

ETIOLOGY OF CHRONIC MURINE PNEUMONIA

by

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### CLAIMS TO ORIGINAL CONTRIBUTION

The etiology of endemic pneumonia in rats has been investigated, and the specific cause of the condition was established. A viral agent was isolated in rat embryo skin primary tissue cultures, and postulates have been fulfilled for identifying this viral agent with endemic pneumonia in rats. Some characteristics of this virus were established. A method for demonstrating the cytopathogenic agent, involving two-step incubation of inoculated tissue cultures at two different temperatures has been described. This technique was successfully employed for the isolation of the virus responsible for endemic pneumonia in rats.

## INTRODUCTION

## INTRODUCTION

Chronic murine pneumonia, comprises two closely associated disease entities, namely "infectious catarrh" and "endemic pneumonia". In laboratories where large numbers of rats and mice are used for experimental work involving toxic inhalants or in chronic toxicity work, the morbidity due to this chronic pulmonary disease is a perpetual problem, since the changes caused in lungs due to experimental manipulations cannot be distinguished from those resulting from, or complicated by, natural disease. Furthermore, the diseased animals might respond in an altered fashion to experimental manipulations and the presence of Pleuropneumonia-Like-Organisms and/or the virus of endemic pneumonia may mask or modify the activity of another agent under investigation, hence resulting in erroneous results. Correspondingly, the recognition and control of chronic murine pneumonia are essential in the maintenance of a breeding colony.

Endemic pneumonia or enzootic bronchiectasis is indigenous to both rats and mice, and most diseased rodents exhibit few if any clinical signs of respiratory disease. The severity of the morbid process is not necessarily

reflected by severe clinical signs and many observers have expressed astonishment at the extent of pulmonary disease in rats which appeared in good condition when alive. The chief feature of endemic pneumonia is peribronchial lymphoid infiltration and much of the histologic picture in endemic pneumonia can be accounted for by the variation in the degree of lymphoid infiltration. These lesions begin very early in life, since microscopic lymphoid collections appeared in some suckling mice.

The etiological agent responsible for infectious catarrh is a pleuropneumonia-like-organism (P.P.L.O.) but the agent causing endemic pneumonia has not been isolated. No single bacterium was implicated and the latest evidence indicates that the agent is filterable and fulfils the postulates for identifying it as the specific cause of experimentally produced enzootic bronchiectasis in mice. As the agent appears to be a frequent resident of rat lung in endemic pneumonia infection, possibly the same virus-like pathogen is involved.

The present investigation was undertaken, first to isolate the etiological agent of endemic pneumonia in a tissue culture system and then to study its behaviour in vitro and in vivo with the aim of fulfilling the essential postulates to identify the pathogen isolated in tissue culture with endemic pneumonia of rats.

LITERATURE REVIEW



## LITERATURE REVIEW

### DISEASE

#### Incidence and Importance

The complex of respiratory diseases called "chronic murine pneumonia" comprises two closely associated conditions, namely "infectious catarrh" and "endemic pneumonia". While chronic murine pneumonia does not completely prevent the use of rats and mice in experimental work, both Cruickshank (1948) and Innes et al. (1956) have suggested that rats might well be discarded for work involving toxic inhalants, and in chronic toxicity work (Foster, 1958) unless derived from a colony free from this condition. Cruickshank in 1948 wrote:

In the course of some tentative experimental work on lung diseases it was found that the stock laboratory rats employed suffered from a spontaneous disease of the lung which made the experiments useless.

The difficulty of determining changes caused by a toxic inhalant from those resulting from, or complicated by, natural disease is insuperable (Innes et al., 1956). In all laboratories in which large

numbers of rats are used experimentally, morbidity (and of lesser importance, mortality) due to chronic pulmonary disease is a perennial problem and may be more striking in commercially obtained rats, for the animals are then of unknown clinical history and reared under conditions not defined by investigators. Nelson (1958) thought that diseased animals because they are abnormal might respond in an altered fashion to experimental manipulations, for example, he previously showed that the presence of Pleuropneumonia-Like-Organisms (P.P.L.O.) may modify or mask the activity of another agent under investigation, and the agent studied is then placed in competition with the contaminating P.P.L.O. (Nelson, 1951). Accordingly, the recognition and control of chronic murine pneumonia are essential in the maintenance of breeding colonies. Innes et al. (1957) reported that over 50 per cent of the rats in their stock colony showed some degree of chronic murine pneumonia, unaccompanied by tangible clinical signs. Both diseases (infectious catarrh and endemic pneumonia) are widely distributed and inter-transmissible in the two species of rodents. Since both diseases occur in wild rodents, it is probable that they are actually of quite ancient lineage (Nelson, 1955). Zucker (1953) presented evidence for individual variations within a given strain of rats and mice. Several investigators, Klieneberger and Steabben (1940),

Passey et al. (1936), Cruickshank (1948) and Ratcliffe (1949) have reported that on autopsy 40 to 75 per cent of rats from established rat colonies showed lesions of bronchiectasis, a characteristic lesion of what has been variously called enzootic pneumonia, endemic pneumonia, bronchopneumonia, lung abscess, enzootic bronchiectasis; while Innes et al. (1956) suggested that macroscopic appearance alone may not lead to the detection of enzootic pneumonia and the numbers considered normal histologically would be reduced if more critical examination was adopted. For example, if microscopic collections of lymphoid tissue at peribronchial locations were considered pathological, the number of so-called normal lungs would be much smaller (Innes et al., 1956).

The manifestations of infectious catarrh develop earlier in both rats and mice and a mortality rate of 20 per cent is not uncommon in weanlings from an infected colony (Nelson, 1955). Nelson, in the same paper, pointed out that the infected mice chatter and the rats snuffle but these signs are often over-looked and the disease may continue to flourish unnoticed. An infected animal with well-established localized areas of inflammation may appear normal but is a poor experimental subject (Nelson, 1955; Innes et al., 1956). Nelson and Gowen (1930) reported that the middle ear

infection, pneumonia and inflammation of the upper respiratory tract are all related conditions, and do not appear in any definite sequence but noted a marked difference in the incidence of middle ear infection and pneumonia between adults and young rats. The incidence of middle ear infection was 69 and 32 per cent, and that of pneumonia, 81 and 2 per cent, in adult and young rats respectively. Although pneumonia is a chronic condition of slow progress there is ample time for the earliest lesions to appear in young rats. Nelson and Gowen (1930) suggested that the high rate of infection in adults may be determined in part by a decreasing state of immunity, either natural or acquired through maternal transmission. They also suggested that in most instances, middle ear infection is not a secondary manifestation of pneumonia and the two conditions show a parallel tendency in adult rats, while in young rats, middle ear infection commonly occurs in the absence of pneumonic lesions. McCordock and Congdon (1924-25) however, reported that middle ear disease was caused by the extension of an inflammatory condition of the upper air passages. Nelson (1948a) reported another disease "Pleuropneumonia" in mice that was also characterized by pneumonia and otitis media. Nelson (1948a) wrote:

During the course of one passage series, in which normal lung was being used as an appropriate control, a secondary disease likewise characterized

by pneumonia and otitis media was indeed encountered.

At autopsy, the recovery of P.P.L.O. from the lungs and middle ear was sufficiently constant in the absence of any other pathogen to warrant the diagnosis of pleuropneumonia. Nelson (1948a) reported that the observations which pertain to the behaviour of P.P.L.O. on nasal instillation in mice and rats added emphasis to their activity and presented as a supplementary appraisal of these organisms. Klienberger and Steabben (1937) were the first workers who showed that P.P.L.O. were associated with pneumonia in rodents. Later, Klienberger and Steabben (1940) recovered the organisms from the lungs, both in the presence and absence of lesions, but were unable to reproduce pneumonia in the rats by the experimental introduction of pure cultures of P.P.L.O. Edward (1940) likewise observed pneumonia in mice following the nasal injection of a normal lung suspension. Horsfall Jr. and Hahn (1940) also encountered P.P.L.O. in the pneumonic lungs of mice during their investigations on the virus of mouse pneumonia. They isolated these organisms from mice both in the presence and absence of virus and did not carry their investigations further on P.P.L.O. The relation of infectious catarrh which was described by Nelson (1937; 1940a) as a native disease of mice and rats to rodent pleuropneumonia is uncertain. Nelson (1948a) successfully produced pleuropneumonia

experimentally in rats and wrote:

A limited number of observations were made on the behaviour of the pleuro-pneumonia-like-organisms in the albino rats. They were successfully established by the nasal instillation of a mixed lung and exudate suspension from infected mice and were subsequently maintained for 6 passages... . P.P.L.O. were not isolated from the pooled lungs of 5 rats in the absence of pneumonia but were obtained from lungs of three animals in which pneumonic foci were present... . The manifestations of the disease were commonly limited to the middle ear and nasal passages from which the specific organisms were reisolated on culture. . . .

Nelson (1948a) pointed out that the behaviour of the P.P.L.O. under natural and experimental conditions in their animal host as well as under artificial cultural conditions was sufficiently different from that of the virus-like agent to warrant definition of the two associated diseases as unrelated entities. In spite of obvious similarities between the coccobacilliform bodies of infectious catarrh and P.P.L.O., there are significant cultural and biological differences which demand further study (Edwards, 1947). Apart from endemic pneumonia or enzootic bronchiectasis which is attributed to a virus-like etiological agent (Nelson, 1946a; 1946b), there are several other specific viral conditions described in the literature. Andrewes and Glover (1945) described a virus disease which produces chronic lesions in the lungs of mice and other rodents. They reported that the

virus possibly exists as a latent infection of laboratory mice and was brought to light by serial passage, but such has not been certainly established. Andrewes and Glover (1945) suggested that emphysema and bronchiectasis may be conspicuous at later stages, and secondary bacterial infection may then occur, causing death. The same authors reported that it is uncertain whether bronchiectasis follows infection or vice versa. Andrewes and Glover (1945) successfully transmitted this virus to mice and to a variety of other rodents, but were unable to infect the cotton rat. A mouse pneumonitis virus was described by Nigg (1942), which differs from grey lung disease virus of Andrewes and Glover (1945). In addition to these, Horsfall Jr. and Hahn (1940) reported the existence of a latent virus in normal mice, which was capable of producing pneumonia in its natural host and they further pointed out that not all mice carried this virus. In the same paper, Horsfall Jr. and Hahn reported that this virus did not become infectious for ferrets even after several serial passages and several other species were also refractory to infection, including rabbits, guinea pigs, rhesus monkeys, voles, deer mice, skunks and hamsters. The grey lung disease virus (Andrewes and Glover, 1945), pneumonitis virus (Nigg and Eaton, 1944; Nigg, 1942) and viral pneumonia of mice virus (Horsfall Jr. and

Hahn, 1940) are separate entities. For details of differentiation reference should be made to the work of Andrewes and Glover (1945).

### Infectious Catarrh

Nelson (1955) reported that chattering is observed as an early symptom in mice, it is a trustworthy indication of respiratory irritation, though it is not pathognomonic of any particular irritant (Nelson, 1955; Nelson and Collins, 1961). Nelson and Collins (1961) also reported that chattering can be duplicated temporarily by nasal instillation of sterile fluids, whereas the absence of chattering however, does not necessarily indicate freedom from respiratory infection and similar remarks were made by Nelson (1955) and Nelson and Collins (1961) about snuffling in rats, unless persistent. Nelson (1955) found that aside from snuffling in rats, which is an unreliable indicator, there may be no outward signs. Later, Nelson (1958) reported that involvement of the inner ear results in labyrinthitis and twisting and is more common in rats than in mice. In the same paper, Nelson also reported that in addition to snuffling, in rats suffering from infectious catarrh, rales may appear if the lungs are extensively involved. The usual signs are loss of weight, rapid respiration, rough hair and inactivity. Occasionally deaths may occur after the first month, but



many animals survive for extended periods (Nelson, 1955; 1958). The same author (Nelson, 1955) reported that a few mice have been autopsied, with positive findings, even after a year of infection. Nelson (1955) reported that the experimental infection closely parallels natural infection with the exception that pneumonia occurs more frequently with the former. Nelson (1958) stated that multiplication of P.P.L.O. is active and attended by a vigorous and persistent inflammation. The resulting exudate may be sufficiently copious to produce an outward bulge in the tympanic membrane, but actual rupture has not been observed (Nelson, 1958). Otitis media is generally detected only at autopsy by exposing the tympanic cavity (Nelson, 1958), but recently Greselin (1961) has described a method where the tympanic membrane is examined by an otoscope. According to Nelson (1958) a state of equilibrium between the host and the invading P.P.L.O. is finally reached and may endure for a period of months. During this time the only outward sign of infection is an intermittent chattering sound, whereas in weanlings even this sign may be lacking and at autopsy, otitis media and rhinitis are the only manifestations. Once initiated, infectious catarrh persists and recovery is rare. Ultimately, P.P.L.O. may reach the lungs via the trachea and a spreading broncho-pneumonia results (Nelson, 1958). Since nasal passages and middle ear are rarely examined at autopsy, diagnosis

of the disease is often missed (Nelson, 1955).

Endemic Pneumonia (Enzootic Bronchiectasis)

Nelson (1955) believed that endemic pneumonia had some characteristics in common with infectious catarrh, and the disease was indigenous to both rodents. Endemic pneumonia is progressive, chronic, communicable and transmissible by nasal instillations. Weanlings and young adults may show neither outward nor inward signs of infection, ultimately a low grade rhinitis occurs, accompanied by snuffling in rats and chattering in mice, which are not reliable as indicators (Nelson, 1955), and most diseased rats exhibit few if any clinical signs of respiratory disease (Nelson, 1955; Innes et al., 1956). Nelson (1955) observed that in the rat there was no involvement of the middle ear, whereas otitis media did occur in mice, but not as severe as in infectious catarrh. Nelson (1955) reported that besides snuffling, rats may show little indication of respiratory distress, even with two lobes completely functionless and early lesions in several other lobes. The severity of the morbid process is not necessarily reflected by severe clinical signs (Innes et al., 1956), and many observers have expressed astonishment at the extent of pulmonary disease in rats which appeared in good condition when alive. However, in advanced stages, unthriftiness may develop, accompanied by rough hair, loss of weight and

as the threshold of safety is reached, breathing becomes laboured and audible (Nelson, 1955; Innes et al., 1956). As the condition progresses, the animal loses weight at an increased rate and finally dies, but rarely before the tenth to the twelfth month following infection. Innes et al. (1957) reported that chronic murine pneumonia in the strict sense might be regarded as a slow rather than a chronic infection. This concept was first developed by Sigurdsson (1954) and is an intriguing one in pathology. Innes et al. (1957) wrote:

As opposed to an acute infection, a chronic one is only slow in the sense of time, it is no different from an acute one in that there is an incubation period, which may be short but after which the course is very irregular. The struggle between the host and the invader may remain undecided for a very long time, and the patient may live to a ripe age and still die from the infection. In chronic infections the immunity mechanisms never, in effect, develop for complete recovery. A slow infection is not merely a slow picture of an acute one; it follows a course which is just as regular as acute infection, for the incubation period, although protracted, does not vary within crude limits. The appearance and progression of the clinical course then follows a strict pattern, and once it has reached a recognizable stage, the course is determined, with continuous progression until death. In the long intervening period before a clinical entity emerges, the disease or sequence of lesions is blossoming (perhaps intermittently) in a subclinical form.

Sigurdsson (1954) stated two criteria for a

slow infection: (1) limitation of the disease to a single host species, and (2) restricted to lesions of a single organ, single tissue, or an organ system. If these criteria are acceptable, good examples stand out in Johne's disease of ruminants, scrapie in sheep and multiple sclerosis and leprosy of man. Innes et al. (1957) suggested that we now can add chronic murine pneumonia as an example of slow infection in rodents.

## **PATHOLOGY**

### **Infectious Catarrh**

In rats and mice, the causal agent of infectious catarrh, a pleuropneumonia-like-organism invades the respiratory tract by way of the external nares and produces a chronic disease. The pleuropneumonia-like-organisms multiply actively on the nasal mucosa and the disease is characterized by a persistent exudative inflammation of the nasal passages, the middle ear and the lung (Nelson, 1955; 1958). The reaction is progressive, chronic and the host responds by producing a copious semi-fluid, mucopurulent exudate, rich in leucocytes. There is no actual discharge from the nares and the exudate is detected only by exposing the nasal passages and aspirating them with a fine capillary pipette. If a true exudate is present, numerous polymorphonuclear leucocytes are found in all fields of a Gram stained smear. P.P.L.O. may also

be seen as small spherical, or slightly elongated, Gram-negative bodies, which may be extracellular or intracellular, within or on leucocytes and epithelial cells. From the nasal passages the organisms spread to the middle ear, through the eustachian tubes. Multiplication is also active in this locality and otitis media ensues. The organisms may invade lungs and produce progressive bronchopneumonia and ultimately entire lobes become consolidated and airless. Nelson (1955) observed that two of the five lobes may be functionless with little or no effect on the host. Under natural conditions of infection, rhinitis and otitis media are the two features most often encountered in weanlings and young adults, while frank pneumonia is generally limited to old adults. There is no reaction in the pleural cavity, except for an occasional adhesion and deaths are sporadic in adults.

Nelson (1957; 1958; 1940b) reported that infectious catarrh is also found in rats with manifestations similar to those in mice, namely, purulent inflammation of the middle ears and nasal passages. The involvement of the inner ear, resulting in labyrinthitis and twisting is somewhat more common in rats than mice. Nelson (1957) pointed out that the involvement of both loci occurs in weanlings as well as in adults. The disease is communicable by direct contact in both rats and mice and may also be produced by nasal instillations

of exudates and cultures of P.P.L.O. Under natural conditions, the organisms are widely distributed in both species of rodents and are interchangeable between them.

### Endemic Pneumonia

Nelson (1955) reported that in the rat the infectious agent does not invade the middle ear, but an otitis media does occur in mice. In both rodents, the infectious agent ultimately reaches the lungs and produces progressive bronchial inflammation. Nelson (1955) and Innes et al. (1956) reported that in rats the advanced pneumonic lesion is particularly characteristic and has led to the term rodent bronchiectasis.

Innes et al. (1956) have discussed in detail the macroscopic and microscopic lung lesions of endemic pneumonia. As a brief remark on some normal anatomical feature of the rat lung, Innes et al. (1956) wrote:

In the rat (also the hamster and mouse) the right lung has three main lobes (apical, cardiac, azygous, and a small intermediate caudal one) exceeding in anatomical mass the left lung, which is a single lobe. There is no functional difference between rodents in the descending arborization of the respiratory tree. The right main bronchus is short and abutts from the trachea at a near right angle, while the left main bronchus descends obliquely into the hilus of the left lung; the right main bronchus immediately gives off a shorter trunk for the most cranial (apical) lobe of the lung - the so-called epi-arterial branch. Cartilaginous plates are found in the walls of secondary bronchi of rabbits

and guinea pigs, but in the rat, mouse, and hamster, cartilage fades from the walls of the bronchi as soon as they enter the lung. The adventitia of the pulmonary veins in the lungs of the rat and mouse show a peculiar normal feature of striated muscle fibres contiguous with those of the heart. These cardiac muscle fibres are present in the larger intrapulmonary branches, but may be seen in small veins. In rabbits and guinea pigs, such fibres are reputed to surround only the short extrapulmonary veins. This might have some physiological significance, but has been given little attention. It might, however, be of some significance pathologically, in the sense that infection with predilection for heart muscle could spread by contiguity to the lung.

Innes et al. (1956) reported that macroscopically the lesions of rodent bronchiectasis may be discrete and affect only a part or all of a lobe, or they may be disseminated. If discrete, the involved area is grey to red, indurated and somewhat depressed (Innes et al., 1956; Nelson, 1955). When a whole lobe is involved, it is shrunken, has a cobbled surface and is rubbery. Disseminated foci are generally small, sharply circumscribed, reddish brown, millet-like masses. These indurated areas cut easily and the exposed surface appears flat, dry and homogenous. There might be cyst-like spaces which are actually dilated bronchi filled with mucoid or mucopurulent material. Nelson (1955) reported also that the primary reaction occurs within bronchi which are distended by a mucopurulent exudate. Furthermore, the bronchial mucosa ruptures, the contents

seep outwardly and adjoining groups of involved alveoli and bronchi coalesce forming circumscribed masses of active and dead leucocytes together with necrotic tissue. These areas may be walled off by connective tissue and not infrequently found culturally sterile (Innes et al., 1956).

Innes et al. (1956) reported that the lesions progress slowly and many months may pass before the lung is affected to the extent of causing respiratory incapacity, which in rats is still difficult to measure in a clinical sense. In older animals, the affected lobe or lobes, are markedly distorted by nodular masses which often appear as pinkish or pearl-grey protuberances. These marked bronchiectatic areas are filled with caseous debris (inspissated exudate), and superficially may resemble abscesses. Innes et al. (1956) noted also that pleural adhesions or emphysema are relatively rare, as is a chest cavity in a rat in which the entire thoracic contents are matted. They reported also that they did not frequently encounter marked bronchiectatic areas filled with caseous debris, since the rats they examined were seldom old animals.

Innes et al. (1956) reported that much of the histologic picture in endemic pneumonia can be accounted for by the variation in the type and degree of lymphoid infiltration. That lesions begin very early in life is



apparent from finding microscopic lymphoid collections in some suckling rats, and is almost a universal finding by the time of weaning. Involvement of the bronchial tree is first noted in its proximal parts, but as the rat ages, the process extends to embrace the peripheral components. The lymphoid tissue implicates all layers of the bronchus and at its height is of a massive kind, in which formation of primary follicles is always found.

Innes et al. (1956) also stated that perivascular collections of lymphocytes occur as early as peribronchial involvement and there need be no quantitative correlation between the two. Perivascular infiltration is most conspicuous about small vessels - arterioles and vessels and frequently large cuffs and sleeves are found. In younger rats eosinophils are fairly numerous and pigment-laden phagocytes sometimes may be present. Parenchymal involvement by lymphocytes tends to be local at first, in the form of patchy chronic interstitial pneumonitis. Then locally diffuse lesions make their appearance and sometimes a whole lobe shows inflammatory thickening of the alveolar septa but inflammatory cells within the alveolar spaces usually are less conspicuous. The changes due to bronchopneumonia seem to be relatively infrequent and more often a picture simulating bronchopneumonia is produced by a combination of atelectasis and interstitial

pneumonia. As the process advances with age, the bronchial tree dilates in a segmental fashion and the accumulation of secretion increases along with more and more atelectasis. Peribronchial fibrosis can be discerned readily, but what at first glance may appear to be extensive pulmonary fibrosis, may turn out to be marked atelectasis. The bronchiectatic process may continue to a stage in which a lobe, or a whole lung consists of multilocular spaces surrounded by collapsed and compacted parenchyma. Innes et al. (1956) reported that it is in the more advanced stages that bronchiectasis may appear. In the same paper these authors have stated that it has not been their common experience to observe squamous metaplasia of the bronchial epithelium. Passey et al. (1936) have pointed out that such metaplasia was mistaken for metastasis, since this change, with keratinization, may be very prominent and superficially resemble epidermoid carcinoma. Innes et al. (1956) reported that there is proportionately less involvement of the bronchioles and the involvement of the bronchial tree varies in degree from a small to a massive aggregation of lymphocytes, infiltrating the deeper parts of the bronchial mucosa and pushing aside and obliterating all structures with cleavage and disappearance of the normal elastic and muscular fibres. The primary reaction occurs within bronchi which are

distended by a mucopurulent exudate and later the bronchial mucosa ruptures with the result that the contents seep outwards. Innes et al. (1956) reported that even examination of the stained sections, simply by the naked eye, showed the bronchial arborizations outlined by broad, blue stained, interrupted sleeves. The same authors reported that these lymphoid nodular lesions, which might well be referred to as follicular bronchitis, may occur in rats even in the absence of bronchopneumonia. Hence to understand the conflicting views of Passey et al. (1936) and Cruickshank (1948) previously expressed about the role of lymphoid nodular lesions in the production of bronchiectasis, Innes et al. (1956) wrote:

An examination, for instance, of the lungs of 50 male and female rats, reputed to be breeders over 15 months old, received from a well known rat colony in England revealed an incidence of 11 with marked macroscopic lesions and 28 with histologic lesions, including excess lymphoid tissue.

Nelson (1946a) also stressed, that the chief feature was peribronchial lymphoid infiltration, which was often filled with a leucocytic exudate. The adjacent lung tissue contained no open air spaces and all the alveoli were compressed and filled with cells, among which the large mononuclear cells were predominant. Comparable lesions of lymphoid hyperplasia have been observed in calves by Jarret (1954) and in pigs by Pattison (1956), in the latter species they are

apparently caused by a virus. Innes et al. (1956) further added that comparisons of results are largely invalid unless comparable detailed histological examinations are made.

The pathogenesis, particularly in regard to bronchiectasis is not very clear and there are several hypotheses forwarded by various investigators. Passey et al. (1936) without any experimental evidence suggested the possibility that lack of exercise deprived the animals of opportunities for full use and expansion of the lungs and this might result in stagnation of normal mucous secretion, which in turn might assume pathological proportions, causing dilation and mild sacculation which later might become infected. In the same paper, they (Passey et al., 1936) indicated that squamous metaplasia of epithelial surfaces and hyperkeratinization of metaplastic and pre-existing squamous epithelium were due to vitamin A deficiency. They (Passey et al., 1936) supported the opinion that vitamin A was essential for the integrity of epithelial surfaces and that infections which occur in its absence invariably originate in defects in these surfaces. Contrary to this, Innes et al. (1956) and Saxton and Kimbal (1941) suggested that the cause of endemic pneumonia was not directly or indirectly related to dietary deficiency factors. Passey et al. (1936) further stated, whether the defective resistance

is manifested by metaplasia or by keratinization - followed by infection, or whether the infection is the first visible phenomenon and the epithelial changes mainly the secondary results of the infection, is a problem about which there has been very sharp division of opinions, whereas Moise and Smith (1928-29) postulated that the bronchiectasis is due to plugging of the bronchi by mucous followed by the growth of microorganisms. While Cruickshank (1948) suggested that mucous secretion is a result and not the cause of bronchial obstruction, the latter being initiated by massive lymphoid aggregates to the extent of forming polypoid masses circumscribing and constricting the bronchial lumen. Thus, it is that a combined viewpoint of obstruction and infection has become accepted as an explanation of bronchiectasis. Innes et al. (1956) stated that this concept does not explain adequately the production of bronchiectasis, because the lymphoid proliferation, even in its most massive proportions, would not constrict the bronchial lumen significantly. The same authors further stated that this process may promote stasis by increasing the rigidity of the bronchial tree, but such stasis, uncomplicated by any other factor would not be expected to produce more than very moderate dilation of bronchi.

### Etiology

The history of work on the etiology of chronic murine pneumonia is reminiscent of that on distemper in dogs. For years this condition was commonly contended to be of bacterial origin, although the exact etiology was uncertain and its pathogenesis not clear. In this connection, Nelson (1946b) wrote:

Endemic pneumonia of the albino rat is commonly regarded as an infectious disease but its etiology is still uncertain, although it has often been investigated and a wide variety of bacteria isolated from the lungs . . . the disease is not primarily bacterial in origin but is referable rather to a non-specific pulmonary irritation with resultant bronchial obstruction.

The most extensive investigations into the etiology and pathogenesis have been those of Nelson (1930; 1931; 1940a; 1940b; 1946a; 1946b; 1948b; 1953), who demonstrated that the disease was not primarily bacterial in origin and has shown that the disorder is initiated in young rats by a "virus-like agent" (Nelson, 1948b).

In 1912, Mitchell studied a highly fatal outbreak of pneumonia in albino rats and isolated Bacillus muris from the lungs, which produced a local abscess on subcutaneous injection and resulted in death with pleural involvement on intraperitoneal injection. Tunncliffe (1916) observed a gram-negative streptothrix in 56 films from the lungs of six white rats with bronchopneumonia

and successfully isolated it from twenty rats. The same authors injected cultures into young rats but only intraperitoneally, with irregular results. Nelson (1930) reported that the bacterial flora of the pneumonic lung was qualitatively similar to that of the ear in middle ear disease, whereas quantitatively, however, the incidence of individual bacteria was different. Klieneberger and Steabben (1940) also noted that rats with pneumonia were more often carriers of Streptobacillus moniliformis than were normal rats. It was suggested by Nelson and Gowen (1930) that pneumonia might be secondary to middle ear infection and Nelson (1930) favoured this suggestion on the basis of high incidence of Bacillus actinoides isolated from these two locations. Later, Strangeways (1933) showed that the streptobacillus (B. actinoides, Nelson, 1930) was apparently a normal inhabitant of the rat nasopharynx, whereas Nelson and Gowen (1931) reported failure to isolate Streptobacillus moniliformis (B. actinoides var. muris) from a special colony free from middle ear disease and thought that the frequent development of pulmonary lesions without middle ear involvement strongly suggested that the primary initiating agent(s) of the two (endemic pneumonia and infectious catarrh) infections were not identical and this refuted the previous hypothesis put forth by Nelson (1930) that pneumonia might be secondary to middle ear

infection. Their findings (Nelson and Gowen, 1931) further indicated that Streptobacillus moniliformis was not the primary etiological agent of rodent pneumonia. The frequent isolation of Actinobacillus muris from both the lungs and particularly the middle ear, coupled with observations that it produced inflammation within the tympanic cavity on intra-aural injection led Nelson (1930) to believe that it was of etiological significance. The subsequent examinations of rats from a selected colony with disease-free breeders clearly indicated, however, that Actinobacillus muris was merely a secondary invader and that the high rate of otitis media observed prior to selection was not associated with pneumonia. Nelson and Gowen (1931) reported that middle ear involvement was reduced by selection and with reduction in otitis media, Actinobacillus muris disappeared from the colony. In the same paper they reported further that all attempts to cultivate it from the lung and occasional middle ear exudate have been unsuccessful though from time to time it has been isolated from rats obtained elsewhere. Nelson (1946b) noted that it was difficult to appraise much of the early work on endemic pneumonia of albino rats because the criteria for judging the significance of a given agent were inadequate. Any unknown agent should meet certain requirements in addition to the original Koch's postulates (Nelson, 1946b) to establish



a causal relation to the disease in question. It is essential that the agent reproduce the basic features of endemic pneumonia when introduced in pure state by way of a natural portal of entry, which in the case of endemic pneumonia would, in all probability, be the upper respiratory tract. Moreover, the agent should be experimentally transmissible by normal passage and naturally so by cohabitation. Nelson (1946b) further added that none of the described bacteria have met the above requirements and it seems justifiable to conclude that the etiology of endemic pneumonia is still uncertain. Some of the associated microorganisms, such as Brucella bronchiseptica and Pasteurella muricida, undoubtedly produce a pulmonary reaction in the rat, but were unrelated to endemic pneumonia and if isolated from the lungs of infected rats were present only as secondary invaders (Nelson, 1946b). In the same paper he indicated that critical study of endemic pneumonia has been particularly handicapped by the lack of suitable rats, since the disease has a universal distribution. Apart from this, an additional source of error has been the coexistence of infectious catarrh which may simulate endemic pneumonia in its initial involvement of the lung. By using specifically selected rats, Nelson (1946b) was able to eliminate infectious catarrh and also ruled out Actinobacillus muris (Streptobacillus moniliformis) as a

significant factor in the etiology of endemic pneumonia.

In 1935, Klieneberger and Nelson independently reported that coccobacilliiform bodies, were the cause of chronic respiratory disease in chickens. Klieneberger (1935) demonstrated the first of the so-called L-forms, in association with Streptobacillus moniliformis and at that time she also introduced the term pleuropneumonia-like-organisms (P.P.L.O.) which is still widely used. Subsequently, amongst others, Klieneberger and Steabben (1937, 1940) and Klieneberger (1935) indicated that P.P.L.O., unrelated to Streptobacillus moniliformis, were the cause of chronic respiratory disease of both rats and mice. Nelson (1957) referred to this disease as infectious catarrh. Some causal connection of the P.P.L.O. (L-3) with bronchiectasis and other lesions of chronic murine pneumonia was reported by Klieneberger and Steabben (1937, 1940). Furthermore, Klieneberger and Steabben (1937) indicated that no worker has ever reproduced the chronic disease experimentally with P.P.L.O., but the predilection of the P.P.L.O. (L-3) to involve the bronchiectatic rat lung was reported again by Klieneberger and Cheng (1955). In this paper they reported that after ligation of a bronchus to cause bronchiectasis there was rapid multiplication of the P.P.L.O. (L-3), which in unoperated lungs were in lesser numbers and stated then that an additional factor might

be necessary to render P.P.L.O. infective. Pankevicius et al. (1957) reported that the role of P.P.L.O. in chronic murine pneumonia is highly questionable and they favoured the opinion that the cause of chronic murine pneumonia is not a single microbial entity, but the secondary role of P.P.L.O., and other microorganisms in enhancing the severity of established chronic pulmonary disease is still a surmise. Nelson (1949b) emphasized that P.P.L.O. were not isolated from the lungs of the mice in which a diagnosis of endemic pneumonia was made. Nelson (1946a) reported that the disease may also appear in younger animals but in general, immature rats fail to show macroscopic evidence of pneumonia under natural or experimental conditions of infection and appear refractory. For these reasons, Nelson (1946b) chose mice as an alternative host and subsequently found that an infective agent was regularly present in the lungs of adult selected rats with pneumonia, which on transfer to mice, likewise gave rise to a pulmonary inflammation, and bacteriological examination of lungs from these mice failed to demonstrate any of the bacteria isolated previously. Because of the high incidence of pleuropneumonia-like-organisms in the pneumonic lungs of rats (Klieneberger and Steabben, 1940), a particular attention was paid to these bacteria. Nelson (1946b) noted that morphologically similar coccobacilliiform bodies of infectious catarrh

were not demonstrable in the experimentally infected mice. Furthermore, Nelson (1946b) made repeated attempts to cultivate pleuropneumonia-like-organisms using adequately enriched media and was uniformly unsuccessful in demonstrating them. Innes et al. (1956) reported that attempts to isolate P.P.L.O. from rat lungs in their laboratory were also unsuccessful.

Nelson (1946a; 1946b) reported that the agent was filterable, and suggested that it was a particle somewhat smaller than the elementary bodies of vaccinia. Like the elementary bodies it was refractory to staining with the usual aniline dyes, but unlike them was not cultivable in embryonated eggs nor able to withstand a temperature of 40° C for a period longer than a week, whereas in the frozen state under dry ice, it remained viable, however, for a period of at least 13 weeks. Nelson (1946b) indicated that these findings, while broadly suggestive of a virus, might likewise be applicable to a P.P.L.O. and the only differential characteristics at present are the inability of the agent to multiply in a cell-free medium and its tinctorial properties. Nelson (1946b) stated that, since these characteristics might be referable to technical inequalities, it seems advisable to regard the agent as virus-like and defer a final decision until additional observations have been made of its behaviour. Nelson (1946b; 1955) reported

that this virus-like agent fulfils the essential postulates for identifying it as the specific cause of the experimentally produced rodent bronchiectasis in mice. He further added that these postulates have not been fulfilled for similarly identifying it with the rat disease and until this was done it can be concluded only that the agent is a frequent resident of the rat lung in endemic pneumonia. Recently, Joshi et al. (1961) have reviewed the literature pertinent to chronic murine pneumonia and have discussed etiological aspects.

#### Transmission

Both the diseases, infectious catarrh and enzootic bronchiectasis, are widely distributed and are intertransmissible between rats and mice (Nelson, 1946a; 1946b; 1940a). Innes et al. (1956) reported that rats are more resistant than mice to the infection, while Nelson (1948b) found that susceptibility of mice to infection was not dependent upon age, since immature mice were as susceptible as adults. The incidence of enzootic bronchiectasis in rats is correlated with age and immature rats were significantly less susceptible than adult animals (Nelson, 1946a; 1946b; 1948b). Nelson (1948b) reported that young rats throughout the third month were essentially as refractory to the virus-like agent passaged in mice as they were to that recovered directly from rats. In the same paper, he further added

that the high rate of pneumonia observed in the six-month-old rats could hardly be regarded as evidence that the injected agent was the initiating factor, as a correspondingly high rate may occur in uninjected rats of the same age. Furthermore, Nelson and Gowen (1930) found that adult and young rats, even from the same colony showed a marked difference in the incidence of middle ear disease and pneumonia. They suggested that the high rate of infection in adults may be determined in part by a decreasing state of immunity, either natural or acquired through maternal transmission, but not demonstrable. Nelson (1955) and Nelson and Collins (1961) reported that nursing rats and mice acquired the virus of enzootic bronchiectasis from their mothers and commonly retain it throughout their life, whereas in infectious catarrh, a direct contact between infected and normal animal was the most common mode of transfer in both rodents. Later, Nelson (1958) and Nelson and Collins (1961) reported that P.P.L.O. were readily transmitted from infected mothers to their nursing young by direct contact via the upper air passages and transmission occurs also when the weanlings are brought together as stock animals.

### Diagnosis

Nelson (1946b) suggested that the most dependable guide in the diagnosis of infectious catarrh was

the microscopic examination of exudate from the middle ears and nasal passages and found that the Gram-stained films regularly showed extra- or intracellular coccobacilli form bodies. Furthermore, Nelson (1946b) pointed out that tissue and exudate films from mice with the experimentally induced disease were repeatedly examined but morphologic units resembling the coccobacilli form bodies were never observed. In the same paper, Nelson stressed the striking difference between the natural and artificial infections. A detailed procedure for the diagnosis of the two conditions (infectious catarrh and enzootic bronchiectasis) was described by Nelson (1955; 1957), which includes artificial infection of susceptible mice. Nelson (1955) reported that the virus of endemic pneumonia does not invade the middle ear and the otitis media which occurs in mice and was less severe than that associated with infectious catarrh. In the same paper, Nelson reported that under natural conditions, enzootic bronchiectasis occurred less in mice than infectious catarrh, and was communicable under both natural and experimental conditions by direct contact. Furthermore, Nelson (1955) reported that enzootic bronchiectasis is readily transmissible in mice by nasal instillation and progresses more rapidly than in the rat. Virus-like agent multiplies first in nasal passages and then invades the middle ear and lungs. In the same paper, Nelson

reported that pulmonary involvement is usually demonstrable at autopsy, four weeks after the nasal injection of exudates or lung suspension. At this stage the right middle and azygous lobes commonly show partial or complete consolidation and later, other lobes also become involved. As in the rat, the pulmonary reaction is basically an inflammation of the bronchial mucosa and in the presence of penicillin, cultures from the nasal passages, the middle ear and the lungs are commonly sterile on serum agar plates. Because of this accelerated reaction, weanling mice from a disease-free colony are valuable aids in the detection of endemic pneumonia (enzootic bronchiectasis) virus in symptom-free rats. The chief pathologic features of the two diseases in mice and rats are given below (after Nelson, 1955).

Disease	Host	Rhinitis	Otitis Media	Pneumonia	Culture on Serum Agar
Infectious Catarrh	Mouse	+	+	+	+
	Rat	+	+	+	+
Endemic Pneumonia	Mouse	+	+	+	-
	Rat	+	-	+	-



Under natural conditions, it was found (Nelson, 1955) that infectious catarrh and endemic pneumonia may co-exist in the same animal, either in the rat or mouse, and in such instances, separation of the two diseases was difficult and also time-consuming. Moreover, in the rat the diagnosis is also hampered by the presence of S. moniliformis (Nelson, 1955), since, in the presence of penicillin, this organism produces variant colonies which may be confused with those of P.P.L.O. and the identification of these colonies admittedly requires considerable experience.

#### OTHER MURINE RESPIRATORY INFECTIONS

##### Rodent Pleuropneumonia

Nelson (1948a) accomplished the experimental production of pleuropneumonia in mice by the intranasal instillation of lung suspension and middle ear exudate from rats, and at autopsy, however, P.P.L.O. were isolated from the lungs and middle ear of infected mice.

Klieneberger and Steabben (1940) recovered the organism from the lung, both in the presence and absence of lesions, but were unable to reproduce pneumonia in the rat by the experimental introduction of the pure cultures. Furthermore, Horsfall Jr. and Hahn (1940) encountered P.P.L.O. in the lungs of mice during their work with virus of mouse pneumonia and these organisms were isolated from

mice both in the presence and absence of the virus. Nelson (1948a) reported that pleuropneumonia which appeared during the course of the normal lung passage was readily established in mice by the nasal instillation of pneumonic lung suspension and middle ear exudate and it was also readily maintained in mice by the nasal transfer of middle ear exudate, a passage being made at monthly intervals. Recovery of P.P.L.O. was sufficiently constant, in the absence of any other pathogenic agent, to warrant the diagnosis of rodent pleuropneumonia. At autopsy, the scope of the disease paralleled that of endemic pneumonia in mice, being limited to the lung, middle ear and nasal passages. However, in comparison with rodent pleuropneumonia, P.P.L.O. have never been isolated from mice infected with the virus-like agent. Furthermore, Nelson (1948a) reported in connection with pleuropneumonia that the involvement of the middle ear was commonly bilateral and indicated by the presence of a copious purulent exudate and pneumonia was generally manifested by the complete consolidation of one or more lobes. It was less regularly restricted to the right middle and azygous lobes than in the disease produced by the virus-like agent. Involvement of nasal passages was indicated by a definite mucopurulent exudate, and the exudate from both middle ears and nasal passages showed numerous polymorphonuclear leucocytes on microscopic

examination. Morphologic forms of P.P.L.O. were often demonstrable in Gram stained films and the presence of Gram negative granules and/or rods within leucocytes was a valuable aid in the differential diagnosis of pleuropneumonia and the disease produced by the virus-like agent (enzootic bronchiectasis). Similar microscopic elements were never observed in exudate from mice infected with the latter disease (enzootic bronchiectasis). Nelson (1948a) also reported that pleuropneumonia was not readily communicable by direct contact and the only manifestation encountered at autopsy in the contact mice was an inflammation of the middle ear from which the specific organisms were isolated.

Nelson (1948a) reported a limited number of observations on the behaviour of the pleuropneumonia-like-organisms in the albino rat. He successfully established P.P.L.O. by the nasal instillation of a mixed lung and exudate suspension from infected mice and were subsequently maintained for six passages. The localization of the specific organisms was similar to that in the mice, being restricted to the respiratory tract and middle ears. Nelson reported in the same paper, failure to isolate P.P.L.O. from the pooled lungs of rats in the absence of pneumonia, but successfully isolated P.P.L.O. from animals in which pneumonic foci were present. Pleuropneumonia is more communicable in

rats than in mice, since in contrast to mice, it was produced by contact in rats (Nelson, 1948a). The manifestations of the disease were commonly limited to middle ears and nasal passages from which the specific organisms were reisolated on culture. Furthermore, Nelson (1948a) reported that the passage of suspensions of macroscopically normal lungs from mice showing no signs of disease ultimately resulted in the development of pleuropneumonia and the recovery of P.P.L.O. from the lungs of the recipients. Nelson (1948b) stated that a virus-like agent associated with endemic pneumonia was commonly carried in nasal passages and lungs of young rats, in the absence of a pathologic reaction and the rhinitis which often attended nasal instillations of the P.P.L.O. (Nelson, 1948a) may have resulted from the combined action of the two agents. The synergistic activity of these agents might also account for the pneumonia described by Klieneberger and Steabben (1937; 1940). Edward (1940) and Sullivan and Dienes (1939) have suggested the presence of pleuropneumonia-like-organisms in a latent form in normal mice and furthermore, Edward (1940) has discussed certain dangers in using intranasal inoculation of mice for the study of viruses. Nelson (1948a) reported that the behaviour of the P.P.L.O. under natural and experimental conditions in their animal host as well as under artificial cultural

conditions was sufficiently different from that of the virus-like agent to warrant definitions of the two associated diseases as unrelated entities. The position of infectious catarrh, which was described as a native disease of mice and rats (Nelson, 1937; 1940a) in relation to rodent pleuropneumonia is much less certain. Edward (1947) wrote in this connection:

In spite of obvious similarities between the coccobacilliform bodies of infectious catarrh and P.P.L.O., there are significant cultural and biological differences which demand further study before a conclusion can be made.

#### Wild Rat Pneumonia

Nelson (1949a) examined the lungs of two wild brown rats (Mus. norvegicus) and observed only minor pathologic changes. Upon intranasal instillation of lung suspension, white mice showed an acute pulmonary reaction which was quite unlike the retarded and chronic response to enzootic bronchiectasis. He isolated the agent and refers to it as "Wild Rat Pneumonia Virus" (W.R.P.). In the same paper he reported that upon intranasal instillation of W.R.P. virus in Princeton mice, the onset of disease was very rapid and the mortality rate was as high as 58 per cent between the 7th and the 14th day in the group of 50 mice and the survivors showed definite signs of disease. By way of comparison with endemic pneumonia, Nelson (1949a) added that the majority

of the mice injected with the virus-like agent survived. Furthermore, he reported that lungs of mice injected with W.R.P. virus showed areas of consolidation with a tendency to coalesce, with survival beyond the second week, but never resulted in the lobar distribution characteristic of endemic pneumonia and he pointed out that the pulmonary reaction involved chiefly the alveolar walls and spaces. Sections of entire lobes regularly showed irregular areas in which the normal structure was obliterated and the alveolar wall was thickened. In this connection, Nelson (1949a) wrote:

The air spaces were filled with a mixture of exudative cells; red blood cells, and granular material, evidently from fluid deposits. The predominant reactive cell was a large mononuclear one and small lymphocytes were also numerous but polymorphonuclear leucocytes were generally few in number. In the same slide it was customary to find other groups of alveoli which contained only red blood cells, granular deposits, or mononuclear cells and adjacent to the areas of consolidation were commonly groups of alveoli which were essentially normal in appearance. Bronchial involvement was inconspicuous and limited to deposits of granular material together with mononuclear cells along the mucosal margin. . . . The autopsy of infected mice prior to the onset of symptoms indicated that a pulmonary reaction was detectable macroscopically as early as the 2nd day after nasal instillation. By the fourth day, when the injected animals were still normal in appearance, the lung reaction was indistinguishable from that in mice killed on the 7th day or later. The disease produced in young mice on

subsequent nasal passage was characterized by rapid onset, high mortality and an infiltrative type of pneumonia which was regularly accompanied by oedema of the lung and occasionally by otitis media.

In the same paper he reported that the transmission of the agent by direct contact was irregular and occurred only after an exposure period of at least three weeks. Furthermore, the nasal injection of mouse lung suspension in white rats resulted in a similar but less acute pneumonia which was transmissible by passage and slightly communicable by direct contact. While the pulmonary reaction after 14 days in white rats was similar in appearance to that in injected mice but was conspicuously less extensive. Edema was not a conspicuous feature of the pulmonary reaction in rats, there being little or no seepage of fluid from the cut ends of the tracheas. The latter observation was substantiated by histological examination of lung sections; granular deposits in the alveoli and bronchi being much reduced in comparison with the condition in mice. The nasal passages and middle ears of the injected rats were uniformly normal. Furthermore, Nelson (1949b) found that a cultivable organism was not concerned in the etiology of wild rat pneumonia, since all attempts at the cultivation of an organism even in embryonating eggs failed, and thought that it was proper to classify the agent as a virus with a special affinity for the respiratory tract.

Nelson (1949b) also stated that high concentrations of penicillin and streptomycin did not reduce the infectivity of this agent even after several hours of exposure to the antibiotics. He indicated that in working with a transmissible disease of this nature the possible association of more than one non-cultivable agent must be given serious consideration. He stated that endemic pneumonia and wild rat pneumonia were caused by two different viruses; on the basis of two different types of response to experimental infection in mice; lobar type of reaction along with otitis media is characteristic of endemic pneumonia and infiltrative pneumonia without otitis media is indicative of wild rat pneumonia. In this connection, Nelson (1949b) wrote:

. . . It is highly probable that the virus-like agent associated with endemic pneumonia was present in the initial suspension from the lungs of the wild rats together with the specific agent of W.R.P. and the subsequent nasal passage in mice was followed by the development of both agents. The fact that manifestations of endemic pneumonia were not apparent in mice killed shortly after the injection may be referable to the slow onset of the disease, though a possible suppressive effect by the specific agent of W.R.P. was not excluded.

His studies (Nelson, 1949b) suggested that the size of the W.R.P. agent was below the range of elementary bodies and the tissue response to the pathogenic action of the agent in the lungs of mice and rats was



predominantly lymphocytic. On this basis Nelson (1949b) classified the causal agent of wild rat pneumonia as a virus, with special affinity for the respiratory tract. Initial sedimentation experiments pointed out that at 18,000 r.p.m., the supernatant only contained W.R.P. virus and mice injected with suspensions which presumably contained only the specific agent showed no modification of the manifestation attributed to W.R.P.

#### Grey Lung Disease

Andrewes and Glover (1945) reported grey lung disease of virus origin and characterized by an interstitial pulmonary reaction together with edema of the lung. Nelson (1949b) reported that in this respect grey lung disease resembles wild rat pneumonia, but seems to be less acute than wild rat pneumonia. The virus of grey lung disease and the lesions it produces persist apparently indefinitely in infected animals (Niven, 1950) and Andrewes and Glover (1945) stated that it produces chronic lesions which remain relatively stationary for many months and rarely lead to death. Furthermore, the virus produces chronic infection in the cotton rat and infects other rodents, though less pathogenic. In the same paper, Andrewes and Glover (1945) mentioned that neither active immunity nor production of antibodies has been demonstrated. In connection with the pathogenicity of grey lung disease virus, Andrewes and Glover (1945) wrote:

. . . Mice killed within three or four days of inoculation rarely showed more than isolated greying red consolidated areas on the lungs. After 6-8 days, lesions were much more extensive, often extending over a large part of the lung surface, but after 14 days an even better and more characteristic picture had developed. Lungs were voluminous, . . . lungs were heavy . . . They had a . . . reddish-grey appearance, looking not nearly as purple as do lungs infected with virus of influenza or mouse pneumonitis (Nigg 1942) . . . Most conspicuous changes are the lesions of interstitial pneumonia, and the collection of rather large mononuclear cells which form thick cuffs around the blood vessels and bronchioles. Other features of the early stages are the diffuse oedema which one would expect from the naked eye appearances; and obliteration of alveoli, without collapse, associated with swelling and proliferation of cells in the alveolar wall. In mice killed after several months the cuffs may be much as before, or the proliferated tissue may have extended to occupy as much as half the lung volume. The alveolar cells may have enlarged still further, giving the appearance of a giant cell pneumonia, or they may be undergoing foamy degeneration.

Emphysema and bronchiectasis may be conspicuous at later stages, and secondary bacterial infection may then occur, causing late deaths. It is uncertain whether bronchiectasis follows infection or vice versa. The bronchial epithelium is at first unaffected, but later the cells form the cuffs; sometimes seem to press in and replace it. Lesions tend to be static or slowly progressive for many months; the end results of the process are not yet known.

Niven (1950) reported that grey lung virus was sensitive to some antibiotics and suggested that it may

belong to a distinct virus group. Differentiation of the grey lung virus from other pneumotropic viruses which have probably originated in mouse stocks, and from murine P.P.L.O., has been discussed by Andrewes and Glover (1945). Niven (1950) stated that the absence of elementary bodies and lethal action distinguishes grey lung disease virus from virus of the Nigg pneumonitis (Nigg and Eaton, 1944); failure to provoke immunity, absence of antibodies and low pathogenicity differentiate it from the pneumonia virus of mice (P.V.M.) (Horsfall Jr. and Hahn, 1940). Niven (1950) reported that an agent of the pleuropneumonia-like-organism group cannot be excluded so definitely. Furthermore, Niven, in the same paper, reported that histologically, grey lung virus behaves quite differently in mouse lung from the Nigg's virus which causes lesions always related to the multiplication of elementary bodies within susceptible cells. In the case of P.V.M., histological differentiation is much less certain and Horsfall Jr. and Hahn (1940) described fluid exudate and mononuclear cells in the alveolar spaces, an increased cellularity of the interalveolar septa and abundant perivascular and peribronchial infiltration. Niven (1950) reported that seriological response in grey lung disease was quite specific, and moreover, a haemagglutinating agent for murine erythrocytes can be extracted from infected lungs. Injected animals succumb in 10 to 14

days following inoculation of material which has undergone a few mouse passages and resemblance was confined to the actual lung changes. Furthermore, the oedema disappears very quickly, the occasional haemorrhages being the result of a further degree of capillary dysfunction and not due to mechanical factors. Niven (1950) has also considered the possibilities that grey lung virus is an agent with neoplastic potentialities, but the lesions could not be considered as new growths within the framework of the lung. Grey lung virus possibly exists as a latent infection of laboratory mice brought to light by the serial passage of mouse lung (Andrewes and Glover, 1945) but such has not been established with certainty.

#### Latent Mouse Lung Virus

Karr (1943) isolated a virus from lungs of apparently healthy mice by serial lung passage, which was capable of producing fatal pneumonia in mice. This agent was filterable through a Berkfeld N filter, could be preserved in 50 per cent buffered glycerine for at least 19 days and on dry ice for at least 14 days. The virus was non-infectious for young white rats and Syrian hamsters. Lung lesions in mice were characterized by the presence of cytoplasmic inclusions and elementary bodies which resemble those of psittacosis and lymphogranuloma venereum viruses. This virus did not induce

the appearance of neutralizing serum when injected into a rabbit. It is not related immunologically to the latent pneumotropic virus of Horsfall Jr. and Hahn (1940), as shown by the cross-immunization and neutralization test. Karr (1943) reported that it is of importance to know that agents morphologically resembling viruses which are infectious for man, are present in mice and may be activated by intranasal inoculation of the animals. The infection thus produced may be erroneously interpreted as an experimentally produced disease or may contaminate another infectious agent isolated or passed by this procedure. Andrewes and Glover (1945) have discussed histological differences between the lesions produced by the grey lung virus and mouse pneumonitis virus described by Nigg (1942). The same authors (Andrewes and Glover, 1945) also discussed the differentiation of grey lung virus, mouse pneumonitis virus (Nigg, 1942), pneumonia virus of mice (P.V.M.) (Horsfall Jr. and Hahn, 1940) and P.P.L.O.

## VIRUS ISOLATION

### Animal Inoculations

The development of the method for intracerebral inoculation of rabbits with rabies virus (Pasteur et al., 1881), marked the first systematic use of a small laboratory animal in the field of virology. Since then several

animal species have been employed for the study of viruses and associated diseases, ranging from human volunteers in the study of yellow fever (Reed, 1902), to the utilization of chick embryos as a source of susceptible tissue for virus growth (Woodruff and Goodpasture, 1931). Andervont (1929) was the first who employed mice for virus titrations, and showed that some strains of herpes simplex virus were pathogenic by intracerebral route of inoculation. Smith et al. (1933) isolated a virus from influenza patients in ferrets and the susceptibility of mice to the viruses of human and swine influenza was reported by Andrewes et al. (1934). Mouse pneumonitis virus belonging to the psittacosis-lymphogranuloma group, generally considered to be indigenous in certain stocks of laboratory mice, has been recovered from mouse lung preparations, passaged serially in mice, and the mouse adapted strain regularly produced pneumonitis leading to fatal termination (Karr, 1943). Intranasal route of inoculation in mice was also employed for the isolation of feline pneumonitis virus, which causes a highly infectious disease in cats (Baker, 1942), and the nature of the infectious agent, a member of psittacosis-lymphogranuloma group was described by Hamre and Rake (1944) and Thomas and Kolb (1943). McKercher (1952) passaged pneumonic sheep lungs intranasally in mice and recovered an agent related to

psittacosis-lymphogranuloma group. In Cocksackie (New York), Dalldrof and Sickles (1948) isolated an agent from two children, suffering from mild poliomyelitis, which was pathogenic for suckling mice, but quite inactive in older animals, and established the Cocksackie group of viruses. Several other members of this group were isolated from human throat excretion and faeces. Schmidt et al. (1961) reported that Cocksackie A21 virus was responsible for mild upper respiratory symptoms in army recruits.

The utilization of chick embryo as a source of susceptible tissue was introduced by Woodruff and Goodpasture (1931), who used small-pox virus on the chorioallantois and subsequently Goodpasture et al. (1932) showed that vaccinia virus could also multiply at this location. Cox (1938) found that rickettsiae of Rocky mountain spotted fever and typhus multiplied in the yolk sac of embryonated eggs. Furthermore, Burnet and Rountree (1935) reported the growth of psittacosis virus in the yolk sac and later Rake and Jones (1942) reported that this was also the site of election for the growth of other members of the psittacosis-lymphogranuloma venereum group of viruses. Thomas and Kolb (1943) reported that the virus of cat pneumonia was easily cultivated in yolk sac of a developing chick embryo. The agent of sheep pneumonitis was also cultivated in

yolk sac of embryonated egg by McKercher (1952). Eaton et al. (1944) isolated the etiological agent of primary atypical pneumonia in chick embryo. Buddingh and Polk (1939) employed amniotic inoculation and Burnet (1940) used this route for the inoculation of influenza virus, which became a standard method for the primary isolation of influenza virus. The technical approach was immensely simplified by the discovery of haemagglutination (Hirst, 1941; McLelland and Hare, 1941). Recently, Marois et al. (1963) reported on an outbreak of Type A influenza in horses and isolated the agent in chick embryo by inoculating infected material into the amniotic sac. Goodpasture (1959) and Buddingh (1959) concluded that many of the fundamental properties of tissue specificity were evident in the miniature and developing animals. For instance, influenza virus destroyed embryo lungs; pseudorabies virus produced widespread nerve tissue damage and the neurotropic strains of influenza virus caused hemorrhagic lesions of the brain.

Amongst other factors influencing the evolution of viral diseases in the organism, Lwoff (1959) has discussed the effect of temperature. Thompson (1938) and Marshall (1959) reported that the survival rate of rabbits inoculated with myxoma virus was increased at higher environmental temperatures than at lower temperatures. Recently, Walker and Boring (1958) reported



that the mice inoculated with Cocksackie virus survived at higher temperatures, whereas, at lower temperature, viral multiplication took place and all animals died. On the contrary, Armstrong (1942) reported that mice infected with herpes simplex virus died when kept at room temperature, whereas the majority of animals survived at higher temperature. Similar results were reported by Sulkin et al. (1960), on rabies infection in Mexican free tailed bats, which survived at lower temperatures (5° C to 10° C) as compared with higher temperature (29° C).

Another important factor influencing the evolution of viral diseases is the effect of adrenocortical hormones, which have been discussed in detail in excellent reviews by Kass and Finland (1953) and by Kass (1960). Kass et al. (1954) reported that cortisone acetate and hydrocortisone acetate depressed the resistance of mice to influenza viral infection, whereas ACTH did not. The enhancing effect of cortisone administration on the development of paralytic poliomyelitis in monkeys by intracerebral, intraspinal and intramuscular routes was reported by Bodian (1956). Recently, Bugbee et al. (1960) demonstrated that cortisone not only permits vaccinia virus to multiply rapidly, but also has some effect on host defence mechanisms. Furthermore, Khoobyarian and Walker (1957) also reported that appropriate treatment of mice with cortisone acetate reduced their

resistance to the acute toxicity of large quantities of intravenously injected influenza virus (PR8 strain), Lee influenza B virus and Newcastle disease virus. The enhancing effect of cortisone is further substantiated by Imam and Hammon (1957a; 1957b) who reported that cortisone administration rendered hamsters highly susceptible to subcutaneous inoculation of Japanese B encephalitis virus. Recently, Eckhardt Jr. and Hebard (1961) reported on a severe attack of herpes ~~zoster~~ during corticosteroid therapy in a patient with rheumatoid arthritis. Kass et al. (1955) and Burns et al. (1960) have reported that corticosteroids markedly depressed the antibody production. Similar findings on the effect of adrenocortical steroids on immunity have been reported by various workers (Newson and Darrach, 1954; Fischel et al., 1954; Robson and Sullivan, 1957 and Imam and Hammon, 1957a, 1957b, 1957c). Apart from the influence of adrenocortical steroids upon antibody production, Thomas (1953) indicated that the increase in mortality from infectious agents in cortisone treated animals and man might be due to a depression of the reticuloendothelial system. Morgan et al. (1954) reported that cortisone administration to rabbits markedly depressed the circulating lymphocytes. Furthermore, Dumm and Ralli (1954) reported that the administration of cortisone produced significant eosinopenia in both intact and

adrenalectomized rats, no matter which diet was fed. In this connection, Denison and Zarrow (1954) wrote that rats required a daily dose of 100 ug cortisone acetate to maintain a depressed level of eosinophiles. Selye (1951) reported that rats administered ACTH or cortisone developed spontaneous pulmonary infections which often end fatally and Kass and Finland (1953) reported that detailed studies on the etiology of these infections were not made. The confusion in the literature regarding the effect of the hormones of the adrenal cortex on immunity is resolved if species differences are appraised. In this connection, Long (1957) made a distinction between two groups of animal species. Those termed cortisone sensitive are unable to maintain body weights, gamma-globulin synthesis and antibody production under cortisone administration. Their resistance to infection is depressed or abolished, while those termed cortisone resistant, maintain body weights, gammaglobulin synthesis and antibody production under cortisone administration. Their resistance to infections is not significantly affected. Rats, mice, rabbits and ferrets are cortisone sensitive, whereas rhesus monkeys, guinea pigs and man are cortisone resistant.

#### Tissue Culture Inoculations

In 1907, Ross Granville Harrison described his new method for studying the development of nerve fibres.

Since then, the development of the Carrel flask technique (Carrel, 1923), of roller tube technique (Gey and Gey, 1936), of perforated cellophane technique (Evans and Earle, 1947), of replicate tissue cultures (Evans et al., 1951), as well as of the single cell isolation technique for establishing pure-cell clones (Sanford et al., 1948-49) has been accomplished. The special applicability of tissue culture in polio-virus investigations was recognized by Robbins and Enders (1950) and rapidly became the standard method for laboratory manipulations. Tissue cultures were employed for the isolation of adenoviruses from the respiratory and intestinal tract of man and animals (Enders et al., 1956). Rowe et al. (1953) were the first who reported the isolation of 13 strains from human adenoids removed surgically and cultivated in tissue culture. Furthermore, Hilleman and Werner (1954) reported the isolation of five agents in tissue culture during an epidemic of acute respiratory disease and pneumonitis among recruits. Brandon and McLean Jr. (1962) have discussed adenovirus in detail and indicated that the human adenoviruses have a very limited host range. Tissue cultures have also been used for the isolation of other respiratory viruses of myxovirus group (Chanock, 1956; Chanock et al., 1958; Price, 1956; Mogabgab and Pelon, 1956). Recently, L'Ecuyer and Switzer (1963) reported that the agent of virus pneumonia

of pigs did not produce any cytopathogenic changes in several tissue culture systems employed. Furthermore, they also reported that the first three tissue culture harvests when inoculated into pigs produced typical gross and microscopic lesions, but no lesions were produced when the sixth and eleventh tissue culture harvests were inoculated. Their attempts at propagating this agent in chick embryos when inoculated by several routes, were equally unsuccessful.

A few of the developments based on tissue culture that have been of importance for the general development of virology are:

- (1) Established Cell Lines - It was desirable to examine whether pure lines could substitute monkey kidney cells that must necessarily be obtained at each stage from the living animal. Two cell lines, L (from mouse fibroblasts, Earle, 1943), and HeLa (from an epidermoid carcinoma of the cervix, Gey et al. 1952) have found an extensive use. Scherer (1953) reported that the herpes simplex and pseudo-rabies virus multiplied on L cells, but HeLa cells have been used widely in medical virology, being of human origin. Scherer et al. (1953) found that poliovirus multiplied, produced gross cytopathogenic changes in HeLa cells and in almost all respects the HeLa cells were equally suitable as monkey kidney

cells. Recently, Salzman (1961) reported that well over 100 cell cultures have been successfully initiated from tissues, both normal and malignant, of a number of species and maintained in a rapidly growing state for prolonged periods. Steps have also been taken in the development of tissue culture growth of viruses for continuous bulk production of vaccines by employing suspended cells (Earle et al., 1954; Grahm and Simonovitch, 1955; Girardi et al., 1956), but there is continuing disinclination to use any cell line which is known or suspected to be of cancerous nature for the production of material for human inoculation.

- (2) Organ Cultures - Cultures of organized fragments of embryonic tissue were first maintained by embryologists for the study of development (Fell, 1951; Martinovitch, 1953; Wolff and de La Forest, 1960). Bang and Niven (1958) pointed out that embryonic tissue grown in this way was ideal for analyzing virus specificity. The study of virus infections in tissue culture has been confused by modern methods of mass culturing and trypsinization of cell lines or entire organs. Although differentiated cells seem to preserve much of their potential for some time after they have grown in a "de-differentiated" state, their morphological relationships and their specific physiological

functions are both largely lost when they are maintained as cell lines or as mass trypsinized cultures (Bang and Luttrell, 1961). These authors stated that primary explants of tissue offer a medium closer to the original host tissue; moreover, in such cultures the species specificity, and in some cases, organ specificity of the parasite, may be studied. Organ cultures of chick trachea have been infected with strains of Newcastle disease virus (Bang, 1961). McGowan and Bang (1960) reported that in embryonic human skin cultures, characteristic cytologic and histologic changes took place following inoculation of herpes simplex virus. Wolff and de La Forest (1960) reported that the specific histopathological inclusions of fowl pox, the Bollinger body consisting of masses of virus within a lipoid matrix, have been produced in organ cultures of embryonic chick skin.

(3) Demonstration of Virus Action on Tissue Cultures -

Although it was known (Maitland and Maitland, 1928) that vaccinia virus could multiply at the expense of fowl kidney cells suspended in a nutrient medium, any effective use of tissue culture in virus research demanded the demonstration of the effects of virus growth in the tissue culture itself. Cox (1936) reported that the Western Equine Encephalitis virus produced necrotic changes in tissue cultures of chick

embryo cells and Huang (1943) was the first to do quantitative elaboration of this method for the titration of virus in tissue culture. Since then the index of virus action in tissue culture has been the cytopathogenic effect (CPE) as observed by low power microscopy. The method for the demonstration of CPE on a quantitative and macroscopic scale has been developed from the excellent work of Dulbecco (1952) and Dulbecco and Vogt (1954) on plaque production by animal viruses. Various metabolic changes take place in healthy tissue culture cells which are modified or cease when cell death results from virus action. There are many possible ways of demonstrating the presence of virus by chemical means and one widely used method depends upon pH change (Enders et al., 1949; Salk et al., 1954). Cultures in which virus growth has caused cell necrosis are less acid than healthy cultures. Furthermore, influenza viruses, most of which have little CPE on standard types of tissue cultures, are detected by "Hemadsorption", whereby a layer of infected cells when flooded with chicken red cells, become coated with firmly held erythrocytes (Chanock et al., 1958).

Lwoff (1959) discussed the factors influencing the evolution of viral infection at cellular level. Lwoff and Lwoff (1958;1959) reported that at 40° C, the



yield of poliovirus in suspension of cells was 250 times smaller than at 37° C over a period of a few hours, and the number of viral particles released per ml. of cells suspension was smaller than the number of viral particles which have been absorbed. Furthermore, in the experiments of Likar and Wilson (1958) the total yield of poliovirus type I (Mohoney) was measured after a few days; the development was much lower at 30° C than at 37° C and lower at 40.5° C than at 39.5° C. Thus temperature markedly affects the virus development, so does also the reaction of the medium. It was shown by Vogt et al. (1957) that some strains of poliovirus can form plaques in media of reduced bicarbonate concentration, that is, in acid medium, whereas some others are unable to do so. Furthermore, Lwoff (1959) reported that a drop in the optimal pH of 7.3 to 6.6, caused a marked decrease in the yield of poliovirus.

The importance of tissue culture nutrition and its effect on virus propagation has been discussed by Morgan (1958). Studies on the growth of psittacosis virus in cultures of minced whole chick embryo maintained in a balanced salt solution, showed that sustained, long-term virus growth did not occur, whereas with the addition of either beef embryo extract or synthetic medium 199 (Morgan et al., 1950) greatly stimulated virus propagation. On the other hand, Burr et al. (1954) reported that the

extracellular medium had little influence on propagation of influenza and mumps viruses in chorioallantoic tissues cultivated in synthetic medium. Even after the tissue cells were depleted of intracellular nutrients by prolonged cultivation in a simple salt solution, good virus propagation was still obtained and the yield of virus was not increased by the addition of synthetic medium 199. Eagle and Habel (1956) while investigating the propagation of poliomyelitis virus in HeLa cell cultures, found that only minimal amounts of virus were formed if the culture medium did not contain glucose and glutamine, even when a wide variety of other nutritional factors were present. The results of the various studies indicate that the contribution of the extracellular medium to intracellular virus propagation may be distinctly different with different viruses, and possibly, characteristic of each virus (Morgan, 1958).

## MATERIAL AND METHODS

## MATERIAL AND METHODS

### ANIMALS

#### Conventional Rats

Rats were obtained from the Department of Nutrition, Macdonald College, who maintain a colony of C.D.F. albino rats from Charles River Breeding Laboratories.<sup>1</sup> In addition a few rats suffering from extreme cases of endemic pneumonia were obtained by the courtesy of Ayerst McKenna and Harrison, Research Laboratories, Montreal. Feed and water were supplied ad lib to these rats which were housed in metal cages which were cleaned routinely every 24 hours. The feed contained:

Wheat . . . . .	45%
Alfalfa Meal . . . . .	5%
Corn . . . . .	20%
Soybean Oil Meal . . . . .	10%
Skim Milk Powder . . . . .	10%
Yeast . . . . .	3%
Dicalcium Phosphate (20/FOS) . . . .	1%
Salt (Iodized) . . . . .	0.5%
Mazola Oil . . . . .	5.5%

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<sup>1</sup>The Charles River Breeding Laboratories,  
Brookline, Massachusetts, U.S.A.

### Caesarean Obtained Barrier Sustained Rats

These rats were obtained from Charles River Breeding Laboratories (C.O.B.S. type ) at six weeks of age and maintained as described above. These rats were housed separately from the rat colony.

### Germ-Free Rats

Germ-free rats (C.D.F. strain of Charles River Breeding Laboratories) were obtained at four weeks of age. Rats were transferred from the shipping container(Fig.A), under sterile conditions into a sterile polyethylene flexible isolator (not shown here) (Trexler and Renolds,1957). The isolator was equipped with air filters, air inlet, air out let, and a transfer door with a transfer sleeve. After heat sealing the seams,all the openings in the isolator were closed, except the air inlet where the air supply was attached. For detecting the site of a leak, 2.0 ml. of ammonium hydroxide were added to the isolator, and the isolator inflated with air under pressure ( 1 1/2 to 2 inches of water). A glass rod dipped in hydrochloric acid was passed over all seams and openings : a leak and its location being indicated by a cloud of white fumes formed by the reaction of ammonium hydroxide and hydrochloric acid. The flexible isolator was sterilized by spraying 2.0 ml. of formaldehyde into the isolator with an atomizer. After two hours the chamber was evacuated, then flushed with air, until free of

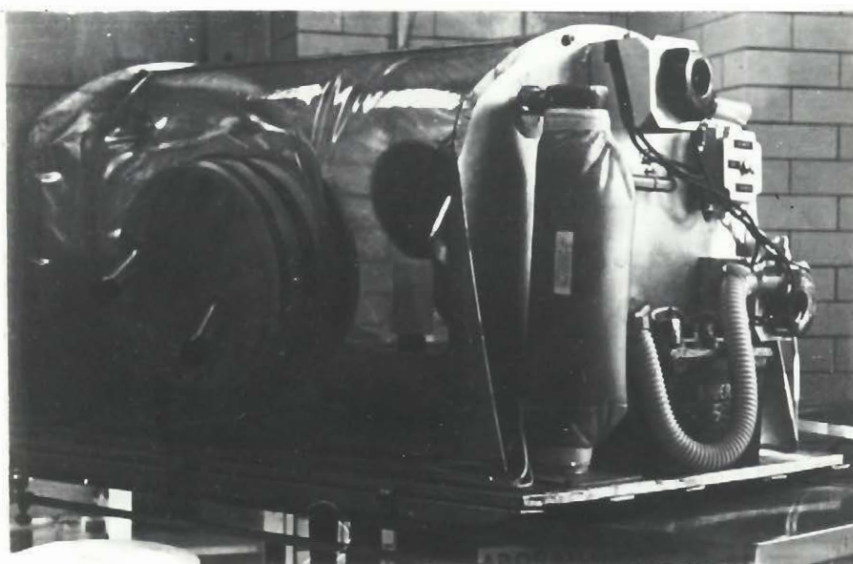


Fig.A. Isolator used for housing germfree rats. Air filter (inlet) and the transfer door are shown.

formaldehyde vapours as evidenced by testing with chromotropic acid by the method of Lambert and Neish (1950) and Neish (1952). The air from the outlet was passed through 5.0 ml. of water in a test tube for five minutes. A 1.0 ml. aliquot was placed in a pyrex test tube and after adding 5.0 ml. of chromotropic acid with a syringe, the tube was heated for 30 minutes in a boiling water bath, in diffuse light. The presence of formaldehyde is indicated by the development of a blue-violet colour. Chromotropic acid is prepared as follows: Dissolve 1.0 gm. of 1,8-dihydroxynaphthalene-3,6-disulfonic acid (chromotropic acid) in 100 ml. of distilled water and filter. Add 300 ml. of concentrated sulphuric acid to 150 ml. of water, cool, and add to the sulfonic acid solution to make 500 ml. Store in a brown bottle and prepare every 2-3 weeks. The pelleted feed placed in autoclavable plastic bags and the drinking water in bottles was sterilized at 15 pounds pressure for 15 minutes. The bedding for the cages used inside the flexible isolator was autoclaved San-i-Cell<sup>1</sup> (regular grade) since this material was found superior to wood shavings.

For intranasal inoculation, each rat was partially anesthetized with chloroform and 0.1 ml. of

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<sup>1</sup>A product of Laurel Farms Inc., White House Station, New Jersey, U.S.A.

inoculum was instilled into the nostril and the rats were made to inhale this quantity. Control rats received maintenance medium, whereas filtered virus suspension or lung suspension was instilled to infect rats. For the inoculation of germfree rats, a screw cap tube containing the inoculum, along with a screw cap bottle containing 20 ml. chloroform, a small jar (with a lid) big enough to hold a rat, and two sterile tuberculine syringes with gauge 24 needles were introduced into the isolator, through the transfer door.

#### Examination of Inoculated Animals

Animals were observed daily for any signs of obvious respiratory distress. At intervals one rat was killed from each group and the lungs examined macroscopically at autopsy. A portion of each lung from both inoculated and control rats was fixed for histopathological examination to detect peribronchial lymphoid infiltration and the remainder of the lung was ground in each case and inoculum for tissue cultures prepared as described below. Tissue cultures were inoculated with 1/10 dilution of filtered inoculum, one series of tissue cultures receiving inoculum prepared from the infected lung and the control series of tissue cultures inoculated with inoculum prepared from the lungs of control germ-free rats. A set of tissue cultures was also included which only received maintenance medium. These tissue cultures



were incubated and examined for cytopathogenic effects as described.

## HISTOPATHOLOGY

### Histological Techniques

The lungs of rats which died or were killed were quickly placed in a fixative solution (5% formalin-saline). After four days fixing at room temperature, the tissue specimens were put through a routine process in an automatic tissue processor<sup>1</sup> as follows:

<u>Step</u>	<u>Time in hours</u>
Distilled Water	1
Distilled Water	1
50% Alcohol	1
75% Alcohol	2
95% Alcohol	2
Absolute Alcohol	2
Chloroform	1
Cedarwood Oil	2
Tissue Mat (Fisher) I	2
Tissue Mat (Fisher) II	2

Chloroform was used for completing the dehydration process and cedarwood oil proved to be a

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<sup>1</sup>Elliot Tissue Processor, Buckland St.,  
Liverpool 17, England.

superior clearing agent and also resulted in a softer lung tissue for final sectioning. All tissue blocks were held at 10° C in the refrigerator to facilitate sectioning. Throughout this experiment, the thickness of the sections was maintained at 7-8  $\mu$ .

### Staining

All lung sections were stained by the standard Hematoxylin-Eosine stain (Harris' modified hematoxylin) and mounted in permount.<sup>1</sup> During staining the slides were slightly overstained with hematoxylin and blued in alkaline water so as to show more clearly the lymphoid infiltration. Representative slides were examined under the microscope and photographs made when desired.

### Determination of Influence of Age on the Development of Histopathological Lesions

Eight litter mates were housed together along with their mother during the weaning period. At regular intervals of 14, 21, 31, 55, 67 and 72 days, one litter mate was killed and lung specimens were quickly placed in a fixative (5% formalin-saline) for histopathological examination. Finally, at the end the mother was also killed and lungs examined histologically for any typical peribronchial lymphoid infiltration. All sections were stained with the Hematoxylin-Eosine (H.E.) stain. This experiment was repeated with three litters.

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<sup>1</sup>Fisher Scientific Company, Montreal.

Determination of Influence of Hydrocortisone Administration  
on the Occurrence of Histopathological Lesions

Ten litter mates were divided into two groups of five each and the test group received daily 1.0 mg. Cortril<sup>1</sup> per 100 grams body weight subcutaneously, whereas the controls received saline injections. Treatments were started at ten days of age and the stress effect of the hormone was measured on the basis of the record of weight gains and whenever the injected group showed severe weight loss, the injections were withheld for a short period of time. At intervals of 14, 21, 55, 72 and 89 days, one animal from each group was killed and a specimen of lung tissue fixed in 5 per cent formalin-saline. Histological sections were stained with H.E. stain and examined for peribronchial lymphoid infiltration. This experiment was repeated four times.

TISSUE CULTURE TECHNIQUES

Glassware

All glassware (Pyrex brand) used for the cultivation of tissue cultures was thoroughly cleaned. New glassware was treated with acid cleaning solution at room temperature for overnight. After this preliminary

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<sup>1</sup>A brand of Hydrocortisone acetate, aqueous suspension of injection, obtained from Chas. Pfizer and Co., Montreal.

treatment, the glassware was boiled in a detergent<sup>1</sup> (one ounce per gallon of water), rinsed five times in tap water, rinsed once in 1 per cent Hydrochloric acid and finally rinsed five times in distilled water (containing less than 0.1 grains salts per gallon). All glassware was wrapped in brown paper, autoclaved at 121° C for 20 minutes and oven dried. Routinely, used glassware was not retreated with acid after every use, but at regular intervals was acid cleaned as described above.

Media and all other solutions used in the tissue culture work were stored in screw capped bottles using screw tops lined with white non-toxic rubber liners. All rubber stoppers used were either made of white non-toxic rubber or silicone rubber.

#### Sterilization of Media and Other Solutions

Growth media, maintenance media and all other solutions used in tissue culture were sterilized by filtration through a Seitz filter, under pressure (five pounds) and stored in sterile screw cap bottles at 4° C in a refrigerator. Growth media and other solutions used were prepared fresh every week and stored as described above.

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<sup>1</sup>Surg-i-Kleen was obtained from Ingram and Bell, Montreal.

### Sources of Tissue for Tissue Cultures

During this investigation, several different types of tissues from different animal species were used for the isolation of the virus responsible for the endemic pneumonia.

1. Bovine Embryo Kidney and Adult Rat Kidney Cell Lines -

These cell lines were obtained by the courtesy of Dr. A. Greig, Animal Diseases Research Institute, Canada Department of Agriculture, Hull, P.Q. These cell lines were maintained as a stock in Roux bottles and subcultures were made as desired.

2. HeLa Cell Line - This cell line was obtained from the Department of Bacteriology and Immunology, McGill University. Its use was abandoned because difficulties were encountered in maintaining a cell line and no great advantages were apparent over primary cultures.

3. Chick Embryo Primary Cultures - Fertile eggs were incubated in a laboratory incubator at 39° C. Arrangements were made to provide free circulation of the air in the incubator and water was placed in a shallow pan to provide the necessary humidity. Eggs were turned by hand five times in 24 hours. After four days incubation, eggs were candled, and those with no embryos were discarded while the embryonated eggs were reincubated. After another

four days of incubation, the outside of the shells were wiped with alcohol and the embryos removed with sterile instruments, using aseptic precautions. The heads of the embryos were cut and the beheaded embryos were washed in two changes of balanced salt solution (BSS, see below). These were then minced with scissors and trypsinized in spinner flasks. Monolayers were prepared as described in a later part of this section.

4. Chick Embryo Heart Primary Cultures - Embryonated eggs were incubated at 39° C for 18 days and embryos taken out aseptically as described above. The hearts of the embryos were removed aseptically, washed in two changes of balanced salt solution. The hearts were minced and trypsinized to obtain a cell suspension which was used in the preparation of monolayers in Leighton type tubes.
5. Rat Embryo Kidney Primary Cultures - Female rats were bred and pregnancy followed on the basis of weight gains. The embryos were removed aseptically from the uterus, between the 17th and 19th days of pregnancy. The embryos were decapitated and their kidneys removed aseptically. The kidneys were washed in two changes of BSS and then minced with a fine pair of scissors. The minced tissue was trypsinized and the cell suspension used for the preparation of monolayers.

6. Rat Embryo Heart Primary Cultures - Female rats were bred and embryos removed as described in the previous section. The embryos were decapitated, their hearts removed aseptically and washed in two changes of BSS. The heart tissue was minced, trypsinized and the cell suspension was used for the preparation of primary monolayer cultures.
7. Rat Embryo Skin Primary Cultures (RES) - Female rats were bred and embryos removed after 17 to 19 days of pregnancy. The embryos were washed in three changes of BSS and their skin removed as free from underlying muscles as possible. The skins were pooled from the litter, washed in two changes of BSS and minced into fine pieces with a pair of scissors. The minced tissue was trypsinized and cell suspension was used for the preparation of monolayers. In this study, rat embryo skin primary cultures were extensively used for reasons of ease in preparation and the large bulk of tissue obtained from one litter.

#### Preparation of Monolayers

Tissue was obtained aseptically and minced into very small pieces with scissors under BSS. The minced tissue was transferred with a sterile wide mouth pipette, into a spinner flask (Bellco)<sup>1</sup> containing a small magnetic

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<sup>1</sup>Bellco Glass Inc., Vineland, New Jersey, U.S.A.

stirring bar. Trypsin solution prepared in BSS, adjusted to pH 7.4 and warmed to 37° C in a water bath, was added to the spinner flask. This flask was placed on the stage of the magnetic stirrer and agitated for one hour at 37° C. The magnetic stirrer was attached to a cut-out device which automatically broke the electrical circuit every seven seconds for a period of seven seconds, thus completing a 14-second cycle. This was done to ensure that the dispersed individual cells do not adhere to the magnetic rod. The spinner flask was not placed directly upon the stirring platform but an asbestos pad was placed in between to avoid excessive heat.

At the end of trypsinization, the cell suspension was passed through four thicknesses of sterilized cheese cloth placed in a funnel so as to remove large pieces of tissue and the filtrate collected in a conical 150 ml. centrifuge bottle. This cell suspension was centrifuged at 1200 r.p.m. for five minutes and the supernatant discarded. The cell pellet was completely dispersed in nutrient medium and the final cell concentration ( $10^6$  cells/ml.) was adjusted by adding more nutrient medium. The nutrient medium contained penicillin (100 units/ml.) and Dihydrostreptomycine sulfate (100 µg/ml.). These suspended cells were dispensed into appropriate glass containers with proper plane surface. The amount of the cell suspension added into various types of glass



containers is as follows:

Containers	Volume of suspension ( $10^6$ cells/ml.)
Leighton type tube with cover slip -	1.5 ml.
Roller tubes . . . . .	1.5 ml.
T 30 flasks . . . . .	5.0 ml.
T 60 flasks . . . . .	10.0 ml.
8 oz. prescription bottle . . . . .	10.0 ml.
Roux bottles . . . . .	50.0 ml.
Dilution bottles . . . . .	8.0 ml.

All cultures were incubated at  $37^{\circ}$  C and examined every 24 hours. Cultures were fed every 48 hours and monolayers were complete depending upon the tissue after three to five days incubation.

#### Growth Medium

The growth medium employed for the cultivation of tissue cultures was T.C. medium 199 x 10 (Difco)<sup>1</sup> of Morgan et al. (1950) modified by Burr et al. (1954) prepared in triple glass distilled water. This medium was enriched by the addition of ten per cent bovine calf serum (Difco) and 0.5 per cent Lactalbumin hydrolysate (Difco). The medium was sterilized by filtration and bottled in sterile screw top bottles in 100 ml. quantities. Freshly prepared medium was stored in the refrigerator at  $4^{\circ}$  C.

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<sup>1</sup>Difco Laboratories, Detroit 1, Michigan, U.S.A.

Maintenance Medium

Medium 199 (Difco) without the addition of bovine calf serum and Lactalbumin hydrolysate was used as a maintenance medium for virus inoculation and further incubation of the cultures. The medium was sterilized by filtration and stored at 4° C in screw cap bottles in 100 ml. quantities.

Balanced Salt Solution (BSS)

Hank's balanced salt solution (Paul, 1960) was prepared as follows:

(a) Sodium chloride . . . . .	8.00 grams
Potassium chloride . . . . .	0.40 "
Disodium phosphate . . . . .	0.06 "
Potassium dihydrogen phosphate . . . . .	0.06 "
Glucose . . . . .	1.00 "
Phenol red . . . . .	0.02 "
Glass distilled water (triple distilled) .	800.00 ml.
(b) Calcium chloride . . . . .	0.14 grams
Magnesium sulphate (.7 H <sub>2</sub> O) . . . . .	0.10 "
Magnesium chloride (.6 H <sub>2</sub> O) . . . . .	0.10 "
Sodium bicarbonate . . . . .	0.35 "
Glass distilled water (triple distilled) .	200.00 ml.

After dissolving (a) and (b) separately, these were mixed and sterilized by filtration. The sterile solution was dispensed in 100 ml. screw cap bottles and stored at 4° C.

Trypsin Solution for the Initial Trypsinization of Tissue

This trypsin solution was employed for obtaining cell suspension for the preparation of primary cultures and contained:

Sodium chloride . . . . .	8.0	grams
Potassium chloride . . . . .	0.2	"
Disodium phosphate . . . . .	1.15	"
Sodium acid phosphate (Monobasic) . . . . .	0.2	"
Calcium chloride . . . . .	0.1	"
Magnesium chloride . . . . .	0.1	"
Glass distilled water (triple distilled) .	1000.0	ml.
Trypsin (Difco) 1/250 . . . . .	5.0	grams
Phenol red . . . . .	0.02	"

After dissolving and sterilization by filtration, the solution was dispensed in 100 ml. screw cap bottles and stored at 4° C.

Trypsin-Versene Solution

This solution was employed for harvesting cells from monolayers to make passages in continuously cultivated cell cultures and contained:

Sodium chloride . . . . .	8.0	grams
Potassium chloride . . . . .	0.4	"
Dextrose . . . . .	1.0	"
Sodium bicarbonate . . . . .	0.58	"
Trypsin (Difco) 1/250 . . . . .	0.50	"

Versene (Disodium ethylenediamine tetra acetate) . . . . .	0.2 grams
Phenol red . . . . .	0.02 "
Glass distilled water (triple distilled) .	1000.0 ml.

This solution was sterilized by filtration and dispensed in 100 ml. quantities in screw cap bottles and stored at 4° C.

#### Staining of Tissue Culture Monolayers on Coverslips

Coverslips were removed from the Leighton type tubes and washed in two changes of Hank's balanced salt solution at 37° C and treated as follows:

1. Slides were fixed for five minutes in absolute methyl alcohol.
2. Stained two hours in 10 per cent solution of Giemsa stain in distilled water.
3. Rapidly dehydrated in:
  - (a) acetone I
  - (b) acetone II
  - (c) acetone-xylene (75:25)
  - (d) acetone-xylene (50:50)
  - (e) acetone-xylene (25:75)
4. Cleared in xylene for one-half hour and mounted in permount.

The stained slides were examined at 100 x magnification for cytopathogenic changes.

### Preparation of Inoculum

Rats were killed by injecting an overdose of Pentobarbital<sup>1</sup> intraperitoneally. The lungs were immediately removed with aseptic precautions and a small piece from both right and left lungs fixed in 5 per cent formal saline for histological sections as described previously. The remainder of the lungs were ground with 10 ml. BSS in a sterile Ten Broeck tissue grinder<sup>2</sup> and the suspension collected in a sterile screw cap bottle. The resulting suspension was frozen in a freezer at -20° C and then quickly thawed at 35° C; this process was repeated twice more. At the end of the last thawing the lung suspension was centrifuged in a refrigerated centrifuge at 9000 x G for one-half hour. After centrifugation the supernatant was very carefully removed with a sterile pipette and filtered through a sterile ultra-fine fritted glass (Morton) filter to remove all bacteria. Tissue culture harvests were also treated in the same manner. This filtrate was diluted 1:10 with maintenance medium containing penicillin (100 units/ml.) and dihydrostreptomycine sulfate (100 µg/ml.) and employed as an inoculum.

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<sup>1</sup>Stevenson, Turner and Boyce Ltd., Guelph, Ontario.

<sup>2</sup>Bellco Glass Inc., Vineland, New Jersey, U.S.A.

### Inoculation of Tissue Culture Monolayers

For preliminary isolation of the virus, monolayers grown on coverslips in Leighton type tubes were employed. Prior to inoculation, the growth medium was removed and replaced with 1.5 ml. of inoculum prepared according to the method described above. In an equal number of tubes maintenance medium alone replaced the growth medium and these served as controls. For the production of larger quantities of virus and for maintaining virus isolates, tissue cultures grown in Roux bottles or T60 bottles were inoculated as described above.

### Incubation of Cultures After Inoculation

After inoculation of the monolayer tissue cultures with the inoculum prepared from rat lungs or the tissue culture harvest, the cultures were incubated at 37° C for four days and examined daily. At the end of this period, all cultures were examined under low power of the microscope for any cytopathogenic changes and transferred to 25° C incubator. At this stage, the cultures were examined every 24 hours for the appearance of cytopathogenic changes and incubated at this temperature for a period of four to five days.

### Examination of Inoculated Cultures

Unstained inoculated cultures were examined every 24 hours under low power of the microscope and

compared with respective controls. One coverslip from each series was also stained with Giemsa stain as described previously and examined in more detail for any cytopathogenic changes, both at low and higher magnification of the microscope.

#### Demonstration of Plaque Formation

Ten-fold dilutions of virus preparation were made in BSS, up to 1/1000. Growth medium was removed from the tissue culture monolayers, grown in T60 flasks, and the cell sheet washed carefully in one change of warm (37° C) BSS. Using one T. flask per dilution, 0.5 ml. of the virus dilution was added and the control T. flask received only 0.5 ml. of BSS. The flasks were rotated gently on a flat surface to spread the inoculum evenly and then incubated at 37° C for one hour. After incubation, the monolayers were washed in two 10 ml. changes of BSS. Then 8.0 ml. of overlay agar medium, kept at 45° C was carefully placed on the cell sheet, and agar allowed to solidify. Overlay agar medium was prepared as follows:

Noble Agar . . . . .	2.0 gm.
Hanks BSS . . . . .	100.0 ml.

Agar was dissolved by heating, dispensed in screw cap tubes in 5 ml. quantities, and autoclaved for 15 minutes at 121° C. Before use, the medium was melted and cooled to 45° C. To this melted agar, 5 ml. of maintenance

medium 199 (containing antibiotics) prewarmed to 45° C was added aseptically and mixed. This medium was used in 8.0 ml. quantities as overlay.

Upon solidification of the agar, flasks were incubated at 37° C for five days, and then transferred to 25° C for an additional three days incubation. The cell sheets were observed daily for any plaque formation and compared with the control sheets. At the end of the incubation period, the cell sheets were also examined microscopically for the formation of plaques.

#### VIRUS TECHNIQUES

##### Preparation of Virus Suspension for Animal Inoculation

At the height of cytopathogenic effects in tissue cultures the maintenance medium was harvested. After freezing (-20° C) and thawing (35° C) three times as described before, this material was centrifuged at 9000 x G for half an hour and the supernatant filtered through a sterile ultrafine fritted glass filter (Morton). The filtrate was pipetted into sterile screw cap bottles, and this served as a material for intranasal inoculation of animals.

##### Examination of the Virus Preparation by Electron Microscopy

Tissue culture harvest was centrifuged at 9000 x G for half an hour and the supernatant filtered through a



sterile ultrafine fritted glass filter (Morton). The filtrate was dialyzed both against two per cent solution of ammonium acetate and distilled water for 48 hours with frequent changes of the ammonium acetate solution and distilled water. The contents of the dialyzing bag were transferred in 10 ml. amounts to tubes and centrifuged in a "Spinco" centrifuge at 100,000 x G for three hours. The supernatant was discarded except the bottom half-inch column of liquid in the tube, this served as the virus concentrate. After shaking the contents of the Spinco centrifuge tube, this virus preparation was again filtered through a fritted glass filter. The filtrate was stored in a sterile screw cap tube, in a freezer (-20° C). The above concentrated and salt-free virus preparation was dispensed in one ml. amount into a screw cap tube. To this an equal amount of a two per cent solution of phospho tungstic acid prepared in distilled water adjusted to pH 7.0 with 1 N NaOH was added and the contents agitated. Droplets of this stained material (phospho tungstic acid and virus suspension) were deposited upon several grids which after drying in vacu were examined at 20,000 x magnification and representative fields were photographed with the courtesy of Mr. J. R. Côté of l'Institut de Microbiologie, l'Université de Montréal, Montreal.

Experiment to Determine the Influence of Chemicals on the Virus

Virus preparation was dispensed in 5.0 ml. quantities into three screw cap tubes and to each tube 1.0 ml. of chloroform or acetone was added. The contents were mixed by agitating and the chloroform mixture left at room temperature for ten minutes, whereas the acetone treated virus was held at 4° C for 24 hours (Greig and Girard, 1963). At the end of this period, most of the solvent was removed by aspiration and the residue evaporated at room temperature by a stream of air. The aqueous virus preparation left at the end of evaporation was carefully transferred into a clean tube and the evaporating dish rinsed with 2 ml. of BSS. This preparation was then filtered through an ultrafine fritted glass filter and the filtrate was pipetted into a sterile screw cap tube and used for inoculation of tissue cultures. Similarly, 2 ml. of maintenance medium was treated and used for inoculating control tubes. Two ml. of chemically treated virus preparation was mixed with 10 ml. of maintenance medium containing penicillin (100 units/ml.) and dihydrostreptomycine sulfate (100 µg/ml.) and 1.5 ml. aliquots were inoculated into three-day-old tissue culture (RES) monolayers in Leighton type tubes.

The cultures were incubated at 37° C for four days and then reincubated for another four days at 25° C.

All cultures were examined every 24 hours and one cover-slip from each series was stained for a more detailed examination. A similar series of eight tubes was inoculated with chemically treated maintenance medium as controls.

#### Experiment to Determine the Temperature Tolerance of the Virus

Virus preparation was dispensed in 2 ml. quantities in four sets of six tubes each. One set of tubes was incubated at each of the following temperatures, 7° C, 25° C and 37° C respectively. Every 24 hours, to one tube from each series, 4 ml. of maintenance medium was added, and 1.5 ml. quantities inoculated into three tissue culture (RES) monolayers and incubated as described previously. An extra set of tubes containing virus preparation was incubated in a water bath at 56° C and every one-half hour one tube was used for inoculating tissue cultures. The viability of the virus was indicated by the development of cytopathogenic effects.

#### Experiment to Determine the Viability of the Virus on Storage

Virus preparation was dispensed in 2 ml. quantities in two sets of six tubes each. One set of tubes with the virus preparation was placed in the refrigerator (7° C) and the other set of tubes containing virus preparation was placed in the deep freeze (-20° C).

Every week, to one tube from each series (the frozen virus preparation was thawed), 4 ml. of maintenance medium was added, and 1.5 ml. quantities inoculated into three tissue culture (RES) monolayers and incubated as described previously. The viability of the virus was judged upon the development of cytopathogenic effects.

#### Demonstration of Hemagglutinating Properties of the Virus Isolate

For these experiments fresh red blood cells were obtained from rats, mice and chickens. The blood was obtained from the heart and then agitated with glass beads to remove all the fibrin. After agitating the blood with glass beads for five minutes, the RBC's were washed three times with saline and at the end of the last centrifugation, the red blood cells were suspended in an equal quantity of saline. The final RBC suspension (2%) for hemagglutination was prepared by adding 2 ml. of 50% suspension to 48 ml. of saline. Seven small tubes were placed in a rack, the first tube received 1.0 ml. of virus suspension and the remaining six tubes received 0.5 ml. of saline. Doubling dilutions of virus preparation were made in these tubes, up to  $1/64$ . To each tube containing virus dilution, 0.2 ml. of 2% RBC suspension were added and after mixing the contents, the tubes were incubated at  $37^{\circ}$  C for one hour and examined twice at 30-minute intervals. The indication of hemagglutination

was the absence of a pellet of RBC's at the bottom of the tubes. The tubes were then left undisturbed at room temperature to be examined every 30 minutes for three hours. This experiment was repeated in triplicate with RBC's from all the three species of animals (rats, mice and chickens).

#### Determination of Antibodies in Rat Serum

Four adult rats, cage mates of a rat which died of pneumonia and whose lungs showed typical peribronchial lymphoid infiltration, were aseptically bled to death from the heart and their blood pooled in a sterile screw top conical centrifuge tube. This tube was left at room temperature overnight and then placed in the refrigerator for six hours. After this, the clot was centrifuged at 3,000 r.p.m. for 15 minutes and the clear supernatant serum collected into a sterile screw cap tube.

Doubling dilutions of this serum were made in sterile tubes with maintenance medium containing penicillin (100 units/ml.) and dihydrostreptomycine sulfate (100 µg/ml.) in two ml. quantities. The final dilution reached was 1/128, starting with one-half dilution. To each tube was added 0.5 ml. of virus preparation which was capable of producing cytopathic effects in tissue cultures at a final dilution of 1/512. This mixture was incubated at 37° C in a water bath for

one-half hour and 1.5 ml. aliquots inoculated into three-day-old tissue culture monolayers (rat embryo skin primary cultures) grown in Leighton type tubes. These tubes were incubated at 37° C for four days and then incubated at 25° C for another four days. All these cultures were examined every 24 hours. With each set of inoculated tubes, equal numbers of control tissue culture tubes were also included which only received 1/2 dilution of rat serum and maintenance medium. One set of eight tissue cultures received only 1.5 ml. of 1/10 dilution of virus preparation in maintenance medium. These tubes were set up in triplicate with each serum pool and in all three serum pools were tested. After each examination, one monolayer on a coverslip was stained by the method described before and examined critically for any protection afforded by the serum dilutions tested.

#### Production of Immune Serum in Rabbits

Virus suspension was prepared as described previously and two six-month-old healthy rabbits were inoculated intravenously in the ear vein and a third rabbit served as the control and was inoculated with the maintenance medium. The immunization schedule was as follows (page 90). Test bleeding from the ear vein was done on the 24th day and after a rest period of three weeks, a booster shot of 1.5 ml. was administered. This was followed by one week rest and then the final

bleeding from the heart. The blood was left at room temperature overnight and then transferred to the refrigerator for six hours. After this period the clot was centrifuged at 3,000 r.p.m. for 15 minutes and the clear serum sterilized by filtration.

<u>Day of Immunization</u>	<u>Injection No.</u>	<u>Dose</u>
1st	1	0.2 ml.
3rd	2	0.2 "
5th	3	0.3 "
7th	4	0.3 "
9th	5	0.3 "
11th	6	0.4 "
13th	7	0.4 "
15th	8	0.5 "
17th	9	0.7 "
19th	10	0.9 "
21st	11	1.2 "
23rd	12	1.5 "

#### Determination of Antibodies in Rabbit Serum

Doubling dilutions of rabbit serum were made in sterile tubes with maintenance medium containing penicillin (100 units/ml.) and dihydrostreptomycin sulfate (100 µg/ml.) in two ml. quantities. To the final dilution of 1/1024. To each tube was added 0.5 ml. of virus preparation known to be capable of producing cytopathogenic effects in rat embryo skin primary tissue

culture monolayers at a final dilution of 1/512. This mixture was incubated at 37° C in a water bath for one-half hour and 1.5 ml. aliquots were inoculated into three-day-old tissue culture monolayers (rat embryo skin primary cultures) grown in Leighton type tubes. These tubes were incubated at 37° C for four days and then incubated at 25° C for another four days. All these cultures were examined every 24 hours. An equal number of control tissue culture monolayers was inoculated with a one-half dilution of serum obtained from the control rabbit. After each examination, one monolayer on a coverslip was stained by the method described previously and examined.

#### DEMONSTRATION OF PLEUROPNEUMONIA-LIKE-ORGANISMS FROM RAT LUNG

Pleuropneumonia-like-organisms (P.P.L.O.) were cultured using a medium suggested by Edwards in 1947 or the medium described by Klieneberger-Nobel in 1962, with slight modifications (see page 92).

To prepare solid medium, 1 per cent agar was added by first melting the agar and then autoclaving at 121° C for 15 minutes. The agar was cooled to 45° C before pouring plates. In addition 1000 units of penicillin, plus 1.0 ml. of either 20 per cent v/v of horse serum (Difco) or bovine P.P.L.O. serum fraction



(Difco) and 0.2 ml. of a 0.2 per cent DNA<sup>1</sup> solution was added.

	Edward	Klieneberger-Nobel
Beef Heart Infusion (Difco)	25 grams	25 grams
Thalium Acetate (Fisher)	0.5 "	0.25 "
Yeast Extract *	100 ml.	100 ml.
Water	800 "	850 "
pH adjusted to	7.8 - 7.9	7.8 - 8.0
Sorensen's Buffer(0.15M)	100 ml.	-
Sterile Defibrinated Bovine Blood	-	50 ml.

(The medium was dispensed in 5 ml. quantities in screw cap tubes and autoclaved at 121°C for 15 minutes.)

\* Fresh yeast extract was prepared by boiling 500 grams of fresh baker's yeast in a liter of water for ten minutes. After cooling to room temperature, the mixture was centrifuged at 6000 x G in a refrigerated centrifuge for 20 minutes. The supernatant was collected and passed through a Seitz filter and filtrate sterilized in screw cap bottles, at 15 pounds pressure for 15 minutes and stored in the refrigerator. This yeast extract was stored for 15 days and then discarded.

<sup>1</sup> DNA (Sigma Chemicals, St.Louis, Missouri,U.S.A.) solution (0.2%) was prepared in water and sterilized by filtration. Sterile DNA solution was dispensed in sterile screw cap tubes in 5 ml. quantities and stored in a refrigerator.

For the primary isolation of P.P.L.O. from rat lungs, liquid medium was employed. The lung specimen was minced with a pair of scissors and a small quantity of the minced tissue dropped into the tube of medium, to which penicillin (200 units/ml.) and serum (20% v/v) had already been incorporated. These tubes were agitated to ensure proper mixing and incubated at 37° C for three days. At the end of this incubation period, the tubes were shaken, allowed to stand for one-half hour, and a sub-culture made by inoculating 0.5 ml. into a fresh tube of liquid medium which was incubated at 37° C for another three days.

For making sub-cultures on solid agar medium, melted medium was cooled and penicillin, serum and DNA solution was added before pouring into disposable sterile plastic petri plates. The poured plates were allowed to solidify and then the surface of the agar was dried by leaving the plates with the lids partially open in an incubator at 37° C for one hour. These plates were inoculated by placing a 0.1 ml. inoculum of liquid culture from the sub-cultured tube and the inoculums spread by tilting the plate in a circular fashion. The surface of the agar was again dried by leaving the plates in the incubator at 37° C for one-half hour and then the lids were closed. These plates were incubated for five days and examined every day for the development

of P.P.L.O. colonies. The efficacy of each batch of medium was tested by isolating P.P.L.O. from chicken tracheas obtained from the Poultry Department, Macdonald College with an inoculum prepared by scraping the lumen of the trachea. The scraped material was mixed with one ml. of saline and 0.5 ml. inoculated in liquid P.P.L.O. medium which when transferred to solid medium contained typical P.P.L.O. colonies.

Demonstration of Haemolysin Produced by P.P.L.O. Isolates for Murine Erythrocytes

A 50 per cent suspension of erythrocytes (rat and mouse) was prepared in saline as described above, and 1.0 ml. of this was incorporated into 24 ml. of P.P.L.O. solid medium (2 per cent erythrocytes v/v), which was melted and cooled to 45° C. A thin layer (2.0 ml.) of this medium was overlayed on the surface of agar showing P.P.L.O. colonies. After the agar had solidified, plates were incubated at 37° C for three days, and at the end of this period the plates were stored at 10° C for eight hours. A clear zone around the P.P.L.O. colonies was considered as evidence of haemolysis of erythrocytes.

## RESULTS

## RESULTS

### Observations on the Symptoms Shown by a Rat at the Acute Pneumonic Stage

During the earlier stages of chronic murine pneumonia there are no outward symptoms of a respiratory distress. However, in old adult rats at later stages of the disease, the hair coat roughens and the body weights may or may not show any decrease. As the condition progresses, with increased respiratory involvement the feed intake decreases and the animal shows a weight loss. Furthermore, the animal shows definite signs of respiratory distress, which are characterized by laboured and audible breathing, arched back and frequent scratching of the nose. The results of observations made at an advanced stage of the disease are shown in Figs. 1 and 2. Most animals showing the symptoms of marked respiratory distress usually die in a period varying from ten days to a month.

### Histopathological Lymphoid Infiltration in the Lungs of Adult Conventional Rats

Histological sections of lungs from rats without any signs of respiratory distress showed marked lymphoid infiltration, both at peribronchial and perivascular



Fig. 1. A rat in advanced stages of respiratory distress, showing arched back and rough hair coat.



Fig. 2. A rat in advanced stage of respiratory distress, scratching nose due to difficulty in breathing.

locations, the former being more frequent. Seventy per cent of the lungs examined showed only the peribronchial lymphoid infiltration, whereas the remainder showed infiltration at both the locations. The histopathological picture presented by the lungs obtained from rats showing respiratory distress, was not different from what has already been reported, except the lesions were extensive (cuffs) and in addition the alveoli surrounding the bronchi with lymphoid infiltration were collapsed. A representative histological section, with lymphoid infiltration is shown in Fig. 3.

#### Influence of Age on the Development of Histopathological Lesions

This experiment was conducted to determine the age at which the lungs of baby rats show typical lymphoid infiltration at peribronchial locations. The results of these experiments showed that there is no peribronchial lymphoid infiltration in the lungs of 14-day-old baby rats (Fig. 4), but at the 22nd day small collections of infiltration make their appearance (Fig. 5). From this stage onwards, the amount of lymphoid mass increases progressively; at 31 days of age the mass of infiltrated material has increased (Fig. 6) and at 55 days the lymphoid infiltration is well marked (Fig. 7). This infiltration reaches enormous magnitude at 67 days (Fig. 8) and at 72 days (Fig. 9) it is comparable with

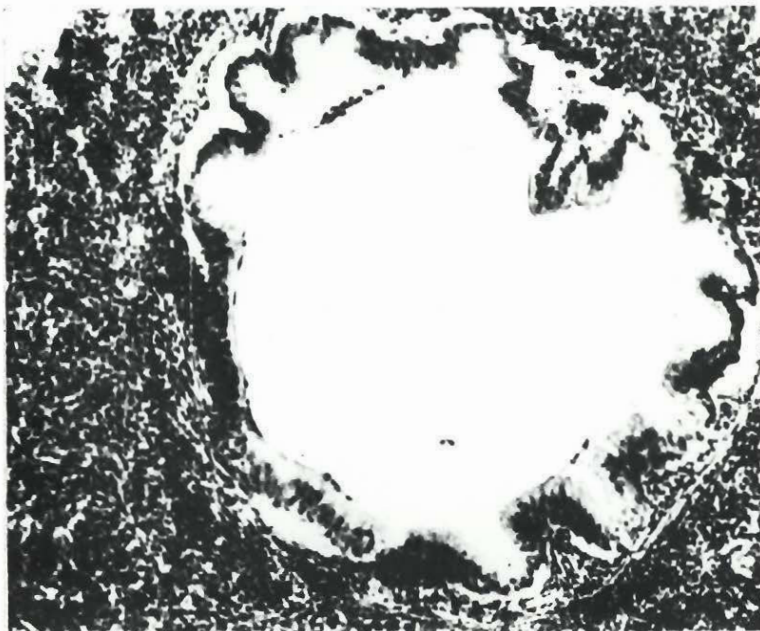


Fig. 3. Lung section, conventional adult rat, showing marked peribronchial lymphoid infiltration. The lymphoid infiltration is massive and has a tendency to push the bronchial epithelium into the lumen. H.E. stain. Magnification x 200.



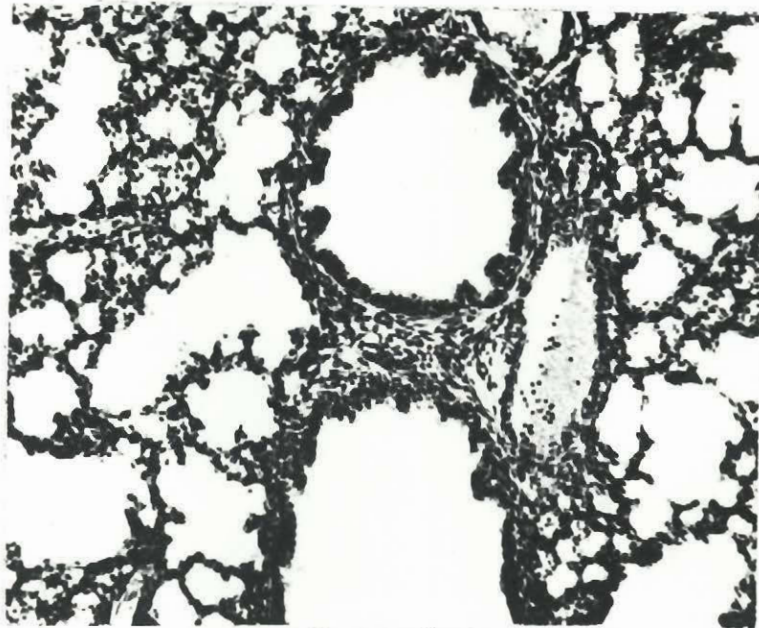


Fig. 4. Lung section, conventional baby rat, 14 days old. There is no peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

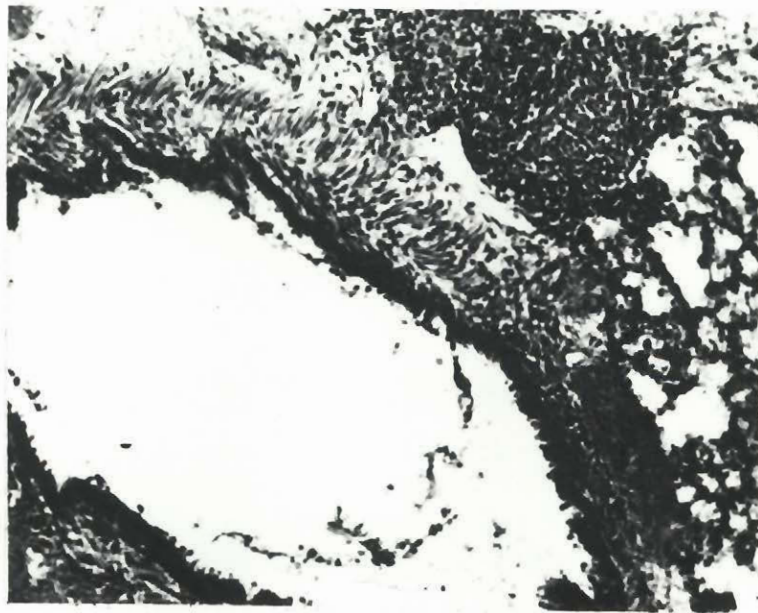


Fig. 5. Lung section, conventional baby rat, 22 days old. Small collection of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

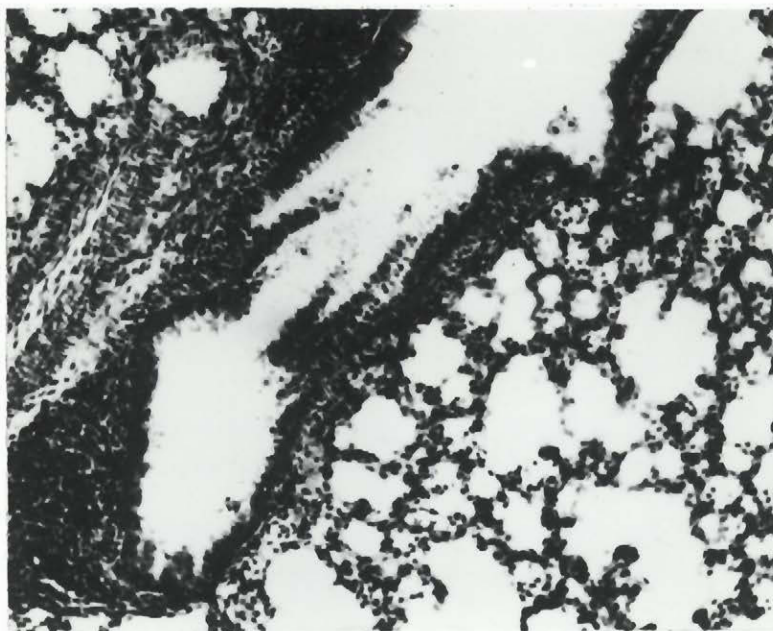


Fig. 6. Lung section, conventional baby rat, 31 days old. Showing increasing peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

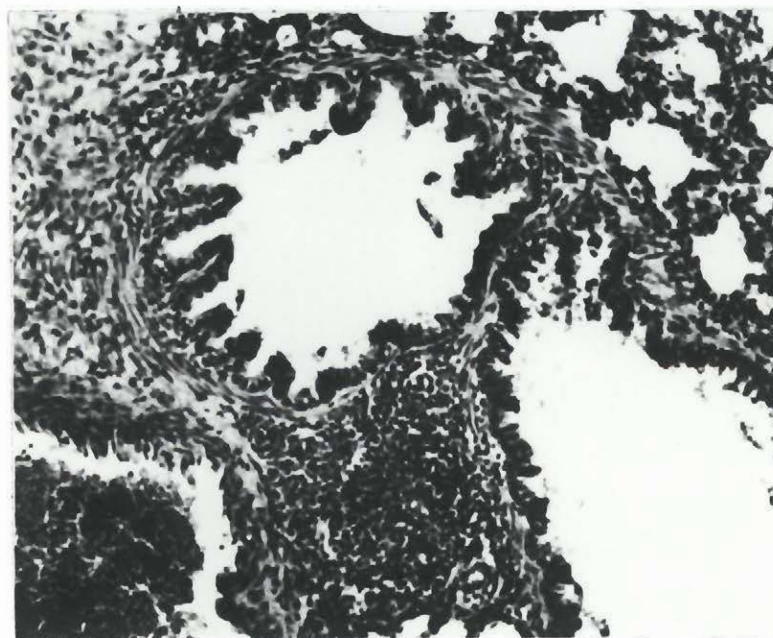


Fig. 7. Lung section, conventional baby rat, 55 days old. Showing well marked peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

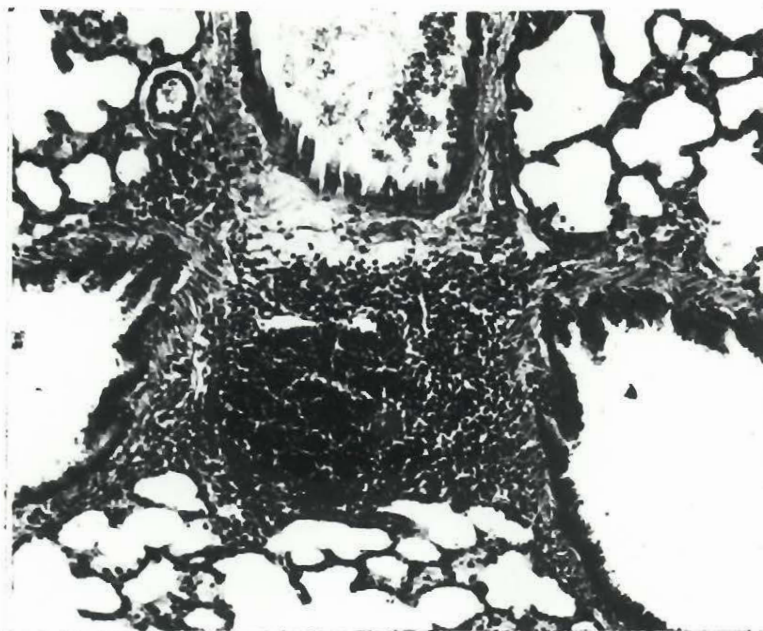


Fig. 8. Lung section, conventional baby rat, 67 days old. The peribronchial lymphoid infiltration has reached enormous magnitude. H.E. stain. Magnification x 200.

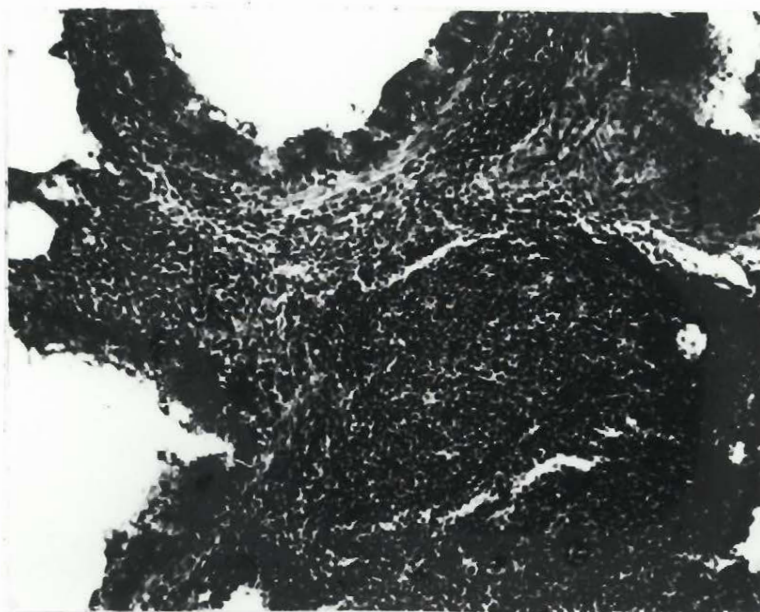


Fig. 9. Lung section, conventional baby rat, 72 days old. The peribronchial lymphoid infiltration is comparable with the amount found in an adult rat. H.E. stain. Magnification x 200.



the amount found in an adult rat. These results indicate that lymphoid infiltration appears at two weeks of age and then increases progressively until maturity.

#### Influence of Hydrocortisone Administration

Baby rats responded to continued administration of hydrocortisone by failure to gain weight and finally by weight loss if the hormone administration was continued. The differences in the weights of control and hydrocortisone treated animals are shown in Fig. 10. While the hydrocortisone treated baby rats in this experiment gained weight, the increase was much less than observed in the control animals.

Another striking effect of hydrocortisone administration was the loss of hair growth resulting in a scanty and rough hair coat. Fig. 11 shows the striking differences between a control and a hydrocortisone treated rat. The majority of the baby rats which received hydrocortisone for a prolonged period (6-7 weeks) developed respiratory distress, had nasal discharge, and died of acute respiratory involvement. Upon autopsy the lungs of such animals showed gross lesions, which covered the surface of the lungs with greyish nodular areas and the cut surface of the lung was dry.

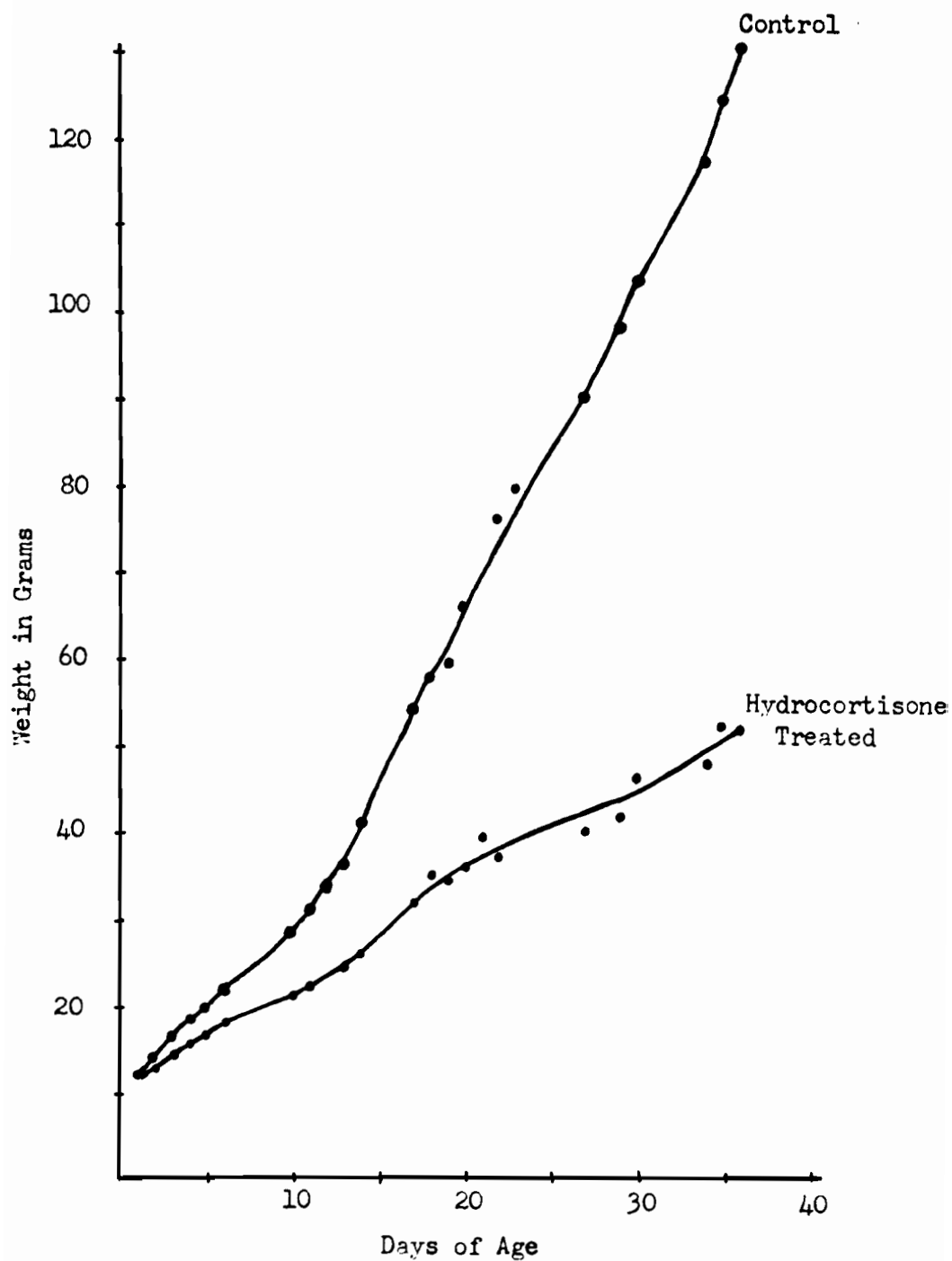


Fig. 10. Influence of hydrocortisone administration on the weight gains of litter mates. (Average of 4 in each group).

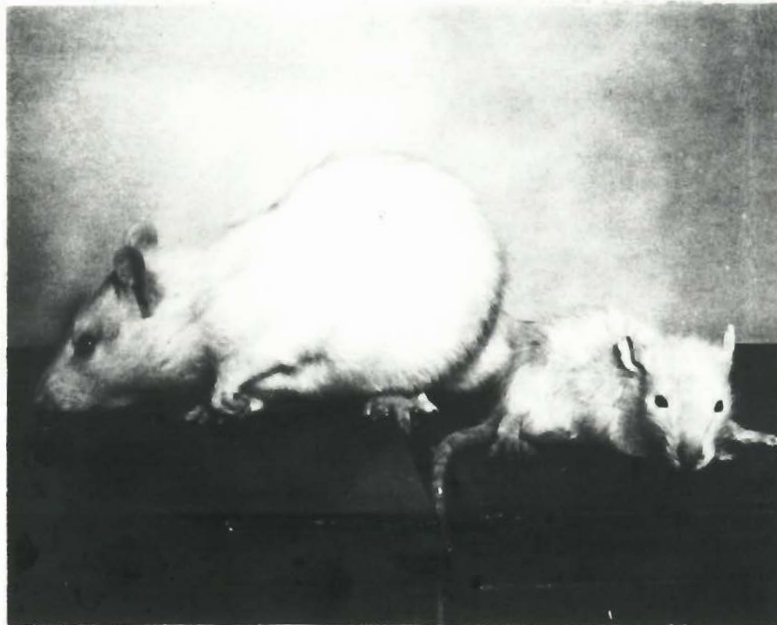


Fig. 11. Two litter mates, one on the right has received hydrocortisone treatment, whereas the one on the left is the control.

Influence of Hydrocortisone Administration on  
Histopathological Lesions (Lymphoid Infiltration)

The lungs from both hydrocortisone inoculated and control rats were histologically examined for peribronchial lymphoid infiltration. The results of this experiment showed that there was no lymphoid infiltration around the bronchi in the lungs of rats which had received hydrocortisone treatment, whereas the control rats had varied degrees of infiltration depending upon the age. A section of the control lung obtained from an 84-day-old rat showed massive peribronchial lymphoid infiltration (Fig. 12), while a similar preparation of lung from a hydrocortisone treated rat showed no lymphoid infiltration around the bronchi (Fig. 13), although the rat showed nasal discharge, accompanied by respiratory distress, and the lungs showed gross macroscopic pathological changes. In the same section (Fig. 13) the evidence is clear that the alveoli around the bronchi were collapsed.

Incidence of Pleuropneumonia-Like-Organisms (P.P.L.O.)

During this study minced lung specimens from all rats killed were inoculated in liquid P.P.L.O. medium which was subcultured once again in liquid medium and finally on solid medium. Throughout this investigation no consistent isolation of P.P.L.O. organisms was found. Furthermore, the isolation of the P.P.L.O. was not related

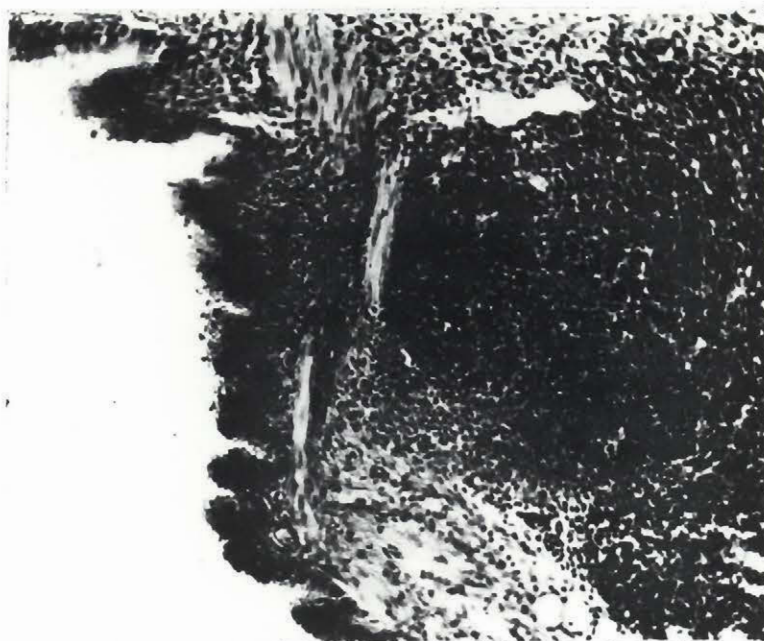


Fig. 12. Lung section, conventional rat, 84 days old, which received saline injections only. The peribronchial lymphoid infiltration is massive. H.E. stain. Magnification x 200.

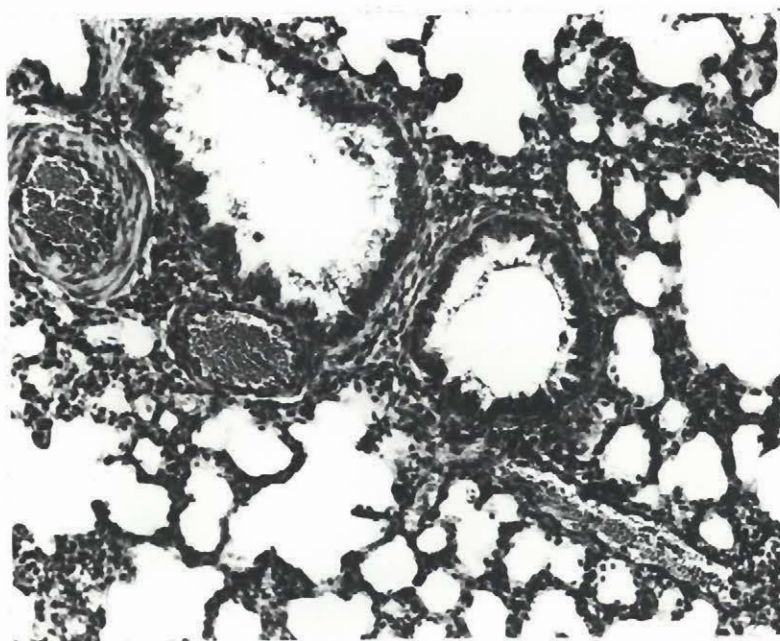


Fig. 13. Lung section, conventional rat, 84 days old, which received hydrocortisone injections. Note the lack of peribronchial lymphoid infiltration as compared to the control. H.E. stain. Magnification x 200.



to the degree of pneumonic lesions, since the P.P.L.O. were isolated from apparently normal lungs, whereas, at times, isolation was unsuccessful from lungs which appeared grossly involved. The P.P.L.O. were isolated successfully from 36 per cent of lungs apparently normal in appearance, but only 30 per cent of the isolations from pneumonic lungs were successful. Similarly, isolations were made from nasal cavity washings of 32 per cent of the rats with apparently normal lungs and 35 per cent from those with pneumonic lungs. These results indicate, however, a higher rate of P.P.L.O. isolations from nasal cavities of rats which showed macroscopic changes in the lungs. The different batches of the P.P.L.O. medium both liquid and solid were tested for efficacy by cultivating P.P.L.O. isolated from chickens; on all batches of the medium the organisms produced typical colonies (Fig. 14).

#### Pathogenicity of P.P.L.O. on Mice and Rats

Ten litter mates (both rats and mice) ten days old were inoculated intranasally with 0.05 ml. of liquid culture of P.P.L.O., each animal receiving a different P.P.L.O. isolate. None of the animals showed any respiratory distress except slight, but not persistent, nasal discharge in the mice a week after inoculation. The histological examination of the lungs at four-day intervals for a period of 21 days, showed neither peribronchial lymphoid infiltration, nor any other pathological

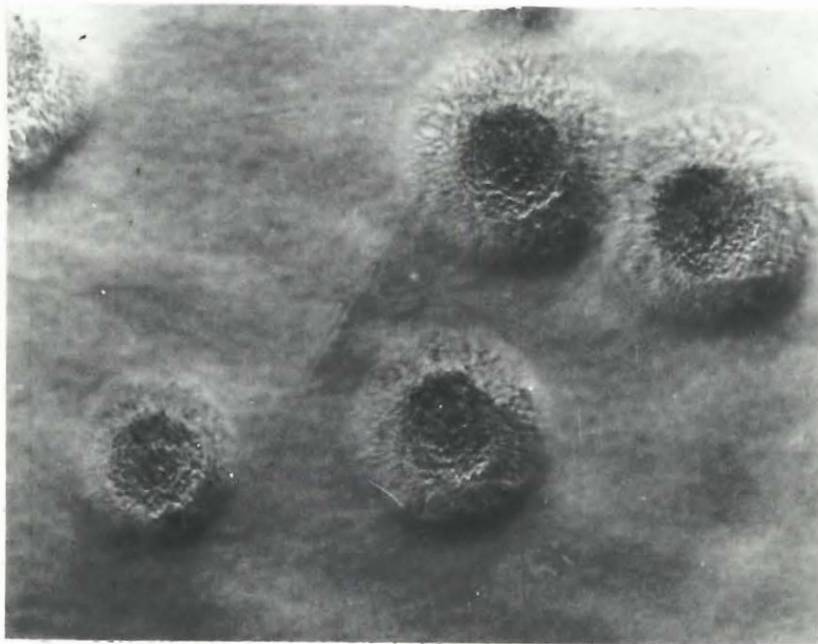


Fig. 14. Colonies of pleuropneumonia-like-organisms isolated from chicken tracheas. Bright field. Phase contrast photograph. Magnification x 200.

changes in the lungs of all rats as compared with the control lungs, whereas only two mice showed some degree of infiltration.

#### Haemolysis of Murine Red Blood Cells by P.P.L.O. Isolates

The result of this experiment indicated that none of the above ten P.P.L.O. isolates produced any degree of haemolysis of either rats or mice red blood cells, when incubated at 37° C for three days. Storing the plates for eight hours at 10° C after this incubation did not cause any haemolysis of the red blood cells.

#### Preliminary Trials for the Isolation of a Viral Agent in Various Tissue Culture Systems

During these experiments various tissue culture systems (bovine embryo kidney cell line, adult rat kidney cell line, HeLa cell line, chick embryo primary culture, chick embryo heart primary culture, rat embryo kidney primary culture, rat embryo heart primary culture and rat embryo skin primary cultures) were inoculated with the rat lung filtrate. All these tissue culture monolayers were incubated at 37° C for seven days and the cultures were examined every 24 hours for any cytopathogenic effects. All these attempts were unsuccessful and no filterable cytopathogenic agent could be demonstrated by this method. On one isolated occasion, a cytopathogenic agent was demonstrated in rat embryo skin primary culture but it was lost after subculturing.

Effect of Incubation Temperature on the Demonstration of  
of Cytopathogenic Effect and the Isolation of a Filterable  
Agent

An accidental observation that inoculated tissue culture monolayers (rat embryo skin primary) left at room temperature (25° C) for two days after previous incubation for six days at 37° C, showed a marked cytopathogenic effect in the inoculated cultures as compared with the controls, this proved to be a most important observation. From then on, all inoculated cultures, with their controls, were first incubated at 37° C for five days and then transferred to a lower temperature (25° C) for another four days of incubation. The results showed that there were no cytopathogenic changes observed in inoculated cultures incubated at 37° C for six days but the cytopathogenic changes became evident after two days of additional incubation at a lower temperature. These cytopathogenic changes were progressive and ultimately the whole cell sheet was destroyed. In addition, no cytopathogenic changes were observed in inoculated cultures when left at lower temperature (25° C) for a total period of eight days. Furthermore, there were no cytopathogenic changes produced if inoculated cultures were first incubated at 25° C for six days and then reincubated at 37° C for an additional two days. Thus, only one combination of temperature treatments was successful and with this method, filterable agents were isolated from 80 per cent of the lungs tested.

Some of the isolates were maintained in the laboratory for well over 40 tissue culture passages in the rat embryo skin primary cultures. Electron photomicrographs (Fig. 15) of the virus particles do not give much information about the virus except to indicate the presence of discrete round particles, approximately  $110 \text{ \AA}$  in diameter. During these preliminary trials for the preparation of virus suspension for electron microscopy, difficulties were encountered in concentrating the virus.

#### Cytopathogenic Changes

The cytopathogenic changes observed in rat embryo skin primary cultures after incubating the cultures at  $37^{\circ} \text{C}$  for six days and then at lower temperature ( $25^{\circ} \text{C}$ ) for three days are shown in Fig. 17 as compared with the control culture (Fig. 16). Examination showed that the cells have fallen from the glass surface in the inoculated cultures leaving cytoplasmic bridges. The cytoplasm was not as abundant as in the control cells. The nuclei of the cells in the inoculated tissue cultures were densely stained and pyknotic. The cytoplasm of the infected cells had numerous vacuoles, but neither cytoplasmic nor nuclear inclusion bodies were demonstrable. Furthermore, the inoculated tissue cultures had a tendency towards giant cell formation as compared with the controls. The pyknotic changes in the nuclei of the infected cultures were also shown by the acridine orange staining, whereby

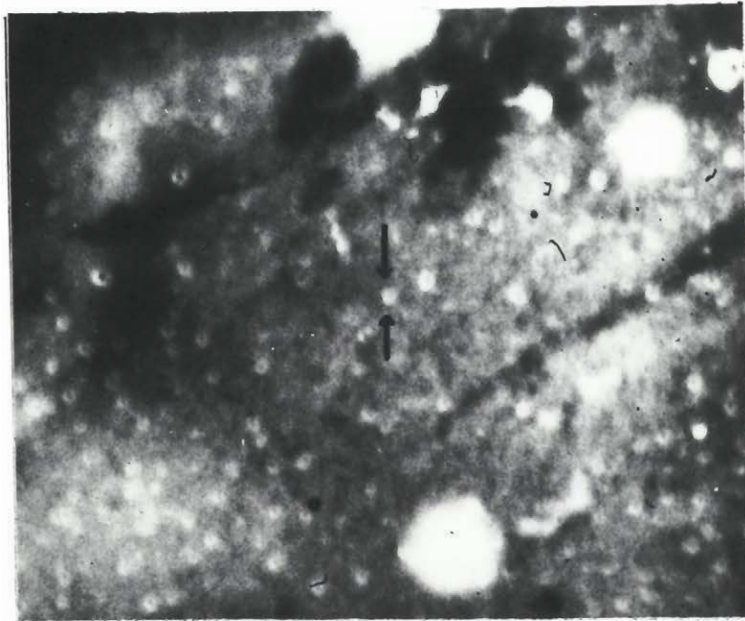


Fig.15. Electron photomicrograph of the virus particles. The particles appear to be round. P.T.A. stain. Magnification x 50,000.

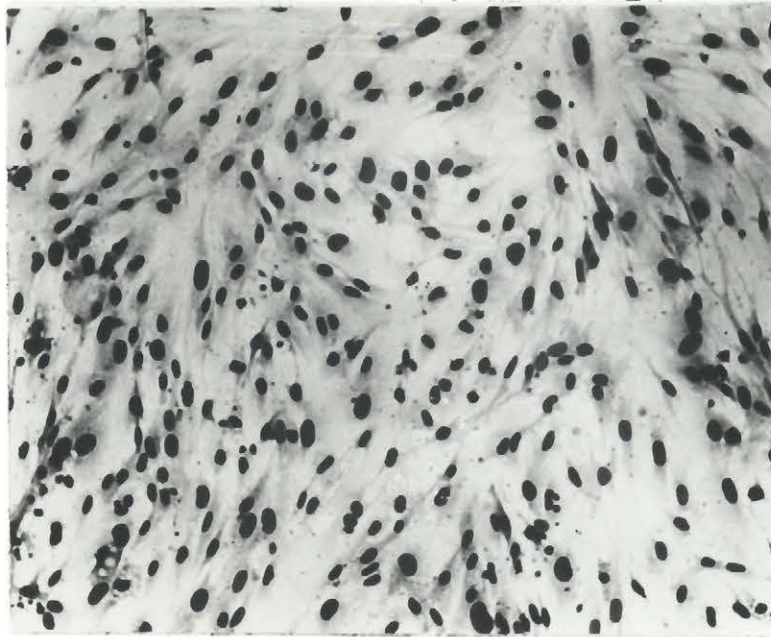


Fig. 16. Normal rat embryo skin primary culture monolayer. Incubated 5 days at 37°C and then reincubated at 25°C for additional 4 days. Giemsa stain. Magnification x 200.

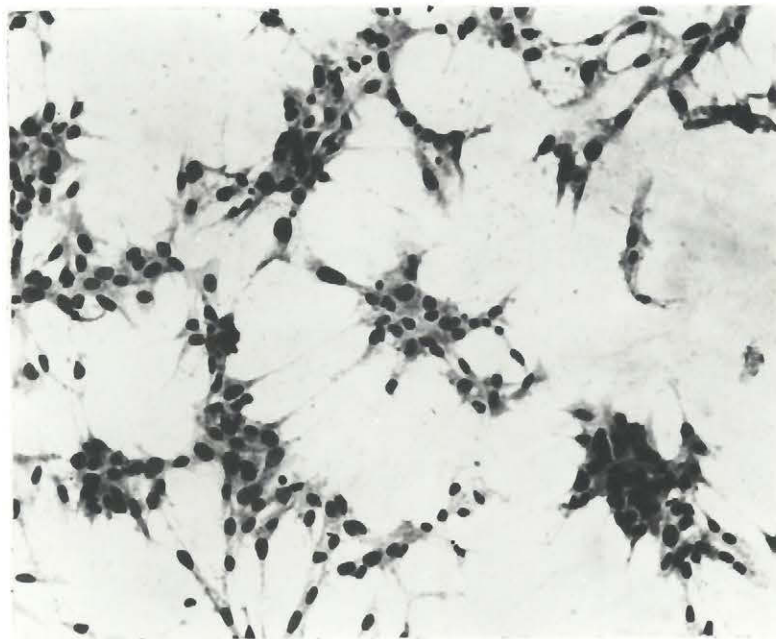


Fig. 17. Infected rat embryo skin primary culture monolayer. Incubated at 37°C for 5 days and then reincubated at 25°C for additional 4 days. Note the necrosis of the cell sheet and the cytoplasmic bridges. The nuclei show pyknotic change. Giemsa stain. Magnification x 200.

the infected cells were found to have a denser nuclear material as compared with the control cells. The viral agent did not produce any cytopathogenic changes in other tissue culture systems tested, eg. HeLa cell line and chick embryo primary cultures.

#### Neutralizing Antibodies in Rat Serum

The results of this experiment indicated that the rat serum had no demonstrable neutralizing antibodies against the viral isolates tested.

#### Neutralizing Antibodies in Rabbit Serum

The results of experiments upon the neutralizing activity of the immune serum produced in rabbits are given below:

Dilution of Serum	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Neutralizing Antibodies in Control Serum	-	-	-	-	-	-	-	-
Neutralizing Antibodies in Immune Serum	+	+	+	+	+	+	+	-

These results indicated that the immune serum completely protected rat embryo skin tissue cultures against cytopathogenic effects of the virus under investigation at the final dilution of 1/128, whereas serum from the control rabbits had no protecting effect.



### Haemagglutinating Properties of the Viral Isolate

The results of experiments conducted on red blood cells (RBC's) obtained from rats, mice and chickens showed that the viral isolate did not possess any haemagglutinating properties. Even when the whole undiluted tissue culture harvests were employed and the mixture (virus suspension and RBC's) was incubated at 40° C and 20° C instead of 37° C, no haemagglutination of the red blood cells was evident.

### Plaque Formation

The results of these experiments indicated that the virus under investigation did not form any plaques on the cell sheet employed.

### Temperature Tolerance of the Virus

Results given below indicate that the virus survived at 7° C for the entire period of the experiment (six days). The virus was non viable after 48 hours at 25° C, 24 hours at 37° C, or 30 to 60 minutes when incubated at 56° C.

Temperature	Days of Survival					
	1	2	3	4	5	6
7° C	+	+	+	+	+	+
25° C	+	+	-	-	-	-
37° C	+	-	-	-	-	-
	Hours of Survival					
	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	
56° C	+	+	-	-	-	

### Viability of the Virus and its Sensitivity to Chemicals

The virus was viable after two weeks of storage at 7°C, but became inactive after three weeks of storage at this temperature. The frozen preparation of the virus stored at -20°C produced cytopathogenic effects after six weeks of storage.

The virus was rendered nonviable and did not produce cytopathogenic changes when treated with chloroform and acetone.

### Pathogenicity of Viral Isolate for Mice

The colony of mice from where the litter mates were obtained, had no previous history of any respiratory disorder. Ten-day-old mice, after intranasal inoculation of the virus preparation showed no clinical signs of respiratory distress or any nasal discharge a week after inoculation. Inoculated mice killed 14 days after intranasal inoculation showed no recognizable lymphoid infiltration as compared with the control lungs. Sections of lungs obtained 21 days after inoculation of mice showed lymphoid infiltration (Fig. 19) whereas the control lungs showed no such infiltration (Fig. 18). Attempts to cultivate P.P.L.O. from lungs as well as from the nasal cavities were unsuccessful, both from inoculated and control mice.

### Pathogenicity of Viral Isolate for Prestressed Conventional Rats

Eight litter mates, ten days of age, were prestressed by the administration of hydrocortisone for two weeks, and inoculated intranasally with 0.05 ml. virus



Fig.18. Section of mouse lung, 31 days old, 21 days after intranasal instillation of Medium 199. Note the lack of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

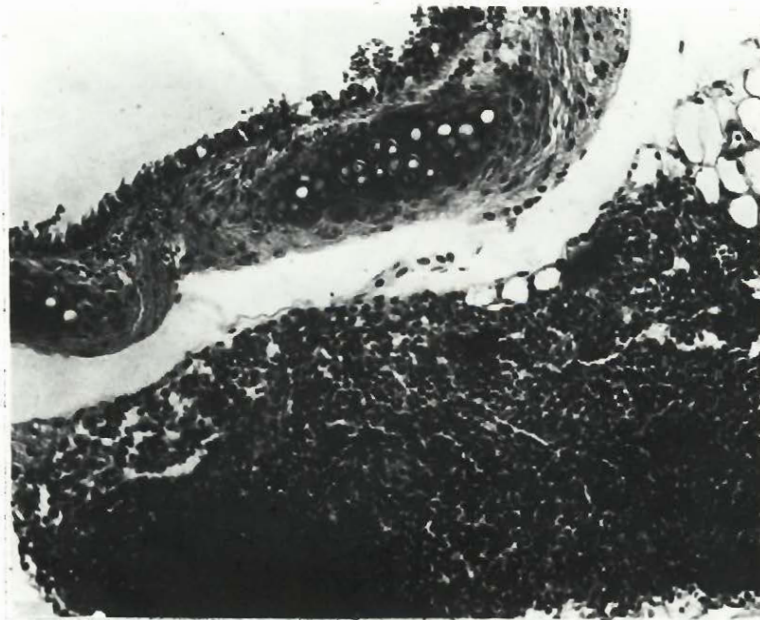


Fig.19. Section of mouse lung, 31 days old, 21 days after intranasal instillation of virus preparation. Note the marked peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

preparation, the controls receiving only maintenance medium 199. Examination of the lungs histologically at weekly intervals showed that after three weeks, lungs from the control animals (Fig. 20) showed no lymphoid infiltration, while the lungs from inoculated rats showed marked peribronchial lymphoid infiltration (Fig. 21).

#### Pathogenecity of Viral Isolate for C.O.B.S. Rats

C.O.B.S. rats were inoculated intranasally with the viral isolate, to determine the pathogenicity of the agent. Observation of histological sections of the lungs indicates that there was no peribronchial lymphoid infiltration in the lungs from control rats (Fig. 22), whereas typical lymphoid infiltration was evident in lungs from virus inoculated rats (Fig. 23). Furthermore, it was observed that C.O.B.S. rats housed with conventional rats for a period of two months developed peribronchial lymphoid infiltration (Fig. 24).

#### Pathogenecity of Viral Isolate for Germfree Rats

Germfree rats were inoculated intranasally with the viral agent to establish its role in the etiology of endemic pneumonia of rats. Examination of the histological sections of the lungs from control and inoculated four-week-old germfree rats at the time of intranasal inoculation indicated that there was no peribronchial lymphoid infiltration in either sample (Figs. 25 and 26).



Fig.20. Lung section, prestressed conventional rat, 45 days old. Received Medium 199 intranasally. Note the lack of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

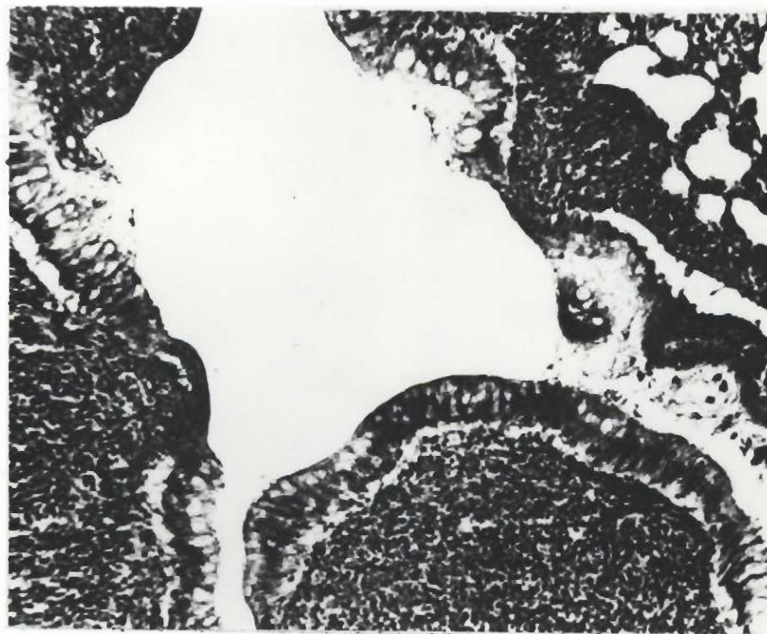


Fig.21. Lung section. prestressed conventional rat, 45 days old. Received virus preparation intranasally. Note the marked peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.



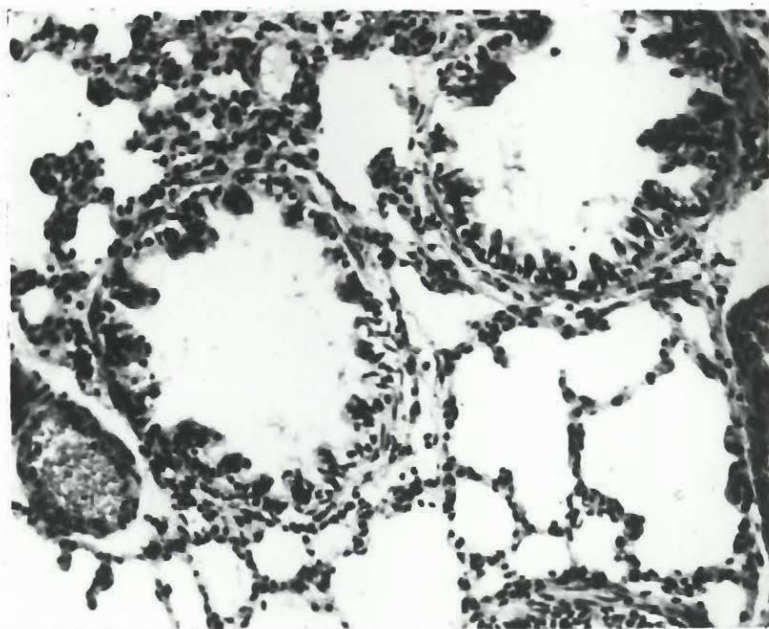


Fig. 22. Lung section, C.O.B.S. rat, 30 days after receiving medium 199 intranasally. Note the lack of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

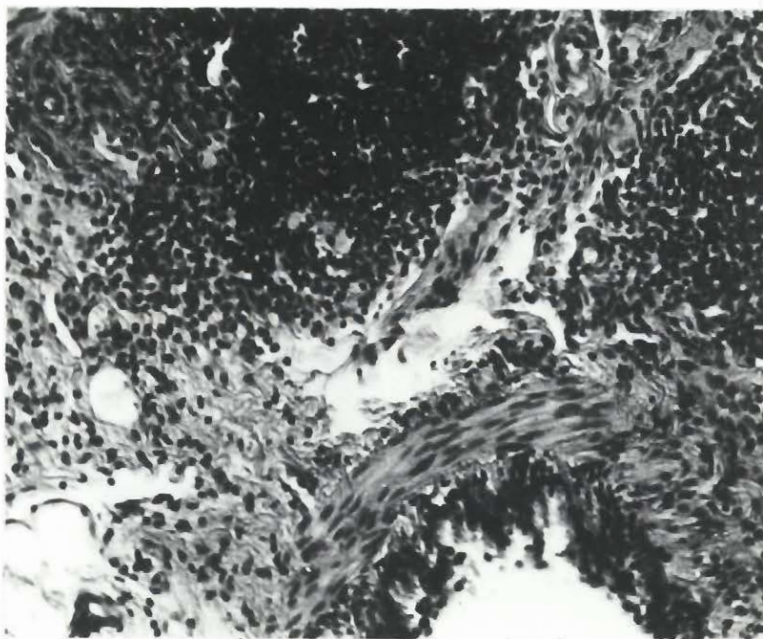


Fig. 23. Lung section, C.O.B.S. rat, 30 days after receiving virus preparation intranasally. Note the presence of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

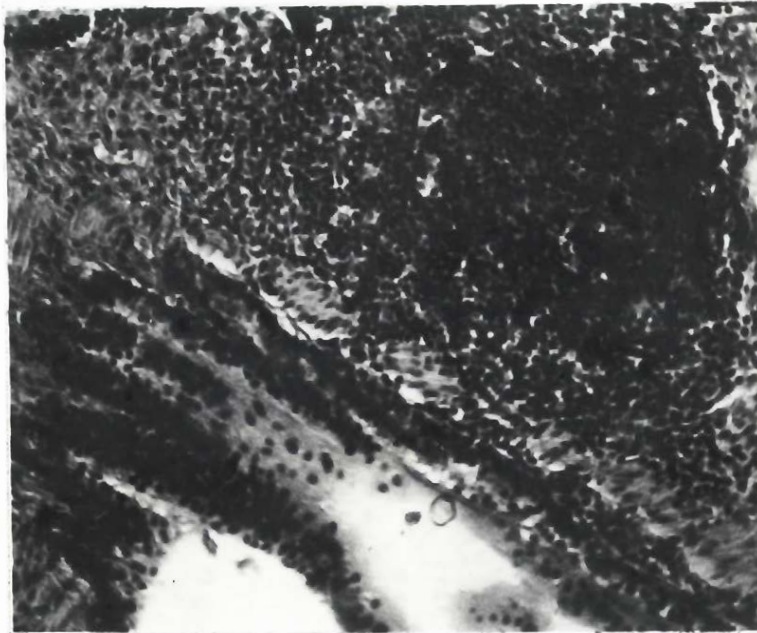


Fig. 24. Lung section, C.O.B.S. rat, after 60 days of cohabitation with a conventional rat. The peribronchial lymphoid infiltration is massive. H.E. stain. Magnification x 200.

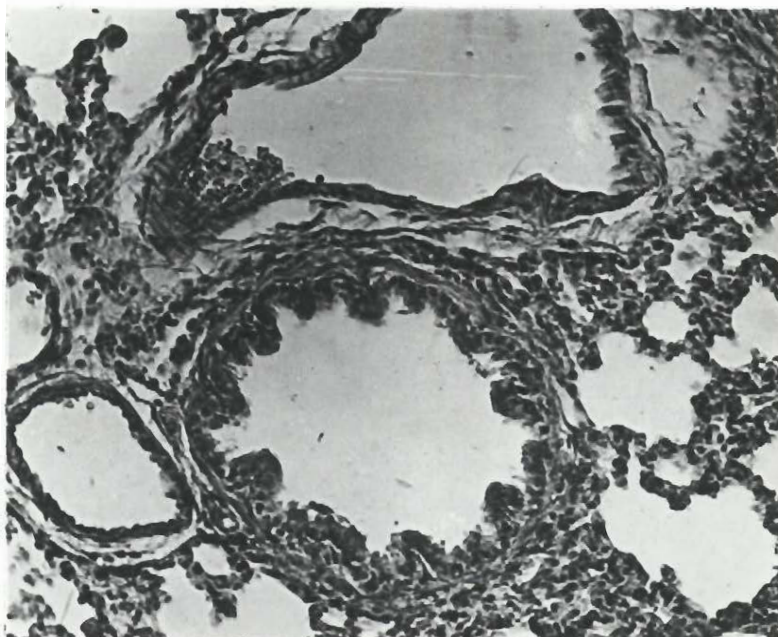


Fig. 25. Lung section, germ-free rat. Control group, 0 day. There is no peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

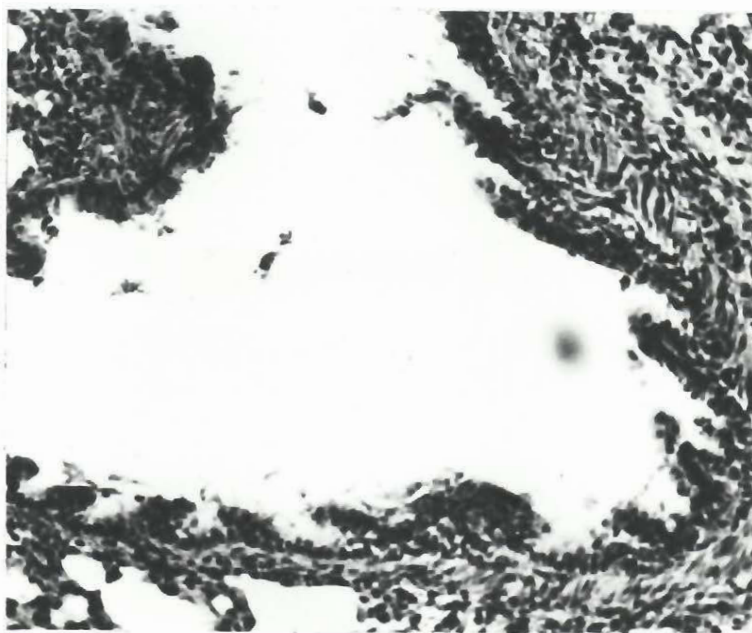


Fig. 26. Lung section, germ-free rat. Inoculated group, 0 day. Note the lack of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.



Twelve days after intranasal inoculation of medium 199, the histological section of the lungs from the control group showed no peribronchial lymphoid infiltration (Fig. 27), while the lungs from the virus inoculated group showed slight peribronchial lymphoid infiltration (Fig. 28). Although the lungs from the inoculated group showed no visible macroscopic changes, the results of the microscopic examination indicated that 21 days after the intranasal inoculation with the virus preparation, the degree of peribronchial lymphoid infiltration had increased progressively (Fig. 30), in comparison with the control lungs which lacked such infiltration (Fig. 29). Furthermore, 25 days after the intranasal instillation of the virus preparation there were small greyish-white protruberances and the histological examination of these lungs showed a marked peribronchial lymphoid infiltration (Fig. 32), whereas the control lungs showed no macroscopic changes nor any peribronchial lymphoid infiltration (Fig. 31). The results of these experiments indicated that the viral agent produced typical peribronchial lymphoid infiltration and also macroscopic changes when inoculated intranasally into germfree rats.

Attempts were also made to reisolate the viral agent from both the control as well as the inoculated lung suspensions. Results of this experiment indicated that no cytopathogenic agent could be demonstrated twelve

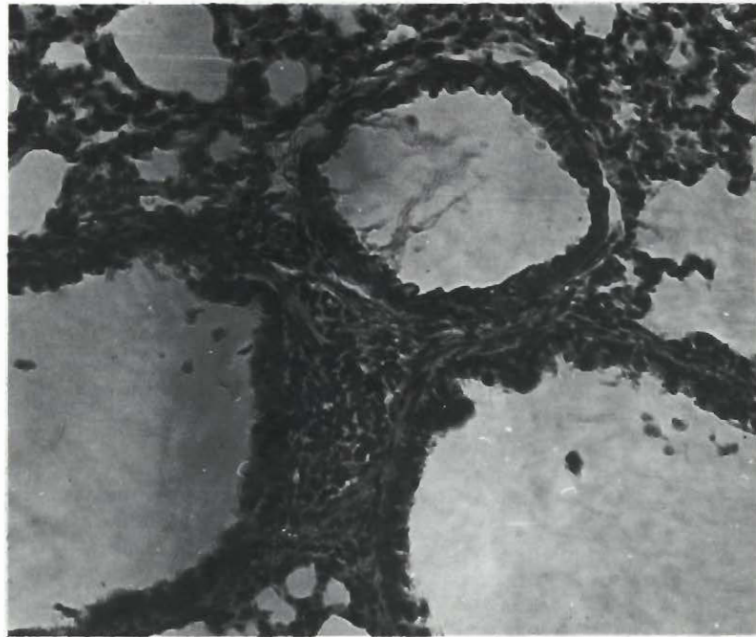


Fig. 27. Lung section, germ-free rat. Control group, 12 days after receiving medium 199 intranasally. Note the lack of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

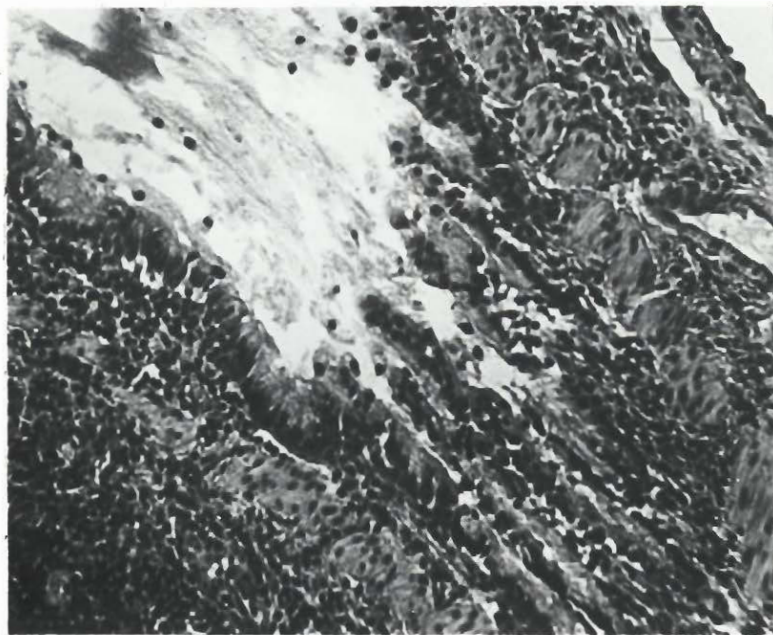


Fig. 28. Lung section, germ-free rat. Inoculated group, 14 days after receiving virus preparation intranasally. There is slight peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

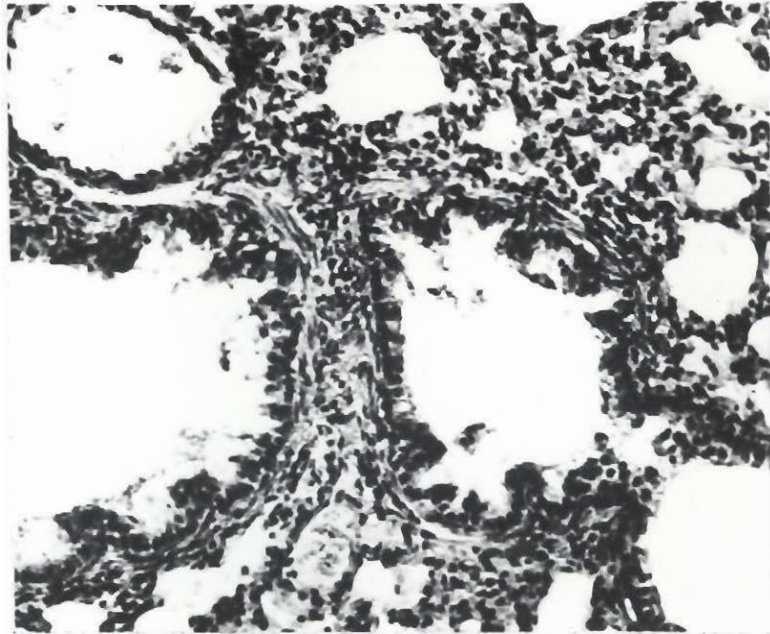


Fig. 29. Lung section, germ-free rat. Control group, 22 days after receiving medium 199 intranasally. The peribronchial lymphoid infiltration is lacking. H.E. stain. Magnification x 200.



Fig. 30. Lung section, germ-free rat. Inoculated group, 21 days after receiving virus preparation. Note the increasing magnitude of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.



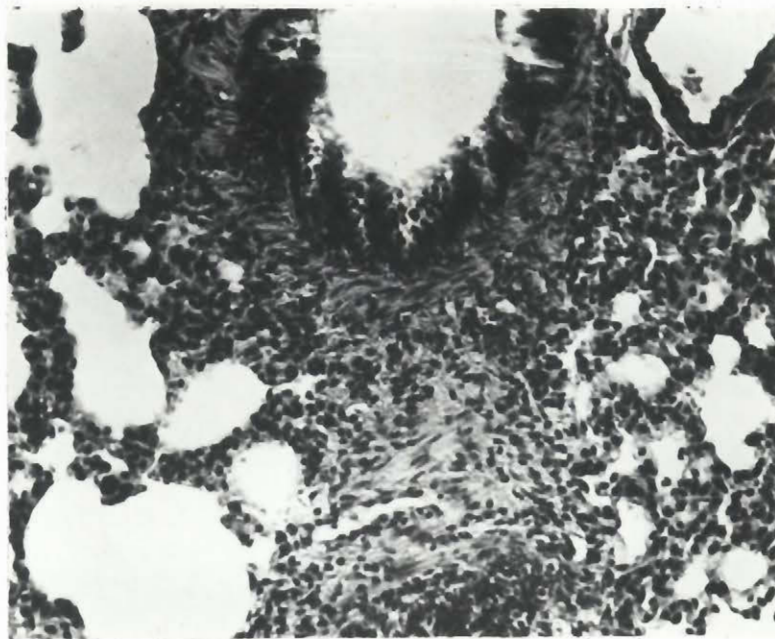


Fig. 31. Lung section, germ-free rat. Control group, 25 days after receiving medium 199 intranasally. Note the lack of peribronchial lymphoid infiltration. H.E. stain. Magnification x 200.

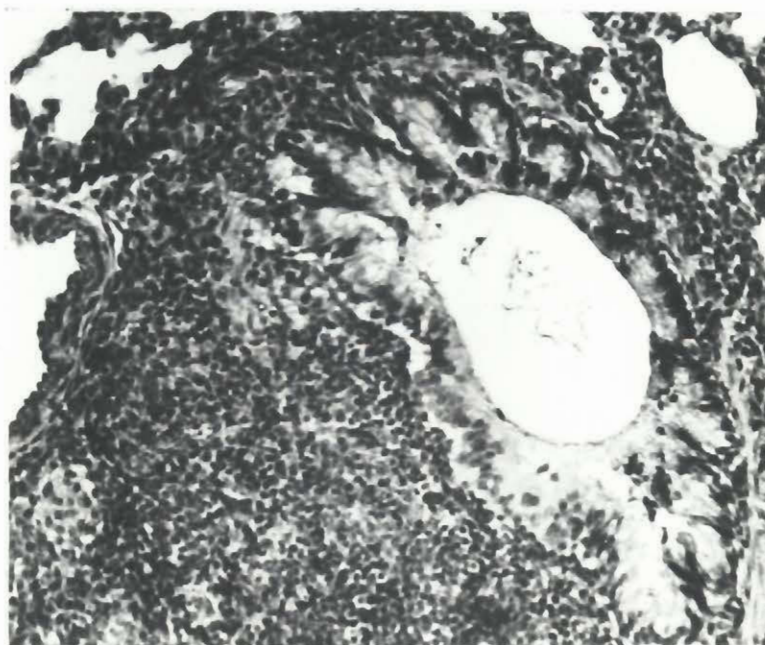


Fig. 32. Lung section, germ-free rat. Inoculated group, 25 days after receiving virus preparation intranasally. The peribronchial lymphoid infiltration is massive. H.E. stain. Magnification x 200.

days after intranasal inoculation of the viral agent, but 21 days after intranasal inoculation the filtrate from the lung suspension produced typical cytopathogenic changes in rat embryo skin primary cultures. No cytopathogenic changes were produced in the rat embryo skin primary cultures by the inoculum prepared from the lungs from the control animals.

Sterility of the isolators was checked at intervals by inoculating faecal samples into brain heart infusion broth (Difco), and thioglycollate medium without dextrose (Difco). Throughout this experiment, no growth was observed in these inoculated media after incubation at 37° C for four to five days.

## DISCUSSION

## DISCUSSION

Previous workers (Nelson, 1955; Innes et al., 1956) reported that most rats suffering from endemic pneumonia exhibit few if any clinical signs of respiratory disease. Many investigators have expressed astonishment at the extent of pulmonary disease in rats who appeared in good condition when alive. However, in advanced stages, unthriftiness may develop, accompanied by rough hair, loss of weight and as the condition progresses, breathing becomes laboured and audible; but the animal rarely dies before the tenth to the twelfth month following infection. The results of the present study indicate that in the earlier stages of the disease adult rats do not show any outward signs and as the condition progresses, the animal shows respiratory distress, as evidenced by laboured breathing, arched back and frequent scratching of the nose.

Histological examination of lungs indicated that lungs from adult conventional rats without any outward signs of respiratory distress showed marked peribronchial lymphoid infiltration, as well as perivascular lymphoid infiltration, the former being more

frequent. Seventy per cent of the lungs examined showed only a varying degree of peribronchial lymphoid infiltration, whereas the remainder showed infiltration at both the locations. Furthermore, it was also observed that histopathological lesions in the lungs obtained from rats showing respiratory distress were not different from what has already been said, except that the lymphoid infiltration was in the form of extensive cuffs and in addition alveoli surrounding these bronchi were collapsed. Similar findings were reported by Innes et al. (1956), who suggested that if microscopic collections of lymphoid tissue at peribronchial locations were considered pathological, the numbers of so-called normal lungs would be much smaller. Innes et al. (1956) also reported that about 80 per cent of adult rats showed excess lymphoid tissue at peribronchial locations. Although the incidence of lymphoid infiltration reported here is higher in comparison with the previous reports (Innes et al., 1956; 1957), this difference might be due to the fact that the rats examined in the present study were older and also obtained from colonies with a history of endemic pneumonia. The influence of age on the development of histopathological changes indicates that the peribronchial lymphoid infiltration is first noticed at the age of 22 days, whereas there is no infiltration at 14 days of age. These findings are substantiated by previous workers (Innes et al., 1956)



who reported that microscopic lymphoid infiltration is almost a universal finding by the time of weaning. It was observed that the peribronchial lymphoid infiltration increases progressively with advancing age. These results indicate that the nursing rats acquire the infection from their infected mothers and retain it throughout their life, a finding substantiated by Nelson (1955) and Nelson and Collins (1961). The aim of these experiments was to establish the age at which the etiological agent would be actively multiplying and provide better chances for its isolation.

Baby rats responded to continued administration of hydrocortisone acetate by failure to gain weight and finally by weight loss if the hormone administration was continued. These findings are substantiated by previous workers (Rupp and Paschkis, 1953; Williams and Davis, 1959; Cavallero et al., 1952). An other effect of hydrocortisone administration was the loss of hair growth resulting in a scanty and rough hair coat. Similar findings were reported by Baker and Schairer (1953) on the influence of hydrocortisone administration on hair growth in rats. The majority of the baby rats which received hydrocortisone for a prolonged period, developed respiratory distress, had nasal discharge, and died of acute respiratory involvement. These findings are substantiated by previous report (Selye, 1951), who reported that rats administered

ACTH or cortisone developed spontaneous fatal pulmonary infections. The histological examination of lungs from hydrocortisone acetate treated rats indicates that continued administration of hydrocortisone acetate abolished peribronchial lymphoid infiltration. Such a finding would be expected in the light of previous report of Morgan et al. (1954) that cortisone administration markedly depressed the circulating lymphocytes in rabbits.

Throughout this investigation no consistent isolation of pleuropneumonia-like-organisms (P.P.L.O.) was found. Furthermore, it was observed that the isolation of P.P.L.O. was not related to the degree of pneumonic lesions, since the P.P.L.O. were isolated from apparently normal lungs, whereas, at times, isolation was unsuccessful from lungs which appeared grossly involved. Similar findings were also reported by Nelson (1946a; 1946b), Pankevicius et al. (1957) and Innes et al. (1956) who reported failure to isolate P.P.L.O. from rats suffering from chronic murine pneumonia. Nelson (1949a; 1949b) also emphasized that P.P.L.O. were not isolated from the lungs of the mice in which a diagnosis of endemic pneumonia was made. These results do indicate, however, a higher rate of P.P.L.O. isolation from nasal cavities of rats which showed macroscopic changes in the lungs. Similar findings were reported by Klieneberger-Nobel and Cheng (1955) who reported a higher incidence of P.P.L.O.

isolations from bronchiectatic lungs. During this investigation, P.P.L.O. were isolated successfully from thirty per cent of the pneumonic lungs. Whereas Pankevicius et al. (1957) reported that P.P.L.O. could be isolated frequently from the lungs of about fifty per cent of rats showing part or all of the complex of chronic murine pneumonia. The lower figures reported here could be explained on the basis that the rats examined were only suffering from endemic pneumonia and moreover, the incidence of P.P.L.O. infection could differ from colony to colony. None of the animals inoculated with P.P.L.O. showed any respiratory distress except slight nasal discharge in mice a week after inoculation, but not persistent, whereas in rats no such discharge was observed. Furthermore, histological examination of lungs from infected rats and mice showed neither peribronchial lymphoid infiltration nor any other pathological changes in the lungs as compared with controls. These findings are substantiated by previous investigators (Pankevicius et al., 1957; Klieneberger and Steabben, 1937).

Although several investigators have been interested in the etiology of endemic pneumonia in rats (quoted by Joshi et al., 1961), no attempts have so far been made towards the isolation of the virus-like agent (Nelson, 1946a; 1946b) in tissue culture systems. In the light of previous reports (Bodian, 1956; Bugbee et al.,

1960; Khoobyarian and Walker, 1957; Imam and Hammon, 1957a, 1957b; Eckhardt Jr. and Hebard, 1961) that the administration of adrenocortical hormones enhanced the viral infections, it was considered desirable to inject hydrocortisone acetate into suckling rats so as to abolish the host defence mechanisms and thus give the agent responsible for endemic pneumonia more favourable environments for multiplication and consequently a flaring up of the disease. It was observed during this investigation that this effect was indeed achieved. The results of preliminary attempts for the isolation of a viral agent in various tissue culture systems, incubated at 37° C for seven days after inoculation, were unsuccessful, except at one isolated occasion when a cytopathogenic agent was demonstrated in rat embryo skin primary cultures, but was lost after subculturing. Later during this investigation an accidental observation was made, that inoculated tissue culture monolayers (rat embryo skin primary cultures) left at room temperature (25° C) for two days after incubation at 37° C for six days, showed marked cytopathogenic changes in inoculated cultures as compared with the controls. The first isolation of the cytopathogenic agent in rat embryo tissue cultures was made from the lungs of a hydrocortisone acetate injected baby rat, by employing this technique of changing the temperature of incubation as

described above. Further isolations were made by this combination of incubation temperatures from both hydrocortisone injected rats as well as from rats which did not receive hydrocortisone acetate treatment, but showed histological collections of peribronchial lymphoid infiltration. The viral agent isolated did not produce any cytopathogenic changes in any other tissue culture system tested, except rat embryo skin, kidney and heart primary cultures. It was also observed that there were no cytopathogenic changes produced if the inoculated cultures were only incubated at 37° C or 25° C for seven days, nor were there any cytopathogenic changes if the inoculated tissue cultures were incubated first at 25° C for six days and then reincubated at 37° C. This two-step incubation of inoculated tissue cultures at different temperatures for the isolation of a cytopathogenic agent has not been described previously. Similar observations in animals have been reported by Thompson (1938) and Marshall (1959) who reported that the survival rate of rabbits inoculated with myxoma virus was increased at higher environmental temperatures than at lower temperatures. Walker and Boring (1958) also reported that the mice inoculated with coxsackie virus survived at higher temperature, whereas at lower temperature, viral multiplication took place and all animals died. A different situation has recently been reported by Sulkin et al.

(1960), who found that Mexican free tailed bats infected with rabies virus survived at lower temperature as compared with higher temperature. As an explanation of this phenomenon of two-step incubation at different temperatures, it is suggested that when inoculated cultures are incubated at 37° C the virus multiplication takes place in the infected cells and does not cause destruction of the infected cells: host and parasite exist in an equilibrium, but as soon as the temperature of incubation is lowered, this relation of equilibrium changes with a shift for the advantage of the virus, thus producing cytopathogenic changes in the tissue culture monolayers. The results of this study indicate the absence of neutralizing antibodies in the rat serum. These results are substantiated by previous workers (Nelson and Gowen, 1930) who found a higher rate of infection in adult rats and suggested that this might be due to decreasing state of immunity, either natural or acquired through maternal transmission, but not demonstrable. Furthermore, it was also found that the viral agent isolated in rat embryo skin primary tissue culture was capable of producing neutralizing antibodies in rabbits. Although the titer of the antibodies was not very high, these results do substantiate the previous suggestion (Nelson and Gowen, 1930) and also indicate that this viral agent is not capable of eliciting a

marked immunological response. The absence of neutralizing antibodies in rat serum could be explained on the basis of these results.

The results also indicate that the viral isolate did not show any haemagglutinating properties towards red blood cells obtained from rats, mice and chickens. Furthermore, under the experimental conditions employed, the viral isolate did not produce any plaques on the rat embryo skin primary cultures. The viral agent isolated was sensitive to adverse temperatures, and was rendered nonviable after 48 hours at 25° C, whereas at 37° C it was rendered nonviable before 24 hours. Furthermore, the virus survived for two weeks at 7° C and for much longer periods at -20° C. The preliminary examination of the viral isolate by electron microscope showed that the particles were round and approximately 110 Å in diameter. Whereas Nelson (1946a; 1946b) reported that the virus-like agent, isolated by him was not able to withstand temperature of 40° C for a period longer than a week. He also reported in the same papers that the agent was a particle somewhat smaller than the elementary bodies of vaccinia. The discrepancies between the results reported here and those of Nelson (1946a; 1946b) can be explained as follows. It is possible that this particular viral isolate does not produce particles as big as those described by Nelson when propagated in tissue culture

system, and it is also possible that what Nelson observed were actually aggregates of smaller particles.

The viral isolate described in this study produced peribronchial lymphoid infiltration when inoculated intranasally into mice, a finding substantiated by Nelson (1946b; 1955). The results of this study showed that the viral agent was capable of producing peribronchial lymphoid infiltration when inoculated intranasally into prestressed conventional rats. Similar findings on the enhancement of susceptibility of animals to viral infections have been reported by various workers - Bodian (1956) on poliomyelitis, Bugbee et al. (1960) on vaccinia and Imam and Hammon (1957a; 1957b) on Japanese B encephalitis viruses. The viral agent also produced peribronchial lymphoid infiltration when inoculated into C.O.B.S. rats. It was also observed that the C.O.B.S. rats, when housed along with conventional rats, developed characteristic peribronchial lymphoid infiltration. Nelson (1946b) suggested that to establish a causal relationship to the disease in question, the agent should reproduce the basic features of endemic pneumonia when introduced in pure state by way of a natural portal of entry and the agent should be experimentally transmissible by normal passage and naturally so by cohabitation. Results of infectivity experiments with germfree rats indicate that the viral agent is capable of producing



peribronchial lymphoid infiltration 12 days after the intranasal instillation of the virus preparation, and 25 days after infection the lungs showed macroscopic changes apart from the microscopic peribronchial lymphoid infiltration. Whereas Nelson (1946b; 1955) reported that the virus-like agent fulfils the essential postulates for identifying it as the specific cause of the experimentally produced endemic pneumonia in mice. He further added that these postulates have not been fulfilled for similarly identifying it with the rat disease and until this was done it can be concluded only that the agent is a frequent resident of the rat lung in endemic pneumonia. As a result of this investigation a virus has been isolated from rat lungs in tissue cultures (rat embryo skin primary cultures), and postulates have been fulfilled for identifying it as the specific cause of endemic pneumonia in rats.

## SUMMARY

## SUMMARY

The etiology of endemic pneumonia in rats was investigated. Rats suffering from this condition do not show any outward symptoms in the earlier stages of the disease but develop respiratory distress as the condition progresses with age. Baby rats responded to continued administration of hydrocortisone acetate by marked suppression of peribronchial lymphoid infiltration, and continued administration of this hormone induced the development of a fatal pneumonia. A histological examination of lungs of conventional rats showed that the majority had peribronchial lymphoid infiltration. Pleuropneumonia-like-organisms have been considered as the cause of endemic pneumonia, but there was no consistent isolation of pleuropneumonia-like-organisms (P.P.L.O.) and furthermore, the isolation of P.P.L.O. was not related to the degree of pneumonic lesions, since the P.P.L.O. were isolated from apparently normal lungs, whereas, at times, isolation was unsuccessful from lungs which appeared grossly involved. Trials on the infectivity of P.P.L.O. isolates for rats and mice, indicated that no histopathological changes were caused in lungs upon intranasal

instillation of these organisms. The viral etiology of endemic pneumonia in rats was also considered and all preliminary attempts for the isolation of a cytopathogenic agent from rat lungs in various tissue culture systems, incubated at 37° C for seven days after inoculation were unsuccessful, except in one isolated instance, and this agent was lost upon subculturing. An accidental observation was, that inoculated tissue culture monolayers (rat embryo skin primary cultures) after incubation for six days at 37° C and then at room temperature (25° C) for two days, showed a marked cytopathogenic effect in the inoculated cultures as compared with the controls. This viral agent produced typical peribronchial lymphoid infiltration when inoculated intranasally into mice, prestressed conventional rats, C.O.B.S. rats, and germ-free rats. Postulates have been fulfilled for identifying this virus with endemic pneumonia in rats. The virus is round, small, weakly antigenic, sensitive to adverse temperatures, nonhaemagglutinating for murine red blood cells, and sensitive to chloroform and acetone.

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