CONTAGIOUS RESPIRATORY DISEASE IN DOGS

by

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The problem of contagious respiratory disease in the dog is
discussed. A wide variety of micro-organisms have been isolated from
dogs with respiratory disease but there was a lack of experimental
evidence to show that most of these agents were capable of inducing
disease. Recently, in addition to canine distemper, several viruses have
been found in the respiratory secretions and tissues of dogs with
respiratory disease.

PART 1 of the thesis dealt with a study of canine herpesvirus
(CHV) respiratory infection. An outbreak of respiratory disease in a
group of kennelled dogs was described and one of the dogs was examined
in detail. At necropsy there was an acute tracheitis and bronchopneumonia
and eosinophilic intranuclear inclusion bodies were present in necrotic
cells of the tracheal epithelium at all levels; inclusions were not
observed in the lungs. Homogenates were prepared from the trachea and
lungs and inoculated individually into monolayers of a canine kidney cell
line. A focal cytopathogenic effect identical to that produced by strains
of CHV isolated from neonatal puppies appeared within 48 hours in all
inoculated cultures. The identity of the cytopathic agent as a herpesvirus
was confirmed by electron microscopy and by a plaque-neutralisation
test using antiserum prepared against a known strain of CHV.

Twelve-week-old puppies were challenged either intratracheally
or by aerosol with the herpesvirus isolate. The dogs infected by the

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intratracheal route did not show any signs of disease but reddish grey areas of consolidation were found in the lungs. Histological examination showed focal areas of necrosis in the bronchi, bronchioles and alveoli. CHV was recovered from the lungs and trachea up to 5 days after infection.

Following exposure to CHV by aerosol puppies developed a bilateral serous nasal discharge. The lungs showed multiple grey foci at necropsy: all lobes were affected equally and the bronchomediastinal lymph nodes were enlarged and hyperaemic. Histological examination of the lungs showed necrosis of the bronchiolar epithelium with subsequent spread into the surrounding alveoli. Focal areas of necrosis were also noted in the nasal mucosa and turbinates but the trachea was histologically normal. Eosinophilic intranuclear inclusions were present only in any number in the nasal and turbinate lesions. CHV was isolated from the lungs up to 10 days after aerosolisation and from the trachea and turbinates up to 7 days. With the electron microscope herpesvirus particles were detected in the bronchiolar epithelium and in alveolar macrophages.

Day-old puppies were found to be highly susceptible to the herpesvirus which was isolated from the adult dog. The main features of the experimental disease in the neonate were necrosis and haemorrhage in many organs.

PART 2 of the work was concerned with a study of canine adenovirus respiratory infection. In SECTION I 8-week-old puppies were infected with canine adenovirus type 1 (CAV-1) either intratracheally or by exposure to aerosolized virus. A severe disease characterised by anorexia, tachypnoea and hyperpnoea was induced in all the puppies by intratracheal administration of the virus. One dog died on the 2nd day after infection and others were killed in extremis on days 4, 7 and 12. At necropsy extensive areas of consolidation were found in the lungs especially in the diaphragmatic lobes. Viral antigen was detected in the lesions by

immunofluorescence and CAV-1 was recovered from the lungs.

Clinical pneumonia with tachypnoea, pyrexia and coughing was induced in experimental puppies exposed to an aerosol spray of CAV-1. Susceptible animals kept in separate pens but having a common air space also developed the infection. A necrotizing bronchiolitis in which many inclusion bodies were present was found at necropsy. Immunofluorescence studies showed large amounts of virus antigen within the bronchiolar lesions and adenovirus particles were detected in bronchiolar epithelial cells by electron microscopy. In the late stages of the disease the main histological feature was bronchiolar hyperplasia. CAV-1 was isolated from the infected lungs, tonsils and turbinates up to 12 days after aerosolisation. None of the infected dogs developed hepatitis.

In SECTION II the isolation of adenoviruses from natural cases of canine respiratory disease was described. The viruses were shown to be indistinguishable from CAV-1 but distinct from canine adenovirus type 2 (A26/61 strain). The growth of the new isolates in tissue culture was studied with electron and fluorescence microscopy. Intravenous inoculation of the viruses into susceptible dogs induced classical acute hepatitis (Rubarth's disease) while, when administered in aerosol form, a severe respiratory disease was produced.

PART 3 of the thesis dealt with a study of reovirus type 1 infection. Sera collected from adult mongrel dogs were screened for antibody to reovirus type 1 and significant haemaglutination-inhibiting (HA-I) antibody levels were observed in 19·7 per cent of the dogs tested.

Eight to ten-week-old puppies were inoculated intratracheally with reovirus type 1 of bovine origin. A clinical illness was not observed but pneumonic lesions were present in the dogs killed at 2, 3 and 7 days after inoculation. The lesion consisted of an interstitial pneumonia with marked thickening of the interalveolar septa. Inclusion bodies were not

demonstrable. Reovirus was recovered from the lungs and bronchial lymph nodes of infected dogs and a rise in HA-I antibody was detected in all the infected puppies killed after the 3rd day.

Clinical signs of respiratory disease were not detected in dogs exposed to an aerosol cloud of reovirus type 1 of bovine origin. Tiny grey foci were found in the lungs and microscopic examination revealed cellular accumulations in the alveoli and interalveolar septa. Reovirus type 1 was recovered from the lungs and bronchial lymph nodes up to 8 days after aerosolisation and a rise in HA-I antibodies was detected in infected dogs.

PART 4 of this study dealt with the application of organ culture techniques to canine respiratory virus infections. Tracheal organ cultures were prepared by the method of Hoorn from adult and 8-week-old dogs. The normal uninfected cultures retained a differentiated ciliated epithelium for at least 14 days and strong ciliary activity could be observed in most cultures up to 20 days.

There was no difference in the effect of CHV on cultures prepared from adult and young dogs. In both cases ciliary activity diminished rapidly and ceased by the 6th day after infection. Foci of epithelial necrosis were noted at the edge of the explants on the 2nd day and spread to involve all the epithelial surface. Eosinophilic intranuclear inclusions were noted in infected cells.

In distemper infected cultures cytopathic changes were characterised by the appearance of phloxinophilic cytoplasmic inclusions, reduction in height of the epithelium and squamous dedifferentiation. These changes developed more slowly in explants prepared from adult dogs.

Two strains of CAV-1 were studied in tracheal explants prepared from adult dogs. Moderate amounts of virus were released by the

infected explants for up to 8 days and viral antigen was detected in the respiratory epithelium by immunofluorescence up to 20 days. The main pathological changes in the respiratory epithelium of the CAV-1 infected cultures were necrosis, proliferation and the formation of basophilic intranuclear inclusions.

It was concluded that organ cultures of dog trachea might be of value in studies on the pathogenesis of canine respiratory viral infections.

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GENERAL INTRODUCTION

Contagious respiratory disease is one of the most important problems encountered in canine medicine. The introduction of an infected dog into a veterinary hospital or kennels may result in an explosive outbreak of respiratory disease. Although in the majority of cases clinical signs are restricted to those of a mild upper respiratory disease with nasal discharge and a dry hacking cough, a proportion of cases do subsequently develop bronchopneumonia which is sometimes fatal. Excluding distemper, the cause of which is now well established, the aetiology of contagious respiratory disease among dogs in kennel populations is still imperfectly defined and a variety of terms such as "kennel cough", "tracheobronchitis" and "influenza" are used to describe the condition.

The literature contains many reports concerning the isolation of bacteria, mycoplasmas, and viruses from dogs with respiratory disease and it is becoming clear that, in any one outbreak, several of these agents may be involved. It is apparent that the mere isolation of a bacterium, mycoplasma or virus is insufficient to justify acceptance of that particular agent as a cause of canine respiratory disease; controlled experimental studies are needed to show that the agent can produce respiratory disease without the assistance of other organisms.

The purpose of this dissertation is fourfold. Firstly, to define the importance of contagious respiratory disease in dogs. Secondly, to discuss the isolation of viral agents from natural cases of canine respiratory disease. Thirdly, to compare and contrast experimental infections due to a number of different viruses and fourthly to assess the importance of tracheal organ cultures as potential models for the study of canine respiratory viral infections.

REVIEW OF THE LITERATURE

1. Distemper

2. Kennel Cough

    (a) Early investigations and confusion with distemper.

    (b) Emergence of a disease distinct from distemper and termed "kennel cough".

    (c) The role of bacteria and mycoplasmas in kennel cough.

    (d) The role of viruses in kennel cough.

        (i) Parainfluenza virus.

        (ii) Herpesvirus.

        (iii) Adenovirus.

        (iv) Reovirus.

Contagious respiratory disease in dogs has been recognised for many years as an important problem in boarding kennels, research establishments and animal pounds throughout the world. Thus, Bjovedt et al. (1969) recorded that respiratory disease resulted in a 50 per cent morbidity and a 20 per cent mortality in dogs maintained in laboratories. Similarly, Rubin, cited by Snow et al. (1969), reported that 17 per cent of 3,650 dogs admitted to an experimental surgery unit died of respiratory disease and that those dogs used in transplantation studies in which immunosuppressive drugs were given post-operatively were particularly susceptible.

Canine respiratory disease is seen in two distinct, but sometimes overlapping, clinical situations. Firstly, respiratory disease is one of the main features of distemper, an extremely common paramyxovirus infection which involves also the lymphoid, alimentary and nervous systems. Secondly, it occurs as a poorly understood condition known as "kennel cough" which, unlike distemper, is restricted to the respiratory system and is widespread in any situation where dogs are maintained in close contact. The following discussion is a review of the relevant literature pertinent to both these syndromes.

1. Distemper

According to Jenner (1809), canine distemper was first recognised in Britain during the 18th century although it had been observed on the Continent much earlier. One of the first detailed descriptions of clinical distemper was provided by Youatt (1845) who reported that the disease was more common in young dogs and was associated with ocular and nasal discharges, coughing, pneumonia, fits and, in some cases, chorea and paralysis.

From 1850 to 1900 many articles were written on the contagious
nature of distemper and its possible bacterial aetiology; these were
reviewed by Torrey and Rahe (1913). In 1905, Carré showed that
canine distemper was caused by a filterable virus and although
Lignières (1906) confirmed this finding it was not generally accepted
and many workers argued that a bacterium, Bordetella bronchiseptica
(Bacillus bronchicanis, Bacillus bronchisepticus, Brucella
bronchiseptica) was the primary aetiological agent (McGowan, 1911;
Ferry, 1911, 1912, 1913; Torrey and Rahe, 1913). This controversy was
settled when G.W. Duncan and Sir Patrick Laidlaw (1926a, 1926b)
published the results of their investigations. These workers had
recognised the difficulties of studying a highly infectious disease
and had employed strict and elaborate precautions to preclude any
accidental infection of their experimental animals. They showed that
the infectious agent could pass through bacterial filters of standard
type and proven quality and that the disease could be transmitted
from dog to dog by bacteria-free filtrates. Furthermore, Laidlaw
and Duncan (1926) showed that ferrets were extremely susceptible to
canine distemper and that the infection was airborne over short
distances, the disease passing from dog to ferret or from ferret
to dog without direct contact.

During the last 70 years many aspects of canine distemper
have been described and the monographs published by Gorham in 1960 and
by Appel and Gillespie in 1972 have adequately reviewed the relevant
literature. The clinical, morbid anatomical and histopathological
features of field distemper have been discussed at length by
De Monbreun (1937), Cordy (1949) and Lauder et al. (1954). The
pathological changes regarded as characteristic of the respiratory

aspects of distemper are essentially those of mononuclear infiltration of the alveolar interstitium and proliferation and hypertrophy of the alveolar epithelium together with eosinophilic cytoplasmic inclusion bodies in epithelial cells throughout the respiratory tract.

In spite of the hundreds of articles written on canine distemper, few serial descriptions of the experimental disease are available. Gibson et al. (1965) reported that experimental distemper in gnotobiotic dogs, which were infected intraperitoneally with virulent virus, was an extremely mild disease and they concluded that secondary bacteria were responsible for the overt clinical signs so much a feature of natural cases. However, the absence of pathological findings in their group of experimental dogs may have been related to the route of infection. Fairchild, cited by Bussell (1970), found that 2 out of 5 specific pathogen-free dogs died following aerosol exposure, whereas, of 5 dogs inoculated intravenously none died.

The problems of isolating and maintaining virulent strains of distemper virus in tissue culture have made controlled experimental infections difficult and in order to simulate natural distemper, Cornwell et al. (1965) infected dogs by contact with diseased ferrets. Respiratory, gastro-intestinal and nervous signs developed in the exposed contact dogs and there was a wide variation in the duration and severity of the clinical disease. One dog died of bronchopneumonia 21 days after infection and a catarrhal and interstitial pneumonia was found in 4 more when necropsied between 29 and 57 days post-infection. In these experiments, the disease was allowed to run its course; consequently, serial data on the development of the pathological changes was not available.

A major advance in our understanding of the pathogenesis of

distemper was made by Appel (1969) who applied immunofluorescence to a study of the distribution of virus in dogs infected by aerosol. Viral replication appeared to commence in the lymphatic tissues of the bronchial lymph nodes and tonsils which contained viral antigen on the day after infection. Small numbers of infected mononuclear leucocytes were found in the blood on the 2nd day post-infection and, by 6-9 days after infection, mononuclear cells laden with virus antigen were seen throughout the body. Dogs which did not develop sufficiently high antibody levels had widespread viral invasion of all epithelial tissues and clinical illness occurred. However, if the neutralising antibody titre developed rapidly, reaching a titre of 1/100 or greater (against 100-300 Egg infective doses (EID50)) within 14 days, the spread of virus to epithelial tissues was limited and these dogs, with a few exceptions, did not show any clinical signs of illness.

There have been many epidemiological studies of canine distemper in dog populations (Gorham, 1960) but little attention has been focussed on the role of the virus in respiratory disease among kennelled dogs. In one study, Bjovedt et al. (1969) investigated this problem in newly acquired laboratory dogs. One hundred dogs of mixed breeds whose ages ranged from 8 months to 10 years were collected at random from diverse sources and were kennelled for a conditioning period of 42 days. It was found that 60 dogs had neutralising antibody titres of 100 or greater against 300 EID50 on arrival. Forty susceptible dogs subsequently developed respiratory disease and of these 13 died; the latter animals were shown to be distemper cases at postmortem examination. Seventeen of the 60 immune dogs also developed respiratory disease and Bjovedt et al. (1969) concluded that agents other than distemper virus must be involved.

2. Kennel cough

(a) Early investigations and confusion with distemper.

For long it had been suspected that canine distemper virus (CDV)
was not the only cause of infectious respiratory disease in the dog
but, unfortunately, many of the earlier writers attempted to differentiate
between canine respiratory diseases on a purely clinical basis and this
caused much confusion. In 1941, Whitney described a syndrome which
he called "canine influenza". Affected dogs were young and the major
presenting signs were running fits, photophobia, anorexia, nasal
discharge, hypersensitivity to noise and "tenderness about the head".
Some dogs died and a pneumonia was found at necropsy. Whitney claimed
that although the clinical picture had much in common with that of
distemper, in his opinion the two could be differentiated on clinical
grounds. This was surprising because he further declared that dogs
vaccinated against distemper were immune to infection with canine
influenza.

Two years later, Whitney (1943) described yet another syndrome
which he believed also was distinct from distemper. Since the aetiology
was not known and the affected dogs were usually household pets, he
proposed tha name "housedog disease". The early signs of this infection
were a retching cough, diarrhoea and pyrexia. The affected dogs usually
recovered within a few days, but in some cases coughing persisted for
several weeks and pneumonia developed. In a significant percentage of
cases, apparent recovery from the respiratory illness was followed by
signs of acute encephalitis. Whitney again attempted to differentiate
this condition from distemper on clinical grounds but this time also
on the absence of inclusion bodies in epithelial cells. He did not
describe the general pathology of the disease in detail but microscopic
examination of brain sections from a dog that died showed an

encephalomyelitis with lymphocytic perivascular cuffing and
demyelination, lesions characteristic of distemper virus infection
(Campbell, 1957).

In 1947, Gustafson attempted to isolate and characterise the
aetiological agent of housedog disease. He collected nasal washings
from 2 infected dogs and passaged this material in series from dog
to dog 11 times. Many of the inoculated dogs died, some developing
bluish corneal opacities and others nervous symptoms, but since they
had been obtained from various kennels and none of them were kept as
controls the possibility that intercurrent viral infection might have
been present cannot be excluded. For example, canine adenovirus
infection of dogs can be associated with corneal opacities (Carmichael,
1964,1965a) and nervous signs are a common feature of CDV infection
(Lauder et al., 1954). In the light of present knowledge Gustafson's
claim to have reproduced housedog disease cannot therefore be accepted.

In England, Townson (1947) reported a condition which clinically
had much in common with housedog disease and which he considered to
be of viral origin. He called this entity "infectious catarrhal fever"
and noted that it was also known locally as "X disease","American
distemper" and "German distemper". Affected dogs developed a cough
and diarrhoea and in some cases, nervous symptoms appeared 14-28 days
after an apparent recovery. Townson differentiated this condition from
distemper on the grounds that many of the dogs had been vaccinated or
had previous histories of distemper infection. Furthermore, inclusion
bodies were not found in those cases submitted for pathological
examination. In 1948 Leask, also in England, described a contagious
disease which was common in dogs that either were in or had recently
been resident in kennels. He recognised two forms of the disease, an

acute form with pharyngitis, tonsillitis and pyrexia and a mild form characterised by a slight cough. Leask (1948) believed that this condition was not caused by distemper virus infection because it occurred in dogs of all ages and did not confer a lasting immunity.

(b) Emergence of a disease distinct from distemper and termed "kennel cough".

The next decade saw the development of safe and effective distemper vaccines and their use in the canine population has done much to control systemic distemper. However, outbreaks of respiratory disease still occurred with some regularity in kennel populations, particularly in boarding and breeding establishments. Because in the majority of cases the dogs were vaccinated against distemper and clinical signs were confined to a mild upper respiratory disease, many veterinarians assumed that agents other than distemper virus must be involved. In recent years these outbreaks of respiratory disease, distinct from distemper, have been given the general term "kennel cough". Dogs of all ages were involved in these outbreaks and morbidity was high. The outstanding clinical sign was a harsh, dry, paroxysmal cough which was aggravated by activity or excitement. It has been suggested that the inhalation of irritating smoke, gases or chemicals together with excessive barking may predispose to kennel cough but the contagious nature of the condition leaves little doubt concerning the importance of infective agents.

(c) The role of bacteria and mycoplasmas in kennel cough.

In the search for the cause of kennel cough many micro-organisms have been recovered from dogs with respiratory disease and some of the bacteria and mycoplasmas which have been isolated are shown in Table 1. Of this wide variety of agents only _Bord. bronchiseptica_, the haemolytic

TABLE 1

Some Bacteria and Mycoplasmas Associated with Respiratory Disease in Dogs

Bacterium	Reference
Bordetella spp.	Ferry, 1911; Torrey and Rahe, 1913; Chappel et al., 1956; Snow et al., 1969; Appel and Percy, 1970; Wilkins and Helland, 1973.
Streptococcus spp. Alpha-haemolytic Beta-haemolytic	Townson, 1947; Chappel et al., 1956; Binn et al., 1968; Snow et al., 1969; Baker and Huebner, 1970; Wilkins and Helland, 1973.
Staphylococcus spp.	Snow et al., 1969; Wilkins and Helland, 1973.
Klebsiella spp.	Ludford and Stevens, 1958; Cole, 1964; Wilkins and Helland, 1973.
Proteus spp.	Wilkins and Helland, 1973.
Pseudomonas spp.	Snow et al., 1969; Wilkins and Helland, 1973.
Pasteurella spp.	Stevenson and Hilbert, 1937; Brennan et al., 1965; Wilkins and Helland, 1973.
Neisseria spp.	Snow et al., 1969; Wilkins and Helland, 1973.
Mycoplasma spp.	Shoentensack, 1934; Grieg, 1954; Razin and Rottem, 1967; Appel and Percy, 1970; Armstrong et al., 1970; Rosendal, 1972.

streptococci and mycoplasmas have been investigated as possible primary agents and the role of the others listed in Table 1 remains to be ascertained.

In 1948, Ray suggested that kennel cough was due to infection with Bord. bronchiseptica and Chappel et al. (1956) claimed to have isolated a similar organism from throat swabs taken from diseased dogs. Snow et al. (1969) recovered Bord. bronchiseptica from the nose of 9 out of 25 dogs with acute respiratory disease but also from 10 out of 80 asymptomatic controls. Bord. bronchiseptica was one of a number of micro-organisms recovered by Appel and Percy (1970) and by Wilkins and Helland (1973) from dogs with mild respiratory illness.

Haemolytic streptococci have also been associated with respiratory infection in kennelled dogs. Thus Townson (1947) stated that beta-haemolytic streptococci were frequently recovered from cases of infectious catarrhal fever and Baker and Huebner (1970) isolated haemolytic streptococci from the pharynx and tonsils of 8 out of 23 dogs with acute upper respiratory disease. Wilkins and Helland (1973) claimed that the bacteria most commonly isolated from the tonsils of dogs with tracheobronchitis were streptococci (85 isolates out of 172 cases) of which the alpha-haemolytic Streptococcus viridans was by far the most common. Similarly, Binn et al. (1968) found that alpha-haemolytic streptococci were frequently present in throat swabs collected from dogs with respiratory disease but these organisms could also be isolated from contact and control dogs.

The presence of Bord. bronchiseptica and haemolytic streptococci in the naso-pharynx of dogs with respiratory disease is in itself far from conclusive proof of their pathogenicity since it is known that the same bacteria occur in the naso-pharynx of healthy dogs (Smith, 1961; Clapper and Meade, 1963; Singh and Parnaik, 1965; Snow et al., 1969;

Brennan and Simkins, 1970). Moreover both Grieg (1954) and Chappel
et al. (1956) were unable to produce respiratory disease in dogs using
either an inoculum of Bord. bronchiseptica alone or combined with
haemolytic streptococci.

Bacteriological studies of dogs with and without respiratory
disease have failed to show any differences in the bacterial flora
of the nose and throat (Binn et al., 1968; Snow et al., 1969) although
there has yet to be a detailed bacteriological examination of the
lower respiratory tract in kennel cough. It has been assumed that
bacteria are not responsible for the primary illness but play a part
in determining the progression of the disease. However, recent work
(Wright et al., 1973) has shown that dogs experimentally infected with
Bord. bronchiseptica by means of an aerosol spray developed a transient
respiratory disease with cough and nasal discharge. A group of puppies
kept in a separate pen but with a communicating air space with the
infected dogs also developed clinical respiratory disease. At postmortem
examination a severe purulent rhinitis and bronchitis was found in both
groups and masses of gram negative bacteria were found trapped in the
mucus of the ciliated tracheal and bronchial epithelium. Bord.
bronchiseptica was recovered in pure culture from the turbinates and
lower respiratory tract. In view of these findings more detailed
investigations are needed to re-assess the role of bacteria, particularly
Bord. bronchiseptica in canine respiratory disease.

Mycoplasmas have been isolated on numerous occasions from the
respiratory tract of dogs but their role in canine respiratory disease
is not clear. Binn et al. (1968) recovered mycoplasmas from throat swabs
of ill, contact and control dogs during an outbreak of respiratory
disease in military guard dogs. Appel and Percy (1970) isolated
mycoplasmas from 181 out of 221 (81 per cent) pharyngeal swabs from

3 to 4-month-old puppies with a mild respiratory disease. However, both investigations showed that other agents, including viruses, were present.

Mycoplasmas were first isolated from dogs by Shoetensack (1934, 1936a, 1936b) who discovered two serologically distinct strains in secretions from the upper respiratory tract. In 1954, Grieg described the isolation of mycoplasmas from dogs with kennel cough but no attempt was made to type these strains. Three years earlier Edwards and Fitzgerald (1951) had reported a detailed study of the mycoplasma flora of normal dogs and three canine mycoplasma species were identified from a number of throat and vaginal cultures. These were subsequently designated Mycoplasma spumans, Mycoplasma canis and Mycoplasma maculosum (Edwards and Freundt, 1956). A throat isolate, PG24, which was found to be serologically distinct from these, was not named. More recently, Razin and Rottem (1967) reported the recovery of a mycoplasma resembling the PG24 strain from the lungs of a dog dying of pneumonia and Tully et al. (1970) proposed that strains of this type should be called Mycoplasma edwardii.

Barile et al. (1970) found that 66 out of 93 (71 per cent) apparently healthy dogs had mycoplasmas in their laryngeal tissues and they noted that 57 of the 66 (85 per cent) positive dogs supported a mixed flora, the four known canine serotypes occurring in roughly equal numbers. Barile et al. (1970) concluded that Mycoplasma spumans, Mycoplasma canis, Mycoplasma maculosum and Mycoplasma edwardii were commensal inhabitants of the upper respiratory tract of the dog. Brennan and Simkins (1970) also came to the same conclusion after finding a high incidence of mycoplasmas in throat swabs from healthy Beagles in a closed breeding colony. Armstrong et al. (1970, 1972) recovered a number of mycoplasmas from lung biopsies and necropsy

specimens of dogs suffering from pneumonia. The isolates were characterised and some were identified as being similar to the species described by Barile et al. (1970). Three species designated groups C, C1 and D were, however, found to be distinct. The group C and C1 mycoplasmas were recovered only from the lungs but their pathogenicity was not assessed experimentally. At about the same time Rosendal (1972) described an outbreak of pneumonia in young kennelled dogs which he believed was caused by a mycoplasma. The affected dogs had been vaccinated against distemper and infectious canine hepatitis and the main clinical signs were anorexia, pyrexia and coughing. Two $3\frac{1}{2}$-month-old dogs which were placed in contact developed similar clinical signs 14 days post-exposure. Both contact dogs developed a significant rise in antibody titre to mycoplasma and pneumonia was found at necropsy in one of the dogs. A mycoplasma which was isolated in great numbers from the trachea, bronchi and pneumonic lesions, was found to differ biochemically from the known canine species.

The pathogenicity of mycoplasmas for the dog has not been clearly established experimentally. Grieg (1954) inoculated dogs intranasally, subcutaneously and intravenously with strains recovered from cases of kennel cough. Transient bouts of sneezing and occasional coughing developed but, in general, the results indicated that these organisms did not possess any striking pathogenicity for dogs. However, Appel and Percy (1970) noted that specific pathogen-free dogs exposed to a parainfluenza virus developed more severe respiratory signs if they were also exposed to an aerosol of mycoplasma and Bord. bronchiseptica. It is possible therefore that mycoplasmas act as secondary rather than as primary pathogens.

(d) The role of viruses in kennel cough.

The economic losses caused by respiratory disease in experimental and military dogs, particularly in North America, provided the stimulus for virological investigations of this problem. Rapid progress has been made and several new canine viruses have been isolated and identified. The viruses associated with canine respiratory disease as a whole are listed in Table 2.

(i) Parainfluenza virus.

A parainfluenza virus has been recovered on several occasions from dogs in the United States of America where it appears to be an important cause of canine respiratory disease. It was first isolated from nasal secretions and postmortem tissues of laboratory dogs with a disease characterised by high morbidity and mortality (Binn et al.,1967). Serologically the new isolate was closely related to a simian parainfluenza virus, SV-5. The significance of the canine parainfluenza virus in the original outbreak was difficult to assess because many of the dogs were infected with more than one viral agent.

In 1966 an epizootic of upper respiratory disease occurred in sentry dogs at the Lackland Air Force base in Texas (Crandell et al., 1968). A haemadsorbing agent was isolated from 2 dogs and identified as a parainfluenza virus similar to the simian virus SV-5. Intranasal inoculation of two 6-week-old puppies with this agent resulted in a slight serous nasal discharge and tonsillitis. Virus was re-isolated from them and an elevation of specific antibody demonstrated in both dogs. Dogs from the Lackland base were transferred for military training to Fort Benning, Georgia and respiratory disease subsequently appeared in dogs kennelled at this base (Binn et al., 1968). The disease was characterised by rapid spread, sudden onset, non-productive

TABLE 2

Viruses Associated with Respiratory Disease in Dogs.

Virus Group	Name	Reference
Myxovirus	Distemper	Duncan and Laidlaw, 1926
		Cornwell et al., 1965
	Parainfluenza SV-5	Binn et al., 1967
		Crandell et al., 1968
		Binn et al., 1968
		Appel and Percy, 1970
		Rosenberg et al., 1971
Reovirus	Reovirus type 1	Lou and Wenner, 1963
		Massie and Shaw, 1966
Herpesvirus	Canine herpesvirus	Motohashi and Tajima, 1966
		Binn et al., 1967
		Karpas et al., 1967
		Karpas et al., 1968a
		Wright et al., 1970
Adenovirus	Canine adenovirus type 1 (Infectious canine hepatitis virus)	Binn et al., 1967
		Campbell et al., 1968
		Wright et al., 1972
		Studdert and Studdert, 1972
	Canine adenovirus type 2 (Toronto A 26/61 strain)	Ditchfield et al., 1962
		Binn et al., 1967
		Appel and Percy, 1970
		Danskin, 1973

cough, serous to mucopurulent nasal discharge and fatigue. An SV-5-like virus was recovered from 4 out of 25 throat swabs and the discovery of a marked rise in SV-5 antibody titre in dogs with clinical signs and also among incontact animals left little doubt that the virus had spread rapidly through the colony. The dogs at Fort Benning had been vaccinated against CDV and canine adenovirus type 1 (CAV-1) and, with a few exceptions, had significant but unchanging CDV and CAV-1 antibody titres.

In a bacteriological and virological study of young dogs, Appel and Percy (1970) isolated SV-5-like agents from 3 of 9 puppies with a non-productive cough and a mucous nasal discharge and from 19 of 191 pharyngeal swabs from 3 to 4-month-old urban dogs with a mild upper respiratory illness. Mycoplasma, Bord. bronchiseptica, CDV and canine adenovirus type 2 (CAV-2) were also recovered.

Although a canine parainfluenza virus has not yet been isolated in the United Kingdom, serological evidence suggests that infection may be widespread in the canine population (Danskin, 1973 - personal communication).

The epidemiology of parainfluenza virus infection in the dog is largely unknown although serological surveys in the USA have provided useful information on the occurrence and distribution of viral antibodies. Parainfluenza virus infection in dogs appears to be widespread in the United States (Bittle and Emery, 1970) but the incidence of infection seems to be related to the size and concentration of the canine population. Bittle and Emery (1970) suggested that the incidence was probably low in pet animals isolated in family dwellings but when dogs were brought together in large numbers, the virus, if present, spread rapidly. Binn and Lazar (1970) observed that the prevalence of parainfluenza SV-5 virus antibodies in laboratory and

military dogs increased from 3 per cent at the time of arrival at kennels to 72 per cent 6 weeks later.

In a preliminary serological survey carried out between July and November 1971, Danskin (1973 - personal communication) found a high incidence (48per cent) of haemagglutination-inhibiting (HA-I) antibodies to canine parainfluenza virus in sera collected from dogs in various parts of the United Kingdom. There was no particular breed or age incidence but there was an increase in the monthly average titre during the period July to November.

Relatively little is known about the pathogenesis and pathology of naturally occurring parainfluenza virus infection in the dog. Most accounts of natural cases have been concerned with the virological and serological aspects of the infection and scant attention has been paid to the pathological changes.

The experimental exposure of dogs to parainfluenza virus SV-5 has resulted in either a mild respiratory disease or a clinically inapparent infection (Crandell et al., 1968; Bittle and Emery, 1970; Lazar et al., 1970; Saona Black and Lee, 1970; Appel and Percy, 1970; Rosenberg et al., 1971). Lazar et al. (1970) found that dogs inoculated by multiple routes i.e. intranasally, orally, intratracheally and peritoneally, developed a slight nasal discharge with mild pharyngitis and tonsillitis. Virus was recovered from throat swabs of all inoculated and contact dogs. Rises in both serum neutralising and HA-I antibodies were demonstrated in all dogs including the uninoculated controls and antibody persisted at peak levels for 42 days.

Appel and Percy (1970) did not observe any illness in dogs following intramuscular and subcutaneous inoculation of the virus but a challenge by aerosol caused coughing and slight nasal discharge. Macroscopic lesions were not present at necropsy but on histological

examination there was a catarrhal rhinitis and bronchiolitis. Virus was isolated from nasal swabs from all dogs between 1 and 8 days post-infection and viral antigen was demonstrated in epithelial cells of the nasal mucosa, trachea, bronchi and peribronchial glands by immunofluorescence.

A detailed study of experimental canine parainfluenza virus infection in the dog was made by Rosenberg et al. (1971). The virus used in their study was isolated from a case of kennel cough and experimental dogs were infected by intranasal instillation of virus. Parainfluenza virus was recovered from all the infected dogs for 5 to 9 days after inoculation and rises in specific antibody titres were noted. The infection was readily transmitted to contact controls, providing exposure was initiated during the virus shedding period. About 60 per cent of the dogs infected by either inoculation or contact developed clinical signs of kennel cough and spontaneous coughing was noted up to the 12th day. The serial pathology of the disease was not studied although a bronchiolitis and an interstitial pneumonitis were observed in 5 of 7 dogs sacrificed between 14 and 20 days after infection.

Saona Black and Lee (1970) have shown that the parainfluenza virus isolated from dogs is capable of multiplying in a wide range of host cells and that cats are as equally susceptible as dogs to experimental infection, but the question as to whether dog to cat transmission occurs under natural conditions is not clear.

Previous studies of parainfluenza viruses have revealed antigenic differences within a given type between isolates from man and other species. Thus the human and bovine strains of parainfluenza virus type 3 can be differentiated by neutralisation, haemagglutination inhibition and complement fixation tests (Ketler et al., 1961; Abinanti et al., 1961). To date there does not appear to be a consistent antigenic

difference between simian, human and canine parainfluenza SV-5 viruses (Lazar et al., 1970). The close environmental relationship between dogs and man has led to the possibility that dogs may be reservoirs of infection for man or vice versa. The risk of cross-infection between dogs and man should be considered although infection in dog handlers was not observed in a parainfluenza SV-5 virus epizootic in military dogs (Crandell et al., 1968).

(ii) Herpesvirus.

Canine herpesvirus (CHV) was first isolated from neonatal puppies (Carmichael et al., 1965b) and was shown to be the cause of a generalised, haemorrhagic and necrotizing disease (Carmichael et al., 1965a; Wright and Cornwell, 1968a). Under both natural and experimental conditions, this fatal infection has only been recorded in dogs less than 3 weeks of age whereas in adult dogs the virus has been recovered from non-fatal infections of the respiratory and genital tracts (Karpas et al., 1967; Poste and King, 1971). The first report of the isolation of CHV from the respiratory tract of dogs was made by Motohashi and Tajima (1966) who recovered a herpesvirus from the lungs of an adult dog suffering from pneumonia. Further isolations have since been made in the United States of America from mature dogs with respiratory disease, CHV being found either alone or with other agents (Binn et al., 1967).

The experimental exposure of young dogs over 3 weeks of age to CHV has produced disease of variable severity. Karpas et al. (1968b) reported that adult dogs inoculated intranasally developed respiratory signs ranging from mild nasal discharge and sneezing to paroxysmal coughing. This was not confirmed by other workers who found that 5 to 12-week-old dogs challenged by intranasal instillation of virus developed only

mild or inapparent infection. Pathological changes were minimal

and only a few cellular foci of mononuclear cells were found in the

lungs (Appel et al., 1969; Wright and Cornwell, 1970).

The isolation of CHV from an adult dog with tracheitis and

pneumonia, the first in Great Britain, stimulated renewed interest in

CHV respiratory infections (Wright et al., 1970). An account of the

isolation of the virus and the experimental disease is included in the

first part of this thesis.

(iii) Adenovirus.

Two distinct canine adenoviruses, CAV-1 and CAV-2, have been

recovered from dogs with respiratory disease. The CAV-2 strain was

first isolated in Canada by Ditchfield et al. (1962) from dogs with

kennel cough. Since then the virus has been isolated from the respiratory

tract of dogs in the United States (Binn et al., 1967) and more

recently, in the United Kingdom (Danskin, 1973). Swango et al. (1970)

showed that dogs infected with CAV-2 by the respiratory route developed

a proliferative interstitial pneumonia with necrotizing bronchitis

and bronchiolitis.

The CAV-1 virus has long been recognised as the causal agent

of the acute systemic infection known as Rubarth's disease or infectious

canine hepatitis (Rubarth, 1947). Recently CAV-1 has been recovered

from dogs with respiratory disease (Binn et al., 1967; Wright et al.,

1972; Studdert and Studdert, 1972) and the second part of this thesis

investigates the pathogenicity of CAV-1 for the respiratory tract.

(iv) Reovirus.

Reoviruses have been associated with canine respiratory disease

in the United States of America where Lou and Wenner (1963) isolated a

reovirus type 1 from the lungs and pharyngeal tissue of a dog which

died 2 weeks after the onset of a respiratory disease. Massie and Shaw (1966) recovered a similar agent from nasal swabs of laboratory dogs with a respiratory illness.

Serological evidence has indicated that reovirus infection is prevalent in the dog population of many areas of the United States of America and serum neutralising and HA-I antibodies against all 3 strains of mammalian reovirus have been found in both clinically ill and healthy dogs (Rosen, 1960; Lou and Wenner, 1963; Massie and Shaw, 1966; Schmidt et al., 1966). In one survey Fairchild and Cohen (1967) found significant levels of HA-I antibodies to at least one of the 3 reovirus types in 90 out of 112 (80 per cent) dogs at a veterinary clinic whereas dogs raised in isolation had little or none.

There have been conflicting reports on the pathogenicity of reovirus type 1 in dogs. Lou and Wenner (1963) found that the intranasal inoculation of this virus into weanling puppies resulted in a disease characterised by a cough and nasal discharge. The experimentally infected puppies developed an interstitial pneumonitis and intracytoplasmic inclusion bodies were found in the bronchial epithelium. Massie and Shaw (1966) also noted coughing and a nasal discharge following the intranasal and intravenous inoculation of puppies with reovirus type 1. In contrast, Holzinger and Griesemer (1966) found no clinical evidence of disease after the intranasal and intraperitoneal inoculation of germ-free and disease-free dogs, although the dogs developed serological evidence of infection.

To date there have been no reports of the isolation of reoviruses from dogs in Great Britain but a preliminary survey showed the presence of HA-I antibodies. Accordingly it was decided to study the response of dogs to experimental infection with reovirus type 1. This investigation forms part 3 of this thesis.

PART 1

CANINE HERPESVIRUS RESPIRATORY INFECTION

SECTION I : The isolation of a herpesvirus from an adult dog with

respiratory disease.

SECTION I : Contents

INTRODUCTION

In 1964 Carmichael et al. described an acute, septicaemic disease of young puppies caused by an agent which was cytopathogenic for cell cultures of canine kidney. Although initially a mycoplasma was thought to be responsible for the condition, further studies showed the causal agent to be a herpesvirus (Carmichael et al., 1965b). Since then, the isolation of CHV from neonatal puppies dying of an acute, haemorrhagic and necrotizing disease has been reported from many other parts of the world (Schwartz and Martin, 1966; Prydie et al., 1966; Cornwell et al., 1966; Geldard et al., 1971; Rondhuis and Lindesipman, 1972).

The clinical and pathological features of both natural and experimental CHV infection in the neonate have been described in detail (Carmichael et al., 1965a; Cornwell et al., 1966; Wright and Cornwell, 1968a; Kakuk and Conner, 1970). Under natural and experimental conditions, the acute haemorrhagic and necrotizing disease characteristic of CHV infection occurs only in puppies less than 3 weeks of age. The reason for the resistance of older animals to this form of the disease is not completely understood but it seems to be related to their ability to stabilise their body temperature to around 39°C; neonatal puppies have a poorly developed thermoregulatory mechanism. (Crighton, 1968; Carmichael et al., 1969) and in vitro studies have shown that while CHV can replicate efficiently at 37°C., it is unable to do so at 39°C. (Aurelian, 1968; Carmichael and Barnes, 1969; Cornwell and Wright, 1969).

The role of CHV in disease of the older dog is less well known. CHV has been isolated from adult dogs with respiratory disease in America and Japan (Motohashi and Tajima, 1966; Binn et al., 1967; Karpas et al., 1967), from a dog with "malignant lymphoma" (Kakuk et al., 1969), from cerebellar cultures of a dog with encephalitis (Cornwell,

Wright and Thompson, 1972 - unpublished observations) and from
apparently healthy dogs (Karpas et al., 1968a). Recently Poste and King
(1971) reported the isolation of CHV from vesicular lesions of the
genitalia of both male and female Alsatians and the virus isolated
from these lesions produced an identical condition in a bitch
inoculated experimentally.

In Great Britain, the first recorded isolation of CHV from an
adult dog with respiratory disease was made by the author and his
colleagues (Wright et al., 1970). This section deals with the isolation
and identification of that virus.

HISTORY

In June 1970, there was an outbreak of respiratory disease in
kennelled dogs near Glasgow. The kennels formed part of an experimental
surgery unit and all the dogs had been purchased from commercial sources.
The dogs were of mixed breeds and the majority were aged between 1 and
3 years.

There was a constant turnover of dogs through the unit and
immediately on arrival, new dogs were vaccinated with a live attenuated
canine distemper vaccine ("Epivax"; Burroughs Wellcome, Berkhamsted).
They were then held in isolation quarters for a period of 3 weeks
before entering the main kennels and any dog which showed signs of
ill health during the quarantine period was culled.

In the kennels the dogs were housed singly, or occasionally in
pairs, in wire mesh pens with concrete floors. They were allowed free
access to individual outdoor runs which were separated by wood and
wire partitions.

The pens were arranged along 3 sides of large rooms and in each
room there were approximately 50 dogs. A feature of the buildings was
a low ceiling which made ventilation poor.

Despite the quarantine precautions and distemper prophylaxis, outbreaks of respiratory disease were a major and recurrent problem in this unit, especially when the accomodation was being utilised to its full capacity. At the time of the June outbreak, 75 dogs were present in the kennels and, of these, 20 developed significant signs of respiratory disease. All of the affected dogs were treated with antibiotics but 16 had to be destroyed and one of these, an adult Collie bitch, was made available for examination in the Department of Veterinary Pathology.

MATERIALS AND METHODS

Histological procedures

After clinical examination the dog was destroyed by the intravenous administration of pentobarbitone sodium ("Euthatal"; May and Baker Ltd., Dagenham). A full postmortem examination was carried out immediately on death and multiple blocks of lung, trachea, bronchial lymph node and urinary bladder were taken for histopathological examination. The tissues were fixed in mercuric chloride - formol for 48 hours, dehydrated in an alcohol-amyl-acetate-chloroform series and embedded in paraffin wax. Sections were cut at 6μm and stained with Mayer's haemalum and eosin; Martius - scarlet-blue for fibrin, Gordon and Sweet's silver stain for reticulin, periodic-acid-Schiff for basement membranes and haemalum-phloxine-tartrazine for distemper virus inclusion bodies (Lendrum, 1947) were also used.

Bacteriological procedures

Blood agar and McConkey plates were inoculated with material from the trachea and lungs and were incubated aerobically and anaerobically. Examination of those organs was also made for the presence of mycoplasmas and, for this purpose, inoculated plates of

Edward's medium and tubes of sloppy agar were incubated for 5 days
at 37°C. Thereafter the sloppy agar was subcultivated onto fresh
plates of Edward's medium which were incubated for a further 5 days
and then examined microscopically.

Virological procedures

Cell culture

A canine kidney cell line (Madin and Darby MDCK) in the 66th
subculture was obtained from Flow Laboratories Ltd., Ayrshire and
reseeded as recommended by the suppliers. The cells were maintained
in 8oz. glass prescription bottles and reseeded in 1oz. glass bottles
as required.

Growth medium consisted of Earle's solution with 0·5 per cent
lactalbumin hydrolysate and 10 per cent foetal bovine serum (Flow Labs.
Ltd.). The antibiotics penicillin and streptomycin were added at
100 units per ml. and 100µg per ml. respectively. The maintainance
medium was identical in composition but contained only 2 per cent of the
foetal bovine serum.

Virus isolation

Samples of trachea and lung were collected so as to preclude
cross-infection and stored at -20°C. until virus isolation was attempted.
The tissues were ground in a mortar and pestle with sterile sand, after
which Earle's balanced salt solution was added to make a 10 per cent
suspension.

Each suspension was inoculated into two 1oz. bottles of MDCK
cultures. The medium was removed from each bottle, 0·5ml of the
inoculum was added and the bottles were then reincubated for 1 hour.

Following removal of the inoculum, the cells were washed with
phosphate buffered saline (PBS) and the maintainance medium was then

replaced. The cultures were incubated at $37^{\circ}C$. and then examined daily using a Prior inverted microscope.

Cytology

For cytopathological studies MDCK cells were grown in 50mm plastic Petri dishes ("Nunclon": Jobling, Staffordshire) each of which contained 6 coverslips. The cultures were incubated at $37^{\circ}C$. in a moist atmosphere containing 5 per cent carbon dioxide and when the cell sheets were confluent they were inoculated with fluid from the infected MDCK cultures. Coverslips were removed at 24 and 48 hours after infection, washed with PBS to remove excess medium and divided into 4 groups. Two groups were fixed for 1 hour in mercuric chloride-formol and then stained by Mayer's haemalum and eosin and by the fluorescent Feulgen technique (Culling and Vassar, 1961). The third group were fixed in methyl alcohol and stained with May-Grünwald Giemsa while the fourth group were fixed in acetone for 10 minutes and stained by the acridine orange method (Mayor, 1964).

The coverslips stained by the fluorescent Feulgen and acridine orange techniques were examined under a fluorescence microscope ("Orthoplan": Leitz, Germany) equipped with a BG12 exciter filter, a barrier filter and a dark ground condenser.

Electron microscope procedures

Negative staining

Infected MDCK cultures showing an advanced cytopathogenic effect (CPE) were rapidly frozen and thawed to release virus from the cells. The latter were then sedimented by centrifugation at 10,000 r.p.m. for 10 minutes and 20ml of the supernatant were spun at 30,000 r.p.m. for 45 minutes in a Spinco Type L ultra centrifuge. The resulting pellet was resuspended in a few drops of PBS and a small sample of the

suspension was placed in a 50mm plastic Petri dish containing several drops of 1 per cent osmic acid hanging from the lid. The suspension sample was fixed in the osmic acid vapour for 10 minutes at room temperature. A carbon coated electron microscope grid was placed on the surface of the suspension sample and after a few seconds contact the grid was removed, drained of the excess fluid and blotted with filter paper. The preparation was stained with 2 per cent phosphotungstic acid at pH 6·0. A drop of stain was placed on a clean glass slide and the surface of the grid, which had been in contact with the sample, was allowed to touch the stain for 5 to 10 seconds. Excess stain was immediately blotted from the grid with filter paper and the specimen was examined in the electron microscope.

Thin sections

At 24 hours after infection, infected MDCK cells were scraped from the surface of plastic Petri dishes with a silicone rubber "policeman". The cells were pelleted by centrifuging at 1,000 r.p.m. for 10 minutes, fixed for 1 hour in 1 per cent osmic acid, dehydrated in alcohols, rinsed in propylene oxide and embedded in Araldite.

Sections were cut with an LKB Mark III ultratome using glass knives and were mounted on uncoated Athene 483 copper specimen grids obtained from Smethurst High-Light Ltd., Bolton, Lancs.

Sections were stained for 10 minutes with uranyl acetate (Watson, 1958), rinsed successively in metanol, 50 per cent methyl alcohol and distilled water and dried off on filter paper. They were then stained for 10 minutes with lead citrate (Reynolds, 1963) and finally rinsed with 0·02N sodium hydroxide and distilled water before drying on filter paper.

The fixative, buffer, resins and stains for electron microscopy were prepared as described in APPENDIX 1.

Serological procedures

Neutralisation tests were carried out by means of a plaque-inhibition test, the conditions for plaque formation being those recommended by Cornwell and Weir (1972 - personal communication).

Antisera to the new isolate and a known strain of CHV, G1, (Cornwell et al., 1966), were prepared in rabbits. Two rabbits were given an initial subcutaneous inoculation of 1ml. of an equal mixture of virus and Freund's complete adjuvant. After 4 weeks, the rabbits were injected intraperitoneally with 1ml. of virus suspension only and this was repeated 10 days later. Blood samples were collected from the rabbits 2 weeks after the final inoculation.

Plaque-inhibition tests were carried out by adding approximately 200 plaque-forming units (PFU) of virus to serial 2 fold dilutions of serum. After the virus-serum mixtures had been incubated at room temperature for one hour, 0·5ml of each dilution was inoculated onto monolayers of MDCK cells. These were prepared in 50mm plastic Petri dishes, each plate being seeded with 3×10^6 cells. The monolayers were washed twice with PBS before inoculation and the virus was allowed to absorb for 4 hours in a humidified atmosphere of 5 per cent carbon dioxide. During the attachment time the plates were shaken manually every 15 minutes to distribute the fluid and prevent drying of the cell sheet.

After the inoculum had been withdrawn, each plate was given 4ml of agar overlay and reincubated at $37^{\circ}C$. in a humidified atmosphere of 5 per cent carbon dioxide. The overlay was prepared by mixing double strength Earle's balanced salt solution containing 0·5 per cent lactalbumin hydrolysate, 5 per cent foetal bovine serum and 200 units per ml. of penicillin, 200μg per ml. of streptomycin and 100 units per ml. of mycostatin with an equal volume of melted 1·8 per cent Difco agar in deionised water, both solutions being held at $44^{\circ}C$. prior to use.

After 4 days, each culture was fixed for 24 hours in mercuric chloride-formol. The fixative was then decanted and the solid overlay was carefully removed. The plates were washed in tap water for 5 to 10 minutes and stained with Leishman's stain for a further 20 minutes. At the end of this time the plates were washed in running tap water and the plaques counted. Serum titres were recorded as the highest dilution which reduced the plaque count by 50 per cent.

RESULTS

Clinical findings

On admission to the Veterinary Hospital, the bitch was dull and in poor bodily condition. The respiratory rate was 60 per minute and, on auscultation, soft rales were heard over both lungs. Spontaneous coughing was not apparent during the period of examination but a retching cough could be induced by gentle pressure on the trachea. The body temperature was $103 \cdot 2^{O}F$.

Macroscopic findings

At postmortem examination the trachea and bronchi were filled with blood tinged frothy fluid. The lungs were diffusely oedematous and plum red areas of consolidation were present in the apical, cardiac and anterior diaphragmatic lobes (Fig. 1). The broncho-mediastinal lymph nodes were enlarged, hyperaemic and oedematous. Significant abnormalities were not found in the other organs.

Microscopic findings

In all sections of the trachea examined, the epithelium was reduced to a low columnar or cuboidal layer and there were localised areas of epithelial hypertrophy and necrosis at all levels (Fig. 2). In these regions Cowdry Type A eosinophilic intranuclear inclusions, suggestive of herpesvirus infection, were present in necrotic epithelial

cells (Fig. 3). The inclusions were round or oval in shape, approximately 8μm in diameter and surrounded by a clear halo. In some affected cells the nucleoli were still present although pushed to one side by the inclusion body. There was a moderate cellular infiltration, composed mainly of lymphocytes and plasma cells, in the lamina propria.

The lungs showed patchy areas of bronchopneumonia characterised by a copious neutrophilic exudate into the bronchi, bronchioles and surrounding alveoli (Fig. 4). In the middle and peripheral parts of the lobules, the alveolar lumina were filled with eosinophilic precipitated oedema fluid and macrophages. Around some bronchioles, a few alveoli showed epithelial hyperplasia and in addition there was prominent peribronchial infiltration by lymphocytes and plasma cells (Figs. 4, 5). The bronchial and bronchiolar epithelium was flattened and infiltrated by lymphocytes. Intranuclear inclusion bodies were not observed in the lung lesions and histological examination of sections of lung and trachea stained with haemalum-phloxine-tartrazine proved negative for distemper virus intracytoplasmic inclusions.

The pulmonary lymph nodes were congested and the sinuses were filled with macrophages and red blood cells. Large numbers of plasma cells were present in the medullary cords.

Bacteriological findings

Bacteriological examination of the lung and trachea failed to reveal the presence of any organism.

Virological findings

A CPE, characteristic of herpesvirus, appeared within 48 hours in all inoculated cultures (Fig. 6) whereas the control cultures showed no evidence of degeneration (Fig. 7). At first, discrete foci of rounded, highly refactile cells appeared throughout the cell sheet (Fig. 8). On further incubation the foci enlarged and the affected cells gradually

detached from the glass until the whole cell sheet was destroyed. The

CPE was readily reproducible on passage and pooled suspensions

had titres in the order of 10^4 to 10^5 PFU per ml. Undilute virus of

high titre produced a CPE within 24 hours.

Small, discrete plaques 0·5mm to 2mm in diameter were produced

in MDCK cell cultures under agar (Fig. 9). The plaques were clearly

defined as transparent circles in the stained monolayers (Fig. 10),

although, on occasions, a few pink degenerate cells remained in the

centre.

In coverslip preparations, the cells in infected foci were

often shrunken, hyperchromatic and piled upon one another (Fig. 11).

The cells stained intensely with haemalum and eosin, while with

acridine orange the nuclei fluoresced light green and the cytoplasm

appeared yellowish-orange (Fig.11). At the edge of the foci, a few cells

contained intranuclear inclusion bodies. The inclusions were oval to

round and occupied the central nuclear area. They were separated from

the nuclear chromatin by a clear halo and appeared eosinophilic with

haemalum and eosin and purple or violet with Giemsa (Fig. 12). The

nuclei of infected cells fluoresced bright golden yellow with Feulgen

indicating the presence of DNA.

The presence of intranuclear inclusions was not, however,

typical of the changes in most infected cells. More characteristic was

a breakdown of the nucleoli and the appearance of small eosinophilic

granules (1-2μm) within the nuclei. These granules fluoresced dark green

with acridine orange. Another feature was clumping of the nuclear

chromatin into masses around the inner nuclear membrane.

Electron microscopic findings

Although amorphous debris was present, virus particles with the characteristic appearance of herpesvirus virions were identified in negatively stained preparations (Figs. 13, 14, 15). Naked and enveloped particles were observed, the former predominating. In some virions the core was completely penetrated by the phosphotungstic acid and in these empty particles the capsid was clearly demonstrated (Fig. 14). The latter appeared hexagonal and composed of elongated, hollow and regularly disposed capsomeres. There were five capsomeres along the edge of each face of the capsid and the naked capsids ranged from 90-100nm in size.

The envelopes varied in shape and in size and in some particles they were ruptured although the nucleocapsid still lay within. The diameters of the envelopes varied from 140-180nm.

Examination of ultrathin sections of infected cells revealed the presence of virus particles in the nucleus and cytoplasm. Within the nucleus, the particles were round or hexagonal in shape, approximately 100nm in diameter with a single limiting membrane, the capsid. The detailed morphology of the internal components of the virus showed considerable variations (Fig. 16). In some, the core was electron lucent giving the impression of a hollow capsid. In others the nucleoid was a small, round, electron dense structure which measured 30-40nm in diameter; this was the form commonly seen in enveloped particles. However, the most striking core structures in the nucleus appeared to be 3 or 4 electron dense aggregates arranged symetrically against the inner capsid wall leaving a clear star or cross shape in the centre (Fig.16).

Enveloped virus particles were seen at 3 sites; in the perinuclear space, in membrane bound vesicles (Figs. 17, 20) and at the cell surface (Fig. 18). At high magnifications the envelope was seen to

TABLE 3

Plaque Inhibition Tests between the Herpesvirus Isolate, CHV-G3,

and CHV-G1.

Virus Strain	Antiserum	Highest dilution which reduced the plaque count by 50 per cent.
CHV-G1	CHV-G3	1:32
CHV-G3	CHV-G3	1:32
CHV-G1	CHV-G1	1:128
CHV-G3	CHV-G1	1:64

consist of 3 layers. Stubby projections were present on its outer surface, a unit membrane composed the middle layer and electron dense amorphous material enclosed the nucleocapsid (Fig. 19).

The most striking cellular changes were in the nucleus. The nuclear alterations consisted of a widening of the perinuclear space, condensation and margination of the nuclear chromatin into masses along the inner nuclear membrane and the formation of long evaginations of the membrane which extended deep into the cytoplasm (Figs. 20, 21). Inclusion body structures were not identified.

The main change in the cytoplasm was the formation of large vacuoles containing enveloped viral particles (Figs. 20, 21). Naked virus particles lay free in the cytoplasm but enveloped particles were never found in this situation. The mitochondria were swollen and disrupted. An uninfected cell is shown for comparison (Fig. 22).

Serological findings

The results of cross neutralisation tests between CHV, G-1 strain, and the new isolate are presented in Table 3. The results indicate that each virus was neutralised to approximately the same degree by both the sera employed.

DISCUSSION

The herpesviruses are characterised by a DNA core, cubic symmetry, sensitivity to lipid solvents, a size ranging from 100 to 150nm and the formation of eosinophilic intranuclear inclusions (Andrewes and Pereira, 1972). Their appearance in the electron microscope, both when negatively stained and when seen in ultrathin sections of infected cells, is highly specific so that, for the characterisation of the isolate described above, it was decided to limit the investigation to electron microscopy and serology.

The fine structure of the new isolate (designated CHV, G-3) as seen in negatively stained preparations appeared similar to that previously reported for CHV (Stranberg and Carmichael, 1965). The basic morphological types described for herpes simplex virus (Wildy et al., 1960; Wildy and Watson, 1962) were observed in this investigation. These were the full enveloped particle, the empty enveloped particle, the full naked capsid and the empty naked capsid. Enveloped particles were infrequently seen, possibly because their envelopes may have been broken and detached during the preparation of the virus.

Electron microscopy of thin sections of infected cells showed that the development of the virus in the nucleus and the mode of release from infected cells was similar to that previously reported for herpesviruses in tissue culture (Darlington and Moss, 1968, 1969). The viral nucleoid had several distinct forms; some capsids appeared to be empty while others had dense aggregations against the capsid wall. In mature virions, the core was invariably round and electron dense and the other forms may have represented stages in the development of the final nucleocapsid. In a study of the replication of CHV, Stranberg and Aurelian (1969) found that coreless capsids or capsids containing pleomorphic cores were the first to appear in the nucleus. Pleomorphic nucleoids of similar appearance to those described above have been seen with equine rhinopneumonitis virus (Reczko and Mayr, 1963), particularly when that virus was grown in swine kidney cells.

The CPE produced in MDCK cultures by the new isolate was identical in many respects to that seen with other strains of CHV (Cornwell and Wright, 1969). The breakdown of the nucleoli and the appearance of eosinophilic granules in the nucleoplasm of infected cells has been described in detail by Johnson et al. (1969). In the present study typical herpetic intranuclear inclusions were only rarely to be

found in tissue culture but the presence or absence of inclusions may depend on the cell type in which the virus is grown. The F205 strain of CHV isolated in the USA (Carmichael et al., 1965b) produced large numbers of nuclear inclusions when grown in a canine thyroid adenocarcinoma cell-line (Johnson et al., 1969) but few inclusions when grown in primary dog kidney cells (Carmichael et al., 1965b).

Antigenically, the new isolate seemed to be closely related to a strain of CHV isolated from neonatal puppies in that there was complete cross-neutralisation between them. It is possible, however, that kinetic neutralisation tests might have revealed slight differences in antigenic structure.

The above case was the first recorded isolation of a herpesvirus from an adult dog in Great Britain and shows that CHV is not confined to the neonate. This fact has subsequently been confirmed by the recovery of a herpesvirus from the genitalia of adult dogs (Poste and King, 1971) and more recently by the isolation of CHV from cerebellar explant cultures of a 5-year-old dog with encephalitis (Cornwell, Wright and Thompson, 1972 - unpublished observations).

Herpesviruses have been isolated from dogs with respiratory disease in Japan (Motohashi and Tajima, 1966) and in the United States of America (Binn et al.,1967; Karpas et al., 1967) but there is little information concerning the pathological changes which they produced. Thus, Motohashi and Tajima (1966) recovered a herpesvirus from a dog suffering from pneumonia but unfortunately did not describe any histopathological changes in the respiratory tract. During their investigation of an outbreak of kennel cough, Karpas et al. (1968a) isolated a herpesvirus from several dogs showing the characteristic clinical signs and also from some apparently healthy in-contact animals. One of the clinically-affected dogs from which the virus was isolated was sacrificed but the results of

histological examinations were brief and confined to the statement that intranuclear inclusion bodies were found in the tracheal and bronchial epithelium.

In the present study, the main pathological findings were a necrotizing tracheitis and bronchopneumonia. Localised areas of epithelial necrosis and hypertrophy were found in the trachea at all levels and in these regions eosinophilic intranuclear inclusions were seen in necrotic epithelial cells, inclusions were not found in the lung lesions.

Although mild alveolar epithelialisation was present, in general the histopathological features of the above case were quite unlike those described for canine distemper (Lauder et al., 1954). In the present case, epithelial necrosis was prominent and there was no evidence of intracytoplasmic inclusion bodies. The changes in the tracheal epithelium were, however, not unlike those seen in other herpesvirus respiratory infections. The finding of intranuclear inclusions and focal epithelial necrosis are characteristic of the respiratory changes in infectious bovine rhinotracheitis (McKercher, 1959), feline viral rhinotracheitis (Crandell and Maurer, 1958; Crandell et al., 1961) and equine rhinopneumonitis (Prickett, 1969). Furthermore similar changes have been reported in the few recorded cases of fatal herpesvirus pneumonia in man (Nash, 1972).

The finding of inclusions in the tracheal epithelium, together with the isolation of a herpesvirus from the respiratory tract, provides strong evidence that CHV was responsible for the clinical respiratory disease seen. However, the part played by bacteria in the development of the bronchopneumonia cannot be dismissed since the dog was treated with antibiotics prior to examination. The question as to whether or not CHV plays a primary role in respiratory disease of the dog cannot be answered until it is shown experimentally that the virus is pathogenic for the respiratory tract of dogs older than a few weeks of age. The next part of this investigation was designed to throw light on that point.

CANINE HERPESVIRUS RESPIRATORY INFECTION

SECTION II : Experimental canine herpesvirus respiratory infection.

SECTION II : Contents

INTRODUCTION

There is little doubt that the resistance of the dog to CHV infection increases with age (Carmichael et al., 1965a; Wright and Cornwell, 1968a). The widespread necrotizing and haemorrhagic lesions which are characteristic of the disease in the very young pup have not been described in dogs older than 3 weeks of age (Carmichael, 1970; Huxsoll and Hemelt, 1970). However, it has been shown that the virus can multiply in the respiratory and genital tracts of older animals (Carmichael, 1965b). Since CHV has been recovered from adult dogs with respiratory disease (Motohashi and Tajima, 1966; Binn et al., 1967; Karpas et al., 1967; Wright et al., 1970), it was considered worthwhile to examine in detail the lesions produced following intratracheal and aerosol challenge of 12-week-old puppies with a strain of CHV recovered from the respiratory tract.

MATERIALS AND METHODS

Virus

The virus strain used in this experiment was isolated from the lungs and trachea of a dog as described in SECTION I. The virus was passaged three times in MDCK cells and a pool was then prepared; the pooled virus had a titre of 10^5 PFU/ml and was stored at -60°C.

Experimental animals

Twenty seven 12-week-old farm bred Collie dogs, comprising 4 complete litters, were used. The dogs were bled on arrival and, from the sera prepared, were shown to be free of antibody to CHV by a plaque-inhibition test. The dogs were infected by the intratracheal route and by means of an aerosol procedure.

Experimental procedures

(a) Intratracheal inoculation of CHV

Seven dogs were infected by the intratracheal route after they had been lightly sedated with diethylthiambutene hydrochloride ("Themalon": Burroughs Wellcome, Berkhamsted). A fine plastic tube was passed down the trachea to the bifurcation of the bronchi. A syringe containing 1ml of the virus inoculum was connected by a needle to the free end of the tube and the virus suspension was slowly injected.

In the same fashion, 4 control animals were each given 1ml of uninfected tissue culture cells.

(b) Aerosolisation of CHV

Twelve dogs were infected by an aerosolisation procedure. Each puppy was sedated with diethylthiambutene hydrochloride and placed in a polythene chamber to which was attached a polythene tube with a face mask. The tube was connected to a nebulizer unit powered by a CFI electric compressor unit (Aerosol Products Ltd., Colchester). The nebulizer was attached to a universal container which contained the infective tissue culture suspension. Each animal was exposed to the aerosolized virus suspension for a period of 10 minutes and in that time approximately 0·3ml of virus suspension was aerosolized.

For the purpose of control, 4 further puppies were aerosolized in a similar manner with uninfected dog kidney tissue culture fluid and housed in a separate room.

All the infected dogs were kept in isolation quarters and the ambient temperature was maintained at 70°F. The animals were examined clinically each day. They were killed at intervals (Tables 4 and 6) from the 3rd day post-infection by rapid intravenous injection of pentobarbitone sodium.

Histological procedures

At necropsy, the trachea, heart and lungs were removed immediately. The left lung was separated and the left bronchus ligated. The right lung was fixed by infusing mercuric chloride-formol slowly down the trachea until the lung was life size. The trachea was tied off and the lung was allowed to fix for 24 hours and then trimmed and fixed for a further 24 hours. The left lung was used for virological studies and blocks were also taken from each lobe for histopathological examination.

The skull was split longitudinally and blocks of the nasal septum, ethmoturbinates and ventral turbinate were taken. Portions of the following organs were also collected; tonsils, trachea, bronchial and retropharyngeal lymph nodes, kidneys, liver, myocardium, adrenals, urinary bladder, thymus, spleen, cerebrum, thalamus, cerebellum and pons. After fixation in mercuric chloride-formol, processing for histological examination was carried out as described in SECTION I.

Electron microscopic procedures

Lung tissue for electron microscopy was taken from 2 dogs infected by aerosol (Nos. 75 and 76) and from three controls. Small blocks of tissue 1 to $2mm^3$ in size were excised from the lung as soon as possible after death. The specimens were placed in drops of chilled 2 per cent glutaraldehyde on blocks of dental wax, chopped into pieces approximately $0 \cdot 5mm^3$ in thickness by means of grease-free razor blades and then transferred to vials containing chilled fixative. The small blocks of tissue were left for one and a half hours in 2 per cent glutaraldehyde at $4^{\circ}C$., rinsed in Sorensen's phosphate buffer and then post-fixed for one hour in 1 per cent osmium tetroxide.

Dehydration was through an ascending series of 70 per cent, 90 per cent and absolute alcohol. The tissue blocks were then rinsed with propylene oxide before being embedded in either Araldite or Epon epoxy resin,

Sections 1 to 1·5μm in thickness were cut on an LKB Mark III ultratome and mounted on glass slides. They were stained with either Mallory's azure 2 methylene blue in borax (Richardson et al., 1960) or 1 per cent toluidine blue in 1 per cent borax (Trump et al., 1961). These sections were used to locate lesions and orientate specimens for electron microscopical examination.

Ultrathin sections were cut and stained as described before (SECTION I) and examined in an AEI 6B electron microscope.

The fixatives, buffers and resins were prepared as described in APPENDIX 1.

Virological procedures

At necropsy, samples of lung, turbinate, trachea and tonsils were taken and stored at -20°C. until virus isolation was attempted. Tissue suspensions were prepared 1:10w/v in Earle's balanced salt solution containing the usual antibiotics. Each suspension was clarified by centrifugation and the supernatant used to inoculate two 1oz bottles of MDCK cells. The cultures were examined daily for up to 14 days for the typical cytopathogenic effect of CHV (Cornwell and Wright, 1969). Samples of lung and trachea were also subjected to bacteriological examination as described in SECTION I.

RESULTS

(a) Intratracheal inoculation of CHV

Clinical findings

Clinical signs of disease were not observed in any of the puppies nor were there any alterations in rectal temperatures.

Macroscopic findings

The macroscopic findings are summarised in Table 4. At necropsy, visible changes were found in the lungs of all infected dogs from the 3rd to the 7th days. Reddish-grey areas of consolidation were observed in all lobes of the lungs in dogs killed on the 3rd and 5th days after infection. The lesions were most marked in the dorsal regions of the diaphragmatic and cardiac lobes (Fig. 23); discrete lesions were also present in the other parts of the lungs. On the 7th day multiple grey foci depressed below the surface of the lung were found but no gross abnormalities were noted in the lungs of the dogs killed on the 10th and 13th days.

The bronchomediastinal and retropharyngeal lymph nodes were enlarged in all of the infected dogs. On the 3rd and 5th days after infection the nodes appeared moist and hyperaemic but subsequently they became firmer in consistency and greyish in colour (Fig. 24).

The tonsils appeared enlarged and hyperaemic in the dogs killed on the 10th and 13th days. No other lesions were observed in any of the dogs.

Microscopic findings

The microscopic findings are summarised in Table 4. On the 3rd and 5th days after infection, the main lesions were to be found in the small bronchi and bronchioles. There was focal necrosis of the

TABLE 4

Experimental Canine Herpesvirus Respiratory Infection

Pathological findings : intratracheal infection

Dog Number	Day Examined	Pneumonia	Bronchial Necrosis	Alveolar Necrosis
66	3	++	++	+
79	3	+	++	+++
64	5	+++	++	++
80	5	++	+	+
60	7	+	+	-
62	10	-	-	-
65	13	-	-	-
57*	3	-	-	-
56*	5	-	-	-
83*	7	-	-	-
84*	13	-	-	-

* Denotes unexposed control animal.

Lesions were judged + to +++ according to severity.

epithelium (Fig. 25) and in some areas the foci merged, producing destruction of the complete epithelial lining. The epithelial cells sloughed into the lumen and many small bronchi and bronchioles were plugged with necrotic epithelial debris. The necrotizing process frequently extended to involve neighbouring alveoli although isolated foci of alveolar necrosis were also present (Fig. 26). Damage to the alveolar septa and destruction of the walls of small blood vessels resulted in haemorrhage and the exudation of moderate amounts of fibrin; many macrophages were also present.

Eosinophilic or faintly basophilic intranuclear inclusions were found in bronchial epithelial cells, occasionally in the bronchiolar epithelium and only rarely in alveolar lesions.

By the 7th and 10th days after inoculation epithelial necrosis was less marked and there was evidence of healing and repair. The fibrin and cellular debris were removed by macrophages, and fibroblasts and lymphocytes were present in the alveoli and alveolar septa. The bronchial epithelium was replaced by a low irregular cuboidal layer of cells and lymphocytes and plasma cells were seen in small numbers in the peribronchial and peribronchiolar lamina propria. Foci of alveolar scarring with fibroblasts and macrophages were the only lesions found in the lungs of the dog killed on day 13, the bronchial and bronchiolar epithelium being completely normal.

Virological findings

The results of virus isolation from the dogs infected by the intratracheal route are summarised in Table 5. CHV was isolated from the lungs and trachea up to 5 days after infection. The virus was recovered from the tonsils of one dog killed on day 3 but was absent from the turbinates.

TABLE 5

Experimental Canine Herpesvirus Respiratory Infection

Virological findings : the recovery of CHV after intratracheal infection

Dog Number	Day Examined	Lung	Trachea	Turbinate	Tonsil
66	3	+	-	-	-
79	3	+	+	-	+
64	5	+	-	-	-
80	5	+	+	-	-
60	7	-	-	-	-
62	10	-	-	-	-
65	13	-	-	-	-
57*	3	-	-	-	-
56*	5	-	-	-	-
83*	7	-	-	-	-
84*	13	-	-	-	-

+ Denotes CHV recovered.

* Denotes unexposed control animal.

Control animals

Clinical, postmortem and histological abnormalities were not observed in any of the control puppies.

(b) Aerosolisation of CHV

Clinical findings

Bilateral serous nasal discharge developed in all infected dogs between the 3rd and 7th days after aerosolisation. The dogs did not cough spontaneously nor could coughing be induced by palpating the trachea. Increased rectal temperatures were not observed at any time and the animals remained bright throughout the experiment.

Macroscopic findings

The pathological findings are summarised in Table 6. At postmortem examination, the nasal mucosa, particularly that covering the ventral turbinates, was moderately hyperaemic in all infected dogs. There was no evidence of a tracheitis at any time and the most striking macroscopic findings were in the lungs. Pulmonary lesions in the form of grey or red foci, 1 to 3mm in diameter, were observed in 8 of the 12 infected dogs. These lesions, which were of maximal intensity on the 5th and 7th days after infection, were distributed equally in all lobes of the lungs (Figs. 27, 28) and were present throughout the substance of the lung tissue. By the 10th day, only minute grey spots were observed in the lungs and in the dog killed 20 days after infection no gross abnormalities were found.

From the 3rd until the 10th day the tonsils of all infected dogs were enlarged and hyperaemic. The bronchomediastinal and retropharyngeal lymph nodes were enlarged and oedematous in those dogs which showed gross lung lesions on the 5th, 7th and 10th days after

aerosolisation. One dog (No. 71) killed on the 10th day after infection showed small grey foci in the cortices of both kidneys. Each grey area was surrounded by a narrow haemorrhagic ring.

Microscopic findings

The microscopic findings are summarised in Table 6. A focal necrotizing rhinitis was noted from the 3rd until the 10th day after infection. Foci of necrosis were evident in both the stratified squamous epithelium of the nasal vestibule and the pseudostratified ciliated columnar epithelium which lines the turbinates. The initial change consisted of swelling and vacuolation of the cytoplasm of the epithelial cells, inclusions being found in large numbers (Fig. 29). This was followed by necrosis with sloughing of the epithelial cells and with extension of the necrotic process into the lamina propria (Fig. 30). The lamina propria was heavily infiltrated by lymphocytes and macrophages, and large amounts of cellular debris containing polymorphonuclear leucocytes were present within the nasal cavity.

There was no evidence of necrosis or cellular infiltration in the trachea of any of the infected dogs.

The earliest histological change observed in the lungs at 3 days after infection was focal hypertrophy and desquamation of cells in the bronchiolar epithelium (Figs. 31, 32). Many of these cells had greatly enlarged pale nuclei and their chromatin was distinctly marginated (Fig. 31). The cytoplasm of the affected cells was clear and swollen and this contrasted with the "beading" of the chromatin along the inner surface of the nuclear membrane. These injured cells degenerated and sloughed from the basement membrane and the lesion progressed to form a focus of necrosis with subsequent involvement of the lamina propria and the adjacent alveoli (Fig. 33). Focal areas of alveolar necrosis were also scattered throughout the lung lobules

TABLE 6

Experimental Canine Herpesvirus Respiratory Infection

Pathological findings : aerosol infection

Dog Number	Day Examined	Pulmonary Foci	Bronchiolar Necrosis	Alveolar Necrosis	Nasal Necrosis
61	3	++	+	+	+
67	3	-	-	-	-
74	3	+	+	+	-
63	5	+++	++	++	++
68	5	-	-	-	-
75	5	+++	+++	++	++
69	7	-	-	-	-
76	7	+++	+++	++	+++
70	10	+	-	-	-
71	10	+	-	-	+
77	10	+	-	-	+
78	20	-	-	-	-
58*	3	-	-	-	-
59*	5	-	-	-	-
81*	10	-	-	-	-
82*	20	-	-	-	-

* Denotes unexposed control animal.

Lesions were judged + to +++ according to severity.

(Fig. 34) and, in these areas, the alveolar septa were thickened due to the infiltration of lymphocytes and polymorphonuclear leucocytes.

Small amounts of fibrin (Fig. 34) and many macrophages containing dense granular material were present in the air spaces (Fig. 35).

In only one dog (No. 76), was necrosis of the bronchial epithelium evident. A few eosinophilic intranuclear inclusion bodies were found in necrotic bronchiolar epithelial cells but they were not detected elsewhere in the lungs.

Bronchiolar and alveolar necrosis had disappeared by the 10th day and fibrin and cellular debris were removed by macrophages. Foci of septal thickening due to lymphocytes and macrophages persisted until 20 days after aerosolisation.

Lymphoid hyperplasia and proliferation and desquamation of sinusoidal macrophages were evident in the bronchomediastinal and retropharyngeal lymph nodes in all infected dogs from the 5th day onwards. Haemorrhage and erythrophagocytosis were present in the medullary sinuses of the bronchial nodes of the dogs with lung lesions killed on the 5th and 7th days. Inclusion bodies were not found in the lymph nodes.

Small foci of tonsillar epithelial necrosis were observed in 2 dogs (Nos. 61 and 74) killed on day 3 but inclusions were not found. From the 3rd day onwards, there was lymphoid hyperplasia of the tonsils in all the infected dogs.

In one pup (No. 71), focal necrosis of the tubular epithelium of the renal cortices was noted and around these necrotic tubules a few lymphocytes and macrophages had gathered. These lesions corresponded to the grey foci visible at necropsy. A focal interstitial nephritis, characterised by the accumulation of a similar cellular infiltrate,

was present in the renal cortices of 2 other dogs but, in these, tubular necrosis was absent (Fig. 36). Inclusion bodies could not be demonstrated in the kidneys of any puppy.

In three puppies (Nos. 68, 71 and 75), there were foci of hepatic cell necrosis and Kupffer cell proliferation. Foci of necrosis were also found in the adrenal glands of puppy No. 71. In neither of these lesions could inclusions be seen.

Electron microscopic findings

The lumina of affected bronchioles contained large numbers of sloughed degenerating epithelial cells, occasional erythrocytes and electron dense granular debris (Fig. 37). Typical herpesvirus particles were detected in bronchiolar epithelial cells and macrophages in dogs which had received aerosolized virus. The naked virions were dispersed in groups irregularly throughout the nuclei of infected cells and were round or hexagonal in shape (Figs. 38 and 40). Occasionally naked capsids were observed free amongst the granular debris in the bronchiolar lumen or enclosed in the cytoplasm of a macrophage. In one such group engulfed by a macrophage, it was possible, particularly in the empty particles, to discern the spoke-like projections of the capsomeres (Fig. 39). The variation in the appearance of the viral cores was the same as that seen in infected cell-cultures (SECTION I).

Some naked viral capsids were seen close to the inner nuclear membrane and enveloped particles were present in the perinuclear space. The 2 layers of the nuclear membrane were often widely separated and there was commonly reduplication of the inner lamellae of the nuclear membrane (Fig. 38). A few groups of enveloped particles were found in vesicles apparently within the nucleus but this may have been an artefact produced by the plane of section or due to invagination of the inner lamellae of the nuclear membrane.

In the cytoplasm, enveloped particles had a fuzzy, electron dense coat and were found in large, membrane-lined vesicles (Fig. 40). Elongated hyphae-like structures were often seen projecting into the vacuoles from the surrounding membrane (Fig. 41).

Cells which contained virus particles were readily recognisable because, in most cases, their nuclear chromatin occurred in discrete clumps along the inner nuclear membrane while the central region of the nucleus was clear (Figs. 37, 38 and 40). These cells corresponded to those with "beaded" nuclei seen with the light microscope. Inclusion body structures were not identified but, in many nuclei, fragments of nucleoli remained as bundles of dense fibrils (Fig. 40). Degenerative changes also occurred in the cytoplasm; the mitochondria were swollen and distorted and the endoplasmic reticulum was dilated.

Virological findings

The results of virus isolation are summarised in Table 7. CHV was isolated from the lungs of infected dogs up to 10 days after aerosolisation of virus and from the trachea and turbinates up to 7 days. The virus was also recovered from the tonsils of a dog killed on the 7th day.

Control animals

The control puppies showed no clinical, gross or histopathological changes and virus was not isolated from any of these animals.

TABLE 7

Experimental Canine Herpesvirus Respiratory Infection

Virological findings : the recovery of CHV after aerosol infection

Dog Number	Day Examined	Lung	Trachea	Turbinate	Tonsil
61	3	-	-	+	-
67	3	-	-	-	-
74	3	+	+	+	-
63	5	+	+	+	-
68	5	+	-	+	-
75	5	+	+	+	-
69	7	+	-	-	-
76	7	+	+	+	+
70	10	+	-	-	-
71	10	+	-	-	-
77	10	-	-	-	-
78	20	-	-	-	-
58*	3	-	-	-	-
59*	5	-	-	-	-
81*	10	-	-	-	-
82*	20	-	-	-	-

* Denotes unexposed control animal.

+ Denotes CHV recovered.

DISCUSSION

The role of CHV as a primary pathogen in canine respiratory.
disease has not previously been established. Karpas et al. (1968a,
1968b), who isolated CHV from dogs with kennel cough, claimed to have
reproduced the disease in dogs by the intranasal instillation of the
virus. Unfortunately, apart from a brief statement that eosinophilic
intranuclear inclusions were found in respiratory epithelial cells, these
American workers did not describe in any detail the pathological changes
in the respiratory tract of the infected dogs. In contrast, other
workers have failed to induce clinical disease in 6 to 12-week-old dogs
inoculated with CHV by the oral, nasal or intraperitoneal routes
(Appel et al., 1969; Wright and Cornwell, 1970). In these cases,
pathological findings were limited to occasional foci of lymphocytes
and macrophages in the lungs and kidneys.

In the present study, the administration of CHV by aerosol to
12-week-old puppies induced a bilateral watery nasal discharge as well
as a focal necrotizing rhinitis and bronchiolitis. Clinical signs of
respiratory disease were not detected when the virus was administered
by the intratracheal route but a necrotizing bronchitis and bronchiolitis
were found on histological examination.

The anomalies between the results obtained in different
laboratories may be related to the dose of virus, the route of infection
or the pathogenicity of the virus strain employed. In the present
investigation, the dose of virus aerosolized was smaller than that used
for intranasal challenge by Appel et al. (1969) and Wright and Cornwell
(1970) so that it would seem that the mechanism of infection not just the
dose of virus is important in determining the outcome. In a similar
fashion Purcell and McFerran (1969) showed that birds infected with the

avian herpesvirus, infectious laryngo-tracheitis, by the intranasal route failed to show clinical symptoms whereas birds infected by aerosol developed a severe form of the disease which was not unlike that seen in the field. In a kennel population, where outbreaks of respiratory disease also spread rapidly, it is probable that aerosolisation of virus is the natural means for the dissemination of infection.

The lesions produced in the lungs by aerosol infection with CHV were more widely distributed than those found with intratracheal challenge. Following aerosolisation, small red foci were scattered evenly throughout all the lobes of the lungs, a picture which contrasted with the larger, more localised areas of consolidation produced by intratracheal infection.

Microscopically, the pathological changes were of the same type in the 2 groups of dogs though they differed in degree and distribution. In the dogs inoculated by the intratracheal route, there was a necrotizing bronchitis and bronchiolitis whereas, in the dogs infected by aerosol, the virus had penetrated the nasal mucosa and the distal airways with consequent rhinitis, bronchiolitis and alveolitis. It is of note that no lesions were found in the trachea of any of the infected dogs although a necrotizing tracheitis was a feature in the natural case described in SECTION I.

Following aerosol administration of virus, an interstitial nephritis was noted in 3 dogs. The interstitial accumulations of lymphocytes and macrophages were similar to those seen in 6 to 12-week-old puppies infected by the intraperitoneal route (Wright and Cornwell, 1970). Foci of hepatic necrosis were also found in several dogs but both the renal and hepatic lesions were extremely mild when compared to those described in the neonate (Wright and Cornwell, 1968a). This confirms the

previous finding that the resistance of the dog to CHV increases with age (Carmichael et al., 1965a; Wright and Cornwell, 1968a).

In general, the presence of eosinophilic intranuclear inclusions is useful in the diagnosis of herpesvirus infections. In the present investigation, however, inclusions were few in number except in the nasal mucosa although, with the electron microscope, virus could be demonstrated in the lungs. It should be noted that with the exception of the nasal mucosa, inclusions are also difficult to find in the neonatal puppy (Wright and Cornwell, 1970). The reason why inclusions develop more readily in the nasal epithelium is unknown.

The morphology of the particles of CHV seen in bronchial epithelial cells was the same as that found in tissue culture. The pattern of virus development from the single membrane structure in the nucleus to the enveloped particle at the nuclear membrane and the mode of release from infected cells closely resembled that described by Johnson et al. (1969) for CHV in tissue culture and by Darlington and Moss (1968) for the herpesviruses of equine abortion, pseudorabies and herpes simplex. Unenveloped viral capsids were occasionally noted in the cytoplasm of macrophages but there was no evidence to suggest assembly of the nucleo-capsids in the cytoplasm. A few naked capsids were also noted free in the lumen of the bronchioles, presumably released from damaged cells.

An interesting feature in some infected cells was the hyphae-like structures which projected into the lumen of the cytoplasmic virus-containing vacuoles. Purcell (1971) described similar structures in tracheal epithelial cells infected with the avian herpesvirus, infectious laryngo-tracheitis.

CHV was reisolated from the lungs of infected dogs up to 5 days after intratracheal inoculation and up to 10 days after aerosolisation. It was also recovered from the nasal turbinates and tracheas of dogs exposed to aerosolized virus. This is in contrast to Karpas et al. (1968b)

who recovered CHV from throat and nasal swabs up to 18 days after
intranasal inoculation and Appel et al. (1969) who found that CHV could
be obtained from the nasal cavity and oral pharynx for a 2 week period
following challenge by the intranasal route but only occasionally
from the lungs at necropsy.

The clinical reaction of the dog to CHV infection was, in general,
not as severe as that seen in some other species experimentally infected
with herpesviruses. Experimental feline viral rhinotracheitis in cats is
characterised by a copious nasal exudate, coughing and sneezing (Crandell
et al., 1961; Hoover et al., 1970), and a similar pattern is seen with
Aujeszky's virus in piglets (Baskerville et al., 1971a).

The distribution of the lesions and the histopathological
changes in canine herpesvirus pneumonia were similar to those seen after
aerosol infection of piglets with Aujeszky's disease virus (Baskerville,
1971). Necrosis of the bronchiolar epithelium was a prominent feature
in both the pig and the dog although the degree of tissue destruction
seemed much greater in Aujeszky's disease.

The clinical and pathological changes following either
intratracheal or aerosol infection with CHV were in no way as severe
as the lesions seen in the natural case. However, there is little doubt
that CHV can cause necrotizing lesions in the respiratory tract of
young dogs, but possibly only in times of stress or in association with
other microbiological agents does the virus induce severe clinical
respiratory disease. At the present time, virological and serological
evidence suggests that the virus is less easily transmitted than is
distemper virus, canine adenoviruses or canine parainfluenza virus
(Binn et al., 1970). Therefore, although CHV is capable of initiating
respiratory disease, its overall contribution to the aetiology of this
complex condition may be a minor one.

CANINE HERPESVIRUS RESPIRATORY INFECTION

SECTION III : Intraperitoneal inoculation of the herpesvirus isolate

into newborn puppies.

SECTION III : Contents

INTRODUCTION

In SECTION I, a herpesvirus isolated from the lungs and trachea of an adult dog was shown to be related antigenically to a strain recovered from neonatal puppies (Cornwell et al., 1966). It was, therefore, deemed of interest to determine whether or not the respiratory isolate could produce a lethal generalised disease in neonatal puppies.

MATERIALS AND METHODS

The virus used in this section of the work was the same as that employed in SECTION II and had a titre of 10^5 PFU per ml.

A 3-year-old pregnant Collie bitch was purchased from a commercial source and kept in isolation. A blood sample was taken from the dog and, from the serum prepared, a plaque-inhibition test against CHV was found to be negative for anti-CHV antibodies.

Six strong, vigorous puppies were born and 4 received a dose of 0·5ml of virus by the intraperitoneal route approximately 8 hours after birth. This procedure has been found to induce 100 per cent mortality due to generalised herpesvirus infection (Wright, 1968). The remaining puppies were kept as controls and were given 0·5ml of uninfected dog kidney tissue culture suspension by the same route. The controls had of necessity to be kept in contact with the infected puppies, one being killed on the 3rd and the other on the 7th day after inoculation.

The puppies were observed twice daily for clinical abnormalities. At necropsy, representative portions of tissue were taken from the major organs and fixed in mercuric chloride-formol. Histological procedures were carried out as described in SECTION I.

All the experimental animals were subjected to detailed bacteriological examination and the liver, kidneys and lungs from each puppy were examined virologically as described in SECTION I.

RESULTS

Clinical findings

For the first 6 days, the puppies progressed normally. They suckled vigorously and appeared to put on weight rapidly. On the morning of the 7th day the situation had changed dramatically. Two of the infected pups died suddenly and the other 2 were in obvious distress, crying and gasping for breath. The controls appeared normal.

Macroscopic findings

The postmortem findings were virtually identical in all of the inoculated puppies. The mucous membranes were pale and a small amount of serosanguineous fluid was present in the abdominal and thoracic cavities. In most of the animals, the lymph nodes were enlarged, moist and hyperaemic and the spleen was greatly swollen and suffused with blood. Petechial haemorrhages were observed on the peritoneal surface of the intestines and in one puppy tiny haemorrhagic lesions were noted in the gastric mucosa. Hepatomegaly was present in all animals; the liver was enlarged and mottled in appearance with small yellow foci about 1mm in size (Fig. 42).

Petechial haemorrhages and congestion were the most common macroscopic lesions of the thymus. The heart appeared pale and, frequently, there were subendocardial and epicardial haemorrhages. The lungs were oedematous and frothy, blood stained fluid was present in the trachea and bronchi. Reddish grey areas of consolidation were present in all the lobes of the lungs (Fig. 43).

The most striking gross finding was, however, the presence of circular haemorrhagic areas in the kidneys (Fig. 43). The renal surface had a mottled appearance and, on cut section, the haemorrhagic lesions, which measured 1 to 5mm in diameter, were confined mainly to the cortex.

Microscopic findings

The microscopic findings were characterised by congestion, necrosis and haemorrhage. Focal areas of necrosis were scattered randomly throughout the hepatic parenchyma (Fig. 44). Some of the hepatic cells at the periphery of these necrotic areas contained single eosinophilic intranuclear inclusion bodies.

In the kidneys, necrosis was largely confined to the cortex and in these areas the tubular epithelial cells were completely necrotic and the glomeruli were swollen, structureless and eosinophilic (Fig. 45). Leakage of blood from damaged capillaries resulted in haemorrhage around the necrotic areas. A few eosinophilic intranuclear inclusions were seen in tubular epithelial cells but none were observed in the glomeruli.

Subserosal and submucosal haemorrhages were commonly seen in the gastrointestinal tract but, in the main, necrosis was limited to the intestinal crypts. Inclusions were observed in the glandular cells.

The most prominent pulmonary lesion was a focal necrotizing pneumonia (Fig. 46). The affected areas were characterised by necrosis of the alveolar walls, loss of structure and the exudation of fibrin into the alveolar air spaces. Eosinophilic inclusions were seen in the nuclei of some alveolar and interstitial cells. In general, the bronchi and bronchioli were unaffected but necrosis occasionally extended into the wall of a bronchiole from an alveolar focus (Fig. 46).

Focal areas of necrosis were also observed in the pancreas, adrenal glands (Fig. 47) and myocardium.

The spleen showed severe congestion, and necrosis of the reticular network was occasionally observed. In the lymph nodes, there was dissolution of the lymph follicles and haemorrhage and erythrophagocytosis in the medullary sinusoids.

The histopathological lesions in the central nervous system were those of a non-suppurative meningio-encephalitis. Focal areas of necrosis were observed, particularly in the grey matter of the cerebellum, and focal gliosis and perivascular cuffing were seen in the cerebellum, cerebrum, thalamus, pons and medulla.

Virological findings

CHV was isolated in MDCK cells from the prepared suspensions of the liver, kidneys and lungs of each infected puppy.

Bacteriological findings

Bacteriological examination of both infected and control dogs proved negative.

Control animals

Clinical, gross and histological abnormalities were not observed in either of the control puppies.

DISCUSSION

In the present study, a herpesvirus which was isolated from the respiratory tract of an adult dog produced a fatal septicaemic disease when inoculated intraperitoneally into newborn puppies. The clinical and pathological changes in the infected puppies were identical to those found by other workers in both natural and experimental CHV infections of the neonate (Carmichael et al., 1965a; Cornwell et al., 1966; Wright and Cornwell, 1968a; Kakuk and Conner, 1970). Other herpesviruses have been shown to behave in a similar fashion and fatal generalised disease often occurs in the very young of a species whereas, in the older animal, lesions are usually restricted to the oral mucosa, the upper respiratory tract or the external genitalia. Fatal infections of the very young occur

in newborn calves infected with infectious bovine rhinotracheitis virus
(Baker et al., 1960), kittens inoculated with feline viral rhinotracheitis
virus (Bittle and Peckham, 1971) and in Aujeszky's disease in piglets
(Gustafson, 1970). Moreover, a similar pattern is seen with herpes
simplex infection in man; there the primary disease in the adult is
mainly confined to the mucous membranes whilst in the newborn the
infection may be generalised, terminating fatally (Bird and Gardner, 1959;
Szögi and Berge, 1966; Haynes et al., 1968).

A great deal has still to be learnt about the epidemiology of
CHV. It is clear from the work of Stewart et al. (1965) that the virus
can cross the placenta and infection can occur in utero. Carmichael (1965b)
reported that the intravaginal inoculation of bitches one or two weeks
before whelping resulted in the deaths of all puppies within 2 weeks
of birth, infection presumably occurring during the passage through
the vaginal canal.

Wright and Cornwell (1968a) observed in-contact transmission
of infection from inoculated to un-inoculated littermates and, in the
latter, pathological changes were first observed in the nasal mucosa,
this suggesting that infection was probably by the intra nasal route.
SECTIONS I and II of this work show that CHV is associated with
respiratory disease in adult dogs and the spread of virus from the
nose and throat of adult dogs with respiratory disease is yet another
possible source of infection for the neonate.

PART 2

CANINE ADENOVIRUS RESPIRATORY INFECTION

SECTION I : Experimental canine adenovirus respiratory infection.

SECTION I : Contents

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INTRODUCTION

The clinical and pathological entity usually known as infectious canine hepatitis (ICH) or Rubarth's disease is now recognised to be but one form of canine adenovirus infection. This condition, in which hepatitis is accompanied by serious circulatory disturbances, has been extensively studied and comprehensive reviews of its epidemiology, pathology, virology and haematology have been published by Henderson (1959), Cabasso (1962) and Cabasso and Wilner (1969).

As often happens, the relevance of findings in a seemingly unrelated field may not at first be apparent and so with canine adenovirus infection the earliest reports were a study by Green and co-workers (1928) of epidemic encephalitis in silver foxes. Green et al.(1930) demonstrated that this disease was caused by a virus and Green and Shillinger (1934) showed that dogs could be infected experimentally with the virus of fox encephalitis. Intranuclear inclusions were found in the vascular endothelium of the experimentally-infected dogs and centrilobular necrosis of the liver was commonly present. Green et al. (1934) concluded that this disease in all probability occurred naturally in dogs.

In 1947 Rubarth published a comprehensive description of a naturally occurring acute disease of the dog. The main pathological features consisted of centrilobular necrosis of the liver with widespread petechial haemorrhages, serous or serosanguineous effusions into the body cavities and basophilic intranuclear inclusions in vascular endothelial and hepatic cells. Rubarth drew attention to the fact that the inclusions were identical in appearance and distribution to those described by Green and his colleagues in fox encephalitis and Siedentopf and Carlson (1949) later confirmed the close antigenic relationship between ICH virus (or canine adenovirus type 1 (CAV-1) as it is now known) and fox encephalitis virus.

Rubarth's disease has been recognised in many countries and can be successfully controlled by vaccination. In recent years, the causal agent has been incriminated in a number of other disease entities in dogs, including encephalopathy (Sályi and Kapp, 1958), iridiocyclitis (Carmichael, 1964), neonatal disease (Wright and Cornwell, 1968b), chronic hepatitis (Gocke et al., 1970), interstitial nephritis (Wright et al., 1971) and respiratory disease (Binn et al., 1967).

The increasing interest in the aetiology of respiratory disease in kennelled dogs has led to the discovery that several viruses other than that of distemper are associated with this condition (Binn et al., 1967; Binn, 1970). The work of Kelly (1968, 1969), who reported a case of canine pneumonia in which the histological findings were suggestive of an adenovirus infection, stimulated further interest in the pathogenicity of CAV-1 and it was not long before the virus was recovered from dogs suffering from respiratory disease (Wright et al., 1972; Studdert and Studdert, 1972).

It is not generally accepted that CAV-1 can spread by the respiratory route. Baker et al. (1951) claimed that, as long as direct contact did not occur, an infected dog could be kept as little as 6 inches apart from a susceptible one without spreading the infection. However, little experimental work has been done to assess the pathogenicity of CAV-1 for the respiratory tract. This is a surprising omission in view of the early work by Green and his co-workers on fox encephalitis. Green et al. (1936) found typical intranuclear inclusion bodies in the tonsil and mucous membranes of the nasopharynx of natural cases and demonstrated that silver foxes could be experimentally infected with fox encephalitis virus following intranasal inoculation. Furthermore, there was strong evidence from field studies that the disease was transmitted by airborne virus when the infection passed from a group of inoculated foxes to a

susceptible group over an open space of about 8 feet.

The aim of the present investigation was to study the disease produced by CAV-1 in dogs following a respiratory challenge.

MATERIALS AND METHODS

Virus

The virus strain used in this study was originally isolated, by means of dog kidney tissue culture, from the liver of a typical case of ICH in a spaniel (No. 47889) (Wright, 1966).

Experimental animals

Twenty nine Collie puppies, approximately 8 weeks of age and comprised of 5 complete litters, were employed. On arrival the puppies were bled by jugular venipuncture and their sera tested for antibody specific to CAV-1 virus by means of an indirect fluorescent antibody test as described in APPENDIX 2.

Experimental procedures

(a) Intratracheal inoculation of CAV-1

One complete litter comprising 7 puppies was used. The dogs were lightly sedated with diethylthiambutene hydrochloride and 5 were given 1ml of the virus (titre $10^6 TCID_{50}$ per ml) by means of an endotracheal tube. The 2 puppies remaining in the litter were each given 1ml of noninfected dog kidney tissue culture suspension by the same method. The dogs were observed daily for clinical manifestations of disease and rectal temperatures were taken each day until the end of the study.

(b) Aerosolisation of CAV-1

Twenty two puppies were used in the aerosol experiment. Sixteen were exposed to aerosolized virus for a period of 10 minutes by the

technique described in PART 1, SECTION II. In that time, each received approximately 0.3ml of virus (titre 10^7 TCID$_{50}$ per ml).

For purposes of control, 3 puppies were aerosolized in a similar manner with noninfected dog kidney tissue culture fluid and housed in a separate room from their infected litter mates. Three animals were kept as contact controls; they were housed in the same room as the infected puppies, with a common air space but in separate pens.

Rectal temperatures were taken daily for the first 10 days after aerosolisation and the dogs were checked clinically each day. The puppies were observed for evidence of spontaneous coughing. Blood samples were collected at the time of necropsy for the titration of antibody by the HA-I test.

Histological procedures

Pathological and histological procedures were carried out as described in PART 1, SECTION II.

For immunofluorescence, small blocks of tonsil and lung were excised and placed on the sides of Pyrex test tubes, rapidly frozen in a dry ice alcohol mixture at -70°C. and stored at -20°C. until examined by the direct method of the fluorescent antibody technique as detailed in APPENDIX 2.

Electron microscopic procedures

For electron microscopy, portions of pneumonic lung, approximately 1mm^3 in size, were taken from dogs killed on the 2nd, 5th and 7th days after aerosolisation. The tissues were placed in either cold 2 per cent glutaraldehyde buffered at pH 7.2 or in a paraformaldehyde/glutaraldehyde mixture (Karnovsky, 1965) prepared in cacodylate buffer, also at pH 7.2. In the case of the dogs killed 2 days after infection, no gross changes were apparent and a number of blocks of lung were therefore selected from

the left apical and dorsal parts of the left diaphragmatic lobe, regions
which consistently showed pneumonic lesions at a later stage of the
disease.

The tissues placed in 2 per cent glutaraldehyde were fixed for
$1\frac{1}{2}$ hours at 4°C., rinsed in Sorensen's phosphate buffer and post-fixed
for 1 hour in 1 per cent osmium tetroxide. The tissue taken in the
paraformaldehyde/glutaraldehyde mixture was fixed for $4\frac{1}{2}$ hours at 4°C.,
rinsed overnight in Michaelis buffer and then postfixed for 1 hour with
osmium tetroxide.

Following fixation, dehydration was through an ascending series
of alcohols and the tissues were embedded in either Araldite or Epon epoxy
resin as described previously (PART 1, SECTION II).

The preparation of the paraformaldehyde/glutaraldehyde mixture,
the cacodylate buffer and the Michaelis buffer is described in APPENDIX 1.

In order to locate specific lesions, thick sections (1 to 1•5μm)
were prepared with an LKB Mark III ultratome, mounted on glass slides and
stained with aqueous toluidene blue. Thin sections obtained in the same
way were mounted on copper grids, double stained with uranyl acetate and
lead citrate (Reynolds, 1963) and examined in an AEI 6B electron microscope.

Virological procedures

Ten per cent suspensions (w/v) of lung, trachea, tonsils and
turbinates from infected and control dogs were prepared in Earle's
balanced salt solution containing the usual antibiotics. The suspensions
were clarified by centrifugation and 1ml used to inoculate each of 2
bottles of a continuous dog kidney cell line (MDCK). The cultures were
examined daily for the typical adenovirus cytopathic effect.

Serological procedures

Haemagglutination-inhibition tests were based on procedures described by Espmark and Salenstedt (1961). The sera were heat inactivated at 56°C. for 30 minutes, absorbed at room temperature with an equal volume of 25 per cent kaolin (Flow Labs. Ltd., Ayrshire) and incubated at 37°C. for 1 hour with an equal volume of a 10 per cent suspension of human type O erythrocytes prior to use. Serial 2-fold dilutions of the sera were prepared in perspex haemagglutination plates and 4 haemagglutinating units of virus added to each well. The virus/serum mixtures were held at room temperature for 1 hour before the addition of a 1 per cent suspension of human type O erythrocytes. Tests were read after 2 hours. The highest dilution of each antiserum that completely inhibited haemagglutination was recorded as the titre of that antiserum.

RESULTS

(a) Intratracheal inoculation of CAV-1

Clinical findings

On the day after inoculation, all the infected dogs were dull and remained so for the duration of the experiment. One dog was found dead on the 2nd day and 3 others were killed when _in extremis_ on days 4, 7 and 12. By the 3rd day, the surviving puppies were tachypnoeic and hyperpnoeic but coughing was not heard.

Macroscopic findings

The macroscopic findings are summarised in Table 8. All 5 inoculated puppies developed an acute necrotizing bronchopneumonia. At postmortem examination, affected animals showed plum-red areas of consolidation in all lobes of the lungs but especially in the diaphragmatic lobes (Figs. 48, 49). These regions were well demarcated

TABLE 8

Experimental Canine Adenovirus Respiratory Infection

Pathological and immunofluorescence findings : intratracheal infection

Dog Number	Day Examined	Pneumonia at Necropsy	Bronchiolar Necrosis	Bronchiolar Hyperplasia	Inclusions			Immunofluorescence	
					Lung	Tonsil	Turbinate	Lung	Tonsil
27	2	+++	+++	-	++	-	-	+++	NT
24	4	++	++	-	+++	-	-	+++	-
26	7	++	++	+	++	-	-	+++	-
23	9	+	+	+	-	-	-	+	-
25	12	+++	++	+	-	-	-	+	-
21*	4	-	-	-	-	-	-	-	-
22*	7	-	-	-	-	-	-	-	-

* Denotes unexposed control animal.

Lesions were judged + to +++ according to severity.

NT Denotes not tested.

from the unaffected tissue and were very oedematous. The bronchi and
trachea were filled with blood-tinged, frothy fluid and the
bronchomediastinal lymph nodes were distinctly hyperaemic and oedematous
from the 4th until the 12th day after infection.

Microscopic findings

The microscopic findings are summarised in Table 8. Histologically
the main lesions were to be found in the bronchi and bronchioles. On the
2nd and 4th days after infection there was severe epithelial necrosis;
the bronchi and many of the bronchioles were completely filled with
necrotic epithelial debris and polymorphonuclear leucocytes (Fig. 50).
In many instances, intranuclear inclusion bodies were found in epithelial
cells (Fig. 51) and in alveolar macrophages. Surrounding alveoli were
oedematous and often contained fibrin and there was a mixed cell infiltrate
of polymorphonuclear leucocytes and macrophages (Fig. 52). The trachea
and large bronchi were histologically normal. By the 7th, 9th and 12th
days, epithelial necrosis was less marked and was replaced to a large
extent by epithelial hyperplasia resulting in a haphazard, irregular
appearance of the epithelial lining (Fig. 53). Multinucleate giant cells
were observed within alveoli and bronchiolar lumina (Fig. 54).

Lymphocytolysis was found in the bronchial lymph node on days 2,
4 and 7 and there was also marked sinusoidal haemorrhage and oedema.
Lymphoid hyperplasia was noted on days 9 and 12.

The liver of the dog which died on the 2nd day showed engorgement
of the portal veins and of the hepatic sinusoids but neither necrosis nor
inclusion bodies were seen. Histological changes were not observed in
the liver of any of the other puppies apart from the presence of a few
focal aggregations of mononuclear cells in one of the dogs.

Virus antigen was abundant in epithelial cells and in alveolar

TABLE 9

Experimental Canine Adenovirus Respiratory Infection

Virological findings : the recovery of CAV-1 after intratracheal infection.

Dog Number	Day Examined	Lung	Trachea	Turbinate	Tonsil
27	2	+	-	-	-
24	4	+	+	-	+
26	7	+	-	-	+
23	9	+	NT	-	-
25	12	-	-	-	-
21*	4	-	-	-	-
22*	7	-	-	-	-

* Denotes unexposed control animal.

+ Denotes CAV-1 recovered.

NT Denotes not tested.

macrophages on the 2nd, 4th and 7th days (Fig. 55) but by the 9th and 12th days only a few isolated fluorescing cells were seen (Fig. 56).

Virological findings

The results of virus isolation are summarised in Table 9. Virus was recovered from the lungs of all infected dogs up to 9 days after infection, from the trachea of one dog killed on the 4th day and from the tonsils of dogs killed on the 4th and 7th days. Virus was not recovered from the turbinates of any of the dogs.

Control animals

Clinical signs of disease were not detected in any of these animals and at necropsy no abnormalities were noted. Virus was not recovered from the lungs or tonsils and viral antigen was not demonstrated by means of the fluorescent antibody test.

(b) Aerosolisation of CAV-1

Clinical findings

The clinical findings are summarised in Table 10. By 3 days after exposure, all infected dogs were dull, partially or completely anorexic, snuffling and showing a watery bilateral nasal discharge. These signs had disappeared in most of the dogs by the 7th day after infection. Twelve dogs, however, also developed spontaneous coughing and tachypnoea by the 3rd day; these symptoms had disappeared by the 7th day but, in 3 animals, coughing could still be induced by pinching the trachea up to the 15th day after aerosolisation. One puppy developed a unilateral corneal opacity which appeared 10 days after infection and which was still present at 12 days when the animal was killed.

The 3 contact puppies developed a spontaneous cough between 5 and 7 days after aerosolisation of the other animals, but otherwise remained

TABLE 10

Experimental Canine Adenovirus Respiratory Infection

Days on which clinical signs were evident after aerosol infection.

Dog Number	Day Killed	Pyrexia	Apathy	Anorexia	Nasal Discharge	Coughing Spontaneous	Induced
98	2	-	-	-	-	-	-
106	2	-	-	-	-	-	-
110	4	3-4	3-4	3-4	3-4	3-4	3-4
99	5	3-4	3-5	3-5	3-5	-	4
107	5	3-4	3-5	3-5	3-5	3-5	3-5
114	5	3-5	3-5	3-5	3-5	3-5	3-5
105	6	3	3-6	3-6	3-6	3-6	3-6
100	7	3-4	3-7	3-5	3-6	3-7	3-7
111	7	3-4	3-5	3-5	3-5	3-7	3-7
109	8	3	3-5	3-5	3-8	3-7	3-8
102	12	3-4	3-5	3-5	3-5	-	-

TABLE 10

Cont.

Dog Number	Day Killed	Pyrexia	Apathy	Anorexia	Nasal Discharge	Coughing Spontaneous	Induced
108✓	12	3-4	3-6	3-5	3-7	3-7	3-8
113	14	3-5	3-4	3-5	3-5	3-7	3-9
104	15	3-4	3-5	3-5	3-6	3-7	3-15
115	20	3-4	3-5	3-5	3-8	3-7	3-15
112	30	3-5	3-5	3-5	3-6	3-7	3-15
117*	6	-	-	-	-	5	5-6
103*	12	-	-	-	-	5-7	5-7
116*	14	-	-	-	-	5-7	5-7
97**	2	-	-	-	-	-	-
119**	8	-	-	-	-	-	-
118**	30	-	-	-	-	-	-

* Denotes contact control animal

** Denotes unexposed control animal

+ Denotes rectal temperature 103.5F. or greater

√ Denotes corneal opacity

healthy. Coughing could not be induced after 7 days. The unexposed
control animals remained clinically normal.

Macroscopic findings

The pathological findings are summarised in Table 11. At necropsy,
infected animals showed pulmonary lesions from the 4th day onwards, these
consisting of firm, diffuse, plum-red or greyish areas of consolidation
(Figs. 57, 58). Although lesions were observed in all lobes, the dorsal
regions of the diaphragmatic lobes were especially affected. From 14 days
after infection onwards, the lung lesions were more discrete and
consisted of multiple small red foci, many of which had pale centres
(Fig. 59).

The bronchial and retropharyngeal nodes were enlarged, oedematous
and hyperaemic from the 4th until the 8th day after infection (Fig. 60).
Subsequently the nodes, although still enlarged, became pale, firm and
hyperplastic and remained so throughout the remainder of the experiment
(Fig. 61). Distinct tonsillar enlargement was prominent from the 4th until
the 15th day after infection. Similar pulmonary lesions were observed in
the contact control animals but the tonsils and lymph nodes were unchanged.

Microscopic findings

On histological examination, the earliest pulmonary lesion
observed was swelling and vacuolation of the bronchiolar epithelium, the
change being evident as early as 2 days after infection (Figs. 62, 64).
A small number of polymorphonuclear leucocytes were present in the
bronchiolar lumina and were infiltrating the epithelium at this time.
These changes were followed on subsequent days by progressive bronchiolar
epithelial necrosis (Fig. 63), occlusion of many of the bronchioles with
necrotic epithelial debris (Fig. 65) and subsequent alveolar collapse;

TABLE 11

Experimental Canine Adenovirus Respiratory Infection

Pathological and immunofluorescence findings : aerosol infection

Dog Number	Day Examined	Pneumonia at Necropsy	Bronchiolar Necrosis	Bronchiolar Hyperplasia	Turbinate Necrosis	Inclusions			Immunofluorescence	
						Lung	Tonsil	Turbinate	Lung	Tonsil
98	2	-	-	-	-	+	-	-	NT	NT
106	2	-	-	-	-	+	-	-	+	-
110	4	++	+	-	-	+	+	-	+	+
99	5	++	++	-	+	++	-	+	++	++
107	5	+	++	-	+	+	+	-	+	+
114	5	++	++	-	+	++	-	+	+++	+
105	6	++	+++	-	+	++	+	-	++	++
100	7	+++	++	-	+	++	-	+	-	-
111	7	++	+	-	-	+	-	-	+	-
109	8	+	+	+	+	+	-	-	-	-
102	12	+	-	+	-	-	-	-	-	-
108	12	+	-	+	-	-	-	-	-	-

Continued overleaf.

Cont.

TABLE 11

Dog Number	Day Examined	Pneumonia at Necropsy	Bronchiolar Necrosis	Bronchiolar Hyperplasia	Turbinate Necrosis	Inclusions Lung	Inclusions Tonsil	Inclusions Turbinate	Immunofluorescence Lung	Immunofluorescence Tonsil
113	14	+	-	+	-	-	-	-	-	-
104	15	+	-	+	-	-	-	-	-	-
115	20	+	-	+	-	-	-	-	-	-
112	30	-	-	+	-	-	-	-	-	-
117*	6	+	+	-	-	+	-	-	+	-
103*	12	+	-	+	-	-	-	-	NT	NT
116*	14	+	+	+	-	-	-	-	-	-
97**	2	-	-	-	-	-	-	-	-	-
119**	8	-	-	-	-	-	-	-	-	+
118**	30	-	-	-	-	-	-	-	-	-

+ Lesions were judged + to +++ according to severity.   NT Denotes not tested.

* Denotes contact control animal.

** Denotes unexposed control animal.

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the bronchi were unaffected. The respiratory bronchioles and the alveolar air spaces contained many macrophages, red cells and, in some cases, polymorphonuclear leucocytes (Fig. 66). Intranuclear inclusion bodies were abundant in bronchiolar epithelial cells and in macrophages up to the 8th day (Figs. 63, 64, 67). By this time necrotizing bronchiolitis had become less marked and bronchiolar epithelial hyperplasia had developed with consequent projection of nodules of proliferating epithelial cells into the lumina (Fig. 68). In addition, the bronchiolar exudate had become partially collagenised and many spindle-shaped fibroblasts were present (Figs. 68, 69). Focal necrosis of the turbinates and, to a lesser extent, the nasal epithelium was evident by the 5th day and persisted until the 8th day after infection. In these animals, the underlying lamina propria was heavily infiltrated by lymphocytes, plasma cells and, in some regions, polymorphonuclear leucocytes. Necrotic debris containing some polymorphonuclear leucocytes was present in the nasal cavity. Inclusions were present in the nasal and turbinate epithelium up to the 7th day after aerosolisation with virus (Fig. 70). They stained bright golden yellow with fluorescent Feulgen indicating the presence of DNA (Fig. 71).

During the same period the tonsillar epithelium showed focal areas of necrosis with inclusions. Lymphocytolysis was also observed but inclusions were few in number in the lymphoid area of the tonsil. From 7 days onwards the tonsil showed lymphoid hyperplasia. Lytic changes were also observed in the follicles of the bronchomediastinal and retropharyngeal lymph nodes where sinusoidal haemorrhage and erythrophagocytosis were marked; inclusions were not found. In the later stages of the disease there was marked lymphoid hyperplasia and plasma cells were found in some quantity in the sinusoids. Lesions were not found in the trachea with the exception of discrete foci of lymphocytic infiltration in the lamina propria in a few cases.

In 2 animals killed at 8 and 12 days after infection, a very small number of inclusions were seen in the hepatic parenchyma but hepatic necrosis was not observed in any animal. Foci of Kupffer cell proliferation were present in most animals after the 8th day. Four puppies killed on the 5th, 7th, 8th and 20th days showed histological evidence of encephalitis in the form of foci of microglial proliferation and distinct perivascular infiltration by mononuclear cells. The lesions were most evident in the thalamus and cerebral cortex but inclusions were not seen.

Histological evidence of infection was also found in the 3 contact control animals. In the puppy killed 6 days after contact with infected litter mates, a few bronchioles showed epithelial necrosis with inclusions. The remaining 2 puppies killed on the 12th and 14th days after contact exposure showed bronchiolar hyperplasia with focal accumulations of macrophages in alveolar air spaces. Lesions were not detected in any other tissue, however. The unexposed control animals showed no histological changes.

Specific CAV-1 antigen was first detected in the lungs on the 2nd day (Fig. 72), increased in amount until the 5th day (Fig. 73) and disappeared by the 8th day after infection. Fluorescent cells first appeared in the bronchiolar epithelium and in the peribronchiolar connective tissue. Subsequently, antigen was observed in alveolar and septal macrophages (Fig. 74). Antigen was not seen in the tonsils until the 4th day after infection when discrete foci of infected cells were found in the epithelium (Fig. 75) and to a lesser extent in the lymphoid tissue; specific fluorescence could not be detected after the 6th day.

A few fluorescing cells were found in the bronchiolar epithelium of one contact control puppy killed on the 6th day after infection but not in the tonsil. Antigen was absent from one of the remaining 2 puppies (the other one was not tested) and also from the unexposed controls.

Electron microscopic findings

On the 2nd day after infection, virus particles were seen in ciliated and nonciliated cells of the bronchiolar epithelium (Fig. 76). The particles were either round or hexagonal and most of them were composed of a dense inner core and a less dense capsid surrounding it (Fig. 77). The diameter of the complete virion ranged from 65 to 70nm.

In infected cells the virus particles were dispersed throughout the nuclei and occurred singly or in small groups (Fig. 76). They were embedded in fine electron dense granular material and the chromatin of the affected nuclei was clumped in an irregular fashion just within the inner nuclear membrane. The combination of virus particles and granular matrix in the central zone of the nucleus appeared to correspond to the intranuclear inclusion which was visible by light microscopy.

Earlier stages of infection were detected by the appearance of osmiophilic granules within the nucleus (Fig. 78). At first, recognisable viral particles were not visible but they developed within or contiguous to condensations of the granular substance. At the same time, the rest of the chromatin was dispersed to the periphery of the nucleus and nucleoli were fragmented and displaced to one side by the developing inclusion.

Degenerative changes were found in the cytoplasm of virus infected cells and in adjacent cells. These consisted of swelling and reduction in the number of filiform projections at the cell surface. The junctions between epithelial cells were disrupted and some cells were detached from the basement membrane. Portions of apical cytoplasm were present in the bronchiolar lumina and lipid granules were noted in the cytoplasm, particularly near the Golgi region. Polymorphonuclear leucocytes were found infiltrating between bronchiolar epithelial cells and were also noted in the lamina propria. At this time, all the cells of the alveolar

epithelium and capillary endothelium were intact and normal.

On the 5th and 7th days after aerosolisation, the bronchiolar epithelium had detached and the lumina of most airways were filled with necrotic epithelial cells (Fig. 79), cellular debris, large numbers of macrophages and occasional lymphocytes, polymorphonuclear leucocytes and erythrocytes. Adenovirus particles were found in epithelial cells and macrophages and many of these cells appeared to be in a late stage of infection. The nuclear chromatin was now clumped in an even dense layer around the inner nuclear membrane while the rest of the nucleoplasm was less dense than normal (Fig. 80). Fine aggregates of electron dense granules were still present in the centre of the nucleus but only a few virus particles were found in contact with this material (Fig. 79). The virus particles were commonly concentrated around the periphery of the nucleus (Fig. 81) and virions were seen escaping into the cytoplasm through breaks in the nuclear membrane (Fig. 81). Virus particles were found free in the bronchiolar lumina and enclosed in cytoplasmic vacuoles within macrophages. Intranuclear paracrystalline formations were occasionally associated with the virus particles (Figs. 77, 80, 82). These crystals varied in size and differed in appearance depending upon the plane of section. In transverse section, hexagonal packing (Fig. 77) of the subunits was apparent whereas, in longitudinal section, a linear pattern was encountered (Figs. 80, 82). The parallel bundles measured approximately 10nm in diameter with a spacing of 70nm.

Degenerative changes in the cytoplasm of virus containing cells in the lumina were characterised by an increase in the number of lipid granules, dilation of the endoplasmic reticulum and breakdown of cell organelles and membranes. In many cells the plasma membrane was ruptured and the nucleus was contained by only a few remnants of the cytoplasm.

Polymorphonuclear leucocytes and macrophages were present in the alveolar air spaces on the 5th and 7th days (Figs. 83, 84). The

former were oval or elongated in outline and their cytoplasm was packed

with lysosomes which assumed a variety of shapes from round to elongated.

The macrophages were much larger with abundant cytoplasm and an

irregular outline (Fig. 84). Numerous pseudopodia extended from the

cell surface and lysosomes and vacuoles of various sizes were frequently

present in the cytoplasm. The macrophages were commonly seen engulfing

particulate debris and fibrin (Fig. 81) and occasionally entire

erythrocytes could be identified within cytoplasmic vacuoles (Fig. 85).

Virus particles were noted in the nuclei of some of the alveolar

macrophages (Fig. 86) but were not detected in alveolar epithelial

cells. However, many type 2 cells showed extensive cytoplasmic vacuolation

and nuclear pyknosis (Fig. 87) and sometimes whole desquamated cells were

noted in the alveolar air spaces. The thin peripheral cytoplasm of the

type 1 cells was often frayed and in some cases fragments of epithelial

cytoplasm had detached from the basement membrane. In the interalveolar

septa there was an increase in mononuclear cells and the capillary

endothelial cells were swollen with pinocytic vesicles increased in size

and number; polymorphonuclear leucocytes were commonly lodged within

the lumen of alveolar capillaries.

Virological findings

The results of virus isolation are summarised in Table 12. At

necropsy, virus was recovered from the lungs, tonsils and turbinates of

infected dogs up to 12 days after aerosolisation. It was also isolated

from the trachea of 1 and the nasal turbinates of another of the contact

control animals. Virological examination of the tissues of the unexposed

control dogs proved negative.

TABLE 12

Experimental Canine Adenovirus Respiratory Infection

Virological findings: the recovery of CAV-1 after aerosol infection

Dog Number	Day Examined	Lung	Trachea	Turbinate	Tonsil
98	2	+	+	+	+
106	2	+	+	+·	+
110	4	-	-	-	-
99	5	+	+	+	+
107	5	+	+	+	+
114	5	+	+	+	+
105	6	+	+	+	+
100	7	+	NT	+	+
111	7	-	-	-	-
109	8	-	-	-	-
102	12	+	NT	+	+
108	12	-	-	-	-
113	14	-	-	-	-
104	15	-	-	-	-
115	20	-	-	-	-
112	30	-	-	-	-
117*	6	-	+	-	-
103*	12	-	NT	+	-
116*	14	-	-	-	-
97**	2	-	-	-	-
119**	8	-	-	-	-
118**	30	-	-	-	-

+ Denotes CAV-1 recovered.   NT Denotes not tested.

* Denotes contact control animal.

** Denotes unexposed control animal.

TABLE 13

Experimental Canine Adenovirus Respiratory Infection

Serological findings: aerosol infection.

Dog Number	HA-I Titre -- Days after aerosolisation											
	0	2	4	5	6	7	8	12	14	15	20	30
98	4	4										
106	4	NT										
110	8		8									
99	4			4								
107	4			NT								
114	4			32								
105	4				128							
100	4					4						
111	8					8						
109	4						16					
102	4							16				
108	4							128				
113	4								256			
104	4									32		
115	4										64	
112	4											256
117*	4				4							
103*	4							128				
116*									16			
97**	4	4										
119**	4						8					
118**	4											4

* Denotes contact control animal.   NT Denotes not tested.

** Denotes unexposed control animal.

Serological findings

The serological findings are summarised in Table 13. Serum samples collected before and during the first 4 days after administration of virus had HA-I titres of up to 1:8. Significant rises in antibody levels were first detected on the 5th day but considerable variation was found in the rate at which individual dogs produced antibody, a 32 fold rise being noted as early as the 6th day and 4 fold increases as late as the 12th day. Significant rises were also found in the sera of the contact controls killed 12 and 14 days after infection, but not in those of the unexposed controls.

Bacteriological examinations of the lungs of the puppies did not reveal any pathogenic bacteria.

DISCUSSION

The present study shows that CAV-1 is capable of producing pneumonia in susceptible puppies provided that the virus is introduced directly into the lungs by means of an endotracheal tube or is inhaled in an aerosol spray. The same strain of virus when administered by the intraperitoneal route produced hepatitis (Wright, 1967) while inoculation of virus into the pharynx resulted in only a mild infection without severe hepatitis or evidence of pneumonia (Wright, 1966). It seems, therefore, that the pathogenic effect of CAV-1 may depend on its route of administration and that pneumonia is induced only if the virus can reach the lungs directly.

Puppies exposed to an aerosolized spray of CAV-1 developed a respiratory infection characterised by nasal discharge, tachypnoea and spontaneous coughing. However, when the virus was administered by endotracheal instillation a more severe, often fatal, respiratory

disease ensued. Of interest was the fact that in both experiments none of the infected puppies developed classical hepatitis although a few inclusions were found in the hepatic parenchyma of 2 animals.

The essential pulmonary lesion in the CAV-1 respiratory infection was a necrotizing bronchitis and bronchiolitis. Necrosis of the small bronchi and bronchioles was followed by occlusion of their lumina with necrotic debris, macrophages and polymorphonuclear leucocytes. Inclusions and viral antigen were present in abundance in the early stage of the disease and virus was readily isolated from the lungs up to 9 days after intratracheal infection and up to the 12th day after aerosolisation. In time, the necrotizing lesions were replaced by hyperplasia of the bronchiolar epithelium and organisation of the bronchiolar exudate with collagen, an end result which is commonly found in a number of respiratory virus infections in man (Bowden and Wyatt, 1970). Foci of necrosis were also to be seen in the nasal turbinates and tonsillar epithelium of the dogs infected by aerosol.

The histological changes in the lungs of the experimentally infected dogs were quite distinct from those found in distemper virus pneumonia and epithelialisation, which is such a feature of the latter infection (Gillespie, 1962), was not observed in any of the experimental puppies. The necrotic changes in the bronchiolar epithelium, however, resembled those seen in canine herpesvirus respiratory infection (PART 1, SECTION II) but, were more severe; bronchiolar proliferative changes were more marked in the adenovirus infection.

In the present study, ultrastructural observations were limited to the acute phase of the disease and the stages of healing and repair were not examined. Adenovirus particles were found in the bronchiolar epithelium at 2 days after infection. The subsequent loss of cilia and the reduction in filiform projections on the cell surface of ciliated

epithelial cells has also been described in herpesvirus pneumonia of the pig (Baskerville, 1972). By the 5th day, infected epithelial cells had detached from the basement membrane and the bronchiolar lumina were filled with granular debris, neutrophils and dead or dying cells. Macrophages appeared in increasing numbers and fibrin and cellular debris were actively removed. Virus particles were not observed in alveolar epithelial cells although nonspecific degenerative changes were noted. The morphology of the CAV-1 particles and the nuclear changes in the infected cells were similar to those described previously in tissue culture (Tajima et al., 1961; Moulton and Zee, 1969). Virus particles were not always confined to the nuclei of infected cells since rupture of the nuclear membrane had allowed their release into the cytoplasm. Morgan et al. (1956) and Lagermalm et al. (1957) observed similar swelling and focal rupture of the nuclear membrane in association with adenovirus release and Moulton et al. (1966) suggested that this was due to pressure exerted on the nuclear envelope by overproduction of DNA. The evidence for this postulate however is indirect, being based on a lack of change in nuclear size in infected cultures treated with a DNA inhibitor. Pereira and Kelly (1957) were of the opinion that toxic degeneration played a major part in the rupture of the nuclear membrane.

The paracrystalline structures observed in the nuclei in association with CAV-1 particles have been described by others both in adenovirus infections in vivo (Givan and Jézéquel, 1969) and in vitro (Morgan et al., 1957; Givan et al., 1967; Matsui and Bernhard, 1967). By using histochemical staining (Morgan et al., 1957; Godman et al., 1960) and enzyme digestion techniques (Givan et al., 1967), it has been established that the paracrystals are protein in nature and contain no detectable nucleic acid. Attempts to identify the origin of the protein have produced contradictory findings. Boulanger et al. (1970) and

Wills et al. (1973) have proposed that the paracrystals represent accumulations of excess viral capsid components whereas Marusyk et al. (1972) found no evidence of capsid components but observed by immunofluorescence specific staining of the crystals by antisera directed against adenovirus core proteins.

CAV-1 was recovered from the lungs, trachea, turbinates and tonsils of the dogs exposed to the aerosolized virus but only from the lungs and tonsils of the dogs infected by the intratracheal route. Titration of the amounts of virus in each of these tissues was not attempted but immunofluorescence showed a peak of virus activity in the lung on the 5th day. Viral antigen then quickly diminished and could not be demonstrated after the 12th day. The elimination of virus from the respiratory tract coincided with the appearance of HA-I antibodies in the blood, significant rises being detected in all dogs after the 12th day.

Contact control dogs kept in separate pens but with a communicating air space to the aerosolized puppies also developed respiratory disease, presumably as a result of airborne transmission from their coughing, artifically-infected litter mates. This is an interesting finding since it reveals a means of transmission of CAV-1 which had previously been discounted for the dog. Earlier workers believed that canine adenovirus infection was only transmitted by direct contact with either an infected animal or contaminated fomites (Baker et al., 1951; Cabasso and Wilner, 1969). It should however be noted that over 30 years ago Green et al. (1936) emphasised the importance of the airborne route of infection in the natural transmission of fox encephalitis.

The role of CAV-1 in naturally occurring canine respiratory disease has still not been established. The virus has been isolated from the respiratory tract of dogs with respiratory infections (Binn et al., 1967;

Studdert and Studdert, 1972) but information has not been available on the pathological findings, if any, in these cases. However, Kelly (1969) has described a natural case of canine pneumonia possibly of adenovirus origin in which the histological changes were very similar to those described above. The fact that CAV-1 can induce pneumonia in experimentally aerosolized puppies suggests that respiratory infections may occur in kennel populations possibly as a result of aerosolisation of infected secretions.

Experimental canine adenovirus pneumonia has many features in common with adenovirus infection in other species but there are some interesting differences. Colostrum deprived calves inoculated intranasally and intratracheally with bovine adenovirus types 1, 2 and 3 (Darbyshire et al., 1966; Darbyshire et al., 1969) developed a relatively mild clinical disease characterised by nasal and ocular discharge and mild diarrhoea. As in the present study, the significant histological findings in the bovine pneumonia were a necrotizing and proliferative bronchitis and bronchiolitis with intranuclear inclusions but, in the calf, epithelial proliferation was the initial change which was followed by necrosis and sloughing, the opposite of the sequence of events in the dog.

In experimental porcine adenovirus pneumonia, clinical signs have been milder than those seen in the dog and lung lesions have consisted mainly of thickening of the alveolar septa with mononuclear cells; bronchiolar epithelial necrosis and proliferation was minimal (Shadduck et al., 1967).

Adenoviruses have been identified as a cause of febrile respiratory and pharyngeal infections in man (Knight and Kasel, 1973). Although they account for only a minority of respiratory illnesses in the general population, epidemics of infection may occur, especially in institutions such as hospitals and orphanages (Van der Veen, 1963). The role of

adenoviruses, particularly types 4 and 7 and less commonly types 3, 14 and 21 in acute respiratory disease of young men recruited for military training has been amply demonstrated. Up to 90 per cent of recruits may exhibit respiratory symptoms by the 2nd week of training (McNamara et al., 1962) and not infrequently 20 per cent of a recruit company may require medical treatment for respiratory disease at some time during training (Rosenbaum et al., 1965). Kennel cough among dogs would seem to be analogous to adenovirus respiratory disease in military personnel in that both conditions follow the gathering together of susceptible individuals in a closed environment, a situation that probably allows the development of high concentrations of viruses.

Only fatal cases of adenovirus pneumonia have been described in man (Becroft, 1967; Dudding et al., 1972) and the opportunity to examine the pathogenesis of human adenovirus infection has therefore been limited to observations on the growth of the virus in organ culture of human foetal and adult trachea (Craighead, 1970) or to the study of cells obtained by tracheal aspiration during acute respiratory tract infections (Naib et al., 1968). The histological features of the experimental canine respiratory disease, namely necrosis, hyperplasia and bronchiolitis obliterans, are similar to changes described in the few recorded cases of fatal adenovirus pneumonia (Chany et al., 1958; Becroft, 1967). This close similarity indicates that experimental CAV-1 respiratory infection could be a suitable model for the study of human adenovirus infections.

CANINE ADENOVIRUS RESPIRATORY INFECTION

SECTION II : The isolation of adenoviruses from natural cases of

canine respiratory disease.

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    (a) Intravenous inoculation of the adenovirus isolates

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DISCUSSION

INTRODUCTION

Until recently, CAV-1 was the only known canine adenovirus and strains of the virus from different parts of the world were found to be of one immunological type (Cabasso and Wilner, 1969). In 1962, Ditchfield and co-workers reported the isolation of a new canine adenovirus, designated Toronto $A_{26/61}$ (or canine adenovirus type 2 (CAV-2) as it is now called) from the respiratory secretions of dogs with kennel cough. Serological tests indicated that the new isolate was antigenically related to CAV-1 but had significant antigenic differences. It was suggested that the new isolate was either a new canine adenovirus or possibly a variant of CAV-1. Since Ditchfield et al. (1962) published their findings, other American workers have also found CAV-2 in association with respiratory disease of dogs (Binn et al., 1967; Appel and Percy, 1970) and the virus has recently been isolated in the United Kingdom (Danskin, 1973).

The biophysical properties of CAV-2 have been extensively studied. Yamamoto (1966) first reported on the growth and physical properties of the virus and showed it to have a typical adenovirus morphology. Detailed comparative studies between CAV-1 and CAV-2 have revealed significant structural differences between the two viruses. The fibre projections on CAV-2 are approximately 10nm longer than those on CAV-1 (Marusyk et al., 1970) and the CAV-1 capsid components carry a smaller net negative charge than the corresponding CAV-2 capsid (Marusyk et al., 1970). Both CAV-1 and CAV-2 agglutinate human type O erythrocytes but the two viruses have different receptor sites on the red cells and the CAV-2 receptor complex is sensitive to the proteolytic action of trypsin whereas the CAV-1 receptor complex is not (Marusyk and Yamamoto, 1971).

The immunological relationship of CAV-1 to CAV-2 has been thoroughly investigated. The charcoal agglutination, serum neutralisation, complement fixation and HA-I tests have all been used to differentiate CAV-1 from CAV-2 (Ditchfield et al., 1962; Fairchild and Cohen, 1969; Swango et al., 1969). Cross serum neutralisation and HA-I tests with hexon and fibre specific antisera have shown that the immunological relationship between the two viruses is predominantly one sided; CAV-1 carries both homotypic and heterotypic antigenic determinants while CAV-2 carries only the homotypic determinant (Marusyk, 1972). DNA hybridisation studies have demonstrated a 70 per cent relationship between the two viral genomes (Marusyk and Hammarskjöld, 1972).

American workers have claimed that CAV-1 and CAV-2 differ not only structurally and antigenically but also in their pathological effects in that CAV-2 is associated primarily with respiratory disease (Fairchild et al., 1969; Swango et al., 1970) whereas hepatitis is the main lesion produced by CAV-1. Swango et al. (1970) observed that the clinical signs following the intravenous, intramuscular, intraperitoneal or intraocular inoculation of susceptible dogs with CAV-2 were slight but respiratory disease, varying in severity from mild rhinitis to fatal bronchopneumonia, resulted when the dogs were challenged with CAV-2 by the intranasal route. CAV-2 was recovered from the lungs and the salient pathological changes in the respiratory tract consisted of a proliferative interstitial pneumonia with a necrotizing bronchitis and bronchiolitis. Intranuclear inclusion bodies were present in nasal and bronchial epithelium and alveolar septal cells.

The present work describes the isolation of adenoviruses from dogs showing clinical respiratory disease and investigates the relationship of the new isolates to CAV-1 and CAV-2.

<center>HISTORY</center>

Case No. 1.

A litter of three 13-week-old mongrel dogs was presented for examination following a history of intermittent spontaneous coughing. They had all been purchased from a commercial source at 9 weeks of age and vaccinated with inactivated ICH and live distemper vaccine within 2 days of arrival. Coughing developed at 12 weeks of age and the animals were destroyed and necropsied 1 week later.

Case No. 2.

The subject was an 18-month-old male Cross Labrador dog. The animal had been admitted to kennels 10 days previously and, at that time, had been vaccinated with live distemper vaccine ("Epivax"). Six days after arrival, the dog became anorexic and was found to have a rectal temperature of 105°F. It was given the antibiotics penicillin and streptomycin by the intramuscular route, but, on subsequent examination, a bilateral, mucopurulent nasal discharge was observed and the dog was therefore destroyed.

<center>MATERIALS AND METHODS</center>

Histological procedures

At necropsy, detailed gross examinations were carried out on each dog in the manner previously described in PART 1, SECTION I. Tissues were fixed in mercuric chloride-formol and histological studies were carried out as indicated in PART 1, SECTION I.

Virological procedures

Samples of lung and trachea were taken from each of the puppies, frozen at -30°C. and maintained at that temperature until used for viral isolation. Blocks of turbinate, tonsil and trachea were collected from the older dog and treated in a similar fashion.

Homogenates of lung, trachea, tonsil and turbinate were prepared separately and inoculated into monolayer cultures of a greyhound kidney cell line, (GHK), which had been derived from the kidney of an apparently normal adult male greyhound. These epithelial cells are resistant to the action of trypsin and can be passed serially, growth and maintainance occurring satisfactorily in the same media as used for the MDCK cell line (PART 1, SECTION I). The karyology, growth characteristics, ultrastructure and viral susceptibility of the GHK cells has been investigated in detail (Cornwell, 1972 - unpublished observations).

Bacteriological procedures

In case No. 1, the lungs of each puppy were submitted to bacteriological examination as described in PART 1, SECTION I. Bacteriological examinations were not carried out in case No. 2.

RESULTS

Case No. 1.

On clinical examination the 3 puppies were bright and alert but they all had a mild, spontaneous, hacking cough. Pyrexia was not demonstrable in any of them.

At necropsy, the lungs of all 3 animals were studded with multiple, tiny grey foci about 1mm in diameter (Fig. 88); all lobes of the lung were affected equally and no other gross lesions were seen there or elsewhere.

Histological abnormalities were confined to the lungs and consisted of focal areas of septal thickening due to infiltration by macrophages and lymphocytes (Fig. 89); in these regions, the alveolar walls were necrotic and alveolar air spaces contained numerous swollen macrophages and small amounts of fibrin. Inclusion bodies were not

detected in the lungs or elsewhere, nor was there any histological evidence of canine distemper virus infection. Bacteriological examination of the lungs proved negative.

Homogenates of lung and trachea were prepared separately from each of the 3 dogs and inoculated into a canine kidney cell line (GHK). After 3 to 4 days a CPE indistinguishable from that produced by CAV-1 (Cornwell et al., 1970) was evident in all inoculated cultures, whereas control cultures of the same age remained normal in appearance. The CPE was readily reproducible on passage, maximum yields being in the order of 10^7 to $10^{7.5}$TCID$_{50}$ per ml. Undilute virus of high titre produced a CPE within 24 hours and limiting dilutions within 7 days. The 3 virus isolates were designated 'A', 'B' and 'C'.

Case No.2.

When the dog was examined, it was seen to be very thin, obviously depressed, and had a copious bilateral mucopurulent nasal discharge. Its rectal temperature at this time was 102°F. Coughing was not heard during the period of examination nor could it be induced by gentle pressure on the trachea.

At necropsy, the turbinates were swollen and congested and a thick mucopurulent exudate was present. The retropharyngeal lymph nodes were enlarged and oedematous. The tonsils were also swollen and tiny haemorrhagic foci were visible. Gross lesions were not seen in the lungs or elsewhere.

Histological examination showed an acute rhinitis. The blood vessels of the lamina propria were congested and packed with neutrophils. There was subepithelial haemorrhage and the connective tissue was separated by oedema fluid. A marked cellular infiltrate composed of lymphocytes, plasma cells, macrophages and polymorphonuclear leucocytes

was present in the lamina propria. Basophilic intranuclear inclusion bodies were found in the epithelium lining the turbinates (Fig. 90) and inclusions were also noted in macrophages and endothelial cells of the lamina propria.

Sinusoidal haemorrhage and oedema were found in the retropharyngeal lymph nodes and numerous basophilic intranuclear inclusions were seen in the reticular cells of the cortical lymphatic sinuses (Fig. 91).

There was lymphoid hyperplasia and focal subepithelial haemorrhages in the tonsil but no inclusions were found. The liver appeared normal except for the presence of an occasional inclusion in Kupffer cells. One small group of macrophages was also found.

Suspensions of the turbinates, tonsils and trachea were prepared separately and inoculated into GHK cells. An adenovirus-like cytopathic effect developed in the cultures inoculated with the turbinate and tonsil suspensions in 2 to 3 days but those inoculated with tracheal material remained normal. The virus isolate was designated strain "R".

CHARACTERISATION OF THE VIRUS ISOLATES

In order to confirm that the 4 isolates were canine adenoviruses electron microscopic, histochemical and serological examinations were carried out.

MATERIALS AND METHODS

Histological and electron microscopic procedures

GHK cells were grown on glass cover slips in plastic Petri dishes and infected with the new isolates and with CAV-1 strain 47889 at input multiplicities of from 4 to 8.

Coverslip preparations harvested at 24 hours after infection were divided into 4 groups, one being fixed in mercuric chloride-formol for 24 hours and then stained by the fluorescent Feulgen method; another group was fixed in cold acetone for 10 minutes and stained with acridine orange. The 3rd group was also fixed in acetone and stained by the direct method of the fluorescent antibody technique as described in APPENDIX 2. The 4th group was fixed in methyl alcohol for 15 minutes and stained with May - Grünwald Giemsa.

At the same time, infected cells were scraped off the bottom of the Petri dishes, sedimented by centrifugation at 1000r.p.m. for 5 minutes, fixed in 1 per cent osmic acid for 1 hour, dehydrated in alcohols and embedded in Araldite. Ultrathin sections were cut by means of an LKB Mark III ultratome, stained with uranyl acetate and lead citrate and examined with an AEI 6B electron microscope.

Suspensions of each of the isolates were clarified by centrifugation and negatively stained with 2 per cent phosphotungstic acid as described in PART 1, SECTION I.

Serological procedures

To determine whether or not the new isolates belonged to the CAV-1 type or were more closely related to CAV-2, HA-I tests were carried out. Hyperimmune mouse ascitic fluids were prepared against each of the new isolates as well as against the canine adenoviruses CAV-1 (strain 47889) and CAV-2 (Toronto strain $A_{26/61}$ which was kindly supplied by Dr. Ditchfield, University of Toronto, Canada). Four mice were used to prepare fluid for each virus strain. Undilute virus was mixed with an equal volume of Freund's complete adjuvant and 0·7ml of the mixture was inoculated intraperitoneally into each mouse. This was repeated 14 days later when the ascitic fluid, which was collected after a further 4 days, varied in amount from 0·5ml to 12ml per mouse. The fluids were inactivated at 56°C. for 30 minutes and then absorbed with an equal volume of 25 per cent acid washed kaolin (Flow Labs. Ltd.). HA-I tests were then carried out at room temperature with 1·5 per cent human type O erythrocytes and 8 haemagglutinating units of virus per well of a standard perspex haemagglutination plate. All dilutions were made in Alsever's solution and end points were taken as the highest dilutions which completely inhibited haemagglutination.

RESULTS

Histological and electron microscopic findings

In all cases, negative staining revealed the presence of numerous particles measuring approximately 80nm in diameter and hexagonal in outline (Fig. 92). Where orientation was favourable, 6 capsomeres could be seen along the edge of each facet, this being in accord with the morphology of adenovirus virions (Andrewes and Pereira, 1972).

All virus strains produced identical cytological changes as detected by light, fluorescence and electron microscopy. In the infected

monolayer cultures stained with Giemsa, intranuclear inclusions were present in large numbers. The inclusions varied in form and 3 main types were recognised. In the first, irregular granules 2 to 5μm in diameter were found throughout the nucleoplasm and these appeared to combine in the formation of the 2nd type of inclusion, a large single body which almost completely filled the nucleus (Fig. 93). Nucleoli were either trapped within this structure or were pushed to the side of the nuclear membrane. The inclusion was vacuolated and stained pink to violet with Giemsa. The 3rd form of inclusion was smaller, more sharply defined and surrounded by a clear halo (Fig. 94). There was distinct margination of the nuclear chromatin and fine strands often extended from the inner nuclear membrane to the edge of the inclusion.

The inclusions stained bright green with acridine orange and golden yellow with fluorescent Feulgen (Figs. 95, 96, 97), thus indicating the presence of large amounts of DNA. Between the inclusions and the nuclear membrane was a clear, unstained peri-inclusion halo.

Distinct inclusions were not demonstrated by immunofluorescence. In most infected cells, virus antigen was present throughout the nucleus but fluorescence was particularly bright at the nuclear periphery (Fig. 98), this strongly fluorescing zone corresponding to the peri-inclusion halo region.

Electron microscopy showed the inclusions to consist of a matrix of fine granular material containing pockets of adenovirus virions. Aggregates of the granular material occurring at random in the nucleus appeared to correspond to the type 1 inclusion (Fig. 99), at this stage, only a few particles were seen either in the centre or at the periphery of these masses.

Clumps of granular material and clusters of viral particles distributed throughout the nucleus appeared to represent the 2nd form

of inclusion (Fig. 100). In the 3rd form virions were less numerous in the central dense granular mass and were concentrated instead in the peripheral zone (Fig. 101) i.e. the peri-inclusion halo region which stained so intensely with fluorescent antibody.

Serological findings

The results of the HA-I tests are presented in Table 14, from which it will be seen that strains A, B, C and R were indistinguishable serologically from CAV-1 but were quite distinct from CAV-2. The mouse ascitic fluid prepared against CAV-1 had a much higher HA-I antibody titre against CAV-1 homologous viruses than against CAV-2 heterologous virus. The converse was found with mouse ascitic fluid prepared against CAV-2. Mouse ascitic fluid prepared against the control GHK culture did not react with either group of viruses.

TABLE 14

Results of HA-I Tests of Hyperimmune Mouse Ascitic Fluids Against

CAV-1 and CAV-2 Viruses

Hyperimmune Mouse	Reciprocal of HA-I Titre with Indicated Viral Strain					
Ascitic Fluid	CAV-1	A	B	C	R	CAV-2
CAV-1	64	128	64	64	64	2
A	256	256	256	256	512	4
B	128	128	128	128	128	2
C	256	256	128	256	128	4
R	128	256	256	128	256	4
CAV-2	8	8	4-8	8	8	128

EXPERIMENTAL INFECTION OF DOGS WITH THE ADENOVIRUS ISOLATES

The next part of this work was designed to establish the behaviour of the respiratory adenovirus isolates _in vivo_.

MATERIALS AND METHODS

Experimental animals

Fourteen 12-week-old farm-bred Collie puppies were used in these experiments. On arrival, the puppies were bled and the sera tested for antibody specific to CAV-1 by means of the indirect method of the fluorescent antibody technique as described in APPENDIX 2.

Experimental procedures

(a) Intravenous inoculation of the adenovirus isolates (see Table 15).

The experimental animals were divided into 2 groups. In the first group, 5 dogs were inoculated intravenously with 1ml of strains A, B, C, R and CAV-1 strain 47889 virus respectively. The titre of the virus used in all cases was $10^7 TCID_{50}$ per ml. Two litter mates were kept as uninoculated controls.

(b) Aerosolisation of the adenovirus isolates (see Table 16).

Of the 2nd group of 7 dogs, 3 were given C strain of adenovirus and one R strain by means of an aerosol spray as has been described elsewhere (PART 2, SECTION I). The dogs were exposed to the aerosol spray for 10 minutes during which time 0·2ml of the virus suspension was aerosolized. In a similar fashion 1 dog was given CAV-1 strain 47889 and 2 dogs from the same litter were used as controls and subjected to an aerosol of uninfected dog kidney tissue culture fluid. The controls were kept in a separate room from the infected dogs to prevent infection passing from their litter mates.

Pathological procedures

All the puppies were examined twice daily for clinical abnormalities and detailed pathological examinations were carried out. The direct method of the fluorescent antibody technique served to demonstrate the presence of CAV-1 antigen in acetone-fixed sections of liver, lung, kidney and tonsil. Blocks of liver from both infected and control dogs in experiment (a) were fixed in osmic acid and processed as described previously (PART 2 , SECTION I) for examination with an AEI 6B electron microscope.

RESULTS

(a) Intravenous inoculation of adenovirus isolates.

The clinical signs observed in all of the inoculated dogs were those of an acute, generalised infection. The dogs were dull, pyrexic and lacked appetite by the 3rd day after infection and on the 4th day 3 were dead and 2 were moribund.

Each of the infected dogs showed the characteristic pathological changes of Rubarth's disease (Rubarth, 1947). At necropsy, excess serous or serosanguineous fluid was present in all of the body cavities (Fig. 102) and there was distinct hyperaemia of all the lymph nodes. The liver was enlarged, mottled in appearance and showed fibrin strands between the lobes (Fig. 103) and on its surface; there was marked oedema of the wall of the gall bladder (Fig. 104). Petechial haemorrhages of the thymus were present in all cases.

Microscopic examination of the liver showed severe hepatitis with focal parenchymal necrosis (Fig. 105) and the presence of numerous intranuclear inclusion bodies. Apart from a few small pulmonary haemorrhages, there were no lesions in the lungs.

TABLE 15

(a) Intravenous Inoculation of the Adenovirus Isolates

Dog Number	Virus Strain	Day Examined	Hepatitis	Pneumonia
1	A virus	4	+	-
2	B virus	4	+	-
3	C virus	4	+	-
4	R virus	4	+	-
5	CAV-1	4	+	-
*6	Control	4	-	-
*7	Control	4	-	-

* Denotes unexposed control animal.

TABLE 16

(b) Aerosolisation of the Adenovirus Isolates

Dog Number	Virus Strain	Day Examined	Hepatitis	Pneumonia
14	C	3	-	+
13	C	5	-	+
8	C	5	-	+
9	R	5	-	+
10	CAV-1	5	-	+
11*	Control	5	-	-
12*	Control	5	-	-

* Denotes unexposed control animal.

Immunofluorescence studies showed virus antigen to be present in large amounts in the liver (Fig. 106) and, to a lesser extent, in the kidneys (Fig. 107) and tonsils. Antigen was not detected in the lungs.

With the electron microscope, adenovirus particles were found in the nuclei of hepatocytes and Kupffer cells; practically every cell showed evidence of infection. The sequence of nuclear changes in the infected hepatic cells was similar to that found in the bronchiolar epithelium (Fig. 108). In the earliest stages of infection, clumps of fine electron dense granules were scattered throughout the nucleus. The clumps coalesced to form an inclusion-like structure and adenovirus particles were distributed throughout this material. In the liver, virions were not found in the cytoplasm of infected cells and intranuclear paracrystalline structures were not observed.

There was a marked decrease in the glycogen content of infected hepatocytes but the mitochondria were generally unchanged or only slightly swollen. The most striking cytoplasmic change in infected cells was a marked vesiculation of the endoplasmic reticulum.

The control puppies did not show any clinical or pathological changes.

(b) Aerosolisation of adenovirus isolates.

The results are summarised in Table 16. By 3 days after exposure, all the infected dogs were dull, pyrexic, partially anorexic, hyperpnoeic and coughing spontaneously. This continued until the 5th day after exposure when most of the animals were killed. All 5 infected animals showed similar pathological findings. Deep red areas of consolidation were found in all lobes of the lungs, but especially along the edges of the apical and cardiac lobes and the dorsal posterior regions of the

diaphragmatic lobes (Figs. 109, 110). These areas were slightly swollen, firm and oedematous. The bronchomediastinal lymph nodes were enlarged and hyperaemic and excess blood-tinged fluid was present in the bronchi and trachea. The tonsils were enlarged and hyperaemic. No other gross changes were present.

Histological examination of the lungs showed, in all cases, focal areas of pulmonary oedema and severe necrotizing bronchiolitis with occlusion of many of the bronchioles with epithelial debris and neutrophilic exudate (Fig. 111). Many intranuclear inclusion bodies could be detected in necrotic bronchiolar epithelial cells and in all of the animals specific CAV-1 antigen was found in large amounts in these cells (Fig. 112). The surrounding alveoli were haemorrhagic and contained fibrin, neutrophils and macrophages. Focal areas of necrosis were also present in the turbinates and tonsils where, additionally, inclusion bodies and virus antigen were present (Fig. 113). The bronchomediastinal lymph nodes were congested and showed some dissolution of lymphoid follicles and proliferation of the sinusoidal macrophages. Only a very few inclusions and some isolated fluorescing cells were present in the liver but there was no evidence of hepatic necrosis.

Virus was re-isolated from the lungs and turbinates of all of the dogs subjected to aerosol-spread infection but bacteriological examination did not reveal any significant findings. The control puppies showed no clinical, gross or histopathological changes and virus was not isolated from either animal.

DISCUSSION

The present work describes the isolation of adenoviruses, indistinguishable from CAV-1, from three 13-week-old puppies suffering from a mild respiratory disease and from a young adult dog with severe rhinitis. The viruses were typical adenoviruses as demonstrated by

electron microscopy and histochemistry; they were shown to be serologically distinct from CAV-2 which has also been isolated from dogs with respiratory disease (Ditchfield et al., 1962).

In tissue culture, all 4 virus isolates produced cytopathic changes identical to those caused by CAV-1. Characteristic Feulgen-positive inclusion bodies were found in the nuclei of a large proportion of canine renal epithelial cells examined 24 hours after infection. Large amounts of virus antigen, as demonstrated by immunofluorescence were also present in these cells at this time, fluorescence being most intense in the peri-inclusion halo area of the nucleus. With the electron microscope, typical hexagonal adenovirus particles were found scattered throughout the nucleus, in pockets within the matrix of the inclusion or concentrated in the peri-inclusion halo region.

As is the case with CAV-1, the new adenovirus isolates produced hepatitis when administered intravenously and pneumonia when given by aerosol to susceptible dogs. The pulmonary lesions induced in experimental dogs were much more extensive than those observed in the 3 puppies, and lesions were detected elsewhere in the respiratory tract, particularly in the nasal and turbinate epithelium. Conversely, the lesions in the upper respiratory tract of the experimental dogs were much less severe than those found in the adult dog in which numerous intranuclear inclusions were observed in the turbinate mucosa and lamina propria. The reasons for these differences is not clear but may be related to the variations in susceptibility of individual dogs or to the stage of the infection.

In the experimental dogs suffering from respiratory disease, only minimal virus activity was detected in the liver. Dogs which died of acute hepatitis following the intravenous inoculation of virus did not show any histological evidence of respiratory disease. Centrilobular necrosis of the liver, as described by Rubarth (1947), occurred in these

puppies and virus particles were found in hepatocytes and Kupffer cells. The ultrastructural findings in the liver of infected dogs were in accord with those of Tajima and Motohashi (1958) and Givan and Jézéquel (1969).

The isolation of the CAV-2 has recently been reported in Great Britain (Danskin, 1973). Despite the claim of American workers that CAV-1 and CAV-2 differ not only serologically but also in their pathological effects, the present results show quite clearly that CAV-1 is capable of producing either hepatitis or respiratory disease depending on the route of infection. It would be wrong therefore to attempt to differentiate the 2 canine adenoviruses on their pathological effects as these may be due to the route of administration and not to the strain of virus.

It is of interest that the 13-week-old puppies had been vaccinated with inactivated CAV-1 vaccine. It could well be that circulating antiviral antibody in the bloodstream does not necessarily preclude multiplication of virus in the respiratory tract. The efficacy of available vaccines against the respiratory form of CAV-1 infection has still to be assessed but preliminary results (Wright et al., 1974) have shown that dogs with maternal antibody and solidly immune to intravenous challenge with CAV-1 were still susceptible to aerosol spread of virus with resultant respiratory disease. Clearly, an evaluation of vaccination procedures in protecting dogs against adenovirus respiratory disease would be worthwhile.

It is now apparent that CAV-1 infection may occur in 2 clinically distinct forms, respiratory infection and hepatitis, and there is little doubt that the spectrum of CAV-1 infection among the canine population is much broader than was originally thought.

PART 3

A SEROLOGICAL SURVEY AND EXPERIMENTAL RESPIRATORY INFECTION WITH

REOVIRUS TYPE 1

INTRODUCTION

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DISCUSSION

INTRODUCTION

The name "reovirus" was adopted by Sabin (1959) for a group of enteroviruses which had previously been classified as ECHO viruses type 10. Strains of reovirus were first recovered from the faeces of healthy children by Ramos-Alvarez and Sabin (1954, 1956) though Stanley et al. (1953) had earlier described, under the name "hepato-encephalomyelitis" virus, an agent which was subsequently found to be a reovirus type 3 (Stanley, 1961). Following these initial reports, reoviruses have been isolated from many mammalian species including chimpanzees (Sabin, 1959), monkeys (Hull et al., 1958), cattle (Rosen et al., 1963), cats (Scott et al., 1970), dogs (Lou and Wenner, 1963) and pigs (McFerran and Connor, 1970). The ubiquity of these agents is indicated by the frequent presence of antibodies in a wide variety of vertebrates (Rosen, 1962; Stanley et al., 1964) and strains recovered from man and various animal species have usually been indistinguishable (Rosen, 1962). The relevant literature on the reovirus group has been reviewed by Stanley (1967), Rosen (1968), Spendlove (1970) and Verwored (1970).

In domestic animals, reoviruses have been associated mainly with respiratory infections but many isolations have been made from the faeces of apparently normal animals (Rosen, 1962). All 3 reovirus serotypes have been isolated from cattle (Rosen and Abinanti, 1960; Rosen et al., 1963; Lamont, 1968) and antibodies to them are widespread in the bovine population. Reoviruses types 1 and 2 have been shown to cause a symptomless pneumonia in calves under experimental conditions (Lamont et al., 1968) and examination of paired serum samples taken from cattle involved in outbreaks of respiratory diseases in Gt. Britain has revealed that 28·9 per cent have experienced reovirus infection.

A type 1 reovirus has been isolated from the faeces of a 14-week-old pig which belonged to a herd in which respiratory disease and post-weaning enteritis were prevalent (McFerran and Connor, 1970). Experimental infection of 4-week-old pathogen-free piglets with this virus did not lead to any clinical signs of disease but cellular foci were found throughout the lungs. Reovirus was recovered intermittently from the respiratory and alimentary tracts and a rise in specific HA-I antibody was detected in the experimentally infected animals (Baskerville et al., 1971b).

A reovirus has also been isolated from the intestine of a cat (Scott et al., 1970). Conjunctivitis, lacrimation and photophobia were seen in kittens inoculated with the isolate and the infection spread rapidly to contact-control kittens.

In the dog, a reovirus type 1 has been found in association with respiratory disease (Lou and Wenner, 1963; Massie and Shaw, 1966) and serological evidence indicates that natural reovirus infections are frequent in dogs in the USA (Fairchild and Cohen, 1967). There is little information about the incidence of reovirus infection in the canine population of this country. The aims of the present study were to seek serological evidence of type 1 reovirus infection and to investigate its pathogenicity for the dog.

The studies reported in this thesis were undertaken with a reovirus type 1 of bovine origin (WBR 26) (Lamont, 1968). This isolate had originally been recovered from a faecal sample from a herd with a respiratory problem and it gave a complete cross-reaction in the HA-I test with a prototype 1 strain of human origin (Lamont, 1968). The type 1 reoviruses which have been isolated from dogs (Lou and Wenner, 1963; Massie and Shaw, 1966) were also serologically indistinguishable from the human strain.

MATERIALS AND METHODS

1. Serological survey

Sera

Eighty six serum samples were obtained from dogs at the Glasgow
Dog and Cat Home, Cardonald. The dogs were electrocuted and bled by
cardiac puncture immediately after death. They were of mixed breeds and
varied in age from young adults to aged animals

Virus

A reovirus type 1 (WBR 26) of bovine origin (Lamont, 1968) was
obtained from Dr. W. Martin of the Moredun Institute, Edinburgh. It was
passaged 3 times in MDCK cultures, the virus suspension having a
titre of approximately $10^6 TCID_{50}$ per ml.

In the MDCK cultures, infected cells adopted a rounded or spindle-
shaped contour, became partially refractile and detached from the glass.
Eosinophilic cytoplasmic inclusions were detected in stained monolayer
cultures and viral particles were found in these inclusions by electron
microscopic examination. Haemagglutinin became detectable only when
disruption of the cell sheet was advanced.

Serological procedures

Antibody levels were assayed by the HA-I technique as described
by Rosen (1960). Serum samples were heat inactivated at $56^{\circ}C$. for 30
minutes, absorbed for 20 minutes at room temperature with an equal
volume of 25 per cent acid washed kaolin (Flow Labs. Ltd.) and incubated
at $37^{\circ}C$. for 1 hour with an equal volume of a 10 per cent suspension of
human type O erythrocytes. Serial 2-fold dilutions of the sera were
prepared in perspex haemagglutination plates and tested against 4
haemagglutinating units of virus per dilution. The virus-serum mixture

was allowed to react at room temperature for 1 hour and then 0·3ml of a 1 per cent suspension of human type O erythrocytes was added. Tests were left for 2 hours before being read and endpoints were taken as the highest dilution giving 100 per cent inhibition of haemagglutination.

2. Experimental Studies

Experimental animals

Nineteen farm bred Collie puppies aged between 8 and 10 weeks were used in the following study.

Experimental procedures

(a) Intratracheal inoculation of reovirus

Eight animals were inoculated intratracheally with 1ml of type 1 reovirus of bovine origin (WBR 26), titre $10^6 TCID_{50}$ per ml. For purposes of control, 4 more puppies were given a similar volume of uninfected tissue culture medium; 2 of these were kept as contact controls and 2 were housed in a separate room with no common air space with their infected litter mates. Two infected dogs were killed on day 2 and a control, contact control and infected dog were killed on day 3, after that, 2 infected dogs were killed on days 7 and 12. The last infected dog and the remaining controls were killed on day 20.

(b) Aerosolisation of reovirus

Five animals received virus by an aerosol procedure as described in PART 1, SECTION II. They were exposed to the aerosol for a period of 10 minutes and, in each case, received approximately 0·2ml of the virus. The 2 control dogs were exposed, in a similar manner, to uninfected dog kidney tissue culture fluid and were housed in a separate room from their infected litter mates. Infected dogs were killed on the 2nd, 3rd 6th, 8th and 16th days after infection. The controls were killed on the 6th and 16th days after aerosolisation.

Serum samples were obtained from all the puppies before administration of virus and at necropsy. The sera were treated as described above and tested against 4 haemagglutinating units of virus per serum dilution.

Histological procedures

At necropsy, representative portions of tissue from major organs of both infected and control dogs were fixed in mercuric chloride-formol, embedded in paraffin wax and sectioned at 6μm. Histological procedures were carried out as described in PART 1, SECTION I.

Bacteriological examinations were carried out on the lung, liver and spleen of all puppies.

Virological procedures

Samples of lung, bronchial lymph node, liver, kidney and intestine were taken from each dog and a suspension was prepared (1:10 w/v) in Earle's balanced salt solution containing antibiotics. The suspension was clarified by centrifugation and 1ml used to inoculate 2 bottles of a continuous dog kidney cell line (MDCK). The cultures were examined daily for 21 days and a positive CPE was confirmed by means of a haemagglutination test with human type O erythrocytes.

<div align="center">RESULTS</div>

1. <u>Serological Survey</u>

The distribution of antibody titres in the 86 dogs is presented in Table 17. Forty six of the 86 dogs (53·5 per cent) tested had HA-I titres of over 1 in 8 and of these 17 (19·7 per cent) had titres of 1:32 or greater. The highest individual titre recorded was 1:256.

TABLE 17

Haemagglutination-inhibition Titres to Type 1 Reovirus in Dogs in

the United Kingdom

Titre	8	8-16	32-64	128-256	Total
No. of dogs (%)	40(45·5)	29(33·7)	12(13·9)	5(5·8)	86(100)

2. Experimental Studies

(a) Intratracheal inoculation of reovirus

No clinical signs were observed, the dogs remaining bright and alert.

Macroscopic findings

At necropsy, visible findings were confined to the lungs and bronchial lymph nodes. On the 2nd, 3rd and 7th days after infection, greyish-red areas of consolidation were present in all lobes of the lung but especially in the posterior area of the diaphragmatic lobes and at the hilus of the lung (Fig. 114). In these regions, the lung tissue was firm and excess fluid was present in the bronchi. The bronchial lymph nodes were enlarged, hyperaemic and oedematous.

By the 12th day after infection the pulmonary lesions were less marked and were often slightly depressed below the surface of the lung (Fig. 115). No lesions were found in the dog killed on the 20th day.

Microscopic findings

At 2 and 3 days after infection, the lungs showed diffuse exudative pneumonia characterised by loss of the bronchiolar epithelium and necrosis of the alveolar walls (Figs. 116, 117). In addition, large numbers of macrophages and polymorphonuclear leucocytes were present in the alveoli and alveolar ducts.

Seven days after infection the lesions were confined mainly to the alveoli and the bronchiolar lumina were relatively free of exudate and cell debris. The alveolar septa were thickened by mononuclear infiltration and there was swelling, proliferation and desquamation of septal cells (Figs. 118, 119); a feature of the disease was the presence of many intra-alveolar syncytia (Fig. 120). Although some irregular swelling of the alveolar epithelial cells was noted, there

was no distinct epithelial hyperplasia. Inclusion bodies were not detected at any stage in either alveoli or bronchiolar epithelium.

By the 12th day, evidence of resolution of the lung lesions was observed and fibroblasts and collagen appeared in the cellular accumulations in the alveoli and alveolar ducts (Fig. 121). At this time, foci of lymphoid cells were present around many small bronchioles and blood vessels. By day 20, the lungs were histologically normal except for a few areas of septal thickening and macrophage accumulation.

Sinusoidal oedema and proliferation and desquamation of reticular cells were the most striking histological features in the bronchial nodes of the dogs killed on days 2 and 3. By day 7, oedema was less marked and there was evidence of lymphoid hyperplasia. The main changes in the node on days 12 and 20 were lymphoid hyperplasia and accumulation of plasma cells in the sinusoids.

Virological findings

The results of virus isolation are summarised in Table 18, from which it can be seen that virus was recovered from the lungs and bronchial nodes of infected dogs on the 3rd and 7th days after infection. Virus was also isolated from the intestine of one of the contact control dogs which had been kept in the same room but in a different pen from its infected litter mates.

Serological findings

The results of serological tests are shown in Fig. 122. It can be seen that the pre-inoculation sera at high concentration (1:4 and 1:8) had a slight inhibitory effect on haemagglutination. Significant responses were detected in the post-inoculation sera of the puppies killed after the 3rd day. The contact control animal killed on the

TABLE 18

Experimental Reovirus Respiratory Infection.

Virological findings: the recovery of reovirus type 1 after intratracheal infection

Dog Number	Day Examined	Lung	Bronchial Lymph Node	Liver	Kidney	Intestine
30	2	+	NT	-	-	-
31	2	+	NT	-	-	-
32	3	+	+	NT	-	+
33	7	+	+	-	+	-
34	7	+	+	+	-	-
35	12	-	-	-	-	-
36	12	-	-	-	-	-
37	20	-	-	-	-	-
38*	3	-	-	-	-	-
39*	20	-	-	-	-	+
40**	3	-	-	-	-	-
41**	20	-	-	-	-	-

* Denotes contact control animal.

** Denotes unexposed control animal.

+ Denotes reovirus type 1 recovered.

20th day after infection, and from which virus was isolated, showed an 8-fold increase in antibody titre.

Unexposed Control animals

Clinical signs were not detected in the control animals nor were any abnormalities noted at necropsy. Histological changes were not found in the lungs and attempts at virus isolation proved negative.

(b) Aerosolisation of reovirus

Clinical signs of respiratory disease were not observed.

Macroscopic findings

At necropsy, gross lesions were not detected in the nasal mucosa, lungs or other organs of any of the dogs killed on days 2 and 3. Tiny grey foci, about 1mm in diameter, were scattered through all of the lobes of the lungs of the dogs killed on days 6 and 8 (Figs. 123, 124). The bronchial lymph nodes in these dogs were slightly enlarged. Lesions were not found in the dog killed on day 16.

Microscopic findings

On the 2nd and 3rd days after infection, focal microscopic changes were found distributed equally in all lobes of the lungs. These lesions consisted of alveolar septal necrosis and infiltration of polymorphonuclear leucocytes (Fig. 125). A few polymorphonuclear leucocytes, macrophages and small amounts of fibrin were present in the alveolar air spaces. Necrosis was also noted in alveolar ducts but the bronchi and bronchioles were not affected. On day 6, the cellular infiltrate consisted predominantly of lymphocytes and macrophages (Fig. 126) and there was swelling and desquamation of alveolar epithelial cells. By day 8, the intra-alveolar cellular aggregates had become invaded

TABLE 19

Experimental Reovirus Respiratory Infection

Virological findings: the recovery of reovirus type 1 after aerosol
infection

Dog Number	Days Examined	Lung	Bronchial Lymph Node	Liver	Kidney	Intestine
42	2	+	+	-	-	-
43	3	+	+	-	-	-
44	6	+	+	-	-	+
45	8	+	+	-	-	-
46	16	-	-	-	-	-
47*	6	-	-	-	-	-
48*	16	-	-	-	-	-

* Denotes unexposed control animal.

+ Denotes reovirus type 1 recovered.

by fibroblasts (Fig. 127) and many foci were converted to dense cellular nodules.

Lesions were not detected in the lungs of the dog killed on day 16.

There was a lymphoid hyperplasia of the bronchial lymph nodes of the dogs killed on days 6 and 8 and hyperplastic changes were also noted in the tonsils and retropharyngeal lymph nodes. Histological changes were not found in any other organ.

Virological findings

The results of virus isolation are summarised in Table 19. Reovirus type 1 was recovered from the lungs and bronchial lymph nodes of infected dogs up to 8 days after aerosolisation. The virus was also isolated from the intestine of the dog killed on day 6.

Serological findings

The HA-I results are recorded in Fig. 128. HA-I antibodies, at a 1:32 serum dilution, were detected in all infected dogs after day 6.

Unexposed Control animals

No significant gross or histopathological lesions were noted in the control dogs. Virus isolation studies proved negative and the HA-I antibodies remained constant at 1:4.

DISCUSSION

Although reoviruses have not been isolated from dogs in the United Kingdom, the present investigation shows that a large number of the dogs tested in the survey possessed HA-I antibodies to reovirus type 1. The 19·7 per cent incidence of titres of 1:32 or higher in the present

study was comparable to the 19 per cent (23 out of 118) reported by

Massie and Shaw (1966) and the 24·4 per cent reported by Binn et al.

(1970) for adult dogs of mixed breeds in the USA. In their serological

study of 112 dogs of all ages and breeds presented at a Veterinary

Clinic in Ohio, Fairchild and Cohen (1967) found 37 per cent with serum

positive at the level of 1:40; serological evidence of reovirus type 2

and 3 infection was also noted. Fairchild and Cohen (1967) have suggested

that an HA-I titre of 1 in 40 was indicative of past reovirus infection.

Since little is known about the epidemiology of reovirus infections in

the dog, one can only speculate that the greater incidence found by

Fairchild and Cohen might have been due to a higher rate of infection

in younger animals; most reovirus strains isolated from man have come

from children (Stanley, 1967).

Reovirus type 1 was first isolated from the lungs of a dog with

respiratory disease by Lou and Wenner (1963) who used their isolate to

inoculate 8-week-old puppies intravenously, intranasally and intra-

peritoneally. The infected puppies developed a mucoid nasal discharge

and at necropsy, an interstitial pneumonitis was present; oval eosinophilic

cytoplasmic inclusions were found in the bronchial epithelium. Holzinger

and Griesemer (1966), who also used this reovirus strain, infected germ-

free and disease-free dogs but did not find any evidence of disease,

either clinically or pathologically and suggested that the pneumonia

described by Lou and Wenner (1963) could have been caused by distemper

virus. The present experimental studies however have shown that young

dogs are susceptible to reovirus type 1 infection, and develop a mild

proliferative pneumonia although no clinical signs may be apparent.

The pathological changes following intratracheal and aerosol

infection were basically the same, differing only in degree and

distribution. After intratracheal inoculation, the lesions were more

severe and localised whereas those seen in the dogs exposed to an aerosol spray of virus were milder but scattered throughout the lungs. A similar pattern has been noted in the experimental herpesvirus and adenovirus infections described in PARTS 1 and 2.

The earliest microscopic changes in the reovirus infection appeared to be necrosis of the walls of the bronchioles and alveoli or alveolar ducts with a polymorphonuclear cell infiltration. This was followed by a proliferative phase in which the alveolar epithelial cells became swollen and macrophages flooded the alveolar air spaces in large numbers. In the later stages of the disease, intra-alveolar fibroblasts were observed and the septa became thickened and distorted. At no stage were inclusion bodies evident.

Reovirus was reisolated from the lungs and bronchial lymph nodes up to 7 days after intratracheal infection and up to 8 days after aerosolisation. Virus was recovered with only limited success from tissues other than the lungs but it was isolated from the intestine of 1 contact control puppy presumably as a result of cross-infection.

The serological results showed a significant rise in HA-I titre after 3 days and this was especially marked in the dogs infected by the intratracheal route. The response obtained in the dogs infected by aerosol closely resembled the reaction of dogs infected intraperitoneally (Holzinger and Griesemer, 1966) whereas the levels following intratracheal inoculation were similar to those seen after intravenous challenge (Massie and Shaw, 1966).

Clinically and pathologically, the experimental reovirus infection was the mildest of the virus infections studied. The complete absence of clinical signs contrasted with the rhinitis seen with CHV and the cough, nasal discharge and pyrexia following CAV-1 infection. Histologically, the reovirus lesions were also mild when compared with

the severe necrotizing bronchiolitis and alveolitis seen in CHV and the necrosis and proliferation observed with CAV-1.

The lesions found in the dogs inoculated intratracheally compared closely to those described by Lamont et al. (1968) in newborn calves infected with reovirus types 1 and 2. In the dog, swelling and proliferation of the alveolar epithelium was not so marked as in the calves and "epithelialisation" was not observed. Furthermore, the reactions of dog lungs to an aerosol infection with reovirus type 1 were similar to those described for experimental reovirus infection in piglets (Baskerville et al., 1971b) although damage to the alveolar epithelium was more prominent in the dog.

Although the present results have shown that reovirus infection in the dog is mild, reovirus type 1 must be considered as yet another agent which can be involved in the canine respiratory disease complex.

PART 4

EXPERIMENTAL STUDIES WITH CANINE TRACHEAL ORGAN CULTURES

Contents

INTRODUCTION

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Virus

Organ cultures

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INTRODUCTION

Organ tissue culture is a technique in which embryonic
rudiments or small pieces of adult organs are maintained _in vitro_
with their organised structure intact. The study of such cultures
prepared from foetal tissues has proved very rewarding in experimental
embryology (Fell, 1951, 1963) and in more recent years it has been
found possible to grow many viruses in organ or explant cultures.
Hoorn and Tyrrell (1969) have reviewed in detail the general applications
of organ culture to virology. Organ cultures of ciliated epithelium
have proved particularly successful in virus studies because the
structural integrity of the differentiated cells can be well maintained
in vitro and the ciliary activity gives some indication of the cellular
function. Carleton (1925) was the first to observe ciliated epithelium
in explants of cat and rabbit lungs but he did not see any ciliary
movement. Strelin (1929) using explants of rabbit bronchi observed
ciliary activity and found it to persist for up to 9 days. Both these
workers used the plasma clot technique which was introduced by Burrows
(1910) and later developed by Fell and Robison (1929). Other investigators
using the same method were able to preserve ciliary activity for several
weeks (Cailleau _et al._, 1959).

One of the first systematic studies of respiratory viruses in
organ cultures of ciliated epithelium was made by Bang and Niven (1958).
They prepared cultures of trachea from human embryos on rafts on plasma
clots and inoculated these with different viruses. At intervals thereafter
they examined the cultures for ciliary activity, collected media for
virus isolation and selected fragments for histological examination.

In 1964 Hoorn showed that organ cultures of ciliated epithelium
could readily be maintained by planting them on scratched areas of a

plastic Petri dish and feeding them with a synthetic medium without serum. This technique had considerable advantages over the plasma clot method. It was technically easier and, in the absence of serum, no outgrowths of dedifferentiated cells occurred. Moreover the fluid medium was easily harvested from the Petri dishes and virus titrations could then be carried out.

Organ cultures prepared from human embryonic trachea by the method of Hoorn have been widely used in the study of human respiratory viruses. The growth of myxoviruses, enteroviruses, adenoviruses, herpesviruses and rhinoviruses has been described in human foetal tracheal explants (Hoorn and Tyrrell, 1965; Tyrrell and Hoorn, 1965; Hoorn, 1966) and the technique has proved useful in the initial isolation of agents which are difficult to grow in monolayer cell cultures (Tyrrell, 1970). For example, coronaviruses were first isolated by this means (Tyrrell and Bynoe, 1965) and, by using tracheal organ cultures in addition to monolayer cell cultures, some workers have doubled the efficiency of isolation of rhinoviruses from nasal washings of patients suffering from the common cold (Higgins, 1966; Tyrrell, 1970).

Many human respiratory viruses have been grown in organ cultures of animal origin. Influenza A destroyed the ciliated epithelium of ferret nasal mucosa cultures (Bang and Niven, 1958) while monkey tissue supported growth of the virus without any histological change (Hoorn and Tyrrell, 1965; Herbst-Laier, 1970). In organ cultures of mouse trachea, influenza A was found to replicate and cause ciliostasis, histological examination revealed progressive damage of the epithelial surface the process resulting in complete desquamation (Westerberg et al., 1972).

Recently there has been an increasing interest in the application of organ culture techniques in veterinary virology. The growth and cytopathology of the bovine respiratory viruses, infectious bovine

rhinotracheitis virus (Shroyer and Easterday, 1968), parainfluenza 3 (PI3) virus (Hoorn, 1966; Campbell et al., 1969; Kita et al., 1969; Reed, 1969) and bovine rhinovirus (Reed, 1969) have all been studied in calf tracheal explant cultures. Using scanning electron microscopy Reed and Boyde (1972) showed that in cultures infected with a bovine rhinovirus degenerating ciliated and non-ciliated cells were rapidly extruded from the epithelium whereas in PI3 infected cultures damage developed more slowly, leaving an epithelium covered with short microvilli. Reed et al. (1971) described the isolation, in calf tracheal organ cultures, of a rhinovirus from a calf with respiratory disease. The isolation of the virus in organ culture was achieved after initial failure to detect the virus in monolayer cultures of bovine kidney cells.

Explants of pig respiratory tissues have been found to be susceptible to infection with swine influenza and hog cholera viruses. Both viruses caused little damage to the epithelium but virus replication was detected for up to 20 days after infection (Reed, 1969; Nakamura and Easterday, 1969; Mengeling and Pirtle, 1970).

Tracheal organ cultures have provided an excellent model system in which to study avian respiratory viruses. Newcastle disease virus was found to cause either proliferative or necrotic lesions on chick tracheal epithelium depending on the degree of virulence of the virus (Bang and Niven, 1958; Butler, 1965). Using fowl embryo trachea, Butler (1965) showed that the various avian viruses, Newcastle disease virus, fowl plague, infectious bronchitis and infectious laryngo-tracheitis affected the ciliated epithelium of tracheal cultures in much the same way as in the intact animal. Colwell and Lukert (1970) reported that tracheal explants prepared from chick embryos were of value in the propogation and characterisation of strains of avian infectious bronchitis virus and it has also been demonstrated that rapid titrations

of infectious bronchitis virus were possible in tracheal cultures (Cherry and Taylor-Robinson, 1970; Johnson and Newman, 1971).

Animal tracheal organ cultures have been used as host system to investigate interactions between viruses and mycoplasmas. Reed (1971, 1972) found that the growth of mycoplasmas in bovine and porcine tracheal organ cultures was enhanced by virus infections. The extent to which the virus infection stimulated the growth of the mycoplasmas was related to the degree of epithelial destruction caused by the virus.

At the present time little information is available on the application of tracheal organ cultures to the study of canine virus infections. Herbst-Laier (1970) found that dog tracheal explants could be readily infected with influenza and parainfluenza viruses of human origin and Heyke (1969) briefly described the growth of an attenuated strain of CAV-1 in dog tracheal cultures. The aim of the present investigation was to assess the suitability of canine tracheal organ culture as an _in vitro_ system for the study of canine respiratory viruses.

MATERIALS AND METHODS

Virus

The two strains of canine herpesvirus studied were CHV-G1 isolated from neonatal pups (Cornwell _et al._, 1966) and CHV-G3, the isolation of which was described in PART 1, SECTION I. Both viruses were passed in MDCK cell monolayers and their titres were 10^6 and 10^5 PFU per ml respectively.

The distemper virus was the Glasgow 841 strain, the isolation of which was described by Cornwell _et al._ (1965). The virus had a history of 17 passages in ferret kidney tissue, 4 interpolated passages in ferrets, 4 passages in secondary dog kidney cultures and finally 2 passages in MDCK cells. The virus titre was $10^{4.5}$TCID per ml.

The following strains of CAV-1 were employed. Firstly, strain 47889 which was originally isolated from the liver of a dog suffering from acute hepatitis and secondly, strain EVI which was recovered from a tracheal organ culture prepared from a clinically normal dog (Campbell et al., 1968). The two strains were distinct from CAV-2 and their titres were $10^5 TCID_{50}$ per ml.

Organ cultures

Organ cultures were prepared by a method based on that of Hoorn and Tyrrell (1965). Tracheas were obtained from 8-week-old puppies and young adult dogs. The dogs were destroyed by the intravenous administration of pentobarbitone sodium and the tissues were removed immediately after death. The skin was reflected from the neck up to the level of the mandible and, after separation of the muscles, the oesophagus and trachea were removed. Their exterior surfaces were then washed and rinsed in PBS containing 1000units per ml penicillin and 1000µg per ml streptomycin and the oesophagus and adventitia were stripped from the trachea which was then divided into individual rings. Each ring was cut into squares about $3mm^3$ in size and 3 fragments were planted in a 60mm plastic Petri dish ('Falcon': Becton Dickinson, England); each dish was previously prepared with surgically incised grids (Fig. 129).

Once the tracheal explants were attached to the grids medium was added (1·5 to 2·5ml) until the surface was level with the epithelial edge of the tracheal fragment. The standard maintainance medium was composed of Eagle's medium supplemented with 0·2 per cent of bovine plasma albumen, 0·88 per cent sodium bicarbonate and penicillin and streptomycin at 100units per ml and 100µg per ml respectively. The cultures were incubated at 37^oC. in plastic sandwich boxes in an atmosphere of 5 per cent carbon dioxide in air.

The ciliary action was observed with a low power stereoscopic microscope using reflected light and only explants showing strong ciliary movement after 24 hours were retained for experimental purposes. The ciliary activity of each fragment was assessed visually and rated as normal, reduced or absent.

Experimental procedures

Organ cultures were inoculated with virus after 24 hours of incubation and this was designated day 0 of the experiment. The explants were infected by dropping 0·1ml of tissue culture fluid onto the ciliated surface of each fragment. After allowing absorption for 1 hour, the cultures were washed carefully and the medium replaced. Medium was replaced every 1 or 2 days, the old medium being harvested and stored at -30°C. until required for viral infectivity titration.

The fluid from control cultures was inoculated onto primary dog kidney cells to check for the presence of adventitious viruses.

Histological procedures

In each experiment samples of infected explants were removed daily for histological examination and non-infected control explants were sampled at the same time.

Sampled explants were washed with PBS, fixed in mercuric chloride-formol, double embedded in paraffin wax and sectioned at 6µm. Haemalum and eosin was used as a general histological stain and for some photographic preparations. Cytoplasmic inclusion bodies appeared best when stained with haemalum-phloxine-tartrazine after mordanting for 30 minutes in 3 per cent potassium dichromate. PAS and alcian blue were found satisfactory for the demonstration of mucin.

Explants infected with the adenoviruses were examined by the direct method of immunofluorescence as described in APPENDIX 2 and

samples of medium were collected at various intervals to determine the titre of extracellular virus. The adenoviruses were studied solely in explants prepared from adult dogs.

Virus titration procedures

Virus titrations were performed in primary dog kidney cell (DKC) cultures. Ten-fold dilutions of virus were made in maintainance medium and 1ml amounts were then inoculated into tubes of DKC to the number of 4 tubes per dilution. The cultures were examined up to 7 days and the end point assessed by the appearance of the typical adenovirus CPE. The median tissue culture infective dose was determined by the method of Reed and Muench (1938).

The DKC cultures were prepared by a modification of the method described by Vantsis (1959). Kidneys were obtained from young adult dogs within 10 minutes after death. The kidney capsule was removed and the cortex was separated and chopped finely. The minced tissue was transferred to an Erlenmeyer flask and rinsed thoroughly with PBS until the washings were clear. About 100cc. of 0·25 per cent trypsin was added to the tissue along with a sterile bar magnet and the tissue was agitated by means of a magnetic stirrer at $4^{o}C.$ for 16 hours. The suspension was then filtered using a sterile gauze-covered beaker and the filtrate centrifuged at 800 r.p.m. for 5 minutes. The supernatant fluid was discarded and the cells were resuspended in Earle's balanced salt solution and transferred to graduated centrifuge tubes. These tubes were centrifuged at 500 r.p.m. for 5 minutes and the packed cell volume read. Cell counts were not performed on the cell suspension because the presence of large clumps of cells rendered the count inaccurate. It was found, however, that when the packed cell volume was diluted 200 times in growth medium and incubated at $37^{o}C.$, good monolayers were formed by the 5th day.

Growth medium consisted of Earle's balanced salt solution plus 0·5 per

cent lactalbumin hydrolysate and 10 per cent foetal bovine serum with

penicillin, streptomycin and mycostatin at concentrations of 300 units,

300μg per ml and 80μg per ml respectively. For maintainance the

concentration of calf serum was reduced to 5 per cent.

Monolayers were grown in roller tubes held stationary and a

number of tubes from each kidney were kept as cell controls to check

for latent virus infection.

<div align="center">RESULTS</div>

1. Control explants

Ciliary activity was readily examined by reflected light under

the low power of the microscope. The area illuminated was relatively

small and the plastic dish had to be rotated in order to examine all

the surface of each explant. The beat of the cilia was easily discerned,

particularly at the edge of the explant, by the rapid, wave-like

pulsations of the thin layer of surface fluid which covered the

cultures. The uninfected control cultures showed good ciliary activity

for up to 20 days, though some variation occurred between explants. In

general a few cultures over 2 weeks of age showed a slight diminution of

activity and most cultures over 3 weeks of age showed reduced ciliary

movement. No difference could be detected between the explants prepared

from 8-week-old puppies and those from adult dogs.

The histological structure of the uninfected control cultures

was well maintained for about 14 days (Fig. 130), though there was a

loss of goblet cells. The respiratory tissue of freshly excised dog

trachea consisted of pseudostratified, ciliated, columnar epithelium with

a large number of goblet cells filled with acid mucopolysaccharide.

Goblet cells were abundant in cultures maintained _in vitro_ for up to

a week but were much less obvious in older cultures. Mucus production
however continued in the subepithelial glands and these occasionally
became blocked with eosinophilic debris. After 14 to 16 days in culture
the epithelium gradually became thinner and changed from a pseudostratified
layer to a more simple columnar epithelium, then to a low cuboidal layer
and finally to a squamous epithelium. Cilia were well preserved and a
ciliated columnar epithelium was still present in some cultures up to 36
days after explanting. A few mitotic figures were observed in the basal
layer of the epithelium and there was a slight outgrowth of squamous
epithelium over the cut edges of older explants.

2. Herpesvirus infection

A reduction in the frequency of ciliary beat in cultures infected
with both herpesviruses was first noted on day 3. Total cessation of
ciliary activity was observed in a few cultures on day 4 and the number
of inactive tissues rapidly increased through day 5 until there was
complete ciliostasis in all cultures examined on day 6.

The serial pathology of the explants was essentially similar
after infection with each of the viral strains. The first histopathological
changes observed appeared to begin at the cut surfaces of the explants
and then spread centripetally. Swollen epithelial cells containing
eosinophilic or amphophilic intranuclear inclusion bodies were seen at
the edge of the explant 1 day after infection (Fig. 131). The nuclei of
the infected cells were round and swollen with margination and clumping
of the chromatin. As the number of infected cells increased, the structural
integrity of the epithelium was lost and the cells detached (Fig. 132).
The epithelium was completely destroyed in all cultures examined after
the 7th day and the destructive effect had extended to the lining epithelium
of the mucous glands. Pathological changes were identical in explant
cultures prepared from puppies and older dogs.

3. Distemper virus infection

In tracheal organ cultures prepared from adult dogs, there was no detectable difference in the rate of ciliary activity between the cultures infected with distemper strain 841 and the uninfected controls. However, in the explants prepared from the 8-week-old puppies, the infected cultures showed reduced frequency of ciliary movement on day 10 and complete ciliostasis by day 15 whereas none of the control explants lost ciliary activity during the 20 day test period.

The histological structure of infected adult dog explants was well maintained for the first 17 days after infection and no differences could be detected when comparison was made with the uninfected controls. Cilia remained clearly distinguishable and the infected cultures did not reveal any significant changes until 18 days after infection. At this time the columnar structure was lost and the long axis of the remaining cells had reorientated itself into a horizontal position, thus giving the epithelium a low squamous appearance. Round or oval phloxinophilic cytoplasmic inclusions were noted on day 18 and persisted until day 32 when the experiment was terminated.

In explant cultures prepared from the puppies, inclusion bodies were detected as early as 6 days after infection (Fig. 133). At first, the epithelium appeared relatively normal but gradually it became thinner and by day 10 was flattened and squamous in appearance (Fig. 134). In the altered epithelium, some of the superficial cells became swollen and desquamated. Multinucleate cells or syncytial structures were not detected at any stage in the course of the infection.

4. Adenovirus infection

Both strains of CAV-1 were easily propagated in canine tracheal organ cultures on first passage and the amounts of virus released from these cultures are shown in Fig. 135. The growth curves indicate that

similar amounts of extracellular virus were produced with each strain and that virus production continued at a steady level over the period studied.

The ciliary activity in infected cultures declined after the 10th day and could not be detected by the 15th day after inoculation. Total cessation of ciliary activity of some virus infected explants was first seen on day 11 and the number of inactive tissues gradually increased through days 12, 13 and 14 until there was complete ciliostasis.

The serial pathology was essentially similar with both viral strains. The earliest cytopathic effect, first noted 2 days after infection of cultures, was necrosis of individual cells in the basal layer of the epithelium, cilia remaining clearly distinguishable (Fig. 136). This was followed by the appearance of larger foci of necrosis in which the normal, orderly arrangement of the epithelial cells was lost; the latter cells were swollen and distorted and a few contained large basophilic intranuclear inclusion bodies (Figs. 137, 139). These areas enlarged and the epithelium became hyperplastic, increasing in places, up to 12 cells in thickness; numerous inclusions were present in these hyperplastic regions until the last sample was taken 20 days post-infection (Fig. 138).

In the later stages of the infection, individual cells in the subepithelial tissues contained intranuclear inclusions. These cells appeared to be fibroblasts or the endothelial cells of collapsed capillaries.

By means of immunofluorescence, specific viral antigen was first detected in the nuclei of epithelial cells on the 2nd day. Viral antigen was encountered in the form either of large granules which were located throughout the nucleus or as a bright staining, formed inclusion (Fig. 140); antigen gradually increased in amount and persisted up to 20 days when the last explant was examined.

DISCUSSION

Explant cultures of dog trachea were technically straightforward to prepare. The structural integrity of the differentiated cells was well preserved, a pseudostratified ciliated epithelium being maintained for at least 14 days and in one case up to 36 days. There was, however, an early loss of mucous secreting cells and, in general, after 14 days the epithelium took on a columnar appearance and finally became squamous in type. The morphological findings on the control cultures were in accord with those of Herbst-Laier (1970) for tracheal explants prepared from 2-month-old puppies. In the present experiment, cultures were kept for a longer period of time and there was some outgrowth of squamous epithelium over the cut surfaces of the explants; Hoorn and Tyrrell (1965) also noted this in explants prepared from human foetal material.

The ciliary activity was easily observed and was present for up to 21 days in uninfected cultures. Heyke (1969) found ciliary movement in dog tracheal cultures as long as 19 days.

The cytology and ciliary activity recorded above is very like that described for other species. Campbell et al. (1969) and Reed (1969) found strong ciliary activity in calf and pig tracheal explants for up to 20 days and a pseudostratified ciliated epithelium was also well preserved.

In the present investigation, control cultures were carefully checked for the presence of latent virus infections and none were found. Earlier work had, however, emphasised the need for vigilance as the EVI strain of adenovirus was originally isolated from tracheal organ cultures prepared from a clinically normal dog (Campbell et al., 1968). A search of the literature has failed to reveal any other reports of the isolation of adventitious viruses from tracheal cultures but this

is probably because most workers have used either foetal or neonatal tissues which are less likely to be contaminated.

Both strains of CHV produced an acute cytopathic effect with rapid ciliostasis. The cytopathology of the infected explants, which was characterised by necrosis and the presence of eosinophilic intranuclear inclusions, resembled that described in the respiratory epithelium of the live dog infected with CHV (see PART 1). Inclusions were more readily found in the tracheal explants than in the living dog and this may be related to temperature. The explants were incubated at 37°C. and it has been shown that CHV grows best at this temperature (Carmichael and Barnes, 1969). It would be of interest to compare the cytopathology and yield of virus in explant cultures infected with CHV and incubated at different temperatures.

The histopathological effects of CHV in tracheal explants in this study were similar to the changes described by Shroyer and Easterday (1968) for infectious bovine rhinotracheitis virus in calf tracheal explants and by Hoorn (1964) for herpes simplex in human embryo and rabbit tracheal cultures. In all of these experiments the ciliary activity stopped within a few days and the virus titre in the medium rose quickly.

The degree of epithelial damage following infection with distemper virus was much less than with CHV. The cytopathological changes in distemper virus infected cultures were characterised by the appearance of phloxinophilic cytoplasmic inclusion bodies, a reduction in height of the ciliated epithelium and reorientation of the cells to give a squamous appearance. In the explants prepared from puppies these changes developed about day 10 after infection but in the cultures from the older dogs they were not seen until day 18. As both cultures were infected in the same manner this difference in response could be related

to the age or the immune status of the donor animal. Cesario et al. (1970)
found no difference in susceptibility to parainfluenza virus infection
between tracheal organ cultures prepared from immune and non-immune rats.
In organ culture studies little attention has been paid to the influence
of the age of the donor animal and this would seem to be a factor worthy
of further consideration.

The cytoplasmic inclusions observed in the epithelium of the
tracheal explants were similar to those seen in vivo with distemper
virus infection (Lauder et al., 1954) but squamous metaplasia has not
been reported in either natural or experimental cases. Multinucleate
cells or syncytial structures were not found and this might be due to
the fact that the strain of distemper virus used in this study had been
passaged many times in tissue culture. Campbell et al. (1969) observed
multinucleate giant cells in calf tracheal explants infected with a
recently isolated strain of bovine PI3 virus whereas this was not
described by Kita et al. (1969) in a similar study using a strain of
PI3 which had been passed many times in bovine kidney cells. A less
attenuated strain of distemper virus might therefore produce a different
cytopathological picture in dog tracheal cultures.

Both strains of CAV-1 multiplied readily in canine tracheal
explant cultures and produced an acute cytopathic effect. There were
no obvious differences in the behaviour of the two viruses in organ
culture although strain 47889 had been originally isolated from a case
of hepatitis whereas EVI was recovered from respiratory tissue. The main
pathological changes in the CAV-1 infections were necrosis, proliferation
and the formation of basophilic intranuclear inclusions. Similar sequence
of events have been found in the respiratory epithelium of dogs
experimentally exposed to CAV-1 either intratracheally or by aerosol
(PART 2 SECTION I).

In general the titres of CAV-1 in the explant medium were lower than those obtained in kidney monolayer cultures (Cornwell et al., 1970). It is likely that much of the newly-formed virus remained in the organ cultures; retention of human adenovirus in foetal explant cultures has also been demonstrated by Hoorn (1966).

The growth curves show that the canine adenoviruses were present in the culture medium up to 8 days after inoculation and with immunofluorescence viral antigen was detected in the epithelium up to 20 days. Heyke (1969), studying the growth of an attenuated strain of CAV-1 in canine tracheal explants, found variations in the amount of virus produced by different cultures but CAV-1 was still present in some cases at 19 days after inoculation. It may be that, in the absence of a humoral response in organ cultures, CAV-1 is capable of prolonged replication. Several workers have described persistent virus infection in tracheal cultures infected with bovine PI3 virus (Campbell et al., 1969; Reed, 1969).

The results from this investigation show that 3 of the viruses which can cause canine respiratory disease produce distinctive lesions in the ciliated epithelium of tracheal explants. In general the changes found resemble those described in the intact animal body, although inflammatory and humoral responses are of course absent in the explants. However, since vascular changes and cellular infiltrations cannot complicate the explant cytology, it should be possible to study more closely the pathology of canine respiratory virus infections at the cellular level.

SUMMARY AND CONCLUSIONS

In his article entitled "Discovering and Defining the Aetiology of Acute Respiratory Viral Disease", Tyrrell (1963) pointed out that the study of any new infectious disease followed a distinct pattern. In the first phase, an agent is discovered, in the second it is shown that the agent can and does cause disease, in the next the full range of disease in which the agent can play a part is defined and lastly over a period of time, the epidemiology of the infection is worked out. The aetiology and pathogenesis of canine respiratory disease is complex. It is now evident that a number of viruses may be involved and for each of these agents the pattern set forth above is beginning to take shape. The present investigation provides new information on 3 of these viruses.

The role of canine herpesvirus (CHV) as a cause of neonatal death in puppies is well documented (Cornwell and Wright, 1969). In America, the virus has recently been isolated from dogs with respiratory disease but there are conflicting claims regarding its pathogenicity for older dogs. PART 1 of this work provides a description of the pathological changes in the respiratory tract of an adult dog with respiratory disease, and then describes in detail the isolation of CHV from this particular case. Experimental studies with the new isolate showed that when the virus was administered by aerosol a mild respiratory disease was produced and foci of epithelial necrosis were detected in the respiratory tract. When inoculated into newborn puppies, the respiratory isolate caused an acute, lethal, generalised infection similar to that reported in natural cases of neonatal herpes infection (Cornwell and Wright, 1969).

Much of the recent interest in the viral aetiology of canine respiratory disease has centred around the canine adenoviruses. It is now recognised that there are two serologically distinct strains, CAV-1 and CAV-2. CAV-2 has been isolated from the respiratory tract of dogs

in North America (Ditchfield et al., 1962) and more recently in the
United Kingdom (Danskin, 1973) and respiratory disease has been
produced with this virus in susceptible dogs (Swango et al., 1970).
So far, the virus has not been shown to be associated with naturally
occurring hepatitis. CAV-1 is the causal agent of canine viral hepatitis
and PART 2 of this work shows that it is also involved in respiratory
disease. Both canine adenoviruses have therefore been isolated from
dogs with respiratory disease and, under experimental conditions, both
viruses can induce necrotizing lesions in the respiratory tract of
experimental dogs following challenge by the respiratory route. It would
seem that, under both natural and experimental conditions, both viruses
can produce a mild respiratory infection in which a transient cough,
nasal discharge and anorexia are the main features. However, if CAV-1 is
administered by intratracheal inoculation, a more severe, often fatal
respiratory disease ensues. Histological examination of the respiratory
tract has shown the main lesion in both virus infections to be a
necrotizing bronchiolitis and focal necrosis of the nasal and tonsillar
epithelium are also observed; typical adenovirus intranuclear inclusions
can be demonstrated in these lesions and large amounts of virus are
found. Following recovery, proliferative changes occur in the bronchiolar
epithelium.

It is clear that when CAV-1 is administered by the respiratory
route, lesions are confined to the respiratory tract and, although virus
can be detected elsewhere in the body, hepatitis does not occur.
Susceptible animals kept in separate pens from the dogs infected with
CAV-1 by aerosol also developed a mild respiratory disease, presumably
as a result of aerosolisation of virus.

The isolation of both strains of adenovirus from outbreaks of
respiratory disease and the relative ease with which infection can be

produced in experimental puppies would seem to establish these viruses as primary causes of canine respiratory disease. Detailed epidemiological studies however are awaited to assess their relative importance.

The efficiency of current vaccination procedures in the control of adenovirus induced respiratory disease is not known. Preliminary studies with CAV-1 have shown quite clearly however that dogs solidly immune to intraperitoneal challenge with adenoviruses are still susceptible to challenge by aerosol (Wright et al., 1974). If it is eventually established that adenoviruses are important agents in respiratory disease, then clearly an evaluation of current vaccination procedures against them would be indicated.

Under experimental conditions, only mild pathological changes were induced in the respiratory tract of experimental dogs following challenge with reovirus type 1. Clinical abnormalities were not detected in the experimental dogs and lesions were confined to the lungs which showed exudation of macrophages into the alveolar airspaces and thickening of the alveolar septa. A limited serological survey showed antibody to be present at significant levels in approximately 20 per cent of the dogs tested.

Parainfluenza virus SV-5 has been frequently isolated from dogs with respiratory disease in the USA but so far has not been isolated from dogs in the United Kingdom. However, Danskin (personal communication - 1973) has demonstrated antibody to the virus in dogs from various parts of England and Scotland and it would seem to be only a matter of time before this agent is discovered in dogs in Gt. Britain.

Parainfluenza SV-5, CHV, CAV-1 and reovirus type 1 can usually be cultivated in monolayer cultures of canine kidney cells. However, not all respiratory viruses can be isolated in this way. In the case

of the human rhinoviruses, some strains can be isolated only with
difficulty if at all in monolayer cultures and organ cultures of human
embryo, nasal and tracheal epithelium have been used to facilitate
their isolation (Hoorn and Tyrrell, 1969). As more attention is paid
to the aetiology of canine respiratory disease, similar problems may
well be encountered. PART 4 of this work shows that dog tracheal organ
cultures are easily prepared and maintained. Furthermore, the pathological
changes produced in the respiratory epithelium of the organ cultures
by CDV, CAV-1 and CHV were not unlike those found in the living animal.
Organ culture systems should prove of value in the study of canine
respiratory disease.

A number of reports in the literature have pointed out that
dual infections with viruses such as adenoviruses and distemper virus
may occur in the same animal (Stookey et al., 1972) and, indeed, in any
one outbreak of disease in kennel populations, a number of different
viruses may be involved (Binn et al., 1967). For that reason it seems
logical that the next step in the study of canine respiratory virus
disease will be controlled, experimental, and multiple, virus infections.

The part that bacteria and mycoplasmas play in modifying virus
infections in the field is not clear. Certainly in outbreaks of
respiratory disease in which viruses have been implicated, a variety
of bacteria and mycoplasmas have also been isolated (Binn et al., 1968).
Although many of these agents are present in the normal bacterial
flora of the respiratory tract of dogs, their presence may be necessary
for the clinical progression of the respiratory disease. It may, however,
be unwise to dismiss respiratory bacteria as being purely opportunistic
since recent work with strains of Bord. bronchiseptica has shown that,
under experimental conditions, the exposure of dogs to an aerosolized
stream of bacteria produces a transient clinical respiratory disease

with coughing and purulent nasal discharge (Wright et al., 1973).
More detailed studies are therefore needed to reassess the importance
of bacteria, in particular, <u>Bord</u>. <u>bronchiseptica</u>, in canine respiratory
disease and also to examine mixed viral-bacterial infections.

Until 1962 CDV was the only canine viral respiratory pathogen
recognised but, in the last decade, 5 new viruses have been recovered.
When one considers that 118 distinct viruses belonging to 7 different
virus groups (Chanock, 1970) have been identified as aetiological
agents of diseases of the human respiratory tract, it is not unreasonable
to assume that further canine respiratory viruses will be discovered.
It is hoped that the present work will provide a pathological basis
against which one can compare and contrast these new virus infections.

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APPENDICES

APPENDIX 1

ELECTRON MICROSCOPY

1. <u>Fixatives</u>. The fixatives were prepared as follows.

   (a) Osmium Tetroxide

      1 per cent osmic acid (BDH Chemicals Ltd., Poole, England) was

      made up in Millonig's buffer at pH 7·2 to 7·4.

   (b) Glutaraldehyde

      A stock solution of 25 per cent glutaraldehyde stabilised at

      pH 5-6 was used (Searle scientific services, High Wycombe, England).

      The fixative was made up to a 2 per cent solution in 0·067M

      Sorensen's phosphate buffer, pH 7·2 to 7·4.

   (c) Paraformaldehyde/glutaraldehyde

      A mixture of 1·3 per cent paraformaldehyde and 1·6 per cent

      glutaraldehyde was prepared in cacodylate buffer at pH 7·2 to 7·4.

      The proportions were :-

Paraformaldehyde	2g.
Distilled water	25ml.
I N Sodium hydroxide (NaOH)	1-3 drops
25 per cent glutaraldehyde	10ml.
Cacodylate buffer	115ml.
Anhydrous calcium chloride (Cacl$_2$)	25mg.

2. <u>Buffers</u>.

   (a) Millonig's phosphate buffer

Sodium dihydrogen phosphate (NaH$_2$PO$_4$ 2H$_2$O) (2·26 per cent	83ml.
NaOH (2·52 per cent)	17ml.
Distilled water	10ml.
Sucrose	0·54g.

      Final pH 7·2 to 7·4.

(b) 0·067M Sorensen's buffer was prepared as follows:-

Di-sodium hydrogen orthophosphate

$(Na_2HPO_4)(9·512gm/litre)$ 3 parts

Potassium dihydrogen orthophosphate

$(KH_2PO_4)(9·118g/litre)$ 1 part

Final pH 7·2 to 7·4.

(c) Cacodylate buffer was prepared from a stock 0·1M solution of

sodium cacodylate (21·4g/litre) as a 0·067M solution of

14·331g/litre. A few drops of concentrated hydrochloric acid were

used to adjust the pH to 7·2 to 7·4.

(d) Michaelis buffer

Barbitone sodium ($(C_2H_5)_2C.Co.NH.C(ONa):N.CO$)   14·7g.

Sodium acetate $(CH_3COONa3H_2O)$ 9·7g.

Distilled water   .   500ml.

3. Embedding resins

Two preparations were used.

(a) Araldite

Equal parts of Araldite resin (CY212)(Ciba-Geigy(UK) Ltd. Cambridge,

England) and Araldite hardener (HY964)(Ciba-Geigy (UK) Ltd.) were

mixed by stirring overnight and stored at 4^oC. This formed mixture (1).

Before use 0·6ml of accelerater (HY960) (Ciba-Geigy (UK) Ltd.) and

2·4ml of Di-n-Butyl-phthalate (BDH) were added to 57ml of mixture (1)

and the whole stirred well for 30 minutes. Hardening was at 57^oC. for

48 hours.

(b) Epon

     85.4ml Epon 812 (Epikote 812, Searle scientific services)

     86.4ml D.D.S A (Searle scientific services)

     28.2ml N.M.A. (Searle scientific services)

The mixture was thoroughly stirred and left at room temperature.

Before use 2 per cent D.M.P. 30 (Searle scientific services) was

added and well mixed. Hardening was at $60^{\circ}C$. for twenty four hours.

4. <u>Stains</u>

  (a) Uranyl acetate

    20 per cent uranyl acetate (BDH) in absolute methanol.

  (b) Lead citrate

Lead nitrate $Pb(NO_3)_2$	1.33g.
Sodium citrate $Na_3 C_6H_5O_7 . 2H_2O$	1.76g.
Distilled water	30ml.
I N NaOH	8ml.

    pH 12 to 12.1.

APPENDIX 2

IMMUNOFLUORESCENCE

1. Preparation of tissues for immunofluorescence

Small blocks of tissue approximately 5mm^3 in size were

snap-frozen by placing them on the inner side of a Pyrex test tube and

partly immersing the tube in a dry ice-alcohol freezing mixture. The

blocks were stored at -20°C. until required for examination.

Frozen sections were cut at 4-6μm on a Slee cyostat.

2. Preparation of globulin for immunofluorescence

Antiserum to CAV-1 was prepared in a dog aged 6 months which

received 1ml of the virus orally and, after a period of 14 days, a

further dose by the intravenous route. Ten days after inoculation, the

dog was bled by cannulation of the femoral artery under surgical

anaesthesia.

The antiserum was fractionated by half-saturation with ammonium

sulphate. Equal volumes of chilled serum and saturated ammonium sulphate

were mixed thoroughly, refrigerated at 4°C. for 10 minutes and

centrifuged in the cold. The supernatant was drained off and the globulin

fraction was resuspended in PBS to one third of the original volume of

serum. The protein solution was then dialysed for about 18 hours at 4°C.

against PBS to remove the ammonium ions. Rabbit anti-dog globulin and

bovine gamma globulin were obtained from a commercial source (Sylvana,

New Jersey, U.S.A.).

3. Preparation of conjugate for immunofluorescence

The globulin preparations were conjugated with 10 per cent

fluorescein isothiocyanate on celite powder (FITC)(Calbiochem, California,

U.S.A.) as recommended by Rinderknecht (1962). Ten mg. of dry FITC was added

to 2ml of the globulin solution and the mixture was left for 1 hour at room temperature to allow the dye to dissolve. Free dye remaining in the solution after this time was removed by passing the conjugates through a column of Sephadex G25 (Pharmacia, Uppsala, Sweden).

The conjugates were further purified by absorption overnight at 4°C. with 100mg of a mixture of dog liver and kidney tissue powder per 1ml of conjugate.

4. Preparation of tissue powder.

Samples of liver and kidney were obtained from young healthy dogs. The tissues were washed in saline and homogenised in a Waring blender. The homogenates were washed several times in large volumes of acetone before the tissue particles were allowed to sediment. The supernatant was removed and the material spread on filter paper to be dried overnight in an incubator at 37°C. It was then ground in a mortar, passed through a sieve to remove fibrous material and stored at -20°C.

5. Direct method of immunofluorescence for viral tracing.

Sections, 4-6μm, were fixed in acetone for 15 minutes and then exposed to the CAV-1 conjugate for 30 minutes in a moist chamber at room temperature. After thorough washing in PBS to remove the unreacted conjugate, the stained sections were mounted in PBS and examined under a fluorescence microscope ("Orthoplan": Leitz, Germany) equipped with an exciter filter (BG12), barrier filter and dark ground condenser.

Control sections consisted of (a) normal uninfected tissue stained as above and (b) infected tissue in which conjugated bovine gamma globulin was used in place of the conjugated canine hyperimmune globulin.

6. <u>Indirect method of immunofluorescence for detection of CAV-1 antibody.</u>

A section of liver from a known case of infectious canine hepatitis was exposed to the test serum for 30 minutes and, after washing in PBS, was stained with a rabbit anti-dog globulin conjugate for a further 30 minutes. Bright, apple green fluorescence in the nuclei of the hepatic and Kupffer cells indicated the presence of specific CAV-1 antibody in the serum.

ADDENDUM

ADDENDUM.

The following publications have arisen from this work.

Thompson, H., Cornwell, H.J.C., Wright, N.G. and Campbell, R.S.F.
(1972) Canine Adenovirus in Organ cultures. Res. vet. Sci. 13, 191.

Thompson, H., Wright, N.G. and Cornwell, H.J.C. (1970) Experimental
Reovirus Infection in Dogs: a Preliminary Report. Res. vet Sci.,
11, 302.

Thompson, H., Wright, N.G. and Cornwell H.J.C. (1972) Experimental Canine
Herpesvirus Respiratory Infection, Res. vet. Sci., 13, 123.

Wright, N.G., Cornwell, H.J.C., Thompson, H., Armitage, A. and Morrison, I.
(1972) Canine Adenovirus Respiratory Disease: Isolation of
Infectious Canine Hepatitis Virus from Natural Cases and the
Experimental Production of the Disease, Vet. Rec., 90, 411.

Wright, N.G., Cornwell, H.J.C., Thompson, H. and Stewart, M. (1970)
Canine Herpesvirus Respiratory Infection,
Vet. Rec., 87, 108.

Wright, N.G., Thompson, H. and Cornwell, H.J.C. (1970) Experimental
Pneumonia in the Dog Due to the Virus of Infectious Canine
Hepatitis, Res. vet. Sci., 11, 385.

Wright, N.G., Thompson, H. and Cornwell, H.J.C. (1971) Canine Adenovirus
Pneumonia, Res. vet. Sci., 12, 162.

CONTAGIOUS RESPIRATORY DISEASE IN DOGS

THESIS

Submitted for the degree of Doctor of Philosophy in the

Faculty of Veterinary Medicine, University of Glasgow

by

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Department of Veterinary Pathology,

University of Glasgow.   March, 1974.

VOLUME 2

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Fig. 1 Natural CHV infection. There are dark areas of

consolidation (arrows) in the apical, cardiac and

diaphragmatic lobes of the lungs.

Fig. 2   Natural CHV infection: tracheitis. The epithelium is

reduced to a low columnar or cuboidal layer and a diffuse

cellular infiltrate is present in the lamina propria.

Note the focal area of epithelial hypertrophy at the

top left hand corner of the section.

Haemalum and eosin.   X 120.

Fig. 3   Natural CHV infection. Intranuclear inclusion bodies

(arrows) can be seen in tracheal epithelial cells.

Haemalum and eosin.   X 1,200.
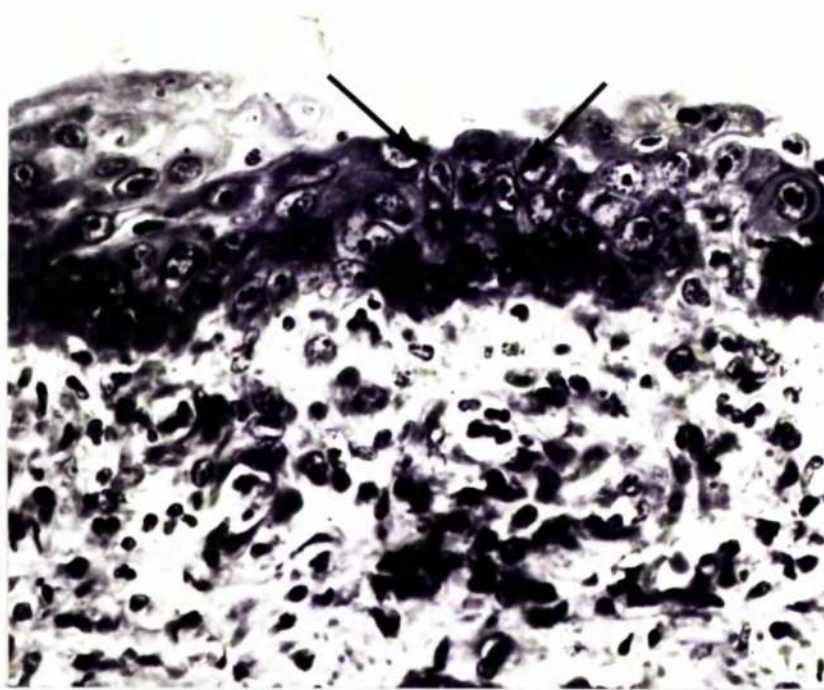
Fig. 4   Natural CHV infection. A purulent cellular exudate is

present in the lumen of the bronchiole and there is

peribronchiolar cuffing with mononuclear cells. A

diffuse cellular exudate is present in the alveoli and

around the bronchiole there is alveolar epithelial

hyperplasia.

Haemalum and eosin. X 150.

Fig. 5   Natural CHV infection: bronchiolitis. The bronchiolar

epithelium is vacuolated and there is infiltration by

lymphocytes and macrophages. The lamina propria contains

many lymphocytes and plasma cells while polymorphonuclear

leucocytes, erythrocytes and macrophages are present in

the lumen.

Haemalum and eosin. X 400.
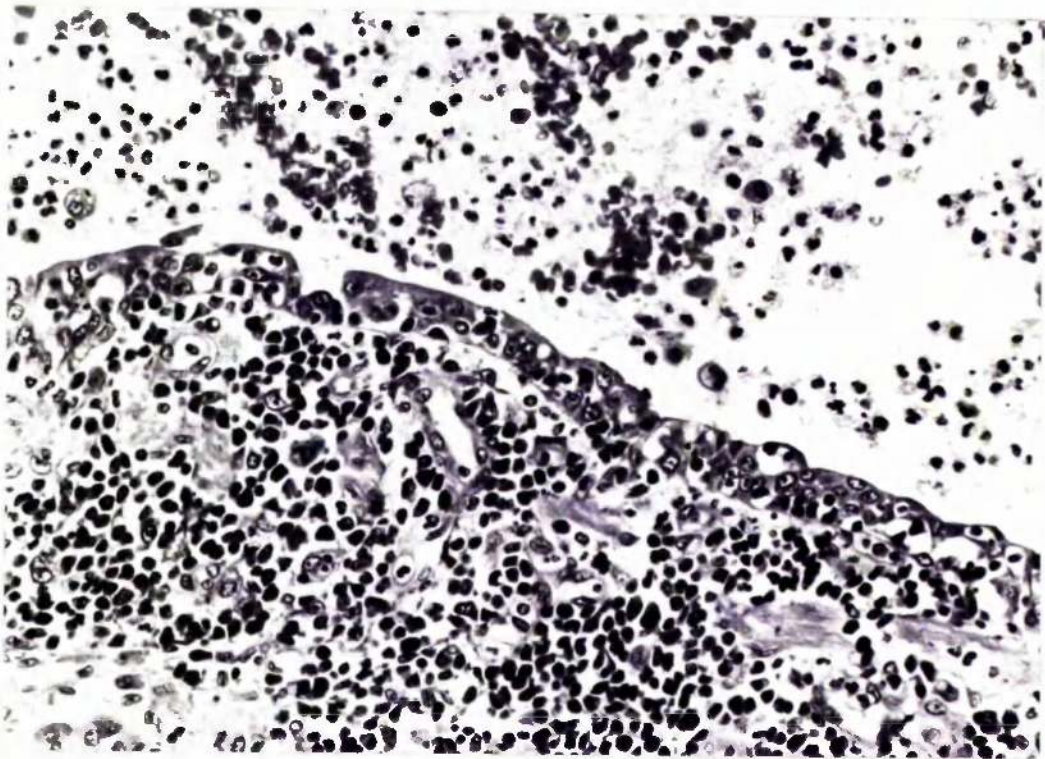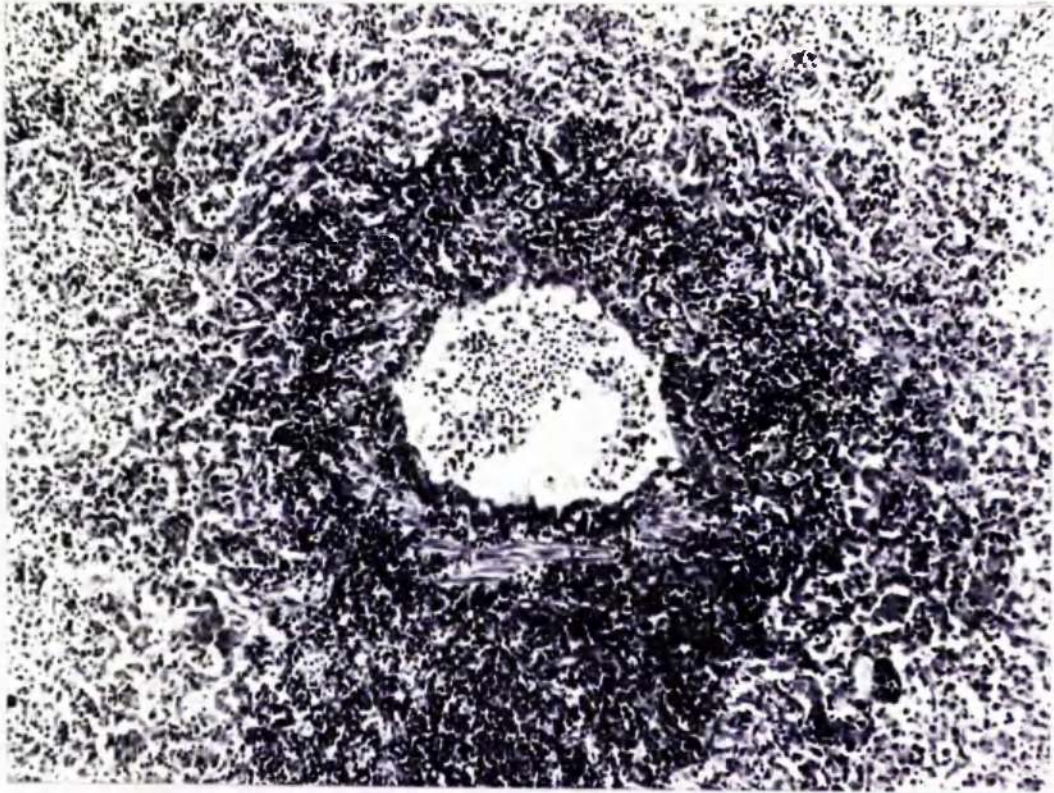
Fig. 6   MDCK cell culture 48 hours after inoculation with

CHV-G3. Note the foci of rounded, refractile cells.

Unstained.   X 80.

Fig. 7   Uninfected MDCK cell culture.

Unstained.   X 80.
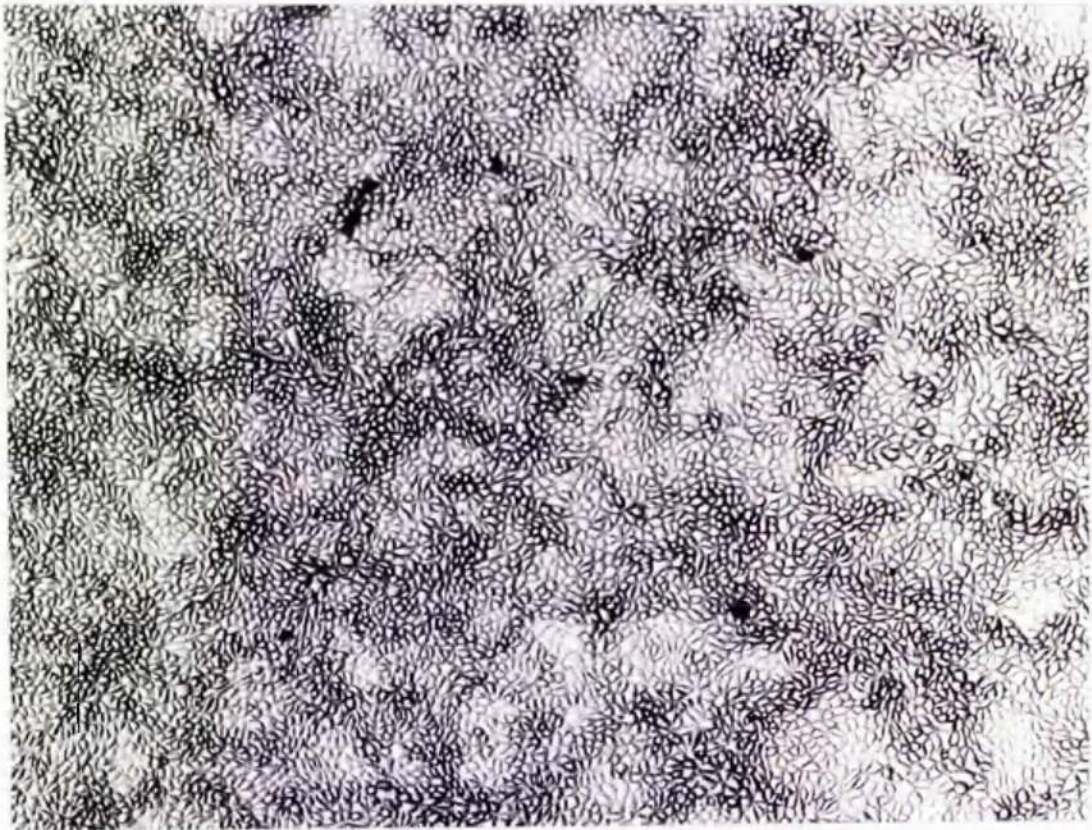
Fig. 8   MDCK cell culture 48 hours after inoculation with

CHV-G3. Focus of rounded refractile cells.

Unstained.   X 500.
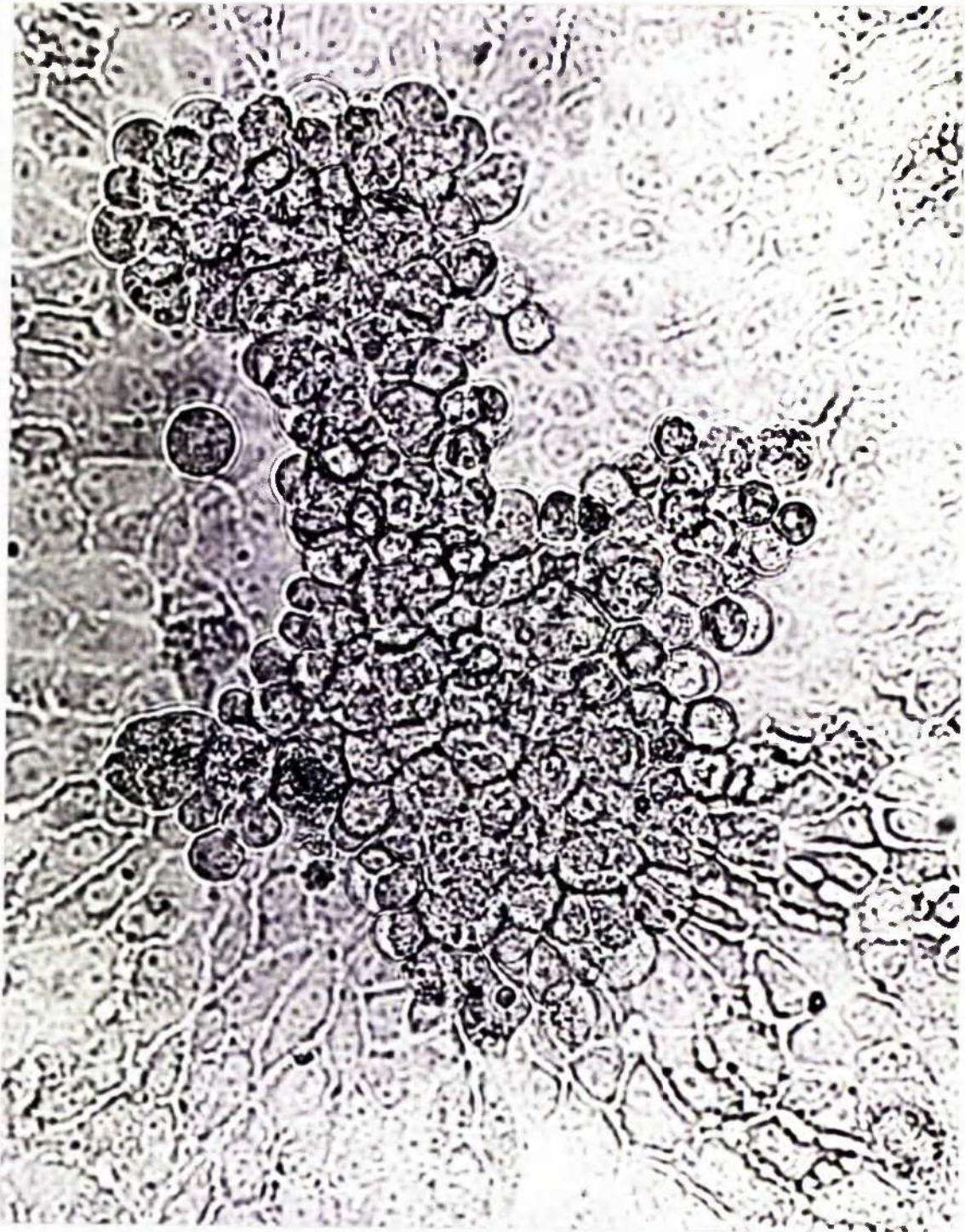
Fig. 9   Plaque formation by CHV-G3 in MDCK cell monolayer,

4 days after inoculation. Serial 10 fold dilutions.

Leishman's.   natural size.

Fig. 10   Plaques produced by CHV-G3 in an MDCK cell monolayer

under agar.

Leishman's.   X 10.
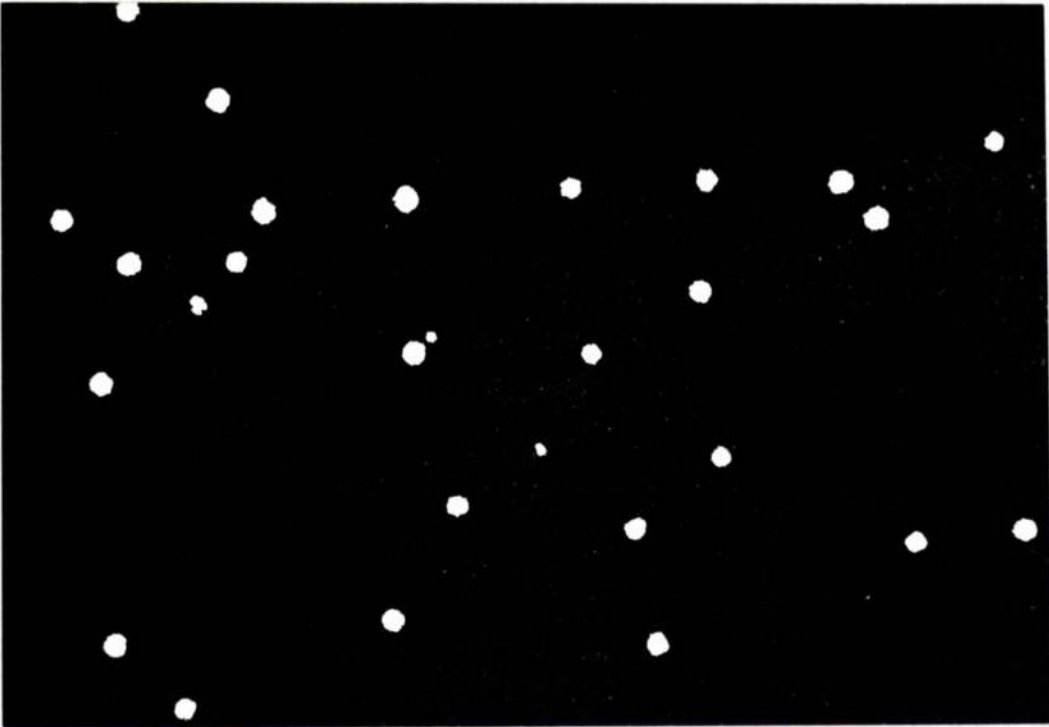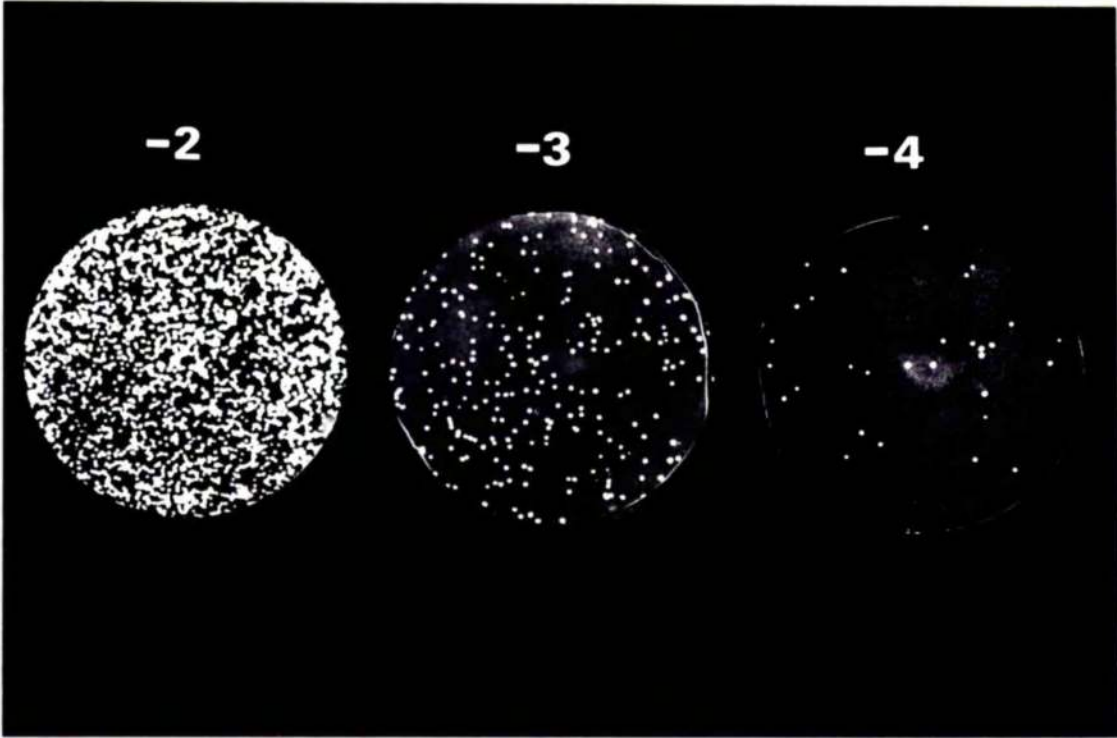
Fig. 11   An infected focus in an MDCK cell culture 48 hours

after inoculation with CHV-G3. The cells in the centre

of the focus have detached while those at the edge are

piled upon one another.

Acridene orange. X 300.

Fig. 12   A single intranuclear inclusion in an MDCK cell, 24

hours after inoculation with CHV-G3.

Giemsa.   X 1,500.
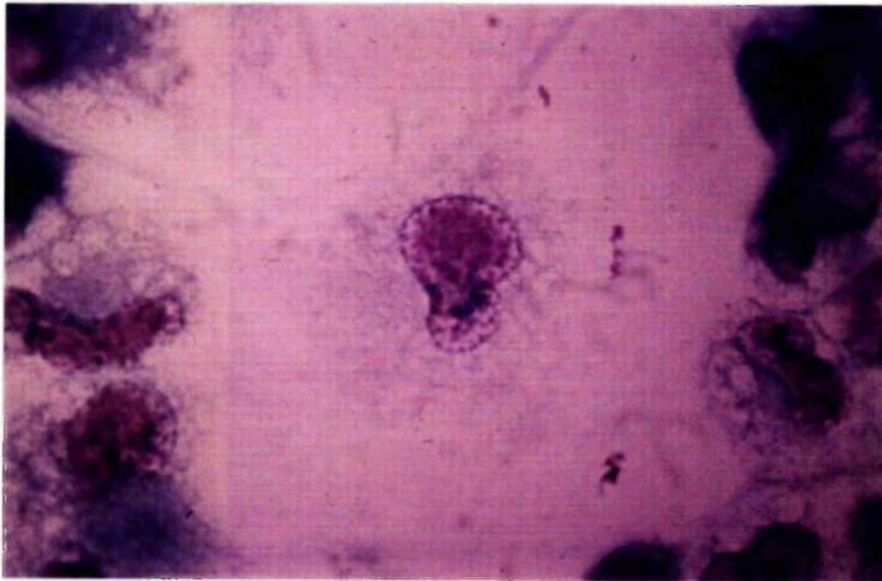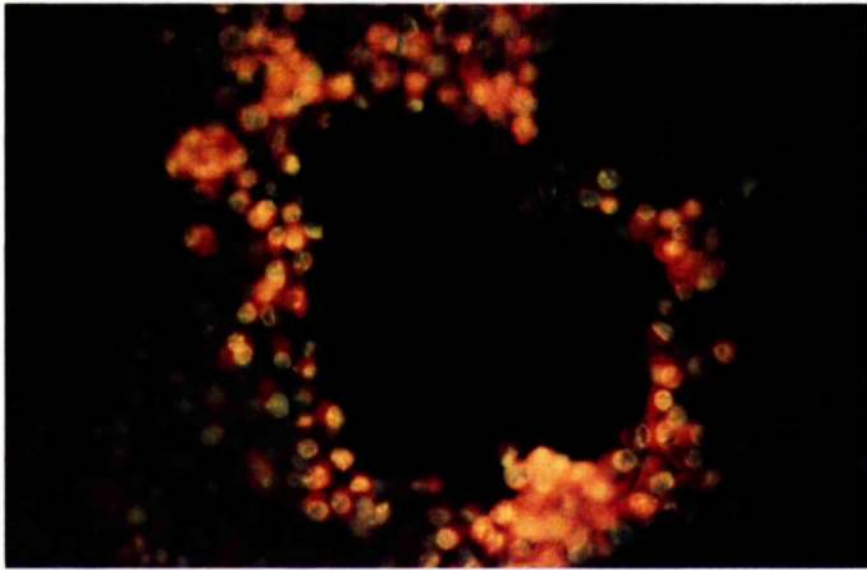
Culture fluid harvested from infected MDCK cell culture.
Virus particles negatively stained with 2 per cent phosphotungstic
acid.

Fig. 13 (top left)   Unenveloped nucleocapsid.   X 120,000.

Fig. 14 (top right) Nucleocapsid showing elongated capsomeres.

X 140,000.

Fig. 15 (bottom)   Complete enveloped virions.   X 150,000.
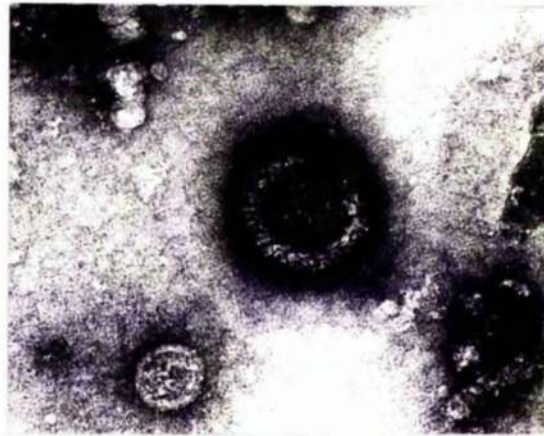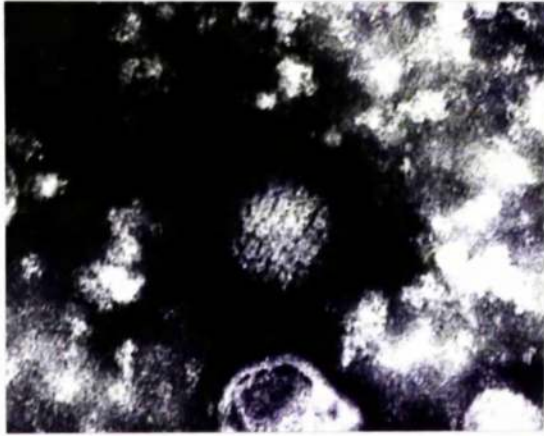
Fig. 16   Thin section of MDCK cell 24 hours after infection with

CHV-G3. Empty capsids (1) and capsids containing

various types of cores (2, 3 and 4) are present in

the nucleus.

X 60,000.
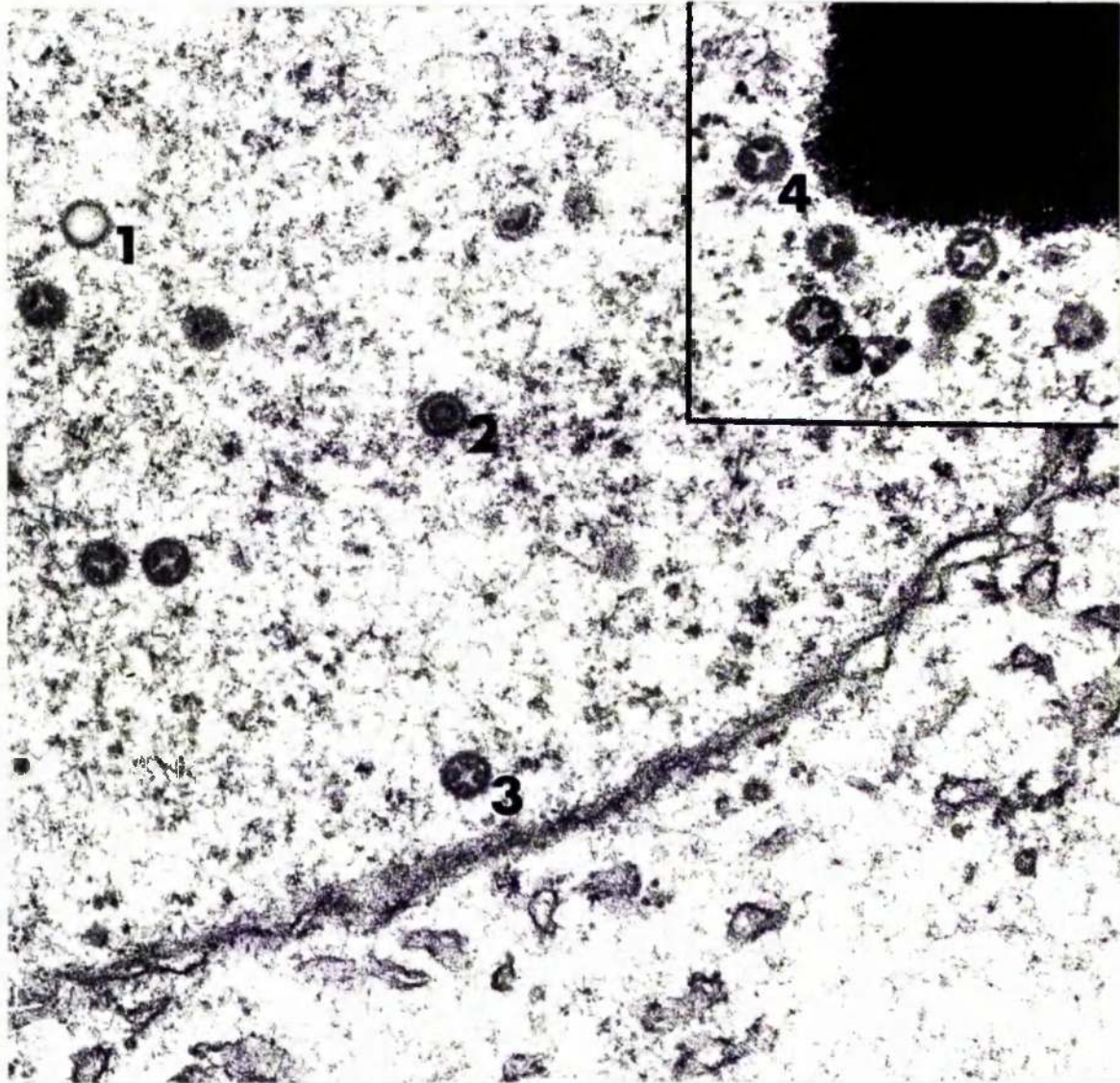
Fig. 17   Thin section of MDCK cell 48 hours after inoculation

with CHV-G3 to show a cytoplasmic membrane bound

vesicle (Ve) filled with enveloped virus particles.

X 60,000.
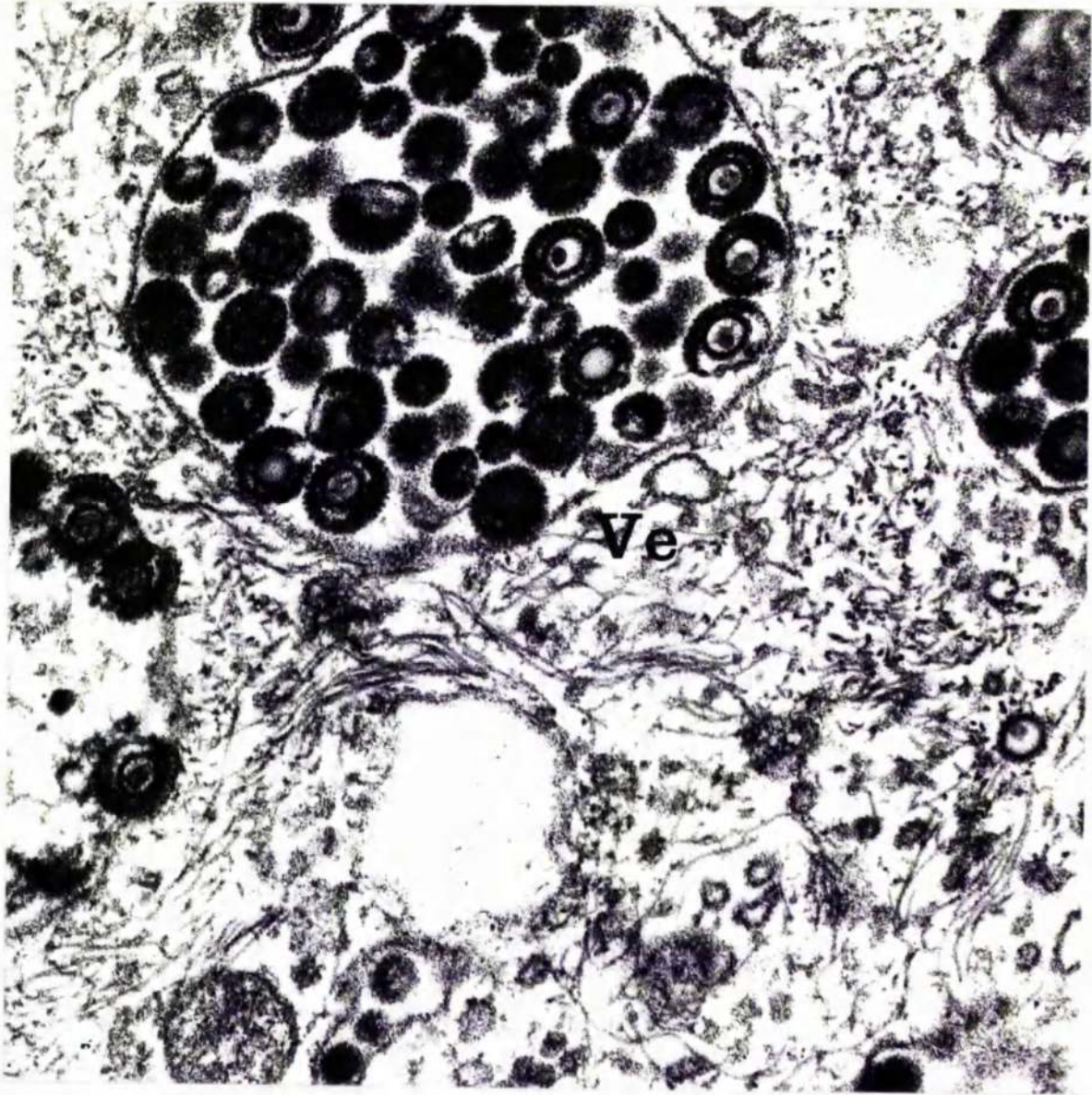
Fig. 18   Thin section of MDCK cell 48 hours after inoculation
with CHV-G3 to show enveloped virus particles at the
cell surface.

X 40,000.
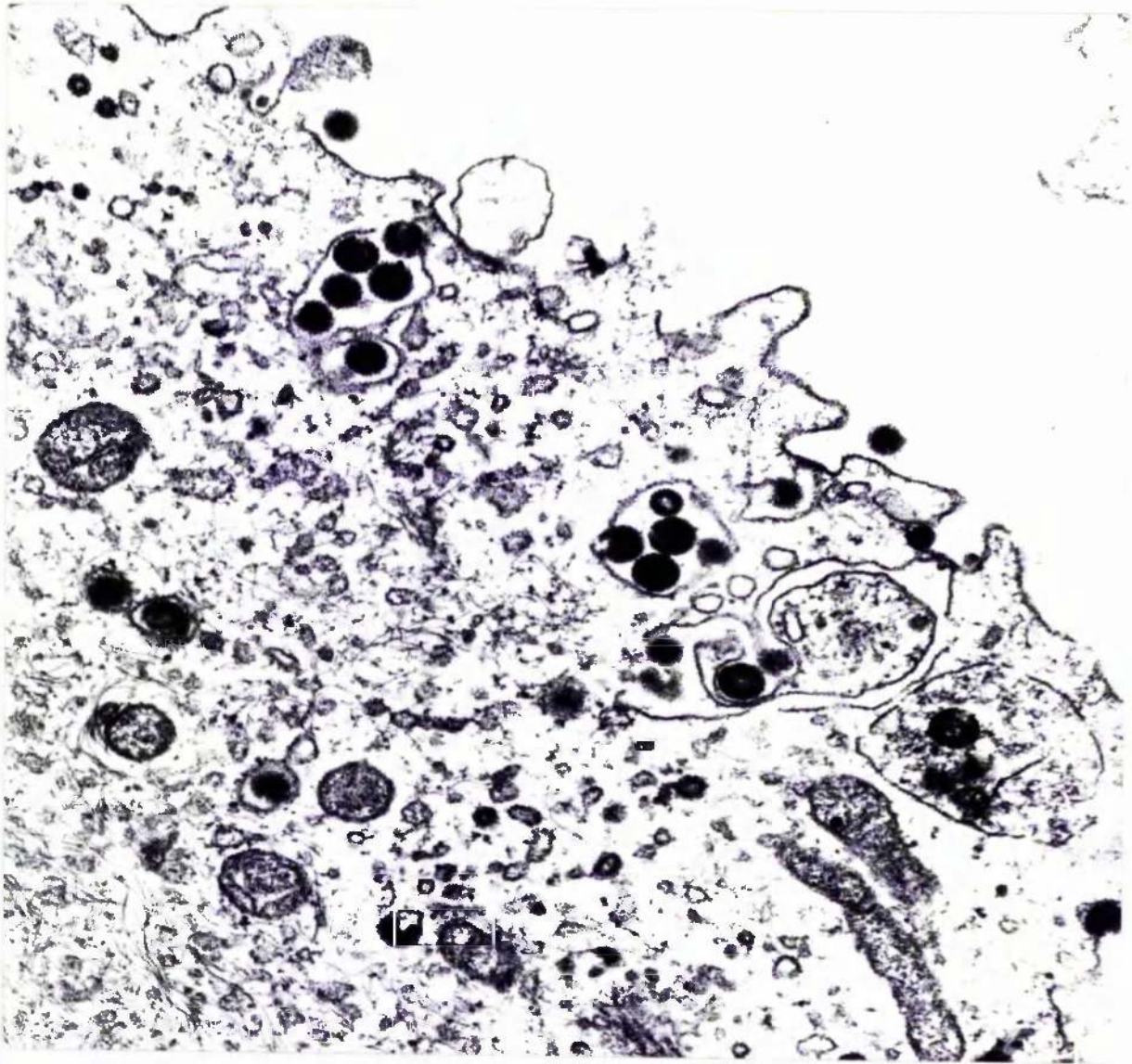
Fig. 19   Enveloped virus particles in the cytoplasm of an MDCK

cell, 48 hours after inoculation with CHV-G3. The

envelope is composed of 3 main layers: (1) stubby

projections, (2) a unit membrane, (3) electron dense

amorphous material.

X 150,000.
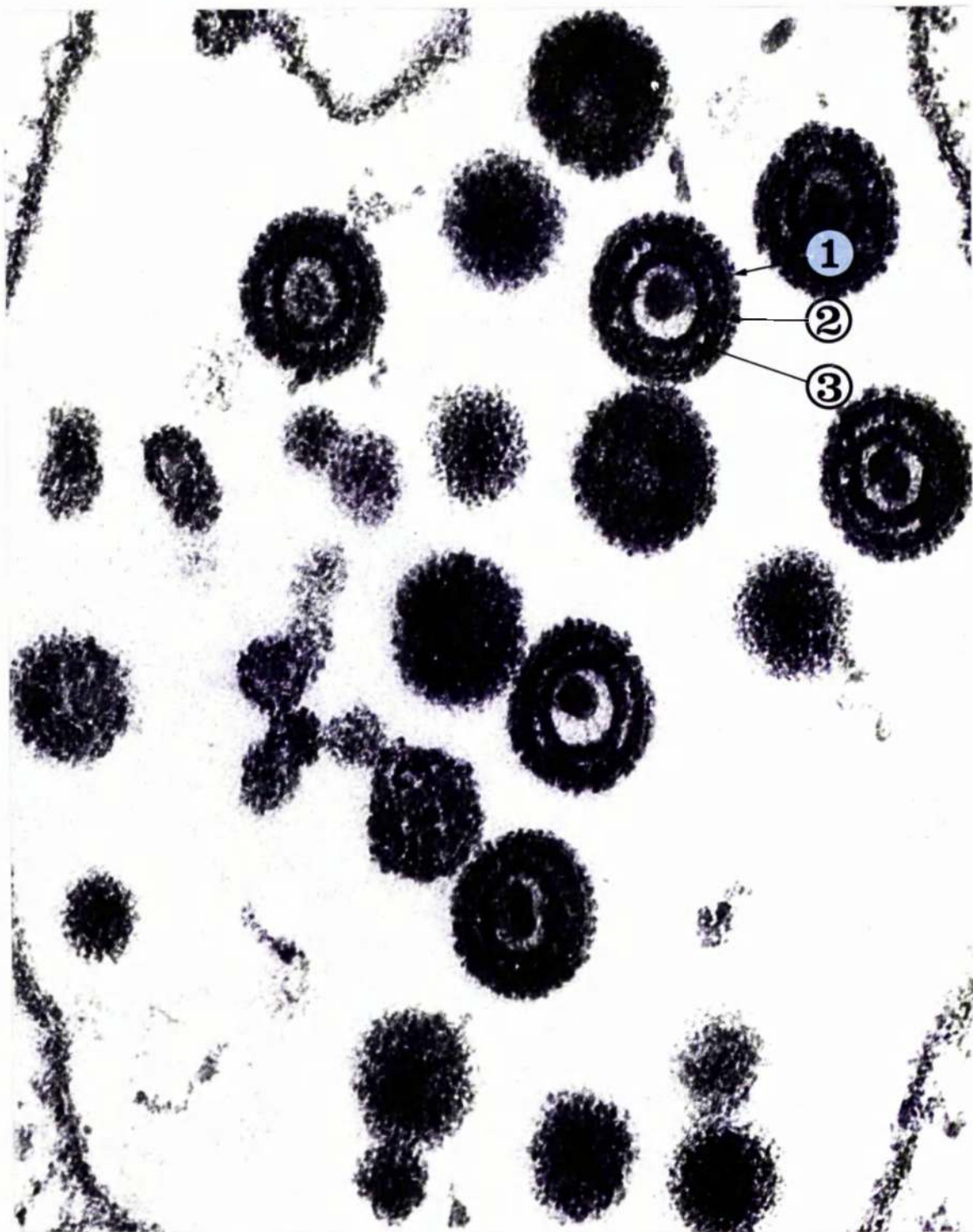
Fig. 20   Thin section of MDCK cell 48 hours after inoculation

with CHV-G3. A long projection (arrows) of the nucleus

(N) extends into the cytoplasm. Note the membrane

bound vesicles (Ve) which contain enveloped virus

particles.

X 20,000.
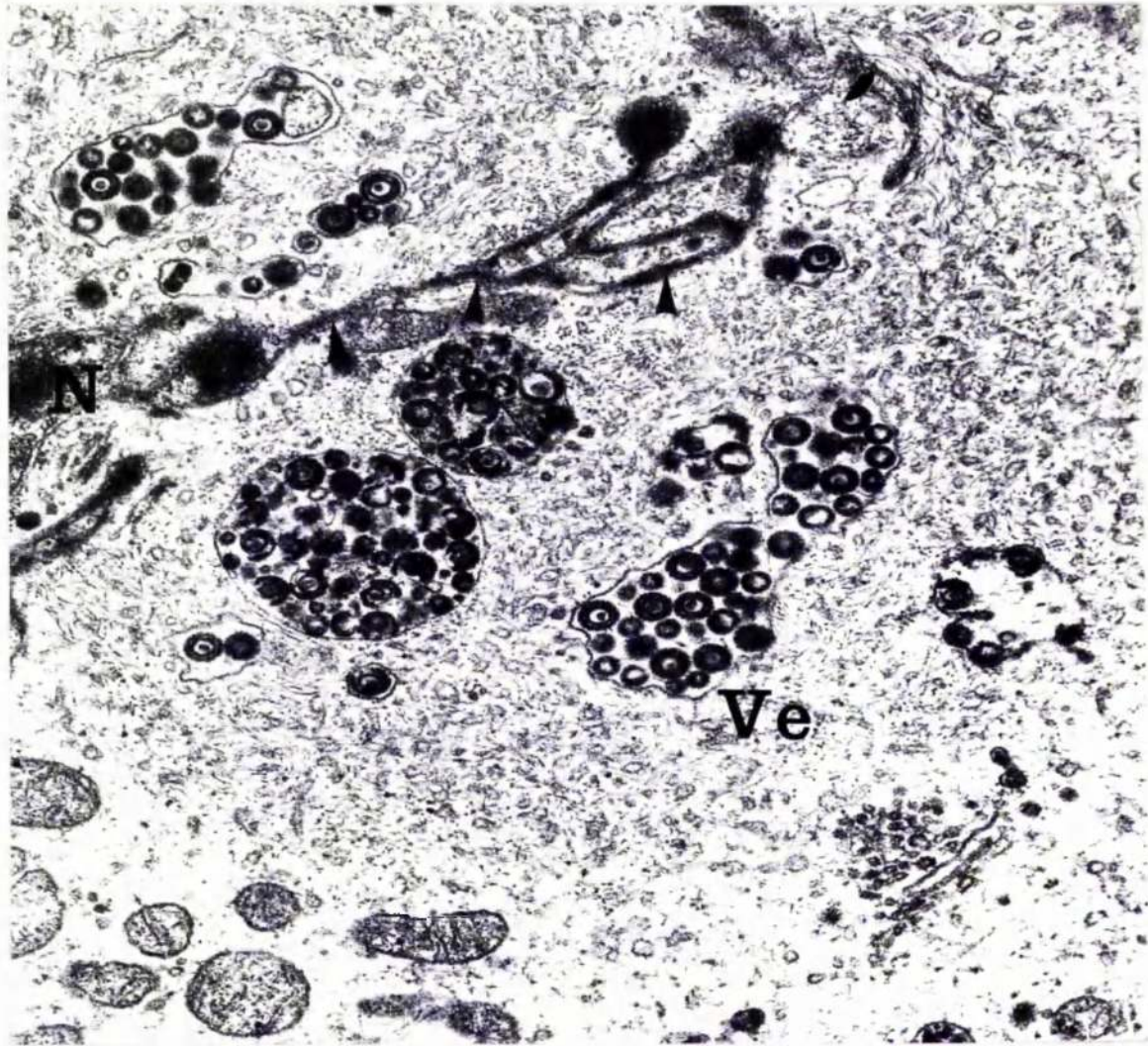
Fig. 21   Thin section of an MDCK cell 48 hours after inoculation

with CHV-G3. The nucleus (N) is disrupted and the

nuclear chromatin is clumped into dense masses (arrows)

along the nuclear membrane. Enveloped virus particles

are present in membrane bound cytoplasmic vesicles (Ve).

X 15,000.
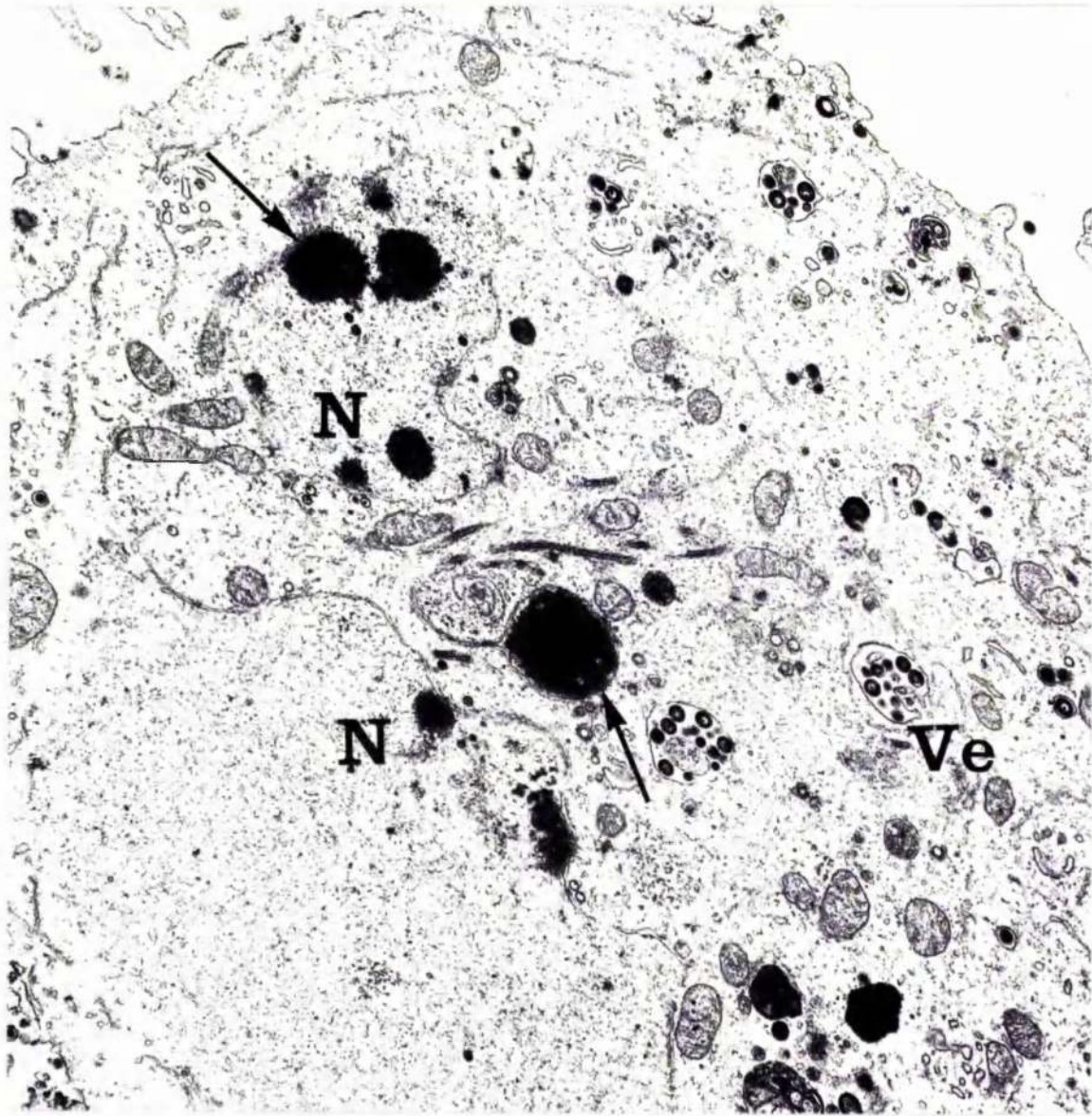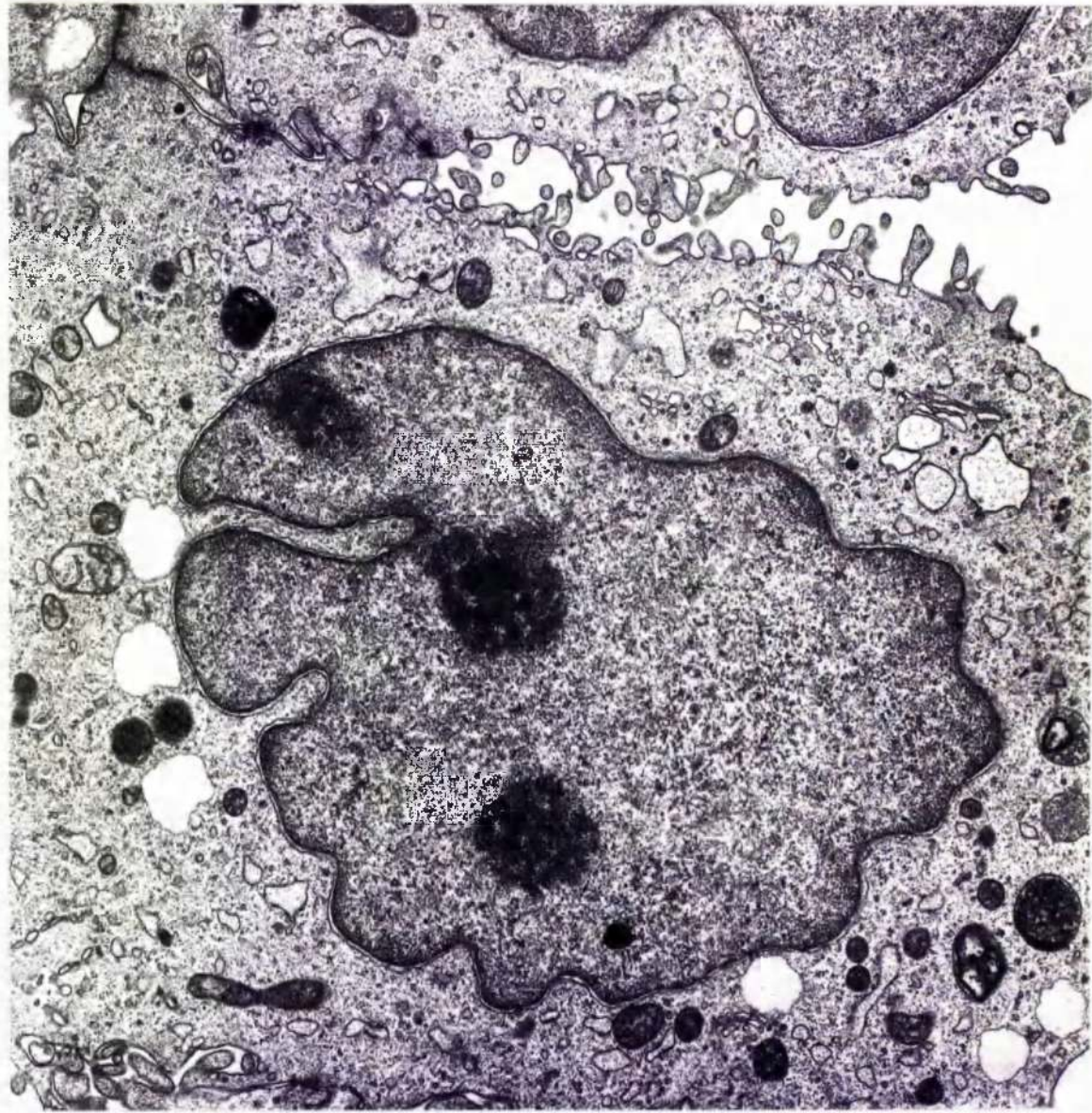
Fig. 23   Experimental CHV infection, 3 days after intratracheal

inoculation. Focal areas of consolidation (arrows) can

be seen in all lobes of the lungs.

Fig. 24   Experimental CHV infection, 7 days after intratracheal

challenge. Note the enlarged bronchial lymph nodes

(arrows).
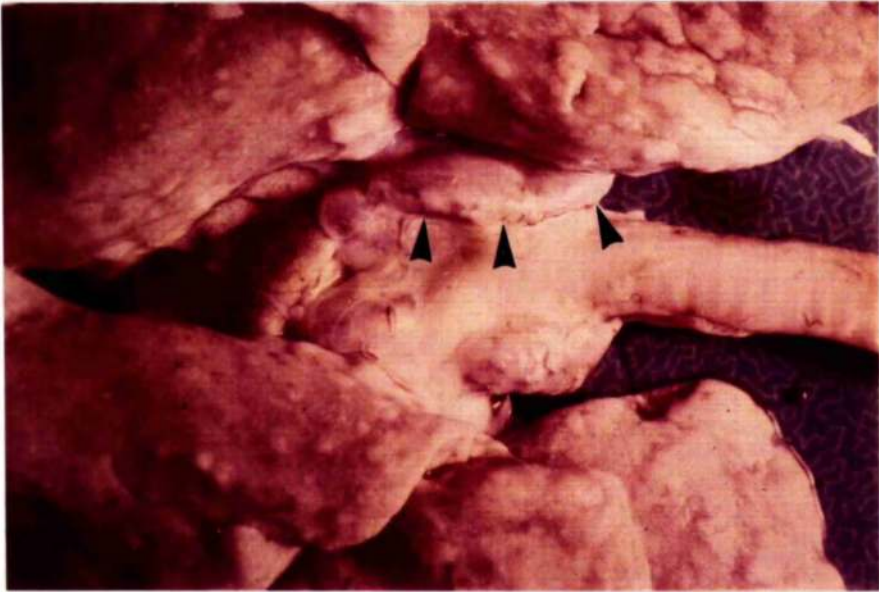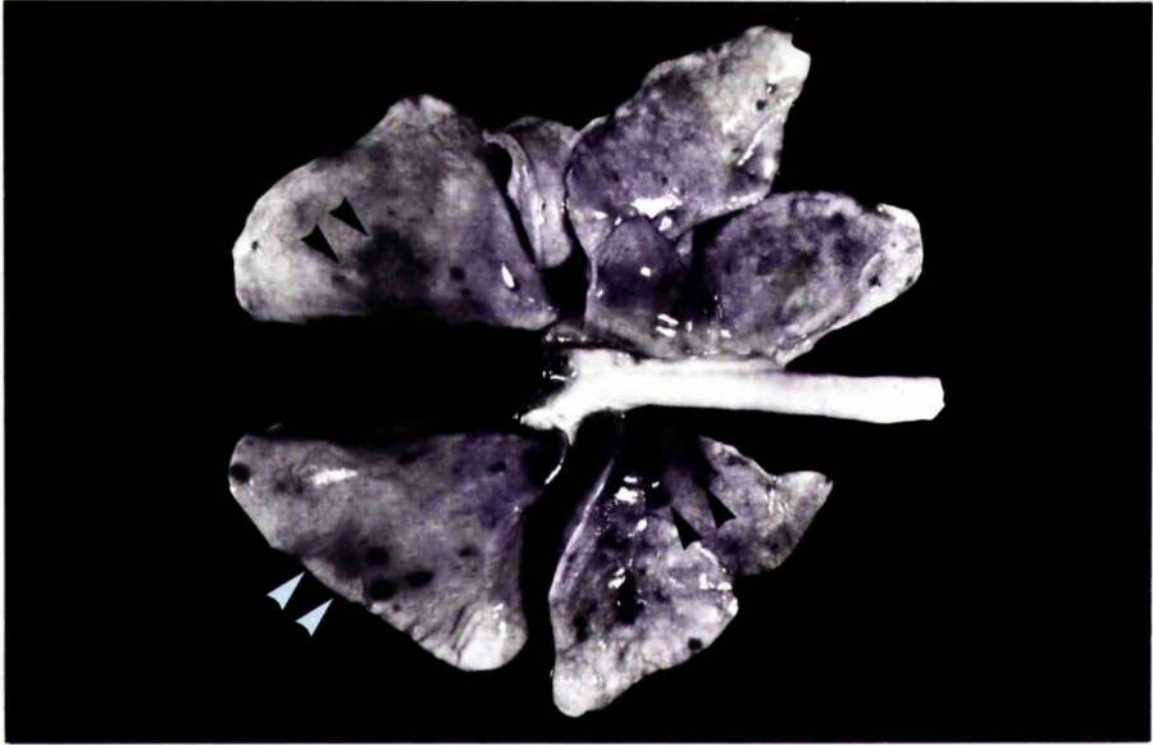
Fig. 25   Experimental CHV infection, 3 days after intratracheal

challenge. There is a focal necrotizing bronchiolitis.

Haemalum and eosin.   X 120.

Fig. 26   Experimental CHV infection, 5 days after intratracheal

challenge. There is necrosis of the alveolar septa and

a fibrinous exudate is present in the air spaces.

Haemalum and eosin.   X 175.
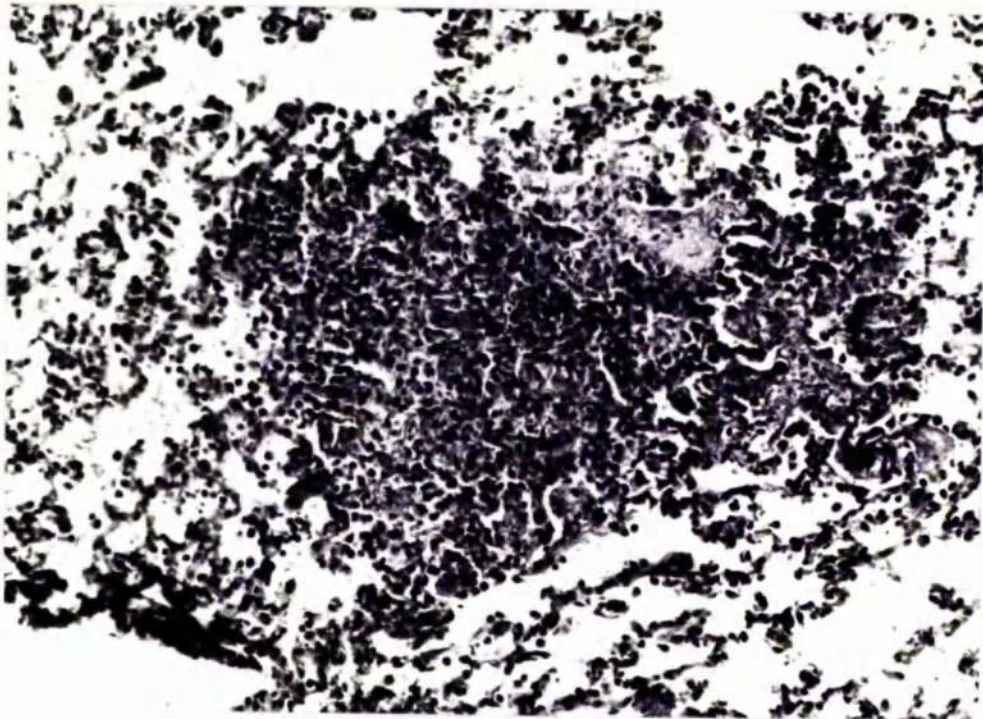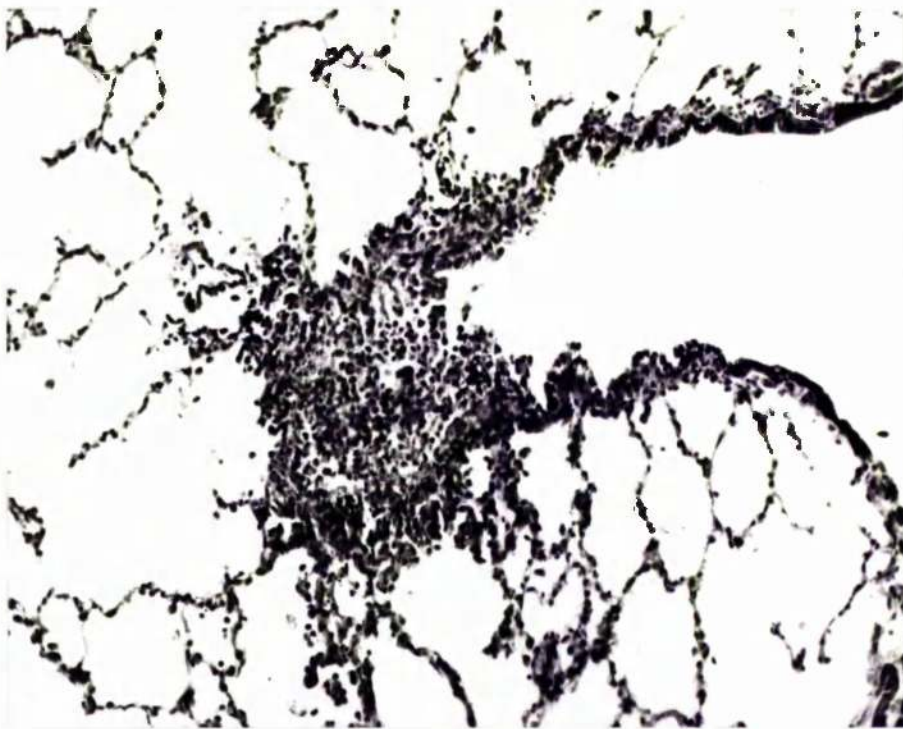
Fig. 27   Experimental CHV infection: lungs of an infected dog,
          5 days after aerosolisation to show multiple foci of
          pneumonia in all lobes.

Fig. 28   Experimental CHV infection. A close-up view of the
          lung of the same dog.
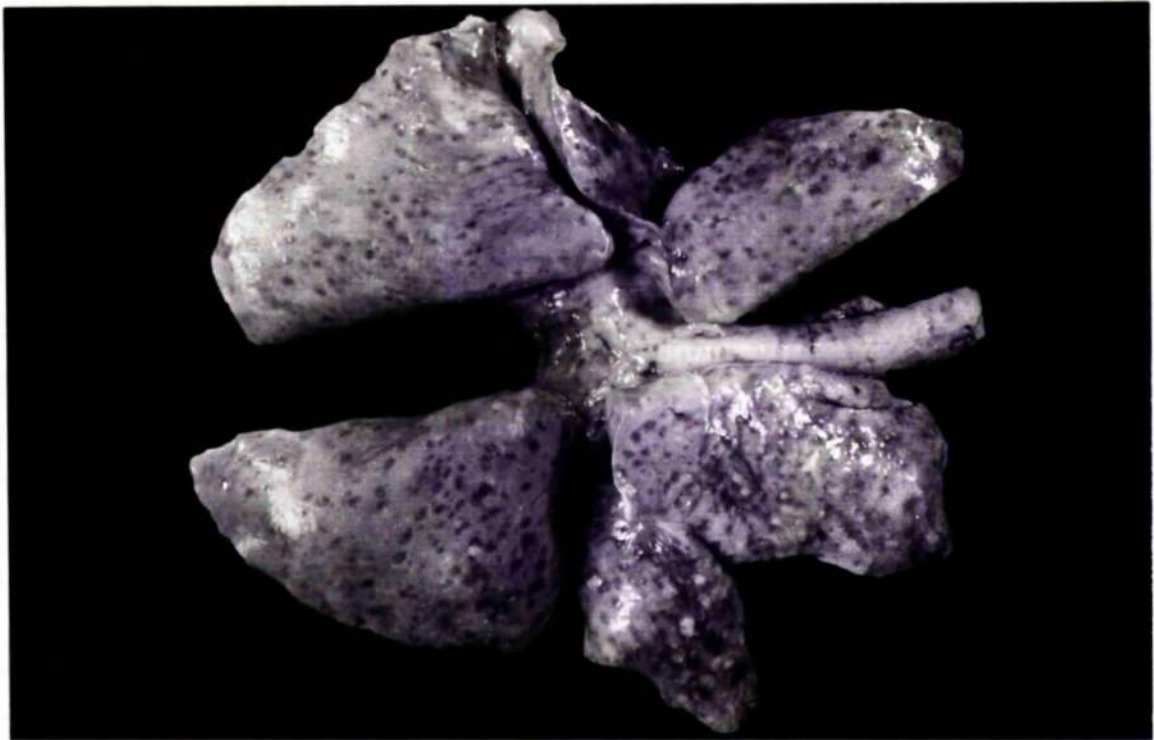
Fig. 29 Experimental CHV infection: necrotizing rhinitis, 3

days after aerosolisation. Many intranuclear inclusion

bodies (arrows) can be seen in vacuolated epithelial

cells.

Haemalum and eosin. X 300.

Fig. 30 Experimental CHV infection: necrotizing rhinitis, 5 days

after aerosolisation. The necrotic process has breached

the epithelium and extends into the lamina propria.

Haemalum and eosin. X 150.
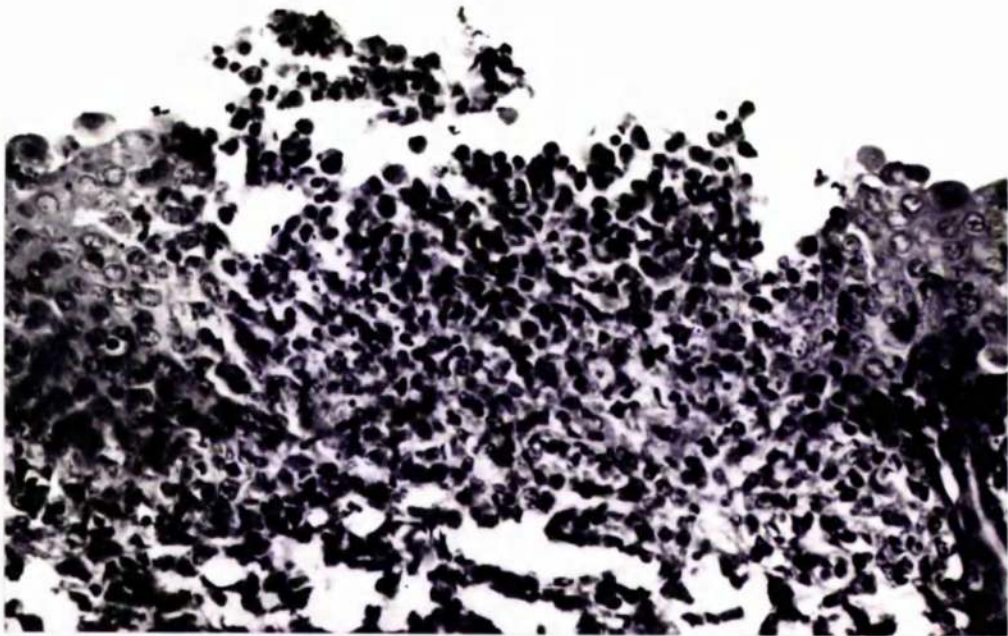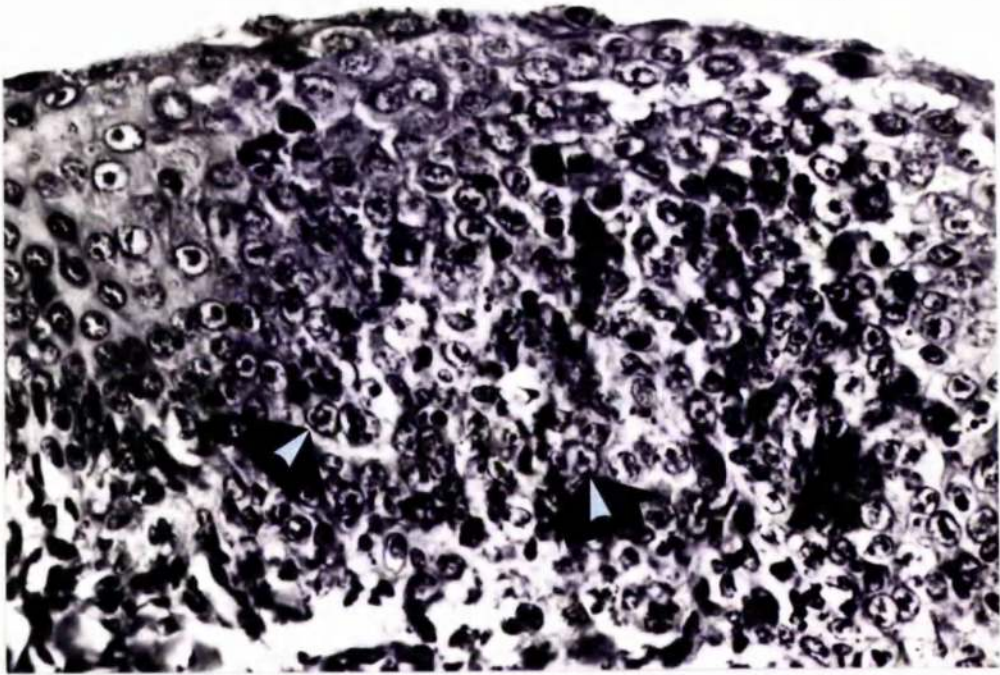
Fig. 31   Experimental CHV infection: bronchiolar epithelium,

3 days after aerosolisation. Infected epithelial cells

(arrow) are swollen and there is "beading" of their

nuclear chromatin. Note the polymorphonuclear

leucocyte (P) infiltrating the epithelium.

Toluidene blue, 1μm section. X 2,500.
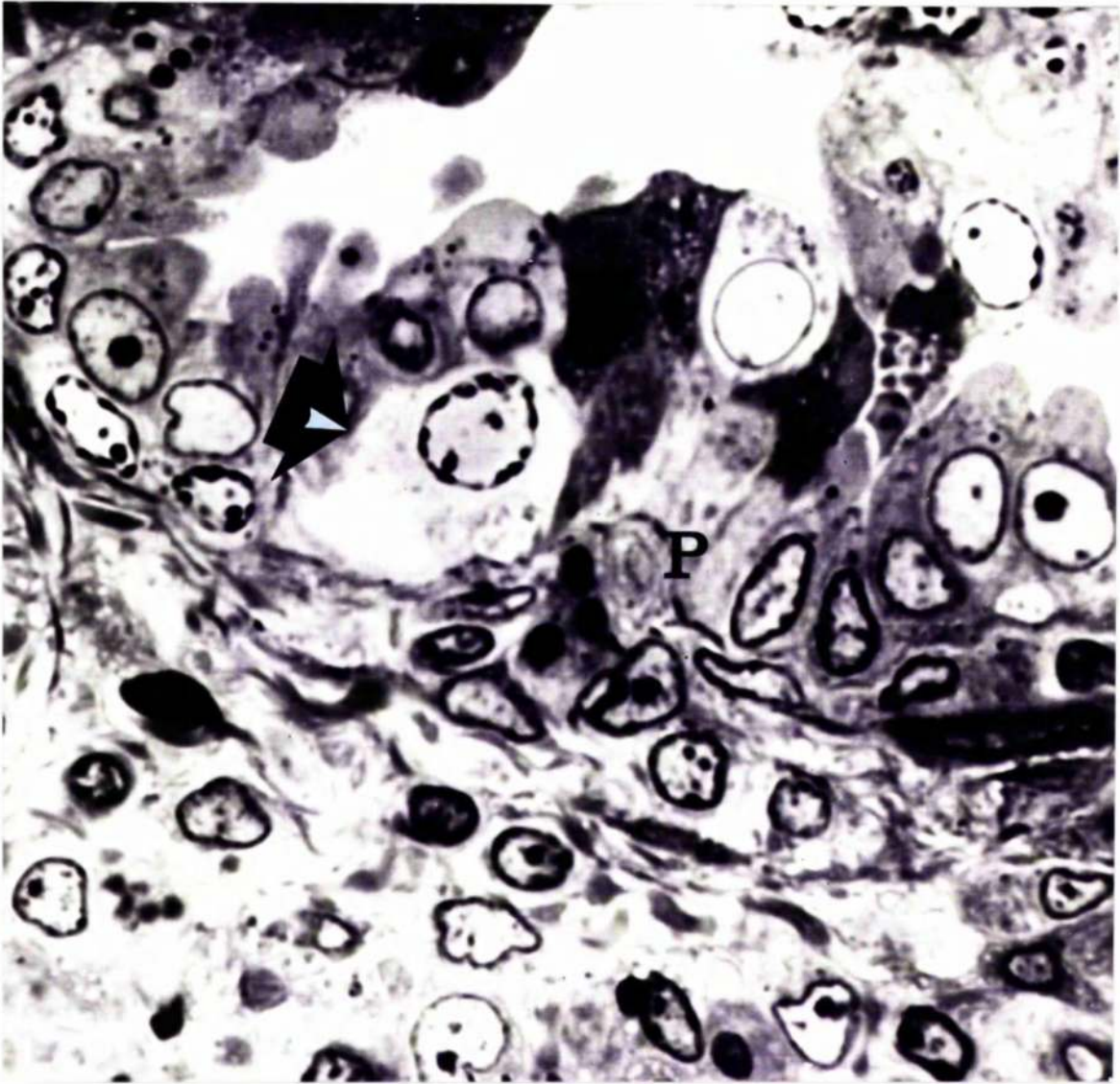
Fig. 32   Experimental CHV infection: bronchiolitis 3 days after

aerosolisation. Early lesion showing focal degeneration

and desquamation of epithelial cells.

Toluidene blue, 1μm section.   X 1,200.

Fig. 33   Experimental CHV infection: bronchiolitis 5 days after

aerosolisation. There is focal necrosis of the

bronchiolar epithelium and spread of the necrotic

process into surrounding alveoli.

Haemalum and eosin.   X 120.
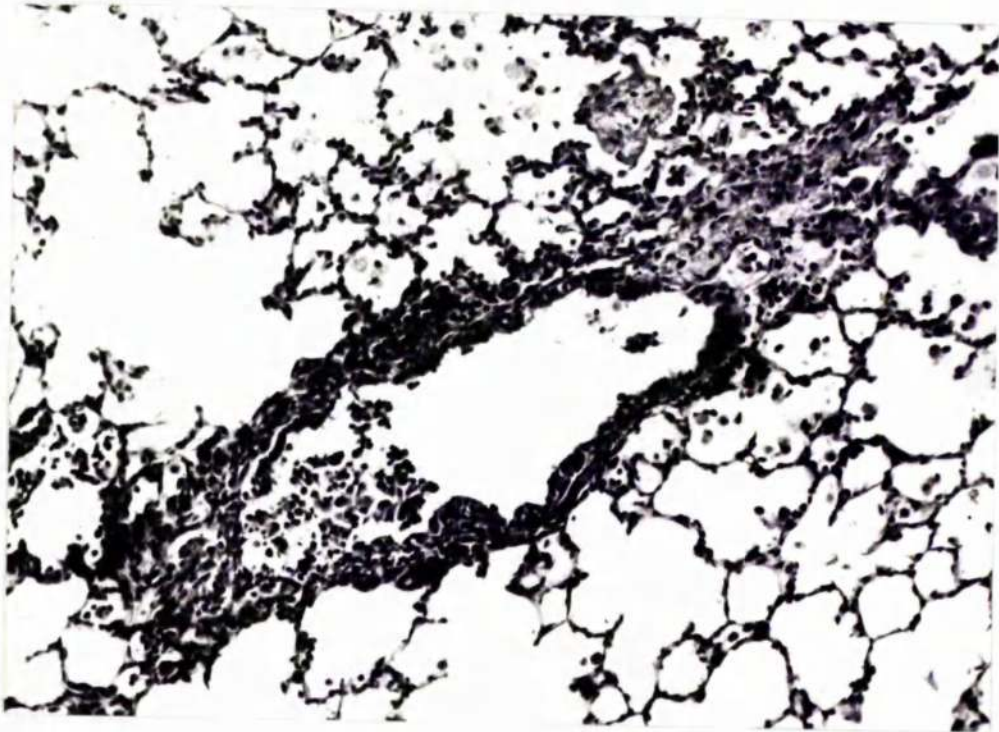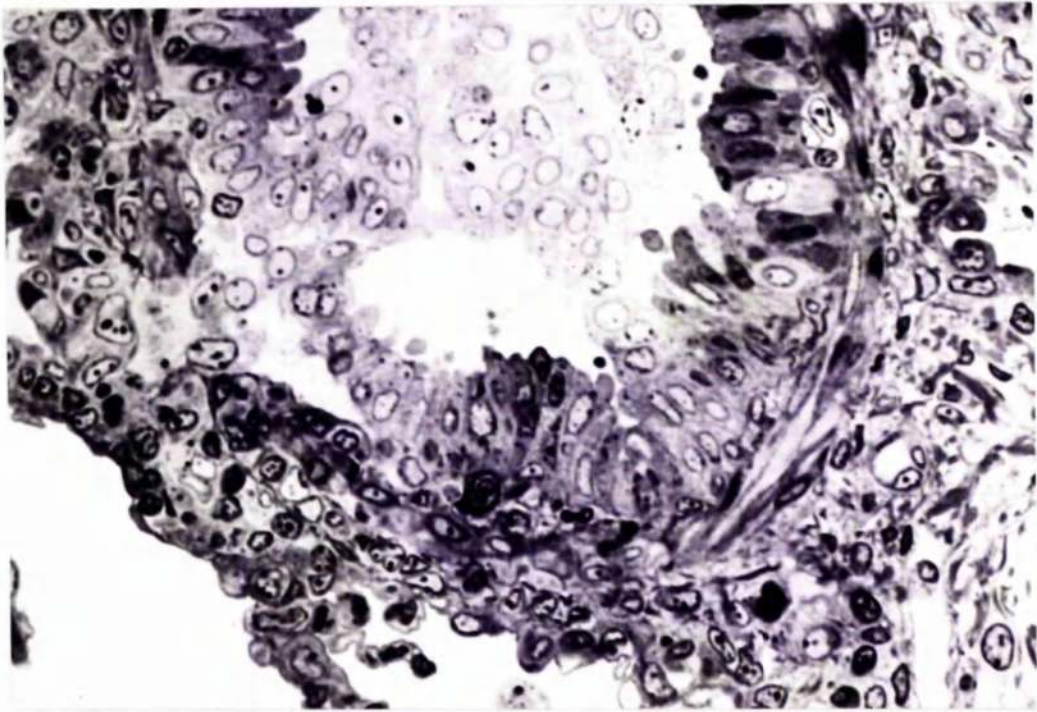
Fig. 34   Experimental CHV infection: necrotizing alveolitis

5 days after aerosolisation. Fibrin is present in

the alveolar air spaces.

Haemalum and eosin. X 350.

Fig. 35   Experimental CHV infection, 7 days after aerosolisation.

The alveolar septa are thickened and macrophages are

present in the air spaces.

Toluidene blue, 1μm section. X 450.
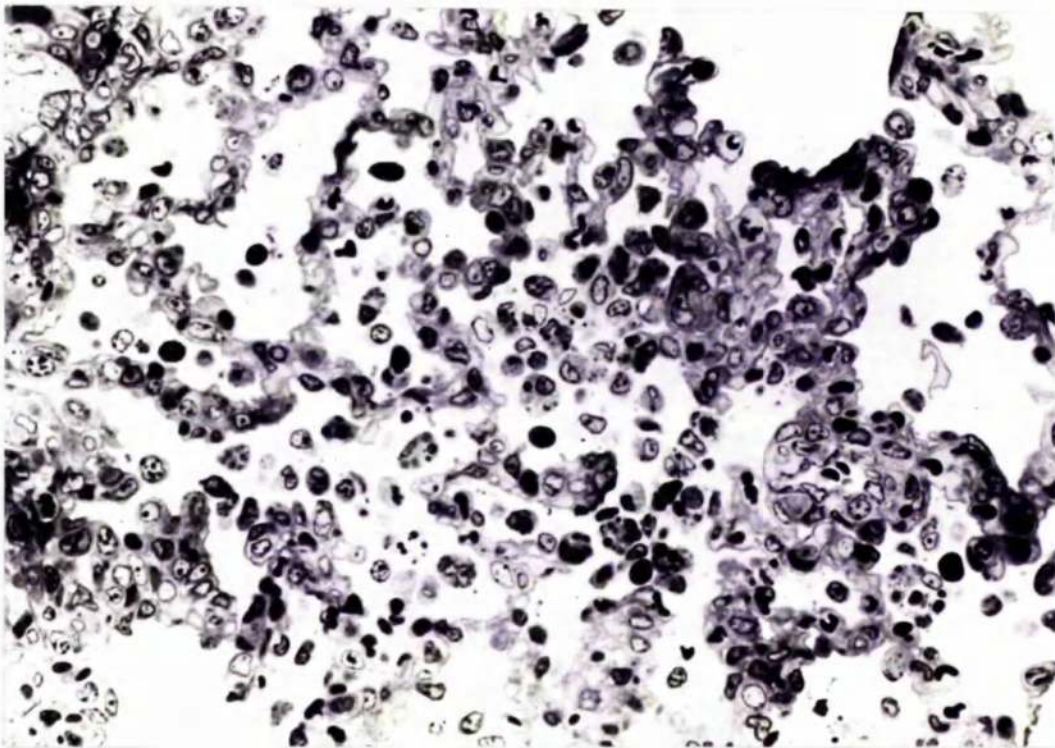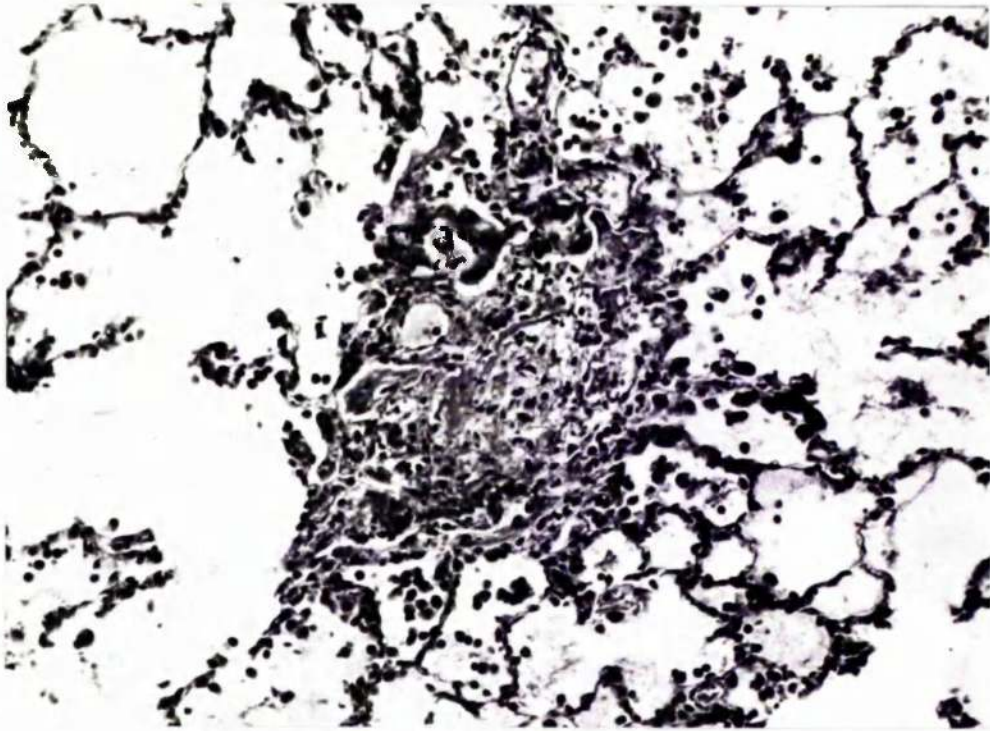
Fig. 36   Experimental CHV infection: focal interstitial

nephritis 10 days after infection. The cellular infiltrate

is composed mainly of lymphocytes.

Haemalum and eosin, X 300.
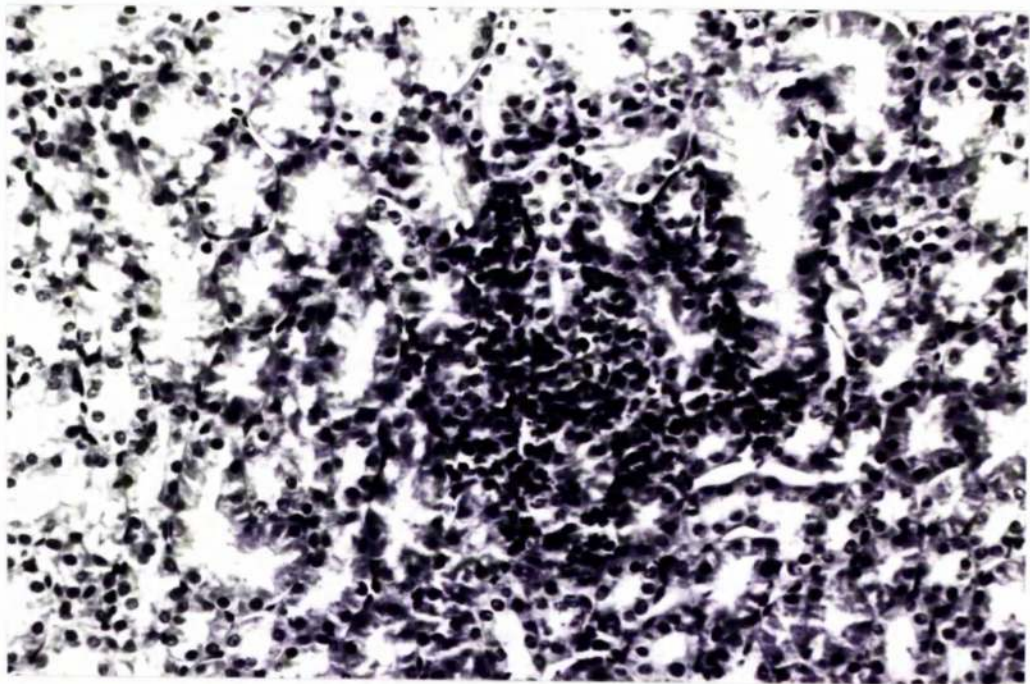
Fig. 37   Experimental CHV infection: low magnification electron

micrograph to show a bronchiole 5 days after

aerosolisation. The bronchiolar epithelium (BE) is

swollen and sloughed degenerate cells and granular

debris are present in the lumen (Ln).

X 10,000.
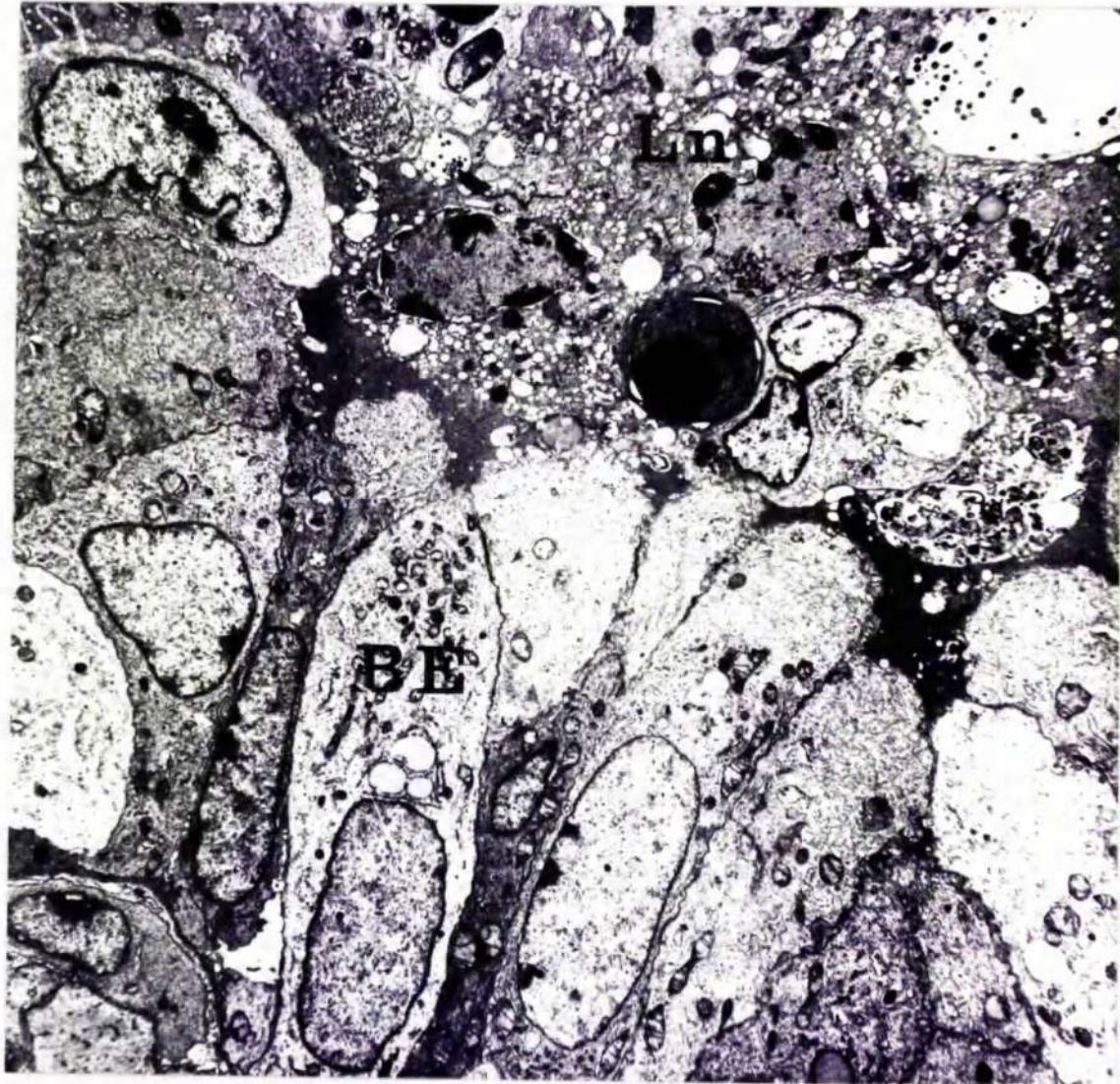
Fig. 38   Experimental CHV infection: bronchiolar epithelial cell,
           5 days after aerosolisation. Naked virus particles (V)
           are present in the centre of the nucleus; note the
           different types of cores. Enveloped virus particles
           are seen at the perinuclear cisternae; note the
           involution and duplication (arrows) of the inner nuclear
           membrane.

                         X 30,000.
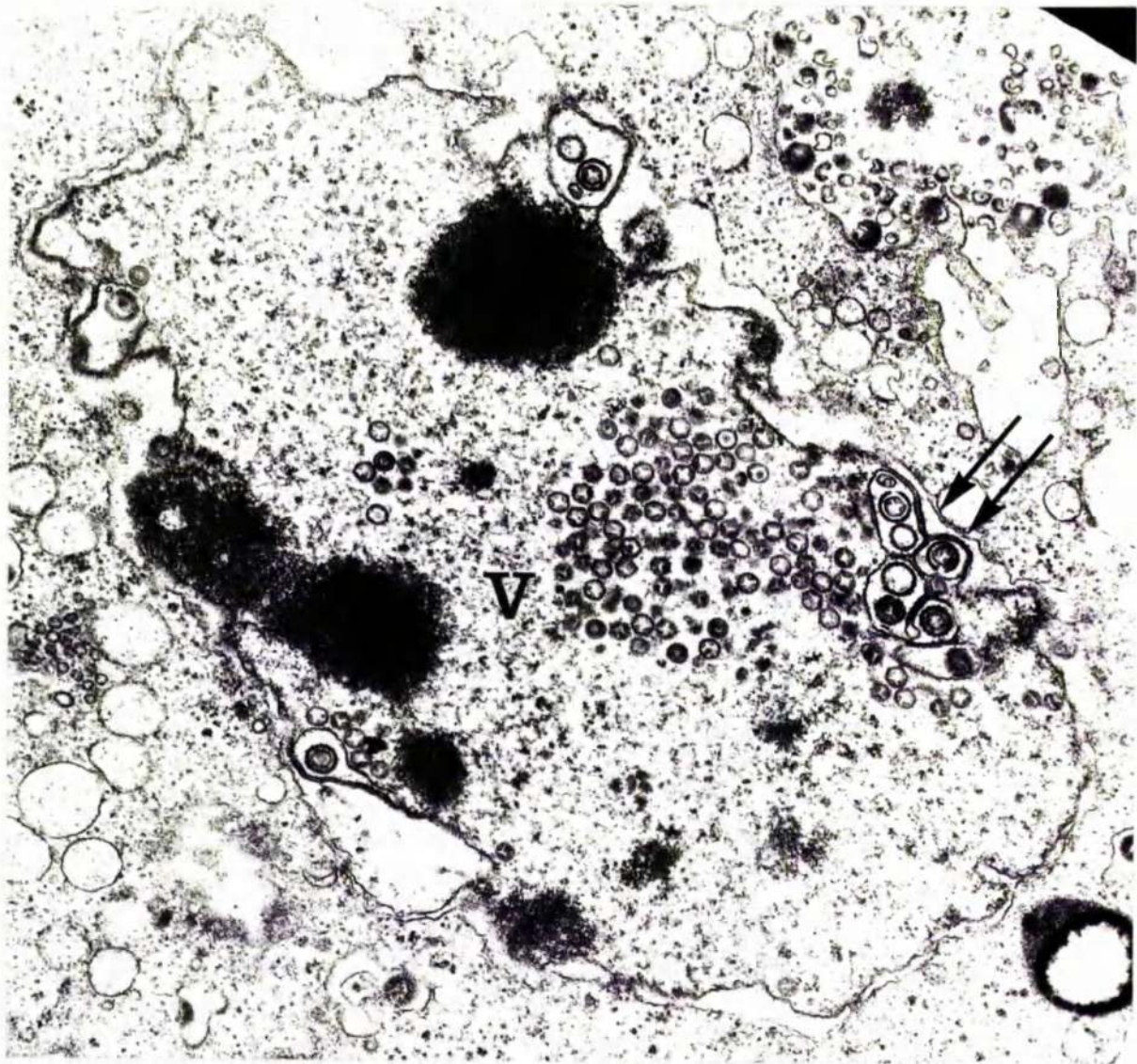
Fig. 39   Experimental CHV infection: naked virus particles in

the cytoplasm of an alveolar macrophage, 7 days after

aerosolisation. The spoke-like projections of the

capsomeres can be seen.

X 75,000.

Fig. 40   Experimental CHV infection: bronchiolar epithelial cell

5 days after aerosolisation. There is margination

of the nuclear chromatin and fibrillar deposits (F)

are present in the central region of the nucleus.

Naked virus particles (arrows) are also seen in the

nucleus while enveloped particles can be observed in

membrane bound cytoplasmic vesicles (Ve).

X 20,000.
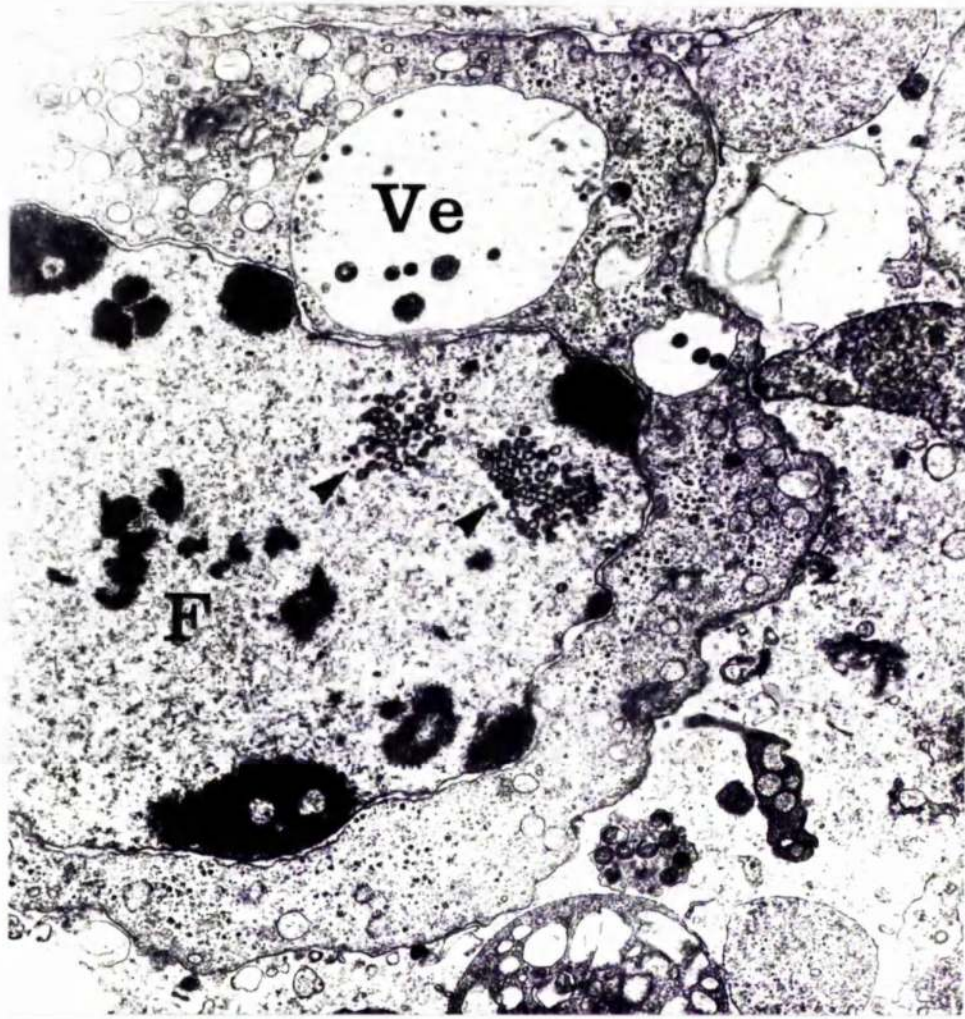
Fig. 41   Enveloped virus particles in a cytoplasmic vesicle,

5 days after aerosolisation. Many hyphae-like

structures project into the lumen of the vesicle.

X 40,000.

Fig. 42   Gross postmortem appearance of a puppy that died 7

days after intraperitoneal inoculation with CHV-G3.

The liver is mottled, the spleen is enlarged and there

are fine haemorrhages in the intestines.

Fig. 43   Gross postmortem appearance of a puppy that died 7 days
          after intraperitoneal inoculation with CHV-G3. Circular
          haemorrhagic foci can be seen in the kidneys and there
          are dark focal areas of consolidation in the lungs.

Fig. 44   Experimental neonatal CHV infection: necrotizing

hepatitis 7 days after intraperitoneal inoculation

with CHV-G3.

Haemalum and eosin. X 150.

Fig. 45   Experimental neonatal CHV infection: necrosis in the

renal cortex, 7 days after infection.

Haemalum and eosin. X 300.
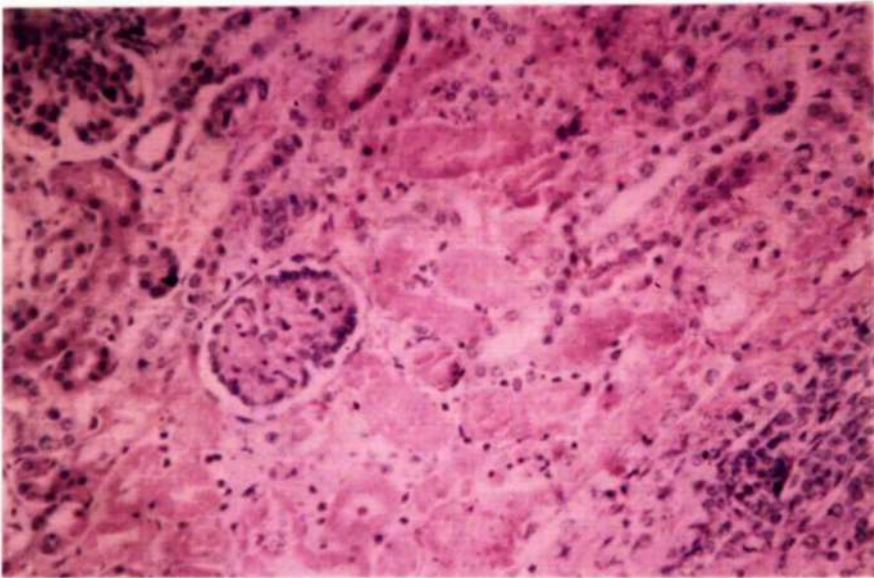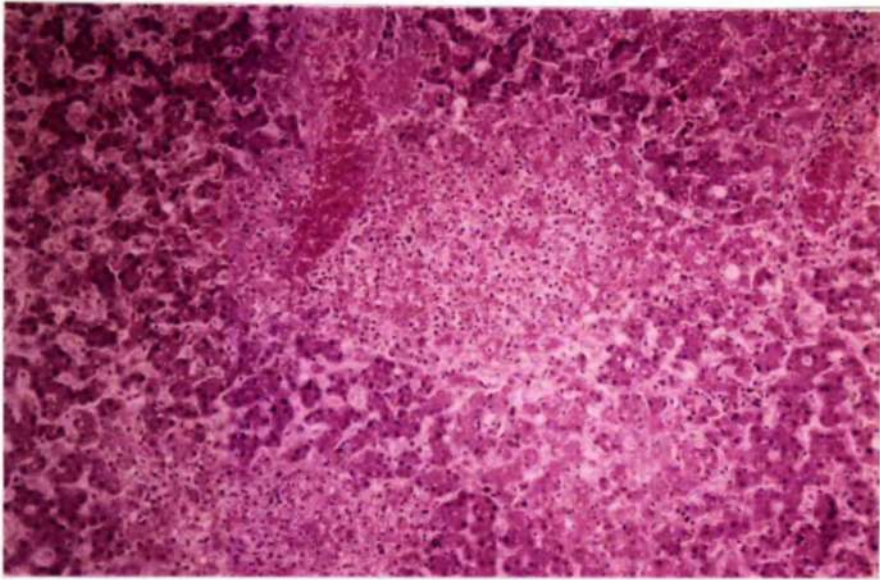
Fig. 46   Experimental neonatal CHV infection: necrotizing

pneumonia. Note the fibrinous exudate in the air

spaces and the necrosis of the bronchiolar wall.

Haemalum and eosin. X 300.

Fig. 47   Experimental neonatal CHV infection: necrotizing

adrenalitis. An eosinophilic intranuclear inclusion

body (arrows) can be seen in a necrotic cell of the

zona fasciculata.

Haemalum and eosin. X 1,500.
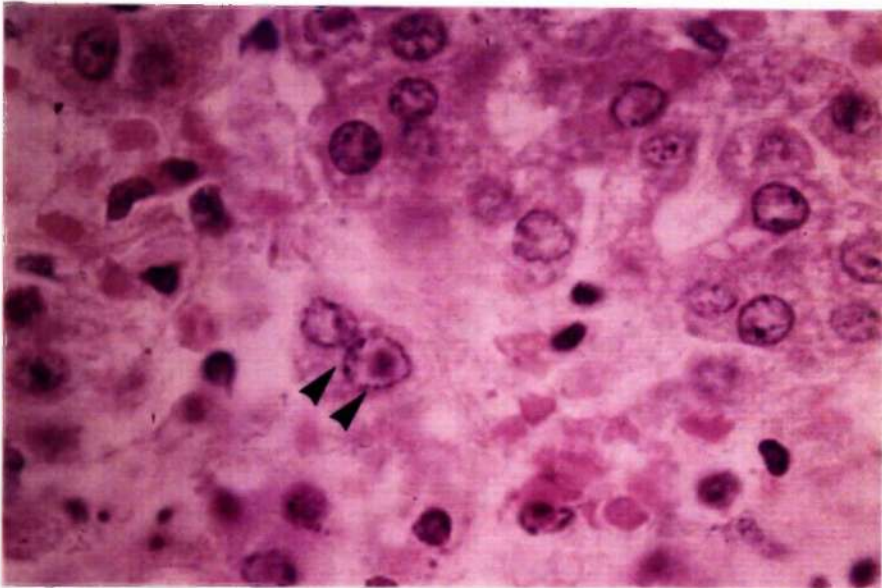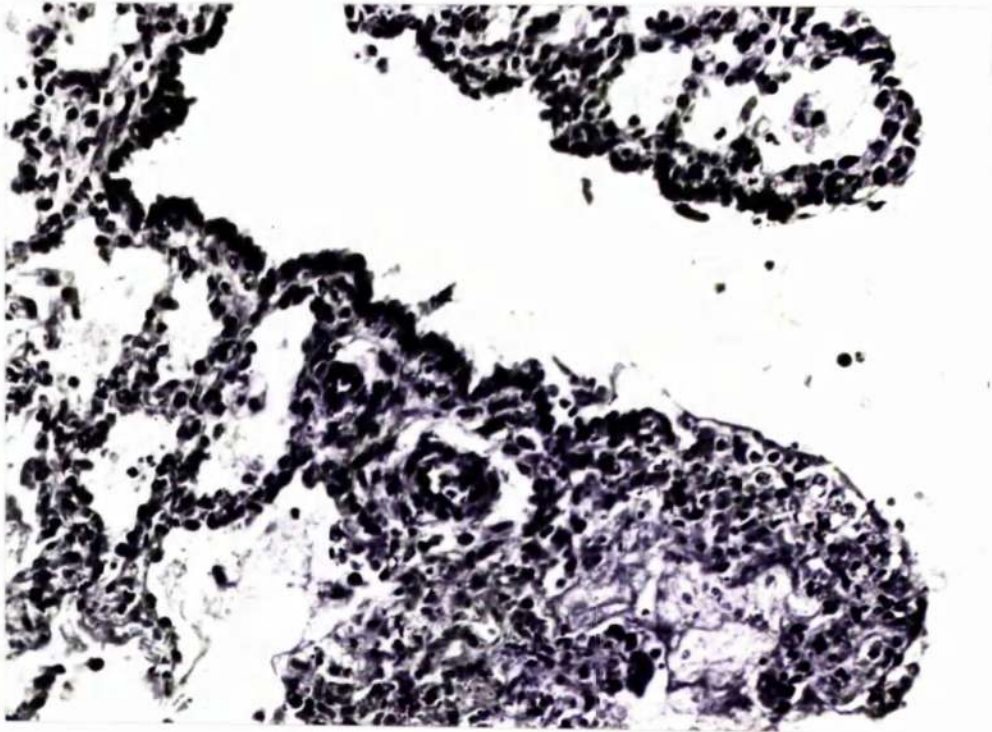
Fig. 48   Experimental CAV-1 infection: lungs of a puppy, 2 days

after intratracheal inoculation. The dark red areas

of consolidation are swollen and oedematous.

Fig. 49   Experimental CAV-1 infection: lungs of a puppy, 4 days

after intratracheal inoculation. There are areas of

consolidation in the apical, cardiac and diaphragmatic

lobes of the lungs.
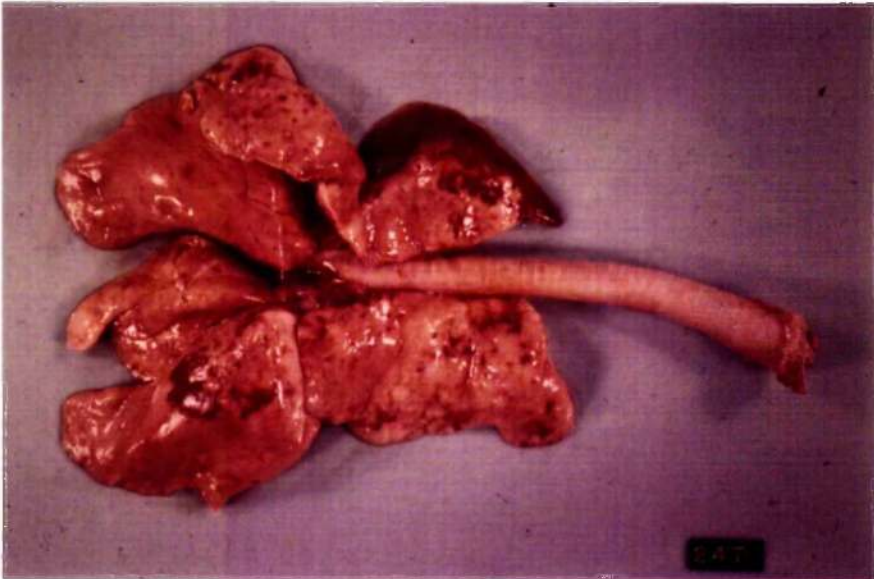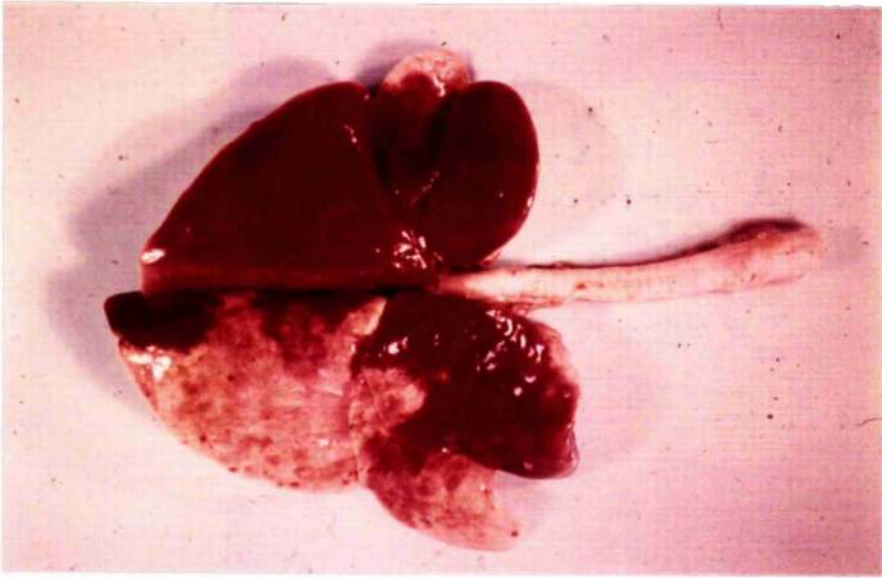
Fig. 50   Experimental CAV-1 infection: a small bronchiole showing

            epithelial necrosis and a catarrhal exudate, 4 days

            after intratracheal inoculation.

                                    Haemalum and eosin. X 150.

Fig. 51   Experimental CAV-1 infection: bronchiole plugged with

            necrotic epithelial cells, 4 days after infection.

            Note the intranuclear inclusion bodies (arrows).

                                    Toluidene blue, 1μm section. X 500.
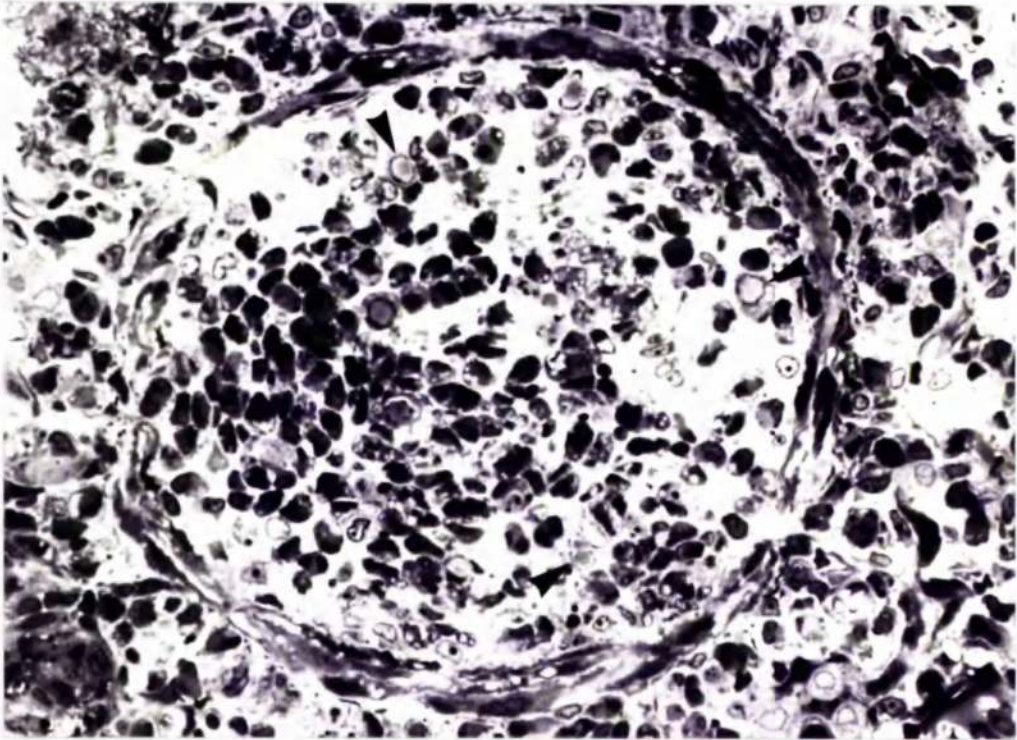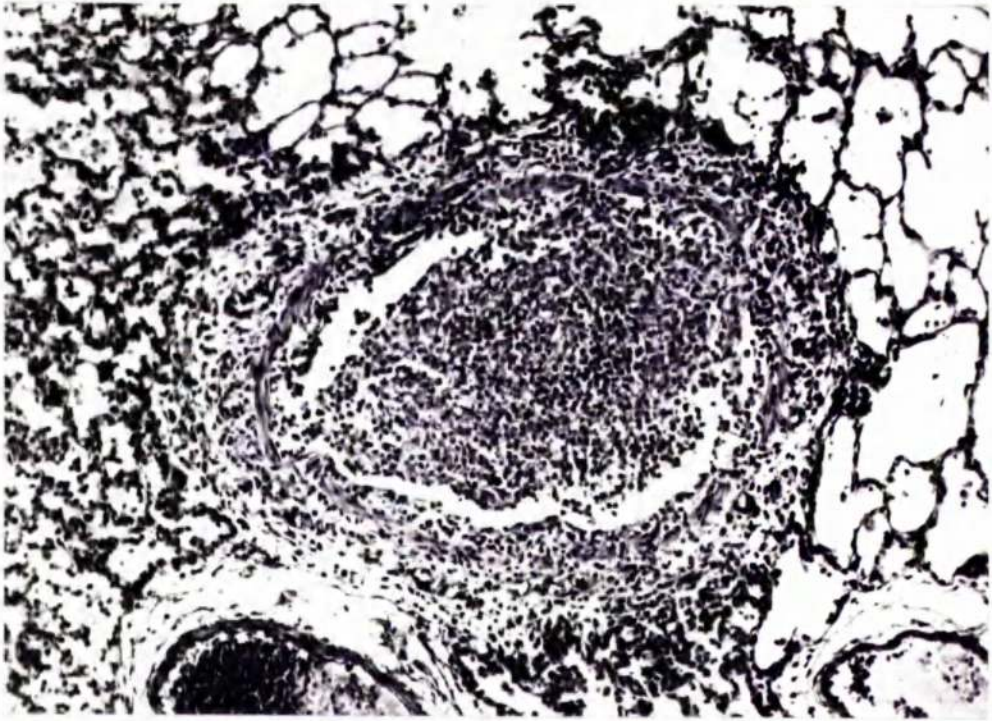
Fig. 52   Experimental CAV-1 infection: low power photomicrograph

of the lung, 7 days after intratracheal inoculation.

There is a necrotizing bronchiolitis and the alveoli

are filled with macrophages, strands of fibrin and

polymorphonuclear leucocytes.

Haemalum and eosin. X 150.

Fig. 53   Experimental CAV-1 infection: proliferative

bronchiolitis, 9 days after intratracheal inoculation.

Haemalum and eosin. X 320.
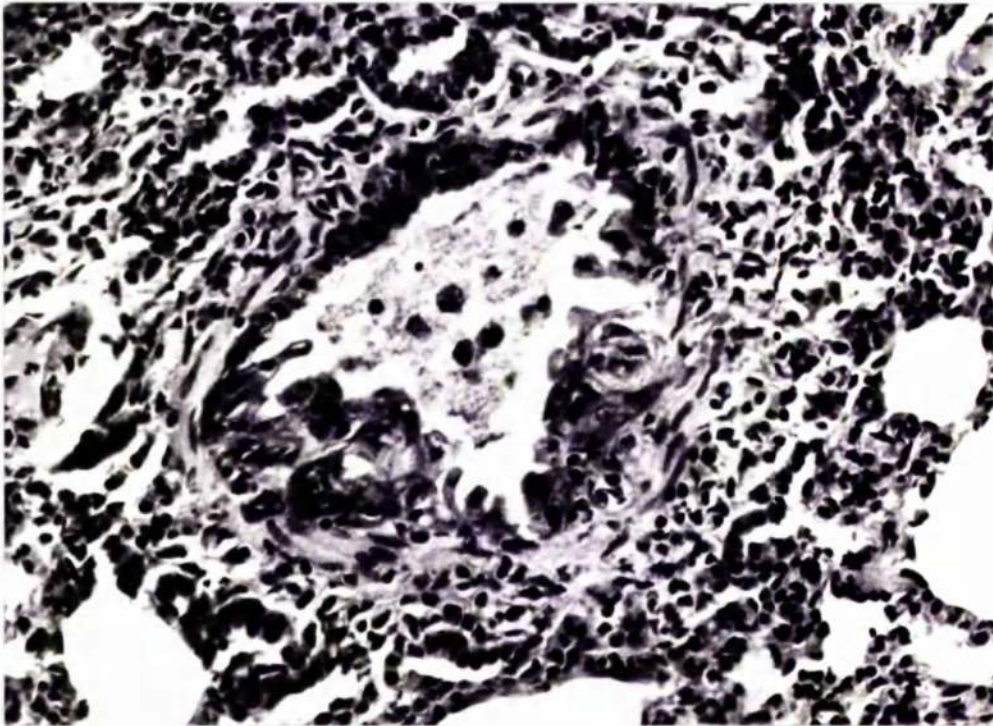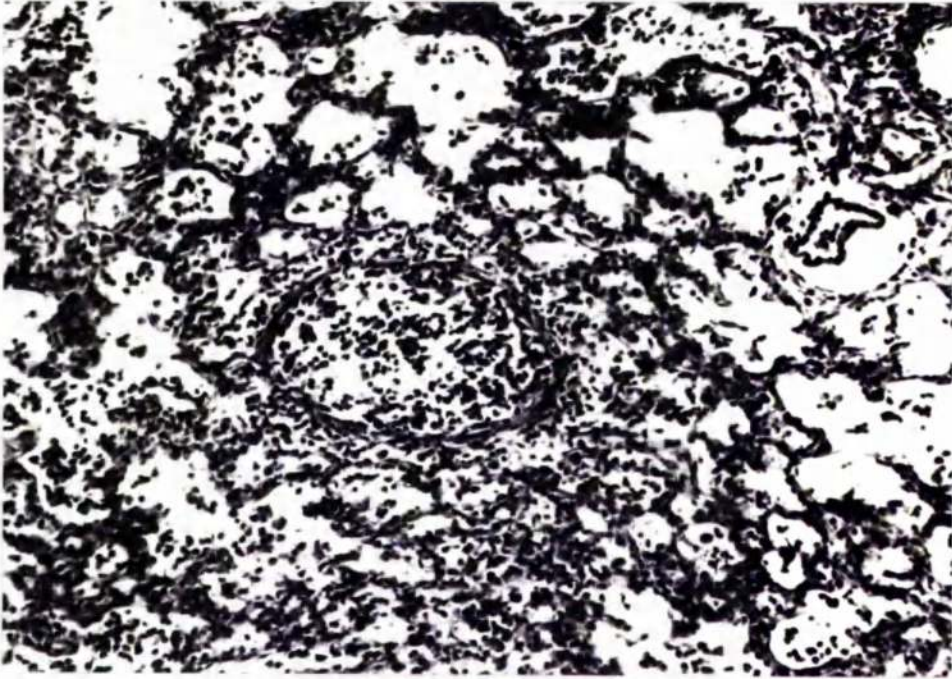
Fig. 54   Experimental CAV-1 infection: proliferative bronchiolitis

with multinucleate giant cells (arrows) in the

bronchiolar lumen, 9 days after intratracheal

inoculation.

Haemalum and eosin.   X 320.
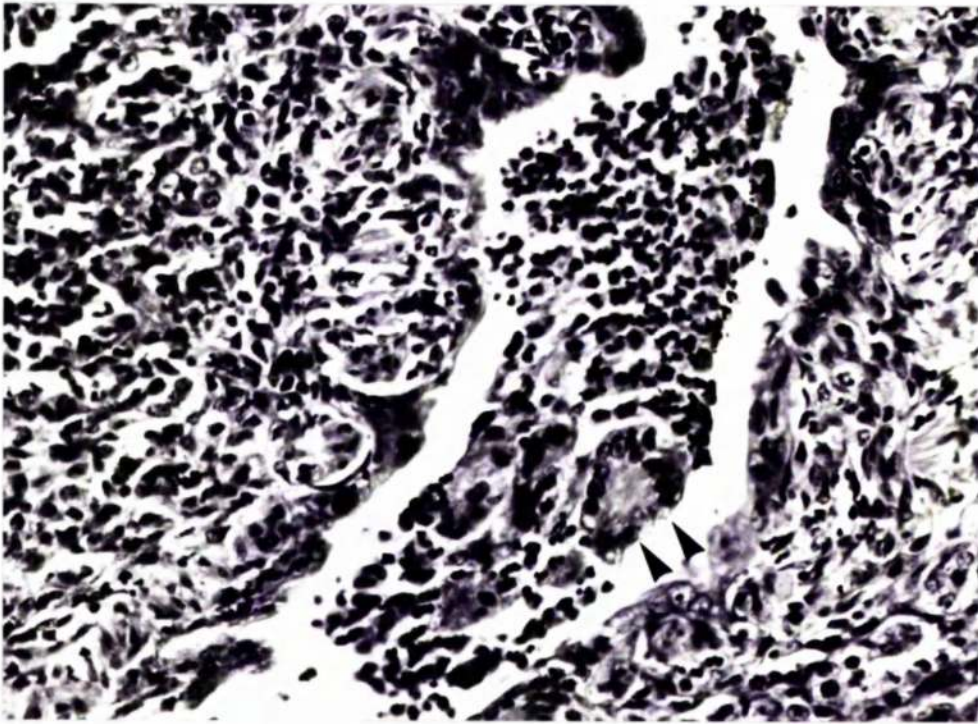
Fig. 55   Experimental CAV-1 infection: CAV-1 antigen in alveolar

and septal macrophages, 4 days after intratracheal

inoculation.

Fluorescent antibody, X 800.

Fig. 56   Experimental CAV-1 infection: CAV-1 antigen in necrotic

epithelial cells within a bronchiole, 9 days after

intratracheal inoculation.

Fluorescent antibody. X 900.
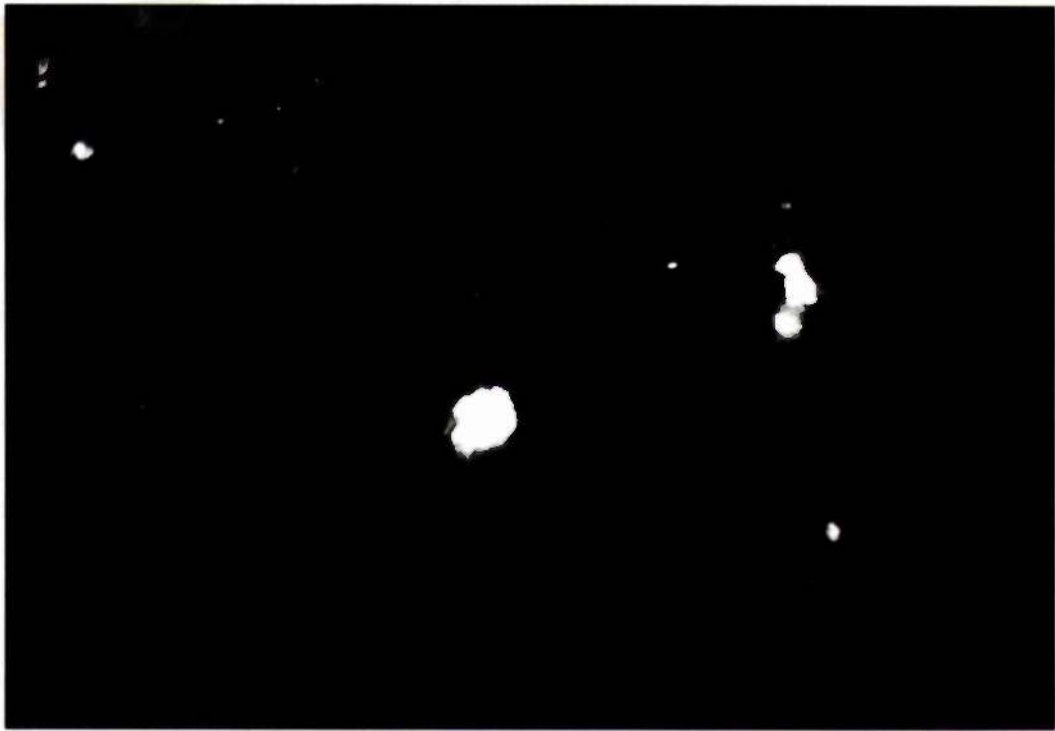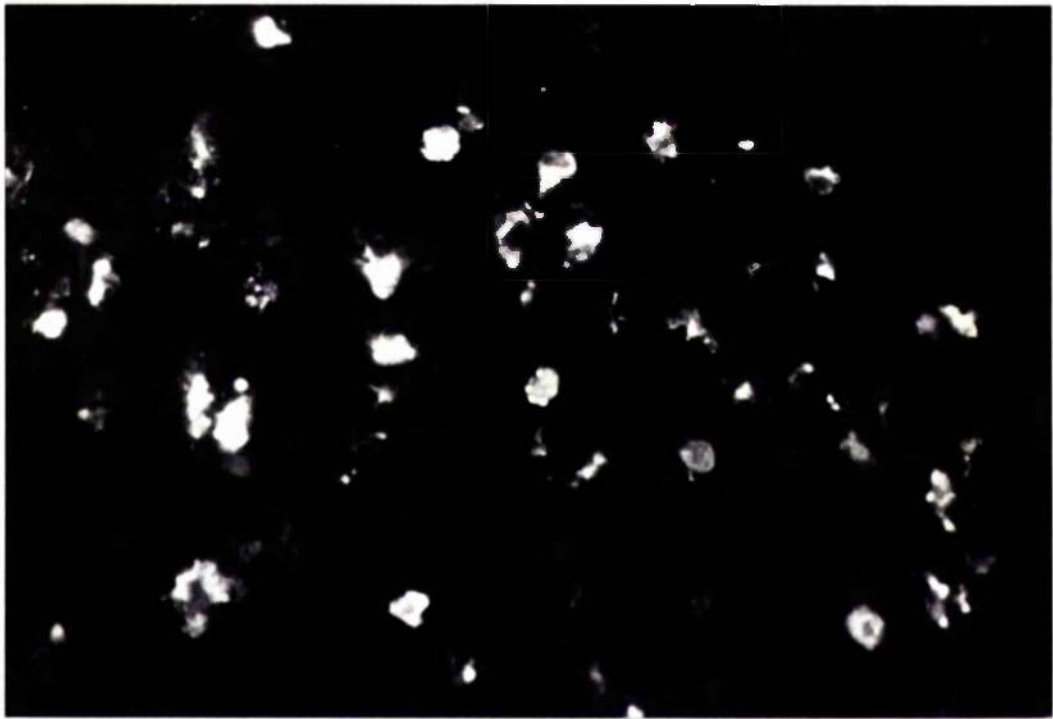
Fig. 57   Experimental CAV-1 infection: lungs of an infected

dog 5 days after aerosolisation. Dark red areas of

consolidation can be seen along the edges of the lungs.

Fig. 58   Experimental CAV-1 infection: close-up view of the

apical and cardiac lobes of the above lung.
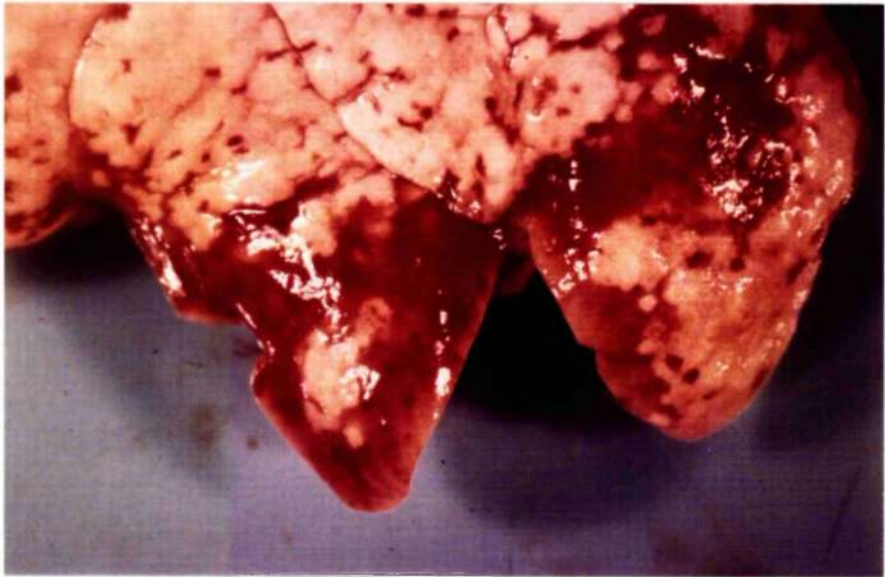
Fig. 59   Experimental CAV-1 infection: lungs of a puppy killed

15 days after aerosolisation to show discrete lesions

(arrows) in the left diaphragmatic lobe of the lung.

Fig. 60   Experimental CAV-1 infection: swollen congested bronchial

lymph nodes, 5 days after aerosolisation.
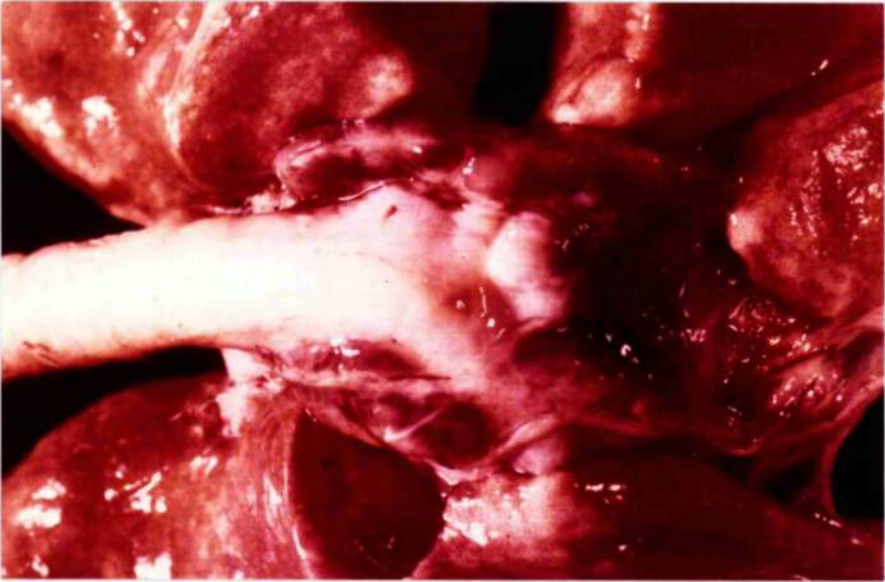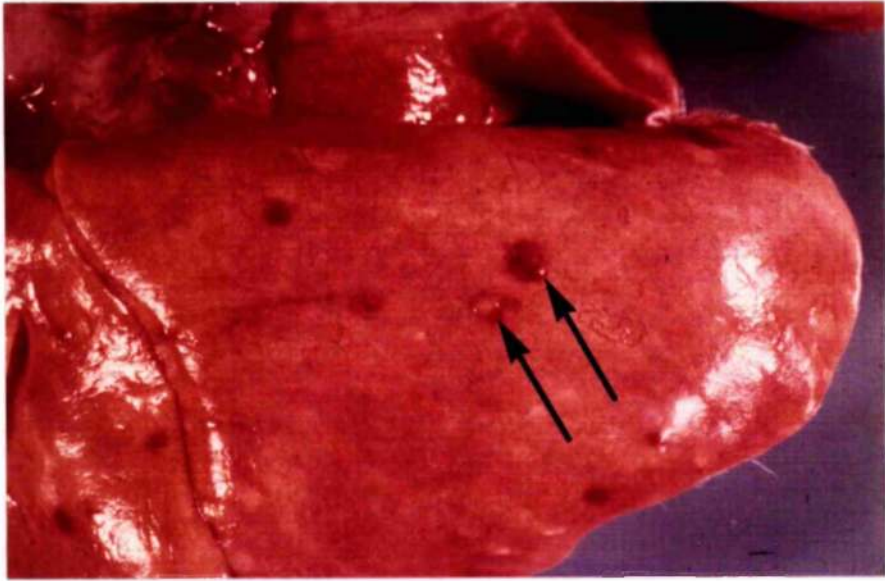
Fig. 61   Experimental CAV-1 infection: lungs of an infected

dog killed 14 days after exposure to CAV-1 by aerosol.

Note the consolidated lesions along the edges of all

the lobes and the enlarged pale bronchial lymph

nodes (arrows).

Fig. 62   Experimental CAV-1 infection: section of a bronchiole

2 days after aerosolisation. The bronchiolar epithelium

is vacuolated and infiltrated by polymorphonuclear

leucocytes.

Haemalum and eosin. X 300.

Fig. 63   Experimental CAV-1 infection: necrotizing bronchiolitis

4 days after aerosolisation. Most of the epithelium

has detached and sloughed into the lumen. Note the

intranuclear inclusion bodies (arrow).

Toluidene blue, 1μm section. X 1,200.
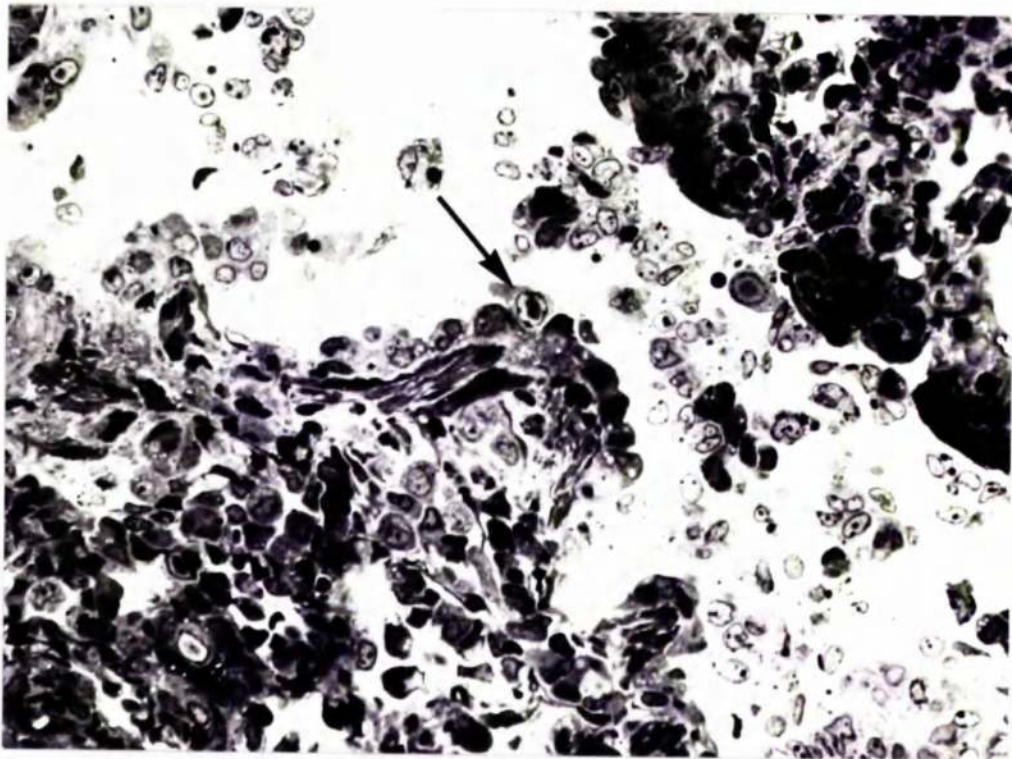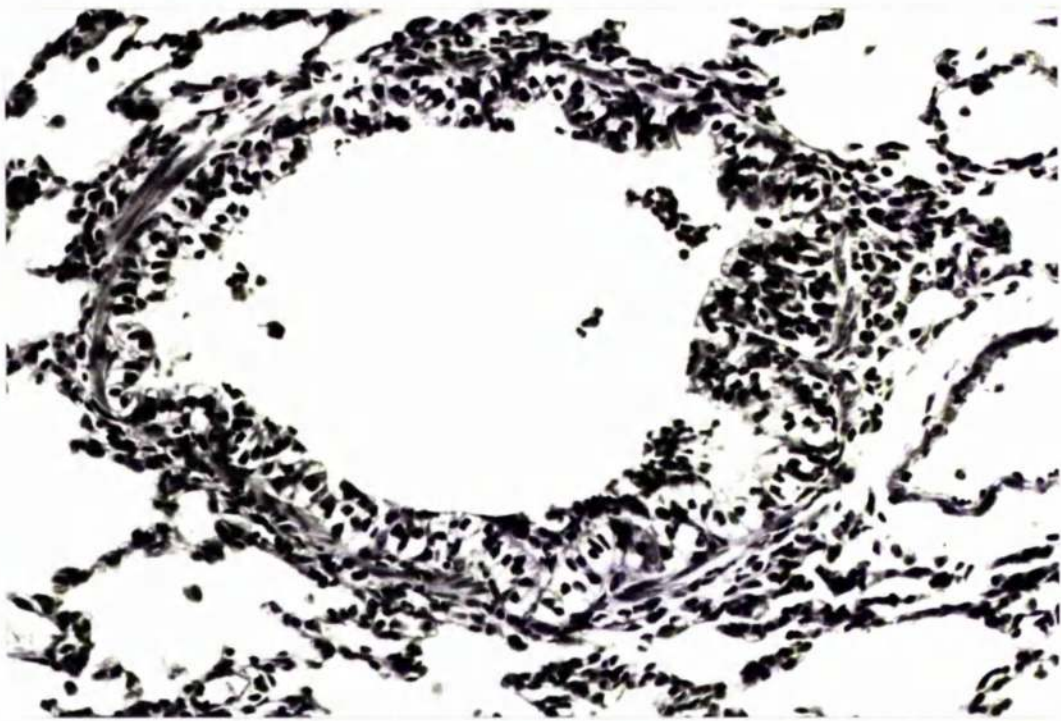
Fig. 64   Experimental CAV-1 infection: high power photomicrograph

of the bronchiolar epithelium 2 days after

aerosolisation. Note the vacuolated epithelium and the

inclusion body (arrow) in extruded epithelial cell.

Toluidene blue, $\frac{1}{2}\mu$m section. X 2,500.
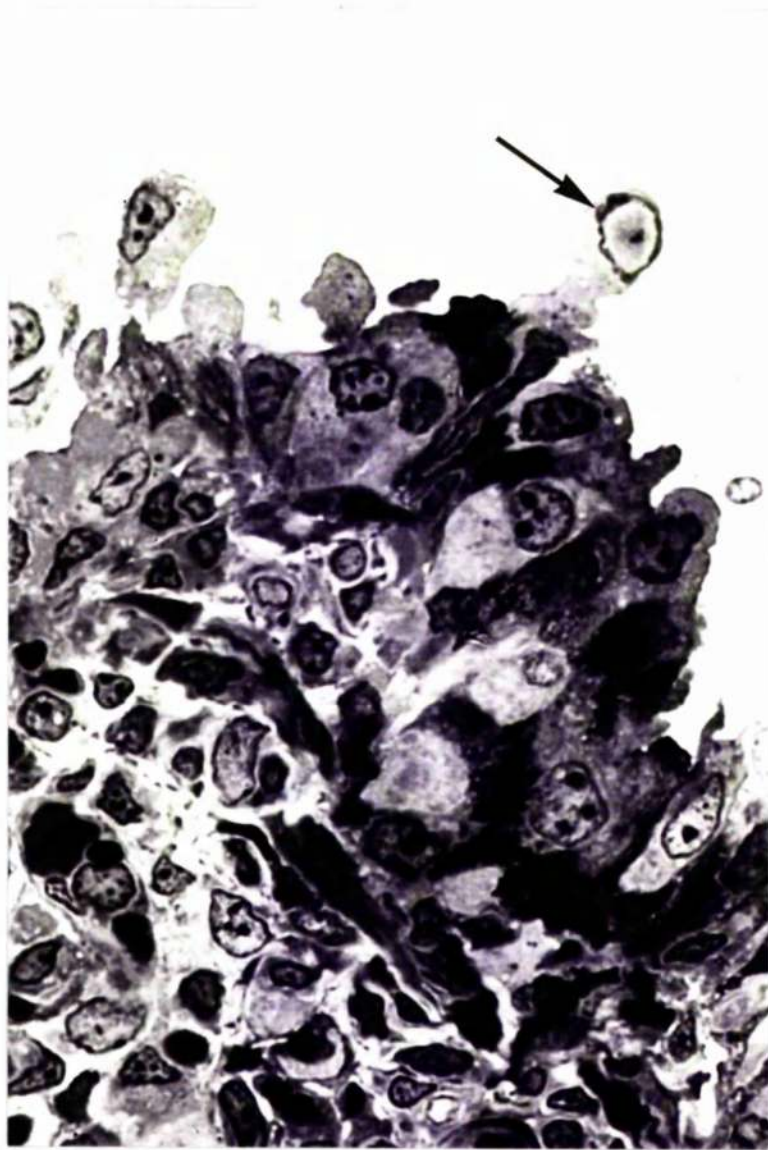
Fig. 65   Experimental CAV-1 infection: necrotizing bronchiolitis

5 days after infection. Note epithelial necrosis and

catarrhal exudate in the lumen. A few intranuclear

inclusion bodies (arrows) can be seen.

Haemalum and eosin. X 300.

Fig. 66   Experimental CAV-1 infection: necrotizing bronchiolitis

5 days after infection. Macrophages and

polymorphonuclear leucocytes can be seen in the

lumen of the bronchiole.

Toluidene blue, 1μm section. X 1,500.
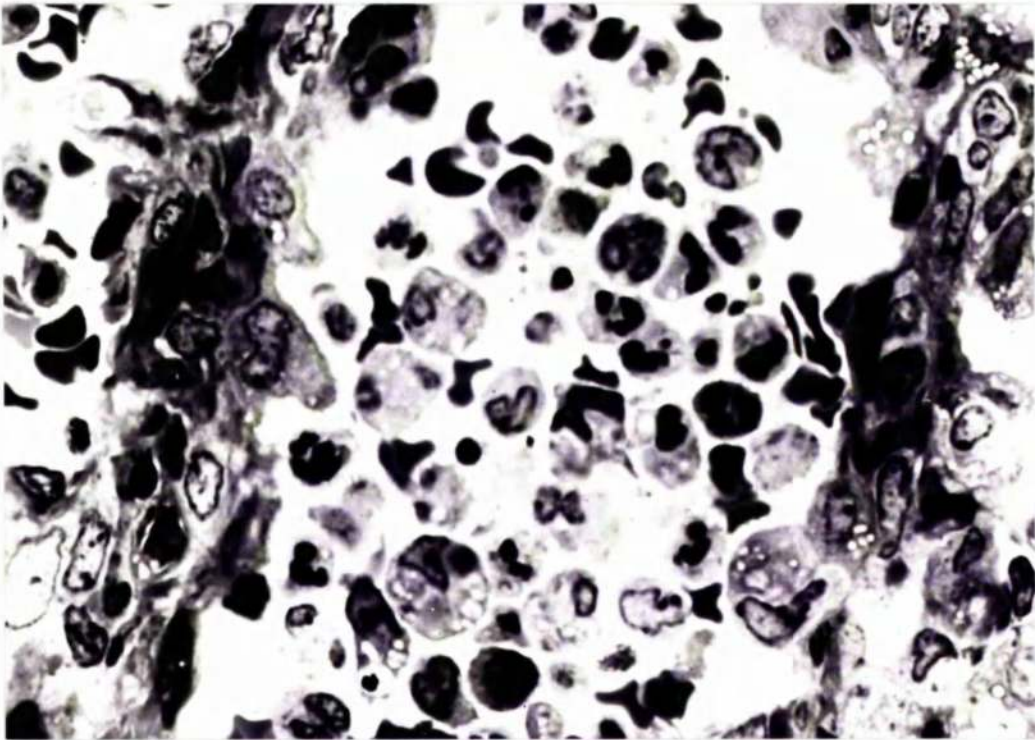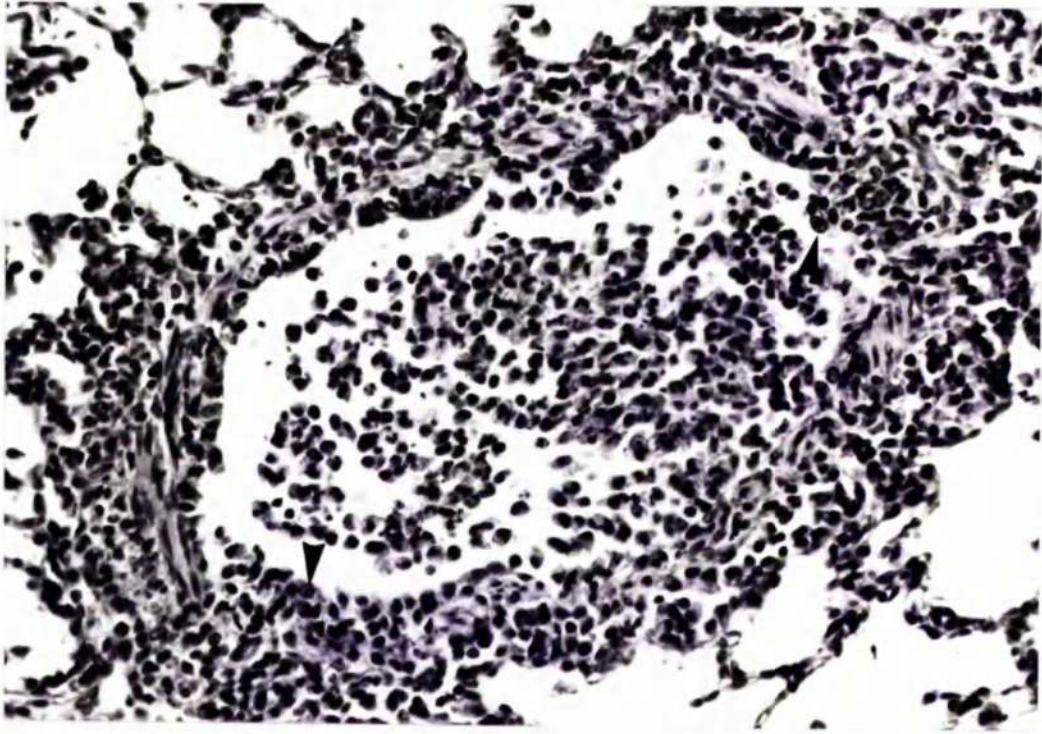
Fig. 67   Experimental CAV-1 infection: necrotizing bronchiolitis,

5 days after aerosolisation. The lumen of the

bronchiole is plugged with necrotic epithelial cells.

Note the intranuclear inclusion bodies (arrows).

Toluidene blue, 1μm section. X 1,200.
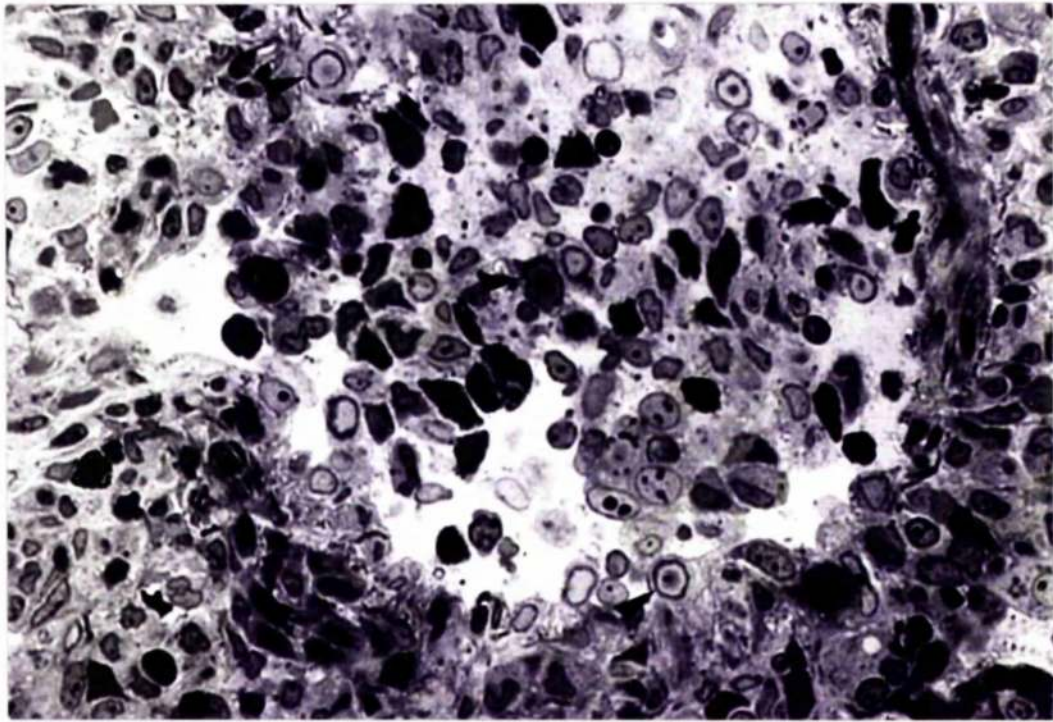
Fig. 68   Experimental CAV-1 infection: organisation of a

bronchiolar exudate, 15 days after infection by

aerosol. Areas of epithelial hyperplasia can also be

seen.

Martius-scarlet-blue. X 300.

Fig. 69   Experimental CAV-1 infection: bronchiolitis obliterans,

20 days after infection by aerosol.

Haemalum and eosin. X 160.
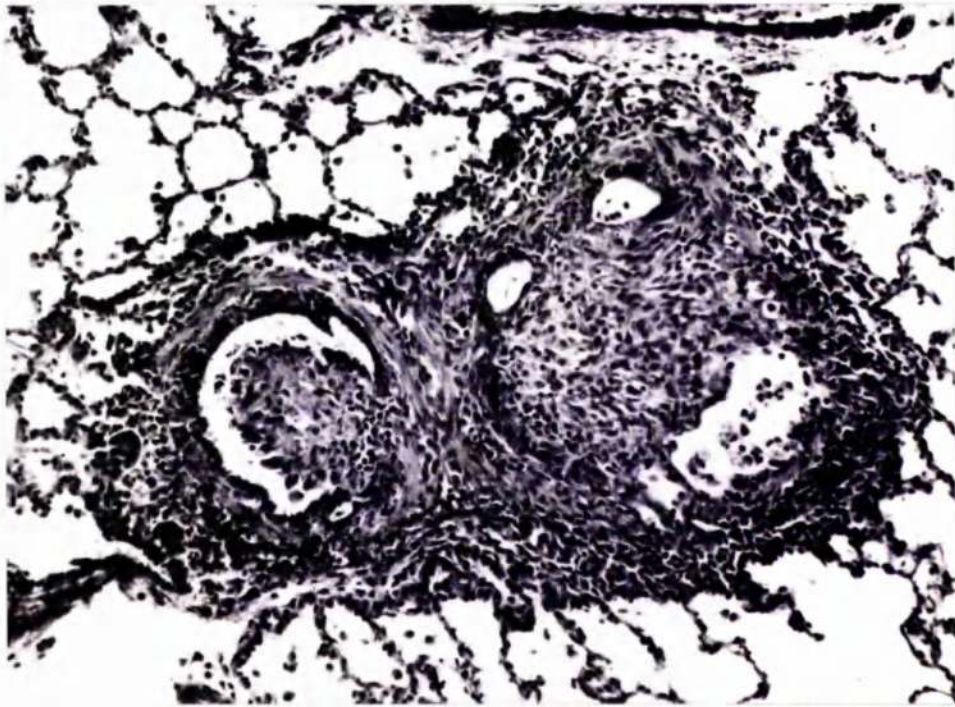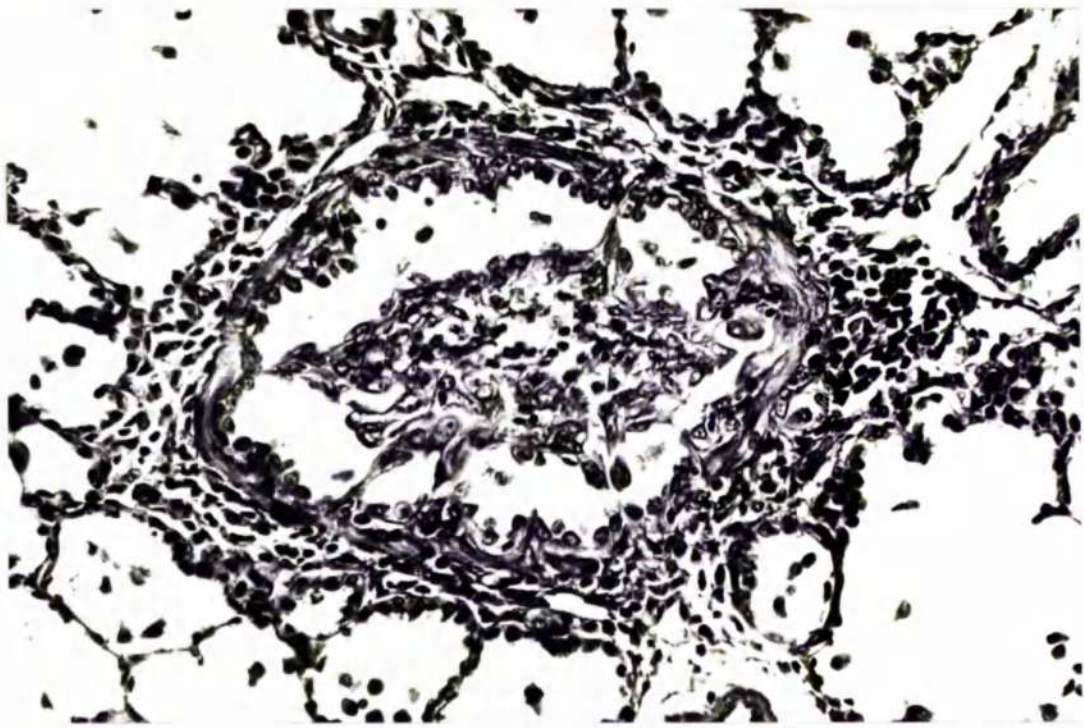
Fig. 70   Experimental CAV-1 infection: intranuclear inclusion

bodies (arrow) in turbinate epithelium, 5 days

after aerosolisation.

Haemalum and eosin. X 160.

Fig. 71   Experimental CAV-1 infection: intranuclear inclusion

body in an epithelial cell of the turbinate mucosa,

5 days after aerosolisation.

Fluorescent Feulgen. X 800.
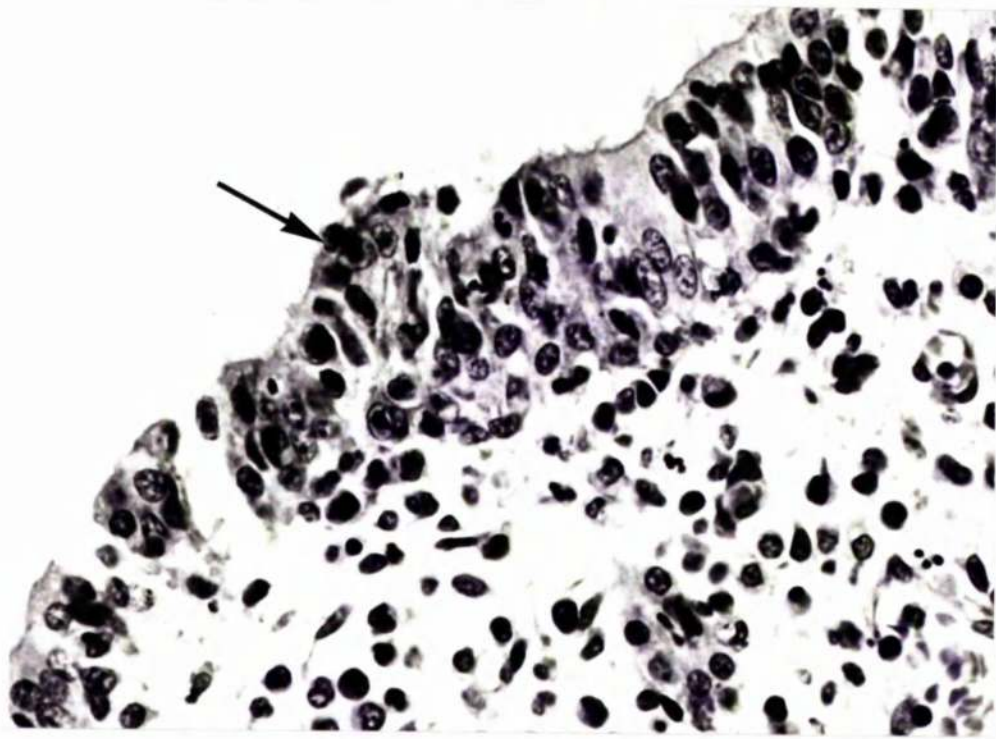
Fig. 72   Experimental CAV-1 infection: CAV-1 antigen in

bronchiolar epithelial cells, 2 days after

infection by aerosol.

Fluorescent antibody. X 800.

Fig. 73   Experimental CAV-1 infection: CAV-1 antigen in

bronchiolar epithelial cells and peribronchiolar

macrophages, 5 days after infection.

Fluorescent antibody. X 800.
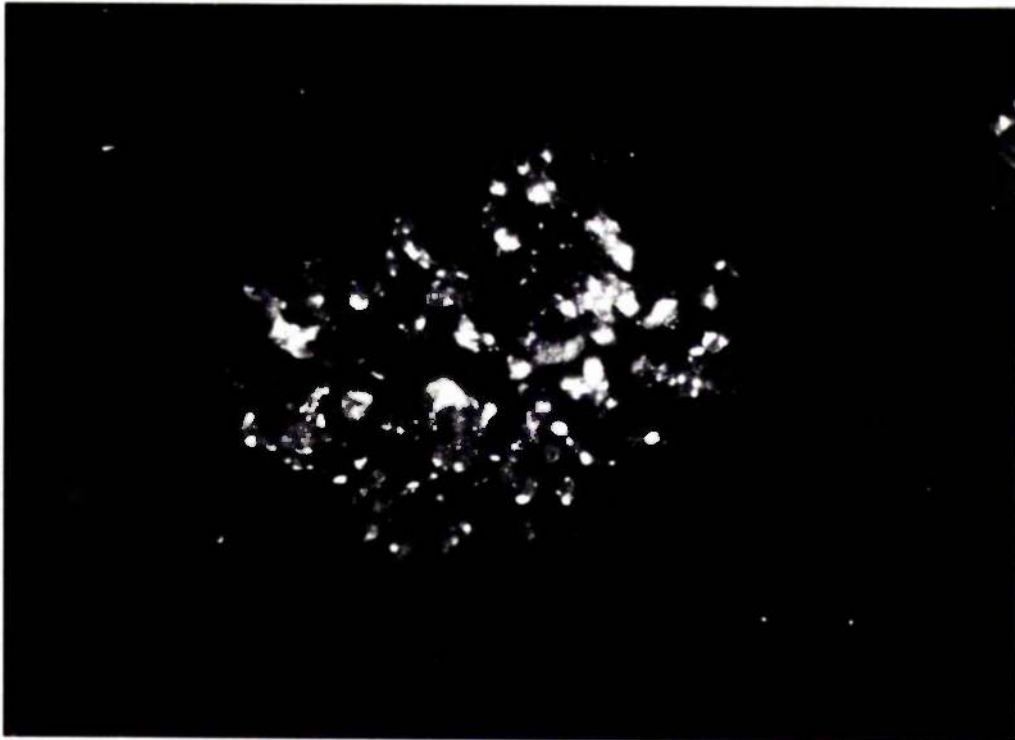
Fig. 74   Experimental CAV-1 infection: CAV-1 antigen in

alveolar and septal macrophages, 6 days after

infection.

Fluorescent antibody. X 800.

Fig. 75   Experimental CAV-1 infection: CAV-1 antigen in

tonsillar epithelial cells, 4 days after aerosolisation.

A brightly staining inclusion body (arrow) can be seen

in the nucleus of one cell.

Fluorescent antibody. X 800.
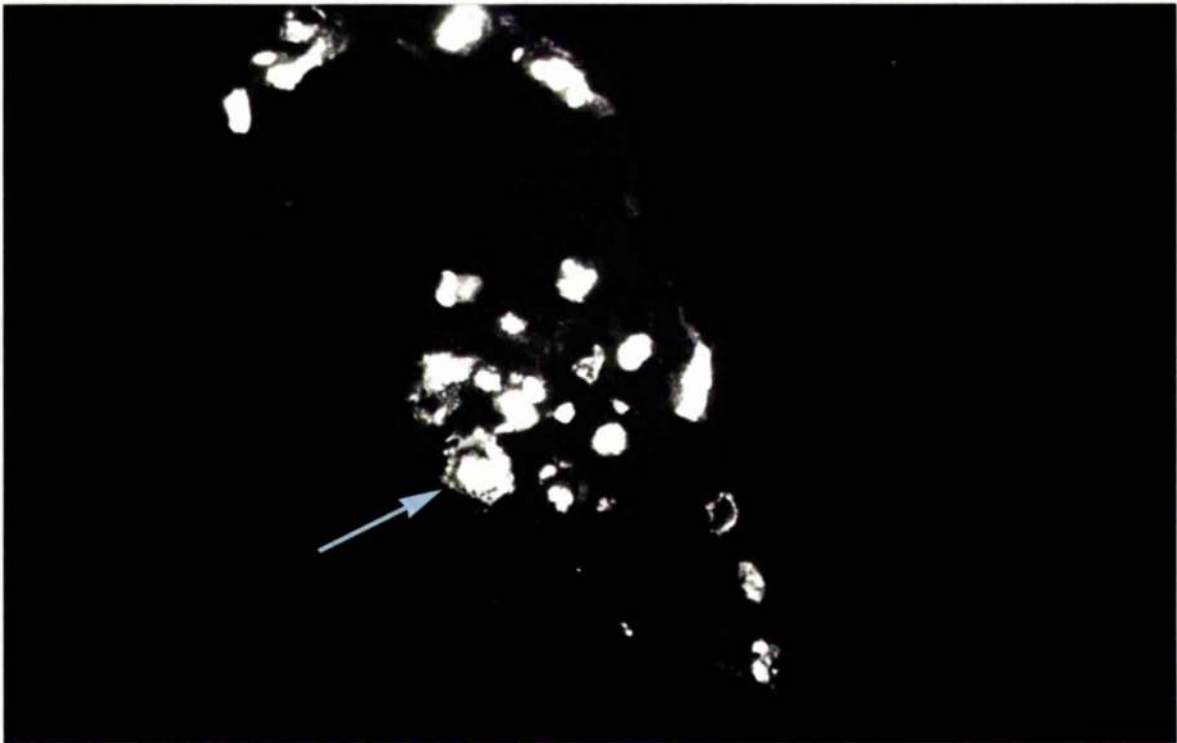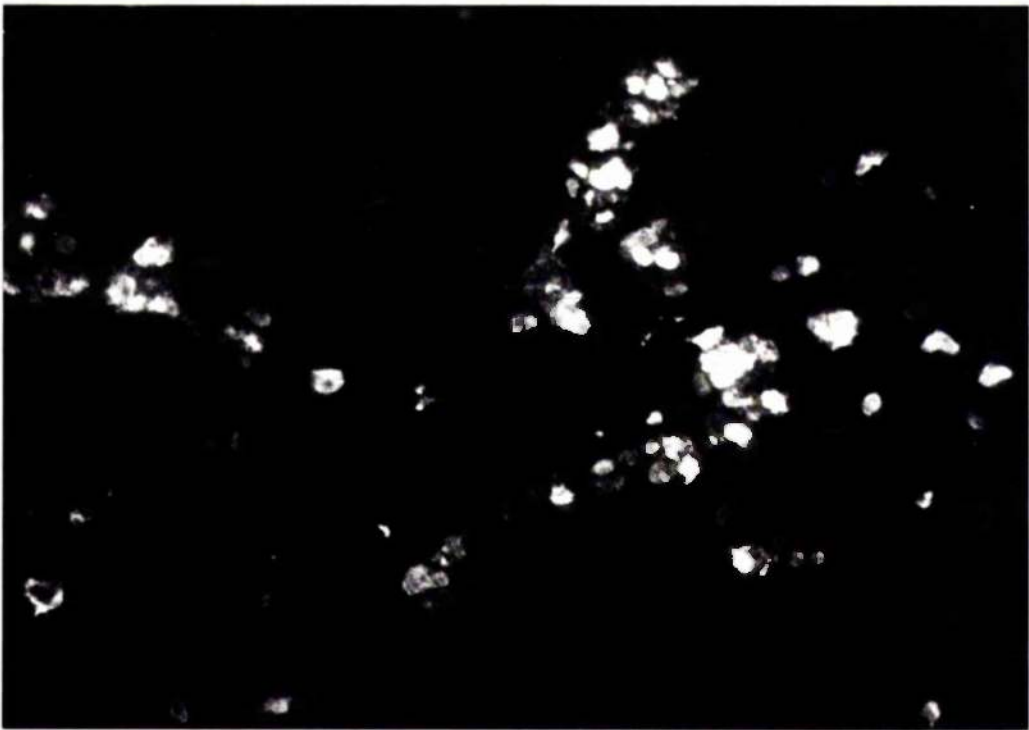
Fig. 76   Experimental CAV-1 infection: bronchiolar epithelium

2 days after aerosolisation. The nucleus of an

epithelial cell contains numerous virus particles (V)

embedded in a granular matrix; the peripheral

chromatin is irregularly marginated.

X 10,000.
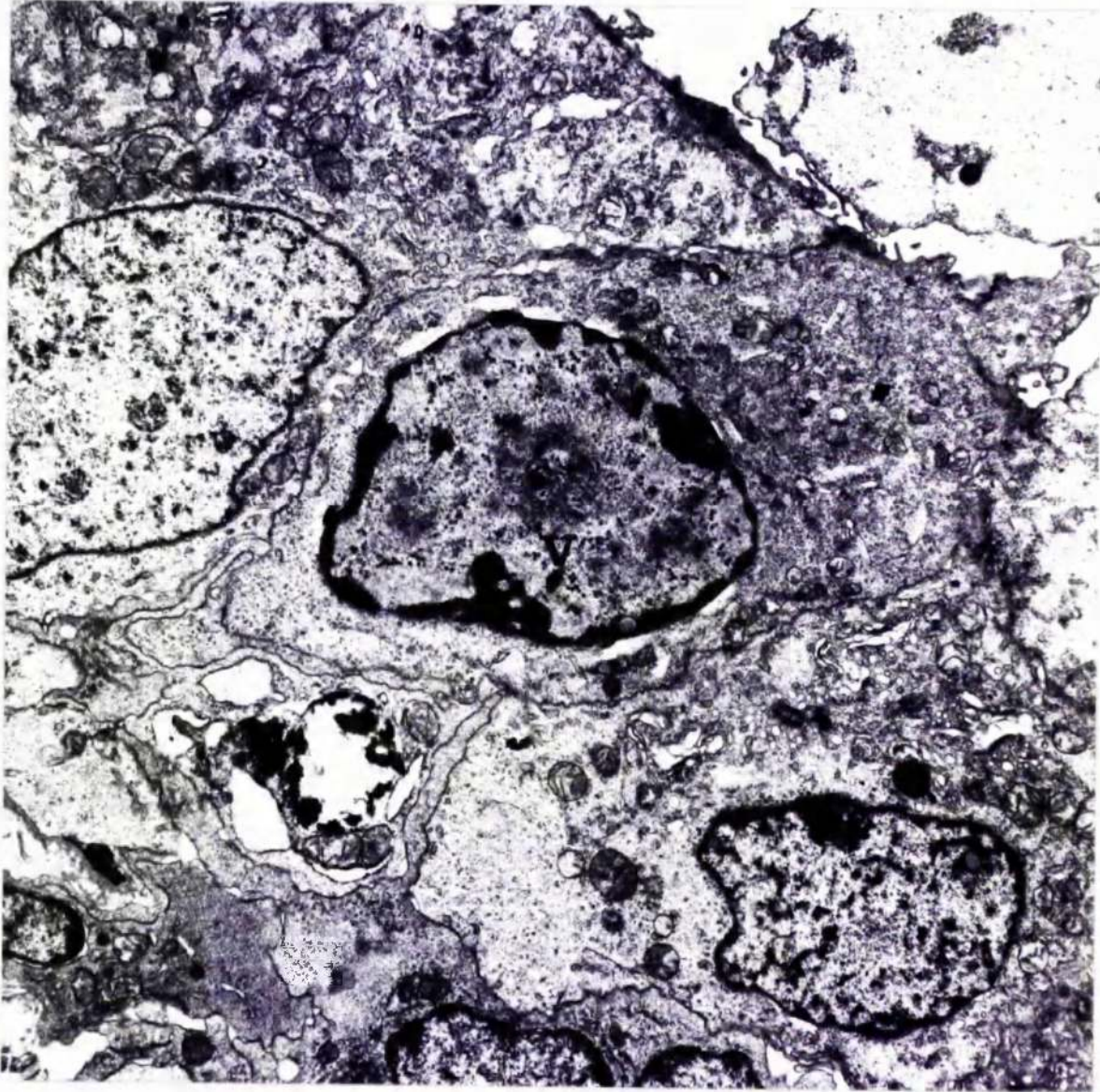
Fig. 77   Experimental CAV-1 infection: part of the nucleus of an

infected epithelial cell to show adenovirus virions (V)

Two crystalline structures (Cy) are present: at the

top a cross section: on the right a longitudinal

section.

X 60,000.
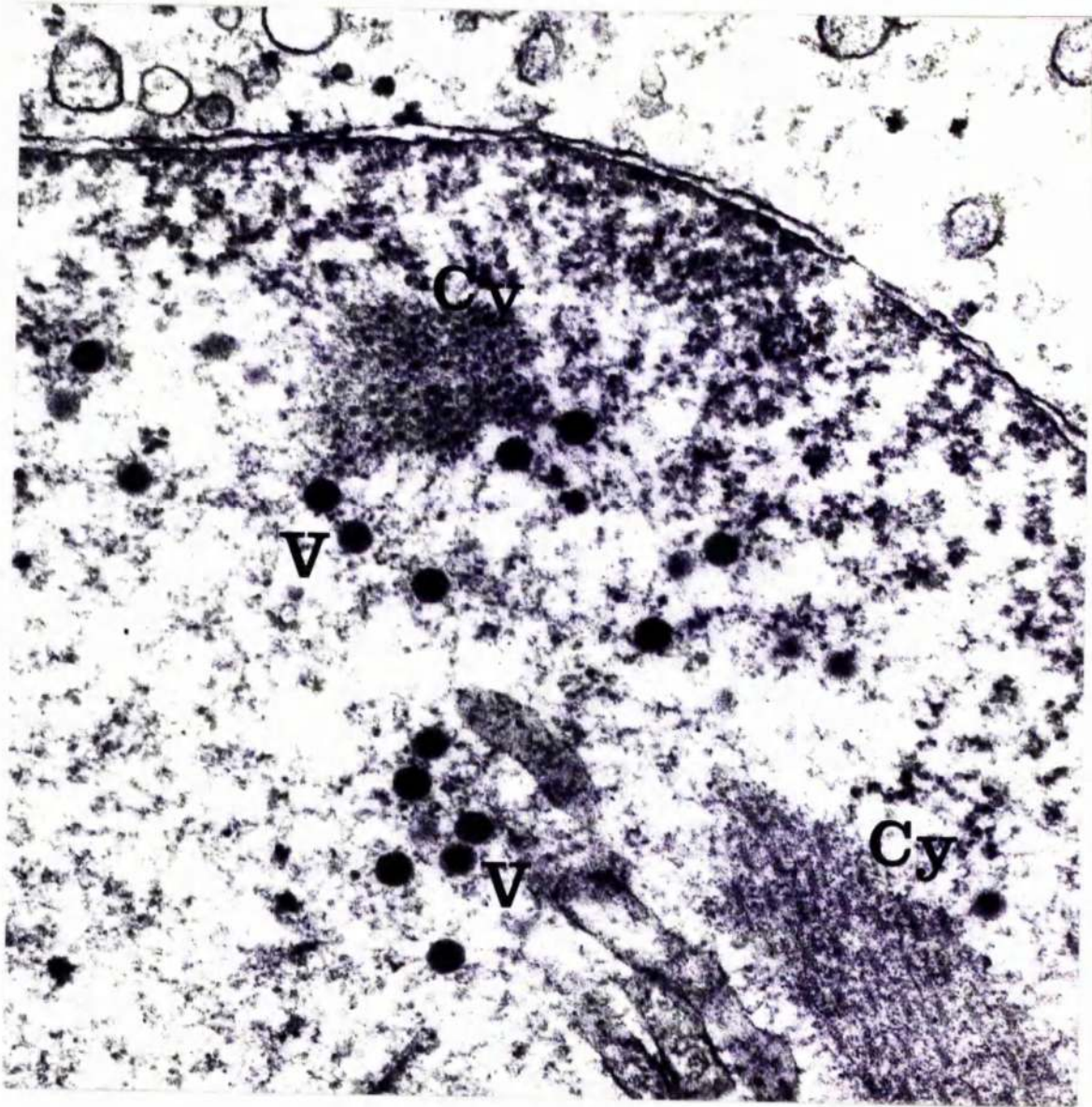
Fig. 78   Experimental CAV-1 infection: bronchiole 2 days

after infection by aerosol. One of the cells

(top left) shows an early stage of infection; virus

particles are not visible but fine granular material

(GM) is present in the centre of the nucleus. A

second cell (lower right) shows a formed inclusion

(In) with virus particles (V) scattered throughout

the nucleus.

X 6,000.
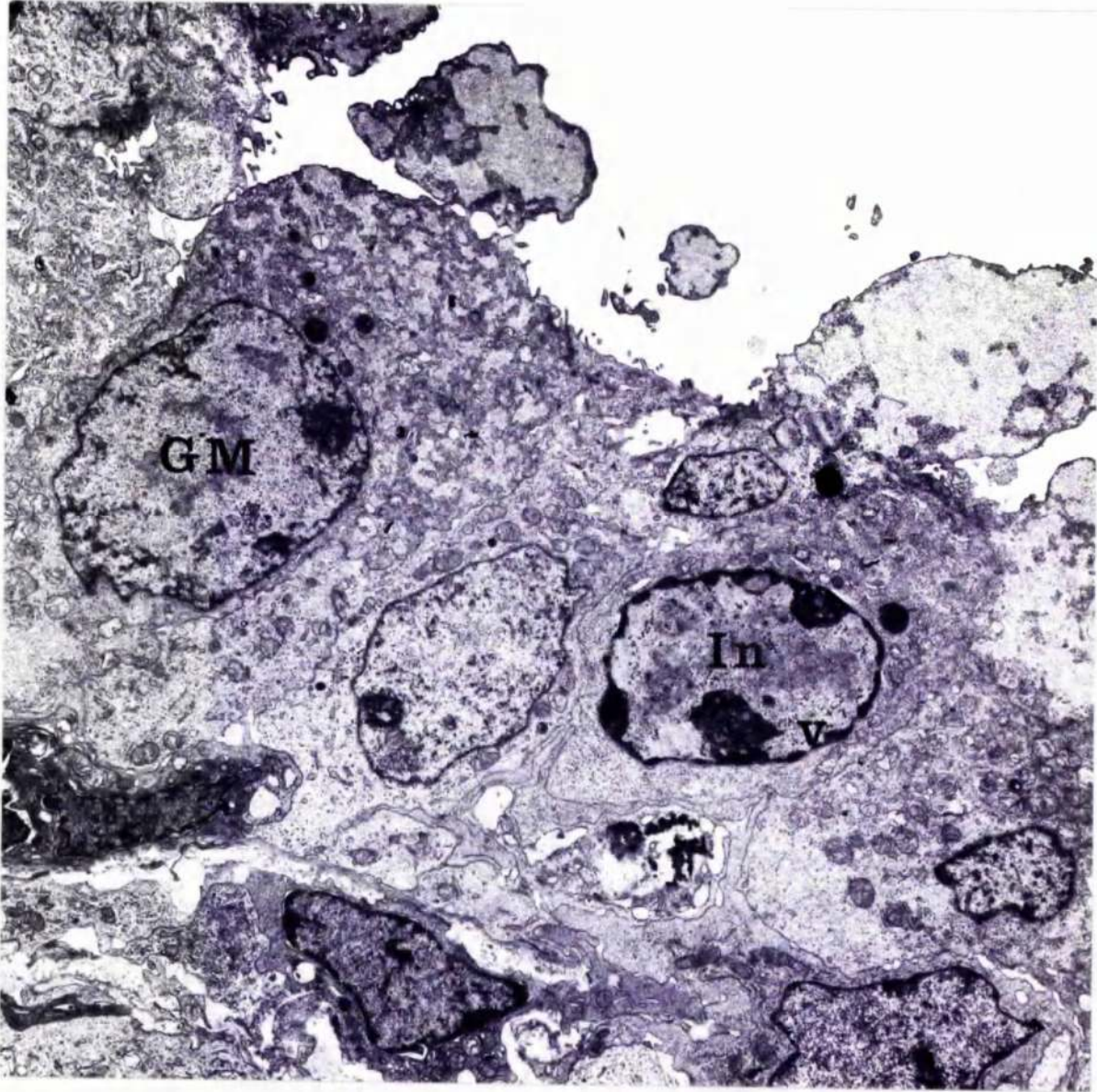
Fig. 79   Experimental CAV-1 infection: necrotic cells in the

lumen of a bronchiole 5 days after aerosolisation.

The inclusion bodies (In) in the centres of the

nuclei are composed of aggregates of fine electron

dense granular material. The virus particles (arrows)

are concentrated around the edge of the inclusion

and large lipid granules are contained in the   .

cytoplasm.

X 10,000.
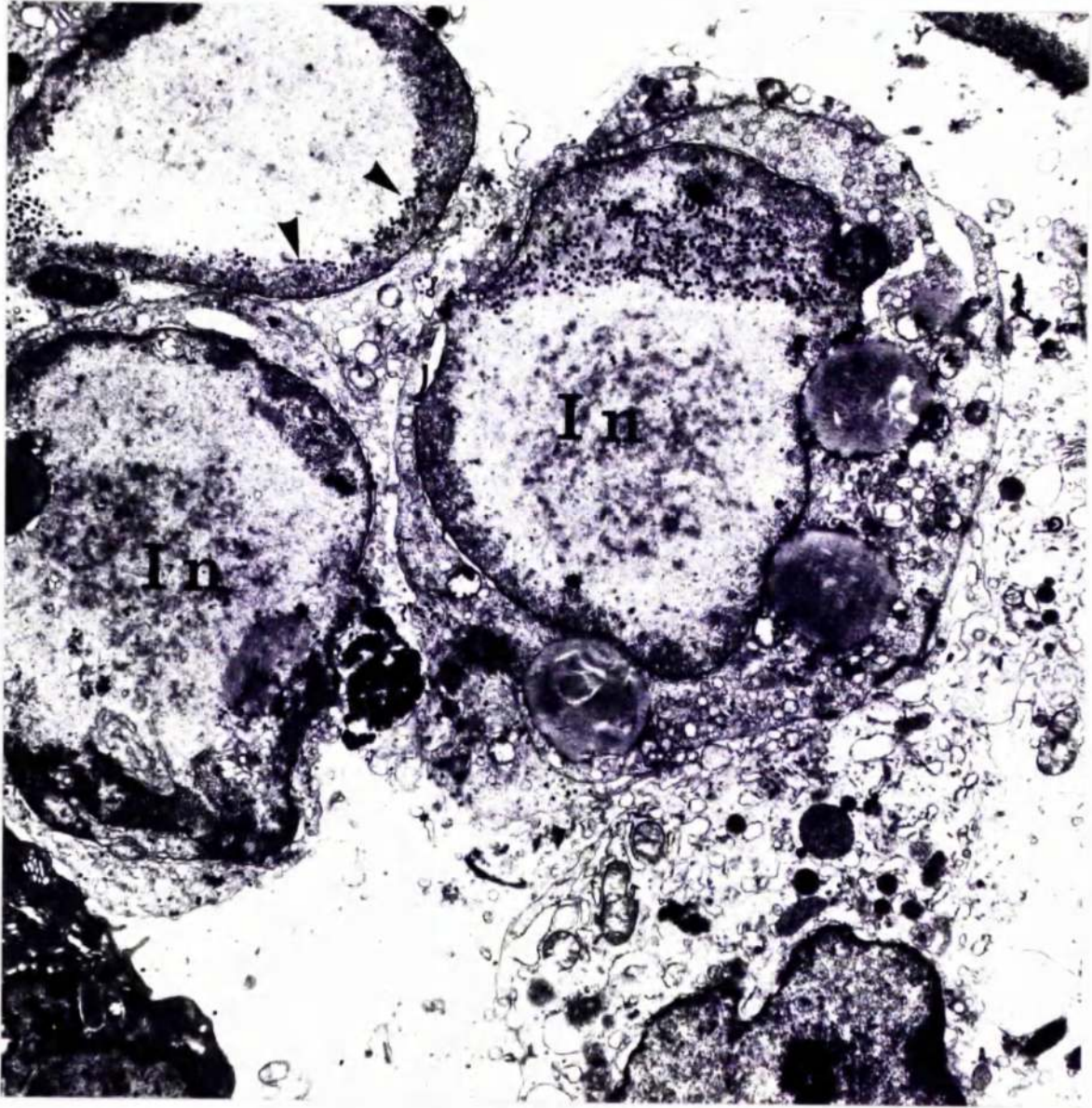
Fig. 80   Experimental CAV-1 infection: necrotic bronchiolar

epithelial cell 5 days after aerosolisation.

Adenovirus virions (V) are concentrated at the

nuclear periphery and are also free in the cytoplasm.

Note the clumping of the nuclear chromatin and the

crystalline formations (Cy).

X 20,000.
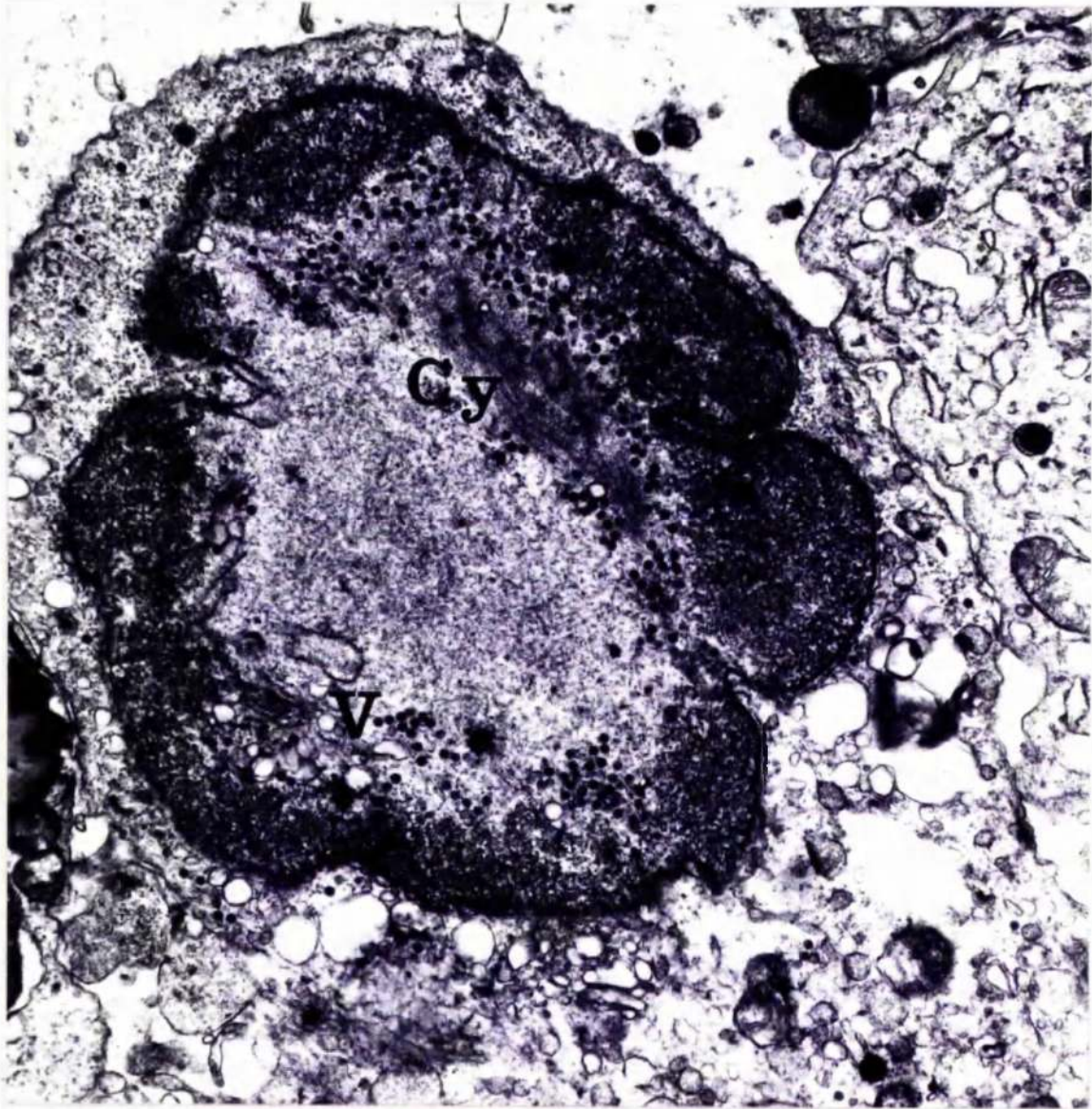
Fig. 81   Experimental CAV-1 infection. Macrophage (M) engulfing

cellular debris and fibrin (Fn) in the lumen of a

bronchiole 5 days after aerosolisation. Note the

adenovirus particles (arrow) released from the

ruptured nucleus.

X 10,000.
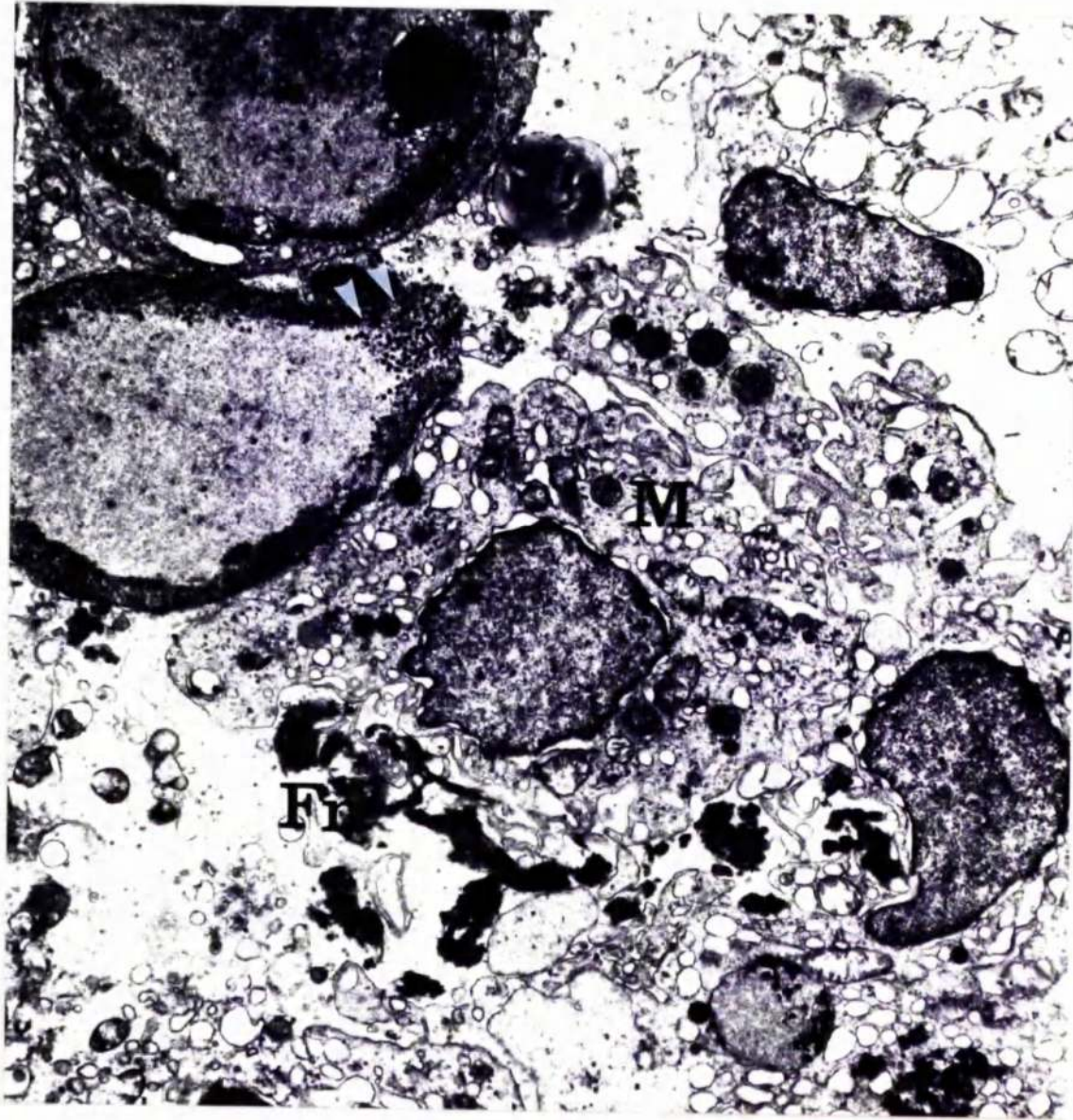
Fig. 82   Experimental CAV-1 infection: portion of an epithelial

cell 5 days after aerosolisation. Adenovirus

particles (V) separated by crystalline formations

(Cy).

X 40,000.

Fig. 83   Experimental CAV-1 infection. Polymorphonuclear

leucocytes (P), erythrocytes and cell debris in an

alveolus, 5 days after aerosolisation.
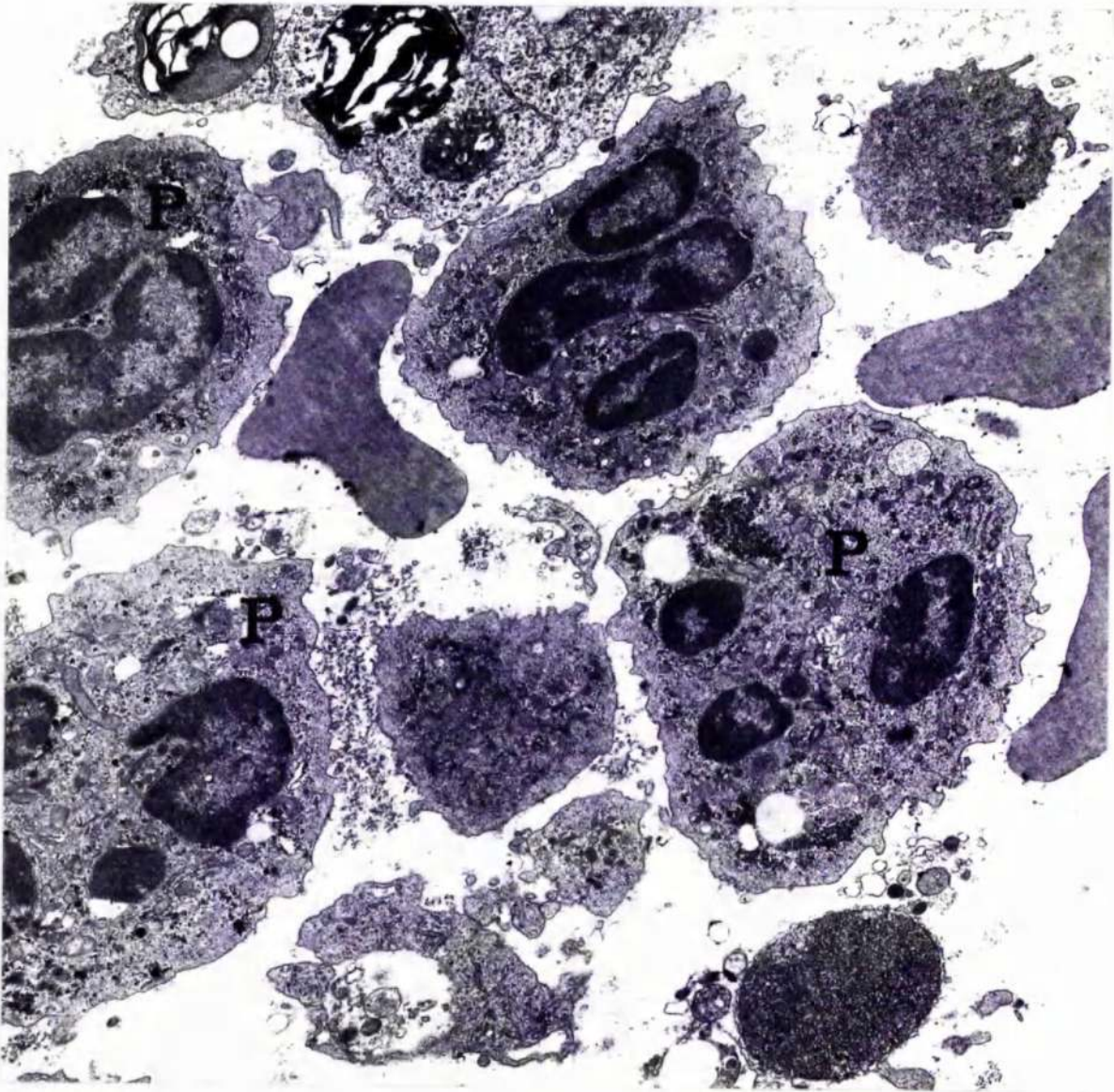
X 10,000.

Fig. 84   Experimental CAV-1 infection. Macrophages (M) and a

lymphocyte (L) in an alveolus, 7 days after

aerosolisation.

X 10,000.
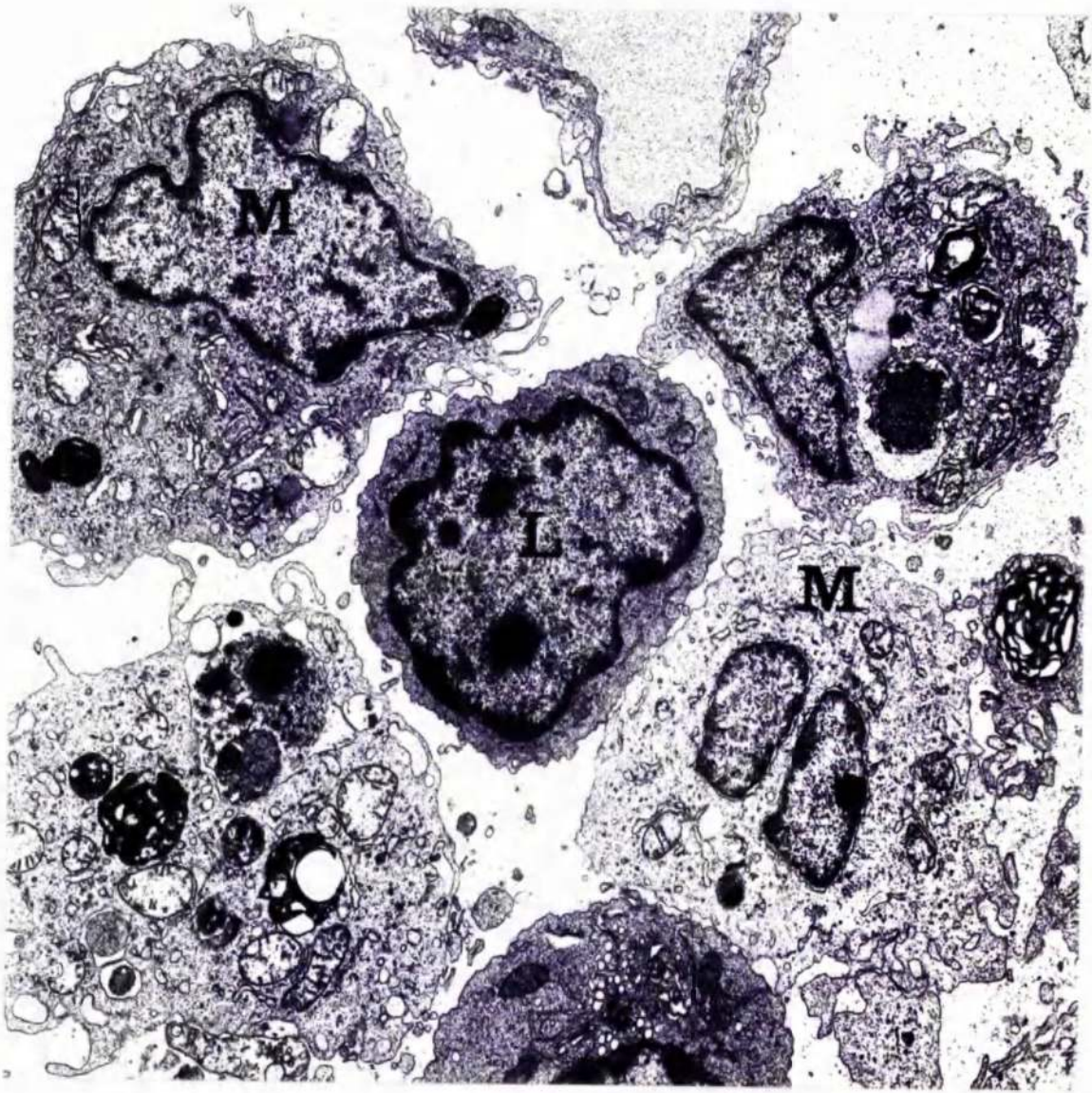
Fig. 85   Experimental CAV-1 infection: an electron micrograph

of part of an alveolar macrophage to show a

phagocytosed red blood cell (RBC).

X 15,000.
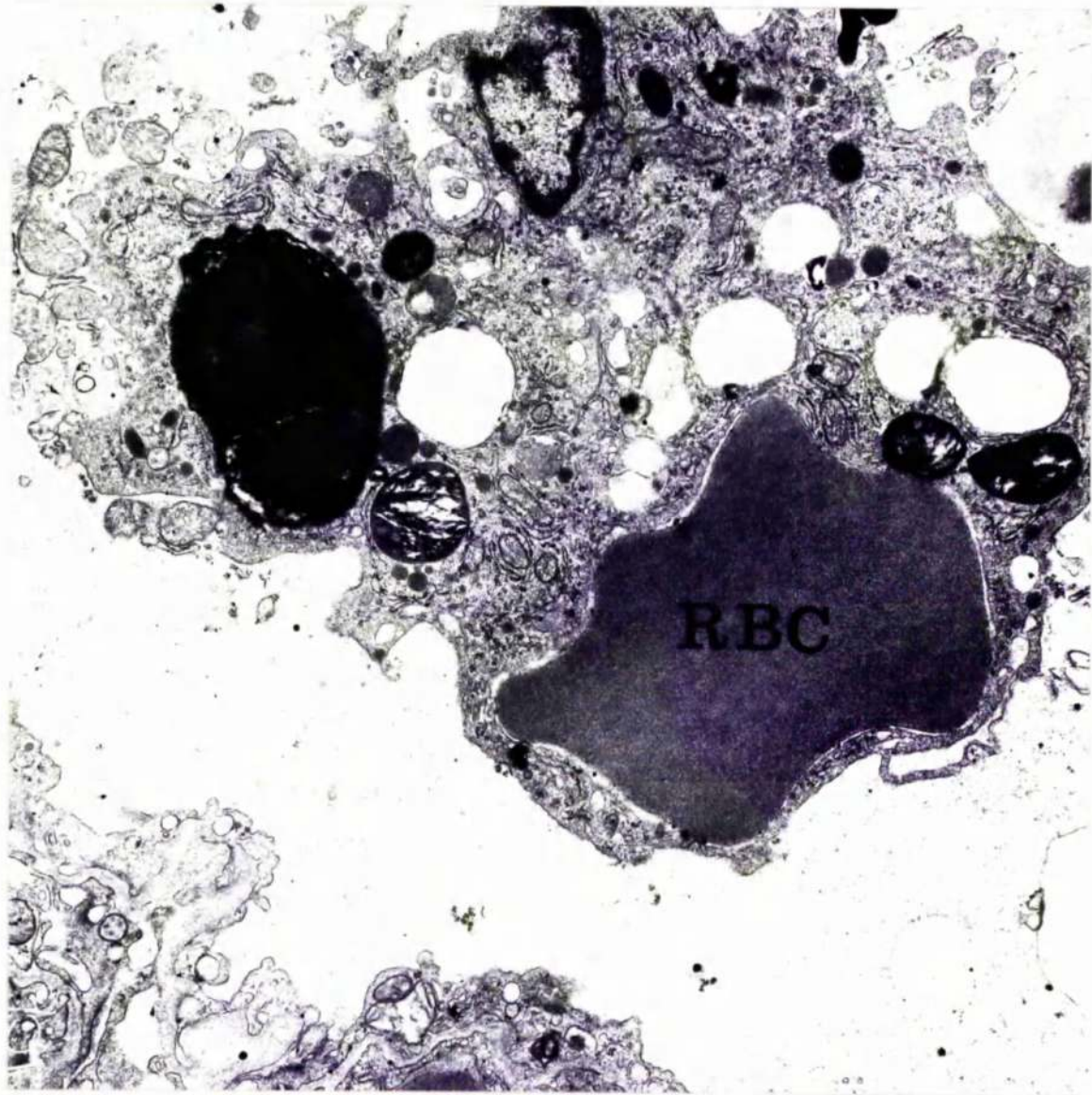
Fig. 86   Experimental CAV-1 infection: macrophage (M) in an

alveolus, 5 days after aerosolisation. Virus

particles (V) are present in the nucleus of one

of these cells.

X 6,000.
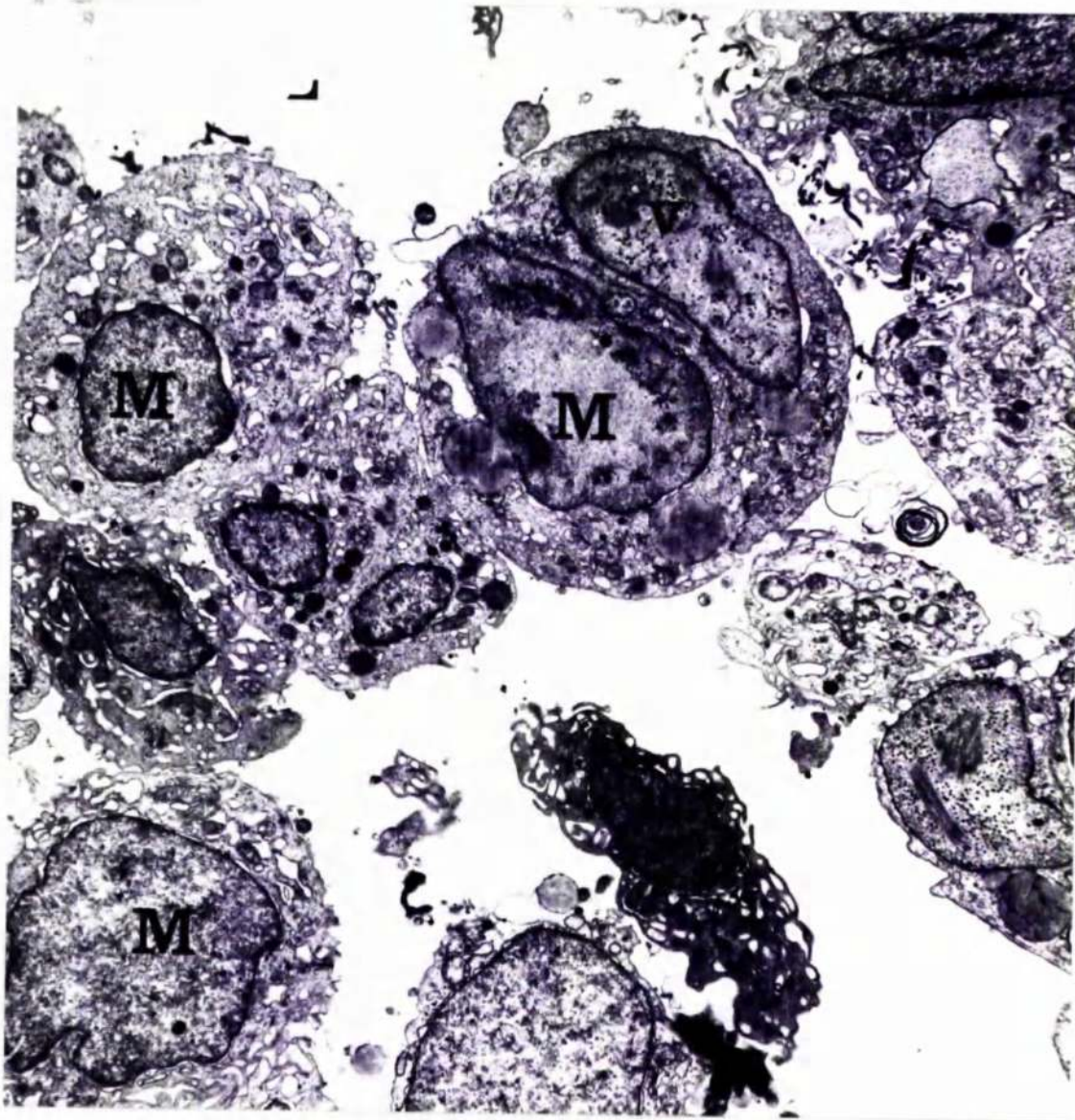
Fig. 87   Experimental CAV-1 infection: an interalveolar septa

5 days after aerosolisation. The nucleus of the

type 2 alveolar cell is pyknotic and the cytoplasm

is vacuolated. Capillary (Cap).

X 10,000.

Cap
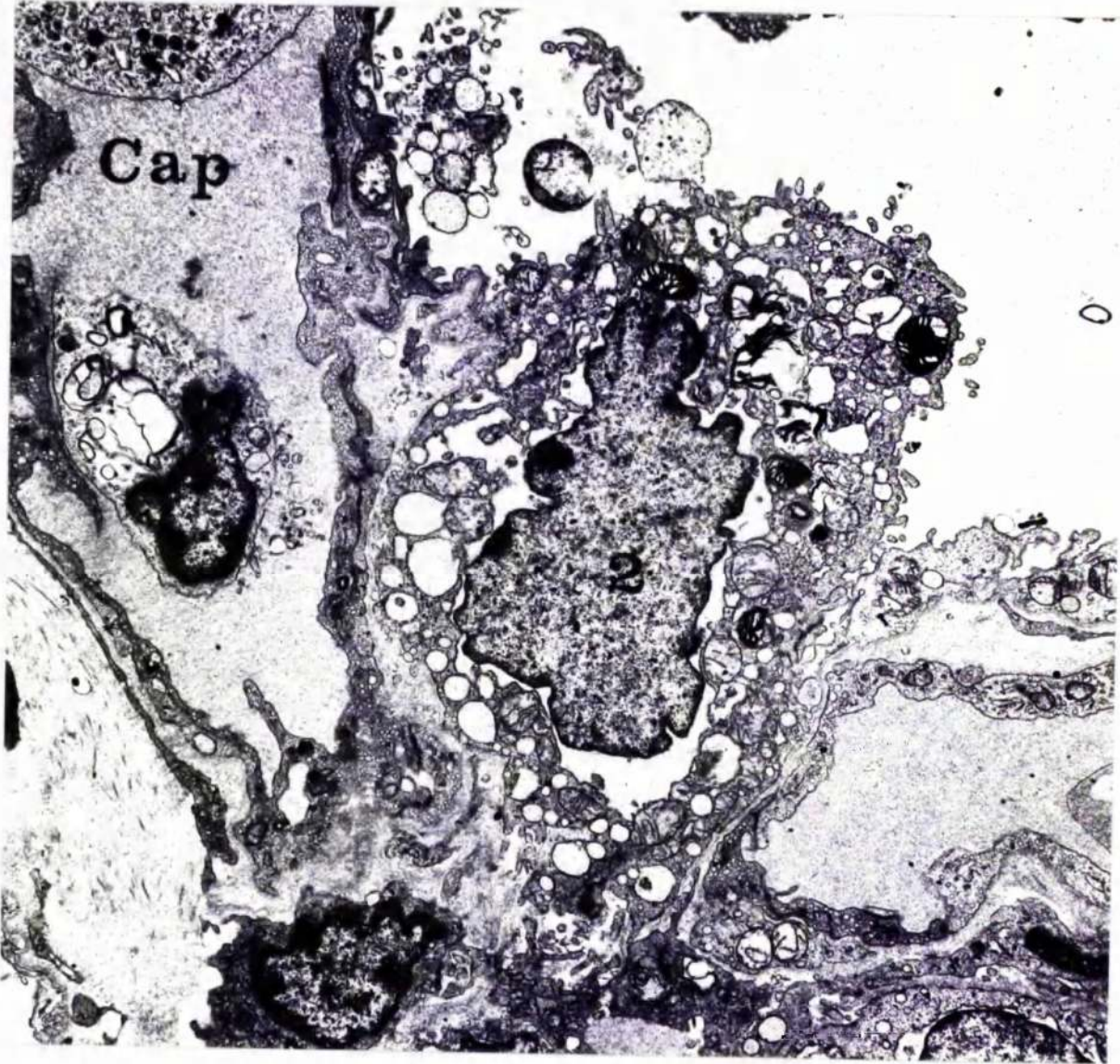
Fig. 88   Natural CAV-1 infection: dorsal aspect of the

diaphragmatic lobe of the lungs to show multiple

tiny grey foci.

Fig. 89   Natural CAV-1 infection: cellular focus in the lung.

Alveolar septa are thickened by infiltration of

macrophages and lymphocytes.

Haemalum and eosin. X 300.
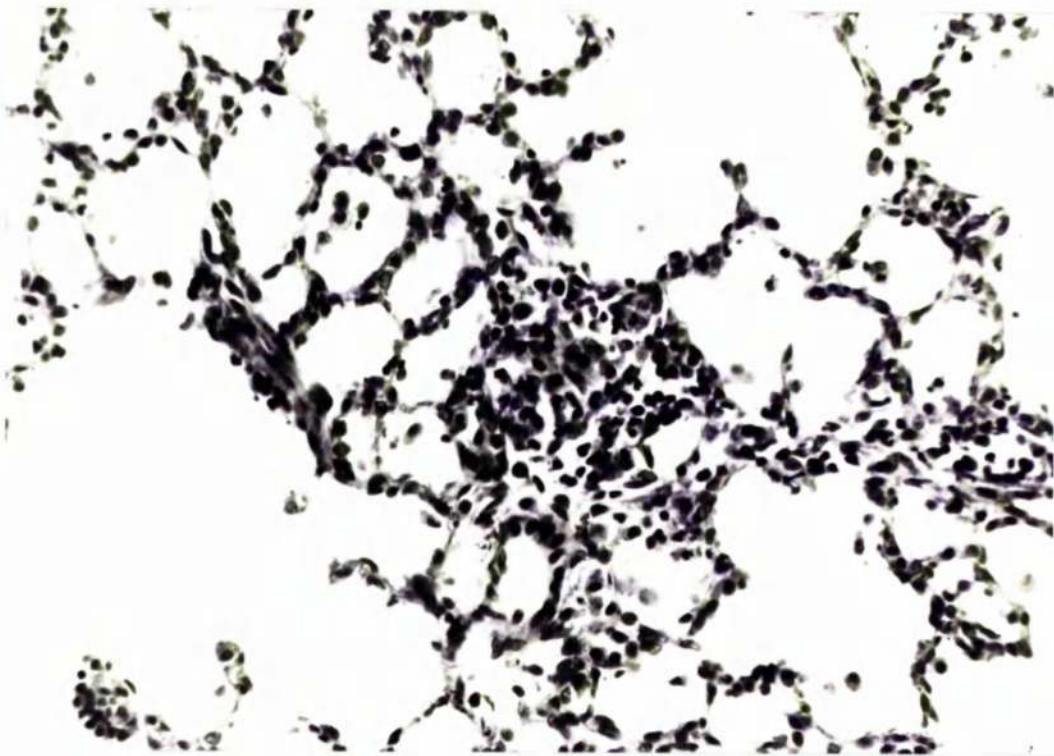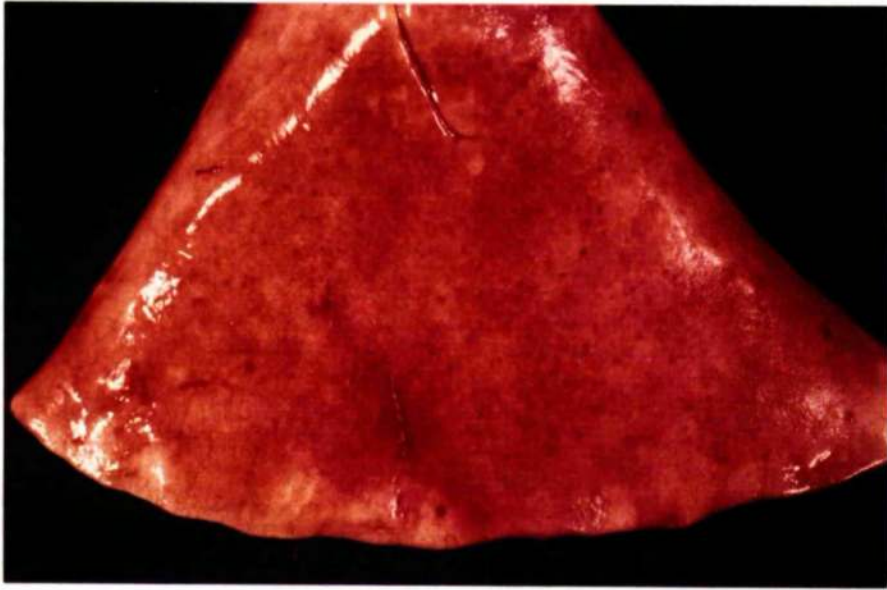
Fig. 90   Natural CAV-1 infection: rhinitis. There is a

pleomorphic cellular infiltrate in the nasal mucosa.

A few intranuclear inclusion bodies (arrows) can be

seen in the epithelium and lamina propria.

Haemalum and eosin. X 600.

Fig. 91   Natural CAV-1 infection: lymphadenitis. The

subcapsular sinus is distended with oedema fluid and

a large number of mononuclear cells are present.

Note the intranuclear inclusion bodies (arrow).

Haemalum and eosin. X 500.
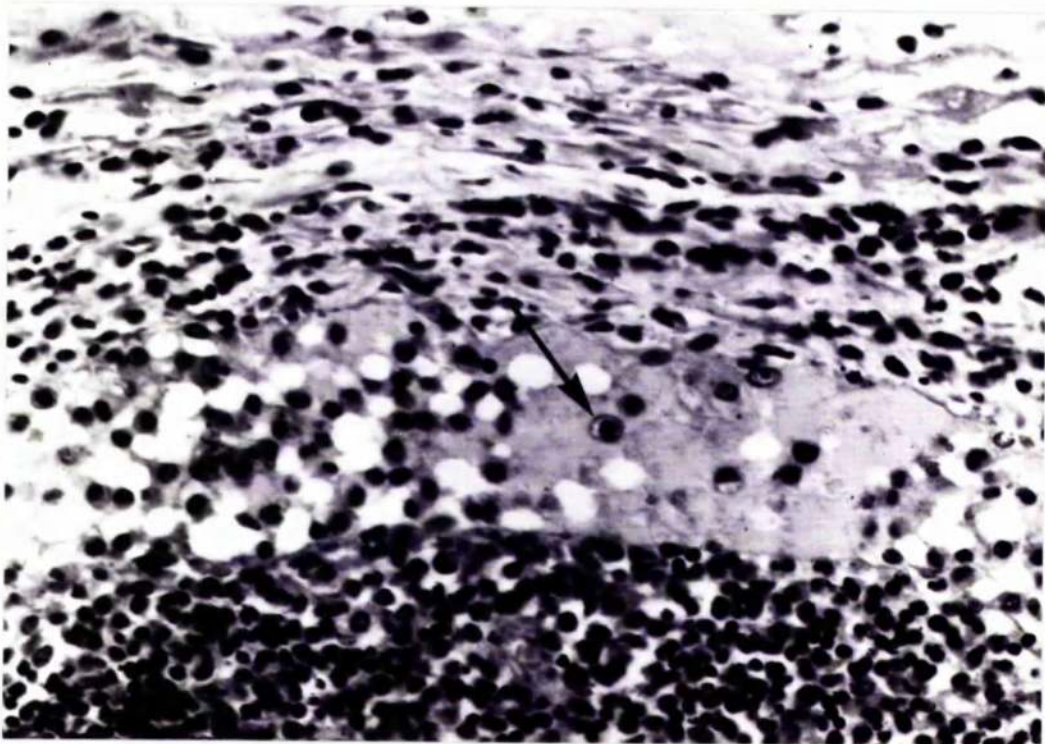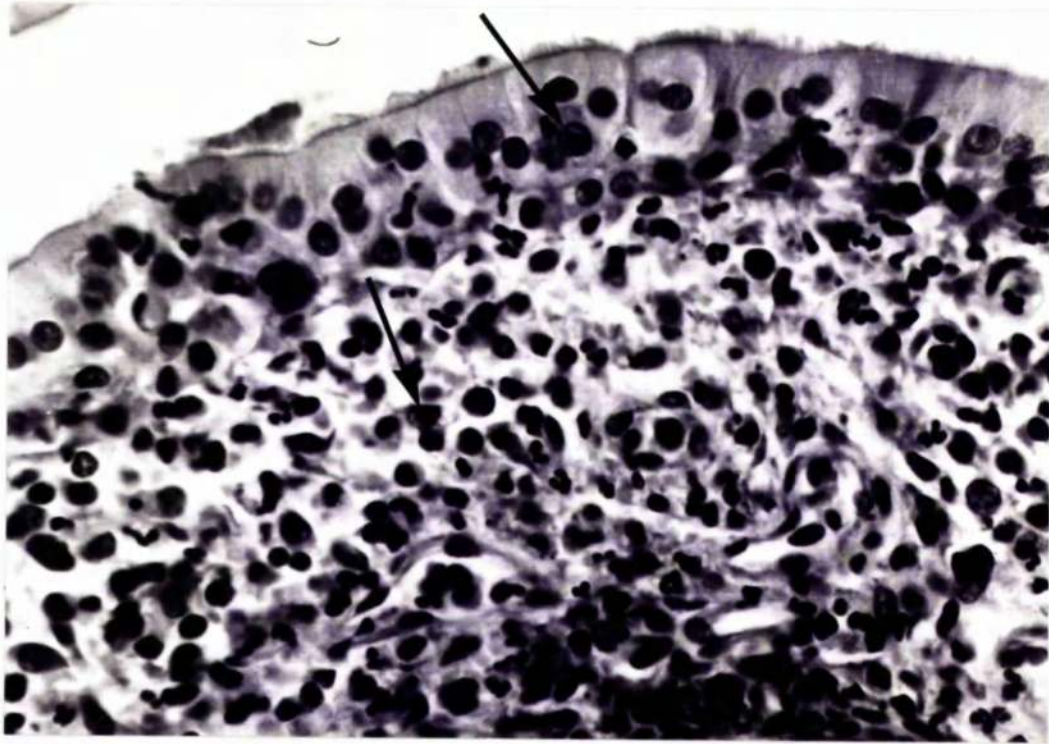
Fig. 92   Culture fluid harvested at 48 hours from GHK cells

infected with "R" virus. Virus particles negatively

stained with 2 per cent phosphotungstic acid.

X 60,000.

A single particle can be seen with capsomeres

arranged in icosahedral symmetry.

X 120,000. (inset)
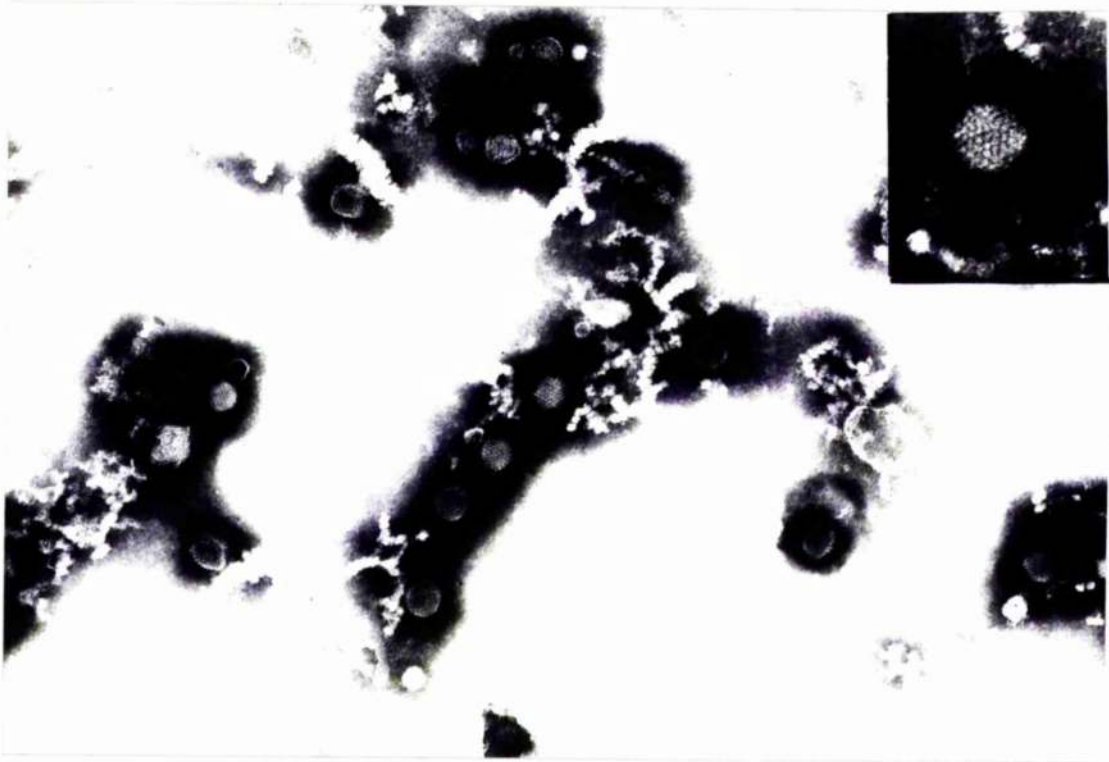
Fig. 93   GHK cells 24 hours after inoculation with "C" virus.

Note the large vacuolated intranuclear inclusion

bodies.

Giemsa. X 1,500.

Fig. 94   GHK cells 24 hours after inoculation with "C" virus.

Note the cell with margination of the chromatin and

the intranuclear inclusion. The inclusion is

surrounded by a clear halo.

Giemsa. X 1,500.
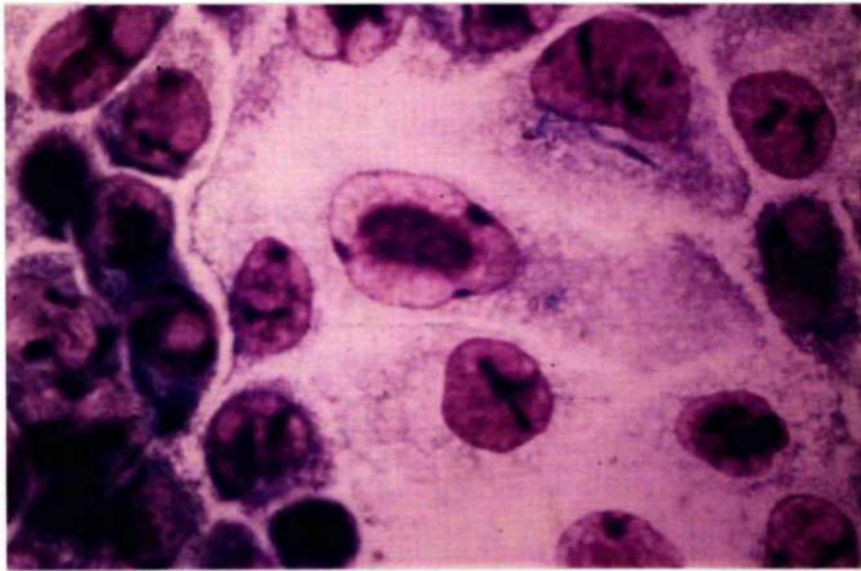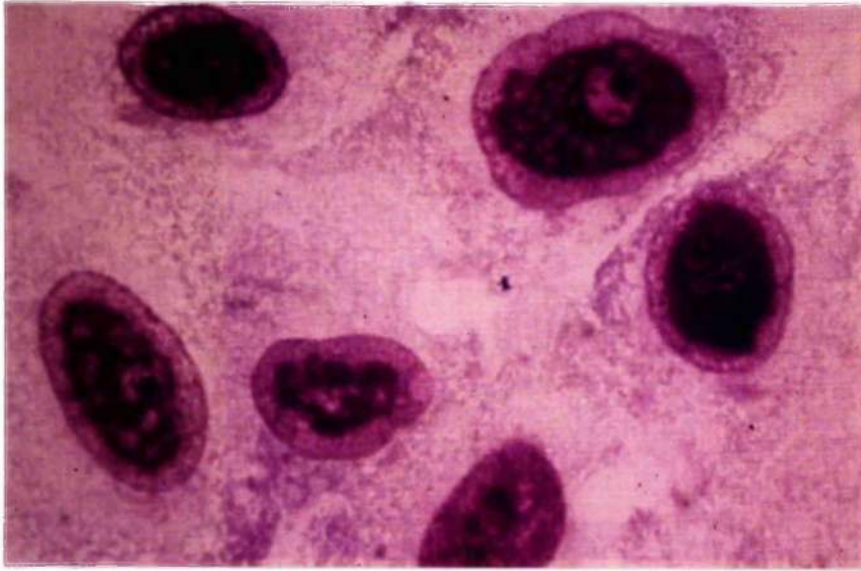
Fig. 95   GHK cells 24 hours after inoculation with "B" virus.

The intranuclear inclusions stain green.

Acridene orange.   X 500.

Fig. 96   Uninfected GHK cells.

Acridene orange.   X 500.
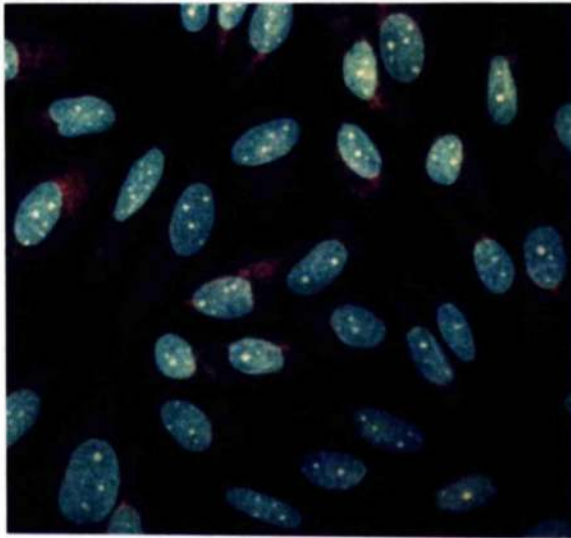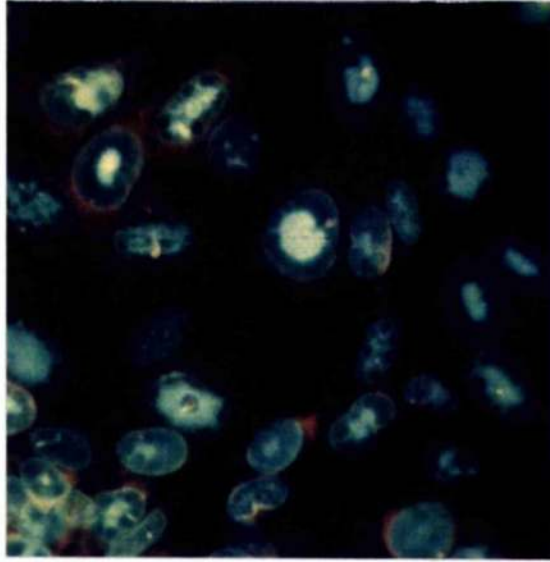
Fig. 97   GHK cells 24 hours after inoculation with "F" virus.
Note the intranuclear inclusion bodies stain yellow.

Fluorescent Feulgen.   X 500.
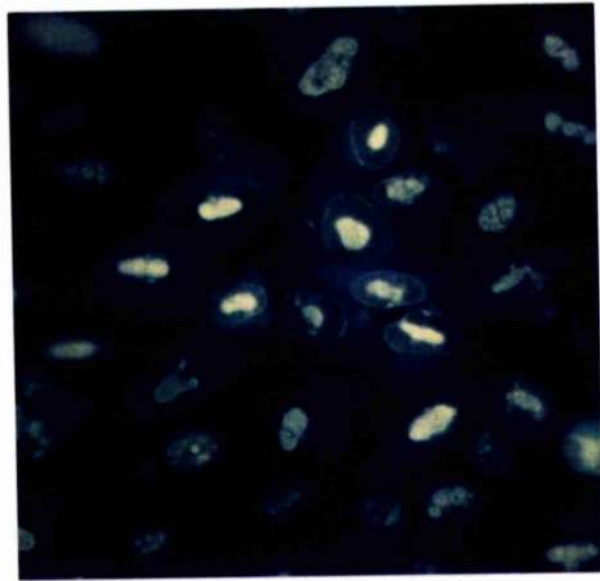
Fig. 98   GHK cells 24 hours after inoculation with "C" virus.

Note granular CAV-1 antigen scattered throughout

the nuclei or concentrated around the nuclear

periphery.

Fluorescent antibody. X 800.

Fig. 99 Electron micrograph of GHK cell 24 hours after

inoculation with "C" virus. Clumps of dense, fine

granular material are found in the nucleus and a

few virus particles are present.

X 20,000.
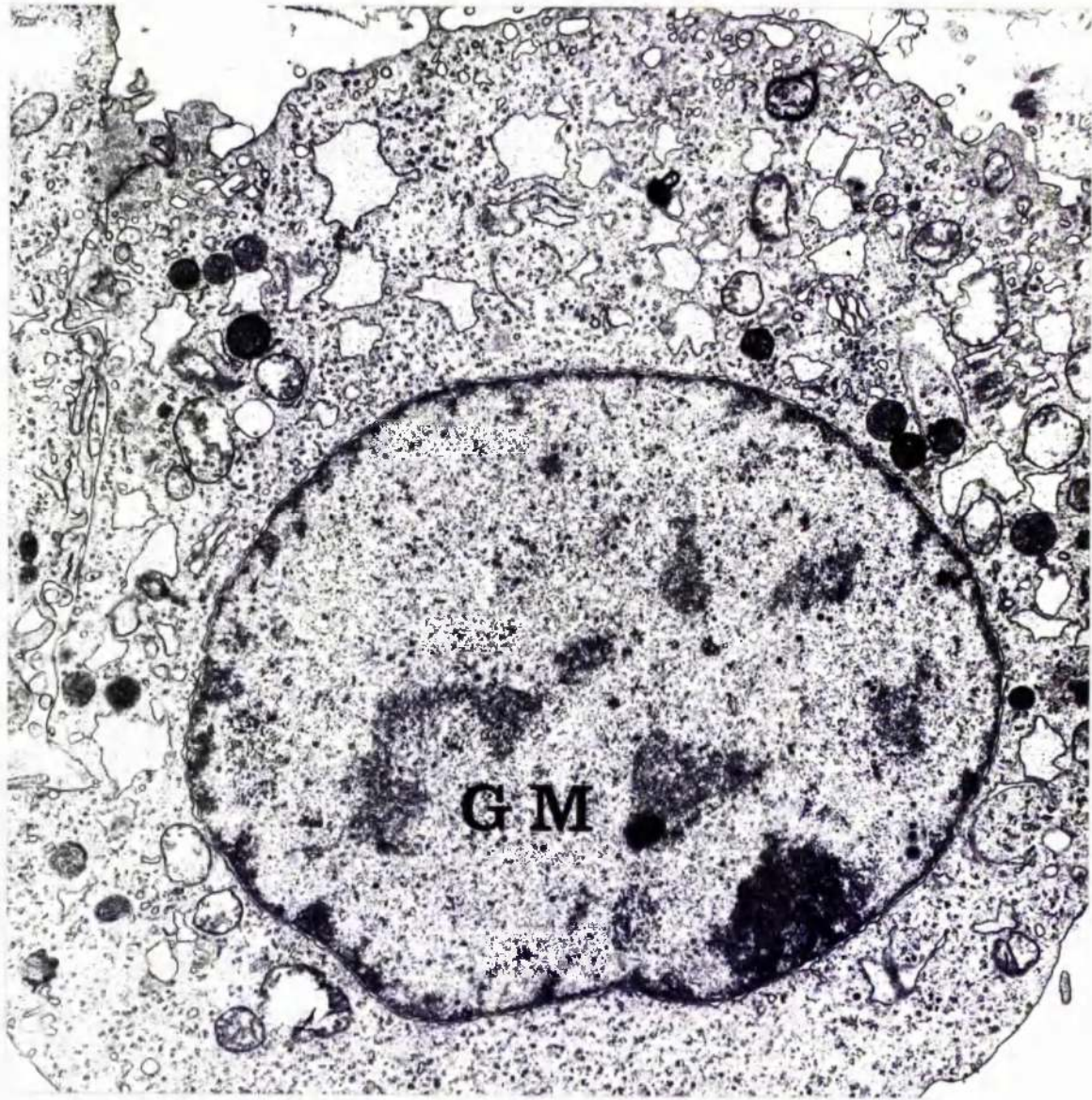
Fig. 100   Electron micrograph of GHK cells 24 hours after

inoculation with "C" virus: adenovirus particles (V)

and granular material (GM) are present throughout

the nucleus.

X 20,000.
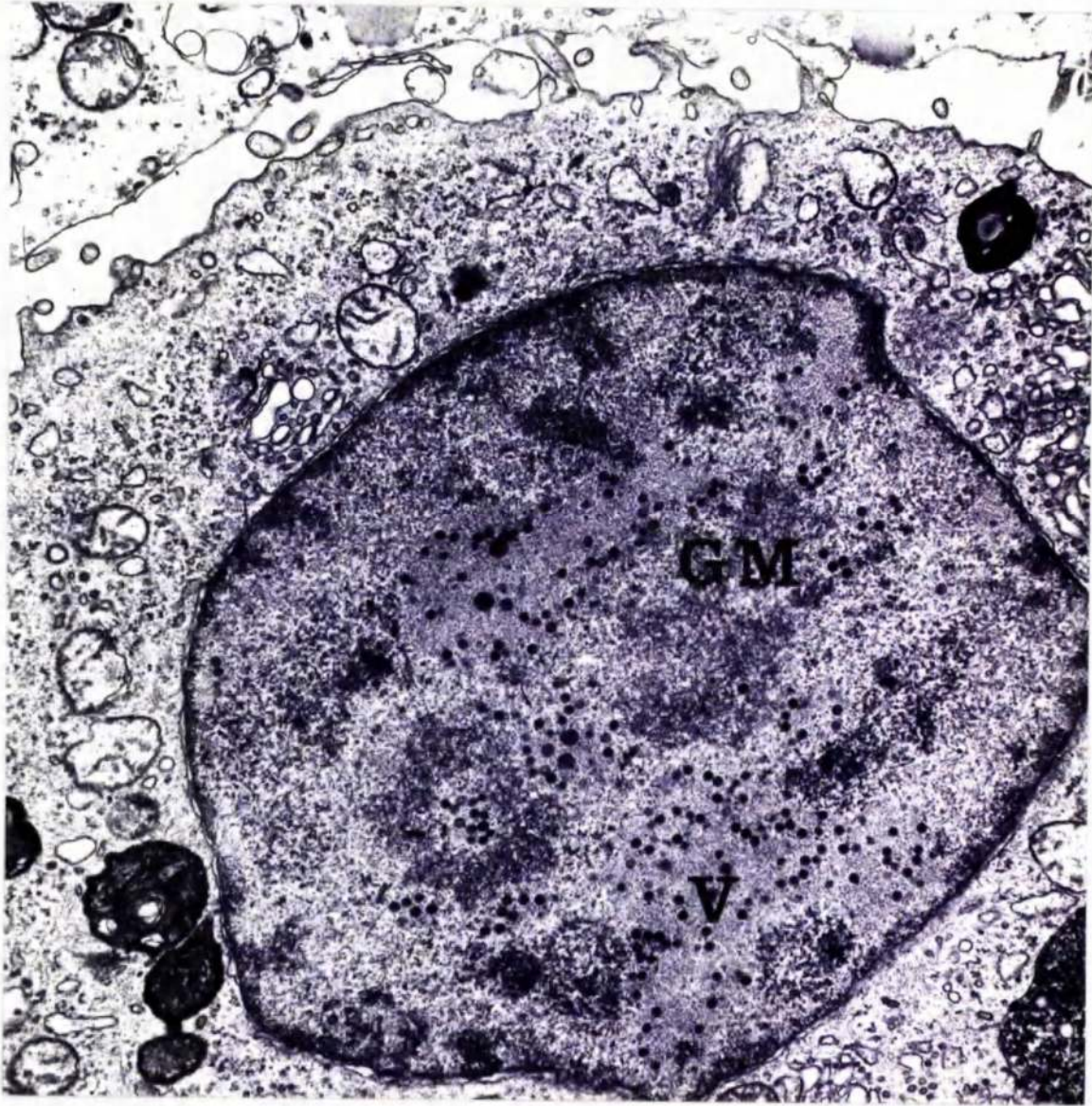
Fig. 101   Electron micrograph of GHK cell 48 hours after

inoculation with "C" virus: the granular material

is clumped in the centre of the nucleus to form a

dense inclusion body (In). Adenovirus particles (V)

are present in the matrix of the inclusion and are

also in the peripheral "halo" region.

X 20,000.
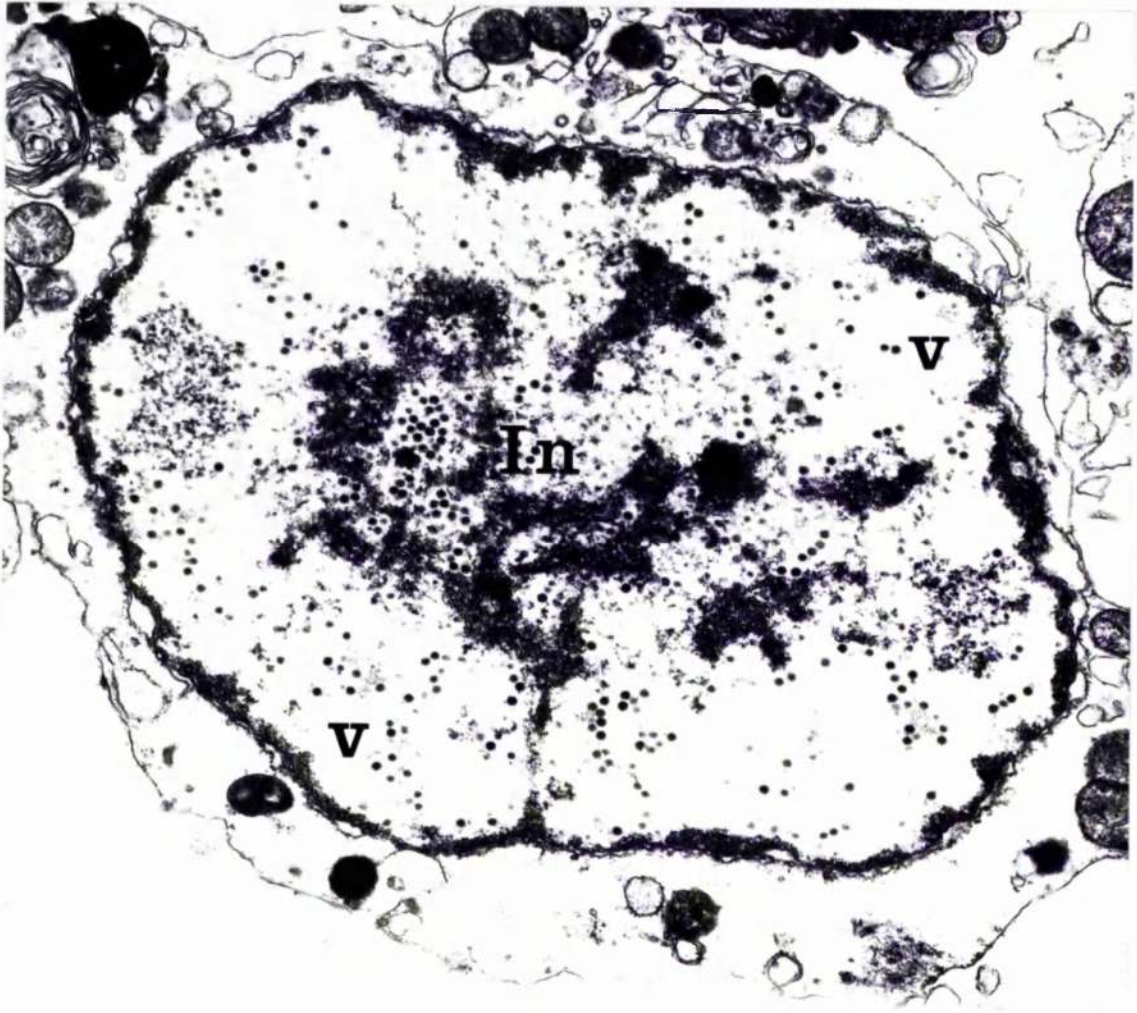
Fig. 102   Gross postmortem appearance of a dog that died 4

days after intravenous inoculation with "A" virus.

The liver is enlarged extending behind the costal arch

and a profuse sanguineous exudate is present in the

abdominal cavity.

Fig. 103   Gross postmortem appearance of a dog that died 4

days after intravenous inoculation with "A" virus.

Strands of fibrin (arrow) can be seen between the

lobes of the liver.
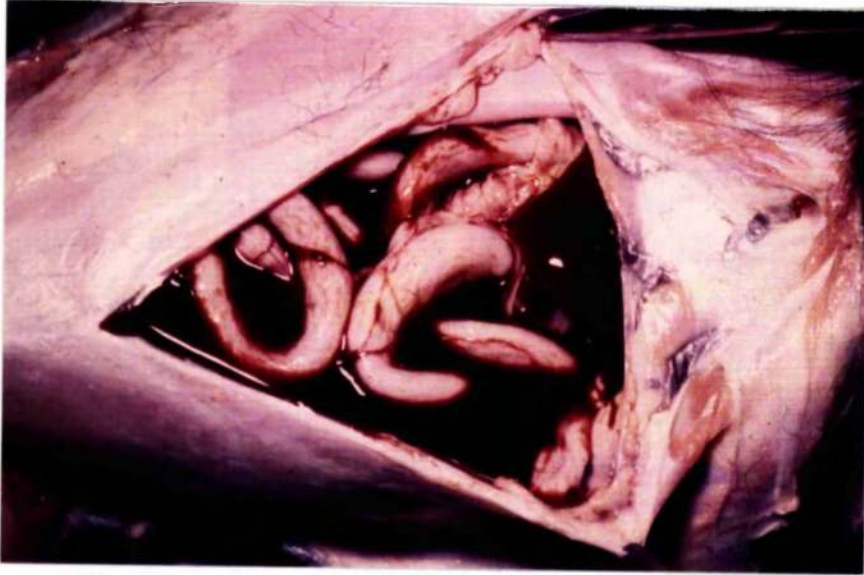
Fig. 104   A section of the liver of a dog infected intra-

venously with "A" virus. Note oedema of the wall of

the gall bladder.

Fig. 105   Liver of a dog infected intravenously with "A" virus.

There is severe hepatic necrosis.

Haemalum and eosin. X 120.

A basophilic intranuclear inclusion body can be seen

in the inset.

Haemalum and eosin. X 1,500.
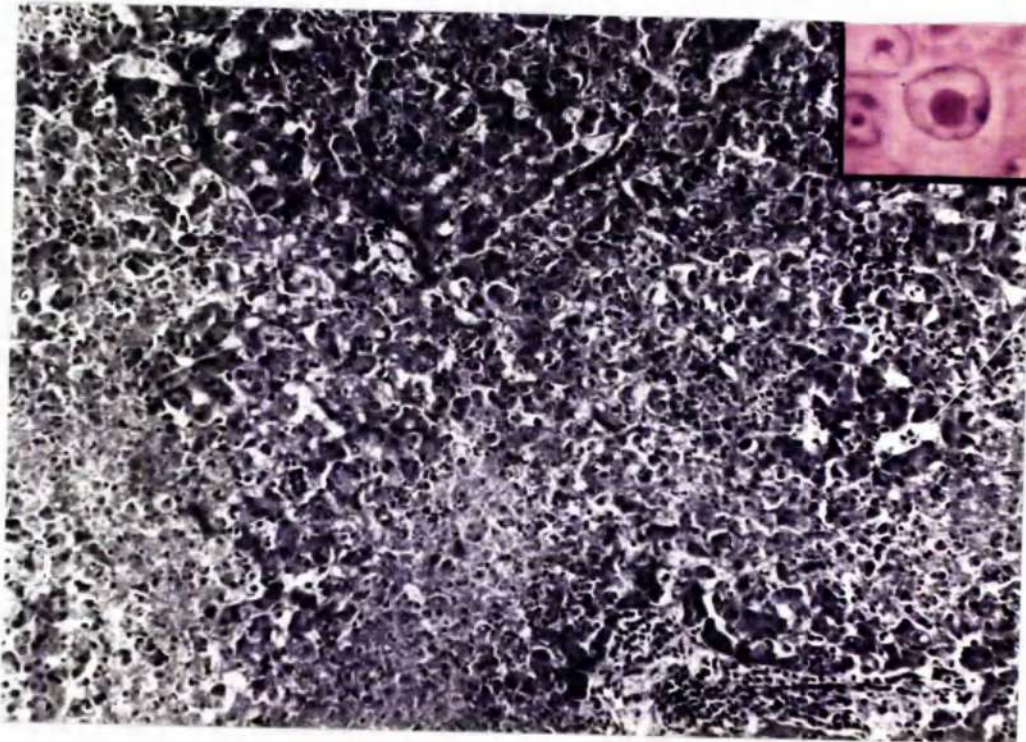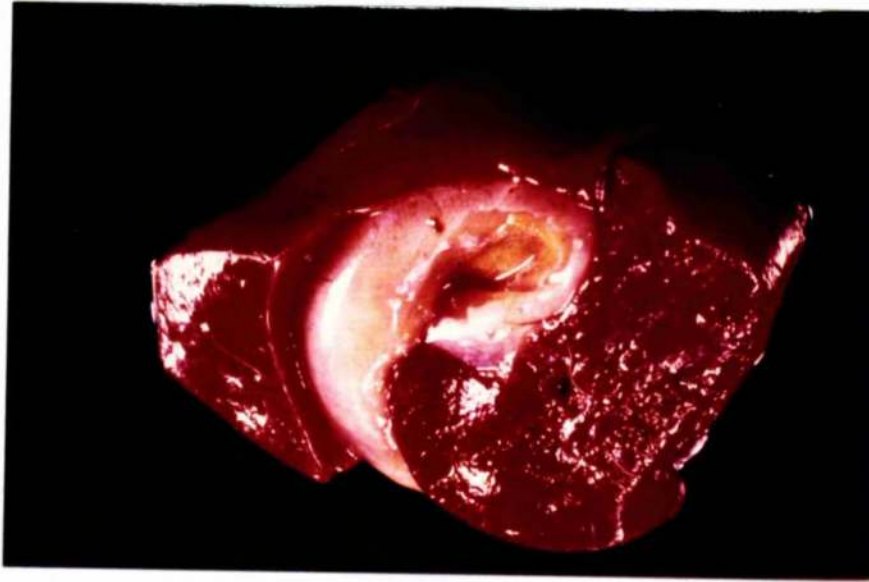
Fig. 106   Liver of a dog 4 days after intravenous inoculation

with "C" virus. CAV-1 antigen is present in the

nuclei of hepatic and Kupffer cells.

Fluorescent antibody. X 800.

Fig. 107   Kidney of a puppy 4 days after intravenous

inoculation with "C" virus. CAV-1 antigen is present

in glomeruli and tubules.

Fluorescent antibody. X 500.
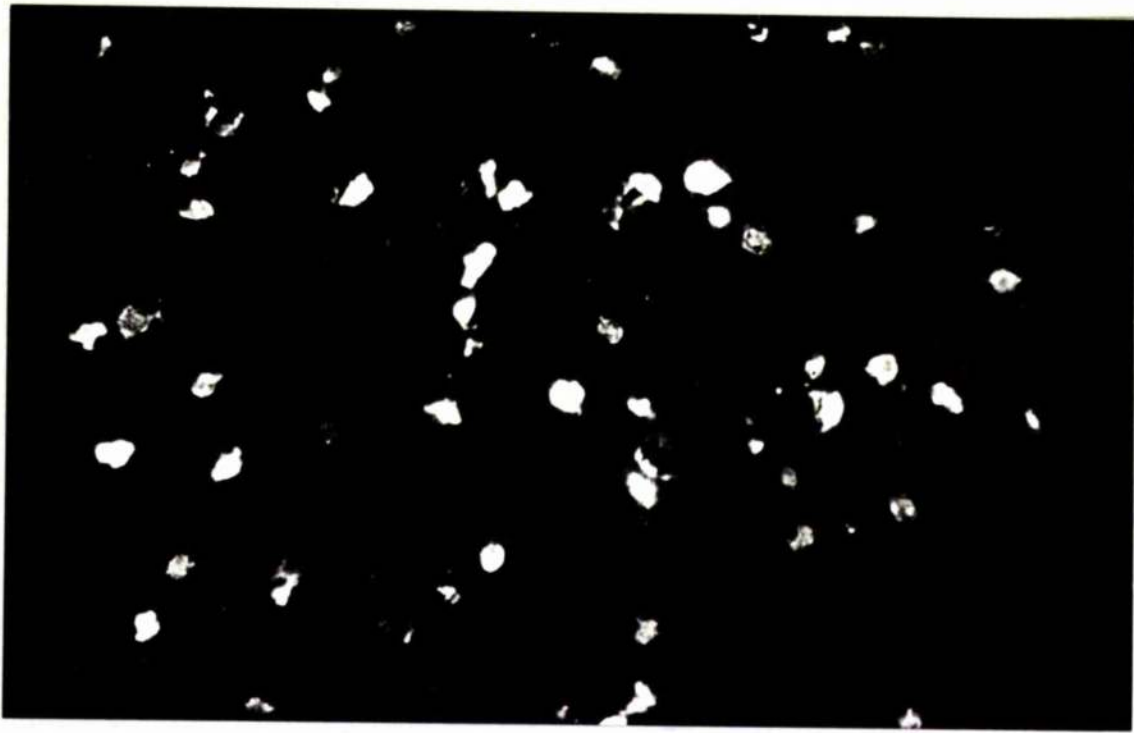
Fig. 108 Electron micrographs of liver to show stages in

CAV-1 infection.

(a) Normal hepatocyte.

(b) Infected hepatocyte: fine electron dense

granules (GM) are scattered throughout the nucleus.

(c) Infected hepatocyte: inclusion body composed of

granular material (GM).

(d) Infected hepatocyte: adenovirus particles (V) are

present in the matrix and peripheral halo region

of the inclusion body (In).
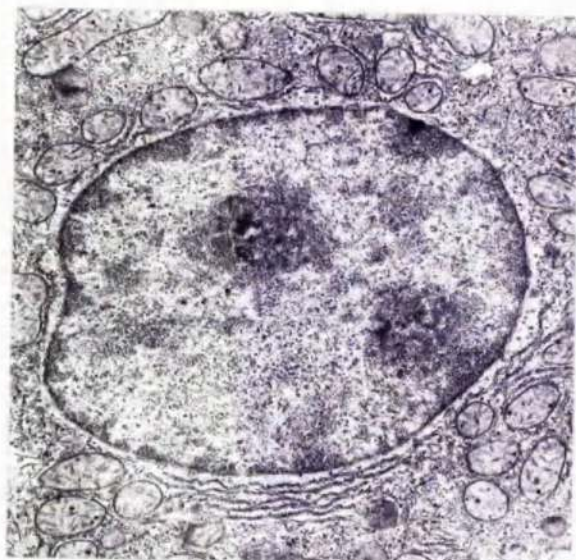
a
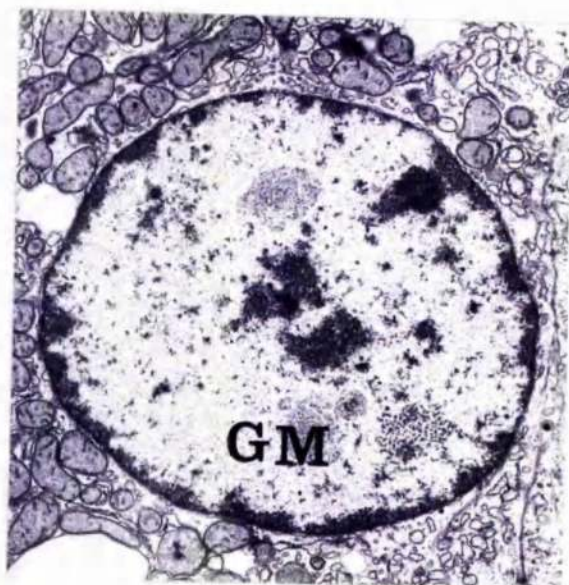
b
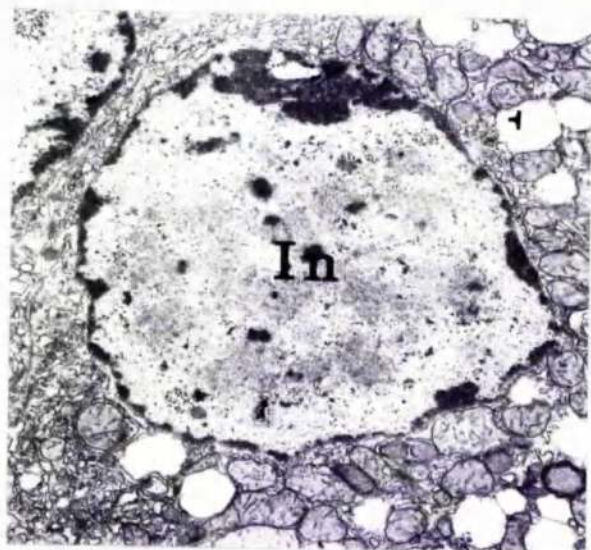
c
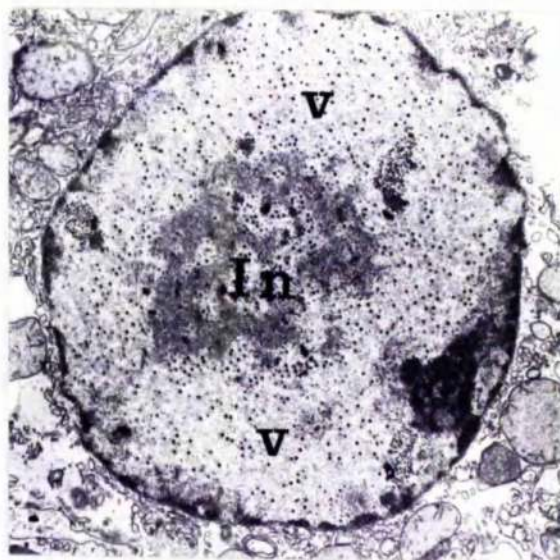
d

Fig. 109   Lungs of a puppy 5 days after exposure to "C" virus
            by aerosol. Note the dark areas of pneumonia along
            the edge of the right cardiac lobe and in the dorsal
            parts of the diaphragmatic lobes of the lungs.

Fig. 110   Lungs of a puppy 5 days after exposure to "C" virus

by aerosol. Dark red consolidated areas are

scattered throughout the lung parenchyma.

Fig. 111   Section of the above lung to show widespread

necrotizing bronchiolitis.

Haemalum and ecsin. X 35.

140.
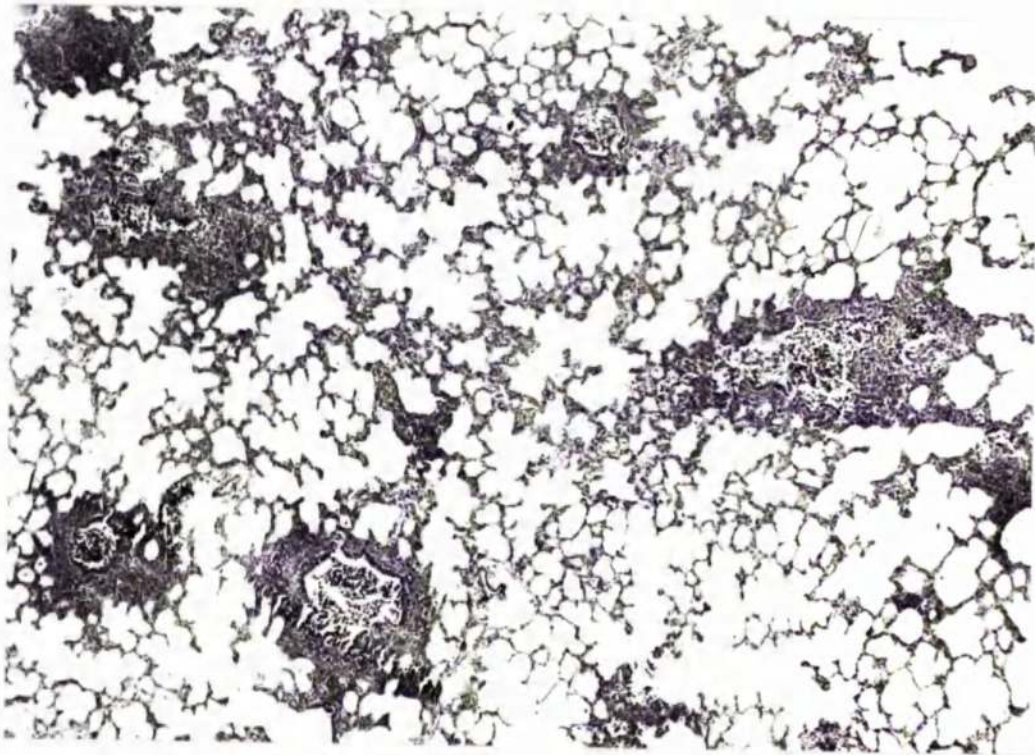
Fig. 112   CAV-1 antigen in bronchiolar epithelial cells and

alveolar macrophage, 5 days after exposure to "C"

virus by aerosol.

Fluorescent antibody. X 300.

Fig. 113   Intranuclear inclusion body (arrow) in tonsillar

epithelial cell 3 days after exposure to "C" virus

by aerosol.

Haemalum and eosin. X 400.
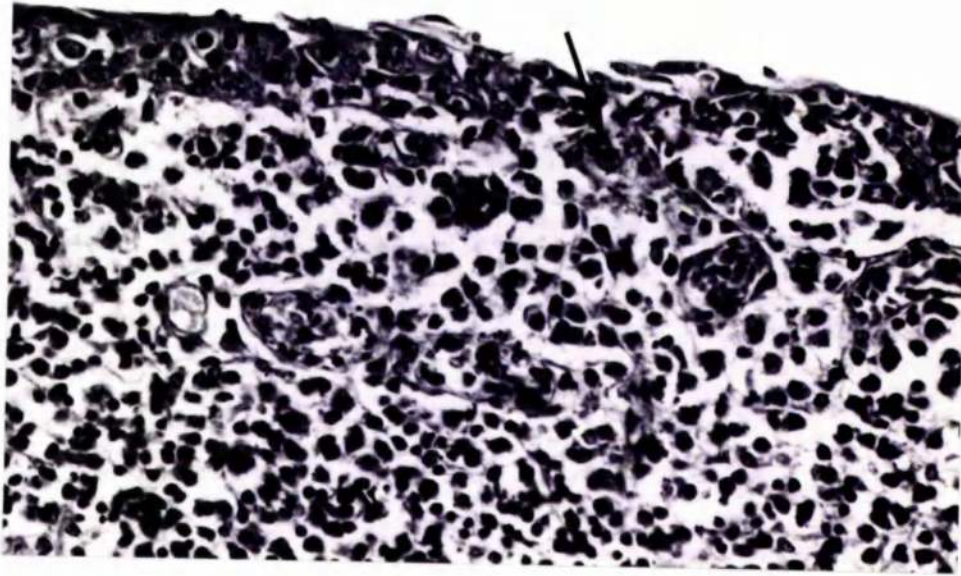
Fig. 114   Experimental reovirus infection: lungs of a puppy

3 days after intratracheal inoculation. Dark red

areas of consolidation are present in all lobes of

the lungs.

Fig. 115   Experimental reovirus infection: depressed lesion

(arrows) in the right apical lobe of the lungs, 12

days after intratracheal inoculation.
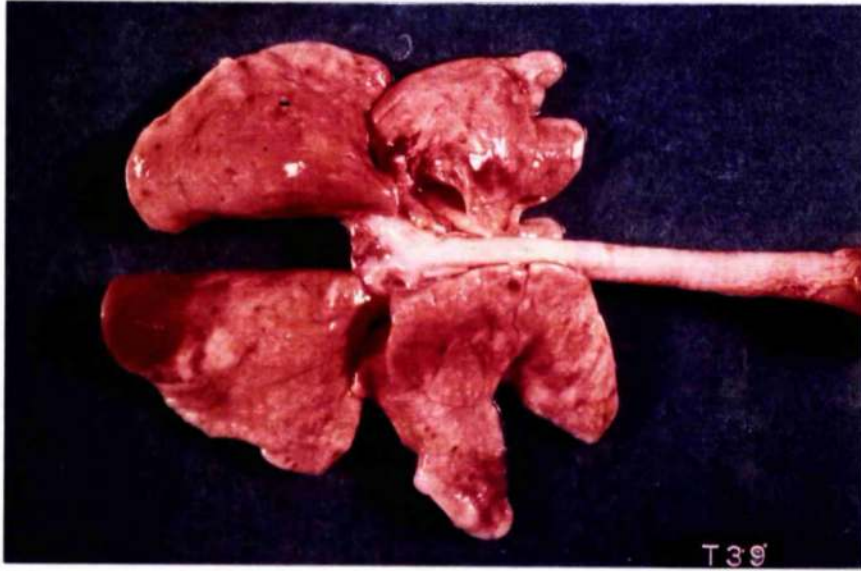
Fig. 116   Experimental reovirus infection: low magnification

photomicrograph of the lungs 3 days after

intratracheal inoculation. There is a diffuse

exudative pneumonia.

Haemalum and eosin. X 120.

Fig. 117   Experimental reovirus infection: high magnification

photomicrograph of the lungs 3 days after intratracheal

inoculation to show alveolar necrosis. Macrophages

and polymorphonuclear leucocytes are present in the

alveolar septa and in the air spaces.

Haemalum and eosin. X 800.
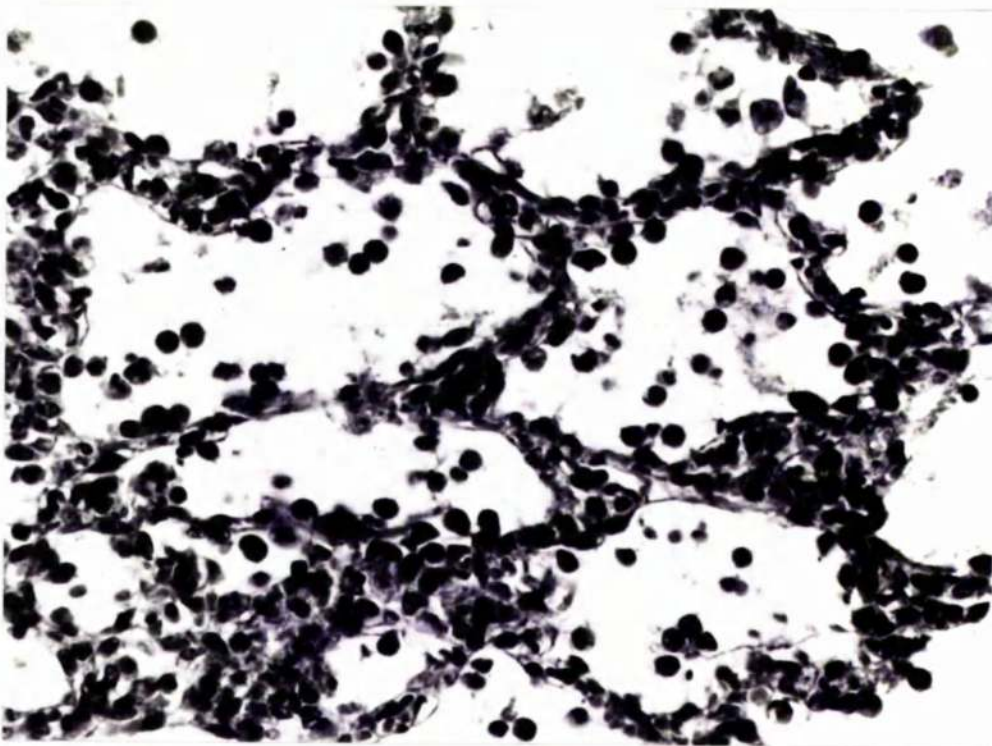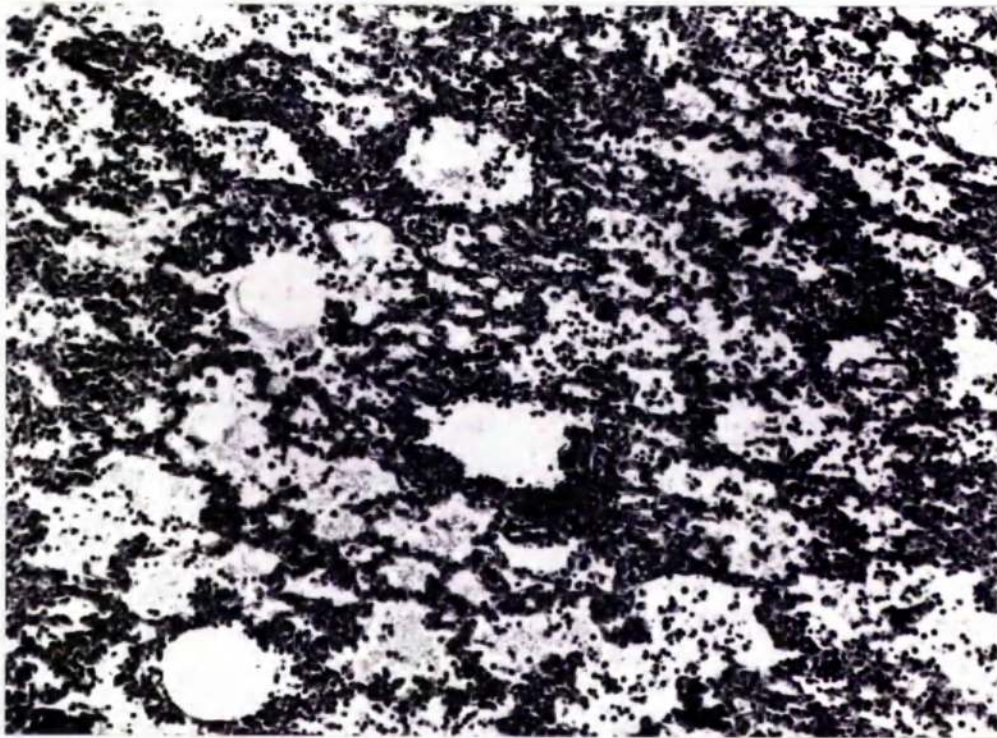
Fig. 118   Experimental reovirus infection: low magnification

photomicrograph of the lung, 7 days after

intratracheal inoculation.

Haemalum and eosin. X 120.

Fig. 119   Experimental reovirus infection, 7 days after

intratracheal inoculation. Note the increased

cellularity of the alveolar septa.

Haemalum and eosin. X 600.
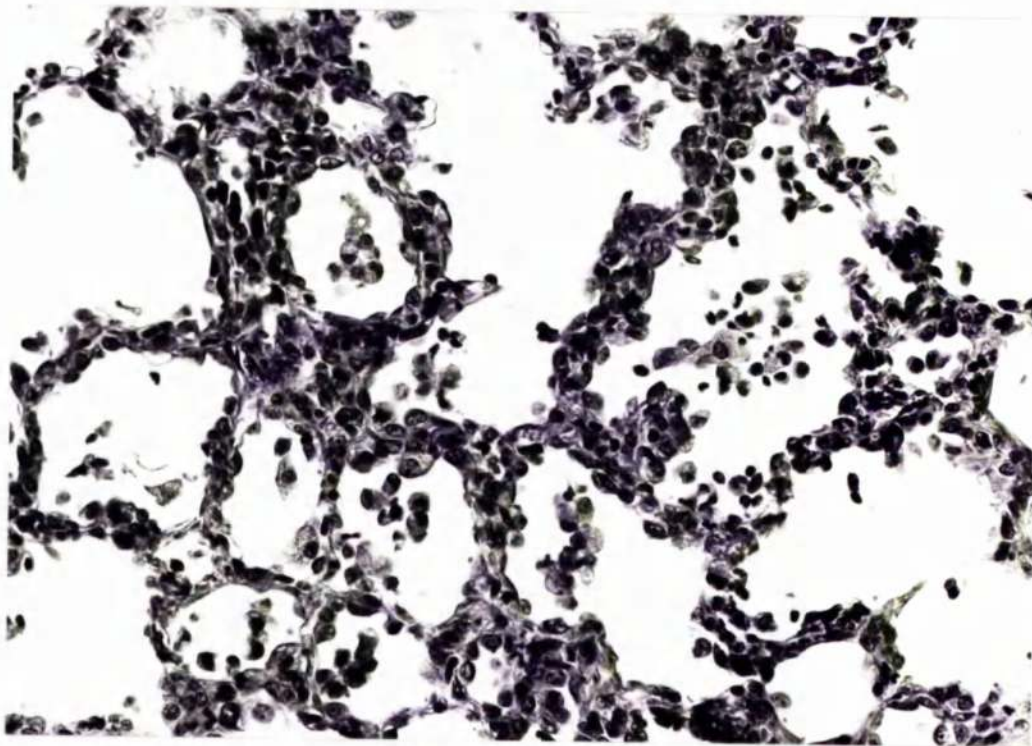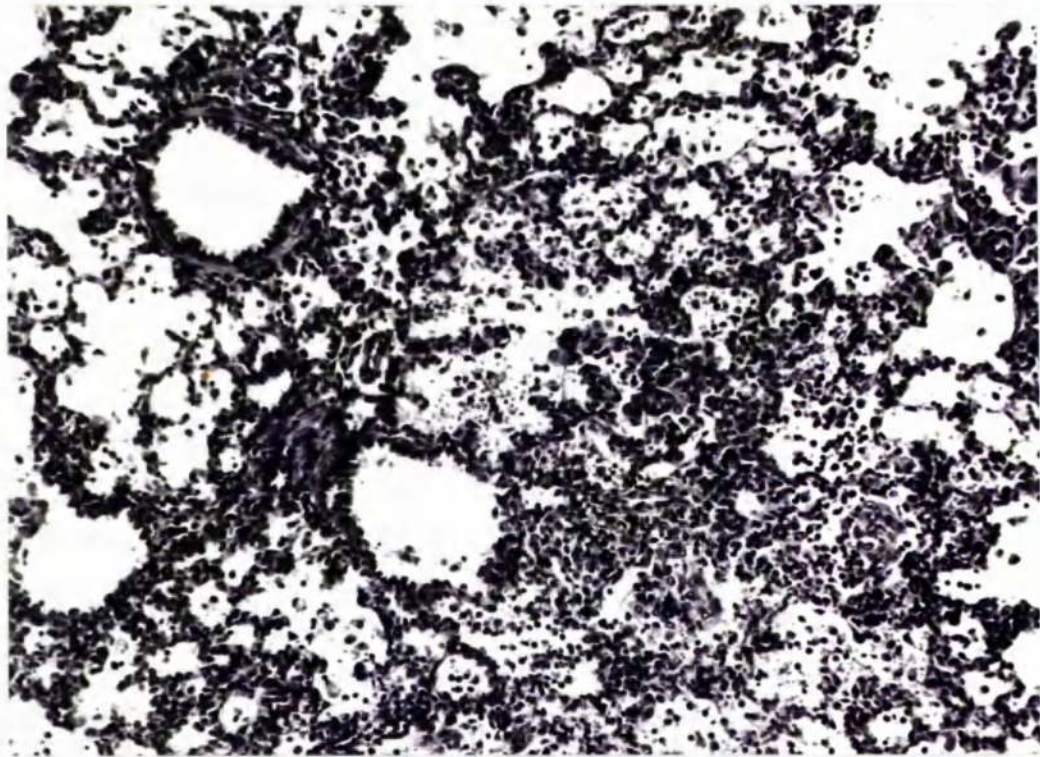
Fig. 120   Experimental reovirus infection, 7 days after

intratracheal inoculation. Note the intra-alveolar

synctia (arrow).

Haemalum and eosin. X 600.
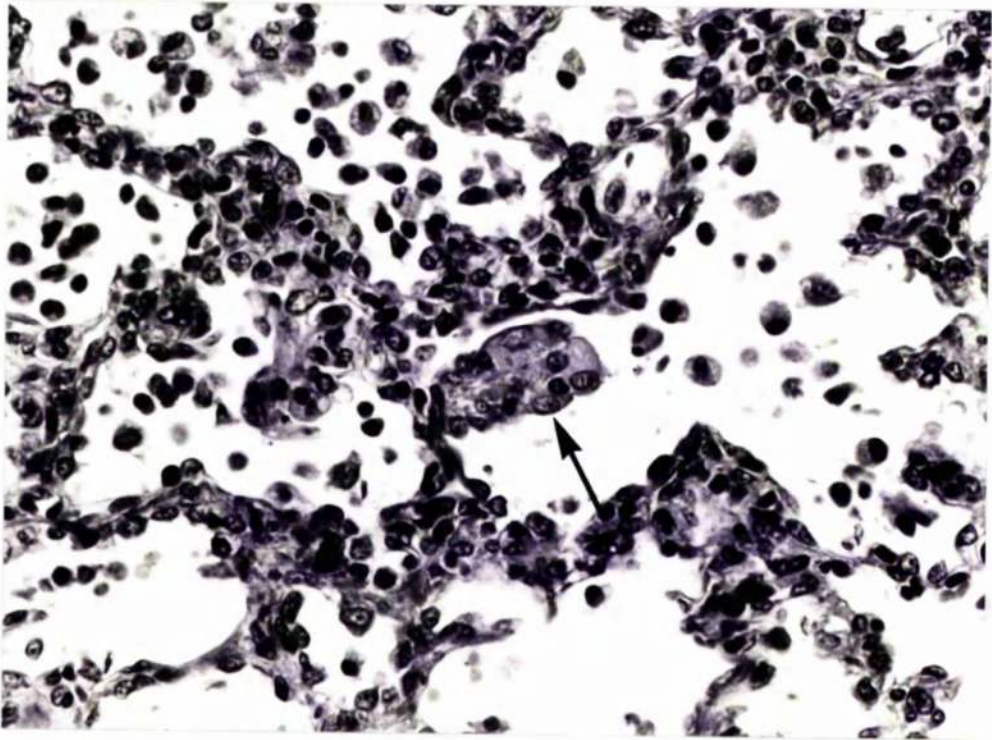
Fig. 121   Experimental reovirus infection, 12 days after

intratracheal inoculation. Note the cellular

connective tissue in the alveoli and alveolar ducts.

Haemalum and eosin. X 300.
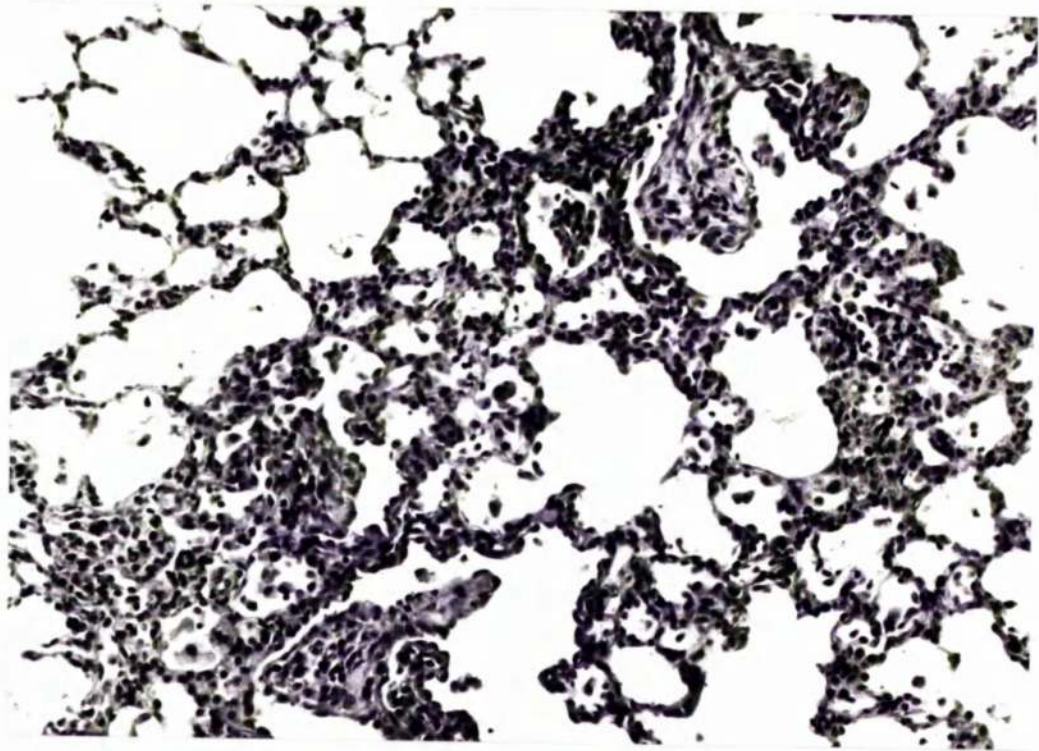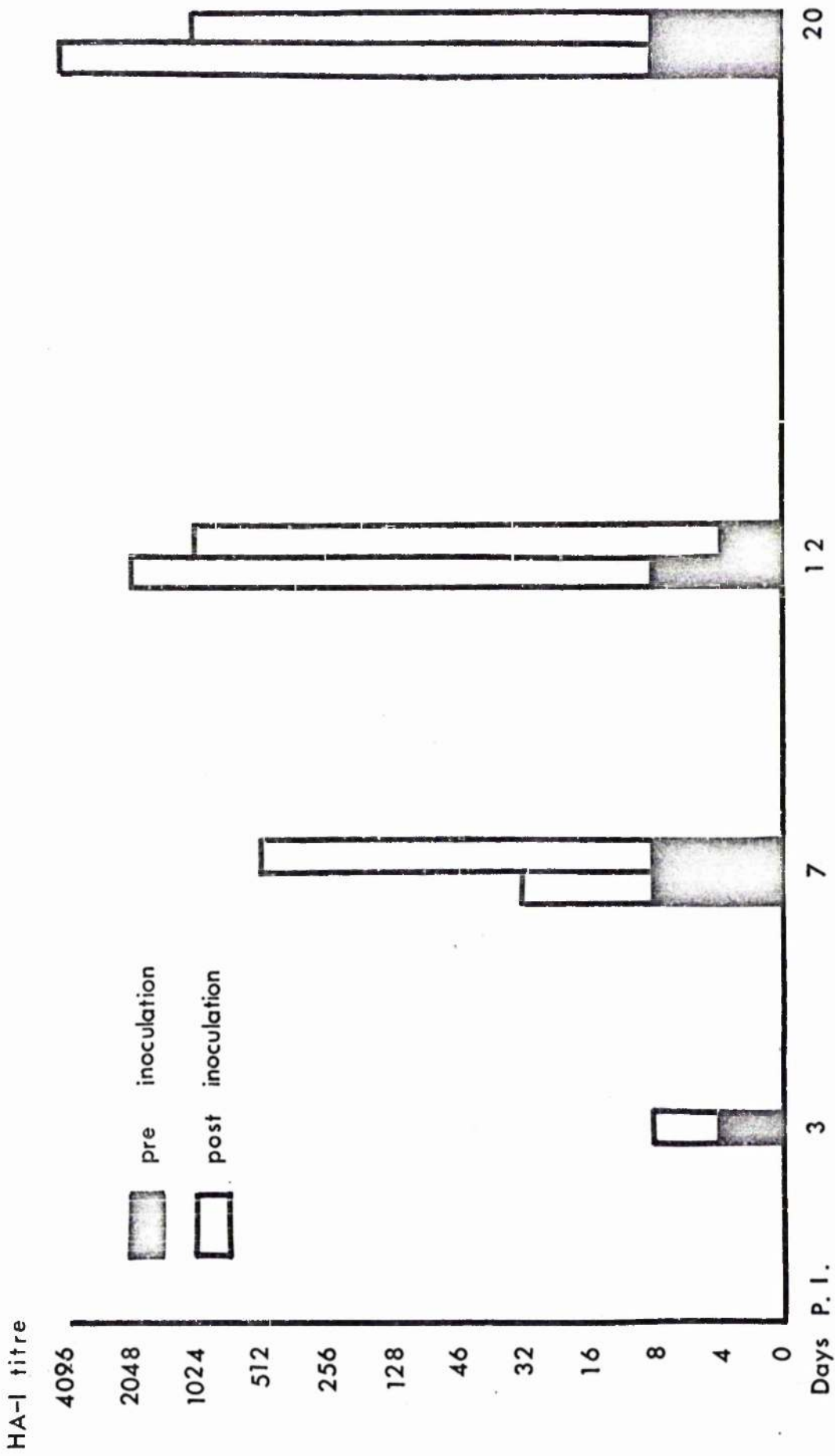
Fig. 122   Serological Response of Dogs to Type 1 Reovirus: intratracheal infection.

Fig. 123   Experimental reovirus infection, 6 days after

aerosolisation. Tiny grey foci are present

throughout the right diaphragmatic lobe of the

lung. Note that these lesions are not unlike those

found in natural CAV-1 infection (Case No. 1. Fig. 88).

Fig. 124   Experimental reovirus infection, 6 days after

aerosolisation. Small cellular foci are present in

the alveolar septa. The bronchioles are not affected.

Haemalum and eosin.   X 35.
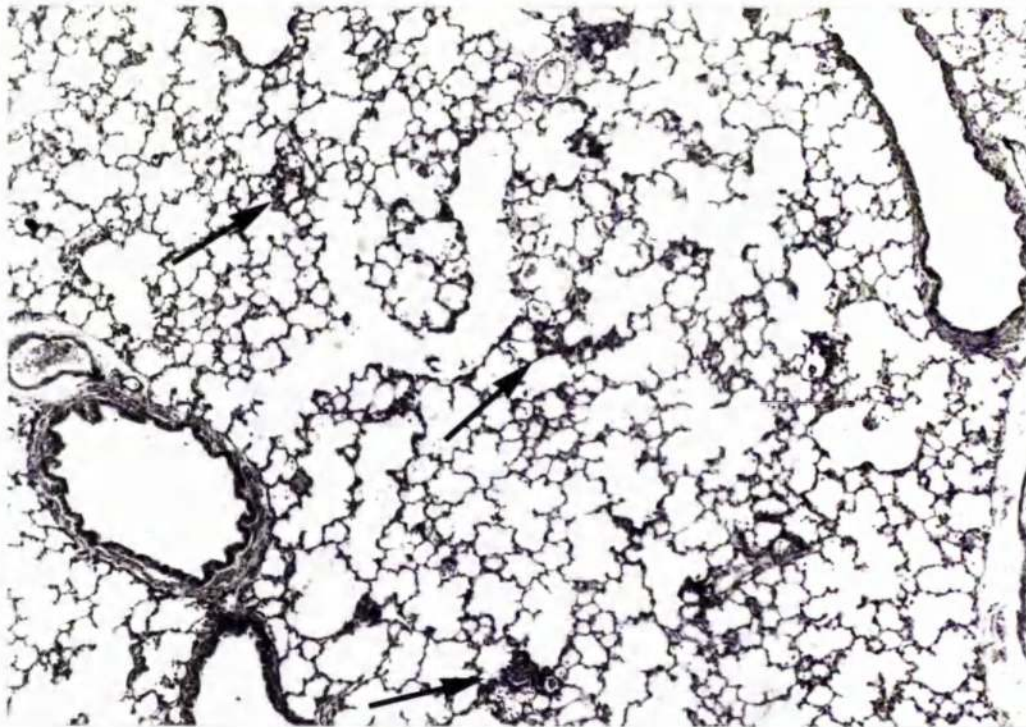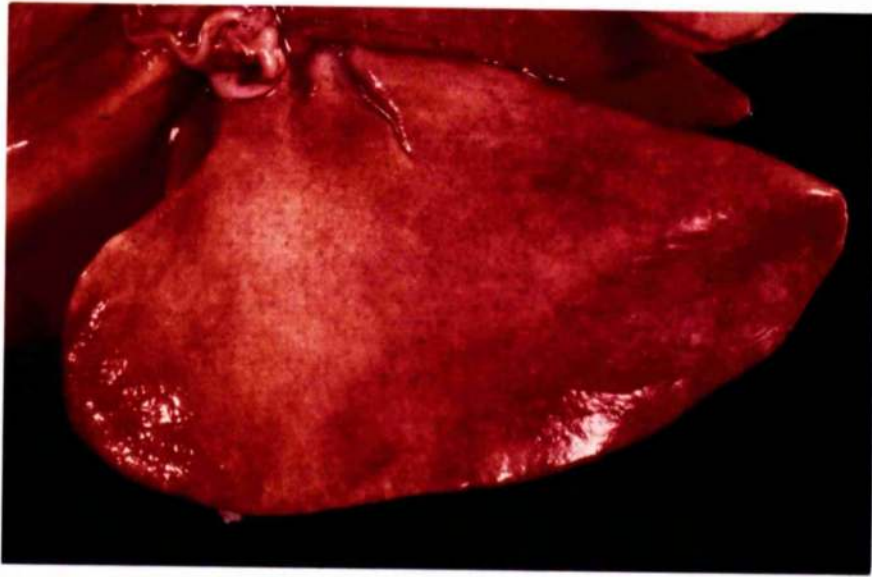
Fig. 125   Experimental reovirus infection, 3 days after

aerosolisation. There is necrosis in the alveolar

septa and polymorphonuclear leucocytes are present.

Haemalum and eosin. X 400.

Fig. 126   Experimental reovirus infection, 6 days after

aerosolisation. A cellular focus composed mainly

of lymphocytes and macrophages is present in the

alveolar septa. Macrophages lie free in the air

spaces.

Haemalum and eosin. X 400.
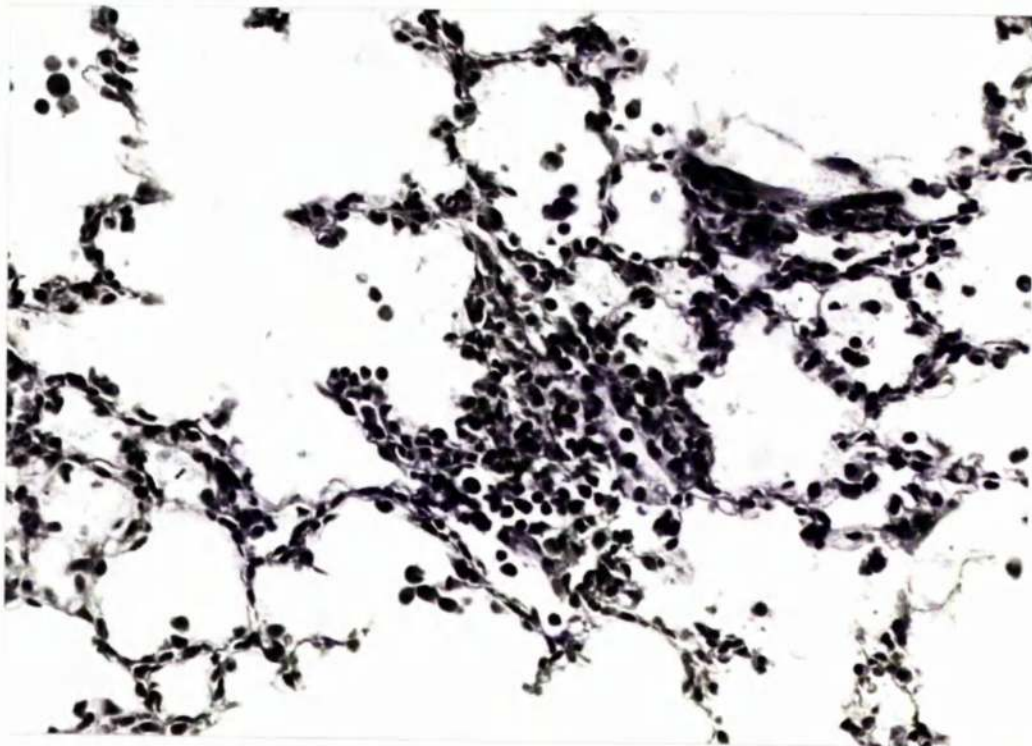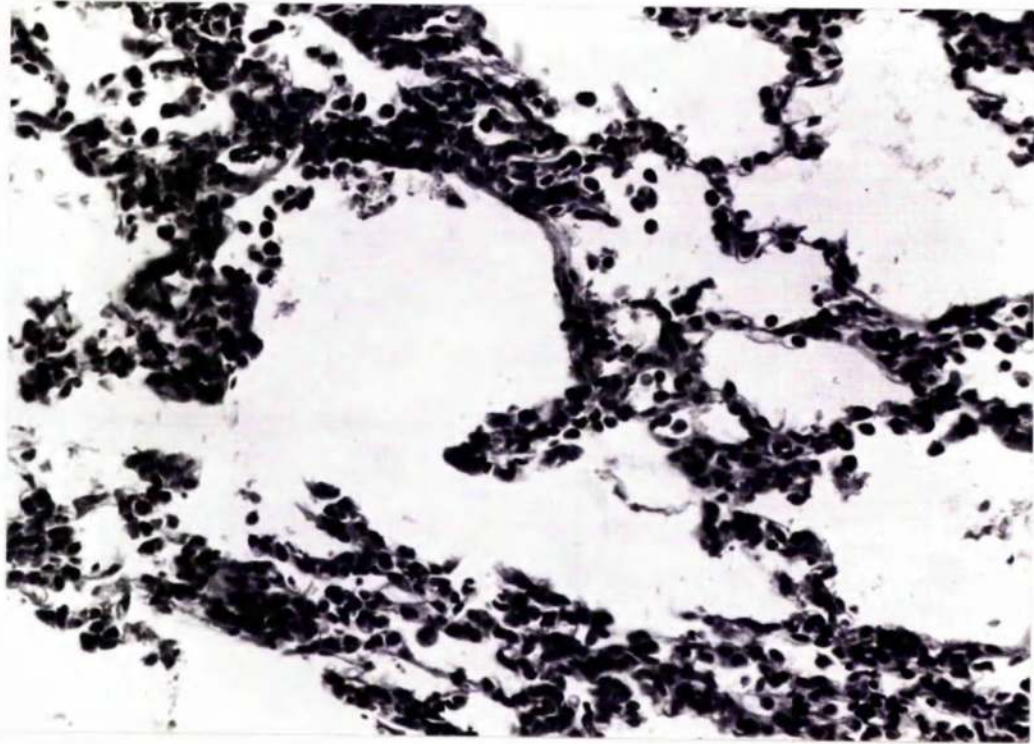
Fig. 127   Experimental reovirus infection, 8 days after

aerosolisation. There is organisation of the

alveolar exudate.

Haemalum and eosin. X 600.
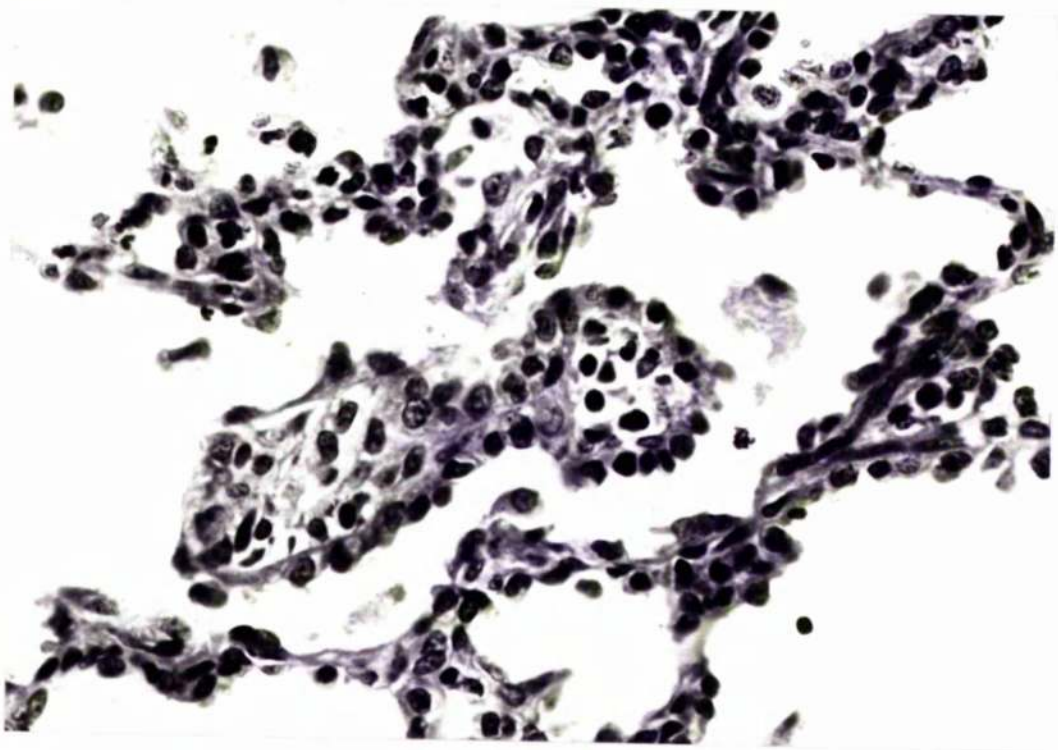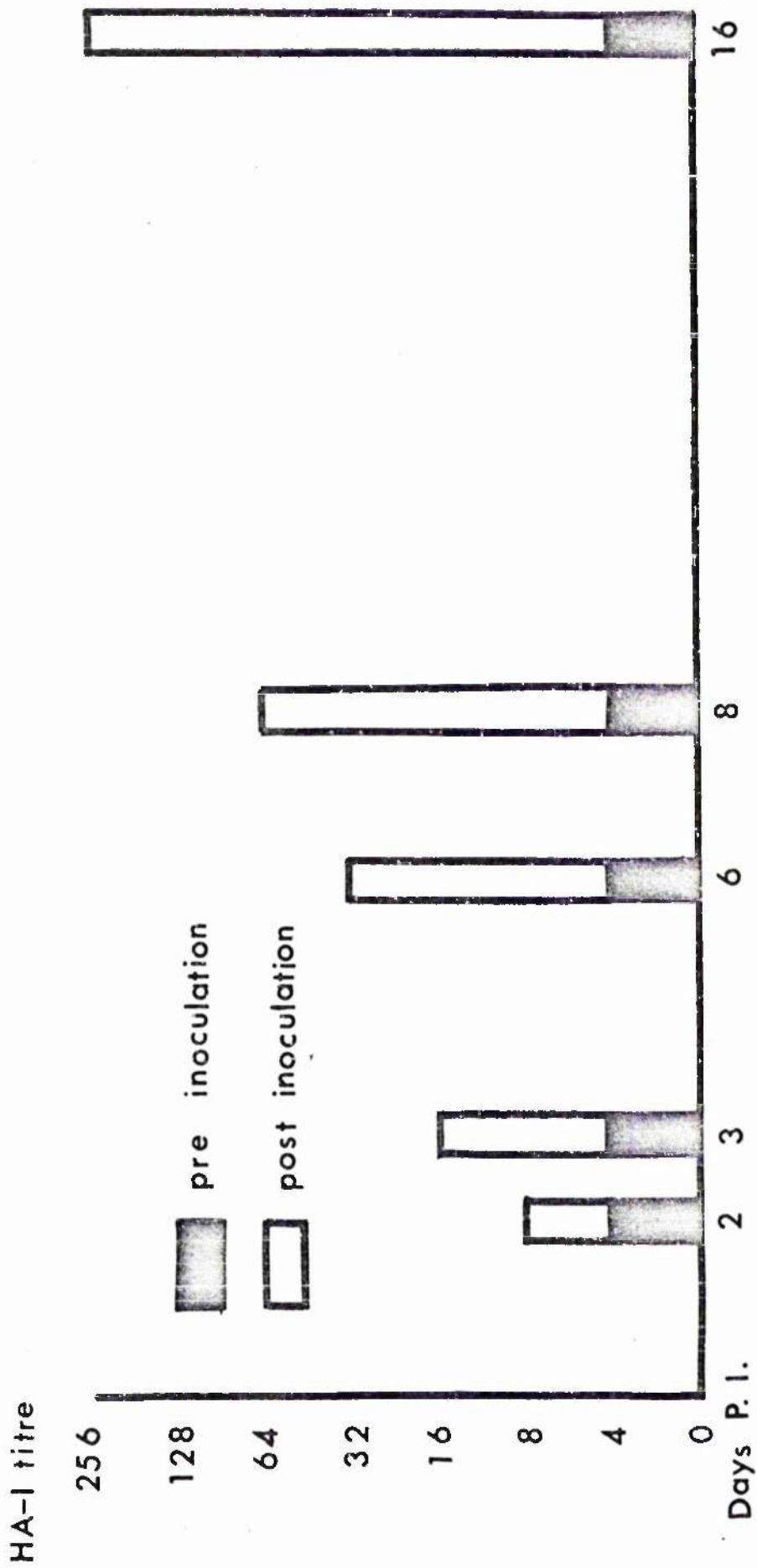
Fig. 128 Serological Response of Dogs to Type 1 Reovirus: aerosol infection.

Fig. 129   Canine trachea explant cultures.

Fig. 130   Uninfected canine tracheal explant maintained for

10 days. Note the pseudostratified ciliated

epithelium.

Haemalum and eosin. X 400.
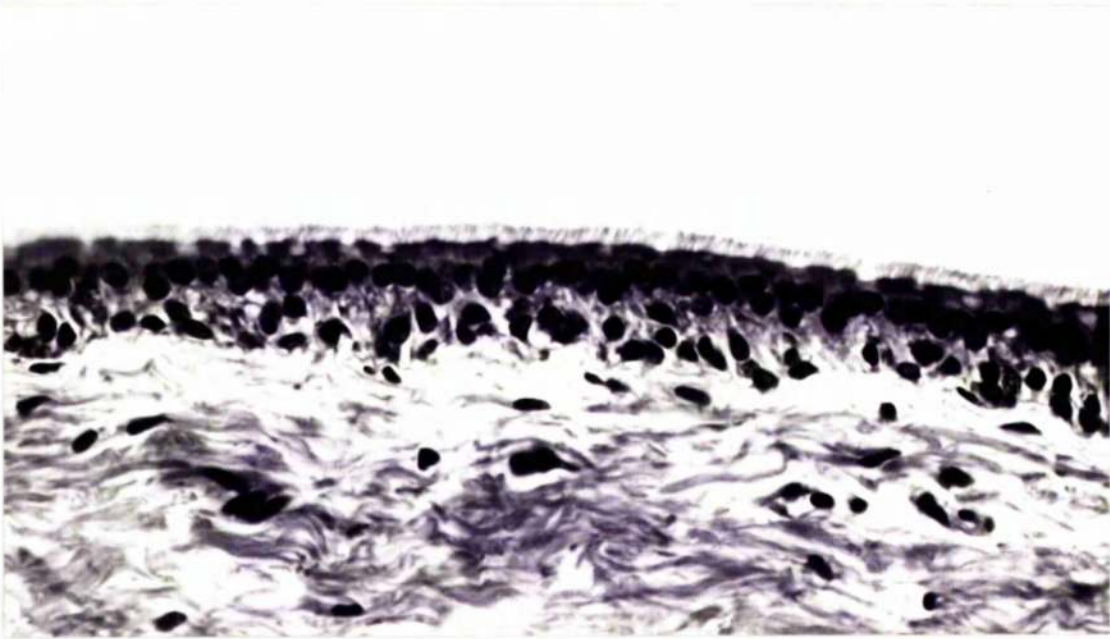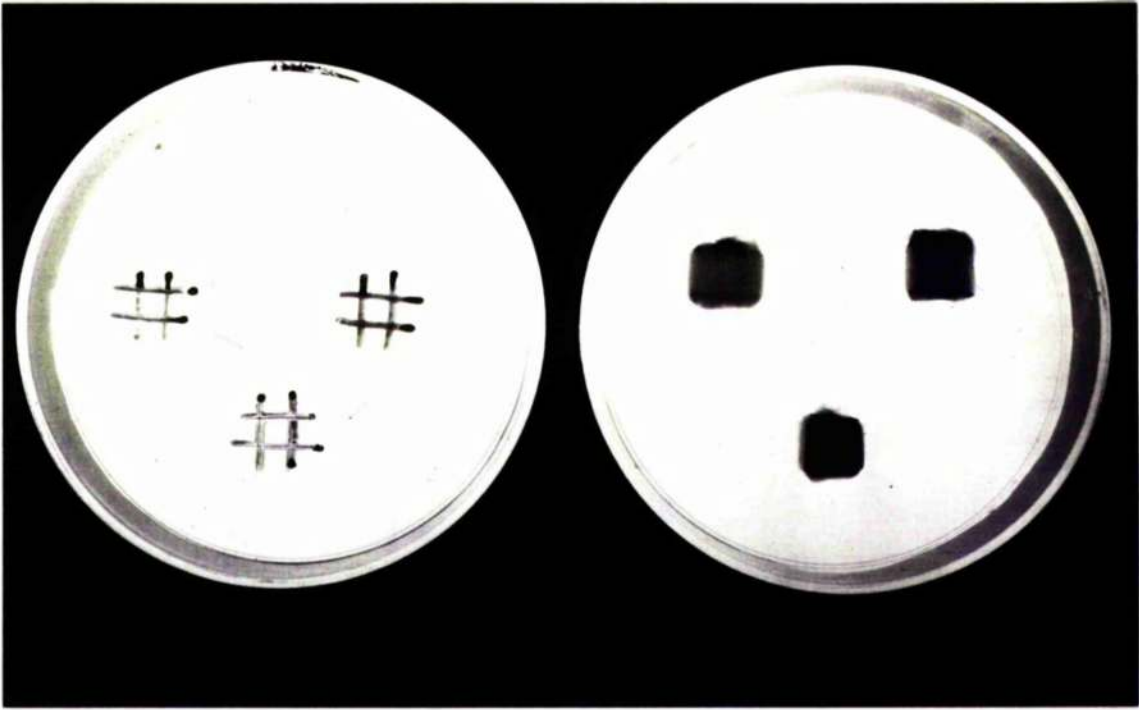
Fig. 131   Canine tracheal explant, 2 days after infection

with CHV. The epithelial cells are swollen and

vacuolated. Intranuclear inclusion bodies (arrows)

can be seen at the left-hand corner of the section.

Haemalum and eosin.   X 550.

Fig. 132   Canine tracheal explant, 5 days after infection with

CHV. There is necrosis and loss of epithelial cells.

Intranuclear inclusions (arrow) are also present.

Haemalum and eosin.   X 400.
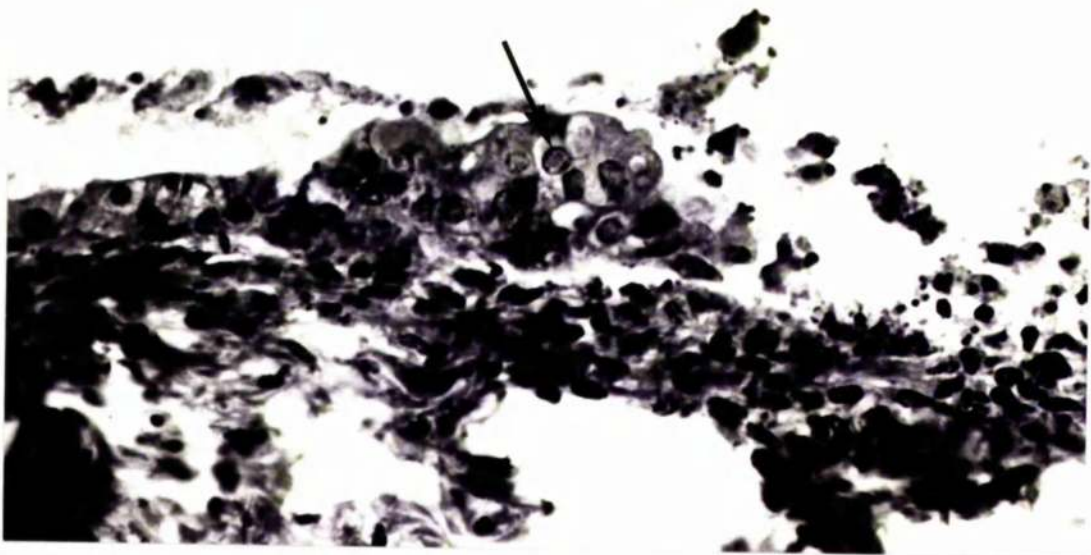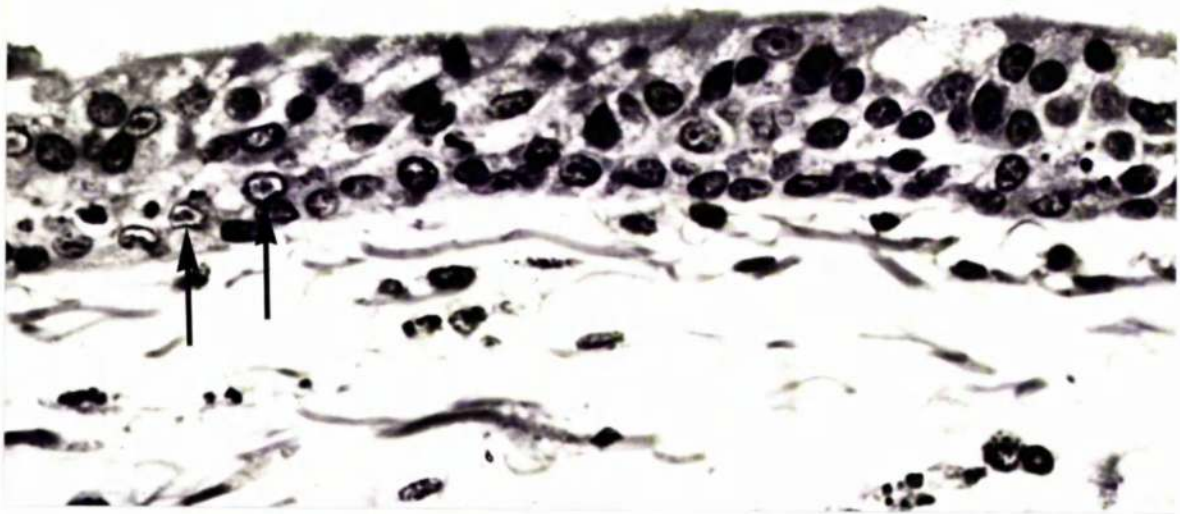
Fig. 133   Canine tracheal explant 7 days after infection

with CDV. Note the cytoplasmic inclusion bodies

(arrow).

Haemalum-phloxine-tartrazine.   X 400.

Fig. 134   Canine tracheal explant cultures 10 days after

infection with CDV. There is squamous epithelial
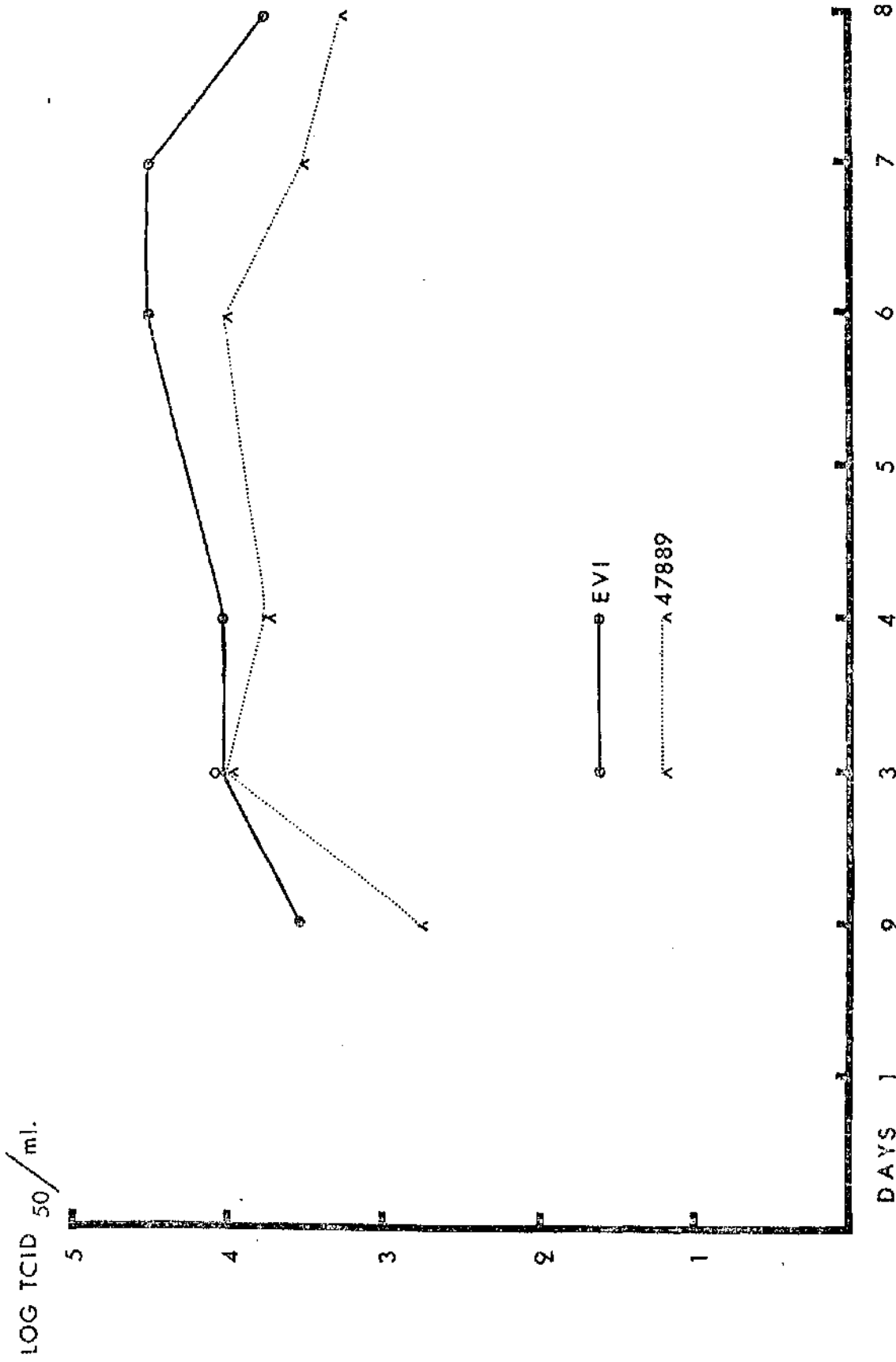
metaplasia.

Haemalum and eosin.   X 300.

LOG TCID $_{50}$/ml.
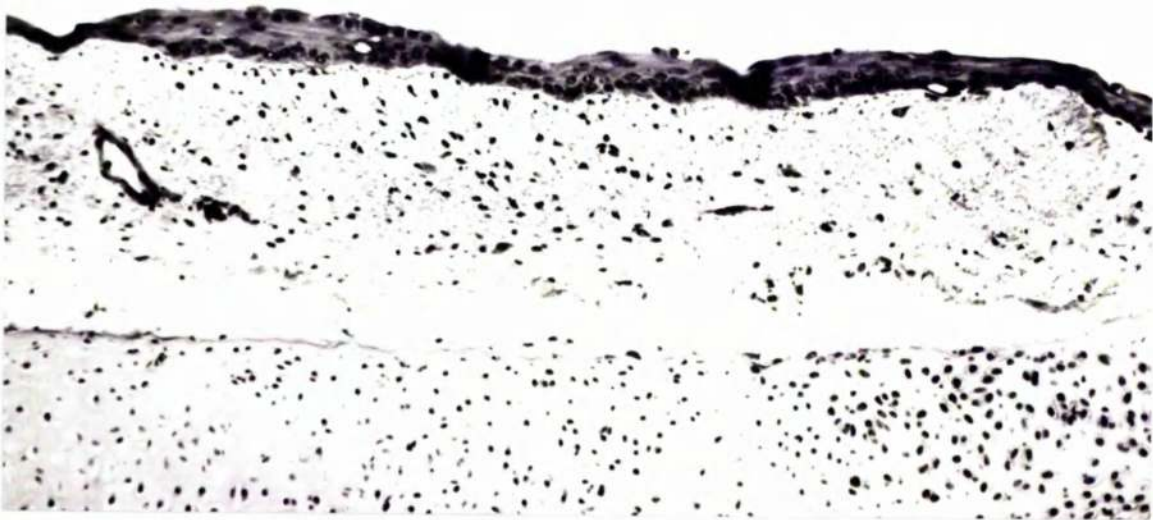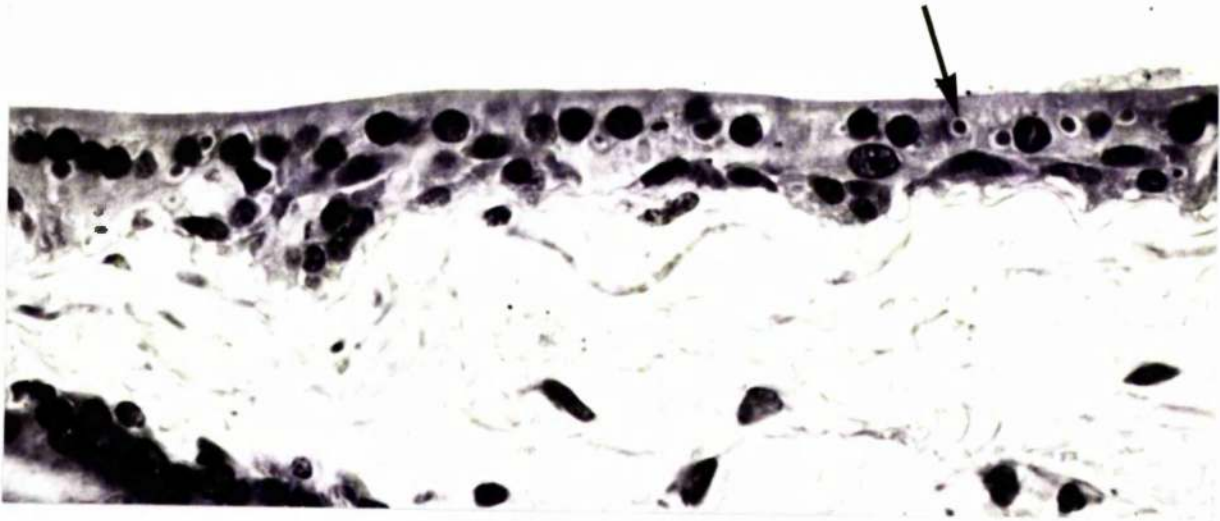
Fig. 135   Growth of Canine Adenovirus in Dog Tracheal Explant Cultures.

Fig. 136   Canine tracheal explant, 2 days after infection with
CAV-1. Note the orderly arrangement of the epithelium
and necrosis of cells in the basal layer.

Haemalum and eosin.   X 550.

Fig. 137   Canine tracheal explant, 9 days after infection with
CAV-1. There is necrosis and loss of the normal
orientation of the epithelial cells. Note the
intranuclear inclusion body (arrow).

Haemalum and eosin.   X 550.

Fig. 138   Canine tracheal explant, 20 days after infection with
CAV-1. There is marked epithelial hyperplasia.
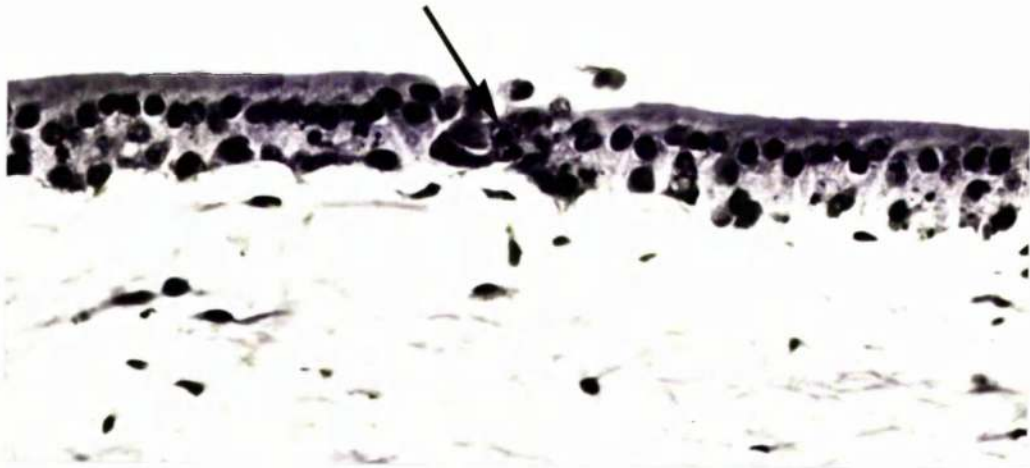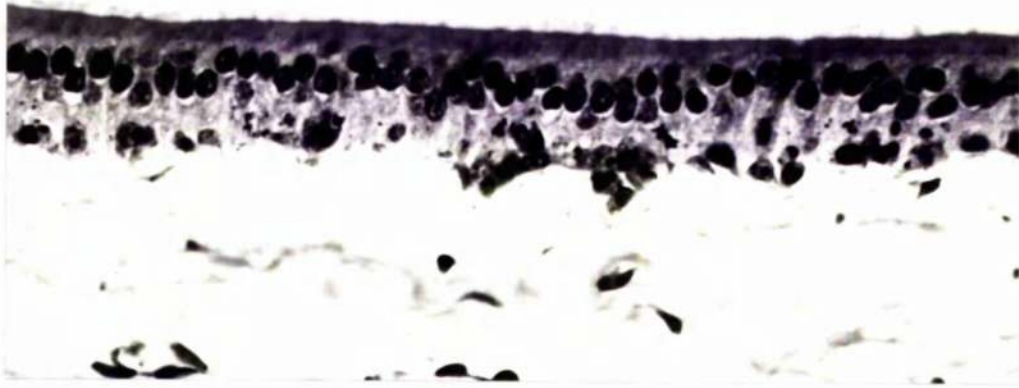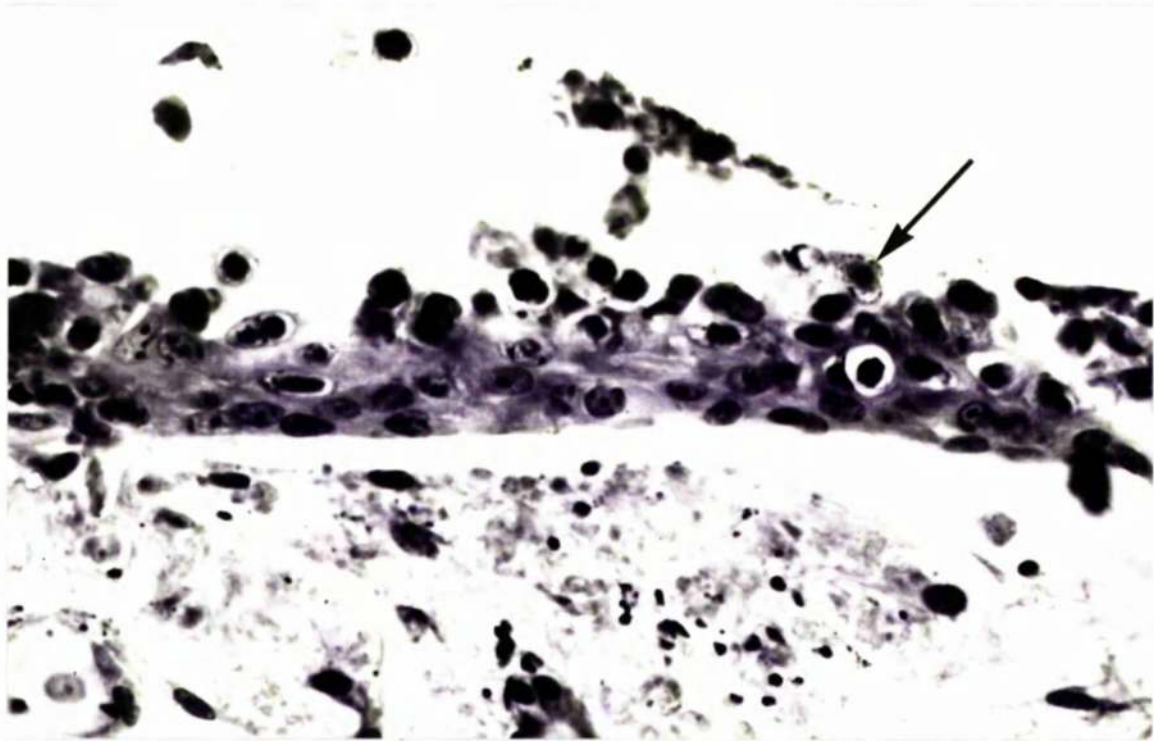
Haemalum and eosin.   X 250.
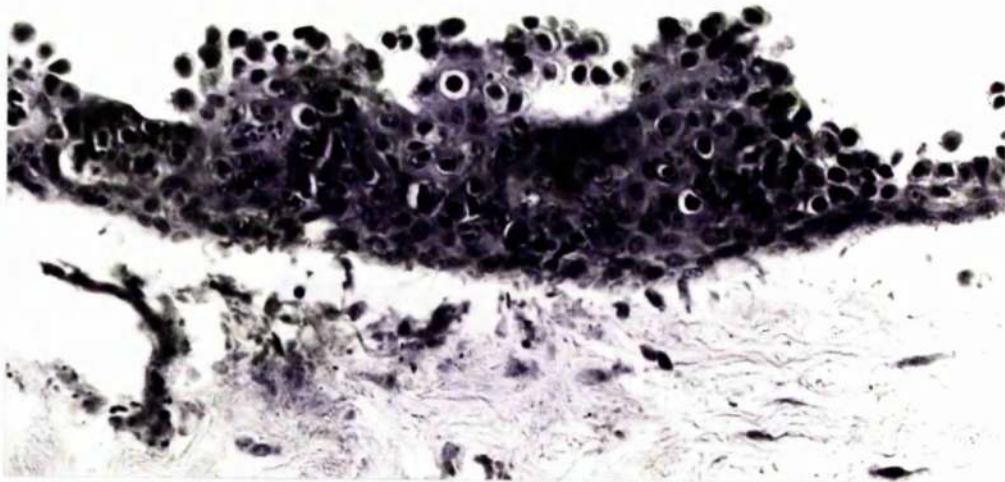
Fig. 139   Canine tracheal explant, 12 days after infection

with CAV-1. There is epithelial metaplasia and

several inclusion bodies (arrow) can be seen.

Haemalum and eosin. X 550.

Fig. 140   Canine tracheal explant 3 days after infection with

CAV-1. CAV-1 antigen is present in the nuclei of

epithelial cells.

Fluorescent antibody. X 1,200.