly given to germfree mice, only M. pulmonis produced lesions. These investigators concluded that these conventional mice which developed pneumonia when either M. pneumoniae or M. salivarium were given already harbored M. pulmonis, and that it was the latter organism which caused the disease. The fact that some control animals which were given sterile broth developed pneumonic lesions from which M. pulmonis was isolated also supported this conclusion. The gross and microscopic lesions produced in germfree mice did not differ significantly from those described in conventional mice, although the course of the disease was more rapid in the germfree mice.

Pasteurella pneumotropica. P. pneumotropica was first isolated and characterized by Jawetz (59, 60). In attempting to adapt a human respiratory agent to Swiss mice, blind lung passages were made. Deaths were not noted until the 7th passage, although pneumonia, as evidenced by grayish consolidation of the upper lobes, was noted as early as the 5th passage. By the 14th passage 85% of the animals died after an illness of 2 to 7 days. The clinical signs of disease were inactivity, lethargy, and ruffling of the coat as early as 18 hr after nasal instillation of mouse lung material. Breathing became labored and a conjunctival exudate glued the eyelids together. Chattering was

heard 24-48 hr after instillation of a lung suspension.

The first gross pathologic changes were observed 18-24 hr after infection at which time the lungs were mottled. Within a day or two there were foci of reddish-gray bronchopneumonia in all lobes, predominantly close to the hilus. Forty-eight to 72 hr after nasal instillation of lung material the consolidation had progressed to form sharply delineated gray foci which later developed into yellow centers of necrosis. In surviving animals, the pneumonic zones regressed whereas the necrotic areas became abscesses filled with yellowish-white viscid material.

Histologically the disease was characterized by a mixed polymorphonuclear and mononuclear exudate in the alveolar spaces and in the bronchi. The lumen of the bronchi subsequently became filled with cells.

A small gram negative, nonmotile rod was isolated from affected lungs. Biochemically the organism was lactose positive, urease positive, indole positive and oxidase positive. On the basis of biochemical and serological evidence, Jawetz placed the organism in the genus Pasteurella, and gave it the species epithet, pneumotropica because of differences in serological reactions with other pasteurella and because of the marked pneumotropism. This designation has been confirmed by other workers (52, 53, 54, 131).

Jawetz (60) found that P. pneumotropica had to be passed serially in mice before pathogenicity could be demonstrated. Immunization against the virulent strain with formalized broth cultures protected mice from later challenge with live virulent organisms.

In further studies on the pathogenesis of infection with P. pneumotropica, Jawetz and Baker (61) found that severe lesions were encountered 2 or 3 days

after intranasal instillation of virulent organisms. They also found that the disease was more easily produced in mice which were latently infected with P. pneumotropica than in mice pre-treated with chloramphenicol to kill the organism. In a survey of rodents from various animal colonies, Jawetz (60) found a 25-100% incidence of inapparent infection. The organisms were also recovered from guinea pigs, rats, cotton rats and hamsters.

An outbreak of fatal pneumonia in a breeding colony of C57/BL6 mice was observed by Gray and Campbell (50). In contrast to Jawetz's belief that experimental stress was an important factor in producing disease, these animals were maintained on a highly nutritive diet and were not subjected to any known experimental stress. The disease was controlled by the use of chloramphenicol and foster mothers.

Hoag et al. (54) were unable to demonstrate any pathogenicity of P. pneumotropica in naturally or experimentally infected mice. Intraperitoneal or intranasal instillation of freshly isolated cultures repeatedly failed to produce disease. They isolated the organism from brains, uteri, livers, testes and spleens as well as the respiratory tracts, and since no lesions were evident, they concluded that P. pneumotropica was a nonpathogenic organism latently infecting mice.

Heyl (53) studied 52 isolates of P. pneumotropica, 48 of which were from pneumonic lungs of mice. His isolates differed slightly from those described by Jawetz in that they fermented xylose. P. pneumotropica isolates from the early phase of the present investigation (11) were similar to those described by Heyl. In these studies P. pneumotropica was also found associated with

pathologic processes such as pneumonia, abscesses, conjunctivitis and metritis in mice, rats and dogs.

P. pneumotropica has been isolated from man in cases of acute and chronic respiratory infections by Henriksen and Jyssum (51). They believed at first that these strains of P. pneumotropica were variants of P. hemolytica, but in 1962 Henriksen (50) concluded that they were P. pneumotropica. Smith and Thal (131) supported this classification using a numerical technique.

Wheater (147) described the accidental infection of a cesarean-derived colony of mice and rats which was maintained under strict aseptic conditions. P. pneumotropica was isolated from breeding rats with mammary abscesses and from the throat and trachea of apparently healthy adult rats. Intranasal instillation of pure cultures of P. pneumotropica into cesarean-derived mice and rats indicated that the infective dose for rats was on the order of 100 viable cells and for mice 100-fold higher. Following intranasal instillation, the organism was never recovered from organs other than the lungs and trachea although spleen, kidney, liver, ovary and uterus were examined. No infection followed intravenous inoculation. Wheater was unable to immunize animals against the infection and the organism was not eliminated by antibiotic therapy.

P. pneumotropica produced no lesions in stock mice and rats. However, when the mice and rats were used in long term drug toxicity studies, 50% of the animals died of pneumonia attributed to P. pneumotropica. Attempts to cause exacerbation in naturally infected animals with sulfur dioxide or superinfection with influenza virus failed. In contrast, Goldstein and Green (48) recently found that the virulence of P. pneumotropica was enhanced

following bilateral nephrectomy. This procedure presumably impairs the pulmonary antibacterial activity.

The availability of specific-pathogen-free and conventional mouse colonies at Argonne National Laboratory, as well as facilities for axenic mice, offered a unique opportunity to study two of these organisms associated with pneumonia, M. pulmonis and P. pneumotropica. These were selected because M. pulmonis and P. pneumotropica had been isolated from the same diseased mouse lungs (Brennan, unpublished result). It seemed probable that one or the other of these organisms was the primary pathogen and the other a secondary invader.

Because confusion exists concerning the pathogenicity of P. pneumotropica, this investigation has been designed to determine if it is pathogenic as suggested by Jawetz (60), Heyl (53) and Wheater (147) or is nonpathogenic as suggested by Hoag et al. (54). M. pulmonis has been studied alone in axenic mice (82), but it has not been studied in conjunction with P. pneumotropica, nor has P. pneumotropica been studied in germfree mice. Pneumonias of viral etiology were not studied because, although pneumonia has often been diagnosed in animals raised in the Argonne National Laboratory animal facility, all colonies are free from PVM, Sendai and K virus infection.

The purpose of this investigation therefore has been threefold: (1) to determine the relative role of each of these organisms in the pathogenesis of murine pneumonia; (2) to determine if P. pneumotropica is pathogenic, as suggested by Jawetz (60), Heyl (63) and Wheater (147) or if it is simply a secondary invader in pathologic processes as suggested by Hoag et al. (54); and (3) to describe the lesions, if any, attributable to each organism in monoinfected axenic mice and to describe the lesions produced when

axenic mice are infected with both *M. pulmonis* and *P. pneumotropica*.

# CHAPTER II

## THE INCIDENCE OF PASTEURELLA PNEUMOTROPICA IN LABORATORY ANIMALS

Flynn, Greco and Jinkins (39) described a respiratory disease of mice raised at Argonne National Laboratory characterized by lung congestion. Microscopically, there was a bronchial exudate consisting of many polymorphonuclear cells and macrophages. Small necrotic areas occurred in the bronchial epithelium. No virus was found associated with this disease, and the only bacteria isolated were those usually considered to be secondary invaders. Flynn (personal communication) subsequently identified these bacteria as Pasteurella pneumotropica.

Two events in the Argonne National Laboratory animal facilities stimulated my interest in P. pneumotropica as a possible pathogen in laboratory animals: (1) an unusually high incidence of pneumonia in one animal room which contained about 750 CF#1/An1 mice 10-12 months of age, and (2) a high incidence of conjunctivitis in weanling mice. In both instances P. pneumotropica was isolated from the lesions. Mycoplasma pulmonis was not isolated from the lungs of these affected mice, nor was M. neurolyticum isolated from the eyes. Nelson (102) found mycoplasma in cases of conjunctivitis which Tully (142) identified as M. neurolyticum.

P. pneumotropica has been associated with pneumonia in rodents by a number workers (50, 53, 59, 60, 61). Gray and Campbell (50) noted conjunctivitis in mice with pneumonia attributable to P. pneumotropica. Nelson (102) attributed conjunctivitis in mice to mycoplasma but he also noted that small gram negative rods were present in conjunctival exudates.

As part of this project, I undertook a long term survey of the pathologic conditions with which P. pneumotropica is associated. I also surveyed the incidence of the organism in apparently healthy mice.

Jawetz (60) screened 13 colonies of mice, rats, guinea pigs, cotton rats and hamsters for P. pneumotropica. He found an overall incidence of 50%. Hoag et al. (54) noted an incidence of 23% in mice but in only nine instances were pathologic conditions associated with the organism; these were all local abscesses. They also observed that 14.6% of DBA/J2 nonproductive breeding mice harbored P. pneumotropica in the uterus.

It is a common belief that cesarean-delivered animals are free of all bacterial pathogens. Foster (43) stated that the fetal placenta acts as an efficient filter and protects the fetus from the bacterial and viral agents carried by the mother. Thus, he believed that cesarean delivery could free a colony of all the common pathogens. Laboratory mice are often described by experimenters or commercial breeders as "cesarean-derived and barrier-sustained"; "specific-pathogen-free" or simply as "pathogen-free". When a cesarean-derived colony becomes contaminated, it is generally assumed that a break in the environmental barrier occurred. Several reports in addition to that of Hoag et al. (54) show this belief to be false.

Tregier and Homburger (141) found Escherichia coli, Aerobacter aerogenes, Proteus species, Corynebacterium species and streptococci in the uteri of adult and immature mice from various sources. Graham (49) isolated mycoplasma from the uteri of 33 of 77 nonproductive female breeder rats. These findings suggest that preparturient, intrauterine infections may occur. Accordingly, as part of this survey, mice of various ages were examined to determine if

their uteri were infected with P. pneumotropica, if the embryos were infected, and if uterine infection had any effect on the ability to maintain conception through gestation. Parts of the work reported in this chapter have already been published in the open literature (11, 40, 41).

## MATERIALS AND METHODS

Animals. Animals submitted for microbiological examination were either raised in the Argonne National Laboratory Division of Biological and Medical Research animal facility or purchased from commercial breeders. Many of these animals were experimental animals in one or another of the Division's projects.

Animals for screening studies were the following:

CF#1/An1. This colony was initiated by Dr. R. J. Flynn of Argonne National Laboratory in 1957-1958. In December, 1957, one litter of axenic "Swiss" mice was obtained from the Lobund Institute, South Bend, Indiana. In September, 1958, 100 gravid CF#1 mice purchased from Carworth, Inc. (New City, Rockland County, N. Y.) were cesarean delivered and the progeny were foster nursed on the Lobund "Swiss" mice. At the time of the present study the colony was free from Salmonella species and Pseudomonas aeruginosa as determined by cultural methods. In September 1958, it was free from ectoparasites with the exception of Radfordia affinis and endoparasites with the exception of Syphacia obvelata. Thirty-five retired breeders were submitted to Microbiological Associates, Bethesda, Maryland in 1965 for serologic determination of antibodies to the indigenous mouse viruses, Reo 3, PVM, GD VII, Polyoma, K, Sendai, Rat, mouse adenovirus and mouse hepatitis virus. All were negative. Most of the clinical cases of pneumonia came from this colony.

Mice from this colony were used for the uterine infection study. The uteri of four groups of mice were cultured for the presence of P. pneumotropica and other bacteria. The groups were arranged as shown below:

| Group | Number of Mice | Age in Days | Reproductive Status |
|---|---|---|---|
| 1 | 30 | 140-160 | Virgin |
| 2 | 30 | 140-160 | Had been bred and produced litters but male removed |
| 3 | 30 | 140-160 | Bred but barren although the male remained |
| 4 | 30 | 140-160 | Gravid in their last week of gestation |

In addition, the fetuses and fetal membranes of 30 animals from group 4 were cultured.

CF#1/An1/SPF-1. This colony was initiated in the fall of 1963 by cesarean delivery of gravid CF#1/An1 mice, foster nursing on axenic HA/ICR mice purchased from A. R. Schmidt Co., Madison, Wis. The young were removed from the germfree isolators in December, 1963 and bred. These mice were free from Salmonella sp. and P. aeruginosa as determined by culture. No P. pneumotropica was detected until late 1966 when it was recovered from several mice. The colony was free from internal and external parasites. Antibodies against Reovirus 3 were present but serologic tests for other indigenous mouse viruses were negative.

CF#1/An1/SPF-2. This colony was instituted in December, 1966. They are the progeny of axenic CF#1 mice purchased from Carworth, Inc. (New City, N. Y.). Mice in this colony are never more than four generations from germfree. When breeders from the fourth generation are retired, they are replaced with mice from the germfree isolators. The colony at present appears to be free from Salmonella sp., P. aeruginosa, P. pneumotropica, and internal and external

parasites. Antibodies to mouse hepatitis virus are present but antibodies to other murine indigenous viruses have not been detected.

SD/An1/SPF-2. This colony was instituted in 1965 by Dr. Flynn from germfree rats he brought from the National Institutes of Health, Bethesda, Maryland. Its status is essentially the same in terms of pathogens as the CF#1/An1/SPF-1 colony. P. pneumotropica has never been recovered from the breeding animals but it has been recovered from stock animals which are segregated from the breeding colony at weaning.

Miscellaneous colonies. Small breeding colonies of miscellaneous strains are also maintained. These strains are designated "SPF" if the breeding nucleus was cesarean-derived and foster nursed on axenic mice.

Commercially produced mice. Mice designated "Lynch-Webster" or "ICR" were purchased from ten commercial breeders. The mice were discarded breeding pairs, at least 8 months of age.

The scientific names of the animal species used throughout these studies are listed below:

Mouse (Mus musculus)

Rat (Rattus norvegicus)

Mastomys (Rattus natalensis)

Hamster (Mesocricetus auratus)

Cotton rat (Sigmodon hispidus)

Kangaroo rat (Dipodomys sp.)

Dog (Canis familiaris)

Rabbit (Oryctolagus cuniculus)

Media. The following media were used: (1) Blood agar (BA) consisting of 5% sterile sheep blood (ARS Serum Co., Madison, Wisconsin) added to a base of Tryptic Soy Agar (Difco); (2) Tryptic Soy (TS) Broth (Difco); (3) PPLO agar with the following composition per liter: 21 g PPLO Broth (Difco), 10 g Trypticase (BBL), 2 g yeast extract (Oxoid), 15 g Agar (Difco). The pH was adjusted to 7.8 with NaOH and the medium was autoclaved 15 min at 121C. When cool, 200 ml sterile horse serum (Grand Island Biologicals, Grand Island, N. Y., lot #24) was added aseptically, and the plates were poured. Penicillin plates were prepared by adding 200 units/ml benzyl penicillin (Parke, Davis and Co., Detroit, Mich.) aseptically before pouring the plates; (4) Brom Thymol Blue Broth Base (Difco) was used for carbohydrate studies. The appropriate carbohydrate was added to a final concentration of 1.0%; (5) Motility Test Medium (Difco) was used to test for indole production; (6) TS broth with 8% gelatin (Difco) was used to test for gelatin liquefaction; (7) OF Basal Medium (Difco) to which 0.5% glucose was added aseptically was used for the OF test; (7) MR-VP (Difco) was used for the Methyl red and Voges-Proskauer test; (9) Simmons citrate agar (Difco) was used to test for citrate utilization; (10) Kligler's Iron Agar (Difco) or lead acetate impregnated strips were used to test for $H_2S$ production, and (11) MacConkey Agar (Difco) for growth on this medium.

Reagents. Kovac's reagent was used to test for indole production. It was prepared by dissolving 5 g p-dimethylaminobenzaldehyde in 75 ml isoamyl alcohol held at 55 C in a water bath. When solution was complete 25 ml concentrated HCl was added.

Urea broth (Difco) was used to test for urea hydrolysis. It was occasionally supplemented with 1.0% horse serum for P. pneumotropica strains which grew poorly in the broth.

A 1% solution of N, N dimethyl-p-phenylene diamine monohydrochloride was used for the oxidase test. In later stages Patho Tec test strips (Warner Chillicot) were used.

The gram stain used in this work is a modification developed by R. S. Benham of the University of Chicago. The formulation of the solutions and directions for their use follow.

A stock solution of 5% crystal violet in 95% ethyl alcohol is prepared. A stock solution of 90% phenol is prepared by dissolving a 1 lb bottle of phenol crystals (ACS reagent grade; Merck, Rahway, N. J.) in 45 ml distilled water. The working solution is prepared by adding 50 ml stock crystal violet solution and 5.5 ml phenol solution to 300 ml distilled water. The volume is adjusted to 500 ml with distilled water, allowed to stand overnight at room temperature and filtered. An aqueous solution of 5% resublimed iodine and 10% potassium iodide is prepared. The working solution is a 1:5 dilution of the stock solution. Equal parts of acetone and ethyl alcohol are used as the decolorizer and 0.25% aqueous safranin is used as the counter stain.

Smears are flooded with crystal violet for 30 sec, then iodine for 30 sec. They are then decolorized for 1-2 sec, washed and counterstained for 1 min with safranin.

The reagents for the Voges-Proskauer test were 5% alcoholic $\alpha$ naphthol and 40% aqueous KOH. Methyl red (0.04% aqueous) was added to a tube of medium

for the MR test. One drop of a 3% $H_2O_2$ solution was used to test for catalase production.

Sampling methods. Tissues, other than lung, and other materials to be cultured were collected aseptically and smeared on a small segment of BA plates and PPLO plates with and without penicillin. The plates were incubated at 37 C for 18-24 hr and examined. PPLO plates were sealed with tape to prevent evaporation, and incubated at 37 C for 7 days. The plates were examined every 2 days for typical mycoplasma colonies. Lungs were ground in a small amount of TS broth in a sterile Ten Broeck tissue grinder and 0.1 ml was plated on a BA plate and on PPLO agar.

Animals from the Anl colonies were killed with chloroform. A pharyngeal culture was taken using a sterile cotton swab prepared on a toothpick. In some cases the nasal passages were opened by dividing the turbinates and a sample of the nasal secretions was taken with a sterile cotton toothpick swab. Both the pharyngeal and nasal swabs were placed together in one tube containing 1 ml TS broth. These swabs were subsequently smeared on BA and PPLO plates.

When uterine cultures were made, the peritoneum was incised with one set of instruments and another set of sterile instruments was used to remove the uterus. The uterine horns and body were incised, the endometrial surface was smeared on BA and PPLO plates and the entire uterus was placed in a tube containing 10 ml TS broth. The fetuses and fetal membranes from the gravid mice were also cultured on BA and PPLO agar and in TS broth. The TS broth was incubated at 37 C for 18-24 hr and then streaked on BA. These plates were incubated for an additional 18-24 hr.

Identification of isolates. P. pneumotropica was identified by its gram reaction, colonial morphology and biochemical reactions.

Mycoplasma species were identified by the typical "fried egg" appearance of the colonies growing on PPLO agar. When colonies were observed growing on penicillin media only, they were subcultured to media without penicillin to determine if the colonies were L forms of bacteria. Other bacteria were identified according to standard methods.

## RESULTS

Characteristics of P. pneumotropica. All of the P. pneumotropica isolates from pathologic processes have been identical in cultural characteristics and remarkably similar in biochemical characteristics. The biochemical reactions are summarized in Table 1. All of the isolates produced acid in xylose and failed to form acid in mannitol and inositol.

P. pneumotropica is a short pleomorphic nonmotile gram negative rod that does not form spores. On blood agar, the colonies are convex, entire, dew drop and grayish-yellow in color. No hemolysis is produced although greening sometimes occurs under the colonies. The colonies are about 1 mm in diameter after 24 hr incubation and attain a maximum size of about 2 mm on prolonged incubation. On PPLO agar with horse serum growth is much more luxuriant. Colonies are white, convex and have a creamy consistency. They attain a maximum size of 4-5 mm in diameter on prolonged incubation.

Association with disease. Table 2 shows the species of animal from which P. pneumotropica and Mycoplasma species were isolated, and the pathologic conditions with which they were associated. P. pneumotropica was recovered from 100% of the mice with pneumonia or conjunctivitis, but it was only recovered from rats with pneumonia 50% of the time. Mycoplasma were recovered from all the rats with pneumonia. In most instances P. pneumotropica was recovered in pure culture. The only exceptions were when it was recovered from the internal organs of dogs with septicemia. Some of these dogs had received $\gamma$ irradiation from $^{60}Co$ so invasion of the tissues with numerous bacteria was expected.

A preliminary survey showed that there was almost a 100% incidence of P. pneumotropica in the respiratory tract of discarded breeding CF#1/An1 mice. Ten out of 84 uteri of these mice were also infected with P. pneumotropica. Samples of each shipment of 6-8 week old mice received from commercial breeders showed an incidence of 96.3% infection in the pharynx.

P. pneumotropica in ANL mice. Results of a survey of the respiratory tract of animals raised at Argonne National Laboratory are shown in Table 3. The CF#1/An1 colony was almost 100% infected with P. pneumotropica. The CF#1/An1/SPF-1 colony had approximately a 10% incidence in weanling and 100 day old stock animals, but the organism was not recovered from retired breeders. The recoveries of P. pneumotropica from weanling and stock mice were made in late 1966, shortly before the colony was abandoned. Prior to that time it was not recovered. On the other hand, there is a consistent recovery of P. pneumotropica from 50% of the SD/An1/SPF rats 100 days of age.

The results of nasopharyngeal and lung cultures of retired breeding mice from commercial suppliers are given in Table 4. A high incidence of P. pneumotropica infection was noted in the nasopharynx of all the mice, regardless of source (87.2%). Mycoplasma were only recovered from four mice, and these were all from the same source. Compared to the high incidence in the nasopharynx, P. pneumotropica was isolated from only 21.9% of the lungs of the same mice. Mycoplasma were recovered from the lungs of one animal. Seven of the 110 animals also had lung infections with P. aeruginosa and three were infected with Klebsiella pneumoniae.

The results of the uterine cultures of the females from these mice are shown in Table 5. P. pneumotropica were the most frequently recovered

organisms; mycoplasma were not isolated. Three of these mice showed the presence in the uterus of lactobacilli, two of corynebacteria and two of micrococci.

Uterine infection in CF#1/Anl mice. Numerous species of bacteria were recovered from the uteri and fetuses examined in this study. The incidence of bacteria in the uteri of gravid females was higher than was observed in nongravid females although the incidence of P. pneumotropica was apparently less. Approximately 20% of the CF#1/Anl virgin females had one or more kinds of bacteria in their uteri (Table 6). During the period of this study 430 female mice were mated. Only five failed to conceive and deliver viable young by the time they were 90 days of age. Thus, mice harboring bacteria in the uterus conceive and can maintain the conception through gestation.

The incidence and the kind of bacteria observed in the recently productive but nongravid females (because they had been kept separate from the male after parturition) is like that of virgin females. The data obtained from previously productive but currently barren females is similar to the other two groups. Fewer mice had P. pneumotropica uterine infection if they had been previously productive but were barren at the time of culture.

P. pneumotropica was isolated from only one gravid uterus and from none of the fetuses and membranes from these gravid females. Mycoplasma were recovered from the fetuses and membranes of one gravid female. No uterus yielded mycoplasma on culture.

TABLE 1. Biochemical reactions of Pasteurella pneumotropica

| Substrate or test | Reaction after 48 hrs at 37 C |
|---|---|
| Glucose | A‡ |
| Lactose | A |
| Sucrose | A |
| Maltose | A |
| Mannitol | - |
| Xylose* | A |
| Inositol | - |
| Indole production* | + |
| Methyl red | - |
| Voges-Proskauer | - |
| Citrate utilization | - |
| Urea hydrolysis* | + |
| $H_2S$ production | |
| Kligler | - |
| Lead acetate paper | + |
| OF + glucose | |
| open* | + |
| closed | + |
| Gelatin hydrolysis | - |
| Catalase | + |
| Oxidase | + |
| Growth on MacConkey | + |

*occasionally delayed beyond 48 hrs; ‡A = acid produced.

TABLE 2. Distribution of Pasteurella pneumotropica and Mycoplasma species

| Animal species | Pathologic condition | Specimen | Number isolates/Number cultured | |
|---|---|---|---|---|
| | | | P. pneumotropica | Mycoplasma sp. |
| Mouse | Pneumonia | Lung | 108/108 | 3*/108 |
| Mouse | Conjunctivitis | Eye exudate | 27/27 | 0/27 |
| Mouse | Metritis† | Uterine exudate | 8/18 | 0/18 |
| Mouse | Urocystitis | Urine | 5/7 | Not done |
| Mouse | Local abscesses (pleural and peritoneal) | Exudate | 17/17 | 0/17 |
| Mouse | Dermatitis | Skin | 3/5 | Not done |
| Rat | Pneumonia | Lung | 22/45 | 45/45 |
| Kangaroo rat | Conjunctivitis | Eye exudate | 2/2 | 0/2 |
| Buffalo rat | Pneumonia | Lung | 0/2 | 2/2 |
| Mastomys | Conjunctivitis | Eye exudate | 2/2 | 0/2 |
| Hamster | Pneumonia | Lung | 1/1 | 0/1 |
| Hamster | Metritis | Uterine exudate | 2/2 | 0/2 |
| Dog | Otitis | Ear exudate | 2/6 | Not done |
| Dog | Abscess | Kidney | 2/7 | Not done |
| Dog | Septicemia | Blood and organs | 15/24 | Not done |
| Dog | Pneumonia | Lung | 4/17 | Not done |
| Dog | Peritonitis | Peritoneal fluid | 1/1 | Not done |

* These were conventionally raised Balb/c mice.

† Two of these isolates were from virgin mice.

TABLE 3. Incidence of Pasteurella pneumotropica in the pharynx of apparently healthy ANL raised rodents

| Strain | Age in days | Number positive/ Number cultured | Percent positive |
|---|---|---|---|
| CF#1/Anl | 365 | 422/443 | 95.5 |
| | 21 | 122/128 | 95.4 |
| CF#1/Anl/SPF-1 | 365 | 0/142 | 0 |
| | 100 | 17/176 | 9.7 |
| | 21 | 16/130 | 12.3 |
| CF#1/Anl/SPF-2 | 365 | 0/132 | 0 |
| | 100 | 0/62 | 0 |
| | 21 | 9/127 | 0 |
| SD/Anl/SPF | 365 | 0/15 | 0 |
| | 100 | 58/110 | 52.7 |
| Miscellaneous conventional strains | 365 | 58/62 | 93.5 |
| Miscellaneous SPF strains | 365 | 0/40 | 0 |
| | 100 | 0/56 | 0 |
| | 21 | 0/25 | 0 |

TABLE 4. Incidence of Pasteurella pneumotropica and Mycoplasma sp. in the nasopharynx of commercially produced discarded breeder mice

| Source | Strain of mouse | Number examined | Number Positive | | | |
|---|---|---|---|---|---|---|
| | | | Nasopharynx | | Lungs | |
| | | | P. pneumotropica | Mycoplasma sp. | P. pneumotropica | Mycoplasma sp. |
| 1 | ICR | 10 | 9 | 0 | 1 | 0 |
| 2 | ICR | 10 | 4 | 0 | 0 | 0 |
| 3 | ICR | 10 | 0 | 0 | 0 | 0 |
| 4 | ICR | 10 | 10 | 0 | 9 | 0 |
| 5 | ICR | 10 | 10 | 0 | 0 | 0 |
| 6 | ICR | 10 | 9 | 4 | 2 | 1 |
| 6 | Lynch-Webster | 10 | 6 | 0 | 5 | 0 |
| 7 | Lynch-Webster | 10 | 10 | 0 | 2 | 0 |
| 8 | Lynch-Webster | 10 | 8 | 0 | 2 | 0 |
| 9 | Lynch-Webster | 10 | 10 | 0 | 2 | 0 |
| 10 | Lynch-Webster | 10 | 10 | 0 | 1 | 0 |
| Totals | | 110 | 96 | 4 | 24 | 1 |

TABLE 5. Incidence of Pasteurella pneumotropica and Mycoplasma sp. in the uterus of commercially produced discarded female breeding mice from commercial suppliers

| Source | Strain of mouse | Number examined | Number sterile | Number infected | |
|---|---|---|---|---|---|
| | | | | P. pneumotropica | Mycoplasma sp. |
| 1 | ICR | 5 | 2 | 1 | 0 |
| 2 | ICR | 5 | 2 | 1 | 0 |
| 3 | ICR | 5 | 5 | 0 | 0 |
| 4 | ICR | 5 | 4 | 1 | 0 |
| 5 | ICR | 5 | 4 | 0 | 0 |
| 6 | ICR | 5 | 3 | 1 | 0 |
| 6 | Lynch-Webster | 5 | 4 | 1 | 0 |
| 7 | Lynch-Webster | 5 | 2 | 1 | 0 |
| 8 | Lynch-Webster | 5 | 4 | 1 | 0 |
| 9 | Lynch-Webster | 5 | 2 | 1 | 0 |
| 10 | Lynch-Webster | 5 | 5 | 1 | 0 |
| Total | | 55 | 37 | 9 | 0 |

TABLE 6. *Pasteurella pneumotropica* and *Mycoplasma* sp. in CF#1/An1 uteri and fetuses

| Group | Material examined | Number examined | *P. pneumotropica* | *Mycoplasma* sp. | Other |
|---|---|---|---|---|---|
| Virgin females | Uteri | 30 | 3 | 0 | 4 |
| Productive but now non-gravid | Uteri | 30 | 5 | 0 | 4 |
| Productive but now barren | Uteri | 30 | 2 | 0 | 6 |
| Gravid | Uteri | 50 | 1 | 0 | 13 |
| Gravid | Fetuses and membranes | 30 | 0 | 1 | 9 |

## DISCUSSION

All 221 isolates of P. pneumotropica from several disease processes in seven species were essentially the same as the strains Heyl (53) isolated from mice with pneumonia. Although it is probable that I have studied fewer than 221 strains, at least 100 came from widely separated geographic areas and different animal species. All of my isolates were lactose and xylose positive and inositol and mannitol negative, as were Heyl's. Wheater's (147) isolates were also lactose and xylose positive and mannitol negative. The strains from mice studied by Hoag et al. (54) were lactose positive although 41 out of 47 cultures were also mannitol positive. Smith and Thal (131) studied three strains of P. pneumotropica, all of which were isolated from man. These strains were lactose and inositol positive and xylose negative. All of the strains studied to date were indole, oxidase and urease positive and all but those studied by Hoag were mannitol negative.

The justification for the classification of P. pneumotropica as a separate species of the genus Pasteurella has been questioned (147). Jawetz (60) visualized P. pneumotropica as being at one end of a sliding virulence scale and P. multocida, P. pestis and P. tularensis at the other. These latter three are highly invasive organisms with a potentially high virulence by any route of administration; P. pneumotropica is of low invasiveness and virulence. Jawetz also regards the tissue tropism exhibited by P. pneumotropica as of some significance in separating it from P. multocida and the other species of Pasteurella. He associated P. pneumotropica only with pneumonia and could demonstrate pathogenicity only by the intranasal route whereas P. multocida causes a fulminating hemorrhagic septicemia in mice regardless of route

of inoculation. Smith and Thal (131), using an Adansonian technique, concluded that P. pneumotropica, although related to P. multocida, is sufficiently different from it to warrant separate species classification. For convenience the differential characteristics of Pasteurella species are tabulated below.

CHARACTERISTICS OF THE PASTEURELLA SPECIES

| Substrate or Test | P. pestis | P. pseudotuberculosis | P. tularensis | P. multocida (septica) | P. pneumotropica | P. hemolytica |
|---|---|---|---|---|---|---|
| OF (glucose) | F | F | F | F | F | F |
| Lactose | - | - | - | - | A | A |
| Sucrose | - | - | A | A | A | A |
| Indole | - | - | - | + | + | - |
| Urease | - | - | - | - | + | - |
| Catalase | + | + | + | + | + | + |
| Oxidase | - | - | - | + | + | + |
| Motility | - | +* | - | - | - | - |
| Hemolysis | - | - | - | - | - | β |
| Growth on MacConkey | + | + | - | - | ± | - |
| Requires blood and cysteine | - | - | + | - | - | - |

* motile at 22 C

Minor differences in carbohydrate utilization and pathogenicity are admittedly poor criteria for the separation of species. However, in the absence of large-scale surveys and a thorough analysis of antigenic relationships, the use of such criteria seems unavoidable at present. Furthermore,

Jones (62) has pointed out that the classification of the pasteurellae rests heavily on pathogenicity. Accordingly, I differentiate P. pneumotropica from P. multocida, because this paper deals primarily with pathogenicity. P. pneumotropica can be described as an organism of low virulence which is lactose, indole, urease, and oxidase positive and mannitol negative. P. multocida is virulent for mice, is lactose and urease negative and is indole and oxidase positive. The majority of strains are mannitol positive. Variable characteristics of the two species include xylose and inositol utilization. Other workers (54, 131) have reported that P. pneumotropica does not grow on MacConkey's agar, however, my isolates do so to a slight extent. This is not regarded as a definitive characteristic.

This is the first report of the isolation of P. pneumotropica from the eyes of mice affected with conjunctivitis. Although other workers have noted gram negative bacteria in the eyes of mice so afflicted (50, 102), they did not identify the organisms. The failure to isolate mycoplasma from these cases of conjunctivitis suggests that P. pneumotropica caused the lesions. However, mycoplasma are notoriously difficult to isolate, especially when they are present in a mixed infection. My inability to recover them from 27 mice does not rule out their presence. The same can be said for the few recoveries of mycoplasma from mice with pneumonia. The only conclusion that can be drawn from this survey is that P. pneumotropica is often associated with pneumonia in mice. The association with pneumonia confirms the findings of Jawetz (60), Heyl (53) and Gray and Campbell (50) and is in sharp contrast to Hoag et al. (54). This association sheds no light on whether P. pneumotropica caused the lesions or was merely a secondary invader in the disease process. In rats this

latter alternative is suggested because mycoplasma were recovered from all the pneumonic lungs, whereas P. pneumotropica was recovered from only half of them.

My results (Table 2) support Hoag's observation that P. pneumotropica can be isolated from tissues other than lung. Although a marked pneumotropism is evident, these results demonstrate that it is common in metritis, urocystitis and local abscesses in rodents. Of particular interest is that the organism was isolated from two virgin mice with metritis. Hoag et al. (54) found that 14.6% of the mice they examined harbored P. pneumotropica in the uterus. This agrees well with my preliminary result of 11.9% infection in the uteri of CF#1/An1 mice. The survey of 110 uteri (Table 6) showed a 10% incidence of infection. Mice from commercial suppliers were similarly infected with P. pneumotropica although the incidence was slightly higher (Table 5). Jawetz and Baker (61) were unable to isolate P. pneumotropica from mouse embryos and concluded that the organism was not transmitted in utero, but rather from the dam to the offspring via the respiratory route. I did not isolate P. pneumotropica from the fetuses or fetal membranes of thirty gravid females (Table 6), however only one of the dams was infected so the significance of this finding is questionable. The fact that approximately 20% of the mice examined had a uterine infection with P. pneumotropica strongly suggests that transmission in utero or during parturition is possible.

This is the first report of P. pneumotropica associated with disease in laboratory dogs. The isolation from cases of otitis, peritonitis, and kidney abscesses in apparently normal stock dogs is of particular interest. It must be emphasized that these dogs are purebred beagles raised at Argonne National Laboratory, rather than pound dogs whose history is unknown. Therefore,

although rodents appear to be the principal host of this organism, dogs may also carry the organism. With the exception of dogs with septicemia, P. pneumotropica was isolated most frequently from the lungs of dogs with pneumonia (Table 2). This demonstrates a tissue tropism of this organism in another species of animal similar to that observed in mice and rats.

The isolation of P. pneumotropica from 93.5-95.5% of conventionally raised mice and rats at Argonne National Laboratory (Table 3) and from the respiratory tract of 87.2-96.3% of the mice from commercial sources (Table 4) reflects the true carrier rate in apparently healthy animals more accurately than results based on the culture of internal organs. This is confirmed by lung cultures where P. pneumotropica was recovered from only 21.9%. Nevertheless this is a high incidence of lung infection and it is not known whether or not any of these lungs exhibited microscopic lesions. Although mycoplasma have not been recovered from healthy mice killed for screening studies, it seems plausible to assume that they are present in numbers too low to be detected by the techniques which are presently available. This is especially likely since mycoplasma were recovered from mice from one commercial supplier and not the others. These mice were probably more heavily infected.

Wheater (147) believes that his rats became infected with P. pneumotropica from the caretakers. He bases this on the isolation of P. pneumotropica from man by Henriksen and Jyssum (51). My experience does not confirm Wheater's belief. The two CF#1/Anl/SPF colonies in this study have been cared for by the same caretaker who also had the responsibility of caring for the CF#1/Anl colony. Furthermore the rooms housing the animals are adjacent to one another. The failure to isolate P. pneumotropica from the SPF colony suggests that

infection from man does not occur to any significant extent. It seems more probable that mouse to mouse infection is more prevalent. It should also be noted that cesarean section served to reduce infection in the CF#1/An1/SPF-1 colony to approximately 10% of what it had been although the organism was not eliminated. Whether those mice became infected from other rodents or whether they became infected in utero is not known at this time.

Data from the uterine survey (Tables 5 and 6) suggests that pregnancy does not permanently alter or eliminate bacteria from the mouse uterus. The same type and incidence of bacteria in the virgin and barren mice support this view. The increased incidence of bacteria in gravid females may be partly due to the problems related to aseptic removal of gravid uteri. While maximum caution aimed at prevention of contamination was exercised, the handling of large, gravid uteri filled with fetuses carried with it a greater chance of contamination than does handling of non-gravid uteri. The concommitant rise in positive cultures may also explain in part the apparent decreased incidence of P. pneumotropica in gravid females. The significance of the single isolation of mycoplasma from the fetuses and membranes of gravid females is unclear, especially since they were not recovered from gravid or non-gravid uteri. In view of these results, however, it seems prudent to assume that all cesarean-derived animals are not free of bacteria.

In summary, 221 isolates of P. pneumotropica from disease processes in laboratory animals were studied. The strains were similar in biochemical and cultural characteristics regardless of host species and resembled those described by Heyl (53).

P. pneumotropica was found in the respiratory tract of almost all conventionally raised rodents but the incidence was much less in cesarean-derived animals. Studies on uterine infection suggest that in utero transmission of P. pneumotropica is possible.

The isolation of P. pneumotropica from so many animals with pneumonia suggests that the organism plays a role in the disease process. The isolation of mycoplasma from three mice and 47 rats with pneumonia similarly suggests that mycoplasma are important in the disease syndrome. The relative importance of each is not clear from these results.

# CHAPTER III

## EXPERIMENTAL INFECTION OF CONVENTIONAL AND SPECIFIC PATHOGEN FREE MICE WITH PASTEURELLA PNEUMOTROPICA AND MYCOPLASMA PULMONIS

In Chapter II it was shown that Pasteurella pneumotropica is associated with pneumonia as well as a variety of other pathologic conditions in laboratory animals. It was associated with pneumonia more frequently in mice than in rats. Mycoplasma pulmonis was found associated with pneumonia in rats but P. pneumotropica was found in the lesions of half the animals studied.

In Chapter I, it was pointed out that most workers have considered the most common cause of pneumonia in mice and rats to be M. pulmonis. Scant attention has been given to P. pneumotropica and one group (54) flatly states that P. pneumotropica is a nonpathogenic organism.

The study by Lutsky and Organick (82) is the only one in which the indigenous flora of some of the experimental mice was known. These were axenic mice. In view of the high incidence of P. pneumotropica in conventionally raised animals it seems likely that rodents used in numerous studies of M. pulmonis infection were concurrently infected with P. pneumotropica. Similarly, the conventionally raised mice studied by Jawetz (59, 60, 61) and by Gray and Campbell (50) were probably infected with M. pulmonis.

Several possibilities are suggested by this line of reasoning: (1) Murine pneumonia is caused by M. pulmonis, and P. pneumotropica is a secondary invader. If this is true, the disease ascribed by Jawetz (60) to P. pneumotropica would in reality be due to undetected mycoplasma; (2) P. pneumotropica is pathogenic and, under the appropriate circumstances, is

capable of producing pneumonia in the absence of M. pulmonis. In this case the pneumonia reported as caused by M. pulmonis in conventional animals would be due to the stress of superinfection with M. pulmonis of mice already infected with P. pneumotropica and (3) Spontaneous murine pneumonia is caused by simultaneous infection with both organisms.

The availability of conventional animals infected with P. pneumotropica and M. pulmonis as well as the availability of specific pathogen free mice presumably free from both organisms presented a means of studying these three alternatives.

Mice were chosen for study because they were more readily available to me and because they are smaller, thereby allowing more animals per experimental group.

## MATERIALS AND METHODS

Media. In addition to the media described in Chapter II, the following were used: (1) PPLO broth formulated as for PPLO agar in Chapter II with the omission of agar; (2) Nitrate broth (Difco); (3) PPLO broth with 0.5% carbohydrate and 1 drop of 1% alcoholic phenol red for carbohydrate studies with M. pulmonis; (4) PPLO broth with glucose and phenol red plus 0.04% agar for the OF reaction; (5) PPLO agar was overlayed with a thin layer of agar containing 1% sheep red blood cells to determine hemolytic activity of M. pulmonis.

Reagents. In addition to the reagents described in Chapter II, 0.8% sulfanilic acid in 5 N acetic acid and 0.5% $\alpha$ naphthylamine in 5N acetic acid were used to test for nitrate reduction. Two drops of each solution were added to a culture in nitrate broth and two to an uninoculated tube of medium. The tubes were shaken vigorously.

Source of cultures. A strain of P. pneumotropica isolated from a diseased mouse, designated 1132, was used in three experiments. The biochemical properties of this strain are shown in Table 1 in Chapter I. The M. pulmonis used in these studies, which I have designated N1, was isolated from the lungs of six Princeton mice supplied by Dr. John B. Nelson of The Rockefeller Institute. These mice were intranasally instilled by Dr. Nelson (passage number 496B) with a lung suspension containing M. pulmonis. The suspension contained 1000 units of penicillin. From the lungs of these mice I also isolated a strain of P. pneumotropica. I have designated this strain 9538. A strain of M. pulmonis (ATCC 14267) was obtained from the American Type Culture Collection and a strain which I have designated "Milwaukee" was

supplied by Dr. Avrum Organick of Marquette University. M. arthritidis (ATCC 13988) was also obtained from the American Type Culture Collection.

Following primary isolation the organisms were subcultured twice on the appropriate solid media to check for purity. A single colony of P. pneumotropica was then inoculated into several tubes of TS broth and incubated 18-24 hr at 37 C. The tubes were centrifuged at 1000 x g in a Servall clinical centrifuge for 30 min. The pellets were washed twice in sterile saline, and then resuspended in 0.2 ml sterile skim milk. These suspensions were dispersed in 0.1 ml amounts in sterile ampules (Bellco Glass Co., Vineland, N. J.). The ampules were frozen in a bath of alcohol and dry ice and then attached to a lyophilization apparatus. When the contents of the ampules were dry, the tubes were sealed under vacuum. M. pulmonis cultures were similarly preserved by lyophilization. The pellets from broth cultures were washed in sterile PPLO broth. After they were lyophilized the cultures were stored at -90 C. Portions of diseased lungs from the original mice were frozen and stored at -20 C.

Working cultures were prepared by emptying the entire contents of an ampule into a tube of the appropriate broth. These tubes were incubated at 37 C for 24-48 hr. Subcultures were then made to fresh media.

Preparation of inocula. A working culture of P. pneumotropica was serially diluted in 0.9 ml sterile saline blanks. Two plates were poured from each dilution using TS agar. The plates were incubated and the number of P. pneumotropica cells in the inoculum calculated on the basis of the dilution which yielded plates with 50-200 colonies. Serial tenfold dilutions of M. pulmonis were made in PPLO broth. A micropipette calibrated to deliver

0.025 ml (Cooke Engineering Co., Alexandria, Va.) was used to inoculate two PPLO agar plates with drops from each dilution. The plates were sealed and incubated for 3 days at 37 C. The number of M. pulmonis colony forming units (CFU) in the inoculum was calculated on the basis of the dilution which yielded 20-50 CFU per drop.

When organisms were to be used in a mixed inoculum the P. pneumotropica was grown in PPLO broth and equal volumes of the two cultures mixed. The remainder of each broth culture was diluted with an equal volume of sterile broth for parallel inoculation with each organism alone.

Light microscopy. PPLO plates were inspected in an inverted position by direct light at a magnification of 100 X. Eighteen hr broth cultures of M. pulmonis were examined for motility with a Zeiss binocular microscope equipped with darkfield phase contrast at a magnification of 1000 X. Tissue sections were examined with a Leitz Ortholux microscope equipped with a 35 mm automatic camera.

Electron microscopy. Electron micrographs of growing broth cultures of M. pulmonis N1 were made. Working cultures were incubated for 24, 48 and 72 hr. Drops of media were placed on Formvar membrane-covered 300-mesh copper grids. After a few seconds the excess liquid was drawn off by touching the side of the grid with filter paper. A drop of 2% sodium phosphotunstate solution (pH 7.0) was applied to the grid and the excess drawn off. The grids were air dried and examined with an RCA 3F electron microscope at an instrumental magnification of 16,000 X.

Antisera production. New Zealand white rabbits were used to produce all antisera. The antigens for the mycoplasma strains were prepared by inoculating

two two-liter flasks of PPLO broth supplemented with 20% rabbit serum with 60 ml of a working culture grown in rabbit serum PPLO broth. The flasks were incubated in an incubator shaker (Lab-line Instruments, Inc., Melrose Park, Ill.) for 48 hr at 37 C. The cells were harvested by centrifuging at 15,000 x g. The pellet was washed three times with sterile saline and resuspended in saline. The absorbance of the suspension was adjusted to 0.350 at a wave length of 450 mμ in a Coleman Junior Spectrophotometer, and merthiolate was added to a final concentration of 1:10,000. After a preimmunization bleeding the rabbits were inoculated according to the schedule described by Bailey et al. (8). Five consecutive daily doses of 1, 2, 3, 3, and 4 ml, respectively, of antigen were inoculated intravenously. On the 12th day the rabbits were given 4 ml more of antigen intravenously. On day 20 the animals were bled from the heart and the blood collected aseptically. The serum from three animals was pooled and heated at 56 C for 30 min to inactivate complement. The sera were either stored frozen at -90 C or stored at 4 C after the addition of 1:10,000 merthiolate.

Antiserum against P. pneumotropica was prepared as follows: one liter of TS broth was inoculated with 60 ml of a working culture. Incubation, harvesting and immunization schedule were the same as for M. pulmonis.

Titration of antisera. The antisera against M. pulmonis strains were titrated using a modification of the method described by Morton (90). Latex particles (Difco), 0.81 μ in a diameter diluted 1:5 in deionized water served as the carrier. The buffer solution consisted of glycine-buffered saline (GBS) pH 8.2 prepared by dissolving 7.505 g of glycine (Schwartz Bioresearch, Inc., Orangeburg, N. Y.) and 5.85 g of NaCl in triple distilled water. The volume

was brought to about 950 ml and the pH was adjusted to 8.2 with 1 N NaOH. The volume was then brought to 1 liter in a volumetric flask. Fraction V of bovine serum albumin (BSA) (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to the GBS at a final concentration of 1:500. Freshly grown mycoplasma strains were harvested as described above and resuspended in saline to give an absorbance reading of about 0.600 on the Coleman Junior Spectrophotometer. The test was performed as follows: To 0.4 ml of 1:5 latex suspension was added 1 ml of mycoplasma suspension. After 10 min at room temperature, 8.6 ml of GBS containing 1:500 BSA was added and incubated another 10 min at room temperature. This antigen preparation in 0.5 ml amounts was added to twofold serial dilutions of serum in GBS plus BSA. The final volume of each tube was 1 ml. The tubes were incubated at 42 C in a water bath for 1 hr and then were centrifuged for 10 min at approximately 1000 x g. The contents were resuspended and examined at a magnification of 100 X to determine agglutination. The titer with homologous antiserum was 1:256.

The M. pulmonis antisera were also used in growth inhibition studies (18) and to identify isolates. PPLO agar plates containing 200 units of penicillin/ml were placed open in an incubator for 1 hr to dry the medium surface. The plates were then smeared with 0.1 ml of a log phase broth culture of the mycoplasma. Sterile 6 mm filter paper discs were saturated with 0.025 ml of undiluted antisera, using a calibrated loop (Cooke Engineering Co., Alexandria, Va.). The plates were incubated 1-4 days and then examined with a low power stereomicroscope for zones of inhibition. Discs impregnated with serum from unimmunized rabbits served as controls on each plate.

P. pneumotropica antiserum was titrated by making serial twofold dilutions in saline. A 24 hr culture of P. pneumotropica was washed three times in saline and resuspended in saline to an absorbance of 0.350 at a wave length of 450 mμ. Formalin (Mallinckrodt, St. Louis, Mo.) was added to a final concentration of 0.6%, and 0.5 ml of the antigen was added to the antiserum dilutions. The tubes were incubated at 37 C for 1 hr in a water bath and then placed in a cold room (4 C) for 4 hr, after which they were read. They were read again after overnight incubation at 4 C. The tubes were inspected macroscopically for agglutination. The titer with homologous antiserum was 1:640.

Sampling methods and animals. The methods for culturing organs were the same as described in Chapter II. The animals used were CF#1/An1, CF#1/An1/SPF-1 and HA/ICR germfree mice. The HA/ICR germfree mice were removed from the germfree isolator immediately prior to inoculation. After inoculation they were housed in filter top cages (Lab Cages, Inc., Hackensack, N. J.).

Experimental design. Experiment 1 was designed to test the influence of the route of inoculation with P. pneumotropica strain 1132 on the localization of the organisms. Three groups of five adult HA/ICR germfree mice of both sexes were inoculated with a broth culture of P. pneumotropica containing $3.8 \times 10^7$ cells/ml. Group 1 was given approximately 0.05 ml intranasally. Group 2 was inoculated with 0.1 ml intravenously. Group 3 was inoculated with 0.1 ml intraperitoneally. A fourth group served as uninoculated controls. One week after inoculation, one animal from group and one from group 3 were killed and necropsied. Two weeks after inoculation all the animals were killed and

the tissues were cultured for P. pneumotropica.

In the second experiment two litters of HA/ICR germfree mice were removed from isolator chambers. One litter was instilled intranasally with $2 \times 10^7$ P. pneumotropica 1132, and the other litter served as uninoculated controls. Five days after inoculation two animals from the inoculated group were killed and the lungs cultured for P. pneumotropica. The remaining mice, both inoculated and control, were stressed with cold by placing the cages in a cold room at 4 C for 1 hr in the morning and 1 hr in the afternoon. Two days later this procedure was repeated. Two weeks after inoculation and one week after the last day of "cold stress" the mice were killed and the lungs cultured for P. pneumotropica.

In experiment 3, P. pneumotropica 1132 was serially passed in weanling CF#1/An1 mice. Three mice were given 0.05 ml intranasally of a broth culture of P. pneumotropica containing $7 \times 10^9$ cells/ml. Two days later the mice were killed, the lungs pooled and ground in a Ten Broeck tissue grinder. A 10% w/v suspension was made in TS broth and the suspension centrifuged at 200 x g for 5 min. Three mice were given an intranasal instillation with 0.05 ml of this suspension. The suspension was cultured for P. pneumotropica and M. pulmonis. This procedure was repeated using groups of three weanling mice every 2-3 days.

In experiment 4, the lungs from the Princeton mice were serially passed in CF#1/An1 mice. The lungs of the original mice were pooled and a 10% w/v suspension made in PPLO broth. Groups of six mice were given approximately 0.05 ml of the suspension intranasally while under light ether anesthesia or Diabutol (pentabarbitol sodium, Diamond Laboratories, Inc., Des Moines, Iowa)

anesthesia. The Diabutol was diluted 1:10 in 10% ethyl alcohol and 0.1 ml/g body weight given intraperitoneally. The lung suspensions were cultured for M. pulmonis and P. pneumotropica.

In experiment 5, four groups of six CF#1/An1/SPF-1 mice were given an intranasal instillation with P. pneumotropica 9538 and M. pulmonis N1 alone or in combination. Group 1 was given 0.05 ml of a broth culture of P. pneumotropica containing $6.1 \times 10^8$ cells/ml. Group 2 was given 0.05 ml M. pulmonis broth culture containing $7.8 \times 10^9$ CFU/ml. Group 3 was given a mixed culture of M. pulmonis and P. pneumotropica at the above concentrations. Group 4 received 0.05 ml of sterile PPLO broth. The mice were housed in filter top cages and observed for 30 days.

In experiment 6, 60 CF#1/An1/SPF-1 mice were given 0.05 ml intranasally of a broth culture of M. pulmonis N1 containing $1 \times 10^7$ CFU/ml. The mice were housed in groups of six in filter top cages. On Monday, Wednesday and Friday of each week, two mice were killed by cervical dislocation and necropsied. The lungs were cultured for M. pulmonis and P. pneumotropica. Portions of lung were fixed in 10% formalin for histopathologic sections. Thirty uninoculated controls were similarly treated.

Histologic technique. The fixed lungs were dehydrated in an Autotechnicon (Technicon Co., Chauncy, N. Y.) through increasing concentrations of alcohol and benzene and embedded in paraffin. Sections were cut at 3 μ and stained with hematoxylin and eosin.

## RESULTS

P. pneumotropica strain 9538 had the same properties as the other strains isolated in my laboratory. This strain also produced acid in galactose, fructose, mannose and trehalose and reduced nitrate to nitrite.

The biochemical properties of M. pulmonis N1 are shown in Table 7 and the appearance of colonies growing on PPLO agar are shown in Fig. 1.

Although motility was observed with young cultures of M. pulmonis when examined by phase contrast microscopy, there were more stationary forms than motile ones in each field. The motility was of two types. Rod shaped forms moved quickly and frequently reversed direction, whereas spherical forms rotated constantly but reversal of direction was seldom observed. Flagella were not observed in the electron micrographs.

Fig. 2, 3 and 4 show the morphology of 24, 48 and 72 hr broth cultures of M. pulmonis N1. The extreme pleomorphism of the organism is obvious. Several budding outpouchings are shown in Fig. 2. There are several internal membranous structures in the large round body which may represent developing cells. There are also densely packed granules that may be ribosomes at the left margin of the large body. Fig. 3 shows the serpentine character of M. pulmonis. Numerous small microtubules are evident, many of them with terminal buds. Pleomorphic saccules frequently observed in older cultures are shown in Fig. 4.

Fig. 5 demonstrates growth inhibition of M. pulmonis N1 by antiserum prepared against the strain and Fig. 6 shows inhibition of strain N1 by antiserum against M. pulmonis ATCC 14267. Absence of growth inhibition is shown in Fig. 7. M. arthritidis is not inhibited by ATCC 14267 strain antiserum. Antisera against N1 and ATCC 14267 also inhibited the growth of

the Milwaukee strain.

Animal experiments. Regardless of the route of introduction, P. pneumotropica 1132 localized in the lungs (Table 8). When the route was intranasal, the eyes were infected. The uterus was found to harbor P. pneumotropica in mice inoculated by the intranasal or intraperitoneal route.

In experiment 2, P. pneumotropica was isolated from the lungs of the two mice killed 5 days after intranasal instillation even though the lungs appeared normal. Clinical signs of disease were not observed in the remaining mice during the next 2 weeks. None showed gross lesions at necropsy. P. pneumotropica was isolated from 9 of the 18 treated mice. P. pneumotropica was not recovered from the lungs of control animals.

Table 9 shows the results of serial passage of P. pneumotropica 1132 in CF#1/Anl mice. Lungs of mice from the sixth and seventh passages showed consolidated areas with gray nodules at necropsy and both P. pneumotropica and M. pulmonis were recovered from these lungs. In an earlier passage M. pulmonis was recovered from the lungs of one mouse, but these lungs appeared normal. Neither organism was recovered after the eighth passage.

The results of serial passage of M. pulmonis are shown in Table 10. P. pneumotropica and M. pulmonis were recovered from all the lungs which had lesions. After the fourth passage of infected lung tissue neither organism was isolated and no lesions were evident.

The results of single and mixed infection with P. pneumotropica 9538 and M. pulmonis N1 are shown in Table 11. Deaths and overt disease were observed in the group which received both organisms. Small areas of patchy consolidation were observed in two mice which were given only M. pulmonis, but

P. pneumotropica was also isolated from these lungs. Lesions were not observed in the lungs of the mice which received only P. pneumotropica although M. pulmonis was recovered from the lungs of one of these mice.

The incidence of macroscopic lesions and the recovery of M. pulmonis and P. pneumotropica from SPF mice infected with M. pulmonis N1 is shown in Table 12. Mice with severe to moderately consolidated lungs consistently yielded both organisms on culture. Although 16 mice infected with both organisms had no macroscopically visible lesions, all had microscopic areas of consolidation. Serial sacrifice did not reveal a pattern of developing lesions. The first macroscopic lesions were observed on the 12th day after infection and gross lesions were present in one or more of the animals examined each day until the 20th day after infection. On the 21st and 23rd days, no gross lesions were seen and thereafter lesions were seen sporadically. None were observed after 40 days. Lesions were demonstrated microscopically from the second to the 47th day after infection in all the mice experimentally infected with M. pulmonis. No lesions were observed in the 30 control animals, and neither organism was recovered from them.

The microscopic lesions in infected animals differed depending on whether gross lesions were present or absent. When no gross lesions were present, the microscopic lesions were characterized by a focal interstitial reaction. This consisted of a thickening of the alveolar septae by mononuclear cells. These cells consisted of macrophages, lymphocytes, histiocytes and hyperplastic alveolar epithelial cells. In contrast, lungs which were grossly consolidated showed microscopic lesions typical of a bronchopneumonia. The lumens of bronchi in the involved areas were completely occluded by a purulent

exudate consisting primarily of polymorphonuclear leucocytes (neutrophils) (Fig. 8). The alveoli were filled with a variety of cell types (Fig. 9). In addition to lymphocytes, neutrophils and macrophages, large foamy mononuclear cells with abundant cytoplasm were observed.

TABLE 7. Biochemical reactions of Mycoplasma pulmonis strain N1

| Substrate or test | Reaction* | |
|---|---|---|
| | 4 days | 7 days |
| Glucose | A | A |
| Lactose | - | (A) |
| Maltose | (A) | A |
| Sucrose | (A) | A |
| Mannitol | A | A |
| Xylose | - | A |
| Inositol | A | A |
| Galactose | - | (A) |
| Mannose | A | A |
| OF + Glucose + serum | | |
| open | ± | + |
| closed | + | + |
| Motility by phase contrast† | - | - |
| Gram reaction | - | |
| Growth inhibition by M. pulmonis ATCC 14267 antiserum | + | |
| Hemolysis of sheep RBC's | β | |

* A = acid produced; (A) = slight acid produced.

† Motility can be observed in 18-24 hr cultures.

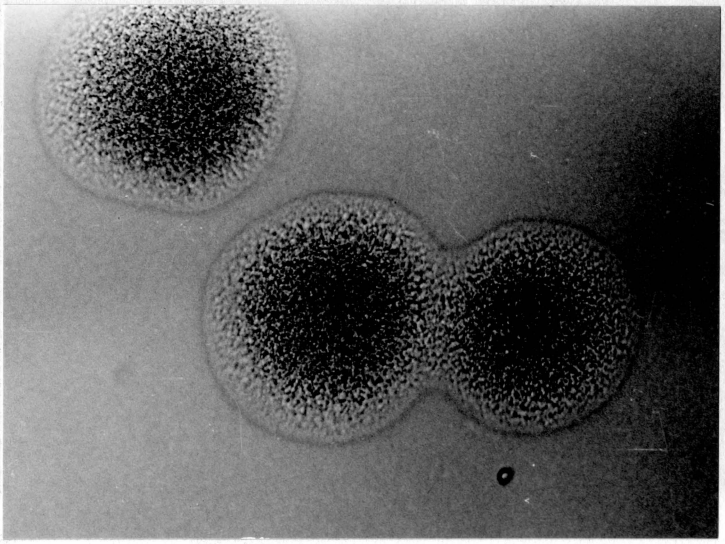

Fig. 1. Colonies of Mycoplasma pulmonis N1 on PPLO agar (x 125). The colonies have a light peripheral area with a more dense center.

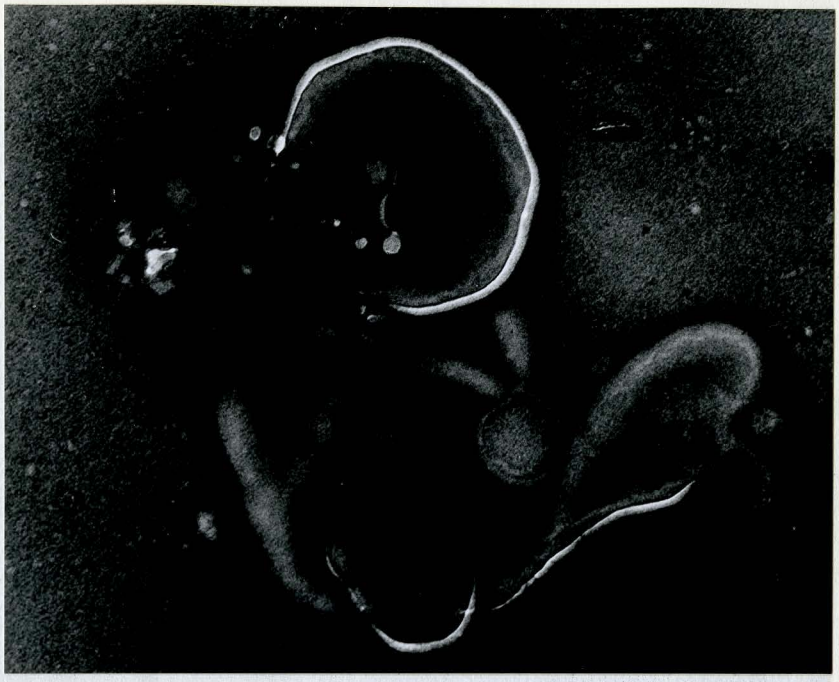

Fig. 2. Cells from a 24 hr broth culture of Mycoplasma pulmonis N1 (x 16,000). Several budding outpouchings can be seen. The large round body has dense membraneous structures inside which may represent developing cells.

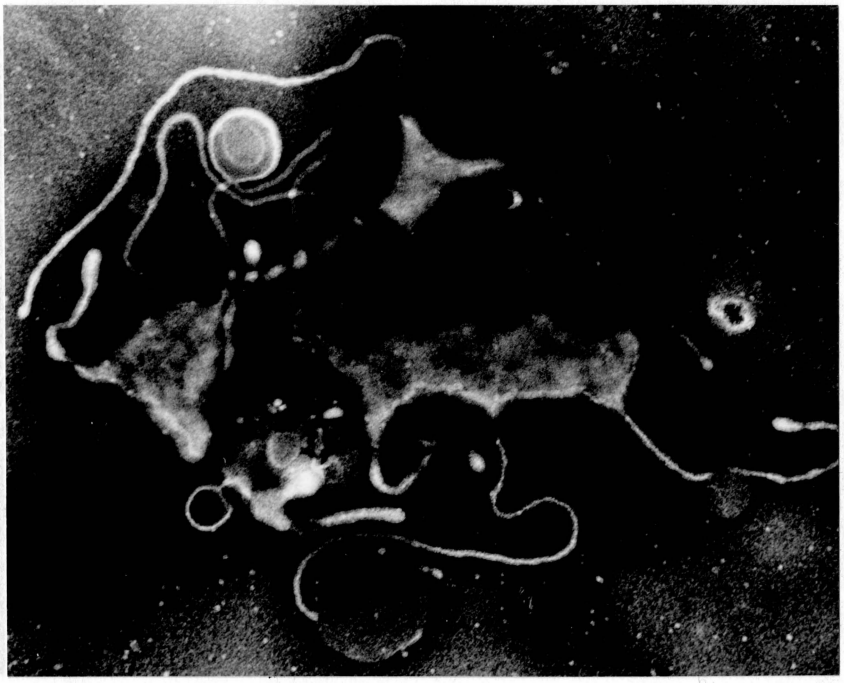

**Fig. 3. Cells from a 48 hr broth culture of *Mycoplasma pulmonis* N1 (x 16,000). The serpentine character of the organism is demonstrated.**

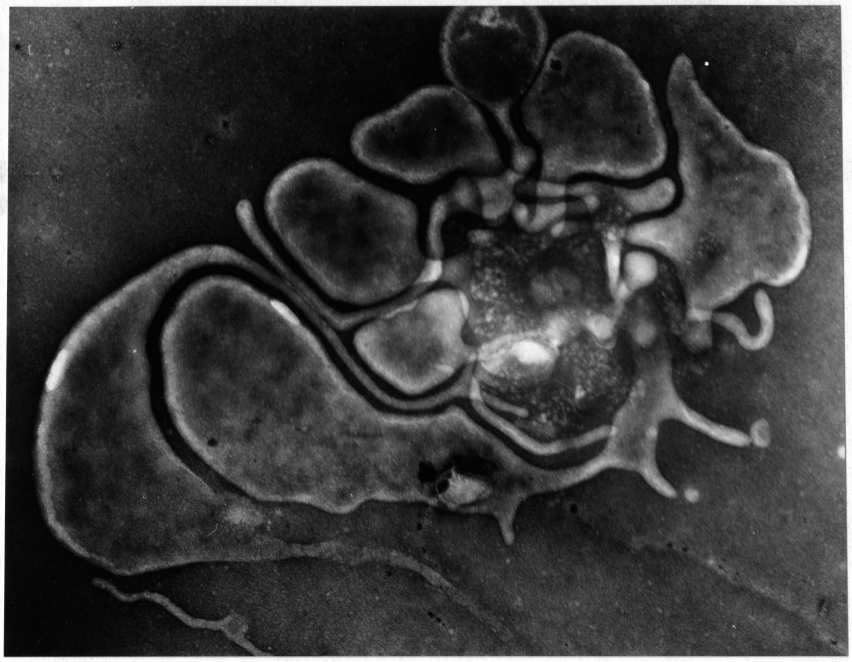

Fig. 4. Cells from a 72 hr broth culture of Mycoplasma pulmonis N1 (x 16,000). Large, pleomorphic sack-like structures are evident.

Fig. 5. Growth inhibition of Mycoplasma pulmonis N1 by homologous antiserum (x 25).

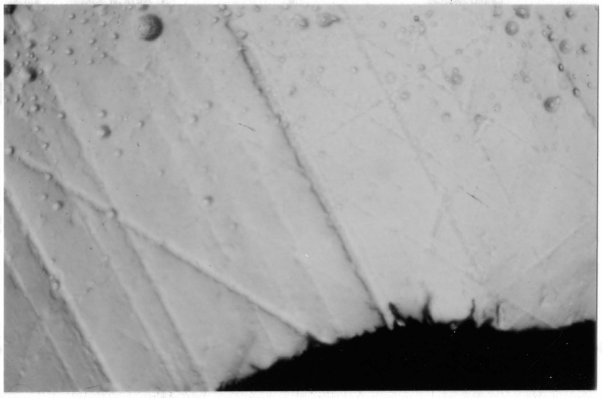

Fig. 6. Growth inhibition of Mycoplasma pulmonis N1 by M. pulmonis ATCC 14267 antiserum (x 25).

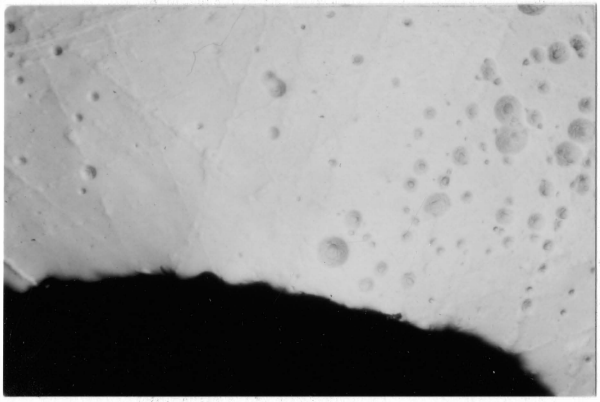

Fig. 7. Negative growth inhibition of Mycoplasma arthritidis by M. pulmonis ATCC 14267 antiserum (x 25).

TABLE 8. Influence of route of inoculation on infection with Pasteurella pneumotropica

| Route of infection | Specimen cultured | Number positive/ number examined |
|---|---|---|
| Intranasal ($1.9 \times 10^6$ cells) | lungs | 3/5 |
| | eyes | 3/5 |
| | uterus | 2/3 |
| Intravenous ($3.8 \times 10^6$ cells) | lungs | 3/5 |
| | eyes | 0/5 |
| Intraperitoneal ($3.8 \times 10^6$ cells) | lungs | 3/5 |
| | eyes | 0/5 |
| | uterus | 1/2 |
| Uninoculated controls | lungs | 0/5 |
| | eyes | 0/5 |
| | uterus | 0/2 |

TABLE 9. Serial mouse passage of Pasteurella pneumotropica in weanling CF#1/An1 mice

| Passage number | Number with lesions/ number examined | P. pneumotropica | M. pulmonis |
|---|---|---|---|
| 1 | 0/3 | 3/3 | 0/3 |
| 2 | 0/3 | 3/3 | 0/3 |
| 3 | 0/3 | 3/3 | 1/3 |
| 4 | 0/3 | 3/3 | 0/3 |
| 5 | 0/3 | 3/3 | 0/3 |
| 6 | 2/3 | 3/3 | 2/3 |
| 7 | 2/3 | 3/3 | 2/3 |
| 8 | 0/3 | 0/3 | 0/3 |
| 9 | 0/3 | 0/3 | 0/3 |
| 10 | 0/3 | 0/3 | 0/3 |

TABLE 10. Serial mouse passage of Mycoplasma pulmonis in CF#1/Anl weanling mice

| Passage number | Number with lesions/ number examined | P. pneumotropica | M. pulmonis |
|---|---|---|---|
| Princeton mice | 6/6 | 6/6 | 6/6 |
| 1 | 6/6 | 6/6 | 6/6 |
| 2 | 6/6 | 6/6 | 6/6 |
| 3 | 4/4 | 4/4 | 4/4 |
| 4 | 2/6 | 2/6 | 2/6 |
| 5 | 0/6 | 0/6 | 0/6 |
| 6 | 0/6 | 0/6 | 0/6 |
| 7 | 0/6 | 0/6 | 0/6 |

TABLE 11. Single and mixed infection with Pasteurella pneumotropica and Mycoplasma pulmonis in CF#1/An1/SPF mice

| | Inoculum | | | |
|---|---|---|---|---|
| | P. pneumotropica | M. pulmonis | P. pneumotropica and M. pulmonis | Sterile broth |
| Deaths/number inoculated | 0/6 | 0/6 | 3/6 | 0/6 |
| Lesions/number inoculated | 0/6 | 2/6 | 4/6 | 0/6 |
| Culture results P. pneumotropica/ number inoculated | 2/6 | 2/0 | 4/6 | 0/6 |
| M. pulmonis/ number inoculated | 1/0 | 2/6 | 4/6 | 0/6 |

TABLE 12. *Mycoplasma pulmonis* N1 infection in CF#1/Anl/SPF mice

| Degree of gross pathology | Number of mice examined | Only *M. pulmonis* recovered | Only *P. pneumotropica* recovered | *M. pulmonis* and *P. pneumotropica* recovered | Other bacteria |
|---|---|---|---|---|---|
| 4+* | 6 | 0 | 0 | 5 | 1 |
| 3+ | 2 | 0 | 0 | 2 | 0 |
| 2+ | 1 | 0 | 0 | 0 | 1 |
| 1+ | 5 | 3 | 0 | 2 | 0 |
| 0 | 28 | 8 | 2 | 6 | 0 |

* 4+ = severe consolidation; 3+ = mild to moderate consolidation; 2+ = severe congestion; 1+ = mild to moderate congestion; 0 = no gross lesions.

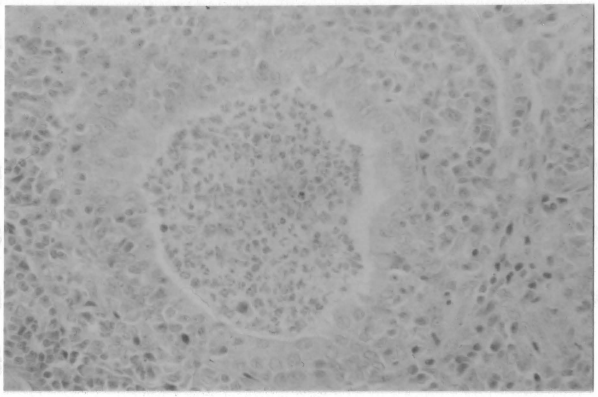

Fig. 8. Occluded bronchus of CF#1/An1/SPF mouse lung (x 250). Hematoxylin and eosin stain. This mouse was infected with Mycoplasma pulmonis N1, but Pasteurella pneumotropica was also recovered from the lung. The bronchus is filled with a polymorphonuclear exudate.

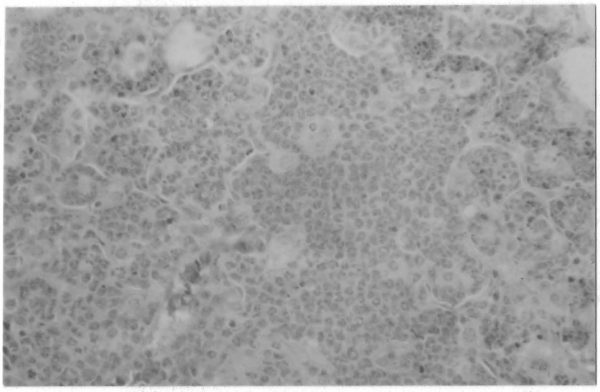

Fig. 9. Alveoli of CF#1/An1/SPF mouse lung (x 250). Hematoxylin and eosin stain. Mouse was infected with Mycoplasma pulmonis N1, but Pasteurella pneumotropica was also recovered. Large pink staining mononuclear cells can be seen as well as lymphocytes and polymorphonuclear cells.

## DISCUSSION

The properties of P. pneumotropica 9538 isolated from Princeton mice are the same as those of the P. pneumotropica strains previously isolated in my laboratory. P. pneumotropica can be identified by its anaerogenic fermentation pattern, indole production and positive oxidase and urease test. The titer of P. pneumotropica antiserum with the homologous strain is comparable to that reported by Jawetz (60).

The biochemical properties of M. pulmonis N1 (Table 7) are essentially the same as those reported by Tully (142) for other strains of M. pulmonis. Growth inhibition by homologous antiserum as well as antisera against the Milwaukee strain and strain 14267 further confirm this organism as a strain of M. pulmonis (Fig. 5 and 6). The growth inhibition method clearly distinguishes the various species of mycoplasma (18, 136). My observation that M. pulmonis N1 is motile confirms the observation of Nelson and Lyons (107). These investigators suggest that mycoplasmas are related to the myxobacters on the basis of their means of locomotion and lack of a restraining cell wall, but Edward et al. (38) propose that a new class be created for the Order Mycoplasmatales. The electron micrographs (Fig. 2-4) are included in this chapter solely to demonstrate the pleomorphic character of M. pulmonis. Those shown are similar to micrographs of other strains of M. pulmonis (2, 107).

The localization of P. pneumotropica in the lungs of mice (Table 8) regardless of the route of inoculation confirms the marked pneumotropism of the organism. The respiratory tract was previously found to be the most usual habitat for the organism under natural conditions (53, 54, 60, 147). The mice used in this experiment were raised in germfree isolators and were not in

contact with P. pneumotropica until they were experimentally infected. They were then housed in filter top cages which prevent cross contamination. This experiment conclusively confirms Jawetz's (60) original observation regarding pneumotropism and negates the idea that it is only a manifestation of the natural infection. Why the organism should exhibit such affinity for the lung is not known. The isolation of the organism from the eyes of mice given the organism intranasally might be a result of contamination of the eyes during inoculation or it might result from an ascending infection of the lacrimal ducts. The uterus was found to be susceptible to infection with P. pneumotropica and this again suggests that a means of natural transmission exists other than from the respiratory tract of the dam.

Cold stress of gnotobiotic mice infected with P. pneumotropica 1132 failed to induce signs of disease. Furthermore, 11 of the animals were successful in eliminating infection with the organisms as determined by culture techniques. Goldstein and Green (48) have found that healthy mice can clear up to 99.5% of P. pneumotropica given in an aerosol in 6 hr after exposure. These workers suggest that some impairment of the intrapulmonary phagocytic system is a necessary condition of pulmonary infection. If this is true, then those mice which still harbored P. pneumotropica two weeks after infection must have had macrophages impaired in some way so that they could not deal with the invading and presumably multiplying organisms. These data suggest that P. pneumotropica 1132 is of limited pathogenicity.

Jawetz (60) found that mice latently infected with P. pneumotropica were more susceptible to superinfection with the organism. My efforts to increase the virulence of P. pneumotropica 1132 by serially passing it in

latently infected mice were unsuccessful (Table 9). Although consolidated lungs with gray nodules were observed in four mice, M. pulmonis was also isolated from these lesions. These mice were probably latently infected with M. pulmonis. Why neither organism was recovered from later passages is not known. The necessity for both organisms to be present for lesions to develop is confirmed by the passage of M. pulmonis (Table 10).

When either organism alone or both organisms together were given to mice believed to be free from latent infection with either organism, both were necessary to produce severe lesions and death (Table 11). Surprisingly, P. pneumotropica was isolated from mice which received only M. pulmonis and and M. pulmonis from those which only received P. pneumotropica. Cross contamination was unlikely because of the method of handling the mice. It seemed possible that M. pulmonis might be an L form of P. pneumotropica. The possibility that mycoplasma are L forms of bacteria has been suggested by other workers (85, 119, 120, 132, 133).

Results from the serial sacrifice of CF#1/An1/SPF-1 mice further strengthen the supposition that M. pulmonis is an L form of P. pneumotropica because P. pneumotropica was recovered from 17 animals believed to be free from latent infection with the organism (Table 12). Rather than clarifying the role of the two organisms in the pathogenesis of murine pneumonia, the experiments presented in this chapter add a new dimension to the problem. Generally, the microscopic lesions observed are similar to those described by Horsfall and Hahn (56), Niven (111), Nelson (94, 95) and Jawetz and Baker (61). Each of these workers attributed the lesions to a different pathogenic agent.

The obvious solution to the dilemma posed by the results reported in this

chapter was to use germfree mice in plastic film isolators. These results will be reported in Chapter V. However, it was essential to determine first whether or not M. pulmonis is an L form of P. pneumotropica. These results are presented in Chapter IV.

# CHAPTER IV

## GENETIC RELATEDNESS BETWEEN *PASTEURELLA PNEUMOTROPICA* AND *MYCOPLASMA PULMONIS*

The name L forms (L for Lister Institute) was originated by Klieneberger (67) when she observed the characteristic colony growing with *Streptobacillus moniliformis*. At that time the pleuropneumonia organism was the only mycoplasma known and its bacterial nature had not been recognized. Klieneberger regarded the L form as a symbiote of the streptobacillus. In 1936 Klieneberger reported that the L form could be isolated from any strain of *S. moniliformis* and that no culture of this organism could be freed of it (68). On the other hand, the L form could be isolated and bred true in artificial culture. Dienes (24, 25, 26, 27, 29) challenged Klieneberger's interpretation that the L form was a symbiote and showed that it was a growth phase of *S. moniliformis*. He also demonstrated L forms of other species of bacteria (26, 28, 29). Klieneberger eventually accepted Dienes' view with regard to L forms of *S. moniliformis* but she strongly opposed the concept that mycoplasma arise from bacteria (74). At the present time the only universally accepted criterion differentiating stable L forms from mycoplasma is the knowledge that the L form was previously derived from a bacterium whereas the origin of mycoplasma is unresolved.

Workers other than Dienes have suggested that mycoplasma are L forms of bacteria. Wittler et al. (148) reported the conversion of a mycoplasma to a diphtheroid. Smith, Peoples and Morton (132) presented evidence that described the reversion of the Campo strain (*M. arthritidis*) to a

corynebacterium on continued cultivation. Smith and Rothblatt (133) showed serologic relationships between the Campo strain and diphtheroids isolated from it. McKay and Truscott (85) suggested that M. gallisepticum is the stable L form of Hemophilus gallinarum. Kelton, Gentry and Ludwig (65) reported the conversion of mycoplasma to streptococci and Neimark and Pickett (92) suggested that the entire mycoplasma group was reminiscent of the streptococci based on the heterogeneity of carbohydrate fermentation.

The most avid proponent of the hypothesis that mycoplasma are L forms of bacteria is Phyllis E. Pease. Based on gel diffusion patterns she reported that S. moniliformis is an intermediate stage between a diphtheroid and M. hominis (119, 123). In 1963 she reported that the Eaton agent (M. pneumoniae) was an L form of Streptococcus MG (120). Pease and Laughton (123) compared H. influenzae, H. vaginalis and Corynebacterium cervicis by gel diffusion and reported that all three were related to M. hominis. Pease believed that the corynebacterium was an intermediate stage between H. vaginalis and M. hominis and questioned the validity of the genus Mycoplasma (121).

The evidence for the relatedness of certain mycoplasmas to bacteria derived from observations of reversions and serologic experiments remains equivocal. Klieneberger (74) points out that frequently adequate controls were not included in the experimental design. McGee et al. (84) state that the presence or absence of similarities in morphology, pathogenicity, physiochemistry and antigenicity are not valid criteria for the confirmation or denial that a Mycoplasma species is an L form of a bacterium. Changes in these properties may occur during transition from bacterium to L form.

This has been reported for Proteus L forms and for the L form of Neisseria meningitidis (127, 146). The criteria chosen must be applicable to the relationship of an L form to its known bacterial parent. The most direct way of demonstrating this relationship is to determine the genetic relatedness of a bacterium and the mycoplasma suspected of being its L form. Such an approach assumes that despite the phenotypic changes which accompany the L form transition, the genetic potential of the organism inherent in the sequence of nucleotide bases of its deoxyribonucleic acid (DNA) remains relatively constant throughout this change. Evidence that this is true have been presented for Proteus mirabilis L 9 and its parent P. mirabilis 9 (84) and for a derived L form of a group A streptococcus (116). The tests used to make these comparisons were the percent guanine-cytosine (GC) content and the extent of homology of base sequences by the agar column hybridization technique (83). Using these two tests, McGee et al. (84) proved conclusively that M. pneumoniae was not genetically related to Streptococcus MG. Rogul et al. (128) similarly showed that H. gallinarum was not genetically related to M. gallisepticum or M. gallinarum.

It has been shown that most organisms which are capable of genetic interchange also have a similar percent GC content (14, 15, 88). The converse of this is not necessarily true and a disparity in GC serves only as a predictive tool aiding in a systematic classification.

The results of the experiments reported in Chapter III suggested that M. pulmonis might be the stable L form of P. pneumotropica. Since the percent GC of these two organisms is not reported in the literature, no prediction as to similarity could be made on that basis. Therefore, experiments were

undertaken to determine these values and to determine if any degree of molecular homology could be demonstrated between the DNA from the two organisms.

## MATERIALS AND METHODS

Organisms and cultural conditions. P. pneumotropica 9538 was grown in TS broth for 24 hr at 37 C in an incubator shaker (Lab-line Instruments, Inc., Melrose Park, Ill.); M. pulmonis N1 was grown in PPLO broth without inhibitors on the same shaker for 60 hr. Radioactive labeling of both organisms was accomplished by adding 5mc $^{3}$H-thymidine (14.0 c/mmole, Schwartz Bioresearch Inc., Orangeburg, N. Y.) per L of broth. Purity of P. pneumotropica cultures was confirmed by cultivation on blood agar, gram stain and the biochemical reactions of the organisms. The purity of M. pulmonis cultures was determined by cultivation on PPLO agar, and by the inhibition of growth by specific antiserum.

DNA extraction. The DNA from both organisms was extracted by using a modification of Marmur's method (86). The organisms were harvested by centrifugation at 5,000 x g for 30 min in a Servall refrigerated centrifuge. Two to 3 g wet packed cells were washed once with 50 ml saline-EDTA (0.15 M NaCl containing 0.1 M ethylenediaminetetraacetate, pH 8). After centrifugation at 10,000 x g for 10 min the cells were resuspended in 25 ml saline-EDTA and lysed by the addition of 2 ml of 25% sodium dodecyl sulfate. The mixture was placed in a 60 C water bath for 10 min and then cooled to room temperature. Sodium perchlorate was added to a final concentration of 1 M to the lysed suspension and the whole mixture deproteinized by shaking with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) in a ground glass stoppered Erlenmeyer flask for 30 min. The resulting emulsion was separated into three layers by centrifuging for 5 min at 5,000 x g in an International PR 2 refrigerated centrifuge. The upper aqueous layer containing the nucleic acids was carefully

pipetted off into a 50 ml graduated cylinder. The nucleic acids were precipitated by gently layering two volumes of 95% ethyl alcohol on the aqueous phase. The layers were mixed with a slender stirring rod and the nucleic acids "spooled" up on the rod. The precipitate was transferred to 10 ml 0.1x saline citrate (SSC). SSC is 0.15 M NaCl containing 0.015 M trisodium citrate, pH 7.0. One tenth x, 2x, 3x, 6x and 10x SSC are 0.1, 2, 3, 6 and 10 times as concentrated. The solution was gently shaken until dispersion was complete and then adjusted to approximately SSC concentration with 10x SSC. This solution was deproteinized with chloroform-isoamyl alcohol as before, centrifuged and the supernatant fluid removed. The supernatant fluid was repeatedly deproteinized until no protein was seen at the interface.

After the last deproteinization, the nucleic acids were precipitated with ethyl alcohol and the precipitate was dispersed in about half the supernatant volume of SSC. Ribonuclease (Crystalline; Armour, Chicago, Ill.) was dissolved in 0.15 M NaCl (2 mg/ml) and heated at 80 C for 10 min to inactivate any residual deoxyribonuclease. It was added to the nucleic acids at a final concentration of 50 μg/ml and the mixture incubated at 37 C for 30 min. Two more deproteinizations were carried out. The nucleic acid in the supernatant fluid from the final deproteinization was precipitated and dissolved in 9 ml 0.1x SSC. When solution was complete 1 ml acetate-EDTA (3.0 M Na acetate containing 0.001 M EDTA, pH 7.0) was added. The solution was rapidly stirred with an electric stirrer fitted with a straight glass rod with spiral grooves and 0.54 volumes of isopropyl alcohol was added dropwise into the vortex to precipitate the DNA. The DNA was washed in increasing concentrations of ethyl alcohol (70-95%) and then dissolved in SSC at a concentration of approximately

0.5-1 mg/ml. Two drops of chloroform were added as a preservative and the solutions stored at 4 C. The concentration of DNA in each solution was estimated from a standard curve correlating $A_{260}$ with μg/ml DNA (Fig. 10).

The standard curve was prepared as follows. Highly polymerized grade A salmon sperm DNA, as the sodium salt (Calbiochem, Los Angeles, Calif.) was dried in a vacuum dessicator over anhydrous $CaSO_4$ (Drierite, Hammond Drierite Co., Xenia, Ohio) for 48 hrs. Two samples, 10.12 mg and 10.06 mg were weighed on an analytical balance (Mettler Micro Gram-atic, Mettler Instrument Corp., Hightown, N. J.) and each was dissolved in 10 ml SSC in a volumetric flask. Dilutions were made in 10 ml volumetric flasks. The absorbance of each dilution was measured at 260 mμ in a Beckman model DU ultraviolet spectrophotometer in 3 ml quartz cuvettes with a 1 cm light path. The Burton (12) modification of the Dischediphenylamine reaction on portions of these dilutions confirmed the amount of DNA/ml. An absorbance of 0.045 at 260 mμ was taken to equal a DNA concentration of 1μg/ml throughout these studies.

Characterization of the DNA. The absorption spectrum of each DNA was determined in the Beckman model DB recording spectrophotometer. Absorbance measurements at 260, 230 and 280 mμ were determined in the Beckman model DU.

Molecular weights of the DNA were determined in a Spinco model E analytical ultracentrifuge. Each sample was diluted in SSC to give an $A_{259}$ of 0.8. $D_2O$ (U. S. Atomic Energy Commission, 99.8% deuterium) served as the supporting medium for the sedimentation. The $D_2O$ was diluted with an equal volume of 2x SSC so that the final concentration was 50% $D_2O$ in SSC. For each run 0.030 ml of sample was carefully layered onto 0.37 ml of the $D_2O$ solution with a Gilmont microsyringe (Cole-Parmer Instruments Co., Chicago, Ill.) in a synthetic

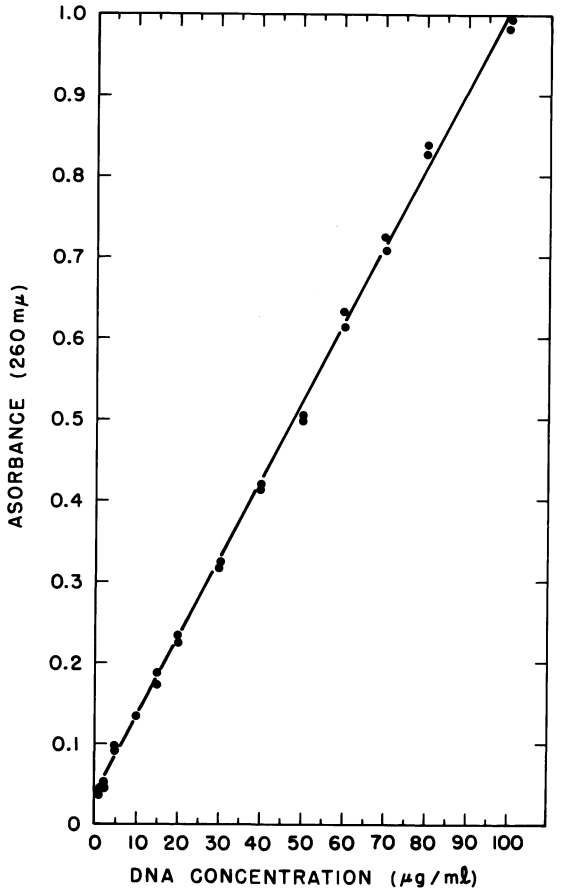


**Fig. 10. Standard curve correlating $A_{260}$ with μg/ml DNA. Samples of salmon sperm DNA were dried in vacuo, weighed and diluted in SSC.**

boundary cell. The samples were centrifuged at 33,450 rev/min. Eight min after the start, and at 2 min intervals thereafter ultraviolet absorption photographs were taken on Kodak commercial process film. The film was developed and tracings were made with a Joyce-Loebl double-beam microdensitometer. The radius of the maximum ordinate was read from the trace. A linear regression was computed on the log of the maximum ordinate versus time on a GE 225 computer. From the slope of the line, the sedimentation coefficient ($S_{w20}$) was computed using the formula $S = Kb$ where $K = 3.1277 \times 10^{-9}$ for 33,450 rev/min, and b = the slope. From the $S_{w20}$ the molecular weights were calculated according to the formula $S_{w20} = 0.116\ M^{0.325}$ (93).

Determination of the GC base ratios. The GC content of the DNA preparations was determined by the thermal denaturation method of Marmur and Doty (87) modified slightly. The DNA solutions were adjusted to a concentration of approximately 20 μg/ml with SSC and placed in a 3 ml cuvette type 6001J (W. H. Kessel Co., Chicago, Ill.) with a 1 cm light path. The solutions were saturated with helium and the cuvettes were tightly stoppered with Teflon plugs. The $A_{260}$ of the solutions against a blank of SSC was followed in a Gilford model 2000 spectrophotometer with a standardized temperature probe. The temperature was raised approximately 1 C every 10 min with a Haake Series F constant temperature circulator equipped with a motor connected to the temperature adjustment knob of the control system. A sharp increase in absorbance occurs in the transition range during which the DNA denatures. When no further increase occurred on raising the temperature the denaturation was considered complete. The absorbance corrected for the thermal expansion of water at each temperature was divided by the value at 30 C and the ratio

(relative absorbance) plotted versus the temperature. The temperature corresponding to half the increase in the relative absorbance was designated as the Tm. The GC ratio was calculated with the equation Tm = 69.1 + 0.43 (GC).

An estimate of the buoyant densities of the DNAs in CsCl was made as a check on the thermal denaturation determinations. The method was a modification of that of Schildkraut, Marmur and Doty (130). DNA solutions were diluted to an $A_{260}$ of about 1.0 in SSC. Each of these samples was then diluted in a stock solution of CsCl (A. D. Mackay, Inc., New York, New York) having a density of 1.710 $g/cm^3$. The final solutions contained between 1 and 2 µg of the DNA to be examined. Approximately 0.75 ml of the final CsCl solutions were placed in a 12 mm cell containing a plastic Kel-F centerpiece and centrifuged in a Spinco model E analytical ultracentrifuge at 44,770 rev/min at 25 C for 15 hr. At the end of that time photographs and tracings were made as before. Densities were calculated by assuming that the density at the radial center (rm) of the liquid column is equal to the density of the original solution. The distance of the band peak from this position was measured and was used to determine the density of the sample DNA. At 44,770 rev/min:

$\rho = \rho o + 0.0092 (r^2 - ro^2)$ $g/cm^3$ where

$\rho$ = density of the sample

$\rho o$ = density of the original solution

r = distance of the sample band peak from the center of rotation

ro = distance of the radial center of the liquid column from the center of rotation.

The density of the sample DNA was used to calculate the GC ratios by the formula $\rho = 1.660 + 0.098$ GC (130).

DNA hybridization. The method used to measure molecular hybridization is a modification of that of Denhardt (23). DNA, either undenatured or denatured by heating to 100 C for 10 min in 6x SSC and followed by quick-cooling, was attached to nitrocellulose membrane filters (HAWP 25 mm, Millipore Filter Corporation, Bedford, Mass.). The solutions were passed (5 ml/min) through a filter prewashed with 6x SSC held on a Millipore microanalysis filter holder. The filters were washed with 5 ml 6x SSC, dried overnight in a vacuum dessicator and then in a vacuum oven at 80 C and 29 inches Hg for 2 hr.

Filters to be used in annealing experiments were preincubated. The preincubation mixture (PM) contained 0.02% each of Ficoll (Pharmacia, Piscataway, N. J.), polyvinylpyrolidione (Sigma Chemical Co., St. Louis, Mo.) and bovine serum albumin (Armour, Chicago, Ill.; Fraction V) in 3x SSC. The filters were rolled up and placed in 1 ml volumetric flasks with 1 ml PM and incubated for 6 hr at 60 C.

The $^{3}H$ labeled DNA preparations for annealing experiments were sheared in 5 ml portions in a 25 ml plastic cup with a Branson sonifier, model LS-75 for 4 min and 45 sec at a setting of 3. At this setting the sonic oscillations occur at a frequency of 20 kc/sec maintained at a medium output of 13 w. Rogul et al. (128) report that the resulting DNA fragments have a molecular weight of about 300,000.

Annealing was carried out by adding sheared $^{3}H$ DNA to the preincubated flasks containing filters with attached unlabeled DNA. The flasks were reincubated for 18-24 hr at 60 C. When the incubation was complete, each side of the filter was washed with 100 ml SSC. The filters were air dried and counted in a Tri-Carb model 314 Scintillation spectrometer (Packard Instruments

Co., La Grange, Ill.) using 15 ml toluene-liquifluor as the counting fluid. The toluene-liquifluor was made up as follows: 750 ml toluene, 210 ml ethyl alcohol, 40 ml Liquifluor (Nuclear-Chicago, Des Plaines, Ill.).

The efficiency of each counting run was determined by adding 50 µl of standard $^{3}H$ toluene to 15 ml toluene-liquifluor. The $^{3}H$ toluene contains $2 \times 10^{6}$ disintegrations/min per ml. The samples were counted 8-16 times for 10 min. Mathematical computation on the counts were performed on a GE 225 computer using a standard program.

The percent label bound was calculated as the number of counts retained on the filter/number of counts $^{3}H$ DNA added to the flask. The relative percent binding was calculated as the [(percent binding of donor DNA to sample) - (percent binding of donor DNA to blank filter)]/[(percent binding of donor DNA to its homologous DNA) - (percent binding of donor DNA to blank filter)] x 100.

## RESULTS

Characterization of the DNA Preparations. The concentration of the DNA solutions, their absorbance ratios and the specific activity of each labeled preparation is shown in Table 13. Using the extraction technique developed by Marmur (86) 1-2 mg of DNA is generally isolated from 1 g of wet packed cells. The absorption spectrum of unlabeled DNA from both organisms are shown in Fig. 11 and 12. Tritium labeled DNA has similar absorption curves, and all four preparations absorb maximally at a wavelength of 260 mμ. There is no shoulder at 280 mμ indicating the virtual absence of protein.

The sedimentation coefficients for DNA from P. pneumotropica and M. pulmonis are 26.2 and 17.6. The molecular weights calculated from these are $17.5 \times 10^6$ and $5.2 \times 10^6$. These molecular weights have no biological significance in themselves because shearing during the extraction procedure reduces the molecular weight. They serve only to characterize the DNA extracted by the procedure used.

Percent GC Content. Figures 13-15 show the temperature-absorbance curves of DNA extracted from P. pneumotropica and Figures 16-19 show the temperature-absorbance curves for M. pulmonis DNA. The percent GC calculated from these curves are 44.4% for P. pneumotropica and 31.1% for M. pulmonis (Table 14). Buoyant density determinations of the DNA from both organisms (Table 15) indicate GC contents in close agreement with those determined by thermal denaturation.

DNA hybridization. Several preliminary experiments were necessary before the extent of molecular homology between DNA from P. pneumotropica and the M. pulmonis could be determined. The effect of drying the nitrocellulose

filters and the effect of preincubation on release of DNA from the filter are shown in Table 16. Approximately 99% of the DNA was retained by the filter during the drying procedure. Less than 5% was released during a 24 hr preincubation at 60 C.

Table 17 shows the effect of varying both the amount of P. pneumotropica DNA attached to the filter and the amount of donor $^{3}H$ DNA. There was no significant difference between 1 μg of input DNA annealed with 20.0 μg on the filter and 0.5 μg input DNA annealed with 6.3 μg on the filter. There was significantly more duplex formation when the ratio of recipient to donor DNA was 20:1 or 10:1 than when it was 30:1. Because the number of counts obtained with 0.5 μg of donor DNA and 6.3 μg of recipient DNA were sufficient to permit counting in a reasonable length of time, these amounts were used in homology experiments.

The effect of varying the ratio of donor to recipient DNA for M. pulmonis is shown in Table 18. Significantly more DNA was bound to the filter when the ratio of recipient to donor DNA was 20:1 or 25:1 than when it was 10:1. However, for the sake of consistency with P. pneumotropica DNA, a ratio of 10:1 was selected for use in the homology experiments.

The extent of molecular homology between P. pneumotropica and M. pulmonis DNA is shown in Table 19. Although DNA from both organisms is capable of duplex formation in the homologous system, no specific duplex formation occurs in the heterologous system.

TABLE 13. Concentration, absorbance ratios and specific activity of Pasteurella pneumotropica and Mycoplasma pulmonis DNA

| Source of DNA | Concentration (mg/ml) | Specific activity* (counts/min/μg) | $A_{280}/A_{260}$ | $A_{230}/A_{260}$ |
|---|---|---|---|---|
| P. pneumotropica | | | | |
| unlabeled | 0.63 | - | 0.539 | 0.451 |
| $^{3}H$ labeled | 1.0 | 2256 ± 27 | 0.520 | 0.482 |
| M. pulmonis | | | | |
| unlabeled | 1.0 | - | 0.535 | 0.408 |
| $^{3}H$ labeled | 0.9 | 1596 ± 41 | 0.541 | 0.425 |

* Determinations were made on 5 separate samples. Each sample was counted 10 times for 10 minutes and the results are reported as the mean ± SE.

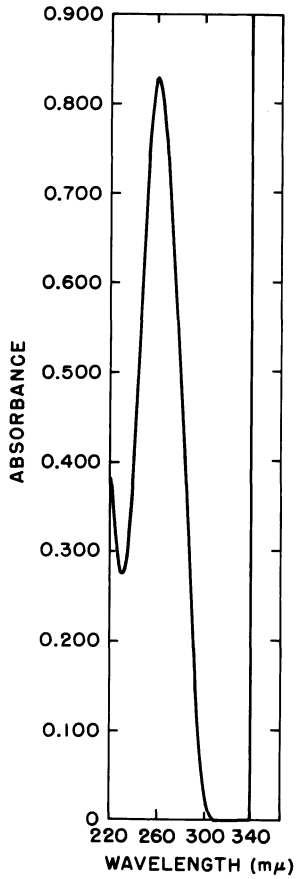


Fig. 11. Absorbance spectrum of DNA from Mycoplasma pulmonis N1.

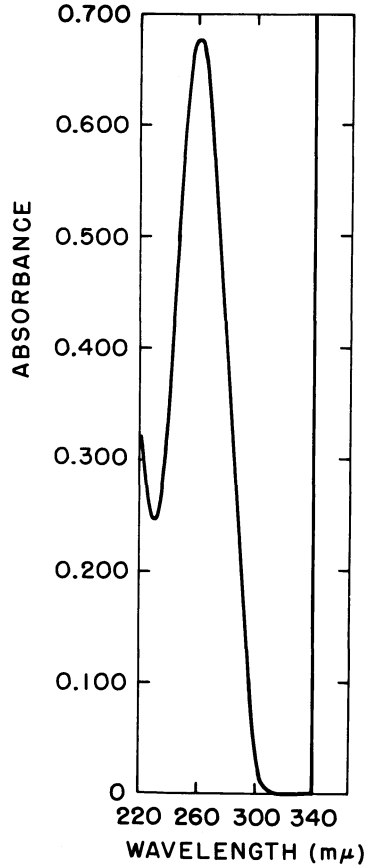


Fig. 12. Absorbance spectrum of DNA from Pasteurella pneumotropica 9538.

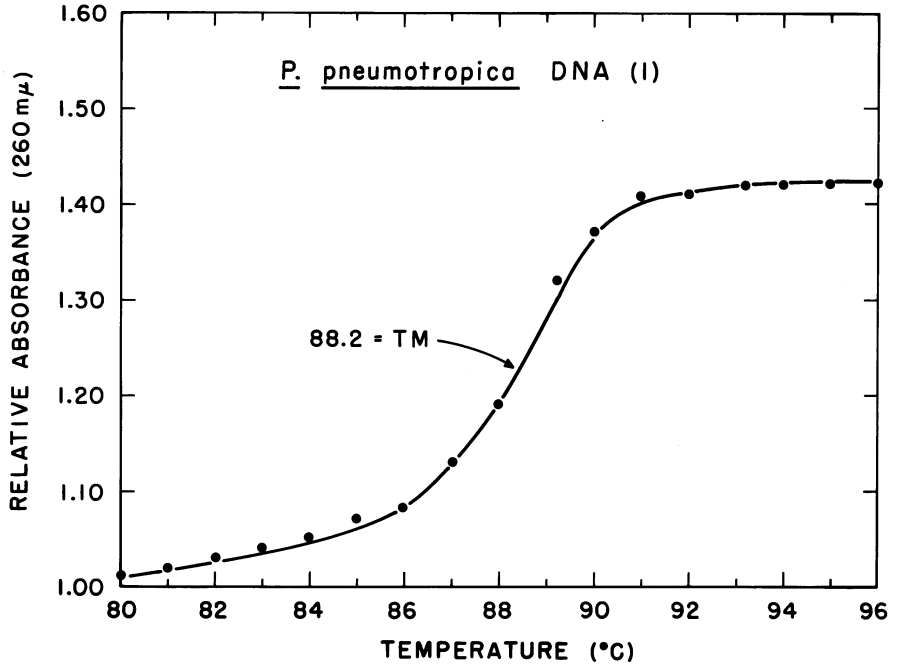


Fig. 13

Figs. 13-15. Temperature absorbance curves of DNA in SSC extracted from Pasteurella pneumotropica 9538. The temperature of the DNA solutions (20 μg/ml) was raised one degree C every min. Absorbance was followed in a Gilford model 2000 spectrophotometer until no further increase in absorbance was observed. Relative absorbance is plotted as the $A_{260}$/at each temperature/$A_{260}$ at 30 C.

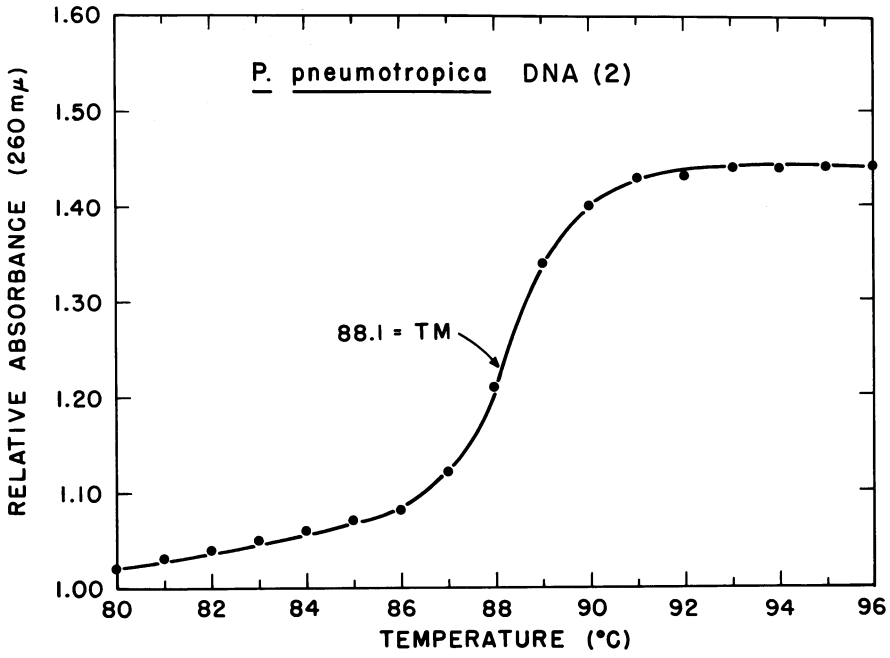
P. pneumotropica DNA (2)
88.1 = TM
RELATIVE ABSORBANCE (260 mμ)
TEMPERATURE (°C)
1.60
1.50
1.40
1.30
1.20
1.10
1.00
80
82
84
86
88
90
92
94
96

Fig. 14

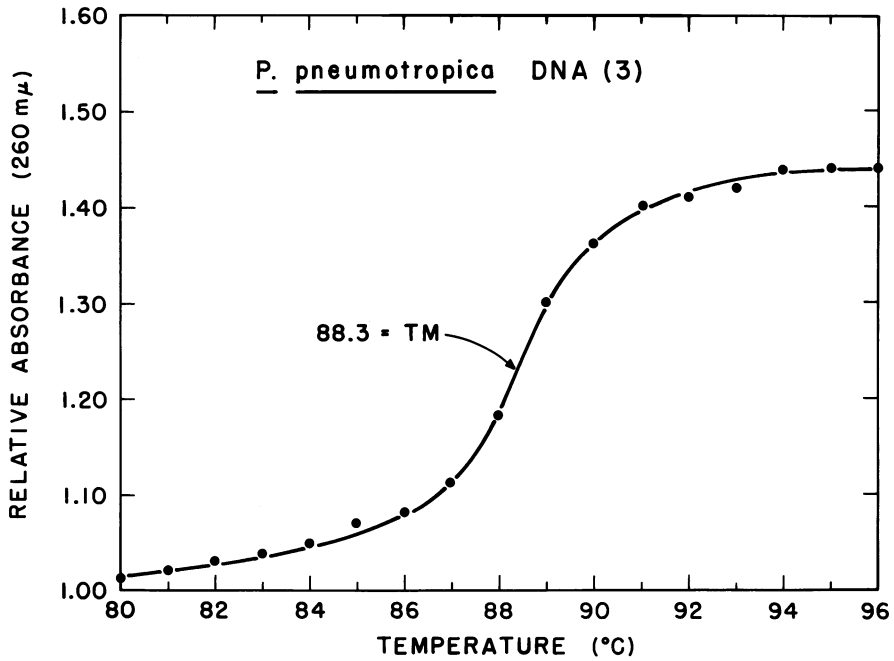
P. pneumotropica DNA (3)
88.3 = TM
RELATIVE ABSORBANCE (260 mμ)
TEMPERATURE (°C)
1.60
1.50
1.40
1.30
1.20
1.10
1.00
80
82
84
86
88
90
92
94
96

Fig. 15

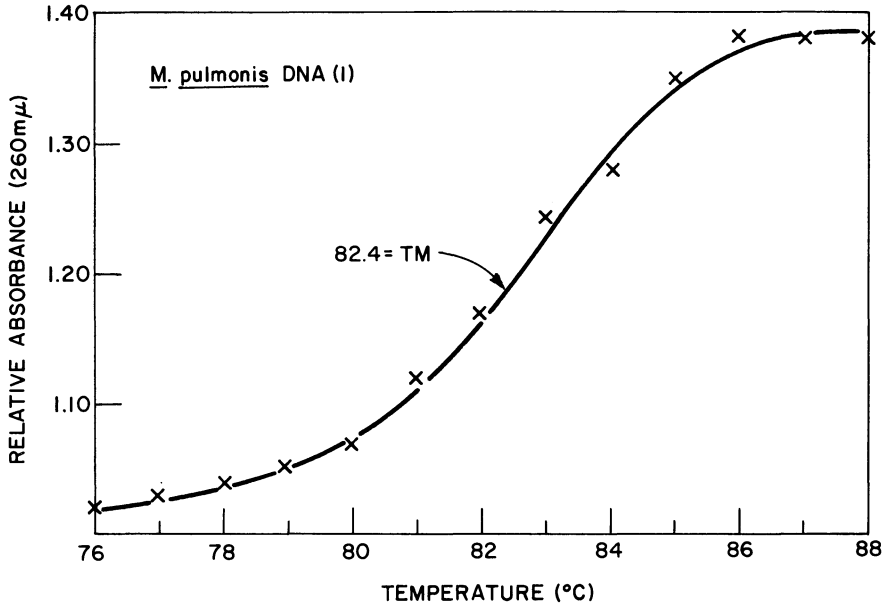


**Fig. 16**

**Figs. 16-19. Temperature absorbance curves of DNA in SSC extracted from Mycoplasma pulmonis N1. The temperature of the DNA solutions (20 μg/ml) was raised one degree C every 10 min. Absorbance was followed in a Gilford model 2000 spectrophotometer until no further increase in absorbance was observed. Relative absorbance is plotted as the $A_{260}$ at each temperature/$A_{260}$ at 30 C.**

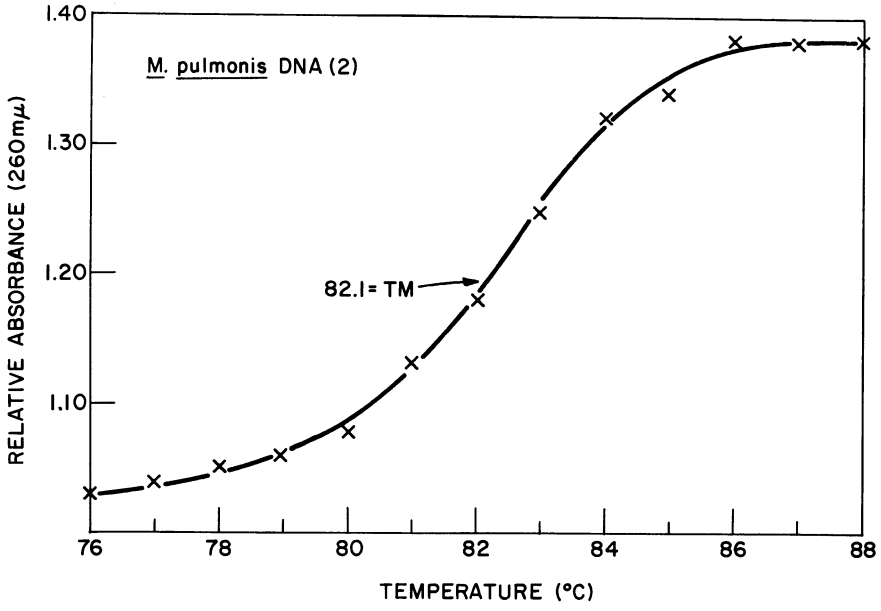
M. pulmonis DNA (2)
82.1= TM
RELATIVE ABSORBANCE (260mμ)
1.40
1.30
1.20
1.10
76
78
80
82
84
86
88
TEMPERATURE (°C)

Fig. 17

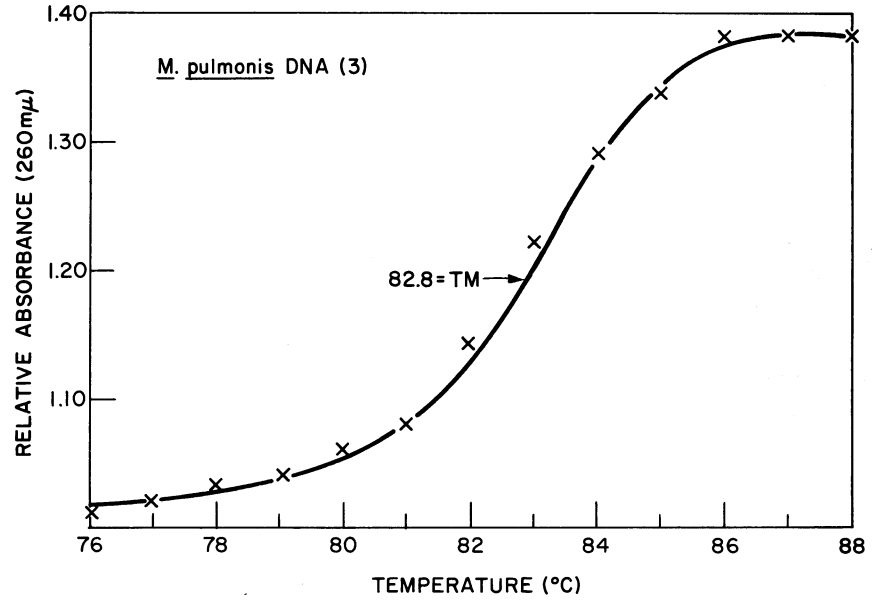
M. pulmonis DNA (3)
82.8=TM
RELATIVE ABSORBANCE (260mμ)
1.40
1.30
1.20
1.10
76
78
80
82
84
86
88
TEMPERATURE (°C)

Fig. 18

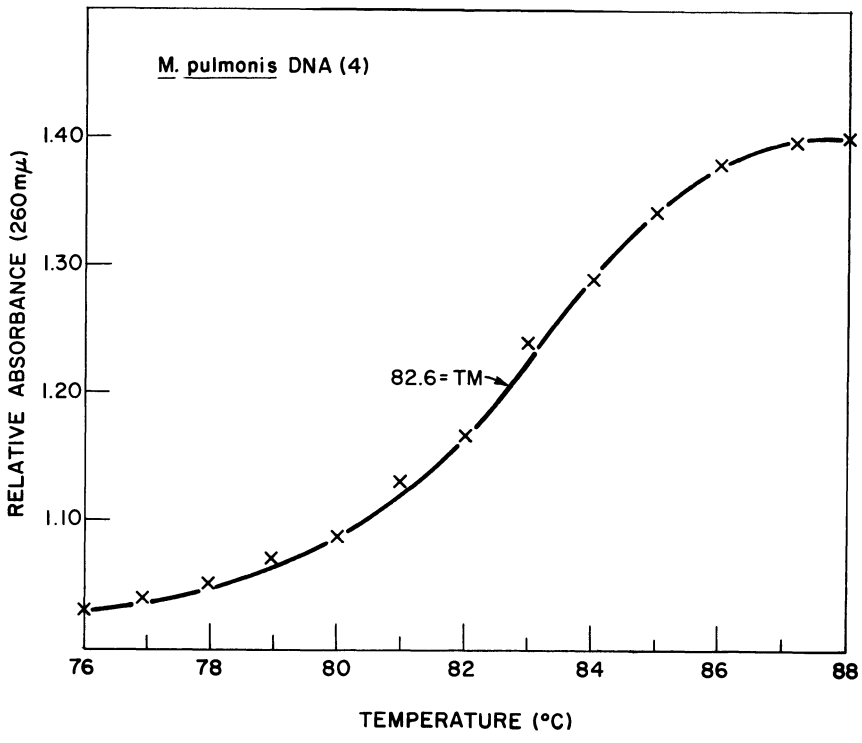
M. pulmonis DNA (4)
RELATIVE ABSORBANCE (260mμ)
1.40
1.30
1.20
1.10
82.6= TM
76
78
80
82
84
86
88
TEMPERATURE (°C)

**Fig. 19**

TABLE 14. GC base ratios of Pasteurella pneumotropica and Mycoplasma pulmonis DNA from Tm

| Source of DNA | Tm(°C)* | % GC | Mean % GC ± SE |
|---|---|---|---|
| P. pneumotropica | 88.2<br>88.1<br>88.3 | 44.4<br>44.2<br>44.7 | 44.4 ± 0.6 |
| M. pulmonis | 82.4<br>82.1<br>82.8<br>82.6 | 30.9<br>30.2<br>31.9<br>31.4 | 31.1 ± 0.6 |

* Determined in SSC.

TABLE 15. Buoyant density of Pasteurella pneumotropica and Mycoplasma pulmonis DNA in CsCl*

| Source of DNA | Distance (cm) of the sample from center of rotation | Density ($g/cm^3$) | % GC |
|---|---|---|---|
| P. pneumotropica | 6.78 | 1.705 | 45.9 |
| M. pulmonis | 6.66 | 1.690 | 30.6 |

* The density of the original CsCl solution was 1.710 $g/cm^3$. The radial center of the liquid column was 6.82 cm.

TABLE 16. The effect of drying and preincubation on $^{3}H$ DNA extracted from Pasteurella pneumotropica

| $^{3}H$ DNA attached to filter (μg) | Treatment* | Corrected mean counts/min retained on the filter | % input# retained on the filter | % released during preincubation |
|---|---|---|---|---|
| 1.0 | Dried | 2227 | 98.7 | -- |
| 1.0 | Dried, PM | 2128 | -- | 4.4 |
| 0.5 | Dried | 1132 | 98.6 | -- |
| 0.5 | Dried, PM | 1079 | -- | 4.7 |
| 0.2 | Dried | 319 | 70.7 | -- |
| 0.2 | Dried, PM | 312 | -- | 2.2 |

* Each filter was dried in a vacuum dessicator overnight and then at 80 C for 2 hr under 29 inches of Hg in a vacuum oven.

\# The input of $^{3}H$ DNA was 2256 counts/min.

TABLE 17. The effect of varying the amount of $^{3}H$ DNA and unlabeled DNA from Pasteurella pneumotropica on percent binding

| Group | µg DNA bound to filter | µg $^{3}H$ DNA added* | Counts/min retained | Percent $^{3}H$ DNA bound | Mean percent $^{3}H$ DNA bound ± SE |
|---|---|---|---|---|---|
| I | 20.0 | 1.0 | 600 | 26.6 | 28.7 ± 7.3 |
| | 0 | | 35 | 1.6 | |
| | 20.0 | | 793 | 35.2 | |
| | 0 | | 65 | 2.9 | |
| II | 6.3 | 0.5 | 379 | 33.6 | 36.0 ± 7.0 |
| | 0 | | 12 | 1.1 | |
| | 6.3 | | 479 | 42.5 | |
| | 0 | | 34 | 3.0 | |
| III | 6.3 | 0.2 | 70 | 15.5 | 19.0 ± 9.1 |
| | 0 | | 5 | 1.1 | |
| | 6.3 | | 138 | 30.6 | |
| | 0 | | 32 | 7.1 | |

* 1.0 µg = 2256 cpm
0.5 µg = 1128 cpm
0.2 µg = 451 cpm

**Statistical Analysis**

1) Comparison of Groups I - II
   $t_{(3)} = 1.9$ (not significant at the 5% level)
2) Comparison of Groups I - III
   $t_{(3)} = 2.3$ (significant at the 5% level)
3) Comparison of Groups II - III
   $t_{(3)} = 4.3$ (significant at the 2.5% level)

TABLE 18. The effect of varying the amount of $^3H$ DNA and unlabeled DNA from Mycoplasma pulmonis on percent binding

| Group | µg DNA bound to filter | µg $^3H$ DNA added* | Counts/min retained | Percent $^3H$ DNA bound | Mean percent $^3H$ DNA bound ± SE |
|---|---|---|---|---|---|
| I | 10.0 | 1.0 | 713 | 47.3 | 43.4 ± 4.8 |
| | 0 | | 19 | 1.3 | |
| | 10.0 | | 643 | 42.7 | |
| | 0 | | 28 | 1.9 | |
| II | 10.0 | 0.5 | 358 | 47.5 | 51.4 ± 8.0 |
| | 0 | | 1 | 0.1 | |
| | 10.0 | | 420 | 55.8 | |
| | 0 | | 3 | 0.4 | |
| III | 5 | 0.5 | 302 | 40.1 | 41.6 ± 3.5 |
| | 0 | | 2 | 0.3 | |
| | 5 | | 330 | 43.8 | |
| | 0 | | 4 | 0.5 | |
| IV | 5 | 0.2 | 143 | 47.5 | 47.7 ± 3.0 |
| | 0 | | 4 | 1.3 | |
| | 5 | | 158 | 52.5 | |
| | 0 | | 10 | 3.3 | |

* 1.0 µg = 1506 cpm
0.5 µg = 753 cpm
0.2 µg = 301 cpm

**Statistical Analysis:**

1) Comparison of Groups I - II; $t_{(3)} = 2.2$; significant at the 5% level.
2) Comparison of Groups I - III; $t_{(3)} = 0.64$; not significant at the 5% level.
3) Comparison of Groups I - VI; $t_{(3)} = 1.6$; not significant at the 5% level.
4) Comparison of Groups II - III; $t_{(3)} = 2.7$; significant at the 5% level.
5) Comparison of Groups II - IV; $t_{(3)} = 1.0$; not significant at the 5% level.
6) Comparison of Groups III - IV; $t_{(3)} = 2.4$; significant at the 5% level.

TABLE 19. Relative percent homology of DNA from Pasteurella pneumotropica and Mycoplasma pulmonis

| Recipient DNA attached to filter | $^3$H donor DNA | % $^3$H DNA bound ± SE | Relative % binding* |
|---|---|---|---|
| P. pneumotropica 6.3 µg | P. pneumotropica 0.5 µg | 32.6 ± 0.8 | 100 |
| Blank | P. pneumotropica 0.5 µg | 1.2 ± 0.9 | 0 |
| P. pneumotropica 6.3 µg | M. pulmonis 0.5 µg | 3.0 ± 0.7 | 6.7 |
| Blank | M. pulmonis 0.5 µg | 0.9 ± 0.7 | 0 |
| M. pulmonis 5.0 µg | M. pulmonis 0.5 µg | 41.7 ± 3.8 | 100 |
| Blank | M. pulmonis 0.5 µg | 1.1 ± 1.1 | 0 |
| M. pulmonis 5.0 µg | P. pneumotropica 0.5 µg | 3.9 ± 0.3 | 5.2 |
| Blank | P. pneumotropica 0.5 µg | 1.8 ± 1.1 | 0 |

* Relative % binding = [(% binding of donor DNA to sample) - (% binding of donor to blank filter)] / [(% binding of donor DNA to its homologous DNA) - (% binding of donor to blank filter)] x 100.

## DISCUSSION

The characteristics of the DNA preparations isolated from P. pneumotropica and M. pulmonis are in good agreement with the characteristics reported by other workers. The spectra are consistent with those reported by Beaven, Holiday and Johnson (9). The absorbance ratios and the molecular weights are very similar to those reported by Marmur (86) and Neimark and Pene (93). The finding that both the P. pneumotropica and M. pulmonis DNAs have high molecular weights is particularly important because a high molecular weight is necessary for the "hyperchomic shift" observed when DNA is denatured (9). This property forms the basis for determining the percent GC from the thermal denaturation temperature.

The GC content of P. pneumotropica is not reported in the literature, and only a few other pasteurella have been studied. McGee et al. (84) state that the GC content of P. multocida is approximately 39-40%, but they give neither data nor references to substantiate their statement. Hill (55) reports the percent GC of P. aviseptica and P. boviseptica which are presently regarded as P. multocida strains as 36.5 and 37.5. Bekker and Kutsemokima (10) report a value of 43.1% GC for P. multocida strains. My result of 44.4% for P. pneumotropica is closer to the value of 46-47% reported for P. pestis. It has been recognized for some time that the genus Pasteurella is composed of a heterogeneous group of organisms and it has been suggested that P. tularensis and P. novicida be formed into a separate genus, Francisella, and P. pestis and P. pseudotuberculosis be placed in the genus Yersinia (125, 131, 135). Smith and Thal (131) believe that the genus Pasteurella should be reserved for species that are oxidase positive, that is, P. hemolytica, P. multocida and

P. pneumotropica, and that the status of species they did not investigate such as P. boviseptica and P. aviseptica requires further investigation. Because of the heterogeneity of the genus and because so few determinations of GC content have been made, it seems wise to postpone any statement regarding the limits of the percent GC of the genus.

Heterogeneity of GC base composition also exists in the genus Mycoplasma. Neimark and Pene (93) reported values of 23.6-24.1% for a calf and goat strain of mycoplasma. M. laidlawii, the saprophytic strain, is reported to have a GC content of 30.9%. Rogul et al. (128) report the range for the genus as 23.6-46.6% GC. Many strains isolated from animals showed a percent GC content varying from 30% to 35% (55, 128) and my result of 31.1% agrees well with these latter data. Other mycoplasma with similar percentages are M. gallisepticum (32.0%) and M. arthritidis (31.9% (113). The range of 23% in GC composition of the genus suggests that several genera should be established, rather than only one.

The DNA homology system selected for use in these experiments has several advantages over the agar column technique devised by McCarthy and Bolton (83). The amount required for use in the membrane system is only 1/10 as much as in the agar system, and there is little loss of DNA from the filter whereas 10% or more is lost from the agar (20). The separation of the bound from the unbound DNA is achieved in the membrane system by simply removing it from the solution and washing it. The only disadvantage of the membrane technique is that the amount of DNA bound in the homologous system is slightly less than that bound in the agar technique. However, since relative rather than absolute values are used for comparisons, this is not considered a serious

disadvantage. The lower amount of binding of the label in the membrane system is believed to be the result of renaturation of the DNA fragments in solution.

It is not known why more M. pulmonis DNA binds in the homologous system than P. pneumotropica DNA. One factor might be the smaller molecular weight of the labeled M. pulmonis fragments. These fragments would bind more readily with DNA attached to the filter than to fragments in solution. It is also possible that more molecules of M. pulmonis DNA can form duplexes at 60 C than can molecules of P. pneumotropica DNA.

The results show conclusively that M. pulmonis is not the L form of P. pneumotropica. This was suggested by the disparity in percent GC obtained. Generally, similar overall DNA base composition is considered a necessary requirement for extensive base sequence homology with the maximum allowable difference in base composition being 4% for homology (13, 15). The DNA base homology studies show that there are few, if any, large sequences of base pairs in common between M. pulmonis and P. pneumotropica. However, as pointed out by McGee et al. (83) these are negative results and the possibility still exists that M. pulmonis is the L form of some other bacterial species whose percent GC is closer to that of M. pulmonis--for example, the streptococci.

In this study the differences in relative percent binding between homologous and heterologous reactions of P. pneumotropica and M. pulmonis are large enough so that there is no need for a statistical test to show their significance. However, when other than gross differences in DNA homology are found, another method of evaluating the results must be found. Such methods have been developed by Reich et al. (126).

DNA homology techniques presently used are unable to detect small changes in nucleotide base sequence. Consequently, it is still possible that a point mutation is responsible for the changes to an L form rather than simply a phenotypic response to environmental conditions. As pointed out in the introduction, however, this method allows for direct genetic comparisons, rather than comparisons on the basis of gene products. It is therefore considered more reliable than comparisons based on immunologic and metabolic studies.

# CHAPTER V

## SINGLE AND MIXED EXPERIMENTAL INFECTION IN AXENIC MICE WITH *PASTEURELLA PNEUMOTROPICA* AND *MYCOPLASMA PULMONIS*

The results of experiments described in Chapter IV show conclusively that *M. pulmonis* is not the stable L form of *P. pneumotropica*. Still unresolved is the question of whether *M. pulmonis* or *P. pneumotropica* alone cause murine pneumonia or whether both organisms are necessary to the development of the disease.

Lutsky and Organick (82) studied pneumonia caused by *M. pulmonis* in conventional and axenic mice. They observed that the disease had a more rapid onset in both types of mice than had been observed by Nelson (94, 95) in conventional mice. The microscopic lesions described by Lutsky and Organick were essentially the same in conventional and axenic mice infected with *M. pulmonis* although bacteria were present in the lesions of conventional mice, presumably as secondary invaders. The most outstanding characteristics of the microscopic lesions attributed to *M. pulmonis* infection in axenic mice were occlusion of the bronchi with a polymorphonuclear exudate and marked lymphocytic infiltration of perivascular and peribronchial areas.

*P. pneumotropica* infection has not been studied in axenic mice. The intimate association of the organism with spontaneous cases of murine pneumonia and its constant occurrence in the pneumonic lesions of mice experimentally infected with *M. pulmonis* indicates that such a study is necessary. The study of *P. pneumotropica* infection in mice free from all other indigenous bacteria and mycoplasma should provide information on the role of *P. pneumotropica* in

murine pneumonia.

If both P. pneumotropica and M. pulmonis can cause a primary pneumonia in mice, a method for differential diagnosis is needed. At present cultural methods are the only ones available for the diagnosis of M. pulmonis infection. For optimum results these methods require 7 days and large amounts of media. Furthermore, because mycoplasma are nutritionally fastidious, particularly on primary isolation, negative culture results are unreliable as an index of infection in spontaneous murine pneumonia. A method which is not dependent on isolation of the organisms is the fluorescent antibody technique originally developed by Coons, Creech and Jones (19). This method has the additional advantage of requiring only a few hours for completion of the test. The indirect fluorescent antibody (FA) technique has been applied with success to the diagnosis and study of M. pneumoniae infection (81, 142). It has not been used to study M. pulmonis and/or P. pneumotropica infection.

## MATERIALS AND METHODS

The organisms, media, preparation of inocula and antisera are the same as described in Chapter III.

Animals. Female CD-1 axenic mice, 21-28 days of age were purchased from the Charles River Breeding Laboratories. On arrival at the laboratory they were transferred into sterile plastic film isolators where they were maintained axenic by supplying autoclaved feed, water and bedding. Samples of feces and bedding were collected and cultured at regular intervals during the course of experiments. No contamination was detected at any time.

Inoculation and sampling methods. Suspensions of organisms and all necessary equipment were put into the isolators through an air lock. The materials were placed in the air lock and the sealed port was sprayed with 2% peracetic acid in a 0.2% Nacconal (Fisher Scientific Co., Fair Lawn, N. J.) solution. After 30 min exposure to the vapor the materials were brought into the chamber. The mice were inoculated intranasally while under ether anesthesia with 0.05 ml of the inoculum using a Microtiter pipette calibrated to deliver 0.025 ml/drop. Fifteen to 20 control mice for each experiment were inoculated with 0.05 ml sterile broth and housed in a separate isolator. At 2 and 3 days after inoculation and at 4-5 day intervals thereafter groups of mice were removed from the chamber. They were transferred to a sterile hood, killed by cervical dislocation, and necropsied. Five to 10 control mice were similarly treated on the first day after inoculation and the remainder were killed the day after the experiment was terminated.

The lungs of all the mice were cultured for M. pulmonis and P. pneumotropica as previously described, and portions from three lungs were

fixed in formalin for histopathologic examination. The lungs of the remaining mice were frozen at -20 C and sections were cut on a Cryostat (International Equipment Co., Boston, Mass.) at 7 μ. The frozen sections were stored at -20 C until they were stained.

Hematoxylin and eosin staining of frozen sections. A modification of the method of Ibanez et al. (57) was used. The frozen sections were fixed in cold methyl alcohol for 2 min. They were then stained according to the following schedule:

> xylene, 5 sec; absolute alcohol 1-2 sec; 95% alcohol, 2 sec; 70% alcohol, 2 sec; tap water, 2 sec; Harris hematoxylin, 1 min; tap water, 2 sec; alcoholic eosin, 30 sec; 95% alcohol, 1 sec; absolute alcohol, 15 sec; xylene, 1 min; xylene, 5 min.

They were mounted in Permount (Fisher Scientific Co.).

Indirect fluorescent antibody (FA) staining. The indirect method of FA staining of tissue sections developed by Liu (81) was modified for use in these studied. The hyperimmune rabbit serum prepared against M. pulmonis and P. pneumotropica in Chapter III had FA titers identical to the agglutination titers and was used throughout this study. The sections were fixed for 10 min in cold methyl alcohol and dried at 37 C for 30 min. A circle was drawn around the section with a marktex pen (Mark-Tex Corp., Englewood, N. J.). Two drops of hyperimmune rabbit serum or normal rabbit serum diluted 1:10 in phosphate buffered saline (PBS; Difco) was placed on the slide and allowed to react at room temperature for 30 min in a moist chamber. The excess serum was washed off with PBS and the slide was soaked in each of two changes of PBS for 10 min. The slide was blotted carefully and two drops of fluorescin isothiocyanate labeled anti-rabbit goat globulin (Difco) diluted 1:10 in PBS

were added to each section. This PBS also contained rhodamine counterstain (Difco) at a dilution of 1:40. The slides were again allowed to react at room temperature for 30 min and soaked in PBS. The sections were mounted under a cover slip with buffered FA mounting fluid (Difco) and examined with a Leitz Ortholux microscope, equipped for fluorescence microscopy. The light source was an Osram HBO 200 W mercury vapor lamp. The ultraviolet exciting filter was a Schoot 15430. The eyepieces were fitted with ultraviolet absorbing filters. Color photographs were taken using Super Anscochrome film. The exposure time was from 1-3 min using the 35 mm Orthomat automatic microscope camera. The fluorescence of each slide was graded according to the following scheme: - = no fluorescence; + = dull fluorescence; ++ = definite fluorescence; +++ = bright fluorescence; ++++ = brilliant yellow-green fluorescence.

Experimental design. In experiment 1 mice were given an intranasal instillation of $2.5 \times 10^7$ CFU M. pulmonis N1 per mouse. In experiment 2 mice were given $4 \times 10^8$ P. pneumotropica cells/mouse intranasally. In experiment 3 mice were given $7.5 \times 10^6$ CFU M. pulmonis and $5 \times 10^7$ P. pneumotropica/mouse intranasally.

In experiment 4 M. pulmonis was given intranasally to another group of axenic mice. In this experiment the mice were examined at 1, 2, 4, 5, 9 and 15 days after infection. The lungs were graded as to the severity of the pathologic changes and cultured, but tissue sections were not made. In a fifth experiment, M. pulmonis, Milwaukee strain, was given to axenic mice. Each mouse received $3 \times 10^7$ CFU intranasally. Six mice were necropsied 9 days after infection and the rest 15 days after infection.

A drawing of the lesions in the lungs of each mouse was made at necropsy on a diagram of mouse lung. A subjective evaluation of the extent of pulmonary involvement was made at necropsy. This evaluation was as follows: 0 = no gross lesions; 1+ = moderate congestion; 2+ = severe congestion with or without 1 or 2 small (1 mm) focal areas of consolidation; 3+ = moderate consolidation; 4+ = severe consolidation.

A subjective evaluation was also made of the severity of microscopic lesions; 0 = no lesions; 1+ = slight perivascular infiltration of lymphocytes with or without peribronchial infiltration; 2+ = marked perivascular and peribronchial lymphocyte infiltration; 3+ = patches of alveolar consolidation; 4+ = large confluent areas of alveolar consolidation.

Statistical analysis. A two-way analysis of variance of the mean scores of the gross and microscopic lesions produced by the three treatments was performed to determine if there was a difference between treatments and between days. To determine if the effects observed in simultaneous infection with both organisms were a result of synergism, a "t" test was performed using the interaction mean square to calculate the standard error of the difference. The computations were performed on the GE 225 computer. Comparisons of culture results, FA results and gross pathologic scores were made using the standard error of the difference of two proportions to compute a "t" value.

## RESULTS

Macroscopic lesions. The lungs of axenic mice are uniformly pinkish yellow in color and consolidated areas are never seen (Fig. 20). Twenty four hr after infection with M. pulmonis, hemorrhagic areas were observed in the left lung and the superior lobe of the right lung (Fig. 21). At that time only half of the animals examined showed such lesions. Figure 22 shows typical lesions observed 2 days after infection with M. pulmonis. There are extensive areas of hemorrhage, and firm red areas of consolidation may be observed in the left lung. Five days after infection, the lungs were extensively consolidated, usually on their dorsal surface (Fig. 23). The mice infected with M. pulmonis N1 and the Milwaukee strain showed similar lesions and although large portions of the lungs were involved no deaths occurred in any of the experiments. The macroscopic lesions were most extensive 5 days after infection after which the severity diminished. Nine to 10 days after infection only 3 of 12 mice infected with M. pulmonis N1 showed the severe lesions seen in Fig. 22 and 23, and only 2 of 6 mice infected with the Milwaukee strain showed such lesions. Few changes were observed 20 days after infection with either strain of M. pulmonis.

When axenic mice were mono-infected with P. pneumotropica the gross pathology observed was similar to that seen in M. pulmonis infected mice for 2-5 days. The lesions were somewhat less uniform in distribution but were more pronounced close to the hilus (Fig. 24). The consolidated areas were a deeper shade of gray than those observed in M. pulmonis infection. In contrast to mice given M. pulmonis those infected with P. pneumotropica developed abscesses. Figure 25 shows P. pneumotropica infected lungs 10 days after

infection. Consolidated areas may be seen in all lobes with grayish nodules on the superior lobe of the right lung. Purulent material was easily demonstrated when these nodules were incised.

Mice simultaneously infected with M. pulmonis and P. pneumotropica developed more severe lesions than mice monoinfected with either organism. Two days after infection (Fig. 26), reddish-gray consolidated areas were visible in all lobes and marked hemorrhage was evident. The reaction was even more severe on the third day after infection (Fig. 27). Two deaths occurred in the mice infected with both organisms on the third day and one death occurred on the fourth day. Figure 28 shows the severely affected lungs of a mouse 4 days after infection. Eight days after infection large consolidated areas were still present in most animals (Fig. 29), and lesions of this type persisted until the experiment was terminated 25 days after infection. The characteristic lesions seen after 8 days were well demarcated gray-white small abscesses.

Microscopic lesions. In general the severity of the microscopic lesions closely paralleled the macroscopic lesions observed in mono-infected and mice infected with both M. pulmonis and P. pneumotropica. The alveoli and bronchi of control axenic mice are shown in Fig. 30. The alveoli are characterized by a lacy open appearance of the cells. Lymphocytic infiltration around the bronchi and vessels is never observed in germfree mice. The microscopic lesions in the lungs of axenic mice infected 1-2 days with M. pulmonis were characterized by capillary engorgement, edema and hemorrhage. Slight to moderate infiltration of the perivascular areas by leucocytes was also observed. Three days after infection the reaction became severe (Fig. 31).

The alveoli were filled with polymorphonuclear cells and lymphocytes, but the lumens of the bronchi remained free from any exudate. This reaction was a constant finding up to 5 days following infection. From 9-10 days after infection, the characteristic lesions were perivascular and peribronchial cuffing with lymphocytes (Fig. 32 and 33). This was observed with varying degrees of severity until the experiment was terminated 30 days after infection.

The early lesions (1-2 days) in mice mono-infected with P. pneumotropica were similar to those observed in M. pulmonis infection. The polymorphonuclear response was more severe and more lymphocytic infiltration was observed. The reaction was most severe 3-5 days after infection and began to subside after 9-10 days (Fig. 34). After this a constant finding was the presence of aggregates of lymphocytes (lymph nodules) throughout the lungs (Fig. 35).

In mice infected with both M. pulmonis and P. pneumotropica, the microscopic lesions were much more severe. Three days after infection large pink staining foamy macrophages and polymorphonuclear leucocytes were the predominant cell types. The lumens of bronchi were filled with a purulent exudate (Fig. 36), which persisted throughout the course of the experiment. In later stages of the disease there was extensive lymphocytic infiltration into the peribronchial and perivascular areas (Fig. 37). Plasma cells were also present in the interstitial areas but in fewer numbers. The large macrophages persisted in consolidated areas throughout the period of observation. All the mice simultaneously infected with both organisms had microscopic areas of pneumonic involvement (Fig. 38) whereas a number of the mice infected with only one type of organism were without these lesions at the end of the experiment. A comparison of the severity of the gross lesions

produced by the three treatments is shown in Fig. 39 and a similar comparison of the microscopic lesions is shown in Fig. 40. A two-way analysis of variance shows that there is a significant difference between treatments and days (Appendix Table 17) in both macroscopic and microscopic lesions. The "t" test of the sum of the grand means of M. pulmonis alone and P. pneumotropica alone and the grand mean of simultaneous infection with both organisms is not significant at the 5% level. Inspection of the distribution of severe lesions (3 and 4+) in the three groups shows that after mixed infection all the mice show severe lesions at 2, 3 and 5 days but only half of the mice show severe lesions at these times when they are infected with only one organism. In later stages of infection none of the mono-infected mice show 3+ or 4+ lesions 15-25 days after infection, whereas 30-50% of the mice infected with both organisms show such lesions at that time.

FA response. Figure 41 shows fluorescence in the consolidated alveoli of lungs infected with P. pneumotropica when they are treated with P. pneumotropica antiserum. Uninfected mouse lungs allowed to react with specific antiserum (Fig. 42) and infected mouse lungs treated with normal rabbit serum (Fig. 43) do not fluoresce. No specific staining was observed when P. pneumotropica infected lungs were treated with antiserum against M. pulmonis or vice versa.

The lungs of mice infected with M. pulmonis show bright fluorescence, primarily in the bronchial epithelium when they are treated with M. pulmonis antiserum (Fig. 44). Controls are negative (Fig. 45 and 46).

The lungs of mice infected with both organisms showed fluorescence similar to that observed in mono-infected mice. When these lungs are treated with

P. pneumotropica antiserum (Fig. 47) fluorescence is observed in the alveoli, whereas when a section from the same lung is treated with M. pulmonis antiserum the fluorescence is confined to the bronchial epithelium (Fig. 48). The controls were negative.

The culture results and fluorescent antibody response of the three treatments are shown in Table 20. There is no significant difference in the percentage of mice giving a positive FA response between mono-infected mice and mice infected with both organisms regardless of the extent of pulmonary involvement. Similarly, when the culture results are compared with the FA response there is no significant difference in the percent positive when macroscopic lesions were present. In mice infected with both organisms which did not show visible lesions, P. pneumotropica was recovered from a significantly larger proportion of mice than was detected by the FA method.

When the recovery rates for each organism are compared, a larger proportion of the animals yielded M. pulmonis when they were infected with that organism alone, than when mice were also infected with P. pneumotropica. P. pneumotropica was also recovered from a significantly larger proportion of mono-infected mice than from those infected with both P. pneumotropica and M. pulmonis. P. pneumotropica and M. pulmonis recovery rates did not differ significantly from each other in mono-infected mice. P. pneumotropica was recovered from a larger proportion of animals without lesions than was M. pulmonis when mice were simultaneously infected with both organisms.

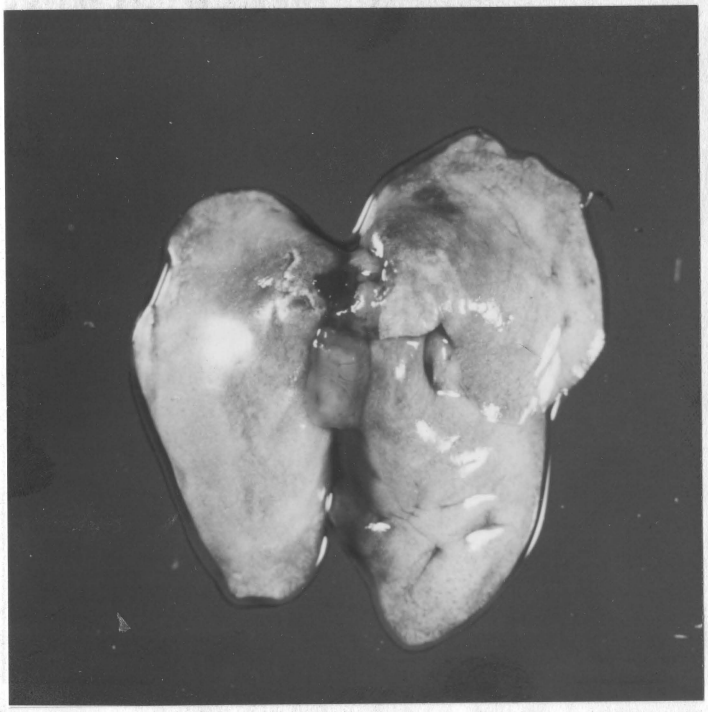

Fig. 20. Axenic control mouse lungs. Note the pale appearance and the absence of consolidation. This mouse was removed from the germfree chamber, killed by cervical dislocation and necropsied within 5 min of removal.

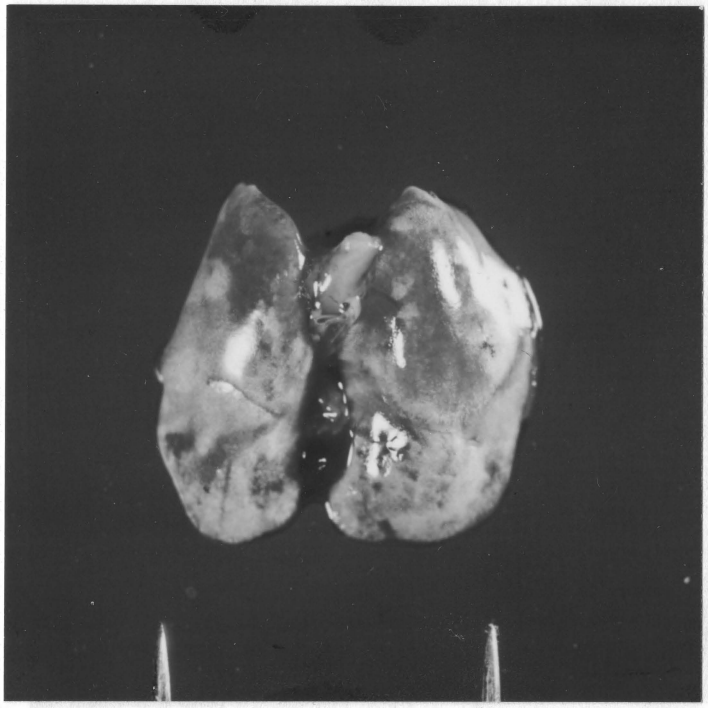

Fig. 21. Lungs from axenic mouse 24 hr after intranasal inoculation with $2.5 \times 10^7$ CFU *Mycoplasma pulmonis* N1. Hemorrhagic areas are seen in the left lung and the superior lobe of the right lung.

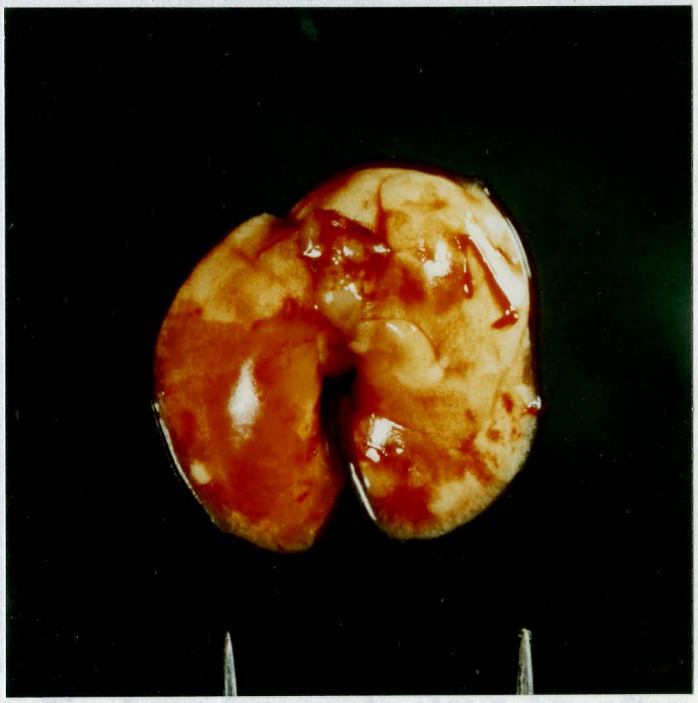

Fig. 22. Lungs from axenic mouse 2 days after intranasal inoculation with $2.5 \times 10^7$ CFU *Mycoplasma pulmonis* N1. Firm red areas of consolidation are observed in the left lung.

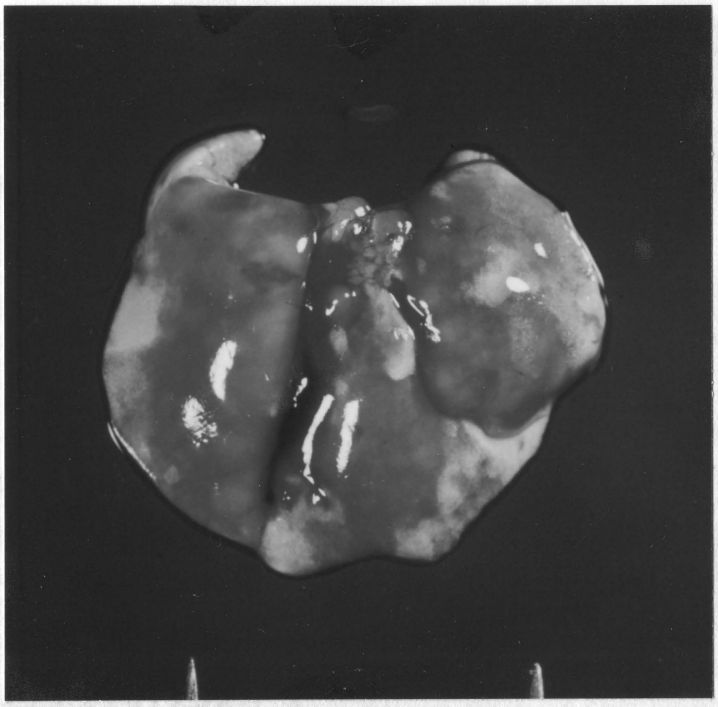

Fig. 23. Lungs from axenic mice 5 days after intranasal inoculation with 2.5 x $10^7$ CFU Mycoplasma pulmonis N1. The lungs show extensive consolidated areas.

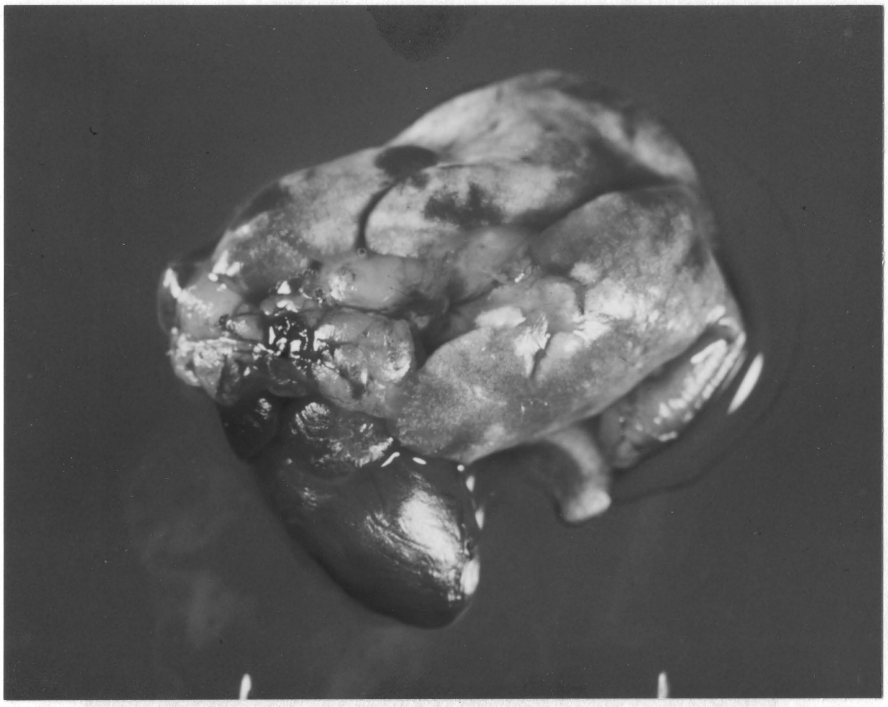

Fig. 24. Lungs from axenic mouse 5 days after intranasal inoculation with $4 \times 10^8$ Pasteurella pneumotropica 9538. The lesions are more pronounced close to the hilus and are gray in color.

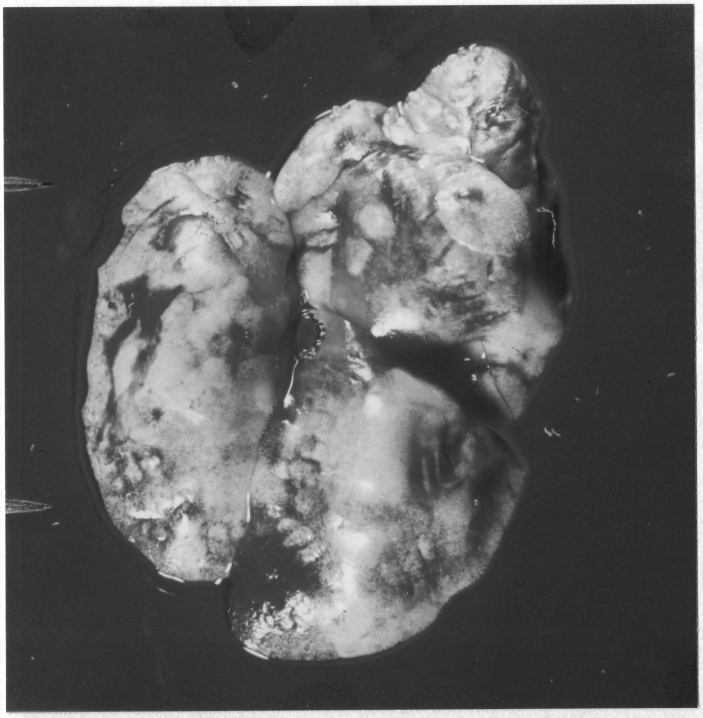

Fig. 25. Lungs from axenic mouse 10 days after intranasal inoculation with $4 \times 10^8$ Pasteurella pneumotropica 9538. Consolidated areas are observed in all lobes and gray translucent nodules are seen on the superior lobe of the right lung.

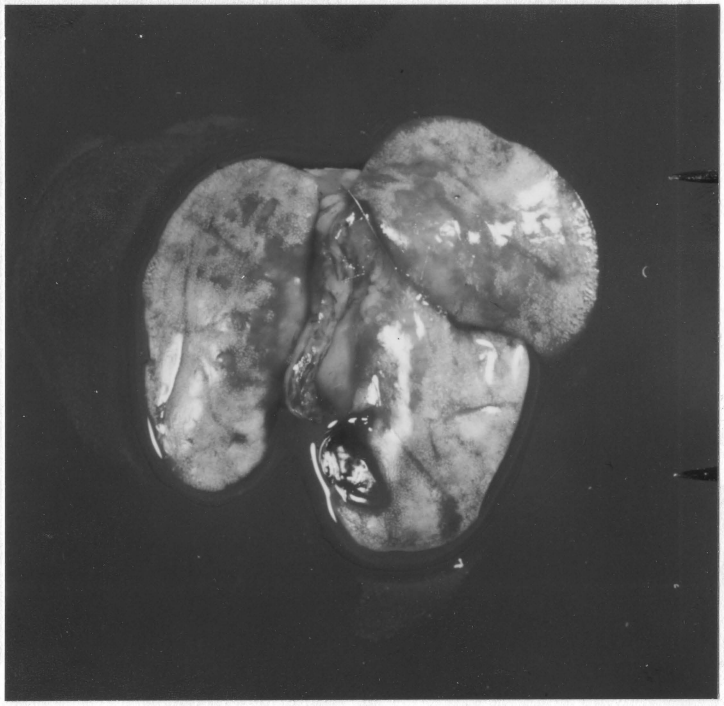

Fig. 26. Lungs from axenic mouse 2 days after intranasal inoculation with $7.5 \times 10^6$ CFU *Mycoplasma pulmonis* N1 and $5 \times 10^7$ *Pasteurella pneumotropica* 9538. Reddish-gray consolidated areas are visible on the inner aspect of all lobes.

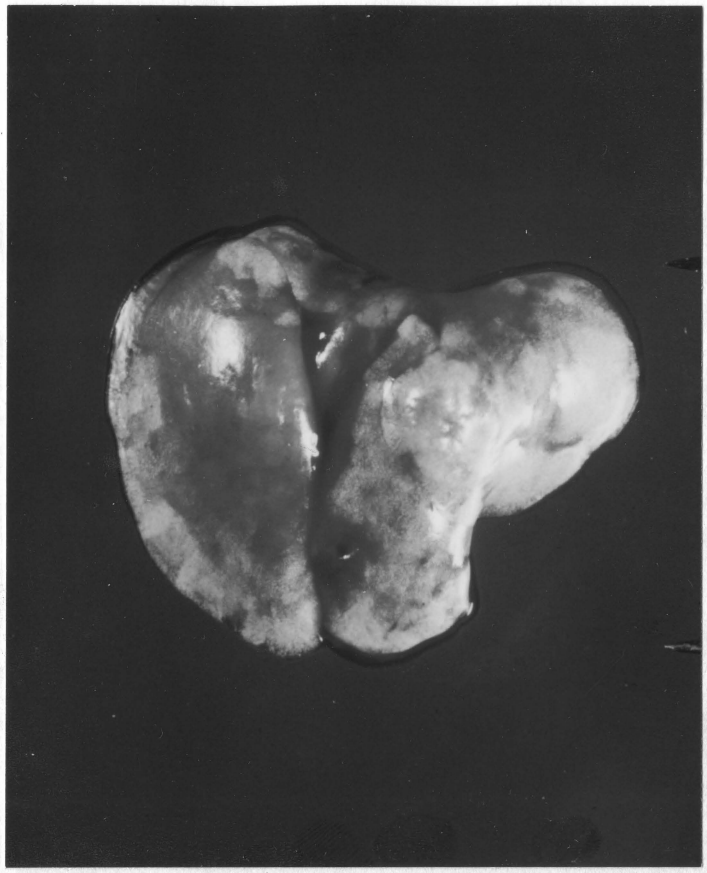

Fig. 27. Lungs from axenic mouse 3 days after intranasal inoculation with $7.5 \times 10^6$ CFU *Mycoplasma pulmonis* N1 and $5 \times 10^7$ *Pasteurella pneumotropica* 9538. The entire left lung is consolidated and areas of consolidation can be seen in the right lobes.

Fig. 28. Lungs from axenic mouse 4 days after intranasal inoculation with $7.5 \times 10^6$ CFU *Mycoplasma pulmonis* N1 and $5 \times 10^7$ *Pasteurella pneumotropica* 9538. The lungs are completely consolidated. This mouse died during removal from the chamber.

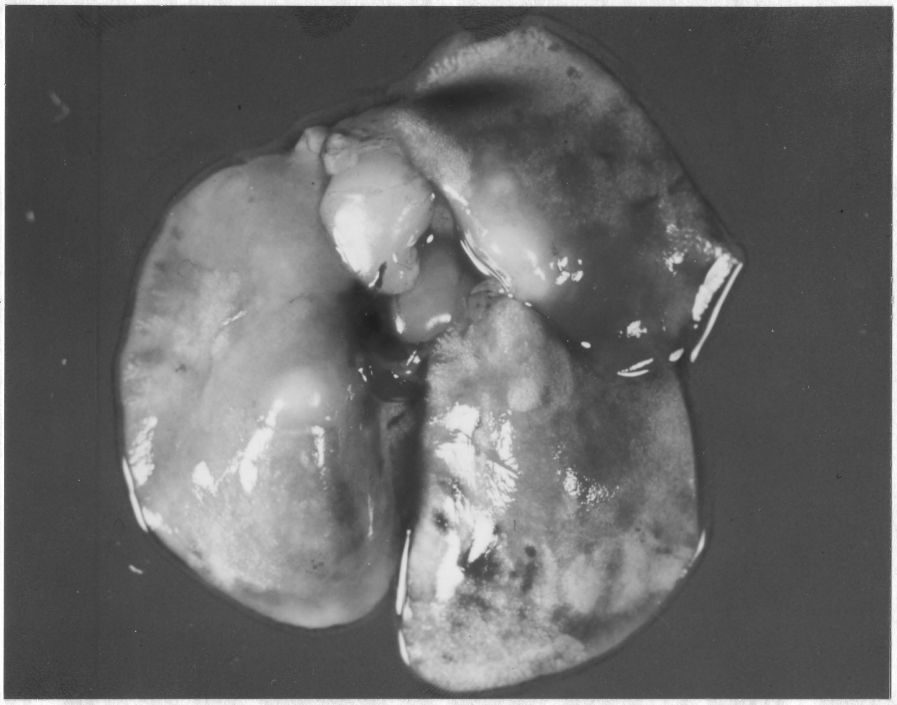

Fig. 29. Lungs from axenic mouse 8 days after intranasal inoculation with $7.5 \times 10^6$ CFU *Mycoplasma pulmonis* N1 and $5 \times 10^7$ *Pasteurella pneumotropica*. Large consolidated areas persist. In addition, abscesses can be seen on the left lung and on the superior lobe of the right lung.

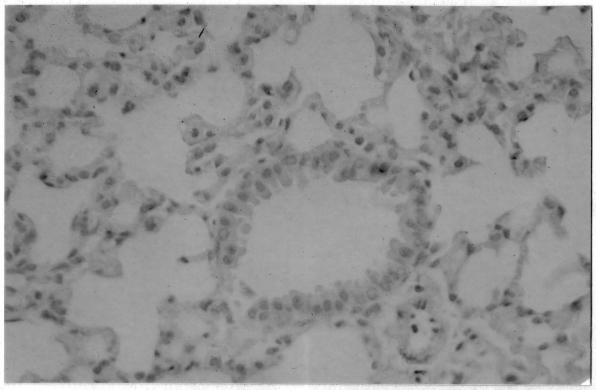

Fig. 30

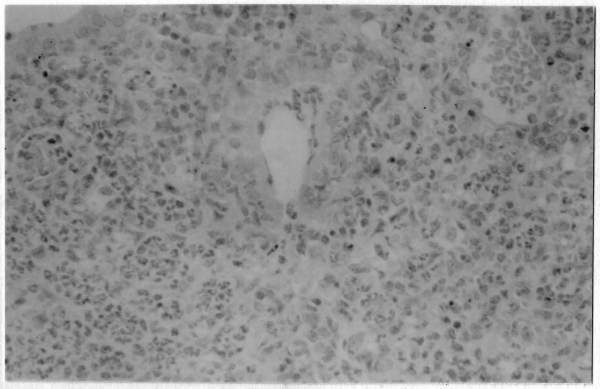

Fig. 31

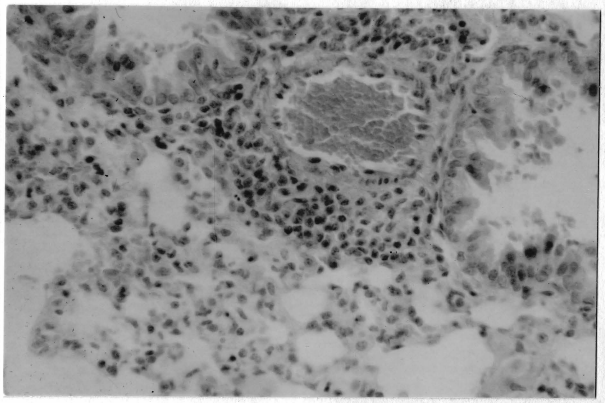

Fig. 32

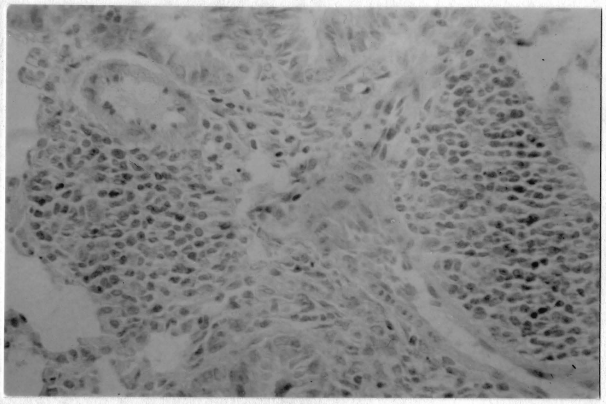

Fig. 33

Fig. 30. Alveoli and bronchus of axenic mouse (x 250). Hematoxylin and eosin stain.

Fig. 31. Alveoli and bronchusof Mycoplasma pulmonis N1 infected lungs (x 250). Hematoxylin and eosin stain. The alveoli are filled with polymorphonuclear cells.

Figs. 32 and 33. Perivascular and peribronchial lymphocytic infiltration in Mycoplasma pulmonis N1 infected lungs (x 250). Hematoxylin and eosin stain.

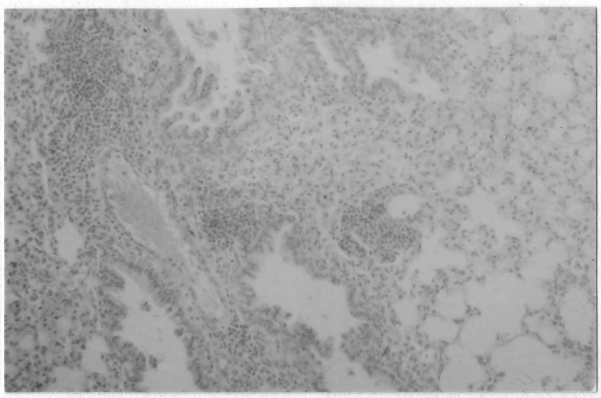

Fig. 34. Vessel and alveoli of lungs 9 days after intranasal inoculation with Pasteurella pneumotropica 9538 (x 100). The alveoli are filled with polymorphonuclear cells and lymphocytes. Infiltration by lymphocytes is marked. Hematoxylin and eosin stain.

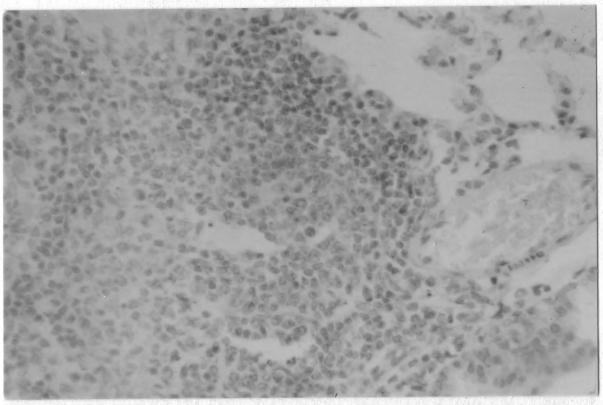

Fig. 35. Lymph nodule in the lungs of an axenic mouse 25 days after intranasal inoculation with Pasteurella pneumotropica 9538 (x 250). Hematoxylin and eosin stain.

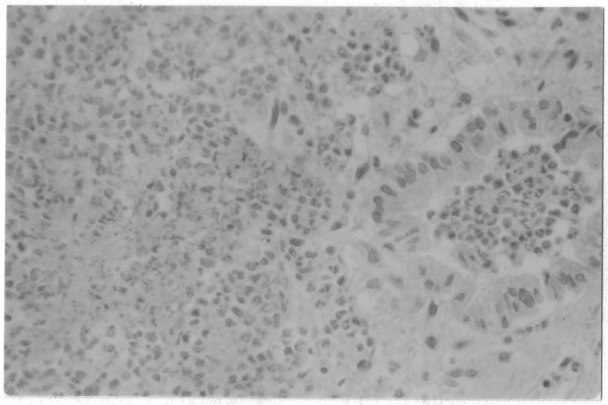

Fig. 36. Alveoli and bronchus of lungs 3 days after intranasal inoculation with Mycoplasma pulmonis N1 and Pasteurella pneumotropica 9538 (x 250). The alveoli are filled with polymorphonuclear cells and the bronchus is occluded by exudate. Hematoxylin and eosin stain.

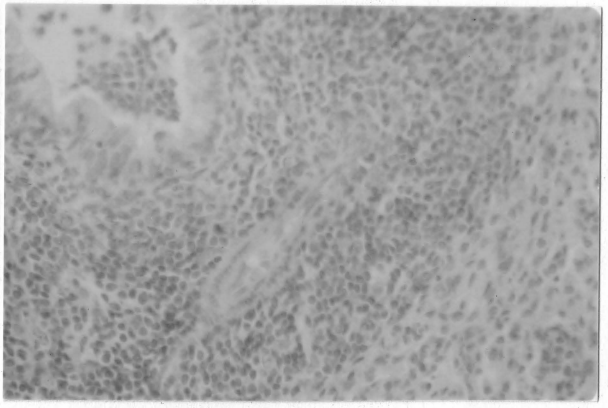

Fig. 37. Peribronchial and perivascular lymphocytic infiltration in mouse lung infected with Mycoplasma pulmonis N1 and Pasteurella pneumotropica 9538 20 days after inoculation (x 250). Hematoxylin and eosin stain.

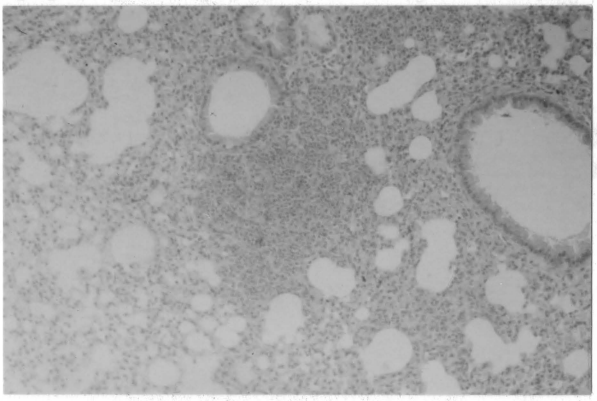

Fig. 38. Consolidated area in mouse lung infected with Mycoplasma pulmonis N1 and Pasteurella pneumotropica 9538 25 days after inoculation (x 100). Hematoxylin and eosin stain.

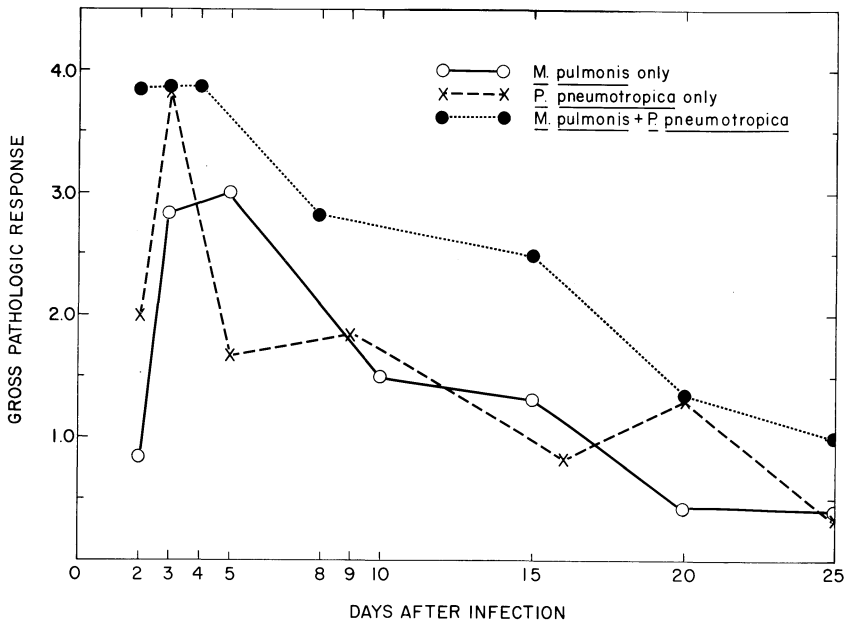


**Fig. 39. Comparison of the mean gross pathologic scores of axenic mice singly and doubly infected with *Mycoplasma pulmonis* N1 and *Pasteurella pneumotropica* 9538.**

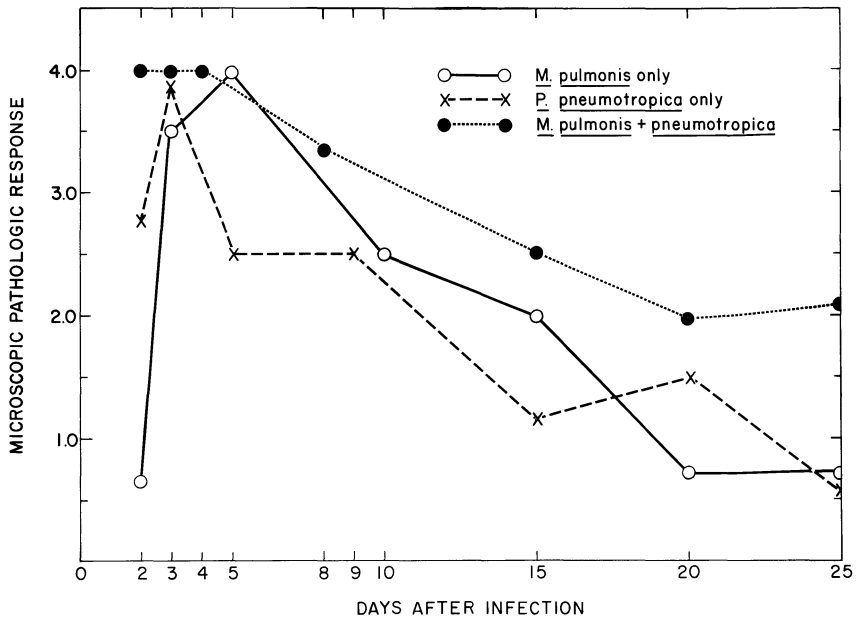


**Fig. 40. Comparison of the mean microscopic pathologic scores of axenic mice singly and doubly infected with *Mycoplasma pulmonis* N1 and *Pasteurella pneumotropica* 9538.**

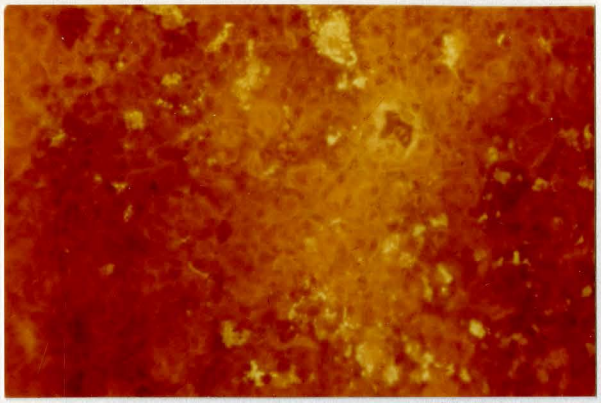

Fig. 41. Fluorescent areas in the alveoli of an axenic mouse lung infected with Pasteurella pneumotropica 9538 (x 250). The section was reacted with P. pneumotropica antisera and then reacted with fluorescein labeled anti-rabbit globulin.

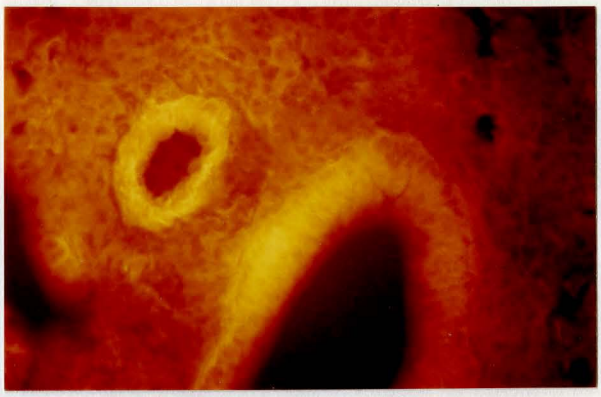

Fig. 42. Control axenic mouse lung reacted with Pasteurella pneumotropica 9538 antiserum and fluorescein labeled anti-rabbit globulin (x 250).

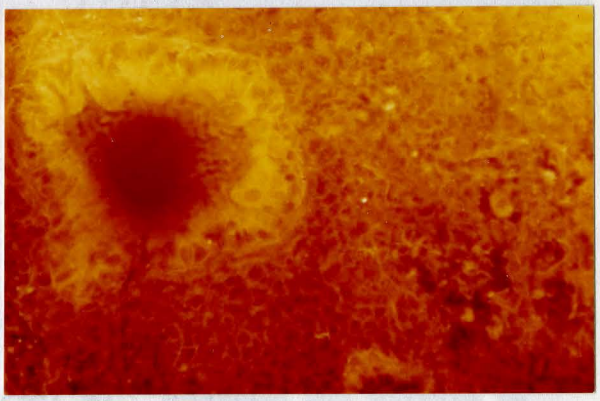

Fig. 43. Bronchus and alveoli of an axenic mouse lung infected with Pasteurella pneumotropica 9538 (x 250). The section was reacted with normal rabbit serum and then reacted with fluorescein labeled anti-rabbit globulin.

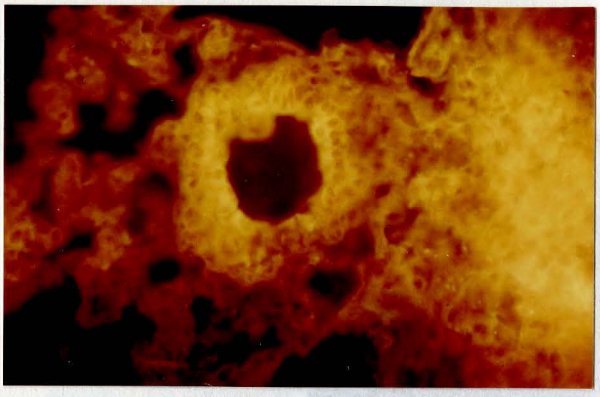

Fig. 44. Fluorescent bronchus of mouse lung infected with Mycoplasma pulmonis N1 and reacted with M. pulmonis antiserum (x 250). The section was then reacted with fluorescein labeled anti-rabbit globulin.

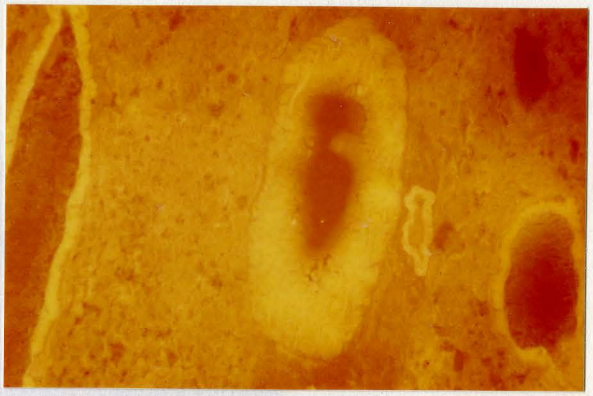

Fig. 45. Bronchus of uninfected control mouse lung reacted with Mycoplasma pulmonis N1 antiserum and fluorescein labeled anti-rabbit globulin (x 250).

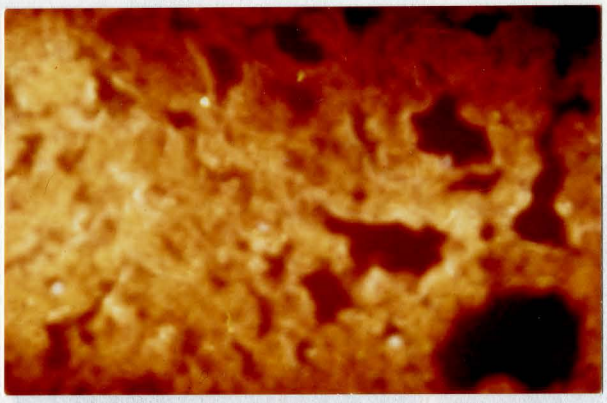

Fig. 46. Bronchus and alveoli of mouse lung infected with Mycoplasma pulmonis N1 reacted with normal rabbit serum and fluorescein labeled anti-rabbit globulin (x 250).

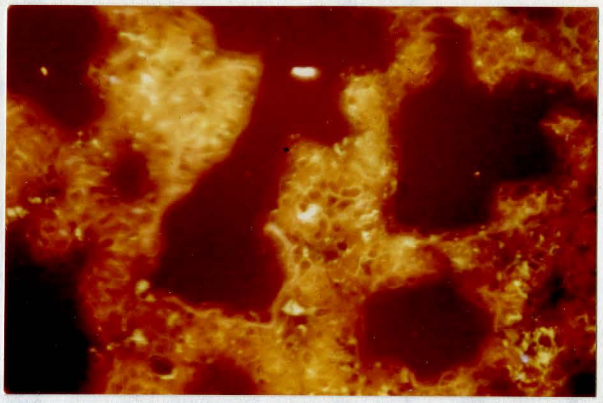

Fig. 47. Alveoli of mouse lung infected with Mycoplasma pulmonis N1 and Pasteurella pneumotropica 9538 reacted with Pasteurella pneumotropica antiserum (x 250). The section was then reacted with fluorescein labeled anti-rabbit globulin.

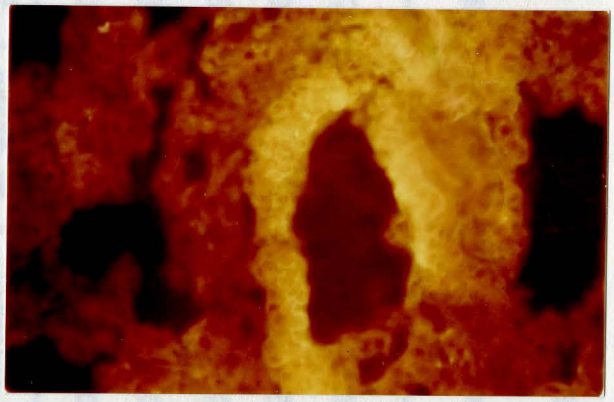

Fig. 48. Bronchus of mouse lung infected with Mycoplasma pulmonis N1 and Pasteurella pneumotropica 9538 (x 250). The section was reacted with Mycoplasma pulmonis antiserum and fluorescein labeled anti-rabbit globulin.

TABLE 20. Culture results and fluorescent antibody reaction of axenic mice infected with Mycoplasma pulmonis and Pasteurella pneumotropica

| Gross pathologic response | M. pulmonis | | P. pneumotropica | | M. pulmonis plus P. pneumotropica | | | |
|---|---|---|---|---|---|---|---|---|
| | Percent positive cultures | Percent positive FA test* | Percent positive cultures | Percent positive FA test | Percent positive cultures | | Percent positive FA test | |
| | | | | | M.† | P. | M. | P. |
| 4+ | 100 (8/8)† | 100 (4/4) | 100 (7/7) | 100 (4/4) | 100 (17/17) | 94 (16/17) | 100 (8/8) | 100 (8/8) |
| 3+ | 100 (2/2) | 100 (1/1) | 100 (6/6) | 100 (2/2) | 50 (6/12) | 66.7 (9/12) | 83 (5/6) | 83 (5/6) |
| 2+ | 50 (3/6) | 60 (3/5) | 90 (9/10) | 100 (7/7) | 33.3 (1/3) | 33.3 (1/3) | 100 (1/1) | 100 (1/1) |
| 1+ | 25 (4/16) | 25 (2/8) | 60 (3/5) | -- | -- | -- | -- | -- |
| 0 | 11.8 (2/17) | 12.5 (1/8) | 30 (6/20) | 33.3 (3/9) | 7.7 (1/13) | 54 (1/13) | 14.3 (1/7) | 14.3 (1/7) |

* FA test results based on animals giving a 3+ response or better.

† M. and P. = M. pulmonis and P. pneumotropica respectively.

† Numbers in parenthesis indicate the number positive/number examined. Complete data statistical analyses are in the appendix. (Appendix Tables 13-15)

## DISCUSSION

Jawetz (60, 61), Heyl (53) and Wheater (147) stated that P. pneumotropica is capable of producing pneumonia in rodents. However the presence of indigenous mycoplasma in the mice and rats they studied could not be excluded. Hoag et al. (54) concluded that P. pneumotropica is not pathogenic for mice, but rather that it was a normal inhabitat of the mouse respiratory tract. Indigenous mycoplasma infection of axenic mice is extremely unlikely (47). The data presented in this chapter show that P. pneumotropica is pathogenic for axenic mice. The lesions produced are similar to, but less severe than those originally described by Jawetz and Baker (61) in conventional mice. They found that the lesions were most pronounced 3-5 days after infection, and my results confirm this observation. Contrary to their observation that the lesions persisted for 26 days, I found that macroscopic lesions did not persist after approximately 10 days. This suggests that some other factor was contributing to the pathologic condition Jawetz and Baker observed. The recovery of P. pneumotropica from 30% of apparently normal lungs of infected mice explains why other investigators believed that P. pneumotropica was nonpathogenic. Finding the organism in the absence of visible lesions may also explain why Nelson (94) considered the pasteurella-like organisms he found in "infectious catarrh" as of no significance in the disease syndrome.

Jawetz believed that mice with inapparent P. pneumotropica infection were more susceptible to superinfection with the organism than those which were free from infection. If this is true, I would also expect axenic mice to be less susceptible to infection than conventional mice. The data do not support this conclusion. It was possible to demonstrate pneumonia attributable to

P. pneumotropica in axenic mice, but attempts to produce disease in conventional CF#1/An1 mice infected with P. pneumotropica were unsuccessful. It might be argued that the immune response of axenic mice is less well developed than that of conventional mice, but several studies indicate that this is not true. Bauer et al. (7) showed that the cellular and serologic response of axenic mice injected with Serratia marcescens equaled and sometimes exceeded that of conventional mice. Olson and Wostmann (112) demonstrated that germfree mice stimulated with either human IgG or Salmonella typhimurium vaccine react in a manner comparable to that of their conventional counterparts. Upton et al. (143) however, reported that germfree RF mice had lower granulocytopoeitic capabilities than conventional mice, and Bauer et al. (7) observed that while phagocytosis was equal in germfree and conventional mice, conventional mouse macrophages had a greater digestive capacity. This lessened digestive capability of germfree macrophages could account in part for the susceptibility of axenic mice to infection with P. pneumotropica. While the total immune response might be the same as in conventional mice, a delay in processing the antigen by the macrophages could delay production of antibodies and thus enhance the pathologic properties of the organism. Furthermore, if there are fewer granulocytes in germfree mice, this could also contribute to the infective process, since the mobilization of polymorphonuclear cells would be one of the first cellular responses to P. pneumotropica infection.

The macroscopic lesions I observed during the first few days following infection in axenic mice infected with M. pulmonis confirms the report by Lutsky and Organick (82). At later periods my results differ from theirs in

the absence of both bronchial exudate and of death in the present study. This was true even when I used their strain of M. pulmonis. Several reasons can be advanced to explain these discrepancies. First, there may be a difference in susceptibility to M. pulmonis infection between the mice I used and those they used. Although both strains originated from the same stock, they were from different suppliers. It is possible that genetic differences were developed by the respective suppliers. It seems more plausible that differences in technique account for the different results. There is a great temptation to begin an experiment with axenic mice as soon as the mice are received because delay may increase the risk of accidental contamination of the chambers. However, the stress of shipping on germfree mice is severe because of the closed containers required to insure that the mice remain germfree. I would expect that if I had inoculated the mice with M. pulmonis as soon as they were received, the course of infection would have more closely resembled that observed by Lutsky and Organick.

The lesions observed in mice simultaneously infected with both M. pulmonis and P. pneumotropica (Fig. 26-29, 36-38) more closely resemble those seen in spontaneously occurring murine pneumonia than to those seen in mice infected with either organism alone. The marked early polymorphonuclear response and the bronchial exudate have been described by other workers (71, 72, 89, 94, 95). The finding that there was no significant difference in the grand means of the pathologic scores between mice infected with both organisms and the sum of the grand means of mono-infected mice is interpreted as evidence that the effects observed in simultaneous infection are additive. It would appear that no synergistic relationship exists between the two organisms. Additional support

for this conclusion comes from the observation that deaths occurred only in the mice infected with both M. pulmonis and P. pneumotropica and that the FA studies indicate two different sites of localization of the organisms in the lungs (Fig. 41 and 44). The disease produced in mice simultaneously infected with P. pneumotropica and M. pulmonis can be considered to consist of two independent infectious agents each having a separate target cell in the same target organ. It is reasonable to assume that the combined effect demonstrated in germfree mice is responsible for spontaneously occurring murine pneumonia from which both organisms are isolated.

Joshi, Dale and Blackwood (64) concluded that chronic murine pneumonia in rats is caused by a virus. The data on which this conclusion rests are open to several criticisms. The inoculum used to produce pneumonia in axenic rats was a sintered glass filtrate from pneumonic lungs from conventional rats. Joshi isolated mycoplasma from these rat lungs and since mycoplasma can pass a filter having an average pore size of 0.45 μ, I doubt if sintered glass would retain all the infective elements. The filtrate was diluted in medium containing penicillin and streptomycin. Penicillin is without effect on mycoplasma and Kleineberger-Nobel (74) states that they rapidly become resistant to streptomycin. Joshi made no attempt to isolate mycoplasma from the germfree mice in which he produced lesions. Electron micrographs of the supposed causative virus were made from the crude filtrate. It would not be surprising to find a number of virus-like particles in conventional rat lungs. Demonstration of such particles does not prove that they are the causative agent of a disease. Interestingly, the lesions described by Joshi are identical to those described for M. pulmonis infection in axenic mice.

By means of electron microscopy, Organick et al. (114) showed that M. pulmonis localized at the surface of bronchial epithelial cells in infected mice forming a layer 3-5 cells deep. My FA results confirm this localization. Organick et al. (114) were able to demonstrate that mycoplasma were also found in the alveoli and that they were engulfed by polymorphonuclear cells. Other investigators (22, 81) were unable to find mycoplasma in the alveoli using FA techniques. I observed fluorescent areas in the alveoli of M. pulmonis infected lungs (Fig. 44), but the fluorescence was more brilliant in the bronchial epithelium. This is undoubtedly a result of the higher concentration of M. pulmonis cells in the epithelial layer.

The indirect FA technique used in this study appears to be a reliable tool for the differential diagnosis of M. pulmonis infection in spontaneous cases of murine pneumonia. The method is as sensitive as cultural methods and the saving in time and materials is considerable. It may also be possible to adapt the technique to screening apparently healthy mice for latent infection with M. pulmonis, but an extensive comparative study is necessary before cultural methods can be replaced.

The use of the FA method will also be useful as an adjunct to culture of P. pneumotropica from infected lungs. The method may be adaptable to screening for P. pneumotropica from tracheal and nasal washing of apparently healthy mice, but more research is needed in this area.

# CHAPTER VI

## GENERAL DISCUSSION

The results of this investigation furnish an accurate evaluation of the relative roles played by P. pneumotropica and M. pulmonis in murine pneumonia. The data in Chapter V show that either P. pneumotropica or M. pulmonis alone are pathogenic for axenic mice, although the lesions produced in axenic mice appear less severe and of shorter duration than those described in conventional mice (60, 61, 94, 95). P. pneumotropica is commonly present in the respiratory tract of conventional mice raised at Argonne National Laboratory and it was isolated from the lungs of all mice with spontaneously occurring pneumonia. Although M. pulmonis was isolated from only a few diseased mice, the technical difficulty of isolating it makes it reasonable to assume that M. pulmonis infection of conventionally raised mice is also common. It can be hypothesized that when the population of M. pulmonis, P. pneumotropica or both exceed the number which can be cleared from the lungs, the mouse is confronted with simultaneous invasion by both organisms. While the animal can successfully resolve the pneumonia caused by either one of these two organisms, the combined assault of two independent infectious agents overwhelms the host's defense and severe pneumonia of long duration or death results. If this hypothesis concerning the etiology and pathogenesis of spontaneous murine pneumonia is correct, a number of observations by other investigators can be explained. Nelson (94, 95, 100, 103, 105) and Edward (33, 34) attributed severe pneumonic lesions in mice to mycoplasma alone. The material used to induce these lesions was a crude homogenate of pneumonic mouse lungs, and probably contained large numbers of P. pneumotropica as well

as M. pulmonis. As a result, the mice were simultaneously infected with both organisms. If a certain threshold infective dose must be exceeded before clinical pneumonia develops, then the often observed foci of lymphoid tissue in the lungs of "normal" rats and mice can be explained. These foci of lymphoid tissue result from the successful containment by the host of constantly invading small numbers of P. pneumotropica, M. pulmonis or both. One would thus expect that a constant small inoculum of M. pulmonis in mice and rats harboring few P. pneumotropica would produce a chronic non-fatal murine pneumonia characterized by perivascular and peribronchial lymphocytic infiltration.

It is tempting, in the light of my results, to speculate that P. pneumotropica may play some role in primary atypical pneumonia of man since this disease is also caused by a Mycoplasma, M. pneumoniae. P. pneumotropica has been isolated from apparently healthy human individuals as well as those with respiratory problems (51, 52, 62). It may be that P. pneumotropica contributes to the severity and long duration of primary atypical pneumonia in man but a large scale survey of the incidence of P. pneumotropica in healthy people and in primary atypical pneumonia is necessary before the role of P. pneumotropica in man can be ascertained.

The importance of defining the indigenous potential pathogens in mice used for the study of infectious diseases is emphasized by this investigation. At the time the CF#1/An1/SPF mice were used they were believed to be free from P. pneumotropica and M. pulmonis infection. Subsequently, P. pneumotropica was found in approximately 10% of the colony. It was undoubtedly present in small numbers in all the mice used in this experiment because it was recovered from

mice challenged with M. pulmonis. The converse of this may be equally true; the mice could have been latently infected with M. pulmonis. Even though an animal is defined as "specific-pathogen-free", it must be constantly borne in mind that this designation is based on negative culture results. An animal is "specific-pathogen-free" only within the limits of the tests employed to define it. Obviously, more sensitive tests are required to screen for M. pulmonis than are presently available. Although the fluorescent antibody test I used did not appear to be more sensitive than cultural methods, with refinements it could be made more sensitive. Further work is required in this area.

No conclusions were drawn from the results of the experiments using SPF mice. Rather, an investigation to determine if a genetic relationship exists between M. pulmonis and P. pneumotropica was undertaken. The lack of DNA base homology and the disparity in percent GC are evidence that M. pulmonis is not an L form of P. pneumotropica. The percent GC of these two organisms is not reported in the literature, therefore their determination adds to the growing list of GC base ratios of bacteria.

Several other lines of research are suggested by this investigation. In my experiments, M. pulmonis and P. pneumotropica were given to axenic mice at the same time. It would be interesting to infect axenic mice with one of these organisms and after the lesions had healed challenge them with the other organism. Such an experiment should provide information on the effect of latent infection with only M. pulmonis or P. pneumotropica and superinfection with the other. Also, my investigation does not provide information on the mechanism of pathogenicity of either P. pneumotropica or M. pulmonis. The possibility that toxic substances are produced by these organisms should

be investigated. Axenic mice could provide a means of studying the production of toxic substances *in vivo*. Another problem raised by this investigation is that of intrauterine infection of mice by *P. pneumotropica* and *M. pulmonis*. The difficulty of studying this problem in conventional mice was discussed in Chapter II. Axenic mice may provide a means for the study of this problem.

In conclusion, both *P. pneumotropica* and *M. pulmonis* cause pneumonia in mice. In naturally occurring murine pneumonia both organisms contribute additively to the pathologic process.

# CHAPTER VII

## SUMMARY

*Pasteurella pneumotropica* was isolated from a number of species and strains of animals including mice, rats, dogs, kangaroo rats and hamsters. The organism was most frequently found in the lungs of mice and rats with pneumonia and in the eyes of mice with conjunctivitis. *Mycoplasma pulmonis* was also found associated with pneumonia in mice and rats. A survey of apparently healthy mice and rats raised at Argonne National Laboratory and mice purchased from commercial breeders disclosed that almost all conventionally raised rodents are carriers of *P. pneumotropica*. Argonne National Laboratory, cesarean-derived, barrier-sustained mice have about a 10% incidence of infection in the respiratory tract. *P. pneumotropica* can also be isolated from the uteri of some mice.

A study of *M. pulmonis* and *P. pneumotropica* infection in CF#1/An1/SPF mice suggested that both organisms were necessary to produce the disease syndrome, murine pneumonia. That *M. pulmonis* might be an L form of *P. pneumotropica* was also suggested by the finding that SPF mice mono-infected with one of these organisms yielded both on culture following necropsy. The latter hypothesis was studied by determining the percent GC and DNA homology techniques. The pathogenicity of the organisms was studied using axenic mice.

The percent GC of *P. pneumotropica* was found to be 44.4% and that of *M. pulmonis* 31.1%, as determined by thermal denaturation. This disparity in percent GC suggests that DNA from the two organisms has few nucleotide pairs in common. This was confirmed by the lack of duplex formation between the two

DNAs although each DNA was capable of duplex formation in the homologous system. M. pulmonis is therefore apparently not an L form of P. pneumotropica.

Both M. pulmonis and P. pneumotropica produced visible lung lesions in mono-infected axenic mice. The lesions produced by either organism were characterized by dark red areas of consolidation. In addition, abscess formation was a common finding in mice infected with P. pneumotropica. Histologically, both organisms produced lesions characterized by a mixed polymorphonuclear and mononuclear infiltration. The bronchi remained free of any exudate in mono-infected mice. The lesions produced in axenic mice similtaneously infected with P. pneumotropica and M. pulmonis more closely resembled those seen in naturally occurring murine pneumonia than did those observed in mono-infected mice. Statistical analysis indicates that the effect of the two organisms is additive. It is concluded that naturally occurring murine pneumonia is the result of simultaneous infection with P. pneumotropica and M. pulmonis.

# CHAPTER VIII

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# APPENDIX

TABLE 1

Determination of specific activity of $^{3}H$ DNA

| μg DNA counted* | Mean counts/min ± SE corrected |
|---|---|
| *P. pneumotropica* | |
| 1.0 | 2212 ± 13 |
| 1.0 | 2253 ± 7 |
| 1.0 | 2242 ± 8 |
| 1.0 | 2293 ± 10 |
| 1.0 | 2228 ± 9 |
| Grand mean of 5 mean counts ± SE = | 2256 ± 27 |
| *M. pulmonis* | |
| 1.0 | 1577 ± 5 |
| 1.0 | 1529 ± 4 |
| 1.0 | 1492 ± 10 |
| 1.0 | 1447 ± 12 |
| 1.0 | 1482 ± 6 |
| Grand mean of 5 mean counts ± SE = | 1506 ± 41 |

* The DNA was placed on membrane filters.

TABLE 2

Data from an experiment to determine the optimum ratio of labeled and unlabeled P. pneumotropica DNA for homology experiments

| µg unlabeled DNA attached to filter | µg $^{3}H$ DNA added | Total counts retained | Mean counts/min retained ± SE |
|---|---|---|---|
| 20 | 1 | 103,616 | 648 ± 8 |
| 0 | 1 | 24,819 | 83 ± 8 |
| 20 | 1 | 134,496 | 841 ± 8 |
| 0 | 1 | 18,000 | 113 ± 6 |
| 20 | 0 | 14,381 | 48 ± 5 |
| 6.3 | 0.5 | 68,528 | 428 ± 7 |
| 0 | 0.5 | 18,276 | 61 ± 6 |
| 6.3 | 0.5 | 87,276 | 546 ± 7 |
| 0 | 0.5 | 13,200 | 83 ± 6 |
| 6.3 | 0 | 14,706 | 49 ± 9 |
| 6.3 | 0.2 | 19,088 | 119 ± 6 |
| 0 | 0.2 | 16,626 | 54 ± 5 |
| 6.3 | 0.2 | 29,840 | 187 ± 6 |
| 0 | 0.2 | 13,024 | 81 ± 6 |
| 6.3 | 0 | 14,706 | 49 ± 9 |
| 0 | 0 | 14,955 | 50 ± 5 |
| 0 | 50 µl Standard scintillator* $2 \times 10^{6}$ disintegrations/min/ml | 179,374 | 17,937 ± 50 |

* Efficiency of counting = 17.9%.

TABLE 3

Data from an experiment to determine the optimum ratio of $^{3}H$ labeled and unlabeled M. pulmonis DNA for homology experiments

| µg unlabeled DNA attached to filter | µg $^{3}H$ DNA added | Total counts retained | Mean counts/min retained ± SE |
|---|---|---|---|
| 10 | 1.0 | 63,088 | 789 ± 12 |
| 0 | 1.0 | 7,564 | 95 ± 9 |
| 10 | 1.0 | 57,494 | 719 ± 12 |
| 0 | 1.0 | 8,340 | 104 ± 9 |
| 10 | 0 | 4,564 | 76 ± 10 |
| 10 | 0.5 | 34,708 | 434 ± 10 |
| 0 | 0.5 | 36,190 | 77 ± 8 |
| 10 | 0.5 | 39,653 | 496 ± 14 |
| 0 | 0.5 | 6,290 | 79 ± 8 |
| 10 | 0 | 4,564 | 76 ± 10 |
| 5 | 0.5 | 30,190 | 377 ± 9 |
| 0 | 0.5 | 6,190 | 77 ± 8 |
| 5 | 0.5 | 32,424 | 405 ± 10 |
| 0 | 0.5 | 6,290 | 79 ± 8 |
| 5 | 0 | 4,517 | 75 ± 11 |
| 5 | 0.2 | 17,428 | 218 ± 9 |
| 0 | 0.2 | 6,340 | 79 ± 8 |
| 5 | 0.2 | 18,664 | 233 ± 9 |
| 0 | 0.2 | 6,761 | 85 ± 9 |
| 5 | 0 | 4,517 | 75 ± 11 |
| 0 | 0 | 4,218 | 73 ± 10 |
| 0 | 50 µl standard* scintillator<br>2 x $10^{6}$ disintegrations/min | 1,127,000 | 18,797 ±182 |

* Efficiency of counting = 18.8%.

TABLE 4

Data on the effect of drying and preincubation on $^{3}H$ DNA from P. pneumotropica

| µg DNA attached to filter | Treatment | Total count | Mean counts/min ± SE |
|---|---|---|---|
| 1.0 | Dried | 366,800 | 2293 ± 13 |
| 1.0 | Dried, PM | 351,200 | 2195 ± 11 |
| 0.5 | Dried | 191,792 | 1199 ± 8 |
| 0.5 | Dried, PM | 183,408 | 1146 ± 9 |
| 0.2 | Dried | 61,728 | 386 ± 9 |
| 0.2 | Dried, PM | 60,672 | 379 ± 6 |
| 0 | Dried | 10,697 | 68 ± 5 |
| 0 | Dried, PM | 10,688 | 67 ± 6 |

TABLE 5

Data from band sedimentation of *P. pneumotropica* and *M. pulmonis* DNA*

| Source of DNA | Time (in min.) | $Log_{10} M_{ro}$ (observed)† | $Log_{10} M_{ro}$ (calculated) |
|---|---|---|---|
| *P. pneumotropica* | 8.08 | 2.1002 | 2.0999 |
| | 10.08 | 2.1016 | 2.1012 |
| | 12.08 | 2.1033 | 2.1033 |
| | 14.08 | 2.1043 | 2.2105 |
| | 16.08 | 2.1070 | 2.1067 |
| | 18.08 | 2.1084 | 2.1083 |
| | 20.08 | 2.1101 | 2.1100 |
| | 22.08 | 2.1114 | 2.1117 |
| | 24.08 | 2.1117 | 2.1113 |
| | 26.08 | 2.1151 | 2.1150 |
| Slope = 0.00838 ± 0.000150 (σ) | | | |
| *M. pulmonis* | 8.05 | 2.0974 | 2.0975 |
| | 10.05 | 2.0988 | 2.0986 |
| | 12.05 | 2.0999 | 2.0997 |
| | 14.05 | 2.1005 | 2.1009 |
| | 16.05 | 2.1019 | 2.1020 |
| | 18.05 | 2.1033 | 2.1031 |
| | 20.05 | 2.1043 | 2.1042 |
| | 22.05 | 2.1053 | 2.1054 |
| | 24.05 | 2.1064 | 2.1065 |
| | 26.05 | 2.1077 | 2.1076 |
| Slope = 0.00562 ± 0.0000492 (σ) | | | |

* The experiments were run at 33,450 rpm in a Spinco Model E ultracentrifuge using 50% $D_2O$ in SSC as the supporting medium.

† The $log_{10}$ of the maximum ordinate magnified ($M_{ro}$) is used for y in the equation $y = a + bx$ so that the magnification factor will cancel out.

TABLE 6

Data from three experiments to determine the extent of molecular hybridization with *M. pulmonis* DNA as the donor and *P. pneumotropica* DNA as the recipient

| Species of DNA attached to filter* | Species of $^{3}H$ DNA added† | Total counts retained | Mean counts/min retained ± SE |
|---|---|---|---|
| *P. pneumotropica* | *P. pneumotropica* | 53,419 | 445 ± 7 |
| 0 | " | 12,100 | 101 ± 7 |
| *P. pneumotropica* | " | 52,114 | 435 ± 9 |
| 0 | " | 9,316 | 78 ± 7 |
| *P. pneumotropica* | " | 54,569 | 455 ± 8 |
| 0 | " | 11,149 | 93 ± 7 |
| *P. pneumotropica* | *M. pulmonis* | 12,077 | 101 ± 7 |
| 0 | " | 11,159 | 94 ± 7 |
| *P. pneumotropica* | " | 13,786 | 115 ± 7 |
| 0 | " | 10,830 | 90 ± 7 |
| *P. pneumotropica* | " | 13,846 | 115 ± 7 |
| 0 | " | 9,418 | 78 ± 8 |
| 0 | 0 | 9,182 | 77 ± 7 |
| 0 | 0 | 9,298 | 77 ± 7 |
| 0 | 50 µl standard scintillator† $2 \times 10^{6}$ disintegrations/min/ml | 2,233,584 | 18,613 ±99 |

* 6.3 µg of unlabeled *P. pneumotropica* was attached to each filter.

† 0.5 µg *P. pneumotropica* $^{3}H$ DNA was added, 1128 cpm.
0.5 µg *M. pulmonis* $^{3}H$ DNA was added, 753 cpm.

† Efficiency of counting = 18.6%.

TABLE 7

Data from three experiments to determine the extent of molecular hybridization with *P. pneumotropica* DNA as donor and *M. pulmonis* DNA as the recipient

| Species of DNA attached to filter* | Species of $^3$H DNA added† | Total counts retained | Mean counts/min retained ± SE |
|---|---|---|---|
| *M. pulmonis* | *M. pulmonis* | 41,316 | 344 ± 7 |
| 0 | " | 9,330 | 78 ± 7 |
| *M. pulmonis* | " | 47,291 | 394 ± 8 |
| 0 | " | 10,828 | 89 ± 7 |
| *M. pulmonis* | " | 50,737 | 423 ± 8 |
| 0 | " | 11,149 | 93 ± 7 |
| *M. pulmonis* | *P. pneumotropica* | 12,875 | 107 ± 7 |
| 0 | " | 12,100 | 100 ± 7 |
| *M. pulmonis* | " | 12,328 | 103 ± 7 |
| 0 | " | 9,316 | 78 ± 7 |
| *M. pulmonis* | " | 12,785 | 107 ± 7 |
| 0 | " | 10,830 | 90 ± 7 |
| 0 | 0 | 9,182 | 76 ± 8 |
| 0 | 0 | 9,176 | 76 ± 7 |
| 0 | 50 µl standard scintillator† $2 \times 10^6$ disintegrations/min/ml | 2,175,804 | 18,132 ± 189 |

* 50 µg unlabeled *M. pulmonis* DNA was attached to the filters.

† 0.5 µg *M. pulmonis* $^3$H DNA was added = 753 cpm.
0.5 µg *P. pneumotropica* $^3$H DNA was added = 1128 cpm.

† Efficiency of counting = 18.1%.

TABLE 8

M. pulmonis infection in axenic mice

| Days after infection | Mouse number | Gross pathology | Microscopic pathology | Culture results | FA results |
|---|---|---|---|---|---|
| 0 | 1-10 | 0 | 0 | - | - |
| 2 | 11 | 0 | 0 | - | |
| | 12 | 2 | 1 | + | |
| | 13 | 0 | 0 | - | |
| | 14 | 1 | 1 | + | +++ |
| | 15 | 0 | 0 | + | +++ |
| | 16 | 2 | 2 | + | +++ |
| | | 0.82 ± 0.41* | 0.67 ± 0.33 | | |
| 3 | 17 | 1 | 1 | + | |
| | 18 | 3 | 4 | + | |
| | 19 | 4 | 4 | + | |
| | 20 | 4 | 4 | + | +++ |
| | 21 | 4 | 4 | + | +++ |
| | 22 | 1 | 4 | - | +++ |
| | | 2.83 ± 0.61 | 3.50 ± 0.05 | | |
| 5 | 23 | 4 | 4 | + | |
| | 24 | 4 | 4 | + | |
| | 25 | 4 | 4 | + | |
| | 26 | 2 | autolysis | + | +++ |
| | 27 | 0 | " | - | + |
| | 28 | 4 | " | + | +++ |
| | | 3.00 ± 0.68 | 4.0 ± 0 | | |
| 10 | 29 | 0 | 2 | - | |
| | 30 | 1 | 2 | + | |
| | 31 | 1 | 2 | - | |
| | 32 | 3 | 3 | + | ++ |
| | 33 | 0 | 2 | + | + |
| | 34 | 4 | 4 | + | ++++ |
| | | 1.5 ± 0.67 | 2.5 ± 0.05 | | |

* Mean ± SE

*M. pulmonis* infection in axenic mice (cont.)

| Days after infection | Mouse number | Gross pathology | Microscopic pathology | Culture results | FA results |
|---|---|---|---|---|---|
| 15 | 35 | 1 | 2 | - | |
| | 36 | 1 | 2 | - | |
| | 37 | 1 | 2 | - | |
| | 38 | 1 | 2 | - | + |
| | 39 | 2 | 2 | - | + |
| | 40 | 2 | 2 | - | + |
| | | 1.33 ± 0.21 | 2.0 ± 0 | | |
| 20 | 41 | 0 | 0 | - | |
| | 42 | 1 | 2 | + | |
| | 43 | 0 | 1 | - | |
| | 44 | 0 | 0 | - | + |
| | 45 | 0 | 0 | - | + |
| | 46 | 0 | 0 | - | + |
| | 47 | 2 | 2 | + | ++ |
| | | 0.43 ± 0.34 | 0.72 ± 0.05 | | |
| 25 | 48 | 1 | 1 | - | |
| | 49 | 0 | 0 | - | |
| | 50 | 0 | 0 | - | |
| | 51 | 1 | 1 | - | + |
| | 52 | 0 | 1 | - | + |
| | 53 | 1 | 1 | - | + |
| | 54 | 0 | 1 | - | 0 |
| | | 0.43 ± 0.22 | 0.72 ± 0.03 | | |
| 31 | 55 | 0 | 0 | - | |
| | 56 | 0 | 0 | - | |
| | 57 | 0 | 0 | - | |
| | 58 | 0 | 1 | - | - |
| | 59 | 1 | 1 | - | - |
| | 60 | 1 | 1 | - | - |
| | 61 | 1 | 1 | - | - |
| | | 0.43 ± 0.22 | 0.43 ± 0.04 | | |
| 32 Controls | 62-66 | 0 | 0 | - | - |

## *M. pulmonis* infection in axenic mice (cont.)

**Scoring Method:**

**Gross pathology:** 0 = no lesions; 1+ = moderate congestion; 2+ = severe congestion with or without tiny areas of consolidation; 3+ = moderate consolidation; 4+ = severe consolidation.

**Microscopic pathology:** 0 = no lesions; 1+ = slight perivascular and peribronchial lymphocytic infiltration; 2+ = larger areas of lymphocytic infiltration; 3+ = patches of consolidation of the parenchyma in addition to lymphocytic infiltration; 4+ = large confluent areas of consolidation of the parenchyma.

**Fluorescent antibody:** - = no fluorescence; + = dull fluorescence; ++ = definite fluorescence; +++ = bright fluorescence; ++++ = brilliant yellow-green fluorescence.

TABLE 9

*P. pneumotropica* infection in axenic mice

| Days after infection | Mouse number | Gross pathology | Microscopic pathology | Culture results | FA results |
|---|---|---|---|---|---|
| 0 | 67-76 | 0 | 0 | - | - |
| 2 | 77 | 3 | 4 | + | |
| | 78 | 0 | 1 | - | |
| | 79 | 3 | 4 | + | |
| | 80 | 4 | 4 | + | +++ |
| | 81 | 0 | 2 | - | + |
| | 82 | 2 | 2 | + | +++ |
| | | 2.0 ± 0.68* | 2.83 ± 0.54 | | |
| 3 | 83 | 4 | 4 | + | |
| | 84 | 4 | 4 | + | |
| | 85 | 4 | 4 | + | |
| | 86 | 4 | 4 | + | +++ |
| | 87 | 3 | 3 | + | +++ |
| | 88 | 4 | 4 | + | +++ |
| | | 3.83 ± 0.16 | 3.83 ± 0.02 | | |
| 5 | 89 | 3 | 4 | + | |
| | 90 | 0 | 1 | + | |
| | 91 | 0 | 2 | + | |
| | 92 | 2 | 3 | - | ++ |
| | 93 | 2 | 2 | + | ++ |
| | 94 | 3 | 3 | + | ++ |
| | | 1.67 ± 0.55 | 2.50 ± 0.04 | | |
| 9 | 95 | 1 | 2 | - | |
| | 96 | 2 | 2 | + | |
| | 97 | 2 | 3 | + | |
| | 98 | 2 | 3 | + | ++ |
| | 99 | 4 | 4 | + | ++++ |
| | 100 | 0 | 1 | - | + |
| | | 1.83 ± 0.54 | 2.50 ± 0.04 | | |

* Mean ± SE

*P. pneumotropica* infection in axenic mice (cont.)

| Days after infection | Mouse number | Gross pathology | Microscopic pathology | Culture results | FA results |
|---|---|---|---|---|---|
| 15 | 101 | 0 | 1 | - | |
| | 102 | 1 | 1 | + | |
| | 103 | 2 | 2 | + | |
| | 104 | 0 | 1 | + | ++ |
| | 105 | 2 | 2 | + | ++ |
| | 106 | 0 | 0 | + | ++ |
| | | 0.83 ± 0.41 | 1.16 ± 0.03 | | |
| 20 | 107 | 0 | 1 | - | |
| | 108 | 1 | 2 | + | |
| | 109 | 3 | 2 | + | |
| | 110 | 0 | 0 | - | ++ |
| | 111 | 2 | 2 | + | ++ |
| | 112 | 2 | 2 | + | ++ |
| | | 1.33 ± 0.49 | 1.50 ± 0.03 | | |
| 25 | 113 | 1+ | 1+ | - | |
| | 114 | 1+ | 1+ | + | |
| | 115 | 0 | 0 | - | |
| | 116 | 0 | 0 | + | - |
| | 117 | 0 | 0 | + | - |
| | 118 | 0 | 0 | - | - |
| | | 0.33 ± 0.21 | 0.33 ± 0.02 | | |
| 33 | 119 | 0 | 0 | - | |
| | 120 | 0 | 0 | - | |
| | 121 | 0 | 0 | - | |
| | 122 | 0 | 0 | - | - |
| | 123 | 0 | 1 | - | - |
| | 124 | 0 | 0 | - | - |
| | | 0 | 0.17 ± 0.03 | | |
| 34 Controls | 125-136 | 0 | 0 | - | - |

Method of scoring--as in Table 8.

TABLE 10

*M. pulmonis* and *P. pneumotropica* infection in axenic mice

| Days after infection | Mouse number | Gross pathology | Microscopic pathology | Culture* results | | FA results | |
|---|---|---|---|---|---|---|---|
| | | | | M | P | M | P |
| 0 Controls | 137-146 | 0 | 0 | - | - | - | - |
| 2 | 147 | 4 | 4 | + | + | | |
| | 148 | 3 | 4 | + | + | | |
| | 149 | 4 | 4 | + | + | | |
| | 150 | 3 | 4 | + | + | +++ | ++++ |
| | 151 | 4 | 4 | + | + | +++ | ++++ |
| | 152 | 4 | 4 | + | + | ++++ | ++++ |
| | | 3.66 ± 0.21** | 4.0 ± 0 | | | | |
| 3 | 153 | 4 | 4 | + | + | | |
| | 154 | 4 | 4 | + | + | | |
| | 155 | 4 | 4 | + | + | | |
| | 156 | 4 | 4 | + | + | ++++ | ++++ |
| | 157 | 4 | 4 | + | + | ++++ | ++++ |
| | 158 | 3 | 4 | + | + | ++++ | +++ |
| | | 3.83 ± 0.16 | 4.0 ± 0 | | | | |
| 4 | 159 | 4 (died) | 4 | + | + | | |
| | 160 | 4 | 4 | + | + | | |
| | 161 | 4 | 4 | + | + | | |
| | 162 | 3 | 4 | + | + | +++ | +++ |
| | 163 | 4 | 4 | + | + | +++ | +++ |
| | 164 | 4 | 4 | + | + | ++++ | ++++ |
| | | 3.83 ± 0.16 | 4.0 ± 0 | | | | |
| 8 | 165 | 0 | 2 | - | + | | |
| | 166 | 3 | 3 | + | - | | |
| | 167 | 4 | 4 | + | - | | |
| | 168 | 2 | 3 | + | + | ++ | ++ |
| | 169 | 4 | 4 | + | + | +++ | ++ |
| | 170 | 4 | 4 | + | + | +++ | ++ |
| | | 2.83 ± 0.65 | 3.33 ± 0.03 | | | | |

* M = *M. pulmonis*; P = *P. pneumotropica*

** Mean ± SE

M. pulmonis and P. pneumotropica infection in axenic mice (cont.)

| Days after infection | Mouse number | Gross pathology | Microscopic pathology | Culture results | | FA results | |
|---|---|---|---|---|---|---|---|
| | | | | M | P | M | P |
| 15 | 171 | 0 | 2 | + | + | | |
| | 172 | 3 | 3 | + | + | | |
| | 173 | 0 | 2 | - | + | | |
| | 174 | 0 | 2 | - | - | ++ | ++ |
| | 175 | 3 | 3 | - | - | ++ | +++ |
| | 176 | 3 | 3 | - | + | ++ | +++ |
| | | 1.50 ± 0.67 | 2.50 ± 0.02 | | | | |
| 20 | 177 | 3 | 3 | - | - | | |
| | 178 | 0 | 1 | - | - | | |
| | 179 | 2 | 3 | - | - | | |
| | 180 | 0 | 1 | - | + | | |
| | 181 | 2 | 2 | - | - | | |
| | 182 | 0 | 2 | - | + | - | + |
| | 183 | 3 | 3 | - | + | - | - |
| | 184 | 0 | 2 | - | - | - | - |
| | 185 | 0 | 2 | - | + | - | + |
| | 186 | 3 | 3 | - | + | + | + |
| | | 1.30 ± 0.60 | 2.20 ± 0.03 | | | | |
| 25 | 187 | 3 | 3 | - | + | | |
| | 188 | 0 | 2 | - | - | | |
| | 189 | 3 | 3 | - | - | | |
| | 190 | 0 | 1 | - | - | - | - |
| | 191 | 0 | 2 | - | - | - | - |
| | 192 | 0 | 2 | - | + | - | + |
| | | 1.0 ± 0.63 | 2.32 ± 0.03 | | | | |
| 26 Controls | 192-202 | 0 | 0 | - | - | - | - |

TABLE 11

*M. pulmonis* N1 infection in axenic mice (2)

| Days after infection | Mouse number | Gross pathology | Culture results |
|---|---|---|---|
| 1 | 202 | 2 | + |
| | 203 | 2 | + |
| | 204 | 2 | + |
| | 205 | 0 | + |
| | 206 | 1 | + |
| | 207 | 2 | + |
| | | 1.50 ± 0.03* | |
| 2 | 208 | 1 | + |
| | 209 | 2 | + |
| | 210 | 0 | + |
| | 211 | 3 | + |
| | 212 | 4 | + |
| | 213 | 3 | + |
| | | 2.8 ± 0.6 | |
| 4 | 214 | 4 | + |
| | 215 | 2 | + |
| | 216 | 2 | + |
| | 217 | 4 | + |
| | 218 | 3 | + |
| | 219 | 1 | + |
| | | 2.68 ± 0.05 | |
| 5 | 220 | 1 | - |
| | 221 | 3 | + |
| | 222 | 4 | + |
| | 223 | 2 | - |
| | 224 | 2 | + |
| | 225 | 2 | + |
| | | 2.34 ± 0.04 | |

* Mean ± SE

M. pulmonis N1 infection in axenic mice (2) (cont.)

| Days after infection | Mouse number | Gross pathology | Culture results |
|---|---|---|---|
| 9 | 226 | 2 | - |
| | 227 | 2 | - |
| | 228 | 2 | + |
| | 229 | 0 | - |
| | 230 | 2 | + |
| | 231 | 3 | + |
| | | 1.86 ± 0.04 | |
| 15 | 232 | 0 | - |
| | 233 | 0 | - |
| | 234 | 0 | - |
| | 235 | 2 | - |
| | 236 | 0 | - |
| | 237 | 2 | - |
| | 238 | 2 | - |
| | 239 | 2 | - |
| | 240 | 0 | - |
| | 241 | 0 | - |
| | 242 | 2 | - |
| | 243 | 0 | - |
| | 244 | 2 | + |
| | 245 | 0 | - |
| | 246 | 2 | - |
| | 247 | 2 | + |
| | | 1.0 ± 0.05 | |

TABLE 12

*M. pulmonis* Milwaukee strain infection in axenic mice

| Days after infection | Mouse number | Gross pathology | Culture results |
|---|---|---|---|
| 9 | 1 | 4 | + |
| | 2 | 4 | + |
| | 3 | 2 | + |
| | 4 | 2 | + |
| | 5 | 1 | - |
| | 6 | 1 | - |
| Mean | | 2.34 ± 0.55 | |
| 15 | 7 | 0 | - |
| | 8 | 0 | - |
| | 9 | 0 | - |
| | 10 | 0 | - |
| | 11 | 0 | - |
| | 12 | 0 | + |
| | 13 | 3 | - |
| | 14 | 0 | - |
| | 15 | 0 | - |
| | 16 | 0 | - |
| | 17 | 0 | - |
| | 18 | 2 | - |
| | 19 | 3 | - |
| | 20 | 3 | + |
| | 21 | 4 | + |
| | 22 | 0 | - |
| Mean | | 0.94 ± 0.29 | |

TABLE 13

Statistical analysis of the fluorescent antibody reaction in axenic mice based on the standard error of the difference of two proportions

| Gross pathologic response | Standard error of the difference | "t" value |
|---|---|---|
| FA reaction in M. pulmonis and P. pneumotropica mono-infected mice | | |
| 4+ | | 0 |
| 3+ | | 0 |
| 2+ | 21.91 | 1.83 |
| 1+ | ND* | -- |
| 0 | 19.58 | 1.06 |
| FA reaction in M. pulmonis mono-infected mice compared with FA response in di-infected mice | | |
| 4+ | | 0 |
| 3+ | 13.60 | 1.1 |
| 2+ | ND | -- |
| 1+ | ND | |
| 0 | 17.66 | 0.10 |
| FA reaction in P. pneumotropica mono-infected mice compared with FA response in di-infected mice | | |
| 4+ | | 0 |
| 3+ | 13.60 | 1.1 |
| 2+ | ND | -- |
| 1+ | ND | -- |
| 0 | 18.47 | 1.02 |

* ND = Not done because of too few animals showing the response.

(See Table 20 in text for raw data for Tables 13-14.)

TABLE 14

Statistical analysis of the FA response compared with culture results

| Gross pathologic response | Standard error of the difference | "T" value |
|---|---|---|
| **M. pulmonis culture compared with FA in mono-infected mice** | | |
| 4+ | | 0 |
| 3+ | | 0 |
| 2+ | 29.94 | 0.34 |
| 1+ | | 0 |
| 0 | 14.07 | 0.05 |
| **P. pneumotropica culture compared with FA in mono-infected mice** | | |
| 4+ | | 0 |
| 3+ | | 0 |
| 2+ | 9.49 | 1.05 |
| 1+ | ND | -- |
| 0 | 18.84 | 0.18 |
| **M. pulmonis culture compared with M. pulmonis FA in di-infected mice** | | |
| 4+ | | 0 |
| 3+ | 21.06 | 1.56 |
| 2+ | ND | -- |
| 1+ | ND | -- |
| 0 | 15.16 | 0.44 |
| **P. pneumotropica culture compared with P. pneumotropica FA in di-infected mice** | | |
| 4+ | | 0 |
| 3+ | 20.5 | 0.80 |
| 2+ | ND | -- |
| 1+ | ND | -- |
| 0 | 19.14 | 2.07(19)* |

* Significant at the 5% level.

TABLE 15

Statistical analysis of the culture results in axenic mice

| Gross pathologic response | Standard error of the difference | "T" value |
|---|---|---|
| **Comparison of M. pulmonis and P. pneumotropica recoveries in mono-infected mice** | | |
| 4+ | | 0 |
| 3+ | | 0 |
| 2+ | 22.51 | 1.78 |
| 1+ | 24.44 | 1.43 |
| 0 | 12.89 | 1.41 |
| **Comparison of M. pulmonis recovered from mono-infected mice with M. pulmonis recovered from di-infected mice** | | |
| 4+ | | 0 |
| 3+ | 14.43 | 3.48(13)* |
| 2+ | 32.04 | 0.49 |
| 1+ | ND | -- |
| 0 | 10.77 | 0.38 |
| **Comparison of P. pneumotropica recovered from mono-infected mice with P. pneumotropica recovered from di-infected mice** | | |
| 4+ | 5.76 | 1.04 |
| 3+ | 13.6 | 2.44(20)† |
| 2+ | 28.82 | 1.96 |
| 1+ | ND | -- |
| 0 | 17.21 | 1.39 |
| **Comparison of P. pneumotropica recoveries with M. pulmonis recoveries in di-infected mice** | | |
| 4+ | | 0 |
| 3+ | 19.83 | 0.84 |
| 2+ | | 0 |
| 1+ | ND | -- |
| 0 | 15.68 | 2.95(25)* |

* Significant at the 1% level.

† Significant at the 5% level.

TABLE 16

Two-way analysis of variance of the gross and microscopic pathologic response mean scores

| Source of variation | Sum of squares | | df | Mean square | | Variance ratio | |
|---|---|---|---|---|---|---|---|
| | Gross | Microscopic | | Gross | Microscopic | Gross | Microscopic |
| Time in days | 19.9769 | 17.8148 | 6 | 3.3294 | 2.9691 | 88.0095 | 61.4720 |
| Treatment | 4.7381 | 6.2401 | 2 | 2.3690 | 3.1204 | 62.6222 | 64.6045 |
| Residual | 4.5397 | 5.7963 | 12 | 0.3783 | 0.4830 | | |
| Total | 29.2540 | 29.8519 | 20 | | | | |

$F_{0.99(6,\ 20)} = 3.87$. Therefore, there are significant differences in day effects.

$F_{0.99(2,20)} = 5.85$. Therefore, there are significant differences in treatment effects.

TABLE 17

**Statistical analysis of the grand mean severity scores of the gross and microscopic lesions**

| Treatment | Grand mean pathologic score ± SE | |
|---|---|---|
| | Gross | Microscopic |
| *M. pulmonis* | 1.5000 ± 0.2267 | 2.0238 ± 2273 |
| *P. pneumotropica* | 1.6429 ± 0.2357 | 2.0952 ± 0.2123 |
| *M. pulmonis* + *P. pneumotropica* | 2.8719 ± 0.2531 | 3.8143 ± 0.1386 |

| Comparison | "t" | "t" |
|---|---|---|
| | Gross | Microscopic |
| 1. *M. pulmonis* - *P. pneumotropica* | 0.2105 | 0.006 |
| 2. *M. pulmonis* - *P. pneumotropica* + *M. pulmonis* | 2.0205 | 2.9595 |
| 3. *P. pneumotropica* - *P. pneumotropica* + *M. pulmonis* | 1.7605 | 2.9039 |
| 4. *M. pulmonis* alone + *P. pneumotropica* alone - *M. pulmonis* + *P. pneumotropica** | 0.674 | 0.758 |

For comparisons 1-3 $t_{0.05}^{(83\ df)} = 1.9600$.

For comparison 4 $t_{0.05}^{(12\ df)} = 2.1788$.

* Formula for "t" = $\dfrac{\bar{X}\ \text{treatment}_1 + \bar{X}\ \text{treatment}_2 - \bar{X}\ \text{treatment}_3}{\sqrt{\text{Interaction mean square}\left(\frac{3}{4}\right)}}$

## APPROVAL SHEET

The dissertation submitted by Patricia C. Brennan has been read and approved by five members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Jan. 18, 1968
Date

Einar Peterson
Signature of Adviser