SHEEP PULMONARY ADENOMATOSIS

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S U M M A R Y

The impetus for research on sheep pulmonary adenomatosis has been the recognition of its contagious and neoplastic nature and, of a possible relationship with certain cancers.

Literature Review:

A detailed review of the literature revealed that the disease has a world-wide distribution. It has been clearly identified as a serious cause of loss on a number of sheep farms in Kenya, South Africa, South West Africa, Bulgaria, Scotland and, in Iceland before it was eradicated. Most countries in which the Merino breed (or those they came in contact with) had been imported have recorded presence of SPA. The important exceptions are Australia and New Zealand. Despite this wide distribution there have been gaps in our knowledge of the aetiology, pathology and pathogenesis of the disease.

Aetiological Studies:

Chicken embryos, bacterial media and cell cultures from SPA lungs were used in attempts to identify the aetiological agent. Serological studies against Chlamydial antigens were also carried out. Infective lung materials from 3 natural and 3 experimental

clinical cases of SPA were inoculated via the yolk-sacs into embryonating eggs. Two specimens from the natural cases and one specimen (lung fluid) from experimental cases were passaged 18, 17 and 27 times, respectively. Constant lesions produced by these 3 specimens were haemorrhages under the skin of embryos and in visceral organs particularly the liver and heart in those dying 3-5 days after inoculation. The specimen passaged 27 times revealed presence of Chlamydiae.

Ten out of 14 lung tumours and fluid from clinical cases of SPA yielded Mycoplasma when cultured on Tryptose Serum Agar (TSA) and in Newing's Tryptose Broth (NTB). More than one isolate were encountered in some cases. Their biochemical and serological behaviour showed that they were different from bovine pleuropneumonia vaccine strain and a strain of caprine pleuropneumonia used for comparison. Four samples did not yield Mycoplasma on culturing. Macrophage cultures from 4 adenomatous lungs maintained for 5-7 days formed many giant cells. Both macrophages and giant cells had no intracellular inclusion bodies.

Although the aetiological studies failed to demonstrate the presence of clear-cut infectious agent, a virus is still considered to be the organism involved. Mycoplasma spp. encountered may be opportunists that take advantage of fluid produced by the adenomatous lesions to proliferate and, not involved in the causation of SPA. Serological investigation showed that Chlamydial infections are widespread in many sheep flocks in Kenya. High antibody titres were found in sera from healthy sheep and, no titres in sera from some sheep with clinical SPA.

Transmission Experiments:

Four transmission experiments were carried out. In the first one, a 25% inoculum was prepared from adenomatous lungs of freshly killed sheep using a pestle and mortar. Eight sheep were each inoculated with 10 ml., 4 intratracheally and 4 into the lungs through the thorax. Three of the sheep at days 65, 118, and 251 after inoculation had lung lesions typical of SPA but no clinical signs. They had been inoculated intratracheally. Sheep inoculated intrathoracically and one intratracheally and, killed 255 days after inoculation had no lung lesions.

Ultra-sonic vibration was used in preparing a 57% inoculum for the second experiment. The infective material was adenomatous lung tissue from freshly killed sheep. Ten sheep were all inoculated intratracheally, 7 of them showed clinical signs (dyspnoea, increased respiration, lung discharge, moist rales, cough) of the disease between 107 and 260 days after inoculation. They all had lung lesions of the disease when examined post-mortem. An 8th sheep without clinical signs also had lung lesions of SPA.

In the 3rd and 4th experiments, infective materials passaged in embryonating eggs and, mixed Mycoplasma isolates were used respectively. Inoculations carried out intratracheally in 8 and 5 sheep respectively produced no lung lesions of SPA. In Experiment 3 the sheep were observed for a year and in Experiment 4 for between 151 and 215 days.

It was demonstrated in Experiment 2 that reproduction of typical lesions and clinical disease of SPA was not as difficult as previously thought. This also showed that the aetiological agent was contained in the tumour cells. The reproduction of clinical SPA would appear to be dose-and susceptibility-dependent

and not time-dependent. The incubation period would appear to be between 6 and 8 months.

Electron Microscopic Studies:

Eight freshly killed sheep with SPA provided adenomatous lung tissue for investigation. Several abnormalities in the tumour cells were observed. These were an enlarged polymorphic nucleus, enlarged Golgi complex, an increase of free ribosomes. Dilatation into vesicules of endoplasmic reticulum was evident. There was also irregularity of cytoplasmic membranes in some adenomatous cells. No intracellular inclusion bodies were seen. In all, the ultrastructural changes of the adenomatous cells had similarity to carcinomas in human beings.

Pathology and Pathogenesis:

The gross lesions encountered in 7 of the sheep in Experiment 2 with clinical SPA revealed that infection is normally via inspired air. Anterior lobes and antero-ventral aspects of the diaphragmatic lobes are the ones which showed the grayish adenomatous tissue typical of SPA. This distribution shows that SPA behaves differently from any other lung cancer.

The histopathological lesions clearly revealed the multicentric origin and the progressive nature of this adenomatous cancer. Within the same lung and even the same section, could be seen adenomatous foci of less than 10 tumour cells and sheets of tumour cells. The earliest adenomatous foci were often located in "centres" of thickened interalveolar walls. This suggests that epithelium of microatelectatic alveoli is the first to be involved in transformation and proliferation in sheep that are affected. From these early beginnings adenomatous foci increase in size until they fuse with adjacent ones to form continuous tumour tissue. Although no metastases were found in the pulmonary lymph nodes of 8 sheep of Experiment 2 with typical lesions, metastases have been found in natural cases by several workers. To date the author has encountered 4 cases with metastases.

This study indicates the need for more research on the aetiology of SPA and, the involvement of infectious agents in the causation of tumours.

CHAPTER 1

INTRODUCTION

Ovine pneumonia is the most important disease of sheep in Kenya, which has so far not been controlled. It has always been a cause of serious losses in large flocks of sheep since the early days of modern sheep farming. Pneumonias in sheep are complicated in their aetiology and pathology. Their terminology is confusing because in many instances the conditions have been described without adequate information available to give a simple description to classify them. The best example of confusing terminology is the "Laikipia Lung Disease" which included many pneumonic conditions of sheep in Kenya (WHITWORTH, 1926, METTAM, 1927, 1929; ANON, 1956), acquired because of the high incidence of sheep pneumonia in Laikipia district of Kenya which then was the chief sheep farming area in the country.

Despite the general similarity in the clinical picture of ovine pneumonia, it is evident that the aetiology and pathology of the condition is complex. WANDERA (1967a,b) classified ovine pneumonia in Kenya on aetiological and histopathological bases as:

"Cuffing pneumonia" - of still unknown aetiological

agent but possibly caused by Chlamydiae;

"Bacterial pneumonia" - being mainly represented by enzootic sheep pneumonia and associated with Pasteurella haemolytica;

"Parasitic pneumonia" - caused by Dictyocaulus filaria and Muellerius capillaris;

and "Chronic progressive pneumonia" - under which are two distinct conditions:

Maedi - progressive interstitial pneumonia

and

Jaagsiekte - sheep pulmonary adenomatosis (SPA).

Both "Maedi" and "Jaagsiekte" have been encountered in Kenya, (WANDERA, 1970) sometimes on the same farm and in one sheep. It is because of this mixed infection in some sheep that SHIRLAW (1959) regarded the two types as just various manifestations of one disease entity.

Jaagsiekte (Dutch: Jagziekte from jagt - to drive, ziekte - sickness, hence driving sickness) was so named as the sick sheep

in advanced stages of the disease showed accelerated respiration as if they had been rapidly driven (Hutcheon, quoted by MITCHELL, 1915). The sheep suddenly stopped when driven hungry for breath and died soon after. The term, Jaagsiekte, is now used synonymously with sheep pulmonary adenomatosis (SPA). When originally applied by South Africa sheep farmers, it could have referred to Maedi (Graaff-Reinet disease) also or both.

Jaagsiekte is a distinct contagious disease manifesting itself as a malignant neoplasm with an incubation period varying from months to 2 years dependent on unknown factors. According to WANDERA (1968) the incubation period in some flocks in Kenya is between 6-12 months. The chief clinical features are progressive emaciation, overt respiratory distress, greatly increased respiratory rate, moist rales, and discharge of mucinous, frothy, and watery exudate from the lungs through the nostrils. It is afebrile.

Most reports of SPA are generally in accord with respect to epidemiology, symptoms and gross pathology. There are variations in histopathology and little on pathogenesis. Little is known about the nature of the aetiologic agent apart from suggestive evidence incriminating a virus because of cytoplasmic

inclusion bodies (ENCHEV, 1966) and/or Mycoplasma. The purpose of this investigation on sheep pulmonary adenomatosis as a distinct disease was to:-

(a) undertake aetiological studies,

(b) carry out transmission experiments,

(c) study in more detail its histopathology and pathogenesis; and,

(d) study the fine structure of adenomatous cells.

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CHAPTER 2

LITERATURE REVIEW

II. INCIDENCE

In the first half of this century, sheep pulmonary adenomatosis (Jaagsiekte) and progressive interstitial pneumonia (Maedi) were never clearly ~~distinguished.~~ differentiated. This confusion was made worse by the use of various terminologies, based on native language of the country concerned though indicative of respiratory embarrassment, such as Lungers, Jaagsiekte and Bouhite. While Bouhite meaning "panting" is non-specific, when first used (AYNAUD, 1926) in France, it applied to a sheep lung condition whose dominent characteristic was adenomatous lesions with metastases to regional lymph nodes. The fact that in some countries (France, Iceland, Kenya, South Africa and U.S.A.) both pulmonary adenomatosis and Maedi were present (WANDERA, 1970) only created more problems. Despite variations in the descriptions of the adenomatous lesions of sheep pulmonary adenomatosis (SPA) it is one disease entity. SPA has a world-wide distribution (TUSTIN, 1969) as it has been recorded in more than 20 countries (Fig.1).

Figure 1

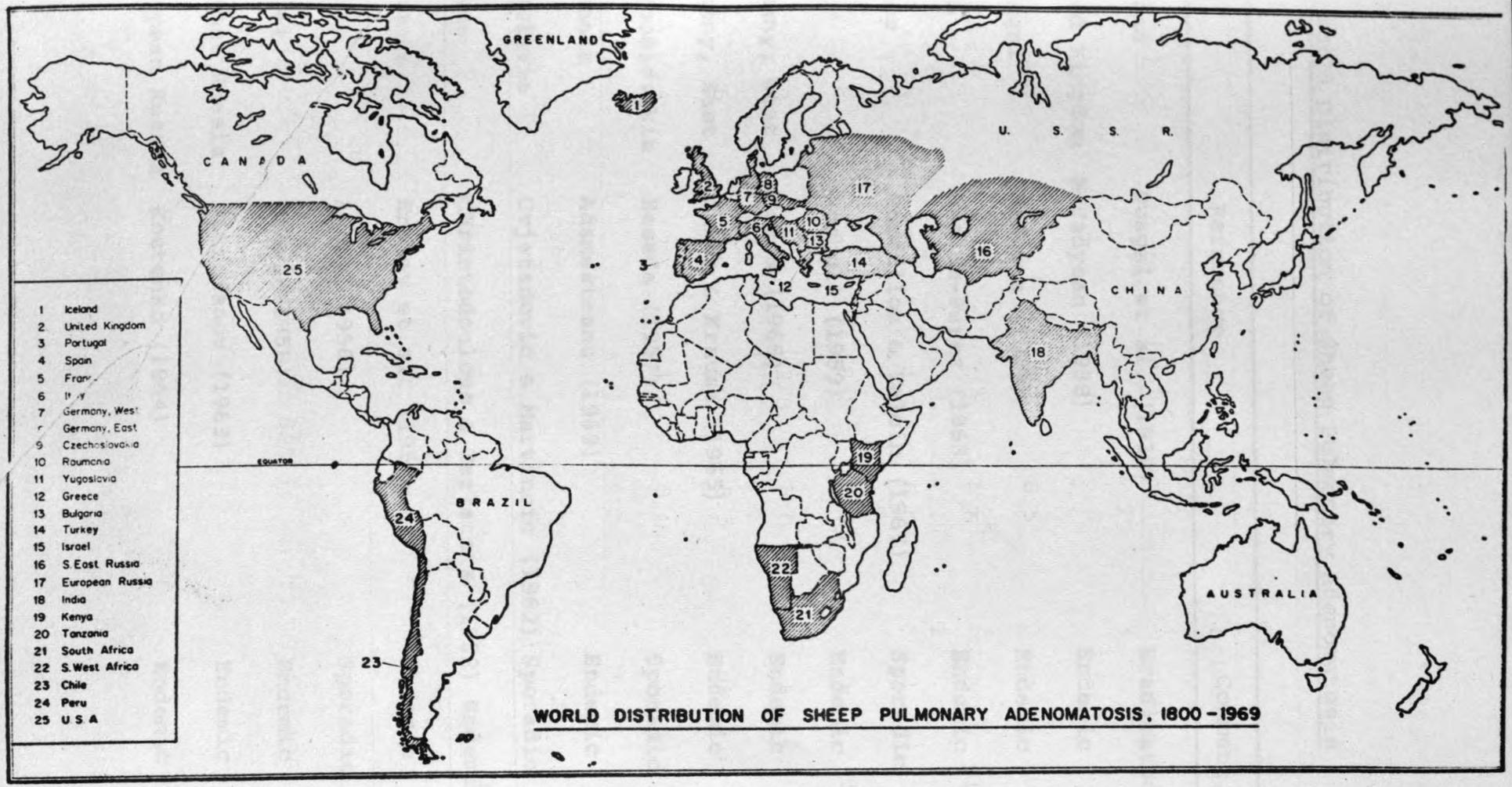
GREENLAND
CANADA
U. S. S. R.
CHINA
BRAZIL
AUSTRALIA
EQUATOR
1 Iceland
2 United Kingdom
3 Portugal
4 Spain
5 France
6 Italy
7 Germany, West
8 Germany, East
9 Czechoslovakia
10 Roumania
11 Yugoslavia
12 Greece
13 Bulgaria
14 Turkey
15 Israel
16 S.East Russia
17 European Russia
18 India
19 Kenya
20 Tanzania
21 South Africa
22 S.West Africa
23 Chile
24 Peru
25 U.S.A
WORLD DISTRIBUTION OF SHEEP PULMONARY ADENOMATOSIS, 1800-1969

Fig.1: World Distribution of Sheep Pulmonary Adenomatosis

Country	Reference	Comment
1. Iceland	Dungal et al. (1938)	Eradicated
2. United Kingdom	McFadyean (1938)	Endemic
3. Portugal	Madeira (1949)	Endemic
4. Spain	Dualde-Perez (1963)	Endemic
5. France	Moraillon & Yalcin (1967)	Sporadic
6. Italy	Romboli (1959)	Endemic
7. Germany, West	Cohrs (1966)	Endemic
8. Germany, East	Jakob & Krause (1965)	Endemic
9. Czechoslovakia	Beseda (1957)	Sporadic
10. Roumania	Adamesteanu (1969)	Endemic
11. Yugoslavia	Cvjetanovic & Martincic (1962)	Sporadic
12. Greece	Christodoulous & Tarlatzis (1952)	Endemic
13. Bulgaria	Enchev et al. (1958)	Endemic
14. Turkey	Akcay (1956)	Sporadic
15. Israel	Nobel (1958)	Endemic
16. S. East Russia	Mitrofanov (1963)	Endemic
17. European Russia	Kostenko (1964)	Endemic

Fig.1: (Continued).

Country	Reference	Comment
18. India	Damodaran (1960)	Sporadic
19. KENYA	Shirlaw (1959)	Endemic
20. Tanzania	(Jakob, H., 1969 - Personal communication)	Sporadic
21. South Africa	Tustin (1969)	Endemic
22. South West Africa	Tustin (1969)	Endemic
23. Chile	Schulz et al. (1965)	Sporadic
24. Peru	Cuba-Caparo et al. (1961)	Endemic
25. U.S.A.	Marsh (1966)	Sporadic

While it was first recorded in scientific publications towards the end of the last century, pulmonary adenomatosis of sheep appears to have been observed by South Africa farmers as early as 1825 (TUSTIN, 1969). It seems to have increased both in incidence and distribution despite the fact that it has completely been eradicated in Iceland, (SIGURDSSON, 1958) and interest in it lost for sometime in South Africa, (DE KOCK 1958). Part of the apparent increase could be explanable on the basis of its recognition as an important disease in commercial flocks. Reports of pulmonary adenomatosis of sheep were made towards the end of the last century in Britain (DYKES & McFADYEAN, 1888; McFADYEAN, 1894). However this was unknowingly so as McFADYEAN at the time considered the lesion to be caused by lungworms. These early reports were later followed by others (McFADYEAN, 1920, 1938; STEVENS, 1957) indicative of interest in the disease in Britain.

In Iceland the disease (DUNGAL et al. 1938) acquired epidemic proportions and was only eradicated (SIGURDSSON, 1958) by a slaughter policy. The Icelanders claim convincingly that the disease was introduced into their country by importation of Karakul sheep from Germany. This is despite the fact that sheep

pulmonary adenomatosis has had a very sporadic occurrence (EBER, 1899; PALLASKE, 1929; SCHULZ & WEILAND, 1968) ever since it was first reported in Germany. Despite illustrations typical of SPA in their work, SEDLMEIER et al. (1966) were not sure that it existed in Bavaria. Its existence was recently confirmed (SCHIEFER & KAST, 1969) in that part of Germany. Though most of the studies were based on slaughter-house materials, a few outbreaks (MOSENFECHTEL, 1937 quoted by Schulz & Weiland, 1968; EYLAU, 1953) have been reported there, and according to COHRS (1966) it is endemic in many parts of Germany.

Other reports of the disease in Europe are by AYNAUD (1926), and MORAILLON & YALCIN (1967) in France; ENCHEV, et al. (1958) in Bulgaria; CHRISTODOULOUS & TARLATZIS (1952) in Greece; AKCAY (1956) in Turkey; NOBEL (1958) in Israel; MADEIRA (1949) in Portugal; ROMBOLI (1959) in Italy; DUALDE-PEREZ (1963) in spain; MITROFANOV (1963) in S.E. Russia; KOSTENKO (1964) in European Russia; BESEDA (1957) in Czechoslovakia, and CVJETANOVIC and MARTINCIC (1962) in Yugoslavia. In Asia, records of the disease have come from India (DAMODARAN, 1960; DHANDA et al. 1963).

The disease has been of a sporadic nature in the United States as viewed from recent statements (MARSH, 1966). This

would appear to conflict with earlier work (COWDRY & MARSH, 1928) in which emphasis was placed on the adenomatous lesions and similarity between "Montana progressive pneumonia" and Jaagsiekte. Materials used for the joint study were apparently not from the same sheep as those handled earlier (MARSH, 1923a). The description and illustrations by DAVIS (1939) on a primary carcinoma of the lung of a sheep are undoubtedly those referrable to SPA, although he was of the opinion that the two were different. It was material supplied by this DAVIS that made MARSH (1966) be convinced that sheep pulmonary adenomatosis existed in U.S.A., especially in parts of Montana and Oregon. "Montana progressive pneumonia" would appear to have been more or less a group name covering both SPA and progressive interstitial pneumonia (WANDERA, 1970). A report by SAVAGE (1926) on pulmonary cancer in sheep emanating from Winnipeg, Canada is essentially that of SPA. It is not clear whether the sheep in question was bred in Canada or imported from elsewhere.

SPA was first reported in Peru in 1945 although it may have existed for a long time before this recognition. This metastasizing adenocarcinoma has been confirmed by CUBA-CAPARO, et al. (1961) and also found in Chile (SCHULZ et al. 1965). One

other country which has suffered from pulmonary adenomatosis is South Africa (COWDRY 1925, DE KOCK, 1929a). According to D. HUTCHEON (Cit. ROBERTSON, 1904), farmers in South Africa, especially in the then Cape Colony, were encountering a serious disease of sheep by 1893 or even earlier. Recent investigations (TUSTIN, 1969) suggest that the disease existed there in early 1800. This disease was called Jaagsiekte. But as reported by ROBERTSON (1904) and MITCHELL (1915) Jaagsiekte was applied to another chronic pneumonia. However the latter was renamed "Graaff-Reinet disease" by DE KOCK (1929a), and differentiated from the present synonym of SPA. The disease was so named because of the number of cases emanating from the now defunct Experimental Station at Graaff-Reinet. It has also been observed on several farms in South West Africa. SPA has recently been reported in Tanzania (Jakob, H., 1969 - Personal communication) and confirmed on the basis of slide examination by the author.

Pulmonary adenomatosis was found in certain flocks of sheep in Kenya, (SHIRLAW 1959). Sheep of all ages and breeds were affected, although some breeds and families within breeds are more resistant than others. One case, (SHIRLAW, 1963 Personal

communication) was encountered in a goat, as were a few others in India (RAJYA & SINGH, 1964), in S.E. Russia (ALIEV, 1967), and in Peru, (CUBA-CAPARO et al. 1961). However none were encountered in goats or cattle in Iceland (DUNGAL, 1946) on farms where they were kept in close contact with sick sheep. These reports of the disease in goats do indicate that this species of animals is also affected. On the other hand they could be erroneous and arise from the apparent inability of histopathologists and others to differentiate between adenomatoid lesions of alveolar epithelium and adenomatosis.

It was in the early 1920's that farmers in the Laikipia district of Kenya became concerned about a lung disease with heavy losses in both lambs and adult sheep. They called it amongst other names, Laikipia lung disease. Although originally this name may have been used to refer to what we now know as pasteurella pneumonia in sheep, the name came to be applied to any type of lung condition in sheep. WHITWORTH (1926) was much more impressed by the lung abscesses and/or fibrinous pneumonia he encountered in the affected sheep and neglected the; ".... numerous greyish spots about a pin's head in size on the cut surface" of the affected lungs. Though both him and

METTAM (1927) did little pathological examination of the cases they were dealing with, their descriptions show that in addition to fibrinous (Pasteurella) pneumonia and pulmonary abscesses due to Corynebacterium pyogenes, some of the lungs had lesions of Jaagsiekte.

A pneumonia of sheep said to resemble that form known as Jaagsiekte in South Africa had previously been reported from two centres (KENYA 1923) by sheep farmers. Unfortunately the complaint was not investigated at the time. Later (KENYA, 1924) more cases of pneumonia in sheep were encountered that were at first thought to resemble those associated with sheep-pox. However, some of the cases were found on farms on which sheep-pox did not exist. Indications are that these early reports could have been dealing with the as yet unrecognised SPA. This is despite the claim of METTAM (1929), that he had not encountered Jaagsiekte after careful examination of many pneumonic lungs.

Mettam cited movement from one farm of 1300 sheep over a distance of 40 miles in 4 days and a subsequent outbreak of a lung disease. Ninety seven of the sheep (7.6%) died in two days but no deaths seen in 4000 sheep found at the destination farm. When the owner examined 15 carcases, he found extensive bilateral

consolidation of anterior lobes of both lungs, and suspected "Laikipia lung disease." Twenty more carcases were later examined by METTAM (1929), and in every case he found identical lung lesions, which he thought were not those of "Laikipia lung disease." No bacteria were isolated in any of the bilaterally consolidated lungs. He incriminated dust as being responsible. Although dust was found in the respiratory tree of some sheep, it is not known to cause fatal pneumonia. This incident does suggest presence of chronic lung disease among some of the sheep prior to their movement. The stress of strenuous exertion due to long driving revealed its presence.

FOTHERINGHAM (1935) showed that Laikipia lung disease was not a single disease entity and that Jaagsiekte in fact did exist in Kenya. It spread rapidly to other areas due to indiscriminate movement of sheep. Because of lack of organised and continuous investigation the confusion regarding lung diseases of sheep continued until much later when SHIRLAW (1959) showed that Jaagsiekte as known in South Africa and Iceland did in fact exist in Kenya. The situation in Kenya was further clarified by WANDERA (1968, 1970) who showed that both Jaagsiekte and Maedi exist in Kenya - sometimes in one flock and in the

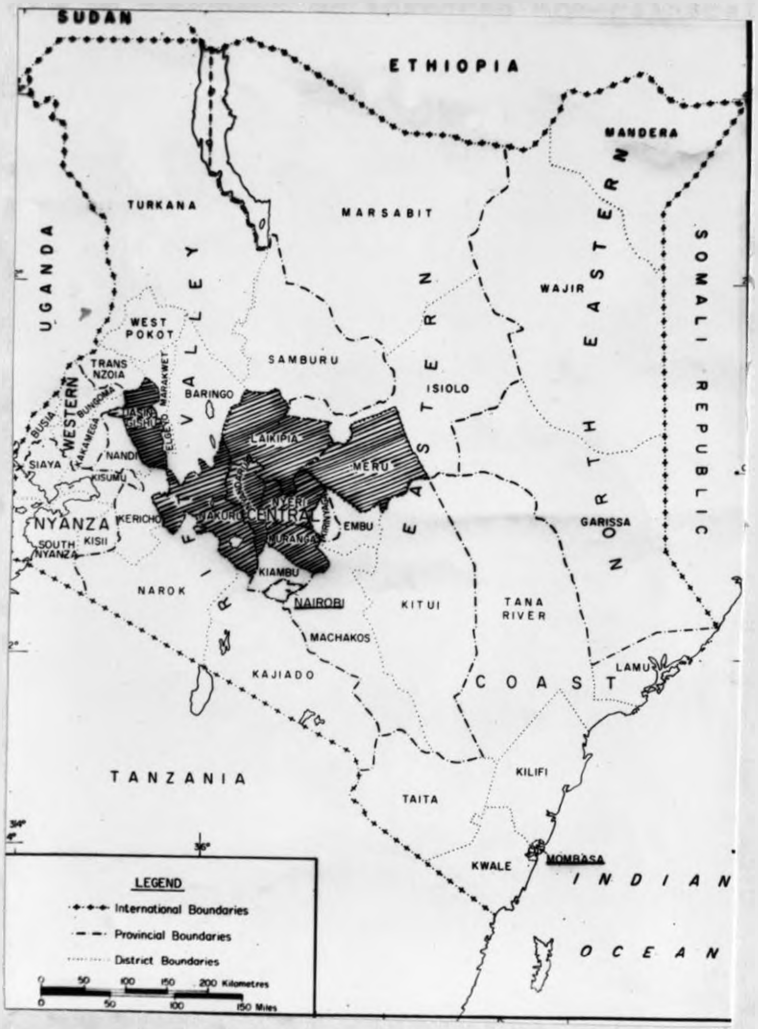

Fig.2: Distribution of SPA in Kenya, 1920 - 1969.

same sheep's lungs. Laikipia, Nyandarua and Nakuru districts of Kenya were the main areas where SPA was a serious problem in some sheep flocks. Sporadic cases in other areas of the country were due to movement of infected non-clinical cases. Fig.2 shows districts of Kenya from which SPA has been reported. The disease is still endemic in some sheep flocks in Nakuru, Nyandarua and Laikipia Districts. The pathological description of the disease by SHIRLAW (1959) was of mixed infections of SPA and progressive interstitial pneumonia.

II. AETIOLOGY AND EPIDEMIOLOGY:

Jaagsiekte is a contagious disease. Its exact aetiology has not been worked out but is probably caused by a virus. Most of the published reports have laid emphasis on clinical signs, epidemiology and pathology. Some suspected aetiological agents have not been subjected to sufficient investigation. Lungworms encountered in several cases (McFADYEAN, 1894, 1920; PALLASKE, 1929; and SEDLMEIER et al. 1966), are no longer regarded as the cause. None of the worms or their larvae were found within the adenomatous lesions. Secondly, there were lungs infected with lungworms but which had no adenomatous transformations and proliferations. ENCHEV (1962) and DHANDA et al. (1963) failed to observe any lung parasites or their ova after careful search. Adenomatoid lesions produced by Muellerius capillaris (WANDERA, 1967a) can be distinguished by experienced investigators from those of Jaagsiekte.

The non-existence of sheep pulmonary adenomatosis in Australia where Muellerius capillaris occur and failure to experimentally reproduce the disease (DUNGAL et al. 1938) with lungworms discount the helminth theory. The significance of Mycoplasma isolated by some investigators (MACKAY, 1966;

ptibility to sheep pulmonary adenomatosis, as with most infectious disease, is influenced by environment. Secondly resistance in affected flocks increases in proportion to the length of time that they had been in contact with the infection.

DUNGAL et al. (1938) repeated contact transmission of DE KOCK (1929b) by housing 8 sheep with diseased ones. One of them died from the disease seven months later. Six had typical lesions and the seventh had no lesions when killed ten months after contact. Clinical disease and death within 8 months were produced by intrapulmonary inoculation of ground unfiltered lung material of SPA in 1 of 3 sheep. However negative results were obtained with a filtered sample inoculated in 5 sheep after 10 months observation. In a later publication DUNGAL (1946) demonstrated in two ways that expired air of an infected sheep contained the infectious agent.

In the first instance sheep were maintained in an elevated compartment 1.5 yards (146 cm.) above the heads of diseased sheep kept in a lower compartment for 4-6 months. Hence other body excretions were excluded. Three of the eight lambs used had adenomatous lesions in their lungs. In the second instance a diseased sheep was made to breathe through a 20% solution of

glycerine in normal saline for 30 minutes. Five millilitres of the mixture were inoculated intratracheally and 2 ml. intrapulmonary in three lambs. Two of the lambs developed lesions of sheep pulmonary adenomatosis of which one showed clinical signs 4 months after infection. In a similar experiment using gradocol filtrate (0.9 μ pore diameter) of SPA lung suspension he inoculated 5 ml. intratracheally into 4 lambs. Clinical SPA disease developed in one of them, 4 months later. The other 3 killed at the same time had no lung lesions.

DUNGAL (1946) further obtained successful production of SPA lesions following intranasal spraying of sheep with a filtrate of bronchial secretion from an affected sheep obtained by filtration through a Chamberland L3 filter. This method of spraying, in addition to contact with clinical cases, was used by MARKSON and TERLECKI (1964) by exposing 6 our-month-old lambs to an aerosol spray consisting of a 10% suspension of affected lung tissue. It was sprayed once a week into the air of the loose-box in which they were kept for 2 years and 15 weeks. Spraying was again applied to another group of 6 lambs without being kept in contact with clinical cases. Scattered adenomatous lesions were present in the second instance in two

animals killed 380 and 864 days, and slightly larger lesions (5-15mm. diameter) in the first instance in one sheep killed at day 1034 after infection.

Typical lesions have also been reproduced by various other routes (DUNGAL et al. 1938; DUNGAL, 1946; ENCHEV, 1966; MARKSON & TERLECKI 1964; SIGURDSSON, 1958; WANDERA, 1968). These were intranasal, intratracheal, intrapulmonary, intrapleural and their various combinations. In South Africa, TUSTIN (1969) used intravenous inoculation of the neoplastic cells, after their growth in tissue culture, and their medium into 2 day-old lambs, to produce clinical disease in one lamb which died 249 days after infection. Advanced lung lesions were also found in the other sheep killed after 253 days.

SHIRLAW (1959) produced typical adenomatous lesions in rabbits. He inoculated 5 rabbits intravenously with tiny infective lung fragments and 5 others with lung suspensions incorporated in an agar plug. In two of the latter and all the former rabbits, lesions suggestive of SPA were observed in the lungs. Two of the rabbits inoculated with lung fragments and kept for a longer time (200 days) had undoubtedly pulmonary adenomatosis lesions. For in addition to

lymphoid hyperplasia around bronchioles and blood vessels observed histologically, there was marked proliferation of bronchiolar epithelium with the formation of papillomatous tufts. These tufts traversed and partially occluded the bronchiolar lumina. The adenomatous process also involved alveoli and alveolar ducts. ZILBER et al. (1962) working in Russia produced, after 4-6 months, multiple cysts filled with serous fluid in the lymph nodes of mice following subcutaneous inoculation of a Seitz-filtered filtrate of a suspension of adenomatous lung.

Taking the above experiments into consideration it is definite that the natural mode of transmission of the disease is aerogenous, and that spread is facilitated by close contact. According to TUSTIN (1969) prenatal infection is of no significance if it ever takes place. Any sheep population is vulnerable, and classic examples of an epidemic are the experiences suffered by farmers in Iceland (SIGURDSSON, 1958) during the latter 1930's - 1940's, in South Africa (TUSTIN, 1969) during the 19th and early 20th centuries, and in Kenya (SHIRLAW, 1959). However after it has appeared in a flock, the rate of infection remains the same for 2-3 years, after which it decreases.

Incidence is increased by overcrowding as during cold periods, or when it is hot by sheep crowding under a shade or where there is none by placing their heads beneath the bellies of others in order to gain some protection from the sun.

III. PATHOLOGY.

The pathology of sheep pulmonary adenomatosis has been described by COWDRY (1925), DE KOCK (1929b), CUBA-CAPARO et al. (1961), STAMP and NISBET (1963), MARKSON and TERLECKI (1964), ENCHEV (1961), and WANDERA (1968). Grossly the disease is characterised by multiple irregularly circumscribed solid nodules scattered throughout a large area of the lungs. These nodules increase in number and size, eventually fusing and giving a continuous grayish-white glandular tissue. Hence the distinction into two anatomical forms: nodular and diffuse used by some workers is arbitrary and erroneous and should be descriptive only.

Affected lungs do not collapse when the chest is opened and are increased in size three or more times their normal weight and volume. There is usually a watery frothy fluid in the trachea, bronchi and bronchioles, and the cut surface is

fleshy. The majority of cases tend to affect the apical and cardiac lobes first. The pulmonary lymph nodes are sometimes enlarged.

Histologically areas of adenomatous proliferations with their protrusion into the alveoli and sometimes the bronchiolar and bronchial lumina are encountered. Metastases to the tracheobronchial and mediastinal lymph nodes first observed by AYNAUD (1926), have also been seen by PAREDES (1953), STAMP & NISBET (1963), MARKSON & TELECKI (1964), CUBA-CAPARO et al. (1961), ENCHEV (1963), SANTIAGO-LUQUE (1963), MITROFANOV (1964), WANDERA (1967b) and TUSTIN (1969). MARTINCIC and CVJETANOVIC (1967) encountered metastases in mediastinal and mesenteric lymph nodes and ENCHEV (1963) observed them in the liver, spleen, kidney and in the heart in one case. NOBEL et al. (1968) recorded 3 extrathoracic metastases - one involving the mesenteric peritoneum, one a psoas muscle and the third, subcutaneous tissue and musculature of the right retrofemoral region. TUSTIN (1969) observed two SPA cases in which extensive spread of the neoplasm to the pleura had taken place. These metastases place the disease in the group of lung tumours. DE KOCK (1929a) mistakenly

classified this malignant tumour as "multiple papilliform cyst-adenoma."

EBER (1899) was the first to recognise SPA as a neoplastic condition: which he regarded as multiple adenomas in the lungs. He observed the clear glandular epithelial forms. The cells were columnar, originating from alveolar ducts and bronchioles. It is now generally agreed (COWDRY, 1925; DE KOCK, 1929a; CUBA-CAPARO et al. 1961), that both cuboidal and columnar epithelial cells are involved, and do arise from alveoli, alveolar ducts, bronchioles, and bronchi. The initial lesion of interalveolar wall thickening observed by COWDRY (1925), COWDRY and MARSH (1927), SHIRLAW (1959), ENCHEV (1966) and WANDERA (1967b, 1968), was not encountered by DE KOCK (1929a), STAMP and NISBET (1963) and MARKSON and TERLECKI (1964). The latter group regarded the minute adenomatous foci as the initial lesions of pulmonary adenomatosis. There is no guarantee that they had the opportunity of observing very early lesions. As WANDERA (1968) stated, minute foci do not necessarily represent beginning lesions.

Generally, in the fast-growing SPA cases, an animal dies before there has been time for secondary fibroplasia. In the slow-growing SPA cases, possibly in the less susceptible sheep, fibroplasia sets in and in addition to crowding out some of the adenomatous cells, there is a tendency for the remaining tumour cells to be much more cuboidal than columnar. Foci of myxomatous tissue have been seen in a small proportion of SPA cases by McFADYEAN (1920), COWDRY (1925), PATTISON (1946), CUBA-CAPARO et al. (1961), and STAMP and NISBET (1963).

Descriptions of Jaagsiekte (ROBERTSON, 1904; MITCHELL, 1915)as a disease characterized by marked thickening of inter-alveolar walls and hyperplasia of lymphoid tissue refer to what is now known as "Graaff-Reinet disease" (DE KOCK, 1929a). This terminology is in reference to the now defunct Graaff-Reinet Experimental Station from where both Robertson and Mitchell obtained their sheep. "Graaff-Reinet disease" is akin in its pathology to Maedi of Iceland, one aspect of "Montana progressive pneumonia" of U.S.A. one form of "Bouhite" of France and Zwoeger-ziekte of Netherlands (WANDERA, 1970).

Lymphoid hyperplasia round the bronchi, bronchioles and blood vessels observed sometimes in SPA cases (WANDERA, 1967b)

some flocks in Central Sierra region. In Britain (MACKAY and NISBET, 1966) the incidence is less than 1%, though becoming a possible hazard of intensified sheep husbandry in parts of that country. In Russia (ALIEV, 1967), the incidence of SPA, especially in Azerbaijan ranged from 1.6 to 7.5%. In Germany, clinical cases and losses in affected flocks show increase, such as 5% stated by PALLASKE (1954) and 22.41% encountered by JAKOB and KRAUSE (1965).

Though no longer present in Iceland SPA was an epidemic in that country. Losses in some flocks (DUNGAL et al. 1938) ranged from 50 to 80% within 1-2 years. The disease is of economic importance in Bulgaria. When first recorded in 1955, ENCHEV et al. (1958) stated that affected flocks were losing between 1-3%. Following spread to other regions of that country (ENCHEV, 1961), an epidemic soon followed with losses on certain farms reaching 35.5%, and is now endemic. Other countries with serious outbreaks also had more losses from the disease, such as 10 to 30% of affected flocks in Greece (CHRISTODOULOUS and TARLATZIS, 1952).

At one time Kenya had up to 30% annual mortality (SHIRLAW, 1959), from the disease alone on certain farms. It has now dropped down to between 1 to 5% (WANDERA, unpublished data) in endemic flocks. This could possibly be due to better husbandry and elimination of overcrowding at night and partly due to the fact that, as with most infectious diseases, susceptibility is gradually reduced in endemic flocks. The figure for South Africa (DE KOCK, 1929b) was less than 5%. But according to TUSTIN (1969) when the disease was first introduced in South Africa in 1800's losses of 30% and more in certain flocks were not unusual. None of the affected sheep ever recover.

V. GEOGRAPHICAL ORIGINS OF THE DISEASE.

It is not known just how the disease started in Kenya. However spread to some countries and within a country (KOSTENKO, 1964) can be traced to importation of healthy latently infected sheep. While the disease may have occured in Scotland for many years, its spread to England is associated with infected but apparently healthy sheep from the former (STEVENS, 1957). Though SPA was and is still largely sporadic in Germany, the Icelandic

epidemic is traced back to Karakul sheep bought from Germany (DUNGAL, 1946) in the district of Halle in December of 1933. Indeed cases recorded in Yugoslavia (CVJETANOVIC & MARTINCIC, 1962) are believed to have been introduced by Merino rams from France and German Federal Republic.

For many years it was believed that in Kenya Jaagsiekte occurred naturally in Masai sheep (SHIRLAW, 1959). However, the available evidence (WANDERA, 1967a) shows that Masai sheep may have been suffering from bacterial and/or parasitic pneumonia and not Jaagsiekte. METTAM (1927) encountered the disease in flocks of Merino sheep in Kenya on farms where no Masai sheep had ever been. Both Jaagsiekte and Maedi could have been introduced by Merino sheep imported into Kenya ever since 1904 from South Africa. TUSTIN (1969) showed that South African sheep farmers had been losing sheep from pulmonary adenomatosis since the 1800's, and further produced evidence to show that the disease was brought in that country with the importation of Spanish Merino sheep.

It is hard to explain how and why the disease has not been recorded in Australia and New Zealand. Both these two countries

imported Merino sheep direct, and, via Britain and South Africa from Spain. It would appear, on the basis of available circumstantial evidence, as if the original home of SPA could be Spain and possible France and Germany. If this is probable, is it likely that the disease could have spread with the movement of the Merino sheep and those that had been in-contact with them to other countries. Of all the sheep breeds, it is only the Merino (RYDER and STEPHENSON, 1968) which has had the widest distribution, ever since it left its Spanish home. The chance of infected symptomless Merino sheep leaving Europe to South Africa, spreading the disease there and later some asymptomatic infected Merino sheep and their crosses being imported into Kenya is quite feasible.

CHAPTER 3

AETIOLOGICAL STUDIES

Despite the fact that SPA is a contagious disease, its aetiological agent has not been isolated and characterised. According to SHIRLAW (1959) adenomatous lung tissue preparation passaged in chicken embryos and put back into sheep produced lung lesions of the disease. He used yolk sac membrane and the whole embryo of 34th passage and yolk sac membrane alone at the 56th passage. On this basis it would appear that, irrespective of its nature the causal agent can propagate in chicken embryos and be transmitted to susceptible sheep.

The aims of the present studies are first to repeat SHIRLAW'S work, and secondly to try and isolate infectious agents propagating in embryonating eggs.

I: STUDIES WITH CHICKEN EMBRYOS

(1) Source and Preparation of Eggs: At the beginning of these studies fertile eggs were obtained from a commercial flock; later, following the establishment of a Specific Pathogen Free (SPF) flock, in the Department of Veterinary Pathology, free of known poultry pathogens including leukosis, such eggs were obtained from an internal source. Eggs were incubated at 37°C., candled

at the 5th day and non-fertile, weak or dead ones removed. On the 7th day, selected embryonating eggs were prepared for inoculation.

(2) Preparation of Infective Materials: Specimens used for passaging in chicken embryos were from sheep with clinical signs and lesions of pulmonary adenomatosis. Lung specimens W_1, W_2, W_3 were from naturally affected sheep killed and immediately prepared for inoculation. Sample W_1 was the same as that used for infecting sheep in the Transmission Experiment 2 of Chapter 4. W_2 and W_3 were from sheep of the same flock as the first one. Specimens W_4, W_5, and W62, used for passaging came from sheep 3616, 3615 and 3645 respectively, with experimentally produced disease (Chapter 4). Lung specimens W_4 and W_5 had been frozen at $-70^{o}C$. for 9 and 6 days respectively before use. Specimen W62 was lung fluid which was collected via the nostrils by lifting up the hind limbs and used immediately.

Inoculum of W_1 was prepared as follows:
One hundred and twenty of representative lung lesions obtained in a sterile way was minced finely with scissors and homogenized in a Silverstone blendor for one minute at full speed. After the first

blending about 60ml. of Eagle's medium containing 100 I.U. penicillin, 100 ug. streptomycin, 100 ug. kanamycin per ml. was added and the suspension again blended for one minute. Following centrifugation in the refrigerated MSE centrifuge for 10 minutes at 2,500 g., the supernatant was removed and stored in an ice-bath. To the sediment 30ml. of the same medium was added and the suspension subjected to ultrasonic vibration with a Branson Sonifier Model S-125 (Branson Instr., Danburry, Conn., U.S.A.) for about 30 seconds at position 8. The suspension was next centrifuged as before, but for one hour. This second supernatant was removed and mixed with the first one, giving it a concentration of over 55%. A sample of this suspension was used for sheep inoculation in Experiment 2 Chapter 4. Another sample of this preparation was diluted to 10% with the same medium and filtered through 0.45 u Millipore, before egg inoculation.

Essentially the same method was used for preparing 10% concentrated inocula of W_2, W_3, W_4 and W_5. Penicillin, streptomycin and kanamycin were included in the medium for preparing W_2 but kanamycin was not used in W_3, W_4 and W_5. These four samples were used unfiltered. Lung fluid making the inoculum of W62 was the third of the 5 fluid samples collected at intervals from

sheep 3645. This one was obtained at day 195 after infection. The fluid was diluted 1:2 with Trisbuffer and filtered through 1.2 microns filter to remove cells and large subcellular elements. No antibiotics were used.

(3) Methods of Inoculation and Sample Collection:

Preliminary trials were made with chorioallantoic membrane, allantoic cavity, amniotic cavity and yolk sac routes. The yolk sac method was found to be most effective and convenient, because of easy administration and the fact that eggs generally died more regularly. The 7-day old embryonating eggs were inoculated with 0.2ml. through the air-cells into the yolk sac using an opening made away from the embryos and large blood vessels after disinfection with iodine. The lobes were sealed with "UHU" glue.

The infected embryonating eggs were again incubated at 37°C. and candled daily. Embryo deaths within 24 hours were regarded as accidental and disregarded while those alive by the 18th day of incubation were killed. The allantoic-amniotic fluid (AAF) was collected from those embryos dying between the 2nd - 11th day after infection. In some cases, liver and heart were also collected. AAF and collected organs were stored at -20°C., after some of it being filtered through sterile Millipore filters of 0.45 u

mean pore diameter for further embryo inoculations. Several passages of W_1 and W62 were titrated in embryonated eggs. Both dead and killed embryos were examined post-mortem for possible gross lesions. Each original and passage samples were routinely checked for bacteria on plates of McConkey's and blood agar, and for Mycoplasma on Tryptose Serum Agar (TSA) and in Newing's Tryptose Broth (NTB).

(4) Results: The data of the six sets of passage of infective materials in embryos are indicated in TABLES: 1, 2, 3, 4, 5, 6. Embryos died more regularly with specimens W_1, W_2 and W62. The average mean death time was about 5.0 days for W_1 and W_2. The earliest average death time was 3.0 days while the latest was 7.0 days in W_1. For W_2 it was 4.0 days and 8.0 days respectively. Passages of W_3, W_4 and W_5 had irregular results in all respects. With the exception of the 1st, 11th, 14th, 15th and 16th passages embryo deaths were consistent in all the 27 passages of W62. The average mean death time was 5.0 days whilst the earliest and latest average death times were 4.0 and 6.0 days respectively.

Constant lesions caused by W_1, W_2 and W62 were haemorrhages under the skin of embryos and visceral organs especially liver and heart in those dying 3-5 days following inoculation. Liver

necrosis was observed in embryos dying between 6-8 days after infection. Other less constant lesions were stunted and/or featherless embryos and enlarged green livers particularly in those dying between 7-11 days after infection. Dwarfed embryos and enlarged green livers were sometimes encountered in those killed at the 18th day of incubation. The other three samples caused variable lesions in embryos.

W_1 was passaged 18 times. Two samples of it at the fifth and fifteenth passages were inoculated into sheep to test their pathogenic effect as shown in Transmission Experiment 3, Chapter 4 of this thesis. W_2 was passaged 17 times, W_3 eight times, W_4 and W_5 seven times each and W62 twenty seven times.

Preliminary determinations of LD50 in chicken embryos of 5 passages of W_1 and 3 passages of W62 revealed the following titres:

4th Passage (W_1)	$10^{6.6}$ ELD50/ml.
5th Passage	$10^{6.6}$ ELD50/ml.
10th Passage	$10^{6.35}$ ELD50/ml.
14th Passage	$10^{7.1}$ ELD50/ml.
15th Passage	$10^{7.6}$ ELD50/ml.
6th Passage (W62)	10^{5} ELD50/ml.
9th Passage	10^{6} ELD50/ml.
11th Passage	10^{6} ELD50/ml.

TABLE 1: Passage of W_1 in Chicken Embryos

Passage	Embryos Inoc.	Accidental Deaths	Deaths	Mean death Time (days)	Earliest Death Time (days)	Latest Death Time (days)
1	7	-	2/7	6.0	6	-
2	12	2	8/10	5.9	4	10
3	10	-	9/10	5.20	3	8
4	5	1	4/4	4.75	3	7
5	6	-	6/6	4.7	3	6
6	6	1	5/5	6.0	3	11
7	8	-	8/8	4.25	3	5
8	8	-	8/8	4.25	2	6
9	12	-	12/12	5.2	3	9
10	10	1	9/9	6.2	3	10
11	10	-	10/10	4.6	4	6
12	10	-	10/10	3.9	3	7
13	10	1	9/9	4.1	3	5
14	9	1	8/8	4.25	3	5
15	14	1	13/13	4.1	3	7
16	4	1	2/3	6.5	4	9
17	7	1	4/6	6.5	4	9
18	6	-	4/6	5.25	2	11
		AVERAGE		5.0	3.0	7.0

TABLE 2: Passage of W_2 in Chicken Embryos

Passage	Embryos Inoc.	Accidental Deaths	Deatns	Mean death Time(days)	Earliest Death time(days)	Latest Death time (days)
1	5	-	3/5	8.33	7	10
2	8	-	4/8	7.0	5	8
3	6	-	3/6	4.0	4	-
4	8	-	5/8	7.0	4	10
5	8	1	6/7	3.5	3	5
6	8	-	8/8	4.0	3	5
7	10	1	7/9	4.86	3	9
8	10	2	8/10	4.1	3	6
9	10	-	10/10	4.3	3	8
10	10	1	9/9	4.1	2	9
11	5	-	5/5	4.0	4	-
12	5	1	4/4	6.5	4	9
13	10	2	3/8	6.67	3	9
14	8	-	2/8	6.5	6	7
15	6	-	5/6	6.0	4	9
16	6	-	3/6	6.67	5	9
17	6	-	0/6	-	-	-
		AVERAGE		5.0	4.0	8.0

TABLE 3: Passage of W_3 in Chicken Embryos

Passage	Embryos Inoc.	Accidental Deaths	Deaths	Mean Death Time (days)	Earliest Death Time (days)	Latest Death Time (days)
1	8	-	2/8	6.5	5	7
2	6	-	2/6	8.5	8	9
3	8	-	5/8	6.6	2	10
4	8	1	4/7	6.0	2	8
5	8	-	8/8	7.25	5	11
6	8	-	2/8	5.0	3	7
7	6	-	2/6	8.5	8	9
8	5	1	1/4	3.0	3	-
		AVERAGE		6.0	4.5	9.0

TABLE 4: Passage of W_4 in Chicken Embryos

Passage	Embryos Inoc.	Accidental Deaths	Deaths	Mean Death Time (days)	Earliest Death Time (days)	Latest Death Time (days)
1	8	-	3/8	5.65	5	7
2	6	-	4/6	3.75	2	8
3	7	-	1/7	6.0	6	-
4	8	-	2/8	5.0	2	8
5	8	-	5/8	6.2	5	8
6	8	1	2/7	7.0	6	8
7	6	-	2/6	5.5	5	6
		AVERAGE		5.5	4.5	7.5

TABLE 5: Passage of W_5 in Chicken Embryos

Passage	Embryos Inoc.	Accidental Deaths	Deaths	Mean Death Time (days)	Earliest Death Time (days)	Latest Death Time (days)
1	8	-	3/8	5.65	3	7
2	6	-	5/6	4.8	2	8
3	8	-	4/8	6.0	2	10
4	8	2	3/6	2.0	2	-
5	8	-	6/8	6.65	4	11
6	8	-	1/8	4.0	4	-
7	6	1	3/5	6.0	2	9
		AVERAGE		5.0	3.0	9.0

TABLE 6: Passage of W62 in Chicken Embryos

Passage	Embryos Inoc.	Accidental Deaths	Deaths	Mean death time (days)	Earliest Death Time (days)	Latest Death Time (days)
1	6	-	1/6	11.0	11	-
2	8	-	8/8	7.0	5	9
3	8	-	8/8	4.5	2	6
4	6	-	6/6	4.5	2	6
5	6	-	6/6	3.5	3	4
6	6	-	6/6	3.8	4	5
7	10	-	9/10	5.3	4	7
8	4	1	3/4	6.0	6	-
9	5	-	4/5	5.3	4	6
10	10	1	9/10	7.0	5	11
11	6	1	3/5	5.0	2	6
12	6	-	6/6	4.7	2	6
13	6	1	5/5	7.4	5	9
14	6	-	3/6	7.0	4	10
15	6	1	2/5	6.0	4	8
16	6	-	2/6	5.5	5	6
17	6	1	5/5	4.8	5	6
18	6	2	4/4	5.0	4	7
19	6	-	6/6	5.7	5	6
20	6	1	5/5	4.0	3	6

Contd. to next page.

TABLE 6: (Continued)

Passage	Embryos Inoc.	Accidental Deaths	Deaths	Mean death Time(days	Earliest Death Time(days)	Latest Death Time(days)
21	6	-	6/6	5.3	5	6
22	6	_	5/6	5.2	5	6
23	6	2	4/4	3.8	3	5
24	6	-	6/6	4.7	3	6
25	6	-	6/6	5.0	3	7
26	6	-	6/6	4.8	4	5
27	10	-	10/10	5.0	3	7
		AVERAGE		5.0	4.0	6.0

Samples of every passage of all 6 cases cultured both on McConkey's and blood agar plates showed no growth of any bacteria. Kanamycin appears to have effectively inhibited the growth of Mycoplasma spp. from W_1 and W_2. These two samples had Mycoplasma in their original tumours (Section II, below). Though kanamycin was not used in preparing W_3, W_4 and W_5, no Mycoplasma were isolated either in Newing's Tryptose Broth or on Tryptose Serum Agar. This is an indication that the original lung tumours had no Mycoplasma. Neither Mycoplasma nor other bacteria were encountered in sample W62 although antibiotics were not used in its preparation. It would appear that at the time the lung fluid was collected, no Mycoplasma had yet invaded the affected lungs. Four Mycoplasma isolates were later made from the lungs of the donor, sheep 3645 (Section II, below).

II. ISOLATION AND CHARACTERIZATION OF MYCOPLASMA SPECIES

Reports have been made of isolation of Mycoplasma from cases of SPA. In Israel NOBEL (1958) encountered Mycoplasma in 2 out of 19 cases. MACKAY (1966) obtained 22 isolates from some of the lungs of 30 sheep with the natural disease in Scotland. He classified his isolates into 2 morphological colony types on solid medium. No isolates were made from lungs of 9 of his sheep.

DEIANA and CERETTO (1967) working in Sardinia, Italy, found antibodies against Mycoplasma isolated from SPA cases.

So far with the exception of DEIANA and CERETTO (1967) only a secondary role has been attributed to Mycoplasma in adenomatous lungs of sheep. It has not yet been conclusively shown that they do not play a role in the causation of SPA. Mycoplasma have been encountered in Maedi lungs (WANDERA, 1970), and nasal fluid (KRAUSS, H. - 1969, unpublished data) from 40 sheep sent for slaughter but free of any lung lesions. Mycoplasma have further been isolated from pneumonic lungs in Australia (ANON, 1964-67) where SPA has not yet been observed. While organisms isolated from Australia may be non-pathogenic, there is no record in literature of respiratory-pathogenic Mycoplasma of sheep. Hence circumstantial evidence suggests that Mycoplasma isolated so far are not involved in causing the adenomatous process of Jaagsiekte.

Mycoplasma were encountered during investigations of the aetiological agent of pulmonary adenomatosis of sheep in Kenya. Apart from COTTEW et al. (1968) in Turkey, little has been done to elucidate the properties of Mycoplasma isolated from sheep.

The present investigation was undertaken to isolate Mycoplasma spp. from natural and experimental cases of Jaagsiekte and study some of their properties.

(2) Materials and Methods:

(a) For isolation and propagation of Mycoplasma: The liquid medium used was modified Newing's Tryptose Broth (NTB) as described by BROWN et al. (1965), made up of:

Bacto-Tryptose (Difco)	2.00%
Dextrose	0.50%
Sodium chloride	0.50%
Anhydrous di-sodium phosphate (Na_2HPO_4)	0.25%
Glycerol	0.50%
Distilled water ....to	100.00%

The solid medium used was Tryptose Serum Agar (TSA) as described by DAVIES and READ (1968), and prepared in two parts as follows:

(i) Tryptose agar

Bacto-tryptose (Difco)	2.00%
Sodium chloride	0.50%
Glycerol	0.50%
Anhydrous di-sodium phosphate (Na_2HPO_4)	0.25%
Distilled water .... to	100.00%

(ii) Pig Serum and Additives

Inactivated (fresh) pig serum	30 ml.
Dextrose	5 ml. (10%)
Bacto-yeast extract (Difco)	1 ml. (10%)
Crystalline penicillin G (Glaxo)	100,000 Units
Thallium acetate	1 ml. (1% Soln.)

Part (i) was prepared and kept in stock until needed. Part (ii) was prepared immediately before use and filtered through Seitz E.K. pads before being added to the melted part (i) in the ratio of 100 volumes part (i) to 36 volumes part (ii). In some cases of solid medium the penicillin and thallium acetate were omitted.

(b) Embryonated chicken eggs were from a SPF-flock. Infective material was inoculated into the yolk sac after 7 days of incubation at 37°C. The eggs were candled daily to detected mortality. Alantoic/amniotic fluid was collected from dead embryos for further passage.

(c) Tissue cultures of chick embryo kidneys from 17 days old embryos, and chick embryo fibroblasts from 10 days old embryos after removing heads, legs and visceral organs were

prepared by trypsinization. Trypsin was at a concentration of 0.25% in buffer. The culture medium used was 50% Eagle's medium in 50% Hanks' medium. For growth, 10% calf serum was added. Lung macrophage cultures were obtained from normal lungs of freshly killed sheep. Such lungs were first filled with Hanks' BSS via the trachea. The opening was stoppered and the distended lungs massaged gently for about 5 minutes. The fluid was next poured out into a sterile container and centrifuged at 400 r.p.m. for 10 minutes. The cells were washed once with Hanks' BSS following sedimentation. The sedimented cells were suspended to a concentration of 1.25% in Eagle's medium in Hanks' BSS containing 10% tryptose phosphate broth, and 10% inactivated horse serum. Antibiotics used were 20 units penicillin, 50 µg. streptomycin and 200 µg. kanamycin per ml. of the suspension. Cultures were inoculated with infective material and checked daily for signs of a cytopathic effect. Medium was collected at the 7th day after infection for further propagation.

(d) Primary isolation of Mycoplasma was from killed sheep with clinical or pathological evidence of SPA. Materials used were either directly from the lung tumours and/or, samples of discharge from the lungs through the nostrils on

(f) Preparation of antisera for serological investigation:

Rabbits were bled for pre-inoculation sera and then inoculated with 100-fold washed concentrate of a 24-48 hour broth culture, 1ml. containing about 10^{11} to 10^{12} living organisms. A series of 4 inoculations were made. The first 3 were done subcutaneously using 2ml. of concentrate mixed with 2ml. of Freund's incomplete adjuvant, at weekly intervals, and the last one intraperitoneally with 4ml. of concentrate only. Seven to ten days after the last inoculation the rabbits were bled for serum.

Hyperimmune sera against T2/33 Vaccine-strain of bovine pleuro-pneumonia previously used in Uganda (SANDERS, 1961) and against M. laidlawii were available for comparative study.

(g) Agar gel double precipitation reaction: The reactants of the tests were incubated in a moist atmosphere at 37°C. for 3 days and the precipitation results recorded. Mycoplasma concentrates, subjected to ultra-sonic vibration at 4°C. with a Branson Sonifier Model S-125 (Branson Instr., Danburry, Conn., U.S.A.) for 3 min. at position 6 were used as antigens. All antisera were absorbed with lyophilized NTB containing 20% (V/V) horse serum by thoroughly mixing 200 gm. of the dry powder into 1ml. immune serum. The absorption was continued for 2 days at 4°C.

The antisera were cleared by centrifugation at 1000g. for 10 minutes.

(h) Inoculated TSA plates were incubated at 42°, 37°, 32°, and 22°C., for 7 days and growth recorded. Isolates growing on serum-free tryptose agar were cloned, propagated in NTB without serum and again grown on serum-free tryptose agar.

(i) Other tests: These included carbohydrate fermentation, production of catalase, H_2S, and reduction of methylene blue, tetrazolium blue, and production of haemolysis, serum liquefaction and haemadsorption. The medium used was NTB or TSA, glucose and yeast being omitted where necessary. Production of indol was tested by use of Kovac's reagent, with an indol producing strain of Escherichia coli as control. Haemagglutination tests were performed with 0.5ml. of serial dilutions of washed Mycoplasma concentrates in phosphate buffered saline and 0.5ml. of a 0.5% horse or sheep erythrocyte suspension. Tests were incubated at room temperature and read when the erythrocytes had sedimented.

(j) A mixed Mycoplasma sample from W_1, W_2, W6/1 and W6/2 were used for sheep inoculation in Transmission Experiment 4, Chapter 4 of this thesis to test their pathogenicity.

(3) Results:

Out of 14 killed sheep with typical SPA disease, 10 had Mycoplasma in their adenomatous lungs and/or lung secretions. Sixteen isolates were made from them; 6 from lung secretions via the nostrils and 10 from tumours directly. Lung tumours and secretions of 4 sheep (cases W_3, W_4, W_5 W62) yielded no Mycoplasma.

The solid medium (TSA) was found superior to liquid medium (NTB) or embryonating eggs for the primary isolation of Mycoplasma. The number of primary colonies could be checked and morphologically different colonies on the same plate could be cloned immediately after outgrowth. Visible colonies appeared within 3 to 10 days after inoculation in numbers that were identifiable by microscopic observation and sometimes with the naked eye. Bacterial contamination was effectively inhibited by the use of TSA medium for primary isolation. Development and passage of possible L-forms of bacteria was avoided by passages in NTB medium without antibiotics or thallium acetate.

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no CPE, but large amounts of Mycoplasma were isolated from culture media. Mycoplasma inoculated in sheep as shown in Chapter 4 of this thesis produced no apparent reaction. The data on the various tests carried out to elucidate the properties of Mycoplasma isolates are tabulated in TABLES 7 and 8.

Three isolates: W7/2, W7/3 and W8 out of 16 grew well at 22°C. They were also passaged on solid and in liquid media without addition of serum. They were therefore regarded as "saprophytic" strains of the Laidlawii type. None of the various isolates tested grew at 42°C., but grew well at 37°C. The saprophytic strains also grew well at 32°C.

Culturally all the isolates could be divided into those growing relatively fast and insensitive to varying environmental conditions, and the relatively slow growers, more delicate and sensitive to environmental factors. The Scottish sheep strain MS, belonged to the latter group. The colonies were further divided morphologically into the "Fried egg" appearance type with a well-developed nipple formation, and the "Lacy" type that did not form a central nipple except in old colonies.

More than one isolate could be made from a single sheep affected with SPA. In one case three (W7/1, W7/2, W7/3) and in another case four (W6/1, W6/2, W6/3, W6/4) Mycoplasma isolates with diffe-

TABLE 7: Properties of Mycoplasma Isolates.

Isolate	Origin	Colony Type	Growth	Turbidity Liq. Medium	Serum Requirement	Growth °C. 32	Growth °C. 22	Carbohydrate Fermentation: Glucose	Dulcitol	Mannose	Lactose	Maltose	Salicin	Fructose	Sucrose	Starch	Reduction: Methylene blue	Tetrazol. blue	Sero Group
W_1	T	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+++	
W_2	T	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W6/3	T	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W6/4	T	L	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W7/1	NF	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+++	I
W10/1	NF	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W10/2	T	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W11	NF	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W12	T	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W13	NF	FE	A	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W6/1	T	L	B	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
W6/2	T	FE	B	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+++	II
W14	T	FE	B	+	+	+	-	+	-	-	-	+	-	-	-	+	-	-	
MS		L	B	+	+	+	-	+	-	+	-	+	-	-	-	+	+++	-	
W7/2	T	FE	A	+++	-	+++	+++	+	-	-	-	+	-	-	-	-	++	-	
W7/3	NF	FE	A	+++	-	+++	+++	+	-	-	-	+	-	-	-	-	-	+	III
W8	NF	FE	A	+++	-	+++	+++	+	-	-	-	+	-	-	-	-	+++	-	
T_1	T	FE	A	+	+	+	-	+	-	+	-	+	-	+	-	+	+++	+++	
VOM		FE	A	+++	+	+	+	+	-	+	-	+	-	+	-	+	+++	+++	

KEY: T - Tumour
NF - Lung secretion recovered via nostrils.
FE - Colony with nipple formation (Fried egg type).
L - Colony without nipple formation (Lacy type).
A - Non-fastidious
B - Fastidious.

TABLE 8: Results of Agar Gel Precipitation Tests.

Group	Antigen \ Serum	I	II	III	T_1	Laidlawii A
I	W_1, W_2, W7/1, W10/1, W11, W12, W13, W6/3, W6/4	+++	-	±	-	-
II	W6/1, W6/2, W14, MS	-	+++	+	-	-
III	W7/2, W7/3, W8	-	-	x +++	-	+
CBPP Vaccine	T_1	-	-	-	+	-
CCPP	VOM	-	-	-	+	+

KEY: CBPP - Contagious bovine pleuropneumonia
CCPP - Contagious caprine pleuropneumonia
x - Antiserum against W7/3 reacted only against its own antigen within the group.
± - Cross reactions produced by individual isolates of the group.

rent properties were recovered. Most of the isolates were non-fermenters of carbohydrates. The 3 saprophytic strains fermented glucose and maltose. Isolate W14 fermented glucose, maltose and starch, comparable with the Scottish strain, MS, but it could be distinguished from the latter by its failure to ferment mannose. The two pleuropneumonia strains (T_1 and VOM) additionally fermented fructose. None of the isolates fermented dulcitol, lactose, mannose, sucrose and salicin.

Alpha-haemolysis of sheep and horse erythrocytes was shown by all types while haemadsorption, haemagglutination, and catalase, indol and hydrogen sulphide production tests were negative.

Two of the 3 saprophytic strains (W7/2 and W8) reduced methylene blue, together with the Scottish strain. The third one (W7/3) reduced tetrazolium blue, together with three other isolates (W_1, W7/1 and W6/2). The two pleuropneumonia strains reduced both methylene blue and tetrazolium blue.

On the basis of precipitation test (TABLE 8) the various isolates could be placed into 3 serogroups. The first group consisted of fast growing non-fermenters, the second of fastidious organisms partly fermenting glucose and other sugars. Group III Mycoplasma are saprophytic and the pathogenicity of groups I and II is as yet unknown, and their role in SPA is not clear. It would appear that

consolidation in cases of pasteurella pneumonia in lambs about 3 weeks old.

In Section II of this Chapter no Mycoplasma were recovered from lungs and lung secretion of four (W_3, W_4, W_5, W62) out of 14 sheep with clinical SPA. These four cases were amongst the 6 (Section I) whose infective preparations were passaged in embryonating eggs. While W_3, W_4, and W_5 killed eggs sporadically, W62 caused more consistent embryo deaths over most of its passages. Samples W_4, W_5 and W62 originated from experimental animals, sheep 3616, 3615 and 3645, previously inoculated with W_1. Mycoplasma had been isolated from the latter sample but not from the former. Hence it is unlikely that Mycoplasma play a significant role in the causation of pulmonary adenomatosis of sheep.

The findings to date reveal that there are other microorganisms other than Mycoplasma which cause SPA. Of the four cases which failed to yield Mycoplasma, W62 was of particular interest. It killed eggs regularly and was passaged more times than the others. It was decided to investigate this sample further for chlamydial agents especially, taking into account SHIRLAW'S (1962) work. It was also planned to test sera from natural and experimental SPA cases, and from several sheep flocks in Kenya both endemic and free of SPA for antibody titres against Chlamydial group antigens.

(2) Materials and Methods:

(a) Embryonating Eggs were obtained from our own SPF-group.

(b) Infective Material: Several passage samples of W62 were used.

(c) Egg Inoculation and Sample Preparation for Electron Microscopy:

Embryonating eggs were inoculated after 10 days of incubation on the chorioallantoic membrane. Following normal incubation the embryos were killed between 3 and 7 days and the chorioallantoic membrane harvested. The membranes were immediately cut into small pieces and fixed for electron microscopy. For negative staining the fixative was 1% osmium tetroxide dissolved in acetate-veronal buffer. Following fixation the specimens were put into 0.5% uranyl-acetate dissolved in acetate-veronal buffer for 2 hours. The blocks were dehydrated in graded acetone (25, 50 and 75% for 15 minutes each, and 90 and 100 each 30 minutes). Embedding and the remaining electron microscopic procedures were as described in Chapter 5.

(d) Serum Samples: Sera from clinical cases of natural SPA and from experimental cases (Transmission Experiment 2 - Chapter 4) provided two sets of samples. Sera were also obtained from several sheep flocks in Kenya: those known to be endemic for SPA, and those from where the disease has not been reported. These were obtained

in the Mau Narok, Molo and Mau Summit areas of Nakuru District; Eldoret area, Uasin Gishu District; Naivasha Government Farm (Nakuru District); sheep at the Faculty of Veterinary Science Kabete; and from Ngobit, Naro Moru, Nanyuki, Timau areas of Laikipia District. The sera were stored at -20°C. until used for CFT. All the sera were inactivated for 30 minutes at 56°C. and diluted in ten steps from 1:10 to 1:160.

(e) Antigens: Antigens for complement fixation tests came from three sources. The first was the Chlamydial group antigen, C. psittaci (PAGE, 1968) obtained from Dr. L.A. Page (of U.S.A.D.A., ARS., NADL, Ames, Iowa) through the services of Prof. L.H. Lauerman, at the time a member of the Veterinary Faculty, Kabete. It was diluted 1:512 before use. The second antigen was prepared from yolk sacs of embryos infected with W62 and dying between the 3rd and 5th day after infection. The third antigen, (control) was prepared from yolk sacs of uninfected chicken embryos killed at the 12th day of incubation.

The second and third antigens were prepared as follows:

The yolk sacs of each set were ground in a pestle and mortar with sand and buffered Hanks' BSS (pH 7.0) to make 25% suspensions. The suspension of each was boiled for 30 minutes, cooled and aqueous 5% phenol added to make a final concentration of 0.5% phenol. The

suspensions were freeze-dried and stored at 4°C. until required for tests. For the complement fixation reaction the two antigens were further diluted 1:30.

(f) Complement: Guinea pigs from our own laboratory animals' colony were used to provide normal serum for complement. The complement used had a titre of more than 1:100. Units of $2\frac{1}{2}$ - 3 of complement were used throughout the tests.

(g) Complement Fixation Tests: These were carried out using a modified method described by FULTON and DUMBELL (1949). Perspex agglutination trays with 8 cavities were used. The reagents: antigen, test sera and complement were mixed in small amounts of drops. The mixtures were kept in a cold room at 4°C. overnight. Next day, drops of the indicator system were added and the mixtures again incubated at 37°C. for 2 hours. All the tests were kept in wet chambers throughout the procedure. The tests were read by placing the agglutination trays flat on a white background with a strong light overhead.

(3) Results: The outcome of complement fixation reactions to serum samples from several sheep flocks in Kenya are tabulated in TABLES 9, 10 and 11.

TABLE 9: Complement Fixation Titres of Sera from Sheep of Transmission Experiment 2 to Chlamydial Group Antigen (C. psittaci)

Sheep No.	Normal Serum	Serum Samples Collected after Inoculation at days									Comment
		65	151	168	178	195	210	224	238	260	
3605	1:10	1:20	-	-	-	-	-	-	-	-	Died of fibnous pneumonia after 120 days: small SPA foci.
3608	1:40	*	1:20	1:40	1:80	1:40	1:40	-	-	-	Died after 221 days; SPA.
3613	0	1:40	0	1:10	1:20	1:10	*	1:40	*	*	Killed after 260 days:SPA
3615	1:20	1:80	1:40	1:160	1:160	-	-	-	-	-	Killed after 178 days; SPA
3616	1:40	1:80	1:40	1:80	1:80	-	-	-	-	-	Killed after 175 days:SPA
3617	1:40	1:40	1:10	1:40	1:40	1:20	*	1:160	1:10	1:80	Killed after 260 days:SPA
3633	0	*	0	1:10	0	-	-	-	-	-	Died after 181 days: No SPA
3645	1:80	1:80	1:40	1:40	1:160	1:80	0	0	-	-	Killed after 228 days:SPA
3648	1:40	1:80	1:80	1:80	1:160	1:160	1:160	1:160	1:80	*	Killed after 260 days:SPA
3711	1:20	1:40	1:40	1:20	1:80	1:80	1:40	0	1:40	1:40	Killed after 260 days: No SPA.

* - Serum sample haemolysed and hence unavailable for the test.

TABLE 10: Complement Fixation Titres of Sheep Sera from Clinical SPA Cases (ex-Farm D) to Chlamydial Group Antigen (*C. psittaci*).

Year of Collection	Serum No.	Titre
1966	276/66	0
	280/66	1:20
	475/66	1:10
	988/66	1:10
	989/66	1:10
	1005/66	0
	1058/66	1:40
1967	W_1	1:40
	W_2	1:80
	W_3	1:40
1968	W8	1:80
	W9	1:40
	W10	1:20
	W11	1:40
	W12	1:20
	W13	1:20
	W14	1:10
1969	WI	1:40
	WII	1:160
	WIII	1:80
	WIV	1:40
	WV	1:10

TABLE 11: Complement Fixation Titres of Sheep Sera to Chlamydial Group Antigen (C. psittaci).

Farm	Area	Breed	SPA Status	Serum Dilutions 0	10	20	40	80	160	Total
A	Mau Narok	R.M.	Absent	0	1	1	4	10	1* 9	26
B	Timau	M/C	"	3	3	9	12	4	1	32
C	Timau	M	Endemic	0	3	5	8	5	1*	22
D	Timau	M/C	Endemic	0	1	0	1	7	1* 4	14
E	Timau	M	?	1	0	3	11	13	3	35
F	Molo	R.M.	Absent	1	0	1	0	0	4* 3	9
G	Molo	R.M.	Absent	1	3	7	8	0	1	20
H	Molo	R.M.	Absent	0	4	5	17	3	3	32
I	Naro Moru	M	?	0	0	0	3	3	2* 7	15
J	Eldoret	R.M.	?	0	0	1	12	7	2* 6	28
K	Mau Summit	C	Absent	0	0	0	1	3	5	9
L	Ngobit	M	?	2	1	6	7	7	0	23
M	Naivasha	M	Endemic	2	6	6	2	0	0	16
N	Kabete	I/C	Absent	0	0	6	9	4	2	21
			TOTAL =	10	22	50	95	66	59	302

KEY:
C - Corriedale
I/C - Indigenous and Corriedale
M - Merino
R.M. - Romney Marsh
? - SPA Status unknown.
* - Titre more than 1/160.

Five of the experimental sheep in TABLE 9 had antibody titres of 1:40 to 1:80 in their normal sera. Though there was a rise in titre in case of sheep 3648 with clinical SPA, other sheep with the disease showed variable results. In TABLE 10, out of 22 sera from sheep with natural clinical SPA, only sheep W_2, W8, WII and WIII had antibody titres of 1:80 and over. Sera of two sheep had no titres.

Sera from apparently normal sheep collected from various parts of Kenya revealed an interesting picture. As shown in TABLE 11, 125 out of 302 samples had titres against chlamydial antigen of between 1:80 and over 1:160. Though the samples were few and some were collected from farms known to be endemic for SPA, there is an indication that many of the sheep flocks in Kenya are infected with chlamydial agents.

The sheep with the natural disease shown in TABLE 10 were obtained from Farm D of TABLE 11. The fact that sera from some of the SPA natural cases had low or no titres at all against chlamydial agents indicates that they do not participate in causing the adenomatous lesions of SPA.

Complement fixation reaction using antigen from W62 had comparable results to that of C. psittaci. The results with the control antigen occasionally had titres of 1:40 or less. This shows that

titres shown with the first 2 antigens were not due to egg material as such. Electron microscopic examination of chorioallantoic membranes from embryos infected with W62 revealed particles similar to those of Chlamydiae.

IV. CELL CULTURES FROM SPA LUNGS.

(1) Introduction: MACKAY (1969) observed a cytopathic effect characterized by the development of intranuclear inclusions in alveolar macrophages cultured directly from sheep lungs affected with SPA. The presence of inclusions was sporadic and found in 14 of the 24 cases. He serially transmitted the agent causing the cytopathic effect in alveolar macrophage cultures from normal sheep lungs.

The purpose of this section is to describe attempts made at repeating MACKAY'S (1969) work.

(2) Materials and Methods: Materials used were SPA lungs obtained from four sheep (WI, WII, WIII, WIV) which had the clinical disease. The sheep were from the same flock (Farm D, TABLE 11) as those used to supply infective material for studies with chicken embryos and the Transmission Experiments 1 and 2 of Chapter 4. Lung tissues of these sheep were also used for electron microscopy in Chapter 5.

The tissue culture medium consisted of Eagle's medium in Hanks' BSS with 10% tryptose phosphate broth (Difco) and 10% uninactivated horse serum. Antibiotics were added to a final concentration of 20 units penicillin G, 50 ug. of streptomycin and 200 ug. kanamycin per c.c.

The method for preparing cell cultures both for alveolar macrophages and mixed cell cultures was a modification of that described by MACKAY (1969). For macrophage cultures, one SPA lung each in the first 2 sheep was tied off and the other one filled with Hanks' BSS via the trachea. The opening was stoppered and the filled lung massaged gently for about 5 minutes. The fluid was then poured into a sterile container and centrifuged at 400 r.p.m. for 10 minutes. The cells were washed once with Hanks' BSS following sedimentation and then diluted so as to form a 1.25% suspension with the medium. Each lung yielded 80-100ml. of cells.

For mixed tissue cultures, affected parts of the tied-off lungs from the first two sheep and from WIII and WIV were collected soon after killing the animals. The specimens were chopped into small pieces about 5 cubic mm. and placed in conical flasks. They were immersed in 10 times their volume in a mixture of equal parts 3.7% trypsin and 1.6% pancreatin at 37^{o}C. and gently agitated for 7 to 10 minutes. The supernatant containing dispersed cells was

next decanted into 200cc. centrifuge bottles placed in an ice-bath containing 20cc. of horse serum to inhibit further enzyme action. The remaining fragments were submitted to further similar cycles of enzyme exposure for gradually shorter times. The supernatants were pooled with those of the first run, filtered through 2 layers of sterile gauze and centrifuged at 100g. at 0°C. for 20 minutes.

The sedimented cells were resuspended in the tissue culture medium and sown at a concentration of $5x10^5$ per cc. into test tubes containing flying coverslips. The test tubes were stoppered immediately, incubated in stationary racks at 37°C. Whenever the pH fell below about 6.8, the growth medium was renewed. Ten to twenty coverslip slides were made for macrophage and mixed cell cultures from each sheep lung and routinely stained with Giemsa.

(3) Results:

(a) Macrophage Cultures: These were set up from alveolar macrophages of sheep WI and WII. Slides were prepared from the 5th to the 8th day. They showed a very high proportion of macrophages. These cells were generally large and rounded. Some had vacuolated cytoplasm. Their nuclei were usually eccentrically located. The majority of the cells, and especially in cases which also had neutrophils, showed many phagocytised nuclear remains or even whole dead neutrophils.

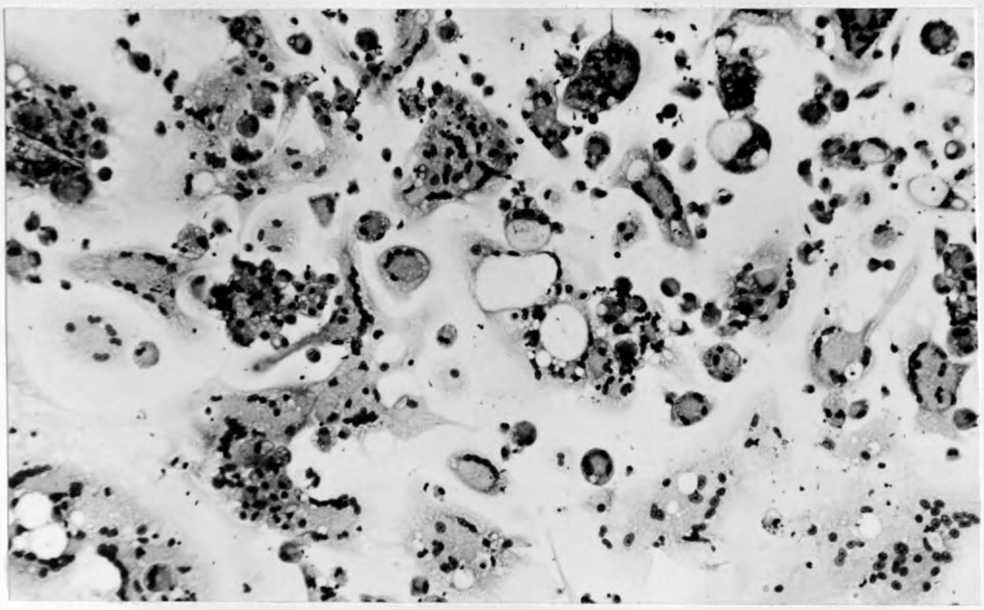


Fig.3: From mixed culture, showing macrophages and giant cells, no evidence of intracellular inclusion bodies. H & E stain. X 125.

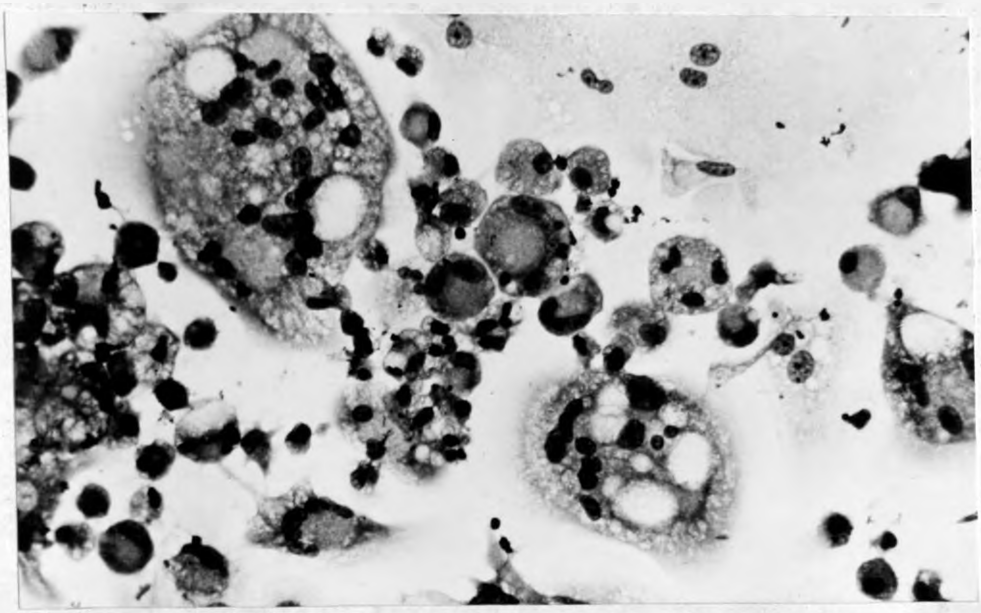


Fig.4: Higher magnification of Figure 3. H & E stain. X 315.

V. DISCUSSION.

Results obtained so far demonstrate that adenomatous lung material passaged in embryonating eggs kills embryos within 4 to 5 days after infection. This was especially evident in sheep W_1, W_2 and W62, indicating that lung material from cases of SPA contain infective agent(s) capable of causing lesions in chicken embryos.

The fact that lung tumours and secretions of 4 sheep with typical SPA yielded no Mycoplasma does point to possible non-participation of these microorganisms in the causation of this sheep disease. While all isolated Mycoplasma could be propagated in embryonating eggs those that killed the embryos did so very sporadically. Their growth in cell cultures of chicken embryo fibroblasts, kidneys, and sheep lung macrophages produced no cytopathic effect. This contrasts the claim of MACKAY (1966) which related the cytopathic effect to the action of Mycoplasma. He had observed plaque formation which resembled a net curtain. GREIG (1955) who isolated Mycoplasma from nasal secretions of sheep failed to isolate the same organisms from the lungs of some of the animals when killed. Pneumonic lungs particularly those associated with Pasteurella spp. infection yielded Mycoplasma much more readily. However not all his pneumonic lung material of the sheep had Mycoplasma on culturing.

It is apparent in our case that in addition to the Mycoplasma, there were other agents in SPA material responsible for the pathological changes seen in dead and some of the killed embryos. The agent(s) could be viral in nature.

The Mycoplasma isolated in our study could be classified into three serogroups, whose pathogenicity is not yet known. But as shown under Transmission Experiment 4, Chapter 4 the encountered isolates are of doubtful pathogenicity for sheep. They may be opportunists that merely take advantage of the oedematous conditions created by the adenomatous lesions to proliferate, rather than being directly involved in the causation of the adenomatous process.

The significance of Chlamydiae in the causation of SPA has not been determined. The fact that SHIRLAW (1962) found intracytoplasmic inclusions resembling those of Chlamydiae in smears made from lamb pneumonia due to Pasteurella spp., and that antibody titres were found in flocks of sheep free of SPA suggest that Chlamydiae are widely spread in some sheep flocks in Kenya. They are unlikely to play a pathogenic role with respect to SPA. Hence while Chlamydiae are capable of killing embryonating eggs and even causing grossly visible lesions, it would appear that sample W62 contained another infectious agent in addition to a Chlamydia.

This other agent may be the one that causes the neoplastic process characterizing SPA. SHIRLAW (1956) used this agent at the 56th passage to reproduce SPA lesions in sheep. It passes through a Millipore filter of 450 mμ. diameter. Though Mycoplasma can pass through such a filter, they do not appear to play a pathogenic role in SPA. SIGURDSSON (1958) discovered that the agent causing SPA could remain viable for over 4 years in the deep freeze.

Failure of the adenomatous cells in the mixed tissue cultures to grow may indicate that the tumour tissue in the three sheep were not actively proliferating. On the other hand the macrophages of the mixed cell cultures were much more active than those of the macrophage cultures alone. These active macrophages as shown in Figure 3 formed numerous syncytia or multinucleate cells. Some of them had phagocytised material. The giant cell formation could be a cytopathic effect indicating the presence of some virus in the cultures.

Many of the phagocytised nuclear material simulate cytoplasmic inclusions at a casual observation. Indeed as JENNINGS (1967) stated, many structures might be found in cells which resemble viral inclusions and which may cause confusion. No intranuclear

inclusions nor other significant changes were observed in the cultured cells or in Giemsa-stained smears made from the cut surface of the adenomatous tissue.

The intranuclear inclusions encountered by MACKAY (1969) indicating a herpes virus and further investigated (SMITH and MACKAY 1969) by electron microscopy, may not be the agent causing pulmonary adenomatosis of sheep. The inclusions were found in macrophages in 14 of the 24 investigated cases. No intracellular inclusions were found in the adenomatous cells. As shown in Chapter 5 of this thesis no inclusions were seen in the adenomatous cells of investigated cases.

CHAPTER 4

TRANSMISSION EXPERIMENTS

A. MATERIALS AND METHODS.

I. EXPERIMENTAL ANIMALS:

Specific Pathogen Free (SPF) sheep were not available in this part of the world and hence could not be used. Day-old colostrum fed or deprived lambs of the right type could not be obtained in sufficient numbers. It was decided to obtain experimental animals from areas that fulfilled certain minimum requirements. The animals were 32 Corriedale and 4 other sheep. The Corriedales were 2 ewes and 30 lambs. The latter's age was between 4 and 15 months. They came from Molo area of Nakuru District in the Rift Valley Province of Kenya from a farm where no cases of pulmonary adenomatosis have been diagnosed since the farm was established, 46 years ago. The other sheep were black-faced Somali type from the Northern part of Kenya where there has been no definite evidence of SPA. Their ages ranged from 6-24 months. The sheep were de-wormed with Thiabendazole immediately they were brought to the Faculty of Veterinary Science at Kabete.

II. DONOR (INFECTIVE) MATERIALS.

One farm, in Timau area of Laikipia District of Kenya (Farm D, TABLE 11) on which SPA was endemic but on which no cases of Maedi have been encountered by the author after examining well over 300 cases of SPA in 2 years, supplied the donor animals. For Experiment 1, the donor, 67/W/1, was one of a group of 30 lambs, 5-6 months old which were being used for fluorosis tests by the Animal Production Department of the Faculty of Veterinary Science. They had previously been acquired from Farm D a month earlier. The donor lamb had clinical signs and lesions of SPA. Infective material 67/W/5 (designated W_1) for Experiment 2 also came from the above original source. The animal was in a group of 32 sheep of which 27 had clinical SPA. Egg-propagated infective material of the 5th and 15th passages of W_1 (W_1/5/38 and W_1/15/38) provided the inoculum for Experiment 3. Fluid containing mixed Mycoplasma isolates was used for Experiment 4.

III. EXPERIMENTS PERFORMED.

Experiment 1: Transmission with Naturally Infected Material.

The object of this experiment was to attempt to produce lung lesions of SPA. The inocula was administered intratracheally or

intrathoracically. For the preparation of the infective inocula, 53.5 gm. of representative SPA lung material, 67/W/1, obtained by sterile methods were weighed out, transferred, and minced finely in a sterile mortar. With sterile sand, the mixture was finely ground into an emulsion using a pestle and mortar. This emulsion was made up to 225 ml. (1:4) with PBS containing antibiotics - 100 I.U. penicillin, 100 µg. streptomycin and 100 µg. kanamycin per 1 ml.

The content was centrifuged at about 2,500 r.p.m. for 30 minutes at +4°C. The supernatant was collected and kept in the refrigerator at +4°C. for 2 hours before use. Ten millilitres of the infective inocula were administered to each of the eight sheep while they were held in a normal standing position. Ten millilitres of PBS containing antibiotics was given intratracheally to each of the 4 control sheep. Temperatures were recorded twice daily for 5 months and then discontinued.

Experiment 2: Repeat Transmission with Naturally Infected Material.

This was a repeat of the above experiment with the purpose of reproducing the clinical disease using differently prepared filtered or unfiltered infective inocula administered intratracheally. The inoculum, 67/W/5, was the same as W_1 passaged in eggs in Chapter 3.

Twenty five millilitres of the supernatant were used for animal trials. Five Corriedale lambs whose normal respirations

had previously been checked for 28 days, were each inoculated with 5 ml. intratracheally. The rest of the supernatant was filtered through a 1.2 μ Gelman filter. This was to get rid of cells, large viruses and any large subcellular structures. It was diluted, 1:2 and 10 ml. of it was inoculated into 5 more Corriedale lambs intratracheally. The 4 control animals were each inoculated intratracheally with 5 ml. of Eagle's medium.

Temperatures were recorded daily at 8 a.m. and 4 p.m. for 3 months, and thereafter in animals that appeared sick. The 10 lambs were bled, for sera before inoculation, then at monthly interval for 4 months and thereafter at 2-week intervals until the experiment was terminated. Those sacrificed were also bled at the time of killing. These sera were kept for serological investigations (Chapter 3). The sheep were examined clinically at each time of bleeding and a record of their respirations, cough (if any) and watery discharge from the nostrils on lifting up the hind legs was made. The infective inoculum was checked for bacteria by culturing on plates of McConkey and blood agar and later in Newing's Tryptose Broth and Tryptose Serum Agar for presence of Mycoplasma spp. The lungs of each sheep were checked for bacteria at slaughter, if this seemed warranted.

Experiment 3: Transmission with Egg-passaged Material.

The purpose of this experiment was to infect animals with SPA material passaged in embryonating chicken eggs. The material (allantoic and amniotic fluid) was filtered through 0.22 μ Millipore filters. Five millilitres were administered intratracheally to each of 8 sheep containing approximately 2×10^6 ELD 50%. The animals were bled for sera at intervals, monthly at first and later at about two weeks. Clinical examination was carried out at the time of bleeding. Temperatures were recorded twice daily for one month.

Experiment 4: Transmission with Mycoplasma-containing Material.

A mixture of Mycoplasma spp. (W_1, W_2, W6/1, W6/2) isolated from adenomatous lungs during the aetiological studies (Chapter 3) were put back into 5 sheep. Five millilitres of the mixture were administered intratracheally to each of 2 ewes and 3, 15-month old wethers.

The mixed culture was 24 hours old. W_1 and W_2 were from natural cases, the former being from the original material of Transmission Experiment 2. W6/1 and W6/2 were from Experiment 2, sheep 3645 with pulmonary adenomatosis. Blood was taken from them before inoculation and at 2-weekly intervals after inoculation. Temperatures were read at 8 a.m. and 4 p.m. each day for a month.

IV. NECROPSY PROCEDURE: COLLECTION AND SPECIMEN PREPARATION.

Sheep were killed by stunning with a humane killer and cutting through one of the brachial arteries. In this way they bled to death without blood aspiration. Post-mortem examination was carried out with the sheep on their backs. Following skinning, the ventral thoracic and abdominal walls were removed to expose visceral organs. Portions of lung with typical SPA lesions were removed and some stored at -70°C. for aetiological studies.

Lungs were then carefully removed and examined for gross lesions, Selected ones were photographed and their lesion distribution recorded on a diagram of the lung outline. Representative specimens from all 7 lobes, cut roughly at 1cc. each were fixed in 10% buffered formal saline. In lobes that had gross lesions, at least three specimens covering portions of complete consolidation, intermediate, and apparently normal lung were taken. The tracheobronchial and mediastinal lymph nodes were routinely preserved. All the fixed specimens were trimmed to about 3-5mm. thick, processed by the paraffin method and sectioned at 5-6 microns. All were stained with haematoxylin and eosin. Selected lung sections were also stained with Lendrum's and May-Grunwald-Giemsa stains for possible inclusions, and with Periodic-acid

Schiff (P.A.S.) to identify the mucin-producing capabilities of the adenomatous cells. Several serial sections were made from each paraffin block. This procedure was used both for experimental sheep and sheep from natural SPA.

B. RESULTS

The data on 4 transmission experiments are summarized in TABLES 12, 13, 14 and 15. Three out of eight sheep in experiment 1 and 8/10 of the sheep in experiment 2 developed pathological lesions of the disease. Only those sheep with significant lung lesions are considered in some detail.

I. Experiment 1. This is summarized in TABLE 12.

(a) Post-inoculation observations:

Record of rectal temperature for 159 days revealed no abnormal change, and ranged between 38-39.4°C. (100-103°F.). However, sheep 3621 showed distinct pyrexia (40.6-41.7°C. or 105-107°F.) about 48 hours before death. Poor quality of hay despite supplementary feeding with bran and dairy cubes led to nutritional oedema in some sheep.

(b) Pathology:

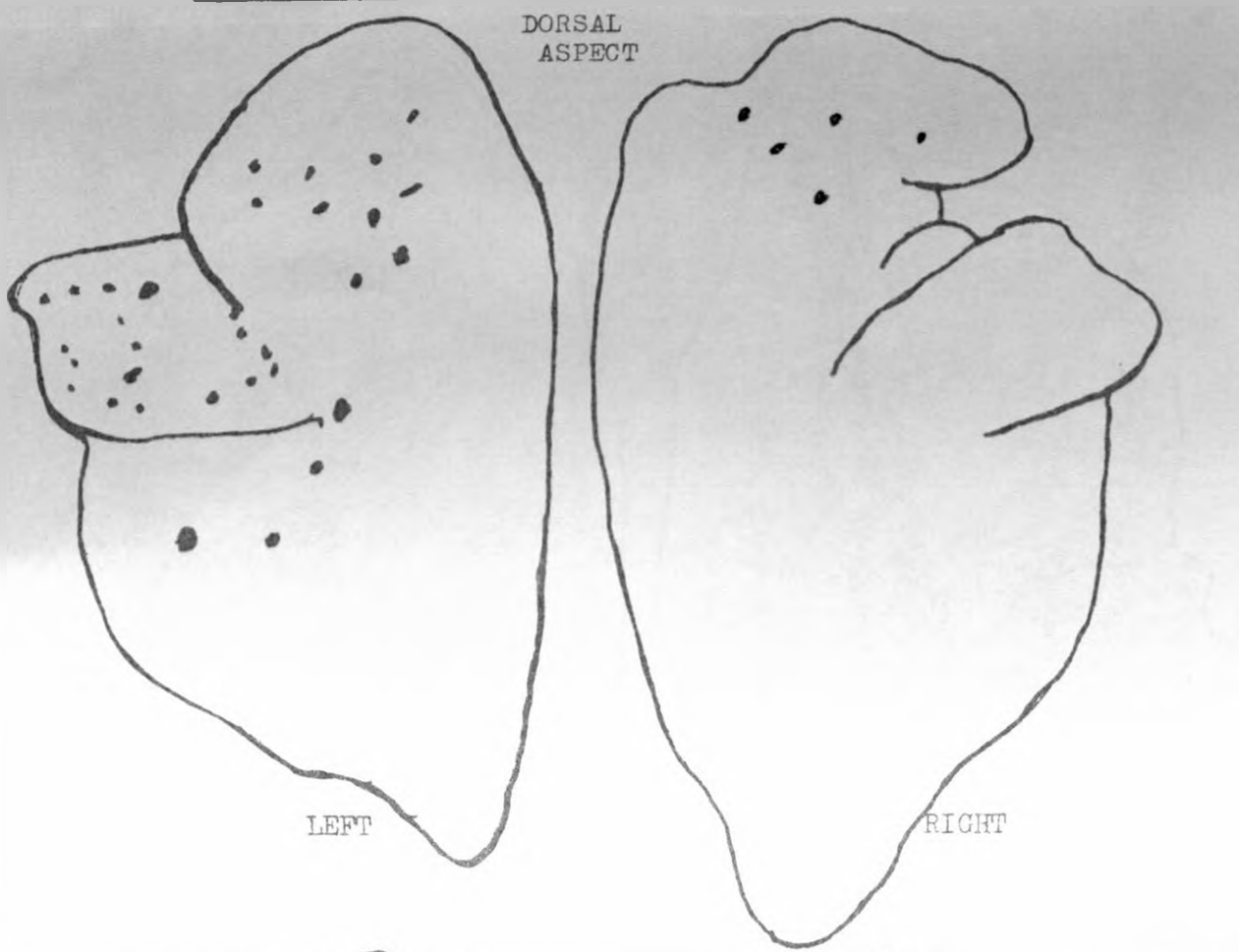
Gross and microscopic lung tissue examinations of sheep 3603, 3612, 3640 and 3644 and, control sheep 3607, 3619, 3646, 3654

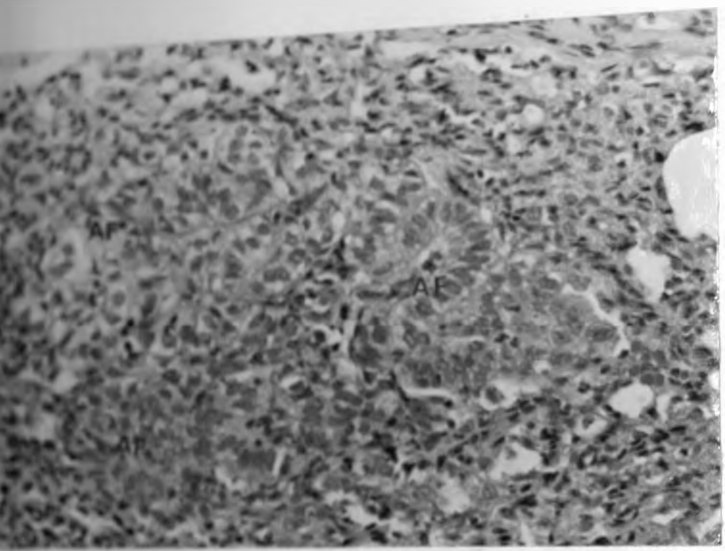
after 311 days of observation revealed no pulmonary changes.
ological changes were seen in any organ.

1631:

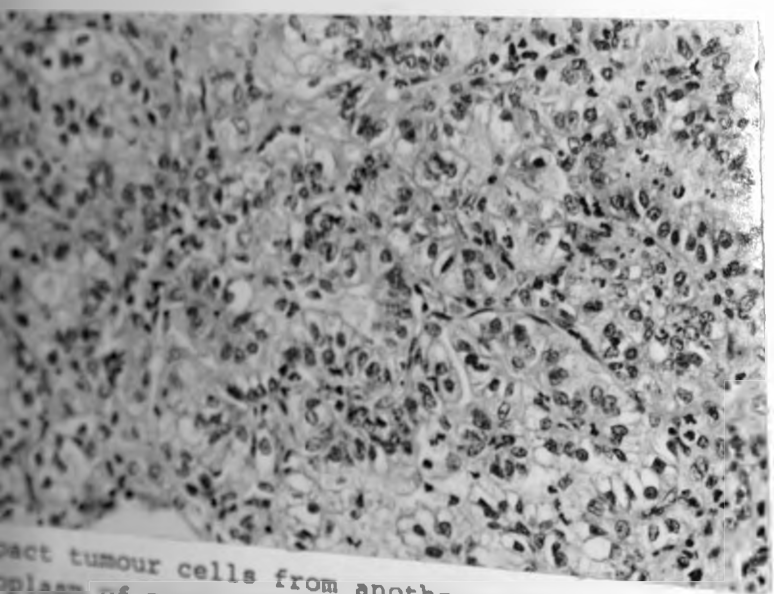
is animal killed, in extremis, 65 days after inoculation,
y emaciated. There was generalised nutritional oedema.
cent gray to grayish-white foci (Fig.5) were visible in the
and cardiac lobes of the left lung, the largest being about
diameter. A few foci were located in the right apical lobe.
ges were seen in the pulmonary lymph nodes. Histologically
ere many areas of interalveolar wall thickening. This was
congestion, oedema and infiltration with mononuclear cells
teralveolar septa. There was as shown in Figures 6-8 sca-
foci of metaplastic, proliferative alveolar epithelium.
lls were cuboidal and/or columnar in type. A few foci were
d in the "centres" of thickened interalveolar septa while
t proliferated from a number of transformed alveolar epithe-
They grew into alveolar spaces in a papillary manner suppor-
loose connective tissue. Lymphoid hyperplasia was in-
icant, and macrophage response absent. No changes were
n the lymph nodes.

Fig: 5. Sketch of Lesion Distribution, Sheep No.3631.





3631. Adenomatous foci (AF) from atelectatic
oil. H. & E stain. X 315.



pact tumour cells from another area of sheep 3631.
plasm of many cells is vacuolated. H & E stain. X 315.

Sheep 3632:

This animal was killed in extremis, 80 days after inoculation. There were no distinct gross lesions in the lungs or other organs apart from generalised nutritional oedema. Microscopic lesions of interalveolar wall thickening due to congestion and infiltration with mononuclear cells were evident. No adenomatous foci of sheep pulmonary adenomatosis were seen.

Sheep 3621:

This animal which died 118 days after inoculation following pyrexia had fibrinous pleurisy and pneumonia. Fibrin deposits were evident on the anterior lobes on either side. Both cardiac and intermediate lobes were completely consolidated. The two apical lobes were consolidated up to 80% of their mass. Small portions of the diaphragmatic lobes in the antero-ventral aspects were also pneumonic. The lesions were dull greyish red, firm and oedematous. Translucent grayish-white foci 1-2mm. in diameter were found scattered in the pneumonic lesions and on their periphery. The rest of the diaphragmatic lobes had compensatory alveolar emphysema. The associated lymph nodes were hyperaemic. Pasteurella haemolytica was isolated in pure culture from the pneumonic lungs.

Microscopic examination revealed fibrin deposition on the pleura, congestion and serofibrinous exudate in the alveoli.

Neutrophils, macrophages and other mononuclear cells were found in the alveoli. Bluish staining bacterial colonies were evident. Adenomatous foci of SPA were found scattered in areas of fibrinous pneumonia. But there were other sections with fibrinous pneumonia and without SPA lesions.

Sheep 3642:

This animal killed in extremis 251 days after inoculation, showed emaciation and generalized nutritional oedema. A few small whitish gray foci approximately 1-2mm. in diameter were found in the apical and cardiac lobes on either side. No other lesions were grossly evident. Microscopically, lesions of interalveolar wall thickening and adenomatous proliferations were present. Some adenomatous foci were located in "centres" of thickened interalveolar septa. There was also intrabronchiolar hyperplasia and moderate lympho-reticular hyperplasia in some sections. Macrophage response was very slight.

II. Experiment 2:

(a) Clinical Findings:

Record of rectal temperature generally revealed no abnormal change, the temperature rarely reaching 103°F. However, sheep 3605 showed distinct pyrexia (40.6-41°C. or 105.4-106°F) about

48 hours before death 120 days after inoculation. Sheep 3613 and 3615 at day 168 after infection had temperatures of 40.6°C. (105°F.) and 41.1°C. (106°F.) respectively. Antibiotic treatment reduced the temperature to normal.

All the sheep were rather emaciated but this cannot all be attributed to the poor hay used, as only 1 sheep had nutritional oedema. As tabulated in APPENDIX A, clinical signs exhibited by 7 sheep became increasingly evident with time. These were dyspnoea, increased respiration rate, marked nostril dilation, watery discharge from the lungs oozing out through the nostrils on lifting up the hind legs and moist rales. Coughing was occasional. The amount of lung discharges in individual animals varied considerably when checked every fortnight.

Sheep 3645 had no cough but produced a profuse watery discharge from the lungs 151 days after infection. Fifty four millilitres of fluid were collected in one minute. Increased respiratory rates were first shown by sheep 3616 and 3645 (40 and 43 respectively) 107 days after inoculation. Sheep 3616 had 59 and sheep 3613 had 63 respirations per minute at rest 168 days after infection but no cough or lung discharge. The normal respiration rate in this group of sheep ranged from 22 to 28. For practical

purposes, respiratory rate below 30 was considered normal, and that of 35 or more respirations per minute as indicating disease. Gurgling sounds indicative of moist rales were detected in sheep 3608, 3615, 3616 and 3645 at certain times. No clinical changes were observed in the controls and in sheep 3633 and 3711.

(b) Bacteriology:

Filtered inoculum was found bacteriologically sterile. The unfiltered sample showed a few colonies of Escherichia coli. The lungs of sheep 3605, 3608 and 3615 which, in addition to pulmonary adenomatosis lesions, had varying degrees of a fibrinous pneumonia and Pasteurella haemolytica was isolated in pure culture. Both the original lung material 67/W/5 and the adenomatous lungs of sheep 3645 yielded Mycoplasma but these organisms were not found in the remains of the inoculum. attempts were made (Chapter 3) to elucidate some of the properties of these and other Mycoplasma species.

(c) Pathology:

The control sheep, 3653, 3717, 3724, 3814, killed after 290 days and sheep 3633 and 3711 were somewhat emaciated but had no gross or histological lesions in their lungs. However sheep 3633 died of severe nutritional deficiency. TABLE 13 gives a summary of this experiment while TABLE 14 records the distribution and extent

of adenomatous lesions in lung lobes of 8 sheep. The lungs were generally increased both in size and weight.

Macroscopic Lesions:

Sheep 3605: The anterior lobes and antero-ventral aspects of the diaphragmatic lobes had red consolidation. The remaining portions of the diaphragmatic lobes were emphysematous. Both in the consolidated and emphysematous parts, except for the right apical lobe, there were distinct translucent grayish to grayish-red nodules typical of pulmonary adenomatosis. They were irregularly rounded, translucent and glandular. The largest nodules were about 5mm. in diameter. A fibrinous pleurisy was evident. Lungs of sheep 3608 and 3615 (Figures 8 & 9) had in addition to lesions of pulmonary adenomatosis, areas of red consolidation and varying degrees of fibrin deposition. The anterior lobes of the lungs in each case and the antero-ventral aspects of the diaphragmatic lobes showed the adenomatous lesions. The rest of the lungs had alveolar emphysema.

Sheep 3616: (Figures 10 & 11).

The anterior lobes as well as the antero-ventral aspects of the diaphragmatic lobes on both sides were transformed into fleshy gland-like tissue in this sheep killed in extremis 175 days after infection. The changed tissue was firm, translucent, grayish-white

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TABLE 12: Summary of Transmission Experiment 1

Sheep	Age in Months	Route of Inoc.	Inocula Amount	Killed after days	Results
3603	4	Intratracheal	10ml.	255	No lesions
3642	4	"	"	251	SPA
3631	5	"	"	65	SPA;Nutritional oedema.
3621	4	"	"	118*	SPA; with F/P.**
3632	5	Intrathoracic	"	80	Thickening interalveolar septa; Nutritional oedema
3612	5	"	"	255	No lesions
3644	4	"	"	255	No lesions
3640	5	"	"	255	No lesions

TABLE 13: Summary of Transmission Experiment 2 using Intratracheal Route.

Sheep	Age in Months	Inocula	Inocula Amount	Killed after days	Results
3613	6	Unfiltered	5ml.	260	SPA
3616	5	"	"	175	SPA
3617	5	"	"	225	SPA
3633	6	"	"	181*	No SPA;Nutrional oedema.
3648	5	"	"	260	SPA
3605	5	Filtered	10ml.	120*	SPA with F/P.**
3608	5	"	"	221*	SPA with F/P.**
3615	5	"	"	178	SPA
3645	6	"	"	228	SPA
3711	5	"	"	260	No lesions.

* - Died

** F/P - Fibrinous pneumonia.

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3632	5	Intrathoracic	"	80	Thickening interalveolar septa; Nutritional oedema
3612	5	"	"	255	No lesions
3644	4	"	"	255	No lesions
3640	5	"	"	255	No lesions

TABLE 13: Summary of Transmission Experiment 2 using Intratracheal Route.

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3613	6	Unfiltered	5ml.	260	SPA
3616	5	"	"	175	SPA
3617	5	"	"	225	SPA
3633	6	"	"	181*	No SPA;Nutrional oedema.
3648	5	"	"	260	SPA
3605	5	Filtered	10ml.	120*	SPA with F/P.**
3608	5	"	"	221*	SPA with F/P.**
3615	5	"	"	178	SPA
3645	6	"	"	228	SPA
3711	5	"	"	260	No lesions.

* - Died

** F/P - Fibrinous pneumonia.

TABLE 14: Experiment 2: Lung Lesion Distribution and Percentage in 8 Sheep.

	Left Lung		- LUNG LOBES -			Right Lung	
Sheep	Apical	Cardiac	Diaphrag.	Apical	Cardiac	Diaph.	Inter.
3605*	1	2	1	-	1	1	2
3608*	3	3	1	2	2	-	2
3613	1	3	1	1	-	-	-
3615	4	4	1	2	3	1	4
3616	2	3	2	4	3	1	3
3617	4	4	1	-	3	1	3
3645	2	3	2	2	3	1	2
3648	3	3	1	3	4	3	4

* - Died

1 - 1 to 25% of lobe mass

2 - 26 to 50% " " "

3 - 51 to 75% " " "

4 - 76 to 100% of " "

Fig.8: Sheep 3615 - Dorsal view showing adenomatous lesion involving the anterior parts of the lungs mainly.

Fig.9: Sheep 3615 - Closer-view of the dorsal aspect of the left lung. Fibrinous strands (FS) can be observed.

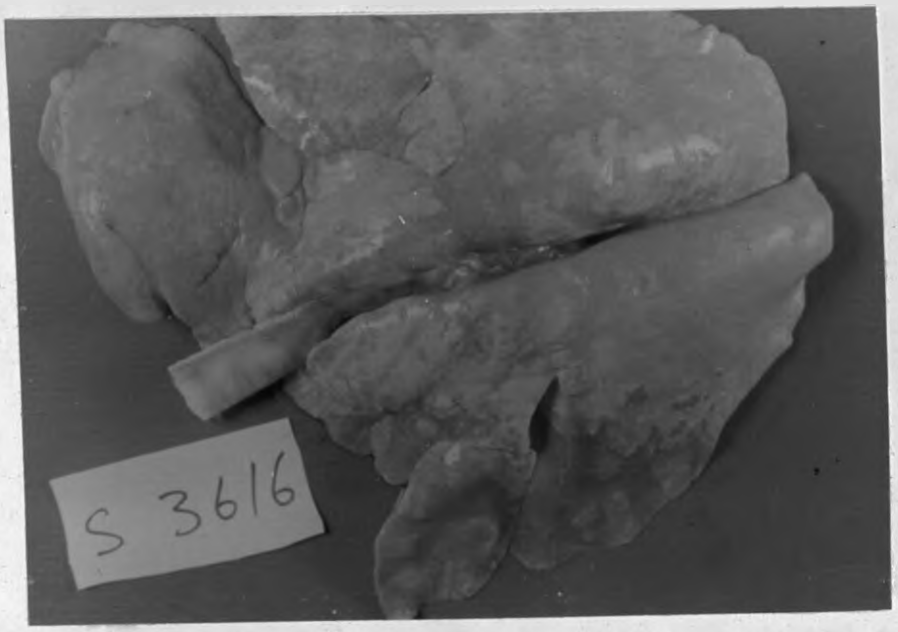

Fig.10: Sheep 3616 - Most of the right apical and cardiac lobes have been transformed into glandular tissue.
adenomatous

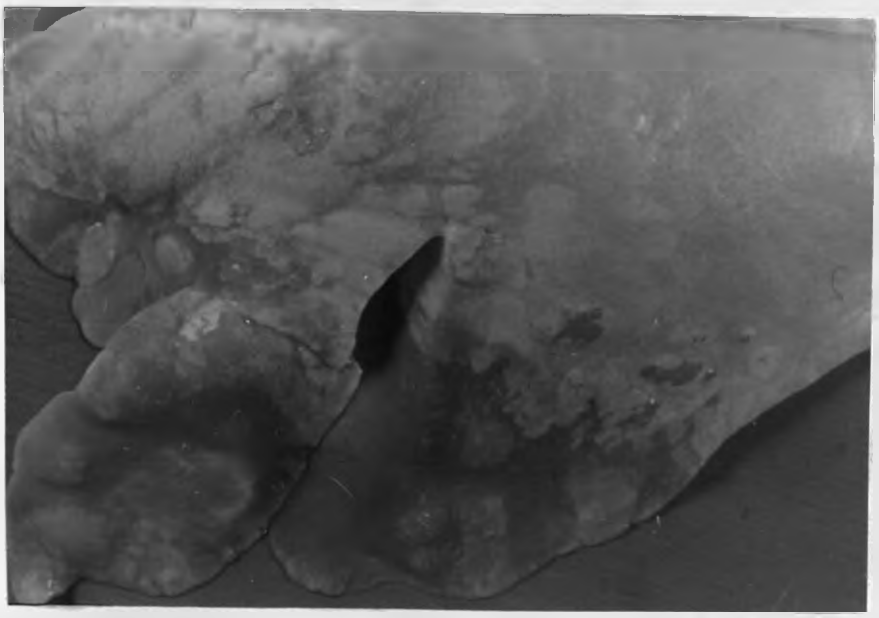

Fig.11: Closer-view of left lung of sheep 3616.

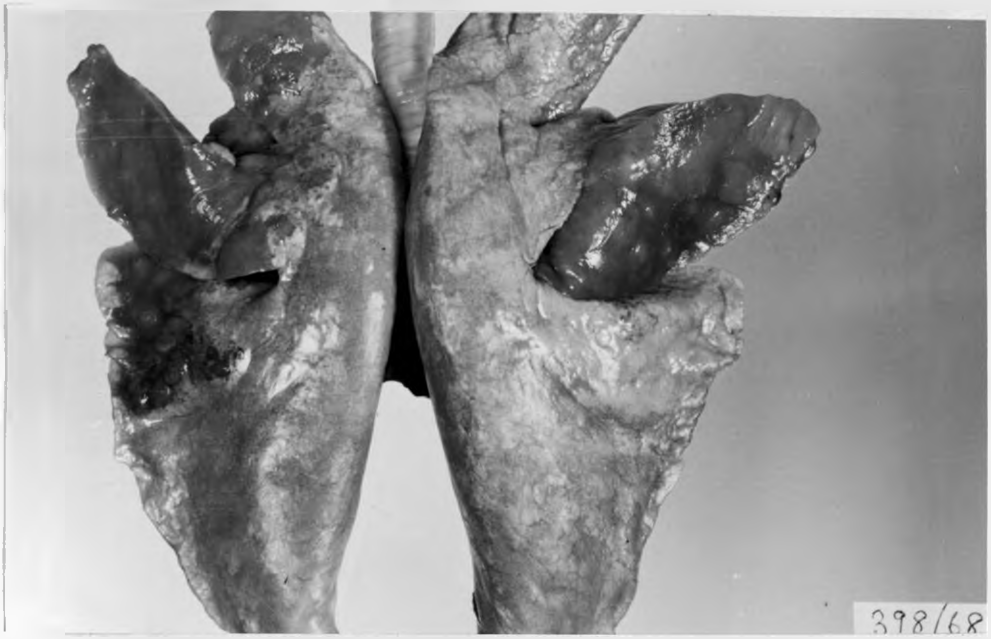

Fig.12: Sheep 3617 - Dorsal surface showing the spread of the adenomatous lesion.

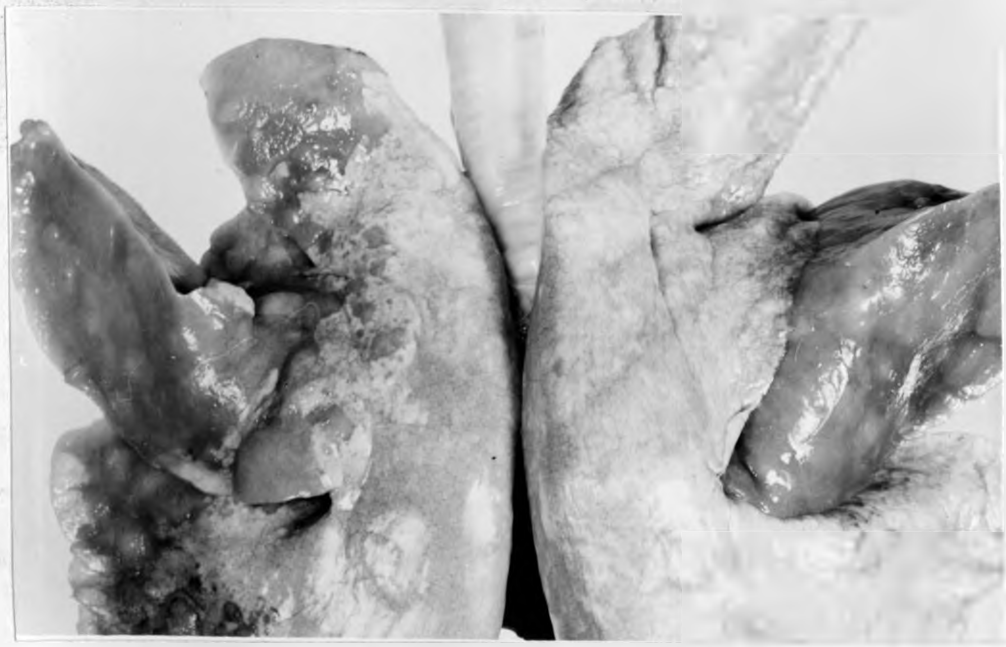

Fig.13: Closer-view of sheep 3617. Minute foci can be observed.

Fig.14: Sheep 3617. The ventral view - the right apical lobe showed no SPA foci.

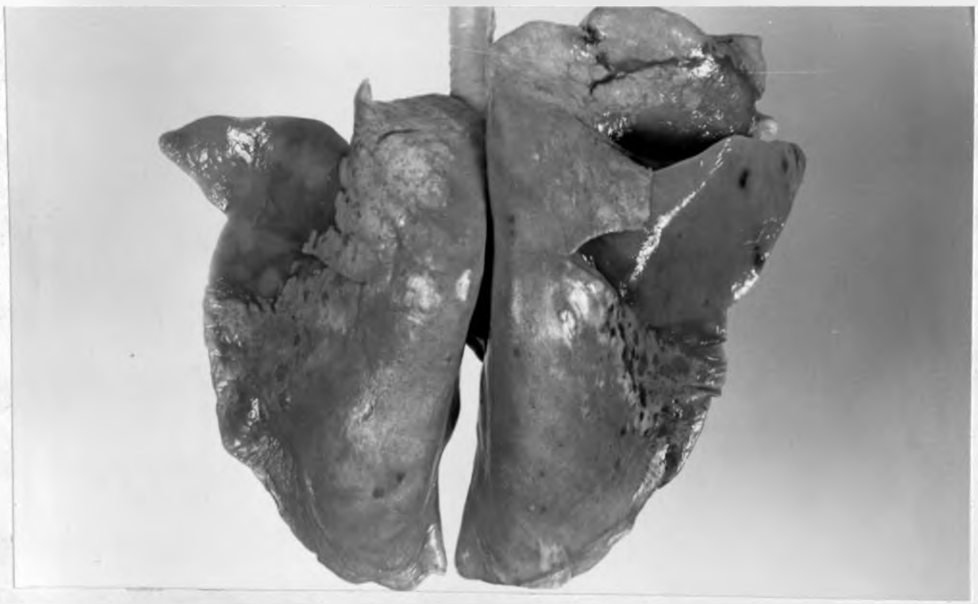

Fig.15: Sheep 3645. Dorsal aspect of the adenomatous lesions. SPA foci of varying sizes are observable.

on the right and slightly grayish-red on the left side. It was moist on the cut surface and friable under pressure. The adenomatous process appeared to have started from the ventral aspects and progressed dorsally. There was no demarcation between the transformed and the apparently normal lung parenchyma. This zone of the lungs was occupied by irregularly rounded, multicentric grayish-white nodular foci. These nodules were of varying sizes ranging from just visible to 1cm. in diameter or larger.

The larger nodules were produced by coalescing of the adjacent smaller ones. They were larger and more numerous towards the completely transformed sections and became smaller and sparser towards the normal-looking parenchyma. Some of the smallest nodules were surrounded by congested zones. These nodules protruded on the cut surface and felt like grains embedded and suspended in soft tissue. Watery fluid oozed out of the cut surface even without pressing. The apparently normal-looking lung tissue, and especially the posterior diaphragmatic lobe aspects, and that between the scattered nodules showed uniform alveolar emphysema. The trachea, bronchi and bronchioles contained white frothy fluid which oozed out through the tracheal opening even without pressure when lungs were placed on a bench.

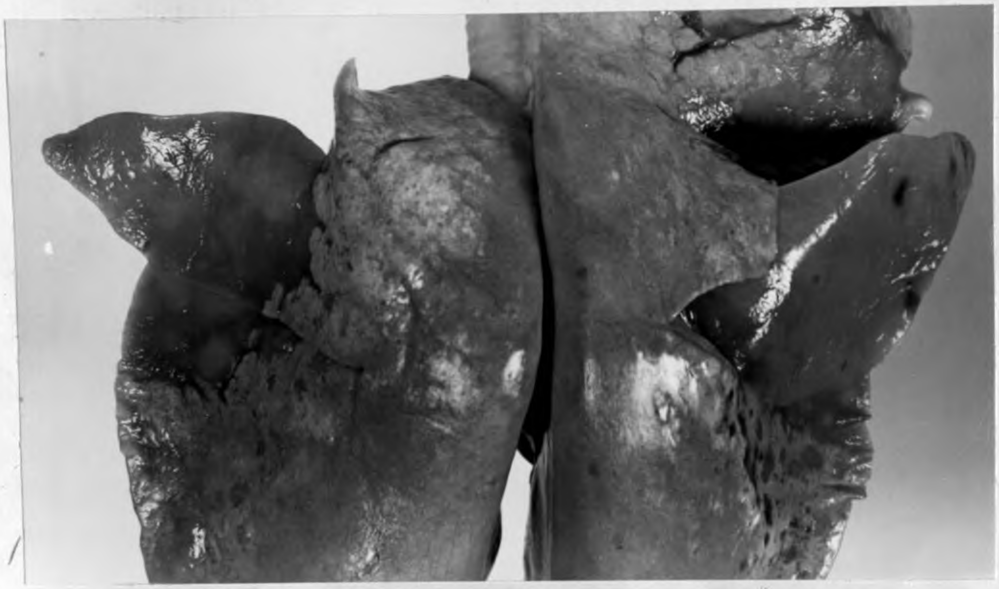

Fig.16: Closer-view of dorsal aspect of sheep 3645.

Fig.17: Sheep 3645. Ventral aspect.

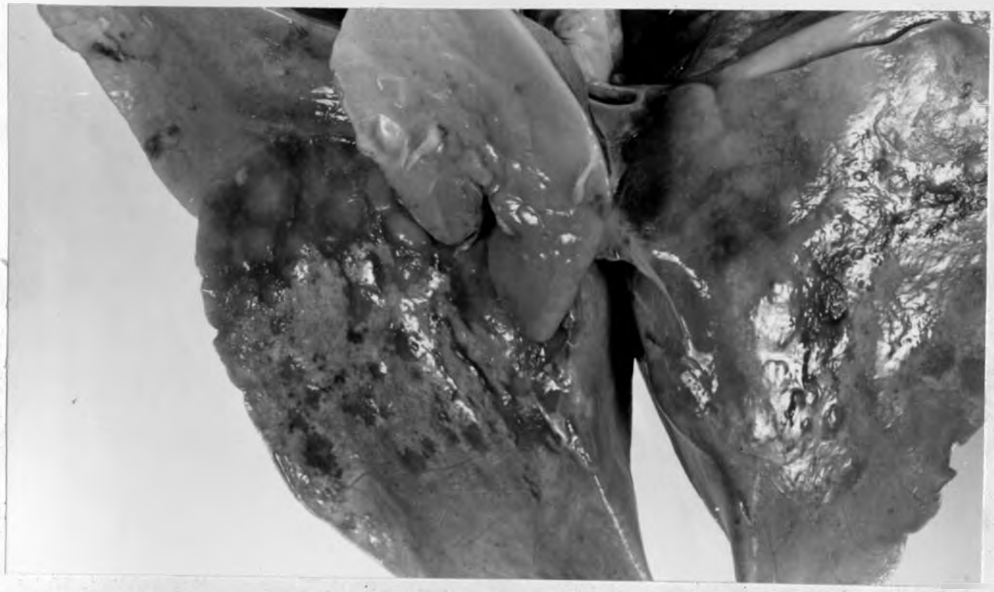

Fig.18: Closer-view of ventral aspect of sheep 3645.

Fig.19: Sheep 3648 - Dorsal view - showing spread of adenomatous lesion.

TABLE 14 reveals that lungs of sheep 3617 (Figures 12-14) killed 225 days after inoculation had no grossly visible lesions in the right apical lobe while those of sheep 3613 (Figures 22 & 23) killed 260 days after infection had transformed lesions involving the left lung mainly. Only very minute foci on the medial aspect of the apical lobe were present in the right lung. Lungs of sheep 3645 (Figures 15 to 18) and 3648 (Figures 19 to 21) killed 228 and 260 days respectively after infection had more involvement of the anterior than the posterior lobes. Their transformed adenomatous lesions were essentially like those of sheep 3616. They showed no secondary bacterial reaction.

Only sheep 3608 showed enlarged tracheobronchial and mediastinal lymph nodes.

Microscopic Lesions:

The specific lesions are shown in Figures 24-35. The histological lesions present in lobes of the lungs of sheep 3613, 3616, 3617, 3645 and 3648 were similar. However, examination of many sections revealed no adenomatous foci in the cardiac, diaphragmatic and intermediate lobes of the right lung of sheep 3613. Lung lesions were generally advanced in the anterior lobes and relatively early (beginning changes) in the diaphragmatic lobes. The

Fig.20: Sheep 3648 - Closer-view of dorsal aspect.

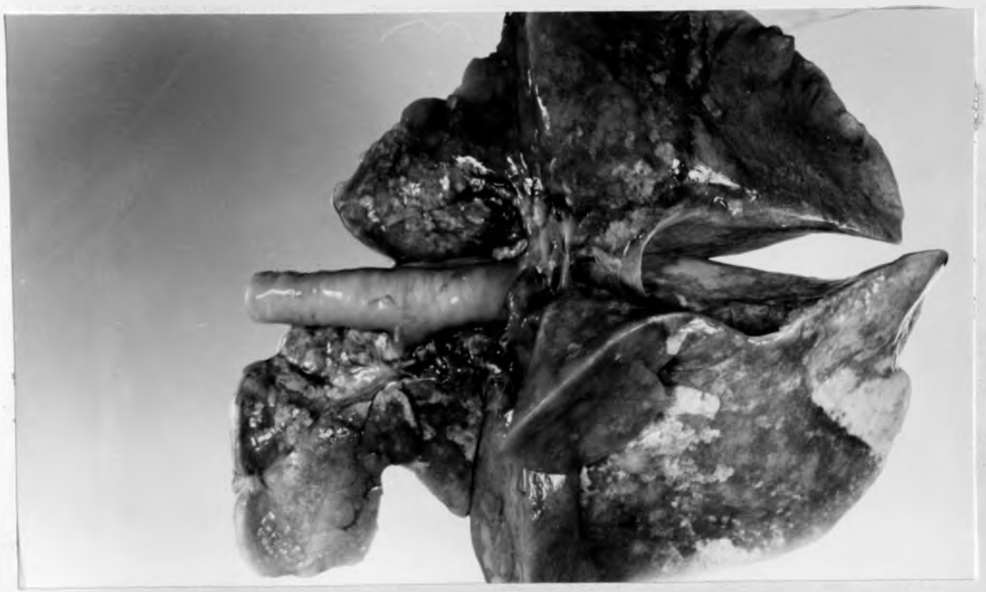

Fig.21: Sheep 3648 - Ventral aspect. Very little of the surface is normal.

Fig.22: Sheep 3613 - Dorsal aspects showing no evidence of SPA foci in the right lung. Several glandular foci are scattered in the anterior lobes of the left lung.

Fig.23: Sheep 3613 - Closer view of the left lung showing scattered adenomatous foci.

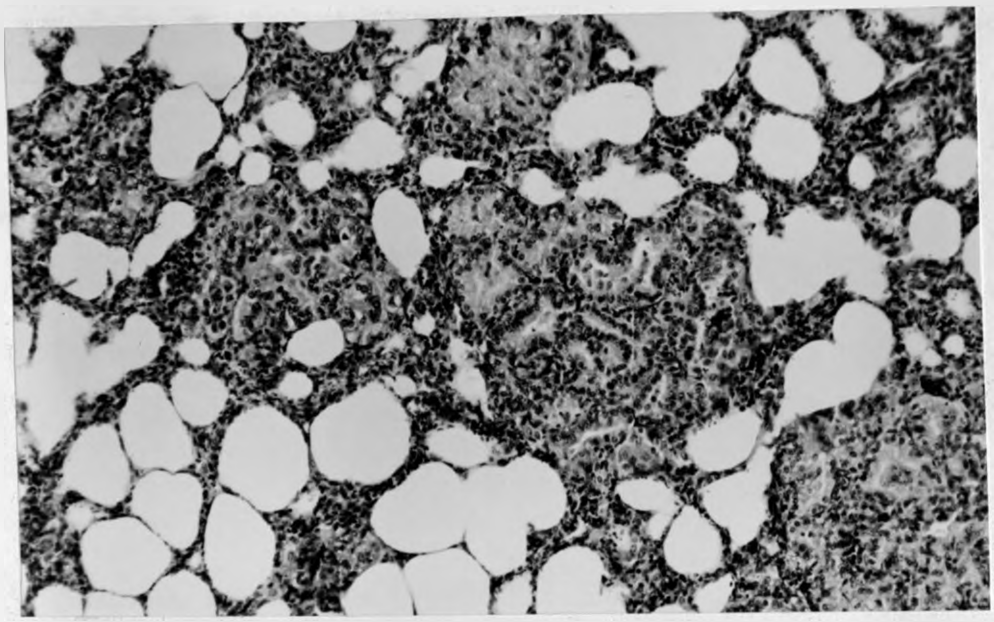

Fig.24: Sheep 3648 - Adenomatous foci located in "centres" of once thickened interalveolar walls. H & E stain. X 125.

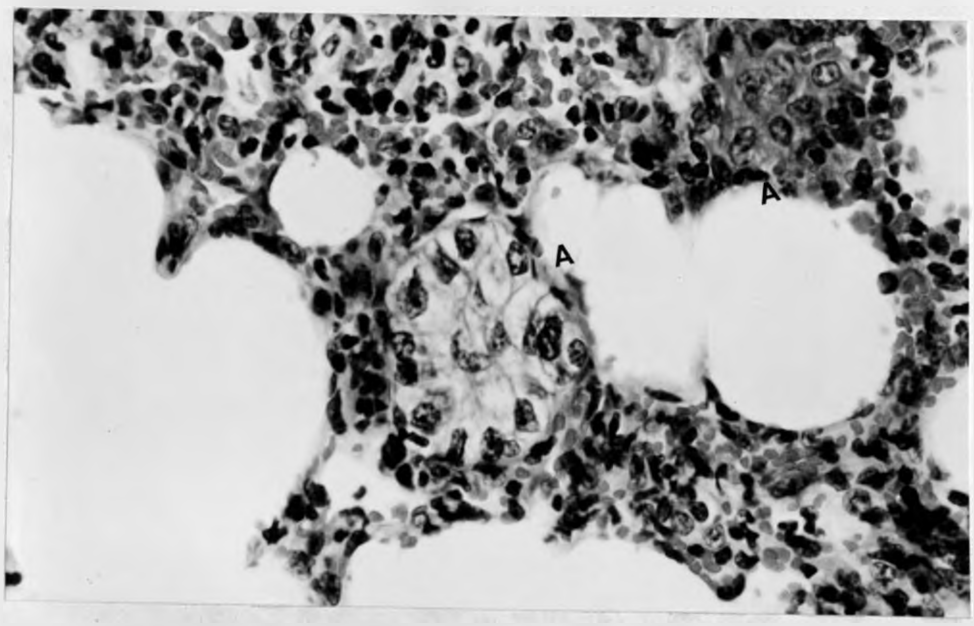

Fig.25: Sheep 3645. Two adenomatous foci (near A) located in "centres" of thickened interalveolar walls. Lendrum's stain. X 500.

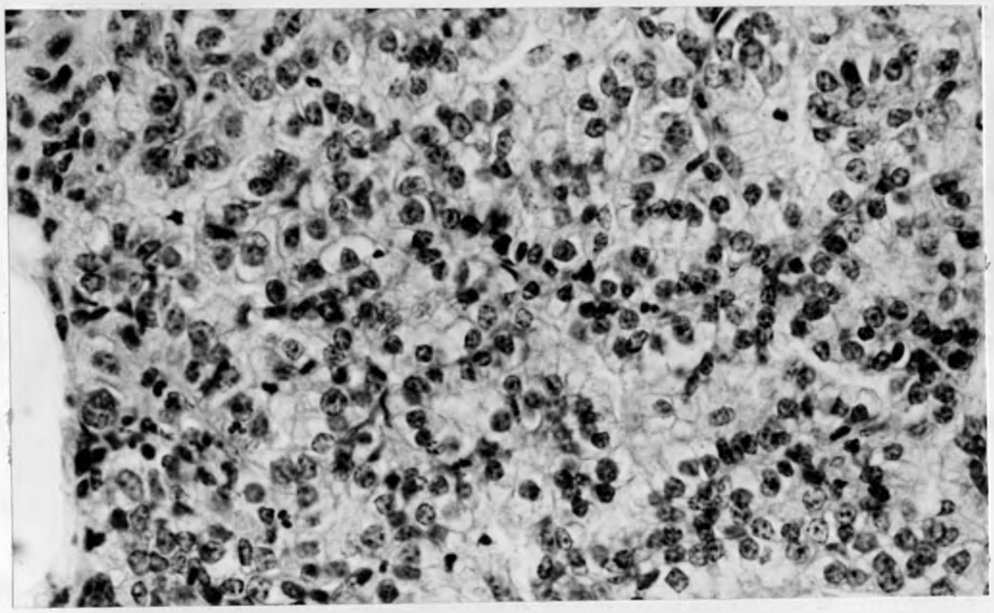

Fig.26: Sheep 3645 - A "Sheet" of adenomatous cells with no marked alveolar spaces. H & E stain. X 500.

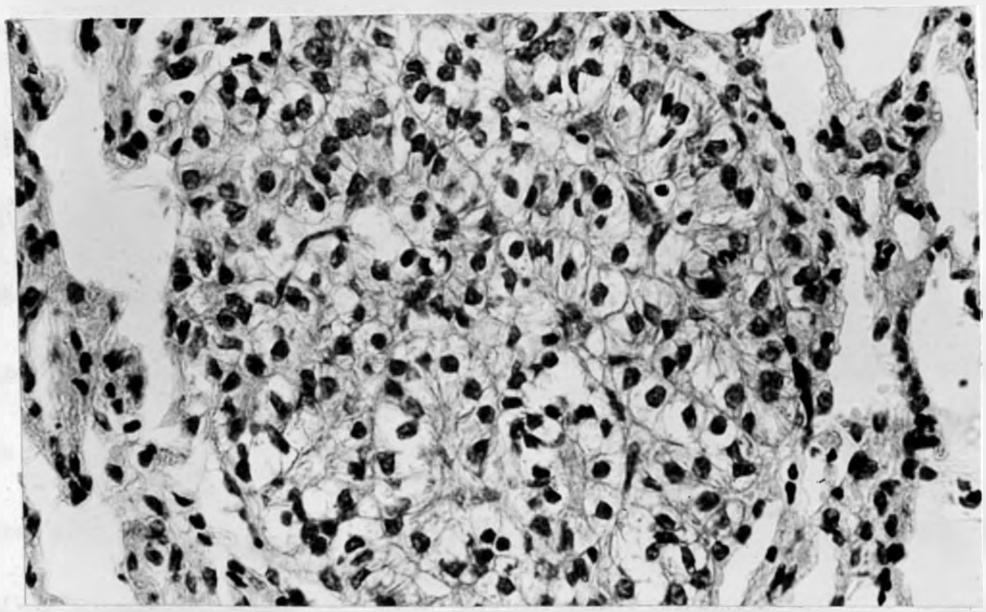

Fig.27: Sheep 3615 - Compact group of adenomatous cells with vacuolated cytoplasm. H & E stain. X 500.

marked cellularity. While the cytoplasm of adenomatous cells was compact in most instances, it was frankly vacuolated (Figure 27) in others. These two features were often evident in the same histological section. The vacuolated structure of the cytoplasm was due to the presence of mucin demonstrated in some adenomatous cells by Periodic acid-Schiff (PAS) staining. Early adenomatous foci located in "centres" of thickened interalveolar septa were especially evident in sheep 3645, 3648 and 3613.

Thickening of interalveolar septa had subsided in slightly long standing lesions (Figures 28 & 29). The adenomatous foci in this case appeared like compact glandular tissue suspended by the "thin" interalveolar walls. The various multifocal lesions grew by expansion coalescing with neighbouring foci to form large grossly visible nodular masses. At this stage, some cuboidal/columnar metaplasia of alveolar epithelium could be seen in isolated foci where there had been no evident thickening of interalveolar walls. In such cases, only a few cells, rarely all of an alveolus, were involved in metaplasia. The proliferating cells supported by loose connective tissue grew in a papillary manner and could be seen breaking through some of the uninvolved interalveolar walls. There was slight alveolar macrophage response in older lesions in

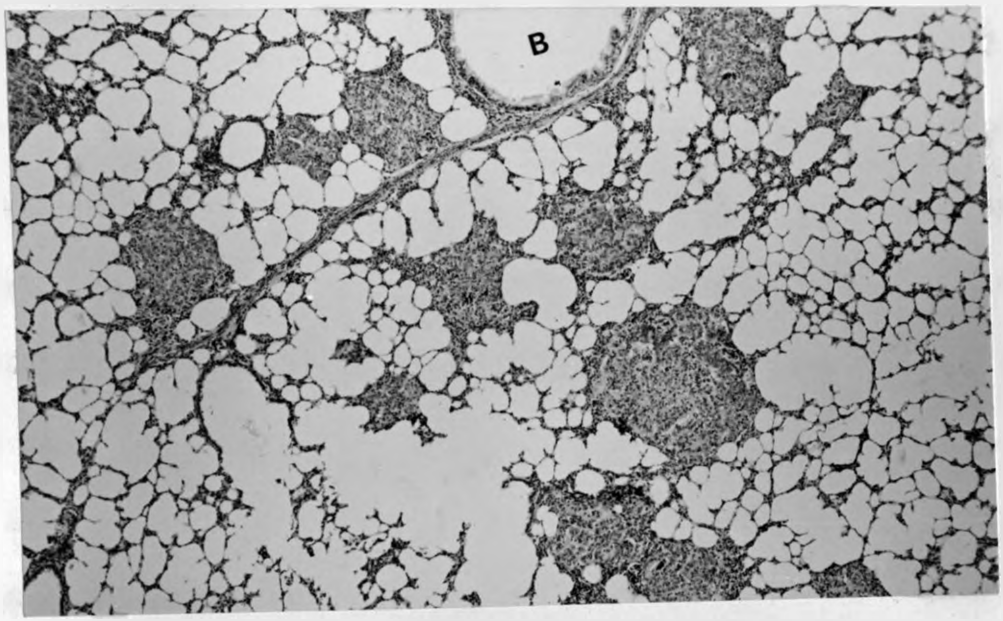

Fig. 28: Sheep 3645 - Several adenomatous nodules "suspended" by interalveolar walls whose transient thickening had subsided. There is no bronchiolar (B) involvement yet. H & E stain. X 45.

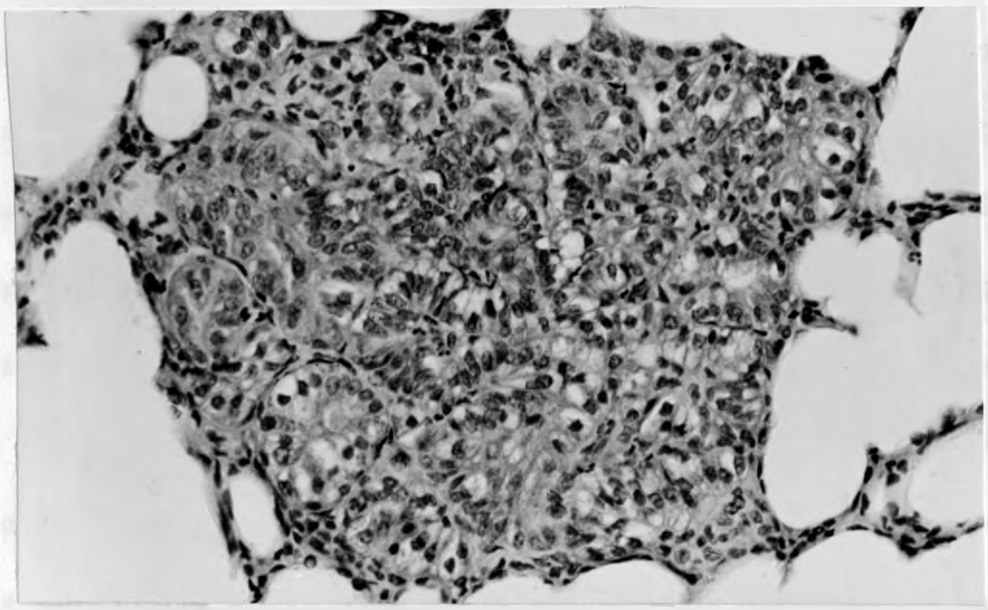

Fig. 29: Sheep 3645 - An adenomatous nodule whose cells are compact "suspended" by normal size interalveolar walls. H & E stain. X 315.

sheep 3608, 3615, 3617 and more evident in the left lung of 3616 (Figure 30). This response was generally confined to alveolar spaces whose lining epithelium was not involved in the metaplastic/ hyperplastic process. They were rare in alveoli formed by adenomatous cells. This would indicate that alveolar macrophages in this disease are not desquamated tumour epithelial cells. There was hardly any significant alveolar macrophage response in sheep 3605, 3613, 3645 and 3648.

In long-standing lesions shown by 8 sheep (TABLE 14) sheets of adenomatous cells, formed alveolar spaces of varying dimensions. In some, the proliferating adenomatous cells, supported by thin connective tissue, appeared to grow in a papillary manner into alveolar spaces (Figure 31). None of the sheep showed any fibrosis and lympho-reticular hyperplasia was occasional. Inflammation was not present in absence of secondary bacterial infection (Figure 32). Epithelial intrabronchiolar proliferations (Figures 33 & 34) were present in the lungs of sheep 3613, 3615, 3616, 3617 and 3648.

Sections of lungs from sheep 3608 and 3615 showed, in addition to the adenomatous foci, fibrinous strands in the alveoli often mixed with mononuclear cells and neutrophils. The fibrinous exudate was more marked in sheep 3608. Sheep 3605 had much more severe fibrinous reaction. It had scattered adenomatous foci, both in

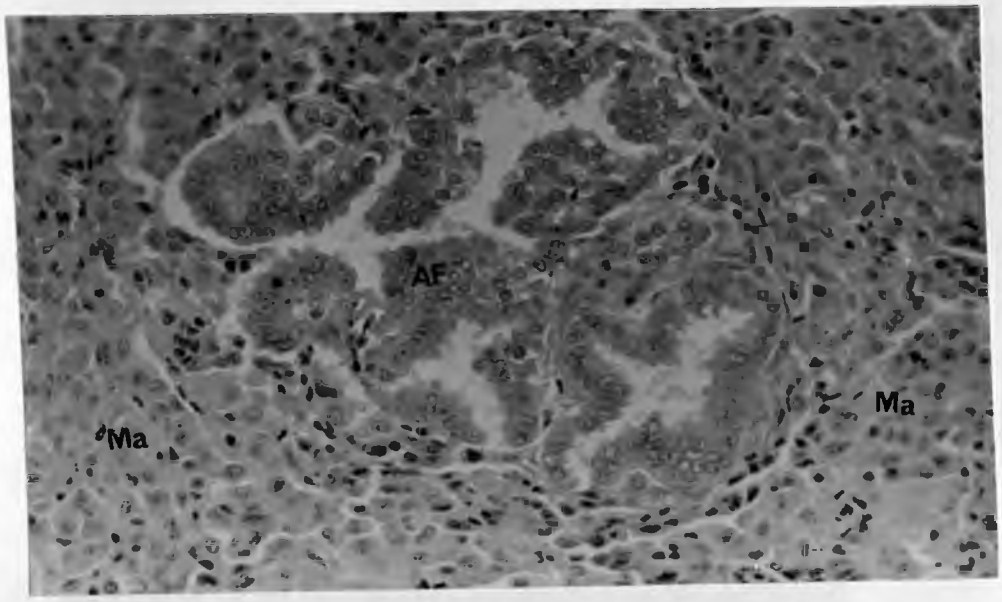

Fig.30: Sheep 3616. An adenomatous focus (AF) surrounded by ~~macrophages (Ma) which had infiltrated alveolar spaces and some fused to form~~ epithelioid cells. H & E stain. X 315.

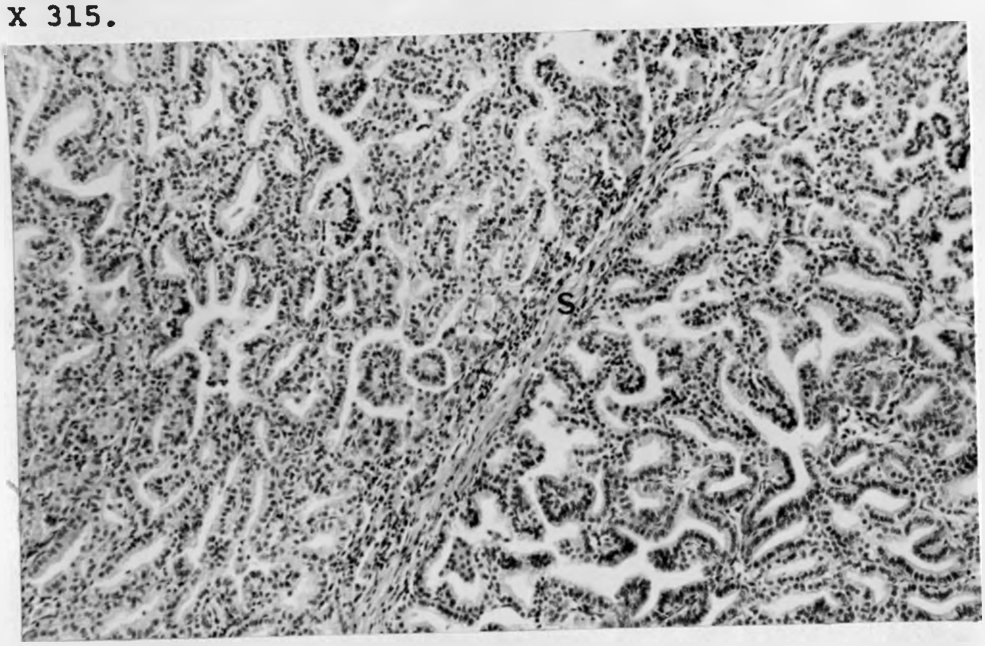

Fig.31: Sheep 3617. An advanced lesion of SPA. Interlobular septum (S) divided by two groups of tumour cells. The latter supported by thin connective tissue grow in a papillary manner into alveolar spaces. H & E stain. X 125.

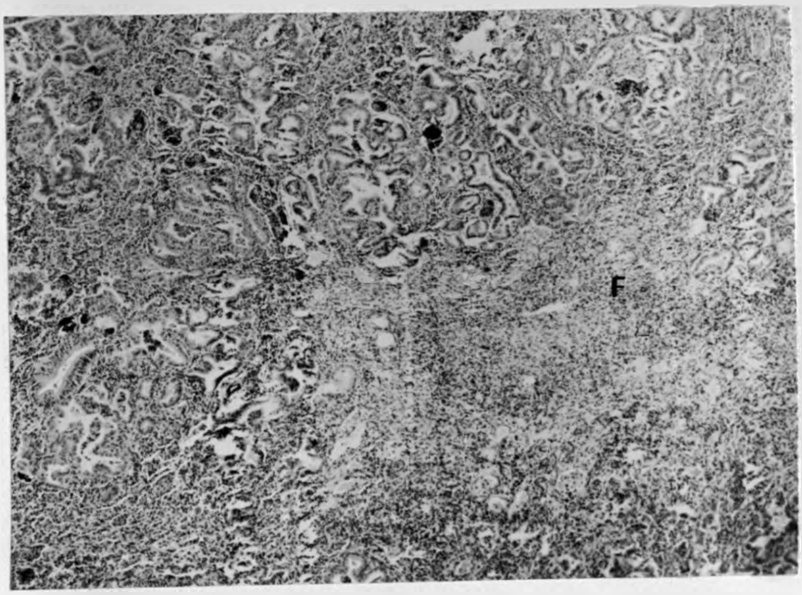

Fig.32: A natural case (WV) showing fibroplasia on the right side (F) in an advanced case with apparent secondary reaction. H & E stain. X 45.

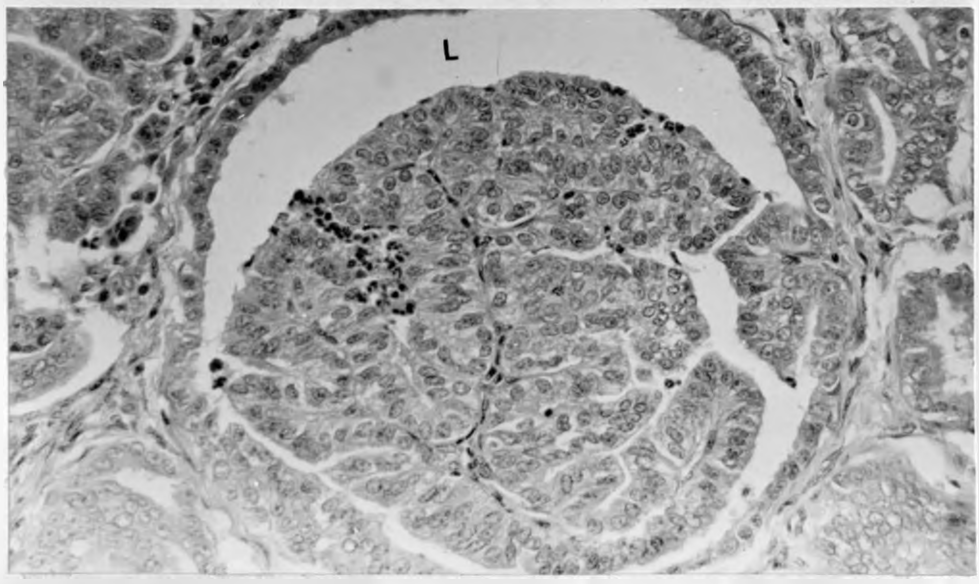

Fig.33: Sheep 3645 - Intrabronchiolar proliferation partially obliterating the lumen (L). H & E stain. X 315.

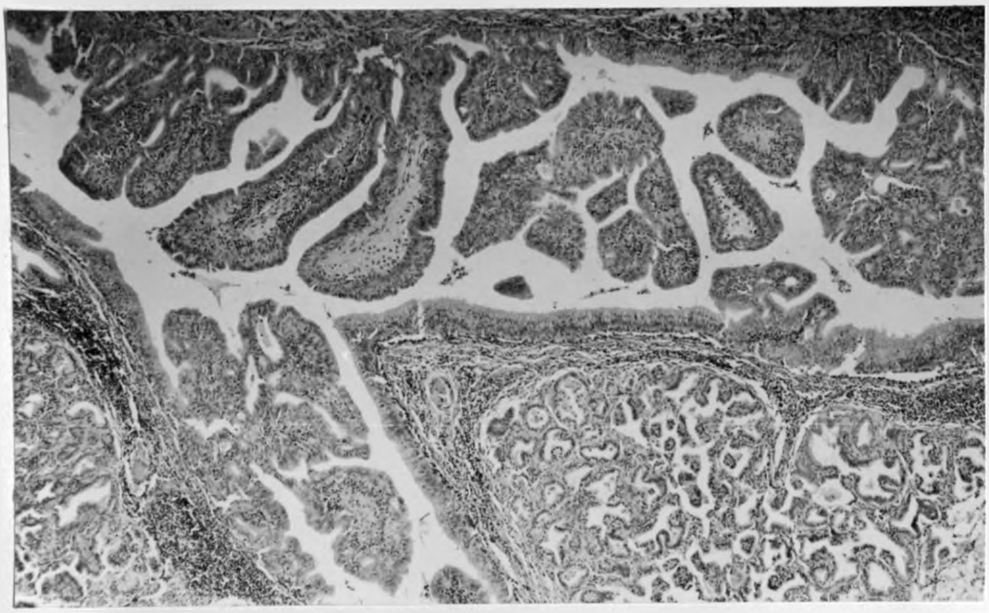

Fig.34: From a natural case (1057/66). Intrabronchiolar proliferation with partial occlusion of lumen. H. & E. stain. X 45.

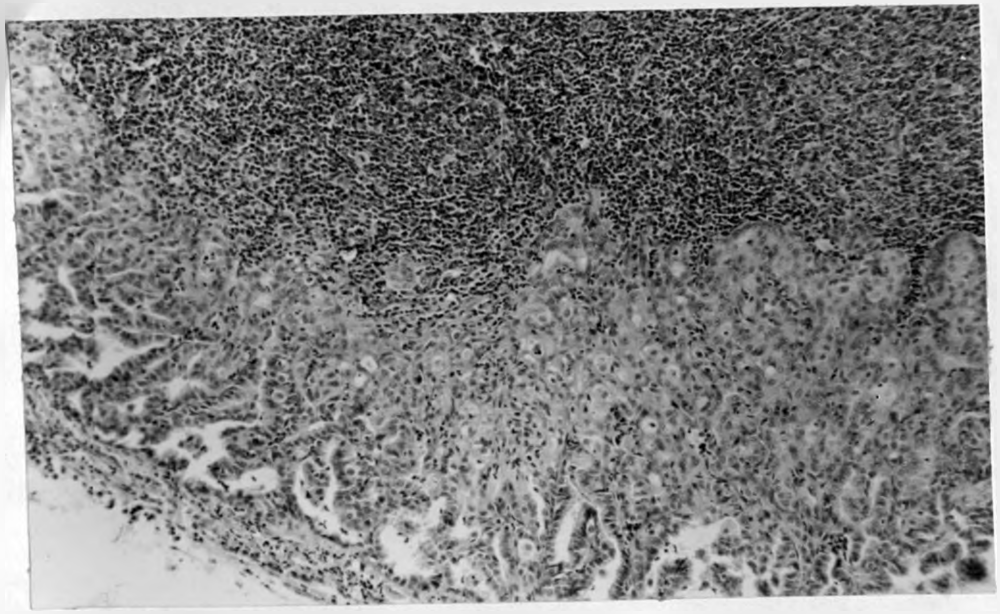

Fig.35: From a natural case (1156/66) showing metastasis to tracheobronchial lymph node. H & E stain. X 125.

pneumonic sections and in areas free from fibrinous pneumonia but showing alveolar emphysema. In some sections of the lungs of sheep 3605 and 3608 only fibrinous pneumonia was present but no lesions of pulmonary adenomatosis.

No metastases, which are sometimes encountered in natural cases (Figure 35), were seen in any of the tracheobronchial and mediastinal lymph nodes. The lymph node enlargement found in sheep 3608 was due to congestion and marked inflammatory cell infiltration, especially in the cortical sinuses. Bacterial colonies were found in the sinuses of some sections.

III. Experiments 3 and 4:

Data on Transmission Experiments 3 and 4 are summarised in TABLE 15.

(a) Post-inoculation Observations:

Records of rectal temperature for one month revealed no change in both experiments. No other clinical signs were observed throughout the period of observation.

(b) Bacteriology:

This was not done routinely to check for common bacteria. But lungs of all the sheep were checked for Mycoplasma spp., and none yielded these organisms.

TABLE 15: Summary of Transmission Experiments 3 and 4.

Experiment	Sheep	Breed and Age (Months)	Inoculum	Killed after days	Results
3	67	Adult Somali	Egg Material	367	No lesions
	68	" "	" "	367	" "
	69	" "	" "	367	" "
	70	6: Somali	" "	71	(Died) "
	3770	10: Corriedale	" "	367	" "
	3792	" "	" "	365	" "
	3795	" "	" "	365	" "
	3796	" "	" "	365	" "
4	87	Ewe Corriedale	Mycoplasma	215	No lesions
	92	" "	"	180	" "
	3763	15: Corriedale	"	151	" "
	3786	" "	"	160	" "
	3821	" "	"	153	" "

(c) Pathology:

Sheep 3795 and 3796 of Transmission Experiment 3 had small parasitic nodules approximately 5mm. in diameter. But gross and histological examination of lungs of these two sheep and those of sheep 67, 68, 69, 70, 3770 and 3792 revealed no lesions of SPA.

Lungs of all sheep in Transmission Experiment 4 showed no lesions of any type.

IV. Discussion:

In this study, infective material was used to try and reproduce lung lesions and/or clinical disease typical of SPA. Mycoplasma mixed cultures in Experiment 4 produced no pyrexia or any other clinical signs. There were no lesions in the lungs. This could be because the Mycoplasma used were non-pathogenic or due to the short time factor following inoculation. The most plausible explanation is that Mycoplasma are not involved in the causation of the adenomatous process of Jaagsiekte. Furthermore they could not be isolated from several SPA cases.

The egg-passaged infective material (AAF) used in Experiment 3 alsc failed to produce definite lesions of pulmonary adenomatosis. While the gross lesions in lungs of two sheep looked suspicious of SPA, they were more like lesions associated with

Muellerius capillaris infection. However, this infective material would appear to have contained some other agent in addition to the Mycoplasma. This agent may have been responsible for the regular embryo deaths in passages of W_1, W_2 and W62 (Chapter 3) compared to the irregular response to Mycoplasma alone. Whether this agent is the one which causes metaplasia of alveolar epithelium of sheep's lungs is not certain. If it were then the apparent failure to produce typical lung lesions of pulmonary adenomatosis could be because the titre of the agent was below the minimum necessary for the stimulation of the adenomatous process.

In Transmission Experiment 2 lung lesions of the disease were produced in 8/10 (TABLE 13) of the animals. Such lesions were similar to those of natural disease in sheep. Clinical signs typical of pulmonary adenomatosis of sheep were exhibited by 7/10 of them as shown in APPENDIX "A". The lung lesions were bilateral, contrary to the findings of CUBA-CAPARO et al. (1961) who encountered only 24/60 of the cases. This finding which is in agreement with the observations of WANDERA (1967b) in natural cases confirms absence of any lobar preference. The symptoms were dyspnoea, increased respiration rate, moist rales, watery discharges from the lungs and occasional cough. Clinical examinations

had been carried out while sheep were at rest. It was not possible to observe cough in many of them for they were not under 24 hours continuous observation for the duration of the experiment. There was no difference in outcome between sheep inoculated with filtered and those with unfiltered material.

None of the 3 sheep in Transmission Experiment 1 with SPA lesions showed any clinical signs as the transformed pulmonary parenchyma was insufficient to interfere with normal lung functioning. The three were out of a group of four sheep inoculated intratracheally. Sheep 3632 with pulmonary interalveolar wall thickening may have developed adenomatous lung lesions had it lived long, and sheep 3631 would perhaps have developed clinical disease had it lived long. The difference in the results of Transmission Experiments 1 and 2 does reflect on the way the inocula were prepared.· In the second experiment, the material was of a high concentration. The adenomatous cells had been subjected to much more disruption by ultra-sonic vibration. Hence a higher proportion of infectious agent was released in the medium. This was not the case in the preparation of Experiment 1 inoculum.

The results of Experiment 2 show that intratracheal route is the most suitable, and that it is the most likely portal of

entry in natural infection. Despite the fact that the number of animals involved is small to be statistically significant, this experiment demonstrates that it is possible to reproduce the clinical disease within 5-8 months after inoculation. The production of SPA lesions in affected sheep would appear to be dose-dependent and susceptibility-dependent and not time-dependent.

Though the sheep were not killed in series, the gross and microscopic lung lesions observed showed changes at different stages of development and hence of significance in the pathogenesis of the condition. From this it was apparent that the transmissible agent having infected alveolar epithelium may have acted as an irritant leading to congestion and infiltration with mononuclear cells into the interalveolar septa. The result of this reaction was thickening of the latter. The micro-atelectatic alveoli were involved in this thickening. This was a transient change and while it may not be specific for pulmonary adenomatosis, the later histogenetic changes appear to be connected with it.

Following a latent period of varying length, the agent induced changes in the normally modified squamous epithelial cells. These cells became endowed irreversibly with the potentiality to initiate the primary stages of cancer formation. The agent next

stimuated or triggered off transformed cells to proliferate. This process first started in epithelium of collapsed alveoli, possibly because they were more susceptible to injury than that of the patent ones. These adenomatous cells formed minute foci which in turn formed large nodules grossly visible by both expansion and confluence. The uninvolved alveoli were later infiltrated with macrophages in some cases. The macrophage response appeared to reduce the rate of hyperplasia of the adenomatous cells and eventually stop it completely, for some of the small adenomatous foci remained "static" and surrounded by the alveolar macrophages. In some areas these "static" adenomatous foci may be surrounded by alveolar emphysema. These minute adenomatous foci have mistakenly been equated with the earliest changes.

To some degree epithelium of bronchioles and bronchi was also involved in this hyperplastic process in some sheep. Macrophage response cannot be regarded as part of this adenomatous process as only a few sheep showed it. However it might be associated with secondary infection by Mycoplasma. Fibroplasia was absent and lympho-reticular hyperplasia ~~was~~ occurred occasionally. Inflammation was only present in some cases (sheep 3605, 3608, 3617) with secondary bacterial infection. None of the sheep had any metastases to the regional lymph nodes. But this does not

disqualify the disease as being essentially neoplastic and transmissible, as metastases have been encountered in some cases of the natural disease by the author of this thesis. In all the cases, the tendency was for the adenomatous process to begin in the anterior lobes and gradually proceed posteriorly.

CHAPTER 5

ELECTRON MICROSCOPIC STUDIES

As discussed in Chapter 3, Mycoplasma of rather doubtful significance were present in some SPA lungs. Because of this and of the fact that agents involved in the causation of pulmonary adenomatosis have not been isolated and characterized, it was decided to carry out electron microscopic studies. Materials to be investigated were adenomatous lungs from sheep with the natural disease of SPA. This study was intended to reveal the presence if any of agents that could be associated with sheep pulmonary adenomatosis and consequently the ultra-structure of the adenomatous cells. Gross and histological examination of the diseased lungs showed them to have typical lesions of SPA.

I. MATERIALS AND METHODS:

Lung specimens from 8 sheep with SPA (WI - WVI, WXI and WXII) were used for the study. Diseased sheep came from the same flock (Farm D, Chapter 3) as those that supplied infective material for Chapters 3 and 4. Lungs from healthy sheep were included as controls. Materials were fixed according to the method of ITO-KARNOVSKY (1968). In this method the fixative was prepared as follows:

4gm. of paraformaldehyde were added to 100ml. of distilled water which was then heated to near boiling. The solution was cleared by adding 1N NaOH dropwise and shaking well. The clear formaldehyde solution was mixed with an equal amount of 0.2 M phosphate buffer, pH 7.2. 0.02% trinitrophenol was added and the solution stored in the refrigerator. For making the final fixative, 2% glutaraldehyde was added to the stock solution just before use.

Immediately after killing the animals and removing the lungs, small pieces of the specimens were fixed in this "Yellow fix" for 2 hours at room temperature. This was followed by 3 changes of 5 minutes each in 0.2 M cold phosphate buffer. They were then post-fixed in cold 1% osmium tetroxide in 0.2 M phosphate buffer for about 4 hours. After rinsing for 15 minutes with 3 changes in physiological saline in the refrigerator, they were dehydrated in acetone at 4°C. through concentrations of 30% for 15 minutes, and 50, 70, 90, 100, and 100 percent for 30 minutes each.

For embedding, DURCUPAN ACM (FLUKA AG., BUCHS SG, Switzerland) was used. The processing was through the following dilutions:

Durcupan I/Acetone in dilution of 1:3 for 1 hour,

Durcupan I/Acetone in dilution of 2:2 for 1 hour,

Durcupan I/Acetone in dilution of 3:1 for 1 hour.

The tissues were then passed through Durcupan I for 4 hours at 56°C. with one change at 2 hours and through Durcupan II for 2 hours at 56°C. The tissues were then placed in polysterene capsules, Durcupan II added and subjected to hardening at 60°C for 48 hours.

Sections were cut with glass knives on the OM U2 (REICHERT) ultramicrotome. Thick sections of approximately 1 u were stained with toluidine blue for light microscopy for orientation of the respective tissue blocks. Thin sections (600-900 A°) for electron microscopy were picked up on formvar-coated copper grids, stained with uranyl acetate for 6 minutes and lead citrate for 2 minutes, and examined in a CARL ZEISS EM 9A electron microscope.

II. RESULTS:

1. Light Microscopy: All the lung sections examined from the 8 sheep with the natural disease revealed the adenomatous cells characteristic of the disease reproduced in Chapter 4. Some of the foci, especially in two animals, appeared to be long-standing, as groups of adenomatous cells were interspersed and even surrounded by increasing number of proliferating fibroblasts. Adenomatous foci of this nature could perhaps be regarded as chronic "static" adenomatous tissue.

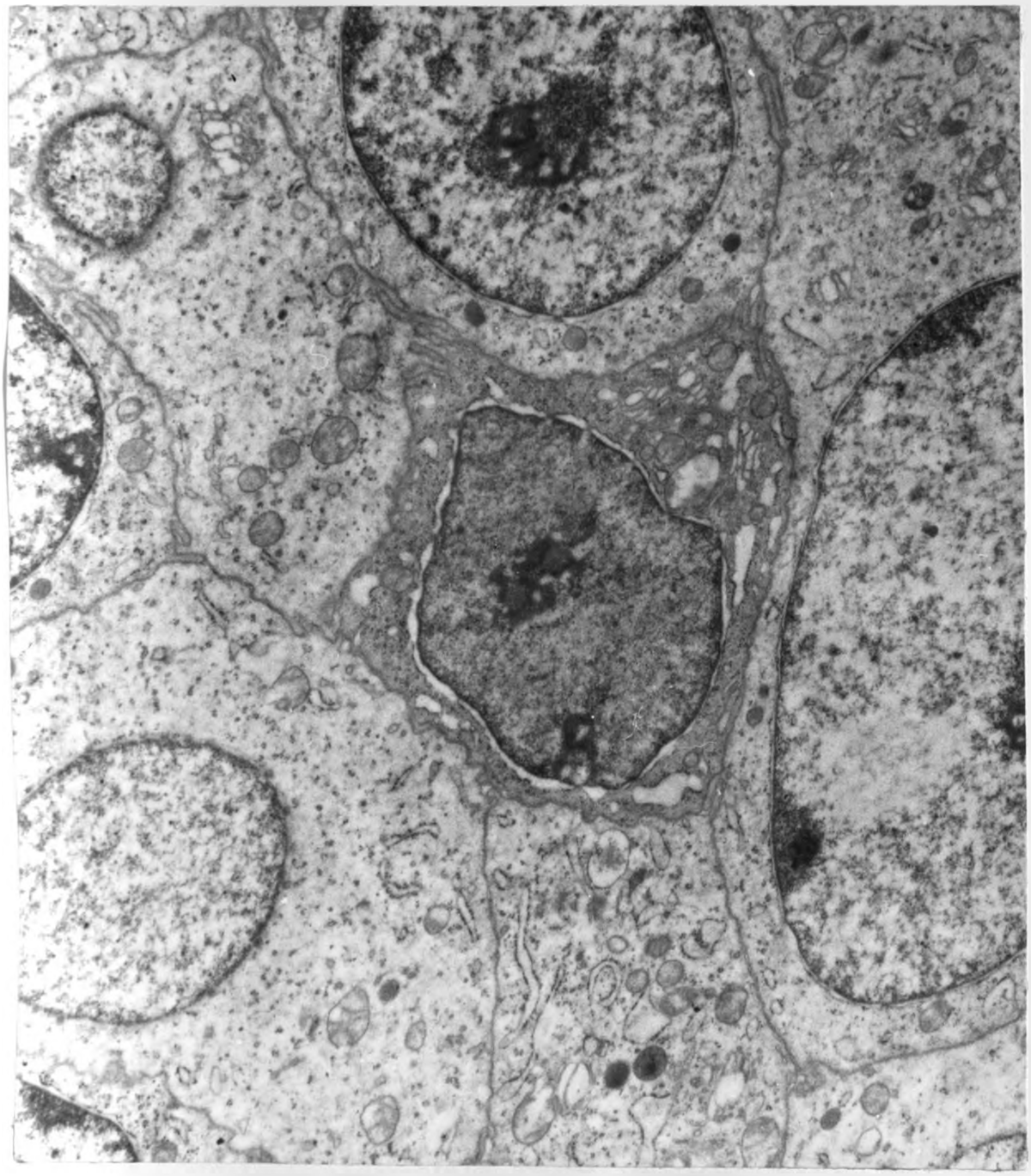

Fig.36: Sheep WII - Adenomatous cells in solid clusters, a dark cell contrally located with an indented nucleus and paranuclear space. Cell organelles are few. X 12,000.

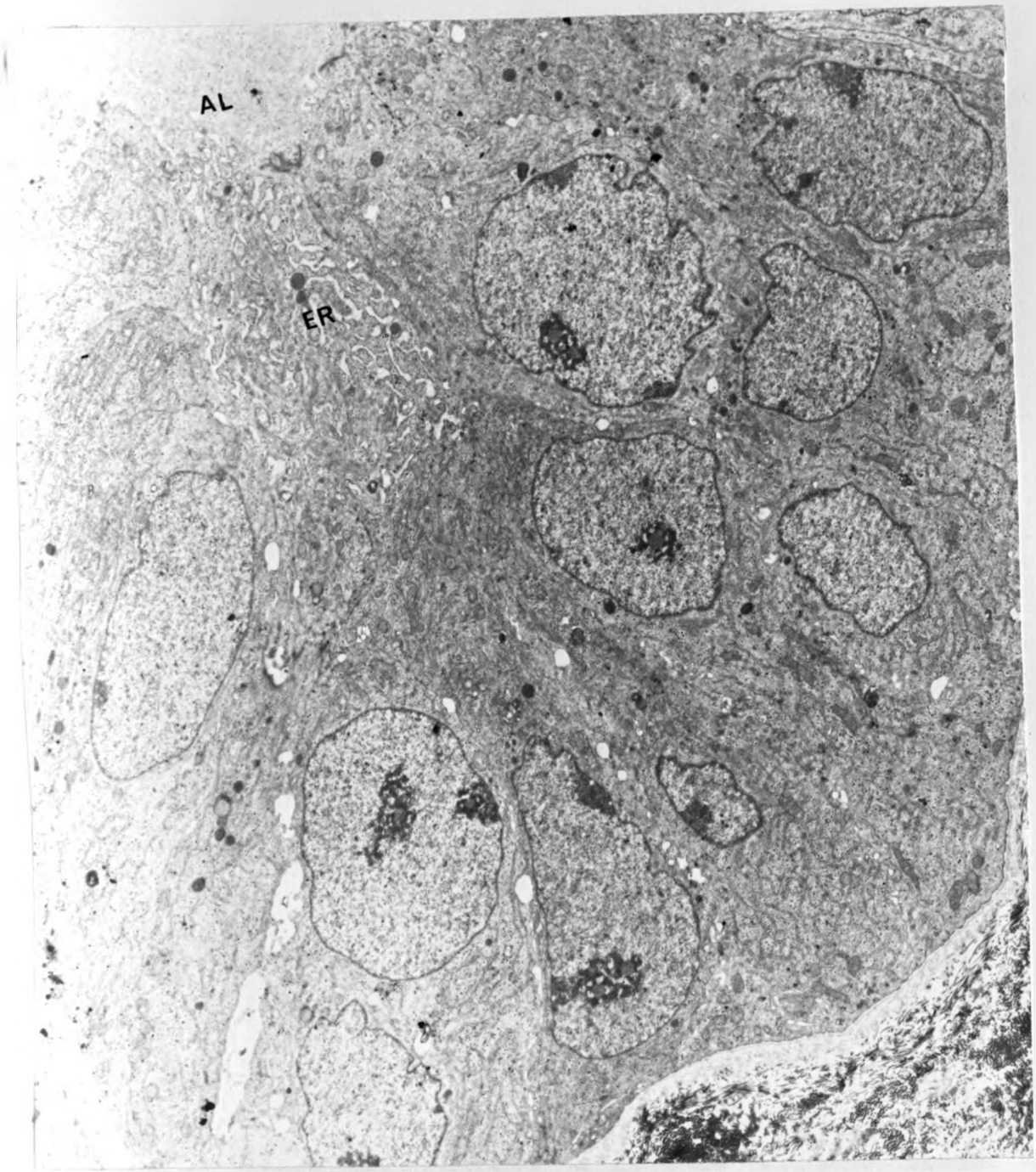

Fig.37: Sheep WXI - Dark and light adenomatous cells around an alveolus (AL) which has secretion. One cell shows irregularly dilated endoplasmic reticulum (ER). Collagen fibres at lower right. X 4,800.

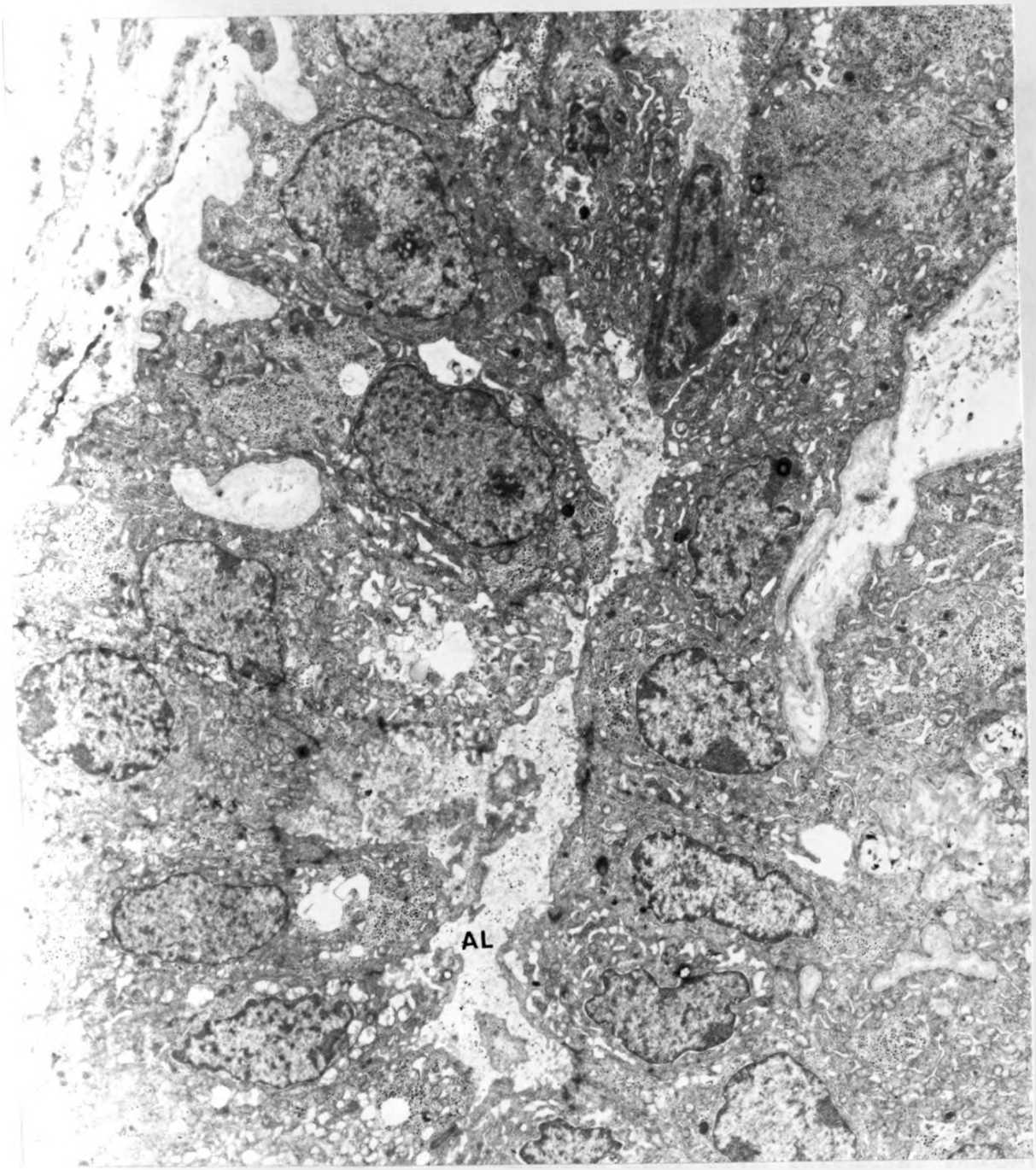

Fig.38: Sheep WXI - Columnar and cuboidal adenomatous cells around an alveolus (AL). X 4,800.

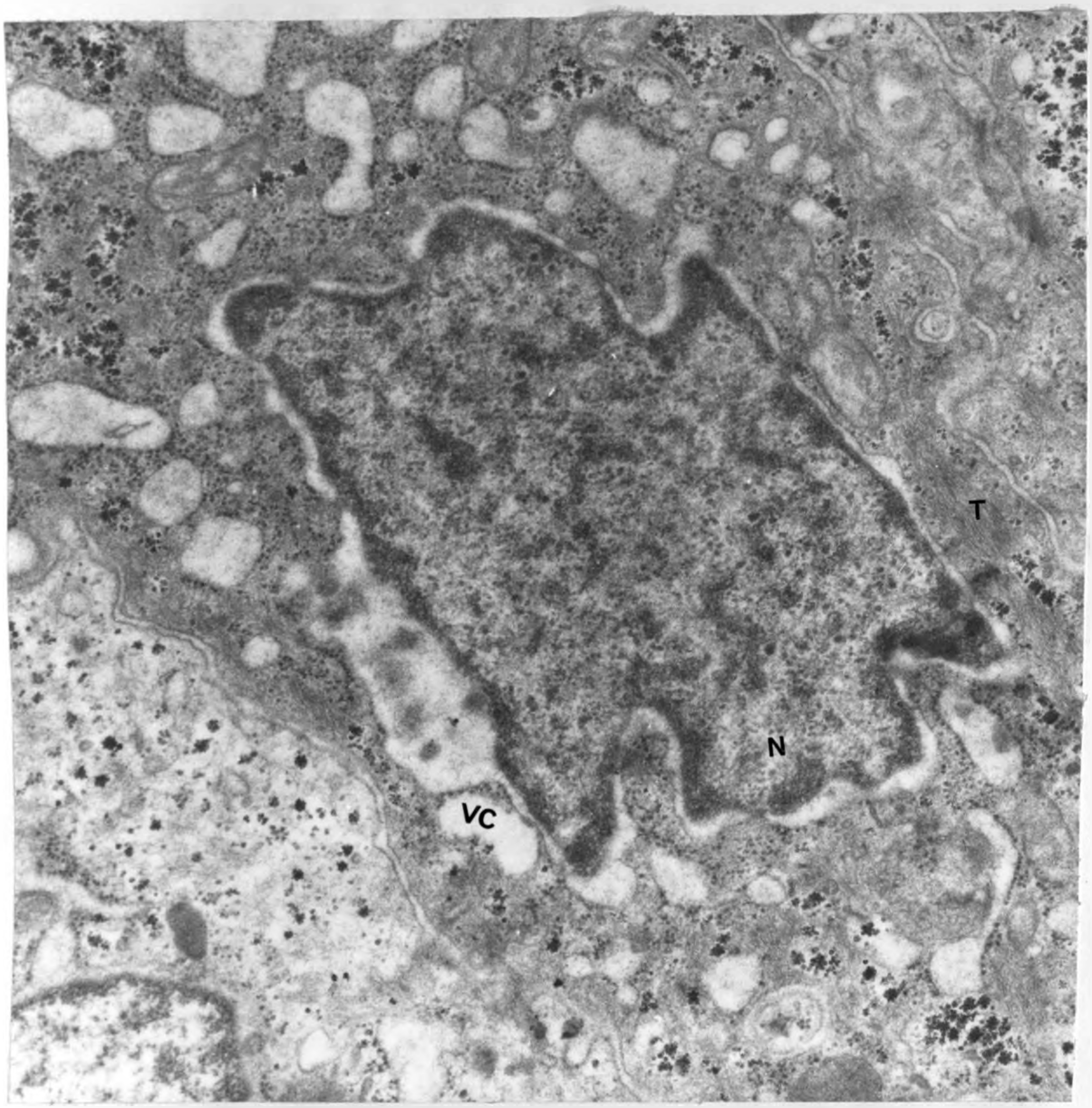

Fig.39: Sheep WXI - Adenomatous cell with irregular border, indented nucleus (N), paranuclear space, tonofilaments (T) vacuolation (VC) and glycogen granules at upper and lower right. X 31,200.

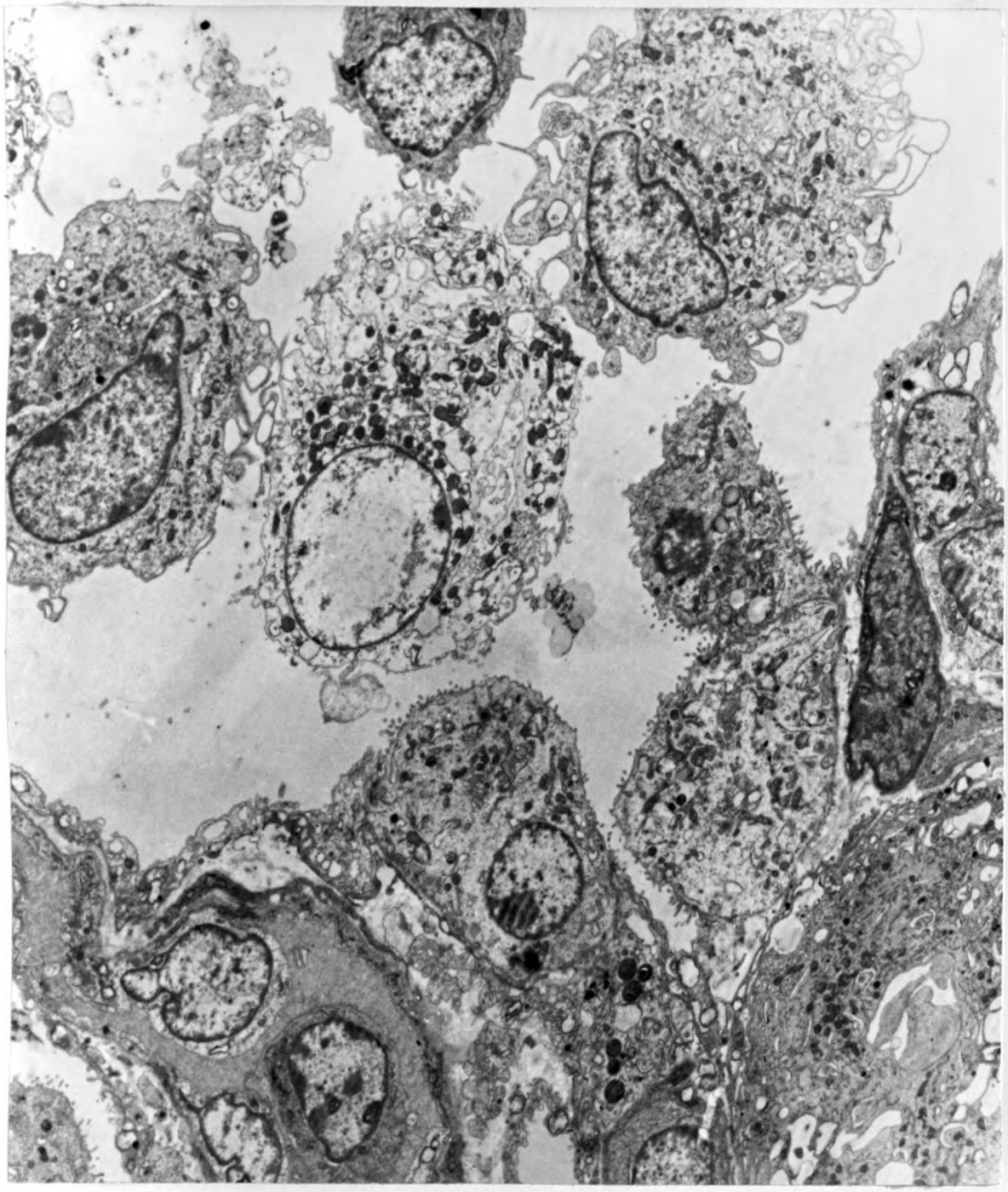

Fig.40: Sheep WII - Adenomatous cells on the lower part lining an alveolus show microvilli. Four macrophages with phagosomes are in the alveolus. They show no intranuclear inclusion bodies. X 4,800.

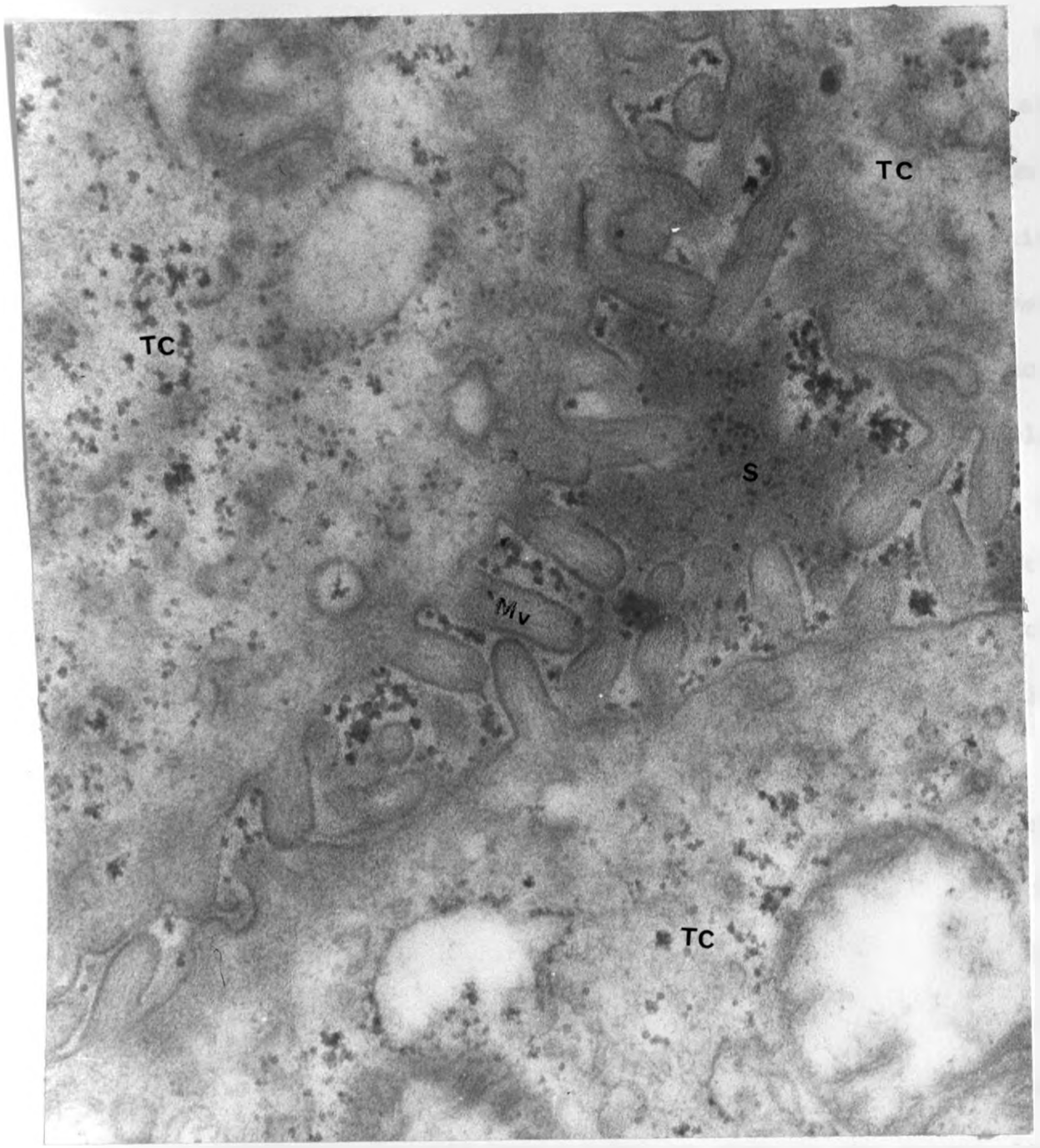

Fig.41: Sheep WIII - Three adjacent tumour cells (TC) with a potential intercellular space (S) in which are many interdigitating microvilli (Mv). X 57,000.

Endoplasmic reticulum were of normal shape in some cells, depleted in others and morphologically abnormal in most cells. The majority of endoplasmic reticulum were greatly dilated into irregularly shaped vesicles (Figure 44) or vacuoles. Some dilated ones were slightly regular and arranged in the direction of the alveolar spaces, forming lacunae. In some cells the vesicles and vacuoles involved both the rough-surfaced and the smooth-surfaced ergastoplasm. Rarely were the two forms of ergastoplasm prominent to the same extent in any one cell. Free ribosomes many of them appearing as polyribosomes were scattered throughout these cells but were more numerous in cytoplasm vacuolated by agranular reticulum. Some vacuoles contained varying amounts of secretion (Figures 45 & 46).

Most of the active adenomatous cells showed hyperplasia of the Golgi apparatus (Figures 43, 47 & 48). These prominent Golgi apparatus were often multiple and seen as convoluted and lamellar membranes of smooth appearance. They were often associated with numerous small vesicles and sometimes vacuoles. A few cells showed well-developed tonofilaments many of them in the paranuclear areas. Glycogen granules were encountered (Figure 48) frequently. Myelin figures were occasionally observed

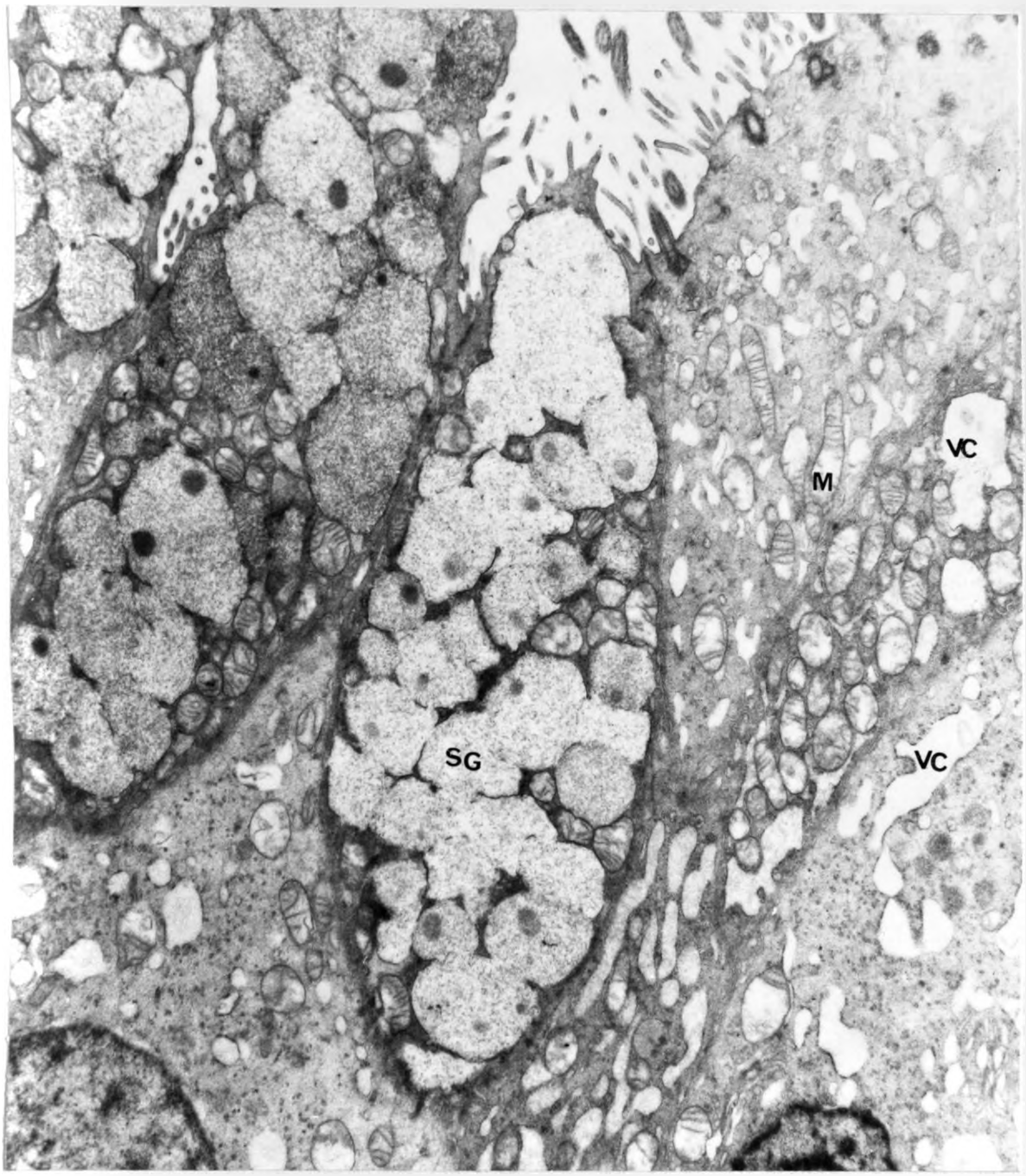

Fig.42: Sheep WIII - Tumour cells showing cilia, microvilli and secretion granules (SG) like those of goblet cells. There are many mitochondria (M) and vacuoles (VC). X 11,000.

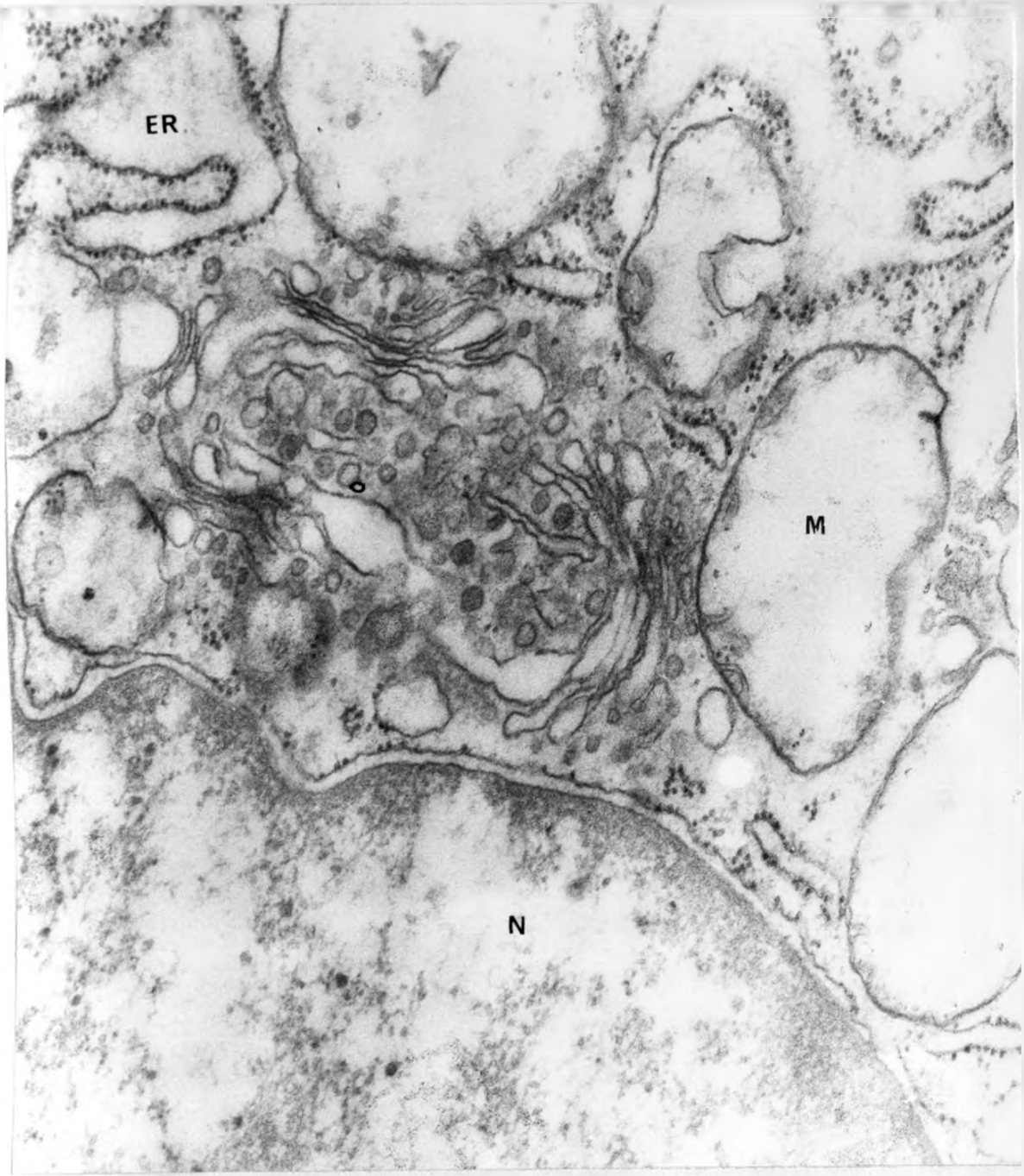

Fig.43: Sheep WI - Tumour cell with prominent Golgi apparatus (G) indented nucleus (N), dilated rough-surfaced endoplasmic reticulum (ER) and mitochondria (M) devoid of cristae. X 57,000.

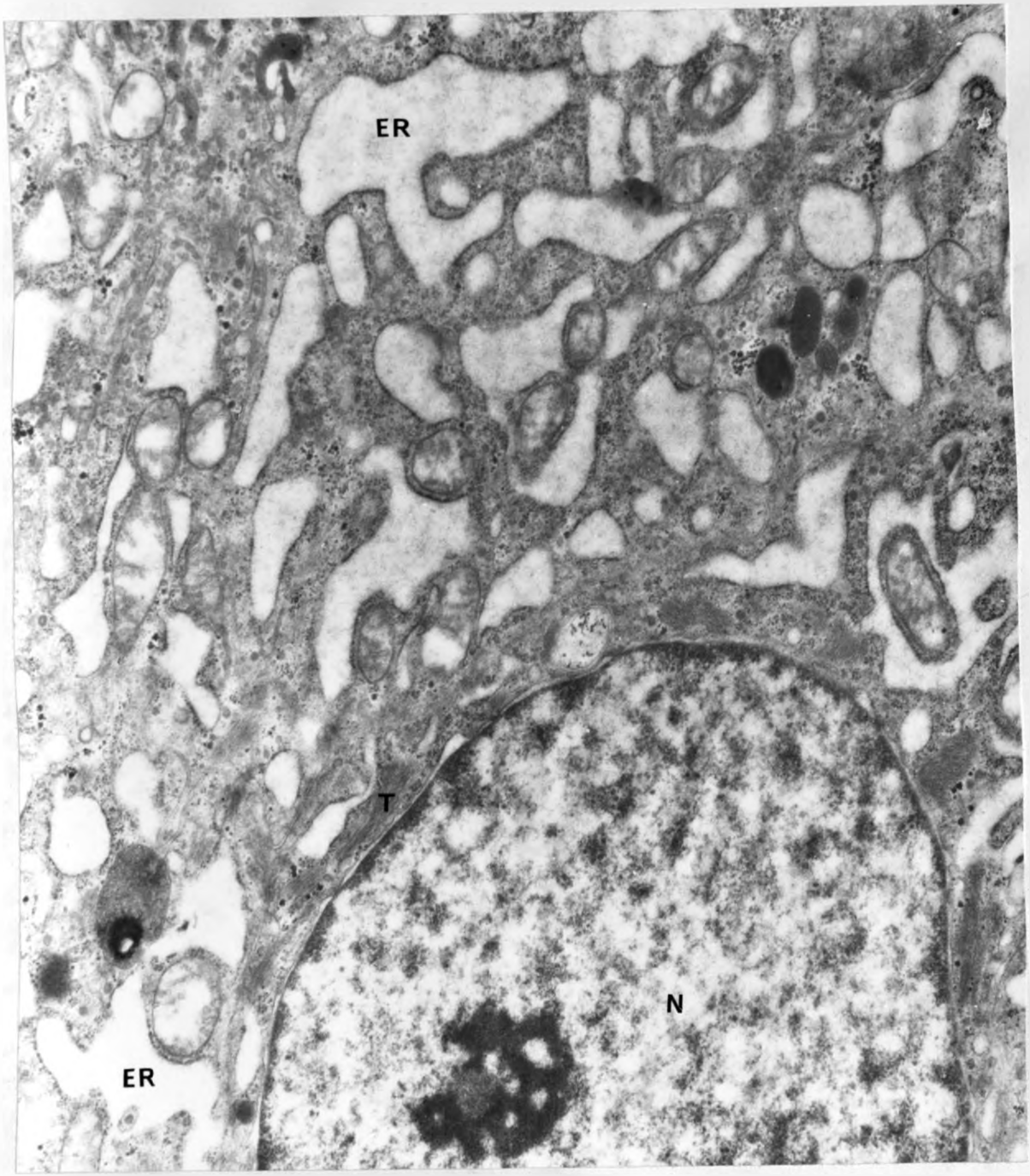

Fig.44: Sheep WXI - Adenomatous cell with large nucleus (N), irregular and markedly dilated rough-surfaced endoplasmic reticulum (ER) and tonofilaments (T) around the nucleu. X 21,000.

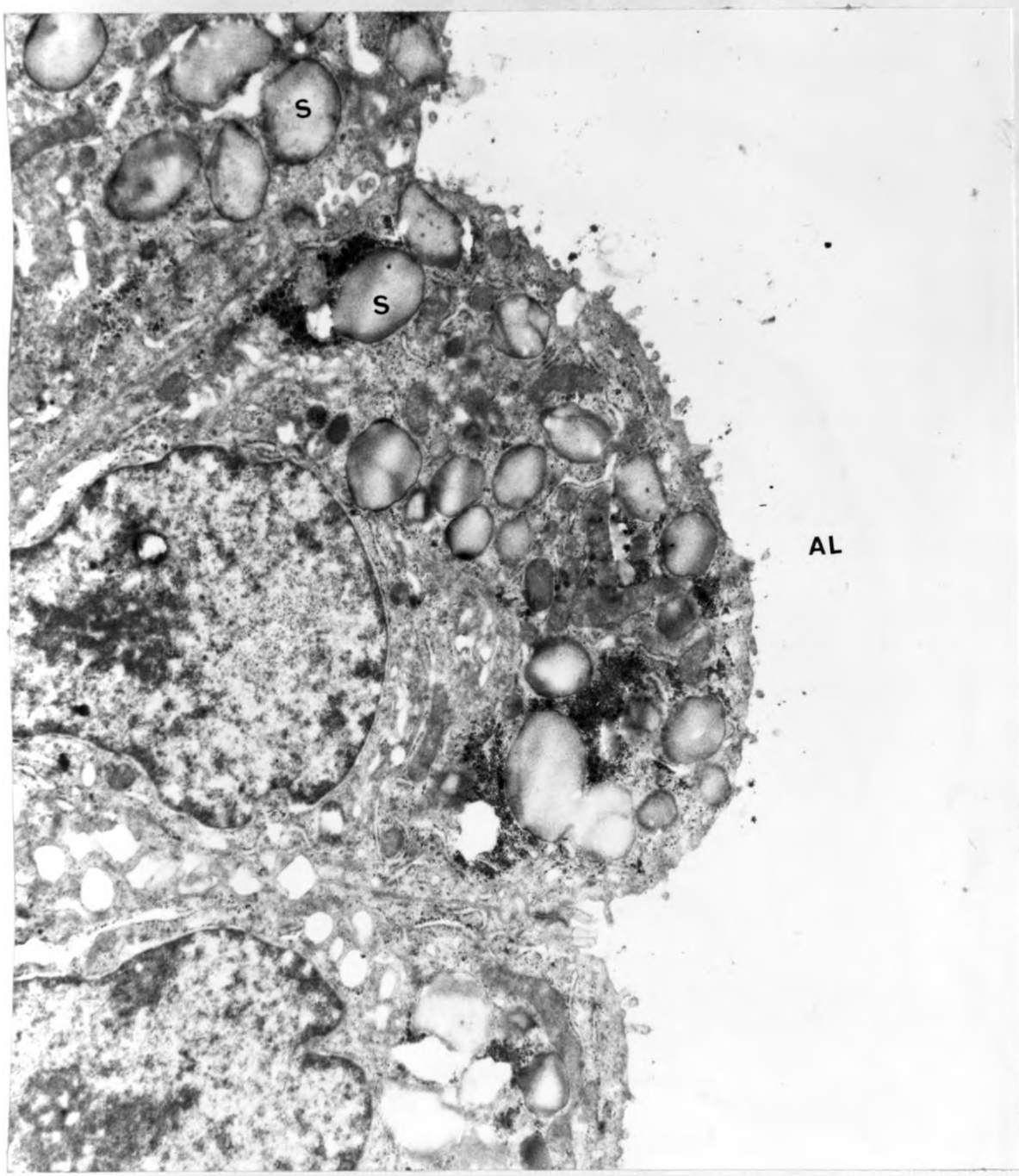

Fig.45: Sheep WXI - Three adenomatous cells lining an alveolus (AL) with many vacuoles full of secretion (S). There are also glycogen granules. X 15,000.

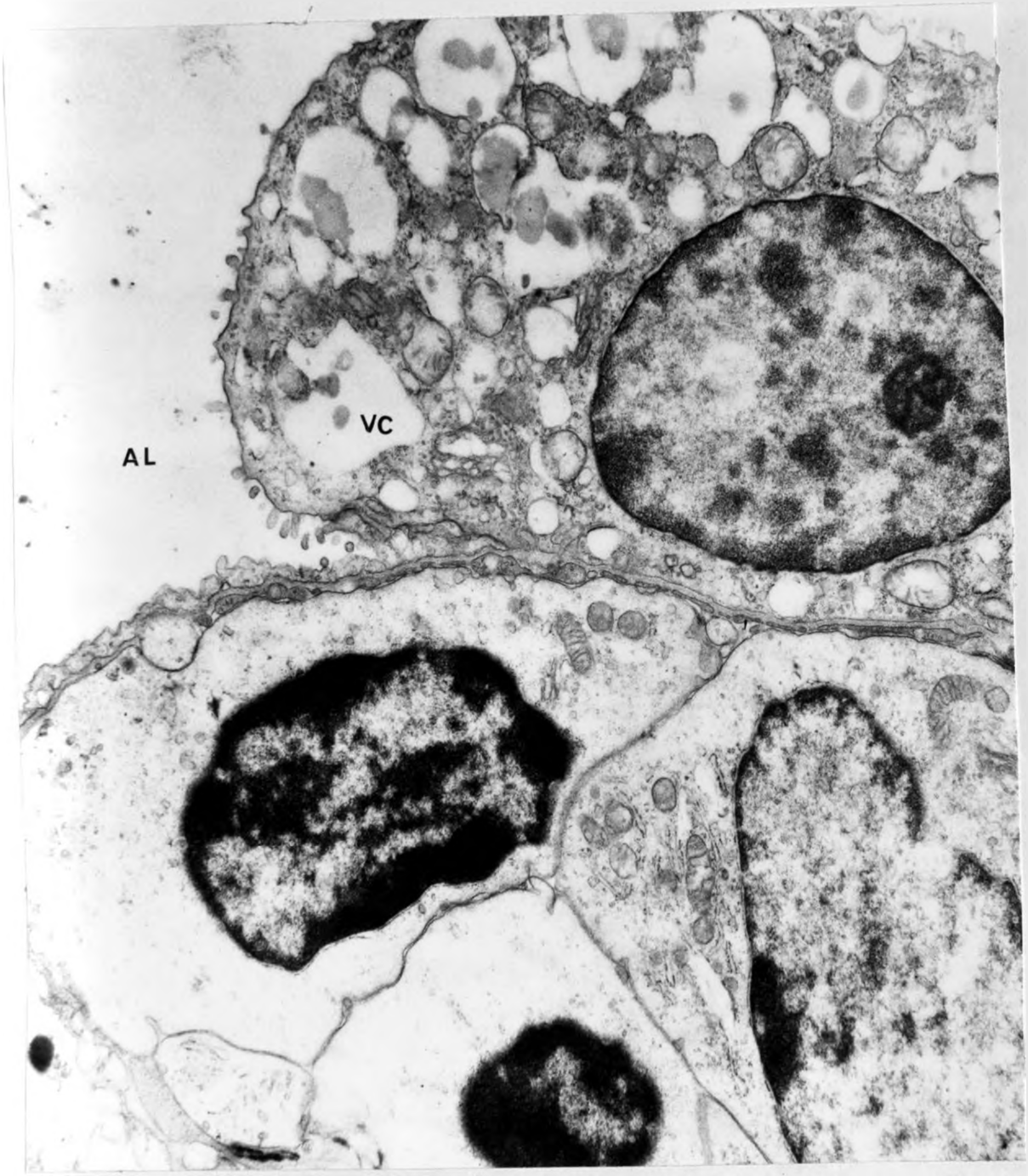

Fig.46: Sheep WV - One transformed adenomatous cell protruding towards the alveolar space (AL) with numerous vacuoles (VC), some of which contain granules. Microvilli are evident. X 13,800.

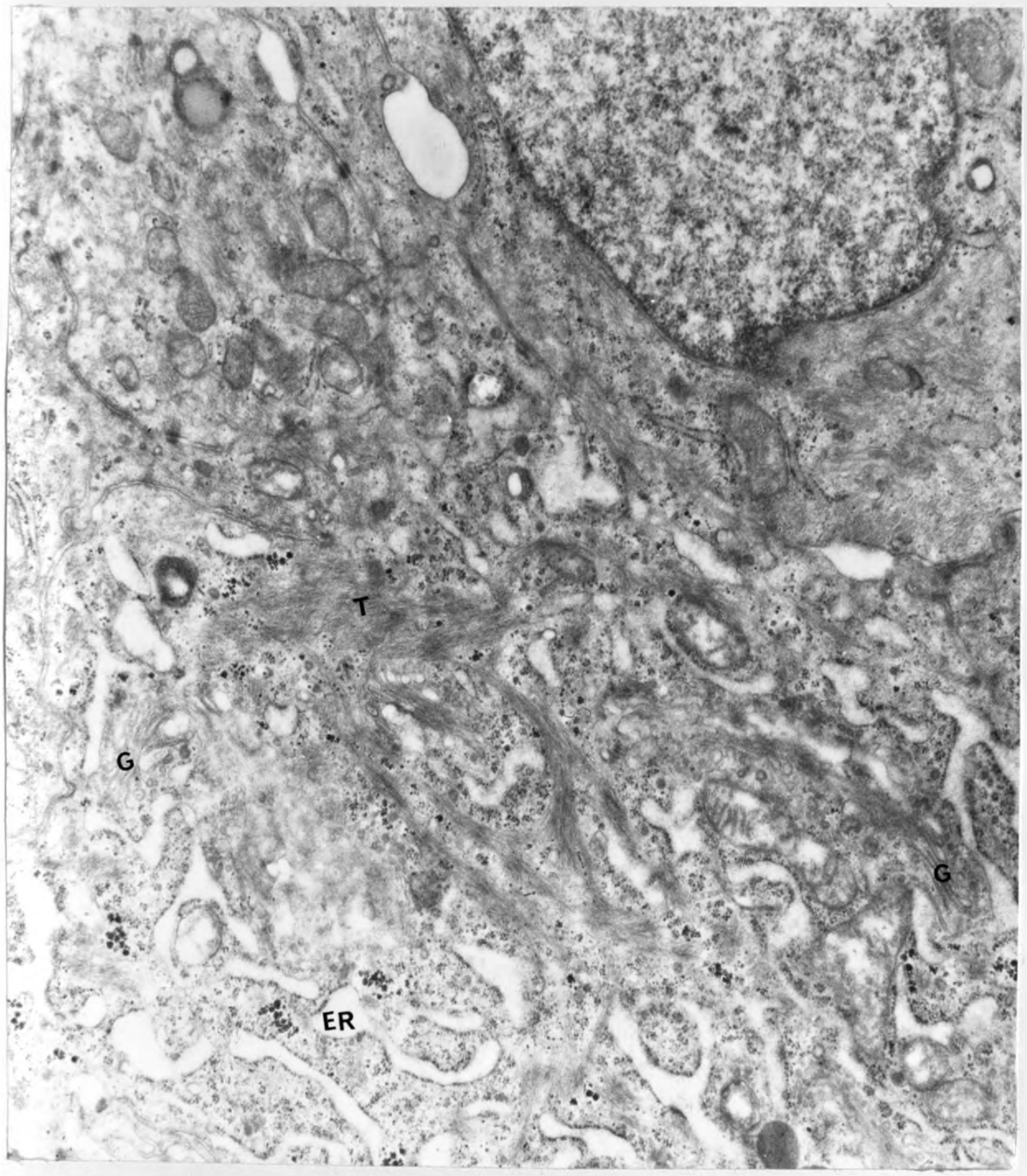

Fig.47: Sheep WXI - Adenomatous cell with multiple Golgi apparatus (G), dilated rough-surfaced endoplasmic reticulum (ER) and tonofilaments (T). X 21,000.

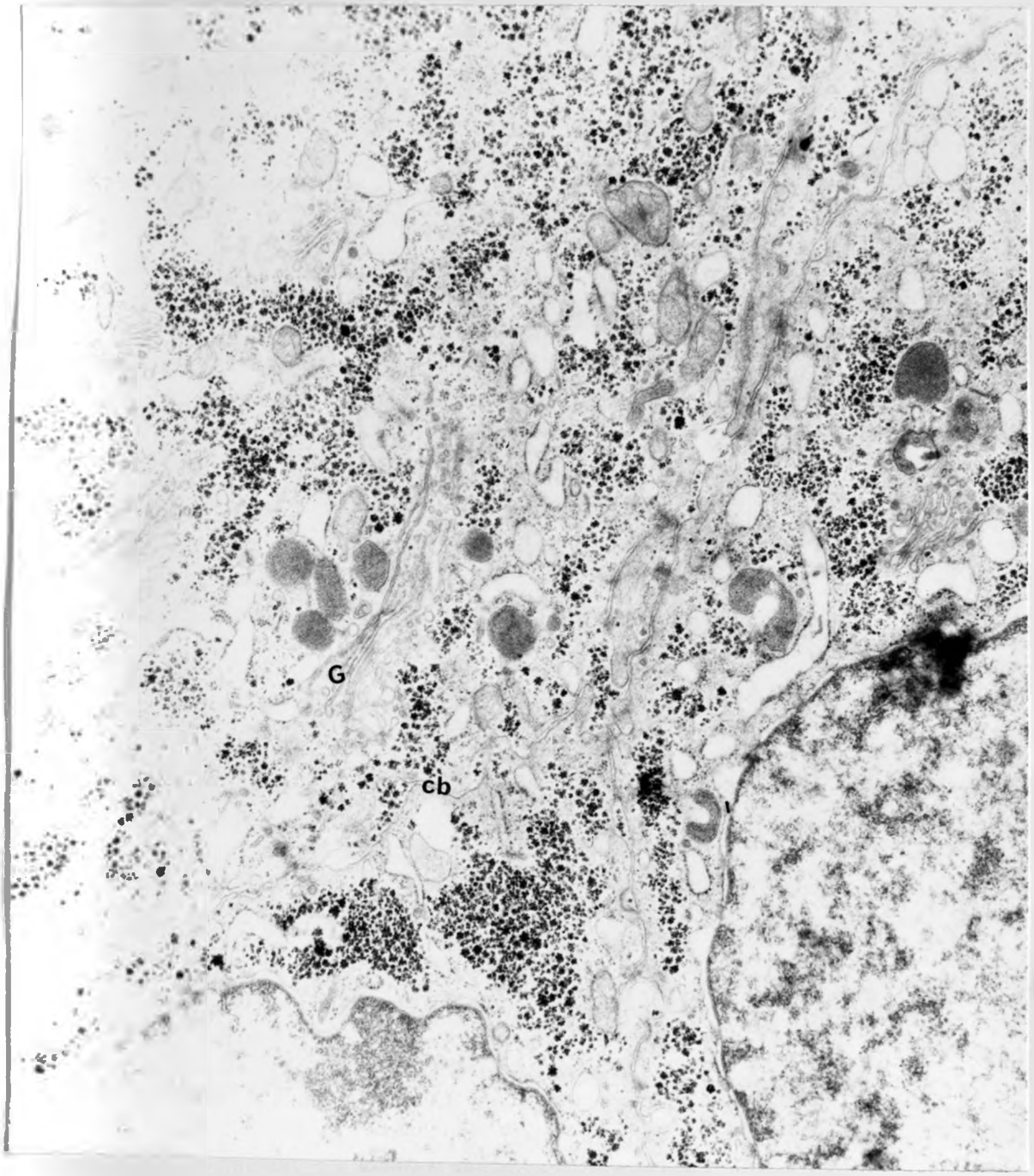

Fig.48: Sheep WXI - Tumour cells with numerous glycogen granules, irregular cell borders (cb) and multiple Golgi apparatus (G). X 21,000.

(Figure 53) especially in disintegrating cells. A few sections showed two adenomatous cell types, light and dark cells (Figures 36 & 37) with some of intermediate type, irrespective of the presence of vacuoles. The dark cells contained more, while the light cells contained fewer cytoplasmic organelles particularly ergastoplasm and ribosomes. Lysosomes were very rare. Where present nucleoli were very prominent.

While some nuclei were eccentrically located the majority were centrally placed. They were large, some ovoid, and others rounded often with scanty peripheral chromatin. The majority had regular nuclear membranes, but a number of cells had indentations in their nuclei with a few having perinuclear spaces.

Chlamydiae were seen in one case (WIII) in a sac-like structure made by two adjacent tumour cells. The various developmental forms were contained in a sac just beneath the plasma membrane (Figure 49), some were oval, and others spherical. They ranged in size from 95 mu to 184 mu. They had some similarity to those illustrated by LITWIN et al. (1961). In addition to these Chlamydiae, there were virus-like particles (Figures 50-54) observed in every grid made from lungs of sheep WXI. These particles were found in the intercellular and in the alveolar spaces.

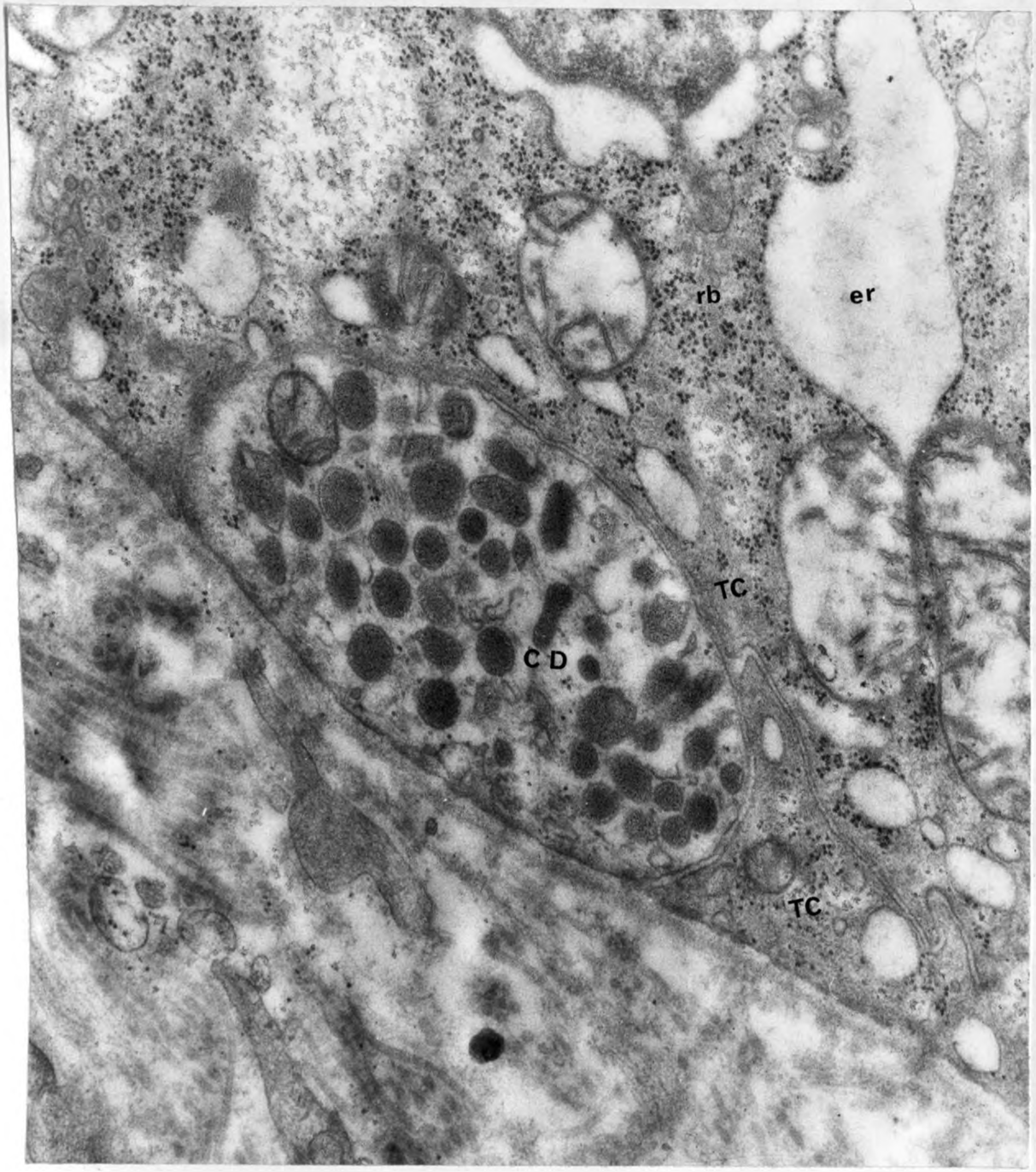

Fig. 49: Sheep WIII - Two tumour cells (TC) forming a sac in which are Chlamydiae (CD). There are free ribosomes (rb) and dilated endoplasmic reticulum (er). X 38,400.

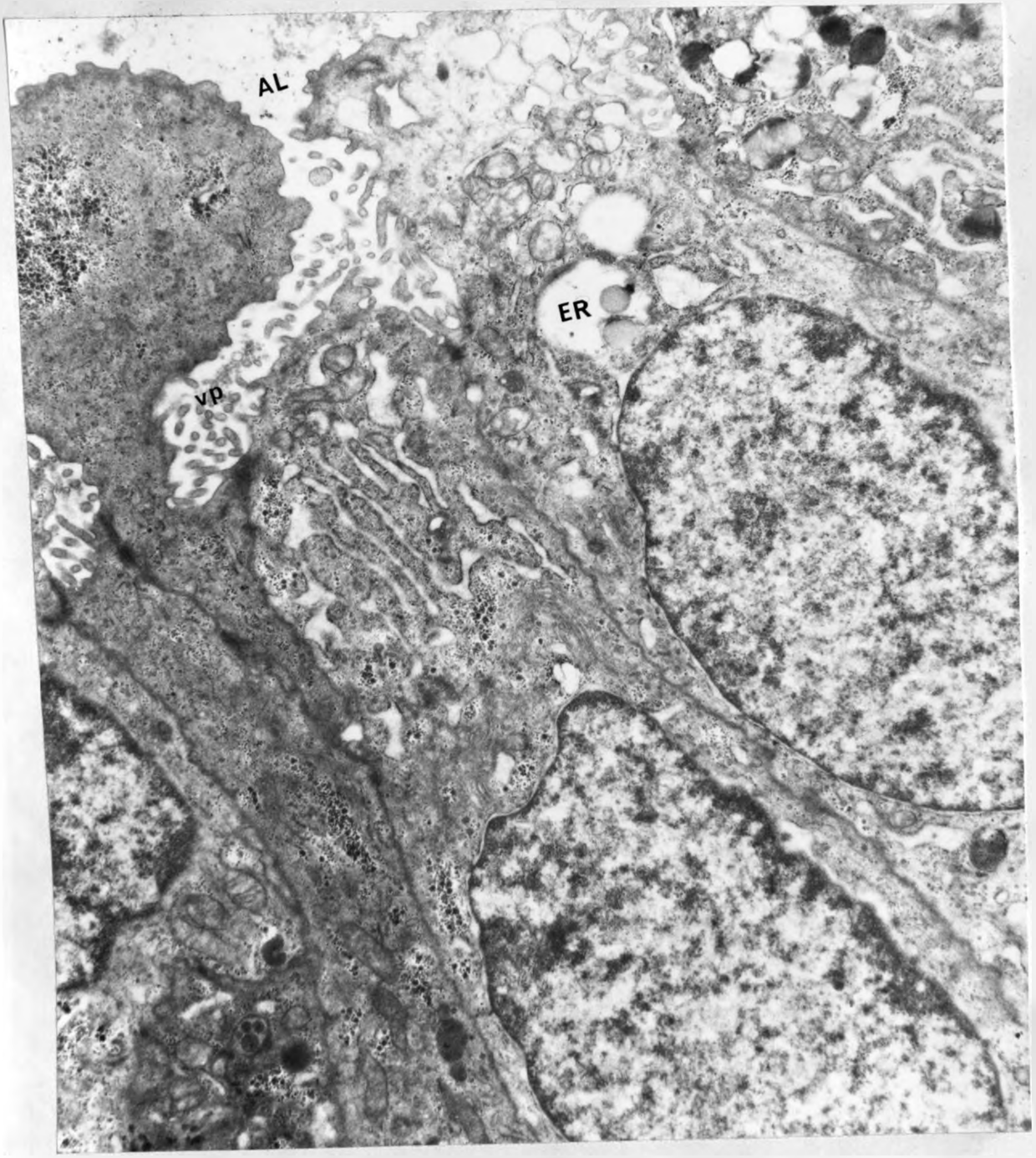

Fig.50: Sheep WXI - Columnar tumour cells around an alveolus (AL) with virus-like particles (vp). There are also glycogen granules and dilated endoplasmic reticulum (ER). X 13,500.

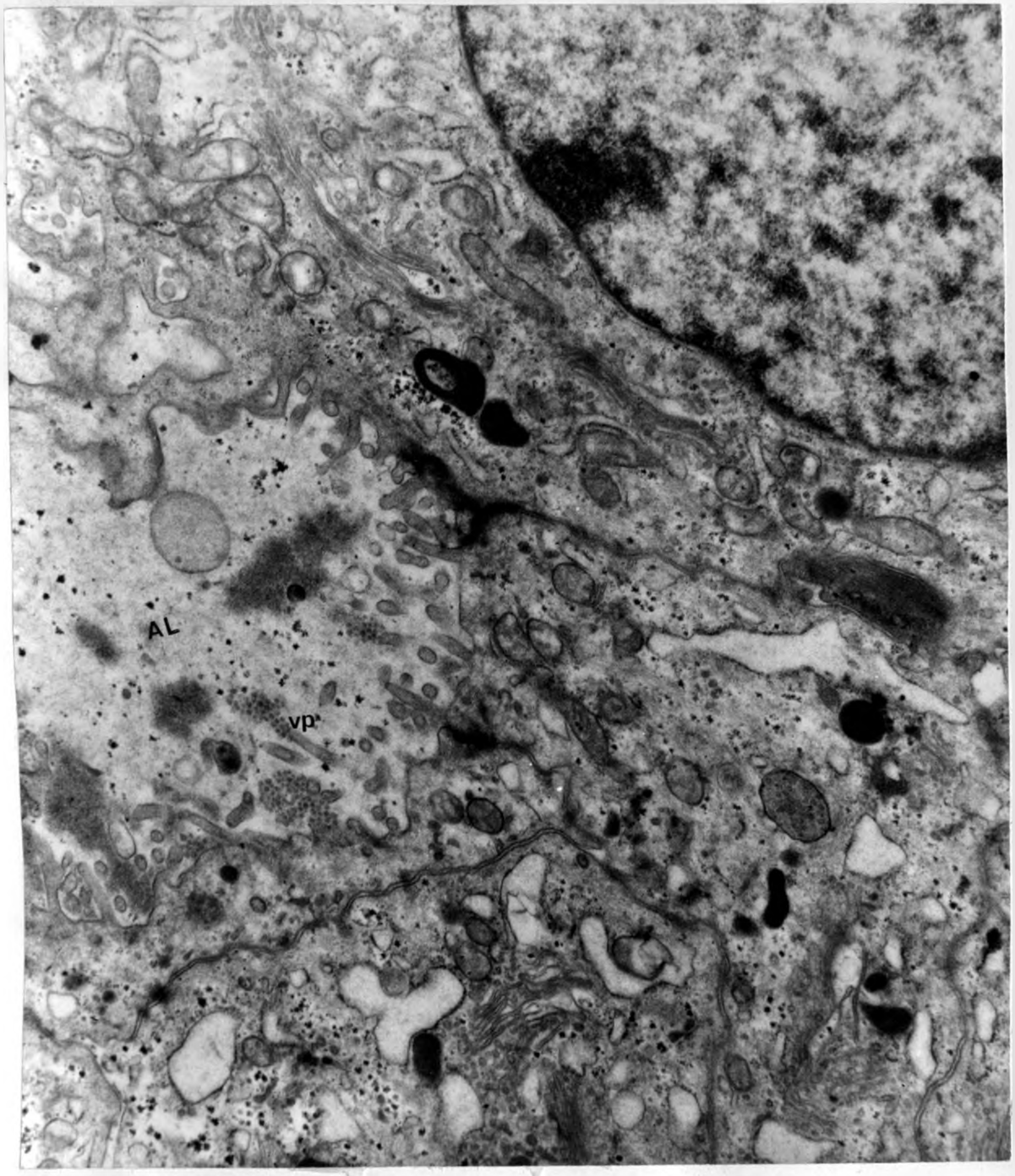

Fig.51: Sheep WXI - Virus-like particles (vp) in an alveolus (AL), close to microvilli of tumour cells. There are a few lysosomes. X 21,000.

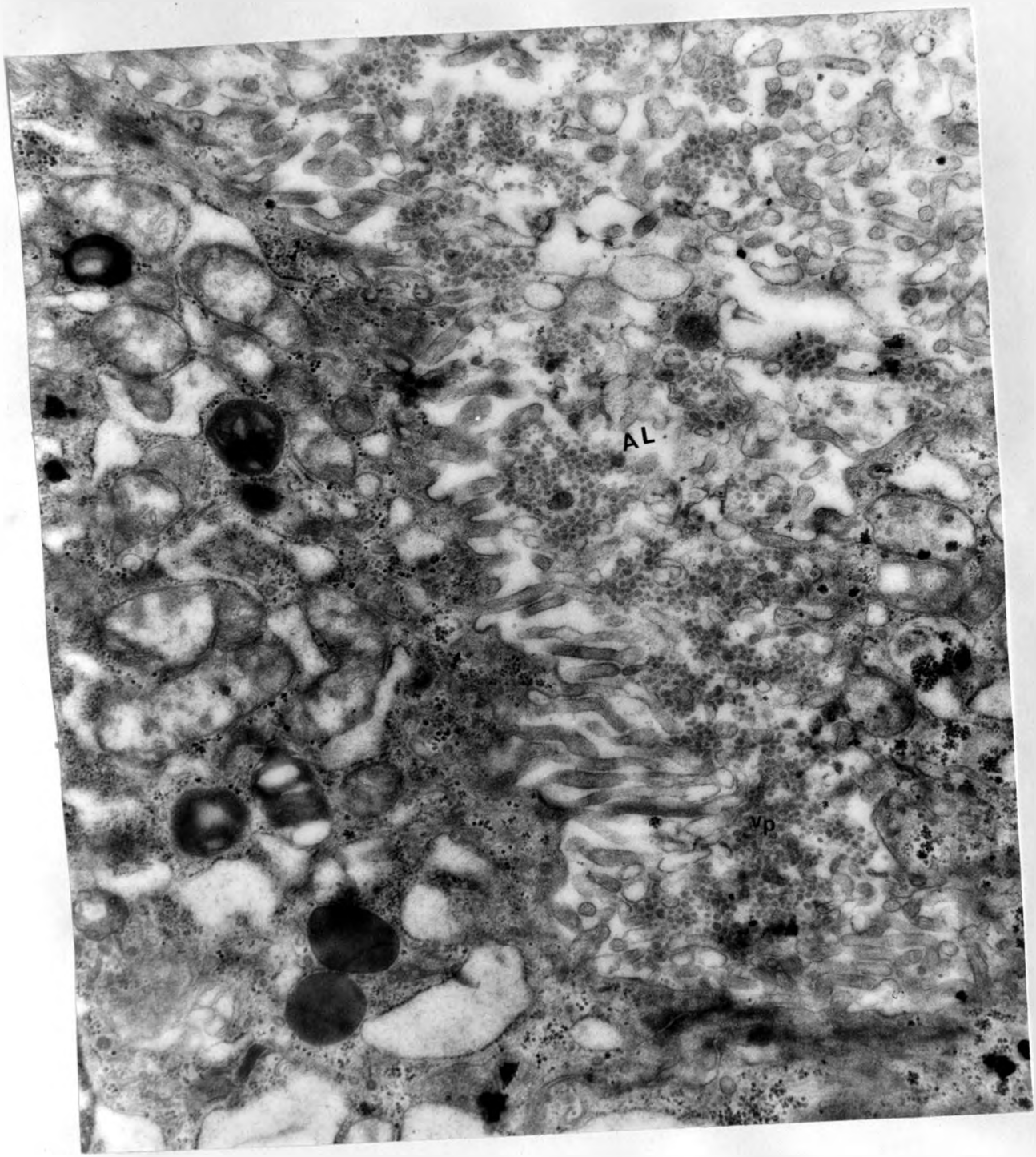

Fig.52: Sheep WXI - Aggregations of virus-like particles (vp) in an alveolus (AL) and close to microvilli of tumour cells. X 26,700.

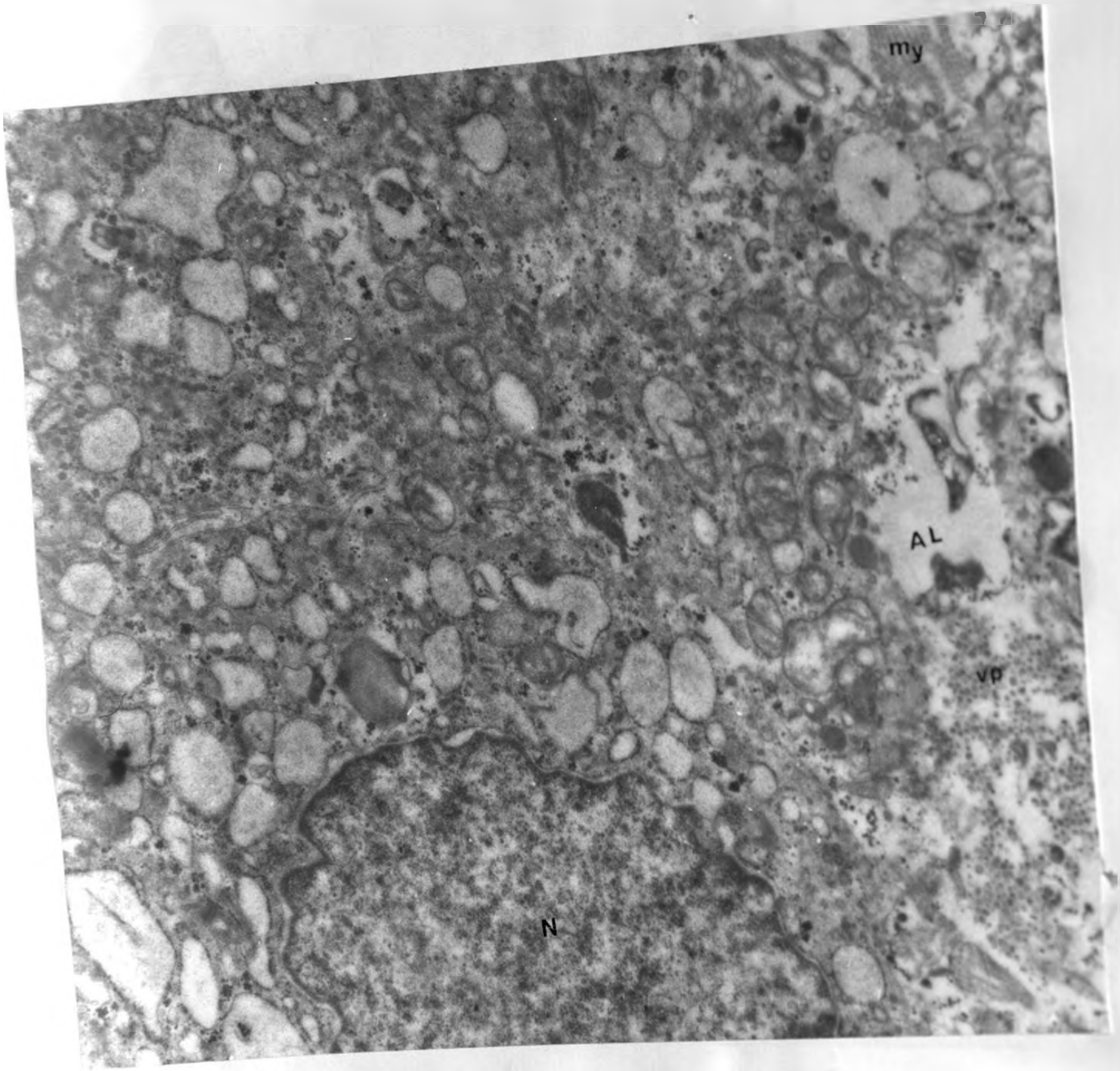

Fig.53: Sheep WXI - A disintegrating tumour cell with indented nucleus (N), vacuoles and aggregations of virus-like particles (vp) in alveolus (AL) - and a myelin figure (My). X 21,000.

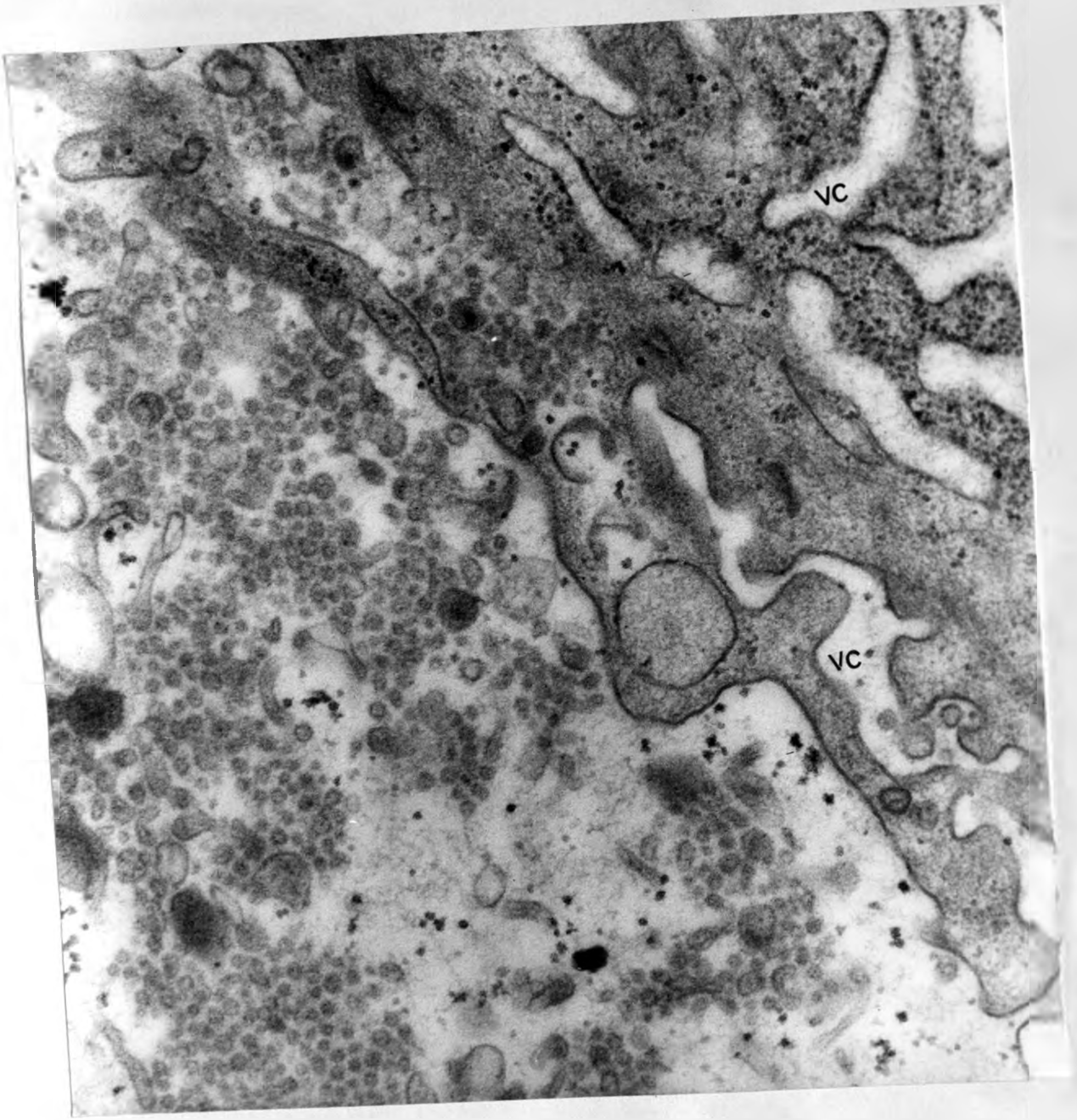

Fig.54: Sheep WXI - Spherical to oval virus-like particles close to plasma membrane. Numerous irregularly shaped vacuoles (VC) are present. X 57,000.

dilated endoplasmic reticulum ? ? ? ?

They were more numerous in the latter where they appeared as aggregations. Many of the virus-like particles found in the alveolar spaces were close to the surface of the plasma membrane. Some of the particles appeared as if they had formed from the tips of microvilli but not budding as such (Figures 51 & 52). In some alveolar spaces the virus-like particles were mixed with what appeared as secretion. These particles were not quite cubic as they appeared to vary in size (Figure 54) at high maginification, ranging in diameter between 46-80 mu.

Fibroblasts and bands of collagen were found in two cases in close association with tumour cells - the so-called chronic "static" foci. In all the tumour cells examined none showed any intracellular inclusion bodies nor Mycoplasma. No clear-cut virus particles were seen intracellularly. The latter was seen in the alveolar spaces of a few cases. Occasionally macrophages were observed in the alveolar spaces, with many vacuoles and phagosomes. None showed any virus-like particles nor inclusions.

III. DISCUSSION:

The submicroscopic observation of the morphology of the SPA lungs shows ultrastructural cellular organization that tallies in most respects with that of other carcinomal cells

(OBIDITSCH-MAYER and BREITFELLNER, 1968). The hypertrophy of granular and agranular ergastoplasm results into greatly distended vesicles. These vesicles correspond to the vacuoles demonstrable with light microscopic techniques and are evidently associated with the P.A.S. positive watery mucinous fluid found in such lungs. The hypertrophy of endoplasmic reticulum explains how the normally attenuated alveolar epithelial cells swell when infected with SPA agent.

The ergastoplasm and other organelles in SPA cells were markedly abnormal in the majority of cases. A few cells were almost devoid of them. Well developed rough and smooth-surfaced endoplasmic reticulum may indicate sites of slowest tumour growth. Adenomatous cells showing this type of cytoplasm were mainly those forming foci of tumour tissue surrounded by fibrous tissue - the "static" ones.

The electron microscopic study does further confirm SPA as a lung tumour with many morphological features similar to those seen in squamous cell carcinoma of the human cervix (BHOWMICK and MITRA, 1968), liver carcinoma (TOKER and TREVINO, 1966) and lung carcinoma (GELLER and TOKER, 1969) in human beings. Apparently cells of this tumour arise as a result of transformation and stimulation by the SPA agent without showing any degenerative changes.

Virus-like particles in this investigation ranged in size between 46-80 mμ. This variation may dictate against these particles being virus. It is not unusual to find virus particles which do not have a cubic morphology showing variation in their sizes as occurs in certain arboviruses like Nairobi sheep disease (TERPSTRA, 1969). Our particles - if they are virus - then the agent is not spherical in shape, but different such as being oval. The virus-like particles were observed outside the cells and hence may have no relation to tumour growth.

Using histochemical means, ALIEV (1967) found a marked increase in ribonucleic acid (RNA) content of tumour cells of SPA compared to that of normal lung tissue. The fact that only one case, sheep lung WXI showed presence of virus-like particles is not remarkable as they are rarely seen even in such virus caused tumours as Marek's disease and avian leukosis.

Whether the observed particles are virus, and if so whether they are causative agents or only secondary in sheep lung tumour is too early to tell. Herpes-like agent investigated in Scotland (MACKAY, 1969; SMITH and MACKAY 1969) was not observed. This problem requires more investigations. Chlamydiae occasionally found are unlikely to be the causative agents.

CHAPTER 6

GENERAL DISCUSSION

I. AETIOLOGICAL STUDIES:

Agents involved in the causation of alveolar epithelial cell transformation with progressive adenomatous process have not been completely elucidated. The lungworm theory has been discarded. In any case DUNGAL et al. (1938) could not find any correlation between lungworm infestation and sheep pulmonary adenomatosis. Several commonly encountered bacteria, especially Pasteurella haemolytica are secondary invaders.

The role of Mycoplasma with respect to the SPA process is doubtful. It is well-known that nearly all domesticated animals harbour benign parasites in the upper respiratory tract, and that some animals may harbour potentially pathogenic Mycoplasma in the same site. Out of well over 50 known species of Mycoplasma (POLAND, 1969) only a very small proportion are frankly pathogenic. The majority are of low pathogenicity or even saprophytes like M. laidlawii. Indeed a number of them have been isolated from cases of human leukemia (FALLON, 1966, HAYFLICK and KOPROWSKI, 1965, LEACH and BUTLER, 1966), and found as contaminants of continuous cell line tissue cultures (TULLY, 1966). Forty

isolates (KRAUSS, 1969 - unpublished data) had been made from nasal discharges of healthy slaughter sheep originating from a district in Kenya where pulmonary adenomatosis is unknown to occur. Mycoplasma have been isolated from sheep lungs with pasteurella pneumonia (GREIG, 1955, WANDERA, 1968) and with Maedi (WANDERA, 1970). The available evidence suggests that certain disease conditions including respiratory infections and certain tumours, produce substances suitable for the rapid growth of Mycoplasma. In the case of SPA, the mucinous fluid produced by the adenomatous cells is utilized by these micro-organisms, but Mycoplasma are not oncogenic agents. In turn, the presence of these Mycoplasma appears to attract macrophage response.

Whether Chlamydiae fall in the same category as Mycoplasma is too early to tell. But the fact that a number of sera from sheep flocks in Kenya where sheep pulmonary adenomatosis has not been reported had high antibody titres would indicate that this organism is not the aetiological agent of SPA. Pathogenic chlamydial agents cause disease (pneumonia, conjunctivitis, and polyarthritis) in young lambs apart from abortion in ewes and cows. In a review, TAMARIN (1969) showed that Chlamydiae cause about seven different conditions, and that the organisms

have been isolated from faeces of apparently normal sheep. Pneumonia is one of the conditions caused, which occurs mainly in calves and lambs. In calves (WILSON and THOMSON, 1968) the pneumonia is proliferative and exudative. Lambs show a proliferative type characterized by interstitial pneumonia often with lymphoid hyperplasia (PAVLOV, 1967; STEVENSON, 1968). On the other hand there is no age preference (CUBA-CAPARO, et al. 1961; WANDERA, 1968) with respect to pulmonary adenomatosis.

A clear aetiologic diagnosis through isolation, identification, and transmission of SPA agent from this respiratory disease cannot be made yet. The virus-like particles encountered in one sheep are of little significance as they were not seen inside tumour cells. However despite the failure to demonstrate causal agents, the available evidence point to a virus aetiologic agent. This agent is not the same as the herpesvirus isolated (MACKAY, 1969; SMITH and MACKAY, 1969) in Scotland. Intranuclear inclusions of herpes type of virus were not seen in 8 cases studied by electron microscopy either in the tumour cells or macrophages.

The causal agent of SPA persists at varying concentration in transformed cells, as well as being continuously liberated into the lung fluid (DUNGAL, 1946), and can pass through a

gradocol membrane with pores 0.9 u in diameter. It can remain viable in affected lung tissue when stored at -20°C. for at least 52 months (SIGURDSSON, 1958).

There is now an impressive list of malignant neoplasms in which a virus entity plays a causal role, for example the leukemias and sarcomas of chicken and rodents and leukemias of cats. Though no clear-cut virus particles were identified in this study this does not mean that no virus is involved in the causation of the disease. For while Marek's disease is known to be caused by a virus (EIDSON, et al. 1969), virus-like particles were not seen by WIGHT (1969). Also LINDBERG (1968) could only see sparse virus-like particles in 3 of 5 Rous rat sarcoma tumours induced by Rous virus strain Schmidt-Ruppin (SR-RSV).

More confirmatory evidence of virus aetiology in case of SPA will add more information on a causal relationship of viruses and certain cancers in large mammals including human beings.

II: CLINICAL RESPONSE OF TRANSMISSION EXPERIMENTS:

As sheep in Experiments 3 and 4 showed no distinct lesions of the disease and those of Experiment 1 had no symptoms, only results in Experiment 2 will be further discussed here.

Pyrexia was not a feature of the disease. Sheep that showed temperature rise above the normal had secondary bacterial pneumonia. This was confirmed by the fact that antibiotic treatment of sheep that had fever reduced the temperature to normal. Secondly, Pasteurella haemolytica was isolated from such cases when they later died or were killed.

Symptoms of rapid breathing, dyspnoea, coughing, secretion from the lungs and moist rales typical of the disease are only manifest when the lung condition is advanced. They were shown by 7 out of 10 sheep of Experiment 2. These clinical signs as tabulated in APPENDIX "A", were observed over a period of months. Increased respirations (40 and 43 respectively) were showed by sheep 3616 and 3645 at day 107 after infection or just over 3 months. By 151 days after inoculation coughing could be detected in sheep 3616, moist rales in sheep 3616 and 3645, and watery discharges from the lungs of sheep 3645. Already by this time 4 of the 10 sheep were showing signs of the clinical disease. Sheep 3616 (at day 175) and sheep 3615 (at day 178) were killed in extremis, suffering from severe effects of the disease such as anoxia. Sheep 3615 had slight fibrinous pneumonia. This may partially have worsened the effects of the

adenomatous process. Sheep 3616 had no fibrinous pneumonia, and no bacteria were isolated from its lungs. Hence its sickness must have been essentially due to the disease of sheep pulmonary adenomatosis.

The immediate cause of death in sheep 3608 appears to be secondary fibrinous pneumonia, although the lung area covered by adenomatous tissue was such that it would eventually have killed it. In the 260 days of observation on sheep died from the disease in absence of secondary infection. However the four sheep (3615, 3616, 3617 and 3645) killed in extremis, would have died before the experiment was terminated. Thus in the experimental transmission, symptoms typical of SPA were observed in 7/10 of the sheep in less than 9 months after inoculation.

This had also been the experience of DUNGAL et al. (1938), DUNGAL (1946) and ENCHEV (1966, 1968). From this the incubation period could be regarded as being between 4-12 months. CUBA-CAPARO et al. (1961) had observed clinical SPA in lambs 3-4 months old. This is an indication of an even shorter incubation period.

III: PATHOLOGY AND PATHOGENESIS.

A proper appreciation of lung epithelial cell reaction to certain irritants calls for a clear understanding of its fine structure particularly of the pulmonary interalveolar walls. These septa are made up of at least three cell-types forming the barrier between air and blood. They are endothelial cells of the capillaries, connective tissue cells and the septal cells which have proliferative and phagocytic potency. The third type is the epithelial cell lining the alveoli. The longstanding controversy regarding the nature of the alveolar lining has been conclusively clarified by electron microscopy. It is composed of continuous extremely attenuated squamous epithelium. This modified cell-lining has been identified in albino rats (WATRACH and VATTER, 1959) and in most other mammals (Schulz, quoted by EPLING, 1964). EPLING (1964) demonstrated such a continuous layer in bovine lungs. The present study has revealed its presence in ovine pulmonary alveoli.

Electron microscopy also reveals that not all pulmonary alveoli are ventilated at any one time - some are artificially atelectatic.

Pulmonary adenomatosis of sheep is a malignant lung tumour brought about by the action of an infectious agent to the alveolar epithelium which would appear to be particularly susceptible to damage. The increase in cytoplasmic volume may in part be caused by an increase in nucleic acids and is indicative of enhanced protein synthesis.

With electron microscopy, it is apparent that the very marked swelling of affected cells was due to changes in the cytoplasmic organelles. One was the vesicular dilatation of the endoplasmic reticulum - in both the smooth-surfaced and rough-surfaced types. Such a change as WIGHT (1969) stated is characteristic of irritated cells. The others were the abundant ribosomes, prominent Golgi apparatus, and mitochondria indicative of increased enzymatic activity and protein synthesis for the internal requirements of the cells and for secretion.

Epithelium of alveolar ducts, bronchioles and bronchi is involved to a varying extent. Metaplasia of alveolar epithelium is preceded by a transient thickening of interalveolar walls. There has been some confusion in the past as to the earliest lesions. DE KOCK (1929a) was convinced that the earliest changes and hence primary lesions were the adenomatous proliferations involving alveolar epithelium. He observed no interalveolar wall thickening claimed by COWDRY (1925). MARKSON and TERLECKI (1964) and TUSTIN (1969) had mistakenly taken the same line of reasoning.

In support of Cowdry's view is the work of several authors (COWDRY and MARSH, 1927; SHIRLAW, 1959; ENCHEV, 1966 and WANDERA, 1968). The "slow pneumonia" - characterized by interstitial pneumonia found in 2/4 of SIGURDSSON's (1958) sheep actually exhibited this earliest reaction. The same inoculum had been used for all the sheep. The work discussed in this thesis agrees with Cowdry's view.

In the present study, several multifocal ~~glandular~~ adenomatous foci appeared to originate in "centres" of thickened interalveolar septa. Even where the thickening had subsided, many adenomatous foci were located, as it were, at intersection of several

interalveolar septa. The latter appeared to be suspending the adenomatous foci. Later metaplastic changes could be seen originating from epithelial cells of alveoli not involved in atelectasis. The various adenomatous foci become confluent, forming sheets of adenomatous tissue. Hence it is erroneous to think of SPA in terms of "nodular" and "diffuse" forms as JAKOB and KRAUSE (1965) and many other workers had tried to do. The various descriptions of SPA demonstrate the progressive nature of this disease, and should not be used to classify it into forms. The multicentric origin is followed by the enlargement and eventual confluence of the various adenomatous foci. With the available findings, it is no longer correct to equate the smallest adenomatous lesions with earliest Jaagsiekte change. Some smallest foci could be temporarily "static" and been like that for many months.

There has been a belief that adenomatous cells lining the alveoli had come from proliferating bronchiolar epithelium. While this is possible the various illustrations in this thesis clearly show an alveolar origin in most cases. There are in fact some sections where adenomatous nodules are adjacent to non-proliferative bronchioles. Proliferative bronchiolar epithe-

lium could be clearly distinguished from adenomatous nodules originating from the alveolar epithelium.

Where intrabronchiolar and intrabronchial proliferation occurs, the epithelial cells tend to form pseudo-stratified and stratified columnar epithelium (WANDERA, 1967b) particularly in advanced cases. Consequently there are varying degrees of luminal occlusion. This reaction while present in some sheep's lungs of the Transmission Experiment 2 was not very marked, like that found in some natural cases.

SPA is a progressive and specific infectious disease, and a special type of malignant lung tumour. Pulmonary fibrosis if any is a secondary reaction and not a precondition to the development of SPA as OMAR (1964) seems to have thought. SPA should not be regarded simply as hyperplasia, and referring to it as an "epithelialisation" ought to be discontinued. "Epithelialisation" is used to refer to the metaplasia of alveolar epithelium. But it is unsatisfactory as it conotes lining by epithelial cells of a previously non-epithelial cell-lined alveoli. What happens in SPA with respect to the extremely attenuated squamous epithelium lining the alveoli, is metaplasia first and followed by progressive proliferation. Besides SPA has a multicentric origin.

Masses of myxomatous tissue have been found in SPA lungs by the author in 2 natural cases. They were located in more advanced sections of cases and appeared to arise from where fibroplasia had crowded out tumour cells. The significance of this tissue is unknown. The tissue may be of secondary significance and possibly fibrous tissue that has been transformed into soft fibroma. The myxomatous tissue is not part of sheep pulmonary adenomatosis as thought NOBEL et al. (1969).

While no tumour metastases were found in the experimental cases, such lesions have been encountered by other investigators in regional lymph nodes (AYNAUD, 1 case, 1926; PAREDES 4 cases, 1953; CUBA-CAPARO et al. 3 cases, 1961; ENCHEV, 12 cases, 1963; STAMP and NISBET, 2 cases, 1963; MARKSON and TERLECKI, 1 case, 1964; SANTIAGO-LUQUE, 4 cases, 1963; NOBEL, et al., 6 cases, 1968; WANDERA, 3 cases, 1967b and one additional case to date) and in extrathoracic locations (ENCHEV, 1 case, 1963; NOBEL et al. 3 cases, 1968; TUSTIN, 2 cases, 1969). Calling it a "multiple" papilliform cyst-adenomata" (DE KOCK, 1929a) is unfortunate and misleading, first, because sheep pulmonary adenomatosis is certainly a malignant tumour, and secondly there are no cyst formations as such.

AYNAUD (1926) was the first to regard it as a lung carcinoma. The classification into adenomatous, fibrous and mixed (scirrhous) types attempted by NOBEL, et al. (1969) is incorrect. They seem to have regarded the secondary fibroplasia and the apparent soft fibroma formation as essential part of the SPA reaction. It should be remembered that in case of metastases of carcinomas, varying quantities of supportive tissue often are found.

Attempts to compare and classify it with certain lung tumours in dogs and man, especially with the so-called alveolar-cell carcinoma or human pulmonary adenomatosis, have been made (BONNE, 1939; SIMON, 1947; SPENCER et al. 1956; ENCHEV, 1963; DAHME, 1966). Although some cases of bronchiolar adenocarcinoma (NIELSEN, 1966) closely resemble histological appearances of SPA this comparison is ill-advised for, in pulmonary adenomatosis of sheep, not only is the alveolar epithelium involved but also that of alveolar ducts, bronchioles and bronchi. In fact, this disease tends to demonstrate the futility of permanent divisions of lung tumours. The arbitrary divisions should be used for descriptive purposes only. For the histology of many of the lung tumours in dogs and man is not conclusively indicative of their site of origin.

IV. CONCLUSION.

The results of Transmission Experiment 2 reveal clearly that pulmonary adenomatosis is a transmissible lung tumour. Of the 7 sheep with clinical disease only 2 sheep survived to 260 days after inoculation before being killed. However they had exhibited clinical signs characteristic of SPA much earlier.

Because clinical cases have generally been recorded in adult sheep, it had been regarded by some workers as a disease with a very long incubation period. Clinical cases observed in lambs 2-4 weeks old (DUNGAL, 1946; SHIRLAW, 1959) possibly suggestive of intra-uterine infection. But as there is no indications that histopathology was done, these reports could be inaccurate. However, DUNGAL et al. (1938) produced clinical SPA within 7 months by contact and after 4 months following intra-pulmonary inoculation. The present work indicate that incubation period particularly in susceptible animals should be 6 to 12 months.

Attempts at the production of typical lung lesions, let alone the clinical disease have not always been successful in the past. While DE KOCK (1929b) was convinced that the disease

was contagious, his contact transmission experiments were inconclusive as he only produced small lesions in the lungs of apparently healthy sheep. Much later (DE KOCK, 1958), on the basis of further observations and transmission experiments carried out between 1929 and 1940, he suggested that susceptibility to the disease might be inherited. While this is not proven, inherited predisposition was shown in Iceland (DUNGAL, et al. 1938) where the Gottorp breed of sheep was the most susceptible, with up to 90% losses (though it was the commonest breed) in some flocks. On the other hand the Adalbol breed was conspicuously resistant, and on some severely affected farms only about 10% were lost.

According to HUTT (1964) natural selection, has produced stocks highly resistant to SPA in parts of the world where the disease has been known for a long time. He believes the high susceptibility of the Icelandic sheep could be attributed to the fact that they had not previously been exposed to Jaagsiekte. Indeed TUSTIN (1969) has observed in South Africa that resistance of affected flocks increases in proportion to the length of time that they had been in contact with infection. This tends to confirm what was observed in

Iceland (DUNGAL, et al. 1938) where mortality remained high in many flocks for the first two years after which it decreased gradually. This is in agreement with the situation in Kenya where originally losses were as high as 30% (SHIRLAW, 1959) in some flocks and have since dropped to between 1 and 5% in endemic flocks.

DUNGAL (1946) observed a few clinical cases in transmission experiments lasting 2-9 months, though SIGURDSSON (1958) in the same country could not reproduce the clinical disease after 14 months of observation. Whether the reason lies in the fact that he used few animals or the material from which the inoculum was prepared had been preserved for a very long time with consequent drop in titre is not known. Or, it may be that sheep used were more resistant to infection.

It would appear that despite a combination of various routes used by ENCHEV (1966) the inoculum applied intratracheally was responsible for the 5/16 of the animals with positive lesions in ~~the sheep's~~ their lungs. Two of the animals, at days 879 and 348 after inoculation respectively, showed clinical signs of the disease. In his more recent work (ENCHEV, 1968), 5/37 lambs developed lesions typical of SPA. Three of the positive cases died at

70, 280 and 322 days post-inoculation presumably from the disease, and 2 were killed in a subclinical stage 412 days post-inoculation.

MARKSON and TERLECKI (1964) using contact, spraying and intrapulmonary inoculation, were able to reproduce characteristic adenomatous lesions of SPA in 7 of 22 sheep observed for between 201 and 1034 days. One of their sheep, showed the clinical disease, 619 days after inoculation. The remaining 6 had small isolated SPA nodules when killed 380, 771, 786, 794, 864 and 1034 days after infection. CUBA-CAPARO et al. (1967) have shown that altitude is not a factor in the causation of the disease unless it is associated with overcrowding.

In the present study, no clinical disease developed in 3/8 sheep of Transmission Experiment 1 with SPA lesions nor any lesions in Experiments 3 and 4. It can, however, be claimed that for the first time, the clinical SPA disease has been reproduced in 70% of the inoculated sheep (Transmission Experiment 2) in less than 9 months. Breed susceptibility may have been a contributing factor. The source (fresh SPA lungs) and the way the inoculum was prepared (ultra-sonic treatment) its quantity and the way adeministered (intratracheally) were other determining

factors. The facts that lambs used were not of SPF category, that pneumonia was encountered in some infected animals and that a few suffered from malnutrition, do not alter the significance of the results.

In previous investigations (DUNGAL, et al. 1938; SIGURDSSON, 1958; MARKSON and TERLECKI, 1964; ENCHEV, 1966; WANDERA, 1968) the inocula were diluted to between 2-30%. In the present work, the inoculum (Transmission Experiment 2) was diluted only up to 57%, and either 5 ml. inoculated at this concentration or the volume doubled before inoculation. DUNGAL, et al. (1938) using 2 ml. of a 30% preparation, via the trachea, could only reproduce clinical disease in 1/3 of the sheep after 10 months; while MARKSON and TERLECKI (1964) using 8 ml. of a 10% preparation could only produce clinical disease in 1/10 of inoculated sheep after 619 days; and ENCHEV (1966) encountered clinical cases at over 300 days observation following 10 ml. intratracheal inoculation of 5% preparation. The sheep that they used may also have been less susceptible.

In this study, the intratracheal route being the natural one for most respiratory infections was preferred for Transmission

Experiment 2. In this way the inoculum spread to the majority of the lobes, their lobules and alveoli, and so a sufficient number of viable infectious agents were in direct contact with the majority of the most susceptible tissue - epithelium of the alveoli and alveolar ducts. The fact that the adenomatous cells were strongly P.A.S. positive indicates that they produce the watery discharge which has a high mucin content.

That metaplasia of the alveolar epithelium can also be caused by a variety of other factors, such as poisons of different plant origin, nematode infections, chronic bacterial infections, influenza and many others were brought out by COWDRY (1925) and GEEVER et al. (1943). This basic reaction of the alveolar epithelium to an irritant, and not infrequently follows prolonged pulmonary oedema, has been referred to as "epithelialisation" by OMAR (1964) and several other workers.

Unfortunately lesions caused by these irritants and encountered in several mammals have been referred to as "pulmonary adenomatosis". For when an interalveolar wall is fibrotic (thickened), there are often accompanying alterations

in the alveolar epithelium, which become metaplastic and hyperplastic. Although the histological appearances are adenomatoid, the lesion being of a limited hyperplasia is not adenomatous. Failure to recognise the distinction has resulted in confusing several conditions especially interstitial fibrosis with alveolar metaplasia, with SPA, a specific sheep disease.

Experience with many natural and the experimental cases under discussion clearly differentiates SPA from "pulmonary adenomatosis" described in cattle (SEATON, 1958, associated with feeding moldy feeds hence with pulmonary emphysema and oedema), in Chinchilla (HELMBOLDT et al., 1958; TRAUTWEIN and HELMBOLDT, 1967) and in many other mammals. The findings in Chinchilla showed that chronic pneumonia was a prerequisite to the development of adenomatoid lesions which is quite dissimilar to the pathogenesis of SPA. The multicentric origin, extent and distribution of SPA lesions and outpouring of mucinous fluids from the affected lungs are very distinct. Besides, the adenomatosis process is not a simple lining of the alveoli with cuboidal/columnar cells.

APPENDIX "A"

Transmission Experiment 2: Clinical Signs Exhibited up to 260 days of Observation Period.

Days Post-Inoc.	Sheep	Respiration and Rate/min.	Coughing	Nasal Discharge	Misc.
	3605	Normal	None	None	-
	3608	"	"	"	-
	3613	30	"	"	-
	3615	Normal	"	"	-
107 (29/2/68)	3616	40	"	"	-
	3617	Normal	"	"	-
	3633	"	"	"	-
	3645	43	"	"	-
	3648	Normal	"	"	-
	3711	"	"	"	-

Days Post-Inoc.	Sheep	Respiration and Rate/min.
	3605	DIED OVERNIGHT 13/3/68
	3608	Normal
	3613	30, Slight dyspnoea
	3615	32, Dyspnoea
137 (30/3/68)	3616	46, Marked Dyspnoea
	3617	Normal
	3633	Normal
	3645	42
	3648	Normal
	3711	"

"A" - Continued

Coughing	Nasal Discharge	Misc.
None	None	-
"	"	-
"	Slight	-
"	Slight	-
None	None	-
None	None	-
"	"	-
"	"	-
"	"	-

APPENDIX "A" - Continued

Days Post-Inoc.	Sheep	Respiration and Rate/Min.	Coughing	Nasal Discharge	Misc.
	3608	Normal	None	None	-
	3613	45, Dyspnoea & Irregular	"	"	-
	3615	35, Dyspnoea	?	Slight	-
	3616	54, Dyspnoea	Occasional cough	Moderate	Marked Nostril dilation. Moist rales
151 (13/4/68)	3617	Normal	None	None	-
	3633	30, Irregular	"	"	-
	3645	40, Dyspnoea	"	Profuse	54ml. Collected at the time. Moist rales.
	3648	Normal	"	None	-
	3711	"	"	"	-

APPENDIX "A" - Continued

Days Post-ino.	Sheep	Respiration and Rate/Min.	Coughing	Nasal Discharge	Misc.
	3608	26, Irregular	None	None	-
	3613	63, dyspnoea very irregular at times	"	"	Temperature 40.6°C. (105°F.) Antibiotic treatment applied.
168 (30/4/68)	3615	33, Dyspnoea	Present	Profuse	Marked nostril dilation; Temp. 41.1°C. (106°F.) Antibiotic treatment.
	3616	59, Dyspnoea	Slight	None	Moist rales.
	3617	Normal	None	"	-
	3633	33, Slight dyspnoea and irregular	"	"	-
	3645	38, Dyspnoea	None	Profuse	Moist rales.
	3648	Normal	"	None	-
	3711	"	"	"	-

APPENDIX "A" - Continued

Days Post-Inoc.	Sheep	Respiration and Rate/Min.	Coughing	Nasal discharge	Misc.
	3608	Normal	None	None	-
	3613	35	"	"	-
	3615	49, Marked dyspnoea	Occasional	Profuse	Moist rales. Killed in extremis 10-5-68.
	3616	56, dyspnoea	None	Moderate	Marked nostril dilation. Killed in extremis 7-5-68.
178 (10/5/68)	3617	Normal	"	None	-
	3633	"	"	"	-
	3645	40, dyspnoea	"	"	Moist rales.
	3648	37, dyspnoea	"	Slight	-
	3711	30	"	None	-

APPENDIX "A" - Continued

Days Post-Inoc.	Sheep	Respiration and Rate/Min.	Coughing	Nasal Discharge	Misc.
	3608	Normal	None	None	-
	3613	35, slight dyspnoea	"	"	-
195 (27/5/68)	3617	Slight dyspnoea	"	"	-
	3633	DIED 13-5-68			
	3645	46, marked dyspnoea	"	Profuse	Moist rales.
	3648	42, dyspnoea	"	Slight	-
	3711	Normal	"	None	-
	3608	Slight dyspnoea	Occasional	Moderate	Moist rales
	3613	33, slight dyspnoea	None	None	-
	3617	34, dyspnoea	"	"	-
210 (11/6/68)	3645	44, marked dyspnoea	"	Profuse	Moist rales
	3648	40, slight dyspnoea	"	Moderate	-
	3711	Normal	"	None	-

APPENDIX "A" - Continued

Days Post-Inoc.	Sheep	Respiration and Rate/Min.	Coughing	Nasal Discharge	Misc.
	3608	DIED 22-6-68			
	3613	36, dyspnoea	None	None	-
	3617	48, marked dyspnoea	"	"	-
224 (25/6/68)	3645	52, very marked dyspnoea	"	Profuse	Moist rales
	3648	43, dyspnoea	None	Moderate	-
	3711	Slight dyspnoea	"	None	-
	3613	36, dyspnoea	None	None	-
	3617	KILLED IN EXTREMIS 26-6-68			
238 (9-7-68)	3645	KILLED IN EXTREMIS 29-6-68			
	3648	48, dyspnoea	None	Moderate	-
	3711	Slight dyspnoea	"	None	-

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