To Hiba, my remarkable wife, for her love, devotion, patience and persistent unfailing encouragement.
STUDY OF THE GROWTH AND THE PATHOGENICITY OF MYCOPLASMA AND ADENOVIRUS INFECTIONS OF AVIAN TRACHEAL EXPLANTS

by

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ABSTRACT

Studies essentially involved examination of the structural details of the pathogenicity of single and mixed infections of mycoplasma and CELO virus in tracheal explants and infected chickens. The first part of the work concerned study of the growth response of the parasites in explants, alone or together. This was followed by study by light and electron microscopy of their effects, and interaction with the explants. Tracheal specimens from infected birds were also examined for any evidence of pathogenicity similar to that found in explants.

In the explants there was evidence for interference between the growth of the parasites but only with Mycoplasma gallisepticum were extensive cytopathological effects detected. Mycoplasma gallinarum also caused some damage to the tissues but by a mechanism which appeared to be different to that associated with M. gallisepticum, which early on developed an intimate relationship with the cells. Structural differences between the two mycoplasmas were also detected in the scanning and transmission electron microscope which probably related to their functional activities. CELO virus was virtually without effects on the explants although viral material was seen in many epithelial cells, and there was little evidence for a synergistic relationship between virus and mycoplasma.
No morphological evidence of mycoplasma or virus was found in the trachea of infected birds and the pathogenic effects, which were detected, seemed to reflect more on the treatment the birds experienced at infection than on the activities of the parasites.
I am grateful to my supervisor, Dr. M. Butler for his suggestions, discussions, criticisms and constant interest throughout the course of this project.

I would like to express my gratitude to the Lebanese Research Council for a long term grant.

I am indebted to Dr. S.M. Aghakhan for providing CELO virus and instruction on preparation of primary chicken kidney cell cultures, the Poultry Department, Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, Weybridge, for supplying the specific pathogen-free eggs and chicken, and Mr. W.H. Allen, Head of the Respiratory Unit for providing all necessary facilities for assay of the virus.

My thanks are due to Mr. M. Hepburn, Structural Studies Unit, Department of Metallurgy, University of Surrey, for his valuable instruction on the manipulation of the transmission electron microscope and for providing all necessary facilities, and also to his colleagues, Mrs. J. Gibbs and Mr. R. Hockham for providing facilities and instruction on the use of the scanning electron microscope.

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M.N. Abu-Zahr.
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INTRODUCTION

Mycoplasmas are found in a wide range of habitats including sewage and soil, human and animal respiratory and urino-genital tracts, joints, tumors and in a variety of cell cultures. Nearly 20 years have elapsed since they were first properly classified (Edward and Freundt, 1956) during which time at least 25 new species have been added to the originally recognised 15 species (Freundt, 1973). These were isolated from domesticated and wild animals, humans, fowls and laboratory animals. A number of mycoplasma species will probably continue to increase and might include in the near future some organisms which are presently referred to as mycoplasma-like organisms and await isolation, propagation and characterization. Examples of such organisms are those frequently seen in transmission electron microscope-examinations of both healthy and diseased plants. Some of these organisms are suspected to be etiologically involved in at least 50 plant diseases (Davis et al., 1972) and similar observations have revealed mycoplasma-like organisms in normal phytophagus insects (Nasu et al., 1970), and recently, Heath and Unestam (1974) reported the presence of mycoplasma-like structures in the aquatic fungus Aphanomyces astaci which lost its ability to produce zoospores in culture and grew more slowly than other closely related strains. In the latter case, if these organisms turn to be mycoplasmas they will be the first described disease-
causing agents of fungi besides viruses.

The list of mycoplasma species might also include in the near future organisms which resemble mycoplasmas in most characteristics but with diverse structure and function, for example, *Thermoplasma acidophilum* isolated from thermal and acidic region of a coal refuse pile (Darland et al., 1970; Bell and Brock, 1972), and *Spiroplasma citri* associated with "Stubborn" disease of citrus (Salgio et al., 1973), and TABLE 2 provides synopsis of the current taxonomic situation.

Although many mycoplasma species from man and animals are evidently harmless, playing the role of commensals and being a member of the natural flora of the host, some are directly or indirectly involved (Hayflick, 1969) in inducing disease, for instance, *M. pneumoniae* causes primary atypical pneumonia in man, *M. Mycoides* var. *mycoides* causes contagious bovine pleuropneumonia in cattle, *M. gallisepticum* causes chronic respiratory disease or infectious sinusitis in fowl, *M. neurolyticum* causes rolling disease in mice, *M. agalactiae* causes mastitis in sheep and goats and *M. suipneumoniae* is thought to cause swine enzootic pneumonia. Other species may play a role in disease for example there have been frequent isolations of several species of human and non-human species from human malignant tissues. Moreover, the exact role of many mycoplasmas remains obscure. The variety and number of organisms that fall within the class Mollicutes...
may require constant revision and reclassification.
REVIEW OF THE LITERATURE

Chronic respiratory disease of chicken and infectious sinusitis of turkeys have been the spur for the efforts of investigators in many countries to characterize the agents involved and to inhibit or control their spread. The major incentive, besides the scientific interest, was the considerable economic problem to the poultry industry which was stressed by Van Rockel et al. (1957) and Calnek & Levine (1957). Yoder (1972) considered it "one of the costliest disease problems confronting the poultry industry" while earlier, Gordon (1967) had stated that in 1962 losses due to *M. gallisepticum* in the U.S.A. were as high as $125 million per annum, and he estimated the losses in the United Kingdom due to the same disease to be not less than £10 million per annum. This avian species of mycoplasma is associated with the above mentioned diseases and with chronic coryza (Adler, 1970). Since the time of its isolation by Nelson (1936) a great deal of information has become available about it, particularly about its role in these diseases and about its pathogenicity. The number of investigators who dealt with the pathogenesis of *M. gallisepticum* in the various species of birds both in the field and in the laboratory is remarkably numerous and almost all of them reported the same results and several reviews exist (Fabricant; 1969, Yoder, 1972; Adler, 1970; Uppal, 1972).
M. gallisepticum has a relatively wide host-range and besides chicken and turkeys it also infects pheasants, guinea fowl, peafowl, pigeons and partridges. Moreover, Van Herik and Eaton (1945) infected cottontail rats with "unidentified PPLO" isolated from chick embryos and it was strongly believed to be an avian mycoplasma. Nearly thirty years later Taylor-Robinson et al. (1972) reported that M. gallisepticum grew in the respiratory tract of immunologically competent CBA mice producing pneumonic lesions.

One way of spread of M. gallisepticum in fowl occurs by egg transmission from infected hens, and contamination usually takes place in the periovarian region and in the upper portion of the oviduct (Fabricant 1968). However, the organisms were also isolated from semen of infected roosters (Yoder and Hofstad, 1964). The mycoplasma multiplies mainly in the respiratory tract of the developing embryo and may or may not cause grossly visible lesions, and the chicks that hatch act as carriers of the organisms. Although this mode of egg transmission occurs at very low rate (1 per thousand eggs) this would be sufficient to spread the organism amongst thousands of birds by contact and cohabitation (Fabricant, 1968). Another mode of transmission which could be of particular importance is through use of mycoplasma-contaminated vaccines employed in the poultry industry, and indeed M. gallisepticum was isolated from a commercial infectious laryngotracheitis vaccine (Benton et al., 1967).
The respiratory disease symptoms have been reported in many countries and it is generally agreed that the incubation period of the disease is fairly long, being one to two weeks in turkeys and one to three weeks in chicken (Yoder, 1972; Fabricant, 1969). Clinical signs in chicken are characterized by respiratory rales, nasal discharge, persistent hacking cough, reduced feed consumption, retarded growth, and decline in egg production, but in young chicken, the disease is often inapparent clinically. The infection may last for several weeks but usually subsides unless provoked by secondary infections (see mixed infections, page 12) or by environmental factors. These include overcrowding, poor housing and poor ventilation, high levels of dust, and ammonia fumes (Fabricant, 1968). In turkeys, infections with *M. gallisepticum* may also result in foaming of the eye secretions, swelling and distention of the paranasal sinuses which sometimes causes partial or complete closing of the eyes. Indeed it is widely accepted that the disease is more severe in turkeys and that subclinical infections are rare.

Gross pathology involves mucoid, mucopurulent and catarrhal exudates in the trachea, bronchi, air sacs, nasal and paranasal passages. This is usually accompanied with marked thickening of all affected epithelial membranes. Sinusitis and air sacculitis are usually more abundant in turkeys than in chicken and in severe cases fibrinous and fibrino-purulent perihepatitis and pericarditis do occur.
Microscopic examination of affected epithelial membranes show hyperplasia and thickening of these membranes with marked infiltration of their basal regions and their lamina propria with mononuclear cells. Hyperplasia of the mucous glands and the lymphoid tissues are commonly found in the submucosa. When the lungs are affected, pneumonic areas, lymphofollicular changes and granulomatous lesions do occur, and polymorphonuclear leukocytes usually fill the alveolar spaces. Occasionally, the bursa, the tendovaginal sheaths, the conjunctiva and the synovial membranes of the joints may also be affected. In turkeys the above lesions may also become exaggerated and the mucosa of the sinuses become petechiated with sloughed epithelium, while the lungs become consolidated.

Besides growth of the mycoplasma in embryonated eggs, the organisms produce marked lesions characterized by hemorrhages of the amnionic and the yolk sac and often results in embryo deaths, particularly when seven-day-old embryos are inoculated via the yolk sac. Gross respiratory lesions may become apparent in chicken embryos during the terminal stages of incubation and caseous exudate in the trachea, bronchi and lungs with opaque and thickened air sac membranes frequently occur. Dwarfing of embryos, generalized edema, liver necrosis, and enlarged spleens are also very common. Microscopic lesions in the livers of affected embryos show haemostasis with lymphocyte concentrations in the sinusoids. Moreover, advanced stages of the infection could lead to
brain neuronal degeneration, edema, thrombosis and diffuse gliosis.

The S6 strain of *M. gallisepticum* was found also to exhibit a toxic effect for turkey pouls, when viable organisms (dose range $10^{10} - 10^{11}$ CFU) were injected through the wing veins (Thomas et al., 1966). This toxic effect was found a speciality of the S6 strain of this mycoplasma which was originally isolated in California in 1954 from the trachea, air sacs, sinuses, and the brains of naturally affected eight-week-old turkeys (Zander, 1961). Injected birds became neurologically ill after an interval as short as thirty minutes and developed neurological signs, drooping wings, ataxia, inability to stand, wry neck and convulsive rolling movements. Injections of smaller doses of the organisms produced the same symptoms but only after intervals of time that prolonged considerably depending on the concentration of the organisms injected (Thomas, 1968; 1970; Clyde and Thomas, 1973 a). Recently, Clyde and Thomas (1973 b), reviewed all their work on this phenomenon and they reported that this pathological reaction was not detected in adult or young chicken, pigeons, mice, rats, rabbits, hamsters, or guinea pigs. Viable organisms were also necessary to produce the above effect, and the birds could be completely protected by pretreatment with antibiotics or antiserum.

The main pathological features as revealed by light
microscopy was polyarteritis which involved the central nervous system and damage to the vessels was manifested by fibrinoid necrosis of the adventitia. However, when death occurred within the first twenty-four hours after injection, no arterial lesions were demonstrated either by light or electron microscopy. When the birds were inoculated with $10^8$ colony forming organisms, they showed early fibrinoid changes in the cerebral arteries forty-eight hours after injection, and examination by immunofluorescence revealed foci of the organisms within the arterial walls or attached to the endothelial surfaces. At seventy-two hours after infection, the organisms were seen predominantly restricted to the cerebral arteries indicating a peculiar cerebral arteriotropism. The capillaries of all organs also contained the mycoplasma organisms which appeared as tiny fluorescent granules associated with erythrocytes. Greater concentrations of the organisms were also seen in reticuloendothelial system including the mononuclear phagocytes of the lungs and liver and the surface of the splenic sinusoids. Moreover, the birds developed chronic progressive polyarthritis one month after infection and acute swelling, redness and motion limitations accompanied gross joint deformity due to destruction of the articular cartilage. Histologic examination of the affected joints revealed hypertrophy of the synovial membranes with round cell infiltration. The organisms were found in the periarticular vessels using immunofluorescence microscopy. Despite the generalized distribution of the organisms via the blood stream after the intravenous injection, the
chronic respiratory tract infection described earlier was not common in these birds.

In contrast to *M. gallisepticum*, *M. gallinarum* is not thought to cause disease. Soon after Edward and Freundt (1956) suggested a new classification for the entire pleuropneumonia group under the genus *Mycoplasma*, *M. gallinarum* was introduced into the 7th edition of Bergey's *Manual of Determinative Bacteriology* as the only avian species listed (Freundt, 1957). During the period between 1956 and 1961 many investigators used the above name for the agents involved in or isolated from chronic respiratory disease of chicken or infectious sinusitis of turkeys (Simbert et al. 1959; Hofstad and Doerr, 1956; Popken et al., 1960; Blanco-Loizelie, 1960; Kiser and Clemente, 1960; Cumming; 1961; Barnes et al. 1961; Dardas and Schoenhard, 1961; Schoenhard and Padgett, 1961). These investigators stated clearly that they used pathogenic avian PPLO isolates. However, Adler and Yamamoto (1957) and Chu (1958) have noted that nonpathogenic isolates were also present. Later, Edward and Kanarek (1960) put an end to this confusion and reclassified 40 strains of avian mycoplasma isolates they received from various laboratories into three major groups, a pathogenic one named *M. gallisepticum* and two commensals named *M. iners* and *M. gallinarum*. Further studies by Fabricant (1960) revealed that *M. gallinarum* was nonpathogenic and belonged in serotype B of Kleckner (1960).
During his investigation on the relationships between serotypes of avian PPLO, Kleckner (1960) have noticed that two isolates, 54-537 and HPR-5 (both belonged to Serotype B), were found nonpathogenic for serologically negative turkey poultis when the latter were inoculated with these isolates into the right infraorbital sinus. The infected birds did not develop sinusitis or air sac lesions as it was found with many other strains tested. Similarly, the above isolates were found nonpathogenic for seven-day-old chicken embryos when they were inoculated via the yolk sac route. Moreover, these isolates did not show haemagglutination activity against chicken erythrocytes.

Exactly similar results to the above were obtained by Yoder and Hofstad (1964) when they used serotype B of Kleckner. This mycoplasma did not show signs of pathogenicity for chicken embryos, young chickens and turkeys and appeared to be limited to the chicken and turkeys except for an isolated report by Taylor-Robinson and Dinter (1968) who found it, together with another avian species M. iners, among porcine mycoplasma isolates.

Unlike M. gallisepticum, M. gallinarum has not attracted the attention of many investigators and relatively little published data is available, such as exists is concerned mainly with its biochemical constitution.
Evidence for the involvement of more than one agent in the aetiology of complex chronic respiratory disease was suggested as early as the 1950s (Van Roekel and Olesiuk, 1953; Fahay, 1955; Chu, 1954; 1958; Adler and Yamamoto, 1956; Van roekel et al., 1957; Jungherr, 1958 and Bankowski, 1961). These investigators and many others intensively studied the mixed infection problems and they used birds from commercial as well as from specific pathogen-free flocks testing them at various ages and after inoculation of various suspect agents by various routes and under different conditions. Unfortunately, there was no serious attempt to standardize methods or correlate observations and this has created some confusion.

Nearly twenty years after Nelson (1938) had reproduced "infectious coryza in chickens" by a combination of Haemophilus gallinarum and coccobacilliform bodies (now believed to be M. gallisepticum) in the domestic fowl, Adler and Yamamoto (1956) confirmed his findings and reported that a combination of H. gallinarum and avian pleuropneumonia-like organisms (M. gallisepticum) in chicken resulted in a disease of rapid onset and long duration similar to that described by Nelson. Then, Gross (1957; 1958) reported his extensive studies on the role of Escherichia coli and the pathological changes due to it in chicken and turkeys when complicated with mycoplasma infections. Three to eight-week-old birds were inoculated in the lesser abdominal air sacs with E. coli (serotype "0") alone, or mixed with mycoplasma (a strain of PPLO
originally isolated from infected chicken) introduced simultaneously. This resulted in air sac involvement whose connective tissue bundles became swollen and enlarged one or two days after inoculation, particularly in birds experiencing mixed infections. Furthermore, this group of birds developed perivascular lymphoid follicles one week after infection and their tracheas showed thickened mucosa, while the lungs had severe lesions due to infiltration of mononuclear and heterophilic phagocytes which obscured the normal architecture of the lobules. In another experiment, as few as ten viable bacteria produced air sac infection when they were introduced with mycoplasma, but 500,000 viable organism were required to produce the disease in the absence of the mycoplasma. It was also noted that birds were most susceptible to E. coli infection six days after the mycoplasma infection. Several other studies of the interaction between bacteria and mycoplasma have been reported but will not be discussed because they add a little to the general conclusion arrived at by Gross. However, the role of such infections must not be underestimated, and together with other mixed infections such as mycoplasma-virus, bacteria-virus and mycoplasma-bacteria-virus interactions, are problems encountered in the field which are often impossible to reproduce in the laboratory.

The majority of studies on such mixed infections relate to poultry, and the mycoplasma most commonly encountered has been M. gallisepticum in association,
in particular, with infectious bronchitis and Newcastle disease viruses. These studies include both field outbreaks and research employing different strains of *M. gallisepticum* with live vaccine strains of these viruses. Indeed it was suggested early on (Van Roekel et al., 1957) that the appearance of chronic respiratory disease might have been precipitated by virus vaccination.

The first report on experimental infection of birds with mycoplasma and virus was by Grumbles et al. (1952) who infected four-week-old chicks by placing them in contact with turkey poultsts that had been inoculated with a strain of mycoplasma (then known as the agent of infectious sinusitis) propagated in eggs. Six weeks later, the chicken were exposed to infectious bronchitis virus and five to six days later symptoms typical of this virus infection developed. However, the symptoms did not subside as expected but persisted for several weeks, mainly in the form of rales, nasal discharge, inflammation of the eyes with edematous swelling occurring around the eyes and infraorbital sinuses. Moreover, both the virus and the mycoplasma were reisolated from tracheal material for an extended period.

Jungherr (1958) examined the effect of mycoplasma and infectious bronchitis administered intratracheally either simultaneously or after an interval of eleven days. Twenty-eight days later, nearly all the birds were normal except the groups inoculated with infectious
bronchitis virus eleven days after the mycoplasma. In these birds severe lesions occurred and it was suggested that in multiple infections the time of entry of the agents was important.

Fabricant and Levine (1962) demonstrated that *E. coli* infection exacerbated the disease associated with infection with *M. gallisepticum* and infectious bronchitis virus, and when the results were evaluated in terms of mortality plus severity and nature of the lesions it was possible to grade the different combinations in the following order; (1) bacteria + mycoplasma + virus; (2) bacteria + mycoplasma; (3) bacteria + virus or bacteria alone.

Adler et al. (1962) tested if *M. gallisepticum* infection could provoke a disease response after intranasal immunization against infectious bronchitis (Massachusetts strain). The birds were first infected intranasally with the mycoplasma 28, 14 or three days before immunization with the virus or simultaneously with the virus. Mycoplasma infection alone caused no clinical signs although there was a serological response and histological examination revealed a lymphoid follicular reaction in the turbinates. However, all the birds which had received the dual infection developed tracheal rales and nasal discharge four days after vaccination which persisted for three weeks with varying degrees of coryza, tracheitis and air sacculitis. The
birds which were exposed to the mycoplasma one month before the virus had least pathologic changes while the other groups all responded about the same. The vaccine virus alone caused only a mild reaction.

Both *M. gallisepticum* and infectious bronchitis were shown by Blake (1962) to affect egg production and dual infection also resulted in higher mortality rates. Moreover, the size of eggs from this group was smaller than those from the birds which were infected with either agent alone.

Uppal (1972) showed that the clinical signs, macroscopic and microscopic lesions in birds inoculated with infectious bronchitis virus and *M. gallisepticum* were more severe after dual infections. Furthermore, in such infections there were higher concentrations of the mycoplasma in the trachea and earlier higher levels of haemagglutination inhibition antibodies developed. However, replication of the virus was not affected in the dually-infected birds and neutralizing antibodies to it were comparable to the controls.

Timms (1972) investigated the effect of infectious bronchitis virus on a low grade and latent *M. gallisepticum* infection. This was achieved by intranasal and sinus inoculation with mycoplasma at six and sixteen weeks of age respectively, and eighteen weeks later the birds were inoculated with the virus by both intranasal and intratracheal routes. The respiratory distress was
most severe in the birds which received the dual
infection, including varying degrees of air sacculitis
accompanied by occasional catarrhal exudate in their
tracheas. Histological examination of trachea revealed
hyperplasia, corrugation of the epithelium and active
proliferation of round cells in the submucosa. An
unexpected finding was that despite the prolonged gap
between infections, haemagglutination and agglutination
antibodies against mycoplasma were greater in the mixed
infection group whereas antibodies against the virus
were greater in the birds with single infection.

McMartin (1965) in an earlier report produced the
only data which contradicted the general trend. He
noted that in a spontaneous outbreak of *M. gallisepticum*
infection in a group of eleven-week-old broilers there
was no increase in severity of the symptoms when
infectious bronchitis virus was deliberately introduced.
Yet prolonged coryza and rales occurred in another group
of adult birds exposed to the virus 31 days after
deliberate infection with the mycoplasma.

Studies with Newcastle disease virus have commonly
been done together with infectious bronchitis virus.
An ambitious study of the effects of sequential infection
of these viruses and mycoplasma was reported by Dunlop
et al. (1964). The organisms were introduced by an
aerosol method and three sequences were tested (1)
mycoplasma followed seven days later with a combination
of the two viruses together, (2) simultaneous inoculation with all the agents and (3) the mycoplasma infection introduced one week after the combined virus-infection. In all the above situations, the three agents were reisolated and the birds developed specific antibodies to each, together with severe gross lesions. However, gross lesions were most severe and persisted longer in the birds which were infected with the viruses before the administration of the mycoplasma.

In contrast, Corstvet and Sadler (1966) did not believe they had enough evidence to suggest that mycoplasma had a significant role in birds infected with infectious bronchitis and Newcastle disease viruses. Their tests differed from those of Dunlop et al. (1964) in that the birds were older, the strains of viruses and the route of infection were different. However, there was evidence for enhanced multiplication of the mycoplasma with a reduced lag phase in the group infected with Newcastle disease virus and the enhancement was thought to result from viral damage to the epithelium. Furthermore, when infectious bronchitis virus was administered fifteen days after the mycoplasma, respiratory signs and lesions recurred and there was a boost in mycoplasma growth.

Another aspect of mixed infection was an effect on weight gain of birds infected with *M. gallisepticum* after immunisation with Newcastle disease and infectious
bronchitis (Heishman et al., 1969). A significant reduction in the final weights was observed in the group of birds which received the two parasites and it was this group which had highest per cent mortality and condemnations.

Another useful study of these interactions was by Omuro et al. (1971) who investigated the interaction of M. gallisepticum with mild vaccine strains of Newcastle disease virus and infectious bronchitis viruses. The mycoplasma was administered intranasally and one week later the viruses were administered either singly or together in the same way. The only significant clinical symptoms developed in birds which received dual inoculations and infectious bronchitis was more damaging than Newcastle disease virus. Gross and histopathological effects were detected in the trachea and infraorbital sinuses from which the mycoplasma also could be reisolated up to seven weeks after infection. Triple infections were no worse than dual infections although the mycoplasma was recovered from the tracheas of this group of birds in larger numbers and over longer periods of time than in the groups of dual infections.

However, Suzuki et al. (1971) did not notice any such enhanced response when they studied the effect of live vaccine strains of Newcastle disease virus and infectious bronchitis on day-old-chicks infected
intratracheally with *M. gallisepticum*. The only synergism noted was that mycoplasma was isolated with the greatest frequency (91%), from the birds which were inoculated with the three agents, 66% from those inoculated with the mycoplasma and Newcastle disease and 61% from the mycoplasma alone-infected group. Also, the agglutinin titers against the mycoplasma were highest in the group of birds which received the triple infection. The application of inactivated Newcastle disease vaccine during the experiment had no significant effect upon the mycoplasma infection.

Where the effect of Newcastle disease alone with *M. gallisepticum* has been tested then results similar to those with infectious bronchitis plus mycoplasma have been recorded. Sato et al. (1970) inoculated birds intratracheally either simultaneously or at different times and observed that the pathological changes and respiratory symptoms were more severe in the mixed infection groups especially when the birds were simultaneously inoculated. Moreover, the mycoplasma was more frequently reisolated and the agglutination titers against it were also higher in these groups.

Nonomura et al. (1971) reporting similar experiments noted increased mycoplasma growth and histological changes in the trachea in the mixed infection groups and concluded that the virus might have caused changes in the respiratory tract which resulted in the enhanced
multiplication of the mycoplasma which was analogous to the earlier observations of Corstvet and Sadler (1966). Extending this work to young birds, Nonomura and Sato (1971) noticed that in the dually-infected group respiratory symptoms were extended to three weeks after inoculation and were accompanied by gross lesions in the trachea and the air sacs. In addition more frequent isolations of the mycoplasma were made and enhanced agglutination titer was detected which was a similar observation to that of Adler et al. (1962).

The route of administration of mycoplasma and virus has a profound effect on the response of birds to these parasites, as does the choice of animal species, thus Cordy and Adler (1965) using turkey pouls, administered both \textit{M. gallisepticum} and Newcastle disease virus at different times either intramuscularly or intravenously. None of the birds given virus reacted, but all birds given mycoplasma, alone or with virus, died. With the dual infection, mortality and the preliminary leg weakness and somnolence developed earlier than those with mycoplasma alone. There were also many more necrotic foci in the brains of the dually-infected animals and muscle lesions were distinctly more extensive.

Apart from the important and valuable studies with infectious bronchitis and Newcastle disease viruses some other viruses inoculated with \textit{M. gallisepticum} have been reported.
Kaji et al. (1970) reported on reovirus acting synergistically with this mycoplasma. Although the extent of the evidence lay in the fact that there was a slight increase in the number of \textit{M. gallisepticum} organisms in the trachea and an increase in the neutralizing antibodies against the virus in the mixed infection group, the significance of the latter was slight.

Influenza but not parainfluenza virus is also thought to act synergistically with \textit{M. gallisepticum} (Ranck et al., 1970). With influenza both serologic reactions and gross lesions were enhanced in turkey poult exposed to mixed infections with \textit{M. gallisepticum}. In contrast birds inoculated with the mycoplasma and the paramyxovirus strain developed neither enhanced serologic reactions nor increased gross lesions.

Up till now chicken embryo lethal orphan adenovirus (CELO) infections in chicken are not thought to cause definite disease. However, the question has been raised as to whether it may exacerbate infections with other pathogens (Du Bose, 1972), particularly after the virus has been shown to be an endogenous contaminant of embryonating eggs and cell cultures of chicken origin (Yates and Fry, 1957; Chomiack et al., 1961; Yates et al., 1962; Taylor and Calnek, 1962). Yates and Fry (1957) were particularly concerned about the possibility of CELO virus becoming a problem as a contaminant in
vaccine production and diagnosis of viral diseases while Bankowski (1961) urged the necessity for evaluation of three "recent" virus isolates of low virulence, including CELO virus (the other two were reoviruses and a paramyxovirus Yucaipa), in mixed infections with other avian respiratory agents, after he demonstrated that infectious bronchitis virus (Massachusetts strain) enhanced a relatively mild mycoplasma infection and produced a severe disease. Bakos (1963) isolated both CELO virus and M. gallisepticum from birds showing mild respiratory symptoms, weakness and somnolence and Yates et al. (1960) reported that serologic tests indicated that CELO virus was widespread among chicken in the United States. McFerran et al. (1971) isolated infectious bronchitis, adeno and reoviruses from 94 flocks of chicken with or without respiratory diseases, and they noticed that there was a higher recovery rate of these agents from the diseased flocks. They also concluded that adenoviruses are important in the etiology of respiratory disease especially in association with mycoplasma or infectious bronchitis virus. A similar conclusion was reported by Luthgen et al. (1967) who carried out serological surveys for CELO and infectious bronchitis virus infections and mycoplasmosis in chicken and noticed that mycoplasma infections could be activated by virus infections. More recently Flir (1969) have noticed in their serological surveys on healthy fowls as well as on
fowls with respiratory diseases, that there was higher incidences of *M. gallisepticum*, CELO and infectious bronchitis viruses in the diseased birds and the importance mixed infections was stressed.

CELO virus alone causes a usually inapparent infection mainly in chicken, and it has been the subject of extensive reviews (Rinaldi et al., 1968; Du Bose, 1972; Aghakhan, 1974). It was first isolated in 1952 (Yates et al., 1954) from chicken embryos inoculated with unrelated pathological material (a serum sample from a cow). Now it is widely known to cause dwarfing and death of chicken embryos within five days after inoculation into the allantoic cavity. Although serologic tests indicated that CELO virus was widespread among chicken and turkeys (Yates et al., 1960; Flir, 1969; McFerran et al., 1971), it was not found highly contagious and no avian disease entity has been ascribed to it (Du Bose, 1972). However, CELO virus infections have been studied by a number of investigators but its role alone or in mixed infections is not yet fully explored. Based either on serological data or on direct isolation of the virus, natural infections were reported to have occurred in a wide variety of wild and domesticated fowls, in particular, it is common in four to twelve-week-old chicken (Taylor and Calnek, 1962).

Although transovarian transmission of CELO virus
was established by Yates et al. (1960), but it was not considered to be of primary importance and mechanical transmission of the virus through virus-loaded faeces contaminating food and water are believed of great importance particularly during the acute phase of infection (Clemmer, 1972; Taylor and Calnek, 1962) as well as through airborne infections (Kawamura et al. 1963).

In naturally infected chicken the virus was usually asymptomatic but several investigators have noticed mild respiratory symptoms, leg weakness, diarrhoea, underdevelopment, nervous symptoms, as well as the occurrence of misshapen and ridged eggs from infected laying hens, and a drop in egg production. Information on lesions in naturally as well as deliberately infected birds is rare and supports the general view that CELO virus produces inapparent infections (Du Bose, 1972). Slight swellings of the upper trachea have been observed which upon microscopic examination revealed a slight proliferation of mononuclear cells in the sub-mucosa (Kawamura et al. 1963). Winterfield et al. (1973) reported tracheitis, inactive ovaries and severe gross lesions of hepatitis in naturally infected chicken with the Indiana C strain of CELO virus. Microscopic examination of the affected livers showed lymphoïyte and fibroblast infiltration, intranuclear inclusions, haemorrhages, necrotic areas and bile duct proliferation. In the affected trachea mononuclear cell infiltration in the lamina propria, hypertrophy in the mucous glands
were seen as well as sloughed-off epithelium in some areas (Gallina et al., 1973).

In deliberately infected chicken most investigators failed to produce clinical symptoms, by intratracheal, intravenous or oral routes (Bakos, 1963; Garside, 1965; Cook, 1968). However, intracerebral inoculation of young chicks produced clinical symptoms and the birds developed nervous signs followed by death (Yates, 1960; Rinaldi et al. 1968; Ahmad and El Sisi, 1969). The latter two groups of investigators also inoculated chicks intravenously (Rinaldi et al. 1968) or intramuscularly and intratracheally (Ahmad and El Sisi, 1969) and they noticed depression, dullness, anoraxia, weight loss, weakness, inappetence ruffling and droopiness in the infected birds. In addition, Winterfield et al. (1973) noticed sneezing, crepitation, and rales in chicken inoculated by various routes with the Indiana C strain of CELO virus.

Gross changes were reported occurring in the liver only out of all other organs of one-year-old chicken inoculated with the Ote strain of CELO virus. This consisted of small greyish white foci spread on the surface and tended to concentrate around the edges of the organ (Kawamura and Horiuchi, 1964). However, microscopic examination of affected birds revealed changes in the kidneys, tracheas and lungs besides the livers. In the kidneys, the epithelium of the tubules
showed mild degeneration and necrosis with intranuclear inclusions, while the interstitial tissues of the damaged areas were infiltrated with histiocytes and lymphocytes. Similar reactions were seen in the tracheas and lung of infected birds but without necrosis or hyperplasia in the epithelium. Infiltration with lymphocytes and heterophils were also noticed in the lamina propria of the primary bronchus and around the cells which showed intranuclear inclusions. In the liver, sites undergoing degeneration and necrosis were seen infiltrated with lymphocytes, heterophils and histiocytes. Inclusion bodies were present in the hepatocytes as well as in the interlobular bile tubules of the same chicken.

Experimental mixed infections with CELO virus and avian mycoplasmas are very limited. Monreal (1966) reported on the simultaneous infection of chicken with *M. gallisepticum* and CELO virus. In the mixed infection group clinical symptoms were mild but histological examination revealed an involvement of the air sac. These same effects were present in birds infected with the mycoplasma alone. Similar experiments on younger birds gave evidence of even milder responses.

Similarly Aghakhan and Butler (1973) found evidence for a slight exacerbation of clinical symptoms in SPF chicken infected by both agents by an aerosol. This was also associated with increased histopathological
reaction, accompanied with a slight increase in the level of mycoplasma in birds infected with the virus, while the activity of the virus was not appreciably altered. Increased immunological response to the mycoplasma was detected in the mixed infection group of birds but not to the virus.

CELO virus and mycoplasma have also been found to influence egg production (Berry, 1969). The slight depression observed after virus infection became greater when M. gallisepticum was present and was further exaggerated when infectious bronchitis was inoculated as a third agent.

Apart from M. gallisepticum some other species of mycoplasma are associated with avian respiratory disease and synergism with viruses is possible. One example of this is M. synoviae. This species was found present in the air sacs of birds condemned for air sacculitis and was capable of causing air sacculitis when given in conjunction with Newcastle disease and infectious bronchitis (Yoder, 1970, cited by Vardaman et al., 1973). This observation was investigated more thoroughly by Kleven et al. (1972) in young chicks which they exposed to aerosols of the mycoplasma five days after virus vaccination via the drinking water and more severe and greater incidence of air sacculitis was observed than in those exposed to the mycoplasma alone. Even more striking effects were noted when the organisms were infected
simultaneously and were paralleled by high HI titers
against the mycoplasma. In contrast, vaccination of
the birds five days after exposure to the mycoplasma
resulted in a reaction no different to that of birds
exposed to the mycoplasma alone.

In a similar experiment, Kleven et al. (1972) came
to the conclusion that the age of birds when vaccinated,
the sequence of infection and route of exposure were
important and in these respects paralleled observations
of M. gallisepticum noted earlier by Dunlop et al. (1964).
Also, as with M. gallisepticum experiments, Kleven (1972)
observed that birds dually infected with M. synoviae
and combined Newcastle disease—infectious bronchitis
vaccine had greatest incidences of air sac lesions with
severest manifestations accompanied with highest HI
titers for the mycoplasma. Furthermore, birds vaccinated
five days before exposure to the mycoplasma experienced
the most severe air sac response.

Studies on other mycoplasma species and viruses
have been reported (Mohamad et al., 1970). M. meleagridis
and influenza A virus in turkey poults did not produce
the enhanced reaction reported for other combinations.
Neither young nor old birds dually infected via the air
sac or the nostrils were affected. Furthermore, there
were no enhanced clinical signs or gross lesions nor were
the patterns of antibodies different to those of the
birds which were inoculated with either agent alone.
These results were in contrast to those reported earlier by Ranck et al. (1970) who employed the influenza virus but in mixed infections with *M. gallisepticum*.

Experimental mixed infection studies have also been performed on ducks (Roberts, 1964) with influenza A and a mycoplasma (*M. anatis*) from a field outbreak of duck sinusitis (chronic respiratory disease), but the agents did not reproduce the disease in the laboratory either alone or together. However, the antibody response to the mycoplasma was stimulated when the two agents were simultaneously used or when the virus was inoculated 14 days after the mycoplasma and it was suggested that there might have been a third stress factor involved in the field outbreaks.

These observations on synergism between mycoplasma and virus in avian respiratory disease have a parallel in various studies of diseases in non-avian animals. In fact, the earliest report on the interaction of mycoplasma and viruses in animals was by Findlay et al. (1938). They noticed that death of mice due to rolling disease agent (*M. neurolyticum*) occurred two days earlier and the virulence of the mycoplasma was raised when either lymphocytic choriomeningitis virus, lymphogranuloma inguinale virus or yellow fever virus were injected with the mycoplasma intracerebrally. Similar observations were reported by Nelson (1957) who studied the interaction between several strains of murine...
hepatitis virus in mice. Some of the mice used were adversely affected by the mixed infection and up to 50% mortality was recorded after intracerebral inoculation of the parasites and the survivors were acutely ill and experienced up to 40% weight loss, spotted livers and brain inflammations.

Kagan et al. (1973) studied the reaction of special strains of mice, to mixed infections with several mycoplasma species and two strains of leukemia viruses. M. laidlawii, M. orale, M. gallisepticum, amongst other species were capable of producing increased spleen weight in infected animals when they were administered simultaneously with the viruses. Likewise, Kasza et al. (1969) noticed synergistic activity in gnotobiotic pigs inoculated simultaneously with M. hyopneumoniae and a pathogenic strain of adenovirus, manifested by severe clinical symptoms, gross pathology in the lungs, and microscopic lesions. The authors questioned whether or not similar situations would occur under natural conditions.

Studies on the interaction of mycoplasma and virus in cell cultures has provided some support for the possibility of synergistic activity. After the first isolation of mycoplasma from contaminated cell cultures (Robinson et al., 1956), the number of isolations made by various investigators from contaminated cultures at numerous laboratories in different countries amounted to
as many as fourteen distinct Mycoplasma and Acholeplasma species (Barile, 1973). Investigators using cell cultures are now aware of the possible role of contaminating mycoplasmas on the results of their studies particularly after the increased knowledge about the nature of these microorganisms and their interrelationships with the cell or the host tissue. Recently, the effect of mycoplasma on cell cultures has been fully reviewed (Stanbridge, 1971; and Barile, 1973) and it became evident that mycoplasmas alter the cell and its functions in many respects including inhibition of cell metabolism and cell growth, alteration of nucleic acid synthesis, cause chromosomal aberrations, alter antigenicity of cell membranes, and disturb other parameters such as sensitivity of cells to viruses, pharmacological drugs and inhibition of interferon production.

Mixed mycoplasma-virus studies in cell cultures is much more complicated than in the animal work and analysis of the available data is difficult because numerous mycoplasma species have been used in many different cell cultures inoculated with various viruses. Furthermore, although many investigators were aware of the presence of mycoplasma as contaminants in their cell cultures, characterisation of these mycoplasmas was frequently lacking. The outcome of these studies is summarized in TABLE 1. This provides an analysis of quantitative as well as qualitative data.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Mycoplasma species</th>
<th>Cell culture</th>
<th>Effect on virus</th>
<th>Investigator(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adeno type 2</td>
<td>Unidentified PPLO</td>
<td>KB, HeLa-53, HEP-2</td>
<td>decreased</td>
<td>Rouse et al. (1963)</td>
</tr>
<tr>
<td>&quot; &quot; 3</td>
<td><em>M. pneumoniae</em></td>
<td>Human embryo kidney</td>
<td>unaffected</td>
<td>Baizhomartov et al. (1971)</td>
</tr>
<tr>
<td>&quot; &quot; 6</td>
<td>Unidentified PPLO</td>
<td>Continuous line of pig embryo kid</td>
<td>decreased</td>
<td>Kagan et al. (1968)</td>
</tr>
<tr>
<td>Adeno types 2, 3 J</td>
<td><em>M. orale, M. hominis, M. hyorhinis</em></td>
<td>HeLa</td>
<td>decreased</td>
<td>Hargreaves and Leach (1970)</td>
</tr>
<tr>
<td>Blue-tongue</td>
<td><em>Acholeplasma laidlawii</em></td>
<td>L strain of mouse Cells</td>
<td>decreased</td>
<td>Rinaldo et al. (1973)</td>
</tr>
<tr>
<td>Coxsackie B3</td>
<td>Unidentified PPLO</td>
<td>Continuous pig embryo kidney</td>
<td>unaffected</td>
<td>Kagan et al. (1968)</td>
</tr>
<tr>
<td>Eastern equine encephalomyelitis</td>
<td>Several species</td>
<td>Chicken embryo fibroblasts</td>
<td>decreased</td>
<td>Yershov and Zadanov (1965)</td>
</tr>
<tr>
<td>Fowl pox</td>
<td>Unidentified PPLO</td>
<td>Continuous pig embryo kidney</td>
<td>unaffected</td>
<td>Kagan et al. (1968)</td>
</tr>
<tr>
<td>Herpes Simplex</td>
<td>Several species</td>
<td>Several cell lines</td>
<td>decreased</td>
<td>Gafford et al. (1969)</td>
</tr>
<tr>
<td>Infectious bovine rhinotracheitis</td>
<td><em>M. gallisepticum</em></td>
<td>Chicken embryo</td>
<td>increased</td>
<td>Slack and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>Influenza</td>
<td><em>M. arginini</em></td>
<td>Human thyroid</td>
<td>decreased</td>
<td>Afshar (1967)</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td><em>M. bovicenitalium</em></td>
<td>Calf kidney</td>
<td>unaffected</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td><em>M. hominis</em></td>
<td>Choriocallantoic membrane</td>
<td>unaffected</td>
<td>Nakamura and Sakamoto (1969)</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td><em>M. hominis, M. orale</em></td>
<td>HeLa</td>
<td>unaffected</td>
<td>Hargreaves and Leach (1970)</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td><em>M. orale</em></td>
<td>Stable porcine kidney</td>
<td>unaffected</td>
<td>Nakamura and Sakamoto (1969)</td>
</tr>
<tr>
<td>Measles</td>
<td><em>A. laidlawii, M. gallisepticum, M. hominis</em></td>
<td>Chick embryo</td>
<td>increased</td>
<td>Smirnova and Kagan (1971)</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>Unidentified PPLO</td>
<td>several heteroploid</td>
<td>decreased</td>
<td>Gori and Lee (1964)</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td><em>M. hyorhinis</em></td>
<td>HEP-2</td>
<td>decreased</td>
<td>Butler and Leach (1964)</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td><em>M. hominis</em></td>
<td>HEP-2</td>
<td>unaffected</td>
<td>Butler and Leach (1964)</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td><em>M. orale</em></td>
<td>VERO</td>
<td>decreased</td>
<td>Romano and Brankato (1970)</td>
</tr>
<tr>
<td>Marek's disease</td>
<td>Unidentified PPLO</td>
<td>Chick embryo fibroblasts</td>
<td>decreased</td>
<td>Simmons and Lukert (1972)</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td><em>M. gallisepticum</em></td>
<td>Chick embryo fibroblasts</td>
<td>decreased</td>
<td>Simmons and Lukert (1972)</td>
</tr>
<tr>
<td>Virus</td>
<td>Mycoplasma species</td>
<td>Cell culture</td>
<td>Effect on virus</td>
<td>Investigator(s)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------------------</td>
<td>--------------------------------------------------</td>
<td>-----------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Mongo encephalitis</td>
<td>Unidentified PPLO, <em>M. pneumoniae</em>, <em>M. hominis</em></td>
<td>L strain of mouse cells</td>
<td>decreased</td>
<td>Brownstein and Graham (1961)</td>
</tr>
<tr>
<td>Newcastle disease</td>
<td>Unidentified PPLO, <em>M. pneumoniae</em>, <em>M. hominis</em></td>
<td>L strain of mouse cells</td>
<td>unaffected</td>
<td>Armstrong and Paucker (1966)</td>
</tr>
<tr>
<td>Polio type I</td>
<td><em>M. hominis</em></td>
<td>Several cell lines</td>
<td>decreased</td>
<td>Kagan et al. (1967)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. hominis</em></td>
<td>Human embryonic lung fibroblasts</td>
<td>unaffected</td>
<td>Herderchee et al. (1963)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. hominis</em>, <em>M. hyorhinis</em></td>
<td>HeLa</td>
<td>increased</td>
<td>Hargreaves and Leach (1970)</td>
</tr>
<tr>
<td>&quot;</td>
<td>Unidentified PPLO, <em>M. hominis</em></td>
<td>Human embryonic lung fibroblast</td>
<td>decreased</td>
<td>Herderchee et al. (1963)</td>
</tr>
<tr>
<td>Parainfluenza type 2</td>
<td><em>M. hominis</em></td>
<td>Monkey kidney</td>
<td>increased</td>
<td>Mahadjer and Kafterians (1973)</td>
</tr>
<tr>
<td>Respiratory syncitial</td>
<td><em>M. orale</em>, <em>M. hominis</em>, <em>M. hyorhinis</em></td>
<td>VERO</td>
<td>increased</td>
<td>Hargreaves and Leach (1970)</td>
</tr>
<tr>
<td>Rhinovirus type 2</td>
<td><em>M. pneumoniae</em></td>
<td>KB</td>
<td>increased</td>
<td>Hargreaves and Leach (1970)</td>
</tr>
<tr>
<td>Rous sarcoma</td>
<td><em>M. arginini</em></td>
<td>Human embryo fibroblasts</td>
<td>unaffected</td>
<td>Baizhmartov et al. (1971)</td>
</tr>
<tr>
<td>Rous sarcoma and Rous</td>
<td><em>M. orale</em>, an unidentified PPLO</td>
<td>chicken fibroblasts</td>
<td>decreased</td>
<td>Milligan and Fletcher (1962)</td>
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<tr>
<td>associated</td>
<td><em>M. hyorhinis</em></td>
<td>Human embryo diploid fibroblasts</td>
<td>decreased</td>
<td>Slack and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>Semliki-Forest</td>
<td><em>M. arginini</em>, <em>M. pneumoniae</em></td>
<td>L strain of mouse cells</td>
<td>increased</td>
<td>Somerson and Cook (1965)</td>
</tr>
<tr>
<td>Sindbis</td>
<td>Unidentified PPLO, <em>M. pneumoniae</em>, <em>M. hominis</em></td>
<td>Human embryo kidney</td>
<td>unaffected</td>
<td>Singer et al. (1972)</td>
</tr>
<tr>
<td>SV-40</td>
<td>Unidentified PPLO, <em>M. pneumoniae</em>, <em>M. hominis</em></td>
<td>Human amnion</td>
<td>decreased</td>
<td>Singer et al. (1969b)</td>
</tr>
<tr>
<td>Tick encephalitis</td>
<td><em>M. pneumoniae</em>, <em>M. hominis</em></td>
<td>VERO</td>
<td>increased</td>
<td>Armstrong and Paucker (1966)</td>
</tr>
<tr>
<td>Vaccinia</td>
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<td>unaffected</td>
<td>O'Connell et al. (1964)</td>
</tr>
<tr>
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<td>several heteroploid cell lines</td>
<td>decreased</td>
<td>Kagan et al. (1968)</td>
</tr>
<tr>
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<td>Unidentified PPLO</td>
<td>continuous pig embryo kidney</td>
<td>unaffected</td>
<td>Gori and Lee (1964)</td>
</tr>
<tr>
<td>&quot;</td>
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<td>Hamster embryo fibroblasts, human amnion</td>
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<td>Kagan et al. (1968)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. hyorhinis</em></td>
<td></td>
<td></td>
<td>Singer et al. (1970)</td>
</tr>
<tr>
<td>Virus</td>
<td>Mycoplasma species</td>
<td>Cell Culture</td>
<td>Effect on Virus</td>
<td>Investigator(s)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------</td>
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<td>--------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>M. arginini</td>
<td>Hamster embryo fibroblasts, human amnion</td>
<td>decreased</td>
<td>Singer et al. (1970)</td>
</tr>
<tr>
<td></td>
<td>M. oralis, M. hominis, M. hyorhinis</td>
<td>HeLa</td>
<td>increased</td>
<td>Hargreaves and Leach (1970)</td>
</tr>
<tr>
<td>Varicella</td>
<td>M. gallisepticum, M. hyorhinis</td>
<td>Human thyroid</td>
<td>unaffected</td>
<td>Slack and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td></td>
<td>M. arginini</td>
<td>Human embryonic lung fibroblasts, human thyroid</td>
<td>decreased</td>
<td>Slack and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>Venezzeulan equine encephalomyelitis</td>
<td>Several species</td>
<td>Chick embryo fibroblasts</td>
<td>decreased</td>
<td>Yershow and Zdanov (1965)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vesicular stomatitis</td>
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<td>Several cell lines</td>
<td>decreased</td>
<td>Kagan et al. (1967)</td>
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<tr>
<td></td>
<td>M. arginini</td>
<td>Mouse embryonic fibroblasts</td>
<td>increased</td>
<td>Armstrong et al. (1965)</td>
</tr>
<tr>
<td></td>
<td>M. hyorhinis</td>
<td>Hamster embryonic fibroblasts</td>
<td>increased</td>
<td>Singer et al. (1972)</td>
</tr>
<tr>
<td></td>
<td>A. laidlawii</td>
<td>Mouse embryonic fibroblasts WI-38</td>
<td>increased</td>
<td>Singer et al. (1969a; 1969b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>primary chick embryo</td>
<td>decreased</td>
<td>Singer and Ford (1972)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rinaldo et al. (1973)</td>
</tr>
</tbody>
</table>
Unlike the effect reported in birds and other animals, which involve clinical, macroscopic, microscopic or serologic reactions, the work in cell cultures is almost extensively concerned with whether or not mycoplasmas increased or decreased virus replication or vice versa and the complex situations have been reported where different species of mycoplasma produced totally different results on the virus yield in one cell system (Butler and Leach, 1964; Nakamura and Sakamoto, 1969; Singer et al., 1970). Different virus yields were also obtained when different viruses were inoculated in one cell system contaminated with one mycoplasma species (Hargreaves and Leach, 1970). Furthermore, two different virus yields could be obtained by inoculating two different cell lines with one virus and one mycoplasma species (Herderchee et al., 1963; Hargreaves and Leach, 1970.
Soon after it was demonstrated that tracheal organ explants successfully supported the growth of mycoplasma (Butler, 1968), a great many mycoplasma species from different animal sources were studied in this system (TABLE 2). The tissue used in these studies was obtained from chicken embryos, human fetuses, pigs, mice, hamsters and from calves. Moreover, infections with mycoplasma were also studied in human embryonic nasal tissue and synovium as well as in human oviduct and in bovine embryo lung (Cherry and Taylor-Robinson, 1973). Many mycoplasma species grew readily in the tracheal explant system and some of them caused damage to the ciliated epithelial tissue while others did not produce noticeable change. Furthermore, many pathogenic strains of mycoplasma caused inhibition of the ciliary activity of the ciliated cells and this particular effect had been used by many investigators as a useful indicator for the pathogenicity of the mycoplasma (Butler 1969a, 1969b, Collier and Clyde, 1969; Collier et al., 1971; Collier and Baseman 1973; Cherry and Taylor-Robinson 1973). However, most of the investigators who used the tracheal organ explant system in their studies on mycoplasmas relied mainly on light microscopy and immunofluorescence (Butler 1969a; 1969b; Collier and Baseman, 1973; Collier et al., 1971; Collier, 1972; Reed, 1972a) which provided relatively limited and poor details concerning the specific sites of activity of the mycoplasma. Recourse to electron microscopy was
<table>
<thead>
<tr>
<th>Source of mycoplasma</th>
<th>Mycoplasma Species</th>
<th>Tracheal organ explant</th>
<th>Investigator(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian</td>
<td><em>M. gallinarum</em></td>
<td>Chicken embryonic</td>
<td>Taylor-Robinson and Cherry (1972); Cherry and Taylor-Robinson (1970b; 1973); Abu-Zahr and Butler (1974)</td>
</tr>
<tr>
<td>&quot;</td>
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<td>Human embryonic</td>
<td>Butler and Ellaway (1971)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. gallisepticum</em></td>
<td>Chicken embryonic</td>
<td>Butler et al. (1973) Butter and Ellaway (1971); Cherry and Taylor-Robinson (1970a; 1970b; 1971; 1973); Taylor-Robinson and Cherry (1972); Abu-Zahr and Butler (1974)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. iners</em></td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. meleagritidis</em></td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. synoviae</em></td>
<td>Human embryonic</td>
<td>Butler and Ellaway (1971)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. synoviae</em></td>
<td>Chicken embryonic</td>
<td>Butler and Ellaway (1971)</td>
</tr>
<tr>
<td>&quot;</td>
<td>WRI</td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>&quot;</td>
<td>IOVa 695</td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>Bovine</td>
<td><em>M. agalactiae</em></td>
<td>Human embryonic</td>
<td>Butler and Ellaway (1971)</td>
</tr>
<tr>
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<td><em>M. agalactiae</em></td>
<td>Chicken embryonic</td>
<td>Butler and Ellaway (1971); Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>M. mycoides var. mycoides</em></td>
<td>Human embryonic</td>
<td>Butler and Ellaway (1971); Cherry and Taylor-Robinson (1970b; 1973)</td>
</tr>
<tr>
<td>Human</td>
<td><em>M. fermentans</em></td>
<td>Human embryonic</td>
<td>Collier and Clyde (1969)</td>
</tr>
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<td>Human embryonic</td>
<td>Collier and Clyde (1971)</td>
</tr>
<tr>
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<td><em>M. hominis</em></td>
<td>Chicken embryonic</td>
<td>Butler (1968; 1969b); Collier and Clyde (1971)</td>
</tr>
<tr>
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<td>Syrian hamster</td>
<td>Collier and Clyde (1969)</td>
</tr>
<tr>
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<td>Human embryonic</td>
<td>Butler (1969b); Butler and Ellaway (1971); Collier and Clyde (1971)</td>
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<td>Butler and Ellaway (1971)</td>
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<td>Collier and Clyde (1969)</td>
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<td>Collier and Clyde (1969); Collier et al. (1969; 1971) Collier and Baseman (1973); Woodruff et al. (1973)</td>
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<tr>
<td>Source of mycoplasma</td>
<td>Mycoplasma Species</td>
<td>Tracheal organ explant</td>
<td>Investigator(s)</td>
</tr>
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<td>---------------------</td>
<td>-------------------</td>
<td>-----------------------</td>
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</tr>
<tr>
<td>Human</td>
<td><em>M. salivarium</em></td>
<td>Human embryonic</td>
<td>Butler (1969b); Butler and Ellaway (1971); Collier and Clyde (1971)</td>
</tr>
<tr>
<td></td>
<td><em>M. salivarium</em></td>
<td>Bovine embryonic</td>
<td>Reed (1972)</td>
</tr>
<tr>
<td></td>
<td><em>M. salivarium</em></td>
<td>Syrian hamster</td>
<td>Collier and Clyde (1969)</td>
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<tr>
<td></td>
<td><em>M. salivarium</em></td>
<td>Chicken embryonic</td>
<td>Butler and Ellaway (1971)</td>
</tr>
<tr>
<td></td>
<td>T-strains</td>
<td>Human embryonic</td>
<td>Cherry and Taylor-Robinson (1970a; 1973)</td>
</tr>
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<td></td>
<td>T-strains</td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1970b; 1973)</td>
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<td><em>M. pulmonis</em></td>
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<td>Westberg et al. (1972)</td>
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<td></td>
<td><em>M. pulmonis</em></td>
<td>Young mice</td>
<td>Cherry and Taylor-Robinson (1973)</td>
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<td><em>M. neurolyticum</em></td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
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<tr>
<td></td>
<td>PC5</td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
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<tr>
<td>Sheep &amp; goats</td>
<td><em>M. mycoides var. capri</em></td>
<td>Human embryonic</td>
<td>Butler (1969a; 1969b); Butler and Ellaway (1971)</td>
</tr>
<tr>
<td></td>
<td><em>M. mycoides var. capri</em></td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1970a; 1970b; 1972; 1973); Butler and Ellaway (1971)</td>
</tr>
<tr>
<td>Swine</td>
<td><em>Acholeplasma granululturum</em></td>
<td>Young pigs</td>
<td>Pijanet al. (1972)</td>
</tr>
<tr>
<td></td>
<td><em>M. hyopneumoniae</em></td>
<td>Young pigs</td>
<td>Pijanet al. (1972a; 1972b; 1974)</td>
</tr>
<tr>
<td></td>
<td><em>M. hyopneumoniae</em></td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td></td>
<td><em>M. hyorhinis</em></td>
<td>Young pigs</td>
<td>Pijanet et al. (1972a; 1972b); Pijanet (1974); Reed (1972)</td>
</tr>
<tr>
<td></td>
<td><em>M. hyorhinis</em></td>
<td>Bovine embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td></td>
<td><em>M. hyorhinis</em></td>
<td>Chicken embryonic</td>
<td>Pijanet et al. (1972a; 1972b)</td>
</tr>
<tr>
<td></td>
<td><em>M. hyosynoviae</em></td>
<td>Young pigs</td>
<td>Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td><em>M. gordonii</em></td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1973)</td>
</tr>
<tr>
<td></td>
<td><em>Acholeplasma laidlawii</em></td>
<td>Chicken embryonic</td>
<td>Cherry and Taylor-Robinson (1970b; 1973)</td>
</tr>
</tbody>
</table>
essential, and some studies which employed this technique proved particularly rewarding in revealing specific cell-mycoplasma relationship (Sobeslavsky et al., 1968; Collier et al., 1971; Collier, 1972; Westerberg et al., 1972; Butler et al., 1973; Abu-Zahr and Butler, 1974).

The only significant studies on mixed infections of tracheal explants is reported by Reed (1971, 1972b), Westerberg et al. (1972) and Taylor-Robinson and Cherry (1972). Reed (1971) studied the interaction of mycoplasma and influenza viruses in tracheal organ explants prepared from eight-week-old piglets as well as from bovine embryos and calves up to three weeks old. The porcine tracheal explants were infected with M. hyorhinis and two to four days later they were superinfected with either swine influenza A virus or influenza A WS. The titers of the mycoplasma and the viruses in the dually-infected explants differed little from the controls. However, ciliary activity diminished rapidly in the cultures which were dually-inoculated with the mycoplasma and swine influenza A virus but not in cultures infected with influenza A WS virus or the mycoplasma. Similarly, histological examination revealed intensive damage of the epithelium in the explants which were infected with the mycoplasma and swine influenza A virus only. Inoculation of the above parasites in bovine explants neither produced any apparent enhanced effects on growth of the parasites nor did it produce histological changes in the respiratory
epithelium. Much the same results were also obtained when bovine explants were infected with a T-strain of mycoplasma (SP 1633) and swine influenza A virus.

Reed (1972b) extended her investigations and examined the interaction between viruses and mycoplasmas in bovine tracheal explants prepared from embryos at various stages of gestation. The mycoplasma species used were *M. hyorhinis* S7, two bovine mycoplasmas (Donetta and D12), and two human mycoplasmas, *M. hominis* and *M. salivarium*. The viruses employed were a bovine rhinovirus Sd—1 and a bovine strain of parainfluenza virus type 3 (PI—3), and they were inoculated between 3 and 9 days before the mycoplasmas. In all cases, mycoplasma titers were higher in virus-infected cultures than in controls though in some cases, especially that of *M. hominis*, the differences were small.

In contrast to Reed's results, Westerberg et al. (1972) noticed no differences in the titers of mycoplasma in their single and mixed infection studies in tracheal explants of five-week-old mice. They used *M. pulmonis* simultaneously inoculated with influenza virus (A/PR—8). Although there was no effect on the growth of the mycoplasma, virus replication dropped off significantly as early as five days after infection. Moreover, the ciliary activity in these cultures was inhibited earlier than in the cultures which were infected with either parasite alone. Histological examination showed complete loss of the epithelium in the dually-infected explants.
five days after infection which was a greater degree of damage compared with single infections.

Taylor-Robinson and Cherry (1972) studied mixed infections of chicken embryo tracheal explants with *M. gallinarum* and *M. gallisepticum* after they have noticed that explants infected with *M. gallinarum* survived better than controls, there being only a 50 percent loss of ciliary activity as late as 41 days after infection while uninfected cultures showed similar loss of activity three weeks earlier. In these studies *M. gallinarum* was shown to inhibit the damaging effects to the epithelium produced by most strains of *M. gallisepticum* particularly when the former was inoculated 24 to 48 hours before *M. gallisepticum*. This dual infection delayed the time required for 50 per cent loss of ciliary activity observed with *M. gallisepticum*-infections (4 - 5 days) to nine days.

The importance of organ explants in both virus and mycoplasma studies stems from its numerous advantages over the cell culture system or animals which made it an ideal laboratory model. In this system the cells of the respiratory epithelium remain differentiated and well organised for long periods of time. Recently, Collier and Baseman (1973) demonstrated by use of radio-autography that the luminal epithelium was the most metabolically active site of hamster tracheal explants.
Tracheal explants are easy to prepare and a procedure for large quantity preparation of these explants is available (Cherry and Taylor-Robinson, 1970a). Like cell cultures and microorganisms, tracheal explants could also be preserved at low temperatures (−196°C) until they are needed (Morris et al., 1973). Of great importance is the fact that many explants could be prepared from one animal, thus they could be used as control and test groups (Collier and Baseman, 1973). Besides these advantages, they could be easily observed by light microscopy, and ciliary activity which reflects on the viability of the cells could be accurately assessed by using a calibrated stroboscope (Collier and Baseman, 1973). Indeed many investigators have used the rate of decline of the ciliary activity in virus- or mycoplasma-infected explants as a measure of injury due to the infective agents.

Since tracheal organ explants could be prepared from specific pathogen-free sources, selected mono- or multi-infections could be controlled and studied even quantitatively away from the interfering secondary infections that are very likely to occur in field experiments. Similarly in this much less artificial system (compared to cell culture system) studies on the pathogenesis and the pathophysiology of respiratory parasites could be performed with minimal concentrations (probably absence) of major defence mechanisms of the intact animal such as the antibodies and the macrophages.
Furthermore, tracheal organ explants are easy to handle. Actually they were employed satisfactorily using various techniques such as immunofluorescence (Reed, 1972a; Collier, 1972;), transmission electron microscopy (Abu-Zahr and Butler, 1974; Reed & Boyde, 1972; Collier, 1972; Blaskovic et al., 1972) scanning electron microscopy (Reed and Boyde, 1972; Butler et al. 1973; Abu-Zahr and Butler, 1974) and finally radioautography (Collier and Baseman, 1973).

The importance of tracheal explants for the isolation of "difficult" or "newer" respiratory viruses from human clinical specimens has been established by many investigators (Hoorn and Tyrrell, 1965; Higgins et al., 1969 and many others). Actually, tracheal explants were found superior to the standard tissue culture methods since many virus isolates (particularly rhinovirus and coronaviruses) were detected in the tracheal system although they were not detected in cell cultures (Almeida and Tyrrell, 1967; Higgins, 1966; Higgins et al., 1969; Higgins and Ellis, 1973). Tracheal explants were even more sensitive than monkey kidney tissue cultures or embryonated eggs for the isolation of an influenza A virus, (Hoorn and Tyrrell, 1969). However, use of tracheal organ explants for mycoplasma studies was equally valuable particularly for pathological studies. Also, they have been shown to be equally sensitive as laboratory media for the isolation of mycoplasma from clinical material (Butler and Ellaway, 1971). Finally both virus and mycoplasma infections in tracheal explants usually take
place in a way nearly the same as that in the *in vivo* situation where the parasites have to pass between the actively beating cilia (Collier and Baseman, 1973) an activity considered to be one of the defence mechanisms in an intact animal.

The experimental mixed infection studies on CELO virus and avian mycoplasma are, as mentioned earlier, few in number despite the fact that they have quite frequently been isolated from the same animal. It was decided to use the tracheal explant system as a model for animal disease and for this purpose avian embryo tracheal organ explants were selected because they offered advantages which have been elaborated on above. The interaction of CELO virus with a pathogenic species of mycoplasma, *M. gallisepticum*, and with a non-pathogenic species, *M. gallinarum*, was tested in single or mixed infections and the growth and pathological reactions were studied using the scanning and transmission electron microscope.
A) Mycoplasma studies

The S6 strain of *M. gallisepticum* used in this work was obtained from Miss L. Timms (Central Veterinary Laboratories, Weybridge) in the form of a fresh liquid culture. The passage history of this specimen was uncertain but it was isolated by Dr. H.E. Adler (Department of Preventive Medicine, University of California, Davis, U.S.A.), passed on to Dr. D.A. McMartin (Veterinary Laboratory, Lasswade, Scotland) and then to Dr. D.H. Roberts (Central Veterinary Laboratories, Weybridge). It is believed to have experienced less than twenty passages. In this laboratory, the specimen was inoculated into 10 ml. liquid growth medium and incubated at 37° for two days. Five millilitres of this culture were used to inoculate 45 ml. of fresh liquid growth medium which was incubated as above and then dispensed in ampoules (0.7 ml. into each), sealed, and then stored in liquid nitrogen. This was the stock culture of *M. gallisepticum* which was used throughout this work.

A lyophilized specimen of *M. gallinarum* (10120) was obtained from the National Collection of Type Culture, London. The contents of the ampoule were reconstituted in 1 ml. liquid growth medium and incubated at 37° for one day. After this, 0.1 ml. of the growing culture was
inoculated on solid growth medium and 1 ml. liquid growth medium was added to the rest and incubated as above. The volume of the culture was doubled every day (for four days) until a stock culture of 32 ml. was obtained, and this was then dispensed in ampoules (0.7 ml. into each), sealed and stored in liquid nitrogen.

**Preparation of media**

Liquid and solid medium base. Liquid medium base was prepared in one litre volumes in distilled water as 2.1% (w/v) Bacto PPLO Broth (dehydrated, without crystal violet, Difco Labs. Detroit, Michigan, U.S.A.). This was dispensed in 70 ml. volumes in 4 oz. bottles and was autoclaved at 15 lbs/sq. in. for 15 minutes. After cooling, the broth was stored at 4°. Agar medium base was prepared by adding 0.75 gm. of Ionagar No. 2 (Oxoid Ltd., London, England) to each of 70 ml. PPLO broth before autoclaving as above. The cooled agar base was stored at 4° until it was required.

Yeast extract. This was prepared from dried active yeast (The Distillers Company (Yeast) Ltd., Morden, Surrey, England). A thick paste was made by taking up 25 gm. dried yeast in 25 ml. distilled water. This was gradually diluted with 100 ml. distilled water while constantly stirring. The mixture was then transferred into a large bottle and brought to a boil as quickly as possible in a boiling water bath, and boiled for 5 minutes.
The resultant solution was dispensed into 1 oz. universal bottles and was centrifuged at 4,000 X g for 30 minutes. After this, the supernatant solutions were pooled together and autoclaved at 10 lbs/sq. in. for 10 minutes. The sterile yeast extract was again centrifuged, this time at 8,000 X g for 30 minutes and the supernatant was stored in 20 ml. volumes at 4°.

Thallium acetate. A stock solution of 10% (w/v) thallium acetate was prepared in distilled water, autoclaved as above and stored at 4°.

Penicillin. A stock solution of penicillin (100,000 units/ml.) was prepared by dissolving the contents of one bottle (Benzylpenicillin (Sodium) B.P., Glaxo Labs. Ltd., Greenford, England) in 10 ml. sterile distilled deionized water and stored at 4°. This was used within three weeks of preparation.

(a) Liquid growth medium.

Liquid growth medium was prepared by supplementing the contents of one bottle of broth base with the following:

- Unheated horse serum (No. 3)* 20 ml.
- Yeast extract 10 ml.
- 10% Thallium acetate 0.25 ml.
- Penicillin 0.20 ml.

* Wellcome Reagents Ltd., Beckenham, England
(b) Solid growth medium

The solid agar growth medium was freshly prepared prior to use by melting the contents of one bottle of agar base, cooling it to about 45° and adding a mixture of serum, yeast extract and antibiotics, prepared as above. Five millilitre volumes were poured into 50-mm. plastic Petri dishes (ESCO (RUBBER) Ltd., London, England), allowed to solidify, and the surface was dried for 15 mins, in a stream of sterile warm air. The Petri dishes were stored at 4° and used within 10 days after preparation.

Preparation of Viable cultures

For the inoculation of growth media (liquid or solid), or tracheal explants, fresh 24 hr. cultures of mycoplasma were used, prepared by rapidly thawing the contents of an ampoule of stock culture and incubating it with 4 ml. growth medium at 37° for one day. Such growth was used either undiluted or diluted by ten-fold steps in liquid growth medium, or in tracheal explant medium (see part on tracheal explants below).

Assay of viability

Serial ten-fold dilutions were prepared in liquid growth medium and drops (3X 0.025 ml.) of appropriate dilutions were put onto the surface of solid growth medium. The inoculated plates were dried in a flow of warm sterile air and the plates were subsequently incubated at 37° for 3 days (M. gallinarum) or 6 days (M. gallisepticum). The colonies were counted using an inverted light microscope at X 10 magnification.
Dynamics of growth

(a) Liquid growth medium.
Fresh cultures were prepared as described above and 0.1 ml. of dilutions in growth medium were inoculated into 5 ml. volumes of growth medium and incubated at 37°C. Samples (0.1 ml.) were taken at intervals and titrated as above.

(b) Tracheal explant medium with or without supplement.
Fresh cultures were prepared as above and decimal dilutions were prepared in explant medium and 0.5 ml. of the dilutions was inoculated into 5.0 ml. volumes of tracheal explant medium, which was incubated at 37°C. Samples (0.1 ml. each) were then removed at intervals and titrated as above. (In this test traces of PPLO medium were carried over with the explant medium. In tests on *M. gallisepticum* this amounted to 1% and with *M. gallinarum* to 0.01%).

Freshly prepared explants were homogenized (Ultra-Turrax, Scientific Instrument Ltd., London, England) aseptically in tracheal explant medium. The resulting suspension contained tissue extracts, cell membranes and other cell debris including fragments of cartilage. It was used either in this form to supplement tracheal explant medium or after centrifugation at 2,000 X g for 5 min. when the supernatant was used either immediately or stored at -20°C. Tracheal explant medium was supplemented with centrifuged or uncentrifuged
homogenate at a final concentration equivalent to the extracts from 2.5, 1.7, and one explant/ml. respectively. The medium was inoculated with *M. gallisepticum* or *M. gallinarum* as in (b) and then incubated at 37°. Samples (0.1 ml.) were removed daily and the surviving mycoplasmas were titrated on solid medium as described earlier.

**Culture of mycoplasma on glass coverslips**

This was necessary for morphological studies by scanning electron microscopy. Small coverslips (1.0 x 0.5 cm) were sterilized by flaming after dipping them in ethanol. They were then placed in plastic 50-mm Petri dishes, overlayed with 5 ml. liquid growth medium which was inoculated with 0.1 ml. of actively growing cultures of *M. gallisepticum* (approx. $10^7$ CFU/ml.), or *M. gallinarum* (approx. $10^9$ CFU/ml.). The inoculated broths were incubated at 37° under conditions described earlier, and samples on the coverslips were taken at different times after incubation. The cultures were removed with Pasteur pipettes and the coverslips were washed twice with PBS, overlayed with 5% phosphate-buffered glutaraldehyde and left overnight at 4°. After short rinses with PBS first, and then with distilled water, dehydration was carried out in graded alcohols, and the specimens were treated in a special way for scanning electron microscopy (see Techniques for scanning electron microscopy). In exactly similar procedures, control (uninoculated) preparations were also prepared and examined.
B) Virus studies

A stock culture of the Ote strain of CELO avian adenovirus was obtained from Dr. S.M. Aghakhan (Central Veterinary Laboratories, Weybridge), containing approximately $10^9$ plaque forming units per millilitre (PFU/ml). The virus was obtained originally from Dr. H. Kawamura (National Institute of Animal Health, Tokyo, Japan) in lyophilized form and was subcultured at Weybridge five successive times in chicken kidney cell cultures. In this laboratory, the stock virus was dispensed in 1.5 ml. volumes and stored at -20°C.

Preparation of cell cultures

Cell cultures were prepared by a modification of the method of Churchill (1965). Chicken kidneys were obtained from four-week-old specific pathogen-free (SPF) chickens by the following procedure: Each chicken was decapitated, drained of its blood, nailed to a dissecting board and opened through the abdominal cavity. The viscera, mesenteries and the surrounding abdominal membranes were all moved outside the carcass and the kidneys were exposed by removing the connective tissue membranes that surrounded them. The kidneys were then removed and immediately placed in 300 ml. PBS at 39°C. As soon as possible thereafter the kidneys were transferred into a sterile Petri dish where the capsule and the large urinary tubules were carefully removed. A few drops of PBS were then added and the kidneys were chopped into small pieces (3 - 5 mm³) which were then put into a sterile 150 ml.
Erlenmeyer flask for trypsinization. A sterile silicone-covered magnet was inserted into the flask to which 75 ml. warm PBS (39°) was added and the contents were stirred on a magnetic stirrer for 3 minutes. The resulting supernatant fluid which mainly contained erythrocytes was discarded and the washing procedure (with warm PBS) was repeated 3 – 5 times until the supernatant was clear of erythrocytes. After this, the main process of trypsinization was initiated using 25 ml. volumes of warm 0.1% trypsin* solution in PBS. The contents were stirred for 3 min., allowed to settle for a few seconds and the supernatant was harvested. A further thirteen cycles of trypsinization were carried out. The first two harvests were discarded because they contained some erythrocytes and the remaining twelve cell harvests were collected separately. Each harvest was put into a universal bottle containing 1.5 ml. foetal calf serum and thoroughly mixed to inactivate the trypsin. The cell suspension was then centrifuged at 185 x g. for 10 min. the supernatant was discarded and the cells were resuspended in 2.5 ml. growth medium. The harvested cells were then pooled together and filtered through a layer of sterile muslin into a 100 ml. Erlenmeyer flask. The filtered cell suspension was dispensed into two graduated conical centrifuge tubes and centrifuged at 185 x g for 10 minutes. The final volume of the centrifuged cells was estimated and each 0.25 ml. packed cells was resuspended in 100 ml. growth medium which was previously incubated at 39°.

This corresponded to a seeding rate of \(5 \times 10^6\) viable cells per millilitre. The cell suspension was then dispensed into 35-mm. multi-Petri dish plates (Flow Laboratories, Scotland), 2.5 ml. into each dish using a sterile Cornwall Syringe. The bottle containing the cell suspension was frequently shaken during the dispensing procedure to avoid the cells settling to the bottom. The cultures were incubated at 37° for 3 or 4 days during which they were examined microscopically for confluent growth.

**Media.**

Hank's balanced salt solution containing Yeastolate* and Lactalbumen hydrolysate** was prepared in 20 litre volumes as a ten times concentrated stock by the following procedure:

**Solution A**

- **NaCl** 800 gm.
- **K Cl** 40 gm.
- **Mg \(\text{SO}_4\cdot7\text{H}_2\text{O}\)** 20 gm.
- **CaCl\(_2\) (anhydrous)** 14 gm.
- **Phenol red (0.2%, w/v) solution** 500 ml.
- **Deionized water** 4.5 litres

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* Difco Labs., Detroit, Michigan, U.S.A.

** V.A. Home Ltd., London, England
Solution B

\[
\begin{align*}
\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O} & \quad 6 \text{ gm.} \\
\text{KH}_2\text{PO}_4 & \quad 6 \text{ gm.} \\
\text{Dextrose} & \quad 100 \text{ gm.} \\
\text{Yeastolate} & \quad 10 \text{ gm.} \\
\text{Deionized water} & \quad 5 \text{ litres}
\end{align*}
\]

Solution C

\[
\begin{align*}
\text{Lactalbumen hydrolysate} & \quad 500 \text{ gm.} \\
\text{Deionized water} & \quad 10 \text{ litres}
\end{align*}
\]

The above stock solutions were autoclaved at 10 lbs/sq. in. for 20 min., after which they were dispensed in small volumes (Solution A and B in 50 ml. each and Solution C in 100 ml.) and stored at 4°.

(a) Growth medium

For cell growth the stock solutions were combined as follows:

- Hank's Solution A 50 ml.
- Hank's Solution B 50 ml.
- Hank's Solution C 100 ml.
- Sterile deionized water 800 ml.
- \(\text{NaHCO}_3 (8.8\% \text{ w/v})\) 4 ml.
- Antibiotics solution (see below) 5 ml.

To every 100 ml. of the above solution was added 10 ml. foetal calf serum and 5 ml. tryptose phosphate broth plus 1.5 ml. 1M HEPES*, 1.5 ml. N/4 NaOH and 2 ml. inactivated calf serum**.

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* 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethane sulphonic acid (HEPES). BDH Chemicals Ltd., Poole, Dorset.
** Wellcome Reagents Ltd., England
(b) Maintenance medium and virus diluent

This was prepared exactly as above but without foetal calf serum and tryptose phosphate broth.

(c) Overlay medium for plaque assay

This was prepared by combining the following:

- Hank's A 50 ml.
- Hank's B 50 ml.
- Hank's C 100 ml.
- Sterile deionized water 600 ml.
- NaHCO₃ (8.8% w/v) 4 ml.
- Antibiotics solution 5 ml.

To every 360 ml. of the above solution the following ingredients were added:

- Tryptose phosphate broth* 15 ml.
- Inactivated calf serum 25 ml.
- 1M HEPES 7.5 ml.
- N/4 NaOH 8.5 ml.

This mixture was incubated at 44° in a water bath before it was supplemented with 4.2% (w/v) Ionagar No. 2 (Oxoid Ltd., England) which was previously autoclaved at 15 lbs/sq. in. for 15 mins. and allowed to cool to about 50°. The supplemented overlay medium was incubated in the water bath at 44° for about 10 min. before it was used.

A mixture of antibiotics was used. Each one was dissolved in appropriate volumes of sterile deionized

* Wellcome Reagents Ltd., England
water to make up the following concentrations before they were pooled:

- Penicillin 100 units/ml
- Streptomycin 100 µg/ml
- Mycostatin 25 units/ml

Where required, HEPES buffer was used and a solution was made in deionized water and sterilized by filtration through 220 µm Millipore filter.

Where phosphate-buffered solution (PBS) was used it was prepared by dissolving one tablet of PBS Dulbecco 'A' (from Oxoid Ltd.) in 100 ml. distilled water and then autoclaved. Its final pH was adjusted to 7.2 with N/4 HCl.

Assay of virus infectivity

Before titration, all samples containing the virus were treated by freezing and thawing (from -20°C to 20°C) for three consecutive times to set free the cell-associated virions. Furthermore, the samples that contained both the virus and the mycoplasma were heated in a water bath for 30 min. at 56°C, to inactivate the mycoplasma. The specimens were then diluted serially by ten-fold steps in the virus diluent. Just before inoculation, the growth medium over the cell monolayers was removed (using a sterile Pasteur pipette connected to a suction pump) and 0.1 ml. of appropriate virus dilution was inoculated. Each dilution was tested in
duplicate and great care was taken to avoid allowing the cells to dry out during the inoculation procedure. The cultures were incubated for virus adsorption at 37° for two hours (Sharpless et al., 1961), before being overlayed with 2.5 ml. of the overlay-medium. The inoculated plates were incubated at 37° and examined after one week for the development of the plaques. After the plaques had developed, the overlay agar was removed and the monolayers were stained with 1% (w/v) crystal violet (in 75% ethylalcohol), for 5 min. and washed with running tap water. Later, the titer of the virus was calculated by the weighted mean method (Reid, 1968).

C) Tracheal explant studies

Explants were prepared from either embryonic or chick tracheas by the method of Harnett and Hooper (1968) modified by Butler (1969). Slight differences occurred in the preparation of the explants from embryonic and chick tracheas and each technique will be described separately.

(a) Embryonic tracheal explants (see Appendix 1)

Fertile eggs from SPF brown Leghorn fowl, Gallus domesticus, were obtained from the Ministry of Agriculture, Fisheries and Food Veterinary Laboratory (Lasswade, Scotland) and were incubated at the Central Veterinary Laboratories (Weybridge). Twenty days after incubation, the eggs were opened and the trachea of each embryo was exposed by cutting the skin covering the neck in a
longitudinal direction from the larynx to the thorax. The connective tissue and the muscles supporting the trachea were disconnected and the trachea was removed by cutting it just below the larynx and slightly above the bronchial junction. Each trachea was then freed from its extra connective tissue and was cut into rings about 1 mm. thick with a scalpel. Each ring was then put into a test tube (16 x 125 mm.), fed with 1 ml. medium (see below) and closed with loose metal cap. The cultures were incubated at 37° in a humidified incubator in an atmosphere of 5% carbon dioxide in air.

(b) Explants from one-week-old chicks

Eggs from brown Leghorn SPF chicken (source as above) were hatched and held under SPF conditions. After one week, the chicks were killed by intraperitoneal injections of 0.2 ml. Expiral (ICI Ltd., Cheshire, England), and the neck and chest of each bird were sterilized by swabbing with 70% (v/v) ethyl alcohol before the trachea was excised. Ring cultures were then prepared as above.

After 24 hr. incubation, the cultures (embryonic or chick) were shaken to clear away the mucus, cell debris and erythrocytes which always accumulated on the surface of the epithelium during this time. The ciliary activity of each explant was then observed and assessed using a Gillett and Sibert Conference microscope provided with quartz-iodine illumination. Only those
explants which showed active and uniform ciliary beat all around the lumen of the explant were used for experiments. The medium in the selected cultures was removed and the explants were either inoculated or fresh medium was added and incubated as before.

Inocula of the virus were prepared by diluting portions of the stock CELO virus, serially ten-fold steps in tracheal explant medium. Just before inoculation, the medium over each explant was removed and 0.2 ml. of the appropriate virus dilution was applied and the culture incubated for adsorption for 45 min. at 37°. Subsequently, surplus inoculum fluid was removed and each explant was washed twice with tracheal explant medium to remove the unadsorbed virions. Explants were incubated as above and samples, 0.1 ml. each, were removed daily and fed again with fresh medium as described for the mycoplasma-inoculated explants. The ciliary activity was assessed as before.

In all mixed infection studies (except one), the virus was inoculated first and then the mycoplasma, following the procedures described above for the single infections. The various combinations used were summarized in TABLE 3. Sampling and examination of the ciliary activity were exactly similar to those made above.
TABLE 3

SCHEDULE OF SINGLE OR MIXED INFECTIONS OF TRACHEAL EXPLANTS

<table>
<thead>
<tr>
<th>Infection Type</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>M. gallisepticum alone</td>
<td></td>
</tr>
<tr>
<td>M. gallinarum alone</td>
<td></td>
</tr>
<tr>
<td>CELO virus alone</td>
<td></td>
</tr>
<tr>
<td>M. gallisepticum + CELO virus</td>
<td>mycoplasma added two days after the virus</td>
</tr>
<tr>
<td>M. gallisepticum + CELO virus</td>
<td>virus added three days after the mycoplasma</td>
</tr>
<tr>
<td>M. gallinarum + CELO virus</td>
<td></td>
</tr>
</tbody>
</table>

Explant medium

The medium was Eagles' Minimal Essential Medium (M.E.M., Wellcome Reagents Ltd.), buffered with sodium bicarbonate. It was free from both antibiotics and serum, and it was prepared by mixing the following ingredients under aseptic conditions.

Eagles' (M.E.M.) 10.0 ml.
Sodium bicarbonate* (4.4%) 2.5 ml.
(final conc. 0.011%)
Distilled, deionized, sterile water 87.5 ml.

This medium was prepared just before use and its final pH was approximately 7.2.

Inoculation of Explants

Inocula of mycoplasma used for infection of tracheal explants were prepared fresh by the procedure described

* Wellcome Reagents Ltd., England
earlier. Explants were first fed with 0.9 ml. fresh tracheal explant medium and then inoculated with 0.1 ml. of appropriate dilutions of M. gallisepticum or M. gallinarum before incubation. This procedure was particularly important since direct inoculation of the mycoplasma onto the drained explant resulted in early detachment of its epithelium from the basement membrane (see histology of the epithelium). Samples, 0.1 ml. each, were then removed daily for 9 days, after shaking the tubes, and 0.1 ml. fresh tracheal explant medium was added to each explant to make up for the loss in volume, before re-incubation. Also, the ciliary activity of the explants was assessed daily before or after sampling.

In all the above experiments, each was repeated at least three times. Experiments employing tracheal explants were repeated using different batches of embryonated eggs and at any one time a set of twelve explants was inoculated with the mycoplasma, the virus, or a mixture of both. Samples taken from each tube in a set were pooled together before titration.

**Special inoculation procedure for scanning electron microscopy**

Tracheal explants for this study were prepared from both embryonic and one-week-old chicks by the procedure described earlier, and they were 2-3 mm. in thickness. Freshly prepared explants as well as one-week-incubated
explants were used throughout these preparations. The fresh explants were inoculated one day after incubation and the other explants had 2 - 3 changes of medium before they were inoculated. Just before inoculation, the explants were shaken several times to clear away the adhering mucus and cell debris and the medium was then removed. Inocula (0.1 ml.) of actively growing cultures of M. gallisepticum or M. gallinarum were applied onto each explant and the tubes were shaken gently to ensure entry of the organisms into the lumen of the explants. After this, 2 ml. of explant medium was added and the explants were incubated as indicated above. Periodic samples were taken and after washing with PBS, explants were fixed with glutaraldehyde and post-fixed with OsO₄. They were then dehydrated in graded alcohols or by freeze-drying procedures, mounted on stubs and treated for examination. (see techniques for scanning electron microscope).

D) Electron microscopy studies

Materials and Methods*

1. Buffers

PBS

This buffer was prepared from the following two stock solutions:

* The procedures used in this part were based on recommendations provided by J. Short (Electron microscope suit, Wellcome Research Labs., Beckenham), and Dr. A. Field and F. Rodgers (Virus Reference Lab., Central Public Health Laboratory, Colindale, London). Complementary methods were based on Pease (1964) and Hayat (1970)
Solution (A)

Dipotassium (sodium) hydrogen phosphate 7.10 gm.
\((K_2HPO_4)\)

Distilled water 500.00 ml.

Solution (B)

Potassium (sodium) dihydrogen phosphate 6.80 gm.
\((KH_2PO_4)\)

Distilled water 500.00 ml.

Both stock solutions were stored at 4° and the buffer was prepared by mixing 7 parts of stock solution (A) with 3 parts of stock solution (B). The pH of the final mixture was about 7.2.

Veronal—acetate

A stock solution of this buffer was prepared by dissolving the following:

Sodium veronal (known also as sodium barbital) 2.89 gm.

Sodium acetate, hydrated 1.90 gm.

Distilled water 100.00 ml.

This buffer was prepared for immediate use and its pH was 7.2.

2. Fixatives

Glutaraldehyde

This fixative was used at 5% (w/v), and was made up in PBS as follows:

Glutaraldehyde EM*, (25% aqueous solution) 2.0 ml.

Phosphate solution (A) 5.6 ml.

Phosphate Solution (B) 2.4 ml.

The fixative was prepared for immediate use and it had a final pH of about 7.2.

* TAAB Labs., England
Osmium tetroxide (OsO₄)

A stock solution of 2% (w/v) OsO₄ (BDH Chemicals Ltd., England) was prepared in veronal-acetate buffer. This was made by dissolving 0.25 gm. OsO₄ in 12.5 ml. of the buffer by the following procedure, carried out in a fume cupboard. The label was removed from an ampoule that contained the OsO₄ crystals, the exterior of the ampoule was washed in cold water and thoroughly dried. It was then placed in a glass stoppered bottle containing the buffer and was broken by shaking the bottle vigorously. When dissolved, the fixative was diluted to 1% by adding 5 ml. veronal-acetate buffer, 5 ml. 0.1N HCl and 2.5 ml. distilled water, to the 12.5 ml. 2% (w/v) OsO₄ just prepared. The final 1% fixative was stored at 4°C in the dark.

3. Stains

Negative stain: sodium silicotungstate (SST).

This stain was prepared at 2% (w/v) by dissolving 2 gm. sodium silicotungstate (TAAB Lbs., England) in 100 ml. distilled water. The pH of the stain was adjusted to 6.3 with 1N NaOH, and it was stored at 4°C.

Lead Citrate

This stain was prepared by dissolving 0.67 gm. lead nitrate and 0.88 gm. sodium citrate in 100 ml. distilled water*. The resultant precipitate was dissolved by

* Distilled water used for preparation of the lead citrate and the uranyl acetate stains was previously boiled and then cooled.
adding 4 ml. of 1N NaOH (Carbonate-free, TAAB Labs.), and then the solution was made up to 25 ml. by adding distilled water. The stain was stored at room temperature.

Uranyl acetate.

A 5% (w/v) of this stain was prepared by dissolving 1 gm. uranyl acetate (BDH Chemicals Ltd., England), in 20 ml. distilled water. The uranyl acetate was dissolved by shaking thoroughly and undissolved material was removed by centrifugation at 8,000 x g. The stain was stored at room temperature.

4. Coating of grids

Copper grids (3.05 mm., 100 mesh) were coated with a thin film of formvar by the following procedure. A clean, dry glass slide was immersed in a solution of 0.4% (w/v) polyvinyl formal (formvar) in chloroform for a few seconds and then lifted up to dry in the air forming two thin transparent membranes on either side of the glass slide. A rectangular scratch was made on one side of the slide with the tip of a scalpel blade and the membrane was floated on the surface of a trough of water by inserting the slide at an angle in the water. Then the grids were placed gently onto the floating membrane and then lifted off on a strip of filter paper which was placed on top of them. The filter paper carrying the grids with the formvar membrane was removed to a Petri dish and left to dry overnight, with the grids on the upper surface. Later, the support film was
stabilized by coating it in vacuo with a thin layer of evaporated carbon while the grids were still on the filter paper.

5. Preparation of Resin

The following embedding formulation was used throughout this work, and was a modification of the formulation reported by Luft (1961).

- Epon C.Y. 812 (Epikote 812)* 20 ml.
- DDSA** (Dodecenyl succinic anhydride 44 ml.
- Araldite Epoxy Resin CY. 212** 12 ml.
- Dibutyl phthalate*** 3 ml.

The above chemicals were measured in a 100 ml. glass measuring cylinder and then transferred into a clean, dry glass bottle inside which they were mixed thoroughly by vigorous shaking. After this, 0.8 ml. of DMP-30* (2,4,6-tri (dimethylaminomethyl) phenol) an accelerator, was added to the above mixture and they were shaken vigorously until homogenous mixing was obvious. The air bubbles trapped in this resin mixture were released by placing the bottle of resin inside a glass dessicator under constant reduced pressure created by a vacuum pump. At this stage the resin was ready for use for both infiltration of tissues and their embedding (see details below). Fresh resin was always prepared just before use.

** Hardner, 964 Practical grade, TAAB Labs., England
*** Plasticizer, BDH Chemicals Ltd., England
6. Technique for scanning electron microscope.

Specimens of trachea, 2-3 mm. thick were used for the scanning electron microscope studies. These were placed in bigou bottles each containing 4 ml. PBS, and were washed thoroughly by vigorous shaking. Several changes of PBS were required until the lumen of each explant appeared smooth and clean. After this, the specimens were fixed with freshly prepared 5% glutaraldehyde overnight at 4°C. Unless otherwise mentioned in the results the preparations were post-fixed with 1% OsO₄ for 30 mins. at room temperature, after an intermediate wash with PBS, transferred into a Petri dish, and the tracheal ring was cut longitudinally into 2 or 3 pieces. The specimens were then rapidly rinsed with distilled water and dehydrated by either of the following techniques:

1) Dehydration in graded ethyl alcohol.

The specimens were transferred into bigou bottles containing 30% (w/v) ethyl alcohol in distilled water and left for five minutes. This procedure was repeated using 50%, 70%, 90% and 100% ethyl alcohol; the latter was used for two successive periods of 10 min. each. Then the specimens were allowed few seconds to dry in the air before they were stuck down on aluminium stubs (specimen holders for the SEM) using sticky tape (Cellotape adhesive on both sides) having the lumenal surface exposed.
ii) Dehydration by freeze-drying

Briefly, the external surface of each piece of specimen was blotted dry on a piece of paper towel and was mounted on a stub as described above. Then the stubs were inserted in a metal stub-holder made of a large mesh gauze with a long handle, and dipped immediately in a tank containing liquid nitrogen. Later, the stubs holding the specimens were freeze-dried overnight, (Refrigerated Chamber Type, Chem. Lab. Instruments Ltd., Ilford, Essex, England).

After dehydration by the above two methods, the specimens were made electrically conductive by coating them in vacuo with a thin (approx. 200 Å) cast of evaporated (60/40) gold/palladium (Johnson Matthey Metals Ltd., London). The specimens were then examined in a Cambridge Stereoscan Electron Microscope, Mark II, operated at 20 KV. The results were recorded on Ilford FP4*, 35 mm. films (ASA 125, DIN 22). Negatives were developed in Ilford D-11 Developer in tap water for 8 min., rinsed with tap water for few seconds and then fixed in 1:3 (v/v) Amfix (May and Baker Ltd., Dagenham, England) for five minutes. Finally, they were washed in running tap water for 30 min. and before drying a few drops of Teepol were added so that the water drained off completely and drops did not remain to dry leaving salt deposits.

7. Technique for transmission electron microscope

Specimens of trachea of variable sizes and tracheal explants for the transmission electron microscope studies were fixed, post-fixed and dehydrated in the graded alcohols in a way similar to that described for the scanning electron microscope preparations. After the second change with 100% alcohol, the specimens were soaked in propylene oxide (1,2-Epoxy propane) for two successive periods of 10 min. each, and they were then ready for infiltration with resin using the following procedure:

- 2:1 (v/v) Propylene oxide/Pure resin 60 min.
- 1:2 (v/v) Propylene oxide/Pure resin 90 min.
- Pure resin 120 min.

At this stage, the specimens were carefully transferred into plastic capsules (TAAB Labs,) which had been previously filled with pure resin. This was done with throat swab sticks that were shaped at one end to provide a thin flat spatula. Each specimen was carefully oriented along the vertical axis of the embedding capsule in such a way as to make it possible to cut transverse sections. Each specimen was identified by a label written in pencil and inserted around the inside of the upper rim of the capsule. The resin was hardened by incubating the capsules in an oven at 60° for two days. The block of hardened resin was removed from its capsule and either stored at room temperature or directly manipulated for the preparation of sections.
Before sectioning, each block was trimmed in an appropriate way so as to expose the desired region of the specimen. Thin sections (approx. 1000 Å-thick) of the exposed specimen were cut on an LKB Ulrotome I using glass knives prepared on an LKB Knife-Maker immediately before sectioning. Sections were collected in a trough of water made by attaching a piece of masking tape sealed with wax along three sides of the sharp edge of the knife. The floating sections were smoothed out by exposing them to chloroform vapour applied by a brush soaked in chloroform which was passed over the sections without touching them. Sections were then picked up on previously prepared grids. The grids carrying the sections were dried on filter paper discs placed inside Petri dishes and the sections were stained with lead citrate (Reynolds, 1963) and uranyl acetate (Watson, 1958). This was done by applying drops of freshly prepared stain on a slide that had been previously coated with paraffin wax, and grids which bore sections were floated face down on the drops. The sections were stained first with lead citrate for 15 min., then they were washed thoroughly in distilled water. Excess water was drained off by touching the edge of the grid to a dry filter paper. Sections were then stained with uranyl acetate for 15 min., washed and dried as before and placed on a filter paper inside a Petri dish. Specimens were examined in a JEOL:JEM 100 B Transmission Electron Microscope operated at 60 or 80 KV. Observations were recorded on 3½ x 2½ in. Ilford plates type E.M-5.
Plates were developed in sets of 20 in 1:4 (v/v) Teclnol Developer (May and Baker Ltd., England) for 3 min., the action of which was stopped by a rapid rinse in distilled water containing 2% acetic acid. The plates were then fixed for 2.5 min. in 1:3 (v/v) Amfix, and washed for 30 min. in running tap water and treated in a similar manner to that described earlier.

Specimens of trachea and tracheal explants from the single infection experiments as well as from the mixed infection studies were routinely processed and examined using the techniques described above.

8. Printing of the negatives

All negatives, whether plates or film, were printed on Ilford photographic paper, Ilfoprint paper, Glossy, Single Weight (Ilford Ltd., Essex, England), processed on an automatic Ilfoprint Processor 1501, which was supplied with Ilfoprint Activator and Ilfoprint Stabilizer 1S-12. Later, the prints were fixed for 2 min. in 1:8 (v/v) Kodafix solution in tap water and washed for 30 min, before they were dried on an Ilford Professional Glazer.

9. Negative staining* 
(a) Virus

Three millilitres of the stock CELO virus suspension

* This technique was recommended by Dr. J.D. Almeida, Wellcome Research Labs., Beckenham, Kent, England.
were centrifuged at 25,000 X \( \text{g} \) for one hour. The supernatant was then discarded and the centrifuge tube was left in an inverted position for nearly one hour. This was important since it allowed the traces of the supernatant to drain off completely, hence minimizing the masking effect produced by crystals of salts that tended to form in these preparations.

The pellet of virus was then resuspended in 3 drops of distilled water using the tip of a Pasteur pipette. Equal volumes of the virus suspension and the negative stain SST were mixed thoroughly on a clean glass slide and drops of the mixture were mounted on formvar/carbon-coated grids using Pasteur pipettes. Excess fluid was removed from the grids by touching them with filter paper and they were allowed to dry for a few minutes on a disc of filter paper inside a Petri dish. The specimens were examined in the transmission electron microscope operated at 80 KV.

(b) Mycoplasma

Staining mycoplasmas by the negative technique was done in a similar manner to that described above. Generally, 3 ml. volumes of \textit{M. gallisepticum} or \textit{M. gallinarum} were centrifuged at 4,000 X \( \text{g} \) for 30 min. and the supernatants were drained off completely before the pellets were resuspended in few drops of distilled water, stained, applied on grids and examined as indicated above.
Group two was infected with CELO virus alone (40 ml. of virus suspension and 10 ml. maintenance medium).
E) Studies in adult birds

This part of the study was done in collaboration with Dr. S.M. Aghakhan (Central Veterinary Research Laboratories, Weybridge). The birds were infected with the S6 strain of M. gallisepticum, Ote strain of CELO virus (both of which were used earlier in this work), and a mixture of both by an aerosol method, Aghakhan (1974).

Birds

A total of 120 brown Leghorn SPF chickens of both sexes, and 7-week-old were used in this experiment. Before and during the experiment, the birds were reared on unmedicated food.

Method of infection

The birds were infected by aerosols administered inside polyethylene tent isolators (Vickers Medical, Basingstoke, Hampshire, England). The birds, after being divided into four groups, 30 each, were treated separately in different isolators. Group one was infected with M. gallisepticum alone (inoculum: 40 ml. mycoplasma culture, containing no less than $10^6$ CFU per ml., and 10 ml. liquid growth medium). Group three was infected with a mixture of M. gallisepticum and CELO virus (inoculum: 40 ml. mycoplasma culture and 10 ml. virus suspension). The fourth group was the control group and it was divided further into three subgroups, 10 each. The first was treated with 50 ml. mycoplasma-

* See opposite page
The first inoculum was mycoplasma-free liquid growth medium; the second, with 50 ml. cell culture growth medium; and the third, with a mixture of 10 ml. cell culture growth medium and 40 ml. mycoplasma-free liquid growth medium. Each of the above inocula was sprayed over the heads of the birds, inside the tent isolators, using a spray-gun (Humbrol Ltd., Hull, England) which produced a thick fog of aerosol. The birds were left exposed to the aerosol for one hour before they were moved to their isolation units.

**Sampling.**

Two birds from each of the infected groups and one from each of the control subgroups were killed each time, 4, 7, 11, 14, 18, 21, 28 and 35 days post-aerosolization, while the rest were used by Dr. Aghakhan for serological and histological purposes. The birds were killed by bleeding out aseptically by heart puncture and intravenous injection with Expiral. Soon after this, the tracheas were dissected out and a piece, approximately 1 cm. long was excised from each trachea, taken usually from the middle portion, and placed in PBS. Each piece of trachea was subsequently divided into two, one was used for the scanning electron microscope and the other for the transmission electron microscope studies.

**Electron microscopy**

The specimens of trachea were treated in similar ways to those described earlier (see sample preparations for scanning and transmission microscopy), before they were examined.
RESULTS

I - Growth of M. gallisepticum, M. gallinarum and CELO virus in tracheal explants.

1. Growth of mycoplasma

The two mycoplasma species grew readily in the explants and the growth curves of M. gallisepticum and M. gallinarum are illustrated in FIGURES 1 & 2. Viable organisms were detectable as early as the second day after inoculation, then rapid multiplication occurred for two to three days reaching a maximum titer of between 1 and 10 million colony forming units per millilitre which was maintained for two or three days after which viability declined. It was also noted that the essential nature of the growth curve was unaffected by the number of organisms originally inoculated and the rate of growth during the exponential or log phase was the same, as were the maxima achieved and the extent of decline.

However, these results represented only the cell free viable mycoplasma and did not include the cell-associated organisms.

In contrast to multiplication in explants the two species of mycoplasma grew much more rapidly and to a higher titer in standard cell-free media (FIGURES 1 & 2) but it was observed that the maximum values reached with M. gallinarum were 100-fold greater than those with
FIGURE 1. Illustration of the differences in the growth curves of *M. gallisepticum* when equal numbers of organisms were inoculated into standard PPLO-liquid growth medium and onto explants maintained in tracheal explant medium. (I and 2 respectively).
Growth of *M. gallisepticum* in PPLO-liquid growth medium and in explants.

Colony Forming Units/ml

Days
FIGURE 2. Differences in the growth curves of \textit{M. gallinarum} inoculated into standard PPLO-liquid growth medium and onto explants maintained in tracheal explant medium. (1 and 2 respectively).
Growth of *M. gallinarum* in PPLO liquid growth medium and in explants.
M. gallisepticum. However, these values were respectively 1,000-fold and ten-fold greater than those found in the fluid of tracheal explants.

Explant fluid alone was unable to support multiplication of the organisms (FIGURE 3). Indeed, inocula with a high viability steadily lost activity and the titer of M. gallisepticum decreased more rapidly than that of M. gallinarum. However, supplementation of explant medium with explant extract provided sufficient enrichment to encourage growth, provided the extract of about two or more explants was added (FIGURE 3). The nature of growth in this medium was different to that in explant cultures, principally in that although quite high colony counts were obtained, viability declined very rapidly afterwards and this situation was more pronounced with M. gallinarum than with M. gallisepticum.

2. Replication of the virus

CELO virus replicated actively in the explant cultures. However, it was not detected before the second day after inoculation and the peak of its replication occurred two days later (FIGURE 4). A maximum viable titer of two to four hundred thousand plaque forming units per millilitre was maintained fairly constantly until the eighth day after inoculation, at which time the experiment was terminated.
FIGURE 3. This shows the rates of survival of M. gallisepticum and M. gallinarum in explant-free tracheal explant medium (1 and 3 respectively) as well as the growth curves of these mycoplasma species in the same medium when supplemented with the extracts of 2.7 explants/ml. (2 and 4 respectively).
Growth of \textit{M. gallisepticum} and \textit{M. gallinarum} in supplemented and unsupplemented explant medium.
FIGURE 4. Replication of CELO virus in tracheal organ explants when inoculated alone (1). (2) represents replication of the virus when it was inoculated two days before *M. gallisepticum*, while (3) illustrates the replication of the virus when it was simultaneously inoculated with the mycoplasma.
Replication of adenovirus alone or with \textit{M. gallisepticum} added simultaneously or later.
3. Growth of the mycoplasmas and replication of the virus in the mixed infection experiments.

Explants were tested for their capacity to support the growth of both virus and one or other mycoplasma. Simultaneous inoculation of virus and Mycoplasma gallisepticum was carried out with different doses of the virus. In fact the virus was always inoculated first and immediately followed by the mycoplasma after an adsorption period (see methods described on page 60). Under these conditions, in the explants which received a low titer of the virus ($10^3$ PFU/explant), the growth of the mycoplasma was similar to its growth in the explants which were inoculated with the mycoplasma alone (FIGURE 5). However, explants inoculated with a higher titer inoculum of virus (approximately $2 \times 10^7$ PFU/explant) supported an apparently slightly more enhanced growth of the mycoplasma (FIGURE 5).

When the virus was inoculated 48 hr. before Mycoplasma gallisepticum, the subsequent growth of the mycoplasma was slightly enhanced (FIGURE 6) in relation to its growth alone but virus replication was virtually unaffected. In contrast to this, virus replication in explants pre-infected with Mycoplasma gallisepticum was completely inhibited although the growth of the mycoplasma was quite unaffected (FIGURE 6).

Growth of Mycoplasma gallinarum in the presence of CELO
FIGURE 5. Growth of *M. gallisepticum* in tracheal organ explants when inoculated alone (1). (2 and 3) represent mycoplasma growth when the explants were simultaneously inoculated with CELO virus (10^3 or 2 x 10^7 PFU/explant respectively).
Growth of M. gallisepticum alone or with different doses of adenovirus added simultaneously.
FIGURE 6. Growth of *M. gallisepticum* in tracheal explants inoculated with CELO virus 2 days before (4), 3 days after (1) or simultaneously (3) with the mycoplasma. (2) represents the growth of the mycoplasma in the control cultures.
Growth of *M. gallisepticum* alone or with adenovirus added before, after, or simultaneously

[Graph showing growth of *M. gallisepticum* with varying time periods for different conditions, labeled with days and colony forming units/ml.]
virus was unaffected (FIGURE 7), while the replication of the virus was greatly inhibited and less than two hundred plaque forming units per millilitre were detected on the second and third days after inoculation but none were detected afterwards.

II - The effect of mycoplasma and virus in tracheal explants.

1. Observations based on light microscopy

The most striking features of healthy uninfected culture was the activity of the cilia which line the lumen of the explant. Variation in the degree and extent of this activity was noted between different batches of eggs. Thus in some batches complete ciliostasis occurred within two weeks. In addition this range of variation sometimes occurred in one batch of explants. However, in general, most explants maintained very active, uniform ciliary beat for ten to twelve days but thereafter the activity declined at varying rates and commonly complete ciliostasis occurred three to seven days later. Observations on explant cultures with frequent changes of medium showed slight improvement and ciliary activity was extended five to seven days longer. During the late periods of incubation, individual ciliated or non-ciliated epithelial cells were seen to detach from the epithelium and drift into the lumen of the explants. Ultimately, when complete ciliostasis had occurred, the epithelium appeared irregular and rough which contrasted with the very regular
FIGURE 7. Growth of *M. gallinarum* in tracheal explants inoculated with the mycoplasma alone (2) or simultaneously with CELO virus (1).
Growth of *M. gallinarum* alone or with adenovirus added simultaneously.
Inoculation of explants with *M. gallisepticum* resulted in early adverse effects on the ciliary activity as well as on the other features of the epithelium and whole explant. A decline in ciliary activity was noticed within a few days after inoculation and this effect rapidly became more distinct leading to ciliostasis (FIGURE 8). The time required for complete ciliostasis varied from one explant to another but generally, it occurred five to eight days after infection. However, in some explants very weak ciliary movement persisted for several more days, but such cultures were very few in number. The ciliostatic effect of the mycoplasma was dose-dependent thus low inocula, for example 6.4 x 10^1 CFU/explant only caused ciliostasis in half the cultures as late as eight days after inoculation whereas this effect occurred within five days in the cultures that were inoculated with 3 x 10^5 colony forming organisms. As ciliostasis developed, extensive damage occurred to the mucosa. For instance, there was extensive stripping off of epithelial cells, singly and in groups, and quite often some of these cells at least temporarily retained their cilia. Eventually, virtually the whole of the epithelium was sloughed-off leaving just scattered cells here and there resting on a bare basement membrane.

Explants inoculated with *M. gallinarum* reacted in
FIGURE 8. The dose-response relationship between ciliary activity and level of *M. gallisepticum* inoculation. Inocula tested (CFU/explant), (1) $3 \times 10^5$; (2) $2 \times 10^3$; (3) $2.8 \times 10^2$; (4) $6.4 \times 10^1$. Control (uninfected) explants (5).
a different manner to those infected with *M. gallisepticum*. There was, indeed, relatively little contrast to the controls. The ciliary activity of inoculated cultures was not affected until, at the earliest, the seventh day post-infected. However, in the majority of the explants ciliostasis did not occur until the eleventh day after inoculation (FIGURE 9). The effect observed with *M. gallinarum* was not obviously dose-dependent because cultures inoculated with as little as $5.7 \times 10^3$ CFU/explant or as much as $8.1 \times 10^7$ CFU/explant behaved in much the same way (FIGURE 9). As with control explants sloughing-off of individual cells was common but it became more pronounced during the later stages of the infection and the final appearance of the epithelium was similar to that infected with *M. gallisepticum*, due to the total loss of cells.

Infection of explants with CELO virus revealed as with *M. gallisepticum* but in contrast to *M. gallinarum* a dose-dependent relationship between inoculum and ciliary activity. For instance, inocula of $10^2 - 10^3$ PFU/explant produced no noticeable effects on the ciliary activity while high titer inocula ($10^8$ PFU/explant) caused complete ciliostasis five to seven days after infection (FIGURE 10). The extent of damage to the epithelium by the virus was much less severe when compared with that produced by the mycoplasmas, and although ciliary activity ceased completely at the later stages of the infection, the epithelium
FIGURE 9. The dose-response relationship between ciliary activity and level of *M. gallinarum* inoculation. Inocula tested (CFU/explant), (1) $8.1 \times 10^7$; (2) $6.3 \times 10^5$; (3) $5.7 \times 10^3$. Control (uninfected) explants (4).
No. of explants showing ciliary activity

Ciliostasis caused by different doses of M. gallinarum
FIGURE 10. The dose-response relationship between ciliary activity and level of CELO virus inoculum. Inocula tested (PFU/explant), (1) $10^8$; (2) $10^6$; (3) $10^4$; (5) $10^2$. Control (uninfected) explants (4).
DAYS

Graph showing the effects of different doses of adenovirus on days 1 to 15.
retained many of its basic structures. For subsequent experiments on single and mixed infection studies, only an inoculum of $1.74 \times 10^4$ PFU/explant was used.

The combination of *M. gallisepticum* and CELO virus infections seemed to be associated with earlier development of ciliostasis as well as other damage to the explants (FIGURE 11). This enhancement was more distinct in the explants which received a high inoculum of the virus. However, in all the explants where the two parasites were present, the final appearance of their epithelia was exactly similar to that of those inoculated with the mycoplasma alone. The mixtures of *M. gallinarum* and CELO virus did not much alter the ciliary activity when this was compared with that of the singly-infected explants (FIGURE 12). The final appearance of the epithelium in the explants where the two agents were inoculated together resembled closely that of those inoculated with *M. gallinarum* alone.

2. **Observations based on electron microscopy**

Transmission electron microscopy of the tracheal epithelium of embryonic and one-week-old chicks revealed pseudostratified columnar ciliated and non-ciliated cells resting on a distinct basement membrane which separated them from the underlaying lamina propria or submucosa (PLATE 1). In the submucosa, there was a relatively thick network of interstitial connective tissue.
FIGURE 11. Ciliostasis in tracheal explants infected with CELO virus and *M. gallisepticum* acting independently or together (simultaneously inoculated). The virus inocula alone (PFU/explant were (1) $10^5$; (2) $10^3$. *M. gallisepticum* alone was inoculated at $3.5 \times 10^3$ CFU/explant (3). Explants inoculated with mycoplasma ($3.5 \times 10^3$ CFU/explant) and virus (4) at $10^5$ PFU/explant and (5) at $10^3$ PFU/explant). Control (uninfected) explants (6).
No. of explants showing ciliary activity

Ciliostasis caused by adenovirus and M. gallisepticum acting independently or together

Days
FIGURE 12. Ciliostasis in tracheal explants infected with CELO virus and *M. gallinarum* acting independently or together (simultaneously inoculated). The virus inoculum alone was $1.74 \times 10^4$ (PFU/explant) (1), and mycoplasma inoculum alone was $4.2 \times 10^3$ (CFU/explant) (3). Explants inoculated with virus and mycoplasma (4) (inocula were the same as (1) and (2)). Control (uninfected) explants (4).
Ciliostasis caused by adenovirus and M. gallinaceum acting independently or together.
infiltrated with a loose network of collagenous and elastic fibres, fibroblasts, lymphocytes, capillaries, occasional free erythrocytes, nerve fibres and mucus-producing glands (PLATES 1 & 17). On the other side of the submucosa, thick cartilagenous tissue predominated (PLATES 2 & 17) with occasional fibroblastic and muscular tissues.

The epithelium consisted of four predominant cell types. The ciliated and non-ciliated cells formed the outermost layer (PLATES 1, 3, 6 & 13) and they were the main types of cells. These cells were supported on another, squat type of cell known as the basal cells (PLATES 1, 3 & 4). In addition, a fourth type of cell, the Goblet cell was scattered among the other types (PLATE 5).

In uninfected explants, from 20-day-old embryos and one-week-old chicks, scanning electron microscopy showed that their epithelia were not uniformly ciliated (PLATE 6); and cells without cilia had a polygonal boundary (PLATE 7). The cells were so closely packed that they formed an apparently impervious plain which upon examination at high magnifications was seen covered with numerous microvilli (PLATE 8). Normally, these microvilli were hidden under dense populations of cilia (PLATE 9). In adult birds, the surface of the tracheal epithelium was fully and uniformly covered with cilia (PLATE 10).
PLATE 1. Transmission electron micrograph (TEM) of a transverse section through the trachea of a chick embryo revealing the mucosa and a part of the sub-mucosa. The mucosa is formed from ciliated cells, non-ciliated and goblet cells (not present here), and the basal cells (B). In the submucosa bundles of collagen fibrils (C) are predominant in the loose connective tissue of the lamina propria with some fibroblast cells (F). The collagen fibrils are cut both transversally (seen as black dots) or longitudinally showing striations. The basement membrane (arrows) separates the mucosa from the sub-mucosa. X 5,400.
PLATE 2. TEM of a section of part of the cartilagenous rings which form the walls of the trachea. It reveals chondrocytes (cartilage cells) embedded in a homogenous interstitial substance which form the body of the cartilage tissue. The cells have short cytoplasmic processes and the cytoplasm contains very distinct endoplasmic reticulum, mitochondria and fat droplets. The nucleus is large and occupies a major part of the cell. X 7,200.

PLATE 3. TEM showing elongated columnar ciliated cells covered on their surface with cilia (Ci). The bases of these cells rest either on the basal cells (B) or on the basement membrane (arrow). Vacuolar structures are apparent as well as numerous mitochondria (M) concentrated in the supra-nuclear region just below the surface of the cells. X 5,700.

PLATE 4. TEM showing dytoplasmic interdigitations which interlace and hold the cells together. Sometimes they are aided by specialized structures, the desmosomes, (small arrow, or inset, X 81,000) which cement cells together or to the basement membrane (large arrow). The nucleus of a basal cell and the cytoplasm of another is apparent. X 20,250.
PLATE 5. TEM of elongated mucus-producing cells containing heavily staining mucus spherules. Their nuclei are situated towards the base of the cell which rests either on the basement membrane or on basal cells. Their cytoplasm contains very elaborate systems of endoplasmic reticulum and Golgi complex, and their surfaces bear some thin microvilli (arrow). X 6,300.

PLATE 6. Scanning electron micrograph (SEM) of the surface of embryonic tracheal epithelium showing ciliated and non-ciliated areas. The rounded but wrinkled structures are individual epithelial cells about to detach themselves from the surrounding tissue. X 935.

PLATE 7. SEM of a de-ciliated area on the surface of the epithelium showing the nature of the close association between the cells forming an apparently impervious layer. X 850.

PLATE 8. SEM of a de-ciliated area at high magnification revealing the irregular polygonal topography of the epithelial cells. The cells, apparently, are cemented together with no spaces between them. Note elevated ridges which distinguish boundaries between cells. The surface of these cells is covered with numerous microvilli. X 4,250.

PLATE 9. SEM of a ciliated and non-ciliated area showing a population of erect cilia masking the much shorter microvilli at their bases. Microvilli are apparent on the non-ciliated portion. X 8,670.
Transmission electron microscopy of transverse sections of the epithelium revealed that ciliated and non-ciliated cells were identical except for the absence of cilia in the latter. They both appeared columnar with their bases sometimes reaching to the basement membrane while their surfaces faced the lumen of the trachea (PLATES 1, 3 & 13). From the apical surface of ciliated cells, numerous cilia projected, which in transverse sections displayed the classical arrangement of nine paired peripheral tubules surrounding a central pair, all enclosed in a unit membrane (PLATE 11). Each cilium originated from a basal body situated within the cytoplasm just beneath the surface of the cell. The cells were connected with each other and to the basement membrane by terminal bars, desmosomes and interdigitations (PLATES 4 & 12). Narrow intercellular spaces were common between the adjacent cells especially in the embryonic tracheal epithelium (PLATES 4 & 13). The epithelium of these cells often contained vacuoles of varying shapes and sizes. Elongated mitochondria were numerous in the cytoplasm of these cells and the majority of them were mainly concentrated in the supra-nuclear region just below the basal bodies of the cilia (PLATES 1, 3 & 13). The mitochondria had well defined cristae and in the cytoplasm a prominent Golgi system was sometimes encountered in the supra-nuclear region (PLATE 13). The tubular elements of a well defined endoplasmic reticulum with numerous single ribosomes attached all along its sides were regularly seen in
PLATE 10. SEM of the surface of tracheal epithelium of adult chicken (12-week-old) which is virtually uniformly ciliated. The cilia are usually covered with a blanket of mucus. X 4,250.

PLATE 11. TEM showing the surface of an embryonic epithelial cell covered with cytoplasmic extensions, the microvilli (Mi) and a part of a cilium. The basal body of the cilium (arrow) is striated and conical, while their filamentous structures run along the length of the cilium which in transverse section (inset, X 65,000) appear tubular displaying the classical arrangement of nine pairs on the periphery surrounding a central pair, all enclosed in a unit membrane. X 54,000.

PLATE 12. TEM taken at high magnification showing the ultrastructural features of a part of the endoplasmic reticulum with numerous ribosomes aligned around it (small arrow). However, individual ribosomes scattered in the cytoplasm are also apparent. Mitochondria with internal structures (cristae), and interlacing interdigitations (large arrow) are also present. X 31,500.
these cells (PLATE 12). Freely occurring ribosomes as well as in clusters were commonly distributed in the cytoplasm.

The nuclei of ciliated and non-ciliated cells were commonly ovoid and somewhat irregular in shape, often occupying the central portion of the cell body (PLATE 3). The inner and outer nuclear membranes were intact and well defined. Thin chromatin material was usually peripherally concentrated close to the inner nuclear membrane and the majority of the nuclei contained one or two nucleoli.

The basal cells rested directly on the basement membrane and they were irregularly round, oval or polygonal in shape with flattened bases (PLATES 1, 3 & 4). They had long cytoplasmic processes which extended across the surrounding intercellular spaces making contact with the adjacent cells and in some regions, the basal cells connected with other cells by desmosomes (PLATE 4). Their nuclei were irregular in shape and occupied most of the cytoplasm usually with only a few mitochondria around them. A few ribosomes were also present but the endoplasmic reticulum and the Golgi complex were not well defined in them.

Goblet cells, or the mucus-producing cells, although much less in number than either one of the
above three types of cells, were readily identified by their numerous mucus spherules which sometimes filled the cytoplasm (PLATE 5). Their basal regions were often touching the basement membrane and their apical surfaces protruded into the lumen. They were attached to the adjacent cells by terminal bars, desmosomes and interdigitations. They bore no cilia but the surface had some microvilli. The nuclei of Goblet cells were irregularly oval and located near the base of the cell. Mitochondria were occasionally seen in the supranuclear region, especially when the cells were not fully packed with mucus. The cytoplasm was rich with ribosomes, endoplasmic reticulum and Golgi complex but all these cell organells were obscured when the cytoplasm was densely filled by the mucus spherules.

Virus infections produced only modest changes in the tracheal epithelium when low titers were inoculated, however, when the explants were infected with high titer inocula say, $10^9$ PFU/ml., there were considerable changes. In the scanning electron microscope, these were first observed three to six days after infection when the epithelium had lost most of its cilia and many areas became covered with numerous, rounded individual epithelial cells (PLATE 14). At higher magnifications these cells were evidently detaching from the epithelium (PLATE 15).

Examination of thin sections of infected explants
PLATE 13. TEM of a thin section of the upper portion of epithelial cells showing clearly the boundaries between cells with thin intercellular spaces. A prominent Golgi apparatus (arrow, or inset, X 34,000) and mitochondria with well defined cristae are also shown. X 11,250.

PLATE 14. A low power SEM showing the epithelial surface five days after inoculation with a heavy inoculum of CELO virus. Ciliated and non-ciliated cells are rounded up and about to slough off into the lumen. X 468.

PLATE 15. A high power SEM showing one cell almost detached from the epithelium leaving an obvious hole in the surface of the epithelium. X 4,680.

PLATE 16. TEM of a non-ciliated epithelial cell infected with CELO virus. The virus appears in clusters of microcrystals situated mainly close to the double nuclear membrane. They are never found in the cytoplasm of affected cells. A distinct basement membrane (arrow) and few collagen fibres of the lamina are also apparent. X 20,250.
by transmission electron microscopy revealed virus microcrystals typical of adenovirus, in both ciliated and non-ciliated cells (PLATES 16, 17, 19 & 23) but not in the Goblet cells. However, the number of infected cells seen was relatively small and these were scattered mainly in the outermost layer of cells.

Development of the virus appeared to be relatively slow and virions were not detected before the second day after infection. The microcrystals which were of varying sizes and shapes, were seen distributed inside the nuclei mainly peripherally in areas which originally contained chromatin material, and they were frequently associated with faintly staining areas (PLATES 16, 17 & 19). Each microcrystal of virus was formed of virions laid on top of each other in a very regular manner forming a crystalline lattice (PLATES 17, 19 & 20). However, some infected cells did not show this regularity in the distribution and arrangement of their virions (PLATE 23). Sectioned virions showed regular hexagonal profile (PLATE 21) which compared closely with that of virions stained by the negative stain (PLATE 22). Virions were never observed in the cytoplasm and they were always bound to the nucleus. However, infected cells were frequently shed into the lumen while retaining their structures and virus crystals (PLATE 23) and clusters of free virions were occasionally seen in the lumen in close proximity to the epithelium (PLATE 24).
PLATE 17. A TEM of a transverse section of an infected tracheal explant showing the relationship of the mucosa and the submucosa to the cartilagenous material (Ca). It also shows one ciliated epithelial cell (cilia indicated to by small arrows) containing large microcrystals of CELO virus filling most of the body of the nucleus (large arrow). In the submucosa bundles of collagen fibres (C), two erythrocytes (very darkly staining structures), two lymphocytes (L), and some fibroblast cells (F) are also present. X 3,500.
PLATE 18. TEM of virus microcrystals present inside the nucleus of an infected cell. The nucleus is surrounded with a distinct and intact double nuclear membrane. The cell also retains a clear cell membrane (arrows) and some distinct tubular structures of the endoplasmic reticulum. A swollen mitochondrion (M) appears in the cytoplasm close to the nucleus. X 13,500.

PLATE 19. TEM showing a swollen cell with a nucleus containing microcrystals of virus as well as virions scattered in the whole body of the nucleus. The faintly staining material (arrow) is often seen in the infected cells with virions associated with it or present around it. This infected cell rests directly on the basement membrane while part of the lamina propria (Lp) is also present. X 9,250.

PLATE 20. TEM showing a large virus microcrystal revealing virions overlayed on top of each other forming a crystalline lattice. Arrows point to at least two layers of virions. X 20,250.
PLATE 21. TEM of monolayers of virions inside the nucleus showing the close and regular arrangement of individual virions which display hexagonal profiles (arrows). X 20,250.

PLATE 22. TEM of CELO virus stained by the negative technique with sodium silicotungstate. The hexagonal profile of the icosahedral particle is apparent. X 41,000. Inset shows the capsomeres of a virus particle at higher magnification. X 233,000.

PLATE 23. TEM of two epithelial cells. One is in situ while the other is sloughed-off retaining the virions within the nucleus (arrow). Many of such infected cells were encountered inside the lumen of the explant culture. In these two infected cells the virions are not arranged in the usual regular manner. X 5,400.
The infected cells were characteristically large and contained swollen nuclei which although atypical still possessed an intact double membrane (PLATES 16, 18 & 23). The other organells of the infected cells were only slightly affected, for example, the mitochondria were slightly swollen and spherical in shape (PLATES 18 & 23) although the cristae retained the usual appearance. The elements of the endoplasmic reticulum and Golgi complex became much less obvious and infected ciliated cells lost many or all of their cilia but the microvilli seemed unaffected.

The cytopathic changes induced by the growth of \textit{M. gallisepticum} were extensive. Transmission and scanning electron microscopy indicated that the organism was extracellularly bound and the use of the two techniques provided information which was complementary. Scanning electron microscopy was found of particular importance in early detection of the mycoplasma when low titer inocula ($10^2 - 10^3$ CFU/ml.) were used to infect the explants, at which stage sections examined by transmission electron microscopy rarely showed any organisms. Soon after inoculation, scanning electron microscopy revealed that the mycoplasmas were resting on the surface of the epithelium especially associated with the non-ciliated regions of the epithelium where the organisms were associated with the microvilli (PLATES 25, 26, 29 & 30). However, some organisms were observed at the
which are sometimes seen on the surface of epithelial cells among microvilli (Mi). Distinct microvilli, endoplasmic reticulum, ribosomes, desmosomes and interdigitations are also present in this micrograph. X 28,000.

PLATE 25. SEM revealing organisms of *M. gallisepticum* resting on the microvilli and surface of a non-ciliated region of the epithelium. Cilia (Ci) belonging to a nearby cell are also present. X 20,400.

PLATE 26. SEM of several mycoplasma cells (*M. gallisepticum*) showing a range of morphology are present. Many examples are also showing the terminal protrusions. X 20,400.

PLATE 27. SEM showing small clusters of *M. gallisepticum* bodies associated with the microvilli and the cilia of a ciliated and non-ciliated region of the epithelium. X 20,400.

PLATE 28. SEM showing mycoplasma cells (*M. gallisepticum*) on and at the bases of cilia. One mycoplasma cell is adhering to the surface of a cilium (arrow). X 20,400.
surface of the ciliated cells as well and some were
even amongst the cilia (PLATES 27 & 28).

At this stage, the mycoplasmas appeared to be
very closely associated with the surface of the
epithelium either being applied with a flat profile
onto the surface (PLATES 25 & 52), or even more
positively by a bleb structure (PLATES 26, 30, 31,
39 & 52 C, E, H). Multiplication of the mycoplasma
was evidently rapid because within three days after
inoculation, very large numbers were seen spread on
the surface occupying extensive areas of the epithelium
individually or in microcolonies (PLATES 31 & 32).

Examination of thin sections of infected explants
by transmission electron microscopy two or more days
after inoculation also revealed mycoplasmas between
the cilia (PLATE 33) and attached to non-ciliated cells
(PLATE 34). This technique also revealed many patholog­
ical changes. Individual as well as groups of affected
epithelial cells were seen detached from the surrounding
and underlying tissues, with numerous mycoplasmas
present between them and on their surfaces (PLATE 35).
Furthermore, at the later stages of the infection (four
to six days post-inoculation), the whole epithelium in
many areas, appeared lifted up from the basement membrane
and many cells were sloughed-off into the lumen (PLATE
36). The basement membrane was invaded by individual
mycoplasmas which at high magnification were seen
PLATE 29. SEM revealing three mycoplasma cells (M. gallisepticum) attached to the microvilli by the bleb structures (arrows). X 19,125.

PLATE 30. SEM of some mycoplasma cells (M. gallisepticum) on the surface of the epithelium. One organism has the bleb structure attached to the microvilli (arrow). X 19,125.

PLATE 31. SEM of an explant three days after inoculation with M. gallisepticum. The mycoplasma are very numerous and they are spread over relatively wide areas of the epithelium colonizing the surfaces of detached and sloughed-off cells (arrow) as well as the surface of the basement membrane. By this time many of the mycoplasma cells appear to have a cocco-bacillary form. X 4,675.

PLATE 32. A high magnification SEM of a colony of M. gallisepticum six days after inoculation showing clearly their cocco-bacillary morphology. Some mycoplasmas have small extensions, probably their bleb structures (arrows). X 21,000.

PLATE 33. TEM of a thin section of a ciliated cell from a mycoplasma-infected explant showing individual M. gallisepticum organisms resting between the cilia and on the surface of the cell (arrows). Also, pathological changes are apparent inside the cell. X 7,200.
mycoplasma cells (M. gallisepticum) on the surface. Intracellular changes such as vacuolization, disorganisation of the cytoplasm, margination of heavily staining chromation in the nucleus and appearance of a faintly staining (fat-like body) structure close to the nucleus are evident. X 8,100.

PLATE 35. TEM revealing numerous M. gallisepticum cells colonizing the surfaces of sloughed-off single cells and attached to the cells by their bleb structures (arrows). X 4,050.

PLATE 36. A low power TEM showing detachment of groups of cells from the basement membrane (Bm). The mycoplasmas are usually cell-associated (arrows), and many others remain attached to the basement membrane (arrows), or penetrate it with the bleb structure (inset, X 45,000), and are directed towards the lamina propria (Lp). X 2,700.
penetrating it with the bleb structure foremost and directed towards the submucosa (PLATE 36). Four to eight days after inoculation numerous mycoplasmas were inside the submucosa (PLATE 37) and the numbers of the organisms increased considerably. Individual mycoplasmas were often seen attached to the striated collagen fibres by their bleb structures.

The mycoplasma appeared as if probing through the surface of the cells and slight depressions at the site of contact were regularly seen (PLATES 38 & 39). In some sections the probing activity appeared very like a burrowing activity (PLATES 40, 41 & 42).

In these locations, the mycoplasma seemed to have multiplied in large numbers inflicting damage on the surrounding tissue sometimes causing its total disintegration (PLATE 43).

From the earliest stages of infection with *M. gallisepticum* intracellular changes of the epithelium occurred. The cytopathological changes involved all the cell organelles. The cytoplasm of the affected cells became highly disorganized and was occupied with numerous vacuoles of varying sizes and shapes (PLATE 33, 34, 35 & 44). The mitochondria which were originally elongated became spherical and swollen, and they also lost their usual cristae and became undistinguishable from the vacuoles that filled the cytoplasm. The
PLATE 37. A TEM showing a situation from the late stages of the infection (5 - 8 days after inoculation) with *M. gallisepticum*. Organisms have invaded the loose connective tissue of the lamina propria causing cytopathological changes (X 4,500). Some of the mycoplasmas are seen associated with the striated collagen fibrils. (inset X 45,000).

PLATE 38. TEM showing the attachment of *M. gallisepticum* to the surface of epithelial cells by the bleb structure. Note that the bleb membrane does not fuse with the cell membrane at the site of contact and that there is a slight depression at the point of contact (arrows). Microvilli (Mi) as well as defined endoplasmic recicum and ribosomes are also seen in this micrograph. X 54,000.

PLATE 39. TEM showing internal details of a mycoplasma cell (*M. gallisepticum*) associated with the surface of a cell by its bleb structure. Inside the body of the mycoplasma there are peripheral striated longitudinally aligned structures (arrows). X 36,000. In the inset a SEM shows a similar mycoplasma attached to the surface of a cell by its bleb structure. X 51,000.
PLATES 40, 41 & 42. High power TEMs of *M. gallisepticum* organisms deeply embedded in the surface of cells and containing the characteristic longitudinal striated structures. X 81,000, X 72,000, and X 54,000 respectively.
on the surface of an explant epithelium. The organisms assume various morphologies, and a part of an epithelial cell is also shown in the micrograph (arrow). X 6,300.

PLATE 44. TEM showing changes in the intracellular organisation of epithelial cells of infected explants. Ciliated cells have lost most of their cilia and the cytoplasm is characteristically rich with vacuoles of various shapes and sizes. The mitochondria lose their cristae after they swell and then become difficult to identify. The ribosomes decrease in number considerably and the endoplasmic reticulum disappears. The nucleus stains less intensely while its chromatin material is localized peripherally and stains densely. The nuclear membrane remains intact but the outer layer becomes irregularly loose. Mycoplasma are never seen within the cells. X 9,000.

PLATE 45. TEM showing changes in the intracellular organisation of the cartilagenous tissue of infected explants. After infection with *M. gallisepticum* the chondrocytes shrink leaving relatively wide areas between the interstitial substance of the cartilage and the cell body. The nuclei of these cells stain densely and the cytoplasm appears dissolved and highly vacuolated while the cytoplasmic organells disappear. Some of these effects were seen in old (14 days) control explants. X 7,200.
endoplasmic reticulum and the Golgi complex also lost their tubular structures, became fragmented and apparently they were transformed into vacuoles. The number of ribosomes decreased considerably and sometimes they were difficult to find. Occasionally a large vacuole developed in the cytoplasm containing faintly staining material typical of fat bodies (PLATES 34 & 39). In the nucleus, which became swollen and spherical in shape, the chromatin material was peripherally situated forming a thin intensively staining band, and nucleolar material was often seen in it. However, the nuclei retained their double membranes but the outer one became distinctly loose and irregular (PLATES 33, 34 & 44).

The surfaces of the epithelial cells were also affected leading to a loss of the attachment system, that is, the interdigitations or desmosomes, which resulted in detachment from each other. Detachment was probably enhanced by the swelling of the cell itself, the surface of which became tightly stretched (PLATES 34 & 36). Apart from this there was a loss of the cilia some of which were seen free in the lumen (PLATES 33 & 44).

Cytopathology was also noticed in the hard tissue of the cartilagenous rings where the chondrocytes were characterized by vacuolization of their shrunken cytoplasm (PLATE 45). The cells lost their marked
boundaries and their short processes, and a clear zone appeared all around each cell. The internal organization of the cells disappeared completely and the mitochondria together with the other organells were damaged while the nuclei stained much more intensely than before. However, the interstitial substance of the cartilage appeared unaffected. These pathological changes were seen, but to a much lesser extent, in old tracheal explants as well as in virus-infected and \textit{M. gallinarum}-infected cultures.

Besides the commonly encountered pear-shaped organisms of \textit{M. gallisepticum}, thin sectioning techniques revealed elongated and spindle-like structures (PLATES 43, 46, 47 & 48). Within the mycoplasma, the body contained densely staining granular material which sometimes filled the whole body of the organisms (PLATES 38, 46 & 47), and which was enclosed by a triple-layered membrane (PLATES 48, 49 & 50). However, many organisms displayed a regular arrangement of this granular material which appeared as uniformly striated longitudinal sections along the long axis of the body of the organism, the above striated structures were seen running along the length of the body of the mycoplasmas and were oriented to gather at the tapered end (PLATE 49). Moreover, transverse sections of these structures indicated that they were hollow and tubular (PLATE 50).

In order to fully interpret the morphological
PLATE 46. TEM showing different morphological manifestation of *M. gallisepticum* organisms associated with the cell surface selectively using their bleb structures. They appear as if directed to the surface by some sort of tropism. Inside the mycoplasma striated structures are also present. X 36,000.

PLATE 47. TEM showing mycoplasma cells (*M. gallisepticum*) stretching themselves to reach the cell surface as if attracted by some force. Striations inside the body of the organism are also obvious. X 27,000.

PLATE 48. TEM showing two *M. gallisepticum* organisms with elongated morphological forms. Uniformly arranged granular structures are clearly seen inside one of them (A). The organisms in (A) and (B) are surrounded by a double unit membrane (arrows). X 72,000 and X 54,000.
PLATE 49. TEM showing one fairly large specimen of *M. gallisepticum*, apparently sectioned longitudinally along the long axis of its body to reveal a double unit membrane (arrows) surrounding the cell body which contains longitudinal striated structures. X 63,000.

PLATE 50. TEM of a transverse section of an *M. gallisepticum* cell showing details of the striated internal structures. Transverse (arrows) and longitudinal views are seen indicating that the striated structures may be hollow and tubular. They are evenly spaced near the periphery of the body of the mycoplasma. X 90,000.

PLATE 51. TEM of negatively stained *M. gallisepticum* cells. They are swollen at one end and narrow to form a slender protrusion at the other end, the bleb structures (arrows). The surface of the body appears smooth in this preparation. X 54,000.
structures of \textit{M. gallisepticum} seen in the tracheal explants, other contrasting and complementary techniques were used. Organisms which were grown in standard broth medium were stained by the negative technique with sodium silicotungstate and examined by transmission electron microscopy. This technique revealed a variety of forms whose surfaces were regular and smooth (PLATE 51). However, pear-shaped bodies with elongated protrusions or blebs, usually occurring only at one end were commonly observed. When \textit{M. gallisepticum} from the log phase growth in standard broth culture was heavily inoculated on the surface of tracheal explants from one-week-old chicks and examined by scanning electron microscopy, the morphology was highly variable (PLATE 52). The organisms which were seen one hour after inoculation were present on the microvilli in various positions and two or more bleb structures were commonly found on each organism. No major differences were observed whether alcohol dehydration or freeze-drying was employed as the preparative technique (PLATES 30, 31 and 53, 54).

However, the morphology of \textit{M. gallisepticum} organisms was different when they were grown as microcolonies in standard broth on the surface of glass (PLATES 56). Besides the elongated or spherical structures which predominated during the first and second days after inoculation, filamentous forms were commonly seen on the third and fourth day (PLATES 56 & 57).
PLATE 52. SEM of epithelium one hour after inoculation with M. gallisepticum (A - L). The mycoplasma cells are resting on the surface of the respiratory epithelium on tops of the microvilli. Various morphological forms are present, and each organism possesses one or more bleb structures (B, D, E, F, H, K, some indicated to with arrows). X 51,000 each.
PLATES 53 & 54. SEMs of infected explants (M. gallisepticum) after dehydration by freeze-drying instead of by alcohol. The morphological features of the explant cells and M. gallisepticum bodies resemble that shown in PLATES 30 and 31. X 9,350 and X 8,500.

PLATE 55. SEM showing the variable morphologies of M. gallisepticum grown on the surface of glass. Their morphology is somewhat different to those grown on explants (PLATE 52). Note presence of bleb structures (arrows). X 20,400 each.

PLATE 56. SEMs of small microcolonies of M. gallisepticum grown on glass. (A) shows two groups of cell connected to each other by an elongated cellular extension. X 51,000. (B) shows a part of a microcolony where cellular extensions are predominant (arrows). X 20,400. Note also presence of bleb structures (arrows).

PLATE 57. SEM of a microcolony of M. gallisepticum grown on glass. Rounded cells are predominant and their elongated extensions are also present (arrows). X 10,030.
M. gallinarum was also found to cause some damage to the tracheal explants but the mechanism appeared to be quite different to that of M. gallisepticum. Thin sections of tracheal explants infected with low titer inocula (10^3 CFU/ml), first showed cytopathological signs three days after infection, by which time, titration studies indicated considerable multiplication of the organisms. The organisms were seen randomly distributed in the lumen near the tips of the cilia but were present in considerable numbers within the lamina propria (PLATES 58 & 59). At this stage the cytopathological changes inside the cells were manifest by the occurrence of one or more densely-staining spherical structures which appeared randomly in the cytoplasm of many epithelial cells (PLATE 60). Moreover, early signs of vacuolization of the cytoplasm was apparent while the nuclei were apparently unaffected.

Two to four days later, cytopathological changes became more extensive and most of the epithelial cells contained the densely-staining structures described above which also increased in size and stained more densely. Some cells contained several of these structures which quite often filled most of the cytoplasm (PLATE 61), and was coincident with the rounding up of the cells. By this time, individual sloughed-off cells were frequently encountered in the lumen some of which were highly degenerate (PLATE 62). In the submucosa
and examined three days later. The tracheal epithelium remains intact and in situ but many organisms occupied areas in the lamina propria particularly between the collagen fibrils and just below the basement membrane (arrow). X 8,100.

PLATE 59. TEM showing *M. gallinarum* organisms at the bases of epithelial cells and some cytopathological changes are obvious. For instance, there is an accumulation of distinct spherical structures of varying sizes within the cytoplasm of these cells accompanied with degeneration of cytoplasm and its organells. X 5,400.

PLATE 60. TEM showing early cytopathological reactions of the epithelial cells to infection with *M. gallinarum*. Spherical, densely-staining structures (arrow) appear two days after infection. X 3,600.
infection with *M. gallinarum* showing sloughed-off cells, also a rounded cell before its complete detachment from the basement membrane. Inside this cell two large and one small darkly-staining structures fill the body of the cell and distort the shape of the nucleus. Organisms are present around a degenerate cell (arrow). X 3,600.

PLATE 62. TEM of detached degenerate epithelium ten days after infection with *M. gallinarum*. Numerous organisms are seen around and among the degenerate cells. The cytoplasm of these cells is highly disorganized and cytoplasmic as well as nuclear organells are not present. A part of the basement membrane with detaching cells are also evident. X 4,500.

PLATE 63. TEM showing numerous *M. gallinarum* organisms filling the spaces between the loose connective tissue of the lamina. The darkly-staining structures are also present with some degenerate cellular components. X 3,600.
numerous organisms filled the areas among the loose connective tissues of the lamina and there was some degeneration of the cellular components (PLATE 63). The densely-staining structures were also seen in the submucosa.

A few days later, patches of the epithelium detached from the basement membrane and sloughed-off into the lumen leaving behind them a bare basement membrane similar to that observed in tracheal explants infected with M. gallisepticum. In the submucosa the mycoplasma practically filled the whole of the lamina (PLATE 65).

Besides the appearance of the densely-staining structures which was particular of M. gallinarum infections, degeneration of the affected cells was remarkable. The cytoplasm and cell membranes of the affected cells appeared highly soft and damaged (PLATES 61 & 62). However, the material which filled the above densely-staining structures was of a different texture from that of the main body of the cells and this was evident in the sections from the rippling phenomenon which was observed indicating a softer texture (PLATES 59, 60, 61 & 63). Moreover, these structures distorted the various cell organelles in the cytoplasm and quite often displaced the nucleus and the mitochondria (PLATE 61). In the highly degenerate cells dissolution of the
around the membrane of a degenerating cell. The organisms do not form any obvious intimate association with the cell membrane, and they do not have the specialized attachment structures seen with \textit{M. gallisepticum}. X 27,000.

PLATE 65. TEM showing the situation of the lamina propria at a late stage of infection with \textit{M. gallinarum}. Numerous organisms are present among the collagen fibrils. A part of the basement membrane (Bm) is also evident. X 6,300. Inset shows that there is no obvious and particular association between the mycoplasma and the collagen. X 22,500.

PLATE 66. TEM showing three \textit{M. gallinarum} cells sectioned at different planes. Number 1 and 2 are believed to be mycoplasmas cut longitudinally while the third is cut transversally. A sloughed-off cell and a distinct basement membrane (arrow) are also apparent in the micrograph. X 15,300.
cytoplasm was more extensive than in the
M. gallisepticum-infected epithelium and sometimes
traces of cell debris were left with numerous myco-
plasmas around them (PLATES 61 & 62). In these cells
the major cell organelles such as the nucleus, mitochon-
dria and endoplasmic reticulum disappeared or became
difficult to identify.

Transmission electron microscopy revealed major
differences in the distribution and mode of local-
ization of M. gallinarum among the cells of the
epithelium when compared with that of M. gallisepticum.
Individual organisms were localized in a loose manner
mainly around the highly degenerate sloughed-off cells
and they were never seen inside them (PLATES 61 & 62).
Also, the organisms were never found forming the
intimate association with the cell membranes that was
do characteristic of M. gallisepticum nor was there any
particularly specialized terminal structures or blebs
(PLATE 64). Similarly, in the submucosa the organisms
were not associated with the collagen fibrils or the
connective tissues of the lamina in any particular
manner (PLATE 65).

The morphology of M. gallinarum, as revealed by
transmission electron microscopy was also quite different
from that of M. gallisepticum. In thin sections,
M. gallinarum displayed two major forms, particularly
illustrated in PLATE 66, which were believed to contain
both longitudinal and transverse sections of the organisms. Various other forms of sectioned mycoplasma were shown earlier (PLATES 61, 62 & 63). However, one major form predominated in the submucosa, and it gave the impression that in this particular location the organisms were mainly irregularly spherical (PLATES 58, 59, 63 & 65). The cell body of the mycoplasma contained finely granular material distributed uniformly in the cytoplasm with heavily staining areas located either uniformly peripherally or at one side and enclosed by a triple-layered membrane (PLATES 64 & 65).

Other techniques were also employed to interpret the morphology of this mycoplasma. Organisms grown in standard broth medium, stained with sodium silicotungstic acid and examined by transmission electron microscopy were mainly spherical in shape, and frequently seen in pairs (PLATE 67). In the latter situation the spherical shape became slightly distorted at the site of contact. Occasionally slender cellular protrusions were also seen but they were not comparable with the bleb structures of M. gallisepticum. A circular structure was frequently observed on the surface of cells which stained less heavily than the rest of the surface and was of variable size (PLATE 67a). However, the surface of the organism was regularly smooth.

Examination of M. gallinarum by scanning electron microscopy was not too rewarding and only rarely were
The cells, either singles or in pairs are apparently spherical with smooth and regular surface. A circular structure is shown in A (arrow) which is stained less intensely than the rest of the surface of the cell. This was frequently seen with this technique. X 28,000 each.

PLATE 68. SEM of small clusters of irregularly spherical forms of *M. gallinarum* on the surface of explant tracheal epithelium. X 17,000.

PLATE 69. SEM showing a colony of doughnut-shaped cells of *M. gallinarum* on the surface of the epithelium of a tracheal explant. X 8,500.

PLATE 70. SEM of a group of *M. gallinarum* cells resting on the surface of the epithelium of a tracheal explant. X 42,500.

PLATE 71. SEM of a microcolony of *M. gallinarum* grown on the surface of glass. The organisms are rod-like or have bacillary forms. X 42,500. Inset is a high power micrograph of one organism. X 85,000.
organisms seen in embryonic explants infected with low inocula ($10^3 - 10^5$ CFU/ml). However, inoculation with heavy concentrations of the mycoplasma ($10^9$ CFU/ml) revealed more organisms but fewer than expected in comparison to the number of $M. gallisepticum$ seen after similar treatment.

Small clusters of spherical (PLATE 68), doughnut-like (PLATE 69), and other structures (PLATE 70) were seen predominantly on the non-ciliated areas of the epithelium. In a further attempt to interpret the morphology of $M. gallinarum$, organisms grown on glass coverslips in the standard broth medium were also examined by the scanning electron microscope. Under this situation, rod-like structures prevailed especially one and two days after inoculation (PLATE 71), while rounded and spherical shapes became more numerous later (PLATE 72). Moreover, swollen, flat, irregularly round or branching structures were occasionally seen (PLATE 73). All the above studies, under the various conditions used, showed that the surface morphology of this mycoplasma was regular and smooth.

Mixed infection studies

(a) Tracheal explants

Tracheal explants infected with mixtures of mycoplasma and CELO virus were examined by both transmission and scanning electron microscopy using the techniques described above. Observations indicated
that no marked characteristics or particular pathological manifestations rendered them distinguishable from cultures infected with mycoplasma alone. Moreover, all the explants which received either one of the two species of mycoplasma with the virus, showed pathological changes typical of that induced by either mycoplasma alone regardless of the time of inoculation of the mycoplasma.

(b) Adult birds

Trachea from healthy uninfected birds which had received sterile medium in the aerosol as well as those which were inoculated with mycoplasma and virus developed the same very characteristic features. After inoculation, the epithelium underwent three main changes. There was a degeneration stage followed by a hyperplasia and finally a return to normal morphology.

Specimens of trachea from birds killed one week after aerosolization and examined by scanning electron microscopy had highly degenerate epithelia. This was manifested by presence of excessive amounts of mucus which was very difficult to wash away completely by the techniques used (see methods). Many sloughed-off cells and dispersed cilia were trapped by the mucus and contributed to the disorganized and rough appearance of the epithelia (PLATE 74 & 75). However, during the second week after aerosolization, a slightly less severe reaction predominated and the epithelia appeared slightly
PLATES 72 & 73. SEMs showing *M. gallinarum* grown on surface of glass and demonstrating morphological variations. X 42,500 and X 14,450.

PLATES 74 & 75. SEMs showing the surface of tracheal epithelium from seven-week-old control birds inoculated with sterile PPL0-broth mixed with virus maintenance media in the form of aerosols. The specimens which were taken one week after inoculation show a highly disorganized surface with collapsed cilia and numerous detached cells. X 1,870 each.

PLATE 76. SEM of the surface of tracheal epithelium two weeks after exposure to the aerosols showing disorganized clusters of cilia. X 425.

PLATE 77. SEM revealing four red blood cells resting on the surface of the ptechiated epithelium among disorganized collapsed cilia. X 1,870.
more organized than before (PLATE 76), although detached cells were numerously encountered. Furthermore, the majority of the specimens examined showed petechia with numerous free erythrocytes resting on the surface among degenerated epithelial cells (PLATE 77).

Examination of thin sections by the transmission electron microscope confirmed the observations made above and revealed features of cytopathological degeneration. In many areas the epithelium had lost its regular columnar arrangement and the lumen was filled with clusters of detached, degenerate ciliated or nonciliated cells (PLATE 78). In some other areas, the basal cells were also undergoing various pathological changes while they were either attached to the basement membrane (PLATE 79), or completely detached from it, leaving a bare and unprotected membrane (PLATE 80).

Intracellularly, cytopathologic changes involved all the cell organelles (PLATES 78 & 81). The endoplasmic reticulum and Golgi complex lost their originally tubular structures and became dilated, fragmented and apparently transformed into swollen vacuoles of various shapes and sizes. Similarly, the mitochondria swelled and became irregularly spherical in shape losing the usual arrangement of their internal cristae. Furthermore, the nuclei of these cells became oval in shape with no distinct chromatin material, but one or
PLATE 78. TEM of the tracheal epithelium sampled one week after the birds were exposed to the aerosols. It shows highly disorganized epithelium with degenerate cells sloughed off into the lumen together with cellular changes in the submucosa. X 2,700.

PLATE 79. TEM showing a few basal cells still in situ on the basement membrane while they are undergoing degeneration. Note presence of vacuoles within these cells. X 4,050.

PLATE 80. TEM showing the basement membrane free of cells. A part of a sloughed-off epithelial cell is also shown (arrow). X 4,050.
two nucleoli were present.

The sloughed-off cells lost their usual elongated appearance and became short and swollen (PLATE 81). Many of the degenerating ciliated cells retained some of their cilia (PLATE 82) but individual, necrotized cilia were also seen dispersed in the lumen among the cell debris (PLATE 81). The plasma membrane of the cilia became loose and stretched while their internal tubular structures were apparently unaffected and clusters of cilia grouped together and surrounded by one common plasma membrane were also encountered in many specimens throughout the whole study (PLATE 82). Individual free erythrocytes were also seen in the lumen or on the surface of the epithelium (PLATE 82) while many others were present among the epithelial cells, presumably on their way to the surface (PLATE 83).

In the submucosa, the lamina propria became highly disorganized and necrotized with a noticeable increase in its thickness while the collagen fibres lost their regular and uniform bundle-like arrangement (PLATE 84).

Scanning electron microscopy on tracheal epithelia from the birds which were killed during the third and fourth weeks after aerosolization showed remarkable changes. Relatively wide areas of their epithelia were covered with huge populations of cilia, with mucus spherules of various sizes resting on their tips (PLATES 85 & 86). However, areas undergoing degeneration
epithelial cell inside the lumen. The cell is short and swollen with few and indistinct cytoplasmic extensions. Intracellularly, the cytoplasm contains many vacuoles, and the cytoplasmic organells cannot be identified. The nucleus is spherical and retains its densely staining irregular membranes. Some detached cilia are also present in this micrograph. X 3,600.

PLATE 82. TEM of an area associated with petechia showing red blood cells (arrows) in the lumen near the surface of the epithelium. Surfaces of degenerate cells are also present. X 1,150. Inside the inset a cluster of cilia enclosed by one large distinct membrane. X 9,250.

PLATE 83. TEM showing infiltration of the basal regions of the epithelium with erythrocytes (arrows) and rounded mononuclear cells. X 1,350.
PLATE 84. TEM of the submucosa showing degenerate cellular components and disrupted bundles of the collagen fibrils. X 1,800.

PLATES 85 & 86. SEMs of samples taken three and four weeks after aerosolization showing a resumption of a more healthy organization. Wide areas of the epithelium are covered with dense populations of cilia on top of which there are globules of mucus. X 1,870 each.

PLATE 87. TEM showing the hyperplastic nature of the epithelium that prevailed during the third and fourth week after infection. The cells are densely packed, elongated and the outermost layer is populated with cilia. Some rounded cells are obvious at the bases of the ciliated cells. X 1,800.
and sloughing-off of cells, similar to that described above were also present.

Examination of similar specimens by the transmission electron microscope also revealed major changes in the cellular and sub-cellular features of the epithelium. All the epithelia of these specimens were characterized by acute hyperplasia (PLATE 87), which in some areas was so characteristic that the basement membrane was loaded with more than ten layers of cells. However, a remarkable feature of these specimens was the presence of dense populations of cilia crowning the tops of the outermost cell layer. Detached individual cells and portions of protruding cells were also seen in the lumen (PLATE 88) while most of the tissue of the epithelium remained attached to the basement membrane.

The hyperplastic epithelium was formed from closely and densely packed cells which appeared as if exerting pressure on each other and holding to each other, thus, intercellular spaces were absent (PLATES 87 & 88). Similarly, desmosomes and interdigitations were absent or rarely seen. The basal cells became difficult to identify due to massive infiltration of the basal regions with rounded cells (PLATE 83), while the ciliated cells were thin and elongated (PLATES 87 & 88).

Intracellularly, the cells of the epithelium were undergoing severe changes (PLATE 88). Their cytoplasm
was full with numerous vacuoles which rendered the mitochondria and the endoplasmic reticulum difficult to identify. The nuclei of the cells were spherical or ellipsoidal in shape with distinct and heavily staining membranes and nucleoli. One large vacuole was regularly seen in the cytoplasm of almost each cell, in close opposition to the nucleus, and apparently exerting pressure on the latter, hence distorting its shape (PLATE 88). The vacuolar structure did not contain any of the familiar cell material and degeneration was more obvious in the cells which were nearest to the surface of the epithelium (PLATE 88).

During the fifth week after exposure to the aerosols, the tracheal epithelia of the birds appeared much more uniform, as revealed by scanning electron microscopy (PLATE 89). The surface of the epithelium which was covered with multitudes of cilia displayed a regular wavy appearance, and at high magnification the cilia had similar lengths and comparable diameters (PLATE 90). Mucus spherules, although encountered, were much less in number than before and small areas undergoing degeneration were rarely seen. Transmission electron microscopy on thin sections from the above samples showed marked reduction in the number of cells of the epithelium, and the epithelial cells which predominated became characteristically columnar (PLATE 92). Intracellularly, the number of vacuoles in the cytoplasm decreased remarkably, and in some sections they were not present.
The cytoplasm is vacuolated and the mitochondria are swollen and degenerate while the endoplasmic reticulum and Golgi complex cannot be identified. A large vacuolar structure in close apposition to the nucleus distorts the shape of the nucleus. This was frequently seen. One desquamated degenerate cell is detaching itself from the epithelium (arrow). X 2,250.

PLATES 89 & 90. SEMs of the surface of epithelium from birds five weeks after infection by the aerosol. The surface is uniformly and densely covered with cilia of uniform length. X 850 and X 4,250.

PLATE 91. TEM showing the ultrastructural features of a tracheal epithelium sampled five weeks after infection by aerosol. The mitochondria are slightly elongated but distinctly less vacuolated. The nuclei appear more regular after having lost the large vacuolar structures that were sometimes associated with them. The cytoplasm is also less vacuolated and more normal-looking. X 6,300.
The mitochondria were more distinct by staining more heavily, and became elongated and regained their specialized internal structures. The nuclei of these cells became slightly irregular in shape and stained more heavily than before while the large vacuolar structures had disappeared from the majority of the cells.

In the submucosa, the lamina gained more order and its collagen fibrils were seen in bundles running parallel to each (PLATE 93).

The epithelia of all the other groups of birds which were exposed to aerosols of the virus, the mycoplasma or mixtures of the two parasites showed reactions similar to those described above which started with a rapid degeneration and followed by hyperplasia and recovery stages. No distinct differences occurred between the controls and the infected birds, or among the various infected groups themselves except for the occurrence of some deciliated areas in the virus-infected group which was revealed by scanning electron microscopy, on specimens from birds killed three weeks after aerosolization (PLATES 94 & 95). However, neither the mycoplasma nor the virus were seen in these studies despite the repeated trials on several different sections from each specimen, which were taken from different areas.
PLATE 92. TEM showing characteristically elongated epithelial cells covered with cilia and microvilli. Although the fine structure of the organelles of some of these cells appears slightly abnormal the pathological reaction is much less severe than before. X 3,500.

PLATE 93. TEM of a section through the lamina propria showing that it is nearly normal five weeks after aerosolization. X 4,050.
PLATES 94 & 95. SEMs showing some de-ciliated areas of the epithelium in specimens taken during the third week after infection with virus. Some cells retained their cilia and the rounded structures are spheres of mucus. X 850 each.
The observations made in this investigation raise a number of interesting speculations. These concern the relationship of either the virus or mycoplasma to the explant tissue and the interaction of these organisms on each other as well as mutually on the tissue. In addition some observations on ultrastructure and overall morphology of *M. gallisepticum* has stimulated speculations about structural and functional relationships.

With regard to the virus-tissue studies, there are no other reports on the ultrastructure of CELO virus in avian tracheal explants. However, there are several studies on CELO virus infections in various other avian tissues. Poteck et al. (1963; 1964) and Jasty et al. (1973) studied CELO virus replication in chick embryo kidney cells and Maeda et al. (1967) studied the morphological and intranuclear changes of these cells after infection. Similar studies were performed on CELO virus-infected chorioallantoic membranes of chicken embryos (Slifkin et al.; 1971) and hepatocytes of virus-infected chicken (Rinaldi et al. 1968).

In my studies, in contrast to that of others, no virus was detected until as late as 48 hr. after infection which was 24 hr. later than that observed by Slifkin et al. (1971) in endodermal cells of chorioallantoic membranes of embryos, and Jasty et al.
(1973) reported mature forms of the virus in the nuclei of infected chicken embryo kidney cells as early as 18 hr. after infection. Furthermore, Jasty et al. (1973) observed the release of virus into the cytoplasm of their chicken embryo kidney cells through breaks in the nuclear membrane or through vesicles derived from the nuclear membrane with subsequent release of the virus into the medium or surrounding cells after disintegration of the cytoplasmic contents.

In my study the virus was strictly associated with the nuclei of the infected ciliated or non-ciliated cells. Indeed the virus was never seen in the cytoplasm of the infected cells and no breaks in the nuclear membrane whatsoever were encountered. This lack of spread of CELO virus in the respiratory epithelium correlated with the limited degree of damage which occurred to the ciliated epithelium of the explants and it has also been noted by Barski et al. (1957; 1959) and Hoorn (1964).

Both *M. gallisepticum* and *M. gallinarum* were evidently successfully parasitic on the explants. Furthermore, there was a large degree of dependence on living cultures, at least in the first instance because they did not survive in explant-free medium unless this was substantially enriched with explant-extracts, and even then growth was relatively transient.

The mechanism by which the mycoplasma assimilated
nutrients in supplemented medium was probably different to that employed when the mycoplasma assimilated nutrients directly from the tissue and certainly, a remarkably intimate association was observed between \textit{M. gallisepticum} and the plasma membranes of the epithelium. Even \textit{M. gallinarum} cells were found fairly closely applied to the epithelial and submucosal tissues.

The importance of an intimate association between the mycoplasma and the host cell was postulated by Stanbridge et al. (1971). They believed that a membrane-membrane association or an intracellular environment was necessary to enable the mycoplasma to obtain necessary nutrients, in particular nucleic acid precursors, but they offered no explanation of the mechanism. They also showed that when medium, containing mycoplasma, was removed from the cell cultures, filtered to remove cell debris and re-inoculated, there was little further propagation of the mycoplasma which died within four days.

The ability of different mycoplasma species to grow in cell-free tissue culture media or in conditioned media, i.e. media left in contact with cell cultures for some time was tested by many investigators and they all agreed that the mycoplasmas failed to grow in such media (Carski and Shepard, 1961; Larin et al., 1969;
Pollock et al., 1963; Powlson, 1961; Stanbridge, 1971). However, supplementation of fresh media with yeast extract or nucleic acid precursors supported the growth of the mycoplasmas (Stanbridge et al., 1971; Pollock et al., 1963; Fabricant et al., 1964).

Furthermore, Stanbridge et al. (1971) found that most of the mycoplasma species that were commonly found in cell cultures could be propagated in Eagle's basal medium provided that it was enriched with 10% calf serum and 5-nucleoside diphosphates. It was believed then, that this result suggested that the mycoplasmas were capable of growing in cell cultures as a result of continuous supply of thymidine precursors resulting from the breakdown of the DNA by the deoxyribonuclease of the mycoplasma.

The ability of mycoplasma to grow in tracheal explant-free, conditioned media was also tested but the results were different to those reported above. Cherry and Taylor-Robinson (1970b) conditioned Eagle's basal medium by contact with chicken embryo tracheal explants for seven days and then inoculated it with M. mycoides var. capri. Viable organisms were recovered for 14 days but there was no growth; that is to say the colony count did not rise. A similar study was also reported by Reed (1972b) who conditioned Eagle's medium by contact with tracheal explants from calves or embryos for one day and then inoculated it with M. hyorhinis. Viable organisms were detected with a titer similar to the
original inoculum for up to five days after inoculation and the author concluded that the organ explants released factors into the medium which stabilized the mycoplasma and allowed limited growth. She even tried to identify the nature of these factors and found that treatment of the conditioned media with trypsin abolished their ability to support growth while heating it to 60°C for 30 min. remarkably enhanced the growth of the organisms. Furthermore, chloroform abolished the ability to support growth while dialysis of the media reduced it but did not completely abolish it. However, all the above investigators agreed that the presence of the explant itself supported growth of the mycoplasmas much more efficiently.

It is interesting that both mycoplasma decreased the yield of CELO virus in the tracheal organ explant system, in particular *M. gallinarum* when simultaneously inoculated with the virus but with *M. gallisepticum* only when the mycoplasma was inoculated three days before the virus. In the latter situation, transmission electron microscopy provided evidence that *M. gallisepticum* had, by this time, inflicted marked damage to the target cells of the virus which presumably prevented its replication. The decreased virus yield in the explants which were simultaneously inoculated with *M. gallisepticum* and the virus might be attributed to direct damage of some of the epithelial cells or to early partial impairment of normal cell activity due to the mycoplasma infection.
Observations regarding decreased yields of virus in mixed mycoplasma-virus infections has been reported earlier. Westerberg et al. (1972) used influenza A/PR-8 virus simultaneously inoculated with M. pulmonis in mouse tracheal explants. The titer of the virus dropped off significantly in the mixed-infected explants starting on the fifth day after infection. Unlike this finding, Reed (1971) did not notice any significant effect on the replication of swine influenza A or influenza A WS viruses in porcine or bovine (embryos or calves) tracheal explants infected with strain S7 of M. hyorhinis, two to five days before the viruses.

Contradictory results on virus yields in cell cultures infected or contaminated with different mycoplasmas are fairly well documented (summarized earlier in TABLE 1). However, yields of different types of adenovirus, in mixed virus-mycoplasma studies were always less than those of the controls. Rouse et al. (1963) noticed a marked inhibition of plaque formation by adenovirus type 2 in monolayers of KB, HeLa-S3 or HEp-2 cell cultures contaminated with an unidentified arginine-depleting mycoplasma. Curing the cultures with kanamycin or increasing the arginine concentration in the media of the contaminated cultures restored the ability of the virus to form plaques. Similarly, reduced titers of adenovirus type 2 and type 31 were recorded in HeLa cells infected with M. orale.
M. hominis or M. hyorhinis (Hargreaves and Leach, 1970), and adenovirus type 6 underwent the same effect when it was grown in a continuous cell line of pig embryo, simultaneously inoculated with the virus and an unidentified mycoplasma (originally isolated from the above cell line).

Arginine has been shown to be necessary for the replication of many DNA viruses such as adenovirus type 5 (Russell and Becker, 1968); herpesvirus (Inglis, 1968); SV40 virus (Goldblum et al., 1968); polyoma virus (Winters and Consigli, 1971); poliomyelitis virus (Ackermann and Wahl, 1966); and cytomegalovirus (Minamisoma and Benyesh-Melnick, 1969). Russell and Becker (1968) found that arginine was not required for the early stages of replication of adenovirus but it was essential for the synthesis of viral coat proteins, lack of which produced incomplete uninfected virus. Long before this, Rouse et al. (1963) established that mycoplasmas decreased virus yields by depleting arginine from the medium, and only recently a few investigators reported similar findings. Singer et al. (1970) noticed decreased yields of vaccinia virus in monolayers of hamster embryo fibroblasts and human amnion cells infected with M. arginini (an arginine-utilizing mycoplasma) three days before the virus. The yield of the virus was brought to near normal by enriching the medium with four times the usual arginine concentration. Similarly, Romano and Brankato (1970) were able to restore the activity of measles virus to produce
plaque formation in M. orale-infected kidney cell monolayers of the African Green monkey, by addition of arginine to the medium.

Recently, Slack and Taylor-Robinson (1973) used cultures of human embryo lung fibroblasts and primary cultures of human thyroid cells infected with M. arginini, and four days later they infected them with infectious varicella virus. The infection resulted in complete or almost complete suppression of microplaque development. However, this inhibiting activity was partially interrupted by feeding the cultures with higher concentrations of arginine in the medium.

Little work has been done to test these effects in the tracheal explant system. Cherry, Powell and Feigin (cited by Cherry and Taylor-Robinson, 1973) noted that M. gallinarum (an arginine-utilizer) infection depleted the arginine content of tracheal explant medium (Eagle's basal medium) within 24 hours. Thus, in the situation which was tested in the present investigation, it is logical to think that both the virus and M. gallinarum competed for a limited amount of arginine present in the medium and that the mycoplasma was more efficient in utilizing it. However, this might not be the exact or only mechanism involved in preventing the replication of the virus because, as already noted, tissue damage may be responsible. Since
M. gallisepticum does not utilize arginine (Stanbridge, 1971), the inhibitory effect it produced on the virus must be attributed to direct damage of the host tissue or to the alterations of basic processes of cell metabolism.

Although M. gallisepticum and M. gallinarum affected the final yield of CELO virus in the tracheal explants by one way or another, the virus did not seem to have affected the growth of either one of the mycoplasmas. This finding is in contrast to the general trend in the intact animals where virus infections are believed to cause considerable damage to the respiratory epithelium which paves the way for more avid growth of the mycoplasma. The slight enhancement of growth of M. gallisepticum recorded when a high dose of CELO virus was inoculated, could have been associated with the presence of the large number of sloughed cells. But, in general, it is not surprising that concurrent virus infection had little effect because of the restricted cell damage incurred.

However, there are some contradictory reports regarding the effect of other viruses on the growth of mycoplasmas in tracheal explants. Westerberg et al. (1972) did not notice any significant changes in the titers of M. pulmonis in mouse tracheal explants simultaneously inoculated with influenza virus and the
mycoplasma, while Reed (1971; 1972b) reported enhanced growth of *M. hyorhinis* in porcine tracheal explants infected with swine-influenza virus two to five days after the mycoplasma infection. Similarly enhanced growth of *M. hyorhinis* was detected in tracheal explants of bovine embryos infected with bovine rhinovirus three days before the mycoplasma.

In my experiments on mixed infections in adult birds, the principle observation was the cytopathology which appeared to result from inoculation trauma rather than effects resulting from infection. Despite the extensive and thorough search for the mycoplasma and the virus, neither was seen, although both were re-isolated (Aghakhan and Pattison, 1974; Butler, personal communication) at low levels.

It would have been of interest to establish which components of the medium were responsible for the cytopathic effects but this was outside the scope of this study.

In both the studies on infected embryonic and adult tissues the transmission and scanning electron microscope provided invaluable comparative information because the two techniques were complementary. Transmission electron microscopy revealed the intracellular cytopathology due to infections as well as the sites of virus replication, and location and ultrastructural details of
the mycoplasma, while scanning electron microscopy revealed the surface changes of the epithelium as well as some details of the overall morphology of the mycoplasmas.

Scanning microscopy has proved very useful for studies in various fields of pathology (Buss, 1973) but only recently has it been used for examination of infections of respiratory epithelia infected with viruses (Reed and Boyde, 1972) or mycoplasma (Biberfeld and Biberfeld, 1972).

The importance of the scanning electron microscope findings presented in my study, mainly relate to the morphology of whole, intact mycoplasma organism grown on the surface of tracheal explant epithelium. The organisms were examined lying on the relatively soft plasma membrane of the tracheal cells without being subject to the mechanical or physical forces necessarily applied when preparations from liquid cultures are examined, that is deposition by centrifugation and washing processes.

Analogous studies have been briefly reported by Biberfeld and Biberfeld (1972) on M. pneumoniae in hamster tracheal organ cultures as well as on monolayer cultures of Chang liver cells. The organisms were shown to be mainly filamentous but when in colonies they were composed of a mixed population of filamentous, pear-
shaped and rounded forms. More recently, Brown et al. (1974) examined LA9, ME180 and BHK cell cultures grown on glass coverslips which had been infected with an unidentified mycoplasma and found mainly spherical forms with dimple-like depression on the surface.

In my studies the scanning electron microscope revealed wide morphological differences as well as differences in size between organisms grown on glass surface and on the surface of the epithelium. The filamentous forms of *M. gallisepticum* and the long cytoplasmic extensions which developed on the surface of glass were never seen on the surface of the epithelium. Similarly, the rod-shaped and deflated pleomorphic structures of *M. gallinarum* in the coverslip preparations were not seen on the surface of the epithelium and it is thought that the morphology of these organisms on the cells is most likely to be close to their actual morphology in nature in the respiratory epithelium of the intact bird since in the two situations the organisms are present on similar tissue.

Although the causes of the differences in morphology of the same mycoplasma species on the surface of glass or the epithelium are not yet fully understood, they might be attributed, fully or in part, to the different conditions of growth used (mycoplasma on the surface of glass was grown in PPLO-liquid growth medium, while those on the surface of epithelium were inoculated in tracheal...
However, if one considers that pleomorphism and the development of long, branched filaments could be induced by lack of unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid, and arachidonic acid) or by decreased concentrations of cholesterol (Razin and Cosenza, 1966; Razin et al., 1966; 1967), then one would expect filamentous forms to prevail on the tracheal explants since they were maintained in a medium which lacked these substances. But the results obtained indicated that the filamentous forms were present almost exclusively on the surface of glass in serum- and yeast- rich medium and not in the tracheal explants. Therefore, it seemed more logical to attribute the various morphological differences to contact of the organisms with the solid surface of the glass especially if the plastic nature of the mycoplasmas and the lack of a rigid cell wall around them are considered.

Preparation of the mycoplasma-infected specimen of explants with minimal application of physical or mechanical forces for scanning electron microscopy minimized creation of artefacts. In fact the variations in the procedures employed in preparation of specimens were held responsible for the contradictory results obtained by various investigators (Freundt, 1969; Boatman, 1973) and the morphology of the mycoplasmas in general is a field of much controversy. For example, *M. gallisepticum* is one of the most extensively studied
mycoplasmas and its pear-shape or tear-drop morphology is fairly well documented. However, many investigators reported many other forms for this mycoplasma which were considered by Freundt (1969) and Maniloff & Morowitz (1972) to be due to deformation caused by the variety of physical forces and chemical agents used or applied on the mycoplasma during preparation. Hence, spherical shapes of \textit{M. gallisepticum} were seen when the organisms were centrifuged before they were fixed chemically (Maniloff et al., 1965a) while filamentous forms appeared when they were exposed to hypotonic solutions during preparation (Bernstein-Ziv, 1971). Also, a variety of distorted shapes resulted when unfixed mycoplasmas were stained by the negative technique (Bernstein-Ziv, 1969; Maniloff et al., 1965a) while various forms with many bleb structures appeared when the mycoplasma was grown on solid surfaces (Maniloff and Morowitz, 1967). Clark (1965) examined the morphology of \textit{M. gallisepticum} sedimented from broth culture on glass coverslips or onto electron microscope grids by centrifugation and noticed predominance of spherical shapes, while observations on living, unfixed cultures using phase-contrast microscopy at various intervals during growth and immediately after settling of the cells on the surface of a glass slide revealed branched filamentous forms (Razin and Cosenza, 1966; Razin et al., 1966; 1967).

The technique and the chemical treatment used for specimen preparation which I used probably had little or
no effect on the morphology of the organisms. Washing of the specimens with PBS, fixation with glutaraldehyde and post-fixation with OsO₄ are routinely used in preparations of mycoplasma for electron microscopy and they are known to preserve well their structures (Anderson and Barile, 1965; Hummeler et al., 1965; Deeb and Kenny, 1967). However, the effect of the very short rinses of the ring explants with distilled water (maximum of 2 min.) on the morphology of the mycoplasma was difficult to assess in this study (short rinses with distilled water were necessary to wash away traces of the PBS which tended to deposit salt crystals on the surface of the epithelium), but Clyde (1969) noticed only slight alterations in the morphology of M. pharyngis after incubation for 1 hour in distilled water.

A possible source of artefact-production in the techniques used in specimen preparation for scanning microscopy was the dehydration procedure in the graded alcohols, particularly the last step in the procedure where the specimens were left to dry in air for a few minutes after the final 100% ethanol rinse and before they were finally metal-coated (see appendix 2). However, no obvious differences in the morphology of M. gallisepticum were seen between specimens dehydrated in graded alcohols and specimens dehydrated by freeze-drying. Actually, M. gallisepticum cells treated by either one of the above techniques, appeared relatively tough because collapsed or ghost cells were never observed. This
preservation of the overall morphology of \textit{M. gallisepticum} was in contrast to the "hexagonally-shaped" and obviously collapsed forms of \textit{M. gallisepticum} reported by Shifrine et al. (1962) who grew the organisms on collodion film on solid medium and shadowed them with uranium metal before examination in a transmission electron microscope. I observed considerable variation in the morphology of \textit{M. gallisepticum} during the first two or three days after inoculation but spherical or rounded forms predominated in older cultures. Furthermore, pleomorphism was obvious during the early stages of infection and were believed to be mainly associated with activity necessarily involved in cell division.

Filamentous forms of mycoplasma are not only associated with \textit{M. gallisepticum}. Biberfeld and Biberfeld (1970) examined \textit{M. pneumoniae} grown on glass or plastic surfaces and noticed rounded, elongated and filamentous forms. The latter forms were thinner at their ends and terminated with knob-like structures, or with short ramifications. Kammer et al. (1970) reported that the morphology of \textit{M. pneumoniae} grown in suspension and fixed \textit{in situ} underwent an orderly and sequential metamorphosis during its life cycle where relatively small spherical forms developed into filamentous forms and then to larger round forms. They also found branching filaments with bulbous elements situated at intervals along the length of the filaments. Similar sequential metamorphosis and
morphological manifestations were also recently reported by Klainer and Pollack (1973) on cultures of *M. pneumoniae* grown in suspension and fixed after repeated washing. Filamentous forms of *M. pneumoniae* were also reported in studies of ultrathin section (Biberfeld and Biberfeld, 1970; 1972; Collier, 1972) and this particular form is considered the major morphology feature of this mycoplasma. In contrast to this, although studies on thin sections revealed some elongated and nearly filamentous forms of *M. gallisepticum* on the tracheal epithelium, they were relatively uncommon.

The prototype mycoplasma species, *M. mycoides* var. *mycoides* exhibits the most pronounced filamentation. Freundt (1969) presented electron micrographs of young cultures (12 - 24 hrs.) of this mycoplasma which was fixed and then centrifuged before shadowing, and revealed branched filaments and filaments showing regularly spaced constrictions. A much less branching, but filamentous mycoplasma was *M. pharyngis* sedimented from broth culture and fixed before shadowing (Clyde, 1969). Recently, Klainer and Pollack (1973) compared the morphology of a number of *Mycoplasma*, *Acholeplasma* and *Uronplasma* species. Strains A and B of *Acholeplasma laidlawii* showed spherical forms. However, strain A displayed a wide variety of sizes while strain B exhibited imbricate surface texture. *Acholeplasma*

* New Classification of *Mycoplasmataceae* "Bergey's Manual" (Freundt, 1974; cited by Razin, 1974)
granularum appeared like deflated balloons of various sizes and were highly pleomorphic. They noticed remarkable differences between certain T-mycoplasmas, thus T-strain 960-19 appeared spherical while T-strain K510-20 displayed rod-shaped and helical-like forms. However, they noticed filamentous forms similar to those of M. pneumoniae in all the cultures of mycoplasma they studied.

Unlike M. gallisepticum, scanning electron microscopy studies on M. gallinarum grown on the surface of the epithelium were not too successful. This was attributed to the fact that the number of organisms detected was much less than that expected and the cells that were seen were mostly located in deep and convoluted areas where the electron beam did not illuminate satisfactorily. It was presumed that the organisms could be lost during the washing procedure particularly after transmission electron microscope observations on thin sections of infected tracheal explants revealed no intimate association with the cell surface as that observed with M. gallisepticum.

The value of tracheal explants in pathogenicity studies has already been noted in the introduction to this thesis and the importance of this system for mycoplasma studies is increasing. Recently, Cherry and Taylor-Robinson (1973) reviewed their own numerous investigations on this subject which included a
comparison of the growth levels and the effects of 58 strains of mycoplasma of various origins on the ciliated epithelium of chicken embryo tracheal explants. The majority of the strains tested grew readily in this system but not all of them affected the epithelium. Those that damaged the epithelium caused inhibition of the ciliary activity and the rate of damage produced accompanied the growth of the organisms. Similarly, Collier et al. (1969) examined the effects of M. pneumoniae and some other human mycoplasmas, but only M. pneumoniae produced pronounced inhibitory effect on the ciliary activity and damaged the epithelium. Almost exactly similar results were detected in human foetal explants infected with M. pneumoniae (Collier, 1972) and with M. mycoides var. capri (Butler, 1969a; 1969b).

Cherry and Taylor-Robinson (1970a) did not detect an adverse effect on the ciliary activity of chicken tracheal explants infected with each of five strains of M. pneumoniae but in a subsequent work (1973) they reported loss of ciliary activity in the same explant system due to infection with the same strains of M. pneumoniae. They claimed that these contradictory results were probably due to their acquisition of a better quality of organ explants in the second experiment.

Butler and Ellaway (1971) compared the growth and the pathogenicity of ten different mycoplasma species in human foetal and chicken embryo tracheal explants and found that
M. gallisepticum and M. mycoides var. capri resulted in severest cytopathic effect in both systems.

In my investigations M. gallinarum as well as M. gallisepticum was found to be pathogenic to chicken embryo tracheal explants, despite the fact that M. gallinarum was widely known as a non-pathogenic species to the intact bird. Although no definite explanation to this effect exists, it could be due to many factors in such an isolated system such as absence of the effective defence mechanisms in the tracheal explant. For example, lack of a thick moving mucus blanket between the organisms and the surface of the cells, absence of macrophages or phagocytes, and antibody circulation, lack of co-ordinated rhythmic ciliary beat as well as lack of a rapid turnover of cells to fill in the places of sloughed cells due to the inability of the basal cells to regenerate and mature to ciliated cells, hence the organisms gain access to the intercellular spaces and to the submucosa. Other factors may also be important in increasing the susceptibility of the epithelial cells to the mycoplasma infection, may be the accumulation of cell- or mycoplasma-metabolites like hydrogen peroxide (Cherry and Taylor-Robinson, 1970b; 1971), or depletion of certain substrates in the medium by the mycoplasma which probably affects an important or a vital activity of the cell. Indeed, Cherry (cited by Cherry and Taylor-Robinson, 1973) have noticed that M. gallinarum was capable of depleting the arginine contents of tracheal explant medium (Eagle's basal
medium) within 24 hours.

The cytopathology induced in the tracheal epithelium due to the infection with *M. gallinarum* might also be a subsequent effect to invasion of the organisms the lamina propria and their rapid multiplication there, since more organisms were detected in the lamina than on the epithelium. The concentration of the mycoplasma or their metabolites might have induced the formation of the darkly-staining structures that were characteristic of *M. gallinarum* infections as early as two days after inoculation. In fact, the presence of *M. gallinarum* organisms in the lamina propria of the infected explants could probably explain the persistence of *M. gallinarum* infection for more than 40 days noticed by Taylor-Robinson and Cherry (1972) despite the fact that the medium was changed frequently.

In contrast to the results obtained in my study, Taylor-Robinson and Cherry (1972) claimed that *M. gallinarum* enhanced longevity of the ciliary activity of the explants in which 50% loss of ciliary activity was noticed 40 days after inoculation as compared with 20 days in control, uninoculated explants. They also noticed that this species protected the epithelium against the damaging effect of *M. gallisepticum* and that it delayed the ciliostatic effect of the latter provided that viable *M. gallinarum* organisms were inoculated 24 to 48 hr. before *M. gallisepticum*. They even obtained some evidence that the cilia-stopping effect of *M. mycoides* var. *capri* was also
inhibited by *M. gallinarum*. At some stage, this effect was believed to be due to reduced growth of *M. gallisepticum* in the mixed infections, but when this was tested they found that actually this was not affected. Another explanation they offered for this effect was that *M. gallinarum* would have formed a barrier between *M. gallisepticum* and the ciliated epithelium. However, they favoured more the opinion that the longevity effect could be due to production of substances by *M. gallinarum* which destroyed peroxides in the tracheal explants. They supported this argument by the fact that they found less peroxide in *M. gallinarum*-infected explants than in the uninoculated controls, which they held responsible, at least in part, for the eventual autolysis of the explants (Cherry and Taylor-Robinson, 1970c). Although the differences between the results obtained in this investigation on *M. gallinarum* infection in chicken embryo tracheal explants and those reported by Taylor-Robinson and Cherry (1972) might be attributed to the frequent changes of medium (8 times) in their experiments or to the possible pathogenicity differences between the two strains of *M. gallinarum* used in the two investigations, yet the differences were great. However, their evidence of lack of pathological effect was based on light microscope observation on living cultures which of course had its own limitations when compared to the evidence obtained by transmission electron microscopy.

The effect of *M. gallisepeticum* which I observed
confirmed and extended the observations reported earlier by Cherry and Taylor-Robinson (1970b; 1970c) and Butler and Ellaway (1971). The organisms consistently caused ciliostasis and damage to the epithelium, regardless of the number of the viable organisms inoculated. However, this effect on the epithelium was found related to the inoculum size and the greater the number of viable organisms the faster this effect occurred. In contrast to this, Cherry and Taylor-Robinson (1970b) did not notice a dose-dependent relationship between M. gallisepticum and ciliostasis, while this relationship was recorded for M. mycoides var. capri in chicken embryo tracheal explants (Cherry and Taylor-Robinson, 1970b; 1973).

The ultrastructural details I observed of the cytopathology induced by M. gallisepticum in the tracheal explants were very similar to those induced by M. pneumoniae (Collier et al., 1969; 1971) in hamster tracheal explants. Like M. gallisepticum, M. pneumoniae caused loss of cilia, cytoplasmic vacuolization, nuclear enlargement with clumping and margination of the chromatin material. Detachment of epithelial cells and breaks in the cell-binding systems such as the terminal bars were also noticed and the final appearance of the damaged epithelium resembled that infected with M. gallisepticum. Similarly, during the early stages of infection with M. pneumoniae, numerous organisms were found among the cilia closely associated to the epithelial cell membranes and a few days later, mycoplasma microcolonies spread into the intercellular spaces between adjacent cells.
However, the above pathologic response was associated with virulent strains of *M. pneumoniae* only and was not detected with other mycoplasmas of human origin. The above investigators suggested that the mechanism of tissue injury is possibly associated with the intimate association between the mycoplasma and the epithelial cell membrane. The presence of numerous organisms among the cilia could interfere physically with normal beating, and injury of the cell membrane could result in disturbance of surface charges needed for synchrony of ciliary motion. They also suspected possible nutritional deprivation of host cells; by membrane injury or metabolic competition between the host and the parasite as additional means of functional impediment.

The invasion of the submucosa of tracheal explants which I found with *M. gallisepticum* and *M. gallinarum* is interesting although it is, perhaps, unlikely to occur in the living animal. However, the apparent probing activity demonstrated by *M. gallisepticum* may be responsible for pathogenicity in the natural host. One could speculate that a burrowing propensity could lead to systemic infections because once the lamina propria is invaded, access to the blood stream could be expected.

The causes of the pathogenicity of mycoplasma and the pathogenesis they induce in animals is poorly understood. Many investigators have concentrated their efforts to demonstrate presence of toxins as products of mycoplasma which could cause or have a role in mycoplasma infections.
and the production of disease. However, the only mycoplasma known to produce a soluble toxin is *M. neurolyticum* which causes rolling disease of mice, (Thomas, 1967; 1970; Kaklamanis and Thomas, 1970 and many others). Other mycoplasmas, for example, *M. pulmonis* and *M. arthritidis*, produced toxic-like effects (Kaklamanis and Thomas, 1970), but the exact nature of the toxic factor is not known. The toxic effect of the S6 strain of *M. gallisepticum* which follows intravenous injection of turkey poults with viable organisms (for details see review of the literature) was thought to be due to an active toxin. However, repeated attempts failed to demonstrate an exotoxin in cultures of this mycoplasma, and more recent research has focused attention on the striking arterio tropism of *M. gallisepticum* organisms which was shown by Clyde and Thomas (1973b) to be limited to the central nervous system and the joints of turkeys.

Cherry and Taylor-Robinson (1971, 1973) attempted to demonstrate a toxin of *M. gallisepticum* which could be responsible for the inhibition of ciliary activity of tracheal explants. They infected explants with medium in which *M. gallisepticum* had been grown and then suppressed by tetracycline. They noticed that the mycoplasma-free medium contained a factor that was slightly toxic to the ciliated epithelium and affected the activity of the explants but not as drastically as when viable organisms were present. More recent studies by Cherry (cited by Cherry and Taylor-Robinson, 1973) have shown
that this "toxin" passed through a 100µm filter that retained the organisms.

Many other factors which could have any bearing on the subject of mycoplasma pathogenicity were tested and explored. Indeed, soon after haemolysin activity of *M. pneumoniae* was shown to be due to hydrogen peroxide production by this mycoplasma (Somerson et al., 1965; Cohen and Somerson, 1967), these investigators, as well as Soberslavsky & Chanock (1968) and Cole et al. (1968) suggested that hydrogen peroxide might be an important factor for the pathogenicity and virulence of various mycoplasma infections and therefore many mycoplasma species were tested for the production of this chemical substance. Among the mycoplasma species which produced hydrogen peroxide were *M. gallisepticum* (Thomas and Bitensky, 1966); *M. pulmonis, M. felis, M. gallinarum, M. arthritidis, M. mycoides var. capri, M. neurolyticum, M. bovigenitalium,* and *A. laidlawii* type B (Cole et al., 1968). These results which were of interest to Cherry and Taylor-Robinson made them test and measure hydrogen peroxide production by *M. mycoides var. capri* (1970b), *M. gallinarum, M. gallisepticum* and many other mycoplasma species (1970c) in the tracheal explant system of chicken embryos, and peroxide production was demonstrated by all the above mycoplasmas. However, explants infected with *M. gallisepticum* or *M. mycoides var. capri* produced more peroxide than the uninfected control explants, while those infected with *M. gallinarum* produced less peroxide than the controls. They also found that mere addition of hydrogen peroxide to uninfected explants decreased their ciliary activity, while addition of catalase,
an enzyme which attacks peroxides, protected the ciliary activity against the cilia-static effect of *M. mycoides* var. *capri* but failed to do so with *M. gallisepticum*. A support to the hypothesis of the role of hydrogen peroxide in the virulence of mycoplasma came from Brennan and Feinstein (1969). These investigators used a strain of mice essentially devoid of catalase activity and infected them with *M. pulmonis*. They noticed that this group of animals experienced more pneumonic reaction 3 days after infection than did wild-type mice, but at 5 days after infection the acatalatic mice had fewer incidences of pneumonia. This latter phenomenon suggested that in the absence of tissue catalase, the accumulated hydrogen peroxide became lethal to the mycoplasma itself. However, peroxide production alone was not considered a determinant of virulence since pathogenic and non-pathogenic mycoplasmas produced it with quantitative differences (Whittlestone, 1972).

The general trend of close association between the various species of mycoplasma and the host tissues reported by many investigators was believed to have some bearing on the problem of mycoplasma pathogenicity. Sobeslavsky et al. (1968) suggested that pathogenesis might follow a sequence of organism adsorption followed by peroxide liberation. Whittlestone (1972) went even further and put the probability that the close association between *M. pneumoniae* and cell membranes noted by Collier and
Clyde (1971) or the attachment at such sites by neuraminic acid receptors (Sobeslavsky et al., 1968) might make the secreted hydrogen peroxide more effective in damaging the host cell particularly if the peroxide attacks the tissue before being destroyed by catalase or peroxidases present in the extracellular body fluids. He also suggested that this would also apply to pathogenic respiratory mycoplasmas of domestic and laboratory animals.

Sobeslavsky et al. (1968) believed that association of mycoplasma with the cell surface should be mediated by specific receptor sites on the cell membranes, and indeed they have shown that *M. pneumoniae* was unique among human mycoplasmas in that they attached to erythrocytes and to cells of tracheal epithelia by means of neuraminic acid receptor sites present on the surface of these cells. A little earlier, Taylor-Robinson and Manchee (1967a; 1967b) noticed that some mycoplasma species adsorbed to spermatozoa and HeLa cells of bovine and human origin and suggested that the receptor sites might be sialic acid sites similar to those reported by Gesner and Thomas (1966) and Roberts (1967) to be present on red blood cells which bound mycoplasmas. However, in a more recent work, Manchee and Taylor-Robinson (1969) tested the ability of neuraminidase-treated erythrocytes and HeLa cells to adsorb to mycoplasma colonies of 17 mycoplasma serotypes and they found that only *M. pneumoniae*, *M. gallisepticum*, *M. synoviae* and mycoplasma WR1 used the neuraminic acid receptors for adsorption. Further work with
HeLa cells with and without neuraminidase treatment showed that the cells readily adsorbed to colonies of *M. mycoides* var. *capri* (Cherry and Taylor-Robinson, 1970b).

The role of such receptor sites was only recently tested in the tracheal organ explant system. Cherry and Taylor-Robinson (1971) incorporated neuraminidase in the medium of chicken embryo tracheal explants infected with *M. gallisepticum*. This treatment did not prolong the time for 50% reduction of ciliary activity, that is, it did not increase or decrease the rate of adsorption of the organisms. Similarly, chicken tracheal epithelial cells with or without neuraminidase treatment did not adsorb to colonies of *M. mycoides* var. *capri* (Cherry and Taylor-Robinson, 1970b). Therefore, these investigators (Cherry and Taylor-Robinson, 1973) concluded that in neither infection was cytadsorption an important pathogenicity factor. However, unlike the above findings, virulent strains of *M. pneumonialae* seemed to require neuraminic acid receptor sites on hamster tracheal epithelium (Collier and Baseman, 1973). Neuraminidase-treated explants showed significantly lowered attachment of the virulent strain of the mycoplasma while the attachment of the avirulent strain was not affected. This particular observation suggested that two different mechanisms of attachment exist. However, these investigators believed that the virulent and the avirulent strains of *M. pneumonialae* definitely have attachment mechanisms, yet unknown, other than sialic acid receptors.
The extracellular distribution and localization of the two avian mycoplasma species *M. gallisepticum* and *M. gallinarum* is an interesting finding of my study. However, of more interest is the fact that the ways these two species localized themselves around the cells were different which might reflect on the mode of behaviour of these mycoplasmas when attacking the host tissue and on their different pathogenicities. *M. gallisepticum* adhered to the cell surface and favoured an intimate association with it while *M. gallinarum* did not show a similar preference to the cell surface and localized a little away from it. Furthermore, *M. gallisepticum* organisms employed their specialized parts, the bleb structures, to attach to the epithelial cells, while *M. gallinarum* did not seem to possess such a speciality. It is apparent that the terminal bleb structure of *M. gallisepticum* is a very important part of the organism and has many functions. This study has shown clearly that the bleb structure is the means of attachment of the organism with the host epithelial cell surface. A finding similar to this has been reported earlier by Zucker-Franklin et al. (1966a, 1966b) in monolayers of HeLa cells and leukocytes in cell culture infected with *M. gallisepticum* where the organisms displayed polarity and attached to the cells using their bleb structures as the binding sites. During the course of the present investigation, Uppal (1972) also presented evidence that *M. gallisepticum* organisms attached to the cell surface of tracheal epithelium of adult chicken by means of their terminal blebs. I have shown that the
organisms were oriented preferentially with their longitudinal axes at almost right angles to the cell surface with their blebs in intimate association with the plasma membrane causing slight or deep invagination in the latter.

The intracellular granular structures of *M. gallisepticum* noticed in my study were also seen by many investigators and they were believed to be ribosomes (Maniloff et al., 1965a; 1965b; Maniloff and Morowitz, 1972), but their exact function is not clear yet. The striking uniform, striated and columnar arrangement of these structures in certain preparations has been observed by some investigators (Maniloff et al., 1965a; 1965b; Domermuth et al., 1964; Bernstein-Ziv, 1969; Allen et al., 1970) and they were considered ribosomal clusters (also known as polysomes) or ribosome superstructures. Maniloff (1971; 1972) and Maniloff and Morowitz (1972) believed that they are not polysome condensations but artifacts produced during preparation, particularly due to centrifugation employed in the pelleting of the organisms (Maniloff, 1971). In contrast to this, these ribosomal superstructures appeared in my studies in preparations that did not experience any centrifugation. The regular and columnar forms of these structures were seen quite often in this study and in many instances, they were directed to meet in the bleb and infra-bleb region. This oriented arrangement of these structures was particularly obvious in organisms attached to or close to
the epithelial cells, and I think that they might have a role in the motility of this mycoplasma particularly if one is allowed to make an analogy with the role of the contractile proteins surrounding the tail of a bacteriophage. Indeed, biochemical analysis of the ribosomes of *M. gallisepticum* indicated that they contained about 60% RNA and 40% protein (Maniloff and Morowitz, 1972). The hollow and tubular ribosomal superstructures seen in my study were also encountered by Maniloff (1965a) and Allen et al. (1970), but in these studies they appeared as helical forms. Analysis of these helical structures by optical diffraction and rotational symmetry indicated that the helix was repeatable (510 Å) and that it was composed of 10 ribosomes in 3 turns and the helices have been shown to be self-assembly structures of 70S ribosomes (Maniloff, 1971). However, so far, these structures are considered unique for *M. gallisepticum*.

*M. gallisepticum* is not unique among the various species of mycoplasma in having terminal structures, but together, with *M. pneumonieae*, which is also endowed with terminal structures form the only two species of mycoplasma which possess these highly specialized structures. The role of the terminal structure of *M. pneumonieae* is apparently similar to that of *M. gallisepticum* and it has been shown (Collier, 1972) that it was the site of intimate association with the surface of the ciliated epithelium of human foetal tracheal explants. The terminal structure of *M. gallisepticum* appeared also to possess an inherent tendency to direct the organism towards the host tissue.
as if attracted to them by some sort of a force which resulted in stretching of the organisms. Similar observations by Zuker-Franklin (1966a; 1966b) suggested a positive taxis exerted by the mammalian cell on the mycoplasma and that the relationship between the two seemed to deserve the term reciprocal chemotaxis. However, the exact nature of this "cell-likeness" or taxis is not yet fully understood.

Another interesting finding about the role of the terminal structure of *M. gallisepticum* noticed in my study is its apparent ability to exert pressure on the plasma membrane of the cell and even to burrow deeply in the cell by pushing the cell membrane inwards or breaking its way through the basement membrane into the lamina. This particular activity of the bleb structure seems to be a speciality of *M. gallisepticum* since similar, high resolution electron microscope studies on *M. pneumoniae*-infected human foetal tracheal explants did not reveal a similar phenomena. In fact my electron micrographs provide very suggestive evidence for a motile nature of this mycoplasma which may lead to the above burrowing phenomenon. Indeed, actual motility of *M. gallisepticum* organisms grown on cover slips and examined by various optical methods including phase-contrast, differential interference contrast and micro cinematography was only recently reported (Bredt, 1972; 1973). In these studies, the terminal structures or the tip-like ends of *M. gallisepticum* (the blebs); *M. pneumoniae* and *M. pulmonis*.

*Bredt (1973) was the only investigator who reported the presence of a terminal structure of *M. pulmonis*. 
were seen leading the rest of the body of the mycoplasma in the direction of movement. This gliding type of motility was a regular property of *M. gallisepticum* and *M. pneumoniae* while *M. pulmonis* lost this property after few passages.

In his recent study, Collier (1972) claimed that "evidence has been presented which suggests that a portion of the terminal structure (of *M. pneumoniae*) may be able to penetrate the unit membrane of the host cell, thus destroying the integrity of the membrane and leading to entrance of extracellular materials or extrusion of intracellular material". Although no direct corroboration of this was observed in my study with *M. gallisepticum*, the evidence did not contradict this view.

The possibility of the terminal structure of *M. gallisepticum* playing a role as an organ for absorption of nutrients is strongly suggested in my study. The fact that the organisms did not grow in the tracheal organ explant medium unless explants were present, and the obvious evidence of intimate association between the host and the parasite mediated by the bleb structure implied a nutritive role. But whether nutrients (intracellular material) are "extruded" by a break through plasma membrane as mentioned above (Collier, 1972) or whether they are "diffused" through the cell membranes across the sites of the intimate cell–mycoplasma association (Zucker-Franklin et al., 1966a) is not clear.
The latter investigators questioned also whether the mycoplasma was able to utilize structural components of the cell membrane which may make available the lipoproteins essential for the mycoplasma in presynthesized form.

Besides the above functions of the terminal structures of *M. gallisepticum*, Morowitz & Maniloff (1966) and Maniloff & Morowitz (1967) presented evidence based on electron microscope studies of thin sections suggesting that these structures were important in the cell division of the mycoplasma. During the life cycle of the organism, two bleb structures were believed to develop at opposite poles before the binary fission cell division took place. However, in more recent studies, Quinlan and Maniloff (1972) found that these structures also contained the DNA growing region and appeared to be involved in DNA replication. Furthermore, the internal regions of the bleb structure were shown to be sites of various enzymic activities. Munkers and Wachtel (1967) performed histochemical studies on *M. gallisepticum* and demonstrated that adenosine triphosphatase activity was localized in the bleb and infra-bleb regions exclusively, while acid phosphatase activity was restricted to the infra-bleb region and was not membrane associated. Maniloff (1972) used similar techniques and showed also that the central core of the infra-bleb region and the periphery of the bleb itself were rich in basic proteins, and using tellurite
as an electron acceptor, Maniloff (1972) localized redox activity in the infra-bleb region.

In conclusion it is quite clear that the specialized terminal structures of *M. gallisepticum* and *M. pneumoniæ* deserve close attention. Understanding their function might well prove very important and contribute definitively to the understanding of the problem of pathogenicity of these mycoplasmas. Apart from high resolution electron microscopy, autoradiography and histochemistry will be regarded to resolve this problem. Indeed, specific enzymic reactions and specific histochemical reactions should be examined in ultrathin sections of unfixed, frozen specimens of infected tissues, now that the technique of cryoultrrotomy has become available.
REFERENCES


Appendix 1

Occurrence of virus-like particles in trachea of non-SPF chicken embryos.

Early during the course of this research, transmission electron microscope studies on thin sections of chicken embryo tracheal explants from non-SPF flocks revealed numerous virus-like particles distributed in the various tissues of the trachea. Two morphologically different types of such particles were found (PLATES 96 & 97). The first type was particularly concentrated extracellularly in the matrix of the cartilage tissue, and to a much lesser extent, in clusters inside vacuoles in the chondrocytes. Although these particles were abundant in some areas, they did not seem to have produced any noticeable cytopathological effect. Each particle appeared spherical with a well defined boundary, and a densely-staining core surrounded by a slightly less dense zone. The second type of particles were more concentrated in the loose tissue of the lamina propria among the collagen fibres. Some were also seen close to the basal cell layer and among the epithelial tissue, but no obvious cytopathological signs were detected. These particles were also spherical but they did not have well defined boundaries and they were of a more diffuse nature, and the whole body stained uniformly.

An attempt to examine these virus-like particles was made in collaboration with Dr. J.D. Almeida by
PLATE 96. TEM of an apparently normal chondrocyte with few virus-like particles in the cartilage matrix close to the cell (long arrow). This specimen was prepared from the cartilage tissue of chicken embryo tracheal explant of non-SPF parents. The virus-like particles are spherical with a dark-staining core. Inside the cell, normal-looking organells such as the nucleus, endoplasmic reticulum, ribosomes and (short arrows) mitochondria are obvious.

PLATE 97. TEM showing few densely-staining spherical structures (presumably virions) distributed among the collagen fibres of a part of the lamina propria. The specimen was prepared from chicken embryo tracheal explants from non-SPF flocks.
crushing tracheal explants in a few drops of distilled water with a conical, blunt-ended glass rod in a glass container, and the tissue exudate was mixed with the negative stain (Phosphotungstic acid), as described earlier and examined in Philips E.M. 300 transmission electron microscope. Numerous virus-like particles were seen of various morphologies but they were all surrounded by evenly spaced spike-like projections of uniform lengths. The particles were either spherical or spherical with a tail at the end of which a knob-like structure was present. Although characterisation of these particles was not attempted, they were believed to be avian leukosis viruses (Almeida, personal communication).
Appendix 2

Special techniques for specimen metal-coating

During the course of this study, it has been noticed that the coating technique employed with the mycoplasma and the tracheal explants in order to make them electrically conductive for scanning electron microscopy was very important and critical. Reproducible results showing clearly the mycoplasma cells on the surface of the epithelium as well as distinct cilia and microvilli were obtained only when the thickness of the metal cast (gold/palladium) used was approximately 200 Å thick. Insufficient coating resulted in charging of the specimens which produced intense glare and prevented resolution and proper image-tracing by photography. However, extra coating covered the mycoplasma cells and the surface structures of the epithelium and obscured their details. Another factor which was found of great importance on the final outcome of the final morphology of a specimen was the effect of heat radiated from the source of evaporation of the metal (heated by electricity until white-hot). To avoid this effect the specimens were placed as far away as possible from the evaporation-source and coated while rotating.