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A PATHOLOGICAL STUDY OF CHRONIC
PULMONARY DISEASE IN THE HORSE

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY IN THE FACULTY OF VETERINARY MEDICINE,
THE UNIVERSITY OF GLASGOW

BY

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ABBREVIATIONS

AB	Alcian blue
AF	Aldehyde fuchsin
BAPNA	Ben zoyl-DL-arginine-p-nitroanilide
CAO	Chronic airflow obstruction
CB	Chronic bronchiolitis
COLD	Chronic obstructive lung disease
COPD	Chronic obstructive pulmonary disease
CPD	Chronic pulmonary disease
DNA	Deoxyribonucleic acid
ECF-A	Eosinophil chemotactic factor of anaphylaxis
Hb	Haemoglobin
H and E	Haematoxylin and eosin
HID	High iron diamine
5HT	5-hydroxy tryptamine
IFAA	Isotonic formol acetic acid
LV+S	Left ventricle plus septum
MFO	Mixed function oxidase
3MI	3 methyl indole
MSB	Martius scarlet blue
PaO ₂	Partial pressure of arterial oxygen
PAS	Periodic acid Schiff
PCV	Packed cell volume (of blood)
PMN	Polymorphonuclear leucocyte
ΔP_{pl}	Change in intrapleural pressure during respiration
RV	Right ventricle
RVH	Right ventricular hypertrophy
SEM	Scanning electron microscopy
STIC	Serum trypsin inhibitory capacity
TEM	Transmission electron microscopy
WBC	White blood cells

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ACKNOWLEDGEMENTS

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DECLARATION

DECLARATION

The work described in this thesis was carried out by myself except for the clinical examination of the horses from the Royal (Dick) School of Veterinary Studies Edinburgh, the estimation of serum protein fractions in the alpha-1 antitrypsin studies in Chapter Six and the experimental infection of the foals with Parascaris equorum in Chapter Eight.

The results of the alpha-1 antitrypsin studies in Chapter Six have been published previously:

Serum antitrypsin activity in horses with chronic pulmonary disease. R.G. Breeze, Julia M. Nicholls, Jean Veitch, I.E. Selman, E.A. McPherson and G.H.K. Lawson (1977). *Veterinary Record*, 101, 146.

As have the results presented in Chapter Eight on experimental Parascaris equorum infection of foals:

A pathological study of the lungs of foals infected experimentally with Parascaris equorum.

Julia M. Nicholls, Hilary M. Clayton, H.M. Pirie and J.L. Duncan (1978). *Journal of Comparative Pathology*, 88, 261.

SUMMARY

SUMMARY

The main part of this thesis describes the detailed pulmonary pathology in 25 cases of naturally occurring chronic pulmonary disease in the horse. Further sections deal with a survey of alpha-1 antitrypsin levels in the horse, the development of an experimental model of the disease and a study of the effects of ascarids on the equine lung.

The main pathological lesion was bronchiolitis, a lesion affecting all the small airways less than 2 mm in diameter and characterised by epithelial hyperplasia, goblet cell metaplasia, peribronchiolar cellular accumulations and an exudate of mucus and cells in the lumen. Rather more than half the cases had pulmonary eosinophilia. Emphysema was confined to small areas in the cranial lobe and periphery of the caudal lobe. The extent of emphysema was assessed by inflating one lung from each case with fixative, slicing it into thin slices and examining it under a microscope. The number and type of goblet cells in the bronchial epithelium of five normal horses were compared with ten horses with CPD; there was no significant difference and in addition there was no hyperplasia of the bronchial submucosal glands. The disease in the horse therefore bears no pathological resemblance to chronic bronchitis and emphysema of man. On the basis of these findings it is proposed that the disease be known as chronic bronchiolitis.

Quantitative measurements of the number of pulmonary mast cells at various sites in the lung showed that seven out of ten horses with CPD had significantly increased numbers of mast cells in all parts of the lung except peribronchially when compared to normal horses; the remaining three had markedly decreased numbers except peribronchially. It was not clear whether the mast cells were part of an allergic reaction or merely markers of inflammation.

Horses with CPD had similar alpha-1 antitrypsin values to normal horses. A deficiency of this in man predisposes to the early development of emphysema.

An experimental model of chronic bronchiolitis was produced in foals by the oral administration of 3-methyl indole and an eosinophilic bronchitis and bronchiolitis was produced by infecting foals with Parascaris equorum larvae.

INTRODUCTION

GENERAL INTRODUCTION

The main object of the work was to investigate in detail the pathology of chronic pulmonary disease in the horse. This, as a clinical entity, has been recognised and described for many years; it is believed that Aristotle in 333 BC gave the first brief clinical description of the signs of disease which he referred to as "heartache". Since this time there have been numerous clinical descriptions of the disease and some rather more limited attempts to define the pathology. More recently lung function tests and tests of allergy have been performed on affected subjects in an attempt to determine the pathogenesis of disease. The available literature to date has been reviewed in the first part of this thesis.

The project was a joint one complementary to a study at the Royal (Dick) School of Veterinary Studies, Edinburgh on the functional aspects of disease. The main part of the thesis deals in some detail with the pathological findings on 25 horses all with clinically diagnosed chronic pulmonary disease; about half of these were referred from the group at Edinburgh and the remainder obtained by myself.

Because the functional disability in the horse affected by CPD resembles to some extent that seen in chronic bronchitis and emphysema in man the disease in the horse has been likened to this pathologically. Chronic bronchitis in man has been extensively investigated because of its high morbidity, particularly amongst smokers; part of this investigation revolved around investigating the mucosubstances of the tracheobronchial tract and the changes that occur in the chronic bronchitic. These methods were applied to investigate the mucosubstances in the equine tracheobronchial tree to see if there was any resemblance to the human disease.

The equine disease has also been attributed to allergy or hypersensitivity to various environmental antigens. In order to ascertain whether there was any pathological basis for this observation the mast cells in the equine tracheobronchial tree were quantified in a number of normal horses and in a number of the horses in the series with chronic pulmonary disease. The mast cell, with its content of vasoactive and bronchoconstrictive amines is one of the cellular mediators of an asthmatic attack.

As a result of the pathological findings a revised terminology for the disease is suggested.

An inherited deficiency of serum alpha-1 antitrypsin is recognised in man which predisposes affected subjects to develop pulmonary emphysema. It was thought that this might have some relevance to the disease in the horse as it appears from clinical observation that certain individuals are more prone to the development of the disease. To test this hypothesis a serological survey of horses with no respiratory abnormality, horses with acute respiratory disease and horses with chronic respiratory disease was carried out and the results are presented in this thesis.

The natural disease in the horse does not lend itself readily to experimental study and a further section is devoted to the evaluation of an experimental model of the natural disease.

Finally, a study on the lungs of foals experimentally infected with Parascaris equorum is included. This induced an eosinophilic bronchitis and bronchiolitis, an effect which is found to some degree in a number of the natural cases.

The Expert Ferrier.

Hippophyl. **V** *What is good to prevent Blowing and Purfiveness in a Horse?*

Hippes. You must first understand Sir, the true nature of this griefe, as also how he came by it; whereby you may the more easily know how to cure the same, for that there be sundry wayes and meanes whereby a Horse may come to be breathlesse and short of winde, and every one of them may be a severall disease; and so require a distinct remedy. But if you meane a shortnesse of winde onely, then know that many Horses are naturally thicke winded, as being *cack-thropled*, narrow *Chawled*, &c. Also shortnesse of winde may come unto him accidentally, as when being *fat* and over-laden with *flesh*, or by being

too rank of *blood*, or by too much glut and foulness in the *body*, then is he subject to shortnesse of breath and purfiveness; so as upon any motion or exercise he will sweat, pant, blow, and heave at the *flanks*; and this cometh upon him by immoderate riding, eating, drinking, and rest. And such like exercise causeth the *panch* of the Horse (if he be put to any sudden motion or exercise) to be so hard and strutted out, as that he must so straine his *lungs*, (*the bellows of the body*) as to cause a dislocation in them, by meanes whereof they cannot execute their office or function as they ought; and if care and remedy be not speedily had, he will in short time be past all recovery; and then hee is brought to that disease which the vulgar doe call *broken-winded*: wherefore to prevent it, administer unto him this ensuing Cure.

Let all the hay he eateth, (nor let him eat all he desireth, for such Horses are commonly great feeders) be sprinkled and moystened with water, which will assuage his excesse of drinking, and very much coole his *blood*, which cannot but be inflamed. Then give him every morning for foure or five dayes together, two egges steeped twenty foure houres in the strongest white wine vinegar you can get: give him (I say) these two egges, and then the vinegar after, then ride him softly an houre after: which done, set him up warme, and three houres after, give him hay sprinkled with water, and at night when you do give him his Oats, wet them in Beere or good Ale, and let his drinke be white water. **¶** Doe this ten dayes together, so that about the beginning of *May*, and about *Michaelmas*, he may be in breath: and so keep him to spare dyet, but with discretion. **¶** This medicine will both purge him, and scowre from him much flegme and filth as well at *nose* as *mouth*, and he will be both sounder and in better health a long time after: provided he be also kept to moderate exercise. And if after you have thus drenched and dyeted him, you doe not perceive his blowing and lifting at the *ribbes* and *flanks* to cease, then be you confident your Horse is past all cure, onely still moysten his meat as before is inculcated, and he will hold out the longer. **¶** Another Receit I have for the same malady, which if he be not past all cure, will infallibly doe him much good, which is this, *viz.*

CHAPTER ONE

REVIEW OF THE LITERATURE

INTRODUCTION

In 333 BC in "The History of Animals" Aristotle described a disease of horses that he called "heartache" which was incurable and characterised clinically by a drawing in of the flank (Smith 1924). It seems probable that he was describing what has since become known as broken wind, heaves or chronic alveolar emphysema, a chronic pulmonary disease of all types of adult horse having an almost world-wide distribution. Since the time of Aristotle much has been written on the disease and many speculations and hypotheses have been voiced as to its pathogenesis based, in large part, upon anecdote, legend, inaccurate comparison to human disease and infrequent, inadequate pathological investigation.

A review of the literature on the subject reveals several major defects and omissions. Publications describe detailed clinical signs with no pathological detail or have detailed pathology with little or no clinical description. Experimental work is often accompanied by completely inadequate description of the animals involved. Alternatively, in an attempt to correlate the disease with one that occurs in man important differences have been ignored.

Thurlbeck and Lowell (1964) and Foley and Lowell (1966) are the only authors in recent years to have published the clinical findings in a group of horses with chronic pulmonary disease along with detailed ^{pathological} findings. Their series comprised 11 animals only.

In the British literature there is only one pathological description of the disease this century (Cook and Rosedale 1963) and this is of two horses only. The disease is a common problem in Britain with an incidence comparable to that of laryngeal hemiplegia (Anon 1965) but very little work has been published on it since the turn of the century.

OCCURRENCE

During the years that working horses were much more numerous and economically important than they are today, chronic pulmonary disease (CPD) was a frequent and well recognised cause of disablement or death, especially in heavy draught horses. This was reflected in the British veterinary literature about the turn of the century - these were reviewed by Smith (1924) with the decline in the number of working horses, the disease was almost totally

neglected by British writers and the only factual record of its modern incidence is included in the results of a British Equine Veterinary Association survey 1962-1963 in which 134 of 43,538 (0.3 per cent.) diseased horses examined by veterinary surgeons in that year were said to be suffering from "emphysema" (Anon 1965). About the same number were affected by laryngeal hemiplegia.

Morgan (1940) considered the disease to be less common in Britain than formerly and attributed this apparent decline to current methods of drying and storing hay, which largely avoided moulding. In our survey we found cases frequently and without difficulty; this would indicate that the disease is still a common problem.

Alexander (1959) claimed that the incidence of disease had decreased markedly in the United States of America during the previous two decades and was thus only rarely encountered - the evidence to support this was not provided. Animals over eight years of age were usually involved, both sexes were equally affected and only stabled horses or those given hay contracted the disease. Udall (1954) on the other hand, claimed that disease had become prevalent in most parts of the U.S.A. following the introduction of timothy, red clover, alfalfa and other cultivated grasses. Before this, Michigan and California had apparently been free of CPD. In his clinic nearly all the cases were over eight years of age, were in draught animals and disease was associated with stabling and hay-feeding.

Switzerland is the only country in which comprehensive surveys have been made of the prevalence of disease. Hug (1937) found that of the 88,367 cases seen in five years at his clinic in Zurich 1.42 per cent. had CPD, but he thought that the overall prevalence in the country was nearer 3 per cent. Most of the cases were at least ten or twelve years old when first referred to him. Gerber (1969) gave more recent information on the situation and concluded that CPD was by far the most common cause of premature loss through disease. The disease was usually first noticed at seven or eight years of age but there was no mention of any sex or work predisposition: 268 cases were examined in his survey. It should be noted that many of his cases were drawn from a local army remount station so it would be dangerous to draw any general conclusions from his survey.

Some figures are also available from Sweden, where horses may be

insured against the disease. In 1939, 466 horses were indemnified but only 21 in 1943 (Alström and Lauritzson 1953). The explanation for the two widely-differing figures was that 1938 was a very wet summer in which bad hay was made whereas 1942 was a very hot, dry summer after which good hay was available; this observation lent support to the view that the disease had an association with the feeding of mouldy hay. Unfortunately no indication was given of the total number insured or the proportion of the total horse population this represented.

TYPE OF ANIMAL AFFECTED

Several authors have made observations on the type of horse generally affected.

Cook and Rosedale (1963) stated that the disease was rare in British racehorses. Cook (1965) observed that ponies were especially likely to develop the condition because they were often stabled during the summer and fed poor quality hay to avoid contracting laminitis and sweet itch. Hunters were also susceptible because they were stabled for long periods and show horses because they were stabled during the summer which is the dustiest part of the year. Carlström and Alegren (1940) found that in Sweden the heavier breeds were often affected whereas thoroughbreds and Northern Swedish horses were rarely affected. Hug (1937) also stated that Swiss thoroughbreds were more resistant to bronchitis, "catarrh" and hence CPD than other breeds but this was later questioned by Amman (1939), working from the same clinic.

EPIDEMIOLOGY

CPD almost always affects individual animals although endemics have been reported from time to time. Morgan (1940) described one farm, where large numbers of horses were stabled throughout the winter, on which it was apparently rare to find a horse over the age of five years free of respiratory disease. More recently Mansmann and others (1975) described an endemic respiratory disease affecting several individuals in a group of 35 horses which were housed with chickens; skin and aerosol provocation tests indicated that this was a form of extrinsic allergic alveolitis to avian proteins.

It is often stated that CPD is a disease of civilisation and is only seen in horses which are stabled or given hay for part or all of their lives

(Udall 1954; Alexander 1959; Cook and Rossdale 1963; Gerber 1973). Morgan (1940) said that the disease was unknown in Venezuela where horses are not stabled; Udall (1954) that it was rare in Spain and Portugal and unknown in California and other warm, dry states of the USA before they were widely cultivated. Moreover disease is said to be associated with the feeding of poor quality or mouldy hay. Insurance settlements in Sweden and Switzerland were found to increase following a wet harvest (Alstrom and Lauritzson 1953; Udall 1954).

Atmospheric factors are said to play a role in exacerbations of the disease. Poor hygiene leading to a build-up of ammonia in the atmosphere (Hug 1937; Carlström and Alegren 1940), damp or cold (Carlström and Alegren 1940) or the Föhn, a seasonal warm wind in Switzerland (Ammann 1939) are among the factors which have been implicated.

The role of intercurrent contagious respiratory disease in the epidemiology of CPD is not clear and there have been no specific studies to identify the viral or bacterial pathogens which might be involved although Gerber (1973) found that 126 of his series of 268 horses (47 per cent.) had had earlier signs of an influenza-like condition. He also noticed that his clinic in Berne had many more referrals for CPD following the 1965 pandemic of equine influenza. Gillespie and Tyler (1969) stated that colds, pneumonia, bronchitis and influenza were not infrequently part of the previous history of CPD cases. If these are important factors it is perhaps surprising that the racehorse population has a very low prevalence of CPD (Mahaffey 1962; Cook and Rossdale 1963) since this is the section of the horse population most susceptible to viral respiratory infections.

By definition CPD is a chronic disease with a variably progressive course typically punctuated by remissions and exacerbations which are largely influenced by the individual management of the case. It does not seem to be a direct cause of death in the present day but there is evidence that it caused the deaths of many working horses in earlier times.

CLINICAL SIGNS

Equine respiratory diseases have for many years been divided into two main categories - acute and chronic. However, whilst the acute forms such as specific viral and bacterial infections have been well-classified and

characterised the chronic respiratory diseases, apart from specific entities like laryngeal hemiplegia, have been grouped under vague non-specific terms such as broken wind or heaves.

Only in recent years have any real attempts been made to divide CPD into distinct categories (Gerber 1973; McPherson and Lawson 1974). Previous to this most descriptions of CPD were substantially alike and did not take into account minor variations from the essential clinical picture of expiratory dyspnoea, cough, exercise intolerance and a chronic course.

Since 1853 when Percivall wrote on the subject it has been acknowledged that not all horses with CPD have emphysema. Despite this, however, many authors continued to call the disease "chronic alveolar emphysema" (Carlström and Alegren 1940; Alexander 1959; Gillespie and Tyler 1969). In an attempt to distinguish between horses with overinflation of the lungs and those without, Cook (1965) and later Sasse (1971) and Gerber (1973) described methods of percussing the horse's chest to determine the position of the posterior border of the lung, a technique earlier described by Carlström and Alegren (1940) who had used it to demonstrate the therapeutic effects of atropine on horses with CPD. More recently though, Cook (1976) has retracted this and now claims that the method is not sufficiently accurate. Gerber (1973) also noted that the presence or absence of overinflation detected by percussion bore little relationship to the morphological findings of emphysema at post-mortem. However, in the course of the clinical investigation of his cases of CPD he administered corticosteroids to the horses and on the basis of the subsequent response he claimed to be able to detect alveolar emphysema. If the lung percussion field failed to decrease following medication this was said to indicate emphysema. Emphysema is, by definition, the destruction and loss of interalveolar septa and is a permanent change. It is now recognised that the accurate clinical detection of emphysema is impossible (Thurlbeck 1976) so all these reports must be treated with caution.

Two forms of the disease were commonly described: 1. an acute onset of respiratory distress which later subsided but usually recurred at intervals, and 2. the usual chronic form with a slow onset of signs and little remission (Carlström and Alegren 1940; Udall 1954; Mahaffey 1962; Cook and Rosedale 1963; Gerber 1973). The acute form often progressed to become chronic

(Carlström and Alegren 1940; Cook and Rossdale 1963). The acute form, according to Cook and Rossdale (1963) was most often seen in thoroughbred brood mares during the summer months when they were brought in to be stabled for the night. Cook and Rossdale (1963) and Gerber (1973) likened these attacks to asthma in man. Lowell (1964) investigated six horses all with CPD and found that he could induce acute attacks by exposing the affected horses to hay, the horses having previously been kept on a hay-free diet. During the course of this present study various horses affected with CPD were exposed to mouldy hay and acute attacks were induced in some of these animals. Further details are given in the clinical section. The distinction between the two clinical forms of the disease may not be as clear as is supposed.

The cough was the most distinctive clinical sign of the disease. Most authors described a cough said to be typical of CPD. This was a short, dry, hollow, nonproductive cough (Alexander 1959; Mahaffey 1962; Muylle and Oyaert 1973) or short and feeble (Fitzwygram 1901). Cook (1965) however said that many types of cough were heard by him in horses with CPD. All writers on the subject agreed that it was rare to find a horse with CPD that did not have a cough.

The pattern of expiration was also said to be characteristic of CPD; it was biphasic comprising an initial passive phase followed by a secondary acute contraction of the abdominal muscle to expel the residual air (Amman 1939; Udall 1954; Alexander 1959; Mahaffey 1962; Cook 1965; Gillespie and Tyler 1969). In advanced cases this led to the development of a "heaves line" along the costal arch (Amman 1939; Alexander 1959) caused by hypertrophy of the musculature (Alexander 1959). A "barrelchested" appearance was noticed in very advanced cases (Cook and Rossdale 1963; Gillespie and Tyler 1969) and the rectum and vagina prolapsed slightly during coughing or the second phase of expiration (Mahaffey 1962; Cook and Rossdale 1963; Gillespie and Tyler 1969).

Reports of auscultation of the chests of horses with CPD are difficult to interpret because what is heard and how it is interpreted is essentially subjective. The American College of Chest Physicians (ACCP) recently published their recommendations on the uses of pulmonary terms and symbols in an attempt to standardise some of the discrepancies occurring in human

medicine (ACCP 1975). Two terms widely used to describe the chest sounds are "wheeze" and "rhonchus". The ACCP recommend that "wheeze" should be replaced by "rhonchus" in most cases and that "rhonchus" be used as a term to describe a short, interrupted, exploded sound (crackle). None of the papers on CPD attempted to define the sounds heard but the consensus of opinion was that adventitious lung sounds and increased breathing sounds were a constant feature of disease. "Wheezing", with or without the aid of a stethoscope, was heard by Cook and Rossdale (1963), Cook (1965), Gerber (1973) and Sasse (1971); "crepitations" on expiration by McPherson and Lawson (1974) and Cook (1976); rhonchi and rales by Carlström and Alegren (1940), Cook and Rossdale (1963) and McPherson and Lawson (1974); "crackles" by Cook (1965) and McPherson and Lawson (1974) and "creaks" by Cook (1965). The sounds heard in one individual varied from day to day (Muylle and Oyaert 1973) and although undoubtedly a wide variety of chest sounds are recognised in diseased horses the ACCP committee are of the opinion that little is gained from subclassification of the sounds auscultated.

A nasal discharge was a common feature of the disease (Udall 1954; Cook and Rossdale 1963; Muylle and Oyaert 1973; McPherson and Lawson 1974; Cook 1976). The nature of the discharge varied from clear serous to mucopurulent (Cook 1976). Cook also reported a "clicking" sound from the larynx in certain cases which may have been caused by an abnormal adduction of the larynx. This may be what Udall (1954) refers to as a "rattling in the head".

The respiratory embarrassment caused the horse to become exercise intolerant and it was often at this stage that a veterinary surgeon was first consulted. Depending on the purpose for which the horse was kept this may have been apparent at an early or late stage of disease.

Flatulence was a clinical sign of horses with CPD that has been consistently mentioned since the symptoms of disease were first described (Fitzwygran 1901; Udall 1954; Alexander 1959). The precise reason for this was not clear but certainly contributed to the belief for many years that poor diet was the main cause of CPD. However most horses with CPD maintained their body weight and some even became fat because the appetite was increased (Cook and Rossdale 1963). Cachexia has been reported but only in advanced cases (Udall 1954; Alexander 1959; Gillespie and Tyler 1969).

RESPIRATORY FUNCTION

Several detailed studies of lung function in normal horses and in horses with CPD have been reported in recent years (Gillespie, Tyler and Eberly 1966; Sasse 1971; Muylle and Oyaert 1973; Bergsten 1974; McPherson and others 1978) but the results must be interpreted with care because the pulmonary lesions in the various series were not identical and different apparatus was used. Gillespie, Tyler and Eberly (1966) utilised horses with marked clinical signs of CPD and post-mortem examination revealed "extensive emphysema" in seven of 15 animals. Sasse (1971) used a large series of horses with varying grades of exercise intolerance and bronchiolitis was the main pathological finding in a limited number necropsied. Muylle and Oyaert (1973) examined 15 horses with CPD but none were sacrificed at the end of the experiment to determine the underlying pathology. Bergsten (1974) looked at 12 horses with symptoms of "chronic alveolar emphysema" the diagnosis being confirmed on four at necropsy. McPherson and others (1978) examined a total of 100 horses and ponies 38 of which had chronic pulmonary disease clinically, the diagnosis being confirmed pathologically on half of these.

All the authors except Bergsten (1974) used a pneumotachograph of varying construction which was attached via cannulae to a mask over the horse's face. To measure intrapleural pressure either a cannula was inserted surgically into the horse's thoracic cavity (Sasse 1971; Muylle and Oyaert 1973) or an intraoesophageal balloon was used (Gillespie, Tyler and Eberly 1966; McPherson and others 1978). Sasse (1971), Bergsten (1974) and McPherson and others (1978) also measured the partial pressure of arterial oxygen (PaO_2), haemoglobin concentration and pH of arterial blood.

All groups found a marked increase in the change of intrapleural pressure during the breathing cycle which indicated increased respiratory resistance and thus more viscous work was done during respiration. It was proposed that this increased work was responsible, at least in part, for the exercise intolerance exhibited by horses with CPD. Sasse (1971) found that both inspiratory and expiratory work values were increased; Muylle and Oyaert (1973) that inspiratory work was equal to or greater than expiratory work; Gillespie and Tyler (1969) that the expiratory work was increased.

Gillespie and Tyler (1969), Sasse (1971) Bergsten (1974) and McPherson and others (1978) all measured a consistently lowered PaO_2 in affected horses. However Sasse (1971) found that the haemoglobin concentration of arterial peripheral blood was within normal limits so that there was no hypoxaemia of peripheral tissues and attributed the lack of stamina entirely to the extra work required for breathing. Gillespie, Tyler and Eberly (1964) measured the blood pH of horses, with CPD, after exercise and found that it dropped markedly as compared to that of horses with no respiratory abnormality. Sasse (1971) repeated this experiment but in his case the same did not hold true and pointed out that most of the Gillespie study results revealed a higher pH than was normally found. Bergsten (1974) found pulmonary hypertension in all the diseased horses indicating an increase in pulmonary vascular resistance.

From the data presented in these papers it is clear that pulmonary function tests are of considerable value in assessing cases of CPD. The measurements most likely to be of use are PaO_2 , ΔPpl , inspiratory viscous work and expiratory viscous work. It must be understood that these measurements give no indication of the underlying pathological process.

HAEMATOLOGY

Consistent significant overall changes in the packed cell volume (PCV), mean corpuscular volume (MCV) and differential white blood cell count (WBC) have not been found in horses with CPD.

Gerber (1969) found a significantly increased PCV in only 18 cases representing 3.4 per cent. of the total. He did, however, find significantly increased MCVs overall, a mean of 51.8 compared to 47.3 in normal horses.

Sasse (1971) did not find any changes from normal in the PCV or haemoglobin content of any of his horses at rest. After 30 minutes work the PCV rose higher than that of comparable normal horses but after 60 minutes work the value dropped once more to normal. No explanation for this was provided.

In contrast to the results mentioned above Gillespie and Tyler (1969) said that elevated PCVs were a fairly common finding in horses with CPD. Haemoglobin content and MCV were not mentioned. They found a mean PCV of 37.0 in "emphysematous" horses as compared to 32.5 in normal horses but as Gerber (1973) has indicated 32.5 is a very low PCV for a normal horse and this casts some doubt on the validity of their results.

Gerber (1973) found a fairly high WBC count in most of his cases but gave no figures to illustrate this. Gillespie and Tyler (1969) failed to find increased numbers of white blood cells in their cases. This may reflect a difference in the underlying pathology of their cases.

Schatzmann (1970) working with Gerber found a significantly higher number of peripheral blood eosinophils in horses with "asthmoid bronchiolitis" than in a group of horses with "non-asthmoid bronchiolitis" but again no figures were given to support this observation. However, as Gerber (1973) pointed out the eosinophil count does not help much in evaluating a horse's condition since individual variations are marked, fluctuations occur from day to day and eosinophilia is influenced by other factors such as parasitic infestation. Lowell (1964) observed six horses with CPD over a period of fifteen months, during which time he induced acute attacks of "heaves" by feeding hay intermittently. He found that during an acute attack the number of eosinophils dropped and then rose dramatically above the normal level once the attack subsided.

SEROLOGY

Farmer's lung and allergic broncho pulmonary aspergillosis are both examples of diseases caused by exposure to bacterial or fungal antigens in which an important diagnostic aid is the presence of precipitins to the relevant antigen in the patient's serum. Pepys and Jenkins (1965) found that 182 of a total of 205 patients (89 per cent.) with farmers lung had precipitins to Micropolyspora faeni a common contaminant of poor hay, whereas none of the 134 sera from urban patients had precipitins. Twentyeight clinically normal farmers, 61 farmers with asthma and bronchitis and 33 farmers with other chronic lung diseases were also tested. Nine (32 per cent.), 21 (34 per cent.) and 13 (39 per cent.) respectively gave positive reactions to M. faeni. Significant numbers of farmers sera gave precipitins to other hay antigens

such as M. vulgaris, Aspergillus fumigatus and mouldy hay extracts. Control sera, from a variety of sources, failed to react. Hapke and others (1968) found that 11 patients with acute farmers lung and 18 of 34 with chronic farmers lung had precipitins to M. faeni; five of 42 co-workers of the acute group (i.e. exposed to the same hay) also had precipitins although none had disease clinically. Sera from 355 normal subjects not exposed to hay did not react.

Precipitins to M. faeni in animals were first reported by Jenkins and Pepys (1965) who found that in hay-fed cattle the incidence was higher in those with respiratory disease than in clinically normal animals. However the presence of precipitins alone cannot be taken as evidence that the animal has clinical signs or pulmonary lesions like those of farmers lung. Wiseman and others (1973) found that in a herd of 54 cattle extensively affected with chronic respiratory disease 45 had precipitins to M. faeni (83 per cent.) and necropsy of 3 animals revealed pulmonary lesions of farmers lung. Pirie and others (1972) found an incidence of precipitins of 76 per cent. in another herd with no clinical evidence or history of chronic pulmonary disease. In cattle, and probably all other species that are regularly exposed to hay the detection of precipitins can only be taken as an indication of exposure to the relevant antigens and not of the presence of clinical disease.

CPD in horses has been anecdotedly associated with the feeding of mouldy or dusty hay for many years (Percivall 1853; Fitzwygram 1901). Acute attacks of "heaves" were induced by intermittent feeding of hay and by exposure to aerosolised hay antigens (Lowell 1964). Therefore a search for precipitins in the serum of affected horses was a logical step. Unfortunately only a limited amount of information on this has been published on the horse; the most obvious deficit being that no large-scale survey of clinically normal horses has been undertaken. Without this it is impossible to assess the significance of the presence of precipitins in horses with pulmonary disease.

Schatzmann and Gerber (1972) looked at the sera of 40 cases of CPD and found that four (ten per cent) had precipitins to M. faeni, none had precipitins to A. fumigatus, Penicillium casei or hay antigens. A later study of eight cases of CPD in which the pulmonary lesions resembled those of farmers lung revealed that all four cases in which sera was available had precipitins to M. faeni (Pauli, Lüginbuhl and Gerber 1971).

Mansmann and others (1975) investigated two cases of suspected chicken hypersensitivity pneumonitis and found that both had precipitins to chicken serum protein and two of 13 horses kept in the same environment but with no clinical signs, also had a low titre of antibody.

Eyre (1972) demonstrated the presence of reaginic skin sensitising antibodies to a number of very different antigens in the sera of eight horses with CPD by means of the passive cutaneous anaphylaxis (PCA) test. This is discussed in more detail below.

SKIN TESTS

Direct intradermal or scratch provocation tests to demonstrate hypersensitivity to various environmental antigens have been used in medicine for many years and detection of reaginic antibodies by passive cutaneous anaphylaxis is an established technique in the rat and other experimental animals. Both these tests can be used as indicators of the presence of hypersensitivity mechanisms although some care must be taken in interpretation since other organs, including the lung, are also sensitised.

Pirie and others (1971) and Wiseman and others (1973) used the intradermal skin test in cattle to detect sensitisation to M. faeni antigens. Skin swellings developed in animals that had been exposed to mouldy hay reaching a maximum size after four to six hours; histopathological examination at this time revealed intradermal lesions compatible with those found in type III (Arthus) hypersensitivity reactions. The skin reactions slowly declined over the next 72 hours. Intradermal skin swellings did not develop in control animals that had not been exposed to mouldy hay and that did not have serological precipitins to M. faeni. The association of precipitins in serum and positive cutaneous reactions in allergic pulmonary disease in cattle has drawn attention to the need for similar studies in the horse. Unfortunately very little information is available about the prevalence of potentially significant precipitins or skin reaginic antibodies in horses with CPD.

Lowell (1964) was the first to investigate skin hypersensitivity in horses with CPD. The test series consisted of six horses with clinically diagnosed CPD although the diagnosis was not confirmed at necropsy, and six clinically normal horses. Commercially available extracts of birch, oak, plantain, timothy and ragweed pollen, house dust extracts, hay dust extract and three

moulds - Penicillium, Alternaria and Hormodendrum species were used as antigens. None of the horses reacted to the pollen extracts. All the horses had intradermal swellings at the sites of injection of hay dust and house dust extract : these consisted of raised oedematous swellings which reached maximum size after three to four hours and persisted for five to six hours. Similar reactions were obtained with the mould extracts but on the whole these were less intense. All animals reacted to at least one of the moulds and five animals reacted to all three. There was no significant difference in sensitivity between the two groups of horses and Lowell concluded that all stabled and hay-fed horses became skin-reactive to various allergenic components in their environment.

Schatzmann (1970) carried out an extensive series of scratch provocation tests on a group of 30 horses nine of which had a chronic cough possibly of "allergic" origin, 12 had a cough which was not thought to be "allergic" and nine animals with no respiratory abnormality acted as a control. Thirteen commercially available antigens were used : moulds (species not specified) shrub, tree and grass pollens, hay, straw, corn and house dust, oats, barley and maize, horse squama and hair and streptococci of Lancefield's group C. 0.02 - 0.05 ml of antigen was injected and the response was considered positive if the diameter of the resultant wheal was greater than 3 mm after 15 minutes; negative reactions left no skin swelling after this time. Apparently the reactions were not checked after four hours for the presence of a type III reaction. The results obtained were equivocal much as Lowell (1964) had found in his series. Schatzmann concluded that most horses were in a state of "latent allergy" and commonly became sensitised to barley, maize and oats. Lowell (1964) had also found that a high percentage of horses were sensitised to feed components.

Eyre (1972) performed skin tests on 15 horses clinically diagnosed as having CPD and on 10 clinically normal horses. One of the 15 was subsequently found to have a neoplastic lesion of the vocal chord so was not a case of CPD - but was included in the results as a case of CPD. The diagnosis of CPD in three of the remaining 14 was confirmed at necropsy. The intradermal skin tests involved injection of 0.1 ml of commercially available antigens including mixed weed pollens, mixed grass pollens, mixed tree-flower pollens, mixed moulds, mixed dust and mixed ragweed pollens. Extracts of single fungal

antigens - A. fumigatus, A. niger, Penicillium notatum, Alternaria and Hormodendrum were also used. The response was measured 30 and 60 minutes after injection when it was said to be maximal and again at 120 minutes when it was subsiding (the response at four hours was not measured; Lowell (1964) found that the reaction was greatest at this time). None of the horses with CPD reacted to weed or tree pollen, one reacted to grass pollen and two gave mild reactions to ragweed. Only one of the ten controls had a very mild reaction to ragweed. All 15 horses with CPD reacted to either mixed or single fungi and six of the ten controls also reacted. Eyre concluded from this that the intradermal allergen or skin test in the horse gave a consistently reproducible reaction in the diagnosis of allergic respiratory disease as in man. However close scrutiny of his published results does not support this assertion. The control group of ten horses was not matched for age with the CPD group and contained several young horses less than six years of age; CPD does not occur in horses less than six or seven years old (Cook and Rosedale 1963). Nine of ten diseased horses reacted to A. fumigatus and this was said to be significant because only one control horse reacted. In fact only three of the controls were actually tested and two of these were three year olds. Only one control horse was tested with the full range of antigens.

Eyre (1972) also examined Prausnitz-Kustner (P-K) and Schultz-Dale (S-D) responses in CPD horses but the results obtained were difficult to interpret and the published tables show several inconsistencies.

PATHOLOGY

Most textbooks of veterinary pathology (Udall 1954; Nieberle and Cohrs 1966; Smith and Jones 1966; Jubb and Kennedy 1973) and many scientific papers (e.g. Amman 1939; Alexander 1959; Cook and Rosedale 1963; Gillespie and Tyler 1969) contain general descriptions of what was said to be the classical appearance of lungs from cases of CPD at necropsy namely: pallor, failure to collapse on opening the thoracic cavity, increased volume, pitting of the surface on pressure and retention of the rib imprints on the pleural surface. These changes were attributed to emphysema, which was assumed to be secondary to bronchitis and bronchiolitis in a proportion of cases. The association of emphysema with CPD was probably first made by Floyer (1717)

but it has been recognised since the middle of the nineteenth century that not all horses that were "broken in their wind" had emphysema (Gloag 1851). Indeed Malkmus (1913) provided a definition of "heaves" that both acknowledged this variation and recognised that the clinical syndrome could be due to a number of pathological syndromes: "a chronic, incurable disease of the lungs or of the heart, characterised by difficult and laborious respiration." This definition is forensic in its sense and includes a number of chronic incurable diseases of the lungs and the heart that are attended by difficult respiration. Therefore, in order to allow differentiation between several possibly similar conditions the pathology of CPD cases must be described more accurately. It is essential to move away from the general to the particular to find out, for example, what lesions other than emphysema are present and how frequently they occur. Unfortunately, very few of the recent reports of CPD contain sufficient detail to allow this analysis; some do not even record the number of animals examined (Hug 1937; Gillespie and Tyler 1969; Goldschmidt 1974). The only authors to describe lesions in a specified number of individuals were Stömmer (1887); Cook and Rosedale (1963); Thurlbeck and Lowell (1964); Foley and Lowell (1966); McLaughlin and Edwards (1966); Sasse (1971) and Gerber (1973). The total number of animals examined was only 76 (half by Sasse 1971). Gerber (1973) did state that 52 of the 300 cases were necropsied but the histopathology was described briefly in only 14.

Stömmer (1887) looked at nine cases of CPD and found bronchitis in two only. Despite this he compared the disease to bronchitis and emphysema of man. He also claimed to find emphysema at necropsy but the extent and type of this was not discussed.

Cook and Rosedale (1963) examined the lungs of two cases of so-called "functional emphysema". Macroscopically the lungs showed no deviation from normal i.e. no macroscopic evidence of emphysema. Microscopically there was bronchiolitis, peribronchiolar infiltration and a marked increase in the mucosecretory cells of the bronchioles. In their description they confused the terms bronchus and bronchiole. The text refers to bronchi but the illustration is a bronchiole. It is vitally important to localise accurately the site of the lesion and this confusion of terminology should not arise.

Thurlbeck and Lowell (1964) and Foley and Lowell (1966) found emphysema to a trivial degree in only one of a series of 11 horses with CPD; the major finding in the remaining ten was bronchiolitis. The one horse with emphysema was said to have centrilobular emphysema affecting mainly the cranial lobe and only up to ten per cent. of the caudal lobe.

McLaughlin and Edwards (1966) looked at the lungs of two horses with CPD; one of these was an apical lobe biopsy. In addition they examined seven lungs from human patients with emphysema. They concluded that emphysema in man was indistinguishable from that of the horse: amongst the lesions said to be present in the equine lungs were panlobular and bullous emphysema, ectasia, atrophy and loss of distal airways, and diminution of the capillary bed.

Gillespie and Tyler (1969) apparently found emphysema in all the cases they examined. Affected lungs deflated irregularly and had rounded edges. Often the entire lung was involved but more frequently the most marked lesions were in the cranial lobes. Various degrees of bronchitis were seen but were not consistently present. They used thick sections of lung to study the extent of emphysema but did not quantify this. The emphysema was usually panlobular, sometimes centrilobular; there was a severe loss of tissue and a diffuse enlargement of nearly all the air-spaces but lesions as severe as found in man with emphysema were not found. Microscopically the bronchial and bronchiolar mucosa were often thickened and hyperplastic but this was not a consistent finding. The bronchial submucosal layer was sometimes thickened and peribronchial scarring was occasionally seen. Using the electron microscope they attempted to quantify the differences between normal and emphysematous lungs (Gillespie and Tyler 1967). Emphysematous septa had fewer capillaries, endothelial cells and red blood cells than normal lungs. For example, normal lungs had 252.4 capillaries, 87.4 endothelial cells and 348.4 red blood cells per centimetre of alveolar wall and the corresponding figures for the emphysematous lungs were 161.8, 57.3 and 242.6. Emphysematous septa had 26.5 pores per centimetre of alveolar wall whereas normals had 17.4. There was an increase in type 2 pneumocytes and "interstitial" cells in diseased lungs. The alveolar septa were wider than normal and contained more collagen especially around the capillaries. Occasionally the septa were very thin in which case they consisted of attenuated epithelium

and collagen fibres with no intervening capillaries. It is however difficult to place much significance on their results as it is only possible to examine very small pieces of tissue with the electron microscope and the pieces of tissue selected were from the most anterior part of the cranial lobe; this area often shows emphysematous change in horses with CPD (see chapter 3) but is by no means representative of the whole lung. The author's main purpose in this study was to elucidate the pathogenesis of emphysema not to describe the pathology.

Sasse (1971) performed respiratory function tests on a large number of horses and 38 diagnosed clinically as CPD became available for necropsy. Twelve had interstitial pneumonia and 26 had what he termed "(chronic) (peri) bronchi (oli)tis". Nine of these had bronchiolitis only and 20 had emphysema which was extensive in 16. Less common findings were: three cases of pulmonary fibrosis, increased collagen deposition in two and one case each of hyperplasia of bronchiolar and bronchial mucosa, thickening of alveolar walls, calcification of the lung and a case that resembled adenomatosis of sheep. Alveolar emphysema without '(peri) bronchi (oli) tis' was not found. Unfortunately he did not expand at any length on his necropsy findings but they do illustrate the fact that a variety of pathological changes may be present in horses that have similar clinical signs.

Gerber (1973) examined 52 of his 270 cases at necropsy. Twenty one of these were said to have a panlobular or bullous emphysema but again the distribution and extent of this was not discussed. All the cases had a moderate bronchiolitis and there was a "diffuse" bronchitis in some cases. Pulmonary eosinophilic infiltration was found in a third of the cases but the nature of this was not described. Fourteen of the 52 cases were examined in detail. Three had hyperplasia of the "bronchiolar mucous glands"; there are no submucosal glands in the bronchioles so the meaning of this is not clear. Six had hyperplasia of smooth muscle and seven had vasculitis. Gerber (1973) took this to indicate that the lesions were similar to those in human asthma. The basis for this will be discussed later. Two of the cases had pathological changes similar to those seen in farmers lung of man and cattle (Pepys 1969; Pirie and others 1971) : bronchiolitis obliterans, cellular infiltration of alveolar septa and epithelial granulomata. A later paper from the same group described eight such cases (Pauli, Luginbuhl and Gerber 1971). So far, this

particular form of CPD has not been recognised in any country other than Switzerland.

It should be clear from this review of the pathological reports to date that there are several major omissions and defects in the work. Firstly in terms of actual numbers very few cases have actually been examined, secondly few reports contain any detail of the findings and thirdly there has been no attempt to quantify or even describe the extent of each pathological change. This is particularly important in considering the extent of emphysema.

BACTERIOLOGY

The tracheobronchial mucus in normal animals and man is usually sterile (Brown and others 1954; Brumfitt, Willoughby and Bromley 1957). The mucus from cases of CPD is widely believed to be sterile (Roost 1950) but in fact very little microbiological work has been done on these cases. Gerber (1973) thought that streptococcal infections played a certain role in his cases but mainly as a secondary aggravating factor. In his clinic at Berne, Switzerland the incidence of CPD seemed to be increasing whereas the incidence of streptococcal infection was decreasing, which would seem to indicate that it was not significant as a causal agent. He did, however, find that some cases of so-called "asthmoid bronchiolitis" were related to streptococcal foci in the upper respiratory tract. No further details were given on this.

Haemolytic streptococci found in the horse almost always fell into Lancefield's group C and within this group five distinct strains are found, one of which is Streptococcus equi the causal agent of strangles an acute usually upper respiratory tract infection of horses (Mahaffey 1962). Bazeley and Battle (1940) considered that one of the other strains was the causal agent of "equine epidemic catarrh", but this was disputed by Mahaffey (1962) who considered it to be the most common opportunistic pathogen in the horse and therefore capable of invasion after any initial inflammatory reaction. He found Streptococci frequently in all respiratory catarrhal conditions of the horse either as a pure culture or as part of a mixed population. In an attempt to prove this hypothesis he inoculated large fresh cultures of group C Streptococci into normal horses via endotracheal tubes but was unable to initiate any pulmonary disease.

Mansmann (1976) cultured transtracheal aspirates from a total of 34 horses with no clinical signs of respiratory disease. He was able to show that the trachea and main bronchi contained representatives of a wide variety of bacterial and fungal species. The number of organisms appeared to depend on the environment as 17 of the horses were housed in an old wooden barn and the transtracheal aspirates from these contained twice as many bacterial species and seven times as many fungal species as those from 17 horses housed in a new equine hospital.

Hajer (1975) also obtained transtracheal samples from a number of horses. Nineteen of these had acute respiratory disease, 10 had subacute disease and 18 had CPD. Of this last 18 six samples were sterile, six contained Streptomyces spp. (a non-pathogenic contaminant), three contained "non-pathogenic bacteria", one contained Klebsiella and two S. zooepidemicus. Hajer concluded from this that bacteria were not important in the maintenance of CPD. Normal horses were not included as a control.

Beech (1975) examined transtracheal aspirates from 27 normal horses and 57 horses with respiratory disease. Each sample was apparently examined cytologically and microbiologically but the results dealt only with the cytological findings.

AETIOLOGY

The aetiology of CPD has not been resolved and has been a matter for conjecture since the disease was first recognised. Many theories have been advanced in an attempt to understand the pathogenesis of disease but due to the fact that the disease is chronic developing over some years, that no satisfactory experimental model has been produced and that very few cases have been studied in depth none of the theories has much evidence to support it.

In discussing the aetiology Gillespie and Tyler (1969) quoted Virchow (1958) to illustrate the problems involved in unravelling the many factors thought to play a part : " How then can one with certainty determine which of two concurrent phenomena is cause and which effect or that both are not simultaneous effects of a third factor, or, indeed, that such is not the effect of two quite distinct causes".

The main theories and the evidence to support them will be discussed separately.

Allergy or mould hypersensitivity

The development of CPD has for many years been associated with or ascribed to bad feeding practices and poor stable hygiene (Fitzwygram 1901; Hug 1937; Amman 1939; Morgan 1940; Udall 1954; Gillespie and Tyler 1969). This theory was based upon largely circumstantial evidence in that disease was seen mainly in stabled horses (Morgan 1940; Udall 1954; Alexander 1959; Cook and Rosedale 1963) especially in those kept in overcrowded, poorly ventilated, dirty conditions (Hug 1937; Carlstrom and Alegren 1940) and in that the overall incidence of disease increased if the hay was made under damp conditions or had been allowed to become mouldy (Hug 1937; Morgan 1940; Alström and Lauritzson 1953; Udall 1954; Alexander 1959; Sasse 1971). Further support was given to this theory by the observation that the clinical disability in horses with CPD was often substantially alleviated by a return to pasture or a change to a dust-free environment (Alexander 1959; Cook and Rosedale 1963; Lowell 1964; Thurlbeck and Lowell 1964; Gillespie and Tyler 1969; Eyre 1972; Schatzmann and Gerber 1972). Lowell (1964) observed a group of six horses with CPD and found that acute exacerbations were associated with either feeding^{of} or exposure to hay. He was unable to identify the constituent responsible but suggested that there were five major antigenic factors possibly present each of which deserved further investigation: moulds, bacteria, grass or other pollen, insects or mites and particles of grass or other plants.

Eyre (1972) was convinced that it was the mould component that was responsible. He carried out various hypersensitivity tests on affected horses using mould antigens but did not try any aerosol provocation tests. He found that hay from an "outbreak" of CPD (no details provided) contained no significant amounts or types of mould over and above that found in two "normal" stables.

Cook (1976) also incriminated fungal spores as the aetiological agent and suggested two means whereby the bronchial mucosa might react to the allergens 1. a simple inflammatory response producing a check-valve effect causing distal air-trapping and 2. an allergic response producing bronchospasm;

he thought that both mechanisms probably operated together. The latter of these two mechanisms is the asthmatic response. Gerber (1973) thought that at least 75 per cent. of all his cases had an allergic origin and he called these cases "asthmoid bronchitis" and "asthmoid bronchiolitis".

"Asthma" refers to the condition of subjects with widespread narrowing of the bronchial airways which changes in severity over short periods of time, either spontaneously or under treatment, and is not due to cardiovascular disease (Ciba guest symposium 1959). It was largely the periodicity of symptoms in many horses with CPD that led people to compare CPD with asthma (Cook and Rossdale 1963; Lowell 1964; Gerber 1973). Attacks were associated with feeding, stabling and exposure to hay (Alexander 1959; Cook and Rossdale 1963; Lowell 1964) and remissions with removal from dusty environments (Cook 1976). However "asthma" is a very broad term and can be applied to a variety of situations. If the disease in the horse is to be likened to extrinsic asthma of man firstly it must be shown that an allergic response is involved and secondly that this induces bronchospasm. Neither of these has been conclusively demonstrated.

The pathology of asthma is poorly documented as uncomplicated asthma is not often a cause of death and pulmonary biopsy is seldom indicated (Thurlbeck 1976). However, the pathology of patients dying of status asthmaticus revealed several pathognomonic features : vastly overdistended lungs which failed to collapse for some time after the thoracic cage was opened; thick, tenacious mucous plugs containing eosinophils, Curschmann's spirals, and Charcot-Leyden crystals in all airways; massive eosinophilia of bronchial walls, mucosa, lumina and interstitium; grossly-thickened basement membrane of bronchi and bronchioles due to deposition of immunoglobulins; areas of shedding of the mucous membrane; hyperplasia of bronchial smooth muscle and an increase in the number of mucous glands and goblet cells (Earle 1953; Dunnill 1960; Messer, Peters and Bennet 1960). In some limited studies carried out on pulmonary biopsies from asthmatics similar changes were found (Glyn and Michaels 1960).

Pulmonary eosinophilia was found in some cases of horses with CPD (Thurlbeck and Lowell 1964; Foly and Lowell 1966; Gerber 1973). Gerber (1973) also found smooth muscle hyperplasia in six of 14 cases, mucous gland hyperplasia in three and vasculitis in seven; changes which he thought were

similar to those of human allergic asthma. As he does not go into much detail about these changes and there are no illustrations it is difficult to interpret this claim. Foley and Lowell (1966) thought that the lungs of one of their four cases of CPD were similar to those of patients dying in status asthmaticus being difficult to inflate with formalin and all bronchioles being occluded with mucus; no other evidence was given to support this statement. On the available pathological evidence therefore there is little convincing evidence that CPD is the same as extrinsic asthma.

Another mechanism whereby the moulds may cause respiratory disease is in causing extrinsic allergic alveolitis (farmers lung). This is a condition well recognised in man and cattle exposed to mouldy hay. It has been suggested that CPD is a disease analogous to this (Thurlbeck and Lowell 1964; Crofton and Douglas 1975). The pathology of the condition is related to the fact that it is a type III or type IV hypersensitivity reaction and the classical features are pulmonary granulomata, alveolar inflammation, vasculitis and bronchiolitis obliterans (Pepys 1969; Pirie and others 1971). Pauli and others (1972) described a series of eight cases of CPD with pathological lesions resembling those of farmers lung. However these formed a small proportion of their total number of cases. There is no other report of equine farmers lung but Mansmann and others (1975) reported a further example of extrinsic allergic alveolitis in the horse. Two horses, housed in stables containing a number of chickens, developed clinical signs of CPD. Chicken hypersensitivity pneumonitis was suspected and various tests were carried out. Both horses developed marked skin reactions to intradermal chicken serum and both had serum precipitating antibodies to chicken serum. One of the horses developed an increased respiratory rate after nebulisation with saline extracts of chicken faeces. A normal horse failed to react. Necropsy examinations were not carried out but on the clinical evidence it is fairly certain that these were cases of extrinsic allergic alveolitis. This demonstrates that environmental antigens will induce respiratory disease in the horse under certain circumstances but it is likely that these cases form only a proportion of the total suffering clinically from CPD.

Similarity to human chronic bronchitis and emphysema

Chronic bronchitis and emphysema of man are diseases that have been recognised for centuries. Sydenham (1717) was probably the first to describe bronchitis in clinical terms and Laennec's classic monograph (1834) gave detailed descriptions of cases of emphysema and speculated on the pathogenesis. The two conditions are defined separately although they commonly occur together in the well-known debilitating chronic respiratory disease whose chief aetiological agent is tobacco smoke. Chronic bronchitis is defined in functional terms as "the condition of subjects with chronic or recurrent excess mucous secretion in the bronchial tree" (Ciba Guest Symposium 1959). The chronic sputum production is associated with a cough. Pathologically the hallmark of chronic bronchitis is hyperplasia of the bronchial submucosal glands (Reid 1960). Other changes include inflammatory changes in the bronchi, goblet cell metaplasia in the bronchioles and excess mucus in the airways.

Emphysema is defined in structural terms as "a condition of the lung characterised by an increase beyond the normal of air spaces distal to the terminal bronchiole due to destruction of alveolar walls" (Thurlbeck 1976). It is clear from this that emphysema cannot be reliably diagnosed in life and correct diagnosis rests on necropsy examination of the lungs, preferably inflated lungs sliced into thin slices so that the true extent of emphysema may be assessed (MRC Committee 1972).

The major clinical signs of CPD - cough and exercise intolerance - are superficially similar to those of chronic bronchitis and emphysema and the main pathological lesions of CPD are said by some to be bronchitis and emphysema (Hug 1937; Amman 1939; Alexander 1959; Gillespie and Tyler 1969). Because of this the disease in the horse has often been said to be analogous to the disease in man (Stömmer 1887; McLaughlin and others, 1965; McLaughlin and Edwards 1966; Cook 1976). The horse has even been suggested as an animal model of the human disease (McLaughlin and others 1965). The bronchitis was attributed to irritation by dusts and moulds in the respiratory tract and emphysema was a sequel to the pathological change (Alexander 1959; Cook 1976). Close examination of the literature on the pathology of the disease fails to give credence to this belief. There is no incontrovertible proof that bronchitis is a major part of the pathology, those that state its presence

either confuse the names of the various levels of the airways (Cook and Rosedale 1963; Gerber 1973) or fail to provide pictorial or quantitative evidence of the lesion (Hug 1937; Amman 1939; Alexander 1959; Gillespie and Tyler 1969; Sasse 1971). Similarly emphysema has rarely been accurately described in cases of CPD. It was undeniably present in a number of cases of CPD but the current evidence points to it being a fairly small part of the entire pathological picture.

Alpha-1 antitrypsin deficiency

Gillespie and Tyler (1969) suggested that there might be a hereditary predisposition to CPD in the horse due possibly to alpha-1 antitrypsin deficiency as occurs in man.

Alpha-1 antitrypsin is a glycoprotein which has the ability to inhibit or neutralise a number of proteolytic enzymes including plasmin, thrombin, chymotrypsin, elastase, collagenase and some bacterial and granulocytic proteases (Kueppers 1971). It is the major serum trypsin inhibitor providing over 90 per cent of the total antitryptic activity (Jacobsson 1955; Schultze Goellner and Heide 1955). There is a hereditary condition in man associated with the development of early-onset panacinar emphysema and CPD in adults (Laurell and Eriksson 1963) which is due to a deficiency of this enzyme. Law (1896) had suggested that horses had a hereditary predisposition to emphysema but Gillespie and Tyler (1969) could find no further evidence of this in the history of cases. The results of a survey of alpha-1 antitrypsin activity in a series of horses are in Chapter 6.

Influence of a previous febrile respiratory disease

Gerber (1973) found that 47 per cent. of 268 horses with CPD had a bout of "influenza" included in their previous history and that after the 1965 pandemic of equine influenza the incidence of CPD in his clinic increased markedly. Gillespie and Tyler (1969) also saw horses that had developed "chronic bronchitis" one or two years after an "influenza" infection.

Picken, Niewohner and Chester (1971) found that viral infections of the respiratory tract in man could be followed by prolonged changes in small airway dynamics which were not apparent clinically. Colley, Douglas and Reid (1973) demonstrated an association between a history of a chest illness

under two years of age and the presence of a chronic winter cough in young adults. Taussig (1977) concluded that children with a past history of croup or of bronchiolitis had an increased prevalence of abnormalities in lung function. In order to investigate any possible association in horses between earlier bouts of respiratory infections and subsequent CPD, long term follow-up studies over a decade or more are required. Experience in history-taking of horses with CPD has shown that retrospective analysis is unreliable.

Overwork and indigestion

It used to be said that working a horse on a full stomach predisposed to the development of CPD (Navin 1869; Fitzwygram 1901) but this theory has now been discredited (Udall 1954). Hard work will merely serve to accelerate the physical disability already present (Carlström and Alegren 1940).

In conclusion it is not likely that the aetiology of CPD will be elucidated by examining natural cases alone because of various difficulties such as the apparently slow development of disease, its sporadic nature and the problems in obtaining accurate case histories. The experimental production of disease should be the aim of any future aetiological study. There is also the very real possibility that the disease is one of multiple aetiology. A variety of agents and mechanisms may produce the same pathological picture or the clinical disease may comprise several different pathological entities each one having a separate aetiology.

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CHAPTER TWO

CLINICAL STUDIES.

INTRODUCTION

The "coughing horse" is a common complaint in equine general practice. The majority of cases make a rapid recovery, some will continue coughing for several weeks and a few will be found to have developed the syndrome of CPD. This is characterised by a chronic dry cough, increasing exercise intolerance, a double expiratory effort and the eventual development of a "heaves line". The purpose of this study was to identify the pathological changes occurring in this particular group of horses. This section describes the clinical signs and some specialist examinations carried out on a group of 25 naturally occurring cases of CPD prior to necropsy.

MATERIALS AND METHODS

Animals

There were two sources of material. The Royal (Dick) School of Veterinary Studies Edinburgh is carrying out an extensive survey of the clinical signs, tests of respiratory function and response to various inhaled antigens in naturally occurring cases of CPD. Eleven of the horses involved in this study were subsequently referred to the Glasgow Veterinary School for pathological examination. A further 14 cases of CPD were directly acquired by the Glasgow Veterinary School.

Clinical examination

As complete a history as possible was obtained from the owner or veterinary surgeon. Specific questions asked were: the duration of disease, nature of onset, history of a previous febrile respiratory disease, expected workload, type of stabling and feed, frequency of coughing, coughing pattern, history of grazing with donkeys.

After purchase the animals were housed individually and fed hay supplemented by concentrates. Usually the bedding was peat moss but some were bedded on straw. Physical examinations were carried out on all animals.

Haematology

Blood was taken from the jugular vein and 2 ml samples placed in sample tubes containing ethylene diamine tetra acetic acid (EDTA). This was used to measure packed cell volume (PCV), Haemoglobin content (Hb), mean

cell haemoglobin content (MCHC), mean cell volume (MCV), total white blood cell count (WBC) and differential white blood cell count.

Serology

Part of the serum was used in the alpha-1 antitrypsin studies (Chapter 6) and the remainder was used to detect the presence of serum precipitating antibodies against antigens of Micropolyspora faeni, Aspergillus fumigatus, Aspergillus nidulans and Aspergillus niger. Double diffusion was carried out in 1.5 per cent. Ionagar No. 2 (Oxoid) prepared with McIlvains citric acid buffer at pH of 7.2. The pattern used was a central antigen well and five peripheral wells; the distance between was 5 mm. The serum was placed, undiluted, in the peripheral wells and an antigen, prepared in the laboratory from cultures of M. faeni, placed in the central well. After five days incubation in a moist chamber the line pattern was read. An identical procedure was used to detect antibodies to Aspergillus spp. except that the buffer used was a borate one at a pH of 8.6.

Radiography

A lateral radiograph of the chest was made with the horse standing and using a stationary grid; tranquilisation was frequently required for this procedure.

Faecal examination

Samples of faeces were examined in each case for the presence of lungworm larvae and strongyle eggs. A Baermann technique was used for the examination for lungworm larvae. For this 50 grams of faeces were suspended overnight in a double layer of gauze in a funnel containing water. The sediment was drawn off and spun at 1500 rpm for three minutes after which the supernatant was drawn off and the remainder examined microscopically for the presence of larvae.

A modified McMaster technique (Gordon and Whitlock 1939) was used in the examination for strongyle eggs.

Endoscopy

On a number of horses an endoscopic examination was made of the larynx and trachea using a fibroptic colonoscope adapted for the purpose

(Olympus CFLB2, Keymed, Southend England). The horse was first tranquilised with acetyl promazine (C-Vet Bury St. Edmunds Suffolk) at a dose rate between 0.25 and 0.5 ml per cwt. A twitch was applied to the upper lip and the colonoscope passed into the lower nares in the same manner as passing a stomach tube. The flexible tip enables detailed and direct examination of the area which is a considerable aid in differential diagnosis. Some of the exudate found lying in the trachea in most of these cases was aspirated via a length of fine tubing passed down the colonoscope and used for bacteriological and cytological studies. For bacteriology, samples were plated out on blood agar and McConkey's agar plates and incubated for 48 hours at 37°C. Smears of the exudate were made, fixed in absolute alcohol and stained with haematoxylin and eosin, Leishmann, Papanicolaou or alcian blue pH 2.6 - PAS. Estimates were made of the type and quantity of cells present.

Provocation tests

A limited number of observations were made of the effect of feeding mouldy hay to these horses. The horse was put in a loosebox with both doors closed and quantities of mouldy hay, obtained from a local farm that had had an outbreak of farmers lung amongst its cattle, were shaken around the floor and mixed with the normal hay in the hay net. Most animals treated in this way ate the hay, although it appeared grossly unpalatable.

The effect of turning out to grass was also studied in a few cases. Horses were put out into the field for variable periods of time and respiratory function tests carried out at intervals to assess the improvement.

Medication with bromhexine hydrochloride

Two animals were treated daily for some weeks with a bronchial secretolytic bromhexine hydrochloride (Bisolvon, Boehringer Ingelheim Crown Chemicals, Lamberhurst, Kent). During this time they were kept inside under the same conditions except for a short period when one was turned out, fed fair quality hay and bedded on peat moss. The medication was in the form of crystals which were mixed in the concentrate ration. One horse found this unacceptable, so it was incorporated first in some molasses. The dose rate was 100 mg twice daily.

Respiratory function tests

The clinical group at Edinburgh carried out detailed respiratory function studies on all the horses referred to them for investigation. Their methods are described in a separate publication (McPherson and others 1978).

RESULTS

History and clinical signs

A summary of the history and clinical signs of the animals acquired by the Glasgow Veterinary School is given in Table 1. All of the horses were more than ten years old at the time of examination. In all those cases in which a history was available the disease had been present for more than a year and in several cases for more than five years. Seven of the animals were ponies, three were hunters and two were stallions.

A chronic cough which became worse on exercise was the most obvious clinical feature and was found in all but one of the 14 cases. All of the animals had double expiratory efforts and had developed "heaves lines". However dyspnoea was very seldom observed. A nasal discharge was often present varying from serous to mucoid but was seldom copious. Increased lung sounds were heard on auscultation of the chest of all fourteen horses. Only one animal was in poor bodily condition, the remainder were in fair to good condition.

Some details of the horses referred from Edinburgh are shown in Table 2. Five were ponies and four were hunters the remaining two being heavy horses. All were affected for more than a year and often for much longer. The clinical signs were similar to those found in the Glasgow horses.

Radiography

All the horses so examined could be said to have abnormal radiographic features. The more obvious abnormal features included "stippling", areas of irregular increase in density and greatly increased bronchial shadowing. These are illustrated in Figures 5 and 6. However it was difficult to obtain clear radiographs and accurate interpretation was not possible. It is perhaps significant that the two cases that had fractured ribs were not identified on radiographic examination demonstrating the lack of clarity obtained.

Faecal examination

Lungworm larvae were not found in the faeces of the nine animals examined for this. Strongyle eggs were sometimes found in the faeces. Two cases had counts of 250 eggs per gram and these were given anthelmintics to eliminate the infection, the remainder had counts of 50 per gram or less.

Haematology

The results are shown in Table 3. Most of the horses had blood values comparable to those of normal horses. In the three horses, 29, 33 and 35, in which the mean red cell volume was recorded several times this was found to be elevated above the generally acceptable level of 40-45 c.u. Only one horse, 11, had a white blood cell count above normal and the rise was hardly significant (13,600 per c.mm). One horse, 35, had a differential eosinophil count of ten per cent. which is above the normal range of 0-6 per cent.

Serum precipitating antibodies

Six out of ten tested horses from Edinburgh had serum precipitating antibodies to M. faeni and four out of ten to Aspergillus species. Of the Glasgow horses only two of 13 tested had precipitating antibodies to M. faeni and three of 13 had precipitating antibodies to Aspergillus species. The results are shown in Tables 2 and 4.

Endoscopy

Horses affected with CPD presented a consistent appearance on endoscopic examination. A pool of creamy-yellow mucus could be seen lying just proximal to the thoracic inlet where the trachea becomes slightly dorso-ventrally compressed. The mucus moved backwards and forwards as the horse breathed. No obvious inflammatory changes were apparent in the trachea or as much of the major bronchi as could be seen.

A number of the larger horses showed mild laryngeal paralysis but this did not cause clinical signs in any of the cases. In these cases the left side of the larynx did not fully abduct on inspiration.

In all cases neutrophils predominated often to the exclusion of other cells. Epithelial cells were present in small numbers and mucus was plentiful. Bacteriological culture revealed no specific bacteria that could be

regarded as pathogenic. In most cases the exudate was sterile but in four cases a gram-negative coccobacillus which resembled *Moraxella J woffi* was found. This was also recovered from the lungs of three cases at necropsy and was subsequently isolated from other equidae at various times, particularly if there was pulmonary infection. Limited studies on this organism performed to date would tend to suggest that it is a non-pathogenic secondary invader of equine pulmonary tissue. I could find no reference to it in the literature. The characteristics of the organism are listed in Table 5.

Provocation tests

A number of provocation tests using mouldy hay were carried out. In five out of six cases the horses responded to this within 24 hours (sometimes within as little as 30 minutes) with an elevated respiration rate, increased nasal discharge and an increase in frequency of coughing. Normal horses, treated in the same way, did not react.

One horse, 11, proved to be of particular interest. Within 24 hours following exposure the resting respiratory rate had risen from 12 per minute to 35 per minute. There were now harsh paroxysms of coughing, the animal becoming quite distressed. Four days after exposure a thick plug of greenish-white mucus was expectorated and this was found to contain many eosinophils,

Blood and serum samples were taken on alternate days throughout the course of the experiment. Fourteen days after exposure to mouldy hay the serum contained detectable precipitating antibodies against Aspergillus species whereas previously there had been negative results. The white blood cell count rose following exposure from 6,000 to 19,000 per c.mm., the differential remaining approximately the same. After seventeen days of exposure the horse was depressed, lethargic, anorexic and slightly diarrhoeic. Because of this she was turned out into the field. A dramatic clinical improvement was apparent within two hours when respiratory rate had returned to normal levels and the horse had become much brighter.

Six weeks later the horse was again housed. Her clinical condition was good, respiration normal with only slight hyperpnoea and coughing being infrequent. Precipitating antibodies to Aspergillus species were no longer detectable. The white blood cell count was still 14,000 per c.mm. of which eosinophils again formed 4-6 per cent. She was again exposed to mouldy hay

in the same way as before and responded within 30 minutes with increased respiratory rate and paroxysms of coughing. Circulating eosinophils increased to 12 per cent. after two days and remained at this level, blood samples being taken on alternate days, until the horse was shot two weeks later. The absolute white blood cell count did not increase beyond 14,000 per c.mm. and precipitating antibodies to Aspergillus were not detected.

The other four responded in similar ways to exposure to mouldy hay although none developed precipitating serum antibodies to either M. faeni or Aspergillus species as 11 had. One horse, 37, failed to respond to mouldy hay in any detectable way whatsoever, even after intensive exposure for four weeks. Likewise, the effect of turning these horses out to grass was quite dramatic. Improvement could be seen within hours and in some cases it was difficult to detect any clinical signs of CPD at rest. Clinical improvement could also be achieved by walking the horse outside for an hour or so.

Medication with bromhexine hydrochloride

Both the horses 33 and 35 treated with this bronchial secretolytic had been severely affected with CPD for some years.

In neither case was there a dramatic clinical improvement but both horses improved mildly after a few days' treatment. In 35, because of the temperament of the horse, this improvement could not be quantified but the other horse, 33, showed improved exercise tolerance and a quicker recovery following exercise tests. There was an impression, although it could not be verified, that the mucus in this horse was not as thick and purulent as before. Increasing the dose or putting the horse out did not increase the slight degree of improvement, nor did prolonged treatment for a month or more. At necropsy 33 had an area of pleurisy associated with a fractured rib. The effect of this was not known but it may have limited the possible effectiveness of the treatment.

Respiratory function tests

The clinical group at Edinburgh have found that two principle measurements serve to identify the horses affected by CPD - the partial pressure of oxygen in the arterial blood (P_{aO_2}) and the maximal change in intrapleural pressure (ΔP_{pl}) measured either by intrathoracic canula or by intra-

oesophageal balloon (McPherson and others 1978). Horses definitely affected by CPD confirmed at necropsy were found to have a PaO_2 of < 82 mm Hg and a ΔPpl of > 6 mm Hg.

The other main diagnostic aid was the response to inhaled antigens. Horses were subjected to an aerosol of a selected antigen and the PaO_2 and ΔPpl measured. A positive response was said to occur if the ΔPpl changed from < 6 mm. Hg to > 6 mm. Hg or if it increased from > 6 mm. Hg by > 15 per cent.

DISCUSSION

The horses in the study exhibited all the classical clinical signs of CPD which have been described many times by many different authors:

- cough of many months to years duration,
- increasing exercise intolerance,
- double expiratory effort,
- presence of "heaves line",
- nasal discharge,
- increased respiratory sounds on auscultation,
- fair to good bodily condition.

As McPherson and others (1978) have shown, these are typical but not diagnostic of CPD. Fourteen horses were admitted to the Glasgow Veterinary School specifically for this study. All were kept for periods of several weeks and examined periodically. Two additional horses were acquired but did not fit in well with the accepted clinical picture; one of these had a chronic cough on exercise but no other symptoms and was subsequently found to have a collapsed trachea; the other had severe dyspnoea, cyanosis and had rapidly lost bodily condition; at necropsy this was found to be a case of chronic pneumonia. One horse, 29, had mild CPD at necropsy, and did not show all the clinical signs expected of a case, in particular there was no cough, but extensive clinical examination failed to reveal any other possible cause for the exercise intolerance, tachypnoea and hyperpnoea. The other 13 cases exhibited all the classical signs.

Radiography of the equine chest remained unsatisfactory. The view on which the maximum lung area is visible is the straight lateral but this involves superimposing one lung on another. Dorso-ventral views would require

general anaesthesia, a technique not without risk in a horse with impaired lung function, and would still give a restricted pulmonary field. The large bulk of tissue to be penetrated necessitates a large kilovoltage with resultant loss of definition. Although the cases had an "abnormal" chest X-ray it was difficult to make an interpretation beyond this and also difficult to correlate the radiographic findings with those at necropsy. Chest radiographs cannot at this time be recommended as an additional aid to diagnosis but may be of some use in differential diagnosis of other cases of respiratory difficulty such as tumours and collapsed trachea.

Endoscopic examination is of value in diagnosis particularly in doubtful or early cases. Various upper respiratory tract conditions may be eliminated such as soft palate paresis, guttural pouch mycosis, sinusitis and laryngeal hemiplegia. In addition aspiration and subsequent bacteriological examination of the sputum will eliminate bacterial conditions that may mimic CPD. In particular infections of Streptococci of group C should be looked for since these may cause prolonged coughing and exercise intolerance if untreated. All cases of CPD confirmed at post mortem had either a sterile tracheal exudate or one containing what appeared to be non-pathogenic bacteria. The gram-negative cocco-bacillus found in the respiratory tract of normal and diseased equidae is of interest, mainly because it has not been described previously. As far as is known only two surveys of the bacteriological flora of equine sputum have been carried out and one of these dealt entirely with clinically normal horses. One, in the Netherlands, involved collecting 60 samples of sputum by transtracheal aspiration. Forty seven of these were from horses with respiratory complaints which were classified as acute (19 cases), subacute (10 cases) and chronic (18 cases) (Hajer 1975). Of this last 18, six were negative bacteriologically, six contained Streptomyces species, two contained Streptococcus zooepidemicus and three contained "non-pathogenic bacteria". Streptomyces species and S. zooepidemicus were also commonly found amongst the acute cases. Streptomyces is an actinomycete which is fairly ubiquitous. Hajer found that on admission to the clinic the sputum of chronic respiratory cases was often negative bacteriologically but by departure infection with Streptomyces species had occurred; that is the horses were picking up the infection at the clinic. His results were very similar in that he found the sputum from most cases of CPD to be

bacteriologically sterile. He did not report any isolates of a bacterium resembling Moraxella J woffi.

The second survey was carried out on 17 clinically normal horses housed in a new university equine hospital and on 17 clinically normal horses housed in an older wooden barn (Mansmann and Strouss 1976). A wide variety of apparently harmless bacteria and moulds was isolated from the transtracheal aspirates in both cases but the horses in the wooden barn harboured twice the number of bacterial species. The discrepancy between their results and those of Hajer (1975) and myself cannot readily be explained; possibly their method of collection was not sterile or their methods of bacterial cultivation were more sensitive. It is difficult to draw any firm conclusions as their methods of collection and culture were not given in sufficient detail.

It may be difficult to determine the exact pathogenic nature of the organism resembling Moraxella J woffi under experimental conditions. If, as is suspected, it is an opportunist invader of pulmonary tissue this task will be further complicated. However, as far as CPD is concerned, it may be concluded with some certainty that by and large the sputum is sterile and that the disease is not one involving bacterial infection at least when the condition reaches the chronic stage.

Cytologically, the findings tend to agree with those of Beech (1975) who examined transtracheal aspirates from 27 normal horses and 57 horses with respiratory disease, 23 of which had CPD diagnosed clinically. She found that the neutrophil was the predominant aspirate cell in the 23 horses and that thick, viscid mucus was also present. She also claimed to see thick plugs which stained histologically for fibrin which she assumed had been sucked out of bronchioles during collection. I did not find these but the method of collection was different in that suction was applied only to the mucus seen lying in the trachea whereas Beech applied suction and pumped fluid in and out of the respiratory tract. Less common findings in her study were eosinophils, squamous cells and macrophages; as in the cases described here. Sputum from human asthmatics is said to contain numerous eosinophils (Crofton and Douglas 1975) but eosinophils are an uncommon finding in tracheal mucus of horses with CPD although they were frequently seen in the bronchial and bronchiolar walls of cases at necropsy. This gives

further credence to the view that CPD is not directly analogous to human asthma.

Culture of aspirated sputum is of value in eliminating a bacterial cause of coughing but cytologically the value of examination is doubtful. In man, of course, cytological examination is extensively used for the diagnosis of lung cancer amongst other conditions and has considerable value. Cytology of the sputum has shown that it does not contribute any more to the diagnosis of the disease.

Haematological examination of the affected horses was of little value in the diagnosis. Jeffcott (1977) has noted a tendency for the MCV to rise with increasing age so that the significance of the measurement is doubtful. The three horses with elevated values were all over 12 years of age.

The observation that the clinical signs in affected horses increased in severity following exposure to mouldy hay merely confirmed what has been realised for many centuries, that mouldy or dusty hay has a certain role to play in the pathogenesis of disease. Similarly it was found that removal of the affected animal to a relatively dust-free environment gave considerable clinical relief, which is another well-documented fact. In a more quantifiable fashion, the clinical team at Edinburgh have demonstrated susceptibility to various purified antigens of mouldy hay. One interesting finding that arose from this work was the ability to induce the appearance of serum precipitating antibodies to Aspergillus species in one animal after 14 days' exposure to mouldy hay. Because of the short time that this took the animal would have had an inherent potential to produce this and it is certain that at some earlier stage of the disease these antibodies had been produced in detectable quantities. It was impossible to induce serum precipitating antibodies in any other of the animals so treated. The team at Edinburgh succeeded in sensitising a horse to avian egg albumen, an antigen not likely to be met in life. The animal was aerosolised several times and in due course developed serum precipitating antibodies to the antigen. The actual significance of these serum precipitating antibodies is not known. Their presence has previously been discussed in the Review of the Literature. It is impossible to determine whether a larger number of horses with CPD have serum precipitating antibodies to M. faeni because this is the causative agent or whether it merely indicates increased exposure of these

horses to mouldy hay in respect to the normal horse population. In order to assess its significance tests must be standardised and a large number of horses, both normal and diseased would have to be examined for serum precipitins. It is interesting that a larger number of Edinburgh horses had demonstrable serum precipitating antibodies to M. faeni and Aspergillus species. This probably reflects a difference in sensitivity of measurement.

Drug treatment of horses with CPD is a controversial topic. Some of the treatments described in the past involved the administration of opium, arsenic, enemata, turpentine, lead shot and bacon fat and an operation to make an artificial connection between the rectum and vagina (Law, 1896). The restriction of food and water and attention to the quality of food is mentioned many times because of the belief that CPD was primarily a digestive upset. The fact that this often effected a remission was most likely because it reduced the dust in the atmosphere rather than improved the digestion. Nowadays it is recognised that the provision of fresh air is the single most important palliative and Cook (1976) expands at some length on this. Antibiotics are of no value as there is no bacterial cause although they are widely employed in practice. Corticosteroids have a limited role and temporarily alleviate symptoms but have too many contraindications to justify prolonged use.

Schatzmann, Bergi and Straub (1973) medicated six horses, five affected with CPD and one control, with 50 mg of bromhexine hydrochloride twice daily for 11 days. No lasting beneficial results were achieved; the viscosity of the mucus decreased only slightly in four out of six horses as assessed subjectively. Two horses were given 250 mg twice daily without enhanced effect. They concluded that bromhexine hydrochloride had little effect on the secretion of horses with CPD. Two horses medicated with bromhexine hydrochloride showed similar results in the two cases in that both seemed to ^{ex-}exp_orate less viscid sputum and there was a minor clinical improvement. Cook (1976) claims that the administration of bronchial secretolytics should be regarded as a placebo only and that their greatest role was in allowing the owner to feel that something positive was being done for the horses.

Lungworm infection is said to be able to be confused with a diagnosis of CPD (Cook 1976). The donkey is said to be the natural host of the parasite Dictyocaulus arnfieldi, and horses will pick up the infection if grazed in the company of donkeys although the infection rarely achieves patency (Round 1976). It was difficult to ascertain, in almost all cases, whether the horses had ever grazed with donkeys. This is however an important facet of the history taking. Faecal examination will not eliminate infection as a cause of coughing as the infections rarely reach patency but at necropsy no evidence of active lungworm infection was found (Chapter 3).

Observation	7	9	11	13	15	20	29	33	34	35	37	38	39	41
Duration if disability	NK	> 1 yr.	> 1 yr.	6 yrs.	> 5 yrs.	> 1 yr.	> 1 yr.	5 yrs.	5 yrs.	NK	5 yrs.	> 5 yrs.	NK	> 2 yrs.
Nature of onset	NK	NK	NK	Slow	NK	Slow	NK	NK	NK	NK	Slow	Slow	Slow	NK
Previous febrile respiratory disease	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK
Work required of horse/Type	Child's Pony	Riding School Pony	Child's Pony	Child's Pony	Grade A Show - jumper	Child's Pony	Riding School Pony	Hunter Hunter Hunter	Hunter Hunter Hunter	Child's Pony	Child's Pony	Stud Work	Stud Work	Stud Work
Stabling and feed available	NK	Fair	Good	NK	Varied	Poor	Fair	Good	Good	NK	Good	Good	Good	Good
Grazing with donkeys	NK	NK	NK	NK	NK	NK	-	NK	NK	NK	NK	-	-	NK
Cough	++	+	++	+	++	++	-	++	++	++	++	++	+	++
Nasal discharge	Serous	-	Mucoid	Serous	Mucoid	Serous	-	Mucoid	Mucoid	Serous	Serous	Serous	Serous	Serous
Double expiratory effort	+	++	++	+	++	++	+	++	+	++	+/++	++	+	++
"Heaves" line	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dyspnoea	-	-	-	-	-	-	-	-	-	-	-	-/+	-	-/+
Increased sounds on auscultation	++	++	++	+	++	++	+	++	++	++	+/++	++	++	++
Abnormal chest radiograph	+	+	+	NK	+	+	NK	+	+	+	+	+	NK	+
Bodily condition	Good	Fair	Good	Good	Fair	Good	Good	Fair	Fair	Good	Good	Good	Good	Poor

TABLE 1. Some historical and clinical details of the horses acquired by the Glasgow Veterinary School.
NK - not known/not carried out.
+ - mild
++ - moderate
- - not present

Observation	1	6	12	14	23	24	25	26	31	36	40
Type of horse	Heavy Hunter	Child's Pony	Hunter	Child's Pony	Child's Pony	Heavy horse	Heavy Hunter	Child's Pony	Child's Pony	Heavy horse	Hunter
Length of time affected	4 yrs.	> 6 yrs.	> 3 yrs.	3 yrs.	> 5 yrs.	1 yr.	Not known	> 2 yrs.	1½ yrs.	Not known	1½ yrs.
PaO ₂ mm Hg.	80.5	Low	Low	Low	Low	Low	Low	Low	Low	Low	58.5
△Pp1 mm Hg.	12.8	High	High	High	High	Not done	High	High	High	High	26
Serum precipitating antibodies against	None	M. faeni	M. faeni	Aspergillus	M. faeni	M. faeni	Not done	M. faeni	Aspergillus	None	M. faeni
Inhalation tests positive to	M. faeni	M. faeni	M. faeni	M. faeni	M. faeni	Not done	Mouldy hay	M. faeni	M. faeni	Mouldy hay	M. faeni
	Aspergillus spp.	Aspergillus	Aspergillus	Aspergillus	Aspergillus	done	Aspergillus	Aspergillus	Aspergillus	Aspergillus	Aspergillus
	Avian Albumen	Avian Albumen						Pollen			
Intradermal tests positive to	Pollen	Moulds	M. faeni	M. faeni	M. faeni	Moulds	Moulds	Moulds	Not known	M. faeni	M. faeni
	Moulds	Nocardia spp.	Nocardia	Aspergillus	Nocardia	Pollens		Pollens	known		
	Aspergillus	M. faeni		gillus	spp.	M. faeni		M. faeni			
	Aspergillus		Nocardia spp.	Nocardia spp.	Nocardia	spp.		Nocardia spp.			

TABLE 2. Selected historical and clinical details of the horses sent from the Royal (Dick) School of Veterinary Studies.

PaO_2 - partial pressure of arterial oxygen.

ΔPpl - change in intrathoracic pressure between inspiration and expiration.

CASE NO.	PCV ml/100 ml.	Hb g/100 ml.	MCV c.u.	MCHC per cent.	Differential white blood cell count				
					Total WBC per c. mm.	Neutrophils per cent.	Lymphocytes per cent.	Eosinophils per cent.	Basophils per cent.
7	34	ND	ND	ND	10900	66	30	4	0
9	40	14.1	ND	ND	11200	55	42	2	1
11	43	ND	ND	ND	13600	54	40	6	0
13	39	ND	ND	ND	5200	52	45	3	0
15	32	ND	ND	ND	6100	57	41	0	2
20	36	13.5	ND	ND	7900	78	20.5	1.5	0
29	32	10.0	52.9	31.2	9700	58	36	6	0
33	40	14.1	51.3	35.2	5600	69	31	0	0
35	45	15.7	48.8	34.9	3200	52	38	10	0

TABLE 3. Haematological data of some of the horses acquired by the Glasgow Veterinary School.

PCV - packed cell volume. MCHC - mean cell haemaglobin concentration

Hb - haemaglobin. WBC - white blood cells.

MCV - mean corpuscular volume ND - not done.

Serological precipitating antibodies to:

CASE NO.	<u>Micropolyspora faeni</u>	<u>A. fumigatus</u>	<u>A. niger</u>	<u>A. nidulans</u>
7	-	-	-	-
9	+	-	-	-
11	-	-	-	-
13	-	+	-	-
15	-	-	-	-
29	-	-	-	-
33	-	-	-	-
34	-	-	-	-
35	+	+	+	+
37	-	-	-	-
38	-	-	-	-
39	-	+	+	+
41	-	-	-	-

TABLE 4. The detection of serum precipitating antibodies to
Micropolyspora faeni or Aspergillus species in the horses
 acquired by the Glasgow Veterinary School.

Isolates

Isolated from tracheal washings of four adult horses with CPD and from tracheal washing of four foals with ascarid infections.

Found in the pulmonary tissue of three cases of CPD and three foals with experimental Parascaris equorum infection.

Also found in the lungs of two cases of acute streptococcal pneumonia diagnosed bacteriologically.

Morphology

Small, stout coccobacillus.

Gram-negative.

Non-sporing, non-motile.

Grows readily on blood agar to form small, non-haemolytic, shiny cream colonies 1-2 mm diameter.

Grows readily on McConkey agar.

Biochemistry

Catalase positive.

Oxidase positive.

Oxidises lactose, maltose, xylose, glucose, trehalose with some fermentation but this is not always constant.

Urease negative.

TABLE 5. Morphological, cultural and biochemical characteristics of a coccobacillus isolated from the equine respiratory tract.



Figure 2. Nostrils of 34. A mild serous discharge can be seen at the left nostril. Even at rest the marked flaring of the nostrils can be seen.



Figure 3. 34. The barrel-chested appearance is obvious and a heaves line is present (arrows).



Figure 4. 38. There is a prominent heaves line visible right along the lower abdomen and thorax (arrows).

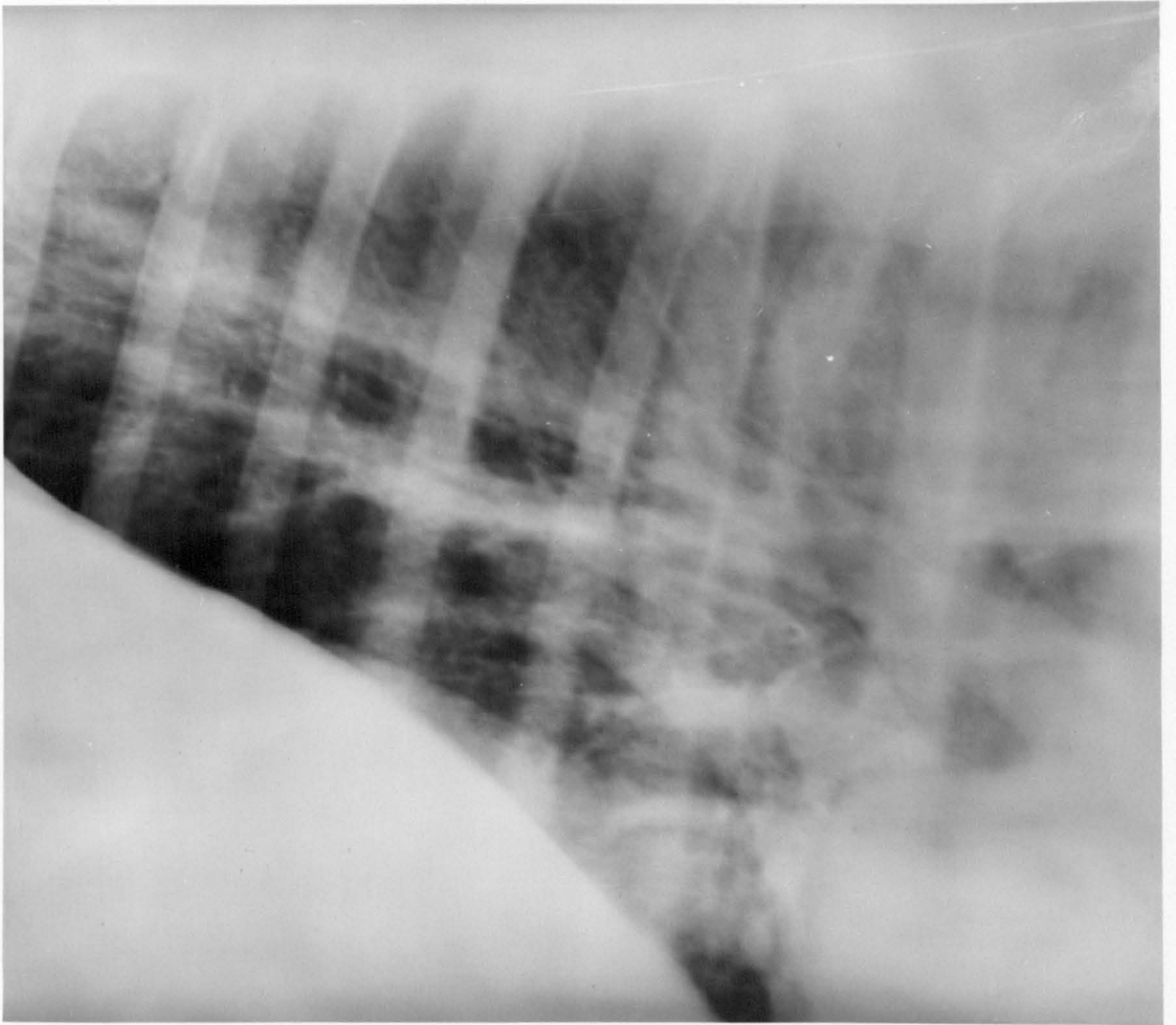


Figure 5. Radiograph of the chest of one of the affected horses lateral view. The bronchi are more radio-opaque than usual and there is a stippled appearance to the dorsal part of the lung.

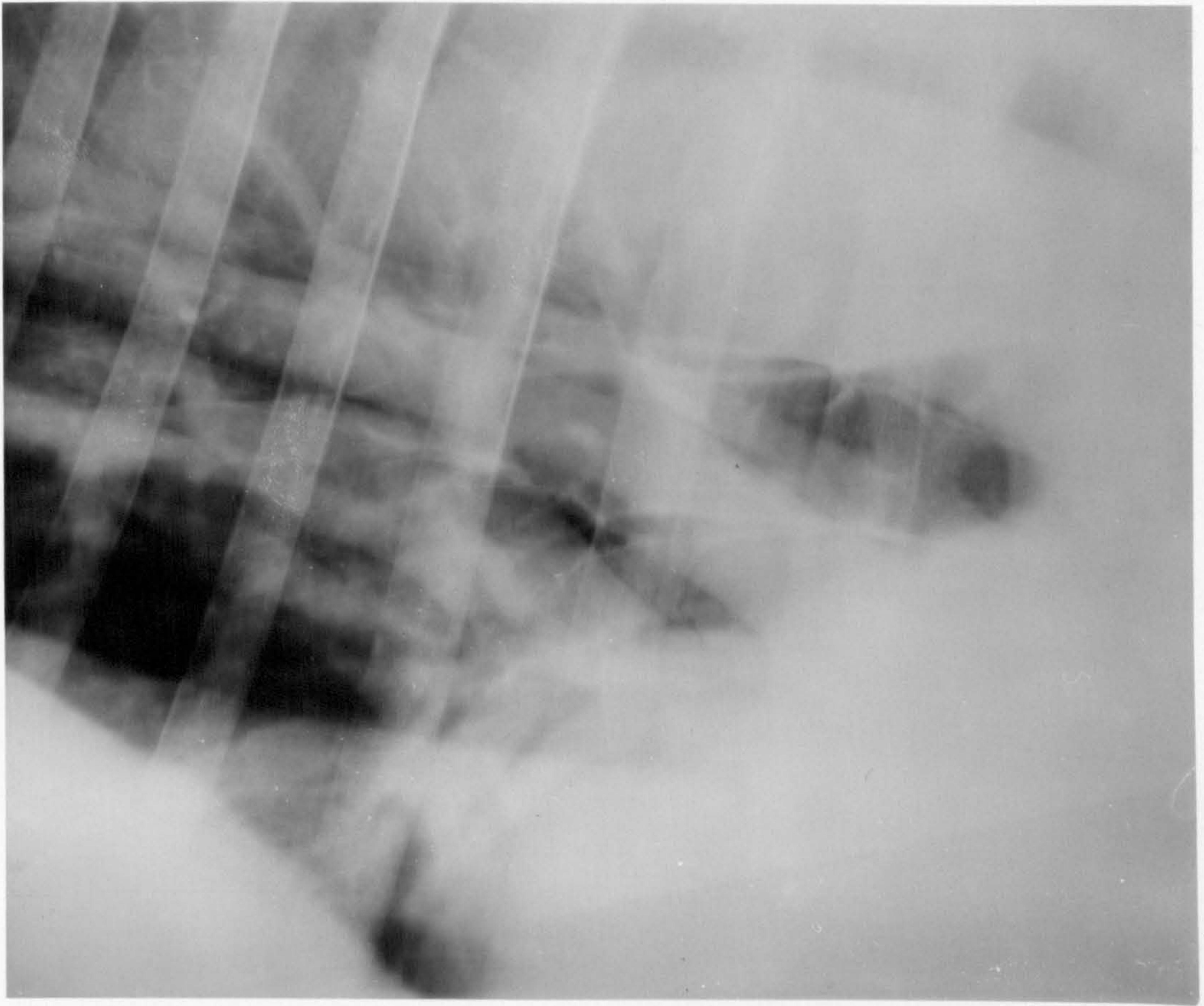


Figure 6. Radiograph of the chest of one of the affected horses lateral view. The bronchi are very opaque and most of the dorsal part of the lung is much denser than normal.

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CHAPTER THREE

PATHOLOGICAL STUDIES

INTRODUCTION

This chapter describes the pathological findings in the horses described in the previous chapter. Eleven of these were derived from the respiratory function/pathogenesis project at the Royal (Dick) School of Veterinary Studies. All of these had PaO_2 s of $< 82 \text{ mm. Hg}$ and ΔPpl s of $>6 \text{ mm. Hg}$ and so satisfied the criteria laid down by the group for identification of horses with CPD. The remaining 14 were horses acquired by the Glasgow Veterinary School. All of these were fully investigated clinically as described in Chapter Two but respiratory function tests were not performed on these animals. As McPherson and others (1978) have indicated the clinical signs alone will not positively identify all cases of CPD. However these fourteen were carefully selected cases and all were observed over long periods of time prior to necropsy. The pathological findings (described below) showed that the clinical methods employed had been sufficient to identify these as cases of CPD.

MATERIALS AND METHODS

Animals

Horses referred by veterinary surgeons were purchased from their owners or gifted to the veterinary schools concerned. The animals were kept for varying periods prior to necropsy, during which time they were either kept inside in individual boxes or were put out to grass. At the Royal (Dick) School of Veterinary Studies the horses underwent various specified tests for allergy and respiratory function and, to a lesser extent, similar tests were performed on some of the horses at the Glasgow Veterinary School. Detailed clinical examinations were made on all the animals going to necropsy. This has been fully described in Chapter Two.

Post mortem and histopathological technique

Clinical cases were slaughtered at various intervals after admission. At Glasgow the larger horses were stunned with a bell gun and the smaller ones with a captive bolt pistol, pithed with a light cane and immediately exsanguinated by jugular section. The lungs were removed as rapidly as possible from the carcase and selected pieces of tissue were excised for electron microscopical and histological examination. In one case a horse which had been dead for two hours was delivered and in a further instance a set of lungs from a horse dead for six hours were picked up from a slaughterhouse.

Usually the horses purchased by the Edinburgh group were transported live to Glasgow for necropsy examination. Horses slaughtered in Edinburgh were given an overdose of barbiturates (Euthatal; May and Baker, Dagenham, Essex) and immediately exsanguinated.

The entire respiratory system was examined macroscopically after which the right lung was excised at the hilus and inflated in ten per cent. formol saline (see below). In some cases this was not possible if the pleura had been inadvertently cut at necropsy, so the left lung was used.

Three blocks of tissue were removed from each site shown in Figure 7 and fixed in separate bottles containing Carnoy's fluid, corrosive formol or ten per cent. formol saline respectively. The tracheo bronchial system was opened completely with scissors and additional material for histological examination was taken wherever necessary. A block from a mediastinal lymph node was also taken.

Small blocks of tissue 1-2 mm in size were excised as soon as possible after euthanasia from the major bronchi and bronchioles, chopped into small pieces not more than 0.5 mm thickness with a grease-free razor blade on a block of paraffin wax and then immersed in small bottles containing chilled paraformaldehyde/glutaraldehyde solution at 4°C. (Appendix 1).

Towards the end of the project scanning electron microscopy became available as a technique for examining the lungs of these horses so that in the last few cases to be necropsied the cranial lobe of the left lung was excised, immersed in a container of paraformaldehyde/glutaraldehyde and inflated under gentle pressure via the main bronchus with the same solution.

The heart was removed from the thoracic organs at the origin of the great vessels, examined macroscopically and then opened to show the valves and to allow measurement of the circumference of the pulmonary arterial trunk and aorta. The method of Fulton, Hutchinson and Morgan-Jones (1952) was used to compare the weights of the right ventricular muscle mass to that of the left ventricle plus interventricular septum. The right ventricle was carefully excised close to its junction with the interventricular septum and the right atrium with extraneous fat removed. The left atrium was separated from the left ventricle and the whole was cleared of excess fat and chordae tendinaea. The right ventricle (RV) and left ventricle plus interventricular septum (LV + S)

were weighed and the ratio $\frac{LV + S}{RV}$ calculated.

Blocks of tissue were fixed for a minimum of 48 hours. After fixation, tissues were dehydrated and cleared in a double embedding series and finally embedded in paraffin wax under a vacuum. Paraffin embedded sections were cut at 5-6 μm on a Leitz rotary microtome and mounted on glass slides. The sections were routinely stained with haematoxylin and eosin (H and E). Selected sections were then stained with a number of special stains designed to help elucidate various aspects of the pathology including Martius scarlet blue, Verhoeff's van Giesson, alcian blue pH 2.6 - periodic acid Schiff (PAS), alcian blue pH 1.0-PAS, Perl's prussian blue, toluidine blue pH 0.3 and 4.0, astra blue-safranin, Biebrich's scarlet pH 9.9, carbol chromotrope, Hogg's and Bank's stain and H and E-toluidine blue. (Appendix 1).

Fixation of lung by inflation

The right lung was encased in double thickness polythene bags and laid on its ventral surface in a fume cupboard. The trachea, cut off to an approximate height of 25 cm. was supported vertically in a clamp and a glass funnel was placed in its open end. The polythene bag was then partially filled with ten per cent. formol saline to allow the lung to float. Formol saline was poured gently into the trachea until the pleura was smooth and the lung was judged to be fully inflated. The intrabronchial pressure did not exceed 30 cm. of water. In many cases this was a long tedious process and in a few instances possibly due to airway's blockage, the formol saline did not flow freely resulting in fixation of some segments only.

After a few days fixation the lungs were stored in bags containing a small amount of formol saline in large plastic dustbins. At a later date the lungs were sliced into thin slices on an electric bacon-slicer. Transverse slices were taken from the sites indicated in Figure 8 and were impregnated with a precipitate of barium sulphate after the method of Heard (1958) to aid identification of tissue, photographed and examined in detail under a dissecting microscope.

Transmission electron microscopy (TEM) technique

All methods for making up the solutions are in Appendix 1. The tissues were fixed for $4\frac{1}{2}$ -6 hours at 4°C and then rinsed several times in 0.1 M

cacodylate solution with added sucrose and left in the rinse overnight. They were post-fixed for one hour in one per cent. osmium tetroxide in Millonig's buffer and then dehydrated through a graded series of alcohols to absolute alcohol followed by half an hour in propylene oxide. Embedding of the tissue was in Araldite epoxy resin (Ciba), contained in gelatine capsules, polymerised for 48 hours at 57°C. 1 µm. sections were cut from the blocks on an LKB III ultratome, mounted on glass slides and stained with one per cent. methylene blue/Azure II solution. After viewing under a light microscope selected areas were cut at 500 Å on the ultratome and mounted on copper grids. These thin sections were stained first with 20 per cent. uranyl acetate and then with lead citrate, blotted dry and viewed with an AEI 6B electron microscope.

Scanning electron microscopy (SEM) technique

The inflated lung was left immersed in fixative for several days at 4°C and then pieces of tissue no larger than 10 mm. square and 3 mm. thick were selected, rinsed several times in 0.1 M cacodylate solution with added sucrose and then left in a solution of the rinse for 24-48 hours. They were then post-fixed in one per cent. osmium tetroxide in Millonig's buffer for one hour and finally dehydrated slowly over 24 hours in a graded series of acetones to absolute acetone. Care had to be taken that they were not in absolute acetone for longer than two hours as this tended to shrink the tissue. The tissues were then dried in a critical point dryer for an hour, mounted on stubs and coated with gold at a thickness of 500 Å in a sputter coater before being examined under a Phillips 500 SEM.

RESULTS

The results for each case are summarised in Table 6.

Macroscopic changes

Figure 9 shows a normal equine lung. In contrast the lungs of nearly all the 25 clinical cases of CPD failed to collapse immediately the thorax was opened and remained inflated or partially inflated after excision. Segments in the cranial lobe, ventero-caudal and the peripheral caudal lobes were most affected by this change (Figures 10 and 11). In the most obvious cases - 6, 12, 14 and 31 this puffy pale overinflated appearance remained for some hours after the lungs were removed from the carcass. In others, such as 1 and 37, the lungs collapsed considerably within five to ten minutes. Cases 26, 29 and 35 had no over-

inflation, the lungs collapsing immediately. Only in 6 were rib-markings seen on the dorso-lateral surfaces of the lung. Apart from the segmental areas of overinflation all the lungs were macroscopically unremarkable, except for pallor and prominent pleural vessels in a few animals.

Cases 7 and 33 had fractured ribs in the mid-thoracic region. Both fractures had rounded calluses and seemed to be of long-standing. The adjacent part of the caudal lobe had adhered to the fractured bone and there was an area of fibrous pleuritic adhesions (Figure 12). In neither horse did the reaction extend into the lung substance nor had any clinical signs been detected or attributed to the fracture.

Case 1 had a large abscess, 10 cm in diameter, in the middle of the right caudal lobe and there was some fibrinous pleurisy associated with this.

Most of the horses had fibrous "tags" originating from the visceral pleura in the posterior caudal regions of the lungs. These were 0.5 to 3 cm long, filamentous and found usually in large groups (Figure 13); similar tags could sometimes also be found on the liver and splenic capsules. There was no apparent explanation for their presence and no clinical or pathological significance was attributed to them.

Parasitic cysts were also a common finding in the lungs of many of the animals but were never numerous. These are not thought to be significant and are usually attributed to migrating parascarids or to aberrant strongyles.

Case 29 had about ten isolated, raised, hemispherical areas projecting above the normal lung surface (Figure 14). On section these were seen to consist of slightly overinflated alveoli with a greenish tinge and consisted of overinflated alveoli with numerous eosinophils present microscopically. Similar lesions were subsequently observed in horses with lungworm infection. No lungworm could be found in this animal but it seems likely that this was the cause.

The trachea of 37 was partially collapsed dorsally (Figure 15). The rings had opened and the dorsal membrane was slightly prolapsed into the lumen over the entire length of the trachea except for a small normal section at the thoracic inlet. The lumen of the trachea was still substantial and the animal had not suffered respiratory embarrassment during life. No clinical signs had been attributed to this and the narrowing was not detected during life.

Hydatid cysts were found in the livers of 1, 14 and 29. Case 7 had a massive mixed parasitic infection of strongyles and trichonemes and also a large adenoma of the right adrenal gland about 15 cm in diameter.

Only one of the 25 animals, 41, had mucus in the main bronchial tree or trachea (Figure 16). In a few cases - 6, 9, 12 and 38 there was pus in many of the subsegmental and smaller bronchi and in these horses the bronchiolar tissue also appeared rather prominent in cross-sections of the lobules.

Figure 17 shows the heart from one of the horses with measurable right ventricular hypertrophy. The right ventricle is thicker and larger than usual. In most cases however no cardiac abnormality was immediately apparent grossly.

Microscopic changes

In all 25 cases the most obvious lesion was bronchiolitis. Inflammatory changes of lesser extent also affected the smallest bronchi proximal to the terminal bronchioles but in no case was there any evidence of generalised bronchitis involving segmental or lobar bronchi. In a normal horse the bronchiolar epithelium is a single layer made up of ciliated cells and dome shaped cells which protrude into the lumen; these are probably Clara cells (Figure 18). Goblet cells are sparse in the smaller bronchi of the normal horse and do not extend into the smaller airways. Typically the bronchiolar epithelium in the 25 cases was hyperplastic having two, three or more cell layers above the basement membrane. The epithelium contained many tall columnar cells such as are found in the bronchi (Figure 19) and a substantial proportion of goblet cells (Figure 20). The goblet cells present in the bronchioles of diseased horses were secreting almost exclusively sulphomucins as determined by alcian blue - PAS staining sequences (see chapter 5). In 11 horses (Table 6) there were obvious plugs of mucus in the bronchiolar lumina (Figure 21) and these could also be seen in some of the smaller bronchi (Figure 22). Ten cases had polymorphonuclear leucocytic exudates in the bronchiolar lumina (Figure 23) including the four cases in which pus could be seen macroscopically.

The lamina propria was often hypercellular and infiltrated by lymphocytes, plasma cells and polymorphonuclear leucocytes (PMN's); the latter could occasionally be seen migrating through the bronchiolar epithelium. The basement membrane did not appear thickened in any of the horses.

There were marked changes in the peribronchiolar tissue. Normally the bronchioles are surrounded by a very sparse sheath of cells and connective tissue (Figure 24) but in most of the CPD cases examined there was a marked peribronchiolar reaction. In 22 cases this consisted of dense aggregates of lymphocytes without germinal centres, PMN's, plasma cells and mononuclear cells (Figure 19). In 3 cases 9, 33 and 41 there was a substantial collagen component as shown by staining with Martius scarlet blue and in 23 and 24 this could be said to be predominantly collagenous in nature with very few cells present (Figure 25). The peribronchiolar cellular accumulations often contained a considerable number of eosinophils and/or mast cells. Eosinophilia of pulmonary tissues - alveolar walls, peribronchial, peribronchiolar and perivascular areas - was found in 16 of the 25 horses. This was of variable extent and intensity but generally peribronchiolar connective tissue and alveolar walls were the areas most affected. When eosinophils were seen in the bronchial tissue they usually occupied the peribronchial connective tissue but were also seen in the epithelium. Two cases, 29 and 37, in particular had marked eosinophilia (Figures 26 and 27). Large numbers of eosinophils had infiltrated in many areas of the pulmonary tissue but especially in the peribronchiolar connective tissue and in all parts of the bronchial and bronchiolar epithelium. Occasionally there was patchy oedema associated with areas of eosinophilia but this was not a common finding.

The number of pulmonary mast cells was found to be increased in ten horses (Chapter 6). This number, however, was not a true proportion of the 25 since in order to preserve mast cell integrity tissue must be fixed adequately shortly after death. In most of the cases derived from the files this had not been done as the tissues were generally poorly preserved as regards definitive mast cell demonstration. If these are excluded ten out of 19 horses had mast cell hyperplasia. The increased number of mast cells was most obvious around the bronchioles (Figure 28). A full description of these changes is in Chapter 6.

Focal alveolar epithelial hyperplasia affecting the alveoli adjacent to larger bronchi and some subpleural areas was seen in five horses all of which had other quite severe lesions. The alveoli were lined usually by a cuboidal epithelium but sometimes this was partially columnar and ciliated. Inter-alveolar septa were thickened by fibrosis focally this sometimes extending to

involve a few acini (Figures 29 and 30). In two of these cases 12 and 33 some of the alveolar spaces were filled with pools of mucus; the change was most widespread and extensive in 33 (Figure 31). Three other cases 14, 23 and 34 also had pools of mucus in alveoli adjacent to bronchi and bronchioles in the absence of focal fibrosis.

Staining with Perl's prussian blue was designed to show up macrophages containing haemosiderin, a break-down product of blood or mucus. In four cases this was found to be a significant feature of the disease but it was a minor observation in many more.

Although bronchiolitis and narrowing was the usual change occasionally the bronchioles retained the cuboidal epithelium and became widely dilated and contained some mucus and macrophages in the lumina. This tended to occur in areas of alveolar overinflation.

The mediastinal lymph nodes were never enlarged beyond normal nor were there obvious microscopic changes in their structure save for mild infiltration by eosinophils in all of the cases with a pulmonary eosinophilia (Figure 32).

Examination of the lung slices

The lungs were found to slice evenly down to a thickness of 1-2 mm and this facilitated the microscopic examination. Not all parts of the lung had inflated fully with the formolín and this variation was clearly shown following impregnation with barium sulphate (Figures 33 and 34). Nevertheless ten cases could be assessed in detail for the extent of emphysema. The gross photographs indicated the approximate extent of this and also provided a permanent record of the slices. The cranial lobe slice was often grossly emphysematous (Figure 35) to a variable extent but never severely so and also the lateral tip of the caudal lobe (Figure 36). The slices were then examined in detail under a dissecting microscope at magnifications of 12 X to 50 X and this confirmed the initial assessment. Only one case, 9, had any evidence of emphysema in the body of the caudal lobe. This consisted of small isolated areas surrounding blocked bronchioles usually in the dorsal area of the lobe (Figures 37 and 38). No other case had any evidence whatsoever of emphysema except in the areas seen as grossly emphysematous. Figures 39 and 40 illustrate the usual appearance of the inflated lung, there is no alveolar wall destruction and the airways are of

approximately equal size.

The emphysema in the cranial lobes was difficult to classify as the lobules in the horse are poorly demarcated. Sometimes it had a centrilobular appearance but generally was unclassifiable. In the edge of the caudal lobe small emphysematous bullae were sometimes seen. In one case, 14, the emphysema was associated with fibrosis (Figure 41).

When the lung of 12 was sliced it was apparent that a cylindrical bronchiectasis was present throughout the lung at all levels (Figure 42). This had not been noticed at necropsy nor at subsequent histological examination. The bronchial epithelium had a normal histological appearance.

As has already been stated the interlobular septa in the equine lung are sparse, incomplete and poorly developed. However, it was interesting to note from observation of the slices that the areas affected by emphysema, the cranial lobe and periphery of the caudal lobe, were also the areas where interlobular septa appeared most frequent and complete (Figures 43 and 44).

Transmission electron microscopy

For various reasons a detailed ultrastructural study of the equine bronchial and bronchiolar epithelium was not possible. As far as could be ascertained the basic cellular structure of the bronchial walls was identical to that found in other mammals (Breeze and Wheeldon 1977) and no difference could be found between the ultrastructure of bronchi of normal horses and those with CPD. However in the bronchioles marked differences were apparent and these reflected the changes found on light microscopy. The epithelium of bronchioles from normal horses was one cell deep and consisted almost exclusively of ciliated cells and nonciliated bronchiolar secretory cells (Clara cells) (Figure 45). Whereas the bronchiolar epithelium of horses with CPD was several cells deep and contained goblet cells in addition to ciliated cells and nonciliated bronchiolar secretory cells (Figure 46). The impression was gained that the goblet cells were present at the expense of the nonciliated bronchiolar secretory cells although nonciliated bronchiolar secretory cells were still seen. Not enough material was examined to confirm this quantitatively. This new population of goblet cells had the typical appearance of mammalian goblet cells as reviewed by Breeze and Wheeldon (1977). The nucleus was oval, basal and sometimes obscured by granules. These cytoplasmic granules were numerous,

of uneven size and density (Figure 46) and gave the cell its characteristic flask or goblet shape. Discharging goblet cells were commonly seen and discharge seemed to be of two different types. Sometimes the cytoplasm was homogeneous as if all the granules had disintegrated, the apical membrane was missing and the contents were being extruded into the lumen (Figure 47). This type of goblet cell discharge was said to be uncommon in other mammalian species (Breeze and Wheeldon 1977) where apocrine secretion of intact granules through apical pores is the usual method of discharge. This type of secretion was also seen in these equine bronchioles and the two forms of secretion occurred in the same specimens. The intact goblet cell cytoplasm contained mitochondria and rough endoplasmic reticulum sometimes in large amounts reflecting the activity of these cells.

The hyperplastic epithelium had a well-ordered appearance with normal cell junctions and no bizarre cells being present. Intermediate cells and basal cells were found and the epithelium had the appearance of an actively secreting normal bronchial epithelium. Mast cells and eosinophils were found amongst the epithelial cells and in the lamina propria of those cases showing hyperplasia of these cells histologically. Neutrophils and lymphocytes were a more common finding particularly in the lamina propria (Figures 48 and 49).

The nonciliated bronchiolar secretory cells had the same appearance as those seen in other mammals (Breeze and Wheeldon 1977) except that the nucleus was more often spherical than deeply invaginated as reported by other workers in various species. The cells were tall, projecting above the ciliated cells and were also much wider than these cells so that the ciliated cells often had a rather spindly squashed appearance. The apical cytoplasm formed a rounded "bleb" and short microvilli with rounded tips projected from the apical cellular membrane. The apical cytoplasm contained numerous mitochondria and an abundant smooth endoplasmic reticulum. The mitochondria in this part of the cell were rounded and sometimes did not contain cristae (Figure 50). Further down the cell they had a more normal appearance being elongated and containing visible cristae. It has been suggested that the bronchiolar secretory cells provide some kind of surface-active layer in the bronchioles (Breeze and Wheeldon 1977) and a thin hypophase in which the cilia may beat normally. It would be interesting to know what sort of effects a change in the secretion to one that is more mucoid has on the action of the cilia.

Scanning electron microscopy

Three animals with CPD, 34, 38 and 42 were used in this study and a comparison was made with several normal equine lungs. The main purpose of the study was to examine the changes occurring in the alveoli of the cranial lobe. By looking at different areas of the lobe in these animals it was possible to build up a sequential order of events in the development of emphysema. The alveoli of the normal equine lung are regular in size and have only a few small alveolar pores of Kohn per alveolar wall (Figures 51 and 52). The first change to occur in the lungs of horses with CPD was that the pores became larger and apparently more numerous (Figures 53 and 54) and at the same time the alveoli became enlarged although at this stage they still maintained a more or less regular appearance and there was no destruction of alveolar walls. A further development seen around this time was that the alveolar macrophages were more numerous, often occurring adjacent to the enlarged pores and "plaques"

were also seen in the alveoli (Figure 55). This latter structure has been observed before in SEM of emphysematous horse lung (Nowell, Gillespie and Tyler 1971) and its significance is not known. Later on in the process alveolar wall destruction occurs and the pores of Kohn enlarge still further. These two changes combine to give the lung an open, irregular almost lacy appearance (Figure 56). The final stages are depicted in Figures 57 and 58 where very little normal alveolar wall remained and large fenestrae were frequent—supported by thin trabeculae of tissue.

Nowell, Gillespie and Tyler (1971) in their studies on emphysematous horse lungs with the SEM could find no changes in the cells of the pulmonary airways of horses with CPD when compared to normal horses although the terminal bronchioles were partially destroyed in the areas of emphysematous change. Similarly in the three horses that were examined for this study it was difficult to correlate exactly the changes seen on light microscopy and TEM with the appearance of the airways on SEM.

The bronchial epithelium in the normal horse lung when viewed under the SEM is shown in Figures 59 and 60. The cilia can be clearly seen projecting above the general level of the cells arranged in dense, regular waves. Individual cells can be seen projecting through this ciliary layer and these are presumably goblet cells. The appearance of the bronchi in the horses with

CPD was identical as it was in the other microscopical studies. Generally the mucous blanket is swept off the airways during the processing of the tissue but occasionally areas of mucus are left undisturbed. The appearance of this mucus is depicted in Figures 61 and 62. Various cell types are caught up in a mesh of irregular haphazard strands of mucus.

The bronchioles of the normal horse present a totally different appearance (Figures 63 and 64). Here, the predominant cell type is the bronchiolar secretory cell the apices of which project up in rounded processes from the main level of the epithelium. These larger cells are surrounded by a fringe of cilia emanating from the ciliated cells. Cilia are much less frequent in the bronchioles than in the bronchi. The bronchioles of the horses with CPD had a roughly similar appearance with large cells projecting above the normal level of cells. It was not possible to tell if these were bronchiolar secretory cells or goblet cells but from the light microscopical studies it would be expected that some would be goblet cells. Any cellular or mucous exudate that may have been present would have been swept off during the processing.

Cardiovascular Measurements

The results from the 17 horses that were measured are displayed in Table 8. Thirty eight horses without respiratory or cardiovascular abnormalities were also examined during the course of this study and the same cardiac measurements were made. These results are shown in Table 7. The value for $\frac{LV + S}{RV}$ is normally within the range 3.306 ± 0.568 as long as the left ventricle is not hypertrophied. Values falling outside this range, particularly lower values, were judged to be abnormal. Only five horses with CPD fell below the normal level although a further one had a marginal value. 11 and 29 both had values of 1.4 indicating severe right ventricular hypertrophy, neither horse had clinical signs of congestive cardiac failure. Despite the fact that only a small proportion of horses had measurable right ventricular hypertrophy 14 of the horses were found to have pulmonary arterial trunks with a larger circumference than that of the aorta. Due to a lack of reliable history in these cases it is not known whether they were affected by CPD for a longer period than the rest.

DISCUSSION

This is the largest study of the pathology of chronic respiratory disease in horses to be published. Other workers (Gerber 1973; Sasse 1971) have recorded greater numbers following their studies on clinical signs and respiratory function but the results of their pathological studies are not published.

The results of the investigation of 25 horses have demonstrated that bronchiolitis is the most consistent pathological change associated with the syndrome of CPD in horses. The most distal bronchi are also involved but to a lesser extent and only if their diameter is comparable to that of the larger bronchioles. Other than this the bronchi do not appear to be involved in the pathological process. The bronchiolar changes have four major characteristics:

1. epithelial hyperplasia the epithelium becoming two to three cells deep.
2. epithelial metaplasia occurs with goblet cells appearing in the epithelium in addition to the ciliated cells and Clara cells
3. peribronchiolar fibrosis and cellular infiltration
4. exudation of mucus or PMN's into the lumina.

This combination of lesions contrives to produce bronchiolar stenosis causing interference with airflow and having various effects on the dependent acini. Initially the acini supplied by a narrowed bronchiole become over-inflated. Laennec (1834) first supplied an explanation of this effect by likening the obstruction to a "check-valve". He proposed that inspiration of air occurred easily but on expiration the change of pressures closed the valve so trapping air in the obstructed portion. This explanation has persisted until the present day. A more sophisticated explanation was afforded by McLean (1958) who recognised that collateral ventilation of acini is possible. When acini are obstructed by a plug of mucus or inflammation in the supplying bronchiole they are ventilated collaterally from adjacent acini, possibly through pores of Kohn or via respiratory bronchioles. Collateral ventilation during inspiration is relatively easy as the pores and air passages are open but during expiration these airways narrow considerably and therefore collateral airflow is more difficult. The degree of overinflation will obviously increase as the collateral pathway becomes longer and more tortuous until the ability to expire air, from that

acinus is lost. Collateral airflow channels in the equine lung are not elucidated but it is said that the equine lung is microanatomically like that of man (McLaughlin, Tyler and Canada 1961). It would seem likely that collateral flow like that in the human lung must have occurred in the overinflated lungs of the horses with CPD.

Build-up of pressure distal to the plug has a slightly beneficial effect in that it may cause the plug to be more readily expelled but this must be balanced against the deleterious effect of increased intraalveolar pressure.

Chronic bronchitis and emphysema, the so-called British Disease, is probably the commonest respiratory condition of middle-aged men causing the loss of 27 million working days per annum (Ministry of Pensions and National Insurance 1955). Chronic bronchitis and emphysema can occur together or separately. In chronic bronchitis the main change is hypertrophy of the mucous glands in central airways with proliferation of goblet cells in small airways. In emphysema, the main lesions are in the acini, although small airways lesions commonly coexist. The relationship of small airway abnormalities to chronic bronchitis and emphysema is not clear but it is believed that these represent a separate effect of air pollution and smoking. In considering human chronic obstructive pulmonary disease, then, as well as chronic bronchitis and emphysema, together or separately, there must be separate evaluation of small airways disease. Disease of the small airways has been described as "a challenge to medicine" (Macklem 1972) and has been the subject of recent intensive research as it has become apparent that acute bronchiolitis is common during so-called upper respiratory tract infections (McLean 1958) and is also frequent as a chronic condition which is asymptomatic, at least in initial stages (Macklem 1972).

It is not surprising that the main lesion found to be obstructing airflow in these horses was bronchiolitis and not bronchitis as has been commonly reported in the literature for many years. This is not the first study to find an absence of bronchitis. Thurlbeck and Lowell (1964) and Foley and Lowell (1966) found bronchiolitis to be the main lesion and other authors, Gerber (1973) and Gillespie and Tyler (1969) only found bronchitis in a small proportion of cases. Despite this, a recent review article (Cook 1976) was entitled "Chronic bronchitis and emphysema of horses". The assumption that chronic

bronchitis was involved appears largely to be based upon a comparison with chronic bronchitis in man and the assumption that because cough and dyspnoea at rest or after exercise are present in man and the horse the underlying pathological lesions must be identical. Such sweeping generalisations are dangerous and have probably impaired investigations into the disease. The disease in the horse does not meet all criteria for the definition of chronic bronchitis which was considered by a symposium sponsored by CIBA in 1959 to refer to the condition of subjects with chronic or recurrent excessive mucous secretion in the bronchial tree. The words chronic bronchitis should not be used to describe the equine condition.

The most striking change in the bronchioles is the presence of goblet cells in the epithelium. It must be assumed that these cells differentiate from precursors already in the epithelium and that there is an external stimulus which initiates this particular line of differentiation which is dormant in the normal individual. From the limited electron microscopical studies carried out on the epithelium it was not clear if the goblet cells proliferated either at the cost of the Clara cells or of the ciliated cells. Certainly Clara cells and ciliated cells still occur in the epithelium in appreciable numbers although there was an impression that Clara cells were less frequent.

The appearance of goblet cells in bronchiolar epithelium is a well documented phenomenon and can easily be artificially induced in animal subjects by causing them to inhale irritants such as sulphur dioxide or tobacco smoke (Reid 1963; Chakrin and Saunders 1974) or by the injection of isoprenaline (Sturgess and Reid 1973; Baskerville 1976). It should be noted, however, that changes in central airways accompany this goblet cell metaplasia whereas this was not a change noticed in our series of horses. Horses have very small submucosal glands as compared to other mammals (personal observation) and this situation is not altered in the horses with CPD. In the rat the Clara cell is said to transform into a goblet cell (Lamb and Reid 1968). It would be unusual however for an already fully differentiated mature cell to transform into another fully differentiated cell with a completely different structure and function. Rather is it more likely that the two cells have the same precursor. At present the origin of these metaplastic goblet cells cannot be stated with certainty because experimental studies of their proliferation have involved procedures stimulating airway cells at all levels. The role of the type 2

pneumocyte as progenitor of type I pneumocytes seems well established now whereas this would have seemed highly unlikely ten years ago. The possibility of a pluripotential differentiating capacity of bronchiolar epithelium is indicated but not yet fully established.

Goblet cell metaplasia, as it is commonly called, is not easy to assess and many workers have not found conclusive evidence of goblet cell metaplasia in peripheral airways of chronic bronchitics although this may be heavily influenced by samples and cases selected (Reid 1960; Greenberg, Boushy and Jenkins 1967; and Bath and Yates 1968). Metaplastic change was, however, more obvious in patients dying of acute respiratory failure following chronic airflow obstruction (CAO). It seems to be more important in contributing to the pathogenesis of CAO or in precipitating acute respiratory failure. The horses in the study had CAO as evidenced by their respiratory difficulties and the results of the respiratory function studies. The animals do not, however, die of respiratory failure under normal circumstances.

The chief importance of the abnormally placed goblet cells is that they secrete mucus into the lumina of the bronchioles and predispose to obstruction unless the mucus is adequately cleared by the cilia or is coughed up into the larger airways. Not all the horses had mucous plugs in the bronchioles and some had no excess mucus in the airways at all, although goblet cells were abundant in the bronchioles. The difference may lie in the relative efficiency of individuals to clear mucus or in the relative volumes or viscosity of the secretions elaborated. A difference detected at autopsy between patients with chronic bronchitis and no emphysema and those with chronic bronchitis and emphysema is that patients with emphysema have excess mucus in the small airways (Thurlbeck 1976). In part this can be explained by the fact that patients with emphysema died of this and terminal infections could have been superimposed in the smaller airways. However it does not fully account for the difference. The patients did not seem to have been hypersecreting mucus, judged by a low Reid Index (relative width of submucosal gland layer), so mucus clearance must have been impaired. Two reasons are proposed to explain this:

1. narrowing of peripheral airways so that maximum volume outflow was reduced lowering the efficiency of coughing.
2. downstream closure during coughing so that mucus was trapped.

The horses with marked mucous plugging also had more severe bronchiolar reactions which would narrow the lumina considerably. The prolonged paroxysms of non-productive coughing that some of the horses were subject to may have been abortive attempts to clear the plugs from stenosed bronchioles. When the plugs were predominantly PMN in nature the same theories would apply. It is not clear whether the so-called "mucous" plugs in patients with chronic bronchitis or emphysema are predominantly PMN or mucus in content. The nature of the plug would considerably affect its viscosity, so it would be interesting to know which type is more readily cleared.

A variable degree of pulmonary eosinophilia was a feature of 16 horses. It was impossible to link this with any particular pathological change in the tissues or with a particular clinical history but nevertheless considerable significance should be attached to this finding. Under natural conditions horses are subject to various parasitic infections. Parascaris equorum in its migrations through the pulmonary tissue causes a transitory pulmonary eosinophilia (Nicholls and others 1978) and it is probable that a considerable proportion of horses are exposed to lungworm infection or reinfection (Dictyocaulus arnfieldi) (Round 1976; Nicholls, Duncan and Greig 1978). Therefore in a series of necropsies subjects with parasite induced pulmonary eosinophilia might be expected to be found. However the number found was too large to be accounted for by sporadic parasitism. Apart from parasites, eosinophils are associated with allergic reactions such as asthma and type I hypersensitivity. Bronchial asthma has been defined clinically by Crofton and Douglas (1975) as a "recurrent, generalised airways obstruction which, at least in the early stages, is paroxysmal and reversible". Bronchitis and asthma often occur together in the same subject and on occasion it is difficult to differentiate the two conditions. Three main factors contribute to the bronchial obstruction:

1. bronchial muscle contraction, sometimes known as bronchospasm. Experimentally this can be induced by specific allergens in guinea-pigs and in human lungs resected from patients with asthma (Schild and others 1951).
2. swelling of the mucous membrane
3. plugging of bronchi and bronchioles with viscid mucus

The first factor, contraction of bronchial muscle, is thought to be the most important of these.

In man it is difficult to define the pathology of asthmatic conditions as material has only been obtained from patients dying suddenly of a severe asthmatic attack (status asthmaticus) an occurrence that is very unusual amongst the population of asthmatics. Most patients are mildly affected and able to live a normal life-span. Cardell and Pearson (1959) necropsied 43 fatal cases of asthma and found several characteristic features at necropsy:

1. distended lungs which did not collapse on opening the thorax.
2. many bronchi plugged with extremely tenacious, viscid mucus
3. thickened, congested bronchial wall with apparent thickening of bronchial muscle. The lining was often shed and goblet cells were abundant.
4. hyperplasia of sub-mucosal bronchial glands
5. basement membrane of bronchi thickened and hyaline.
6. eosinophil infiltration of all parts of the wall.

Similar changes occurred to a lesser degree in the bronchioles. Bronchial asthma seems to be a disease of the bronchi and so is difficult to correlate with our cases. In fact there seem to be very few demonstrable parallels pathologically, the only common denominator being the presence of eosinophils.

Clinically some of the cases resemble asthma, being worse in hot weather, exacerbated by dust and precipitated by aerosols of specific antigens. However, the resemblance cannot be said to continue on pathological examination. Features such as an increase in bronchial goblet cells, submucosal gland hypertrophy and thickening of the basement membrane must be present in non-fatal cases of asthma as well and this is borne out by the results of a limited number of biopsies (Glyn and Michaels 1960). None of these features was shown in the cases and it is therefore concluded that chronic bronchiolitis in the horse is not directly comparable to asthma in man.

Pulmonary eosinophilia in man is an ill-defined condition characterised by blood eosinophilia and shifting pulmonary shadows radiographically. It is classified by Crofton and Douglas (1975) into:

1. simple pulmonary eosinophilia (Lofflers syndrome).
2. prolonged pulmonary eosinophilia.
3. asthmatic pulmonary eosinophilia.
4. tropical pulmonary eosinophilia.
5. polyarteritis nodosa and Wegener's granulomatosis.

Simple pulmonary eosinophilia is a mild, transitory illness possibly associated with infection with Ascaris lumbricoides larvae although it may also be produced by drugs such as para-amino salicylic acid.

Prolonged pulmonary eosinophilia is relatively uncommon and various aetiological agents have been blamed, including moulds, parasites and Brucella abortus infections. The illness is accompanied by fever and a high blood eosinophilia.

Asthmatic pulmonary eosinophilia is the commonest of the five, the majority of cases being associated with hypersensitivity to Aspergillus fumigatus and others to Candida albicans. A few cases have come to autopsy but the pathology did not resemble that seen in cases of CPD, nor are the clinical signs identical.

Tropical pulmonary eosinophilia is commonest in the north-west part of the sub-continent of India and is thought to be related to filarial infestation. Pathologically the reaction consists of focal granulomata and alveolitis.

Polyarteritis nodosa is a rare disease characterised by foci of necrotising arteritis throughout the body and in a third of cases the lungs are involved. Clinically the disease is severe, with weight loss, malaise, fever and bouts of pneumonia. Pathologically there are necrotic, caseous foci, infarcts and bronchiectasis in the lung. Wegener's granulomatosis may be thought of as a variant of this disease localised in the lungs and upper respiratory tract. The aetiology of polyarteritis nodosa is uncertain but because Wegener's variant is becoming commoner it has been suggested that it is related to hypersensitivity to antibiotics.

None of these five syndromes fits the picture of CPD built up from our cases. Allergy has been thought to play a major role in the aetiology of CPD for many years (see Review of the Literature) and severe attacks of dyspnoea can be induced in these horses by exposing them to dust or specific antigens

particularly M. faeni and A. fumigatus. Given that some of the horses at least are "allergic" to substances in their environment in that these substances exacerbate the disease it is difficult to equate this convincingly with the lesions found in the lungs. Apart from eosinophilia which was really only severe in two cases, none of the other features of pulmonary eosinophilia as seen in man were present. It should also be noted that the differential blood eosinophil level in these horses was never above ten per cent. and most of the time it was down at the one or two per cent. level.

It has been observed that eosinophil cells are frequently attracted to immune complexes, especially in the lung (Litt 1964) and it could be that the eosinophilia is not a manifestation of allergy in the classical sense but merely an immunological phenomenon yet to be defined. It is also possible that eosinophilia is not immunologically mediated at all. Interstitial eosinophilia is a feature of fog fever in cattle, a disease which is not known to have a hypersensitivity basis but which appears to be caused by a toxic chemical produced in the rumen.

The significance of the increase in mast cells has been discussed in Chapter 5.

It has been suggested that CPD is a disease analogous to extrinsic allergic alveolitis in man (Thurlbeck and Lowell 1964; Crofton and Douglas 1975), a disease caused by a type III or IV delayed hypersensitivity reaction to fungal spores or bacteria. The chief aetiological agent of this is M. faeni a thermophilic actinomycete found in mouldy hay and straw. Other authors have blamed the disease on hypersensitivity to fungal spores without specifying the precise mechanism (Lowell 1964; Eyre 1972; Cook 1976). The basis for this is the well-documented exacerbation of disease that occurs if mouldy hay is fed, "outbreaks" of disease when mouldy hay is fed habitually and the positive skin tests and serum precipitins to mould antigens that occur in cases of CPD. Pauli, Luginbuhl and Gerber (1972) described a series of seven horses with lesions very similar to those of farmers lung in man and cattle but these are the only well-documented cases in the literature and formed only a small proportion of the large number of horses examined in the same survey. None of the cases showed any of the classical features of extrinsic allergic alveolitis, such as pulmonary granuloma, alveolar inflammation and

infiltration and extensive bronchiolitis obliterans. Bronchiolar reactivity to fungal or bacterial antigens cannot be discounted because of the overwhelming circumstantial evidence to support the theory. All that can be said at the present time is that there is no analogous reaction reported in the literature in any species.

The term "cor pulmonale" tends to convey a slightly different meaning to members of different medical disciplines. As far as the pathologist is concerned it means measurable right ventricular hypertrophy (RVH) which can be precisely delineated. Some clinicians do not use the term until a patient is in heart failure characterised by distended jugulars or peripheral oedema which is confusing it with congestive cardiac failure. Thurlbeck (1976) suggested that the term be dropped altogether, but recognised that it remained a convenient shorthand method of conveying a complicated definition. The World Health Organisation (1963) proposed the following definition which has been widely accepted. Cor pulmonale is defined as "hypertrophy of the right ventricle resulting from disease affecting the function and/or structure of the lung, except where these pulmonary alterations are the result of diseases that primarily affect the left side of the heart or of congenital heart disease". This therefore restricts the term to diseases in which RVH exists or may reasonably be assumed to occur. If there is any uncertainty, terms such as pulmonary hypertension or increased vascular resistance should be used (Thurlbeck 1976).

Extrapolating this to our cases it is correct to use the term cor pulmonale as this might reasonably have been expected to occur in the series of cases. Cor pulmonale is a common consequence of small airway narrowing in man (Bignon, Andre-Bougaran and Bouet 1970). RVH is more common in patients with emphysema than in the general population and more common in patients with severe emphysema than mild emphysema (Thurlbeck 1976). This latter observation has been backed up by several necropsy studies. Hasleton (1963) found that 44 per cent. of patients with panlobular emphysema and 50 per cent. with centrilobular emphysema had RVH, and Otto, Zeilhofer and Reissinger (1969) found that 63 of 244 patients with moderate to severe emphysema had RVH. RVH is uncommon in patients with chronic bronchitis and no evidence of emphysema assessed at autopsy (Thurlbeck 1976).

The series is too small to make dogmatic statements about the incidence of RVH in horses with CPD. However, considering the nature of the clinical signs in many of the horses and the severity of the pathological findings it is surprisingly low. This could be compared to the situation in man, where the incidence of RVH is mild if emphysema is mild despite widespread bronchitic lesions being present. Dilation of the pulmonary artery was an almost constant finding even if measurable RVH was not present, probably indicating a degree of pulmonary hypertension, obviously not severe or perhaps prolonged enough to cause cor pulmonale in most cases. Salutini (1951) and Alexander (1959) both described deaths in horses with CPD resulting from cor pulmonale although it is not clear in the latter instance whether this was from personal experience or review of the literature. This may have been more common in the past when horses with CPD were worked beyond their capacity but nowadays as understanding of the disease develops the animals are not stressed in this way and heart failure is correspondingly uncommon. Only one horse in our group, 12, presented with some clinical signs of heart failure namely ascites and jugular distension. None of the other animals had, to our knowledge, clinical evidence of heart failure at any time.

It is believed that the method chosen to assess RVH is the most consistently reliable available at this time. Various other methods of assessment have been described, such as weighing the septum and ventricles separately which is difficult to do accurately; measuring the thickness of the right ventricular wall, which is subject to gross variability due to post mortem cardiac contraction and variations from site to site; and measurement of cardiac muscle fibre diameter on histological section, which is again subject to variability and shrinkage on fixation. In man, where many measurements are made standard absolute weights for the muscle masses have been cited (Fulton, Hutchinson and Morgan-Jones 1952), although Thurlbeck (1976) claimed that these are actually subject to quite a wide variation dependent upon sex, stature and body-weight, a point already made by the MRC Committee (1972). It is concluded that the ratio $\frac{LV + S}{RV}$ is in fact the most accurate way to assess RVH. The disadvantage is that 'normal' is subject to considerable variation in stature, weight and workload necessitating caution in interpreting normal values, such as left ventricle size. There is need for detailed studies of these normal values in the horse.

Dissection of the heart is relatively quick and easy and, despite the reservations of the MRC Committee (1972) and Thurlbeck (1976) it was not found that the trimming of fat and vessels was a lengthy process. In the light of this experience it would seem to be a useful exercise for cardiac measurements to be carried out on all animals presented for necropsy with evidence of a chronic pulmonary disease.

Cylindrical bronchiectasis was found in one case, 12. The significance of this finding is not known, since bronchiectasis has not been recorded in the horse. In man bronchiectasis is only important clinically if there is bronchial infection or if it gives rise to bronchial haemorrhage (Crofton and Douglas 1975). The condition usually arises in childhood following infections such as bronchiolitis or pneumonia or obstruction of a bronchus by a tumour or foreign body (Crofton and Douglas 1975). It is therefore possible that the bronchiectasis may have been present in this horse before CPD developed. Bacterial complications of equine influenza are common but as little history of this horse was available it is not known if she had suffered such an episode. On the other hand this was one of the cases with marked alveolar epithelial hyperplasia and fibrosis around all the major bronchi and bronchiectasis may also arise from a fibrous condition of the lung (Crofton and Douglas 1975). This horse was also the only one in the series to show clinical signs of heart failure. Until more of such cases are found the pathogenesis and functional significance will be difficult to interpret.

That emphysema is not an important part of the pathology of the disease has been shown by the studies on the lung slices. All of these cases examined had been affected for many years and the fact that even at this stage there was relatively little emphysema would seem to indicate that its development is a terminal feature of the disease. Overinflation, potentially reversible, is the main alveolar change and to call the disease chronic alveolar emphysema is quite incorrect.

Studies, using the SEM, on the small areas of emphysema that do occur in the cranial lobe have shown that the development is associated initially with an enlargement of the alveolar pores of Kohn followed by gradual thinning or loss of alveolar walls. The type of emphysema was difficult to classify accurately; in some cases the pattern appeared centrilobular but was

generally irregular.

In making a detailed study of the pathology it was hoped to be able to make an analogy with a comparable disease in man or other animals. This might then, by comparison, have helped to elucidate the aetiology and pathogenesis of the disease. As has been shown however the disease does not resemble any single condition recognised in man although some features in common with chronic bronchitis, emphysema and asthma of man have emerged. The condition it most resembles is the so-called small airways disease and, if classification of CPD be needed, it may conveniently be amalgamated into this already broad category. It must be remembered that the horse has unique pulmonary features distinguishing it from man and other animals (McLaughlin, Tyler and Canada 1961). The microanatomy of the equine lung has been likened to that of man (McLaughlin and Edwards 1966; Tyler, Gillespie and Nowell 1971) but it must be remembered that the lung of the horse has many important distinguishing features such as the size, lack of lobation and lobulation, horizontal posture of the horse, well developed bronchial arteries and, perhaps most significantly, long terminal bronchioles with few or no respiratory bronchioles. As the lesion is bronchiolar this last may be an important factor in the pathogenesis of disease.

The predominant lesion in this series of horses was bronchiolitis and in the sections that follow this particular syndrome is referred to as chronic bronchiolitis (CB) to distinguish it from other forms of CPD which may occur.

Case	Bronchioles		Peribronchiolar		Alveoli		Pulmonary tissue				
	Inflam- mation.	Mucous plugs	PMN plugs	Cellular Infiltration	Fibrosis	Epithelial hyperplasia	Over- inflation	Mucus in alveoli.	Eosino- philia.	Mast cells	Haemo- siderin.
1	++	+	-	++	-	-	++	-	-	-	-
6	+++	-	++	+++	-	++	-	-	-	++	+++
7	++	+	-	++	-	-	++	-	+	++	-
9	+++	+	++	+	++	++	-	-	+	++	+
11	++	++	-	+	-	-	++	-	+	++	-
12	+++	-	++	++	-	++	+	-	-	++	-
13	++	-	-	+	-	+	-	-	++	-	-
14	+++	+	-	+++	-	-	-	-	+	-	-
15	++	-	-	+	-	-	-	-	-	+	-
20	++	++	-	+++	-	-	-	-	++	-	-
23	++	+	-	-	++	-	-	-	++	-	-
24	+	-	-	-	+	-	-	-	++	-	-
25	++	-	-	+	+	-	-	-	+	-	-
26	+++	-	+	++	-	-	-	-	-	-	-
29	+	-	-	-	+	-	-	-	++	-	-
31	++	+	-	++	-	-	-	-	+	-	-
33	+++	-	-	++	-	-	+	+	++	-	-
34	++	-	+	-	-	-	-	+	++	+	-

Table 6 continued overleaf

Case	Bronchioles			Peribronchiolar		Alveoli		Pulmonary tissue			
	Inflam- mation.	Mucous plugs	PMN plugs	Cellular infiltration	Fibrosis	Epithelial hyperplasia	Over- inflation	Mucus in alveoli	Eosino- philia.	Mast cells	Haemo- siderin
35	+	-	-	+	-	-	-	-	+++	-	-
36	+	-	-	+	-	-	++	-	-	-	-
37	++	+	-	+	-	-	++	-	+++	+	-
38	+++	-	+	++	-	-	++	-	-	-	-
39	+++	+	+	++	-	-	+	-	-	++	+
40	+++	-	+	++	-	-	+	-	-	-	+
41	+++	+	++	++	+	+	+	+	+	+	-

TABLE 6. Summary of the pathological findings in 25 horses with chronic pulmonary disease.

Case	Circumference of (cm)		Weight of (g)		Ratio
	Pulmonary artery	Aorta	Right ventricle	Left ventricle + septum	$\frac{LV + S}{RV}$
1	7.5	7.5	85	300	3.529
2	8.2	8.2	120	370	3.083
3	8.8	9.3	120	420	3.500
4	10	10	170	520	3.059
5	8.8	8.8	138	470	3.406
6	7.5	7.5	150	440	2.933
7	7.0	7.0	80	290	3.625
8	7.4	7.4	105	380	3.619
9	8.8	8.8	130	440	3.385
10	8.8	8.8	150	550	3.667
11	7.4	7.4	75	310	4.133
12	7.4	7.4	80	350	4.375
13	7.4	7.4	80	290	3.625
14	7.4	7.4	123	395	3.202
15	7.4	7.4	100	350	3.500
16	5.0	5.0	67	220	3.300
17	6.0	6.0	60	250	4.167
18	6.0	6.0	50	210	4.200
19	7.5	7.5	80	320	4.000
20	7.5	7.5	80	310	3.875
21	12	17	906	2816	3.108
22	7.5	9.0	228	768	3.368
23	12	11	350	1164	3.326
24	18	18	880	3330	3.784
25	14	14	55	1740	3.164
26	8.5	8.5	76	260	3.421
27	7.5	7.5	66	192	2.909
28	3.5	4	47	147	3.128
29	8	7.5	76	230	3.026
30	7	9	84	260	3.100
31	6	8	78	228	2.923

Table 7 continued overleaf

Case	Circumference of (cm)		Weight of (g)		Ratio
	Pulmonary artery	Aorta	Right ventricle	Left ventricle + septum	$\frac{LV + S}{RV}$
32	16	16	670	2230	3.328
33	7.5	7.5	54	168	3.210
34	7	7	43	128	3.010
35	14	14	370	1220	3.297
36	14	15	470	1365	2.904
37	7	7	50	176	3.520
38	7	7	58	200	3.448

TABLE 7. Cardiovascular measurements in 38 normal horses.

Case	Circumference of (cm)		Weight of (g)		Ratio
	Pulmonary artery	Aorta	Right ventricle	Left ventricle + septum	$\frac{LV + S}{RV}$
1	20	15	860	2300	2.674
6	18	17	670	2045	3.052
7	12	11	320	1142	3.569
9	13	13	360	1200	3.333
11	15	11	460	650	1.444
12	16	14	1032	2172	2.105
14	15	13	410	1468	3.580
15	15	11	830	2320	2.795
29	11.5	11	790	1130	1.430
31	13.5	14	475	1410	2.968
33	7	7	685	2090	3.051
34	15	13	450	1430	3.178
35	15	15	815	2020	2.478
36	14.5	13.5	720	2205	3.062
37	16	13	390	1330	3.410
38	13	11	470	1420	3.021
39	18	11.5	580	1810	3.121

TABLE 8. Cardiovascular measurements in a group of 17 horses with chronic pulmonary disease.

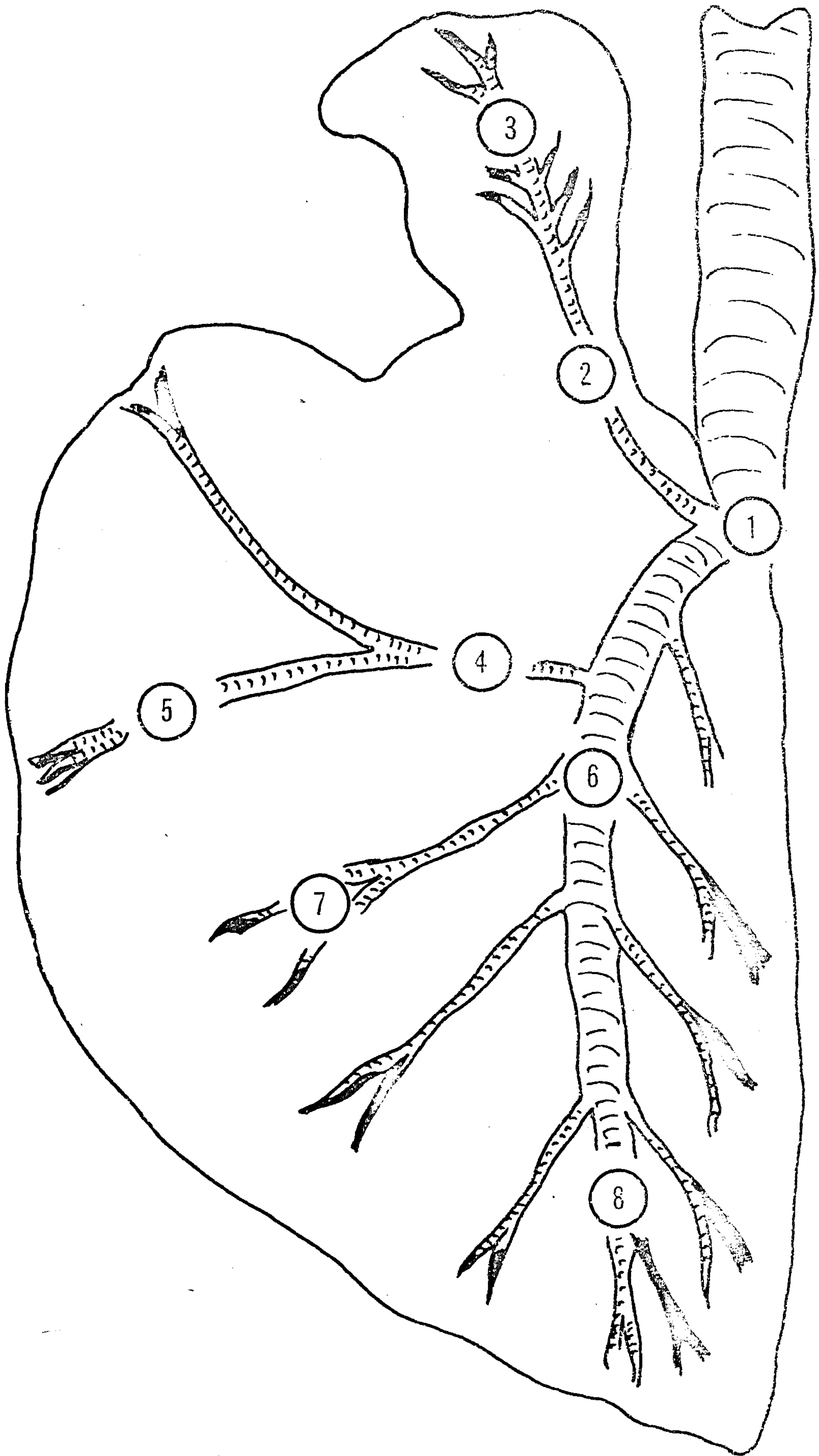


Figure 7. Diagrammatic representation of the left lung of a horse showing the eight standard histological sampling sites.

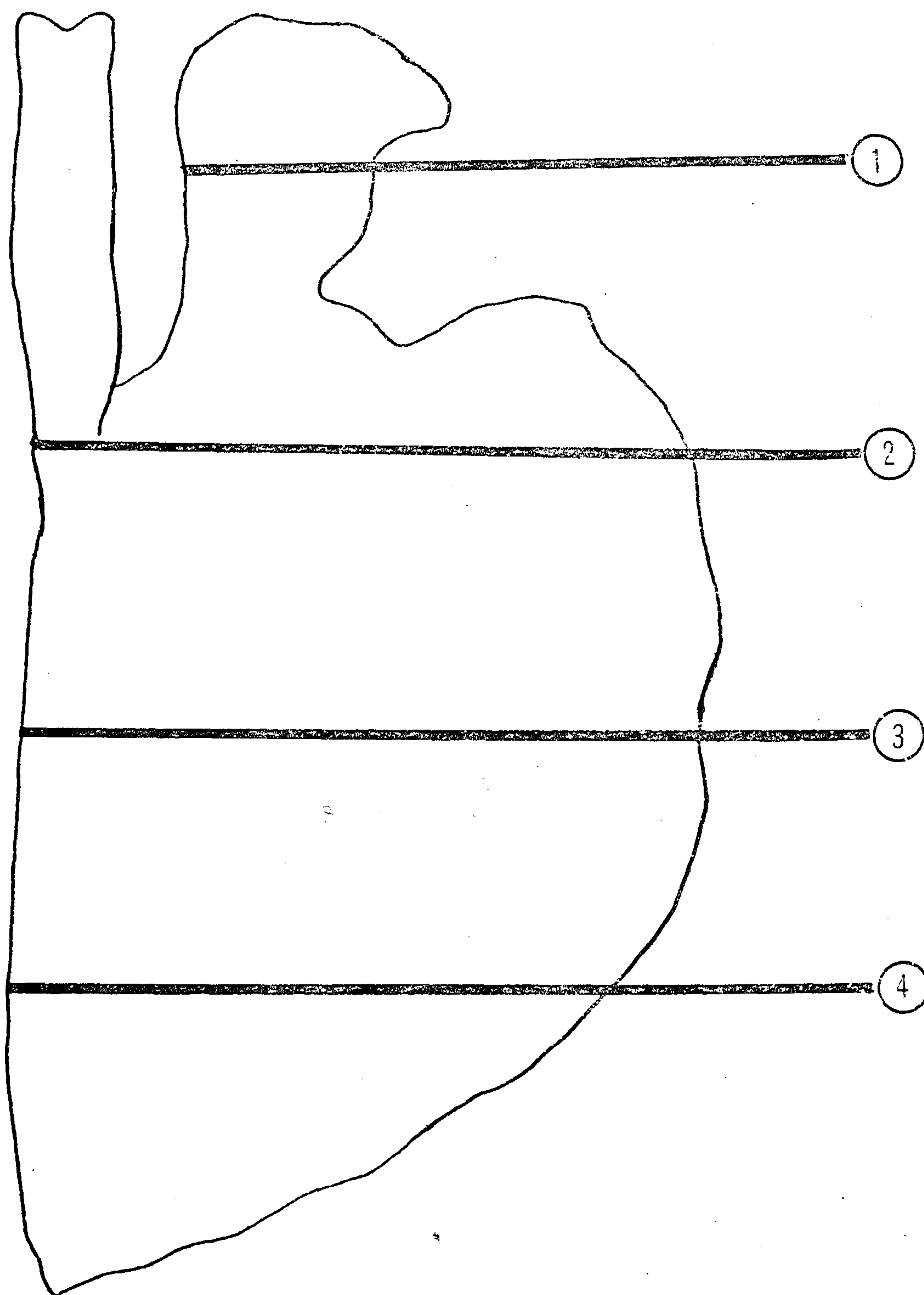


Figure 8. Diagrammatic representation of the right lung of a horse showing the four standard sites at which a thin slice was taken.

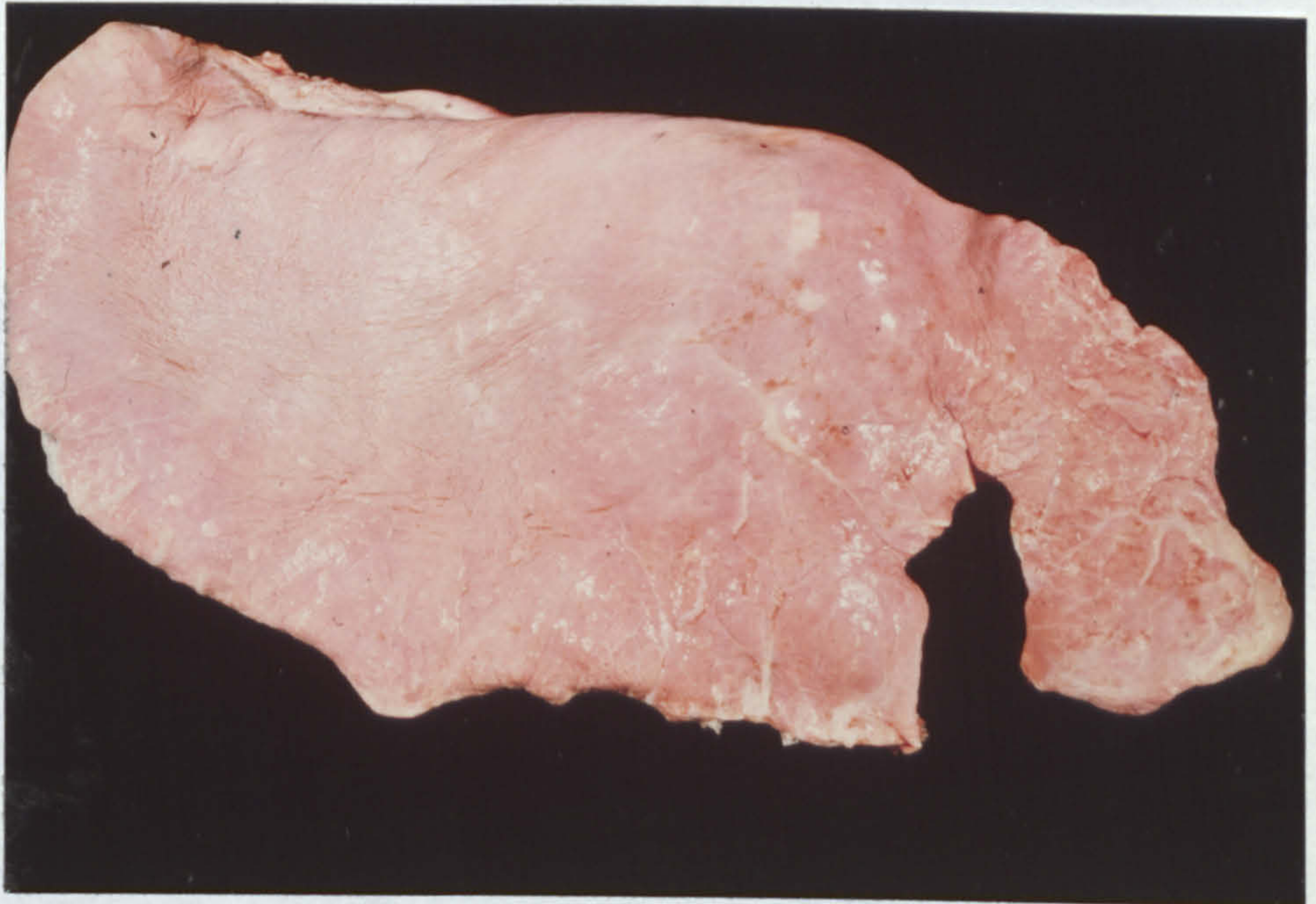


Figure 9. Appearance of normal equine lung at necropsy. The lung is collapsed and deep pink in colour.



Figure 10. Appearance of a lung from a case of CPD. The lung is pale, overinflated and the cranial lobe has a puffy appearance.



Figure 11. Lung from a case of CPD. In this case the body of the caudal lobe is collapsed but the periphery of the caudal lobe and the cranial lobe are puffy and overinflated. Finger-prints can be seen in the cranial lobe indicating some degree of emphysema.

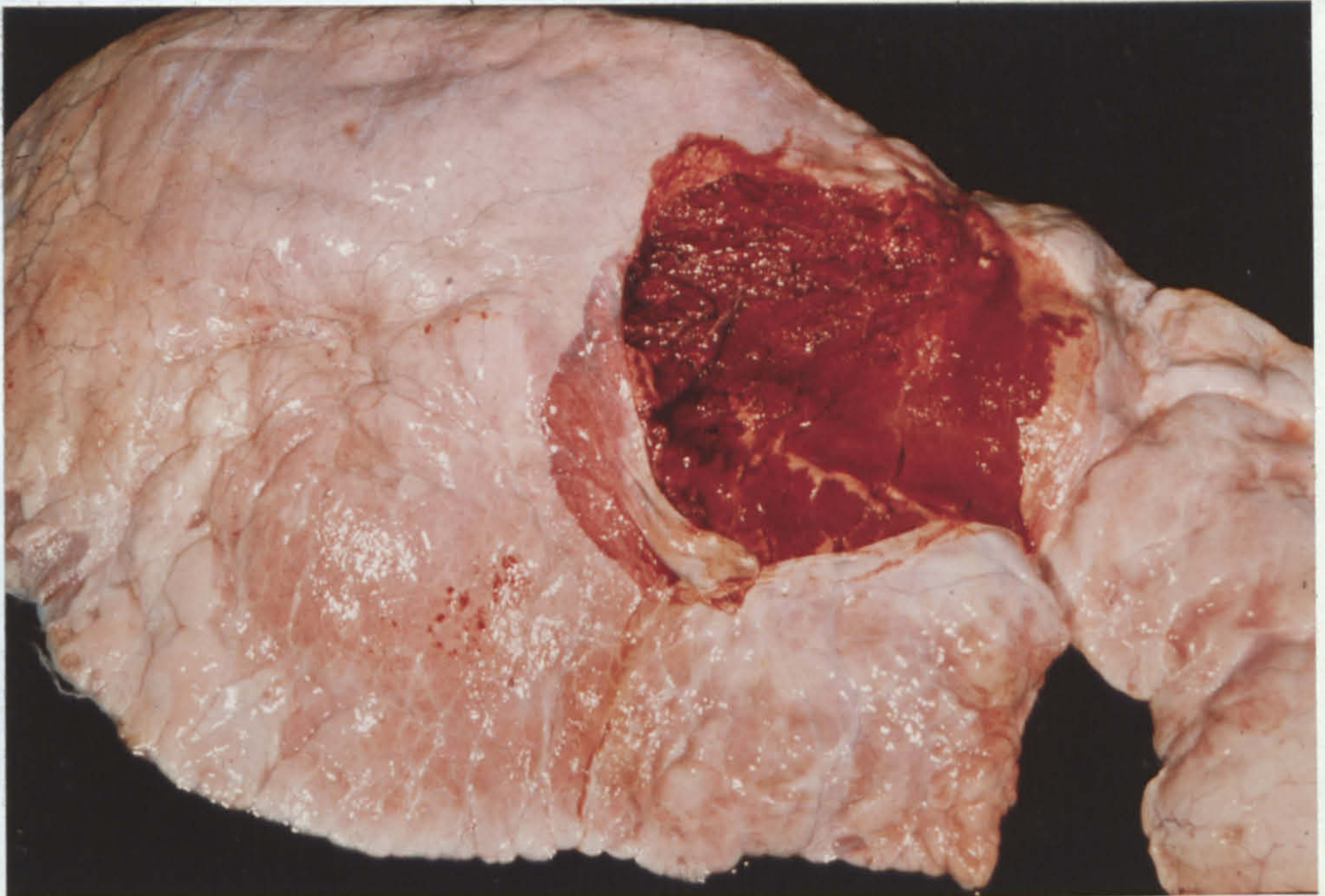


Figure 12. Right lung from 33. The lung is pale and overinflated. The large area of adhesion to the fractured ribs can be seen with some torn pleura still attached.

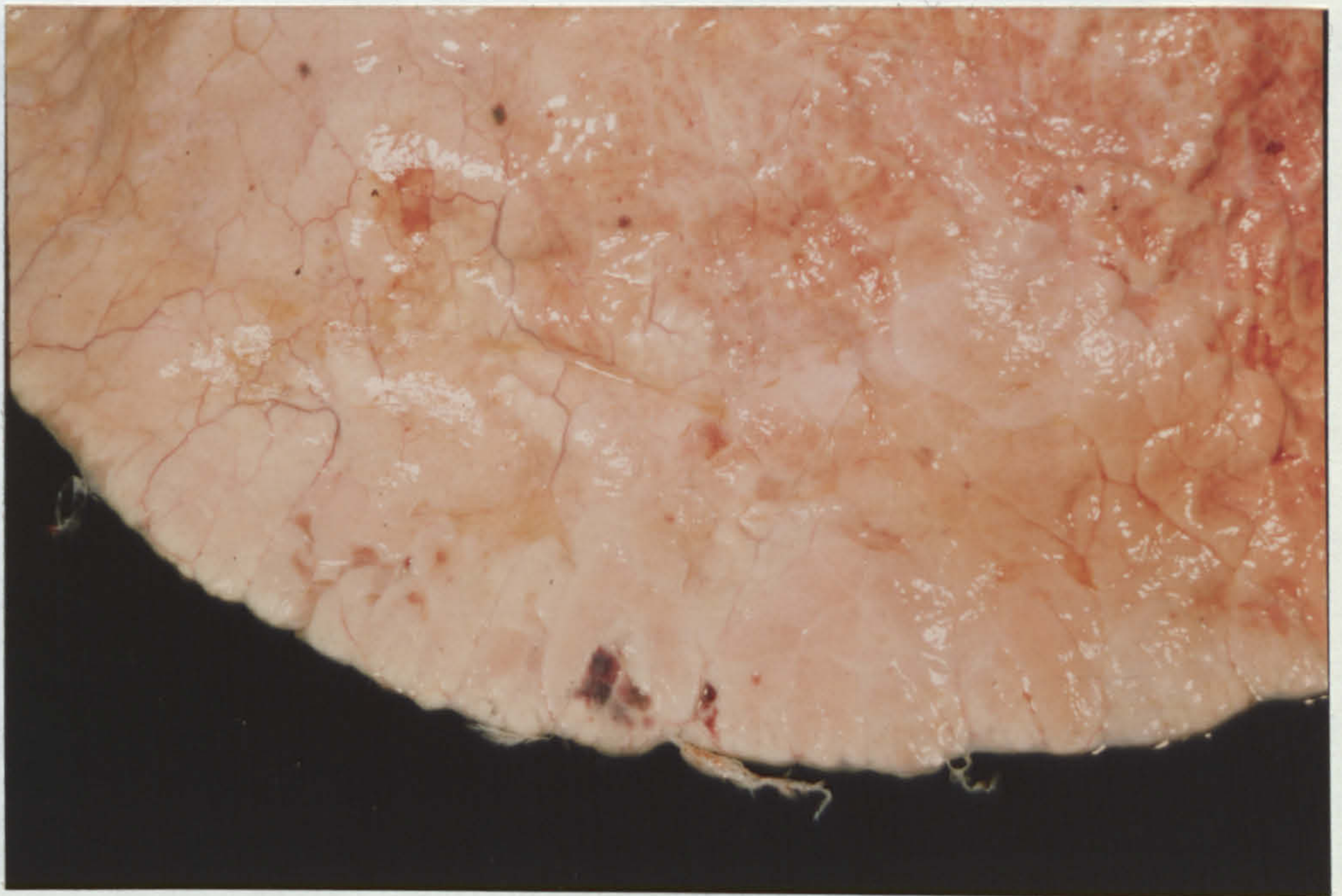


Figure 13. Pleural tags extending from the edge of the caudal lobe of one of the cases.

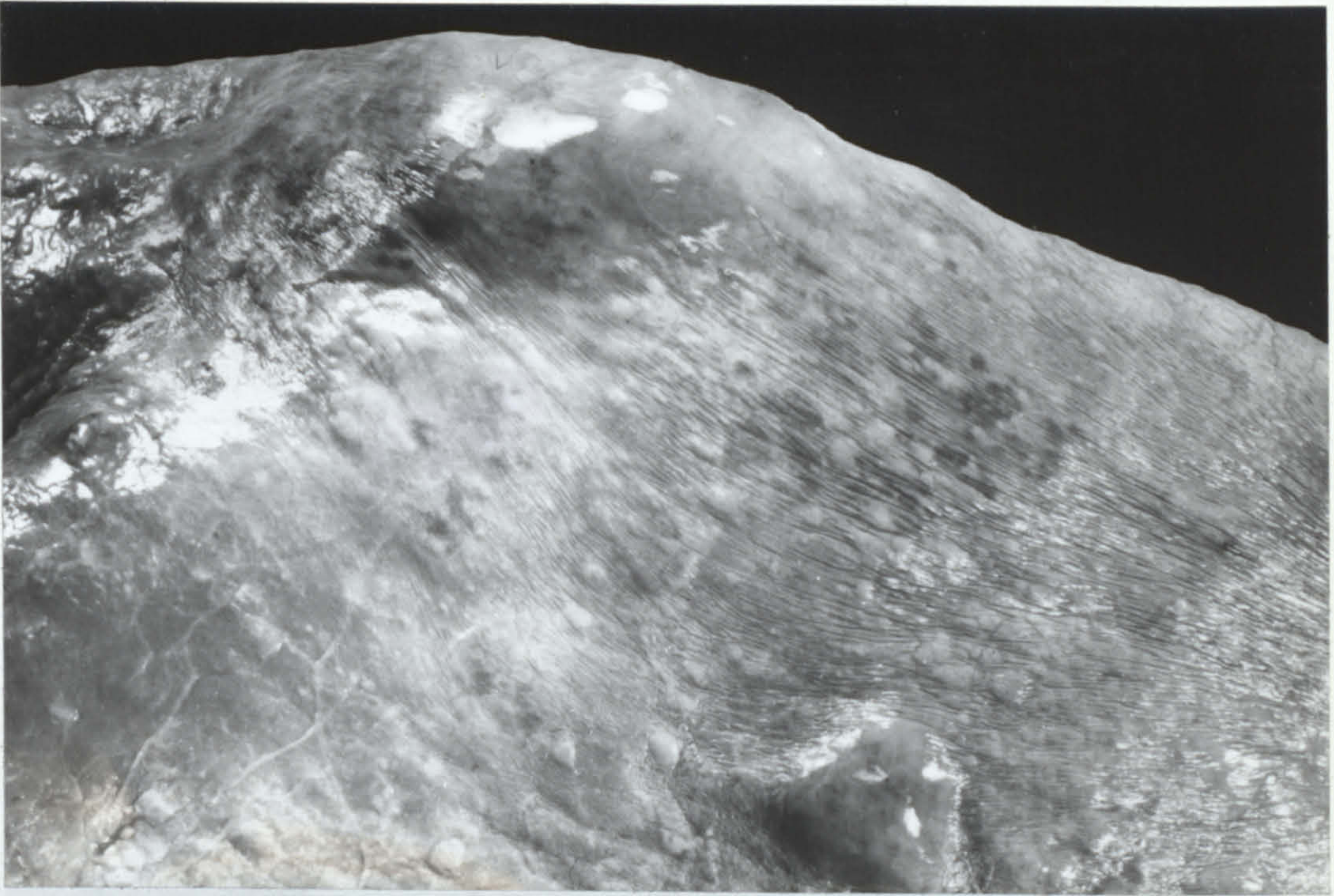


Figure 14. Close-up of two of the raised hemispherical areas on the surface of the lungs from 29, approximately actual size.



Figure 15. Part of the trachea from 37. The cartilaginous rings are opened and the dorsal membrane is stretched between them.

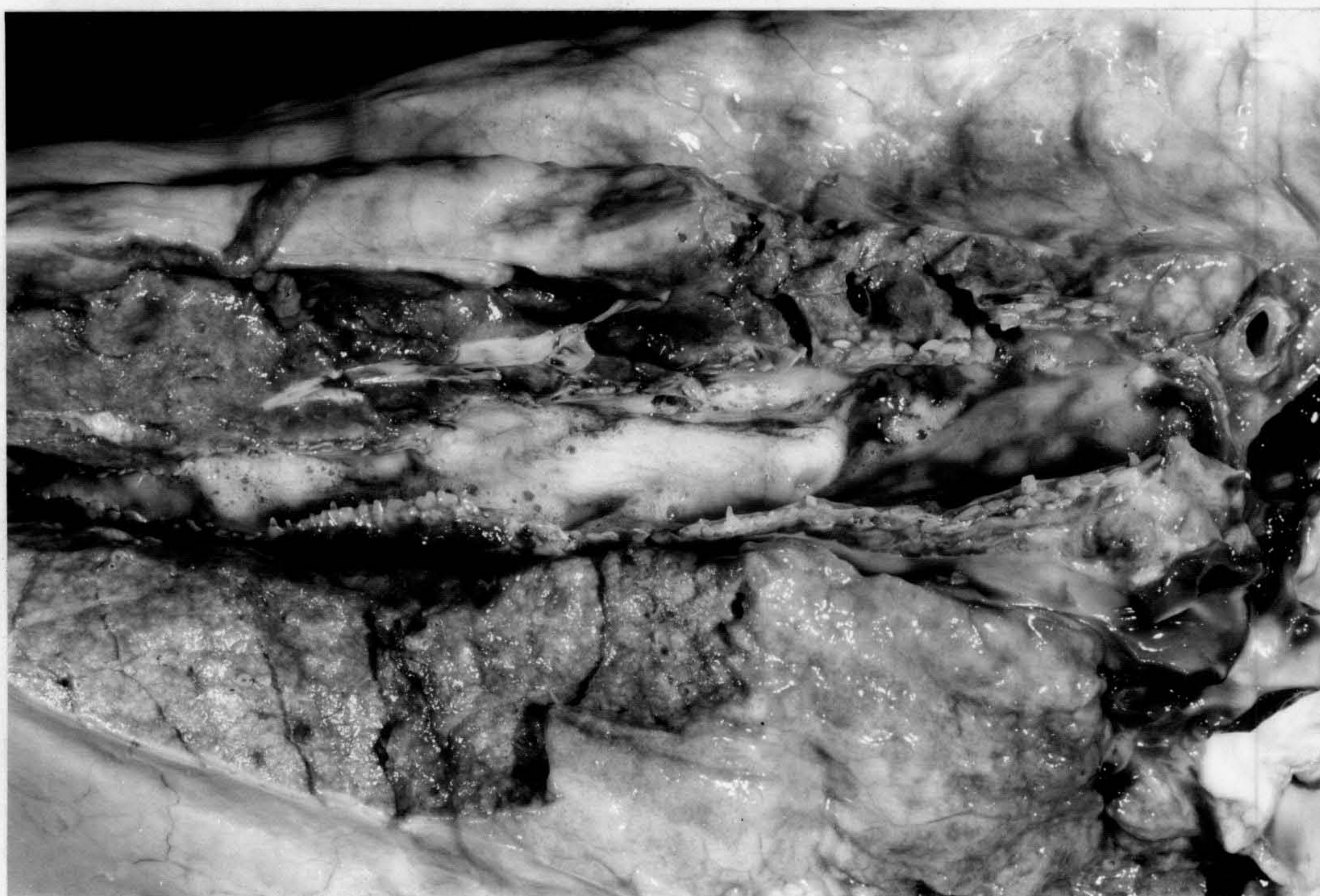


Figure 16. Part of the opened bronchial tree of 41. Free mucus can be seen lying in the lumen.

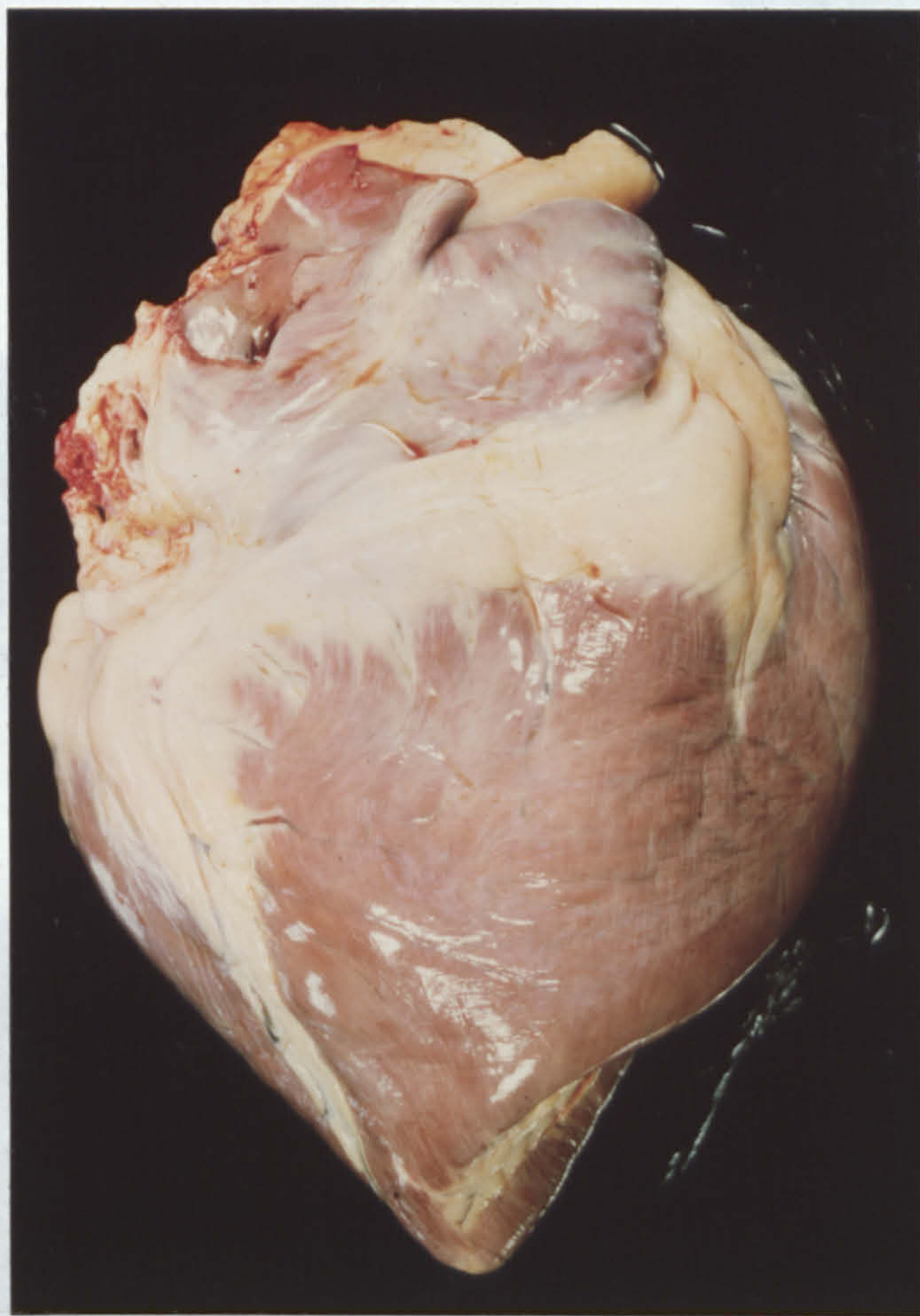


Figure 17. This heart from case 111 shows right ventricular hypertrophy. The ventricle wall is bulging and looks thicker than normal.

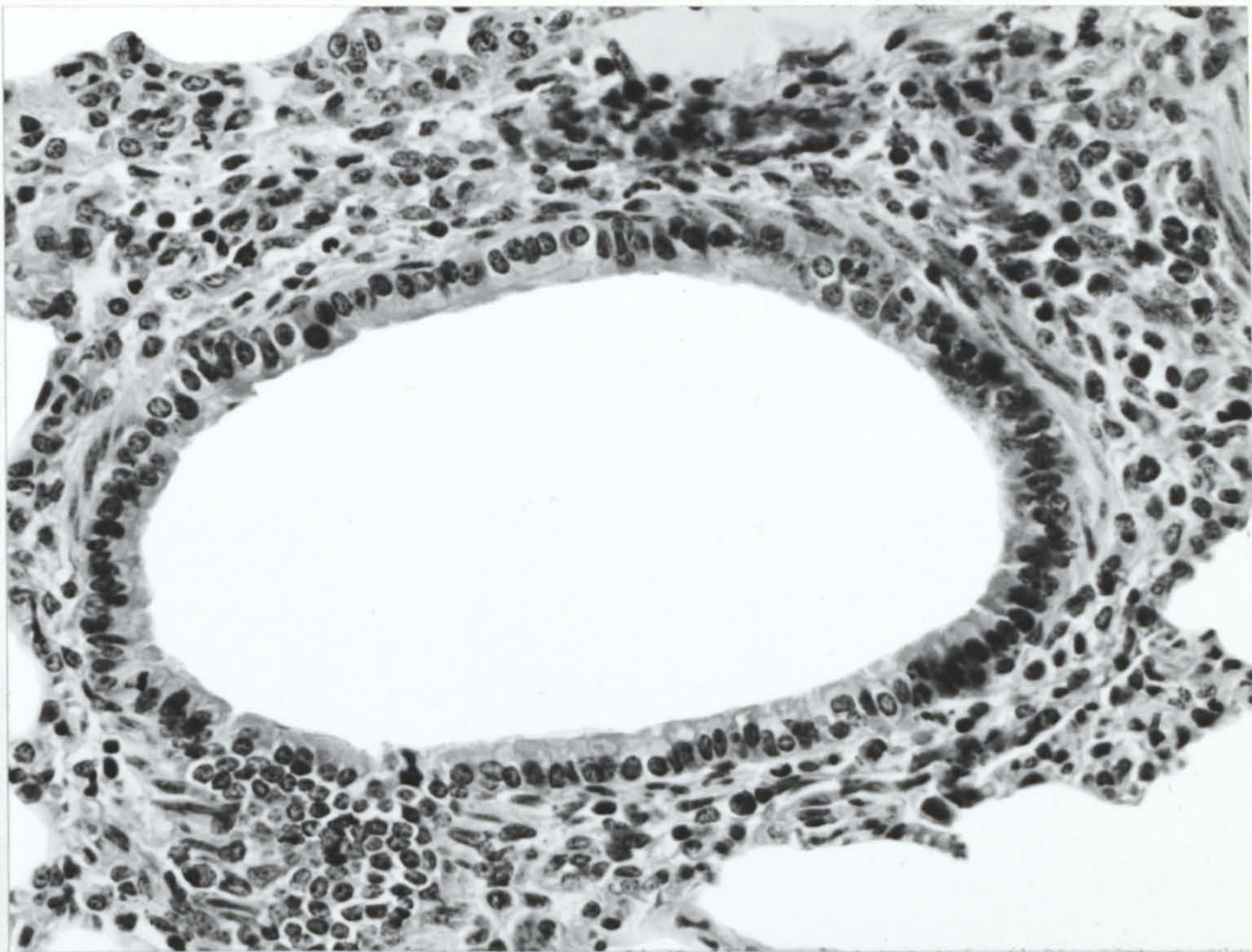


Figure 18. Cross-section of a bronchiole from a normal horse. The epithelium is one cell thick, cuboidal and does not contain goblet cells. X250.

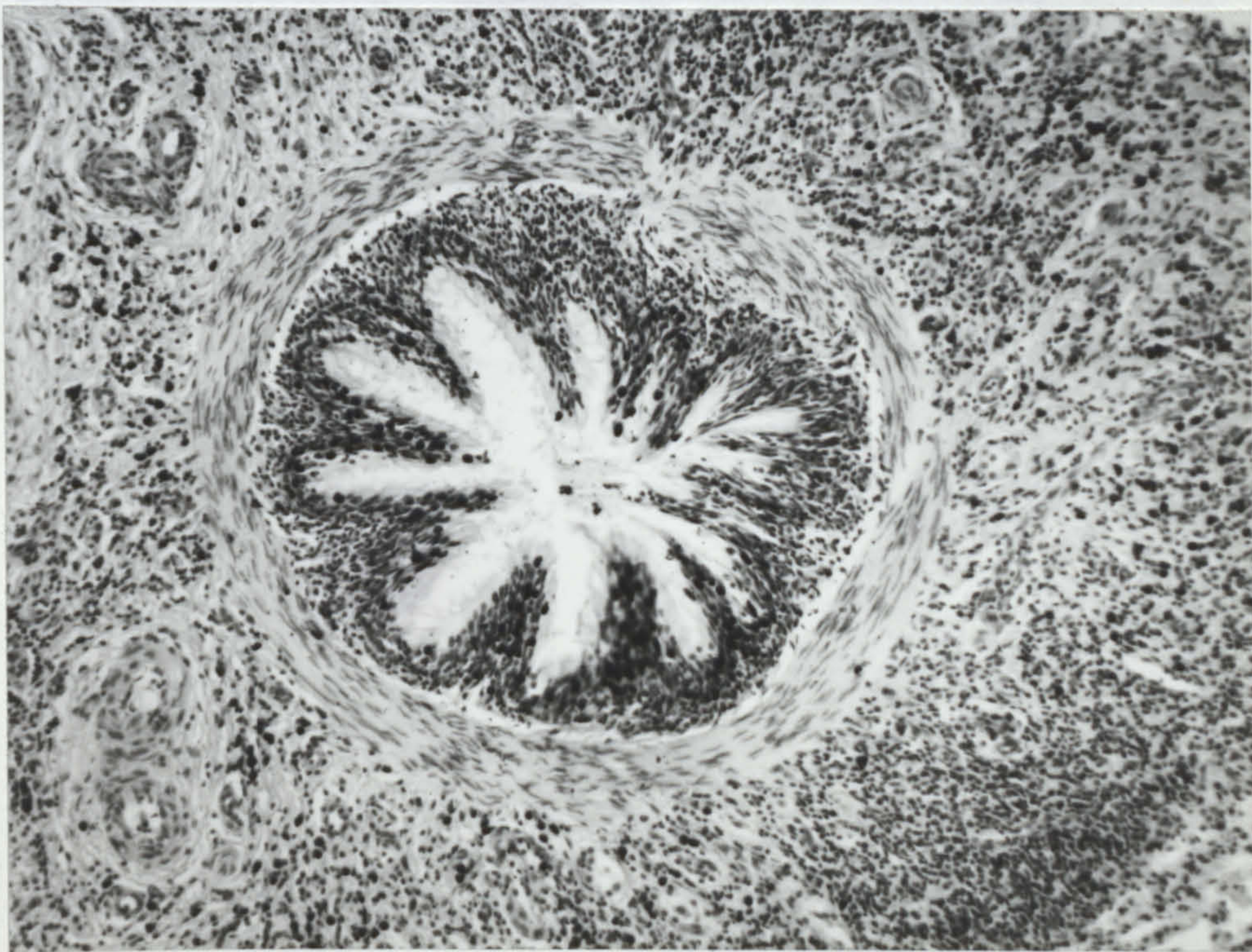


Figure 19. Cross-section of a bronchiole from a horse with CPD. The epithelium is hypertrophied, columnar and infiltrated with inflammatory cells. The peribronchiolar tissue is hypercellular this infiltrate consisting mainly of mononuclear cells. X 110.

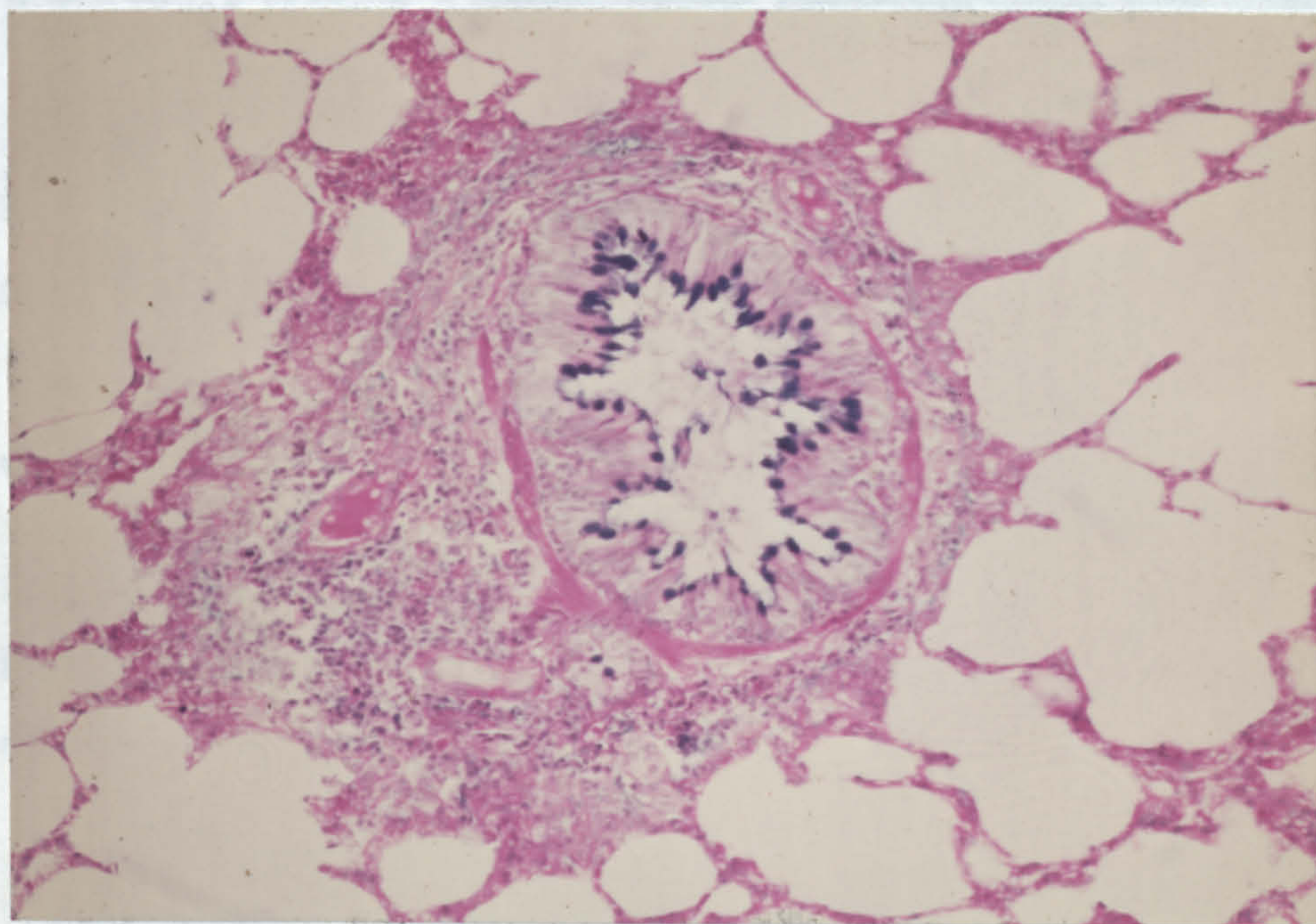


Figure 20. Cross-section of a bronchiole from a horse with CPD stained with AB-PAS. The goblet cells in the epithelium stain dark blue. X 110.



Figure 21. Cross-section of a bronchiole from a horse with CPD stained with AB-PAS. The goblet cells in the epithelium stain dark blue. X 110.

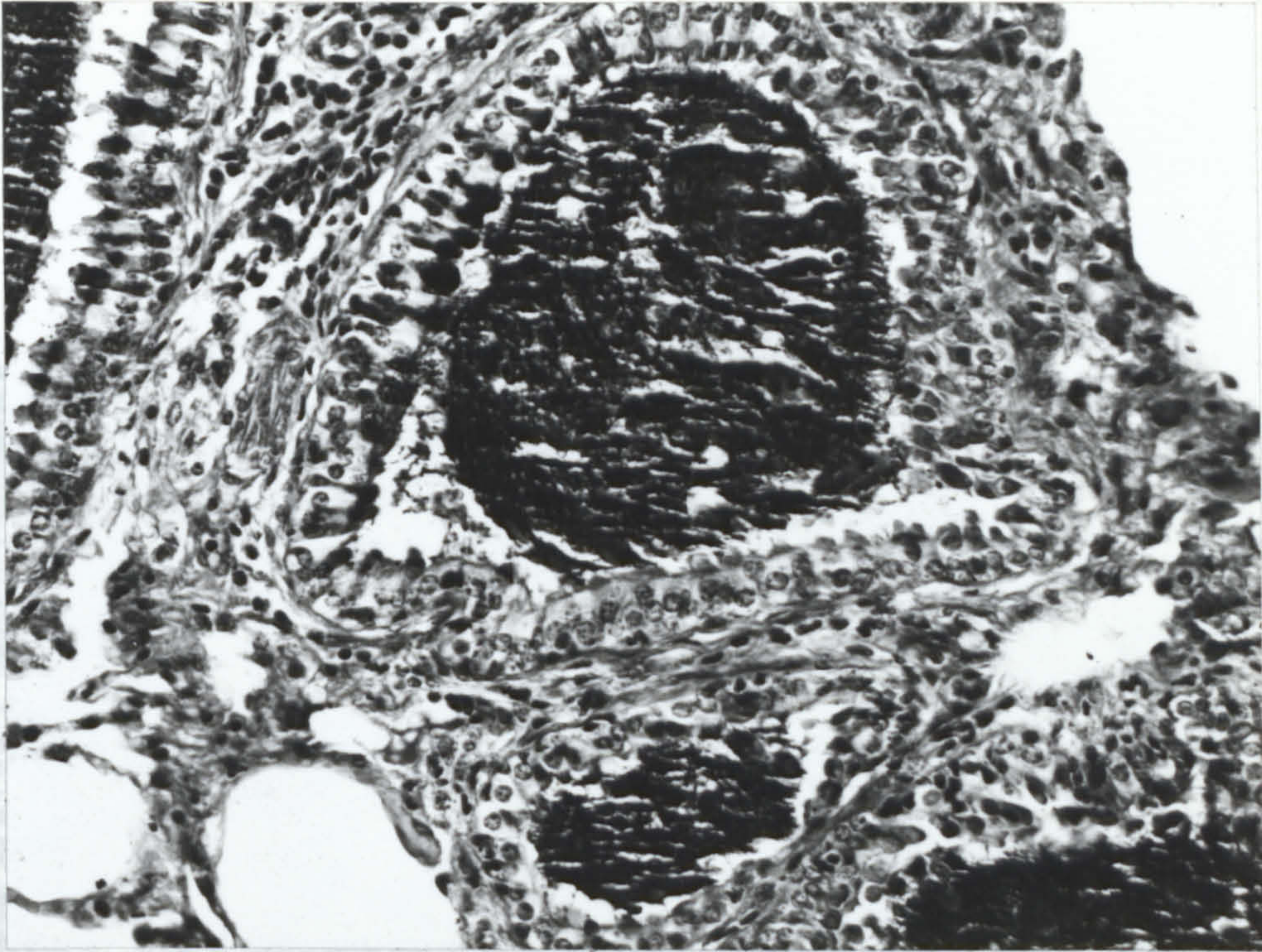


Figure 21. Cross -section of two small bronchioles from a case of CPD. Both are plugged with mucus and the larger one has goblet cells in the epithelium. X 400.

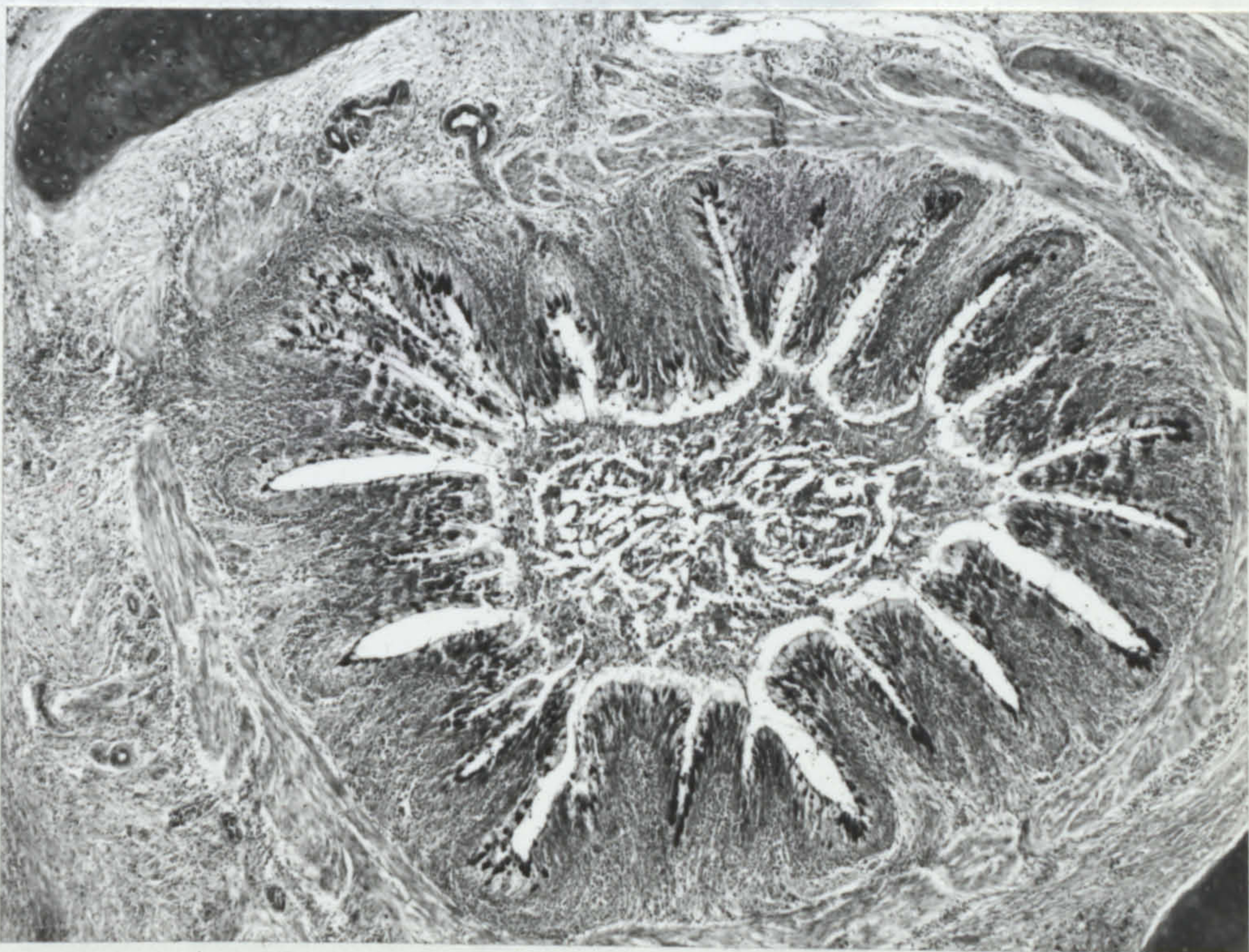


Figure 22. Cross-section of a bronchus from a case of CPD with a mucus plug in the lumen. X 35.

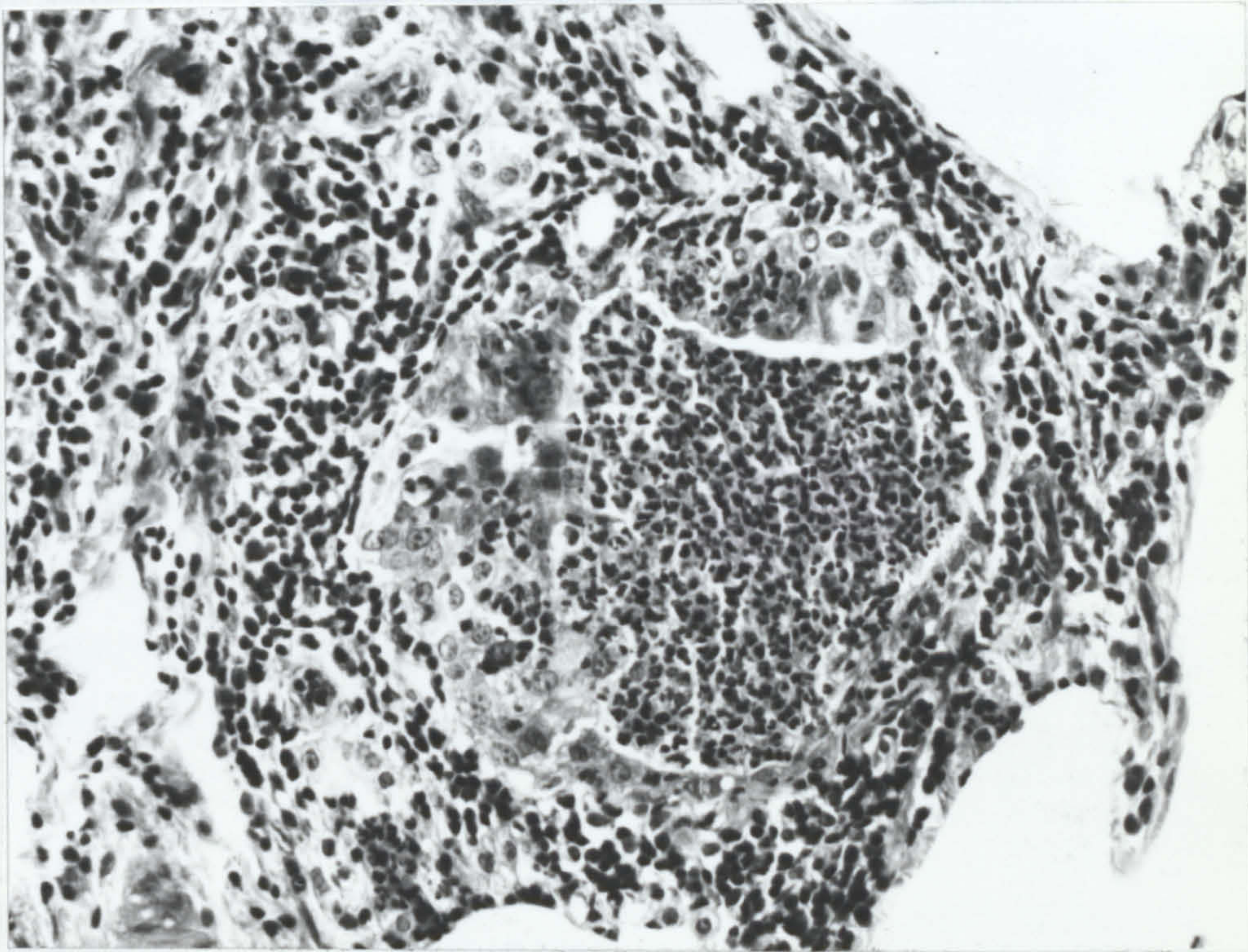


Figure 23. Cross-section of a small bronchiole from a case of CPD. The lumen is blocked with an exudate of polymorphonuclear leucocytes. X 250.

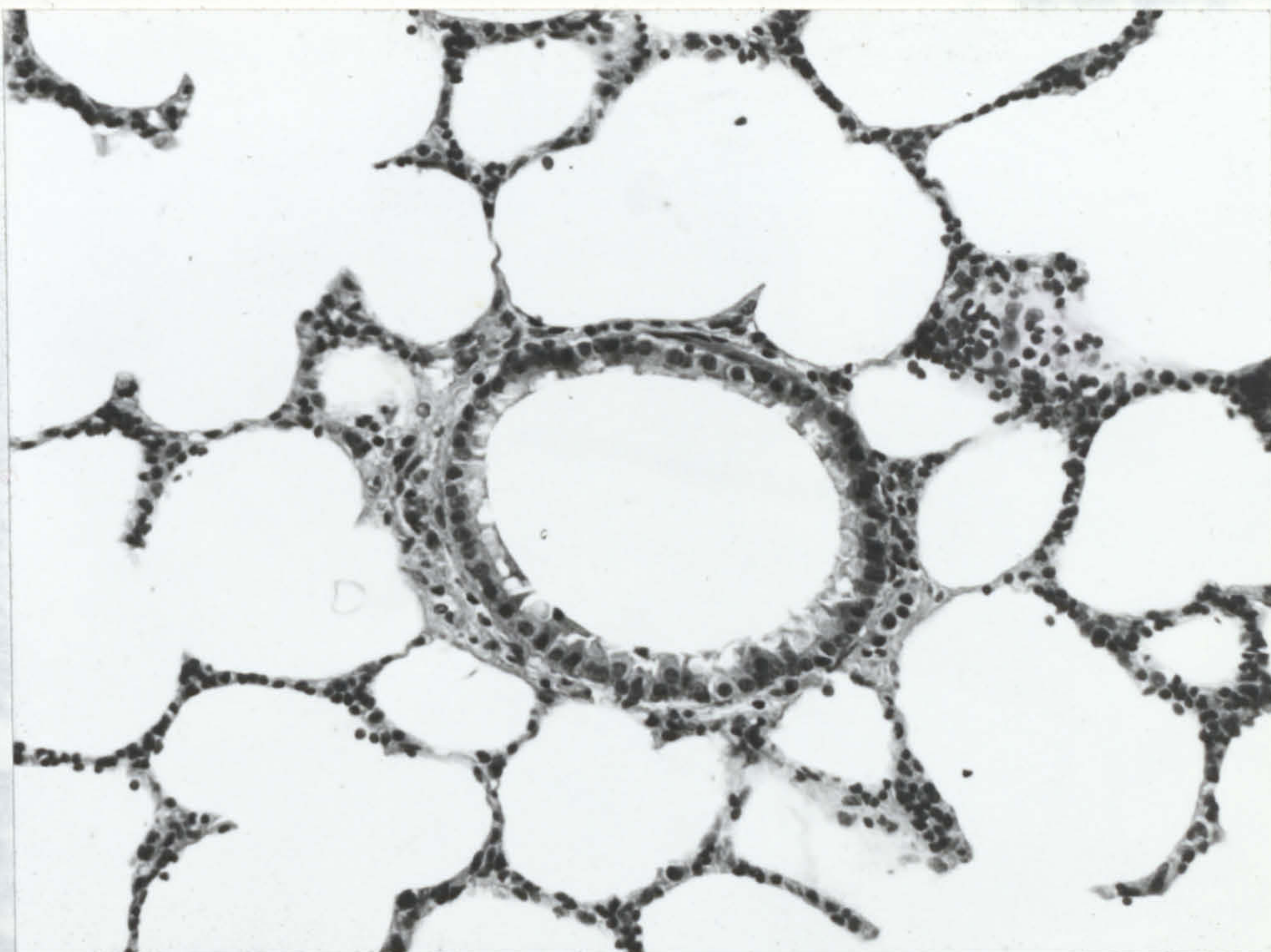


Figure 24. Cross-section of a bronchiole from a normal horse. There is very little peribronchiolar tissue. X 250.

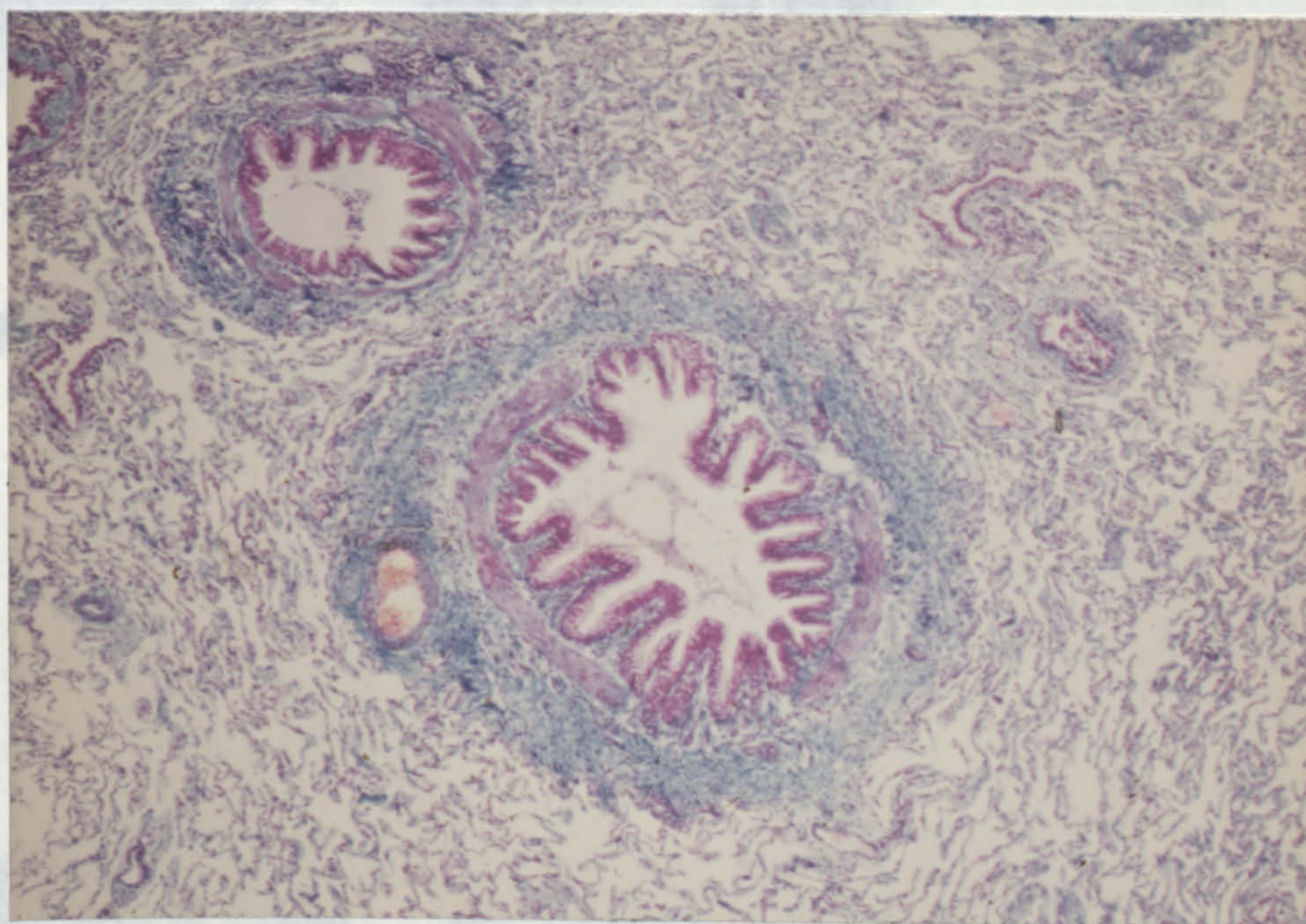


Figure 25. Cross-section of two bronchioles from a case of CPD stained with Martius Scarlet Blue. There is excess fibrous tissue around the two airways. X 35.

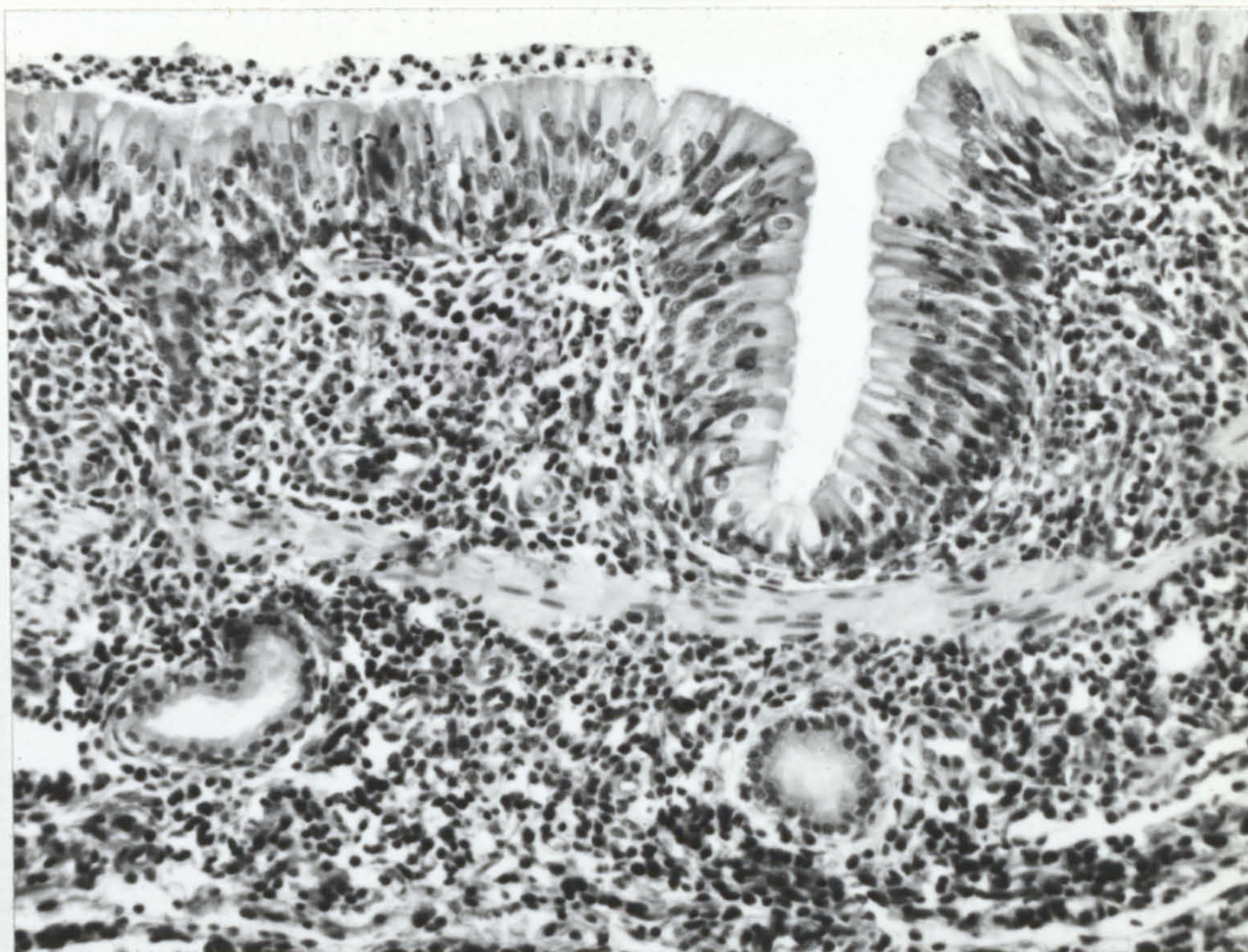


Figure 26. Section of bronchial wall from 29. Eosinophils can be seen as large dark cells infiltrating all parts of the tissue. X 250.

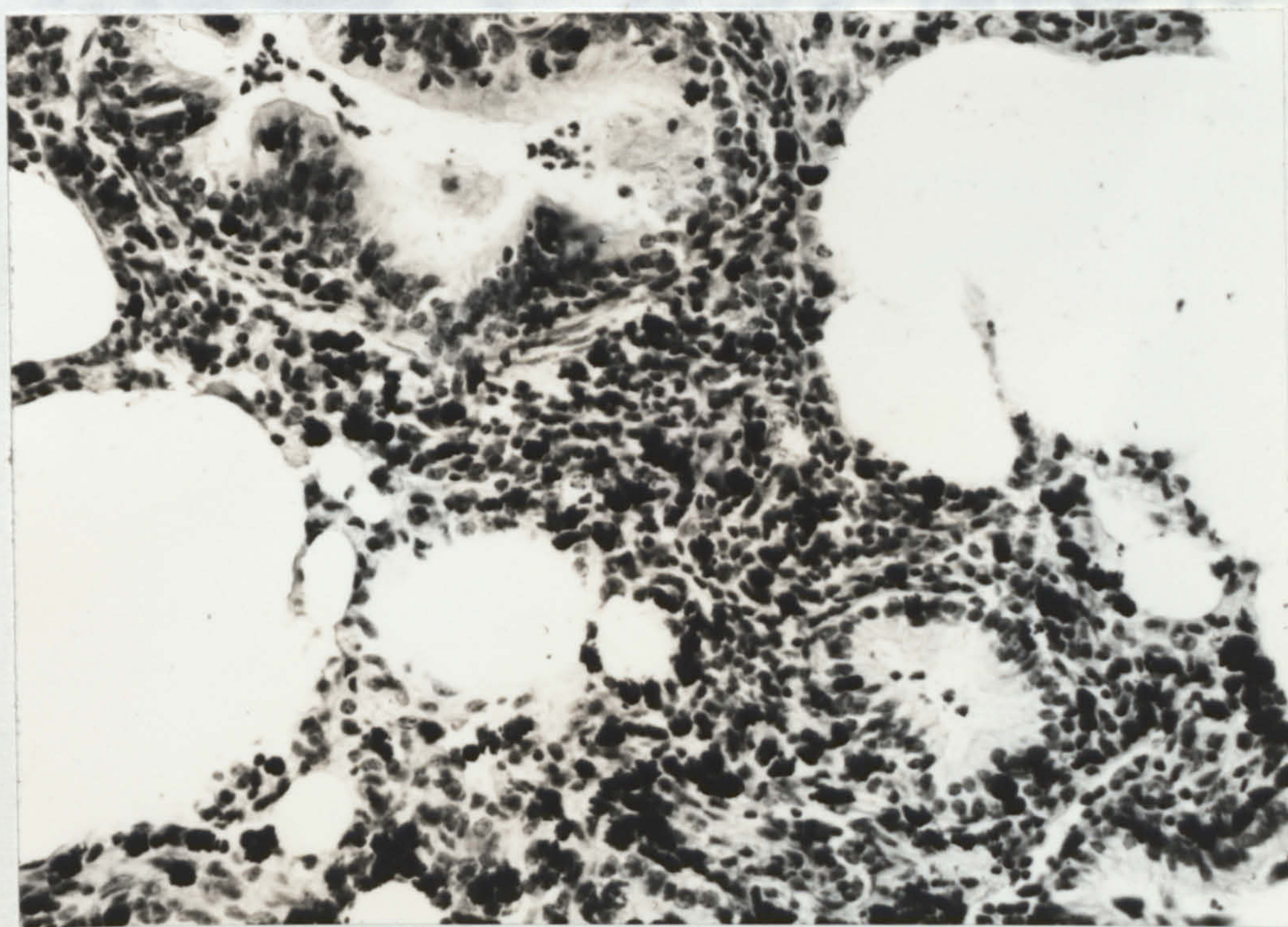


Figure 27. Cross-section of two bronchioles from 37. Large numbers of eosinophils can be seen congregating around these two airways. X 250.

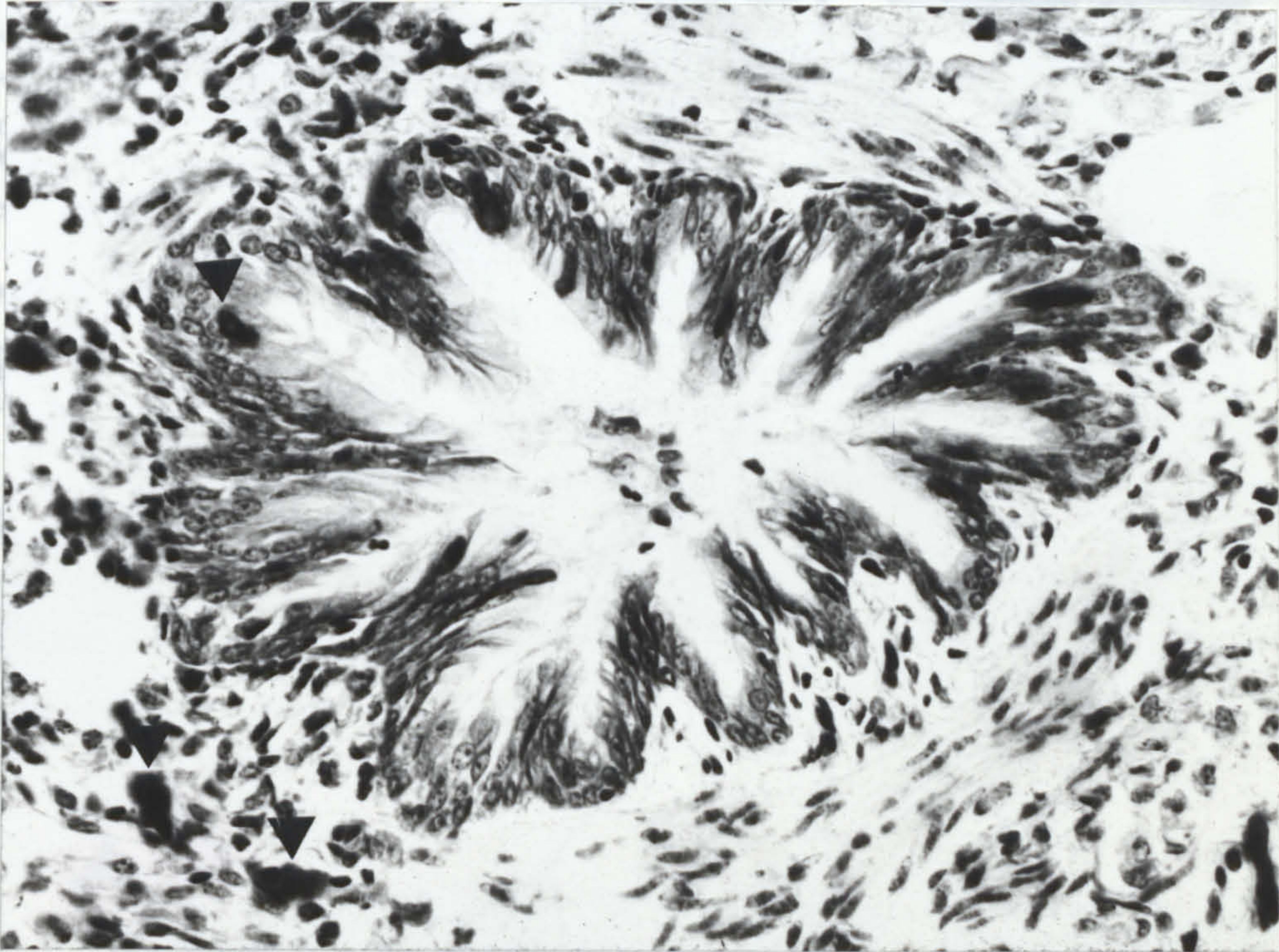


Figure 28. Cross-section of a bronchiole from a case of CPD stained with toluidine blue. Several mast cells can be seen as large dark hazy cells around the airway. (arrows)X 250.

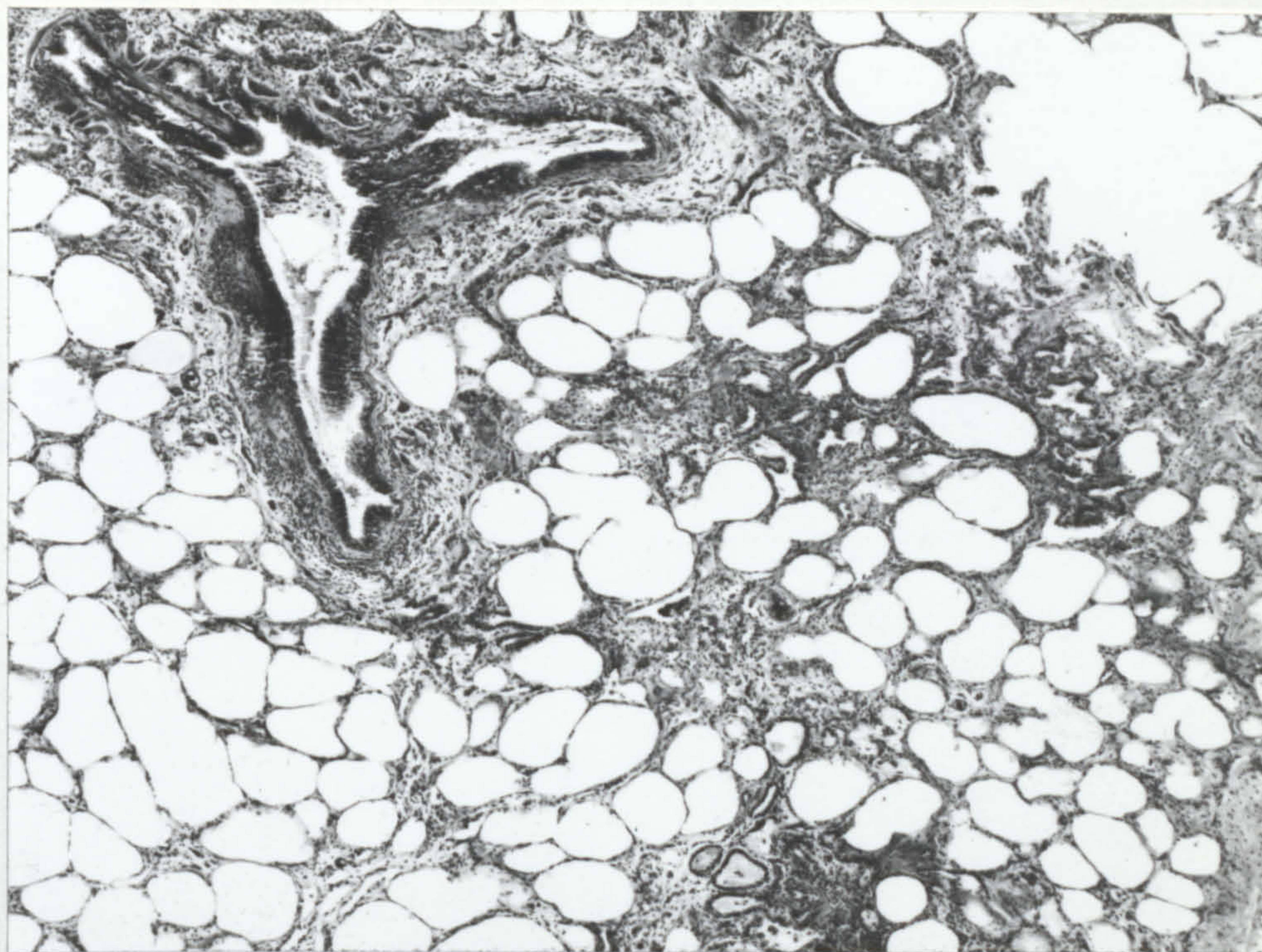


Figure 29. Focal areas of alveolar epithelial hyperplasia are shown on this section from a case of CPD. X 35.

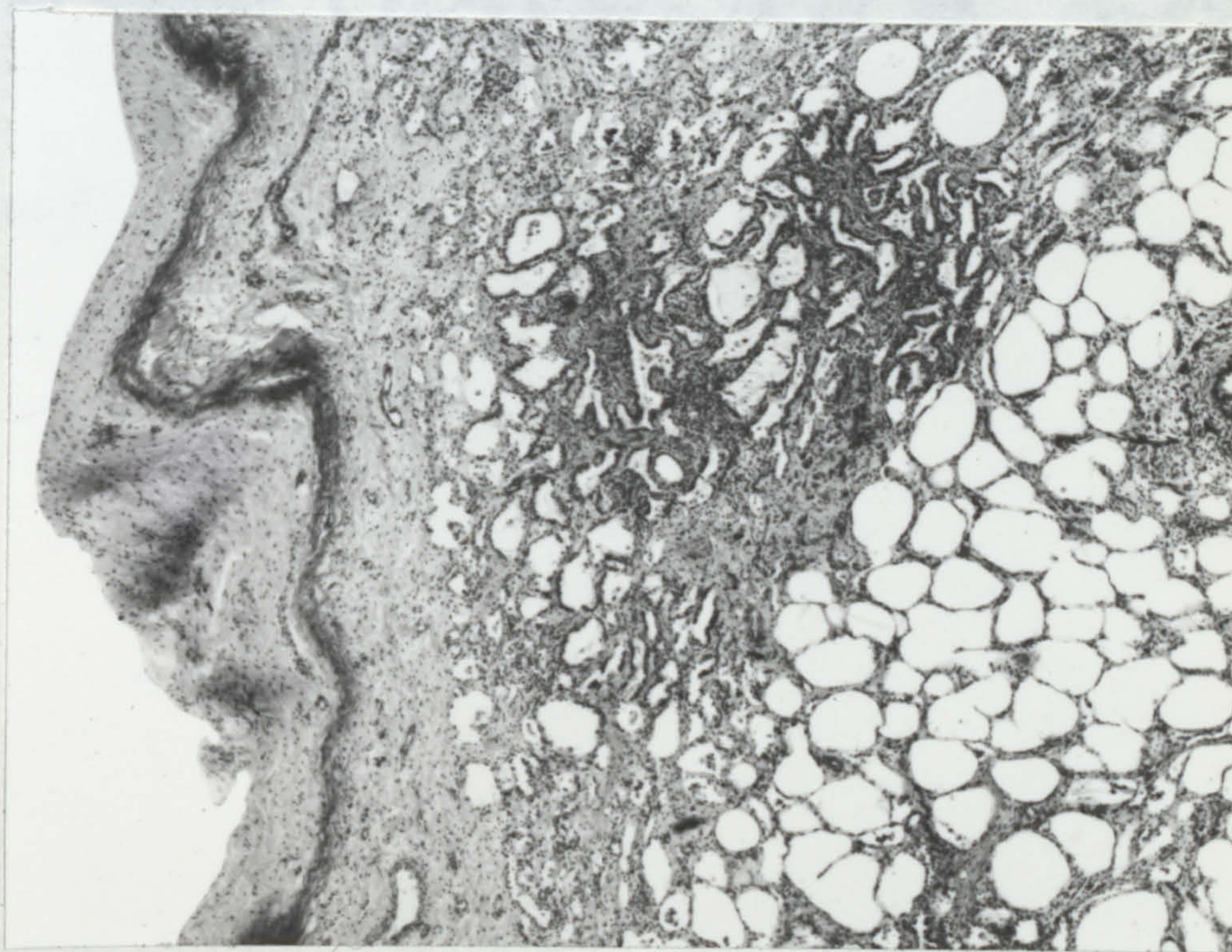


Figure 30. An area of subpleural alveolar epithelial hyperplasia and fibrosis from a case of CPD. X 35.

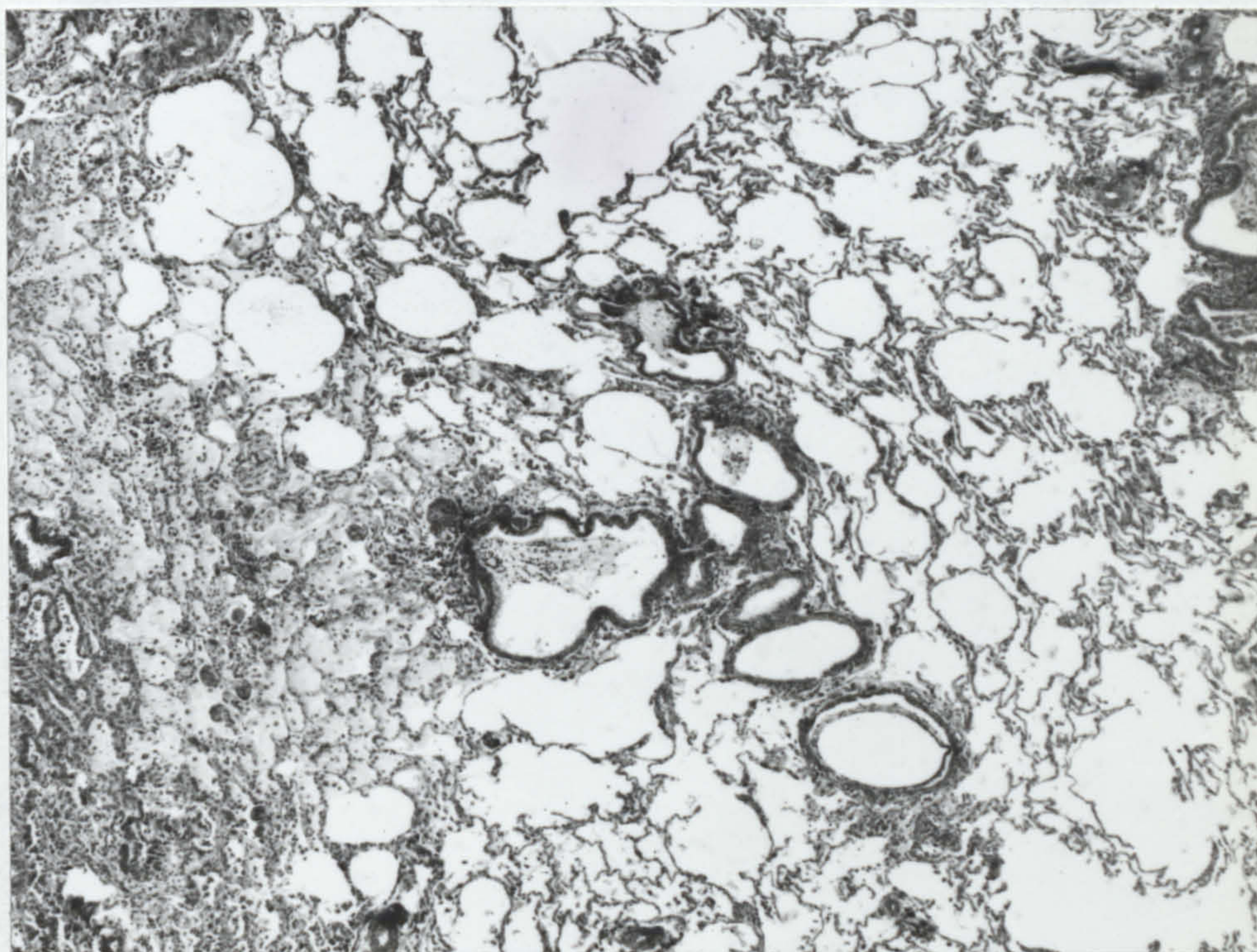


Figure 31. Alveoli filled with mucus can be seen to the left of the group of bronchioles on this section from a case of CPD. X 35.

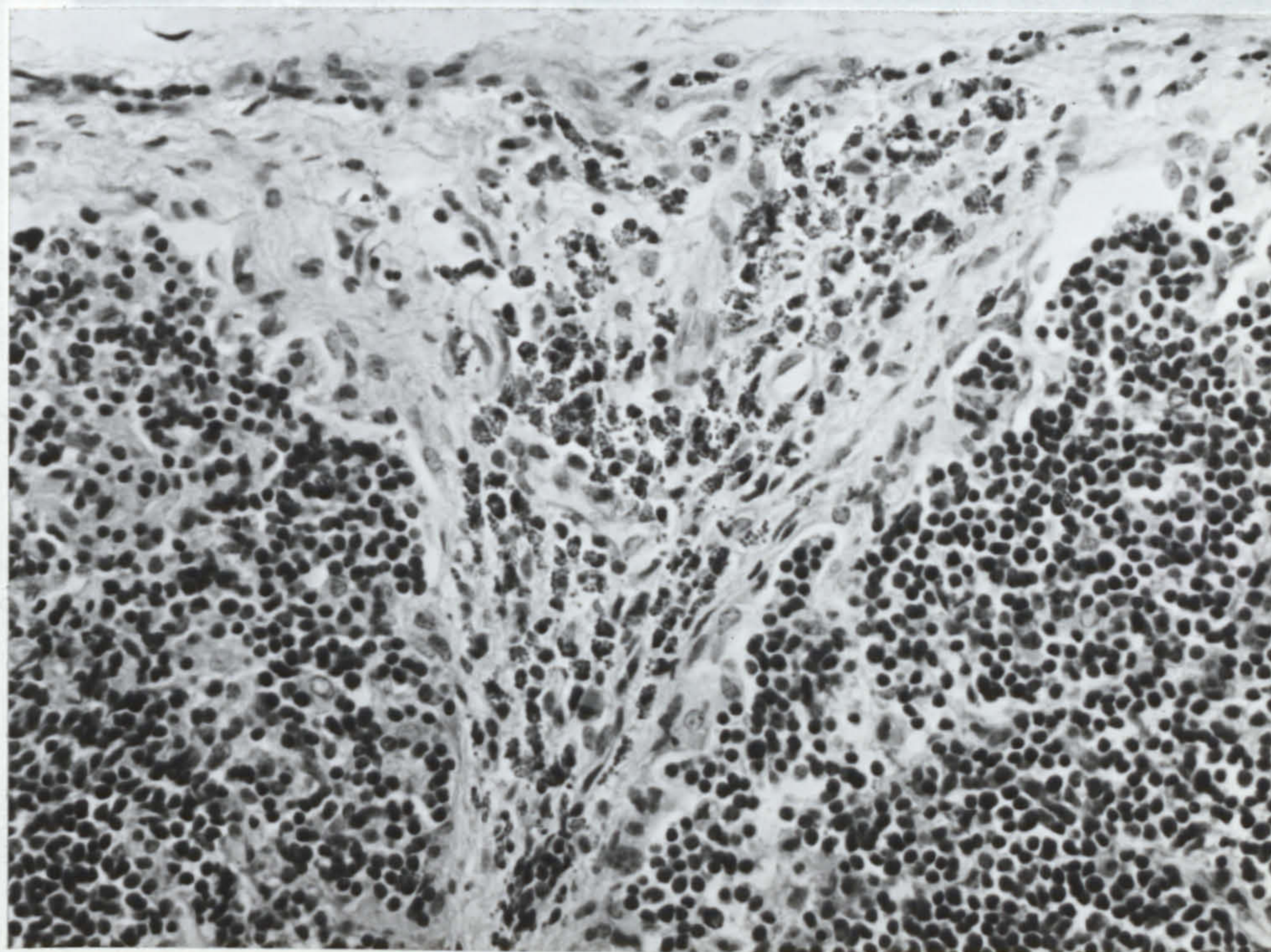


Figure 32. Section of a mediastinal lymph node from a case of CPD. Eosinophils are visible in the sinus. X 250.



Figure 33. Slice taken from level 2 of an inflated lung showing the uneven fixation that has occurred. The pale areas are inflated but the darker areas have failed to inflate.



Figure 34. Similar slice to one shown above showing sharply demarcated areas of inflation and non-inflation.

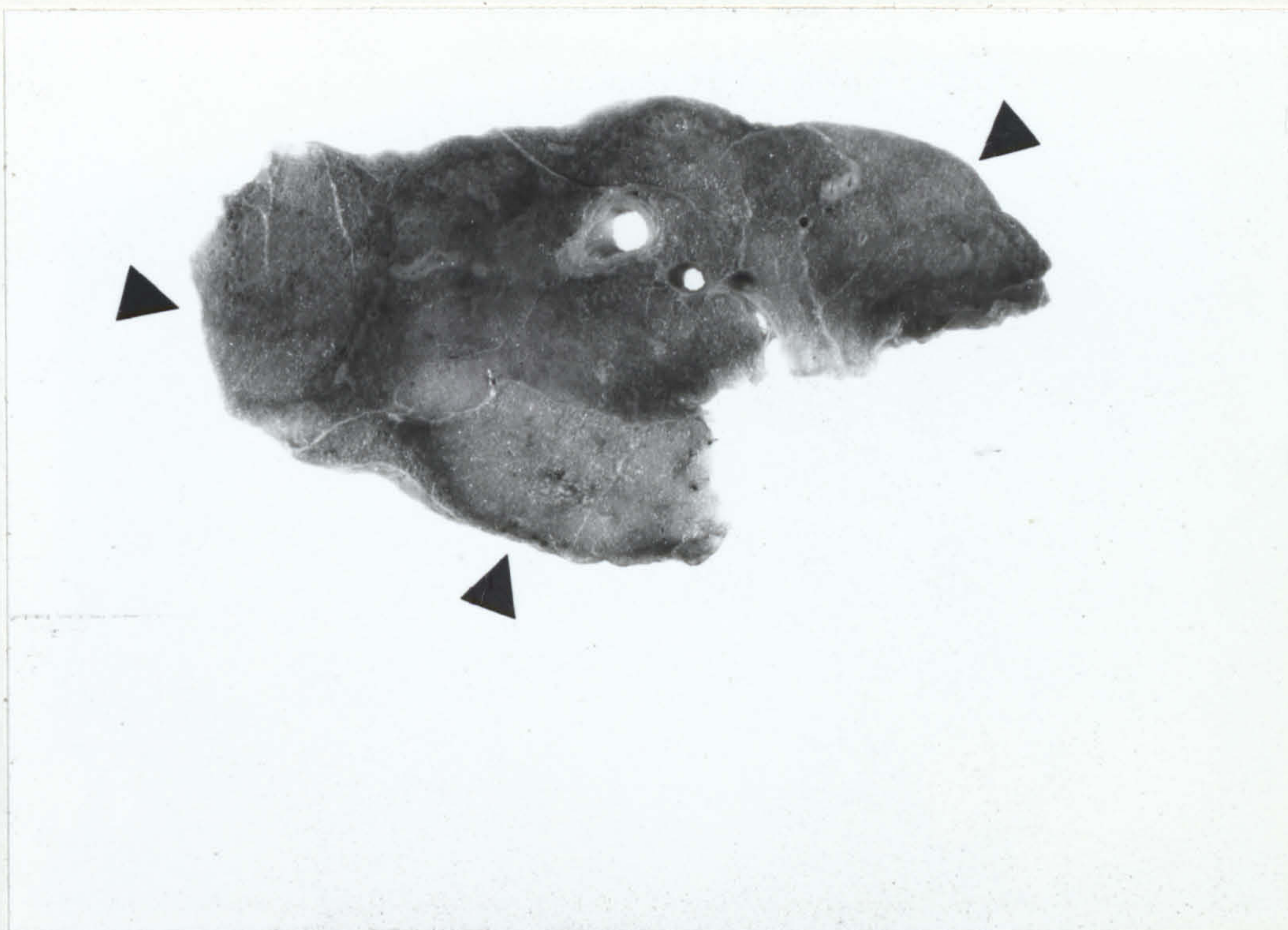


Figure 35. Slice taken from level 1 impregnated with barium sulphate. Emphysematous areas are shown by arrows.



Figure 36. Slice taken from level 2 of same case. The tip of the lobe has a more lacy appearance and is mildly emphysematous. The black spots are areas of anthracosis.

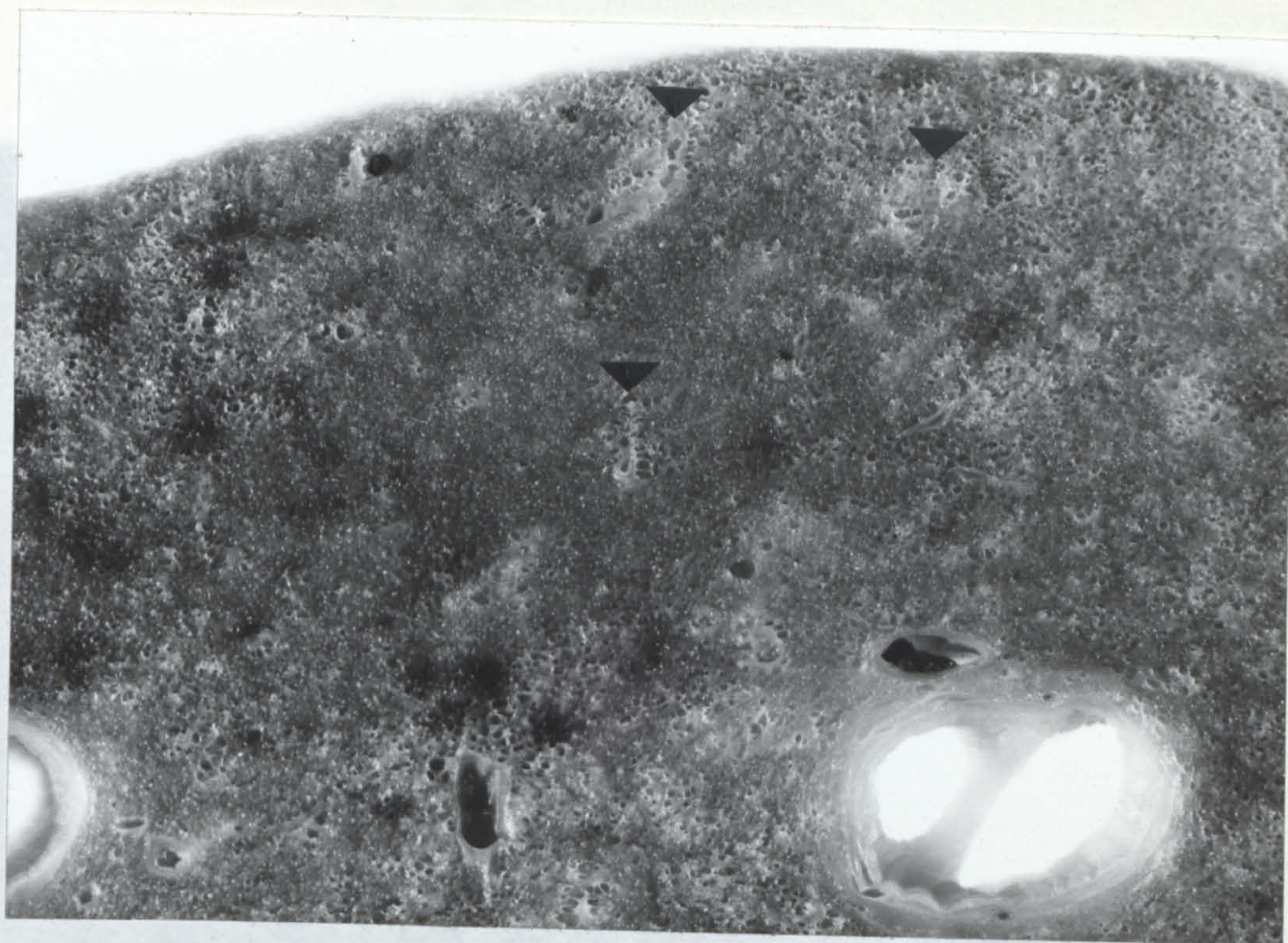


Figure 37. Close-up of barium sulphate impregnated slice of lung from 379. The small bronchioles (arrow) are surrounded by overinflated alveoli. X 15.

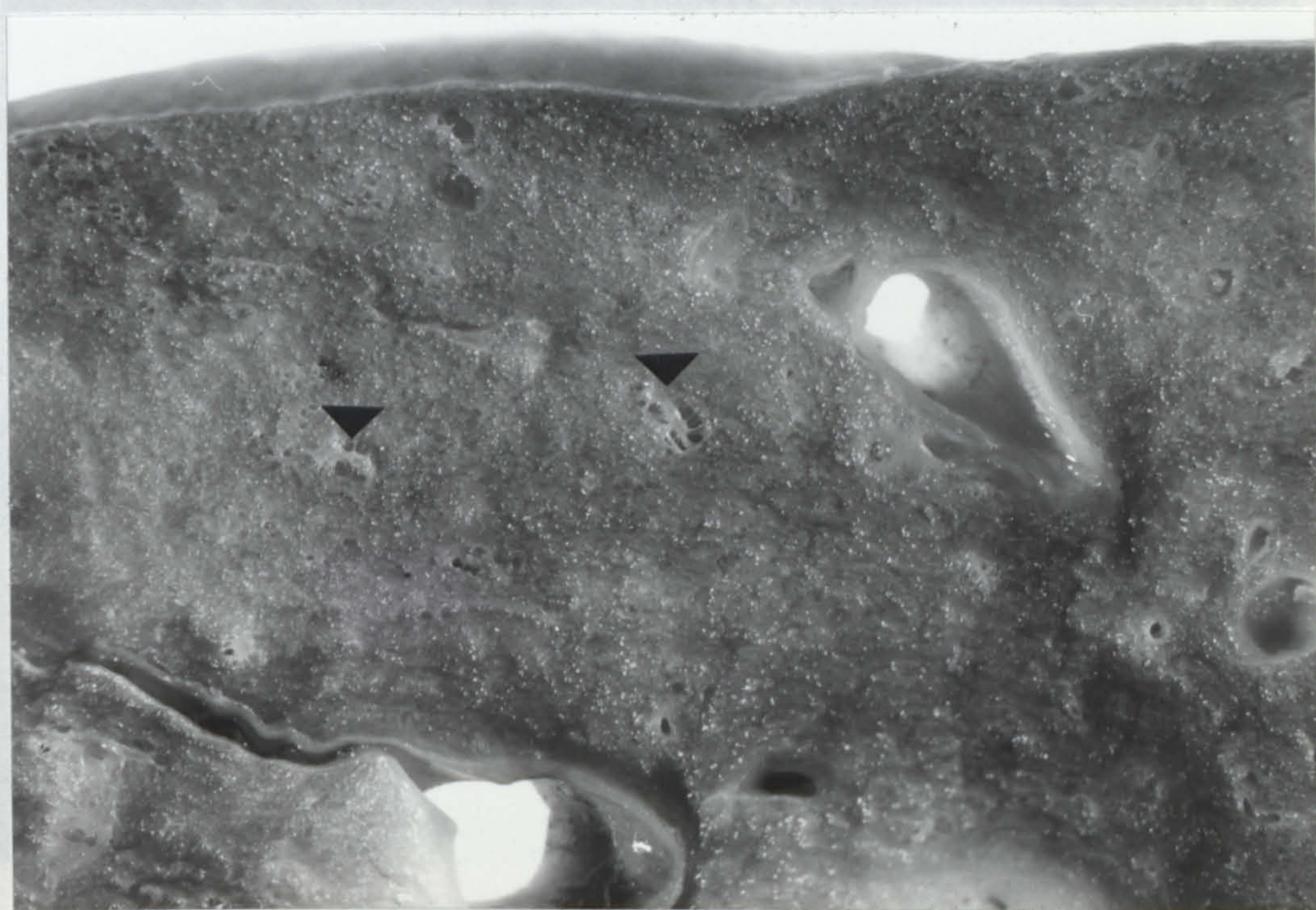
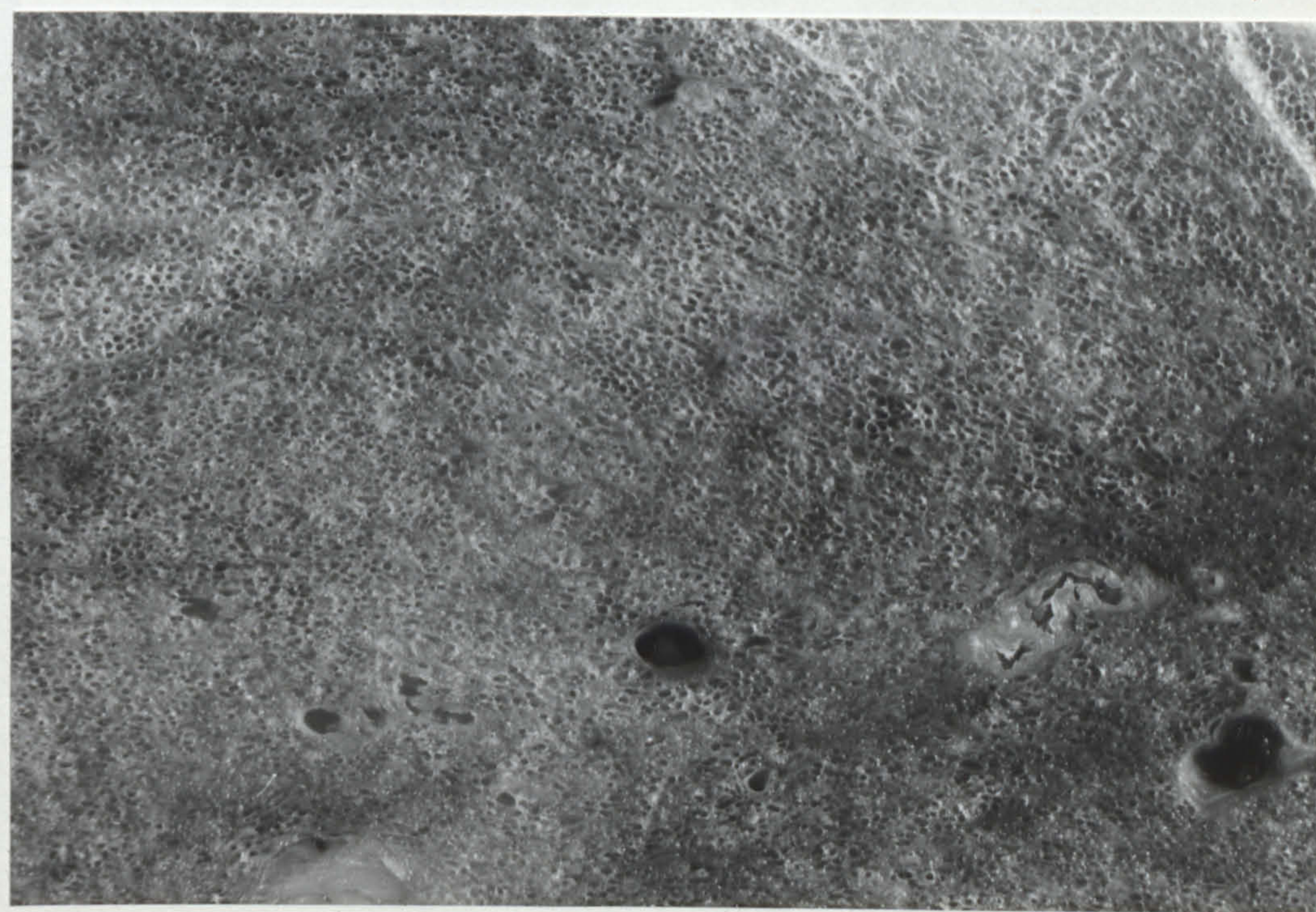
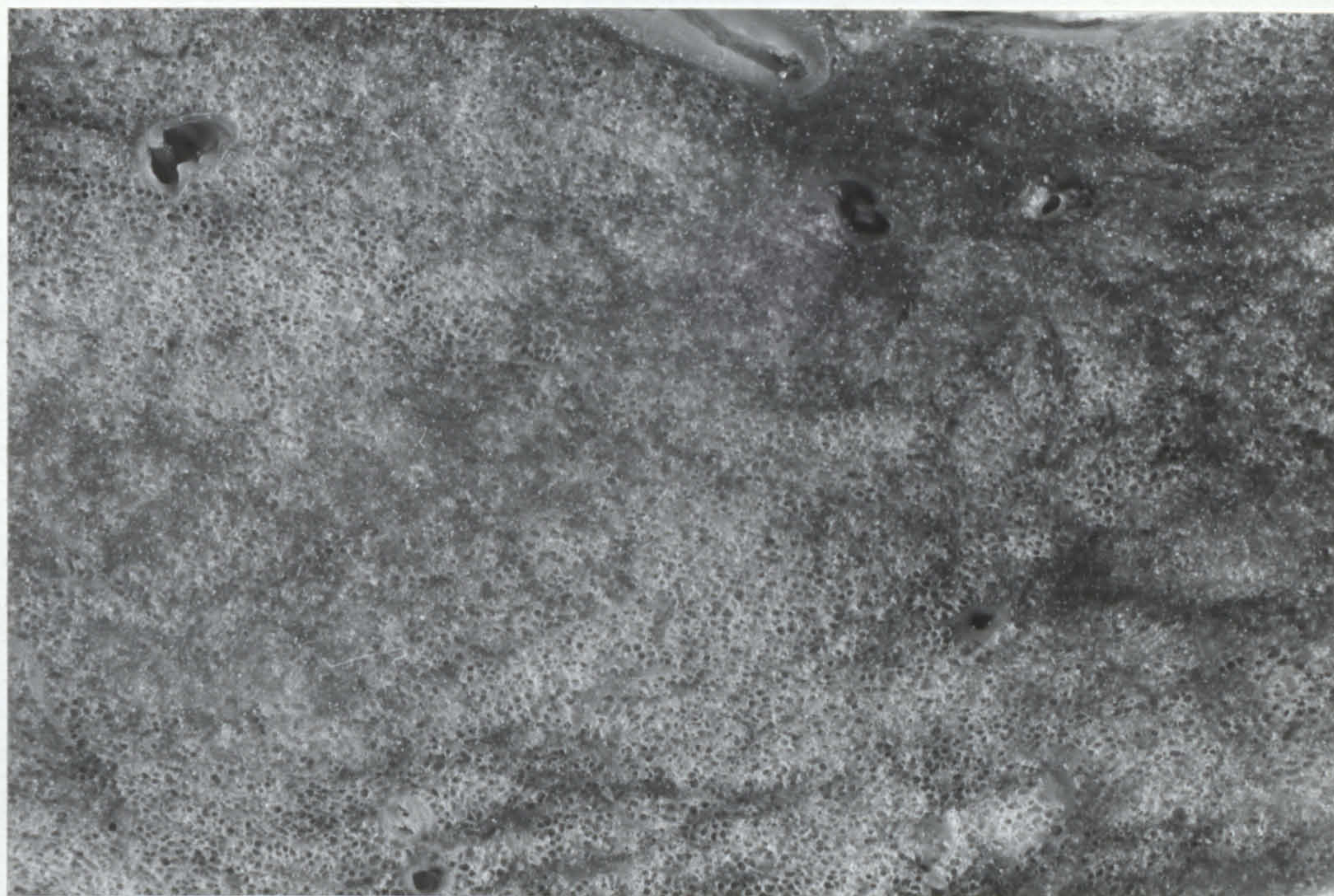


Figure 38. Similar area to that shown above X 15. Slices from two cases of



Figures 39 and 40. Barium sulphate impregnated slices from two cases of CPD. The alveoli are all of equal size and there is no alveolar wall destruction. X 15.



Figure 41. Barium sulphate impregnated slice from 14 showing the tip of the caudal lobe where fibrosis of areas of alveoli (F) was associated with emphysema (E). X 20.

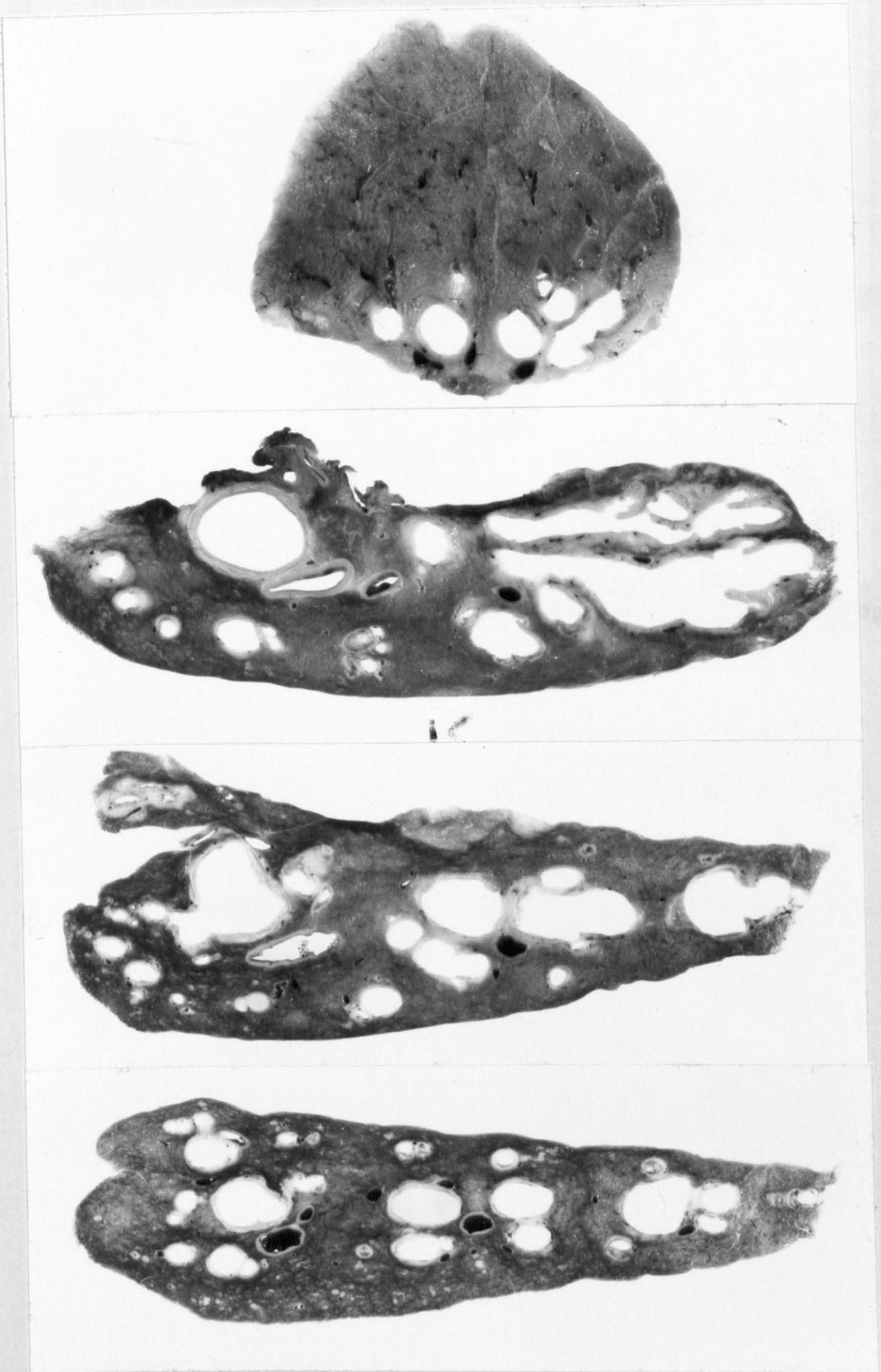


Figure 42. Barium sulphate impregnated slices from all four levels of the lung of BW12 showing the cylindrical bronchiectasis that was found at all levels.

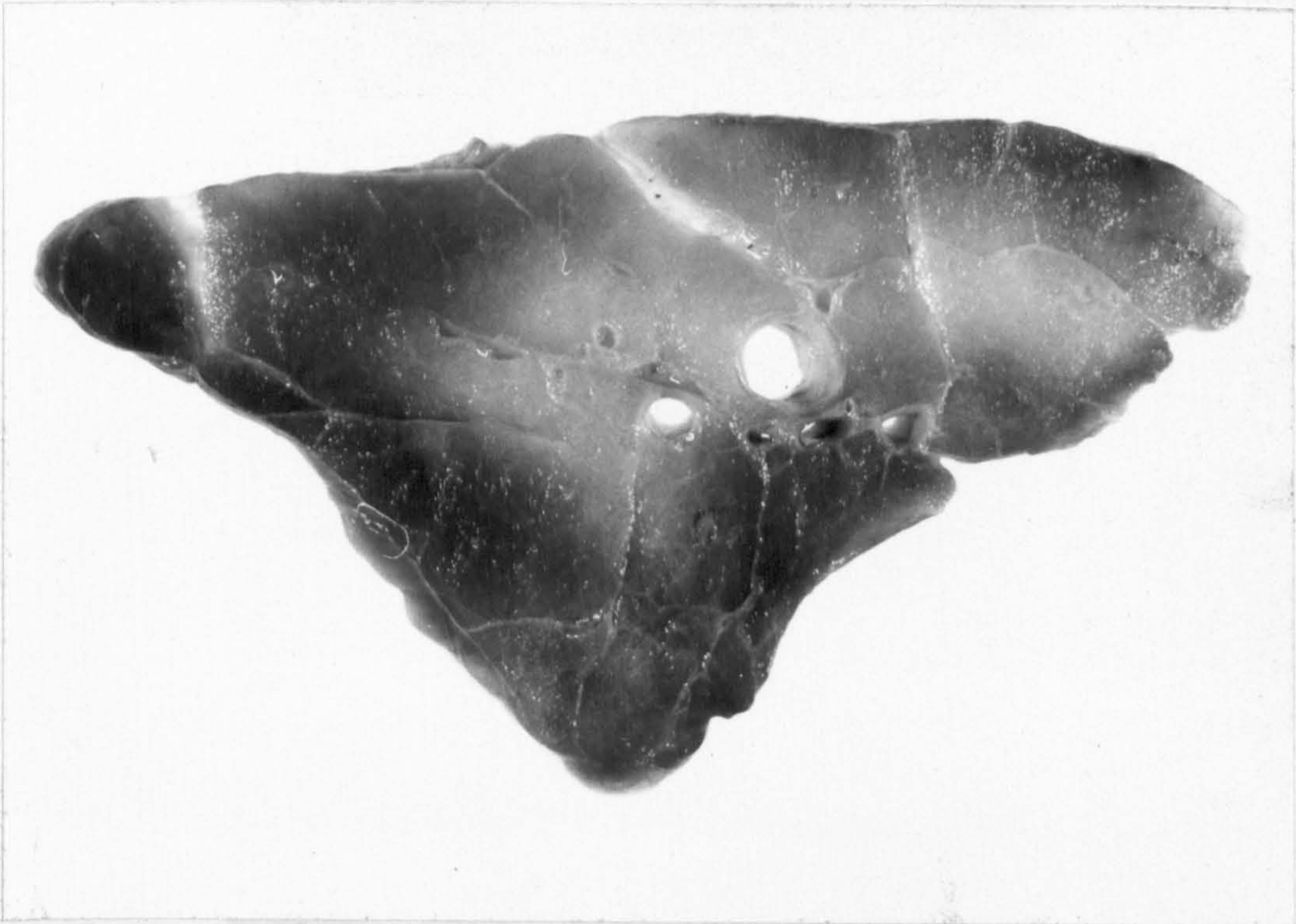


Figure 43. Barium sulphate impregnated slice from cranial lobe showing many complete interlobular septa.



Figure 44. Slice taken from level 2 of same case. Interlobular septa are infrequent in the main body of the caudal lobe but several can be seen at the edge of the lobe.

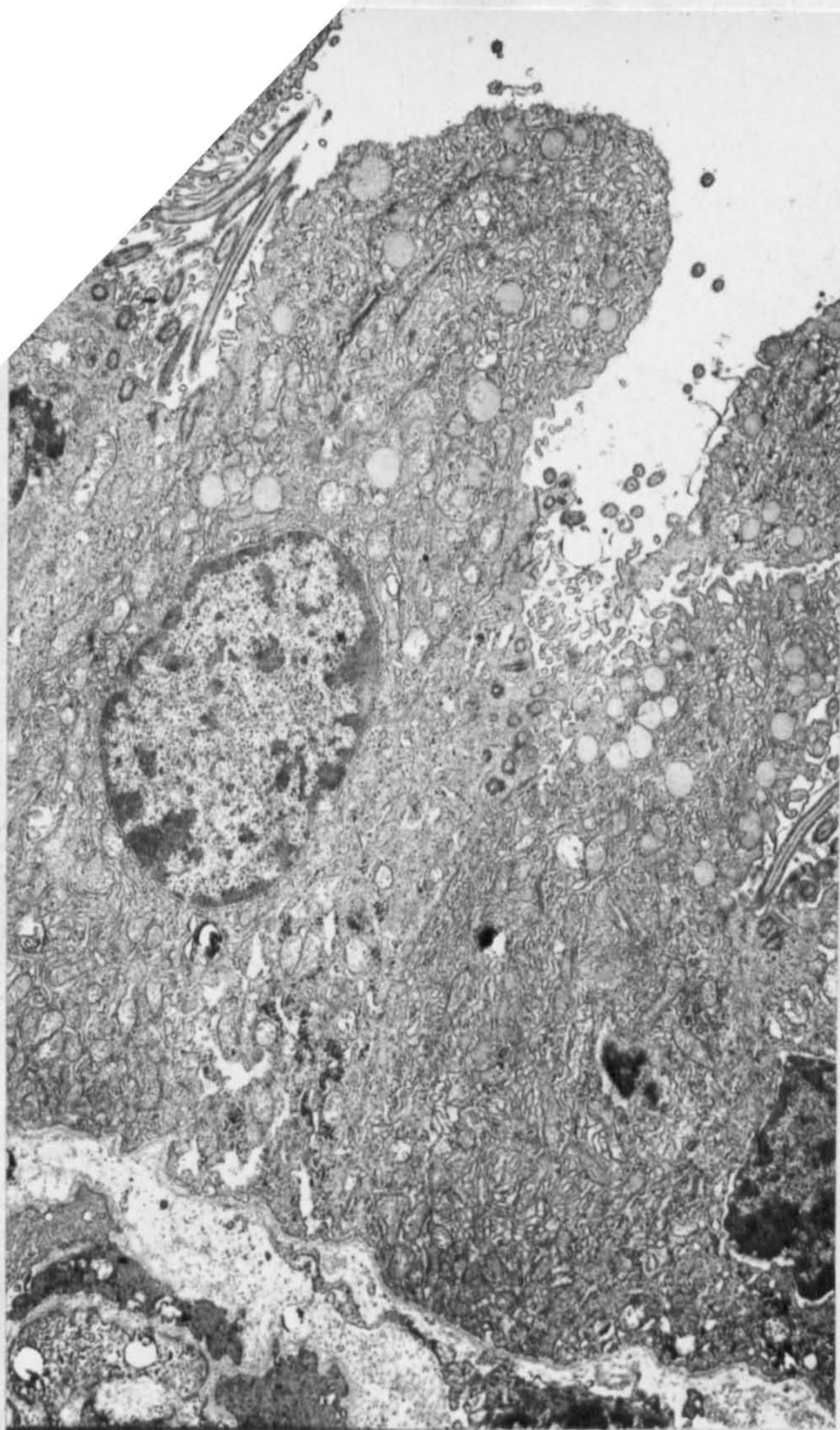


Figure 45. Electron micrograph of normal equine bronchiolar epithelium. The epithelium is one cell deep and consists of large nonciliated bronchiolar secretory cells (Clara cells) and smaller, narrow ciliated cells. X 3,000.

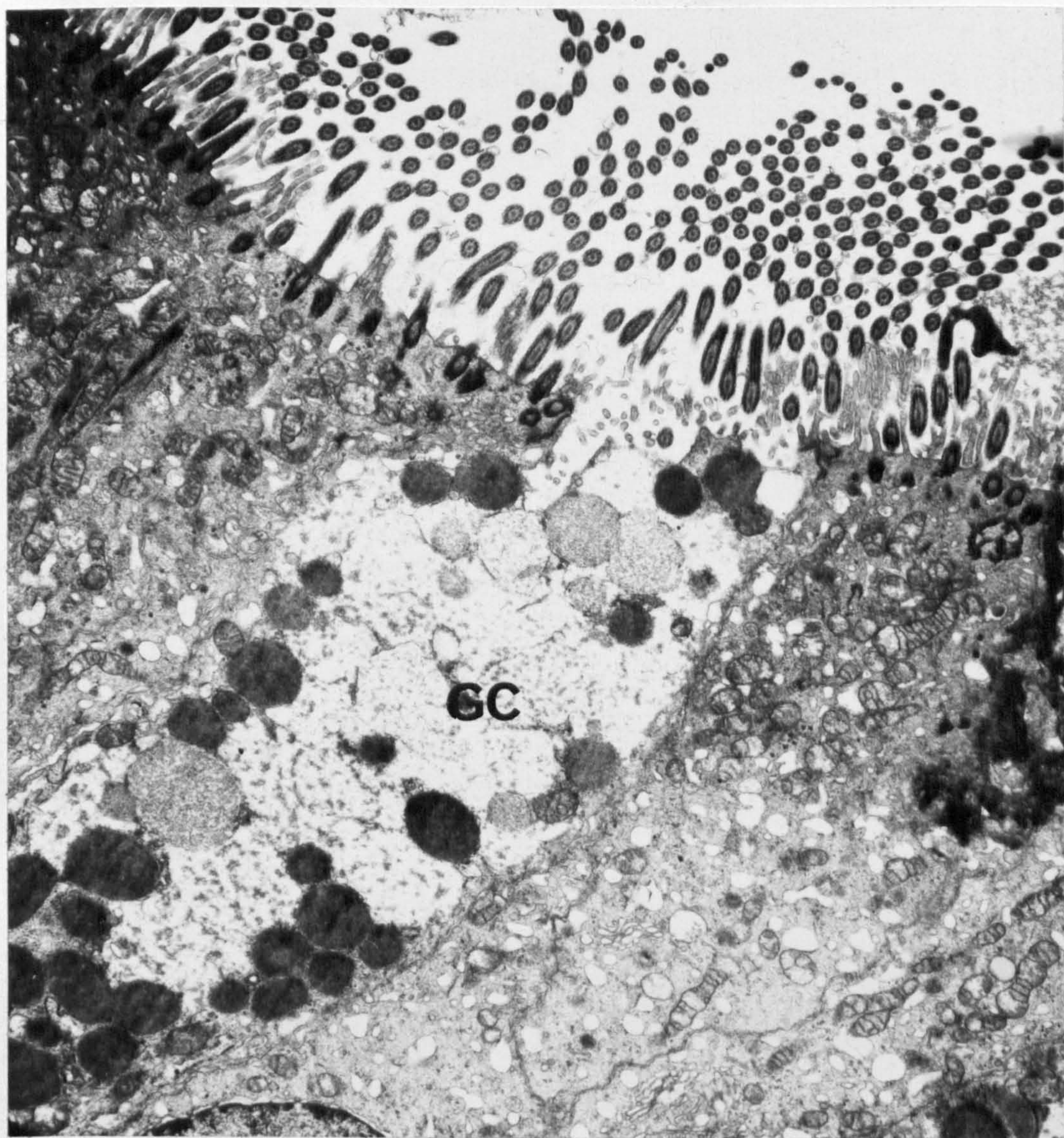


Figure 46. Electron micrograph of bronchiolar epithelium from a horse with CPD. A well-developed goblet cell (GC) is present amongst the ciliated cells. X 5,000.



Figure 47. Electron micrograph of a discharging goblet cell in the bronchiolar epithelium of a horse with CPD. The mucous granules (MG) seem to be coalescing, the apical membrane is disrupted and the contents are being extruded. X 5,000.

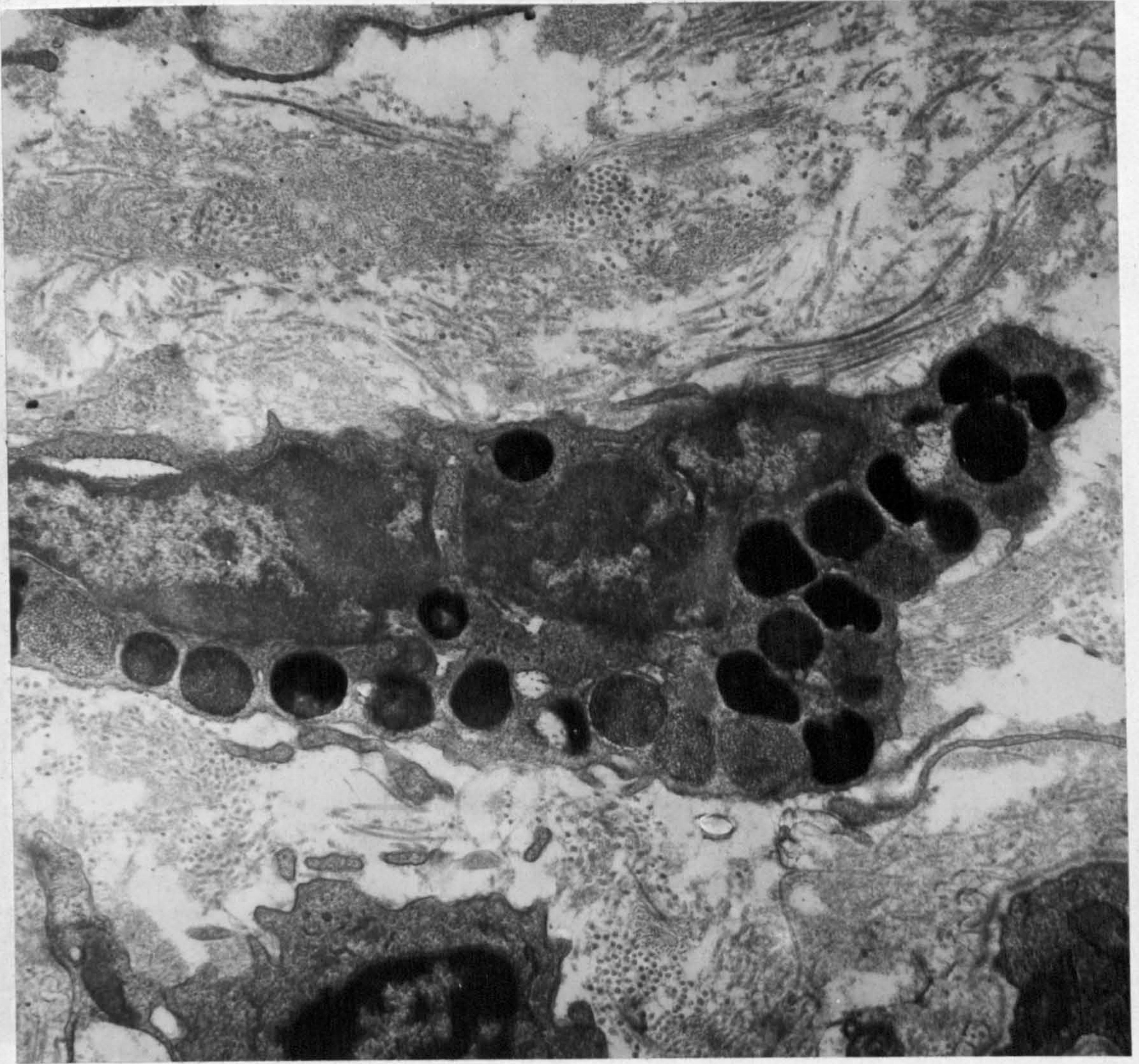


Figure 48. Electron micrograph of a mast cell in the bronchiolar lamina propria of a horse with CPD. The cell is elongated, has a bi-lobed nucleus and contains granules of varying electron-density. X 5,000.

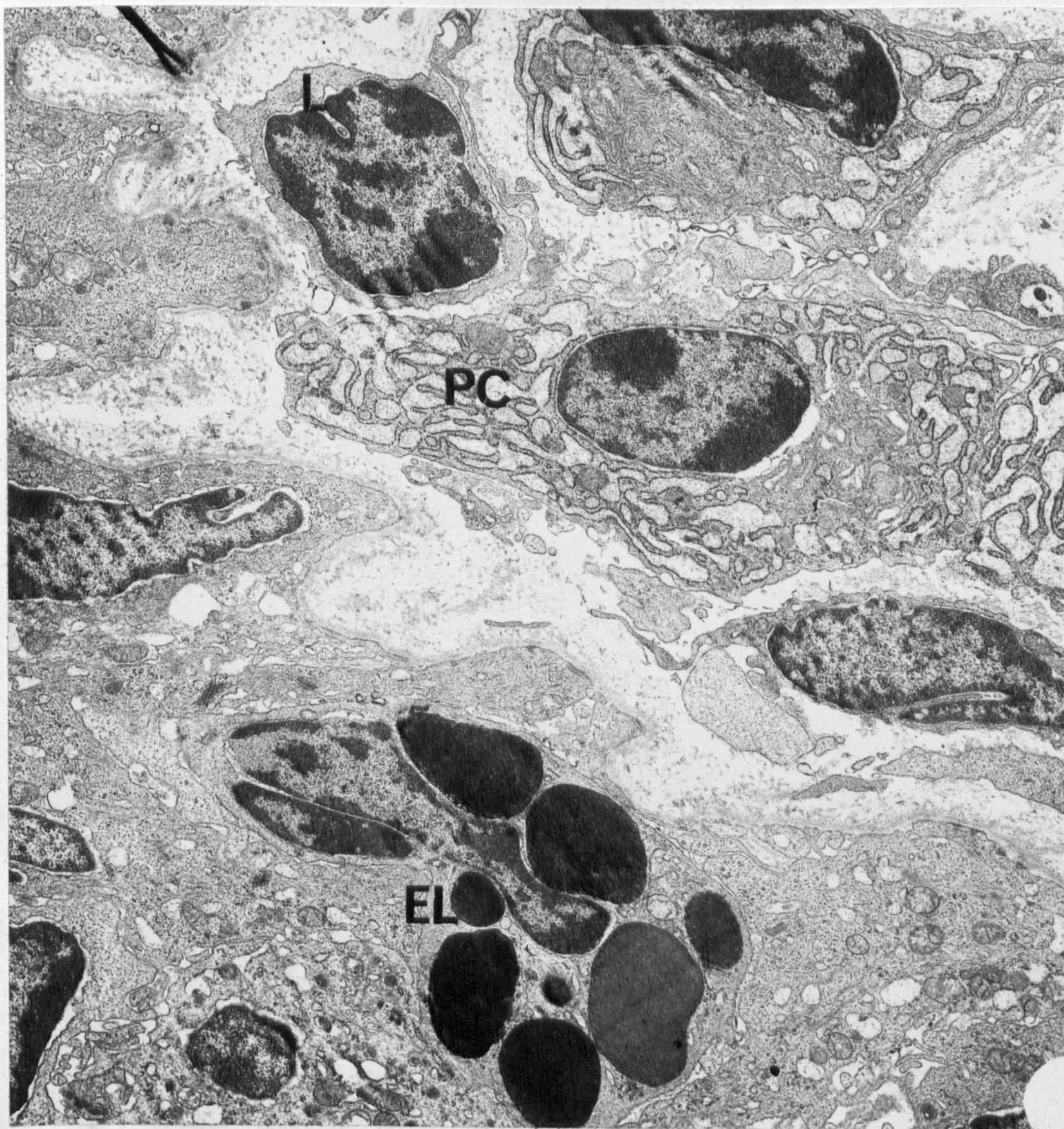


Figure 49. Electron micrograph of bronchiolar lamina propria from a horse with CPD. A plasma cell (PC), eosinophil (EL) and small lymphocyte (L) are shown. X 3,000.

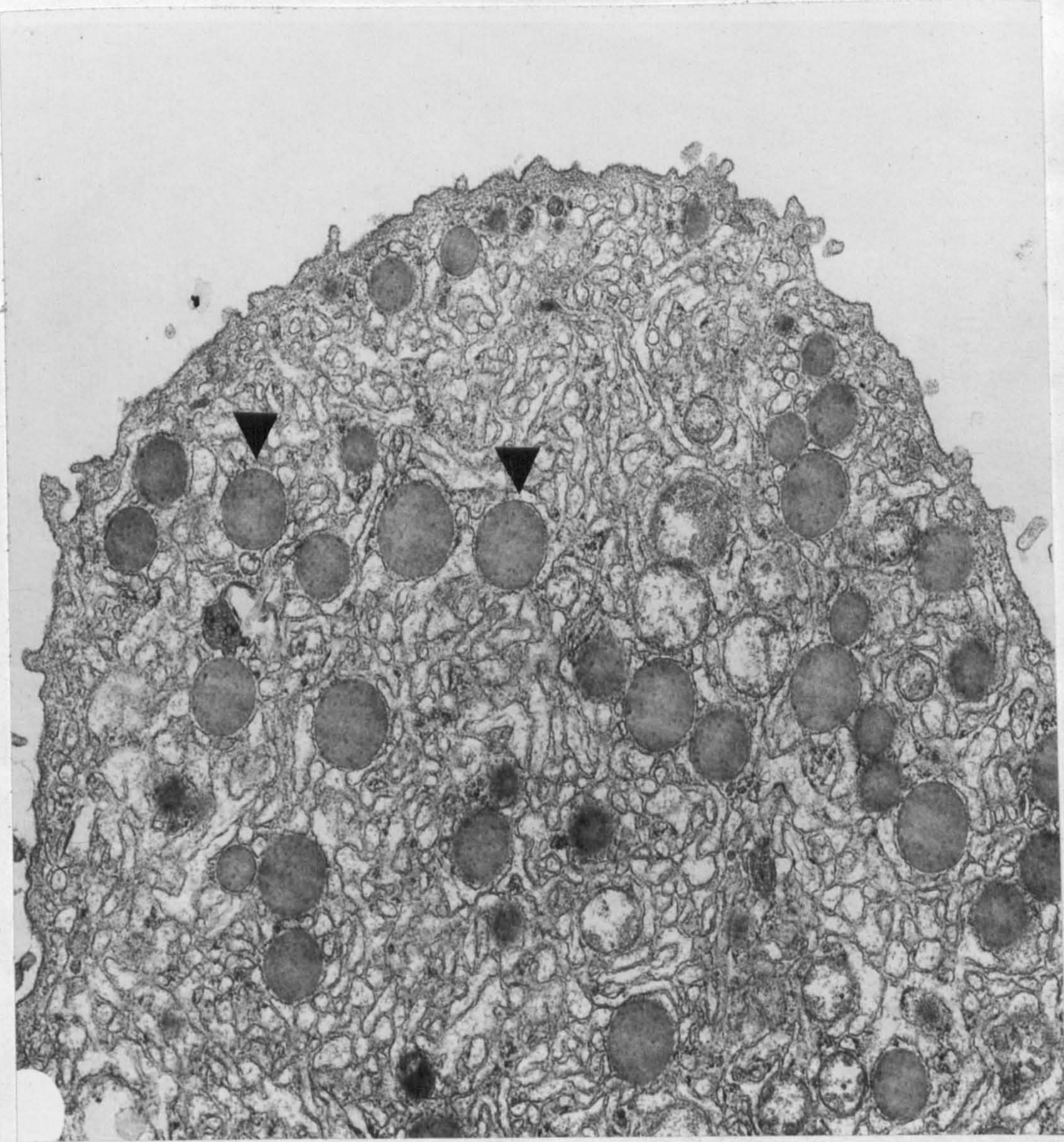
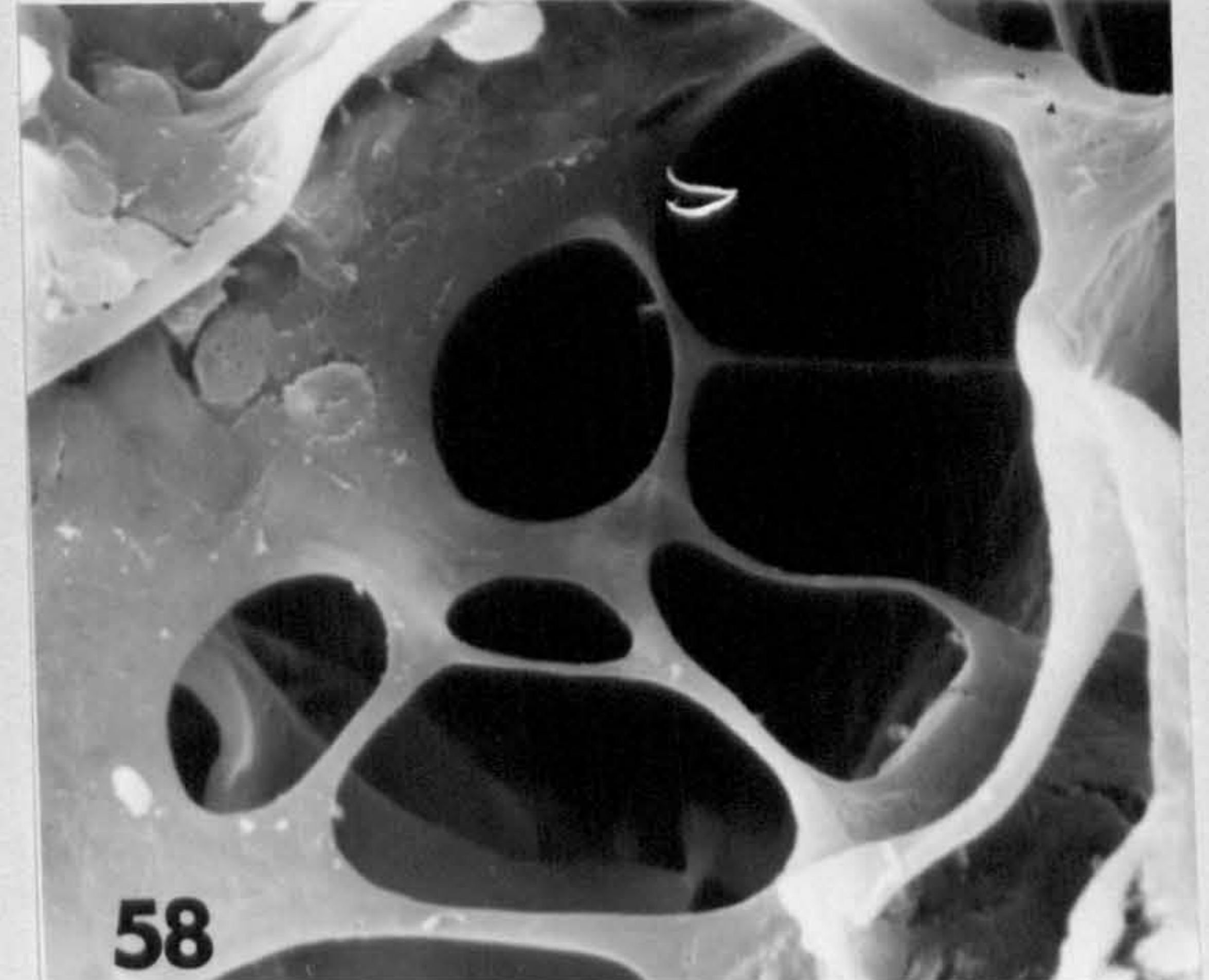
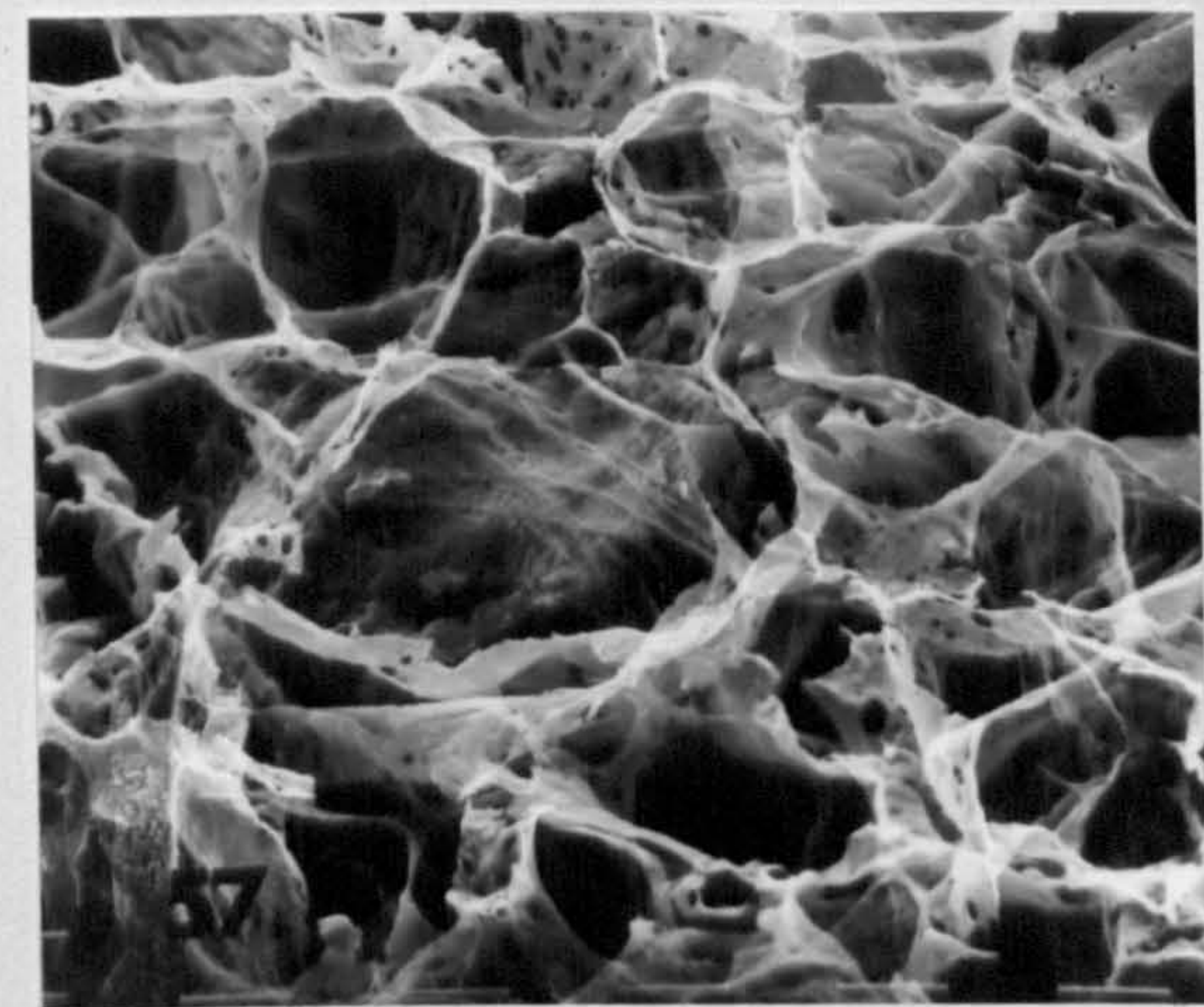
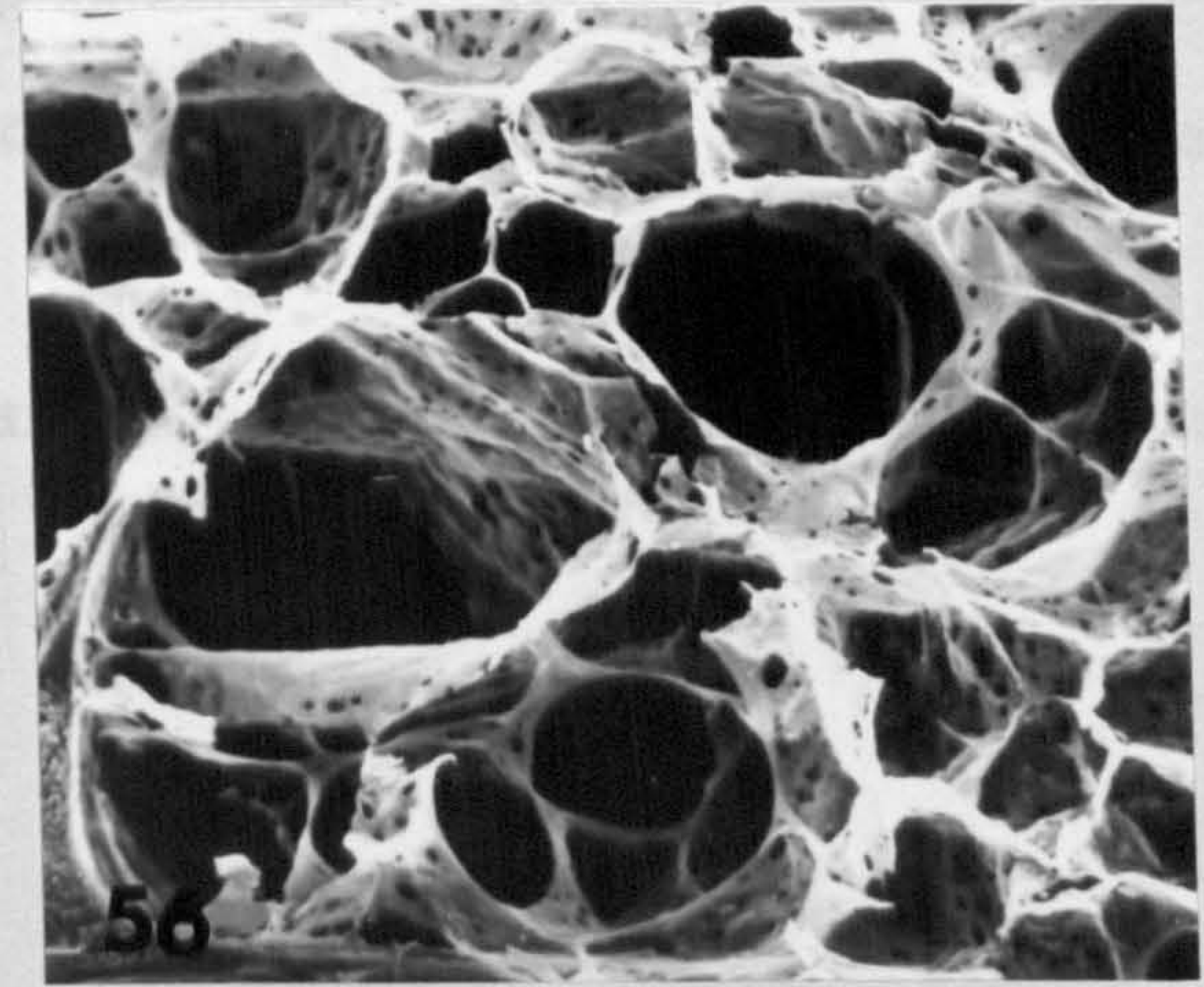
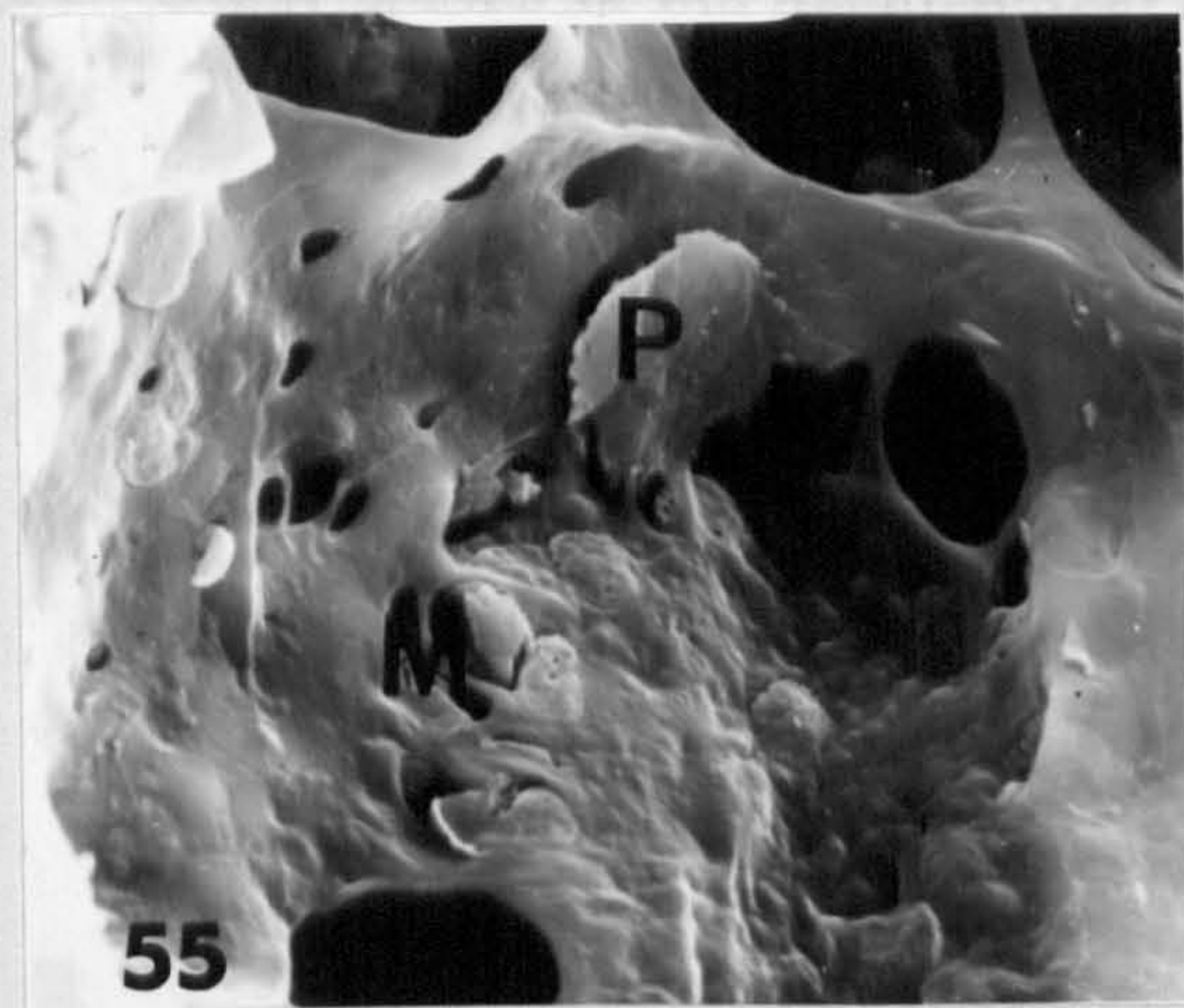
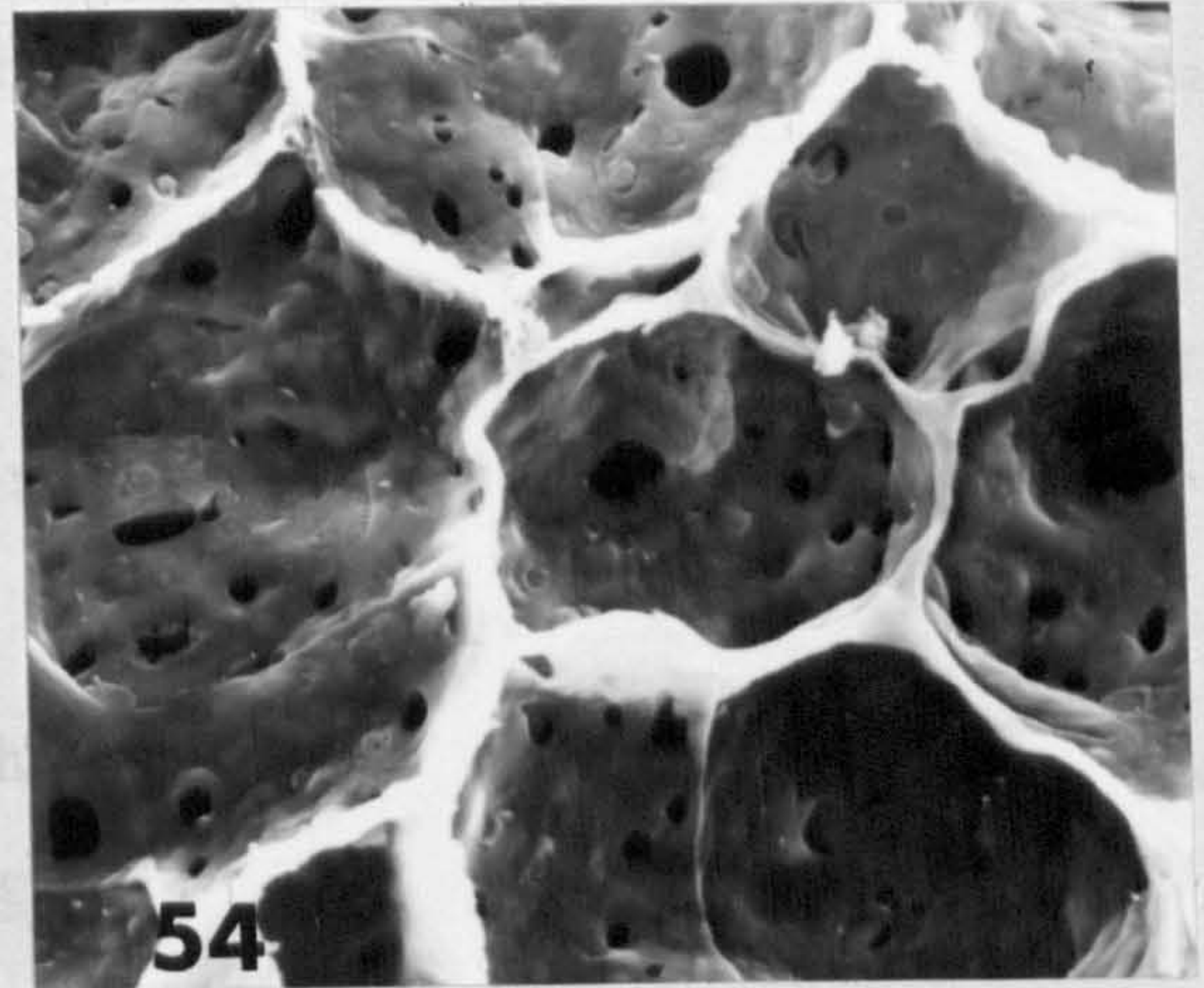
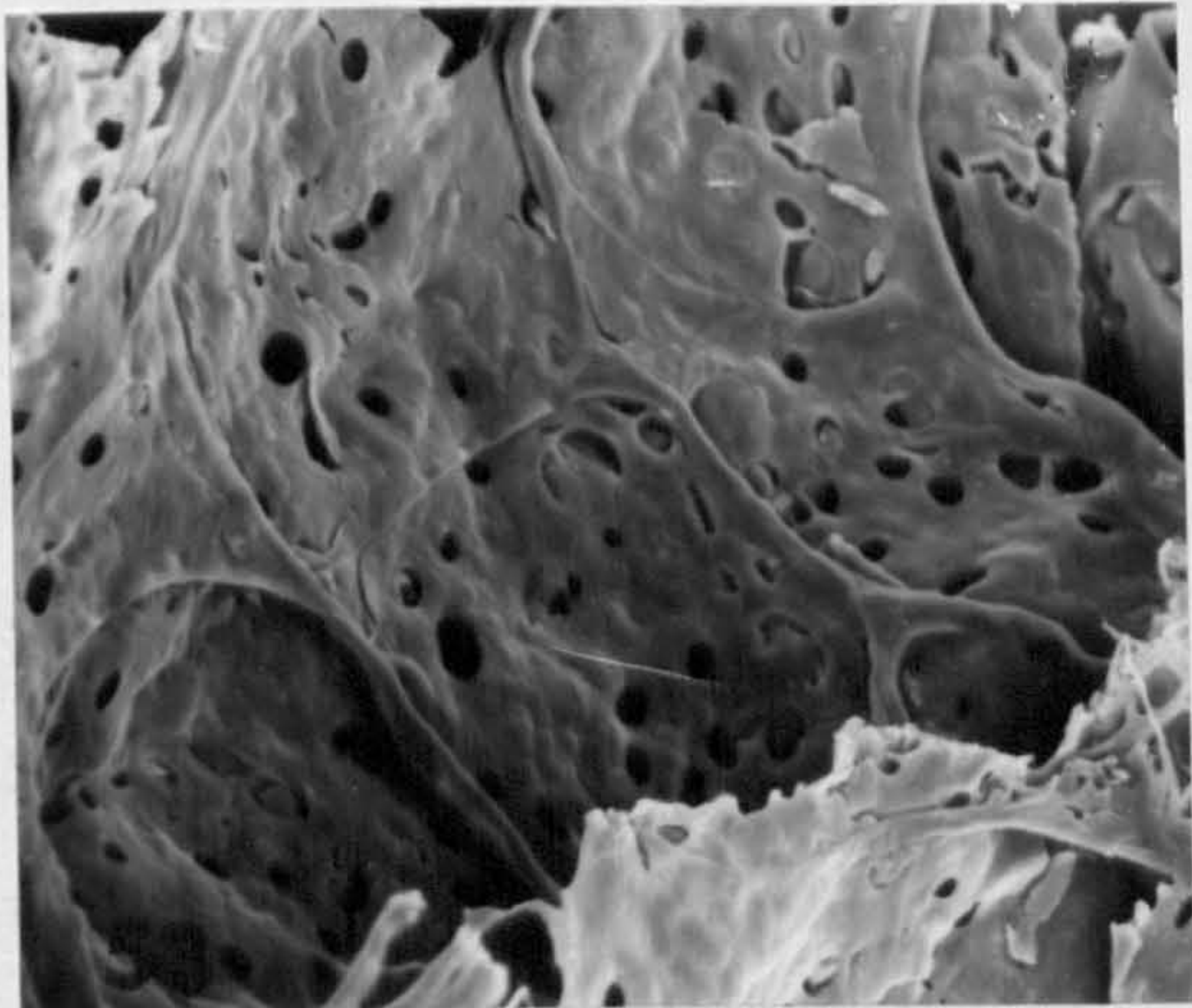
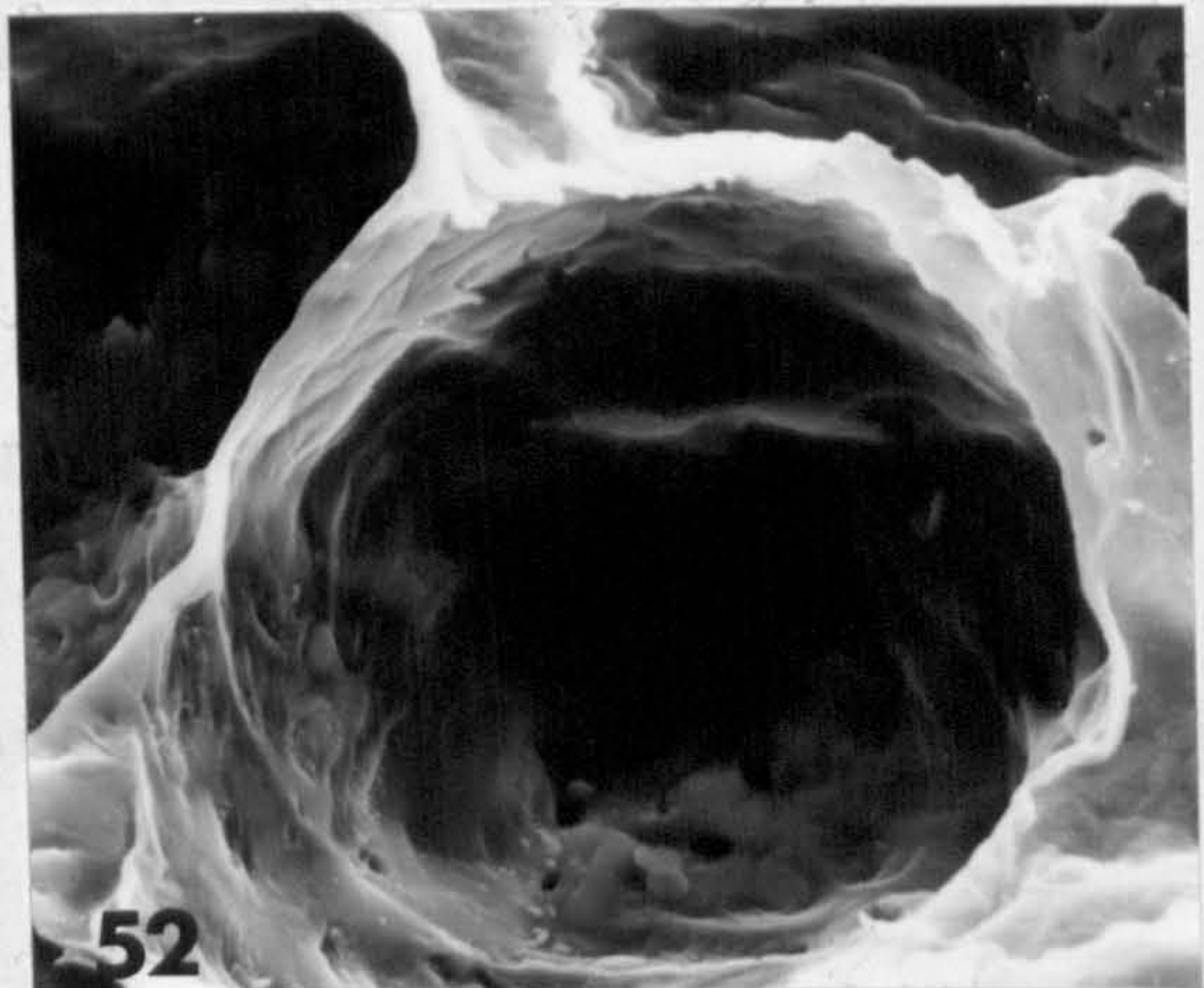
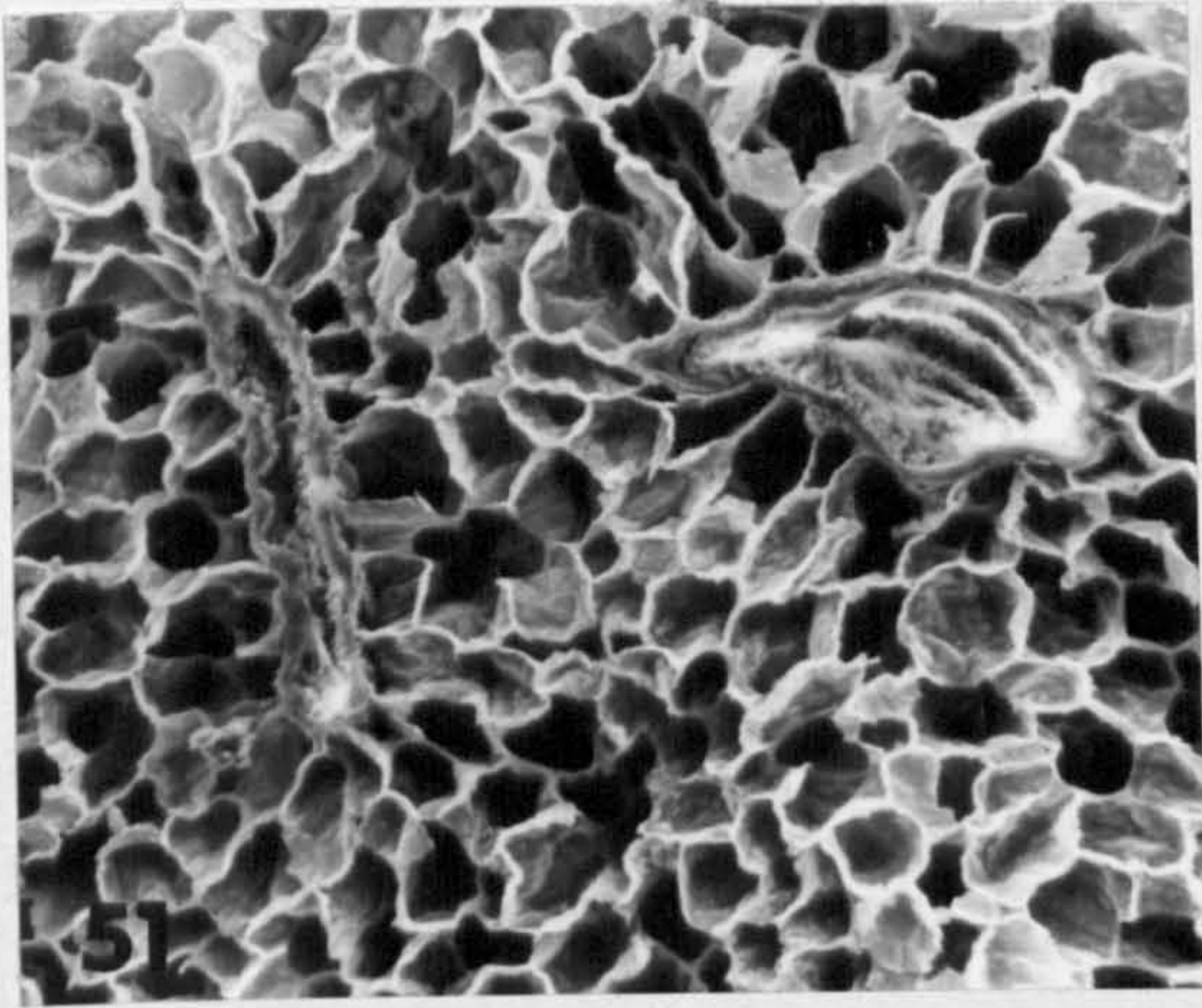


Figure 50. Electron micrograph of an equine nonciliated bronchiolar secretory cell (Clara cell).

The apex of the cell is rounded and bulging and projects above the ciliated cells. The apical mitochondria (arrows) are spherical and contain very few cristae. X 10,000.



Figures 51 - 58. Scanning electron micrographs of equine pulmonary tissue showing the development of emphysematous changes in the cranial lobe of horses with CPD.

51. Low power view of normal equine lung. The alveoli are all of fairly regular size and shape. An arteriole is on the left and a bronchiole on the right. X 40.

52. Normal alveolus, the interalveolar septa are intact and there are a very few, small pores of Kohn. X 1250.

53 and 54. Early changes in the development of emphysema. The alveoli are slightly enlarged but still regular in shape. The pores of Kohn are larger and more numerous. X 640.

55. Early changes in the development of emphysema. The pores of Kohn are fairly large. Alveolar macrophages are more common (M) and plaques (P) appear on the septa. X 2500.

56. More advanced emphysematous change. The alveoli are irregular, the septa thin and disrupted giving the lung a lacy appearance. X 80.

57. Final stages in the development of emphysema. Many alveoli are now only supported by thin trabeculae and others have merged into one another to form bullae. X 80.

58. Close-up of an alveolus from an area similar to that in Figure 57. X 1250.

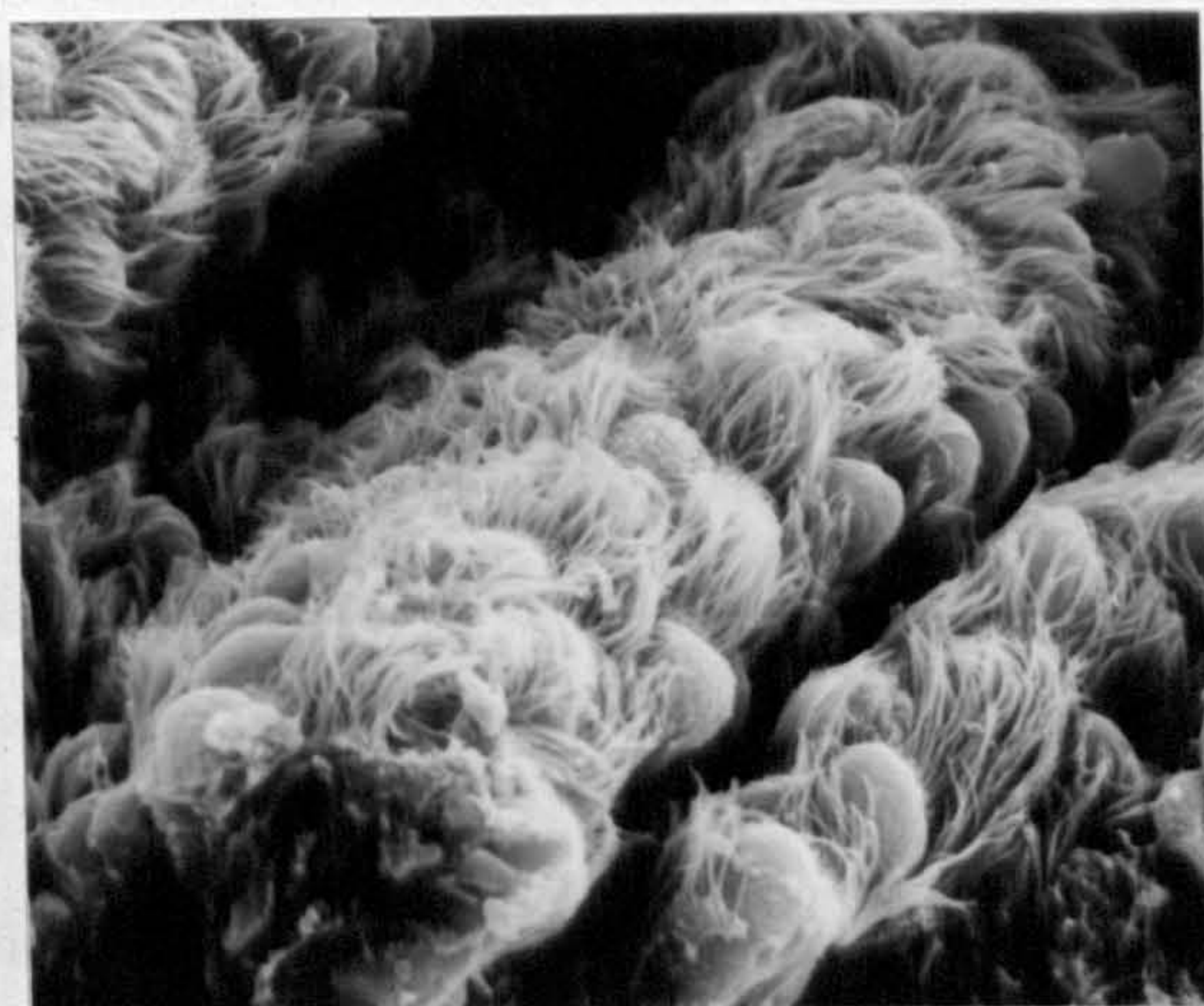


Figure 59. SEM of normal equine bronchial epithelium. The cilia are arranged in dense, regular waves. Two red blood cells are at the bottom of the picture. X 80.

Figure 60. Closer view of bronchial epithelium. The nonciliated cells are probably goblet cells. X 1250.

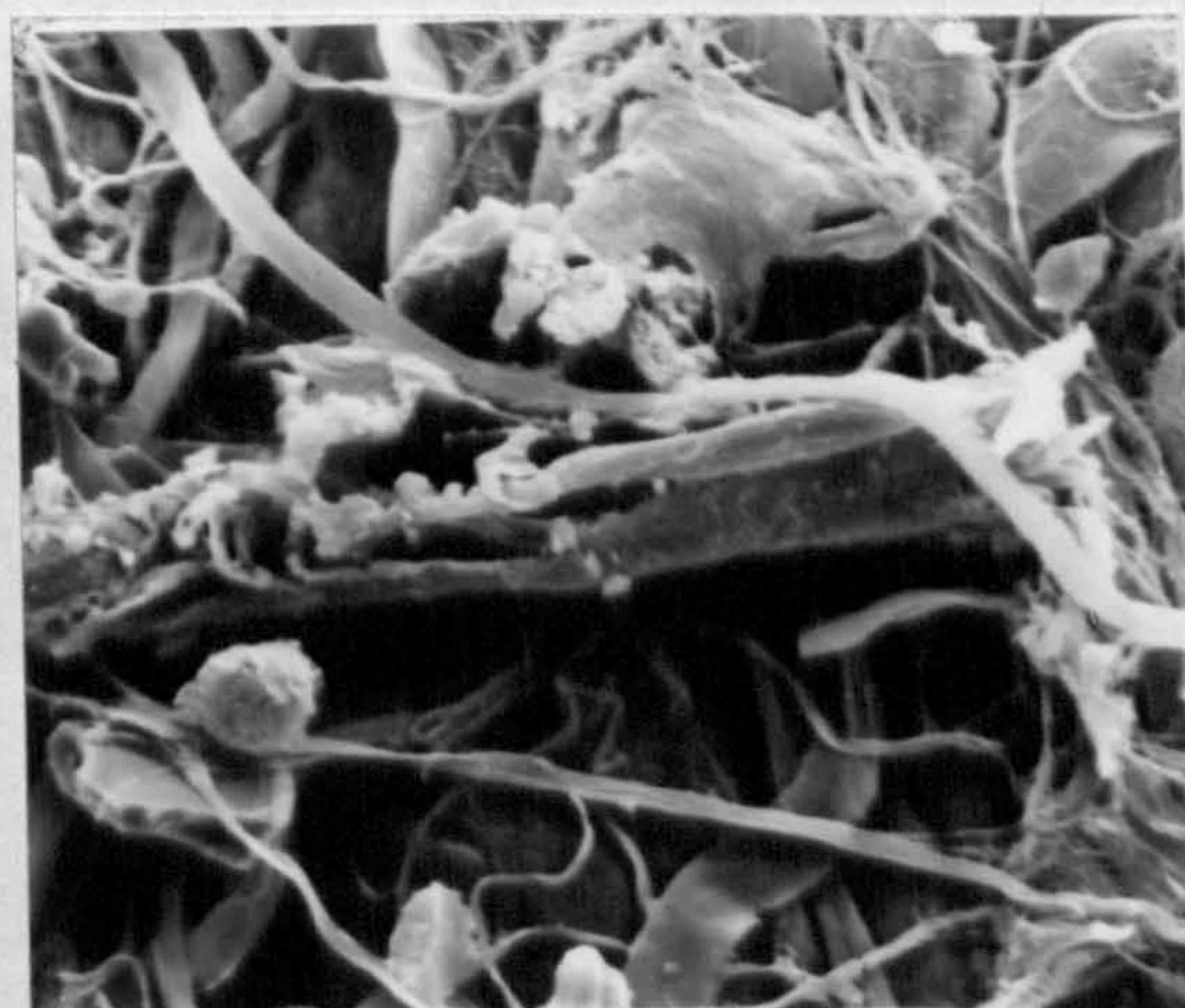
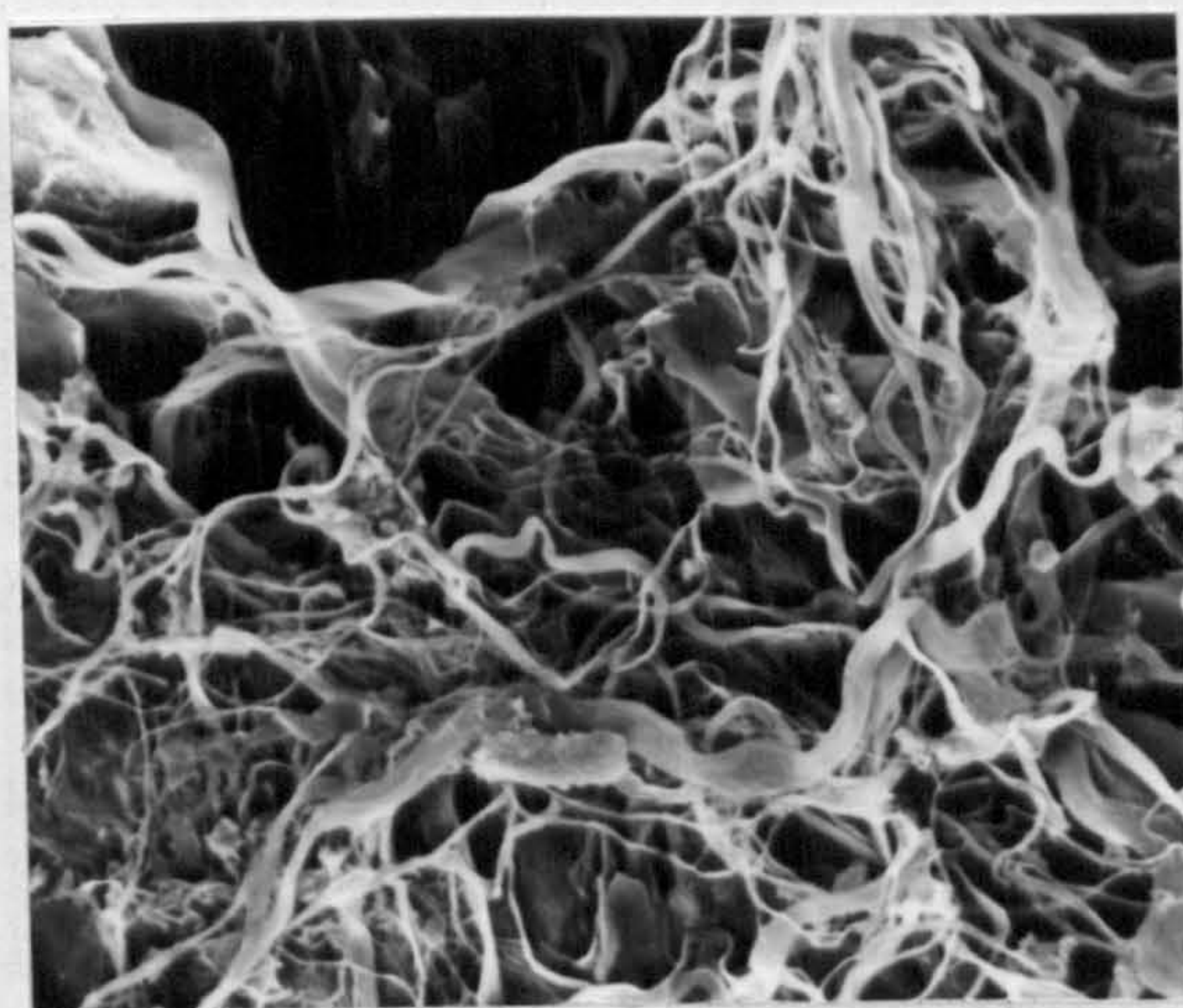


Figure 61 and 62. SEM of equine bronchial mucus. It consists of a thick irregular mesh with various cells caught up in it. X 650 and 1250 respectively.

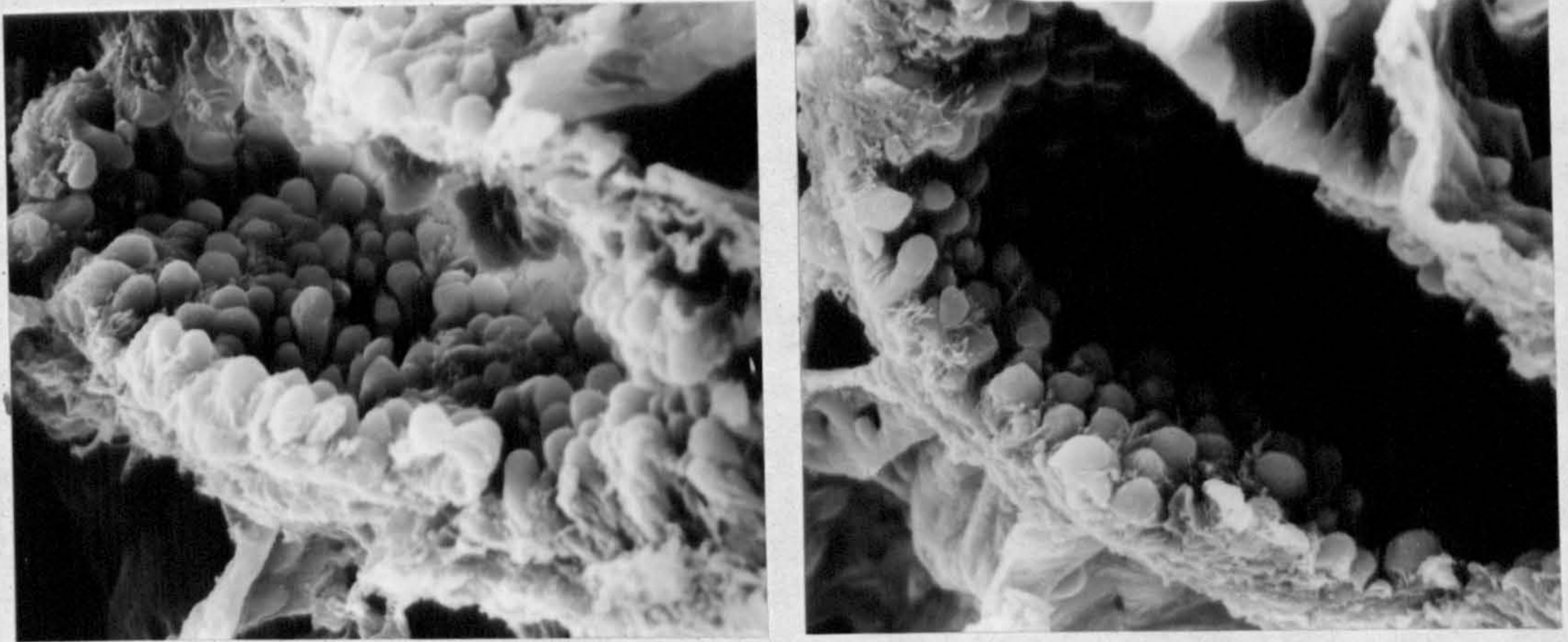
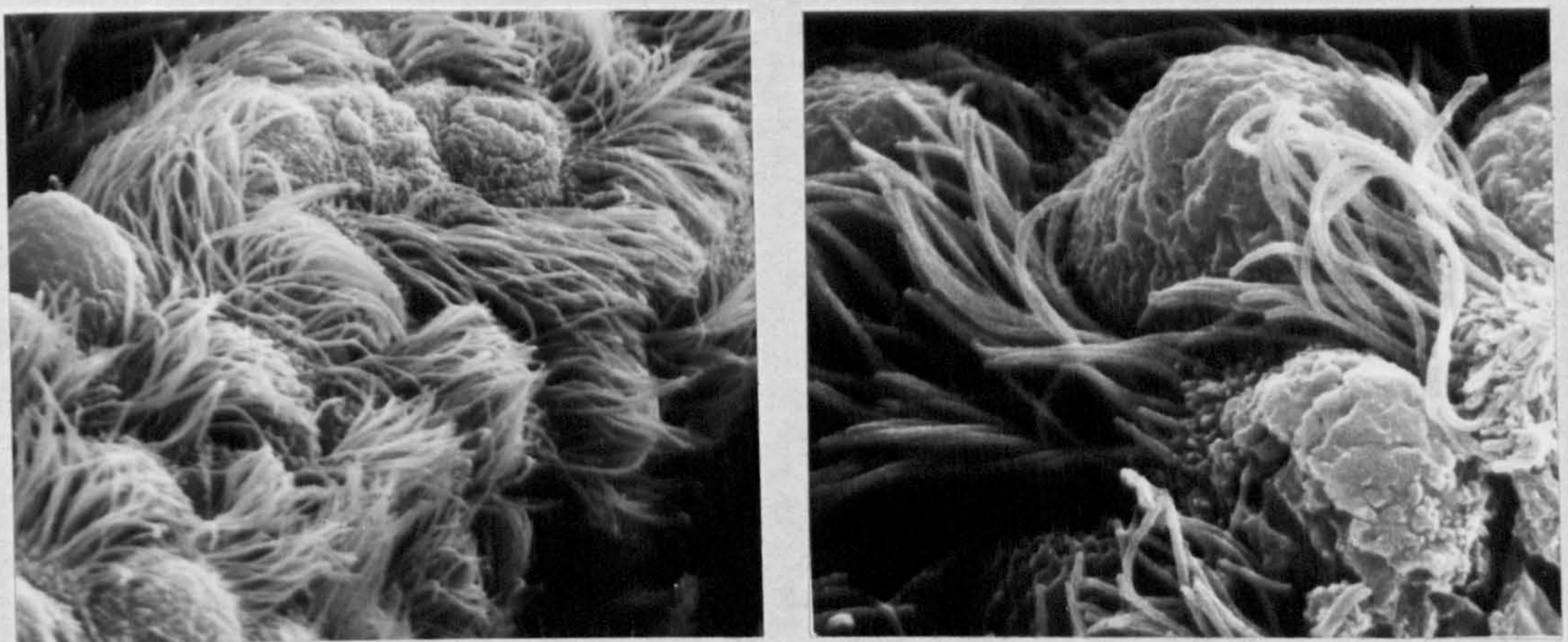


Figure 63 and 64. SEM of normal equine bronchiole. The nonciliated bronchiolar secretory cell is the predominant cell and projects well above the level of the cilia.



Figures 65 and 66. Close-up of bronchiolar epithelium. The nonciliated bronchiolar secretory cells are surrounded by a fringe of cilia. The apical membranes of the former cells are irregular and folded. X 2500 and 5000 respectively.

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CHAPTER FOUR

A HISTOCHEMICAL STUDY OF THE TRACHEOBRONCHIAL MUCOSUBSTANCES IN NORMAL HORSES AND IN HORSES WITH CHRONIC BRONCHIOLITIS

INTRODUCTION

In the chapter on pathological changes it was shown that the main pulmonary lesion in the affected horses was bronchiolitis. Bronchitis was not judged to be present as there were no inflammatory changes in the bronchi and the bronchial submucosal glands were not enlarged. In order to test this observation further a histochemical study of the tracheobronchial mucosubstances was carried out, firstly on a series of normal horses and then on horses suffering from chronic bronchiolitis.

It has been shown that in human chronic bronchitics the mucus secreted by the epithelial goblet cells and submucosal glands changes both qualitatively (Reid 1965; de Haller and Reid 1965; Lamb and Reid 1969) and quantitatively (Reid 1960; Restrepo and Heard 1963; Dunnill, Massarella and Anderson 1969). Similarly in chronic bronchitis in the dog (Wheeldon, Pirie and Breeze 1976) there is a change in the quantity and type of mucosubstance secreted. In enzootic pneumonia of pigs which is caused by mycoplasma infection the submucosal glands were found to be hypertrophied and there was a shift in the type of mucosubstances produced by the goblet cells and the submucosal glands (Jones, Baskerville and Reid 1975). More recently Allan, Pirie and Wheeldon (1977) have shown that in calves with mycoplasma pneumonia there was an increase in the number of goblet cells which also extended into the bronchioles and the type of mucus secreted by the submucosal glands was altered.

The normal mucosubstances in the equine tracheobronchial tree have only been looked at by Goco, Kress and Brantigan (1963) who did not characterise the mucosubstances present and also, erroneously, thought that the horse did not have submucosal glands. This is the first study designed to characterise and quantify the tracheobronchial mucosubstances in the normal horse. Studying horses with chronic bronchiolitis was also intended to localise the disease process in these animals.

REVIEW OF THE LITERATURE

The epithelial goblet cells and bronchial submucosal glands of the tracheobronchial tree secrete mucus into the lumina of the airways, the main function of which is to lubricate and protect the tracheobronchial epithelium. The main constituent of mucus is a high molecular weight glycoprotein containing over 50 per cent. carbohydrate consisting mainly of fucose, galactose, N-acetylglucosamine and sialic acid (Clamp 1977). It is these monosaccharide residues which largely determine the physical and chemical properties of mucus. These different chemical properties can be utilised to differentiate the various mucosubstances with histochemical stains and other techniques.

Much of the early work on classifying connective tissue mucopolysaccharides and mucosubstances was based on connective tissue studies by Spicer and colleagues (Spicer 1960; Spicer, Leppi and Stoward 1965; Spicer, Horn and Leppi 1966 and Spicer and Henson 1967). There has been some confusion over the terminology of the connective tissue carbohydrates largely because of the different investigative methods used by separate scientific disciplines. To rectify this Spicer, Leppi and Stoward (1965) suggested that the term "mucopolysaccharide" be used only when referring to acidic carbohydrates in connective tissue which have been identified by biochemical techniques, the term "mucosubstance" to be used only for carbohydrate components of an unknown nature and that the term "glycoprotein" be used only for those substances that have been analysed biochemically. "Mucin" is used to denote mucopolysaccharides in epithelial sites.

Initially the various carbohydrates were analysed by a battery of techniques including autoradiography, basic dyes used singly or in combination, oxidation, chemical modification of tissue, enzymatic digestion and fluorescent antibody (Spicer and Henson 1967). These methods have been used by a number of workers to examine mucosubstances in a variety of tissues including multiple tissue sites of rabbit, rat and guinea-pig (Spicer 1960), bronchial mucus (Reid 1965), tracheobronchial glands in man (Lamb and Reid 1969, 1970; Spicer and others 1971), canine tracheal pouch (Chakrin and others 1970; Spicer and others 1971), pig bronchial epithelium (Jones, Baskerville and Reid 1975), canine tracheobronchial epithelium

(Wheeldon, Pirie and Breeze 1976), epidermis and gills of plaice and rainbow trout (Fletcher, Jones and Reid 1976) and bovine tracheobronchial epithelium (Allan, Pirie and Wheeldon 1977). The various methods initially used have now been simplified and modified so that the mucosubstances in the tracheobronchial tree may now be adequately identified using a few simple histochemical stains.

Spicer, Leppi and Stoward (1965) proposed a histochemical classification of the connective tissue mucosubstances which divided them up into three broad categories, neutral, sulphated acidic and non-sulphated acidic. A modification of this classification is given in Table 9. Neutral mucosubstances include neutral glycoproteins and fucomucins, all of these being periodate reactive, that is they stain with periodic acid Schiff's (PAS) stain. The remainder of the mucosubstances are acidic and are classified as either sulphated or non-sulphated. Sulphated mucosubstances include substances such as chondroitin sulphate, a major constituent of cartilage, and may be visualised by autoradiography, basic dyes at controlled pH's, colloidal iron stain and aldehyde fuchsin. Non-sulphated mucosubstances are divided up into hexuronic acid mucopolysaccharides such as hyaluronic acid which do not seem to occur in epithelial tissue (Spicer 1971) and sialic acid rich mucopolysaccharides. The two types of acidic mucosubstance found in epithelial tissue are therefore known as sulphomucins and sialomucins. Sialomucins may then be further classified dependent upon their susceptibility to digestion by the enzyme neuraminidase which is derived from Vibrio cholerae. The sialomucins are either highly susceptible to digestion, slowly digested or completely resistant to digestion. There is no known biochemical basis for this. All sialic acid groups are released by acid hydrolysis (Lamb and Reid 1969) which is used as a means for differentiating them.

Until recently very little work had been done on the tracheobronchial mucosubstances of animals other than man, rodents and a short study on dogs (Spicer 1960; Goco, Kress and Brantigan 1963; McCarthy and Reid 1964; Lamb and Reid 1969; Spicer and others 1971). Goco, Kress and Brantigan (1963) looked at the lungs from man, rat, guinea-pig, dog, monkey, sheep, pig and horse to compare the goblet cells and mucous glands. They only used PAS to stain the mucin so that no comparison of the types of mucosubstances could

be made. They found that the horse and the rat had no bronchial mucous glands, guinea-pig, dog and monkey had few bronchial mucous glands, the sheep had a moderate number of bronchial mucous glands and the pig had numerous bronchial mucous glands similar to the situation in man where there is a wide band of bronchial glands in the submucosa of bronchi. The cat also has numerous bronchial mucous glands (personal observation). The horse does in fact have tracheal and bronchial mucous glands although these are sparse and poorly developed (personal observation).

Similar results were obtained by Korhonen, Holopainen and Paavolainen (1969) who examined the mucous glands of the mouse, rat, guinea-pig, rabbit and pig. In addition they used a wide range of histochemical stains and found that the mucous glands of the pig also resembled those of man histochemically.

In the normal human tracheobronchial mucous gland four types of acidic mucin have been identified in addition to neutral mucopolysaccharide which forms only a small proportion of the total (Lamb and Reid 1969). These authors used the following histochemical methods : alcianblue-PAS (AB-PAS), sialidase digestion and acid hydrolysis to identify sialomucins; aldehyde fuchsin - alcian blue (AF-AB), AB in aluminium sulphate-PAS and high iron diamine - AB (HID-AB) to identify sulphomucins. In addition autoradiography was employed to identify sulphomucins which failed to stain with the histochemical stains. The four types were:

- 1) sialic acid susceptible to sialidase and to acid hydrolysis
- 2) sialic acid resistant to sialidase and susceptible to acid hydrolysis
- 3) sulphate which stained with AB after acid hydrolysis and took up radioactive sulphate stains but did not stain with sulphomucin stains
- 4) sulphate which stained with AB after acid hydrolysis, took up radioactive sulphate and stained with sulphomucin stains.

Acid hydrolysis is a drastic procedure involving treatment of tissue with sulphuric acid at high temperatures. This must cause tissue damage and so it would be preferable if another method of sialomucin identification could be used. Jones and Reid (1973a) treated various epithelial mucous sites from different species with AB at various pH levels from 2.6 to 0.5. The relative contents of sialomucins and sulphomucins normally found at these sites were already known. They found that tissues containing sialomucin alone consistently

failed to stain with AB at pH 1.5 and below. Sialomucin sensitive to sialidase stained between pH 2.6 and 1.7 and sialidase resistant sialomucin stained to pH 1.5. AB at a pH of 1.0 was specific for sulphomucins. They also found, in the canine submaxillary salivary gland, a sulphomucin which stained only at the lower pH levels. Such a sulphomucin was then detected in the human bronchial mucous gland (Jones and Reid 1973b), and in the pig bronchial mucous gland (Jones, Baskerville and Reid 1975). This adds a third sulphomucin to those already detected. In addition Jones and Reid (1973a) concluded that the presence of sialomucin did not modify the AB staining of sulphomucins over a pH range and that the presence of sulphomucins did not explain the difference in the resistant and sensitive forms of sialomucin indicating that this difference must lie in the ionisation of these two types of sialomucin.

Since the publication of these findings, investigations into the types of mucosubstance present at a site have been much simplified. A sequence of three staining combinations will identify four types of mucosubstance - neutral, sulphated, sialidase sensitive and sialidase resistant. The stains are AB pH 2.6 - PAS, sialidase AB pH 2.6 - PAS and AB pH 1.0 - PAS. This method of classification is explained in Table 10.

The point was first made by Reid (1965) that no abnormal mucosubstance had yet been identified in disease but rather a change occurred in the relative proportions of mucosubstance secreted. The observation was meant to apply to diseases of man but has held true for the small number of investigations carried out on disease states in animals.

In human chronic bronchitics there is an increase in the volume and number of bronchial mucous glands (Reid 1958). The qualitative changes have also been investigated (Reid 1965; de Haller and Reid 1965; Lamb and Reid 1969) and it is known that subjects with chronic bronchitis have a larger proportion of mucous cells which are completely or partially resistant to sialidase. This was attributed to a relative increase in the amount of this mucosubstance but as Wheeldon (1974) has pointed out this may alternatively have been due to an absolute or relative decrease in sulphomucin.

The relative proportions of mucins secreted by the pig bronchial mucous glands are similar to those of man except that there is a greater proportion of neutral mucosubstance (Jones, Baskerville and Reid 1975). After intra-nasal administration of Mycoplasma hyorhinis which causes enzootic pneumonia the mucous glands were found to be hypertrophied and there was a shift towards the production of more acid mucosubstances with an additional shift towards sulphomucins and sialidase sensitive sialomucins at the expense of sialidase resistant types. In contrast the number of epithelial goblet cells was decreased, the cells had become taller and thinner containing depleted amounts of mucus. In the normal pig the cells contain mainly acid mucosubstances consisting of sulphomucin and sialidase resistant sialomucin; in pigs with enzootic pneumonia many more cells contained neutral mucosubstances only.

Wheeldon, Pirie and Breeze (1976) studied the tracheobronchial mucosubstances of normal dogs and dogs with chronic bronchitis. In normal dogs sulphomucin was the predominant mucosubstance in the epithelial goblet cells and a mixture of sulphomucin and sialomucin was found in the bronchial mucous glands, the latter forming a small proportion only and consisting of equal amounts of sialidase sensitive and sialidase resistant sialomucins. In dogs with chronic bronchitis there was a marked shift towards the production of sialomucins in both sites. Most epithelial goblet cells contained sialomucins, the same being true of the bronchial mucous glands, both types of sialomucin again being found. This is analagous to the situation in the human chronic bronchitic (Reid 1965).

In a similar study Allan, Pirie and Wheeldon (1977) looked at the tracheobronchial mucosubstances in a series of normal calves and calves with cuffing pneumonia caused by Mycoplasma dispar infection. In non-pneumonic calves the epithelial mucosubstances of the goblet cells were almost all sulphomucin containing. Neutral mucosubstances, sialomucins and sulphomucins were produced in approximately equal numbers by the bronchial mucous glands. In the pneumonic calves there was an increase in the number of epithelial goblet cells in the large bronchi and these extended down into the bronchioles where they are not normally found. Sulphomucin was still the predominant type of mucosubstance but small amounts of sialomucin were also found, equally distributed between sialidase sensitive and sialidase resistant. An increase

in the number of gland tubules was found in some calves with pneumonia and there was an increase in the production of sulphomucins and sialomucins.

Experimentally isoprenaline, a sympathomimetic amine, has been found to cause an increase in the number of goblet cells in the rat bronchial epithelium (Sturgess and Reid 1973), this increase affecting the cells producing acid mucosubstances causing a relative increase in the production of acidic mucosubstances. Their number also extended down to the bronchioles (Jeffery 1973). Similar findings have been reported in the pig (Baskerville 1976). There is also mucous gland hypertrophy in the pig and a shift towards the production of sialomucins. Previously it was thought that the secretion from tracheobronchial mucous glands was under nervous control and that goblet cell number was influenced by irritative factors (Florey, Carleton and Wells 1932). These results imply that the number of goblet cells may also be altered by various sympathetic stimuli. Many different irritant factors have been found to cause goblet cell hyperplasia - intra-tracheal formalin in cats (Florey, Carleton and Wells 1932) sulphur dioxide in rats (Reid 1963; Mawdesley-Thomas, Healey and Barry 1971), tobacco smoke in rats (Lamb and Reid 1969) and sulphur dioxide in dogs (Chakrin and Saunders 1974). It is therefore possible that a number of external factors may influence the amount of secretory tissue and type of mucins secreted, some acting directly and some via a nervous stimulus.

Classification of the mucosubstances

(after Spicer, Leppi and Stoward 1965)

1. NEUTRAL MUCOSUBSTANCES

Neutral glycoproteins, immunologically reactive glycoproteins,
Fucomucins, mannose-rich mucosubstances

All stain red with PAS stain.

2. ACID MUCOSUBSTANCES

A. SULPHATED

a) Connective tissue mucopolysaccharides (periodate unreactive)

i) resistant to hyaluronidase

ii) susceptible to hyaluronidase

b) Epithelial sulphomucins (hyaluronidase resistant)

i) periodate unreactive

ii) periodate reactive

B. NONSULPHATED

a) Hexuronic acid-rich mucopolysaccharides e.g. hyaluronic
acid.

b) Sialic acid-rich mucosubstances

i) Connective tissue mucopolysaccharides containing
sialic acid

ii) Epithelial sialomucins

1) Highly susceptible to Vibrio cholerae sialidase
periodate reactive and metachromatic

2) Slowly digestible with Vibrio cholerae sialidase

3) Resistant to Vibrio cholerae sialidase

- metachromatic and susceptible following
saponification

- sialidase resistant after saponification

Table 9. Classification of mucosubstances.

TRACHEOBRONCHIAL MUCUS-SECRETING CELLS

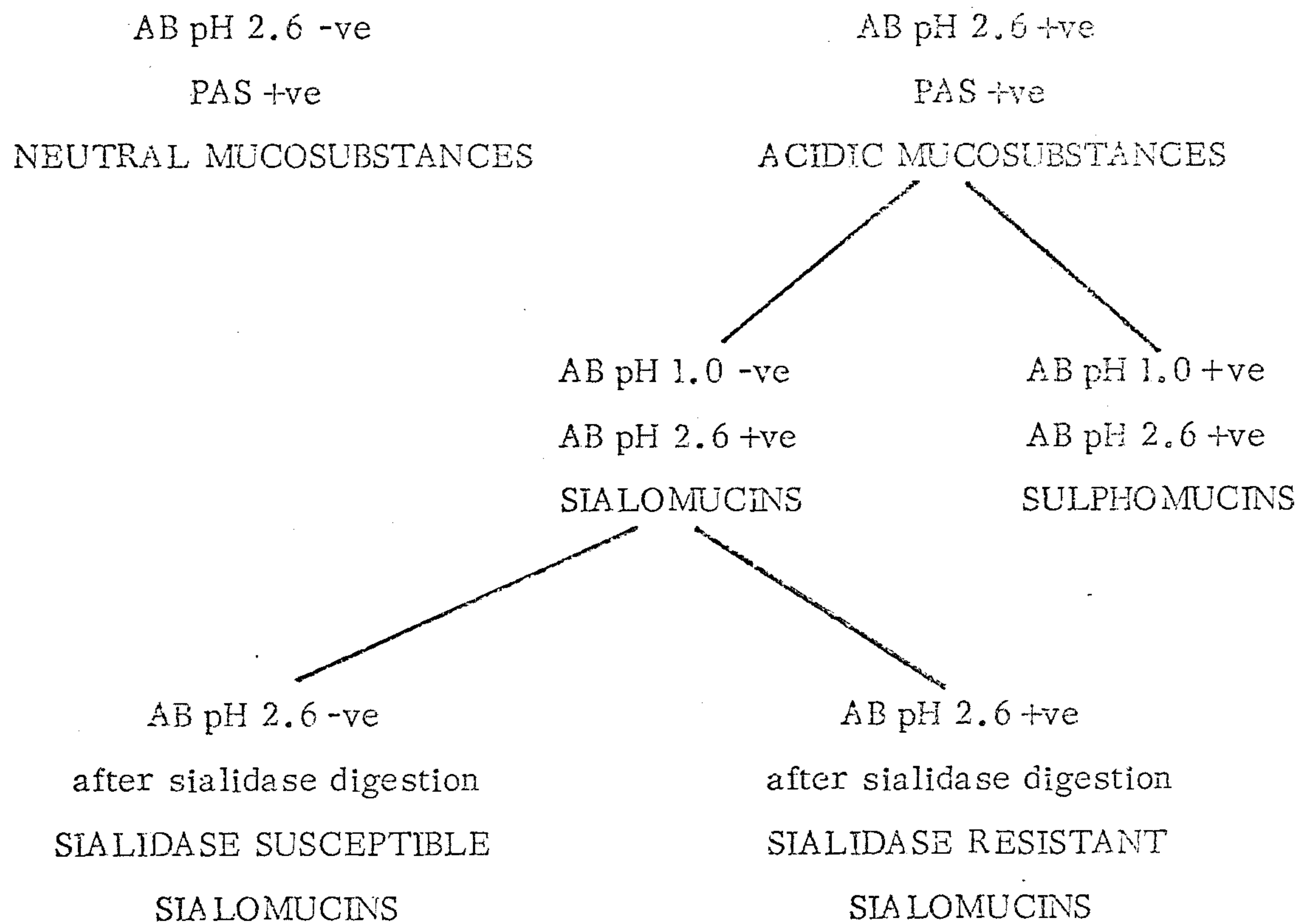


Table 10. Histochemical method of classifying the tracheobronchial mucosubstances.

MATERIALS AND METHODS

Animals

Samples were taken from ten horses with neither clinical signs nor recent history of respiratory disease. These horses were of varying types and ages but as the diseased horses were in the older age range there was a tendency to choose controls that were five years old or above. Samples were also taken from the series of twenty five horses with CB previously described.

Post-mortem technique

Blocks fixed in ten per cent. formol saline from the eight sites shown in Figure 7 were used for this study. Necropsy technique and the method of processing these blocks have been described in the chapter on pathology. As far as possible the same sites were sampled in the control horses.

Histochemical methods

In addition to the standard technique of haematoxylin and eosin, 6 - 8 μ sections were stained by the following methods to demonstrate mucosubstances. Two serial sections were cut from each block and stained using the combined AB-PAS stain of Mowry and Winkler (1956) in the following sequence: 1. AB pH 2.6 - PAS 2. AB pH 1.0 - PAS. The first stain differentiated acid and neutral mucosubstances, acid mucosubstances staining blue with AB and neutral mucosubstances staining red with the PAS (Mowry 1956). The second stain combination further classified the acid mucosubstances, those staining blue with AB were sulphomucins (Jones and Reid 1973a), sialomucins stain red with PAS at this pH. Neutral mucosubstances stain red with PAS at both pH 2.6 and pH 1.0. In certain cases three serial sections were cut the third one being pre-treated with neuraminidase (sialidase, receptor destroying enzyme RDE, Burroughs - Wellcome Ltd; Beckenham, Kent) before staining with AB pH 2.6-PAS to localise sialidase sensitive sialomucins (McCarthy and Reid 1964). Other sections were stained by the HID-AB method of Spicer and others (1971); HID stains sulphomucins brown or black, AB counterstains sialomucins. Serial sections were stained with HID-AB pH 2.6 and HID-AB pH 1.0. This

helped to further characterise the different sulphomucins into those three different types recognised by Jones and Reid (1973b) in the human tracheo-bronchial submucosal gland. The first of these stained with AB at pH 2.6, at pH 1.0 and with HID; the second with AB at pH 2.6, at pH 1.0 but not with HID; and the third one only with AB at pH 1.0.

Sections were also treated with PAS alone, before and after diastase, to identify glycogen.

Four sections representative of the larger lobar bronchi - cranial lobar bronchus of cranial lobe, first ventral lobar bronchus of caudal lobe, mid-caudal bronchus and fourth ventral lobar bronchus of caudal lobe, sites 2, 4, 6 and 8 on Figure 7, were selected from each of five control and five diseased horses. The goblet cells along the entire circumference of bronchus on the slide were counted and classified as staining either red or blue with AB pH 2.6 - PAS and with AB pH 1.0 - PAS two serial sections being used for this. The length of the bronchial epithelium was measured by projecting the slide onto a board and measuring the circumference with a map measure. With the projector a constant distance from the board it was possible to calculate the number of goblet cells present along a one centimetre length of bronchus.

At pH 2.6 those staining predominantly red were said to contain neutral mucosubstances and those staining blue acid mucosubstances. At pH 1.0 red staining cells were considered to contain a mixture of neutral and sialic mucosubstances and those staining blue to contain solely sulphomucins.

Mucosubstances in the tracheobronchial mucous glands were assessed as far as possible in the same way.

Details of the staining methods are given in Appendix 1.

RESULTS

Using these histochemical techniques, it was possible to identify the mucosubstances present in the tracheobronchial system of normal horses and also to demonstrate that there were no significant changes in the proportions or amounts of these mucosubstances in the main airways of horses with CB.

Normal horses

Goblet cells in the trachea and bronchi of normal horses occurred more frequently in the epithelial crypts and in some areas were absent from the upper parts of the epithelium (Figure 67). With a few exceptions the goblet cells had a marked alcianophilia with both AB pH 2.6 - PAS and with AB pH 1.0 - PAS indicating a large proportion of sulphomucin (Figure 68). In some cases a large number of red-staining goblet cells were found at pH 2.6 but in the serial section at pH 1.0 these became alcianophilic (Figures 69 and 70). This particular type of sulphomucin was found in isolated loci in both normal horses and horses with CB. Neutral mucosubstances, staining red with PAS at pH 2.6 and pH 1.0 were less commonly found and were mostly confined to isolated groups of goblet cells. Single cells that were stained with PAS alone were uncommon; there was almost always an additional alcianophilic component, indicating a mixed mucosubstance content (Figure 71). The PAS positive portion of the cell was often at the apex or could be seen being extruded into the lumen.

Sialomucins would seem to be infrequent in most horses. Using the HID-AB stain all the goblet cells stained dark brown at both pH 2.6 and pH 1.0 (Figure 72). The submucosal glands sometimes showed an alcianophilic component but this was also present at pH 1.0 indicating that it was a different type of sulphomucin not sialomucin (Figure 73). Similarly there was very little loss of alcianophilia in the sections stained with AB pH 2.6 - PAS as compared to those stained with AB pH 1.0 - PAS. Consequently prior digestion with neuraminidase appeared to have no effect on the alcianophilia of either the goblet cells or the mucous glands. However if sialomucins are occurring in combination with sulphomucins in the cells the presence of the sulphomucins may mask the sialomucins using these staining techniques. This possibility cannot be entirely excluded.

Submucosal mucous glands in the normal horses were sparse (Figure 68) and often were not seen in sections of bronchi. Some acini contained few or no

mucus-secreting cells and free mucus was very seldom present in the acinar lumina. The mucins again seemed to be almost entirely sulphomucins. It was impossible to count sufficient acinar mucus-secreting cells in every animal as the numbers were so small but as a whole the proportions were sulphomucins 88 per cent; sialomucins three per cent. and neutral mucosubstances nine per cent.

Horses with CB

Mucosubstances in the trachea or bronchi of horses with CB did not differ significantly from the pattern found in normal horses, either qualitatively or quantitatively. Table 11 gives the results obtained on the four major bronchi of five normal horses and five horses with CB. Using the analysis of variance test (Goldstein 1964) there was no significant difference either between the number of goblet cells per centimetre or of their composition in the two groups ($f = 0.21$). In the two individual groups the number and composition between the four sites and the five horses also did not vary significantly (f site = 0.3 and 0.24; f horse = 1.68 and 2.8) (Figures 78 and 79).

No appreciable increase in the number and volume of mucous glands in horses with CB was found although this could not be assessed morphometrically.

In normal horses, epithelial goblet cells were rarely found in smaller bronchi and never found in bronchioles. Submucosal glands were absent from these sites (Figure 74). In horses with CB there was goblet cell hyperplasia in the small bronchi and extension of goblet cells into the bronchioles (Figure 75). The airways were sometimes plugged with mucus which presumably had its origin in these newly formed goblet cells (Figure 76). The dramatic changes in the bronchioles have already been described in the chapter on pathological changes. The epithelium was columnar and hyperplastic and the epithelial cells comprised mainly goblet cells. These goblet cells were strongly alcianophilic at pH 2.6 and at pH 1.0 and stained with HID at pH 2.6 and at pH 1.0 indicating a high content of sulphomucin (Figure 77). No sialomucin-containing cells, as determined by selective pH staining and HID-AB pH 2.6, were found in the bronchioles. There was also no loss of alcianophilia following neuraminidase digestion.

DISCUSSION

In chronic bronchitis of man (Reid 1965) and the dog (Wheeldon, Pirie and Breeze 1976) there is a detectable shift in the type of secretion produced by the tracheobronchial mucus-secreting apparatus so that sialomucins are the predominant mucosubstance secreted. A similar change has been illustrated in mycoplasma infection in pigs (Jones, Baskerville and Reid 1975) and calves (Allan, Pirie and Wheeldon 1977) and in Bordetella bronchiseptica infection in dogs (Wheeldon and others to be published). In each case, significant lesions are found in the larger airways and it seems likely that the histochemical changes in mucus are a reflection of insult to the tracheobronchial mucosa. In the horses with chronic bronchiolitis there was no shift to production of sialomucins by the tracheobronchial epithelium, nor any increase in the number of goblet cells. There was no excess of mucus in the large airways and hypertrophy of the tracheobronchial submucosal glands, the sine qua non of chronic bronchitis, was not found. In both normal horses and horses with CB mucous glands are small, sparse and irregularly distributed. It therefore seems likely that most of the mucus covering the tracheobronchial tree has its origin in the epithelial goblet cells. This is certainly true of the mucus found in the bronchioles in horses with CB as there are no mucous glands at all in these airways.

In the normal horse most of the epithelial goblet cells contained sulphomucin. Neutral mucosubstances were found in much smaller amounts and sialomucins were very infrequent. The sulphomucin was of two kinds. The predominant one corresponded to the first type described by Jones and Reid (1973b) and stained with AB at pH 2.6, at pH 1.0 and with HID at pH 2.6 and pH 1.0. The second type corresponded to the third variation described by Jones and Reid (1973b) and stained only with AB at pH 1.0. Jones and Reid (1973b) described a further variety of sulphomucin which stained with AB at pH 2.6 and at pH 1.0 but not with HID. This did not seem to occur in the epithelial goblet cells but was found in the bronchial mucous glands. The mucous glands then contained the three varieties of sulphomucin and these occupied the majority of the glandular acini; neutral mucosubstances and sialomucins were very infrequent.

The significance of the different types of mucin is not clear. Most domestic animals and man seem to have a preponderance of sulphomucins in the tracheobronchial mucosa. In certain disease states this changes so that there is a shift to production of sialomucins (vide supra). Gibbons (1959) using bovine cervical mucus collected at different stages in the oestrus cycle has demonstrated that variations in the amount of sialic acid can be related to changes in the internal viscosity of the mucus. Thus an increase in production of sialic acid in tracheobronchial disease could indicate that the mucus being secreted is more viscous than normal. Certainly chronic bronchitis in man and dog is associated with hypersecretion of a very viscid mucus (Reid 1965; Wheeldon 1974). The increased viscosity could be an attempt to offer increased protection to the injured epithelium. However many other factors may be involved in determining the viscosity of mucus such as, transudation from the capillary bed, ion content, protein content, even IgA content as IgA itself contains a "mucus-like" stretch in the hinge region which may act as a local concentration mechanism (Clamp 1977). No shift to sialomucins was found in the horses with CB, the mucus secreted being sulphomucin as it was in the normal horse. There is therefore only a quantitative change, as far as can be determined by histochemical methods, and this extra volume is secreted by a new population of epithelial goblet cells in the bronchioles. The functional significance of this has already been discussed.

There is no evidence histochemically of a bronchitic condition in these horses with chronic pulmonary disease due to chronic bronchiolitis and the disease lies solely in the small airways. The excess mucus secreted by the new population of goblet cells in the peripheral airways qualitatively resembles normal equine mucus.

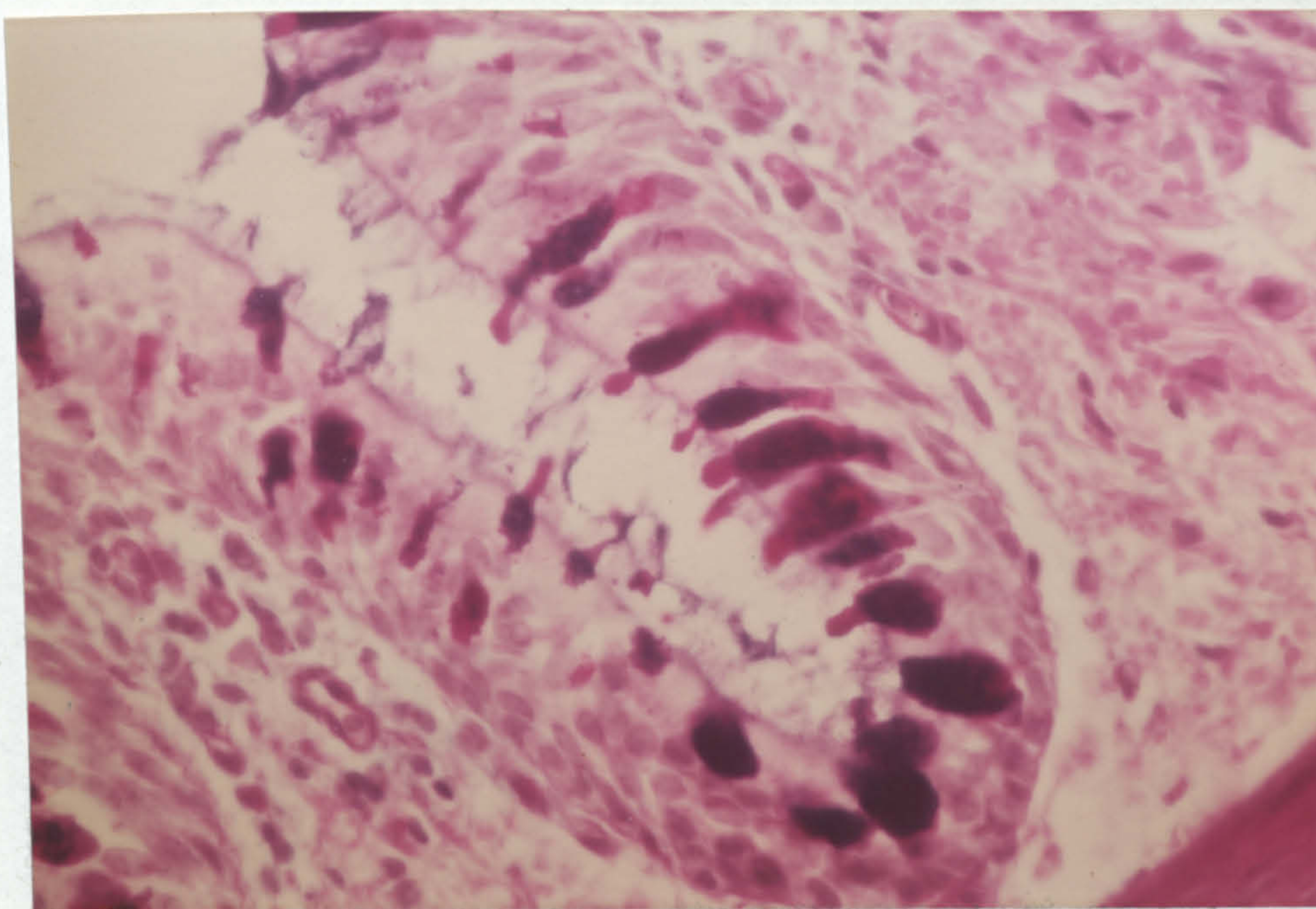


Figure 67. Section of bronchial wall stained with AB-PAS showing larger more numerous goblet cells further down the crypt. X 400.

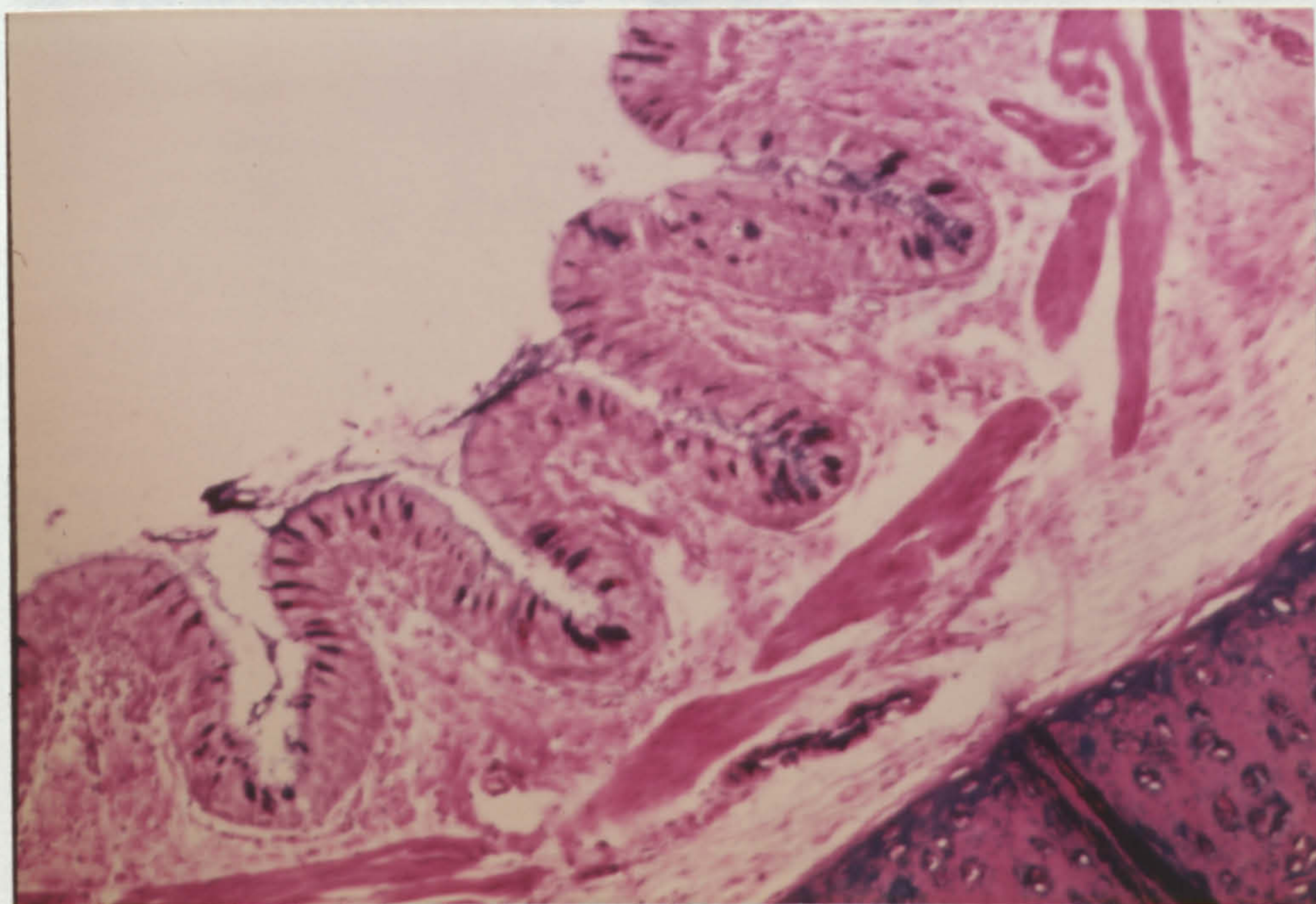


Figure 68. Section of bronchial wall stained with AB pH 1.0 - PAS. Most of the goblet cells have stained dark blue showing a high content of sulphomucin. X 110.

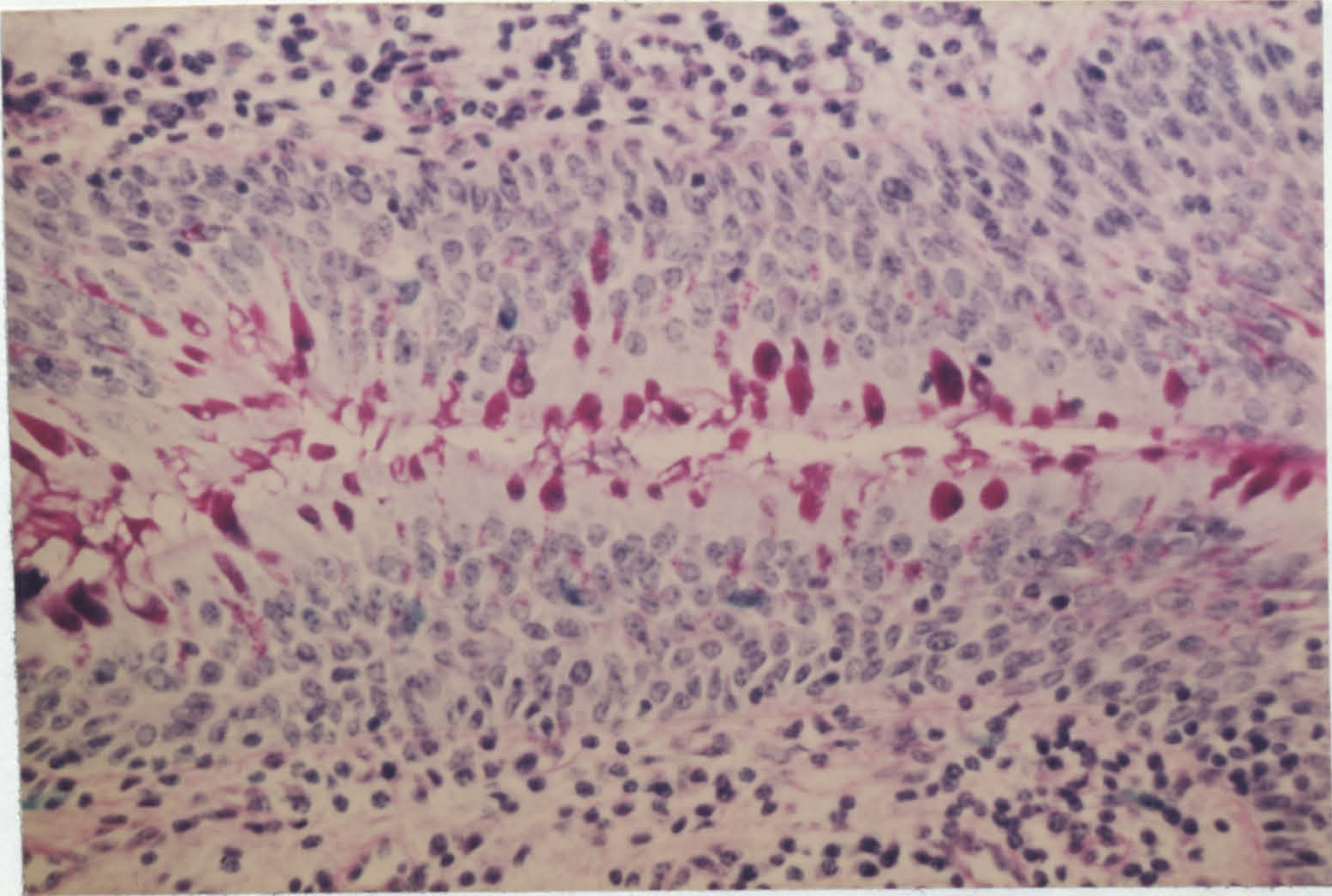


Figure 69, Section of bronchial wall stained with AB pH 2.6 - PAS. The goblet cells stain red with the PAS. X 250.

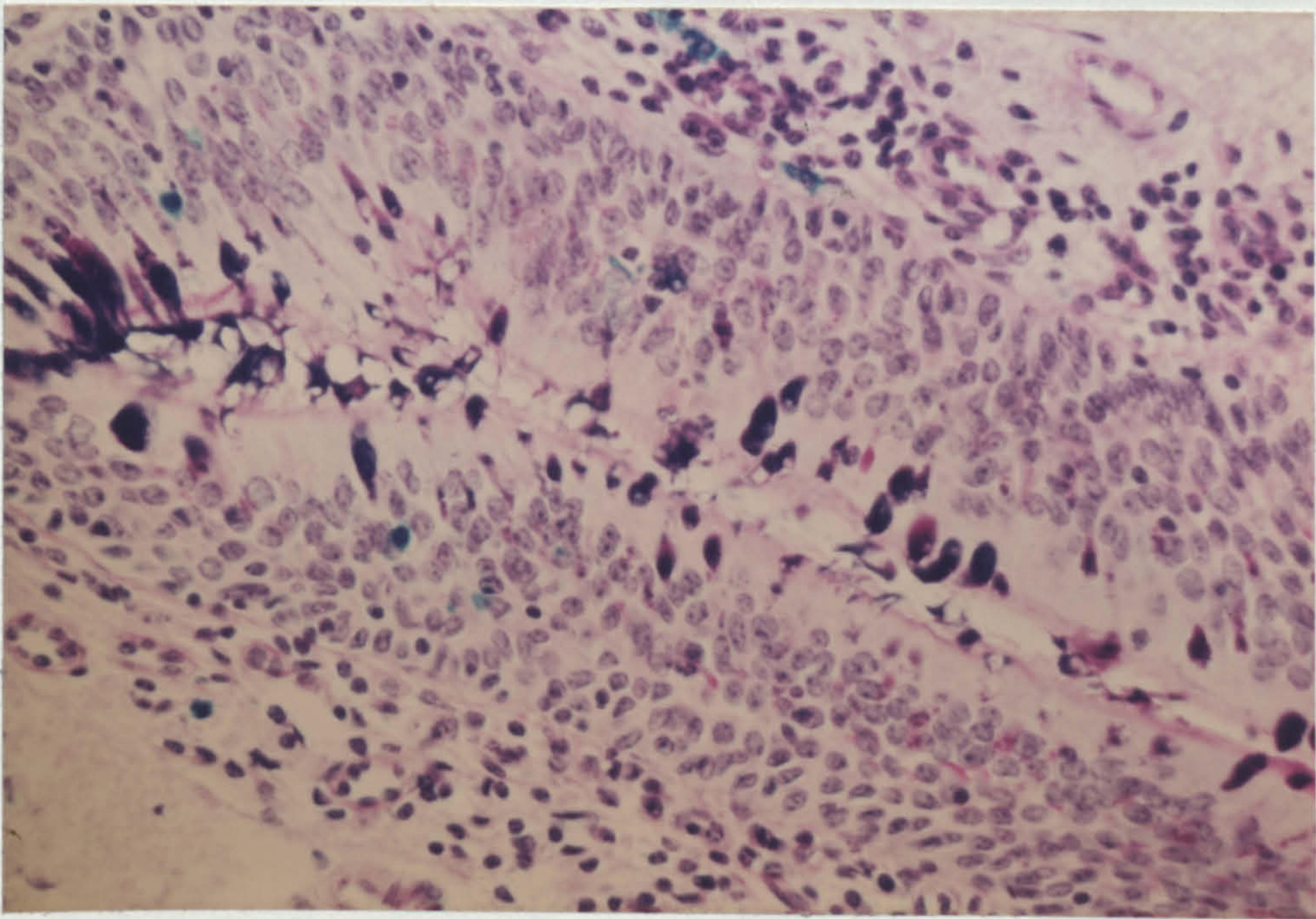


Figure 70, Section of bronchial wall adjacent to above section stained with AB pH 1.0 - PAS. The goblet cells stain blue with alcian blue indicating that they contain sulphomucin. X 250.

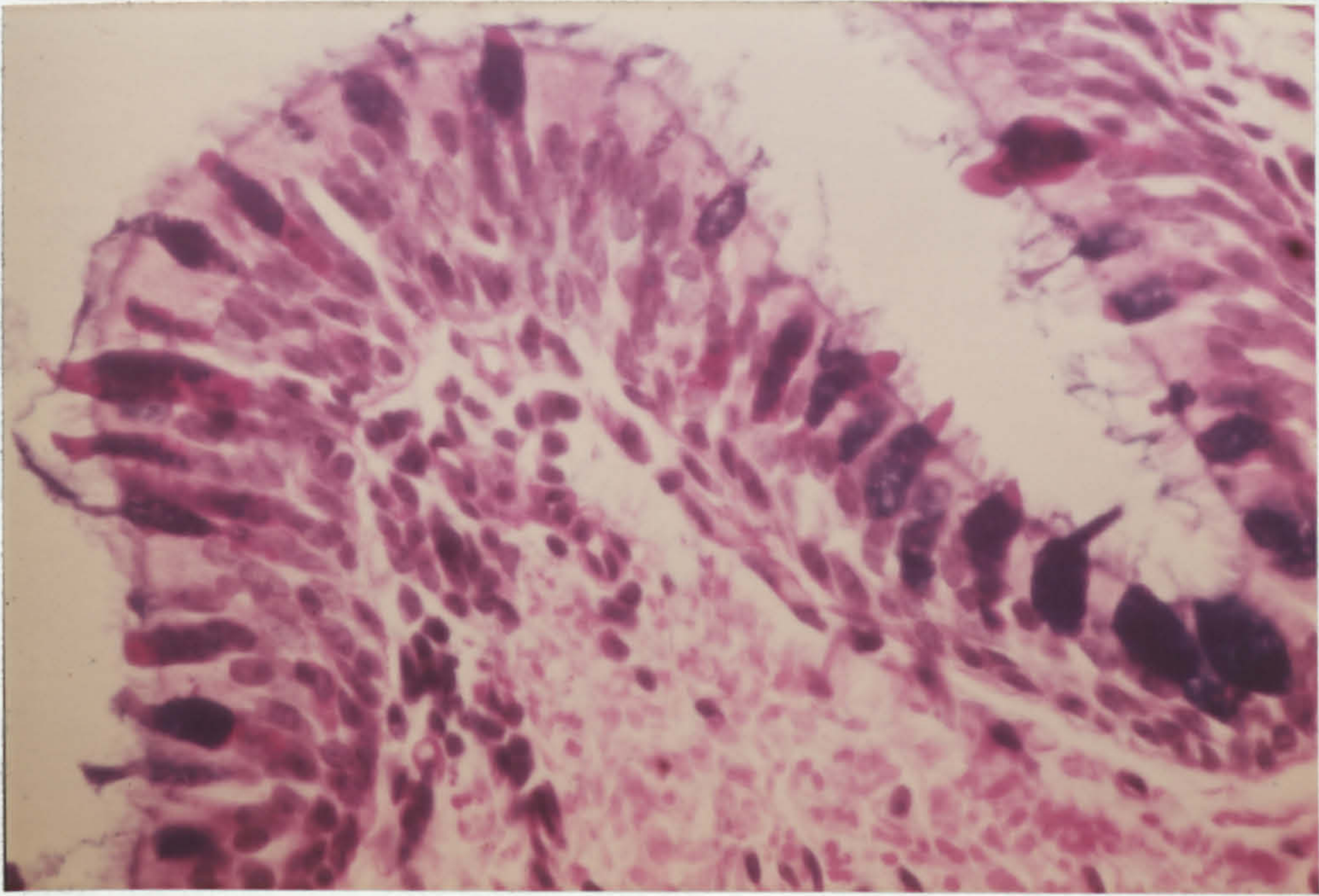


Figure 71. Section of bronchial wall stained with AB pH 2.6 - PAS. The goblet cells show a mixed reaction to the stain indicating a variety of mucosubstances in each cell. The extruded portion is usually PAS positive. X 400.

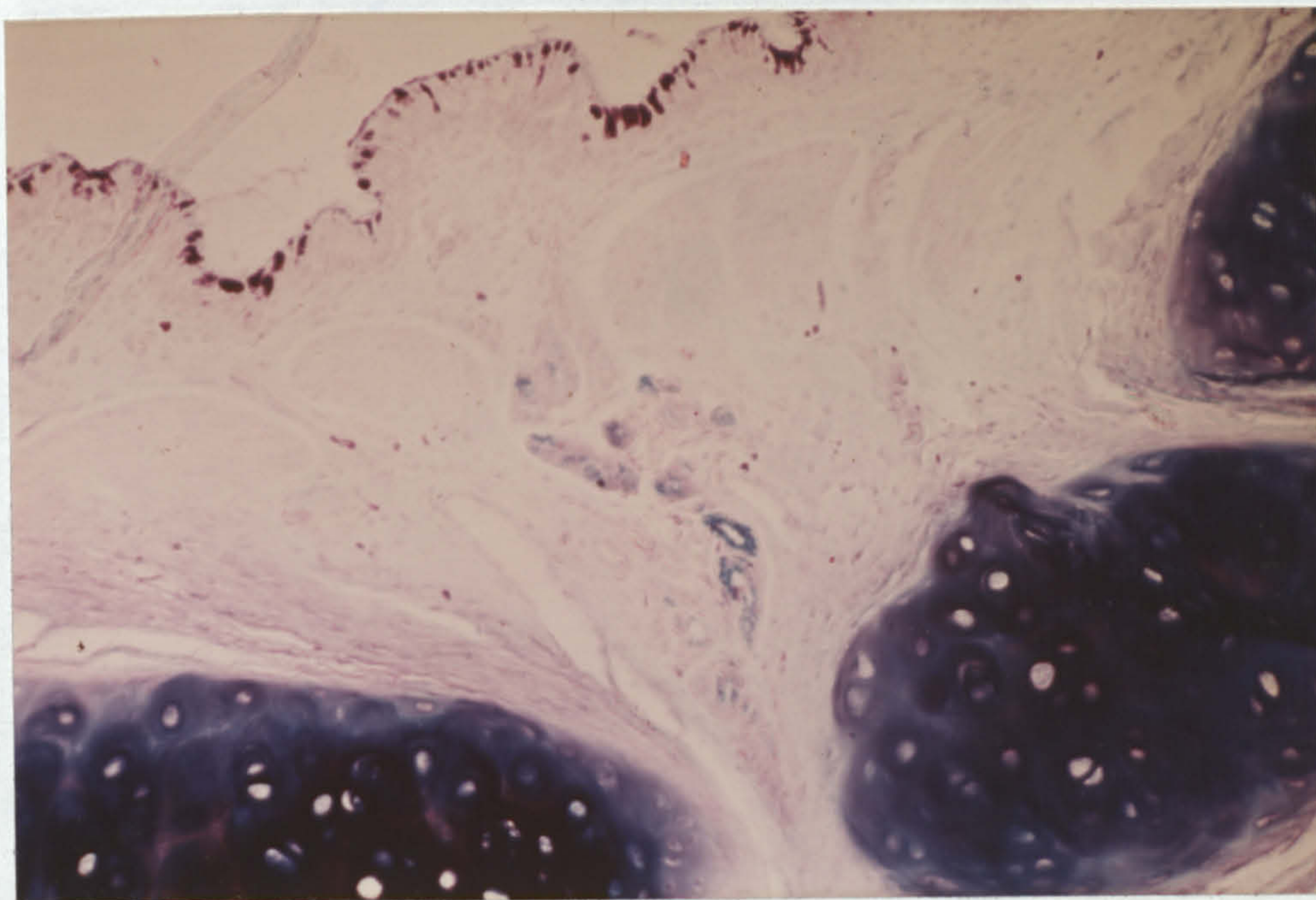


Figure 72. Section of bronchial wall stained with HID-AB. The goblet cells stain dark brown with HID indicating a high content of sulphomucin. The submucosal gland has not taken up much stain but some acini seem to contain sialomucin as they stain blue with AB. X 35.

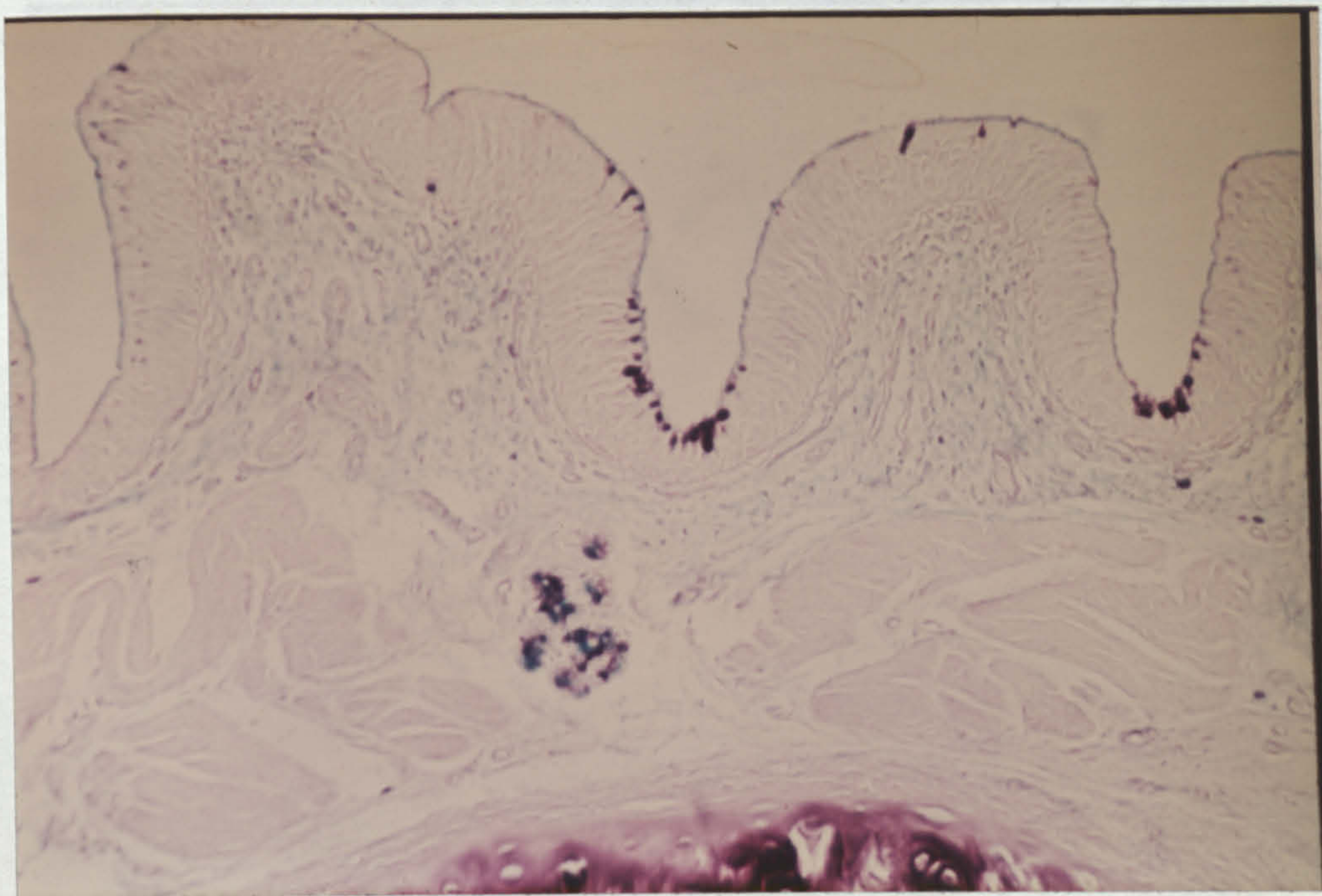


Figure 73. Section of bronchial wall stained with HID-AB. The submucosal gland has blue and brown components indicating it contains both sialomucin and sulphomucin. X 110.

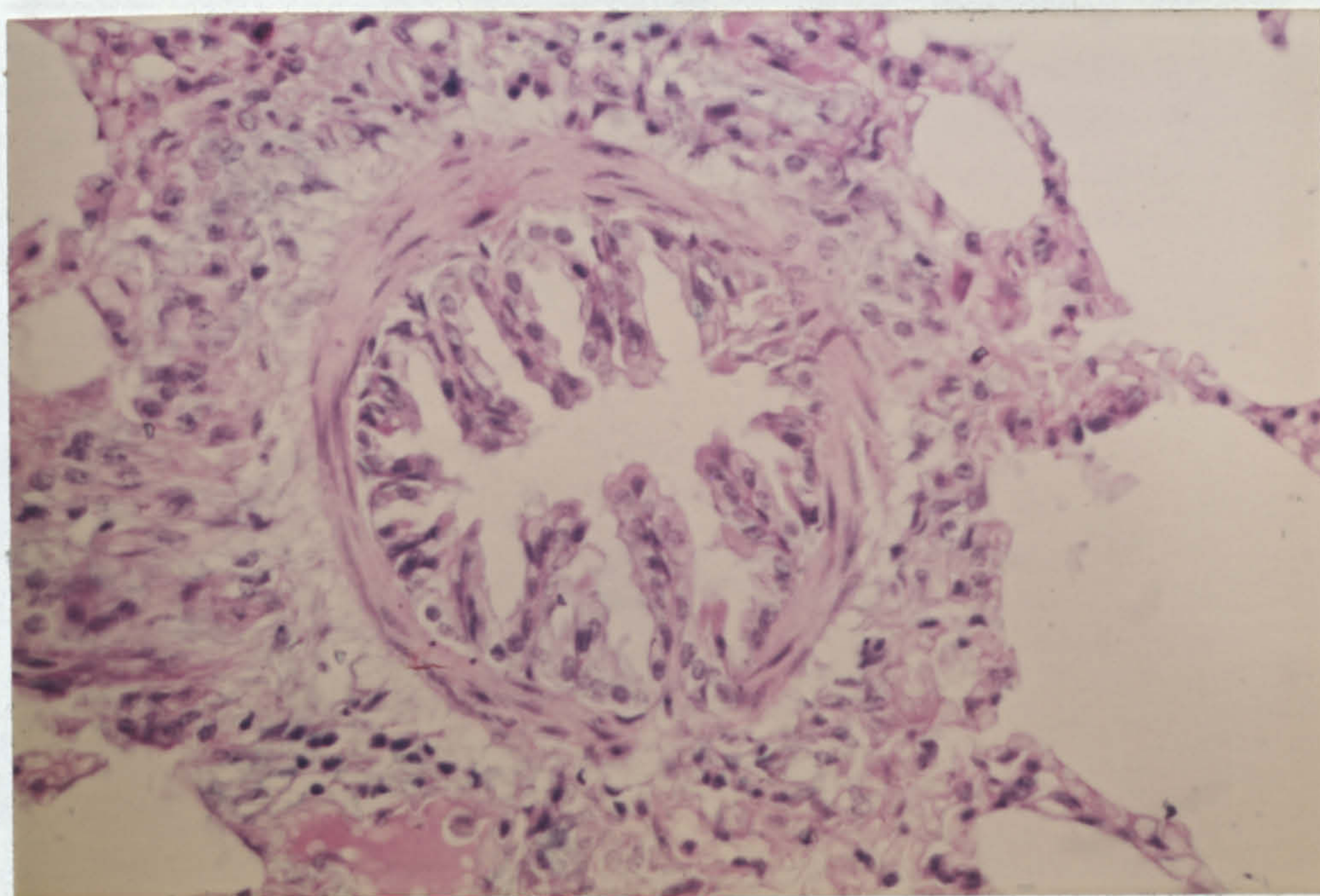


Figure 74. Section of a bronchiole from a normal horse stained with AB-PAS. There are no goblet cells or submucosal glands present. X 250.

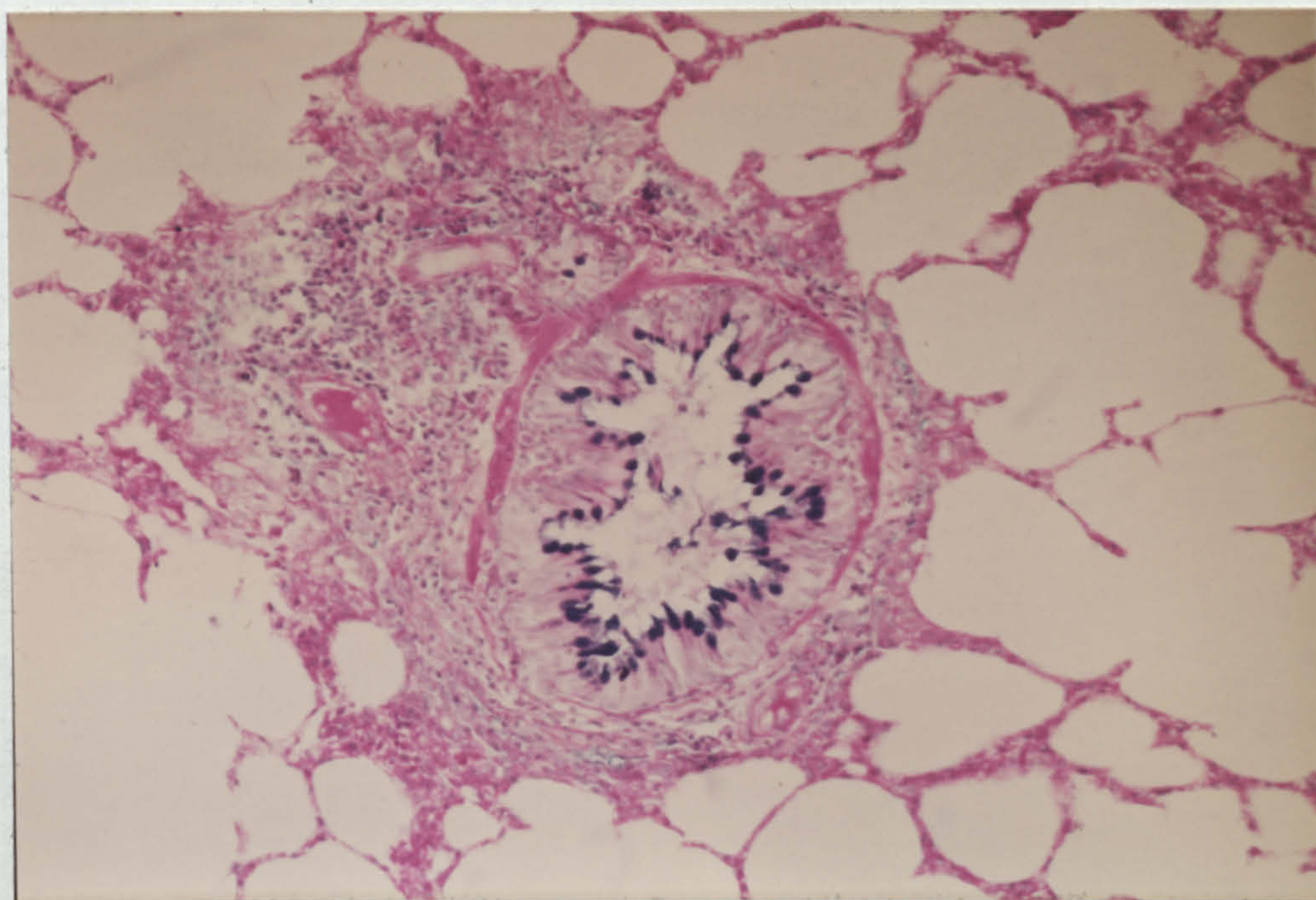


Figure 75. Section of a bronchiole from a horse with CPD. Goblet cells are present in the hypertrophied epithelium. X 110.

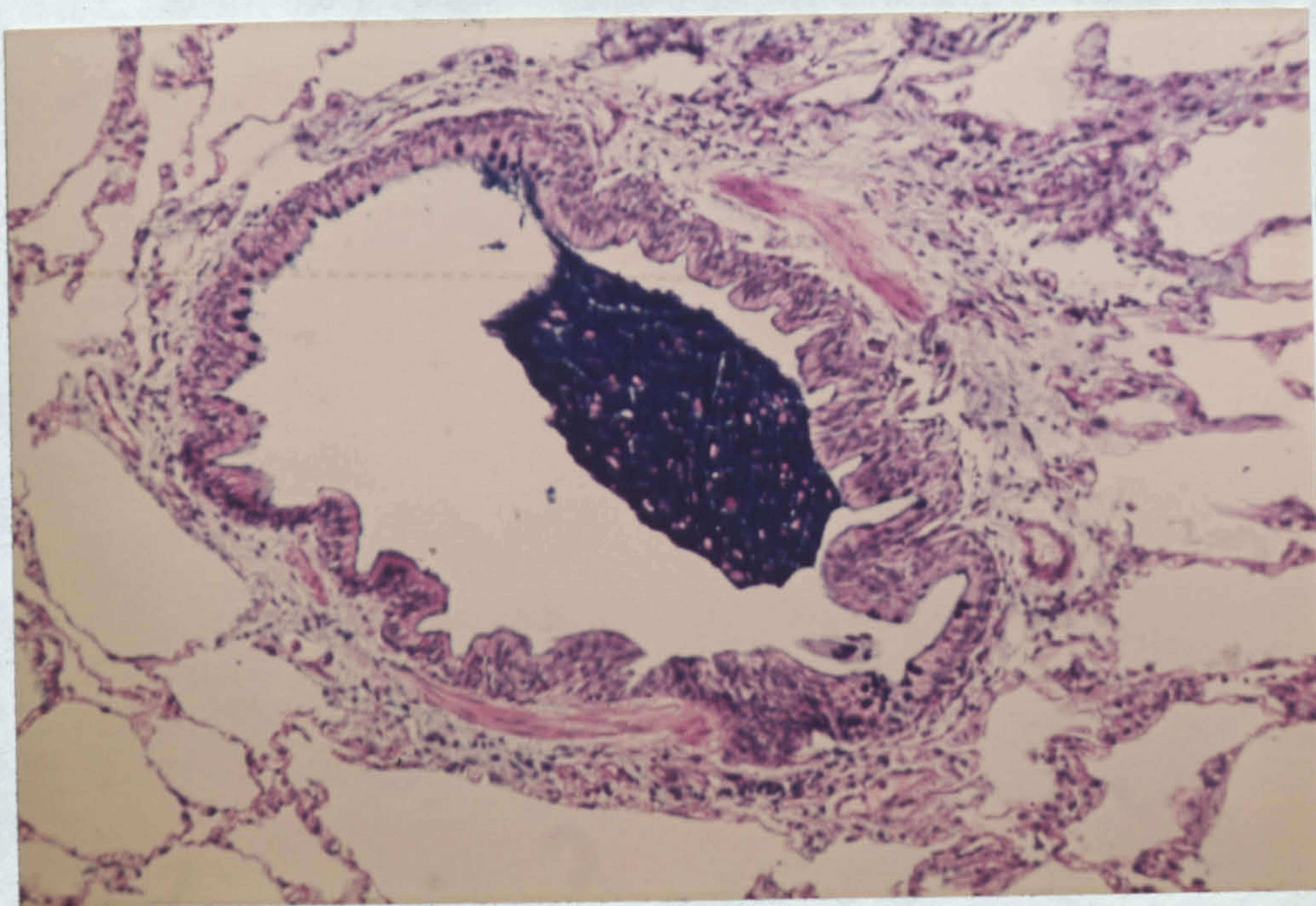


Figure 76. Section of a bronchiole from a horse with CPD. There is mucus in the centre of the lumen. X 110.

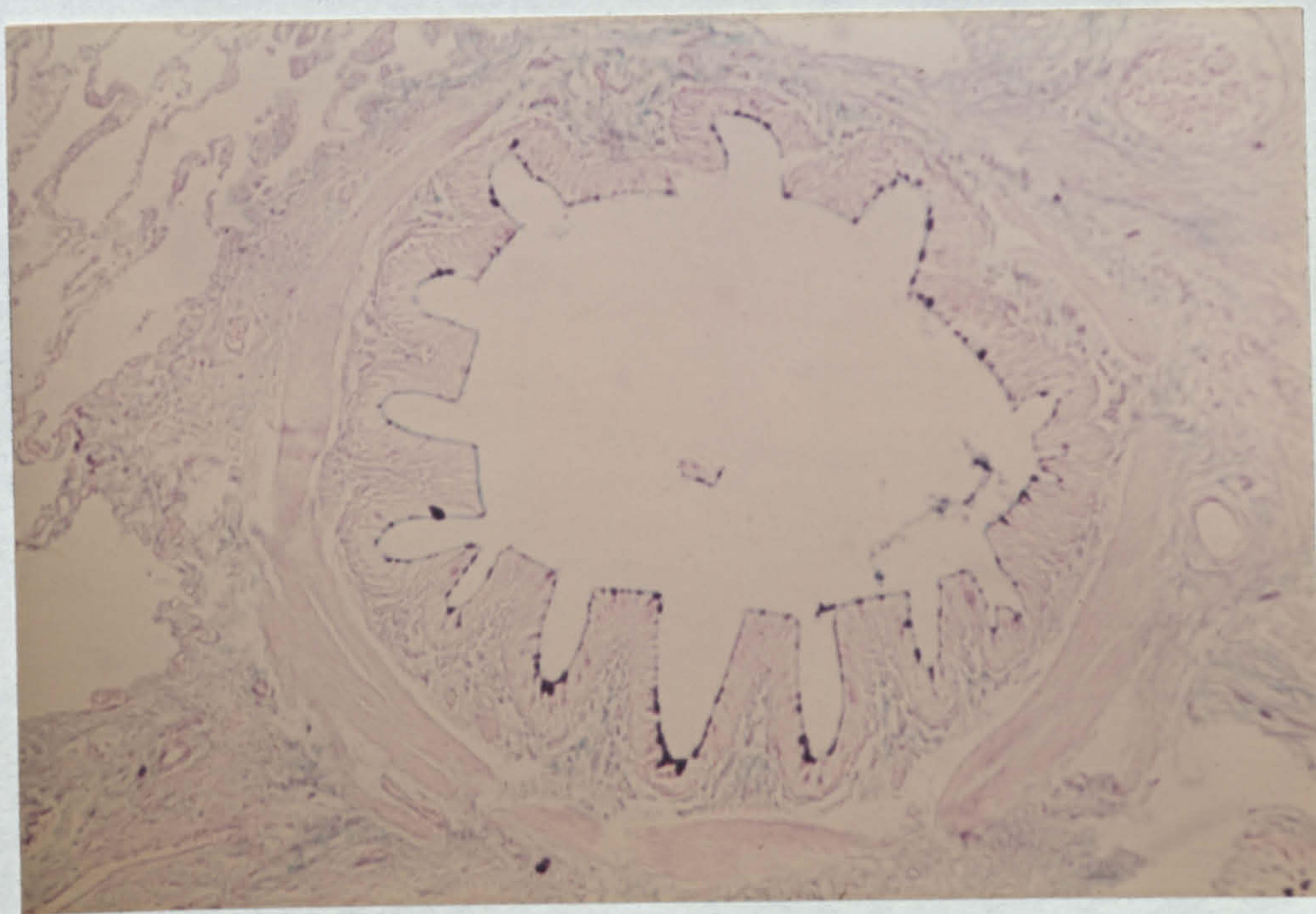


Figure 77. Section of a bronchiole from a horse with CPD stained with HID-AB. The goblet cells stain dark brown indicating the presence of sulphomucin. X 110.

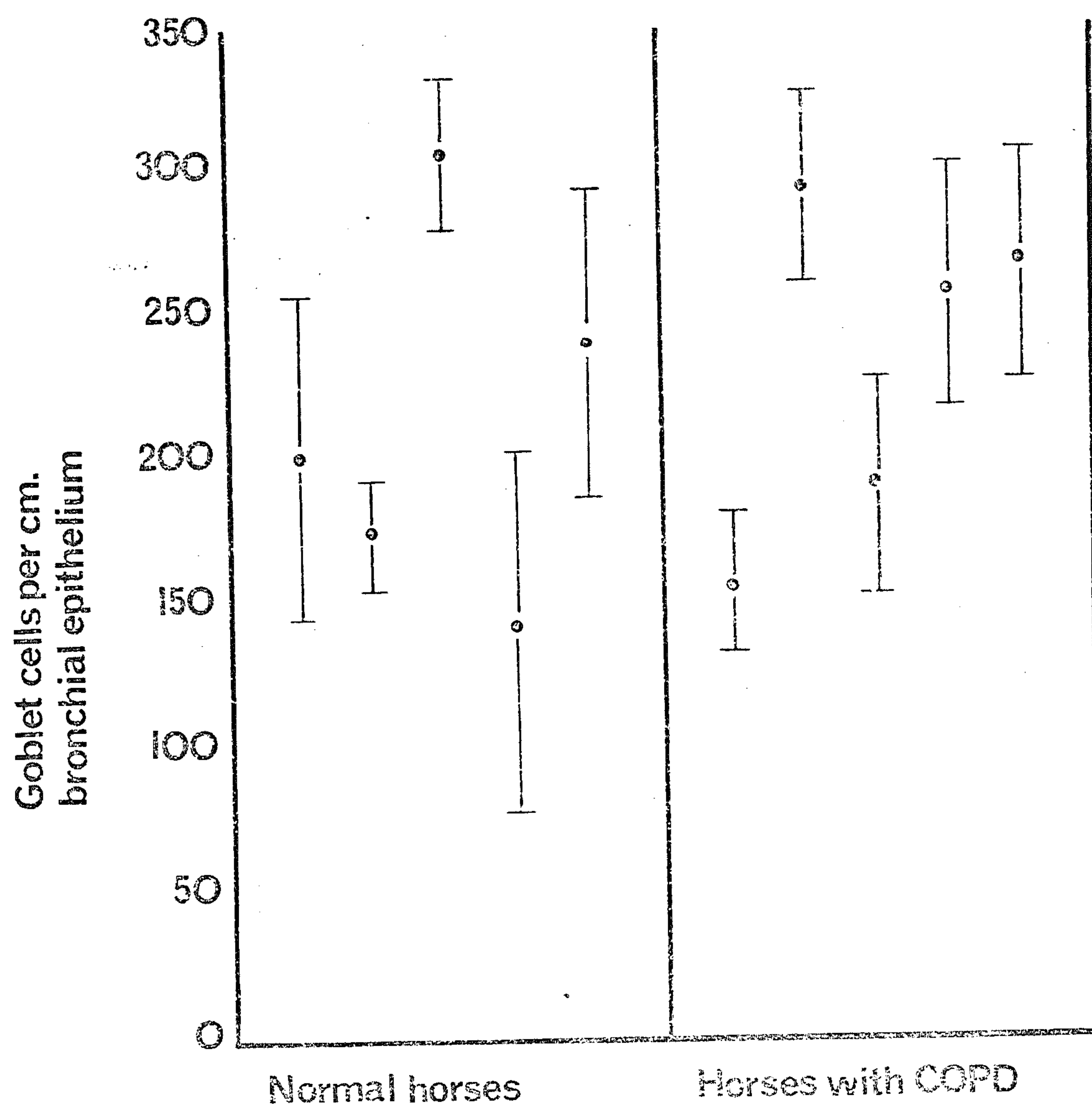


Figure 78. Number of goblet cells per centimetre of bronchial epithelium in five horses with no respiratory abnormality and five horses with CB.
 \pm standard deviation.

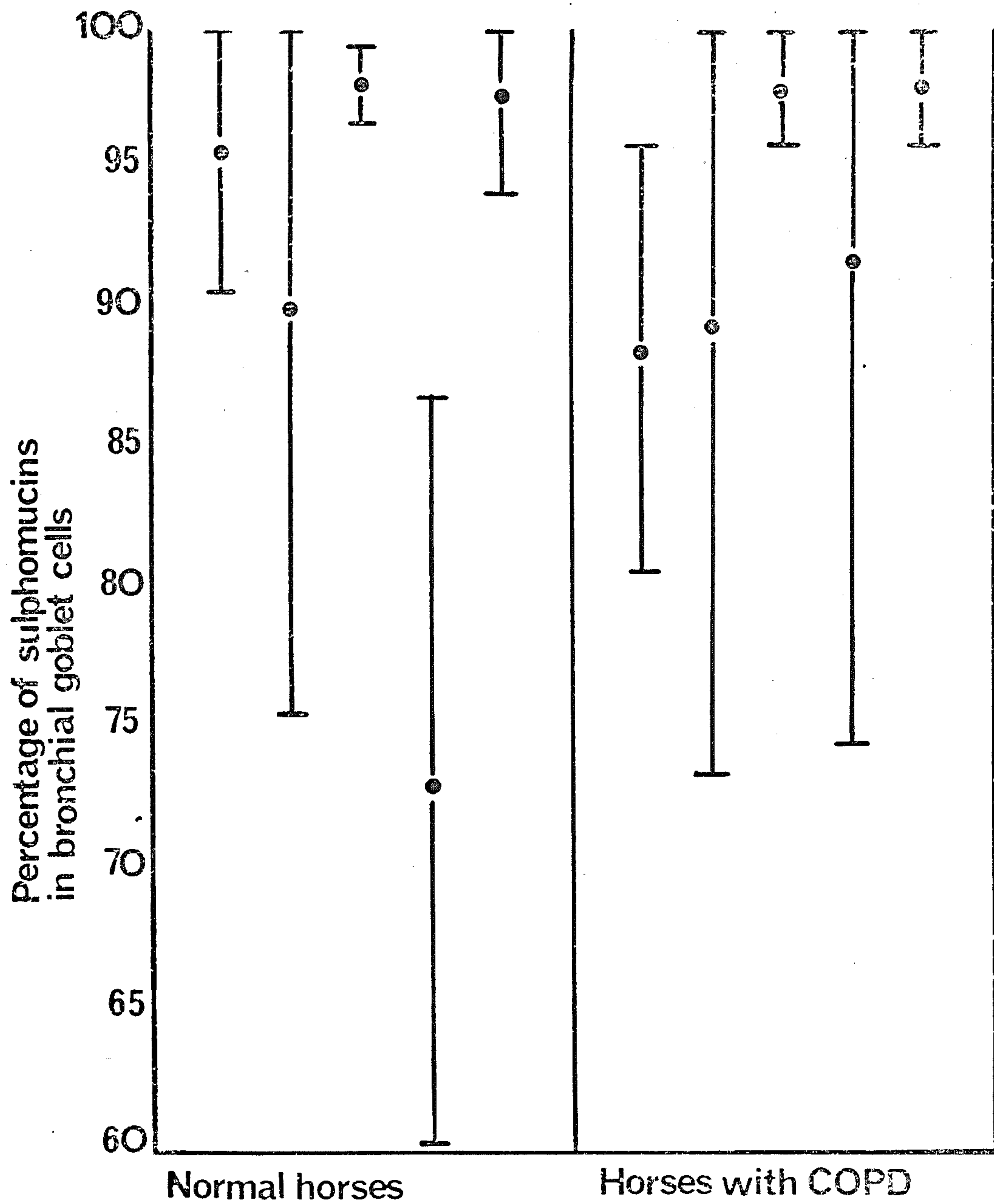


Figure 79. Percentage of chiefly sulphomucin containing cells in the bronchial epithelial goblet cells of five horses with no respiratory abnormality and of five horses with CB \pm standard deviation.

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CHAPTER FIVE

HISTOCHEMISTRY AND QUANTIFICATION OF PULMONARY MAST CELLS IN NORMAL HORSES AND IN HORSES WITH CHRONIC BRONCHIOLITIS

INTRODUCTION

The mast cell, possibly because of its unique and distinctive histochemical features is a cell that has been the subject of many investigations. However despite the large volume of work published on the subject and its well-documented association with certain disease conditions neither its origin nor its precise function are known.

Previously most investigations of mast cell distribution and action centred around the mast cells of the gastro-intestinal tract. Because of their content of potent vasoactive amines the potential role of mast cells in the pulmonary system is an important one and the pulmonary mast cell has been the subject of more recent investigations.

This study of the pulmonary mast cells in the horse was undertaken for three main reasons:

1. to establish by histochemical means the nature of the equine pulmonary mast cell,
2. to see whether mast cell hyperplasia occurred in the lungs of horses with chronic bronchiolitis and if so at what sites this was found,
3. to compare the findings in the horses with CB with those of other investigations in other species especially with regard to allergic responses and the development of pulmonary hypertension.

REVIEW OF THE LITERATURE

The feature which distinguishes mast cells from other connective tissue cells is the presence of many uniform, dense, spherical metachromatic granules in the cytoplasm. The granular content is often such that the cytoplasm appears to contain little else and this was the origin of the term "mast" cell from the German meaning "well-filled". The granules stain metachromatically with certain dyes - notably the thiazine derivatives. A metachromatic reaction is one in which the substance stains a different colour from that of the dye used in the stain. Mast cells assume many different shapes, possibly dependent upon their location, being round, spindle-shaped or even stellate. The nucleus is often obscured by the granules but generally it is rounded or ovoid, often with a slight indentation; there is margined chromatin but no nucleolus. Small microvilli are often seen projecting from the cell surface (Miller, Murray and Jarrett 1967; Saini and Breipohl 1977; Ts'ao and others

1977) but apart from this there does not appear to be any direct connection with other connective tissue elements.

Under the electron microscope the cytoplasmic granules are generally of uniform shape, size and electron density although this can be influenced by fixation. There is considerable variation in granule size. They are 0.7 μm diameter in the rat, 0.9 μm in the cow (Miller, Murray and Jarrett 1967), 0.25 - 0.5 μm in rhesus monkeys (Saini and Breipohl 1977), 0.3 - 1.1 μm in man (Barnett 1973) and 0.6 μm in the cat (Ward and Hurvitz 1972). Granules are bound by a smooth-surfaced trilaminar unit membrane (Miller, Murray and Jarrett 1967). Sometimes granules have a "reticulated" appearance (Miller, Murray and Jarrett 1967) or are "granular" or "empty" (Saini and Breipohl 1977). Miller, Murray and Jarrett (1967) suggested that this was either a fixation artefact or reflected actual changes in the granules. Ts'ao and others (1977) found scrolls, crystals and lamellar elements in alveolar mast cell granules of man but not in mast cells from bronchial lavage or in the bronchial mucosa. The possible significance of these morphological variants was not discussed.

The mature mast cell has very few cytoplasmic organelles apart from a small Golgi zone which usually is more obvious in immature cells and in globule leucocytes (Miller 1969). Granules appear to be elaborated from the Golgi zone (Combs 1966; Miller 1969) as are the granules of neutrophils, eosinophils and basophils. During maturation mitochondria are prominent around the Golgi which would imply that the process requires energy (Miller 1969). However once granular maturation is complete, mitochondria become scarce and there is little or no rough-surfaced endoplasmic reticulum present, indicating that the mature cell has a low metabolic rate and secretes very little protein (Benditt 1968). Microfilaments, lipid droplets and small vesicles are also occasionally observed in the cytoplasm (Murray 1968; Barnett 1973; Ts'ao and others 1977).

The globule leucocyte is a distinctive cell found in the epithelium of mucosal surfaces being most apparent and numerous during parasitic infections (Taliaferro and Sarles 1939; Kirkman 1950). For many years the origin of the cell was in dispute because of its unique histochemical and cytochemical features. Then Miller, Murray and Jarrett (1967) demonstrated by cytochemical and ultrastructural means that the globule leucocyte is a mast cell that has

migrated from the submucosa and is in the process of discharging the contents of its granules. The main distinguishing feature of the globule leucocyte is the presence of large eosinophilic granules in the cytoplasm. Under the electron microscope granules are seen to be of varying size and electron density, bounded as are those of mast cell granules by a trilaminar unit membrane (Miller, Murray and Jarrett 1967). These authors found a range of morphology of the globule leucocytes varying from some that were indistinguishable from subepithelial mast cells to others containing very large coarse granules sometimes up to 5 μ m in diameter. More recently, writers have not distinguished between globule leucocytes and mast cells referring to all forms of this cell as mast cells (Barnett 1973; Saini and Breipohl 1977; Ts'ao and others 1977). This is probably because globule leucocytes are not distinguished from intra-epithelial mast cells in man. This is not the case in cattle for example where there is an obvious difference in haematoxylin and eosin sections between globule leucocytes and mast cells.

The basophil bears many of the mast cell's morphological characteristics and is often regarded as its circulatory counterpart. Most mammals have a very small number of basophils and larger numbers of tissue mast cells.

The origin of the mast cell has been a matter of much speculation for some years and the question is still not fully resolved. The circulating basophil, which is morphologically identical to the mast cell, is derived, as are all white blood cells, from the bone marrow.

Mast cells are a stable population and cell division is a rare occurrence (Benditt and Lagunoff 1964). The stimulus for mast cell proliferation is not known but following antigenic stimulation mast cells have been seen to differentiate in a number of lymphoid tissues (Ginsburg and Lagunoff 1967; Miller and Cole 1968). Most authors agree that mast cells differentiate from precursors rather than by multiplication of an existing population, although Miller and Cole (1968) thought that both mechanisms might operate in the lymph node.

Miller (1969) in common with Taliaferro and Sarles (1939) saw mast cells differentiating from precursors in the intestinal epithelium of cats. The precursor cells had a typical blast-like appearance and seemed to be of lymphoid origin. They contained a well-developed endoplasmic reticulum, a large Golgi

zone and numerous mitochondria. Some of the cells were observed in mitosis. Precursor cells were sometimes found in lymphatics and blood vessels, implying their migration into the region.

More recently, using a tritiated thymidine labelling technique Takeoka, Ashihara and Tada (1976) showed that multiplication of mast cell precursors could occur in the subepidermal regions of the skin following painting with a carcinogen and that the newly formed mast cells then migrated into the deeper layers of the skin. Two to three days were thought to be required for the transformation. Their results also suggested that the precursor cells continually multiplied in the subepidermal layer and remained there to be later transformed into mast cells.

Miller (1969) and Takeoka, Ashihara and Tada (1976) both saw precursor cells but did not discuss the origin of these cells. Kitamura and others (1977) have shown that mast cells can be derived from bone marrow cells in irradiated mice. Using marrow cells derived from the Beige mouse, which has giant granules in its granulocytes, they were able to show that most of the mast cells under glandular epithelium, e.g. in stomach and caecum, were derived from the donor bone marrow whereas most of the mast cells under squamous epithelium and in the mesentery were of host origin. Donor-type mast cells took at least 40 days to appear which would imply that a precursor cell was involved in the process. Normal bone marrow contains less than 0.01 per cent. mast cells, which would be too few to repopulate epithelial surfaces. The differences in the populations could be explained in two ways: either mast cells beneath glandular epithelium are of a different type, and there is histochemical evidence to support this, or the turnover under stratified epithelium is different. Presumably differentiation of precursors is under local control and their kinetics vary at different sites.

Burnet (1975) formulated a hypothesis that mast cells might represent a post-mitotic form of T lymphocyte development analagous to the relationship of the mature plasma cell to the B lymphocyte. Essentially, the T lymphocyte liberates pharmacologically active substances after specific antigen stimulation, as indeed do mast cells. Lymphoblastlike cells that develop mast cell granules in situ have been observed in rat intestine (Murray, Jarrett and Jennings 1971). A common antigen has been found on the cell surface of rabbit basophils and thymic lymphocytes but apparently not on mast cells (Day, Singal and

Bienenstock 1975).

At the present time it is fairly well established that mast cells differentiate locally from mesenchymal precursors which are either of lymphoid or bone marrow origin or both. The major question remaining is the nature of the stimulus or stimuli for proliferation and differentiation of such precursors.

The granules have been shown by histochemistry, biochemistry and formaldehyde-induced fluorescence to consist of a matrix of a highly basic cationic protein linked ionically to polyanionic heparin (Benditt 1968). The vasoactive amine histamine is also a major constituent of the granules and is retained within the matrix by ionic bonding (Aborg, Novotny and Uvnas 1967). In the rat and mouse 5-hydroxytryptamine (5HT) is also found (Benditt and Lagunoff 1964). Falck and others (1964) demonstrated the presence of a catecholamine probably dopamine in the mast cells of cattle and sheep. These substances combine with various histochemical dyes to give distinctive staining reactions. The most striking of these, metachromasia with thiazine dyes, has already been mentioned.

Choice of fixative has been shown to considerably alter the stability of the granules and their staining reactions. Fish, birds, rabbit and man are said to possess water-soluble mast cell granules, so aqueous fixatives should not be used, whereas rat, hamster, mouse and dog have comparatively water-insoluble granules (Selye 1965). An optimum fixative must be found for each species to attain a compromise between good cellular detail and integrity of the cytoplasmic granules. Miller, Murray and Jarrett (1967) found that Carnoy's fluid (a non-aqueous fixative) gave the best results in the rat, but in cattle and sheep globule leucocyte granules were not adequately fixed by this method, corrosive formol giving the best results. Spicer (1963) had also found Carnoy's fluid to be the most acceptable in the rat. Montagna, Eisen and Goldman (1954) used Helly's fluid as the fixative of choice for human skin biopsies. Csaba and Kovacs (1975) used 10 per cent. formalin as the fixative of choice in their studies on the rat. As the fixative may alter the intensity of staining of many of the commonly employed histochemical dyes the fixative of choice must be taken into account when comparing work by different authors. In fact, Pearse (1968) recommends the use of fresh, thin, cold microtome sections in studies on metachromasia: sections to be cut in pairs, one to be rapidly fixed in a recommended fixative and the other to be used fresh to check how much water-

soluble material has been lost in the fixation.

The best known, and possibly widest used, dyes for visualising mast cells in tissue are toluidine blue and azure A, both thiazine dyes. These combine with anionic groups in the granules to give a fairly unstable salt-type linkage. The linkage is broken by ethanol, acids and alkalis, so a water-based dye is generally employed. The different colours produced are due to the formation of polymers. Toluidine blue has an absorption spectrum consisting of three bands, α , β and γ . Alpha is blue and is emitted by the monomer, β is violet and formed by the dimer and the metachromatic red is the γ form emitted by the polymer. In practice, β metachromasia is usually formed by a mixture of α and γ (Pearse 1968). Often much of the red γ metachromasia is lost in dehydration and mounting, so that violet β tends to predominate (Spicer 1960; Spicer Horn and Leppi 1966).

A second major group of dyes is based on copper phthalocyanine, such as alcian blue and astra blue. These dyes differ from the thiazines in that they are non-metachromatic and bind more strongly to the tissue polyanions by an electrostatic linkage (Spicer Horn and Leppi 1966). Dehydration and mounting does not affect the intensity of staining. However the dyes do not have the same affinity for highly-sulphated mucopolysaccharides as are found in mast cells and often will not stain the granules unless used at a low pH (Lev and Spicer 1964). The addition of magnesium chloride to the solution will also increase the intensity of staining. The dye molecule has four cationic sites each one capable of linking with a single anionic group in the tissue. The presence of the magnesium chloride is thought to suppress dissociation of the dye molecule into its four parts so that each large molecule binds with only one anionic site. This will give a much greater concentration of dye in the tissue.

Safranin, an azo dye, can also be used to stain acid mucopolysaccharides. When used in combination with a phthalocyanine dye it tends to stain the polymers left unstained by these dyes. The addition of 0.1 M hydrochloric acid to the solution gives a metachromatic shift from pink towards an orange-red. The combination of astra blue or alcian blue with safranin is a useful stain to study mast cell distribution and type, since granules are stained blue or red.

Acid groups in mast cell granules have an affinity for ferric ions and this is utilised in the colloidal iron stains. The addition of ferric chloride in large

amounts to iron diamine gives a stain known as high iron diamine (Spicer Horn and Leppi 1966) which selectively stains connective tissue and epithelial sulphated mucopolysaccharides. Mast cells are stained dark brown against a pale brown background.

In addition to the sulphated mucopolysaccharides, mast cell granules contain a highly basic protein which can be stained by dyes such as Biebrich scarlet, a ~~di~~^{azo}/polyazo dye, used at high pH e.g. 9.9. Eosinophil leucocyte granules and Paneth cell granules are also stained in this reaction.

Finally, the catecholamines which have been demonstrated in the mast cell granules of some species can be condensed with formaldehyde following freeze-drying of the tissue and may be subsequently viewed by fluorescence microscopy (Falck and others 1964). The fluorescence is usually bright yellow to apple green. Enterochromaffin cells also fluoresce brightly after condensation with paraformaldehyde. Pre-treatment of animals with L-dopamine or incubation of fresh tissue with L-dopamine increases the specific mast cell fluorescence. The fluorescence induced is highly specific and fades on exposure to ultraviolet light. Any non-specific fluorescence of the tissue may be eliminated as it does not fade as rapidly. It is generally accepted that apple-green fluorescence indicates the presence of catecholamines and yellow fluorescence the presence of 5HT.

Because mast cells contain vasoactive amines and are often found grouped around blood vessels they are thought to have a role in controlling the tissue microcirculation. They play an important part in the inflammatory process, degranulation being stimulated non-specifically by fragments of complement (mainly C₃ and C₅) and lysosomal cationic proteases or specifically by antigens. Mast cells have surface membrane receptors for the Fc fragment of the IgE molecule enabling them to act as specific mediators of anaphylaxis and inflammation. IgE also appears to be intracellular in the granules of subepithelial mast cells of rats infected with Nippostrongylus brasiliensis (Mayrhofer, Bazin and Gowan 1976). Release of the vasoactive amines results in local vasodilation, transudation, exudation and oedema. If there is release generally throughout the body the result is anaphylaxis. Histamine and 5HT act to increase vascular permeability but their effect is transient, blood vessels becoming refractory to further stimulation.

In the tracheobronchial system mast cell degranulation and subsequent release of amines results in bronchoconstriction which is one of the manifestations of an asthmatic attack. Bronchoconstriction has been said to occur in horses with CB and examination of the numbers of mast cells and their integrity around bronchi might indicate if this is the case.

Horses with chronic bronchiolitis frequently have low arterial partial pressures of oxygen (McPherson and others 1978). Williams and others (1977) confirming the earlier results of Kay, Waynire and Grover (1974) and of Mungall and Barer (1975) found that hyperplasia of mast cells occurred around the pulmonary blood vessels of rats subjected to chronic hypoxia and that the intensity of mast cell hyperplasia was related to the degree of right ventricular hypertrophy. Few of the horses with CB showed right ventricular hypertrophy at necropsy but many had evidence of early pulmonary hypertension in that the circumference of the pulmonary artery was greater than that of the aorta (Chapter 3). Mungall (1976) working on chronically hypoxic rats found that right ventricular hypertrophy occurred before the mast cell hyperplasia which led Williams and others (1977) to suggest that the proliferation of the mast cells was in fact a protective mechanism to limit the severity of the pulmonary hypertension.

It has become increasingly evident that many parasitic infections are ended by an immunological reaction on the part of the host, mediated at least in part by mast cells. The hypothesis has been advanced that release of histamine and other vasoactive amines from mast cell granules causes an increase in epithelial permeability in the vicinity of the parasites allowing specific anti-parasite antibodies to pass into the lumen (Jarrett, Miller and Murray 1967). In more recent years it has been suggested that mast cells are not the specific cells involved in worm expulsion (Ogilvie and Jones 1971; Wells 1977). Nevertheless increased numbers of mast cells have been found in a wide variety of parasitic infections of different species and elevated levels of serum IgE are found in the sera of parasitised subjects (Ogilvie 1964; Sadun and others 1967). The idea that mast cells either carry immunoglobulins to the epithelial surface or allow their free passage by opening cell junctions is one that has considerable import when considering allergic responses in the lung. IgE is also known as reaginic antibody and elevated amounts are found in the serum and bronchial secretions of atopic subjects (Johansson 1967; Waldman, Virchow and Rowe

1973; Yunginger and Gleich 1973), more IgE is also bound to basophils of atopic subjects (Ishizaka, Soto and Ishizaka 1973) than non-atopics.

MATERIALS AND METHODS

Horses and ponies of varying ages from neonatal to old age, with no clinical or pathological signs of respiratory disease, were utilised. At necropsy tissues were selected from trachea, bronchi, alveoli and pulmonary vessels in all cases and also from gut, tongue, skin, thymus and thyroid in a few cases to act as a comparison. All the tissues were fixed in a variety of fixatives. The series of fixatives used by Miller (1969) was used, that is, ten per cent. formol saline, isotonic formol acetic acid (IFAA), calcium acetate formalin, corrosive formol, mercuric chloride and Carnoy's fluid. (Appendix 1). Tissues were fixed for 48 hours, dehydrated, cleared and finally double embedded in paraffin wax under a vacuum. Sections were cut at 5-6 μ m and stained with a variety of stains, including toluidine blue at pH 4.0 and at pH 0.3; astrablue counterstained with safranin; alcian blue alone and with varying concentrations of magnesium chloride (Scott and Dorling 1965); high iron diamine; aldehyde fuchsin; Biebrich scarlet at pH 9.9 (Spicer, Horn and Leppi 1966); a combined haematoxylin, eosin and toluidine blue stain (H and E - tol. blue) (Conroy and Toledo 1976) and a combined alcian blue, carbol chromotrope stain (Hogg and Banks 1977). Combinations of the individual stains were also tried on selected sections (Appendix 1).

Several young adult rats were killed by cervical dislocation and pieces of tissue from the tongue, gut, skin, lung and trachea were immediately removed and placed in the same series of fixatives. Stained with the same series of stains these acted as controls for the equine tissues, since the results with these methods had been described in detail by Miller (1969).

For the quantitative studies five adult normal horses with no respiratory abnormalities were selected. Tissues were taken from eight sites in the lung as shown in Figure 7. Almost every block contained part or all of a small bronchus, many bronchioles and accompanying blood vessels. The tissues were fixed in Carnoy's fluid, dehydrated, cleared and double embedded under a vacuum in paraffin wax. One 5 μ m section was cut from each block and stained with toluidine blue at pH 4.0. Using the X 25 objective on the microscope and with a 10 X 10 mm grid divided into 100 grid squares in one of the eyepieces it was

possible, by moving the grid systematically horizontally and vertically, to count every mast cell in the section and to classify its position, either bronchial, bronchiolar, arterial, venous or alveolar. Subpleural and interlobular mast cells were classified, for the purposes of this experiment, as extrapulmonary and were not counted. If it was not exactly clear what structure the mast cell was related to it was not counted. In fact this applied to very few as the mast cells were usually clearly related to the various structures. By recording the total number of grid squares covered in each section it was possible to work out a mast cell density for each site by dividing the number of mast cells by the total number of grid squares. In practice the density per 100 grid squares was used as this gave a more manageable number. This technique was first described by Williams and others (1977).

The procedure was repeated for ten horses affected by CB. The results were analysed by students' T test and the analysis of variance test (Goldstein 1964).

RESULTS

Fixation

All the fixatives employed maintained adequate cytological detail in the tissue. This was particularly good with ten per cent. formol saline, excellent with corrosive formol but with IFAA some fine detail was lost. Tissues fixed in IFAA remained very soft and were difficult to trim properly, However once embedded in paraffin wax no problem was encountered in sectioning. Tissues fixed in formol sublimate became exceptionally hard and consequently were easy to handle, This was also true, although to a lesser extent, of those tissues fixed in Carnoy's fluid. Both fixatives are rapid fixatives and care had to be taken that small blocks were used to allow adequate penetration. Tissues fixed in mercuric chloride were crumbly and hard and this proved to be the least satisfactory fixative. Carnoy's fluid was somewhat unpleasant to work with having a strong odour, corrosive properties and being irritant to the hands. Care also had to be taken when using mercuric chloride and corrosive formol as these were extremely corrosive to metals. Ten per cent. formol saline would be the fixative of choice if the quality of staining was not important since it gave good fixation, the tissue was firm and it was less unpleasant to work with.

Staining

The tissues of the rat acted as controls in this study utilising the same stains as Miller (1969). Almost identical results were obtained. The mast cells stained well with alcian blue, astra blue, toluidine blue at pH 4.0 and at pH 0.3 and with Biebrich scarlet. Miller (1969) did not have much success with Biebrich scarlet whereas here it worked consistently and specifically on both subepithelial and connective tissue mast cells.

In addition the use of high iron diamine gave good results and aldehyde fuchsin was less satisfactory. This latter stain was subject to wide variations in staining intensity and quality. It was therefore abandoned as a technique.

Generally poor results were obtained on calcium acetate formalin-fixed material and with corrosive formol as a fixative staining was often pale. This was also occasionally a problem with ten per cent. formalin-fixed material although generally good results could be obtained with this fixative. The reason for variation in quality was not apparent. Mercuric chloride seemed to disrupt the membrane of a proportion of mast cells and in calcium acetate formalin the mast cells had a "rounded-off" or constricted appearance with no granular detail apparent.

Similar results were obtained on the normal equine tissues. The pulmonary mast cells did not differ in their staining reactions from mast cells in other organs or from the mast cells of the rat. The staining reactions of the pulmonary mast cells will now be considered in more detail.

Biebrich scarlet, especially if dehydrated post-staining (Appendix 1), gave very striking results (Figure 80). The background tissue was almost completely unstained but the mast cells showed up clearly as bright orange cells with clearly defined outlines. Unfortunately, two problems arose with this stain. Firstly, the location of the mast cells was difficult to determine as the background was so pale and, secondly, eosinophils also stained. If not dehydrated post-staining the background remained a pale pink or orange but the mast cell detail was not so well preserved and red blood cells took on a striking orange. Eosinophils were a common invader of equine pulmonary tissue and although their granules were very large the cells were still difficult to consistently distinguish from mast cells which usually contained

much smaller granules. Biebrich scarlet did not work well on Carnoy's fixed material nor on calcium acetate fixed material.

However all other stains, alcian blue, astra blue, highiron diamine and toluidine blue, worked very well in Carnoy's fixed material. The most useful of these were toluidine blue at pH 4.0 (Figure 81) and astra blue-safranin (Figure 82). In these the background tissue could be adequately identified and the mast cells were immediately obvious. Good results were also obtained with IFAA-fixed material in which, especially with toluidine blue, very clear granular detail was perceived. High iron diamine (Figure 83) gave good results with all the fixatives employed although the granules did not always appear very prominent.

Toluidine blue at pH 0.3, whilst differentiating mast cells well, suffered from the same drawback as Biebrich scarlet in that the background was very poorly differentiated (Figure 84). Toluidine blue stains cartilage and goblet cells metachromatically. It was therefore difficult to distinguish epithelial mast cells from goblet cells (Figure 85). This was also a problem with alcian blue, astrablue, high iron diamine and Biebrich scarlet.

Astrablue counterstained with safranin gave good clear results with all fixatives. The mast cell granules almost all stained with astrablue although occasionally some stained with safranin; the nuclei always stained with safranin. Unfortunately granular detail was not well preserved and the cytoplasm usually appeared to be homogeneous (Figure 92). Background tissues stained clearly with safranin.

Metachromasia, especially with toluidine blue at pH 4.0, could be very pale with mercuric chloride and corrosive formol and occasionally with ten per cent. formol saline as mentioned above for the rat tissues. This was unfortunate as these three fixatives gave the best histological detail of all fixatives. Calcium acetate formalin-fixed material tended to pick up a background stain with greater avidity than material fixed in other tissues and this, combined with the paler staining of the mast cells, made distinction difficult.

Alcian blue at critical electrolyte concentrations stained pulmonary mast cells from concentrations of 0.2 M to 1.0 M. At 0.2 M $MgCl_2$ results were sometimes indistinct as the background stained very darkly. At 1.0 M some of the clarity of the mast cell staining was lost. Best results were

obtained between 0.5 and 0.8 M MgCl_2 where mast cells showed up clearly as bright turquoise against a pale background.

Because of the relationship observed between the occurrence of mast cells and eosinophils and the high incidence of eosinophils in pulmonary tissue it became apparent that there was a need for a stain to demonstrate both mast cells and eosinophils selectively in the same tissue. Attempts were made to combine Biebrich scarlet staining with carbol chromotrope staining. These two stains could be used together but carbol chromotrope stained eosinophil granules red and Biebrich scarlet stained mast cell granules red to orange so it did not provide satisfactory differentiation. A stain for mast cells that stained the granules a contrasting colour to red was required. Toluidine blue was not suitable because the pH of the buffer used in carbol chromotrope would interfere with this stain. High iron diamine combined with Biebrich scarlet was found to be a satisfactory staining regime. The mast cell granules stained with both stains so that they appeared a reddish-brown (Figure 86) and the eosinophils stained only with the Biebrich scarlet. This only worked if the sections were blotted rather than dehydrated so that the eosinophils were not as clear as they might have been.

At this time, a simultaneous stain for mast cells and eosinophils was described, utilising eosin and toluidine blue (Conroy and Toledo 1976). This was employed on several suitable sections but in my hands the results were disappointing, varying staining intensities being observed. Sometimes toluidine blue was hardly taken up at all; at other times it was bound so strongly that it masked the haematoxylin and eosin reaction. Eosinophils did not stain well.

Subsequently a combined alcian blue and carbol chromotrope method (Hogg and Banks unpublished work) was adopted in which mast cells and eosinophils were well differentiated (Figures 87 - 96). This is known as Hogg's and Bank's stain in this thesis. Best results were obtained on corrosive formol or formol saline-fixed sections. Carnoy's fluid was not suitable for this stain.

The staining results in various fixatives are given in Table 12.

Pulmonary mast cell morphology and distribution

The equine pulmonary mast cell did not differ from mast cells found in other mammalian species. Both subepithelial and connective tissue mast

cells stained readily with dyes used to locate highly sulphated mucopolysaccharides and with Biebrich scarlet which stains basic protein. Equine mast cells stain very readily with Biebrich scarlet perhaps indicating a high content of basic protein. The mast cell granules do not appear to be particularly water-soluble as similar results were obtained with both a aqueous and non-aqueous fixatives.

Morphologically the mast cells of the pulmonary tissue presented a variety of appearances ranging from spherical (Figure 81) to dendritic. Cellular processes were common, although usually fairly short (Figure 85). The nucleus was often obscured by the granular content but when visible was ovoid occasionally with an indentation (Figure 81). The granules generally filled the cytoplasm and were of uniform size.

Mast cells were most frequently seen around blood vessels (Figures 90 and 91) and the smaller airways (Figure 92) and in the interlobular septa and subpleurally (Figure 93).

Epithelial mast cells or globule leucocytes are very scarce in the normal horse. This applies to all the epithelial tissues examined, stomach, gut and airways; on H and E staining the distinctive eosinophilic globule leucocyte described in cattle, sheep and the rat was not found and with selective mast cell stains, although detail was often obscured by the presence of goblet cells, very few mast cells were found actually in the epithelial cell layer.

Mast cell densities in normal horses

The complete results are shown in Tables 13 and 14. Initial observations had indicated that mast cells occurred most frequently around small airways in equine pulmonary tissue and this impression was borne out by the mast cell counts in the five normal horses. A mean of 45.51 ± 11.16 mast cells per 100 grid squares occurred around the bronchioles compared to 107.08 ± 23.97 in the whole area. That is 42.5 per cent. of all mast cells in the normal equine lung occurred around bronchioles. Some 22.43 ± 10.84 and 20.58 ± 6.41 respectively of the total were associated with alveoli and pulmonary arteries and arterioles. Relatively few, (13.6 ± 1.10) were associated with the bronchi i.e. in the peribronchial tissue and lamina propria. The mean number around pulmonary veins was 6.23 ± 5.05 . This may have been an artificially

low figure since the pulmonary vein is situated further away from the bronchus than the pulmonary artery and large bronches were not present. Nevertheless, in sections where both the main artery and vein occurred together and could be clearly distinguished there were fewer mast cells associated with the vein than with the artery.

The analysis of variance test applied to the mean number of mast cells per 100 grid squares at each of the main sites and for each of the five horses showed that there was no significant difference between the mean numbers for each site and for individual horses ($f_{\text{horse}} = 0.63$, $f_{\text{site}} = 1.87$). The same test applied to the mean numbers at each location for each individual horse again showed no significant difference between individual horses as regards numbers at each location ($f = 2.83$) but did show a significant difference ($f = 23.95$) between locations. The studentised range test applied to this result to clarify the significance showed that the bronchioles had significantly more ($p = < 0.05$) and veins significantly less ($p = > 0.05$) mast cells than at other locations. All other comparisons were non-significant including one where the total perivascular mast cells were utilised.

Occasionally sections contained very few mast cells (Table 13). This was not a staining fault as the mast cells that were present stained with a normal intensity. Rather this seemed to be a localised phenomenon occurring apparently with equal frequency in each of the horses.

Mast cells were most frequently found in the connective tissue elements surrounding bronchioles, arteries and veins, rarely being seen in smooth muscle or bronchiolar epithelium. Bronchial mast cells were found in the peribronchial connective tissue, around the submucosal glands or adjacent to the tips of the cartilaginous plates; they were seldom found in the muscle or the bronchial epithelium. Alveolar mast cells were frequently associated with the alveolar capillaries.

Mast cell densities in horses with CB

The results are shown in Tables 15 and 16. The first five horses examined showed a range of 103.36 - 257.73 cells per 100 grids. This lowest result was almost identical to the mean normal result. The analysis of variance test showed that as for the normal horses there was no significant difference between the different sections examined for each horse but, as

expected, there was a highly significant difference between the individual horses. As a result of this a further five horses with CB were counted, but in these only four sections from each were used as tests of significance had shown that this would give a valid result. On the basis of the additional results it was found that these ten horses affected by CB could be divided into two major groups on the basis of their pulmonary mast cell densities. Group One horses had counts in excess of, or equivalent to, those of normal horses (usually much higher). Seven horses fell into this category with a mean value of 130.07 ± 166.63 per 100 grid squares as compared to 107.08 ± 23.97 for normal horses. Group Two horses had counts very much below those of normal horses and three of the horses fell into this category, mean 33.43 per 100 grid squares. The results are depicted in the histogram, Table 17.

Statistical analysis, by means of Students "t" test of the mast cell densities in Group One horses compared to the densities in normal horses is summarised in Table 18. Counts for each site and the total densities are significantly higher ($p = < 0.05$) in the Group One horses than in the normal horses with the exception of the bronchial densities where the difference was only significant at the 0.1 probability level. The arterial and venous results were pooled because of the difficulties discussed above and the sampling bias that may have occurred.

The number of horses in Group Two is too small a sample to apply statistical analysis to, but it is apparent from the numerical results that the number of mast cells in this group is depressed and that this reduction in density affects all locations equally, except for the bronchial density in two of the three cases which remained at the normal density level. However, in a sample of only three, great care must be taken not to regard this result as typical of the whole population.

Qualitatively there was wide variation in the mast cells of horses with CB, both of Groups One and Two. Frequently the granules took up little stain and were difficult to enumerate. This paleness of staining was associated with an irregular, hazy cell outline (Figure 94). In other instances staining was extremely vivid. Numerous morphological forms were seen as in the normal horses. Mast cells occupied the same relative positions as in the normal horses with one exception, in that in areas of alveolar epithelial hyperplasia

there were large numbers of mast cells. This accounts for the high alveolar densities in three of the horses. There was no increase in the number of epithelial mast cells nor were globule leucocytes found in either the bronchi or bronchioles. Figures 95 and 96 illustrate a bronchiole surrounded by a large number of mast cells and a bronchiole surrounded by a fairly normal number of mast cells.

An attempt was made to correlate the density of mast cells around pulmonary blood vessels with the degree of right ventricular hypertrophy in these animals. Right ventricular hypertrophy was assessed by means of the ratio $\frac{LV + S}{RV}$ where $LV + S$ is the weight of the muscle mass of the left ventricle plus interventricular septum and RV is the weight of the right ventricular muscle mass. In a large series of normal horses the average value for this ratio was found to be 3.306 ± 0.568 (Chapter 3). The results are shown in Table 19. Correlation was extremely poor even using logarithms. It may have been better to correlate partial pressure of arterial oxygen values with the mast cell density but unfortunately this data was not available for all the subjects.

The four horses with the highest mast cell densities, 33, 11, 12 and 15, were all necropsied during the summer months i.e. May to August. The three with very low counts, 7, 6 and 9 were all necropsied in the months of February and March. The three intermediate results 14, 29 and 31, were found in horses necropsied in October, November and December respectively. This striking relationship of total mast cell density with season of necropsy is depicted in Table 20.

DISCUSSION

Carnoy's fluid was found to be the best fixative to use on equine tissue to preserve both the granules and the integrity of mast cells and yet retain adequate background cytological clarity. Carnoy's fluid is a non-aqueous fixative but mast cells could be readily visualised, if with a slight diminution in definition, in most aqueous fixatives. The granules of equine mast cells are therefore regarded as relatively water insoluble.

Using a variety of histochemical stains, equine mast cells were shown to contain both a highly sulphated mucopolysaccharide and a basic protein, as in most other species. All the recognised mast cell stains readily demonstrated equine mast cells. Toluidine blue at pH 4.0 was chosen as the stain for enumerating the mast cells for a number of reasons; the granules stained metachromatically magenta or purple against a pale blue background, which made it easy to pick them out and to identify the structure they were associated with. The stain was specific and it was simple to use, and consistent results were obtained with consecutive batches.

The combined special stains, particularly Hogg's and Banks' stain, were useful in certain situations and could be used in preference to the well-established mast cell stains on routine material as they give much more information on the background tissue and cell types without detracting from the singular mast cell staining properties.

Morphologically, equine mast cells are similar to those of other species. The cytoplasm is packed with numerous granules of fairly uniform appearance, the nucleus when visible is ovoid or round and often indented. The shape of the cell has many variations possibly depending on a particular situation. The different forms may also represent variations in maturation and activity. Attempts have been made in the past to classify mast cells into distinct morphological categories (de Vinals 1954) but it is now recognised that there is no basis for this classification (Selye 1965). There was no discernible pattern as regards staining intensity or morphology in the equine mast cells and wide variations in both were observed. There was a general impression perhaps that very pale mast cells were more frequently seen in horses with CB but this was not quantified.

In the pulmonary tissue mast cells were found most frequently beneath the pleura, around bronchioles and blood vessels and in alveolar tissue in normal horses. Smaller numbers were associated with bronchi. There have been relatively few previous studies of the mast cells in the lung of any species. Selye summed up the literature until 1965 and concluded that the greatest concentration of mast cells was found in the trachea and large bronchi particularly beneath the epithelium and that large numbers were found sub-pleurally. Williams and others (1977), did not distinguish between bronchi and bronchioles when assessing mast cell density. They found the highest density perivascularly with less peribronchially and only about ten per cent. of the total in the interalveolar septa. Other quantification studies on the rat pulmonary tissue have also ignored the distinction between bronchi and bronchioles. In view of the fact that an appreciable difference was found in mast cell densities between these two airways in the horse future studies in all species should endeavour to distinguish the two.

This would appear to be the first study of the distribution of mast cells in equine pulmonary tissue or indeed the first study in any species to completely classify mast cell densities involved with a number of separate pulmonary structures.

In the horse the highest density of mast cells is found around the bronchioles and, with respect to the postulated functions of mast cells, could have profound implications in the pathogenesis of disease. Mast cell degranulation releases compounds like histamine, 5HT and heparin. The net result of their action on bronchioles would presumably be a constriction of the smooth muscle, possibly swelling and oedema of the bronchiolar lamina propria, opening of the epithelial cell junctions and resultant transudation of cells and fluid into the lumen.

The main defect in this study was that it proved more difficult than originally anticipated to differentiate between pulmonary arterioles and veins and in the end the differential counts between the two had to be regarded as unreliable. It was, however, true that in the larger pulmonary vessels at least more mast cells were associated with pulmonary arteries than with pulmonary veins.

The mast cell density survey in the horses with CB provided some very interesting results. Dividing the horses into two main groups may be premature as it is quite possible that if a large enough number of horses with CB is assessed a complete range of mast cell densities will be found including more cases having values around the normal range. However most of the ten horses studied had results either far in excess of or far below those of normal horses. This increase or decrease was reflected equally in the densities around bronchioles, blood vessels and alveoli. Two of the horses in Group Two, 6 and 9, had bronchial densities around the normal level and, except for two horses with rather high counts, 12 and 15 the horses in Group One had counts not much above that of normal horses. This, although only a small sample was assessed, adds further weight to the view that the bronchi do not appear to be primarily involved in the pathogenesis of CB in the horse.

To a considerable extent the increase in alveolar mast cell density in Group One horses was due to the large numbers of mast cells associated with areas of alveolar epithelial hyperplasia. The mechanism of this pathological change is still largely unknown but mast cells are associated with alveolar epithelial hyperplasia in cattle with diffuse fibrosing alveolitis (Breeze and others 1975).

An observed increase in mast cell numbers around pulmonary blood vessels as occurred in Group One horses has also been seen in rats submitted to chronic hypoxia. These developed pulmonary hypertension accompanied by right ventricular hypertrophy and an increase in the number of "pulmonary muscular arteries" (Kay, Waymire and Grover 1974; Mungall and Barer 1975; Mungall 1976). Horses affected with CB develop early pulmonary hypertension but right ventricular hypertrophy is infrequently found at necropsy (Chapter 3). However, it proved difficult to demonstrate a correlation between either pulmonary blood vessel mast cell density or total pulmonary mast cell density and the degree of right ventricular hypertrophy.

Kay, Gilland and Heath (1967) fed Crotalaria spectabilis seeds to test rats to induce pulmonary and cardiovascular changes resulting in profound pulmonary hypertension. The main lesions were right ventricular hypertrophy and an increase in the number of pulmonary muscular arterioles. Only a proportion of these rats, however, 14 out of 21, had a proliferation of the pulmonary

mast cells, and this was in no way correlated with the degree of hypertension. They postulated that the increased number of mast cells seen in the test animals was in fact due to the chronic inflammation and exudation induced in the lungs by Crotalaria and, indeed the number of mast cells correlated with the degree of this change. A similar explanation could be used to explain the increased mast cell densities in the horses. Chronic bronchiolitis, pathologically, is essentially a chronic inflammatory change so this may be the simplest explanation for the hyperplastic changes observed.

The increase in mast cell density around small airways but not particularly around bronchi in horses of Group One is perhaps the most significant finding of this study. Small airways have been shown previously to be the main site of pathological changes in CB, the disease being characterised by chronic bronchiolitis consisting of epithelial hyperplasia, of goblet cell metaplasia and exudation of mucus and pus into the lumen. The fact that increased mast cell numbers may be associated with this change offers an opportunity to discuss further the pathogenesis of the disease. Mast cells are thought to perform a function in three major areas of disease - inflammation, allergy and parasitism. There is no evidence that parasites are associated with the development of CB, so this reaction can be excluded. Allergy, on the other hand is said to play a major part in the development of CB and it is possible to demonstrate pulmonary allergic reactions to many environmental antigens in these horses (McPherson and others unpublished data). Knowing that in the normal horse there is a large number of mast cells around bronchioles and that in horses affected with CB there can be a considerable increase in mast cell numbers it is possible to hypothesise that the bronchioles are the site of an allergic reaction. Equally though some other explanation must be found for bronchiolitis in the horses with no observable mast cell density increase.

No plausible explanation for the low mast cell densities of Group Two horses could be found. All three horses had exhibited marked clinical signs of CPD and at necropsy had extensive changes in the bronchioles identical to those of the horses in Group One. Salvato (1961) found less mast cells in the bronchi of asthmatics than non-asthmatics, his explanation for this being that the mast cells originally present had discharged their granules. It could be that these three horses were killed at a time when a massive antigenic onslaught

had caused widespread mast cell degranulation but there is no clinical or pathological data to support this. Numbers of mast cells in horses with CB could, in fact, fluctuate in a wave-like manner as degranulation and dedifferentiation succeed each other. This theory would be impossible to prove without doing successive pulmonary biopsies. In addition these three were all slaughtered at about the same time of year - in the winter. It has been shown that there is a summer rise in circulating basophils of human asthmatics (Hirsch and Kalbfleisch 1976) and the highest mast cell densities were recorded in horses killed in the summer months. Obviously more horses need to be investigated as this could be a coincidental finding but is worthy of investigation.

Several interesting results arose from this survey and it is apparent that the subject deserves further study.

1. Equine mast cells resemble histologically and morphologically the mast cells of other species.
2. In normal equine pulmonary tissue 42.5 per cent. of the total number of mast cells are found around the bronchioles, 21 per cent. are in interalveolar septa, 26.8 per cent. are perivascular and 12.7 per cent. are around bronchi.
3. In horses with CB two main patterns were found. Some, Group One, horses had significantly raised levels of mast cells at all the sites and locations equally, except for the peribronchial mast cells which were only significantly elevated in a few cases. Others, Group Two, had depleted numbers of mast cells down to 20 per cent. of the normal level. This reduction affected all sites and locations equally except the bronchi.
4. The degree of mast cell hyperplasia was not associated with right ventricular hypertrophy.
5. It is suggested that the increased numbers of mast cells are associated with an allergic or inflammatory reaction rather than with pulmonary hypertension.

HISTOCHEMICAL STAIN EMPLOYED					
Fixative	Quality of Fixation	Toluidine blue pH 4.0	Toluidine blue pH 0.3	Astrablue safranin	Acian blue + 0.5M Mg Cl ₂
Ten per cent. formol saline.	Good	Metachromatic purple Blue	Metachromatic purple Blue	Blue/orange Pink	Turquoise Blue
Carnoy's fluid	Good	Metachromatic purple Blue	Metachromatic purple Unstained	Blue/orange Pink	Turquoise Blue
Corrosive Formol	Excellent	Pale purple Blue	Pale purple Unstained	Blue/orange Pink	Pale Turquoise Blue
Mercuric chloride	Good/ difficult to cut.	Pale purple Blue	Pale purple Unstained	Pale blue/orange Pink	Pale Turquoise Blue
Isotonic formol acetic acid	Fair	Metachromatic purple Blue	Metachromatic purple Unstained	Blue/orange Pink	Turquoise Blue
Calcium acetate formalin	Good	Metachromatic purple Dark blue	Metachromatic purple Unstained	Blue/orange Dark pink	Turquoise Dark blue

TABLE 12(a) Histochemical staining of the equine pulmonary mast cell, tissues fixed in various fixatives.

HISTOCHEMICAL STAIN EMPLOYED

Fixative	Quality of Fixation	High iron diamine	Aldehyde fuchsin	Biebrich scarlet pH 9.9	Toluidine blue H and E	Hogg's and Banks
Ten per cent. formal saline	Good	Dark brown	Few stained purple Yellow	Orange/red Unstained	Purple blue Pink/Blue	Turquoise Blue
Carnoy's Fluid	Good	Dark brown	Not stained	Not stained well Unstained	Purple blue Pink/Blue	Turquoise Blue
Corrosive Formal	Excellent	Dark brown	Not stained	Orange/red	Pale purple blue Pink/Blue	Turquoise Blue
Mercuric chloride	Good/ difficult to cut	Dark brown	Not stained	Orange/red	Pale purple blue Pink/Blue	Turquoise Blue
Isotonic formal acetic acid	Fair	Dark brown	Not stained	Pale orange	Purple blue Pink/Blue	Turquoise Blue
Calcium acetate formalin	Good	Dark brown	Few stained purple Yellow	Very pale orange Unstained	Purple blue Pink/Blue	Turquoise Blue

TABLE 12(b). Histochemical staining of the equine pulmonary mast cell, tissues fixed in various fixatives.

MAST CELL DENSITIES PER 100 GRID SQUARES IN:

Horse No. and Site	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total Perivascular	Total
No.1	1	8.21	19.17	9.81	13.47	4.79	55.48
	2	10.95	31.93	12.35	23.78	10.72	89.74
	3	23.98	23.98	10.18	21.94	4.07	84.16
	4	14.00	52.11	14.98	30.94	6.51	118.57
	5	31.34	67.06	17.86	25.00	8.73	150.00
	6	13.70	29.01	11.19	15.46	10.60	79.97
	7	2.03	0	0	1.35	0.33	3.71
	8	0.30	1.53	0	0	0.92	2.76
Mean	12.97	27.07	9.71	16.56	6.40	22.96	72.73
No.2	1	12.46	94.36	83.68	40.65	8.01	239.17
	2	27.60	107.81	36.98	17.97	13.54	20.39
	3	14.08	69.01	41.12	18.87	18.03	161.1
	4	27.33	82.28	39.04	45.94	8.41	203.0
	5	2.30	0	1.97	0	0	4.28
	6	9.54	21.20	3.53	3.89	1.41	39.57
	7	5.90	12.79	5.24	4.26	0	28.20
	8	0	12.75	11.11	6.58	0	30.45
Mean	13.4	54.28	29.83	18.31	14.86	33.17	122.72

Table 13 continued overleaf.

MAST CELL DENSITIES PER 100 GRID SQUARES IN :

Horse No. and Site	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total	
						Perivascular	Total
1	21.22	41.22	40.81	7.75	2.04	9.79	113.06
2	4.80	55.80	20.20	14.39	0.25	14.64	95.45
3	38.81	61.18	19.74	8.55	3.28	11.83	131.58
No. 3 4	14.71	63.42	30.01	16.10	2.58	18.68	126.84
5	16.91	44.96	29.50	14.39	5.39	19.78	111.15
6	0	12.55	17.00	4.45	0	4.45	34.00
7	22.41	72.41	38.22	21.55	6.60	28.15	161.21
8	13.73	55.28	30.28	27.46	1.76	29.22	128.52
Mean	13.41	52.95	28.70	15.25	2.73	17.98	114.63
1	74.0	80.66	89.33	72.0	0	72.0	316.0
2	0	94.27	112.74	82.16	5.73	87.89	294.90
3	1.42	13.70	16.11	7.58	0	7.58	48.81
No. 4 4	0	24.84	12.10	22.93	0	22.93	59.87
5	22.50	54.09	17.73	31.59	1.14	32.73	127.04
6	0	17.44	13.56	14.43	1.55	15.89	46.90
7	18.60	47.91	22.32	18.14	5.12	23.26	112.09
8	12.65	71.43	27.35	28.16	9.80	37.96	149.39
Mean	15.49	50.14	32.30	31.26	2.89	34.15	132.10

Table 13 continued overleaf.

MAST CELL DENSITIES PER 100 GRID SQUARES IN :

Horse No. and Site	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total Perivascular	Total
1.	16.45	49.11	3.03	15.19	8.86	24.05	92.66
2	5.75	33.70	12.88	28.22	0.82	29.04	81.37
3	21.97	39.02	9.51	9.84	5.57	15.41	85.90
No. 5 4	22.80	98.34	28.50	42.52	8.79	51.31	200.95
5	4.48	4.97	2.49	2.49	0	2.49	14.42
6	3.14	0.90	0.45	2.69	0	2.69	7.17
7	13.02	26.56	10.94	26.04	3.64	29.68	80.20
8	0	56.66	17.22	27.22	0	27.22	101.11
Mean	12.73	43.09	11.60	21.50	4.28	25.78	93.21

TABLE 13. Mast cell densities per 100 grid squares at eight sites in five horses with no clinical or pathological signs of pulmonary disease. Uninflated pulmonary tissue fixed in Carnoy's fluid and stained with toluidine blue at pH 4.0

MAST CELL DENSITIES PER 100 GRID SQUARES IN:

Horse Number	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total Perivascular Tissue	Total
						Arteries and veins	
1	12.97	27.07	9.71	16.56	6.40	22.96	72.73
2	13.40	54.28	29.83	18.31	14.86	33.17	122.72
3	13.41	52.95	28.70	15.25	2.73	17.98	114.63
4	15.49	50.14	32.30	31.26	2.89	34.15	132.10
5	12.73	43.09	11.60	21.50	4.28	25.78	293.21
Mean	13.60	45.51	22.43	20.58	6.23	26.81	107.08
Standard	±	±	±	±	±	±	±
Deviation	1.10	11.16	10.84	6.41	5.05	6.85	23.97

TABLE 14. Mean mast cell densities per 100 grid squares uninflated pulmonary tissue fixed in Carnoy's fluid and stained with toluidine blue at pH 4.0.

MAST CELL DENSITIES PER 100 GRID SQUARES IN:

Horse No. and Site	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total	
						Perivascular	Total
No. 14	1	70.56	133.89	131.67	46.11	11.11	57.22
	2	21.94	40.74	57.83	36.18	5.13	41.31
	3	24.80	72.40	53.40	44.80	10.60	55.40
	4	36.16	33.58	44.28	12.55	1.11	13.66
	5	13.46	98.08	35.77	11.92	1.92	13.84
	6	13.14	98.15	132.20	64.83	2.12	66.95
	7	0.71	67.86	38.30	18.79	2.84	21.63
	8	15.84	60.41	34.02	26.10	0	26.10
Mean		22.64	71.13	60.14	32.76	4.63	37.36
No. 33	1	51.51	134.94	203.61	40.66	4.42	45.08
	2	12.50	156.91	89.14	39.14	1.32	40.46
	3	20.45	92.80	50.00	13.64	0	13.64
	4	28.57	101.02	108.16	35.20	3.57	38.77
	5	19.77	108.14	71.40	29.53	0.47	30.00
	6	10.11	97.27	42.90	23.50	6.83	30.33
	7	23.70	107.23	58.09	25.43	4.62	30.05
	8	21.74	107.69	64.75	27.48	2.55	30.03
Mean		23.37	114.39	87.40	29.49	3.08	32.57

MAST CELL DENSITIES PER 100 GRID SQUARES IN :

Horse No. and Site	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total Perivascular	Total
No.29	1	31.10	32.78	46.92	56.57	6.97	174.53
	2	1.30	0	0.65	0.65	0	2.60
	3	50.36	45.00	15.71	59.29	2.86	173.21
	4	20.31	57.50	46.88	46.25	10.00	180.94
	5	12.61	57.98	34.24	55.25	6.09	166.18
	6	24.38	61.33	26.00	35.22	4.93	151.48
	7	28.25	41.23	13.31	36.04	4.22	123.05
	8	20.43	49.61	23.78	45.65	5.16	144.62
Mean	23.15	43.91	27.49	42.25	5.18	47.43	141.92
No.31	1	4.41	37.44	16.30	31.28	10.13	99.56
	2	2.26	58.06	18.39	32.58	8.06	119.35
	3	0.85	53.69	9.94	16.19	5.11	85.80
	4	53.38	87.16	25.68	46.62	1.35	214.19
	5	2.67	0.67	2.67	0	2.00	8.0
	6	4.44	1.67	0.56	13.89	0	20.56
	7	23.21	35.71	13.69	28.57	0	101.19
	8	28.11	55.02	35.74	32.53	13.25	164.61
Mean	12.33	43.95	15.92	25.34	5.83	31.77	103.36

Table 15 continued overleaf.

MAST CELL DENSITIES PER 100 GRID SQUARES IN :

Horse No. and Site	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total Perivascular	Total	
No.15	1	54.90	161.96	151.76	78.82	4.71	83.53	452.16
	2	124.78	94.25	101.77	44.25	9.29	53.54	374.34
	3	13.10	85.12	57.14	11.90	0	11.90	167.26
	4	0	48.93	98.29	51.92	6.01	57.94	205.15
	5	13.90	52.88	42.37	51.19	0	51.19	160.34
	6	49.43	47.51	21.07	67.05	1.92	68.97	186.97
	7	89.84	89.69	126.77	61.54	7.24	68.44	443.52
	8	31.67	48.22	31.72	64.30	0	60.08	235.99
Mean	42.70	80.88	78.03	53.41	3.62	57.03	258.62	
No.11	1	0	144.40	80.69	83.01	1.54	84.55	310.04
	2	0.47	0	1.88	0.94	0	0.94	3.30
	3	25.07	62.82	33.52	36.90	6.48	43.38	164.79
	4	55.45	69.55	30.00	25.91	4.09	30.00	185.00
	5	1.32	75.88	55.70	29.82	0	29.82	162.72
Mean	16.88	72.45	41.21	37.13	2.83	39.96	170.49	
No.12	1	93.20	36.40	68.50	66.80	3.20	70.00	254.40
	2	92.95	5.13	86.54	30.77	0	30.77	215.38
	3	6.06	58.31	70.69	56.30	2.72	59.22	194.26
	4	85.60	81.07	98.35	20.16	2.06	22.22	287.24
Mean	61.84	49.90	76.02	46.02	2.24	48.26	236.02	

MAST CELL DENSITIES PER 100 GRID SQUARES IN :

Horse No. and Site	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total Perivascular	Total
No. 7	1	0	0	0	0.01	0.1	1.27
	2	22.0	12.43	5.95	0	5.95	48.65
	3	0	14.29	5.19	0	5.19	37.01
	4	0	0	0.43	0	0.43	1.30
	Mean	5.50	6.19	2.75	0.27	3.02	20.91
No. 6	1	33.03	15.38	6.33	0	6.33	55.66
	2	11.53	5.92	4.05	0	4.05	24.92
	3	11.51	0	2.88	0	2.88	15.83
	4	29.69	2.04	4.94	0	4.94	37.50
	Mean	21.44	7.78	4.55	0	4.55	33.04
No. 9	1	32.33	4.22	3.88	0	3.88	53.88
	2	7.33	0	0.33	1	1.33	9.33
	3	22.46	14.83	16.53	0	16.53	84.32
	4	15.70	4.13	7.02	0	7.02	47.93
	Mean	18.61	5.84	6.53	0.3	6.56	46.34

TABLE 15. Mast cell densities per 100 grid squares in ten horses with CB. Uninflated pulmonary tissue fixed in Carnoy's fluid

and stained with toluidine blue at pH 4.0.

MAST CELL DENSITIES PER 100 GRID SQUARES IN:

Horse Case Number	Bronchial	Bronchiolar	Alveolar	Arterial	Venular	Total Perivascular Tissue	Total
14	22.65	71.13	60.14	32.76	4.60	37.36	190.00
33	23.37	114.39	87.40	29.49	3.08	32.56	257.73
29	23.15	43.91	27.48	42.25	35.18	41.43	141.97
31	12.33	43.95	15.92	25.34	5.83	31.17	103.36
11	16.88	72.45	41.21	37.13	2.83	39.96	170.49
12	61.84	49.90	76.02	46.02	2.24	48.26	236.02
15	42.70	80.88	78.03	53.41	3.62	57.03	258.62
7	5.50	6.19	6.19	2.75	0.27	3.02	20.91
6	21.44	2.20	7.78	4.55	0	4.55	33.04
9	18.61	15.05	5.84	6.53	0.3	6.56	46.34
Mean	28.99	68.09	30.93	38.06	3.91	41.11	130.07
Group One	±	+	+	±	+	+	+
Standard Deviation	17.3	25.28	56.47	9.83	1.33	9.05	166.63
Mean	15.17	7.81	6.60	4.59	0.19	4.71	33.43
Group Two							
Sample too small to calculate standard deviation.							

TABLE 16. Mean mast cell densities per 100 grid squares uninflated pulmonary tissue, fixed in Carnoy's fluid and stained with toluidine blue at pH 4.0.

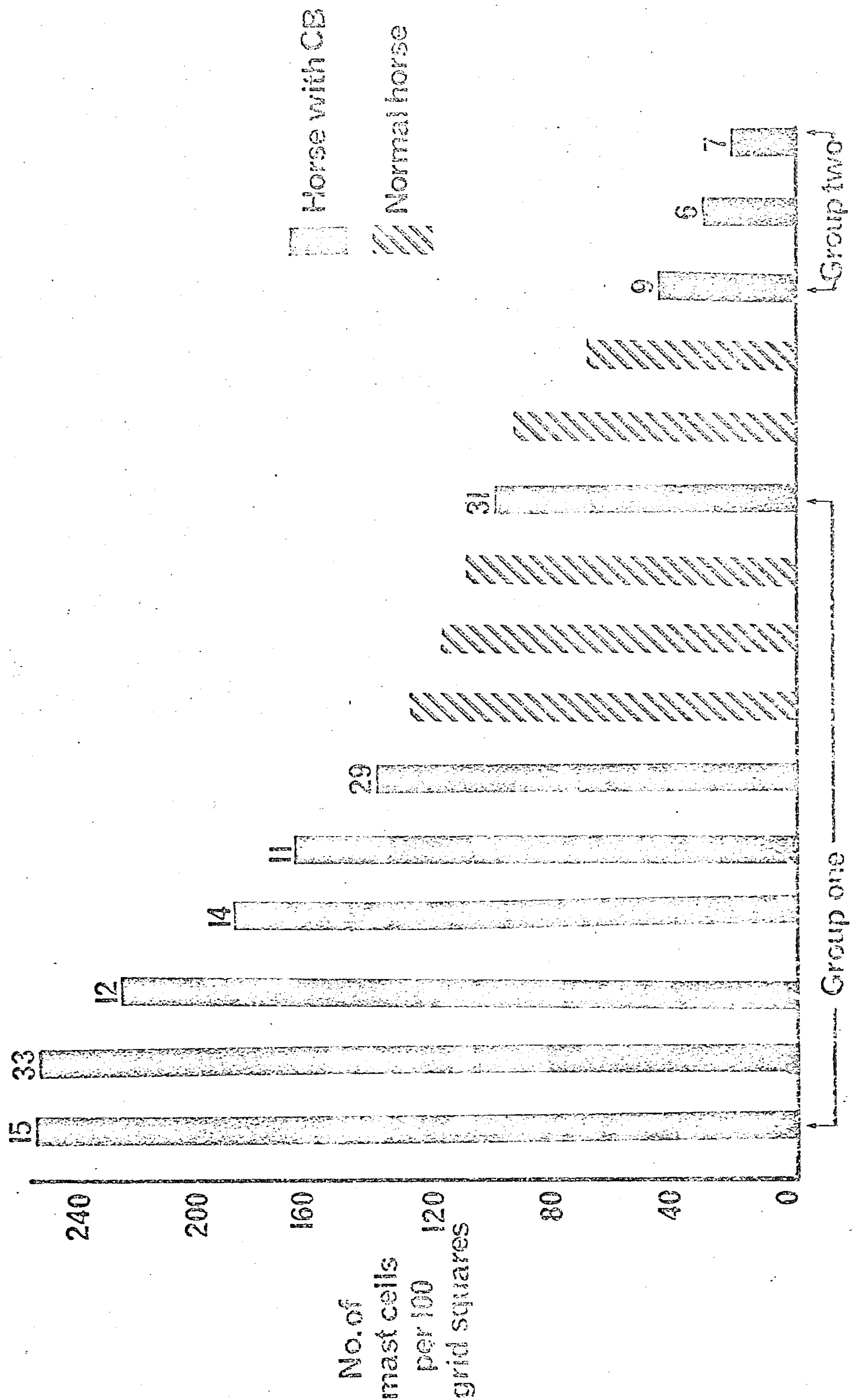


TABLE 17. Histogram showing total mast cell densities in ten horses with CPD and in five normal horses.

Sample	Value for t	Probability	
Total	3.04	< 0.02	> 0.01
Bronchial	1.96	< 0.1	> 0.05
Bronchiolar	2.11	< 0.05	> 0.02
Alveolar	2.50	< 0.05	> 0.02
Perivascular	3.06	< 0.01	> 0.001

TABLE 18. Mean mast cell densities of five normal horses compared with those of seven horses with CB in Group One analysed by students 't' test to find the significance ($p \leq 0.05$).

Horse Case Number	Perivascular Mast Cell Density	Left Ventricle + <u>Septum</u> Right Ventricle
15	57.03	2.795
12	48.26	2.105
29	47.43	1.430
11	39.96	1.444
14	37.36	3.580
33	32.57	4.000
31	31.17	2.968
9	6.56	3.333
6	4.55	3.052
7	3.02	3.569

TABLE 19. Perivascular mast cell densities per 100 grid squares in sections of uninflated pulmonary tissue from ten cases of CB, fixed in Carnoy's fluid and stained with toluidine blue at pH 4.0; arranged in decreasing order of magnitude and compared to the ratio $\frac{LV + S}{RV}$ for each horse.

There is no obvious correlation between the two values.

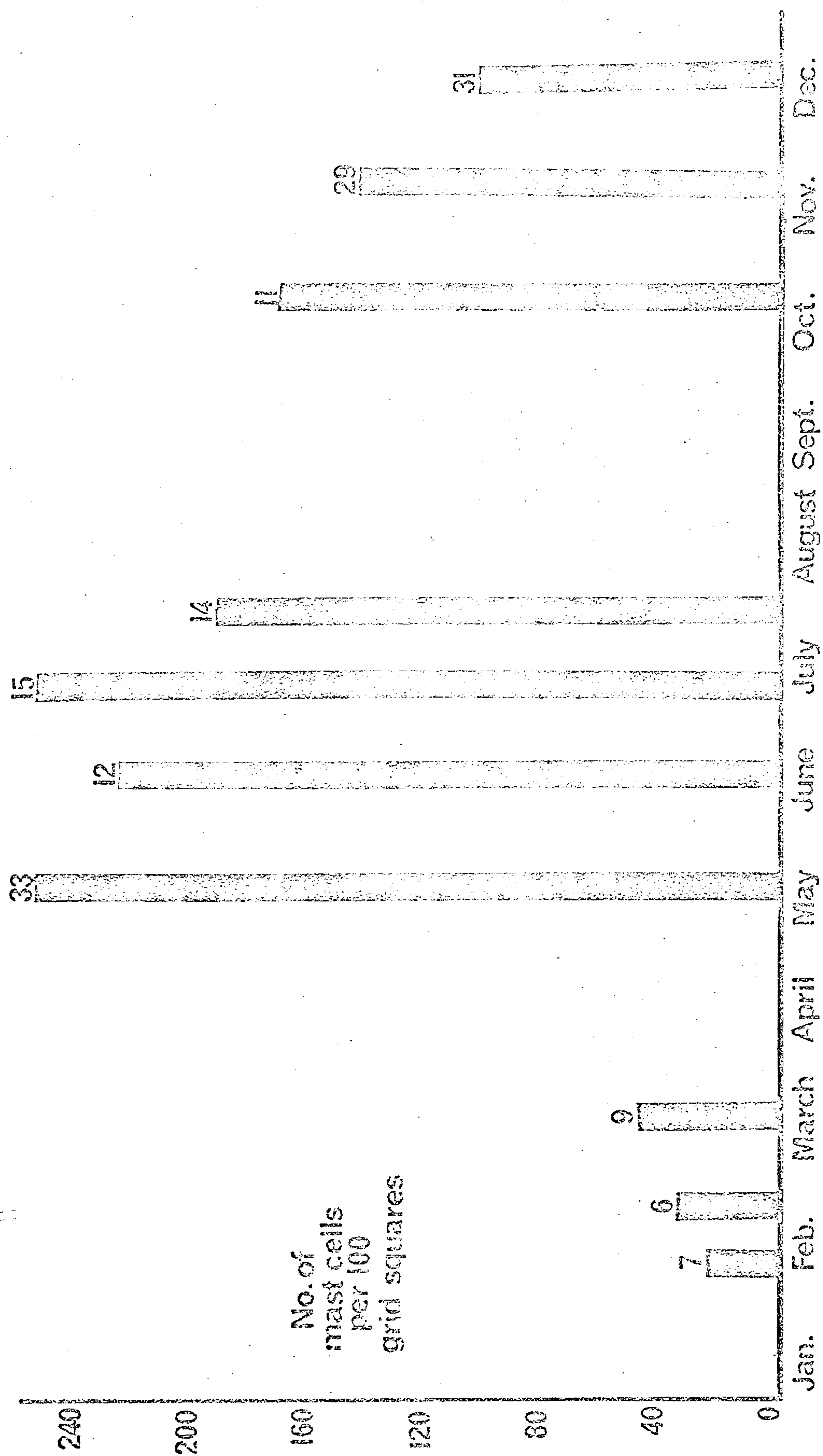


TABLE 20. Histogram showing the relationship between total mast cell density and time of year of necropsy of the horses with CB.

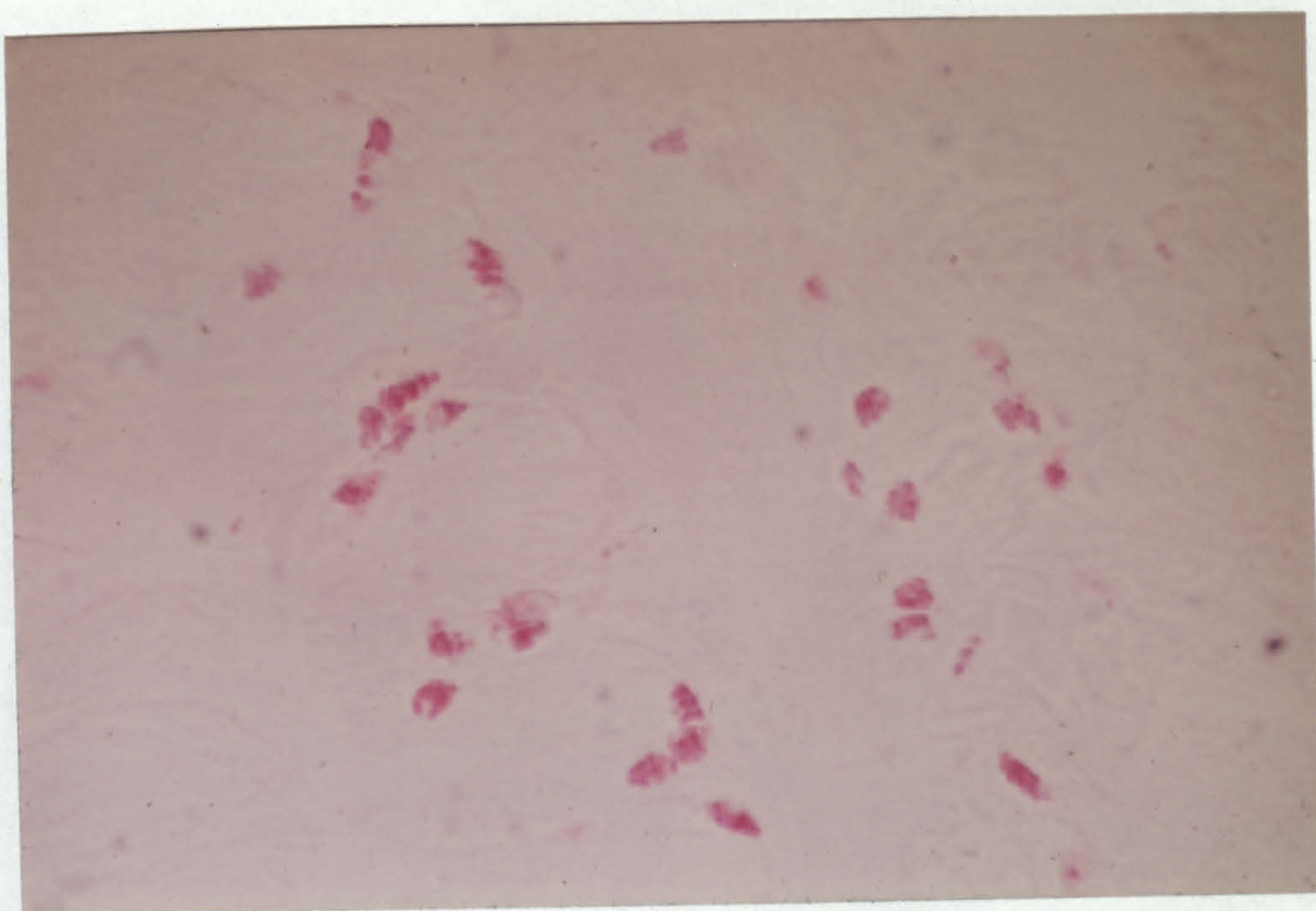


Figure 80. Biebrich scarlet staining of normal equine pulmonary tissue fixed in formol saline. A mixture of mast cells and eosinophils are stained and it is difficult to distinguish the two cell types. The background tissue is not stained. X 400.

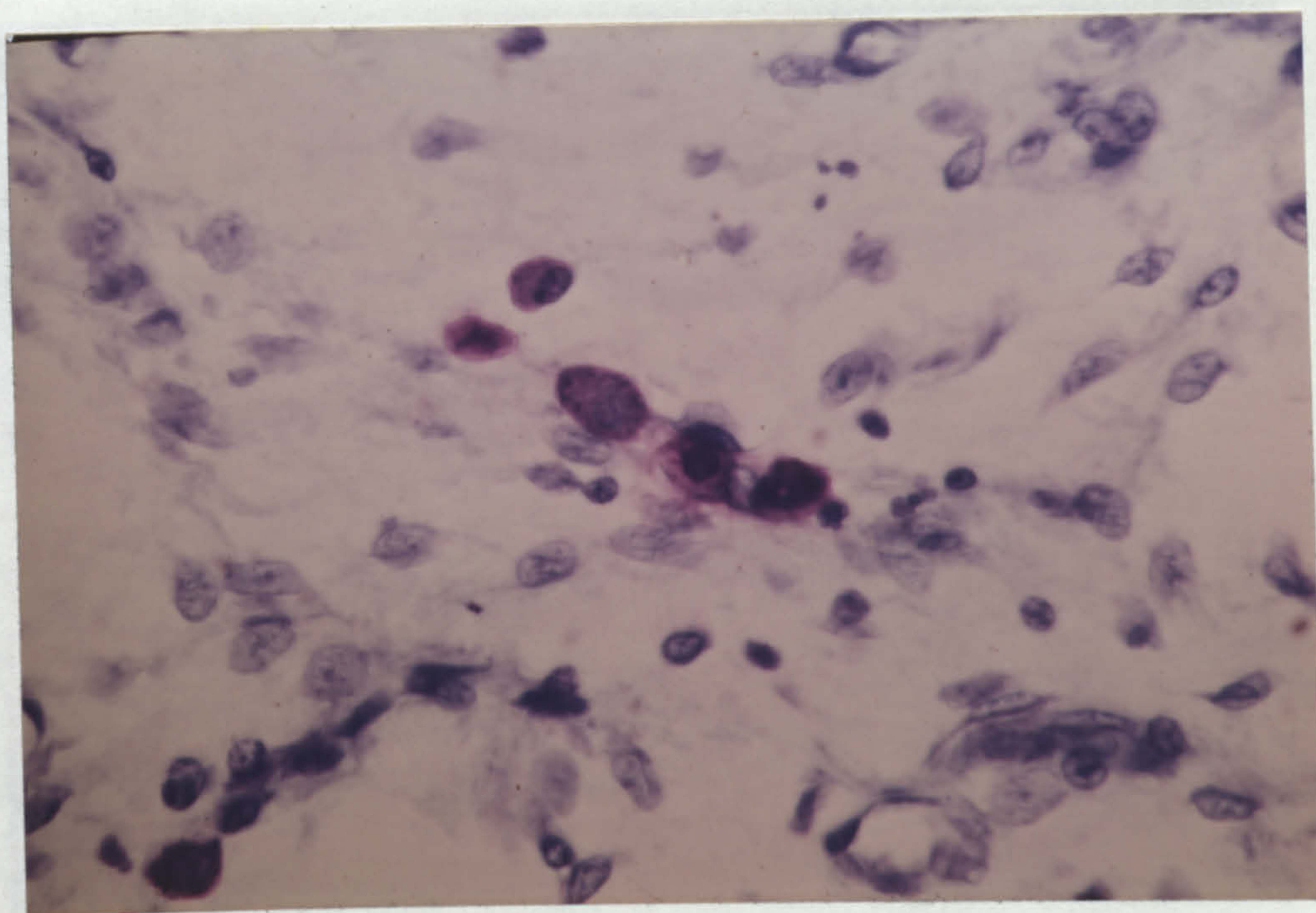


Figure 81. Toluidine blue pH 4.0 staining of normal equine pulmonary tissue fixed in IFAA. The morphology of the mast cells is well preserved and the granules have a deep easily seen purple colour. The background tissue stains blue and is easily discernible. This group of mast cells are spherical and the indented nucleus is seen in the uppermost one of the group. X 400.

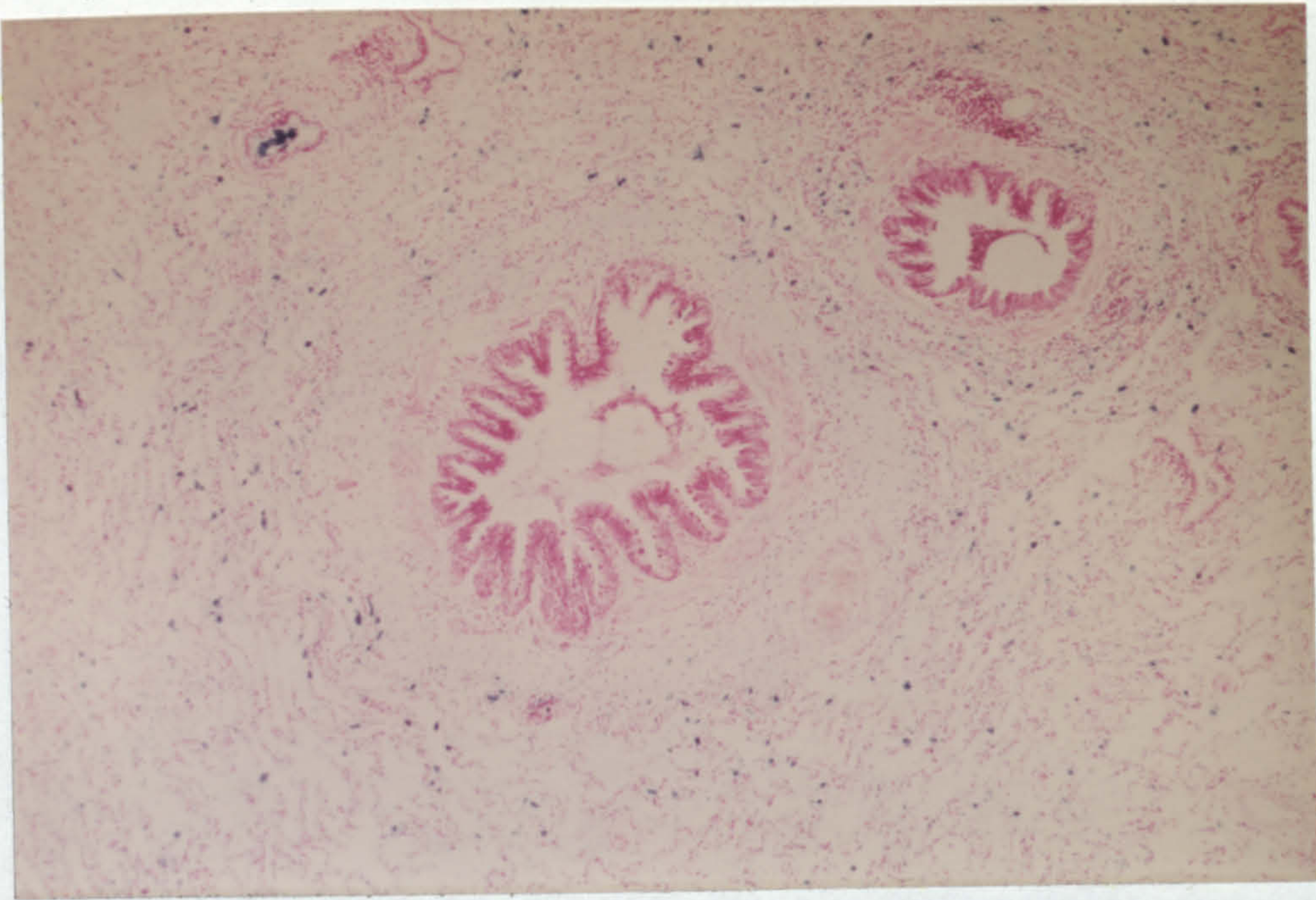


Figure 82. Astra blue-safranin staining of normal equine pulmonary tissue fixed in Carnoy's fluid. Even at this low power numerous blue mast cells can be seen around the two small airways. This is an area of unusually high mast cell density. X 110.

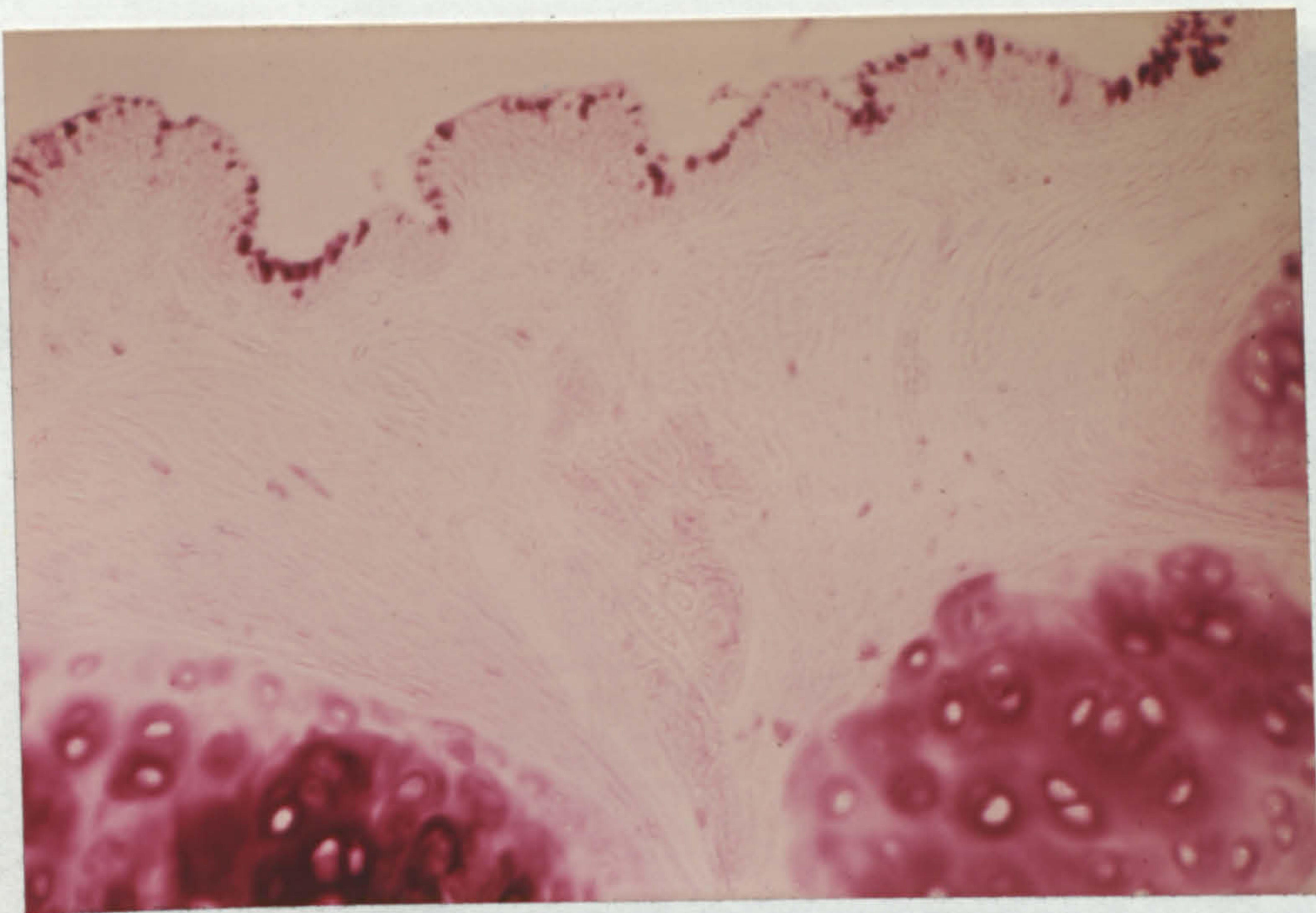


Figure 83. High iron diamine staining of normal equine bronchus. The cartilage and goblet cells have stained strongly with the stain. Numerous mast cells staining a paler brown are present in the subepithelial tissue. At higher magnifications mast cells are easily distinguishable from other cells. X 110.

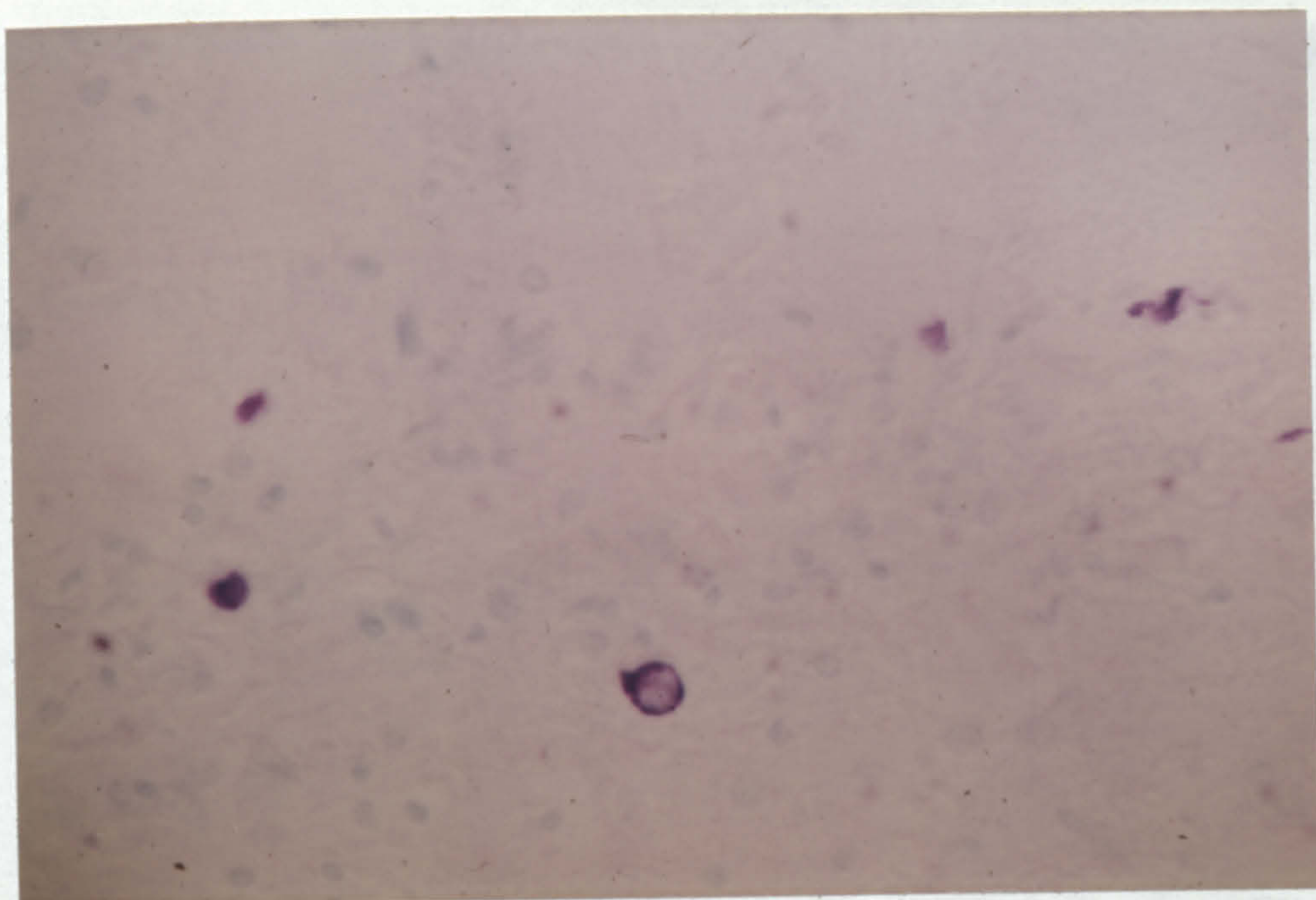


Figure 84. Section of normal equine pulmonary tissue stained with toluidine blue at pH 0.3. The mast cells stain vividly but the background tissue stains very poorly and is difficult to identify. X 400.

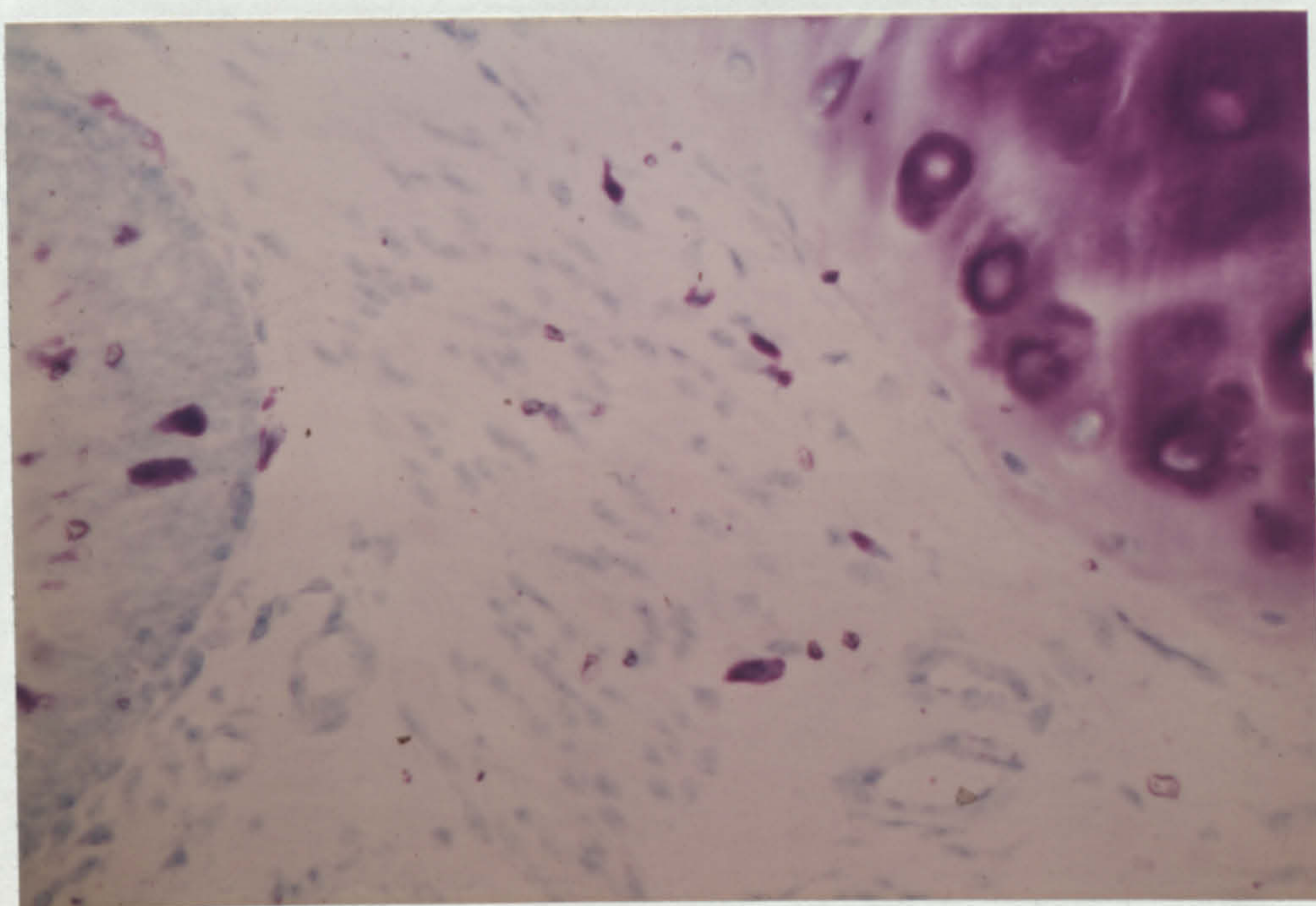


Figure 85. Section of normal equine bronchus fixed in Carnoy's fluid and stained with toluidine blue at pH 4.0. The cartilage and goblet cells are also strongly metachromatic and whilst sub-epithelial mast cells are identifiable epithelial mast cells cannot be definitely distinguished from goblet cells. Note the cellular processes on some of the mast cells. X 250.

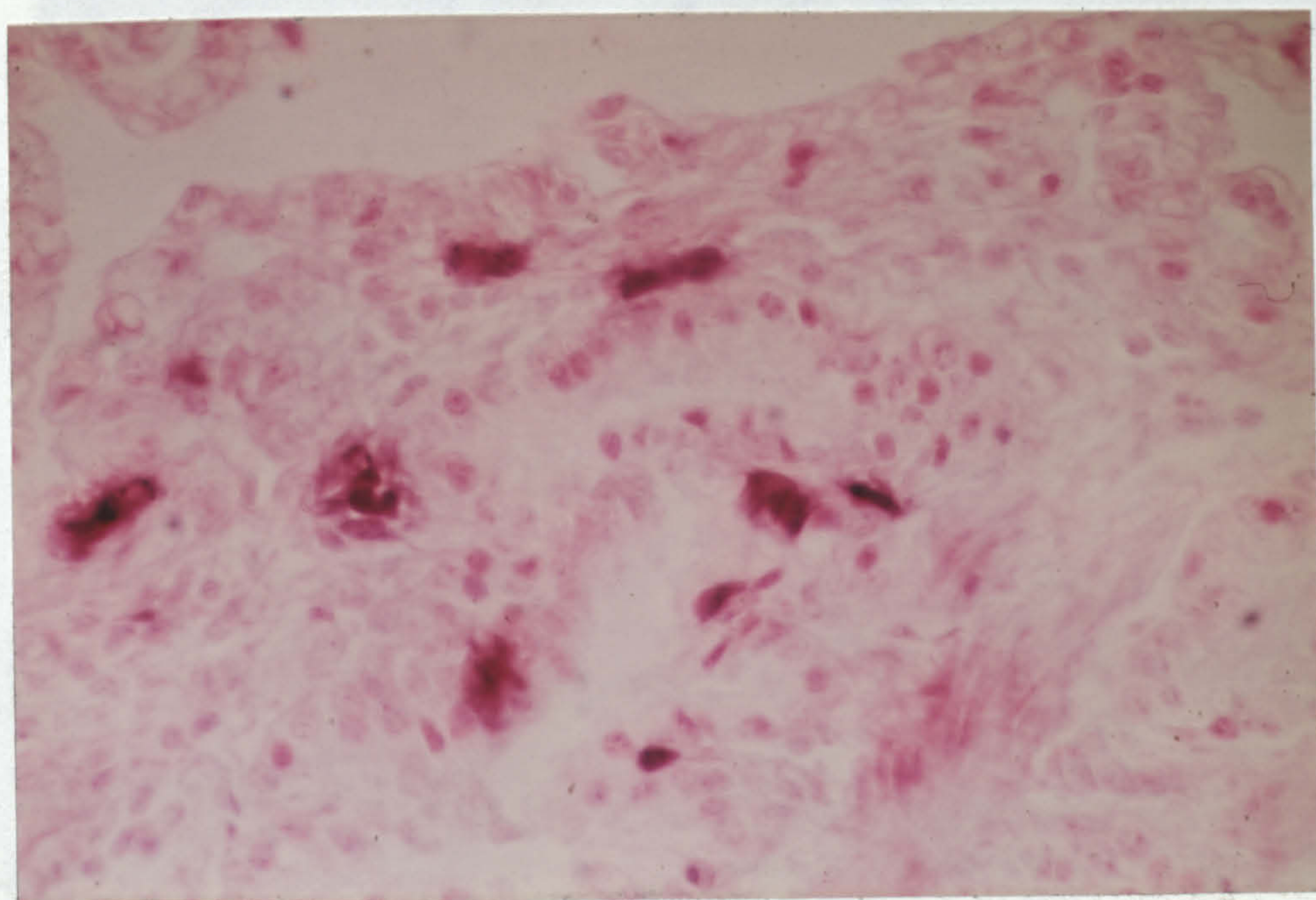


Figure 86. Section of normal equine pulmonary tissue stained with a combination of high iron diamine and Biebrich scarlet. Mast cells are grouped around the bronchiole. The cytoplasm shows a mixture of brown and red staining. Eosinophils in the same tissue would stain red only. X 250.

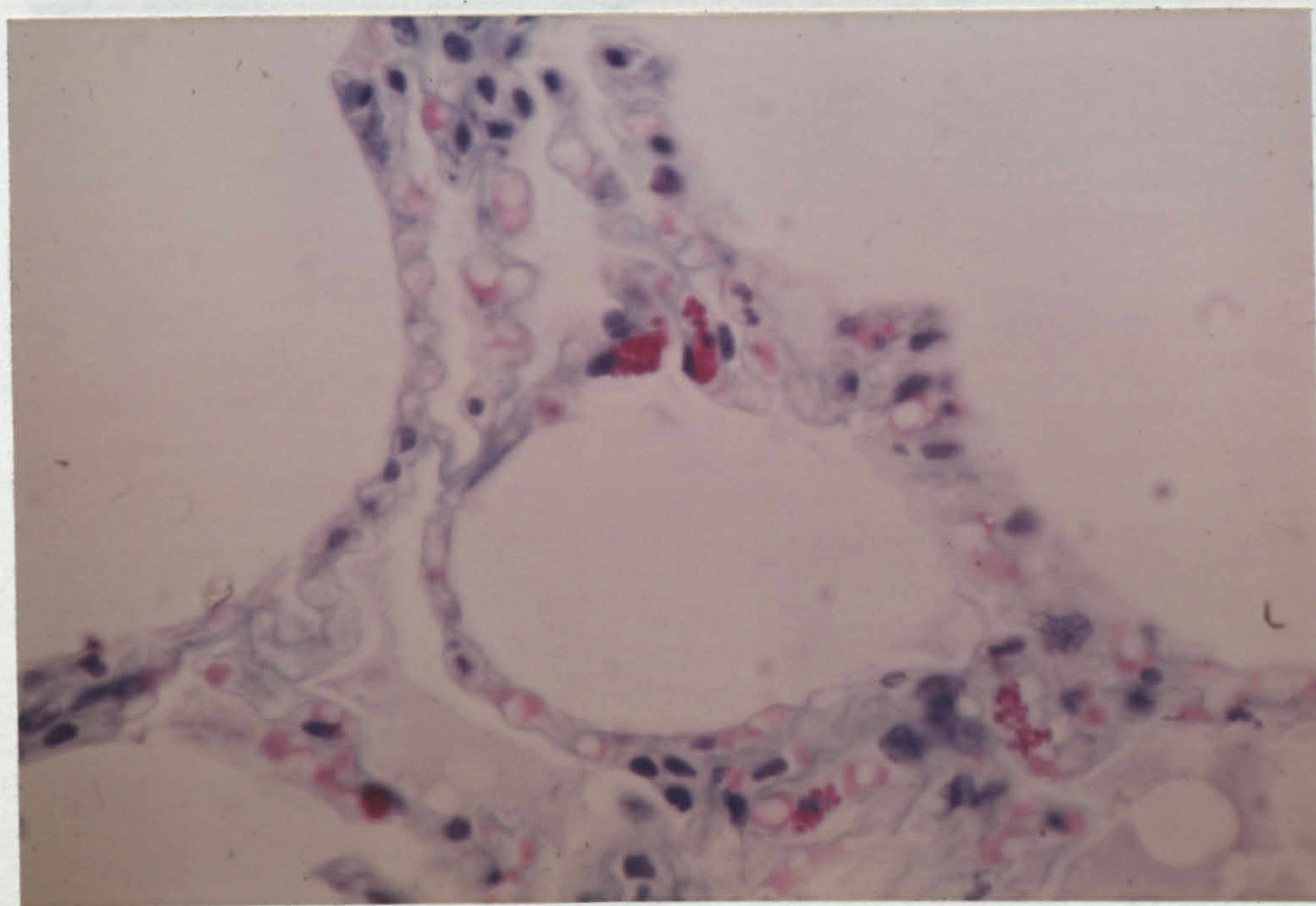


Figure 87. Alveolar tissue from normal horse fixed in formol saline and stained with Hogg's and Bank's stain. Eosinophils staining red with large granules are strikingly obvious and the smaller deep blue mast cells adjacent to the two upper eosinophils are also clearly stained. X 400.

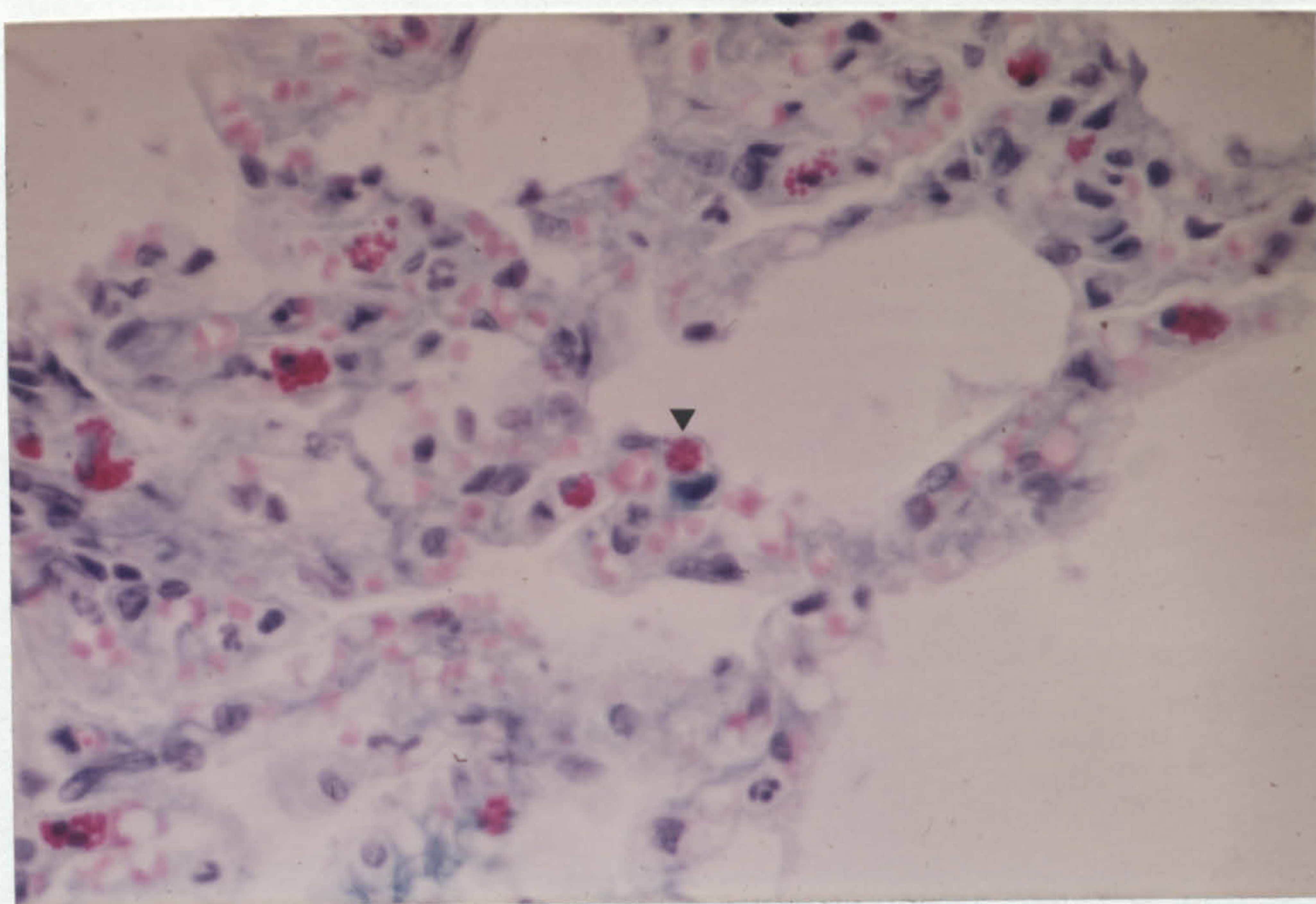


Figure 88. Similar section to the preceding one. Several eosinophils are visible, one of which is closely associated with a mast cell (arrow). X 400.

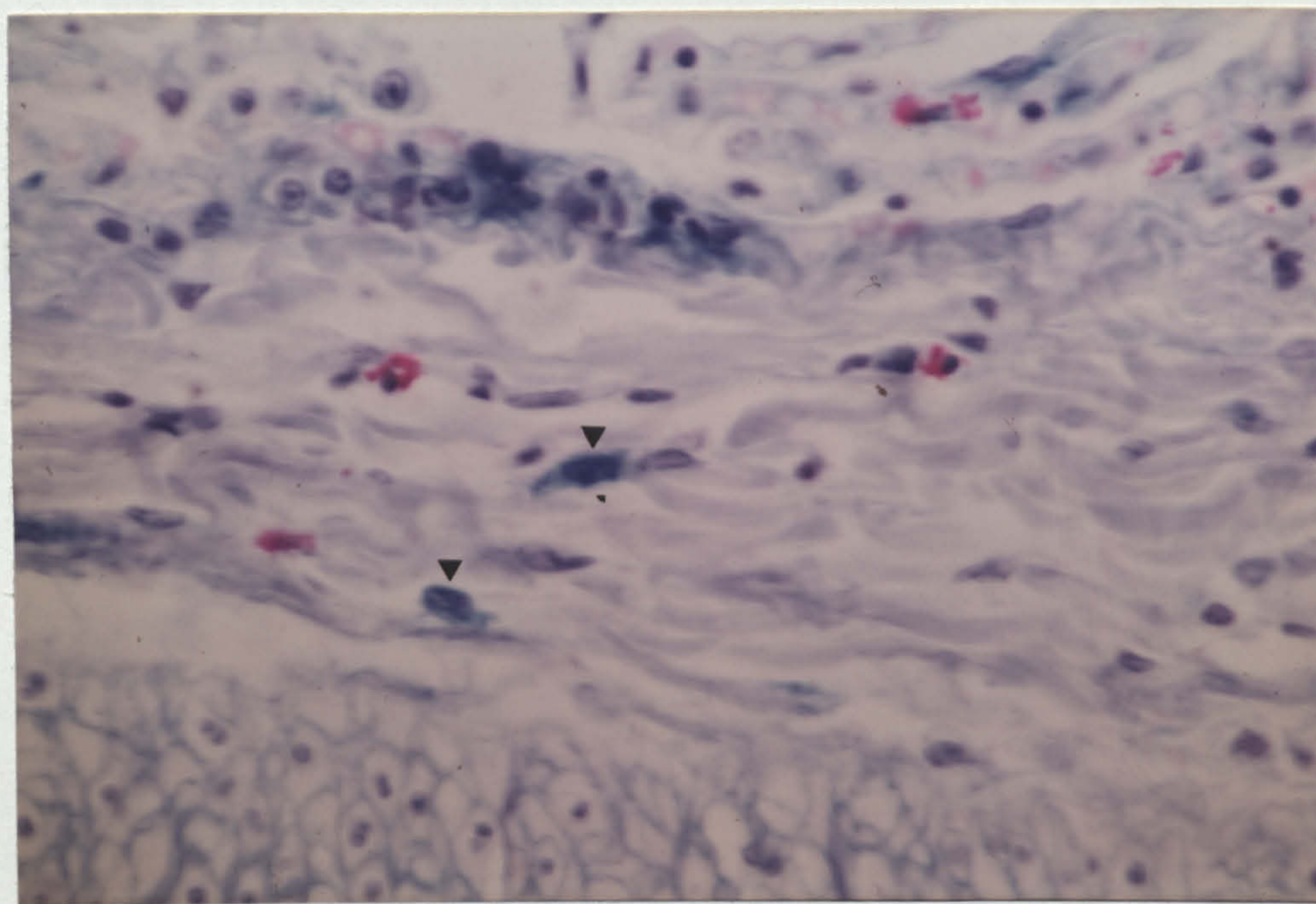


Figure 89. Subepithelial bronchial tissue stained with Hogg's and Bank's stain. Two large mast cells are visible (arrows).

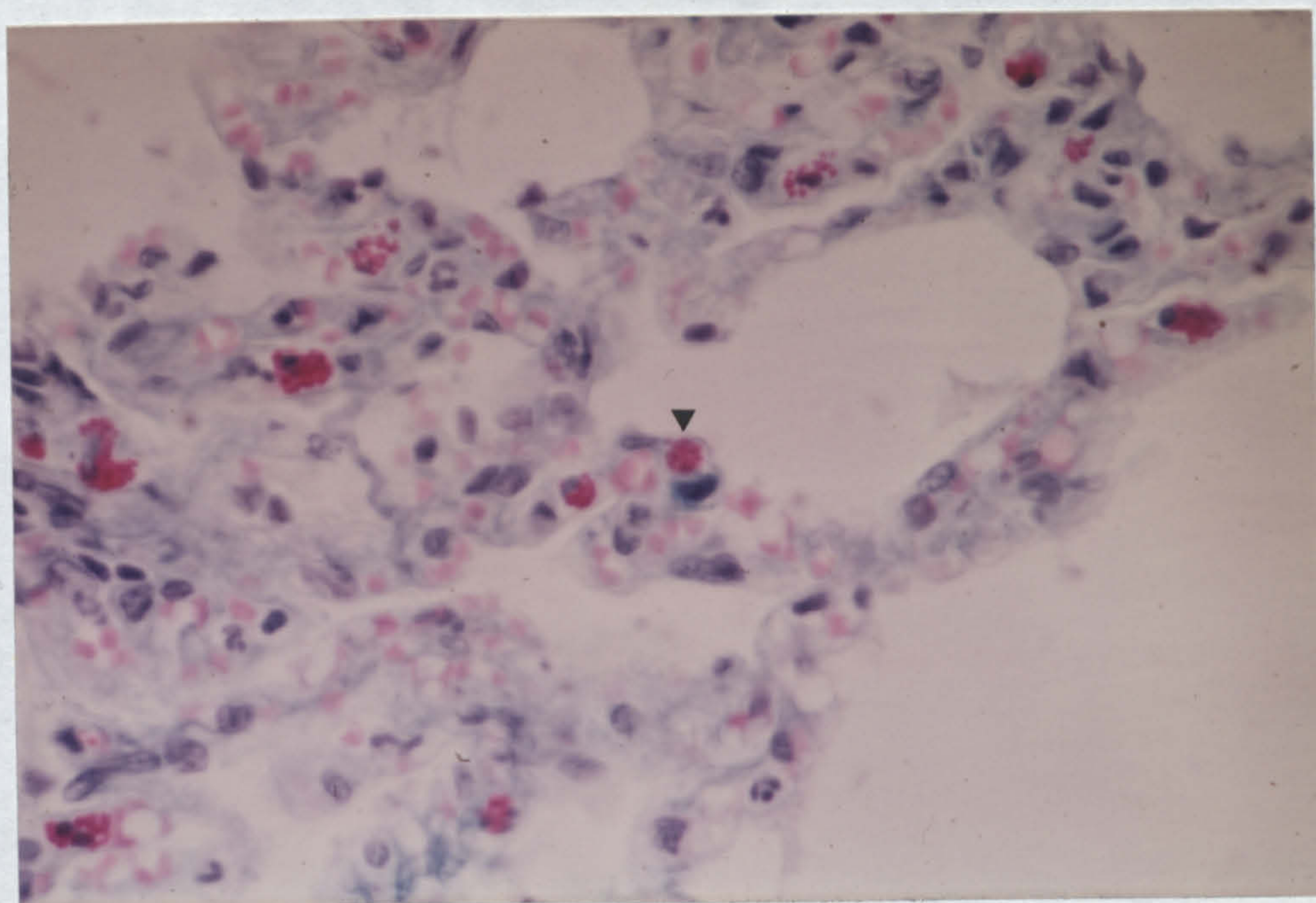


Figure 88. Similar section to the preceding one. Several eosinophils are visible, one of which is closely associated with a mast cell (arrow). X 400.

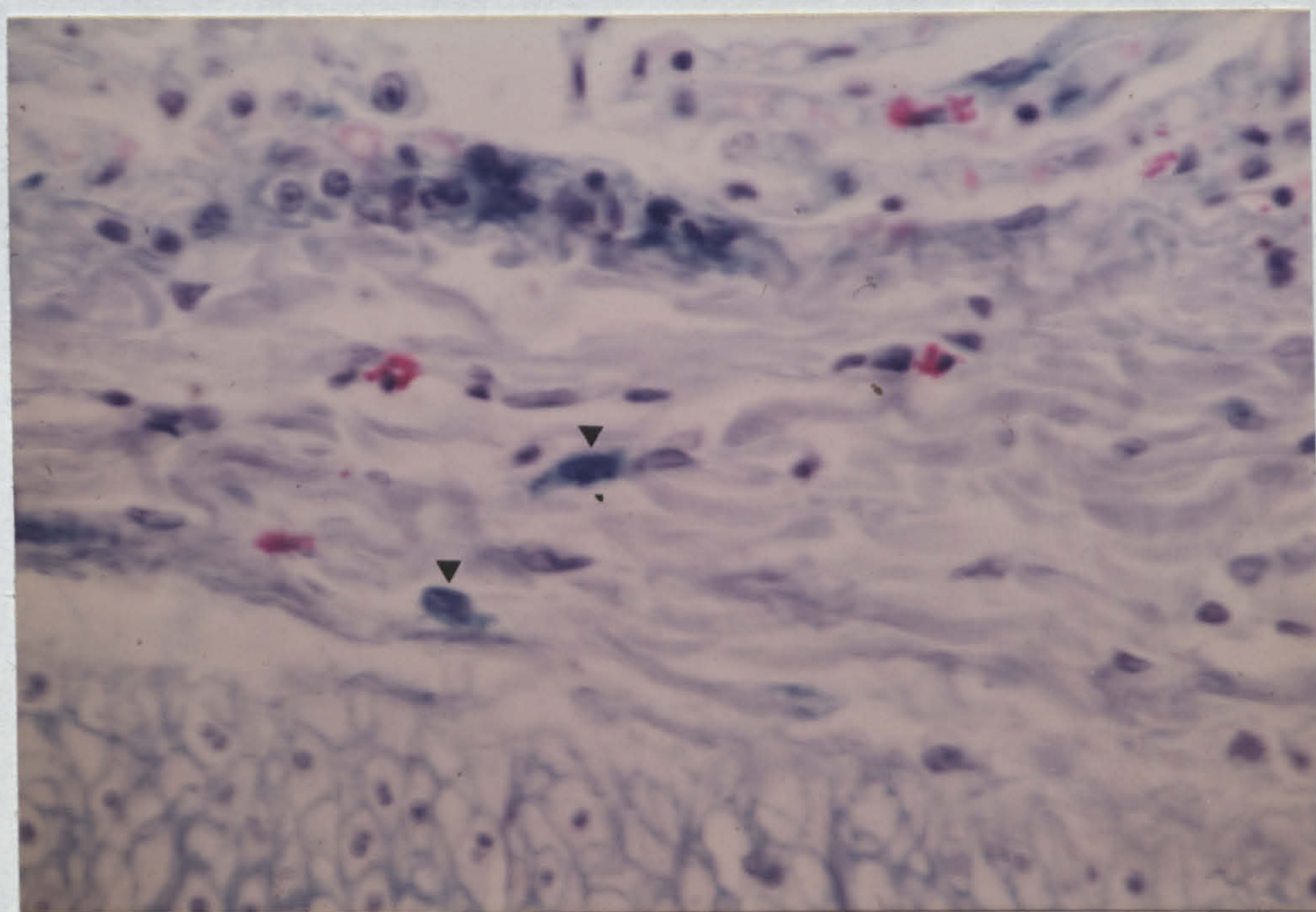


Figure 89. Subepithelial bronchial tissue stained with Hogg's and Bank's stain. Two large mast cells are visible (arrows).

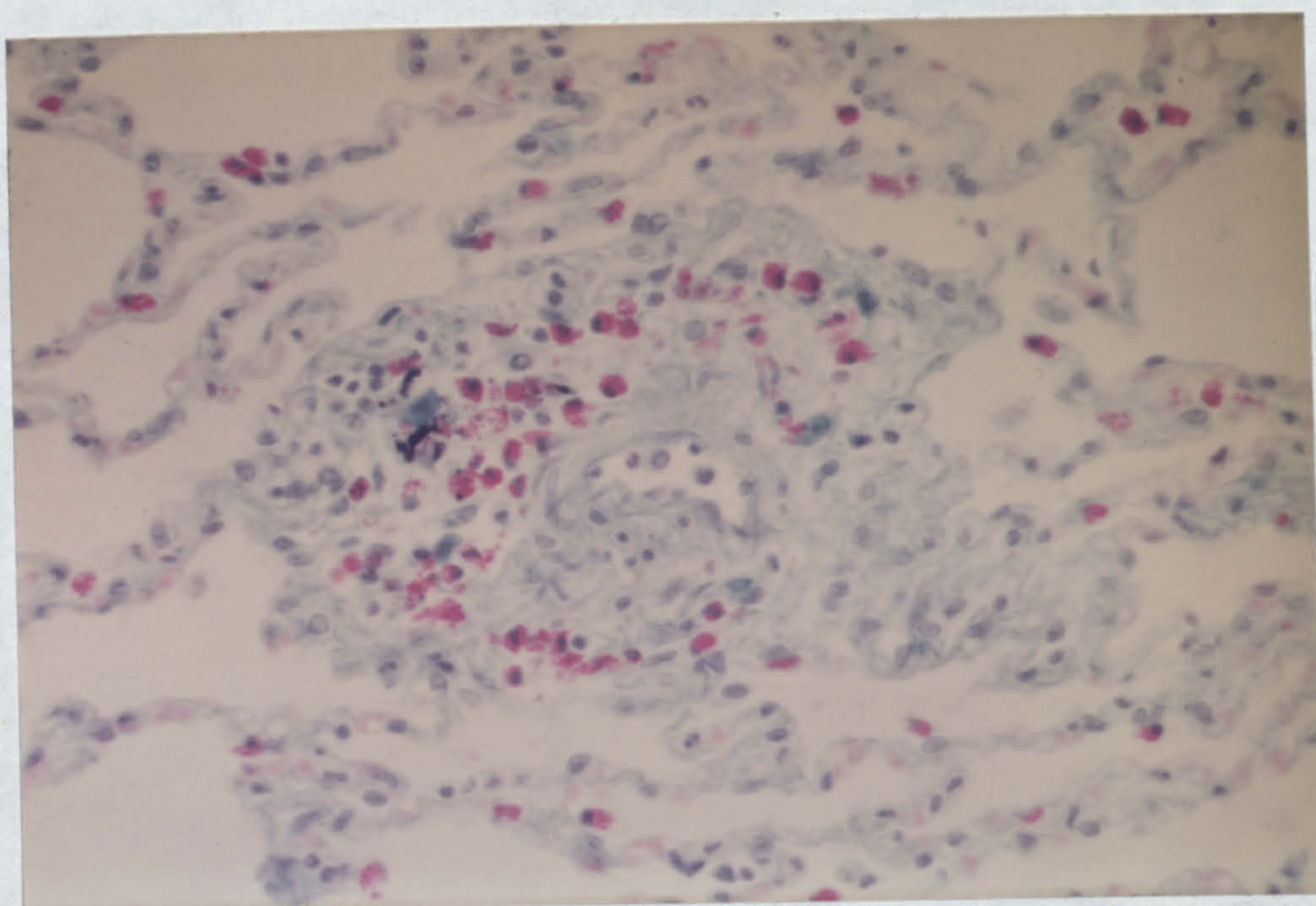


Figure 90. Small pulmonary blood vessel from a parasitised horse stained with Hogg's and Bank's stain. Numerous eosinophils are present but several mast cells are also grouped around the vessel. X 250.

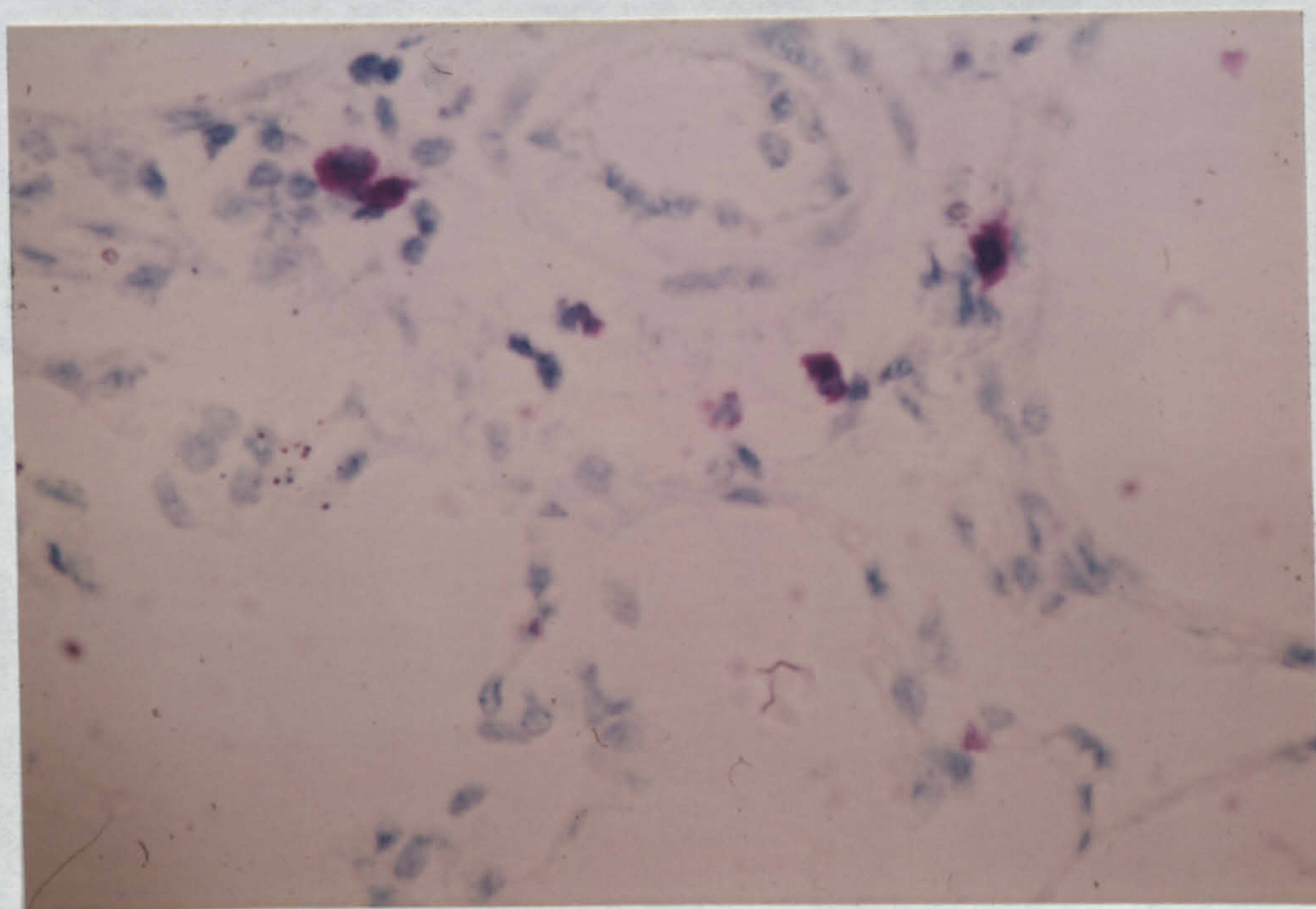


Figure 91. Small pulmonary blood vessel in a normal horse fixed in Carnoy's fluid and stained with toluidine blue at pH 4.0. Six mast cells are grouped around the periphery. X 250.

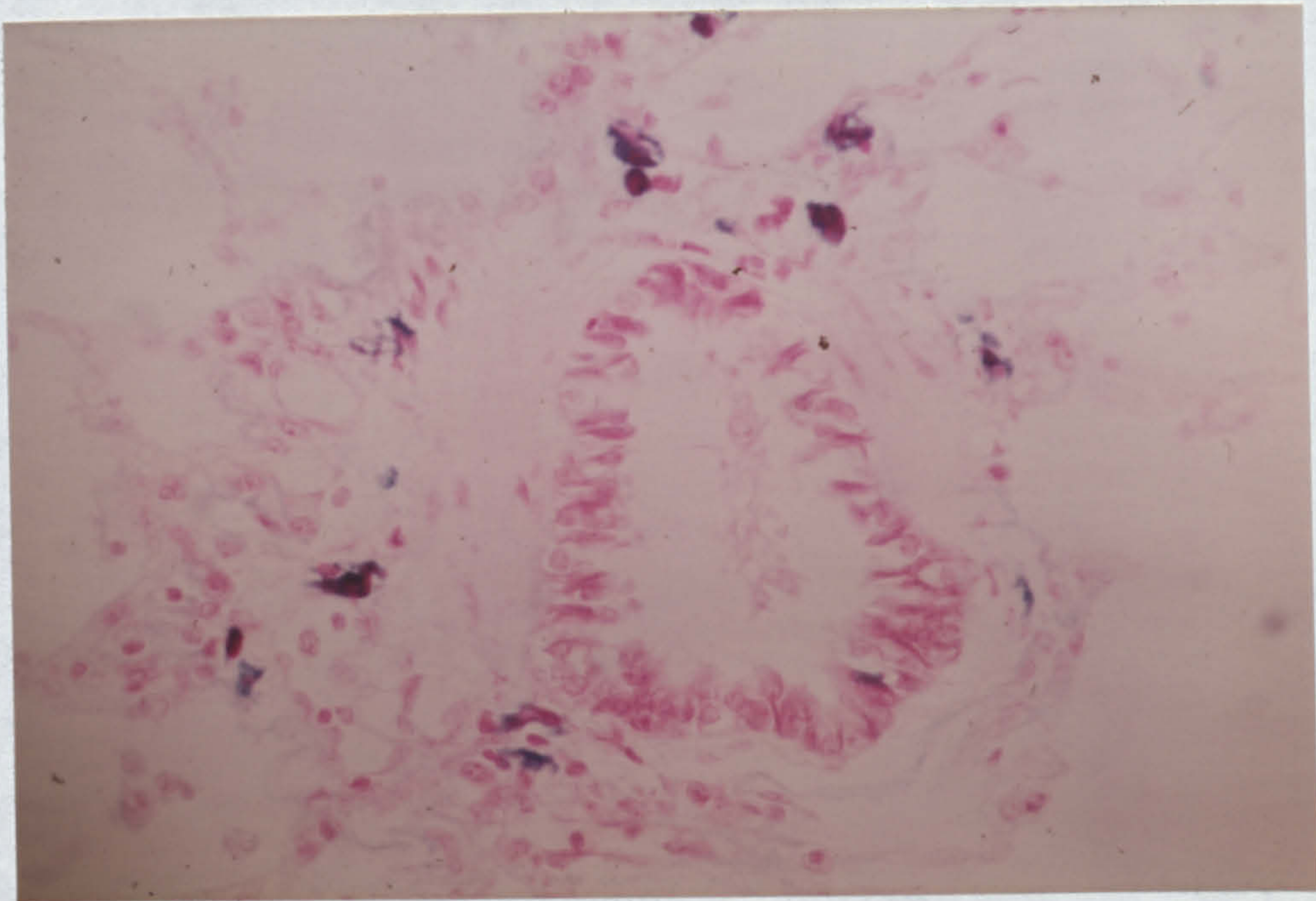


Figure 92. Bronchiole from a normal horse fixed in Carnoy's fluid and stained with astra blue-safranin. Many mast cells are shown around the airway some of which have stained with safranin rather than astra blue. This is uncommon and reflects their granular contents. X 250.

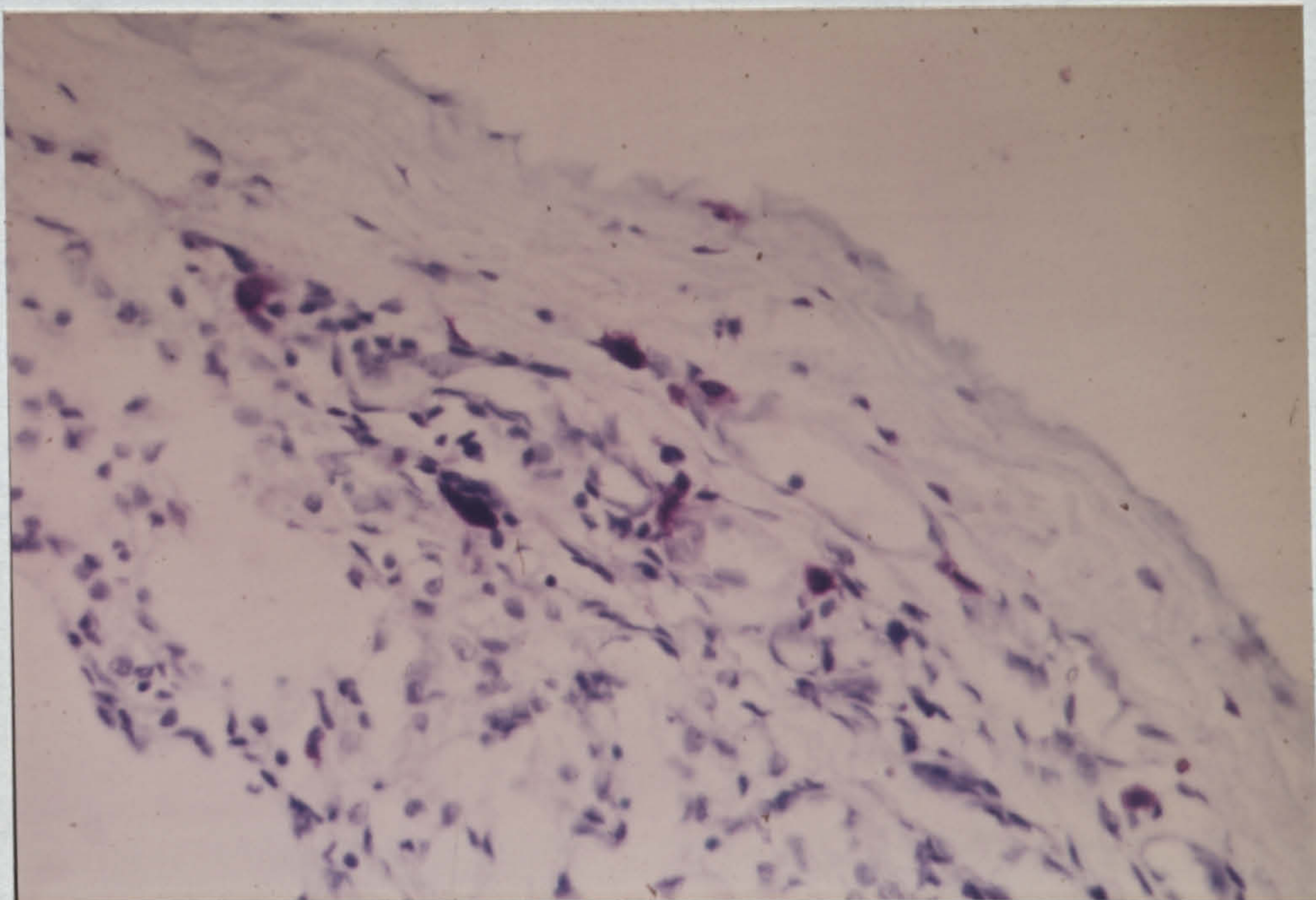


Figure 93. Subpleural mast cells stained with toluidine blue at pH 4.0. The mast cells are numerous and have assumed many morphological shapes. X 250.

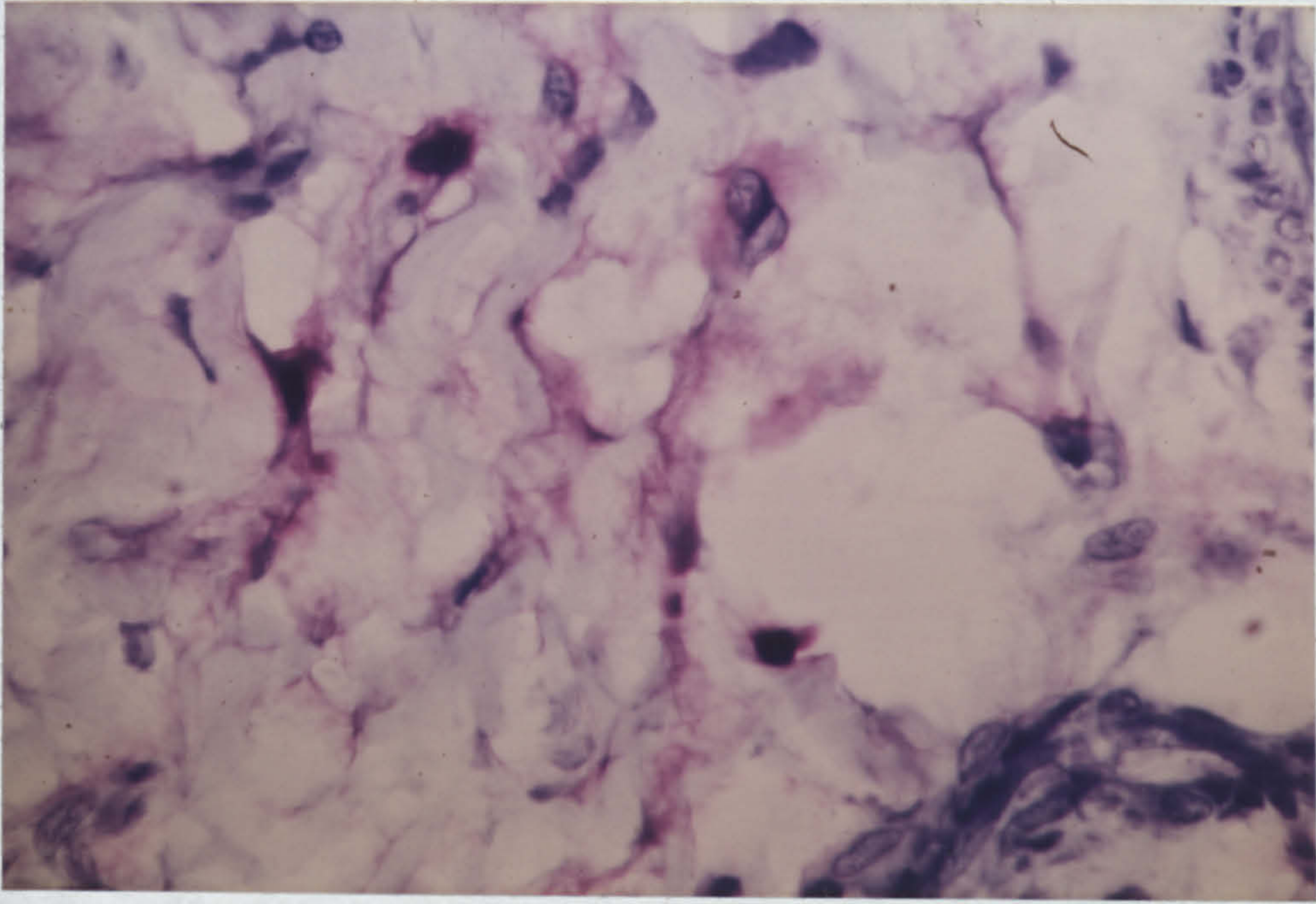


Figure 94. Section of bronchial tissue from a horse with CB stained with toluidine blue at pH 4.0. The mast cells have a very hazy irregular appearance. X 400.

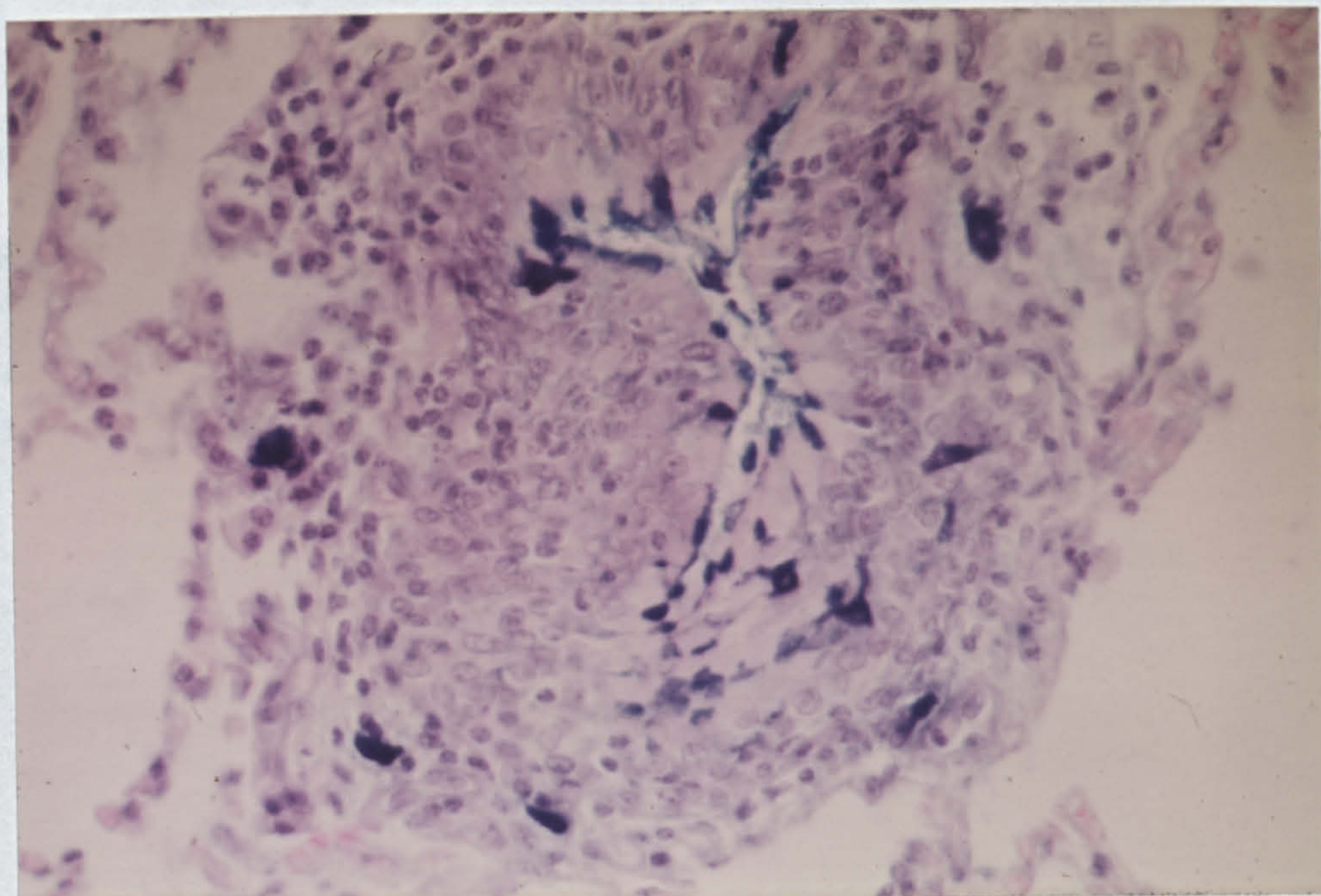


Figure 95. Section of a bronchiole from a horse with CB stained with toluidine blue at pH 4.0. There is no obvious increase in the numbers of mast cells although the bronchiole itself is markedly altered having a hyperplastic goblet cell-containing epithelium. X 250.

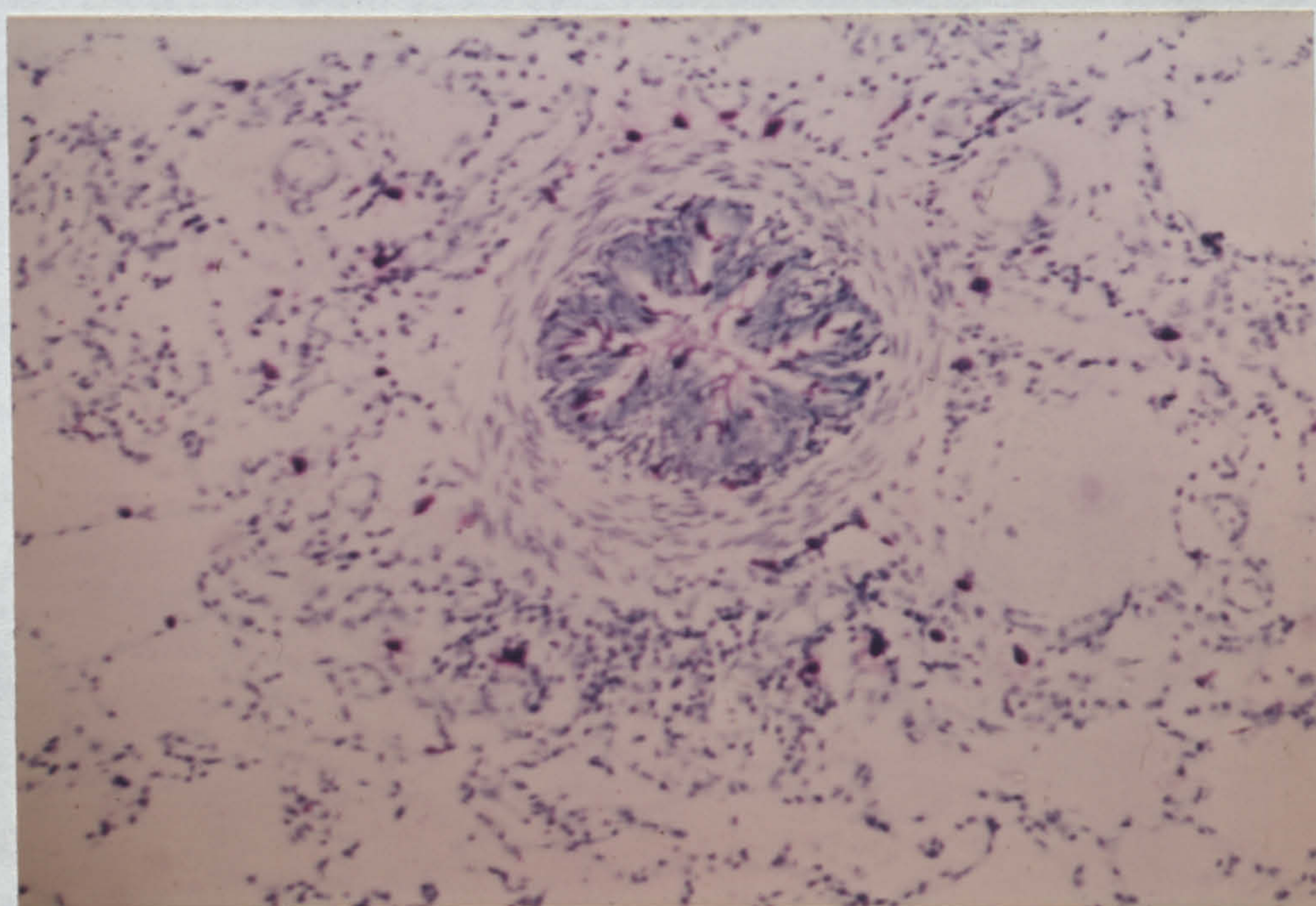


Figure 96. Section of a bronchiole from a horse with CB stained with toluidine blue at pH 4.0. A large number of mast cells are grouped around this airway and the smooth muscle is rather prominent. X 110.

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CHAPTER SIX

SERUM ANTITRYPSIN ACTIVITY IN HORSES WITH CHRONIC PULMONARY DISEASE

INTRODUCTION

The glycoprotein alpha-1-antitrypsin has the ability to inhibit or neutralize a number of proteolytic enzymes, including plasmin, thrombin, chymotrypsin, elastase, collagenase and some bacterial and granulocytic proteases (Kueppers 1971). Alpha-1-antitrypsin, the main component of the electrophoretic alpha-1-globulin fraction, is also the major serum trypsin inhibitor, providing over 90 per cent. of the total antitryptic activity (Jacobsson 1955; Schultze and others 1955); alpha-2-macroglobulin, alpha-1-antichymotrypsin and the inter-alpha-trypsin inhibitor make up the remainder (Johnson and Alper 1970). Deficiency of alpha-1-antitrypsin is a hereditary dysproteinaemia which is associated with the development of early-onset panacinar emphysema and chronic obstructive pulmonary disease in adults (Laurell and Eriksson 1963; Eriksson 1964) and of liver disease in children (Sharp and others 1969) and adults (Berg and Eriksson 1972; Eriksson and others 1975). The pathogenesis of the emphysema in alpha-1-antitrypsin deficiency is uncertain, but it is possible that lack of inhibitor allows proteolytic enzymes in inflammatory exudates, or released from leucocytes sequestered in the pulmonary capillary bed, to destroy the pulmonary parenchyma (Eriksson 1965; Kueppers and Bearn, 1966).

Gilliespie and Tyler (1969) could find no evidence to support the idea that horses with chronic pulmonary disease had an inherited predisposition to emphysema, as had been suggested by Law (1896), but drew attention to the recent discovery of the association between hereditary alpha-1-antitrypsin deficiency and early-onset emphysema in man and the parallels that might be drawn in the horse. The results of an investigation of serum antitrypsin activity in a series of horses and ponies with chronic pulmonary disease that were examined clinically and at necropsy are described.

MATERIALS AND METHODS

Animals

There were three groups of animals. Group One consisted of nine horses and ten ponies with confirmed chronic pulmonary disease. These animals had been examined clinically by the methods outlined by McPherson and Lawson (1974) and the clinical signs, histories and post-mortem findings have been described in chapters 2 and 3. Group Two was made up of 17 horses and 11 ponies with a history of cough and respiratory disease of several weeks or months duration; these horses were not examined at necropsy. The third group contained 28 horses, 16 ponies and two donkeys that had no clinical signs or recent history of respiratory disease; five of these animals were examined post mortem.

Sera

Human serum from a healthy young adult male was used as control; this serum had been stored at -20°C for three years. Serum was taken from each animal and examined fresh or after storage at -20°C ; in the animals of group one, the period of storage was up to two years.

Serum trypsin inhibitory capacity

Serum trypsin inhibitory capacity was measured by the method of Erlanger and others (1961) using benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate in tris (hydroxymethyl) aminomethane buffer without CaCl_2 , as suggested by Troyer and Moskowitz (1968). The relative amount of BAPNA hydrolyzed was determined by measuring the absorption at 410m μ on a Unicam SP600 spectrophotometer. The serum trypsin inhibitory capacity (STIC) was expressed as mg trypsin inhibited per ml of serum.

Twice-recrystallized, salt-free trypsin and BAPNA were obtained from Sigma Laboratories and tris (hydroxymethyl) aminomethane through British Drug Houses.

Electrophoresis

Electrophoresis of serum samples was performed on cellulose acetate strips (Cellogel, Reeve Angel Scientific Ltd.) for 70 min at 200 V in barbitone buffer at pH 8.6. Strips were stained with Ponceau S and the distribution of dye density in the strips was measured using a Chromoscan (Joyce Loebble Ltd.) and the percentage of total protein in each peak was calculated. Total serum protein was estimated by the biuret method of Weichselbaum (1946).

Post-mortem and histopathological examinations

All the horses in Group One and five of those in Group Two were examined in detail post mortem. The procedure is described in Chapter 3. In addition, several portions of tissue, each about 2 x 1 x 0.5 cm, were taken routinely from representative sites in the liver of most animals. These samples were fixed in 10 per cent. formalin and processed by routine methods. Sections cut at 6-8 μ m and stained by haematoxylin and eosin, PAS before and after diastase and picro-Mallory were examined by two independent observers.

RESULTS

Serum trypsin inhibitory capacity

Individual STIC values are shown in Figure 97 and the mean STIC values in the horses and ponies of the three groups are set out in Table 21. Statistical analysis using the "student's" t test revealed no significant differences between the mean STIC values of horses and ponies within a group. Using the same method, no significant differences were found between mean STIC values of groups one and three or of groups two and three.

The STIC of the control human serum was 1.1 mg per ml.

Serum proteins

Electrophoresis of control human serum demonstrated a prominent alpha-1-globulin peak (Figure 98c) but electrophoresis of horse serum under the same conditions failed to separate alpha-1 and alpha-2 globulins in almost half the animals (Figures 98a and 98b). A measurable alpha-1-

globulin peak was found in nine animals of Group One, range 1.9-4.1, mean 2.73 g per l (standard deviation 0.72). The corresponding figures in 15 animals of Group Two were 1.7 - 4.9, mean 2.9 g per l (standard deviation 0.99) and in 26 animals of Group Three 1.0 - 4.5, mean 2.70 g per l (standard deviation 1.05). No significant differences were found between the mean values in Groups One and Three or Groups Two and Three, using the t test.

The results of measurements of total serum protein and serum albumin, alpha, beta and gamma globulins are set out in Table 21. There were no significant differences in mean values of each of these measurements between horses and ponies within a group except in Group Three, where there was a small difference in mean levels of serum albumin ($0.05 > p > 0.02$), and beta globulin ($0.05 > p > 0.02$). Mean values of total serum protein, alpha and gamma globulin did not differ significantly between Groups One and Three or Two and Three. Comparison of mean albumin and beta globulin levels in horses of Groups One and Three or Two and Three revealed no significant differences, nor were any found when a similar comparison was made of values in the ponies of these groups.

Histopathology of liver

Adequate samples of liver were available from five horses and eight ponies of group one. No PAS-positive, diastase-resistance inclusions were found in the cytoplasm of liver parenchymal cells of any animal.

DISCUSSION

The concentration of serum alpha-1-antitrypsin is controlled by alleles of a pair of fully penetrant codominant genes that produce variants of the normal antitrypsin molecule (Fagerhol 1967; Fagerhol and Laurell 1967). Certain variants are associated with low serum alpha-1-antitrypsin levels and in these cases it appears that deficiency is the result of reduced activity of the particular molecule. These different molecules have characteristic but slightly variable electrophoretic mobilities and are designated by letters that correspond to the various alleles, which

are known collectively as the Pi (protease inhibitor) system (Fagerhol and Braend 1965; Fagerhol and Laurell 1967). Most normal individuals are homozygous for the Pi^M gene (phenotype $PiMM$) and their serum alpha-1-antitrypsin molecules have a medium (M) electrophoretic mobility. Variants with faster mobility have letters preceding M in the alphabet and variants with slower mobility have been assigned letters after M. The slowest moving protein is the Z variant and it is this allele, Pi^Z , which is most often associated with severe alpha-1-antitrypsin deficiency. Individuals homozygous for the Pi^Z gene (phenotype $PiZZ$) have serum concentrations of alpha-1-antitrypsin corresponding to 12 per cent. or less of those found in individuals with the Pi^M genotype (Kueppers 1972). Heterozygotes have approximately 60 per cent. or less of the normal alpha-1-antitrypsin concentration and most of these people are phenotypically $PiMZ$ (Eriksson 1965; Fagerhol 1967; Lieberman 1975).

To date, at least 24 different identifiable phenotypes and 15 different codominant alleles have been identified, but early-onset emphysema has only been clearly associated with severe alpha-1-antitrypsin deficiency in Pi^Z individuals (Kueppers and Black 1974; Eriksson, Moestrup and Hagerstrand 1975). There is still some argument about the incidence of chronic obstructive pulmonary disease in $PiMZ$ heterozygotes (Kueppers and Black 1974), since an increased prevalence has been found by some workers but not by others. Much of this apparent disagreement has resulted from the use of different methods for identifying heterozygotes and different criteria for assessing respiratory disability or pulmonary lesions. However, the present consensus of opinion is that intermediate deficiency in $PiMZ$ individuals contributes to the development of emphysema (Lieberman 1975). The prevalence of chronic obstructive pulmonary disease in subjects of other Pi phenotypes (e.g. $PiSS$, MS) is even less clear (Eriksson, Moestrup and Hagerstrand 1975; Lieberman 1975). Thus, it is sufficient to recognise : normal individuals ($PiMM$) with STIC of 0.85 - 1.40 mg per ml; heterozygous individuals (mostly $PiMZ$) with STIC of 0.4 - 0.8 mg per ml; and homozygous deficient individuals (mostly $PiZZ$) with STIC of less than 0.40 mg per ml

(Crofton and Douglas 1975). Total lack of alpha-1-antitrypsin is a very rare form of deficiency that has been reported in individuals with a null or inactive gene, Pi- (phenotype Pi blank) (Talamo and others 1973; Martin, Vaudeville and Ropartz 1973).

The results of our study demonstrate that pulmonary disease in the horses and ponies of groups one and two was not associated with deficiency of serum trypsin inhibitory capacity. The lowest STIC value of group one (1.2 mg per ml) or group two (1.25 mg per ml) was greater than the lowest of group three (1.00 mg per ml). No significant difference was detected between the mean STIC values of animals in groups one (1.64 mg per ml) and three (1.61 mg per ml) or between the mean values of groups two (1.73 mg per ml) and three. None of the animals in groups one and two had STIC in the range characteristic of human homozygous deficient individuals (PiZZ) and it may be inferred from this that none of the horses had a genotype corresponding to Pi^Z.

It is not possible to determine the heterozygote (PiMZ) state by examination of STIC alone, since persons deficient in alpha-1-antitrypsin retain the capacity to respond to various inflammatory and hormonal stimuli by elevation of the alpha-1-antitrypsin concentrations into the normal range (Kueppers 1968; Lieberman, Mittman and Kent 1971). Thus, heterozygotes can be accurately identified only by antigen-antibody crossed electrophoresis in combination with starch-gel electrophoresis (Laurell 1965; Fagerhol 1972), a procedure which determines the phenotypic variant of the antitrypsin molecule. However, in PiZZ individuals and in carriers of the Pi^Z gene there is an accumulation of PAS-positive, diastase-resistant inclusions within liver parenchymal cells (Sharp 1971). These inclusions contain alpha-1-antitrypsin molecules without a terminal sialic acid moiety; the incomplete alpha-1-antitrypsin accumulates in dilated portions of endoplasmic reticulum and is not released into the circulation (Lieberman, Mittman and Gordon 1972; Feldman and others 1974; Eriksson and Larsson 1975). Such PAS-positive inclusions occur in PiZZ individuals irrespective of the presence

or absence of liver disease (Gordon, Dixon and Rogers 1972) and are also found, in lesser numbers, within liver parenchymal cells of carriers of the Pi^Z gene, such as $PiMZ$ individuals (the most common heterozygotes), but not in association with any other phenotype within the Pi system (Gordon, Dixon and Rogers 1972; Morin and others 1975). The genetics of the equine Pi system are unknown, but the absence of PAS-positive, diastase-resistant inclusions in liver parenchymal cells of 13 animals in group one suggests that these 13 animals do not correspond to the common heterozygous ($PiMZ$) individuals with an intermediate level of alpha-1-antitrypsin deficiency. In other words, as far as we are able to determine at present, there was no demonstrable association between serum alpha-1-antitrypsin deficiency and chronic pulmonary disease in the group one animals of our series. This statement does not mean that alpha-1-antitrypsin deficiency should be discounted in studies of the pathogenesis of equine chronic pulmonary disease, since only about 17-25 per cent. of humans with chronic obstructive pulmonary disease are heterozygotes (mostly $PiMZ$) (Lieberman 1969; Kueppers, Fallat and Larsen 1969) and only 10 per cent. or less are homozygously deficient ($PiZZ$) (Tarkoff, Kueppers and Miller 1968; Lieberman 1969).

The prevalence of alpha-1-antitrypsin deficiency states in the horse will only be determined by a large-scale screening programme. Until genotyping is developed for the horse, the most simple and useful test for this purpose is measurement of STIC, provided it is noted that this is a measure of the total serum antitrypsin capacity, which is made up of several fractions. Routine serum electrophoresis on cellulose acetate provides a distinct alpha-1-globulin peak that can be used for initial screening in man (Lieberman 1975) but this was not found to be of value in our series. Negative immunoelectrophoresis has also been used as a screening procedure to detect alpha-globulin deficiency in turkeys with round heart disease (Meirom and others 1974). In this condition there is diminished STIC, hypoproteinaemia, alpha globulin deficiency and an accumulation of PAS positive, diastase-resistant inclusions in liver parenchymal cells (Meirom and others 1974; Rattner 1976). Hypoproteinaemia and alpha globulin deficiency were not found in any of the horses in our study.

	GROUP ONE			GROUP TWO			GROUP THREE		
	Horses and Ponies			Horses and Ponies			Horses and Ponies		
	Horses	Ponies	Horses and Ponies	Horses	Ponies	Horses and Ponies	Horses	Ponies	Horses and Ponies
Serum trypsin inhibitory capacity (mg per ml)	1.4-2.0	1.2-2.0	1.2-2.0	1.25-2.5	1.3-2.5	1.25-2.5	1.0-2.1	1.0-2.0	1.0-2.1
	1.68	1.61	1.64	1.68	1.81	1.73	1.60	1.62	1.61
	0.24	0.32	0.28	0.38	0.42	0.40	0.31	0.30	0.30
Total serum protein (g per l)	59-76	56-82	56-82	55.6-72.4	59.2-78.1	55.6-78.1	54.8-81.6	47.4-86.6	47.4-86.6
	65.1	68.3	66.8	64.1	66.9	65.2	64.7	65.2	64.9
	5.1	8.4	7.0	5.4	5.3	5.4	5.8	11.3	8.1
Serum albumin (g per l)	17.7-24.8	16.8-27.6	16.8-27.6	17.1-28.9	18.9-26.4	17.1-28.9	18.3-30.8	17.2-33.0	17.2-33
	22.5	22.6	22.5	22.8	23.1	22.9	24.3	21.6	23.3
	2.5	3.9	3.2	4.60	2.9	3.5	3.0	4.3	3.7
Serum alpha globulin (g per l)	8.5-12.4	9.6-15.8	8.5-12.8	9.2-13.3	8.5-13.3	8.6-17.0	8.6-17.0	8.2-14.4	8.2-17.0
	10.6	11.8	11.2	10.5	10.4	10.45	10.9	10.7	10.8
	1.2	2.3	1.9	1.1	1.1	1.1	1.9	1.8	1.8
Serum beta globulin (g per l)	14.5-22.6	13.7-21.4	13.7-22.6	7.8-20.2	8.6-25	7.8-25	7.8-19.5	13.2-32.0	7.8-32
	16.4	16.8	16.6	14.2	15.7	14.8	14.9	18.8	16.3
	2.7	2.6	2.6	3.5	4.6	4.0	3.4	5.9	4.8
Serum gamma globulin (g per l)	13.6-20	12.1-30.4	12.1-30.4	11.0-24.8	12.9-29.0	11.0-29.0	9.0-26.1	8.2-26.5	8.2-26.5
	15.9	17.1	16.6	16.6	17.6	17.0	14.7	13.9	14.4
	2.4	5.3	4.1	4.2	5.1	4.5	3.6	4.8	4

TABLE 21. SERUM PROTEIN VALUES OBTAINED FOR THE THREE GROUPS OF HORSES

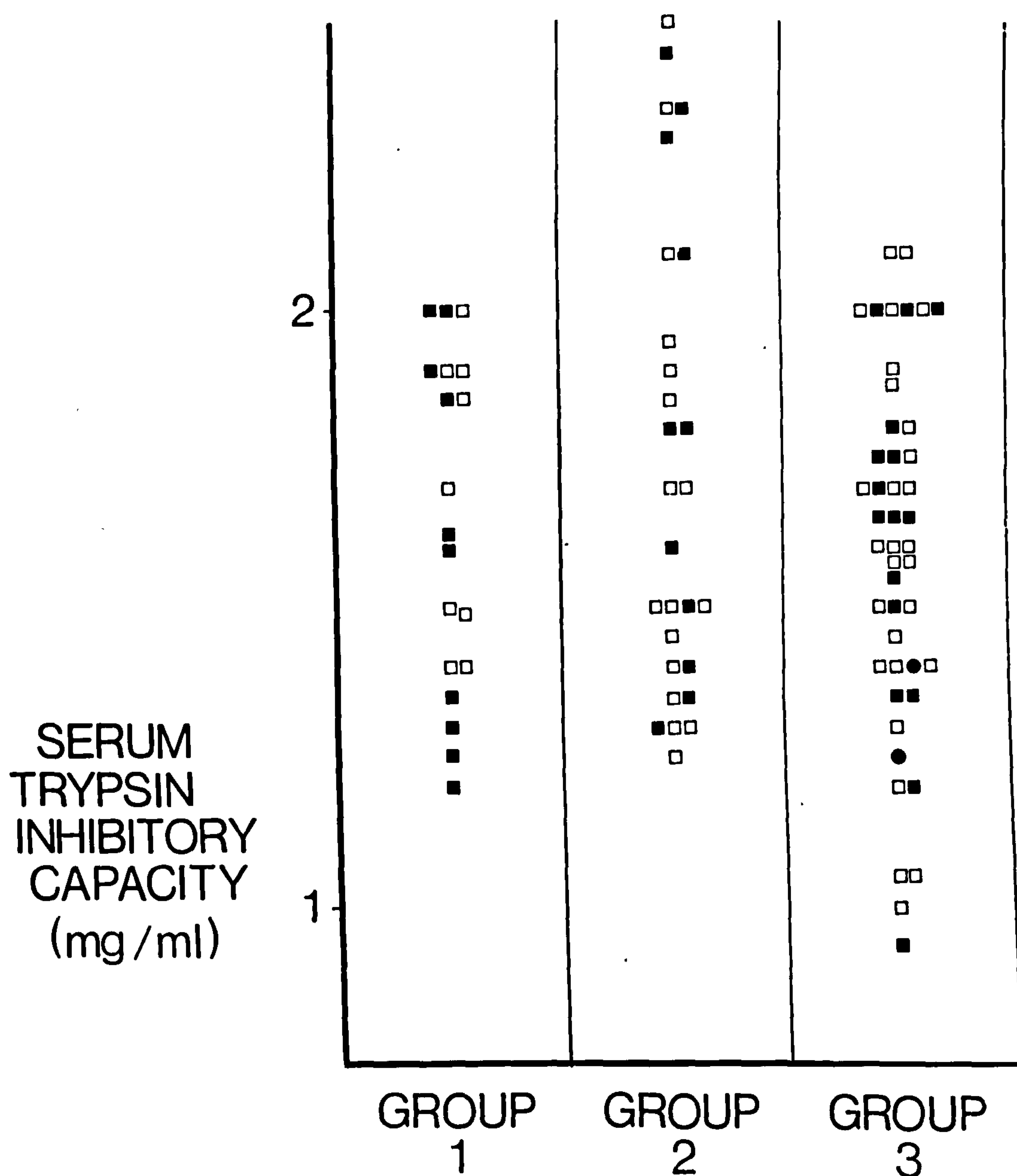


Figure 97. Scatter diagram of the mean STIC values in the three groups of horses.

Group 1 : Horses with confirmed CPD.

Group 2.: Horses and ponies with respiratory disease of a few weeks to months duration.

Group 3 : Horses, ponies and donkeys with no clinical signs or recent history of respiratory disease.

■- horse

□- pony

●- donkey.

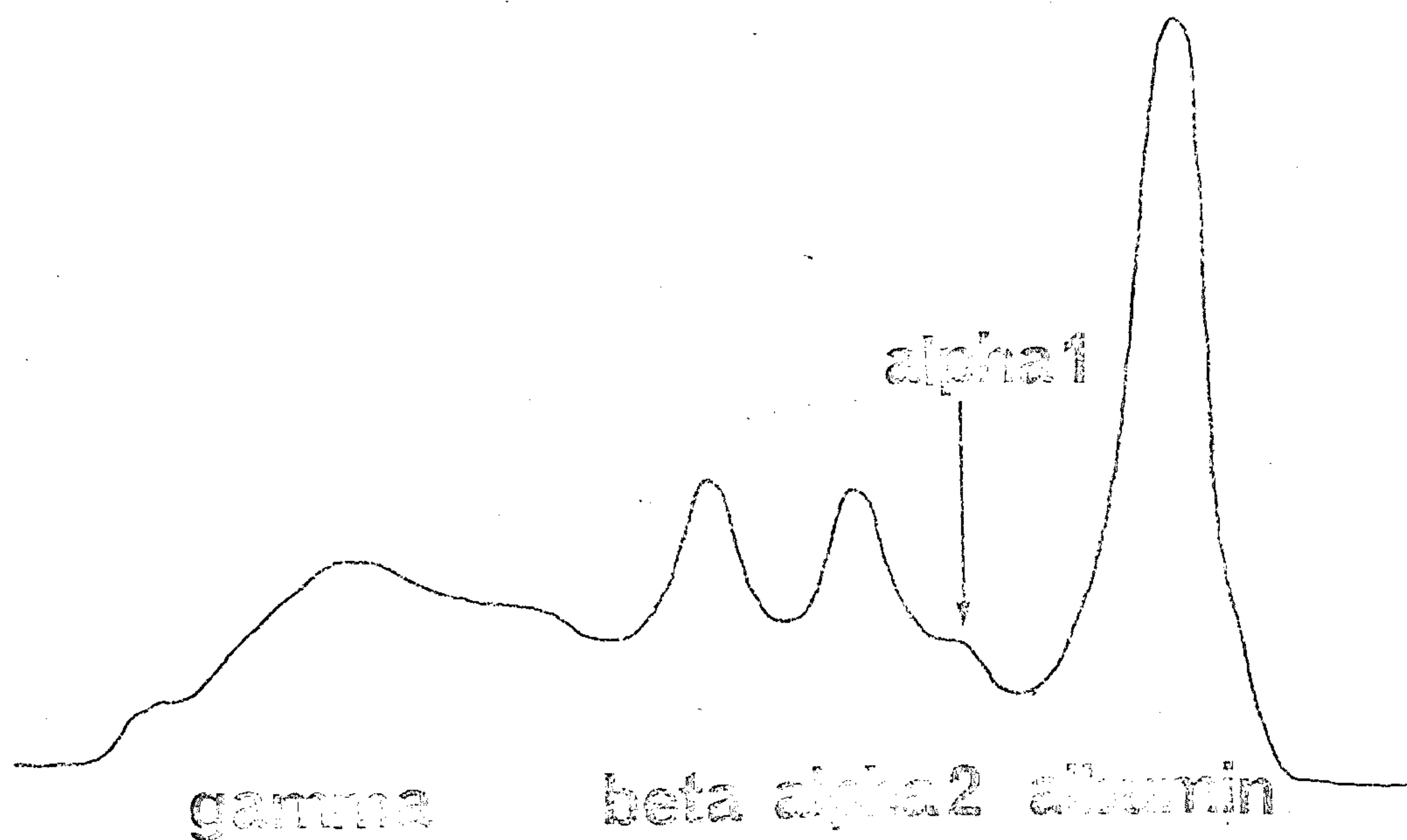


Figure 98a. Electrophoretic pattern of equine serum showing small alpha-1 peak.

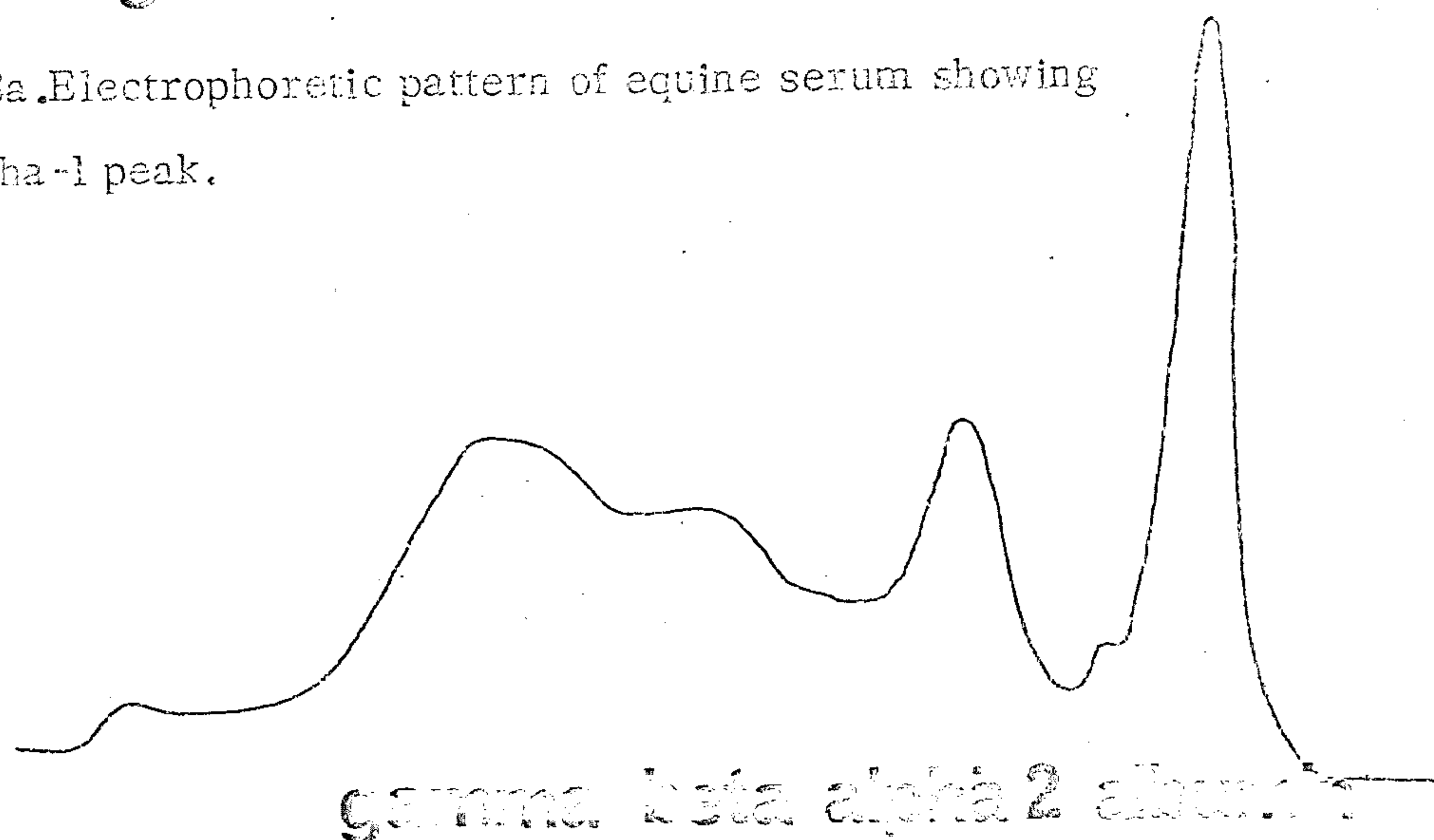


Figure 98b. Electrophoretic pattern of equine serum showing absence of alpha-1 peak.

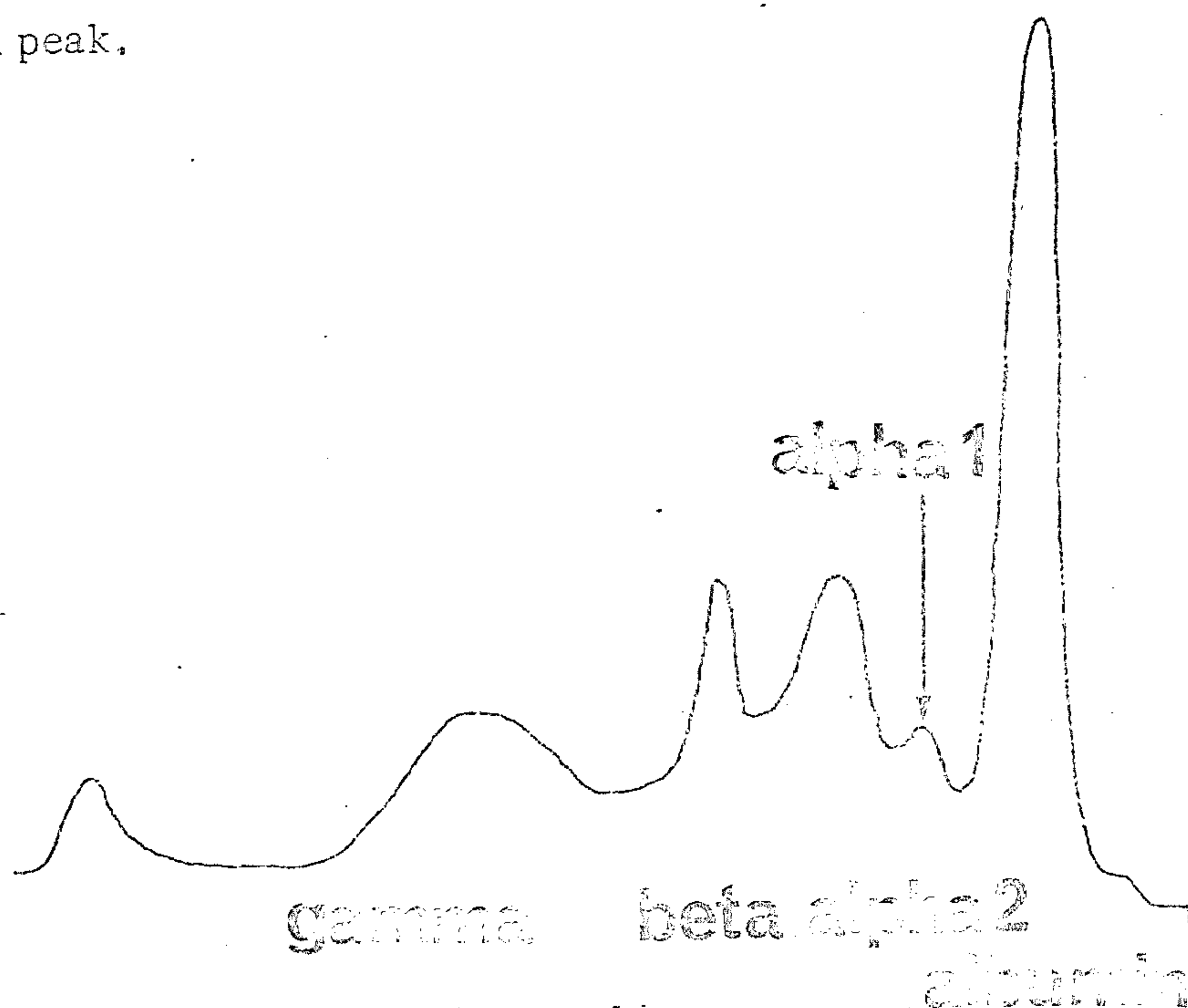


Figure 98c. Electrophoretic pattern of normal human serum showing prominent alpha-1 peak.

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CHAPTER SEVEN

THE PRODUCTION OF AN EXPERIMENTAL MODEL FOR BRONCHIOLAR DAMAGE

INTRODUCTION

It is not likely that the aetiology and pathogenesis of CB will be fully elucidated by studying field cases only. This is for various reasons, mainly due to the fact that the disease is of insidious onset, is chronic persisting for many years and past experience has shown that it is difficult to associate the onset with any specific event or type of husbandry. Even the feeding of mouldy or dusty hay, said for centuries to have been the root cause, is not a constant feature in the history of disease (McPherson and others to be published). Therefore there is a need for an experimental model of the disease.

The model animal employed should be equine because of the unique anatomical features of the equine lung. Ideally the means of production will be simple, easily reproducible and directly applicable to the field situation. There has only been one previous recorded attempt to artificially produce the disease in the horse and the main object of this was to evaluate the horse as an experimental model of emphysema in man (McLaughlin and others 1965). Chlorpromazine was injected into the bronchial arteries of a series of ten normal horses. Two horses, which survived for three and 11 months respectively (the remainder having died during or very soon after the operative procedure) were said to have lesions resembling naturally occurring emphysema of man and the horse. However, these lesions were chiefly of a necrotic nature affecting all the tissues of the lung with severe sclerosis of airways, blood-vessels and pleural areas. This lesion is not seen in the naturally occurring disease in Britain. Moreover emphysema is not the main lesion in the horse; the disease is a chronic bronchiolitis. Experimental production of the disease should aim to produce this rather than emphysema.

There have been numerous reports of attempts to produce an animal model of the human disease chronic bronchitis and emphysema. These may largely be divided into two categories; those involving the administration of irritants such as sulphur dioxide, ammonia and cigarette smoke and those involving the intratracheal administration of enzymes such as papain, elastase and leucocyte homogenates. The pathological changes produced by any of these methods would not satisfy our requirements for an equine model of CB.

There is an important disease of cattle known as fog-fever or acute bovine pulmonary emphysema which occurs when cattle are moved from poor pasture onto new rich pasture. Recent research has shown that similar lesions can be reproduced by the administration of 3-methyl indole the chief fermentation product of L-tryptophan, an amino-acid found in lush grass (Carlson, Yokoyama and Dickinson 1972). The main pulmonary lesions induced are pulmonary oedema and emphysema. Experimental administration to goats (Dickinson and others 1976) also resulted in severe pulmonary oedema but a later electron-microscopical study of goat lungs following administration of a sub-lethal dose of 3MI (Huang and others 1977) showed that the first changes that occurred in these lungs were that the membranous pneumocytes of the alveoli and the bronchiolar epithelial cells became swollen and contained large vesicles. Within eight hours the membranous pneumocytes had been shed from the basement membrane and by 24 hours the bronchiolar epithelium was almost completely denuded. 3MI is therefore a substance which will specifically damage bronchiolar epithelial cells. Although the experiment was not continued to study the reparative process it nevertheless seemed worthwhile to test the effects of low doses of 3MI on experimental ponies.

MATERIALS AND METHODS

Experimental ponies, all less than one year old, were dosed orally with 3MI in a small amount of water, hay and water having been withheld overnight. The dose was administered via a stomach tube directly into the stomach. Dose rates of 0.1 mg per kg and 0.2 mg per kg were used and the foals were killed at varying times following dosing as follows:

- | | | |
|-------|-------------------|----------------|
| (i) | One at six hours, | 0.2 mg per kg. |
| (ii) | Two at 24 hours, | 0.1 mg per kg. |
| (iii) | One at 72 hours, | 0.1 mg per kg. |
| (iv) | Three at 6 days, | 0.2 mg per kg. |
| (v) | One at 10 days, | 0.1 mg per kg. |

The ponies were shot with a captive bolt, exsanguinated and the thoracic organs immediately removed from the carcase. In most cases samples were taken for transmission electron microscopy and scanning electron microscopy and in all cases blocks for histological examination were taken from the

standard eight sites and fixed in formol saline, Carnoy's fluid and corrosive formol. After processing, as described in Chapter Three, sections were stained with H and E, Martius scarlet blue, Verhoeff van Giesson, Unna-Pappenheim, Feulgen, astra blue-safranin, AB pH 2.6 - PAS and AB pH 1.0-PAS.

Processing of tissues for viewing with SEM and TEM were as described in Chapter Three.

RESULTS

Six hours following administration

Clinical signs

This pony had clinical evidence of respiratory disease prior to dosing, it was tachypnoeic, coughed and rhonchi were heard on auscultation. Within one hour of dosing it became dull and recumbent and was in severe respiratory distress. Respiratory rate was 70 per minute and there was frequent harsh coughing.

Pathological changes

Both lungs were overinflated, pale pink and had a "rubbery" consistency. There were fairly extensive areas of exudative pneumonia cranially. Culture of the affected pulmonary tissue revealed infection by streptococci of Lancefield's group C.

Microscopically the areas of exudative pneumonia consisted of collapsed, consolidated tissue heavily infiltrated by polymorphonuclear leucocytes. A large number of eosinophils were also seen in the reaction and large foamy macrophages were present in the remaining air-spaces.

In the grossly non-pneumonic areas there was nevertheless a marked bronchial and bronchiolar reaction with a polymorphonuclear leucocytic reaction around the airways and a muco-purulent exudate in many of the airways. A diffuse pulmonary eosinophilia was seen. The only change that could be directly attributed to the action of 3MI was that some of the smaller bronchioles and alveolar ducts had lost part or all of the epithelium and this was lying in the lumina as a disordered mass of epithelial cells (Figure 99).

Twenty four hours following administration

Clinical signs

Both ponies became tachypnoeic half to one hour post-administration respiratory rate increasing to 60 per minute and remaining at this level until death. Diarrhoea occurred overnight, the faeces smelt strongly of 3 MI and were very mucoid. Apart from this, the ponies remained bright and ate well.

Pathological changes

At necropsy the carcasses had very little of the characteristic smell of 3 MI. The lungs were grossly overinflated with many splash and petechial haemorrhages visible on the surface (Figure 100) and throughout the pulmonary substance. There was no gross pulmonary oedema nor was there excess froth in the trachea and large airways. There were also petechial haemorrhages throughout the gut.

Microscopically there were many small areas of alveolar haemorrhage and oedema. The alveoli in these areas were lined by thick discrete hyaline membranes and a proportion contained an amorphous highly basophilic material (Figure 101). Special staining with Unna-Pappenheim and Feulgen stains showed that the basophilic mass contained DNA and so was probably degenerate nuclear material (Figure 102). The hyaline membranes did not pick up any DNA stain and were PAS positive. With Verhoeff van Giesson stain each hyaline membrane could be seen to have a single thick elastic lamina at its base (Figure 103). Hyaline membranes have not been observed in any other equine pulmonary condition to our knowledge except in neonatal foals with respiratory distress.

Throughout the entire lung all the bronchioli and alveolar ducts had lost their epithelium and in a lot of cases all that remained was a ring of smooth muscle (Figure 104). In other cases a strip of epithelium could be seen lifting off the basement membrane (Figure 105). The separated epithelial cells were often lying free in the lumina completely occluding them (Figure 106). There was very little associated bronchiolar reaction but plasma cells, as seen by use of Unna-Pappenheim stain, were grouped around most of the affected bronchioles.

The bronchi were relatively unaffected by this change although a few of the smaller ones contained small amounts of cellular exudate possibly cells

carried up from the bronchioles and some smaller ones were losing their epithelium.

Scanning electron microscope studies of the cranial lung lobes also demonstrated these changes. The alveoli were oedematous in places and focal haemorrhages could be seen. The bronchioles contained plugs of cellular debris and their peribronchiolar tissue was oedematous and disordered. Higher magnifications demonstrated that the Clara cells being desquamated had a degenerate, flattened, irregular appearance. These changes are illustrated in Figures 107 to 114.

Transmission electron microscopy showed that many of the epithelial cells of the bronchioles of all types contained large vacuoles (Figure 115). Epithelial cells often lay free in the lumen and the underlying lamina propria was of normal appearance. Abnormal, stunted cilia were seen occasionally (Figure 116); these are not usually seen in normal equine bronchiolar epithelium.

Seventy two hours following administration

Clinical signs

The pony became dull and tachypnoeic after about half an hour and this lasted for several hours. The tachypnoea persisted for the rest of the time before slaughter (40 per minute) but the animal's general demeanour was bright and lively and he ate well. There was no diarrhoea or any sign of abdominal discomfort.

Pathological changes

The carcase did not smell of 3 MI. The lungs were pale pink, rubbery and overinflated. A very few petechiae were found on the surface. Microscopically the lesions found in the airways were intermediate between those found at 24 hours and those found at six days. All the small airways were surrounded by sheaths of polymorphonuclear leucocytes, lymphocytes and plasma cells. A variety of epithelial changes was seen. In some there was no epithelial cell layer and the lumen was blocked with cellular debris and macrophages. There was evidence of regenerating epithelium in the majority of bronchioles. These new epithelial cells were cuboidal, with basophilic round nuclei and an abundant cytoplasm. They were arrayed irregularly along the basement membrane at first but in other airways a later stage of regeneration was seen where the cells were more columnar and regular in appearance. There were no goblet cells present in the epithelium of the bronchioles. Inflammatory exudates were commonly present in the lumen and macrophages were a frequent component.

Occasionally the double epithelial effect found in the ponies killed at six days was found but this was uncommon.

Small focal areas of alveolar epithelial hyperplasia were found and this was sometimes associated with pulmonary oedema. Hyaline membranes were no longer present.

There was a diffuse eosinophilia of the pulmonary tissue.

Six days following administration

Clinical signs

All the ponies showed some degree of respiratory abnormality prior to dosing. Clinically there was harsh coughing, nasal discharge and harsh respirations on auscultation. However all were bright, lively, eating and had normal rectal temperatures. After dosing they became dull and showed signs of abdominal discomfort with acute colic occurring in one. Tachypnoea became evident within a few hours the respiratory rates rising to 40 per minute. Within 48 hours the abdominal signs had disappeared and all three were bright and eating. Tachypnoea was noticeable until slaughter and all of them had spontaneous coughs.

Pathological changes

The carcasses had very little taint of 3 MI on them. The lungs were all pale pink, overinflated and rubbery (Figure 117). All areas of the lung were equally affected except for some small areas cranially which were pneumonic. There was no evidence of emphysema.

Microscopically all the sections had a lacy, open appearance due to the extreme overinflation of the alveoli. Despite this, there was no convincing evidence of alveolar wall destruction. The main lesion was a bronchiolitis which affected all the bronchioles. These airways were surrounded by large numbers of lymphocytes and plasma cells and many also had a substantial fibrous element in this inflammatory reaction. Three distinct reactions were seen in the bronchiolar epithelium. Most commonly the epithelium was merely hyperplastic, columnar and several cells deep. Polymorphonuclear leucocytes and lymphocytes could be seen migrating through the epithelium and some of these bronchioles contained a cellular exudate (Figures 118 and 119). Other airways had been completely obliterated being blocked with massive cellular accumulations (Figure 120). These cells appeared to be mainly epithelial cells and polymorphonuclear leucocytes. The third reaction, which was fairly common, was one resembling bronchiolitis obliterans. The extent of this varied from a small tongue of tissue projecting into the lumen (Figure 121) to a large plug of oedematous tissue filling the lumen completely (Figure 122). A peculiar effect of an apparently double epithelium was often seen in these cases. A fairly normal, though hyperplastic, epithelium was present on the

basement membrane and then on top of this was a mass of undifferentiated cells which projected into the lumen and then capping this was a further layer of epithelial cells (Figures 123 and 124). Goblet cells were sometimes found in the hyperplastic epithelium but these were poorly developed and only stained for mucus at the very apex of the cell (Figure 125). The type of mucus secreted was sulphomucin staining with alcian blue at pH 2.6 and pH 1.0.

The submucosal glands of the bronchi had a dilated appearance but contained little mucin and there was little evidence of bronchitis. Eosinophils were frequently found in all the pulmonary tissues. There were a few small areas of alveolar epithelial hyperplasia associated with polymorphonuclear leucocytes and giant cells (Figure 126).

Microscopic examination of sections of kidney showed that there was vascular congestion in the cortex and medulla. Some of the glomeruli looked degenerated and disorganised and there were protein casts in a few of the tubules.

There were focal accumulations of lymphocytes in the lamina propria of some bronchi and these sometimes could be seen migrating into the epithelium. Some distinctive cells were present in the basal lamina area of the bronchial epithelium. These were small roundish cells with small dark round basophilic nuclei. PAS positive granules were present in the cytoplasm, these were of varying sizes. In H and E these cells were difficult to see but the granules seemed to be fairly pink. These were probably Russell body cells. They were present in only small numbers and were only seen on detailed studies of PAS stained material.

Electron microscopical studies were not carried out on these animals.

Ten days following administration

Clinical signs

Within a hour the foal became dull and tachypnoeic. An occasional cough was heard. This foal had no sign of respiratory disease prior to dosing. There was no apparent abdominal discomfort and within a few hours she was much brighter and eating well. The tachypnoea persisted at levels of 40-50 respirations per minute for a week when the clinical condition suddenly

worsened, respiratory rate increasing to 60 per minute and a thick mucopurulent nasal discharge being present. The appetite remained fairly good although she had a very dejected appearance.

Pathological changes

The lungs were large, heavy, pale and gross alveolar emphysema could be seen, especially anteriorly. There were focal areas of alveolar collapse in the cranial lobe but these did not extend deeply into the pulmonary substance. On section the pulmonary tissue was spongy and had a speckled appearance.

Microscopically alveolar emphysema was present in addition to alveolar overinflation. The interalveolar septa were thin and in many places disrupted. The lesions seen in the bronchioles could be regarded as a progression of the changes found at six days. All the small airways were surrounded by sheaths of inflammatory cells and many had inflammatory exudates in the lumen. A few were obliterated with connective tissue, the rest had a hyperplastic epithelium. Many of the bronchioles had more than one lumen. This varied from two, up to six. When a large number were present these were arranged around a central larger airway which presumably was the original. All were lined by epithelium and separated from each other by connective tissue. The overall effect was that the bronchioles were very prominent and larger than usual which would account for the speckled appearance of the lung grossly. Goblet cells were present in the hyperplastic epithelium of a number of bronchioles but very little mucus was found in the lumina of the airways. The most usual exudate was solely of polymorphonuclear leucocytes. Focal alveolar epithelial hyperplasia was a fairly common finding especially in the lower caudal lobe. The alveoli were lined by a regular cuboidal epithelium and macrophages were often present in the lumen. These areas could not be related to any specific structure in the lung.

There was a mild bronchial reaction which consisted of infiltration of the bronchial epithelium and lamina propria with plasma cells and lymphocytes and exudation of small amounts of mucus into the lumen.

DISCUSSION

These experiments have shown that it is possible to produce specific bronchiolar damage in the horse by the toxic action of 3MI.

The experimental lesions produced after six days resembled to some extent those seen in naturally occurring chronic bronchiolitis. Following the loss of the normal epithelium, at 24 hours, it was replaced in many cases by a hyperplastic, multilayer, columnar, goblet cell containing epithelium identical to that seen in CB. In addition the alveoli were overinflated throughout the lung but true emphysema would seem to be rare. Again this is what was found in the naturally occurring cases. However the other experimentally induced bronchiolar lesions, the total obliteration of small airways and bronchiolitis obliterans were not found in the naturally occurring disease. Despite this it seems that the experimental lesion bears sufficient resemblance to the naturally occurring disease to be used as an experimental model of the pathological changes and possibly also the functional changes although this has not been investigated as yet. By ten days post-administration true emphysema had developed and the bronchiolar lesions were less typical of CB. This may still be a useful functional model however, especially to assess the functional effects of true emphysema.

The mechanism of cellular damage by 3MI is still a matter of some conjecture but it seems that indoles may intercalate between the lecithin fatty acid chains near the polar head of the molecule and thereby reduce the water mobility in this area (Bray, Magnuson and Carlson 1974). This alteration in membrane permeability is thought to be the initial event in the toxic action of 3MI (Huang and others 1977). It does not explain the specificity of action to two major cell types the nonciliated bronchiolar secretory cell (Clara cell) and the type I membranous pneumocyte. These two cell types are involved in the xenobiotic activity of the lung (Boyd 1977; Huang and others 1977). Xenobiosis is the biotransformation of foreign chemical compounds and the enzyme system for this is the mixed function oxidase (MFO) system which occurs in the smooth endoplasmic reticulum (Boyd 1977). 3MI may be specifically converted by this system to electrophilic compounds which are potentially damaging to the cells.

The toxic action of 4-ipomeanol has also been shown to specifically affect Clara cells in the first instance and a highly reactive metabolite of this substance has been shown to be preferentially formed and bound in these cells (Boyd 1977) In vitro studies have shown that the metabolic conversion is carried out by the MFO system. The formation of the metabolite results in necrosis of the Clara cells.

Reid and others (1973) first observed that naphthalene injected intraperitoneally into rats specifically damaged the Clara cells in the lung. Mahvi, Bank and Harley (1977) examined these changes by electron microscopy. They found that initially the Clara cells dilated and then exfoliated. Following this, abnormalities appeared on the surface of the ciliated cells. Regeneration of the Clara cells occurred within 48 hours and once apical projections reappeared on these new cells the ciliated cells regained their normal appearance. The authors interpreted this to mean that loss of Clara cells meant that no hypophase was being secreted into the bronchioles so that the cilia were exposed to the thicker mucoid layer which is thought to lie above the hypophase. This change in environment resulted in changes in the ciliated cells. They also concede that the difference in timing of damage to the two cell types could lie in differing susceptibilities of the two cell types to naphthalene.

It was not really possible to say from our experiments whether it was primarily the Clara cells that were affected or not although comparison with the goat studies would suggest this. In the transmission electron microscopical studies carried out at 24 hours both cell types, Clara cells and ciliated cells were shed or were in the process of exfoliating and both had large vacuoles in the cytoplasm. The cilia were missing, stunted or deformed in most of the epithelial cells but it is not possible to say whether this was due to a direct injury to them or if it merely reflected the generalised cell necrosis that was occurring.

It is interesting that none of the foals was affected severely by the toxin and that none died as a direct result of the experiment. The one foal to be killed in extremis had a widespread exudative pneumonia. It is possible that the last foal, killed at ten days post-administration may have died eventually as the pulmonary signs were gradually worsening. Nevertheless cattle and goats given doses such as this die in a few days (Carlson, Yokayama and Dickinson 1972; Dickinson and others 1976). The main cause of death was extensive pulmonary

oedema and emphysema. Emphysema was most noticeable in the cattle where it was mainly interstitial. Emphysema develops easily in cattle who have wide, loosely arranged fibrous interlobular septa. In the goats the main lesion was pulmonary oedema.

Pulmonary oedema and hyaline membranes were found in the two foals killed at 24 hours but this resolved rapidly and was not a fatal development. In cattle and goats the fluid leak into the alveoli is thought to be transcellular as even in desquamated cells the tight junctions are intact (Huang and others 1977). Electron microscopy of the type II pneumocyte was not carried out to see if these were less damaged in the horses than in the goats. It may be that the horse is better able to resist pulmonary oedema than other animals.

Focal alveolar epithelial hyperplasia was a feature in all the horses from 72 hours after administration. Initially this was associated with pulmonary oedema. Alveolar epithelial hyperplasia was found in a number of the naturally occurring cases of CB where it was chiefly present around the large airways and subpleurally. In the experimental lesion the development was not associated with any particular structure. At ten days after administration the lower lung was most affected by this although changes were not apparent macroscopically. Diffuse alveolar epithelial hyperplasia as a result of the proliferation of type 2 pneumocytes was found in cases of fog fever in cattle that were recovering from the acute disease (Breeze and others 1975). There was no opportunity to investigate the experimental alveolar epithelial hyperplasia by electron microscopy but this was probably also due to proliferation of type II membranous pneumocytes following their initial damage by the action of 3MI.

One striking feature of this experimental lesion was the rapidity with which regeneration of epithelium occurred. Mahvi, Bank and Harley (1977) also found that in the rat lung damaged by naphthalene extensive regeneration of both bronchiolar and alveolar epithelium occurred within 72 hours. The stem cell involved in the regeneration of the bronchiolar cells is not known. In most cases all that was left following necrosis of the epithelium at 24 hours was the ring of smooth muscle. In naphthalene-damaged rat bronchiolar epithelium Mahvi, Bank and Harley assumed that the Clara cells were replaced primarily through division of surviving cells, but may also have been derived from ciliated cells. In addition they pointed out that recruitment from other

areas must be considered. In bleomycin induced alveolar injury in mice it was proposed that bronchiolar cells grew down to heal certain alveolar injuries (Aso, Yoneda and Kikkawa 1976). Further detailed electron microscopical work must be done on this aspect of cell regeneration.

The bronchiolar damage and subsequent hyperplastic regeneration of the epithelium induced by the administration of 3MI in the horse has resulted in a potentially useful model for the study of the naturally occurring disease chronic bronchiolitis.

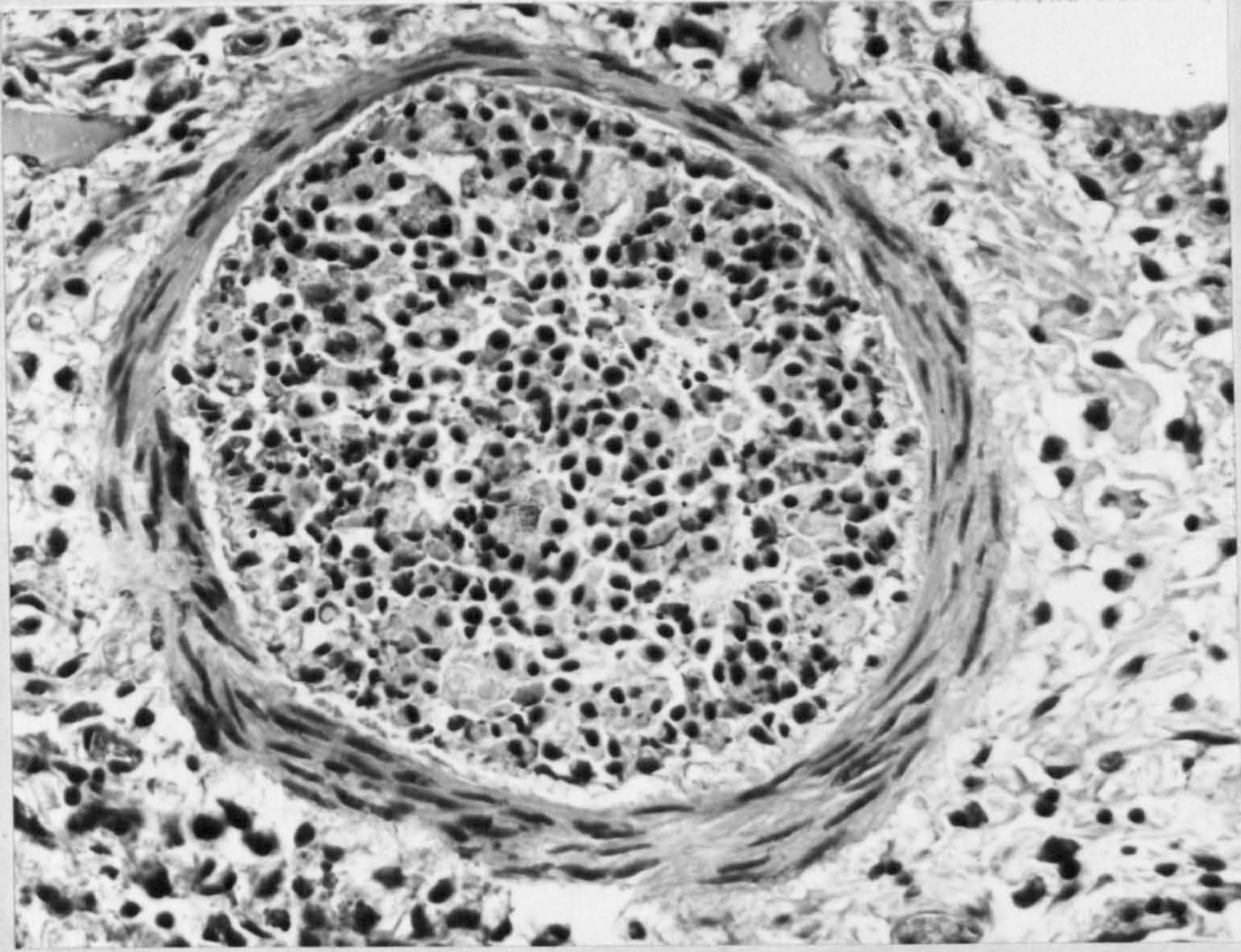


Figure 99. Foal killed at six hours following administration of 0.2 mg per kg 3 MI. The epithelial cells of the bronchiole have separated from the lamina propria and are lying in the lumen as a plug of desquamated cells. H and E X 250.

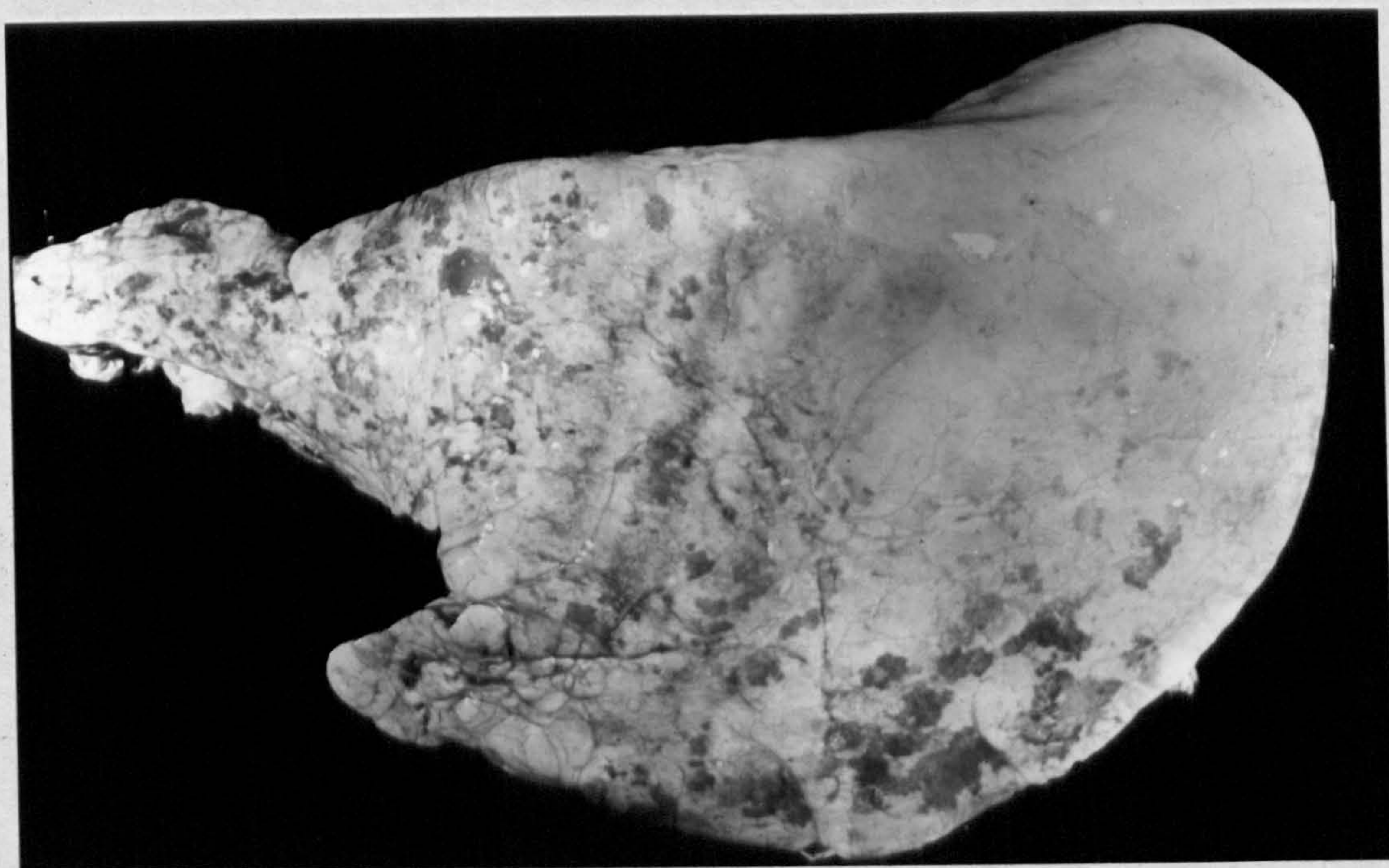


Figure 100. Lung from one of the foals killed at 24 hours following the administration of 3 MI. Many petechial and ecchymotic haemorrhages are visible on the surface anteriorly and the lung is overinflated.

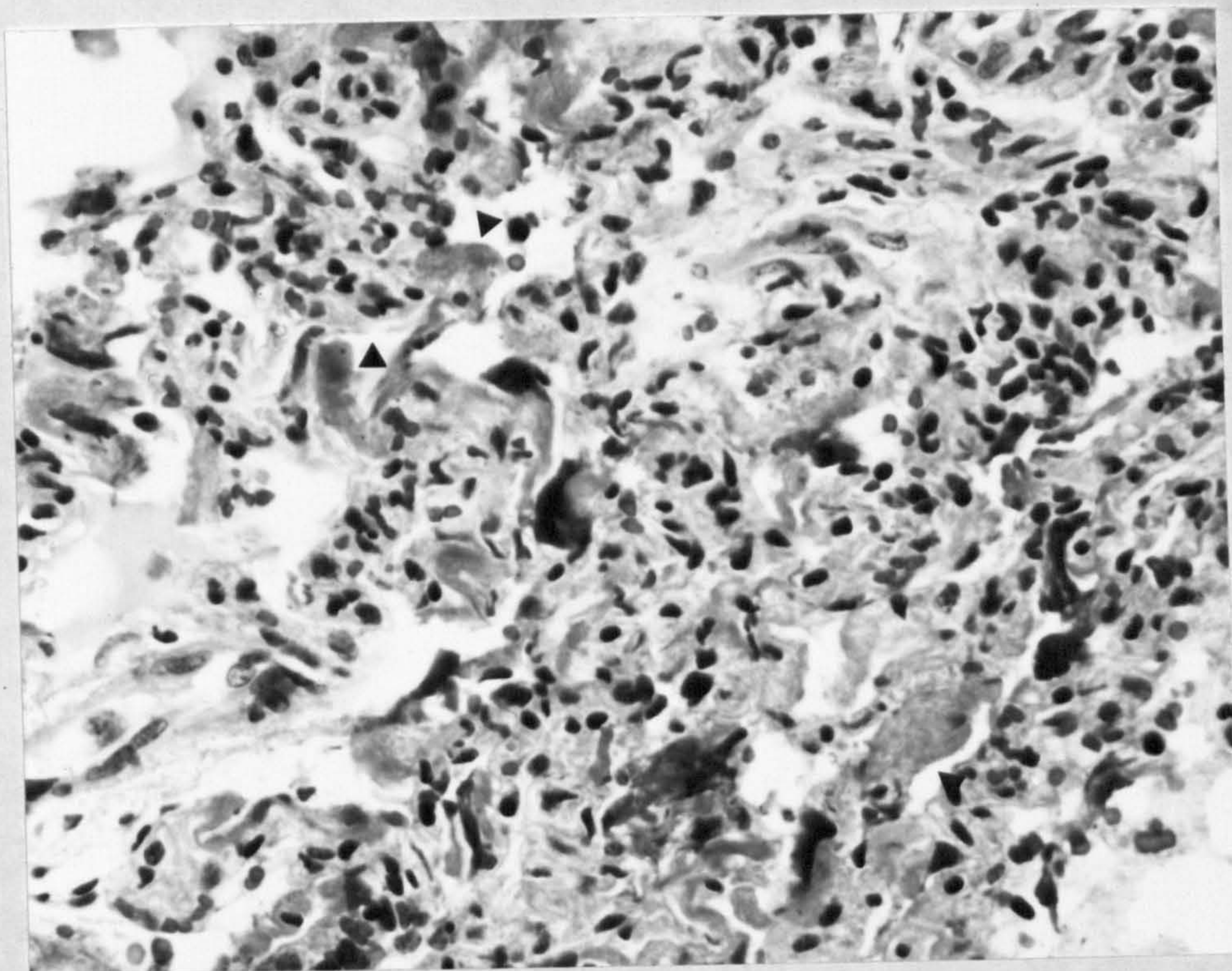


Figure 101. Foal killed at 24 hours following administration of 0.1 mg per kg 3 MI. The alveoli are lined in places by hyaline membranes (arrows) and there are clumps of basophilic amorphous debris in the lumina. H and E X 250.

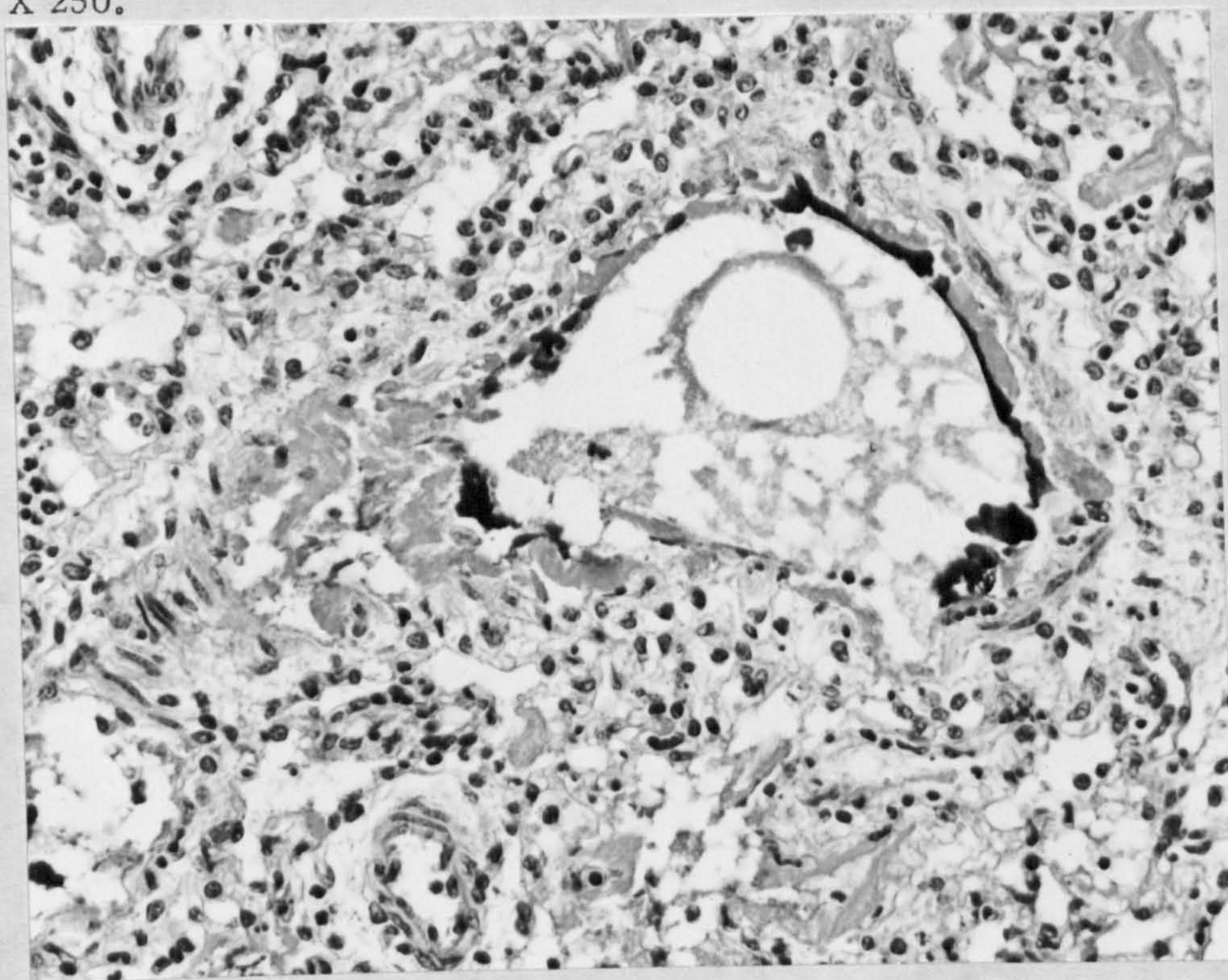


Figure 102. Similar area to that shown above stained with Feulgen. The debris, which stains darkly, seems to comprise DNA material and the hyaline membrane is principally RNA containing. X 250.

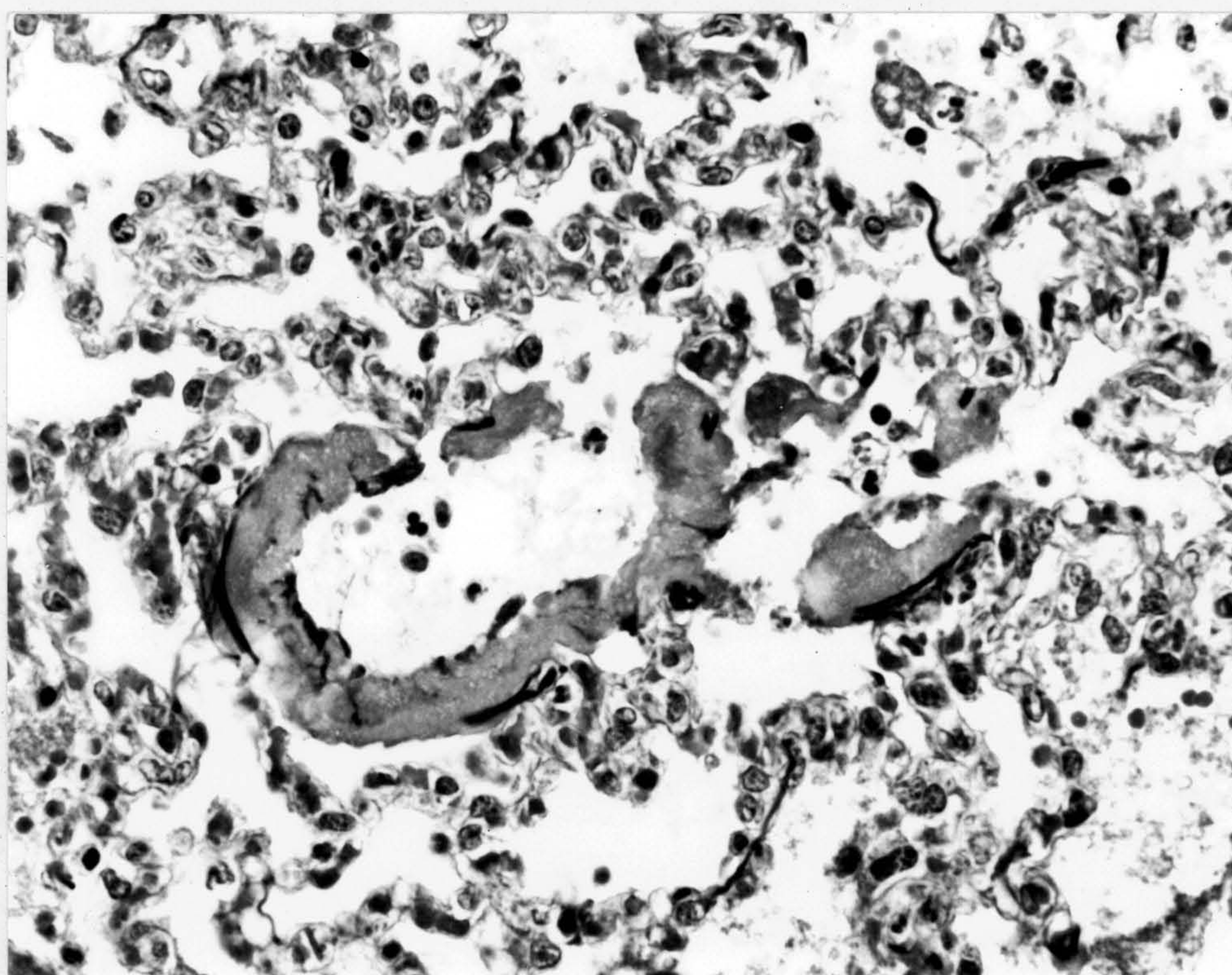


Figure 103. Similar area to that shown in preceding figure stained with Verhoeff's van Gieson. A single thick black elastic lamina is seen under areas of the hyaline membrane. X 250.

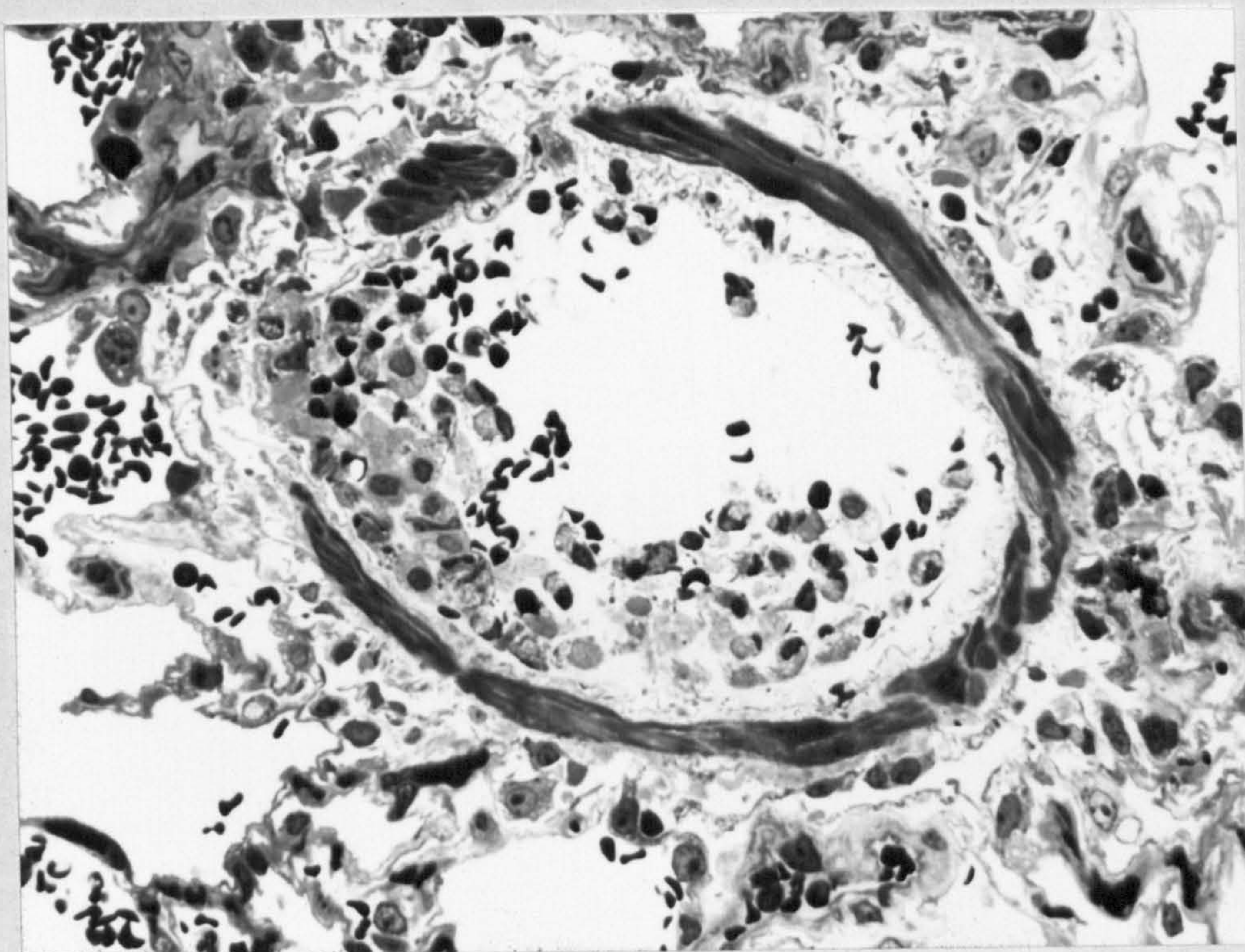


Figure 104. Section of a bronchiole from a foal killed at 24 hours following administration of 0.1 mg per kg 3 MI. The epithelial cells are lying detached in the lumen. Areas of alveolar haemorrhage are seen at the edge of the section. Section cut at 1 μ m stained with methylene blue/Azure II. X 250.



Figure 105. Section of a small bronchus from a foal killed at 24 hours following administration of 0.1 mg per kg 3 MI. The epithelium is lifting off the lamina propria in a continuous strip. In the other areas the epithelial cells are becoming detached from each other and lifting off the lamina propria. H and E X 110.

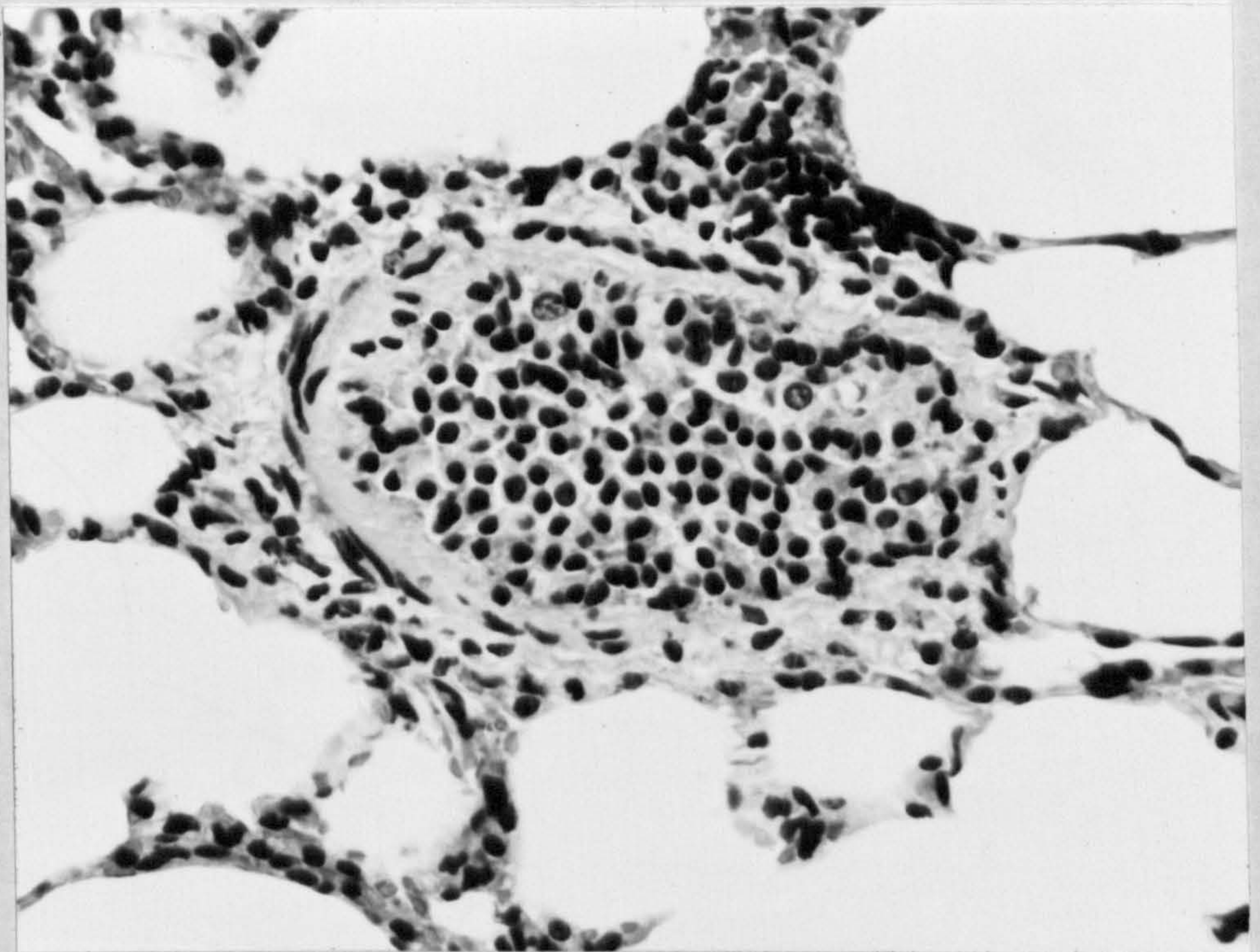
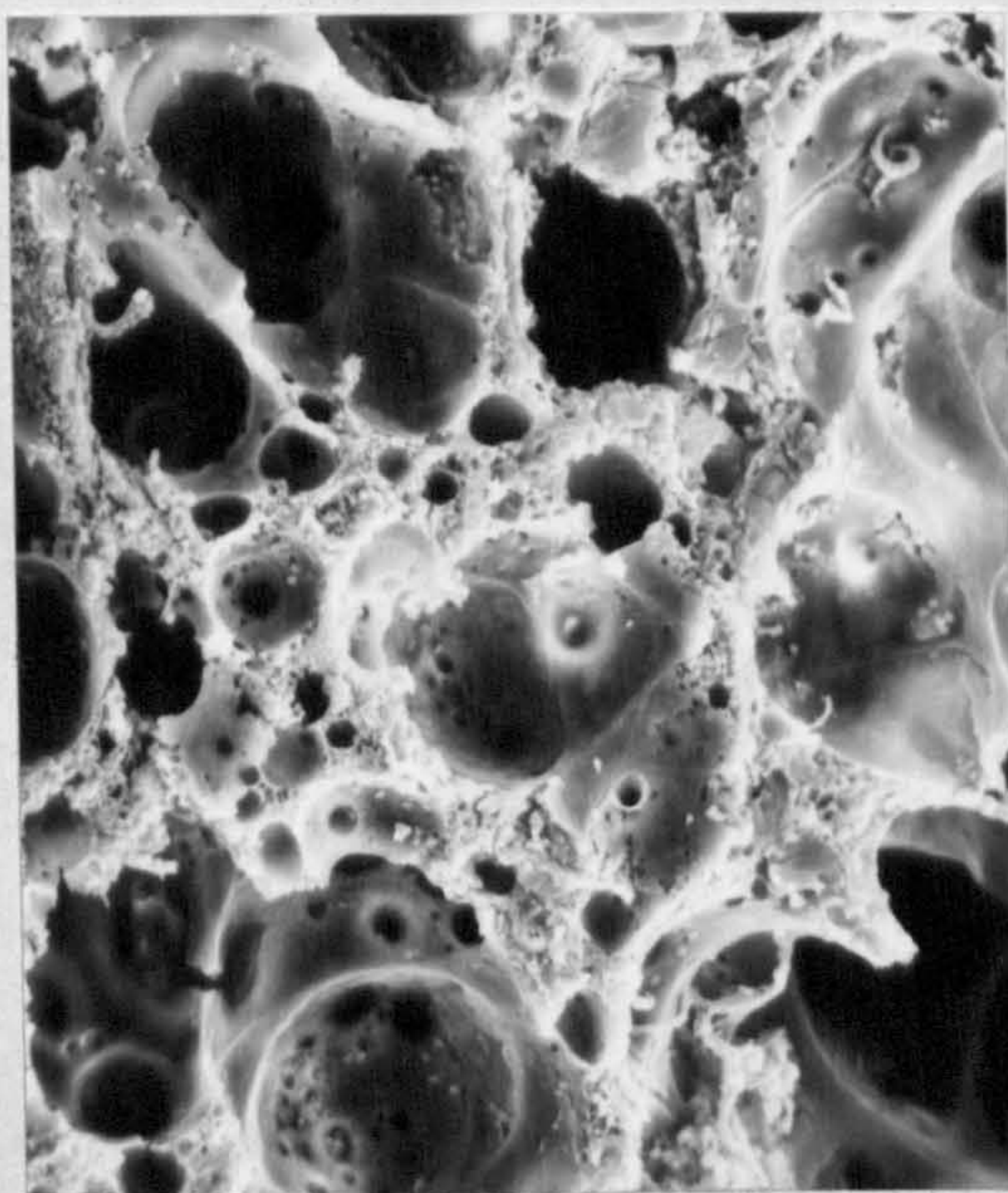
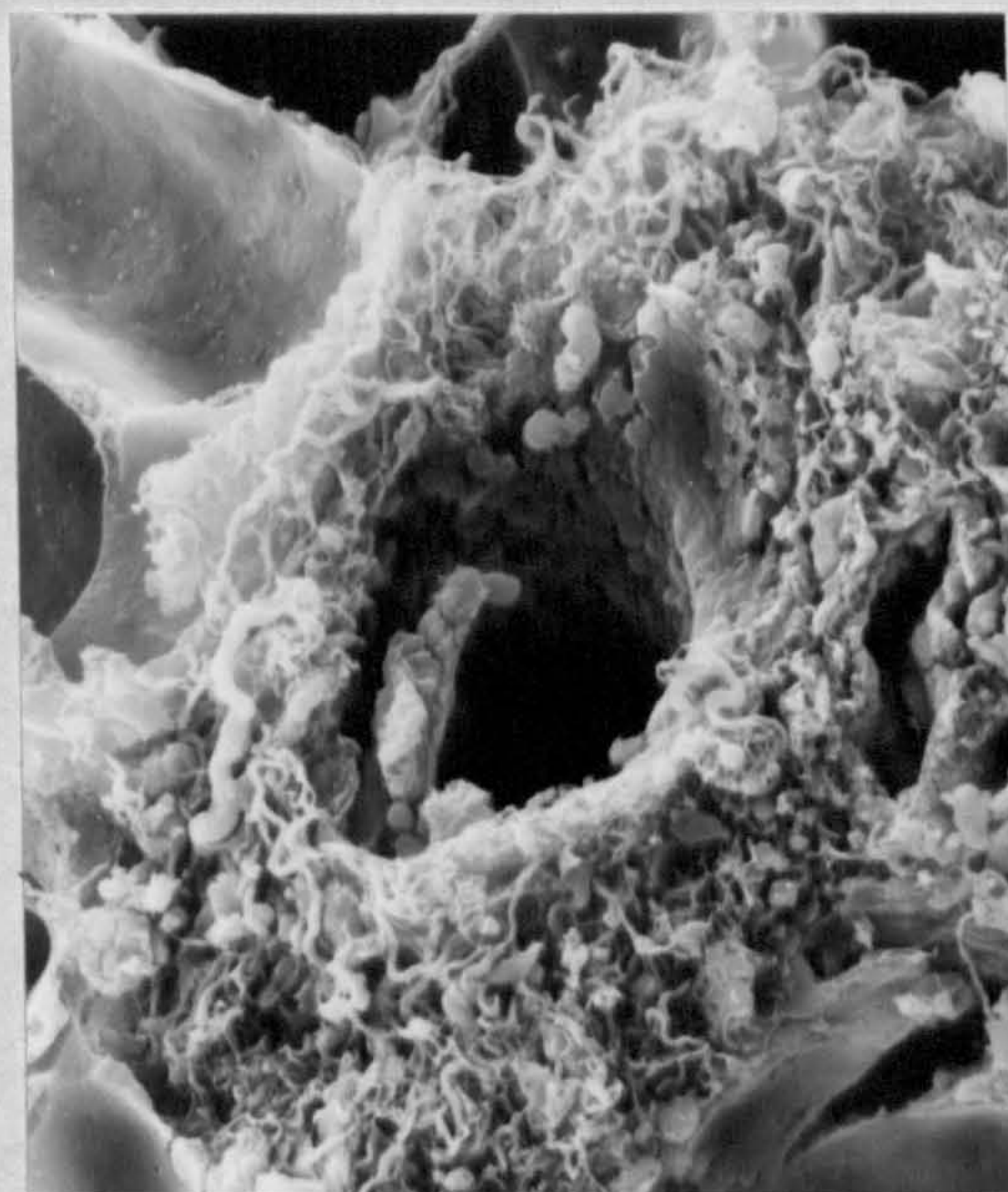
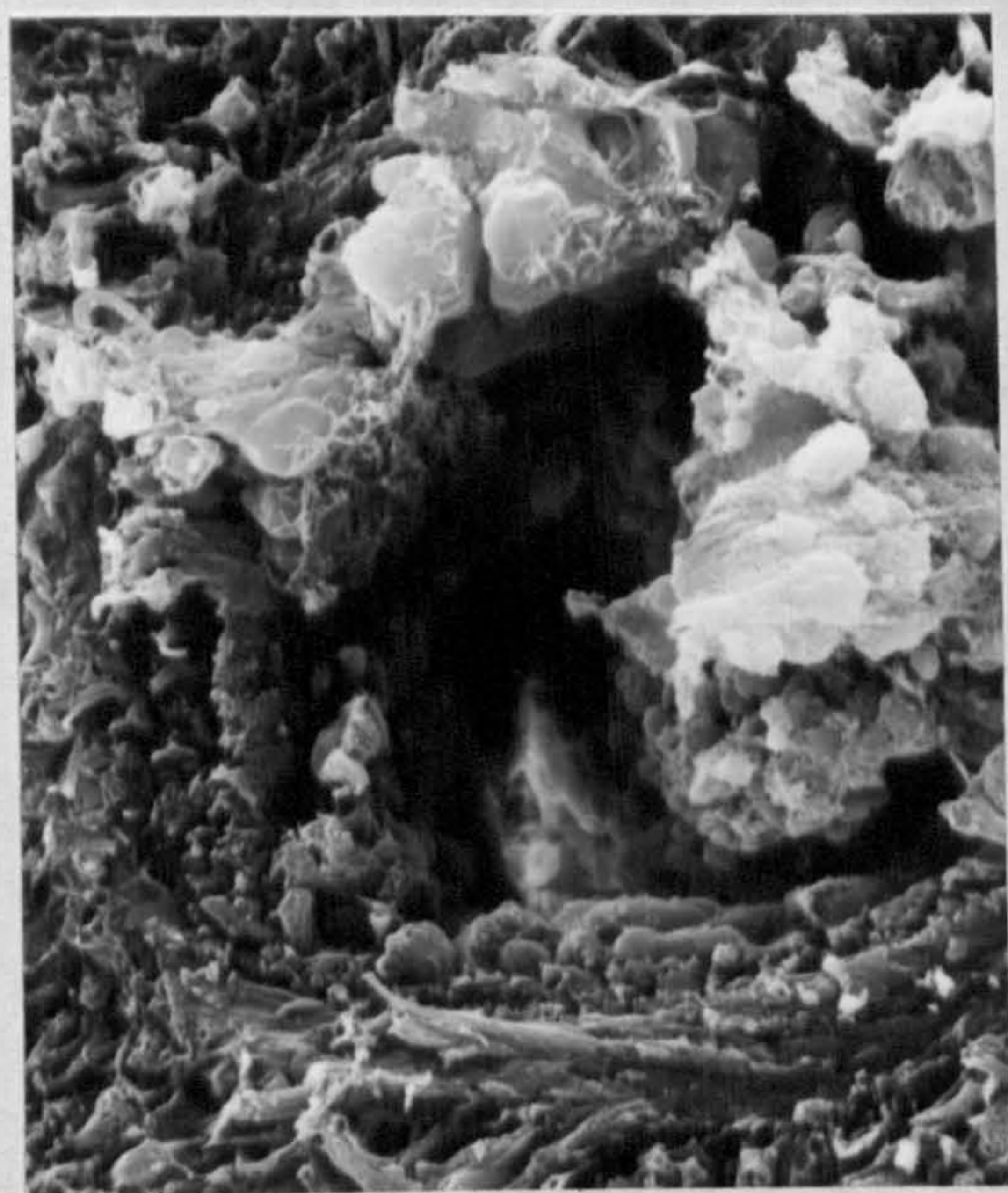


Figure 106. Section of a small bronchiole from a foal killed at 24 hours following administration of 0.1 mg per kg 3 MI. The lumen appears to be totally occluded with a plug of desquamated epithelial cells. H and E X 250.



Figures 107 and 108. SEM of terminal bronchioles and alveolar ducts of horse 24 hours after the administration of 3 MI. The epithelium has been lost and there is haemorrhage and oedema of the tissue. X 80.



Figures 109 and 110. Small bronchioles from same horse as above. The epithelial cells are degenerate and have lifted off the lamina propria. There is oedema of the peribronchiolar tissue in Figure 110. X 640.

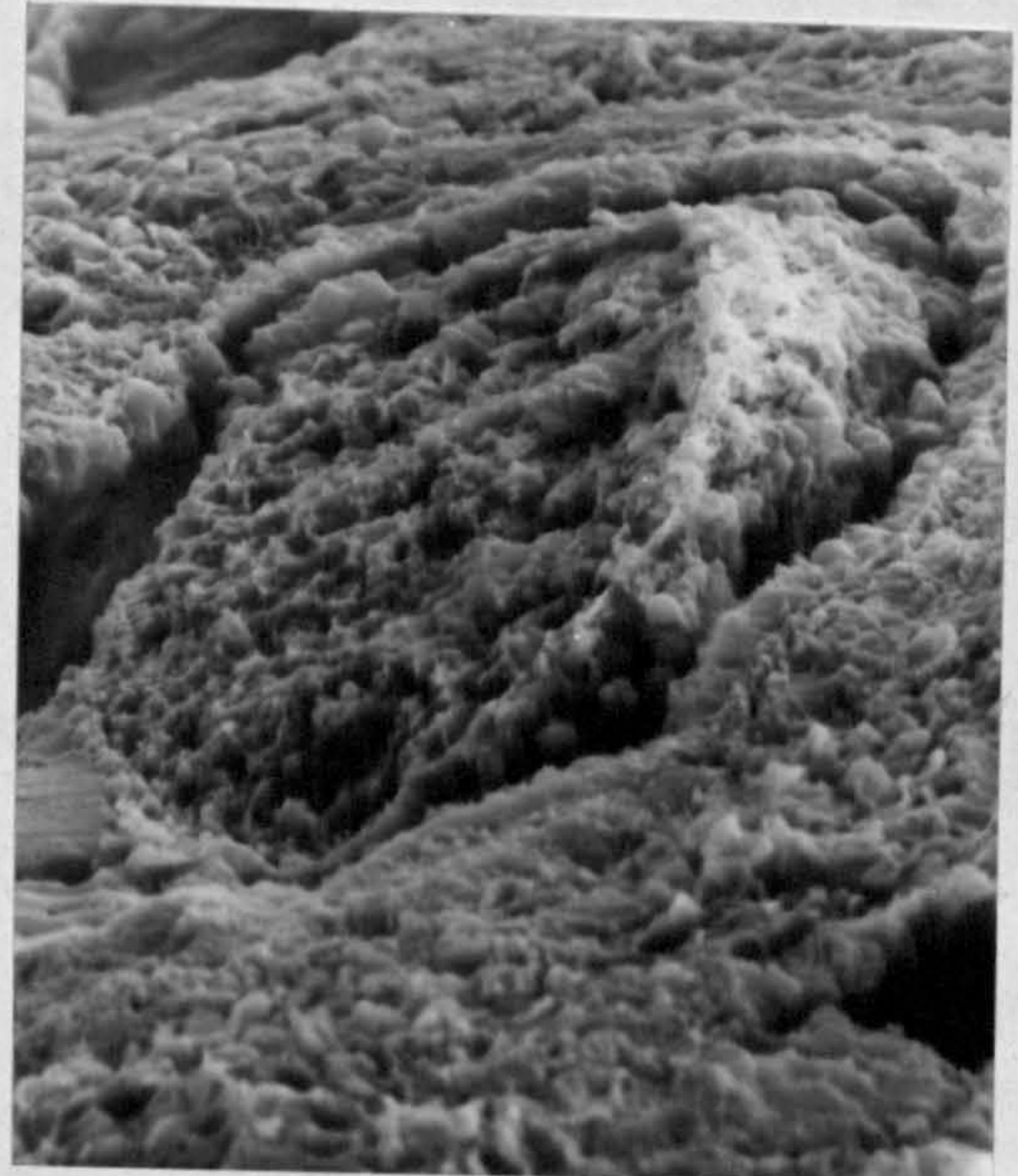
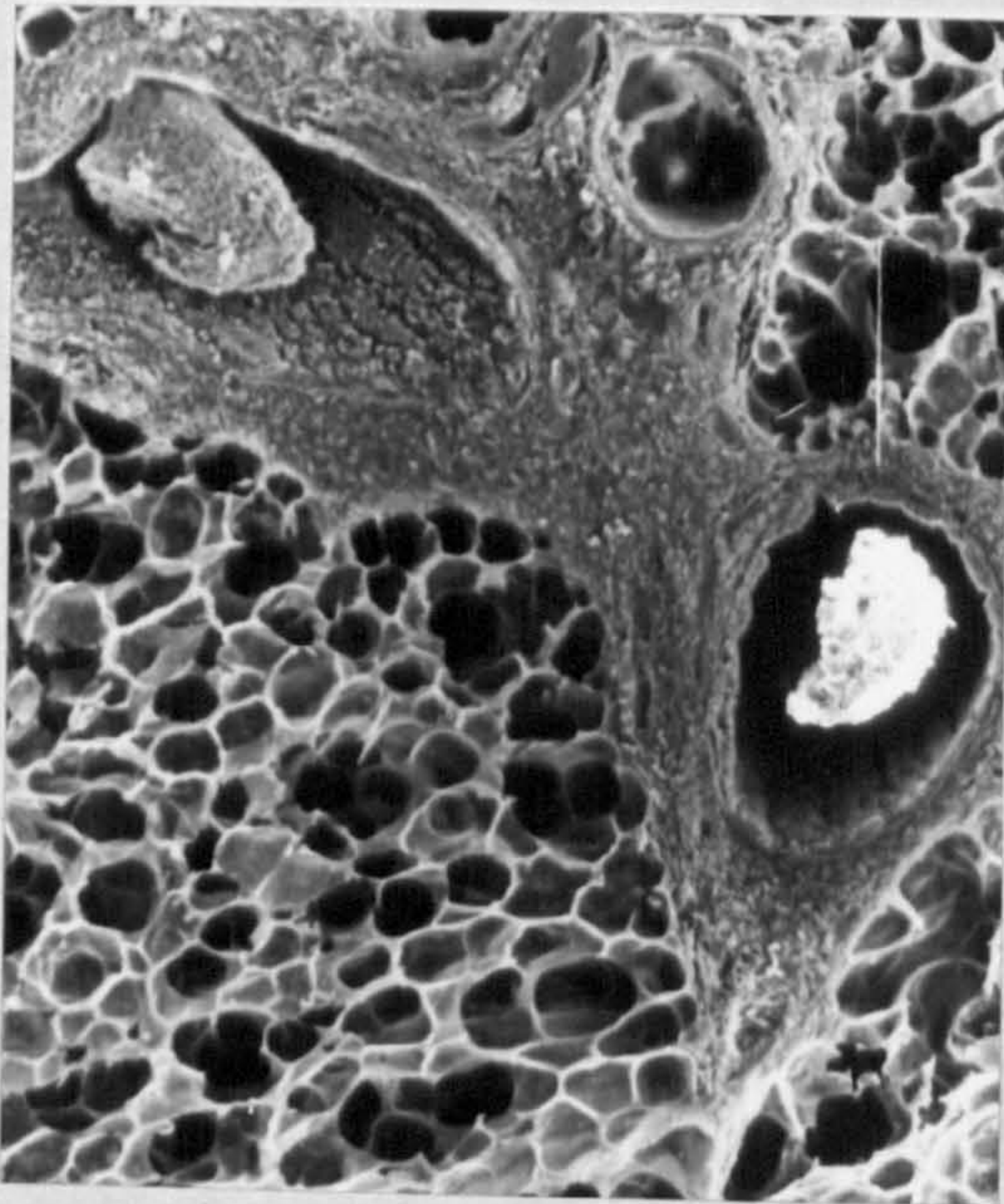


Figure 111. Low power of equine lung 24 hours after administration of 3 MI. Two bronchioles are shown and both contain plugs of epithelial tissue. The alveoli in this area appear relatively normal. X 40.

Figure 112. Close-up of similar bronchiole. X 320.

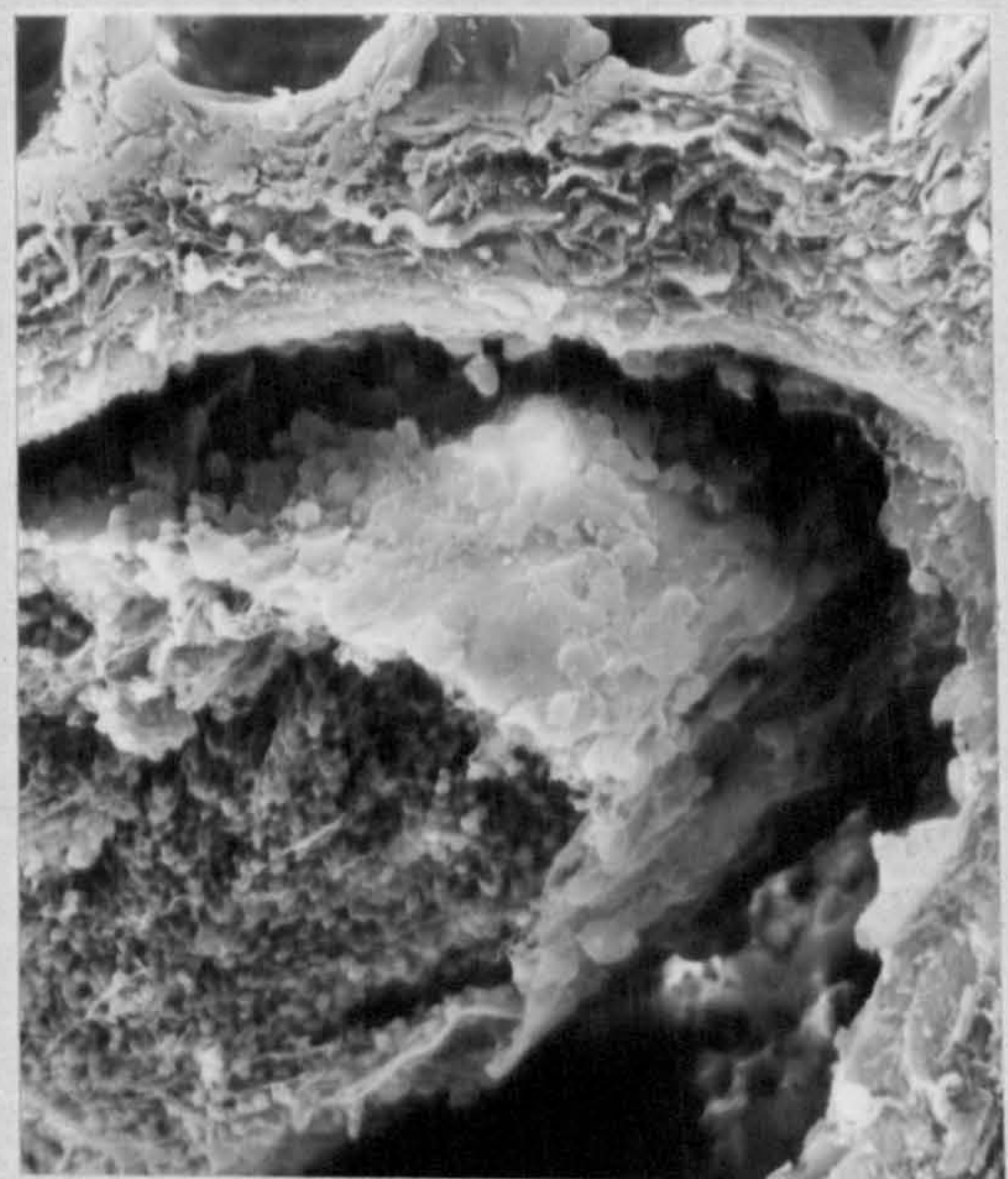
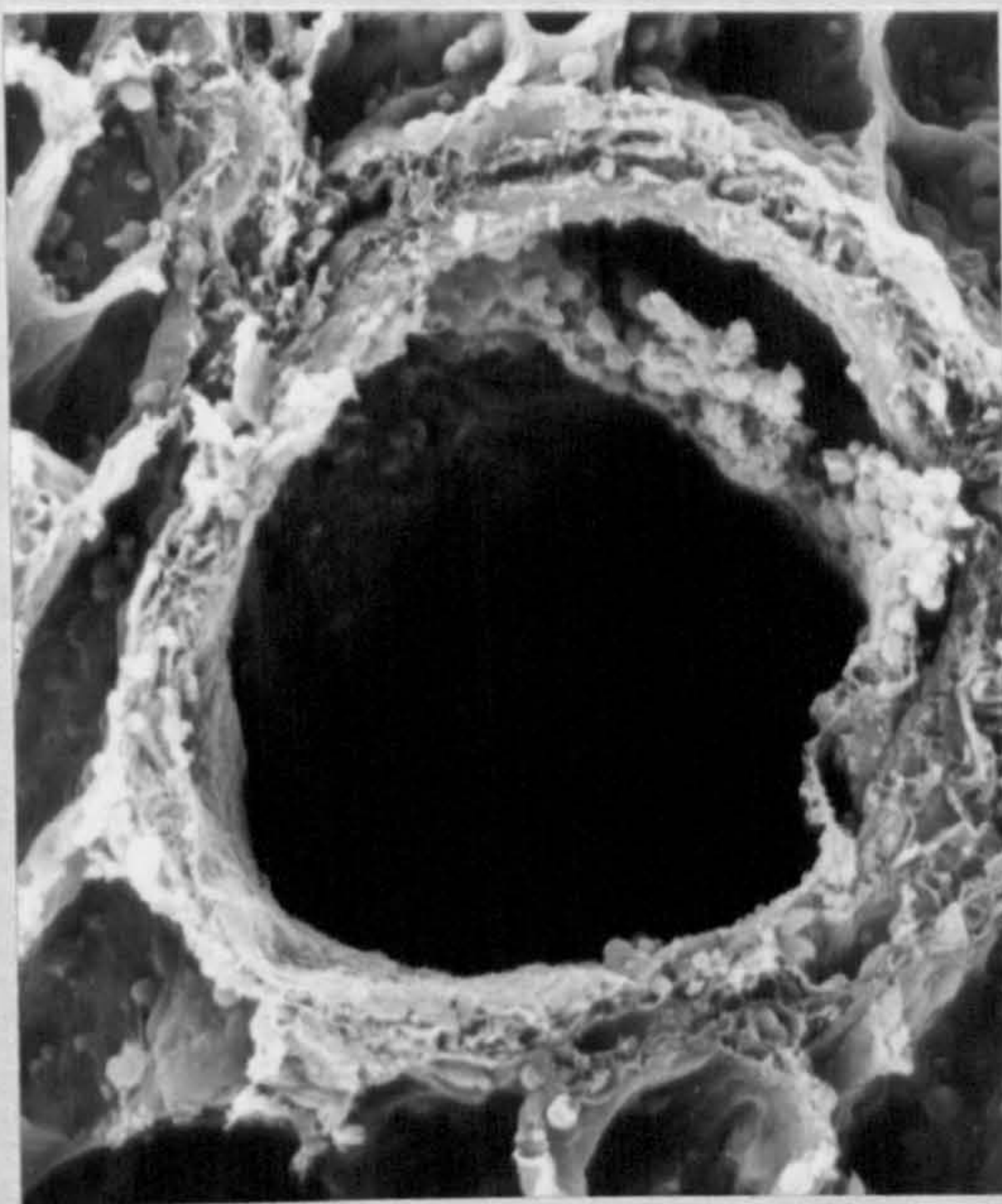


Figure 113 and 114. As for Figure 112. The epithelium can be seen lifting off the lamina propria in a continuous sheet. Haemorrhage is also present in the lumen of 114. X 320.

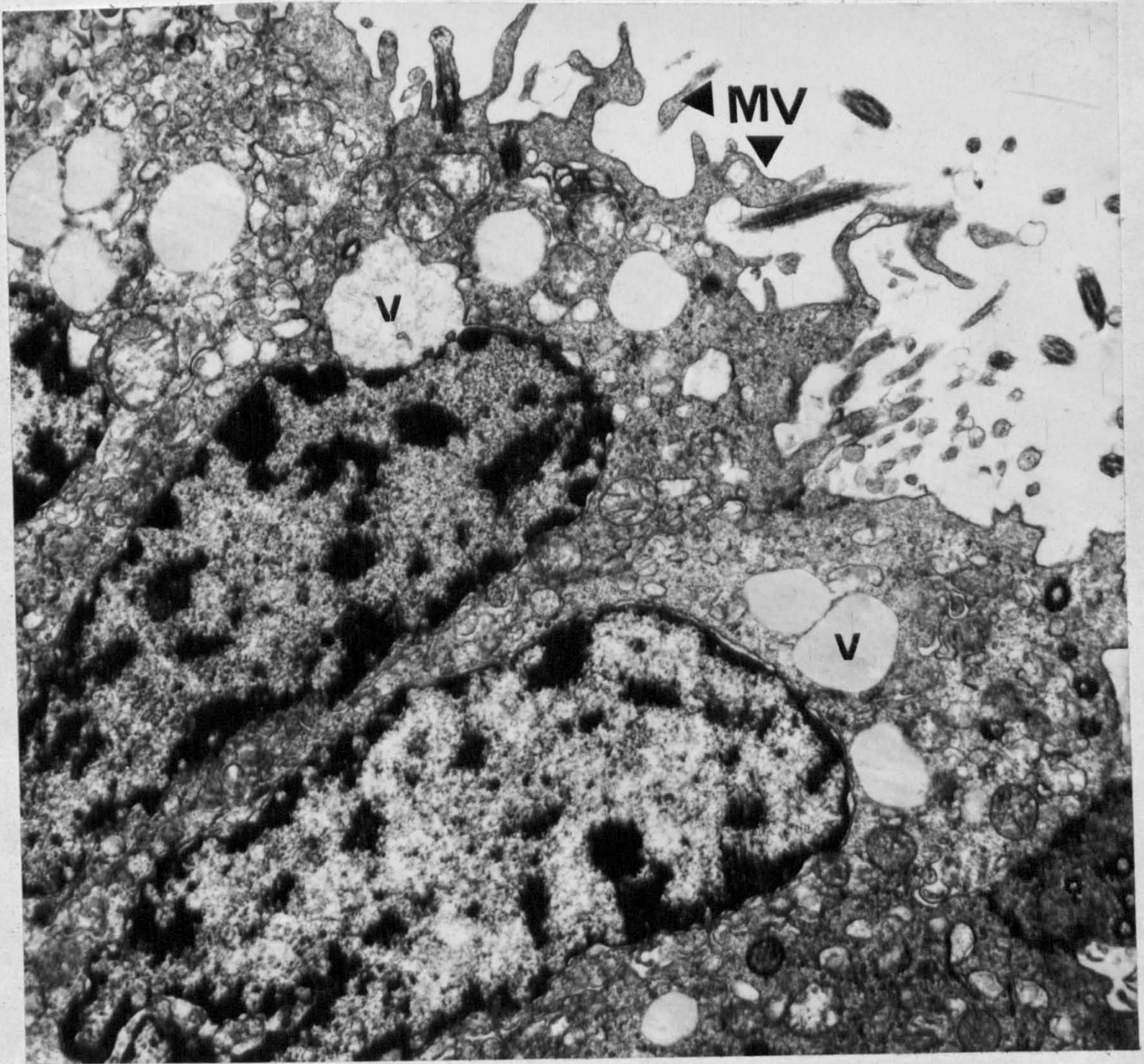


Figure 115. Electron micrograph of equine bronchiolar epithelium 24 hours after administration of 3 MI. The cells are stunted and the apical cytoplasm contains large vacuoles (V). Most cilia have been shed and the microvilli (MV) are stunted. X 10,000.

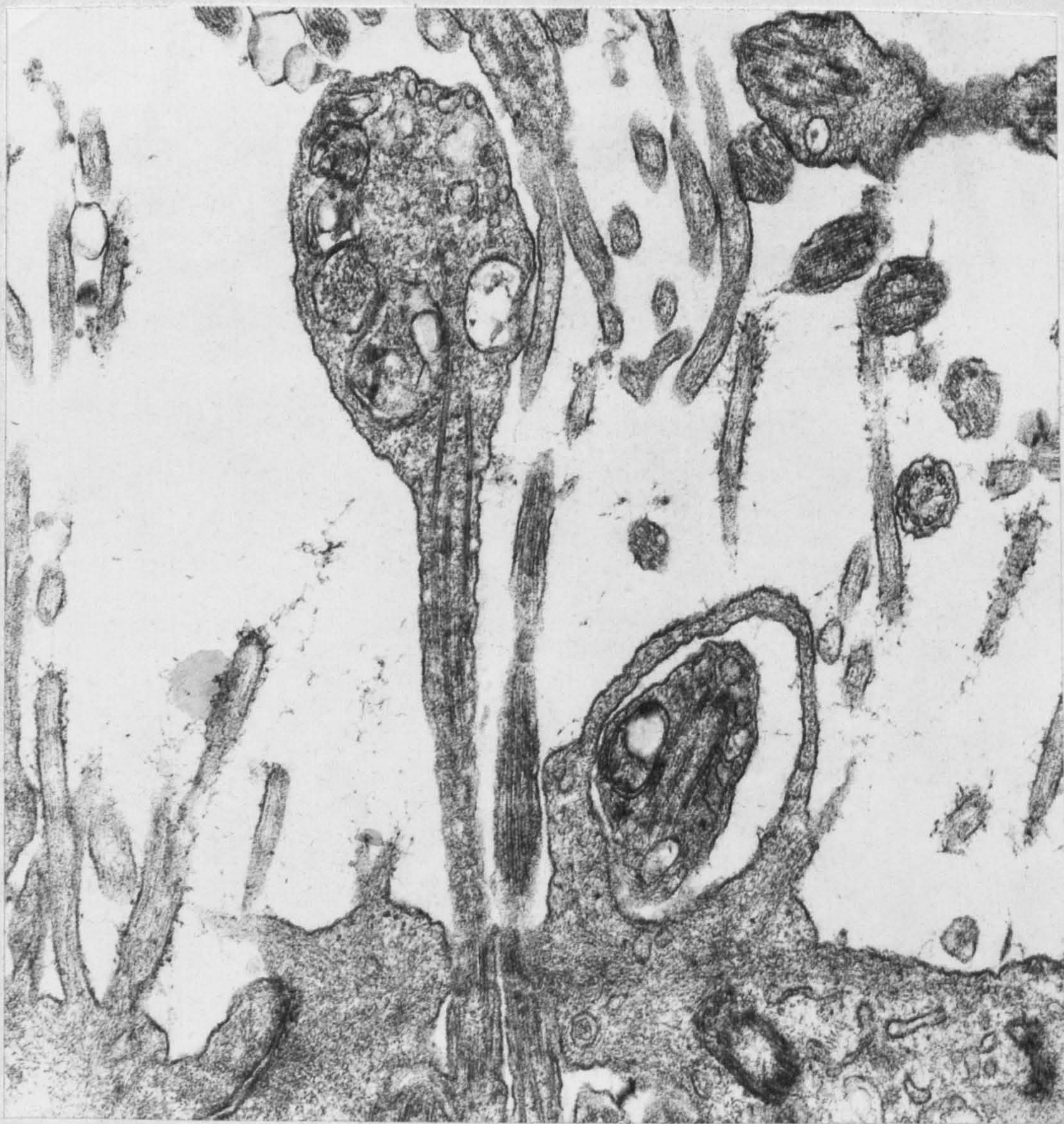


Figure 116. Electron micrograph of cilia in the bronchiole of a horse 24 hours after the administration of 3 MI. The cilia have coalesced. X 20,000.



Figure 117. Set of lungs from one of the foals killed at six days following administration of 3 MI. The lungs are very pale, puffy and overinflated.

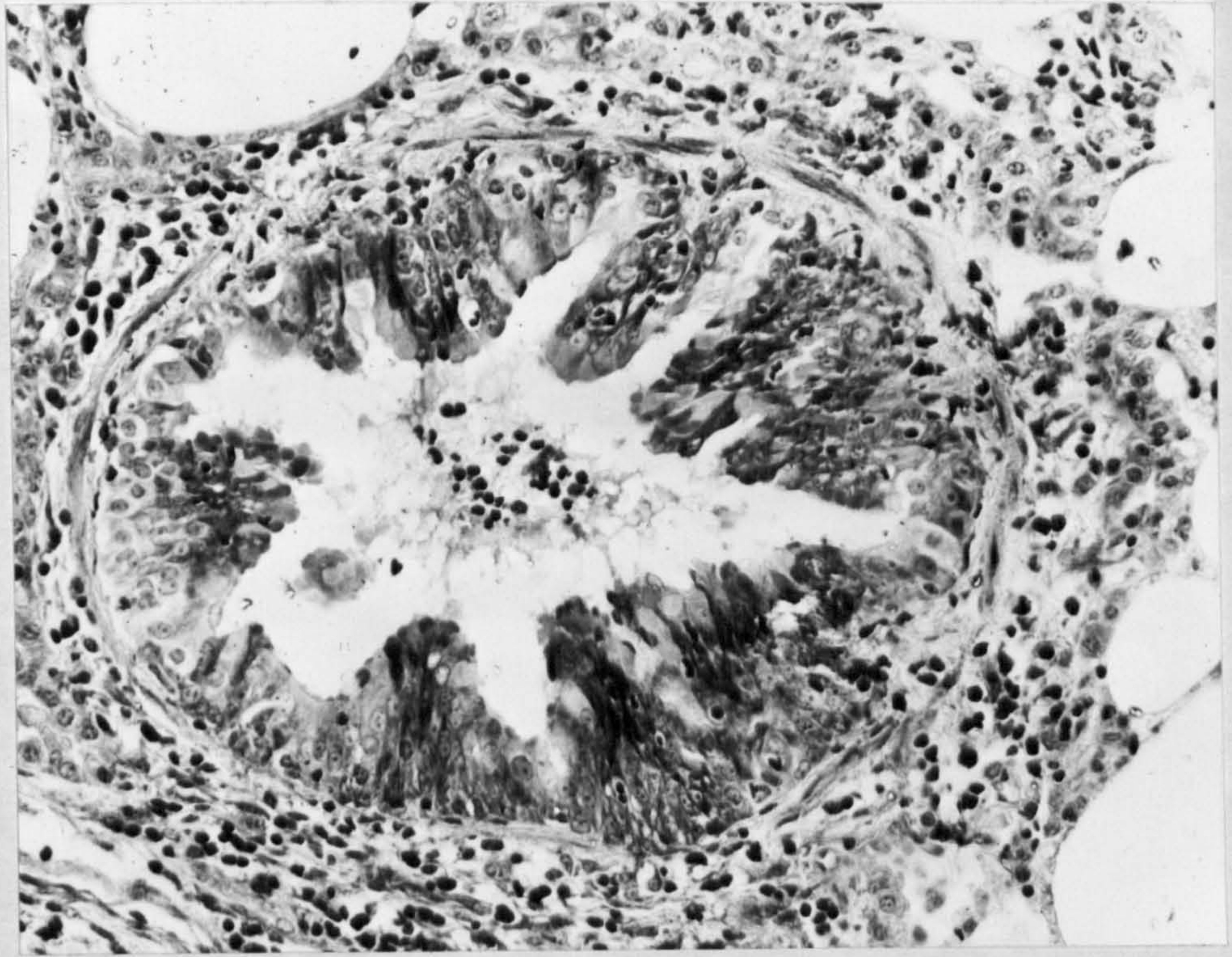


Figure 118. Section of a large bronchiole from a horse killed at six days following administration of 0.2 mg per kg 3 MI. The epithelium is hyperplastic and the cells are columnar. An inflammatory exudate is present in the lumen and inflammatory cells are present around the airway. H and E X 110.

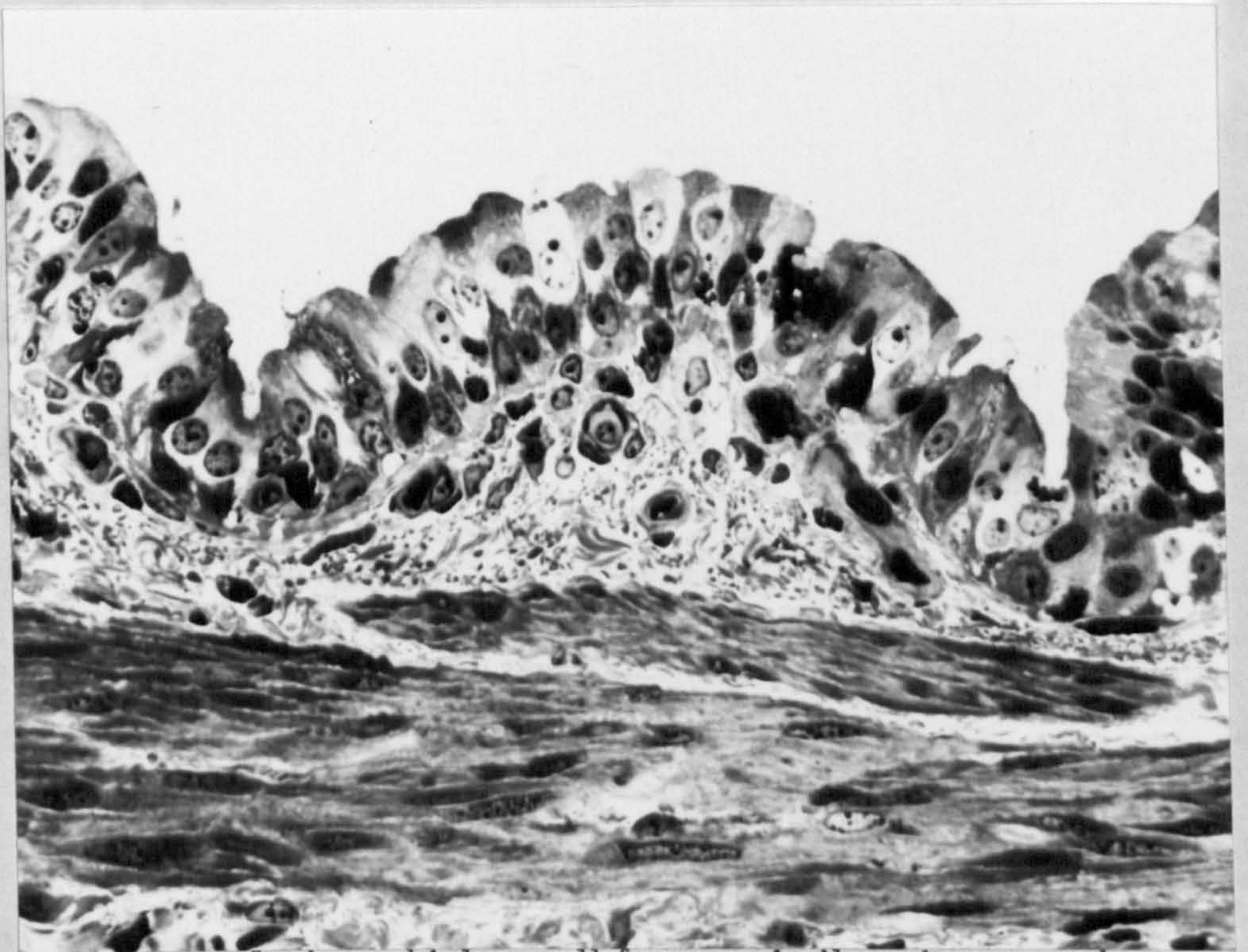


Figure 119. Close-up of a bronchiolar wall from a similar airway as that shown above. The epithelium is two cells deep, the upper layer being columnar. The lamina propria is slightly oedematous and the muscle layer is rather prominent. Section cut at $1\ \mu\text{m}$ stained with methylene blue/Azure II X 1200.

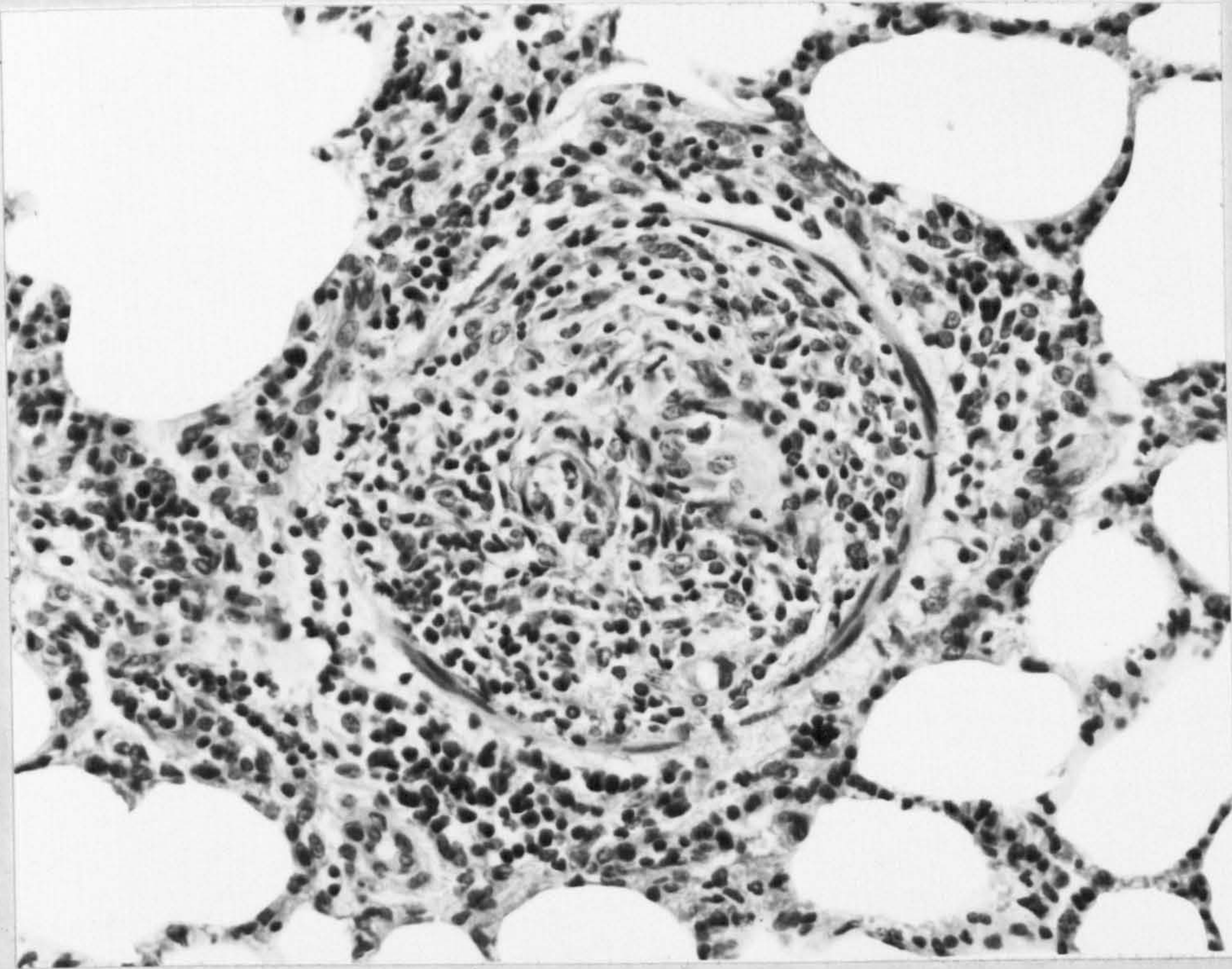


Figure 120. Small bronchiole from a horse killed six days following administration of 0.2 mg per kg 3 MI. The lumen is totally occluded with a mass of epithelial and inflammatory cells. H and E X 250.

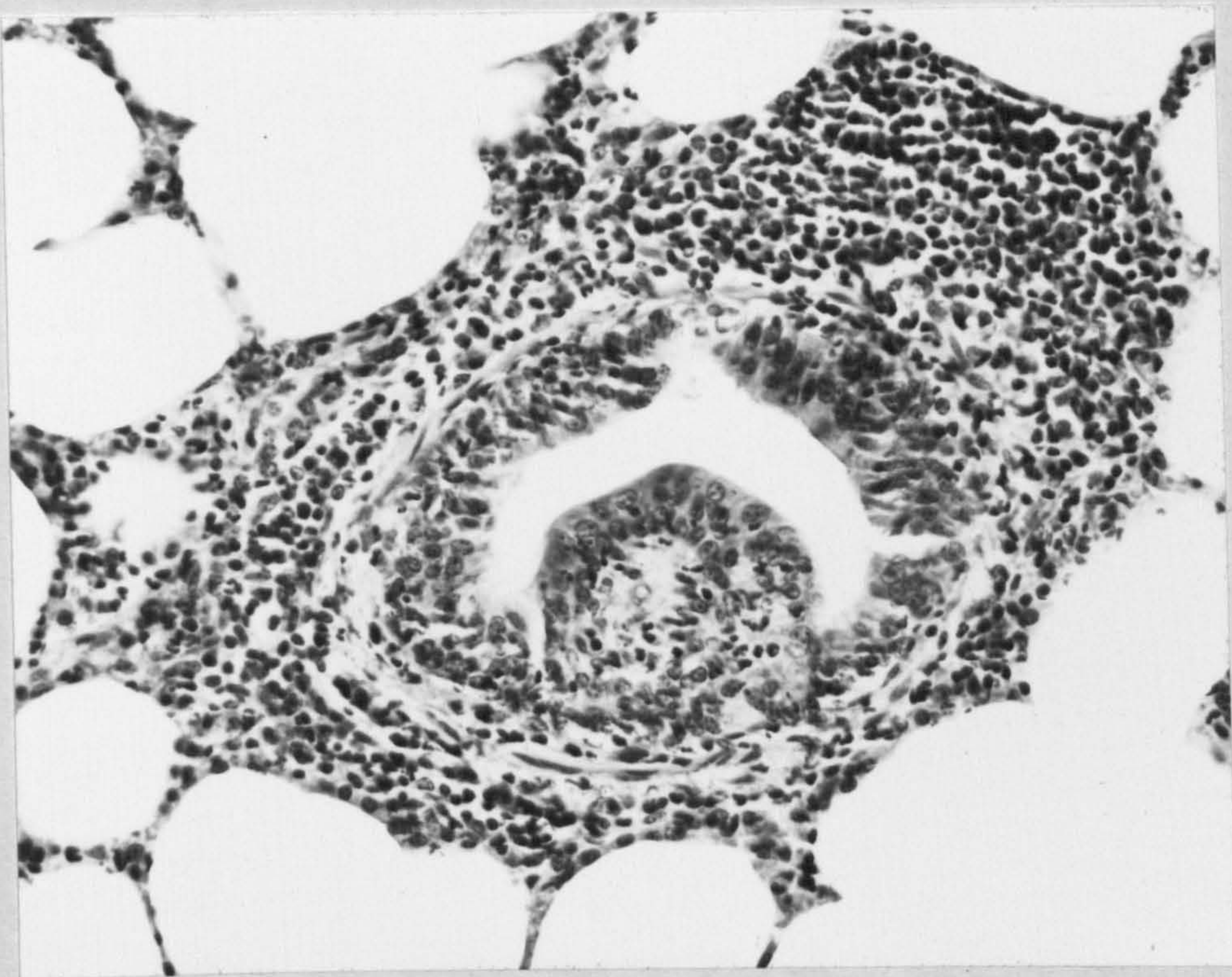


Figure 121. Small bronchiole from the same case. A small tongue of epithelial tissue is projecting into the lumen. The epithelial cells are hypertrophied and columnar. There is an inflammatory exudate around the airway. Hand E X 250.

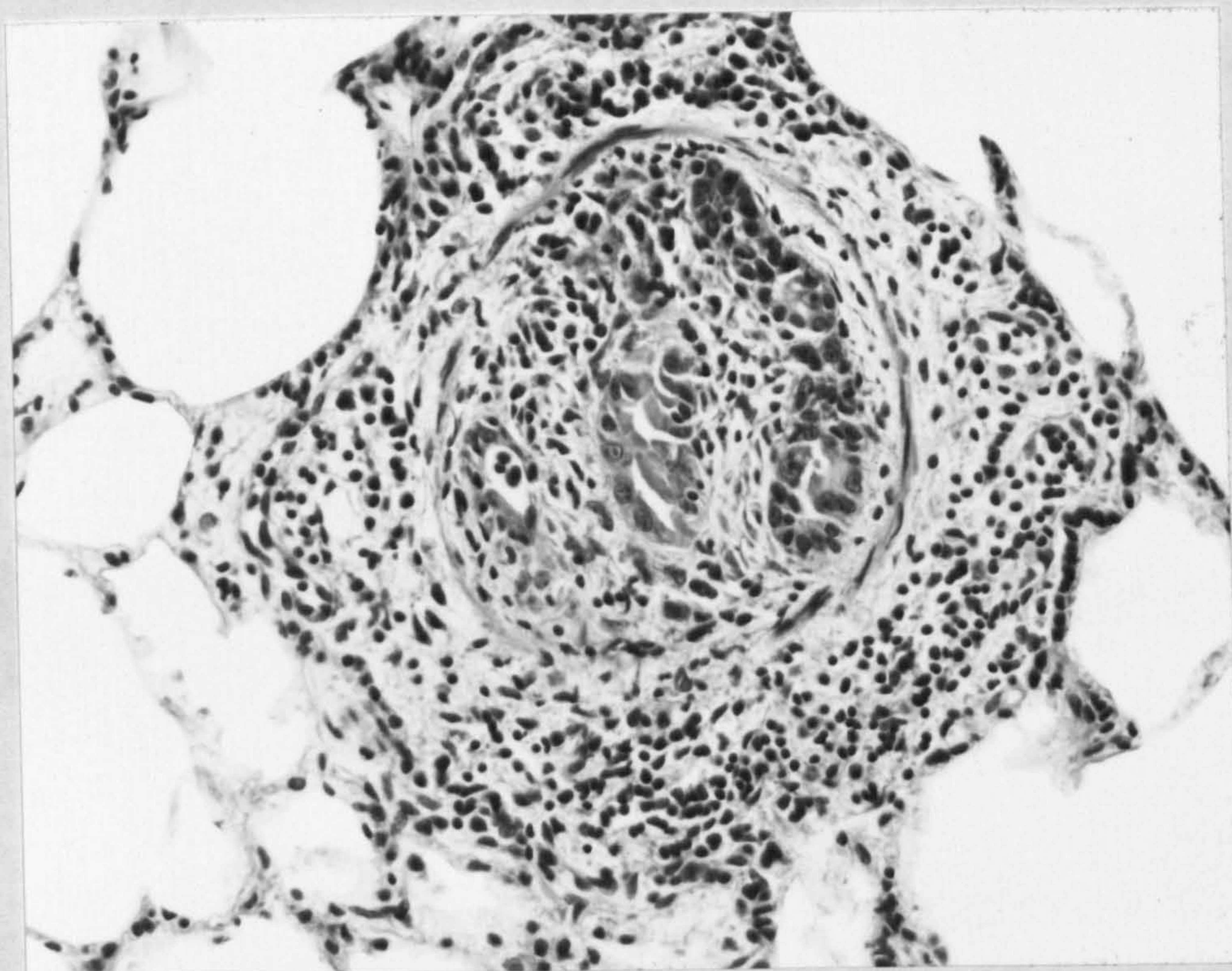


Figure 122. Small bronchiole showing a more advanced form of bronchiolitis obliterans. The airway only remains patent through several small epithelial lined ducts. The remainder of the lumen is filled with connective tissue. H and E X 250.

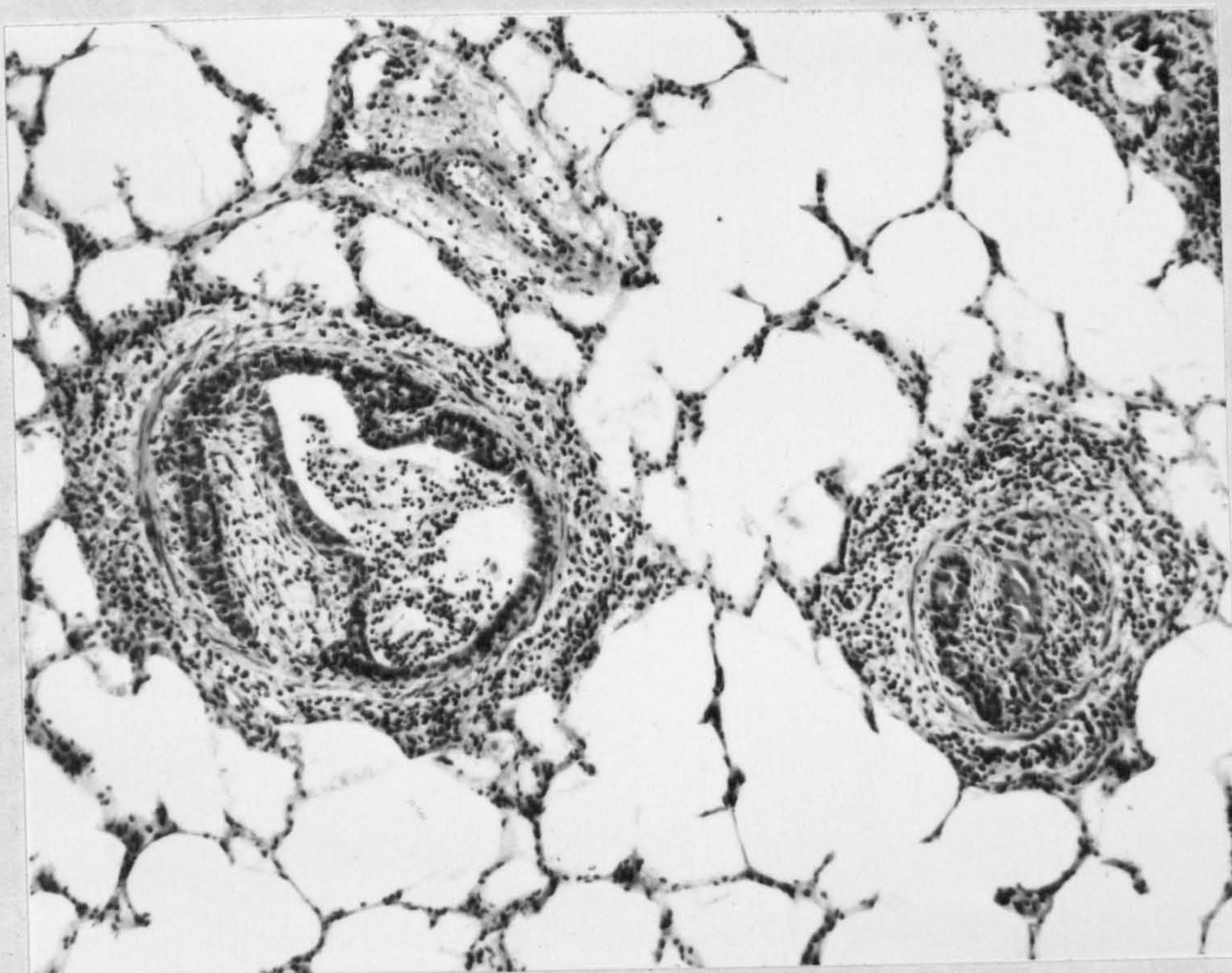


Figure 123. Foal killed at six days following administration of 0.2 mg per kg 3 MI. Two bronchioles are shown. The smaller one is the same as shown in Figure 122. The larger one shows the double epithelium that has developed around the plug of tissue. An inflammatory exudate is present in the lumen. The alveoli are overinflated and the perivascular tissue (top left) is oedematous. H and E. X 35.



Figure 124. Close-up of large bronchiole shown above. H and E X 250.



Figure 125. Section of a bronchiole from a foal killed at six days following administration of 3 MI. Small dark-staining goblet cells can be seen in the epithelium. AB-PAS X 110.

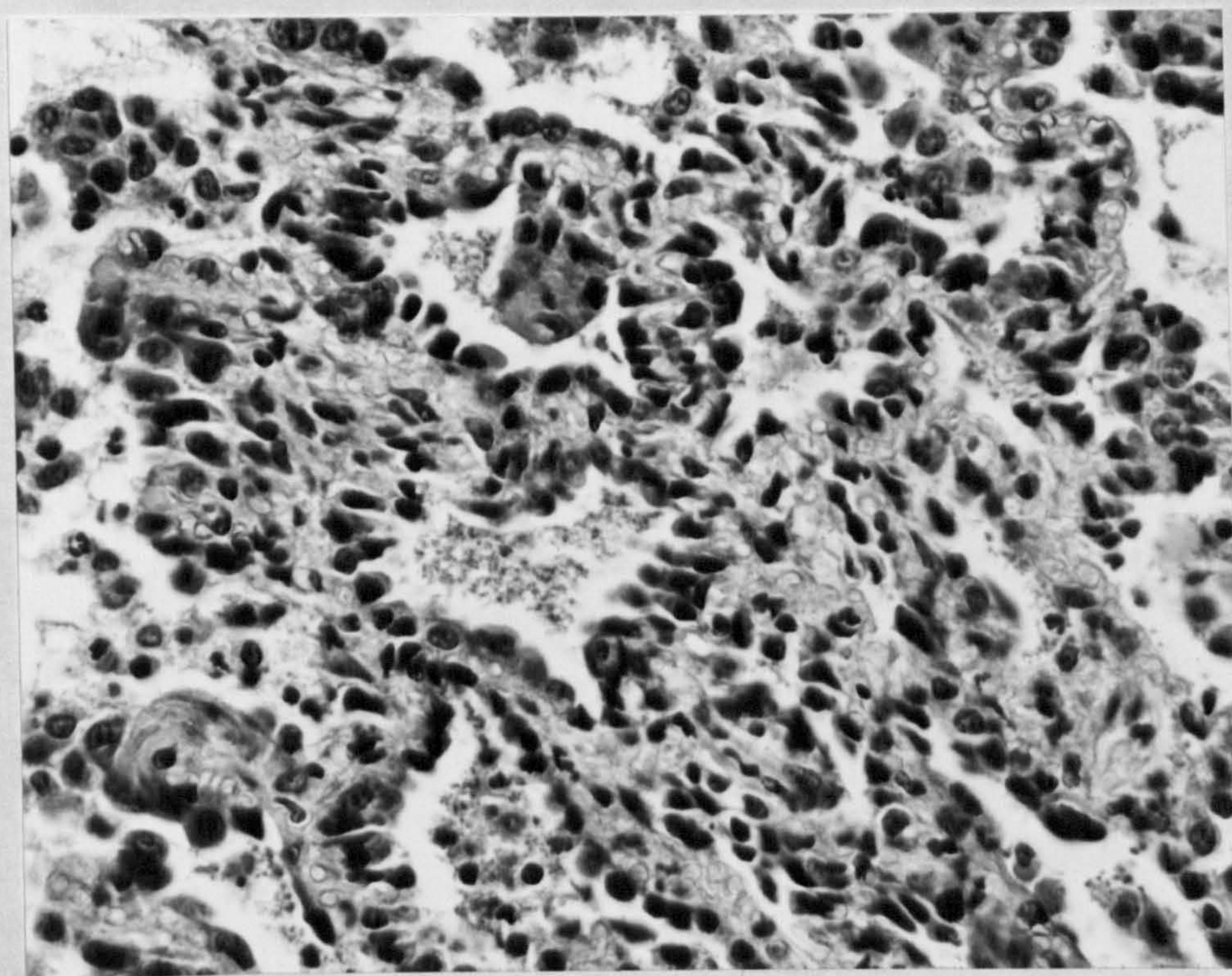


Figure 126. Area of alveolar epithelial hyperplasia in a foal killed at six days following the administration of 3 MI. H and E X 250.

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CHAPTER EIGHT

A PATHOLOGICAL STUDY OF THE LUNGS OF FOALS INFECTED
WITH A SINGLE DOSE OF PARASCARIS EQUORUM LARVAE.

INTRODUCTION

Many of the horses in the field study had pulmonary eosinophilia. The significance of this was not clear. The opportunity arose to study the lungs of foals infected with Parascaris equorum larvae. Parasitism, particularly by ascarids, is known to evoke an eosinophilic response in the host. This study therefore provided a model on which to examine the effects of parasitic eosinophilia on the equine lung.

Infection with the ascarid P. equorum is common in both foals and yearlings. The main source of infection is believed to be the faeces of the mare or of other foals in which the thick-shelled eggs containing second-stage (L2) larvae are found. Following ingestion by the host the eggs hatch in the gastrointestinal tract and rapidly migrate to the liver and then to the lungs. Larvae gradually move up the airways eventually reaching the trachea from where they are coughed up into the larynx and then swallowed to re-enter the gastrointestinal tract. Patency is reached in the small intestine, the whole life-cycle taking 80 to 106 days (Clayton and Duncan 1977).

Clinical signs of coughing and nasal discharge which are often seen in young horses have for many years been attributed to the effects of ascarid larvae migrating through the lungs (Hadwen 1925). Experimental infection of foals has shown a correlation between the occurrence of these clinical signs and the presence of larvae in the pulmonary parenchyma and airways (Lyons, Drudge and Tolliver 1976; Clayton and Duncan 1977). However, whilst there have been some detailed pathological descriptions of the lesions provoked by Dictyocaulus viviparus larvae in calves (Jarrett, McIntyre and Urquhart 1957), Metastrongylus elongatus infection in pigs (Mackenzie 1958; Pirie 1965) and experimental infections of calves and of sheep with ascarid larvae (Greenway and McCraw 1970; Fitzgerald 1962) there has not been a pathological study to determine the nature and extent of the lesion accompanying the migration of P. equorum larvae in the horse.

This study was undertaken to examine the pathological changes in the lungs which were found after a single infection of foals with P. equorum larvae.

MATERIALS AND METHODS

Experimental Design

Eight worm-free foals aged two to four weeks were given a single infection of 8,000 P. equorum eggs through a stomach tube. They were killed at intervals of 2, 7, 14, 23, 37, 73, 106 and 146 days after infection.

Experimental Animals

Pony foals were reared and maintained worm-free in the following manner. Mares foaled indoors in clean looseboxes. They were dosed every ten days with pyrantel embonate (Strongid - P Granules, Pfizer Ltd.; Sandwich Kent) at a rate of 19 mg per kg which prevented faecal contamination of the stables with parasite eggs. The stables were cleaned out daily and hosed weekly with a high pressure steam washer (Warwick 5000S, Warwick Pump and Engineering Co., Oxford) after which they were sprayed with five per cent. lysol which has been shown to be lethal to P. equorum eggs (Nevenic 1952). At eight weeks of age the foals were weaned and were housed in pairs in clean loose boxes which were also maintained in the manner described above.

Culture and administration of infective eggs

Mature female P. equorum worms were collected from the small intestine of naturally infected ponies immediately after death and placed in warm saline at 37°C for 24 hours. The sedimented eggs produced by these worms were collected, mixed with silver sand in petri dishes and incubated at 27°C for 10 days. The sand containing eggs was then covered with a filter paper soaked in 5 per cent. copper sulphate to prevent fungal contamination and stored at 4°C. Eggs were harvested by adding a 0.1 per cent. lissapol solution to the petri dishes, allowing the sand to sediment and pouring off the supernatant. This procedure was then repeated, and the eggs thus recovered were examined microscopically to confirm that a high percentage contained second stage larvae. Only those batches of eggs in which over 60 per cent. of the eggs were larvated were retained for use.

Infective doses of 8,000 larvae were prepared by dilution from a single batch of eggs and suspended in 30 ml of a 0.1 per cent. lissapol solution. Administration was by stomach tube the infective dose being flushed

through with water.

Faecal examination

Faecal samples were collected from the rectum and examined for parasite eggs by a modified McMaster method (Gordon and Whitlock 1939). 2 gm of faeces were thoroughly mixed with 30 ml of water, 30 ml of a saturated salt solution were then added as flotation fluid. 0.15 ml of the supernatant was run into a specially made counting chamber mounted on a slide and the eggs contained in an area bounded by a grid counted under a microscope.

Clinical examination

Rectal temperatures were taken at the same time each day.

General clinical examinations with particular reference to the respiratory system were performed and the animals were observed for a few hours each day to determine whether colic or nervous disturbances occurred. During these times the appetite, general demeanour and frequency of coughing of individual foals were monitored.

Body weights were measured three times weekly.

Pathology

Tissue blocks selected both from areas of pathological change and from areas of apparently normal lung were fixed in ten per cent. formol saline, Carnoy's fluid or corrosive formol. After dehydration and clearing in a double embedding series tissues were finally embedded in paraffin wax under a vacuum. Sections cut at 6 to 8 μ were stained with haematoxylin and eosin and in addition selected sections were stained with carbol chromotrope, toluidine blue at pH 4.0 and 0.3 and Martius scarlet blue.

RESULTS

Faecal egg counts

A patent infection was established in the last two foals to be killed on days 106 and 146 with egg counts of 150 and 550 eggs per gm respectively. The foal killed at 146 days post infection passed large quantities of eggs, 17,500 eggs per gm being found on day 124.

Clinical signs

Rectal temperatures of all animals were between 37.4°C and 38.9°C which is within the normal range. All surviving foals developed a cough around 13 days post-infection which persisted for one to five days. The cough was accompanied by a bilateral muco-purulent nasal discharge of varying severity which persisted for about ten days. These were the only signs of disease at this time and uninfected controls appeared completely normal.

After weaning uninfected controls gained weight at the rate of 0.5 kg per day; rates which were never matched by the infected controls although appetite was unaffected. During the late pre-patent period weight gains in the two foals remaining were reduced to 0.17 kg per day. From day 60 post-infection onwards loss of condition became apparent and the last foal to be killed at 146 days post-infection was in a state of marked emaciation. Infected foals became increasingly dull and lethargic from day 50 onwards.

Pathology

Lesions of two distinct types were found in the lungs; the first was attributable to the migration of larvae and the second was a diffuse pneumonia of the cranial lobes. Microscopic changes were also noted in the mediastinal lymph nodes. The results are summarised in Table 22.

1) Pulmonary pathology associated with P. equorum migration

Larvae could not be recovered from the lungs of the foals killed two and seven days after infection and there were no pulmonary lesions that could be attributed to parasite invasion.

14 days post-infection

Petechial and ecchymotic haemorrhages were scattered over the entire lung surface (Figures 127 and 128) being particularly numerous in the cranial part of the lung. On the cut surfaces of the lung interlobular septa were more prominent than usual.

Microscopically the main change was marked pulmonary eosinophilia. The interlobular septa and subpleural connective tissues were thickened by oedema fluid and by large numbers of eosinophils. Focal areas of alveolar

collapse and eosinophilic alveolitis together with occasional areas of intra-alveolar haemorrhage were seen (Figure 129). There was widespread bronchitis and bronchiolitis in which the main cell involved was the eosinophil sometimes also with a few eosinophils in the lumina of the airways (Figure 130). Apart from small numbers of polymorphonuclear leucocytes associated with foci of alveolar collapse, the eosinophil was the only cell involved in the observed pathological changes. Large numbers of eosinophils had accumulated around many of the pulmonary arteries and veins (Figure 131). Cross-sections of parasitic larvae were seen in some of the smaller bronchi (Figure 132). These were associated with a localised copious mucous exudate and there were small numbers of eosinophils and lymphocytes in the lamina propria. One slide showed a cross-section of a parasite in an alveolus but this was not associated with any reaction (Figure 133). Large numbers of larvae were recovered at necropsy from the bronchi by opening all major airways and then floating the lung in warm water at 37°C.

23 days post-infection

Six raised, spherical, translucent subpleural nodules of 0.3 to 1 cm diameter were found scattered over the lung surface (Figure 134). Microscopically these consisted of a mass of lymphocytes surrounded by a delicate fibrous capsule and a rim of eosinophils (Figure 135). A few eosinophils could also be seen within the nodule. Some nodules contained small pieces of strongly eosinophilic amorphous material which represented disintegrating parasitic larvae whilst others had within them collections of large pale-staining cells which seemed to be remnants of bronchial epithelium. Focal areas of eosinophilic alveolitis were present as in the previous animal but more lymphocytes and macrophages were now involved in this reaction.

37 days post-infection

Numerous sub-pleural nodules were scattered over the lung surface. Multiple slices of the lungs were made but no nodules could be detected in any part of the lung other than subpleurally.

Microscopically there were narrow perivascular sleeves of lymphocytes in the areas occupied by the eosinophils in the earlier reaction. The interstitium still contained eosinophils but there were some accumulations of lymphocytes and these were also found in the bronchiolar lamina propria.

73 days post-infection

There was one lymphoid nodule in a caudal lobe. Microscopically there were a few focal areas of alveolar collapse, sometimes accompanied by a lymphocytic reaction and some prominent follicular lymphocytic accumulations around blood vessels and bronchioles.

106 days post-infection

Four lymphoid nodules were found on the surface of the lung. Microscopically the lungs had the same appearance as those of the foal killed at 73 days post-infection. In both these animals the lymphocytic nodules had developed germinal centres (Figure 136).

146 days post-infection

Twelve sub-pleural nodules were the only macroscopic abnormalities. Microscopically there were a few areas of mild eosinophilic bronchitis and bronchiolitis. Only a few accumulations of lymphocytes were found mainly in the bronchial lamina propria and these were much reduced in size from those found at 73 days post-infection.

2) Cranial areas of pneumonia

In all the foals except those killed at seven and 146 days post-infection there were areas of reddened, collapsed, consolidated lung tissue in the cranial and accessory lobes. These were particularly extensive in the foal killed at 106 days post-infection.

Microscopically this consisted of collapsed and consolidated pulmonary tissue with bronchitis and bronchiolitis. Large numbers of polymorphonuclear leucocytes had accumulated in the airways and interstitium. Eosinophils were found within this lesion in all of the animals except that killed two days post-infection but they were not particularly numerous and they were confined mainly to the inter-alveolar septa. Moderate numbers of lymphocytes had accumulated in the lamina propria of some of the affected airways (Figures 137 and 138).

3) The mediastinal lymph nodes

Sections were taken from the mediastinal lymph nodes of each animal. At 14 days post-infection the nodes were enlarged, reactive and

contained large numbers of eosinophils especially in and around the medullary sinuses, subcapsular area and blood vessels (Figure 139). There were also large numbers in the perinodal fat. Twentythree days post-infection the nodes were still reactive with enlarged and prominent germinal centres and with moderate numbers of eosinophils. By 37 days the eosinophils had largely dispersed but many active germinal centres were present. These changes were most marked in the foal killed at 73 days post-infection (Figure 140) and had subsided by 106 days post-infection.

DISCUSSION

In this experiment the larvae of P. equorum reached the lungs between seven and 14 days after infection. Large numbers of larvae were recovered from the lungs at 14 days whereas by day 23 only small numbers of much smaller larvae were found. The larvae appear to break through into the alveoli leaving a focal area of haemorrhage and eosinophilia similar to the lesion described by Jarrett, McIntyre and Urquhart (1957) in calves infected with D. viviparus larvae although they also found foreign body giant cells in that reaction which were not present in our foals. From the alveoli the parascarid larvae presumably migrate through the alveolar ducts into the bronchioles and up the bronchi into the trachea where they are coughed up and swallowed.

The predominant reaction provoked by the migrating larvae was eosinophilia. This was especially marked in the interlobular septa and subpleural areas of the lung where there was also considerable oedema. Hadwen (1925) saw this in foals that he experimentally infected with P. equorum larvae and it is also seen in calves infected with D. viviparus larvae (Jarrett, McIntyre and Urquhart 1957).

When larvae were found in the sections of small bronchi there was surprisingly little associated reaction in the lamina propria. The larvae were always associated with large amounts of mucus but there was very little cellular reaction. However, an eosinophilic bronchitis and bronchiolitis affected most of the pulmonary airways at this time so it would be reasonable to conclude that there is a delayed response to the presence of larvae in the airways. Ascarid larvae contain a potent mast cell degranulator in their

cuticle (Uvnas and Wold 1967). One of the components of the mast cell granules is the so-called eosinophilic chemotactic factor of anaphylaxis (ECF-A) which attracts eosinophils to an area of anaphylaxis (Warren 1976). Extracts of ascarids have been used frequently to induce experimental anaphylaxis and eosinophilia (Vaughn 1961). If all helminths had the ability to degranulate mast cells this might explain the presence of eosinophils in helminthic infections. The functional role of the eosinophil is not yet fully resolved. Traditionally their presence is associated with helminthic infections and allergic reactions. Eosinophil granules do in fact contain an enzyme which acts as an antihistaminase enabling them to modulate allergic phenomena due to the release of histamine from mast cells (Warren 1976). More recently however evidence has accumulated that the eosinophil may be involved in immunological processes. Eosinophils appear to be the effector cell in acquired resistance to Schistosomal infections (Butterworth, Sturrock and Houba 1975; Mahmoud, Warren and Peters 1975). Mice, depleted of eosinophils by injection of specific anti-eosinophil serum, showed a reduced immunity on secondary exposure to Schistosoma mansoni cercariae. James and Colley (1976) found that eosinophils derived from S. mansoni infected mice were able to damage isolated S. mansoni eggs when they were maintained in culture together. The same effect could not be obtained with eosinophils derived from non-infected hosts, or with macrophages, neutrophils or lymphocytes derived from infected and non-infected hosts. The damage was dependent upon the surface antigens of the schistosome eggs being left intact. Eosinophils derived from hosts infected with Trichinella spiralis also failed to show a significant reaction to the schistosome eggs. These results imply that the eosinophil is capable of acting as a specific effector cell able to recognise specific antigens.

It proved difficult to demonstrate the presence of mast cells and globule leucocytes in our material. Normally Carnoy's fluid as fixative and stains such as toluidine blue at pH 4.0 will demonstrate metachromatic mast cells in the tissue but such was the intensity of the eosinophilia in the material that it completely masked the reaction.

It was interesting to note that the eosinophilia was rapidly resolved and by 23 days after infection had been completely replaced by a lymphocytic reaction. By this time there were very few larvae left in the lungs.

Subpleural lymphocytic nodules have been reported in horses by Theiler (1918), Hadwen (1925) and Nieberle and Cohrs (1967). Identical nodules are seen in M. elongatus infection of pigs (Mackenzie 1958) and D. viviparus infection of cattle (Jarrett and Sharp 1963). In pigs the nodules are said to be more numerous in long-standing infections and are a feature of post-patent disease (Mackenzie 1958). In cattle they are more likely to be found following vaccination with X-irradiated larvae (Jarrett and Sharp 1963; Michel and Mackenzie 1965), treatment with anthelmintics (Jarrett, McIntyre and Sharp 1962) or reinfection (Jarrett and others 1960; Breeze and others 1975) demonstrating that the presence of such lymphocytic nodules in the lungs of cattle is an indication of an immune response to D. viviparus (Pirie and others 1971; Breeze and others 1975).

In the foal killed at 37 days after infection numerous sub-pleural nodules were found, but in the next one killed, 73 days after infection, only one nodule was found. In other experiments with P. equorum we have found nodules under a range of circumstances. A foal dosed ten times on alternate days with 160 eggs and killed 100 days after the first infection had a large number of sub-pleural nodules. Another foal similarly treated and killed 40 days after the first infection had only a few. A naturally infected foal challenged with a dose of 8,000 eggs and killed 30 days after infection also had large numbers of nodules whereas two similar foals killed 120 days after challenge had only a few nodules. Small numbers of nodules are often observed in naturally reared foals and in adult horses that we have necropsied. Theiler (1918) also found nodules in adult horses.

In calves nodules persist for at least six months following a double dose of X-irradiated larvae (Pirie and others 1971). They are not found following a single experimental infection. Our experimental design ensured that there was no reinfection from the foals themselves and therefore it would seem that nodules develop readily in foals given only one dose of larvae. Our numbers are too small to draw any firm conclusions from but in most foals the nodules persist only a few weeks.

Betts (1954) found that a massive dose of Ascaris lumbricoides given to virus pneumonia-free pigs failed to induce lesions that in any way resembled enzootic pneumonia. Underdahl and Kelly (1957) found that pigs given Ascaris

suum larvae plus an agent isolated from a case of enzootic pneumonia developed ten times as much pulmonary consolidation as pigs given enzootic pneumonia alone, whilst pigs given A. suum alone did not develop any pulmonary consolidation. They concluded from this that migrating ascarid larvae could enhance lesions of pre-existing enzootic pneumonia. A similar mechanism could be proposed to explain the presence of considerable pulmonary consolidation in some of the foals.

Clinical signs of a cough and muco-purulent nasal discharge were first apparent around 13 days after infection and persisted up to five days (Clayton and Duncan 1977); worm-free foals maintained in the same air-space were not affected. This corresponds approximately to the period when the larvae were actually migrating through the respiratory system to the gastro-intestinal tract. The pathological findings at this time were severe enough to account for some clinical signs in the animals but the fact that there was also cranial lobe consolidation cannot be ignored. Paired serum samples were examined for the presence of a rising antibody titre to several of the common viral pathogens of the equine respiratory tract. There was no serological evidence of infection with equine influenza 1 (Prague) or 2 (Miami), equine rhinovirus type 1, equine rhinopneumonitis or adenovirus. We did not attempt to isolate mycoplasmata from this material although several mycoplasmata have been isolated from the respiratory tract of horses (Allam and others 1973). Two seem to be specific to horses and these have been identified as Mycoplasma equirhinis (Allam and Lemcke 1974) and the unnamed N3 (Allam and Lemcke 1975). A large number of horse sera were examined for the presence of complement-fixing antibody to these two organisms and 78 per cent. were found to be positive (Hooker and Butler 1976), activity against M. equirhinis being much more common than activity against N3. Experimental infections of adult ponies with the two mycoplasmata failed to induce any clinical signs. N3 could not be reisolated from the ponies but M. equirhinis infection persisted at high levels for six weeks and spread readily throughout a group of ponies (Hooker and Butler 1977). No young foals were infected in these experiments; nor was any pathological study carried out on them. More work is needed to find if this form of pneumonia is widespread amongst young foals, since this might explain the sometimes severe clinical reactions accompanying P. equorum migration.

The lesions found in these foals in no way resembled the lesions found in natural cases of chronic bronchiolitis. The eosinophilia had a different distribution being subcapsular and interstitial as well as peribronchiolar. In addition no larvae were found in the cases of chronic bronchiolitis and few of the animals had subpleural lymphoid nodules. The natural disease, chronic bronchiolitis, is therefore unlikely to be caused by chronic ascarid infection and the experimental eosinophilia produced by artificially infecting foals with P. equorum does not give a useful model of the disease.

Number of days post-infection	Cranial lobe pneumonia	Larvae recovered from lungs	Pulmonary eosinophilia	Pulmonary lymphocytic accumulations	Subpleural lymphocytic nodules	Mediastinal lymph node	
						Eosinophilia	Germinal Centres.
2	++	-	-	-	-	-	++
7	-	-	-	-	-	-	+
14	++	+++	+++	-	-	+++	++
23	++	+	++	+	+	++	++
37	++	-	+	++	++	+	++
73	++	-	-	++	+	-	+++
106	+++	-	-	++	+	-	+
140	-	-	-	-	++	-	+

+ indicates mild reaction

+++ indicates severe reaction.

TABLE 22. THE PULMONARY LESIONS FOUND FOLLOWING ORAL INFECTION OF FOALS WITH PARASCARIS EQUORUM LARVAE.



Figure 127. Right lung from foal killed at 14 days post-infection. Petechial and ecchymotic haemorrhages are scattered over the entire lung surface.

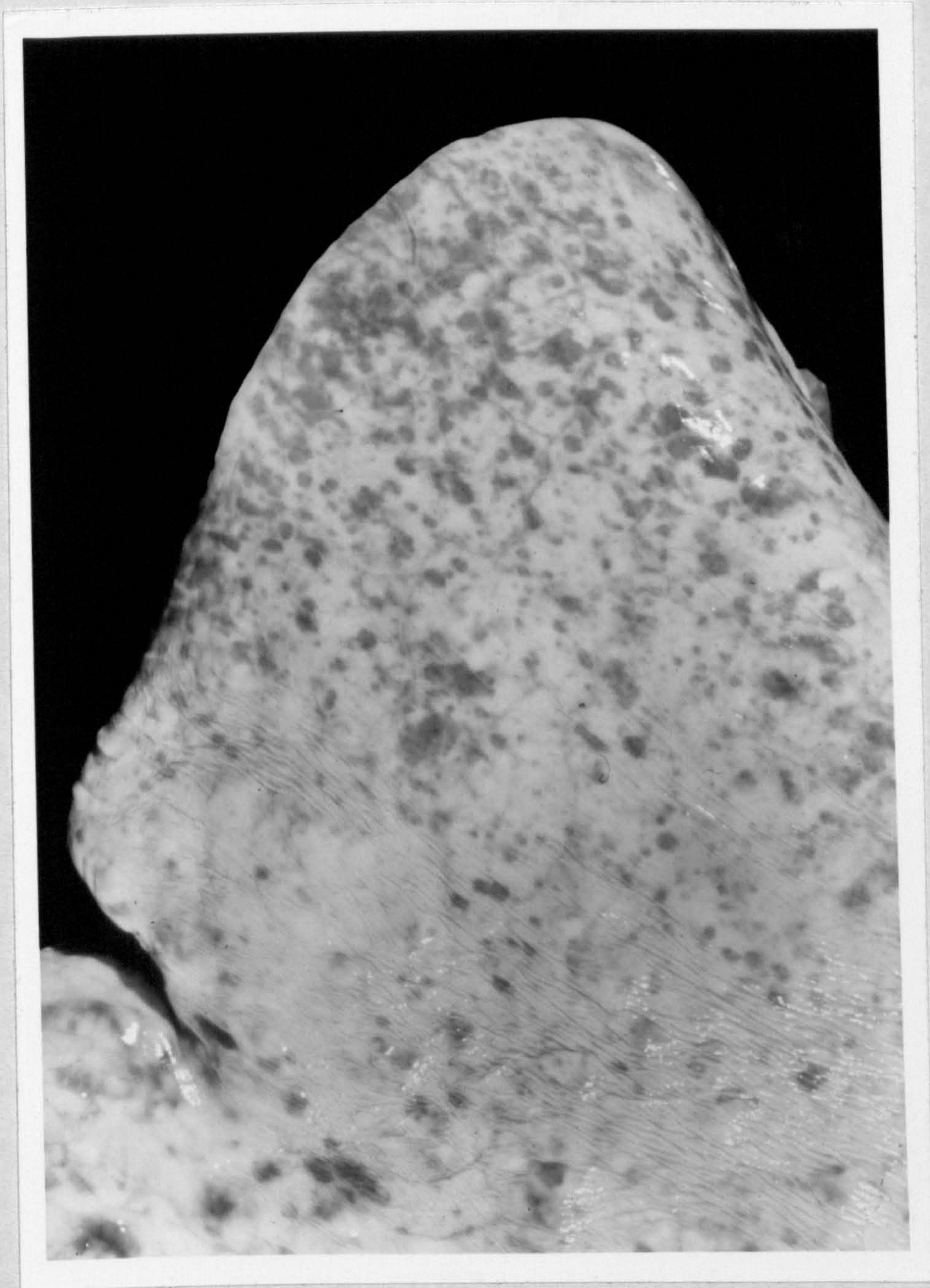


Figure 128. Detail of a part of the lung shown in the preceding figure.

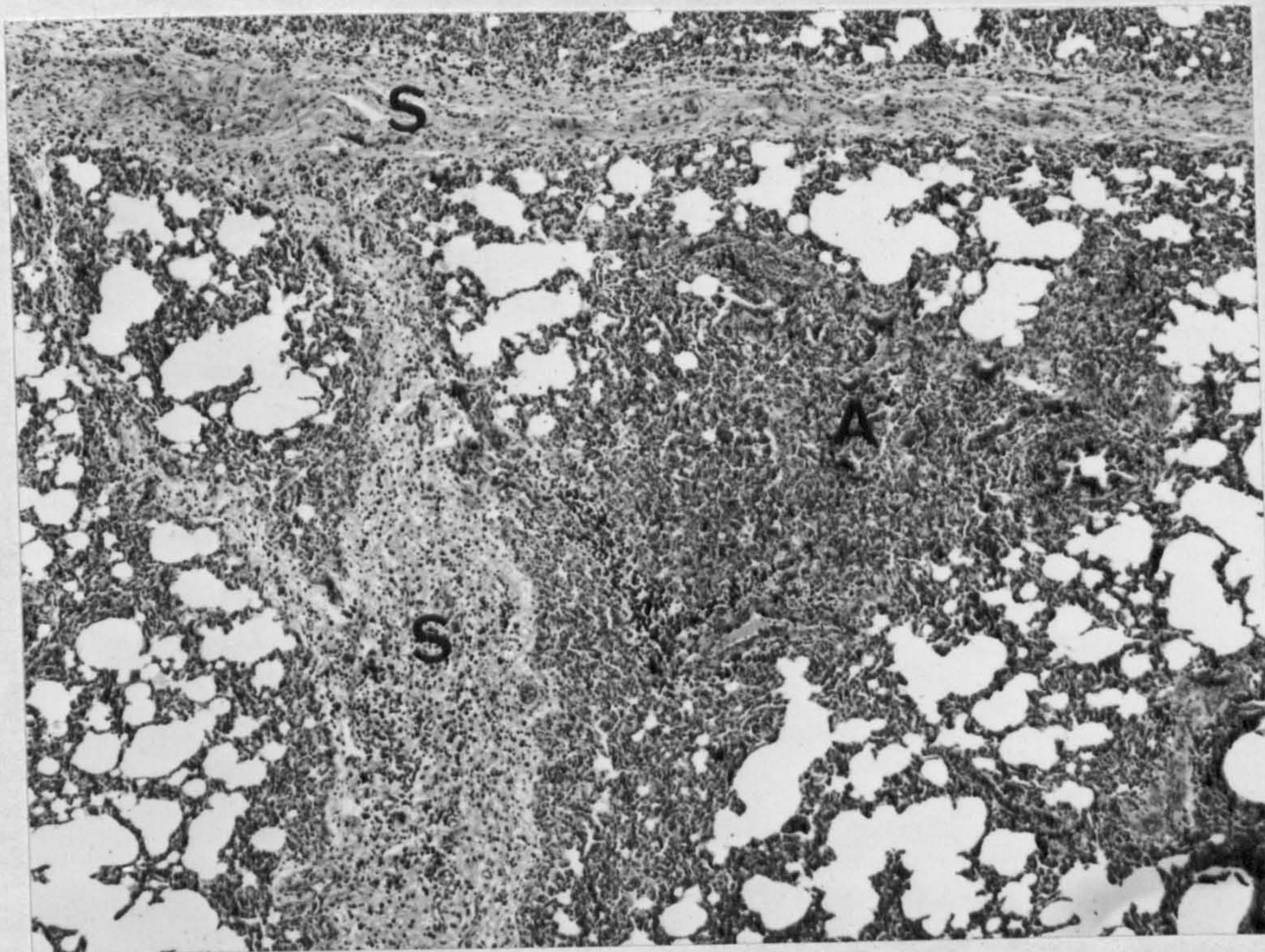


Figure 129. Foal killed 14 days post-infection. The interlobular septa (S) are widened by oedema and eosinophilic infiltration. An area of alveolar collapse and haemorrhage (A) is also present. X 35.

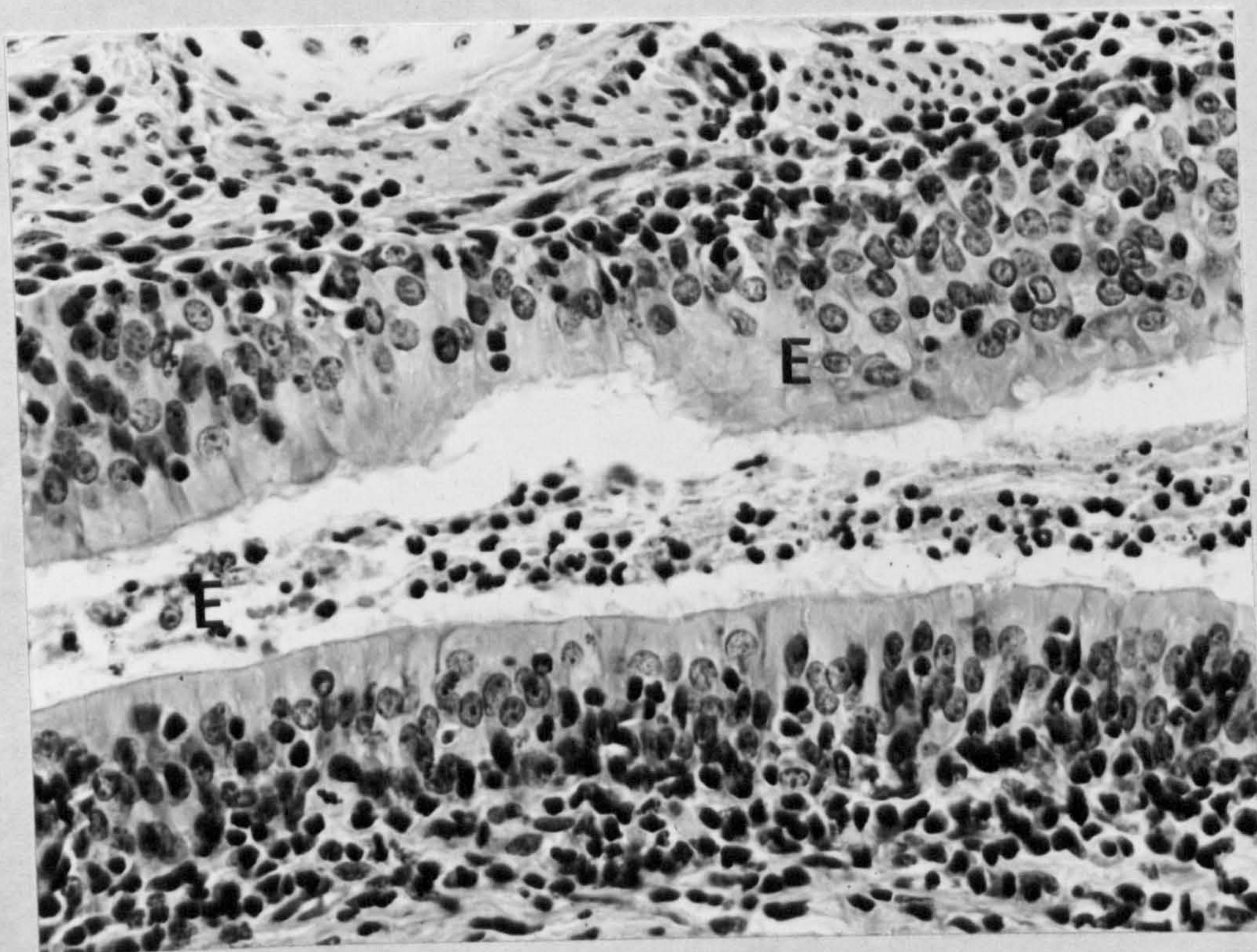


Figure 130. Foal killed 14 days post-infection. Longitudinal section of a bronchus showing eosinophils (E) in the epithelium and in the airway. X 300.

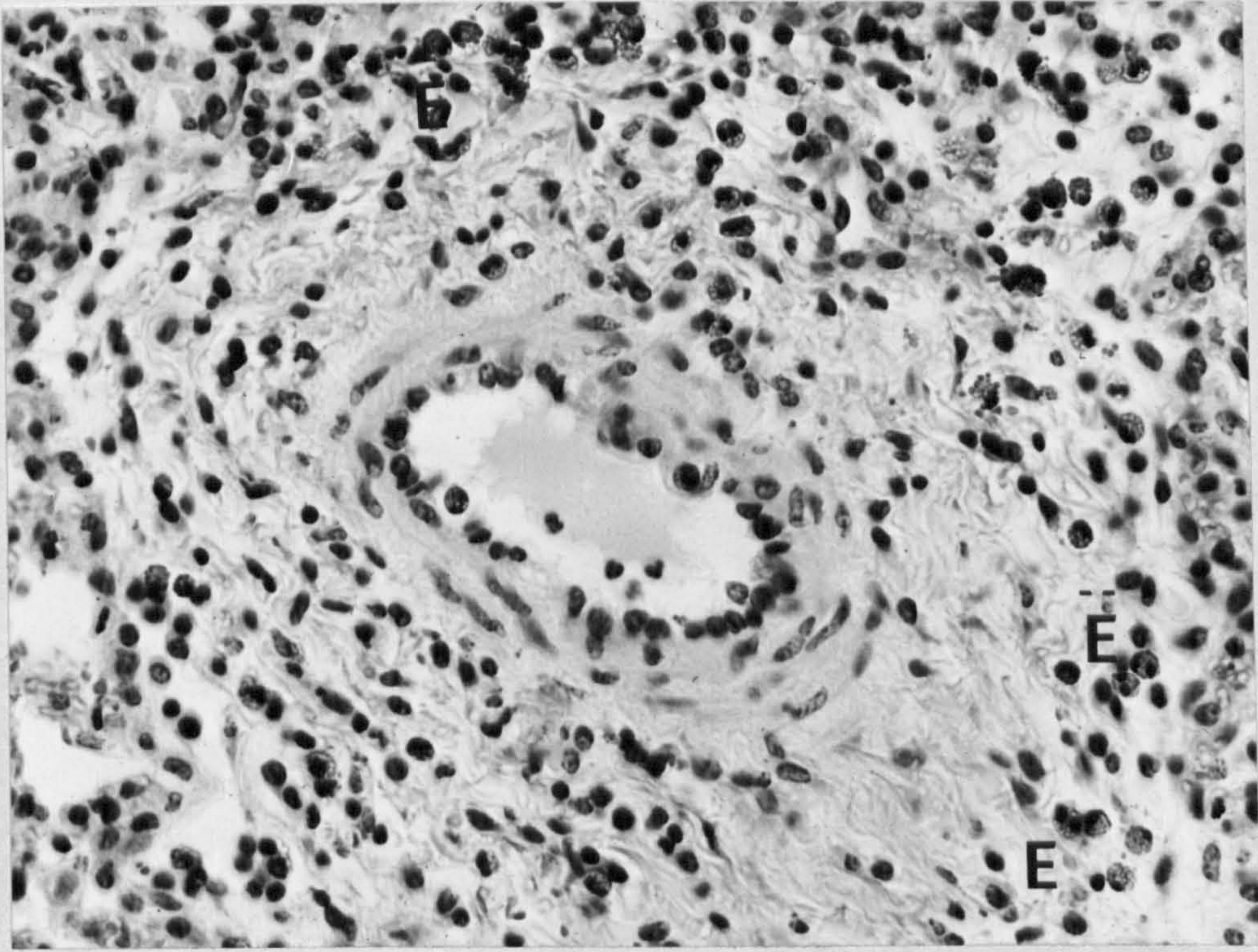


Figure 131. Foal killed at 14 days post-infection. Section of small pulmonary arteriole surrounded by numerous eosinophils (E). X 250.

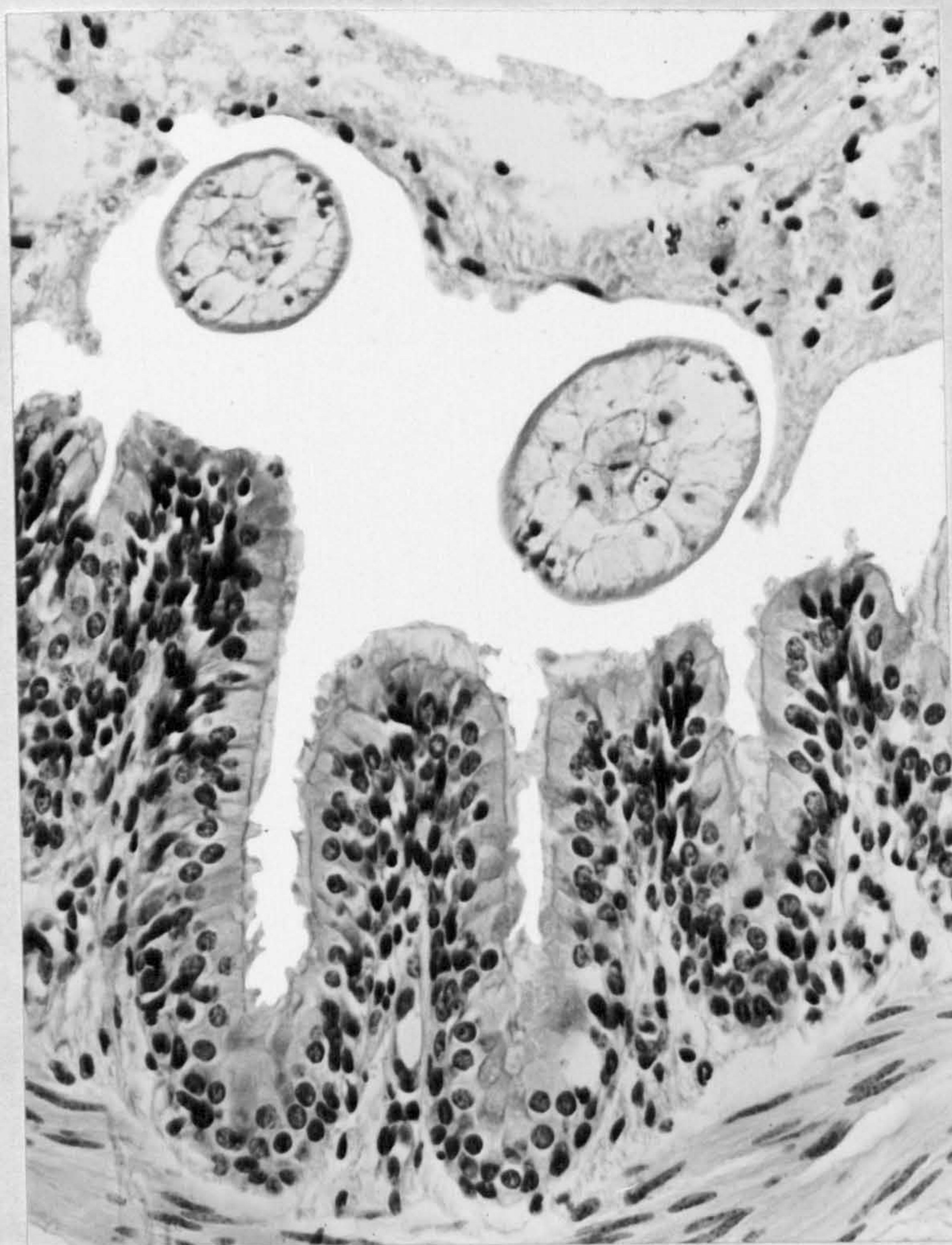


Figure 132. Foal killed at 14 days post-infection. Small bronchus containing two cross-sections of parasitic larvae. X 250.

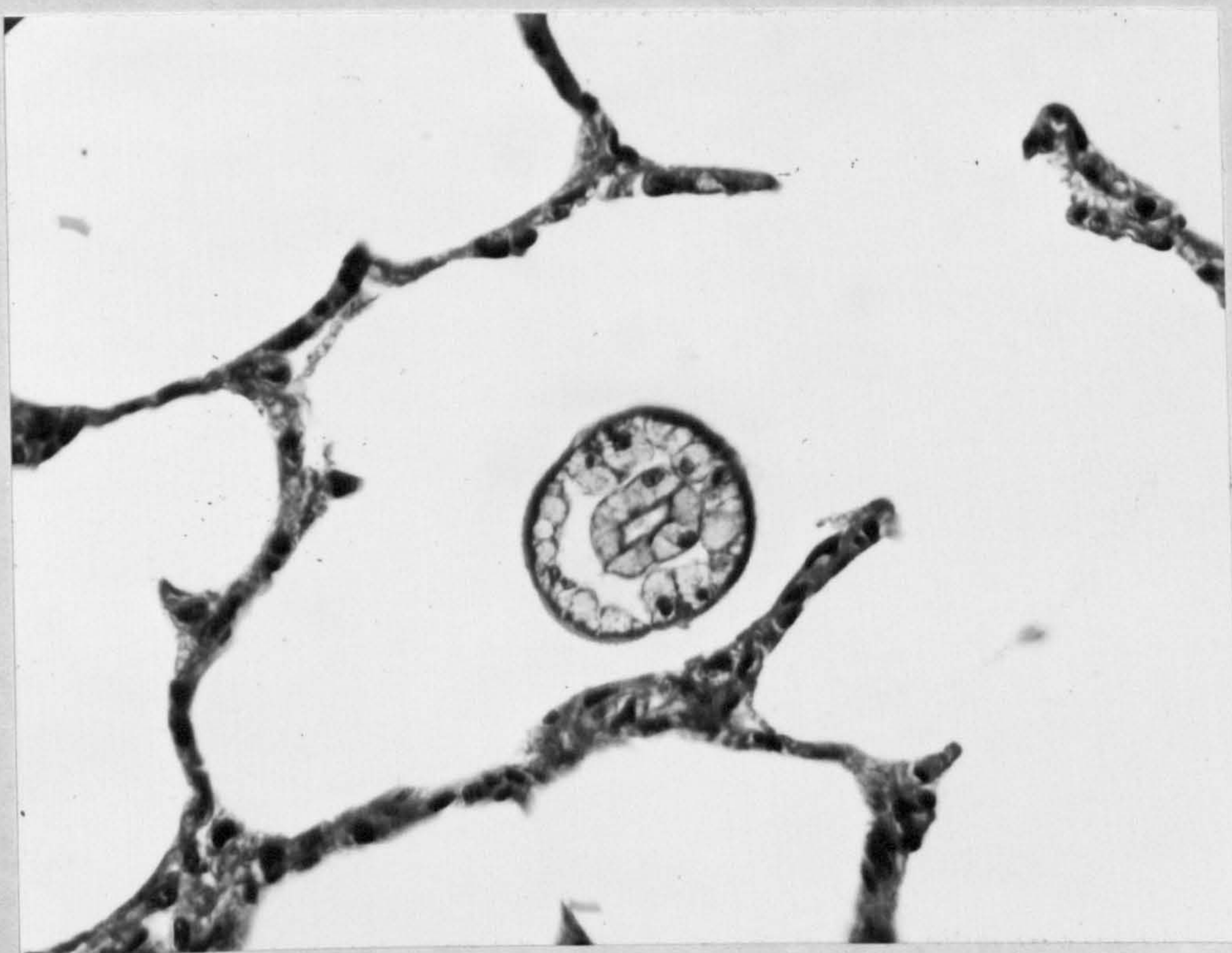


Figure 133. Foal killed at 14 days post-infection. Cross-section of a parasite in an alveolus. X 400.

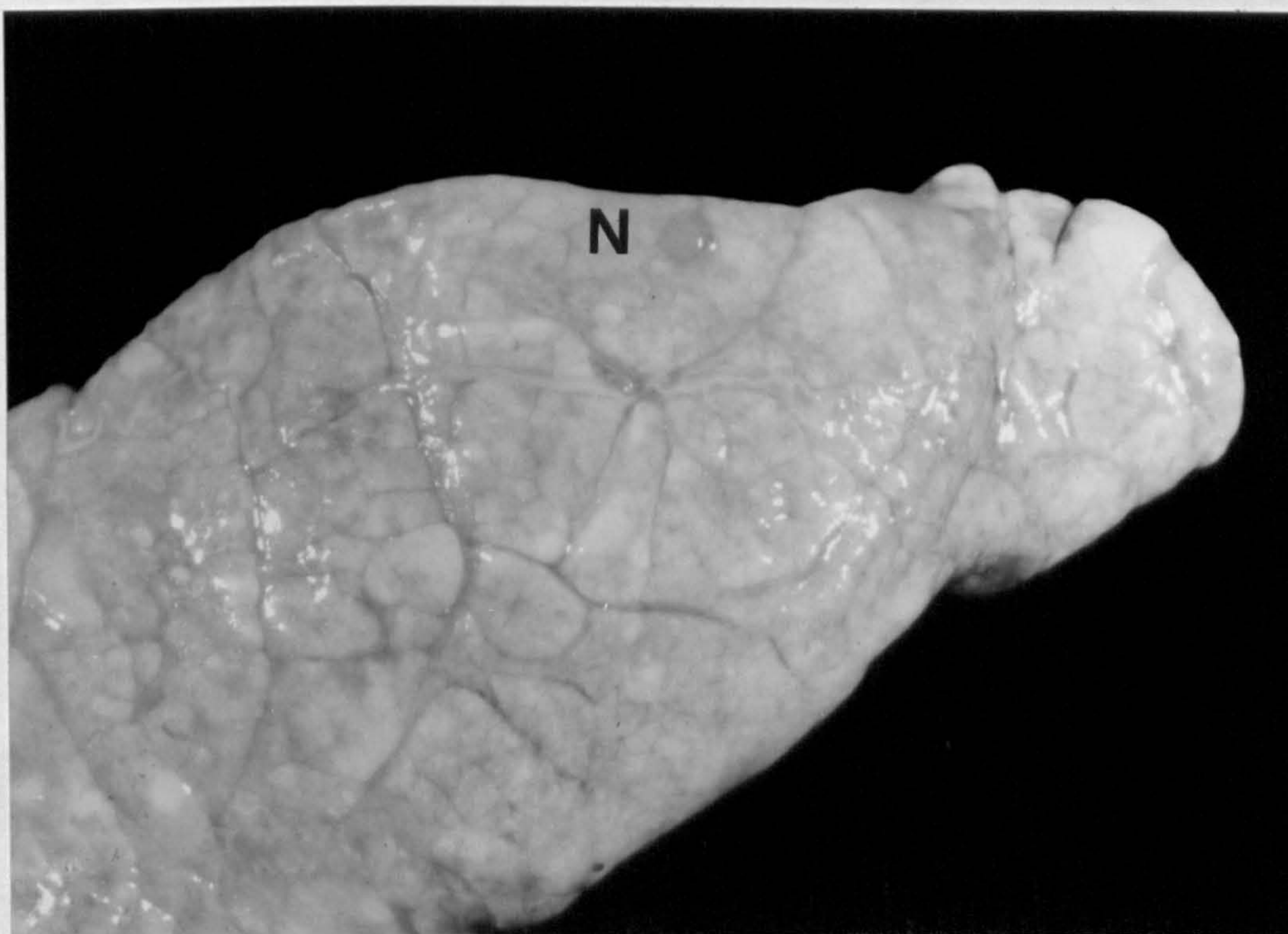


Figure 134. Foal killed at 23 days post-infection. Sub-pleural parasitic nodule (N) in the cranial lobe of the left lung.

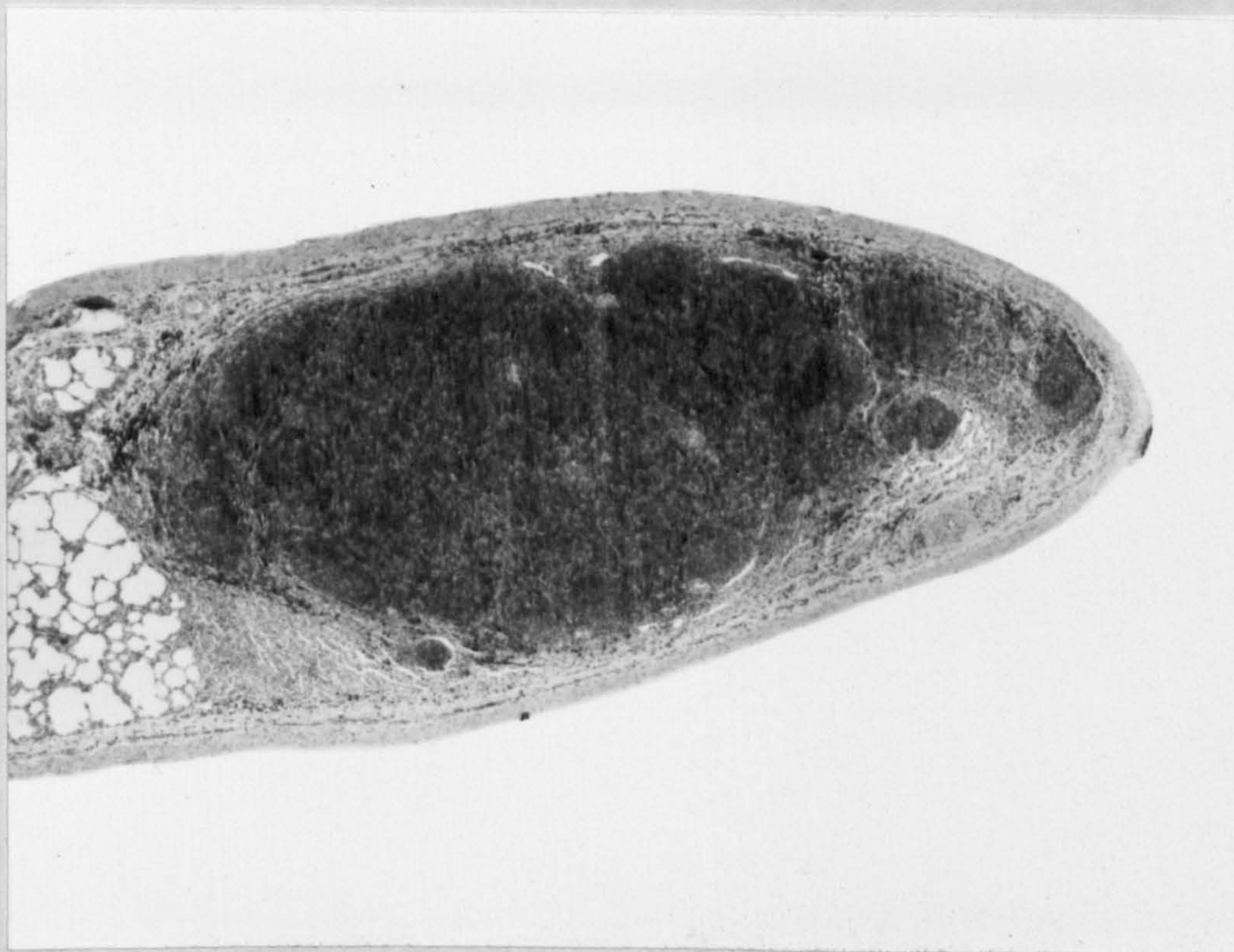


Figure 135. Foal killed at 23 days post-infection. Cross-section of a similar nodule to that shown in the above picture. Microscopically it consists of a mass of lymphoid cells. X 12.

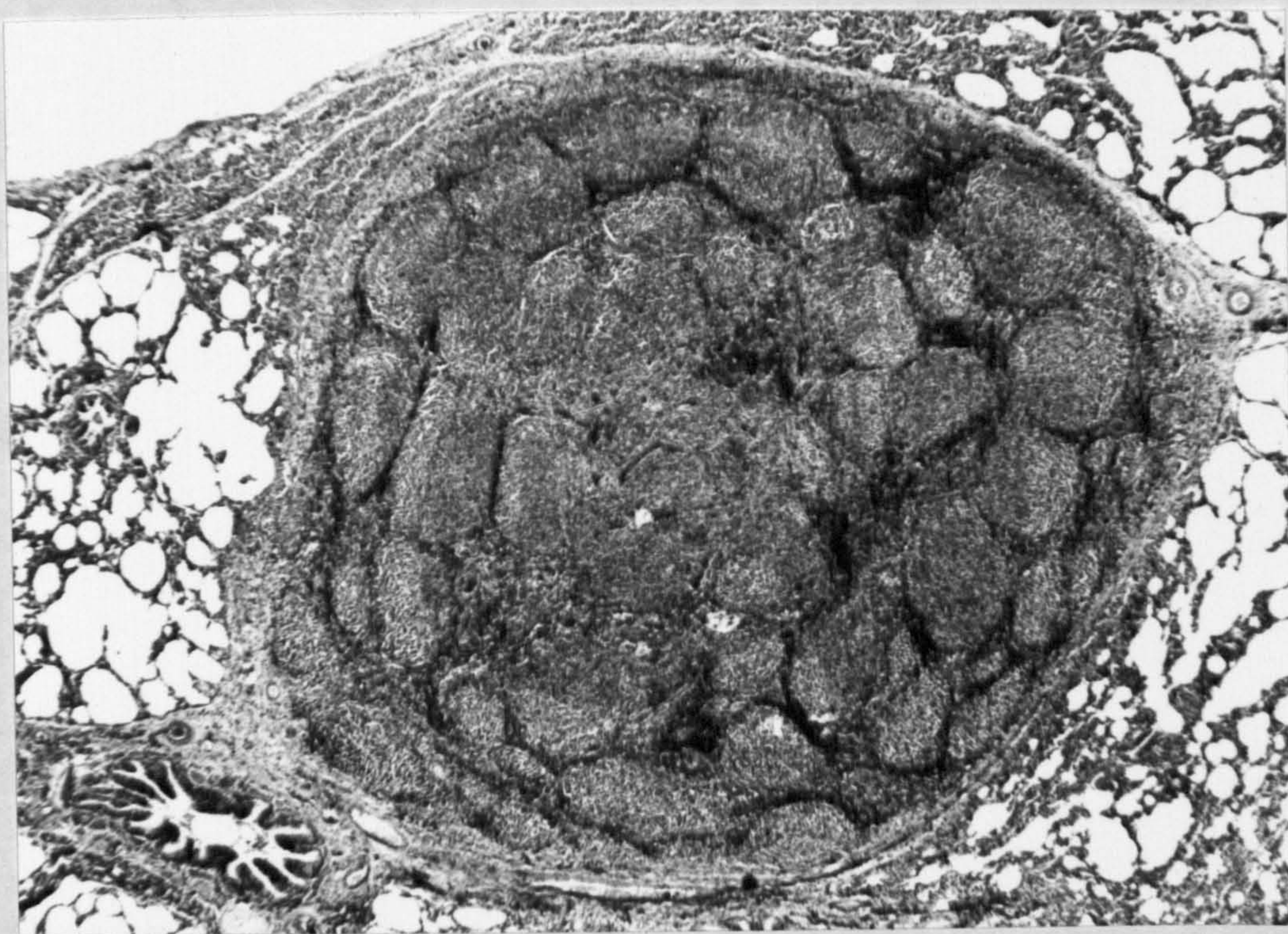


Figure 136. Foal killed at 106 days post-infection. Cross-section of a sub-pleural nodule. Many germinal centres are arranged concentrically around the periphery. X 12.

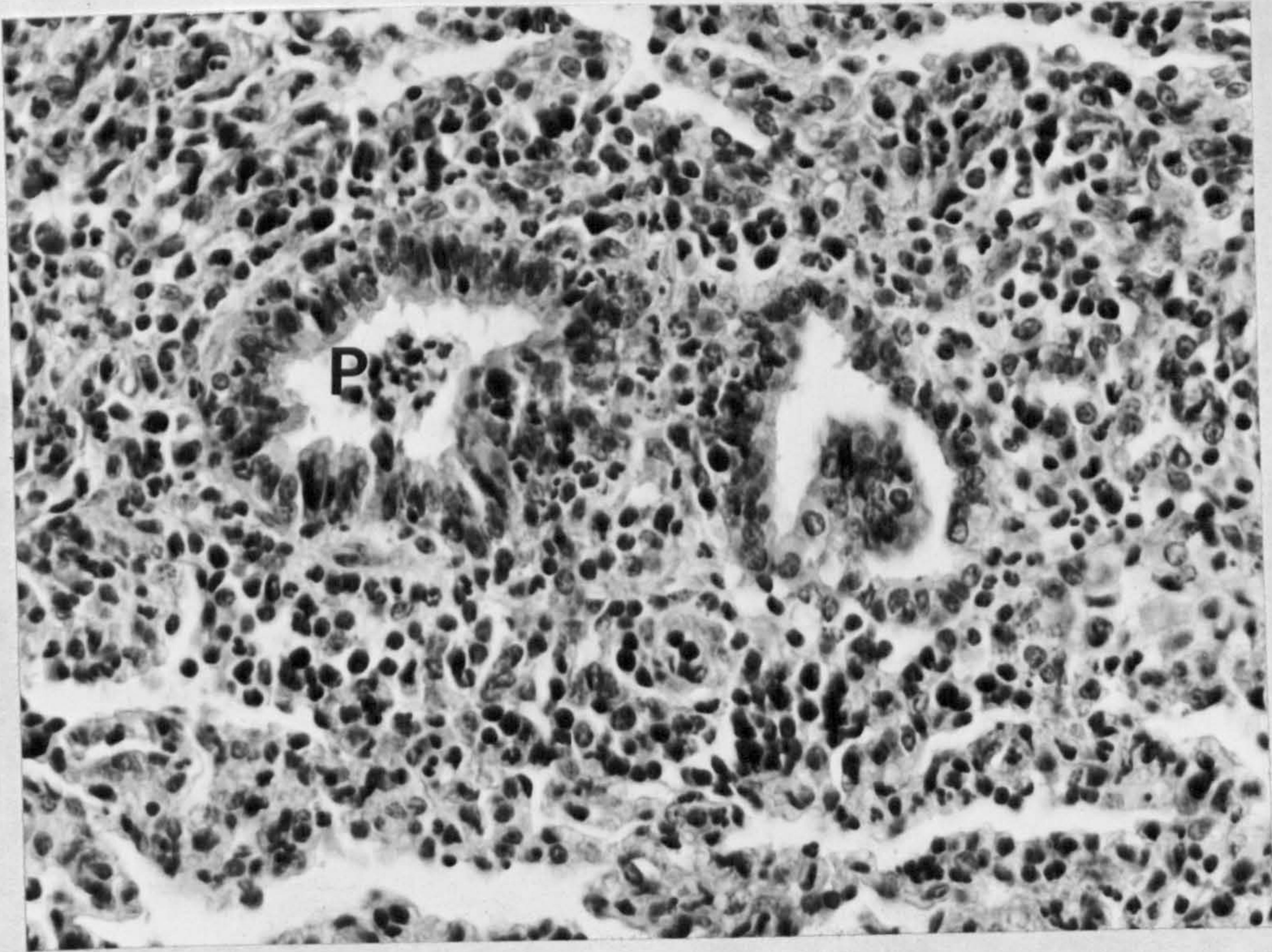


Figure 137. Section taken from an area of pneumonia in the cranial lobe. Two bronchioles are shown with a PMN exudate in the lumen of one (P). The area is surrounded by a moderate inflammatory reaction. X 250.

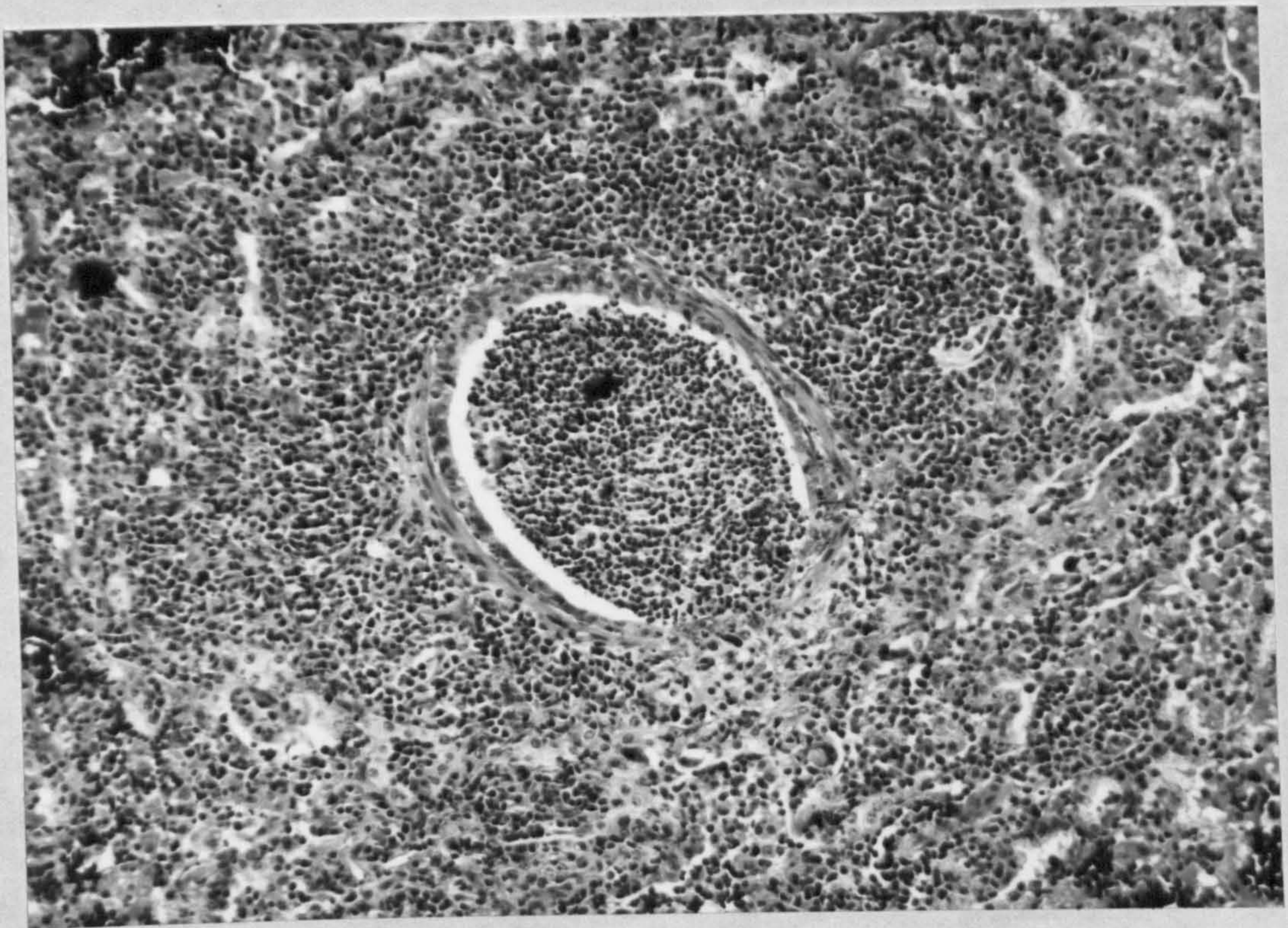


Figure 138. Section taken from same area as the above picture. The bronchiole has lost its epithelium and is plugged with a collection of PMN's which are also accumulated in the peribronchiolar tissue. X 110.

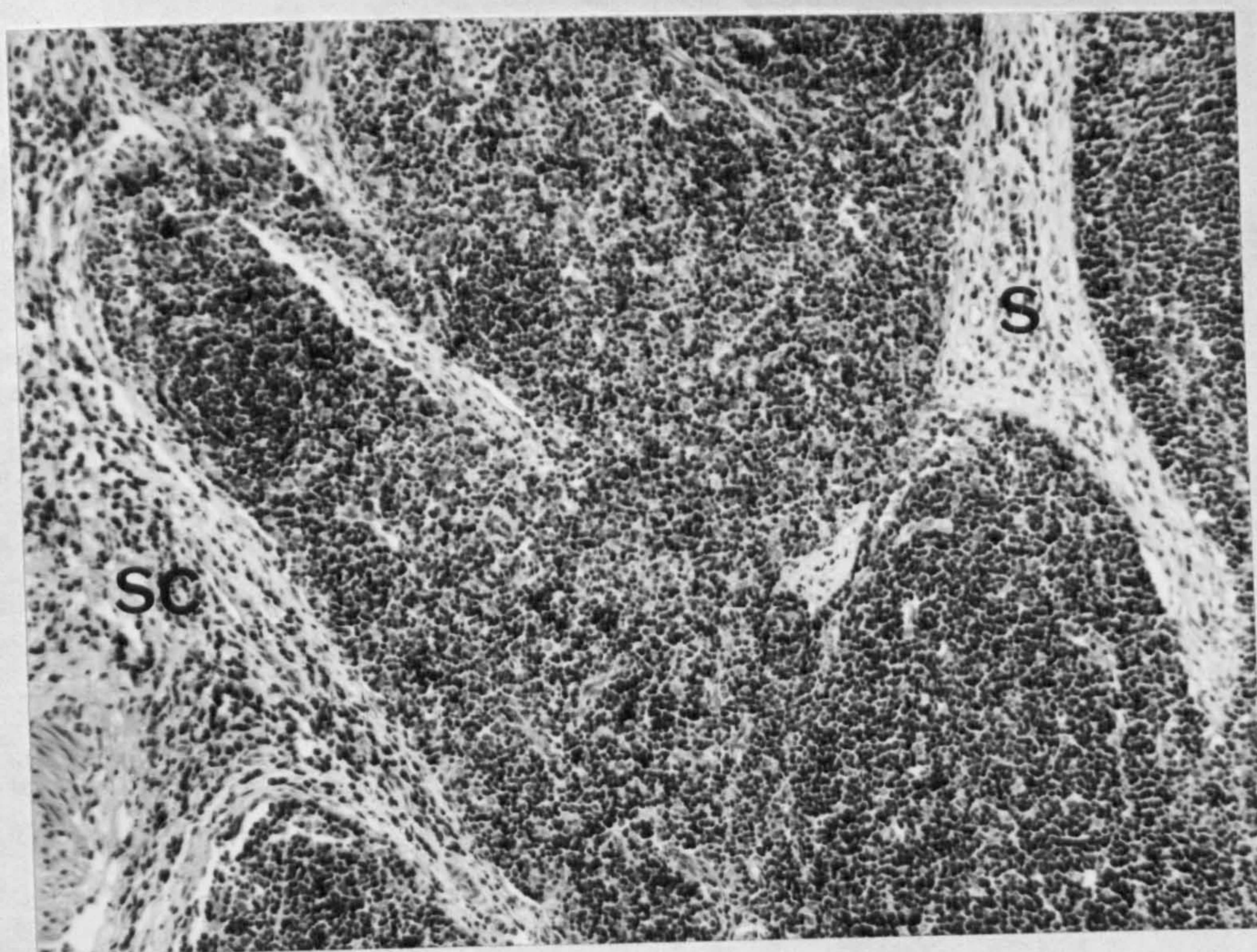


Figure 139. Section taken from a mediastinal lymph node of the foal killed at 14 days post-infection. The subcapsular area (SC) and sinus (S) are widened and contain large numbers of migrating eosinophils. X 110.

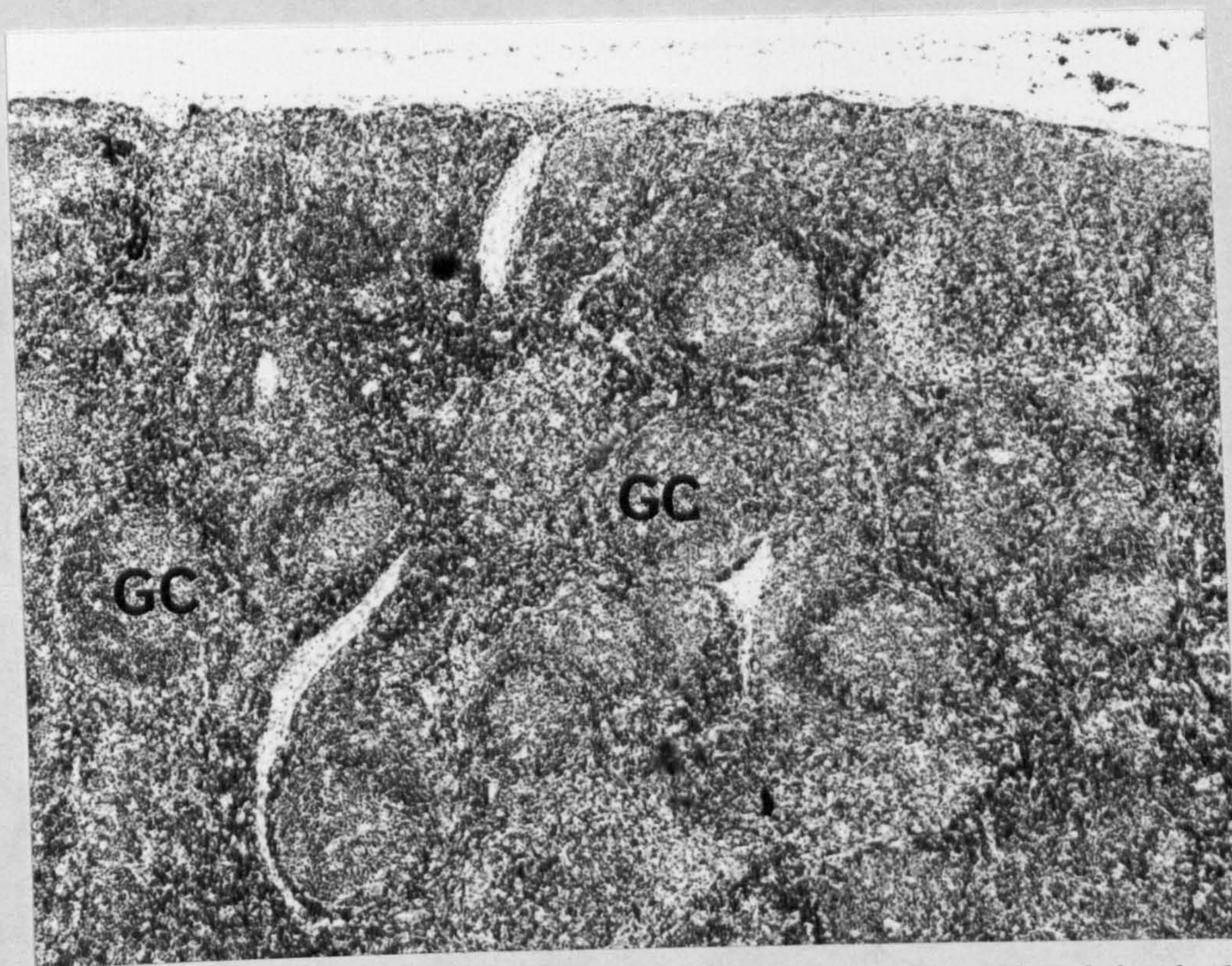


Figure 140. Section taken from a mediastinal lymph node of the foal killed at 73 days post-infection. An increased number of germinal centres (GC) are present. X 35.

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CHAPTER NINE

DISCUSSION ON TERMINOLOGY AND THE DEFINITION OF THE DISEASE

The pathological findings in the clinical cases to some extent belie the previously held conceptions as to the basis of disease. A distinctive pathological syndrome was found to be present in every animal diagnosed to be clinically suffering from "broken wind", the only variation being apparently due to differences in severity. It is not the first time that a bronchiolar lesion has been found to be the main pathological change in CPD (Thurlbeck and Lowell 1964; Foley and Lowell 1966; Gerber 1973 and Sasse 1971) but the equine world still tends to refer to the disease in terms that had their origin centuries ago and as a result there are many misapprehensions as to its true nature. In order to both put descriptions of the disease on a more scientific footing and to clear up these anomalies in the terminology it was decided to suggest an alternative simpler more correct name to replace the older terminology and the more recent names that have been borrowed from the human literature.

A disease is defined by Scadding (1959) as referring to "those abnormal phenomena which are common to a group of living organisms with disturbed structure or function, the group being defined in a stated way".

A disease is often first described in clinical terms as a set of symptoms shown by a patient. Later, as knowledge of the disease evolves, it may become known by a name based on the pathological findings and finally it is known on an aetiological basis. Scadding (1959) gives tuberculosis as a useful example of this evolution of terminology. Initially the various manifestations of disease were recognised clinically as "consumption", "scrofula", "Pott's disease" and "tabes mesenterica", but then it was discovered that one primary lesion, the tubercle, was common to all the diseases so it then became classified on a morbid-anatomical basis. Finally, the causative organism the tubercle bacillus was discovered and the disease is now known on this aetiological basis.

As far as CPD in horses is concerned the disease is still being defined in the least preferable way, on a clinical-descriptive basis, this despite the fact that several authors have investigated the pathology of disease. This has then led to a whole farrago of terms being used to describe and name the disease. The common terms "broken wind" and "heaves" are names that are evocative of the clinical signs. Broken wind is a term that has been used,

mainly in Britain, for hundreds of years (Floyer 1717; Gloag 1851; Percivall 1853; Mayhew 1887 and Fitzwygram 1901). That this term may have also had an anatomical basis in describing the supposed alveolar emphysema has been disputed by Percivall (1853) who thought that the name originated from the unfortunate predisposition of a broken winded horse to pass excessive flatus especially on exercise. If this is so, the usefulness of the term to describe the disease is long since past. "Heaves" is a term that has arisen mainly in the United States (Udall 1954; Lowell 1964) and, quite obviously, describes the movement of the flanks of affected animals during respiration.

Terms such as chronic alveolar emphysema (Stommer 1887; Carlstrom and Alegren 1940; Alexander 1959; Gillespie and Tyler 1969 and Bergsten 1974) and equine pulmonary emphysema (Amman 1939 and Eyre 1972) came into use following a few pathological investigations of the disease when it was thought that pulmonary emphysema was present in the lungs of these horses (Floyer 1717; Gloag 1851). Emphysema is a morbid-anatomical term and care must be taken in its usage as clinicians cannot reliably diagnose it in life (Thurlbeck 1976). This caution has not been exercised by most equine clinicians using the term. They use the term by and large on a clinical-descriptive basis which is a misapprehension. It has, in fact, been recognised for many years that not all of the horses so affected have emphysema and that even in those that do it is often fairly localised (Gloag 1851 and Percivall 1853).

More recently the disease has become known as chronic obstructive pulmonary disease (COPD) (Sasse 1971; Muylle and Oyaert 1973 and McPherson and others 1978). This is an attempt to define the disease physiologically and manages to avoid a precise morbid-anatomical definition. The basis for this in all three authors' opinions is that judged by respiratory function tests, there is obstruction to airflow, particularly on expiration. Their usage derives from the ACCP-ATS Joint Committee's recommendations (1975) that COPD should be used in preference to chronic obstructive lung disease (COLD) and chronic airflow obstruction (CAO) to refer to diseases of uncertain aetiology characterised by persistent slowing of airflow during expiration. Thurlbeck (1976) disagreed with the use of this term and uses the term CAO in referring to this complex of diseases. His argument is that it defines the condition more precisely and avoids the use of the term disease. Scadding's (1959)

definition of disease (see above) however allows wide scope for the term and, since my series of horses is suffering from a disease COPD is quite permissible as a term. In human medicine, COPD mainly encompasses chronic bronchitis, emphysema, asthma, bronchiectasis and possibly small airways disease (Thurlbeck 1976). It is argued that chronic bronchitis, asthma and bronchiectasis cannot be shown to be present in these animals and that emphysema does not develop until a late stage of disease and is seldom if ever extensive. It may therefore be necessary to revise this terminology.

The morbid-anatomical basis for the disease in horses was a chronic bronchiolitis and following the recommendations of Scadding (1959) and the ACCP-ATS Committee (1975) this should now be incorporated in the definition and name of the disease. It is therefore proposed that the disease be known morbid-anatomically as chronic bronchiolitis (CB). The concept of disease is inherent in this. It is anticipated that once the aetiology of CB is known the name will be changed once more to encompass this.

There is a great deal of confusion and misunderstanding about chronic pulmonary diseases in the horse, principally because of the different bases for disease definition (see above). On the basis of the clinical signs and history alone, it is only possible to diagnose heaves or broken wind, terms taken to be synonymous. It was shown that horses so diagnosed could have several different lesions as the cause of such clinical signs such as chronic pneumonia, collapsed trachea. However the most common pulmonary lesions in horses with heaves are either chronic bronchiolitis with overinflation or chronic bronchiolitis with overinflation and emphysema. At the present time it is more probable that three bases for the definition will be used concurrently.

There seems little reason why the practitioner should not use "heaves", the physiologist COPD if this has been demonstrated and the pathologist "chronic bronchiolitis" (CB) provided each is aware of the meaning of these terms.

The disease description and diagnostic criteria may embrace characteristics determined by any method of study and, particularly in the case of diagnostic criteria, these will have a tendency to be clinical-

descriptive. The practitioner however should not assume that all his cases of heaves have the same morbid anatomical lesions.

On clinical grounds the diagnostic criteria are less well defined in that none are specific to the disease CB but the whole complex occurring together in a horse is highly suggestive of CB. These clinical signs are well known and include -

Animal over five years old,
disease usually of several months to years duration,
poor work performance,
cough, especially on exercise, in the stable and at feeding time,
history of previous febrile respiratory disease,
double expiratory effort,
increased respiratory sounds on auscultation.

The diagnostic criteria of CB have been defined in physiological terms. If apparatus for measuring respiratory function is available measurement of the PaO_2 and ΔPpl will suffice. McPherson and others (1978) found that horses suffering from CB had a PaO_2 of less than 82 mm Hg and a ΔPpl of more than 6 mm Hg.

If the disease is to be given a morbid-anatomical name its definition must also be stated in morbid-anatomical terms (Scadding 1959). As a basic working definition the following is thus proposed: Chronic bronchiclitis (CB) is a generalised chronic respiratory disease of adult horses which affects airways of less than 2 mm external diameter; the airway epithelium is hyperplastic being transformed from one layer of cuboidal cells into a multilayered columnar epithelium which contains mucus secreting cells in addition to the normal cellular constituents. Affected airways are surrounded by inflammatory cells and in advanced cases by fibrous connective tissue, an exudate of mucus or pus is frequently present in the lumina.

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CONCLUSIONS

CONCLUSIONS

This is the first published comprehensive pathological investigation of a series of horses affected clinically by the classical syndrome known as broken wind or heaves. The horses involved in the series were investigated fully prior to necropsy and in many cases this involved the application of respiratory function tests.

The principle lesion in all 25 horses was chronic bronchiolitis, consisting of a hyperplastic bronchiolar epithelium, goblet cell metaplasia, peribronchiolar cellular infiltration and exudation of mucus or pus into the lumen. This combination produced stenosis of the airway and resultant alveolar over-inflation. Emphysema, estimated by examining slices of inflated lung, was infrequent, occurring only in the cranial lobe or the periphery of the caudal lobe and seemed to be a late development in the course of the disease. There has been much confusion in the past as to the terminology of disease and in order to clarify this situation it is suggested that the name be known in pathological terms as chronic bronchiolitis.

The absence of a bronchial lesion in the disease was confirmed by quantitative histochemical studies on the tracheobronchial mucosubstances. There was no obvious hyperplasia of submucosal bronchial mucous glands, no increase in the number of bronchial epithelial goblet cells and no change in the type of tracheobronchial mucus secreted in horses with chronic bronchiolitis as compared to horses with no respiratory abnormality. The disease therefore bears no resemblance to chronic bronchitis and emphysema of man.

The mast cells of the equine tracheobronchial tree had similar histochemical properties to those of mast cells of other mammals. Quantitative studies revealed that in horses with no respiratory abnormality 42 per cent. of the total complement of pulmonary mast cells were found around the small airways. In horses with CB the number around bronchioles, blood vessels and alveoli were significantly increased in seven out of ten animals and in the remaining three the numbers were depressed. There was no obvious explanation for these findings but it was interesting that the horses with the highest numbers of mast cells were necropsied in mid-summer and those with the lowest numbers in the winter months. No evidence of an allergic reaction could be found pathologically; the lesions in no way resembled those of human

asthmatics or those of subjects with hypersensitivity pneumonitis. It is suggested that the mast cells may merely be markers of a chronic inflammatory reaction.

There was no evidence of a serological deficiency of alpha-1 antitrypsin in a series of horses with chronic pulmonary disease. This is an inherited condition which occurs in man and predisposes to the early development of pulmonary emphysema.

Oral administration of 3-methyl indole to foals initially resulted in necrosis of the bronchiolar epithelium which was replaced in 72 hours by a hyperplastic bronchiolar epithelium. By six days the lesions bore a striking resemblance to those of naturally occurring chronic bronchiolitis. At ten days emphysema was present throughout much of the pulmonary tissue. This is a potentially useful experimental model of the naturally occurring disease.

Experimental infection of foals with larvae of the ascarid Parascaris equorum resulted in clinical respiratory signs after about fourteen days' infection. Pathologically the larvae had induced an eosinophilic bronchitis and bronchiolitis and some focal alveolar damage. In addition there was a cranial lobe bronchopneumonia. No infective cause could be found for this but it is suggested that an infective agent, possibly mycoplasma, was involved in its pathogenesis and that the migrations of the parasite potentiated its development. The lesions produced in no way resembled the lesions found in natural cases of chronic bronchiolitis.

APPENDIX I

HISTOPATHOLOGICAL, HISTOCHEMICAL AND
ELECTRON MICROSCOPICAL TECHNIQUES

FIXATIVES

Ten per cent. formol saline

42.5 g sodium chloride
500 ml formaldehyde (40 per cent. gas in water)
4,500 ml water

Carnoy's fluid

30 ml chloroform
60 ml absolute alcohol
10 ml glacial acetic acid

Corrosive formol

4 per cent. formaldehyde in a saturated aqueous solution of mercuric chloride (approximately six per cent.).

Mercuric chloride

Saturated solution of mercuric chloride (approximately six per cent.) buffered with 1.3 per cent. sodium acetate.

Calcium acetate formalin

4 per cent. formaldehyde
2 per cent. calcium acetate

Isotonic formol acetic acid

0.6 per cent. formaldehyde
0.5 per cent. acetic acid

STAINING TECHNIQUES

Haematoxylin and eosin

Take sections to water
Lugol's iodine two minutes
Rinse in water, five per cent. sodium thiosulphate, rinse in water
Haemalum five minutes
Rinse in water, blue with Scotts tap water substitute, rinse eosin two to three minutes
Dehydrate through alcohols to xylene and mount.

Martius Scarlet Blue

Take sections to water

Celestin Blue ten minutes

Haemalum five minutes

Wash in water, blue in STWS, rinse in water

Rinse in 95 per cent. alcohol

0.5 per cent. Martius Yellow in 95 per cent. alcohol containing two per cent. phosphotungstic acid two minutes

Rinse in water

One per cent. Brilliant Crystal Scarlet in 2.5 per cent. aqueous acetic acid ten minutes

Rinse in water

Treat with one per cent. phosphotungstic acid five minutes

Rinse in water

0.5 per cent. Soluble Blue in one per cent. aqueous acetic acid ten minutes

Rinse in water and dehydrate through alcohol to xylene and mount.

Result: Red blood cells - Yellow

Fibrin - Red

Collagen - Blue

Verhoeff's van Giesson

Take sections to water

Stain for fifteen minutes in a solution of:

five per cent alcoholic haematoxylin 20 ml

ten per cent. ferric chloride 8 ml

Verhoeff's iodine 8 ml

Wash in water

Differentiate two per cent. ferric chloride

Wash in water

Wash in 95 per cent. alcohol to remove iodine staining

Wash in water

Counterstain van Giesson three to five minutes

Dehydrate through alcohols to xylene and mount.

Result: Elastic - black

Collagen - red

Muscle - brown

Fibrin - yellow

Red blood cells - brown

Nuclei - blue

Perl's Prussian Blue

Take sections to water

Transfer to a fresh solution of approximately equal parts of four per cent. aqueous solution of potassium ferrocyanide and four per cent. hydrochloric acid. Heat this to 60°C and filter through a double filter paper onto the sections.

Leave two minutes then wash well in water

Counterstain in carmalum twenty minutes

Wash in water

Dehydrate through alcohols to xylene and mount.

Result: Haemosiderin - blue

Nuclei - red

Carbol chromatrope

Take sections to water

Stain in haemalum five minutes

Rinse in water

Blue in STWS

Wash in water

Stain in carbol chromatrope 30 minutes

Rinse in water

Dehydrate through alcohols to xylene and mount

Result: Nuclei - blue

Eosinophil granules - red

Red blood cells - red/orange

Unna - Pappenheim

Take sections to water
 Stain with Unna-Pappenheim ten minutes
 Wipe off excess stain
 Rinse in butanol
 Rinse in cellosolve
 Rinse in xylene and mount

Result: DNA - green
 RNA - red
 Plasma cell cytoplasm - purple

Feulgen reaction

Take sections to water
 Rinse in cold normal hydrochloric acid
 Warm normal hydrochloric acid at 56°C ten minutes
 Rinse in cold normal hydrochloric acid
 Rinse in distilled water
 Stain with Schiff's reagent for at least 30 minutes
 Rinse one minute in three changes of metabisulphate
 Wash in tap water five to ten minutes
 Stain in light green 15-30 seconds
 Dehydrate through alcohols to xylene and mount

Result: DNA - red
 RNA - green

Alcian blue pH 2.6 and pH 1.0 - PAS

Take sections to water.
 Stain in freshly filtered one per cent. alcian blue in three per cent. acetic acid (pH 2.6) or in 0.1 normal hydrochloric acid (pH 1.0) 30 minutes.
 Wash in water
 Oxidise for five to ten minutes in one per cent. of aqueous periodic acid.
 Wash in running water for five minutes and rinse in distilled water.
 Treat with Schiff's reagent 30 minutes.

Wash for ten minutes in running water.

Counterstain with haemalum and tartrazine in cellosolve

Dehydrate through alcohols to xylene and mount.

Result: Sulphomucins stain blue at pH 2.6 and pH 1.0.

Sialomucins stain blue at pH 2.6 only.

Neutral mucins stain red

Sialidase digestion

Bring two serial sections to water and dry.

Flood one section with neuraminidase enzyme solution, cover and incubate at 37°C overnight.

Flood control section with four per cent. calcium chloride, cover and incubate overnight.

Wash carefully in distilled water.

Stain both sections by the combined AB pH 2.6-PAS method.

Enzyme solution: obtained from Wellcome Research Laboratories, Beckenham, Kent and is a filtrate of vibrio cholerae stored in 25 ml bottles at 4°C until use.

The working solution is composed of eight parts of enzyme preparation to one part of four per cent. calcium chloride solution.

High iron diamine - alcian blue

Take sections to water.

Stain in fresh diamine solution at room temperature for 24 hours.

Rinse quickly in water.

Stain in one per cent. alcian blue in three per cent. acetic acid (pH 2.6) for 30 minutes.

Rinse in water.

Dehydrate through alcohols to xylene and mount

Result: Sulphomucins - brown/black

Sialomucins - blue.

Toluidine blue pH 4.0

Take sections to water.

Stain in 0.5 per cent. toluidine blue in McIlvane's buffer for 45 seconds.

Rinse in water.

Blot dry.

Dehydrate through alcohols to xylene and mount.

Result: Background tissue - blue

Mast cell granules and mucin - purple/red.

Toluidine blue pH 0.3

Take sections to water.

Stain in 0.1 per cent. toluidine blue in 0.7 normal hydrochloric acid for ten minutes.

Rinse in 0.7 normal hydrochloric acid for ten minutes.

Rinse in distilled water.

Dehydrate rapidly to xylene and mount.

Result: Mast cell granules and mucin - purple.

Astra blue - safranin

Take sections to water.

0.1 per cent. astra blue in 0.7 normal hydrochloric acid for 30 minutes.

0.7 normal hydrochloric acid for 30 minutes.

Wash in water.

Stain in 0.5 per cent. safranin in 0.125 normal hydrochloric acid for 10 minutes.

Rinse in water.

Dehydrate through alcohols to xylene and mount.

Result: Mast cell granules - blue

Mast cell nuclei - red

Background tissue - pink

Gomori's aldehyde fuchsin

Take sections to water.

Stain in half strength Lugol's iodine for ten to 15 minutes.

Wash and bleach in 0.5 per cent. hypochlorite.

Wash in water and dehydrate to 90 per cent. alcohol.

Stain in Gomori's aldehyde fuchsin for ten to 20 minutes.

Rinse off excess stain in 90 per cent. alcohol.

Counterstain in tartrazine in cellosolve.

Rinse in cellosolve then alcohol.

Clear in xylene and mount.

Result: Mast cell granules, elastic fibres - blue

Background tissue - yellow.

Biebrich scarlet

Take sections to water.

Stain in a solution of one per cent. Biebrich scarlet in glycine buffer at pH 9.9 for twenty minutes.

Wash in water or blot and dehydrate in absolute alcohol.

Clear in xylene and mount.

Result: Mast cell granules and eosinophil granules - red

If rapidly dehydrated nucleus stains orange

If washed in water background stains pale orange.

Alcian blue with graded increases in magnesium chloride

Take sections to water.

Stain in 0.1 per cent. alcian blue in 0.05 molar sodium acetate buffer at pH 5.7 with magnesium chloride added to a level of 0.1 M, 0.2 M, 0.6 M, 0.8M, 0.9 M and 1.0 M for 30 minutes.

Wash in water for five minutes.

Rapidly dehydrate through alcohols to xylene and mount.

Result: Sulphated mucosubstances gradually lose alcianophilia with increasing magnesium chloride concentration.

Toluidine blue - Hand E

Take sections to water.

Stain in haemalum for $\frac{1}{2}$ to 1 minute.

Wash in water and blue in STWS

Stain in alcoholic eosin for four minutes.

Rinse in 95 per cent alcohol.

Rinse in tap water.

Stain in 0.1 per cent. aqueous toluidine blue pH 6.8 - 7.2 for 7-10 seconds.

Rinse rapidly in distilled water.

Blot and dehydrate through alcohols to xylene and mount.

Result: Mast cell granules and mucin - blue/purple.

Collagen - pink

Nuclei - blue

Red blood cells - bright red

Fibrin - light blue.

Hogg's and Bank's stain

Take sections to water.

Alcoholic acetic acid one minute.

Stain in alcian blue pH 2.6 for 15 minutes.

Wash in tap water.

Stain in alum haematoxylin for $\frac{1}{2}$ - one minute.

Wash and blue in STWS.

Wash well in running tap water.

Stain in carbol chromotrope for 30 minutes.

Wash rapidly and blot.

Dehydrate through alcohols and clear in xylene.

Result: Mast cell granules - turquoise.

Eosinophil granules - scarlet

Nuclei - dark blue

Background - pale blue.

ELECTRON MICROSCOPY

Paraformaldehyde/glutaraldehyde (Karnovsky (1965) Journal of Cell Biology 27, 137A).

2g of paraformaldehyde is dissolved in 25 ml. of distilled water. The solution is heated to 60° - 70° C shaking continuously. Add 1 - 3 drops of 1N NaOH still shaking until the solution is clear or slightly turbid. Allow the solution to cool then add 5 ml. of 50% glutaraldehyde solution (or 10 ml. of 25% solution) and make up to 50 ml. with 0.1M cacodylate buffer - pH 7.4 - 7.6. Final pH should be 7.2. 25 mg. of anhydrous calcium chloride are added. Dilute this solution with another 100 ml. of buffer.

The above solution is a mixture of 1.3% paraformaldehyde and 1.6% glutaraldehyde.

Method:- 1. Fix for $4\frac{1}{2}$ - 6 hrs. at 4° C.
2. Rinse for 2 mins in 0.1M cacodylate rinsing solution and then leave overnight in fresh rinse at 4° C.

0.1M cacodylate with sucrose rinse

0.1M solution of sodium cacodylate (21.4g/l.) adjusted to pH 7.4 - 7.6 by addition of a few drops of concentrated hydrochloric acid.

Add 0.1M sucrose (34.2 g/l.) and adjust pH to 7.2 - 7.4.

Millonigs Rinsing Solution

NaH ₂ PO ₄	83 ml.
NaOH	17 ml.
Dist. H ₂ O	10 ml.
Sucrose	

Adjust to pH 7.2 - 7.4.

Osmium tetroxide (in Millonig's phosphate buffer).

Stock acid solution - monosodium phosphate 2.26%.

Stock alkali solution - sodium hydroxide 2.52%.

Buffer:-	NaH ₂ PO ₄	83 ml.
	NaOH	17 ml.
	Dist. H ₂ O	10 ml.
	Sucrose	0.54 g.
	pH 7.2 - 7.4	

1g of Osmic Acid is added to this buffer.

Araldite

Stock mixture:- Equal parts of Araldite Resin (CY212) and Araldite Hardener (HY 964).

Heat both to 55°C and mix by hand or leave at room temperature and mix overnight on the mixer. This mixture can be left indefinitely.

<u>Before use:-</u>	Mix - Stock mixture	58 ml.
	Accelerator (HY960)	0.6 ml.
	Di-n-Butyl-phthalate	2.0 ml.

This mixture should be mixed well for at least $\frac{1}{2}$ an hour before use.

Methylene blue/Azur II

Rinse briefly in water.

Stain for 5-15 mins. (or as necessary) in a freshly prepared solution of equal parts of:-

1% Azur II
1% methylene blue
1% borax

Heat - but do not allow to dry.

Rinse in water.

Dry and mount.

Uranyl acetate (Watson, M.L. (1958) Journal of Biophysical and Biochemical Cytology, 4, 475.

20 per cent. solution in absolute methanol. Store in a dark bottle in the refrigerator.

Lead citrate (Reynolds, E.S. (1963) Journal of Cell Biology, 17, 208.

Lead nitrate $\text{Pb}(\text{NO}_3)_2$	1.33 g.
Sodium citrate $\text{Na}_3(\text{C}_6\text{H}_5\text{O}_7) \cdot 2\text{H}_2\text{O}$	1.76 g.
Distilled water	30 ml.

Each salt is dissolved in 15 ml. of distilled water and when dissolved completely mixed together in a 50 ml. volumetric flask. The resultant precipitate is shaken for about 1 minute and then left to stand for 30 mins. with intermediate shakings to ensure complete conversion of lead nitrate to lead citrate.

8.0 ml. of N sodium hydroxide is added and the suspension is diluted to 50 ml. with distilled water and mixed by inversion. The lead citrate dissolves and the staining solution is ready for use. pH 12.0 ± 0.1 . Store in refrigerator.

Allow to heat to room temperature before use.

APPENDIX 2

INDIVIDUAL CASE REPORTS OF THE HORSES WITH CPD.

Number	Origin	Age	Sex	Type
BW1	Edinburgh, necropsy at Glasgow.	12	CM	Heavy Hunter

Gross findings

Cranial portions of lung overinflated. Abscess right middle caudal lobe with associated pleurisy. Edges of caudal lobes overinflated, some pleural tags on mediastinal surface. Hydatid cysts in liver.

Microscopic findings

Bronchiolitis, goblet cells prominent in epithelium, mucus in lumina. Infiltration of peribronchiolar tissue with lymphoid cells. Moderate to mild vasculitis of some of the smaller blood vessels. All mucus secreted stained with alcian blue at pH 2.6 and at pH 1.0. One bronchiole contained Langhan's giant cells.

Number	Origin	Age	Sex	Type
BW6	Edinburgh, necropsy at Glasgow.	30	CM	Pony

Gross findings

Lungs pale and overinflated especially anteriorly and peripherally. Rib markings on dorsal surface. Areas of thickened pleura at periphery of lungs. Many visceral and parietal pleural tags which were also found on the diaphragm and liver.

Microscopic findings

Widespread bronchiolitis, many bronchioles plugged with muco-pus. Cellular infiltration into lamina propria, mainly consisting of lymphocytes. Goblet cells in epithelium of most bronchioles, secretion mainly sulphomucin staining with alcian blue at pH 2.6 and at pH 1.0. A few bronchioles were dilated and had a low cuboidal epithelium. Alveolar epithelial hyperplasia around many of the large bronchi, occasionally fibrosed. Macrophages containing haemosiderin were numerous in most tissues and in the lumina of some bronchioles

Number	Origin	Age	Sex	Type
BW7	Glasgow	20 +	CM	Pony

Gross findings

Lungs pale and overinflated especially cranially and peripherally. Ribs 5 and 6 on right side fractured and badly healed. Well-developed callous and associated area of parietal pleurisy. One largish strongyle granuloma in caudal lobe of left lung. Many pleural fibrous tags. Mesenteric artery massively thrombosed with associated peritonitis. Tumour of right adrenal gland. Stomach contained bots (*Gasterophilus*) and there were many intestinal strongyles.

Microscopic findings

Widespread bronchiolitis with goblet cells in the epithelium of most. Excess mucus in the lumina of many small airways. This was sulphomucin staining with alcian blue at pH 2.6 and at pH 1.0. Mast cell numbers seemed increased especially around the bronchioles and blood vessels. Some areas of alveolar consolidation with foamy macrophages in the air spaces, in other areas the alveoli seemed disrupted and overinflated. Increased numbers of eosinophils in all the pulmonary tissues.

Number	Origin	Age	Sex	Type
BW9	Glasgow	10	CM	Riding school pony

Gross findings

Lungs pale, bulky, overinflated especially cranially. A few dark red consolidated lobules peripherally. Pleural tags on diaphragmatic surface of caudal lobes. One or two parasitic nodules found. Cut surface of lung showed many small airways blocked with purulent material.

Microscopic findings

Marked widespread bronchiolitis with massive cellular infiltration of peribronchiolar tissue. Goblet cells found in most bronchioles, epithelium very thick in places. Many polymorphonuclear leucocytic (PMN) plugs in airways and PMN's were visible migrating through the epithelium. Increased numbers of mast cells especially around the bronchioles and in the perivascular tissue. Excess fibrous tissue around some small airways. Increased numbers

of eosinophils present in focal areas of the lung. Mild alveolitis with pockets of PMN's in the walls. Alveolar epithelial hyperplasia commonly seen especially around the larger bronchi and blood vessels. Sometimes this newly formed epithelium was columnar and the associated air-spaces were filled with PMN's and cellular debris. There was also fibrosis in these areas.

Number	Origin	Age	Sex	Type
BW11	Glasgow	11	F	Highland X Pony

Gross findings

Pale overinflated lungs with a very puffy appearance anteriorly. The surface blood vessels were very prominent. Two hydatid cysts in the liver. Mesenteric artery thrombosed. Fibrous tags on the spleen. Small airways seemed prominent when the lung was incised.

Microscopic findings

Widespread bronchiolitis, all contain goblet cells and some have mucus in the lumina. Eosinophils prominent around many of the bronchioles as well as a lymphoid infiltrate. Increased numbers of mast cells around some of the bronchioles. Eosinophils were more prominent in the cranial areas of the lung, mast cells in the caudal areas. Alveoli sometimes looked rather disrupted.

Number	Origin	Age	Sex	Type
BW12	Edinburgh, necropsy at Glasgow	21	CM	Light Hunter.

Gross findings

Markedly pale overinflated lungs, most prominent in cranial lobes, anterior caudal lobes and periphery of caudal lobe. Small area of necrotising bronchopneumonia left anterior caudal lobe. Mild pleurisy ventral left caudal lobe. Many pleural tags ventrally and dorsally on both caudal lobes some of which had haemorrhagic areas around them. Incised surface of the lung showed very prominent cuffed small airways with purulent material in many of them. Fibrous tags on liver and splenic capsules.

Microscopic findings

Widespread obstructive bronchiolitis. Most had hypertrophied epithelium containing goblet cells but some were dilated and had a low cuboidal epithelium. Muco-pus in the lumina of many. A great increase in the number

of mast cells was seen especially around the bronchioles. Alveolar epithelial hyperplasia around most of the large bronchi and blood vessels, large numbers of mast cells were found in these areas.

Number	Origin	Age	Sex	Type
BW13	Glasgow files	23	F	Unknown

Gross findings

Marked overinflation of whole lung especially cranial areas and periphery of caudal lobe. Very pale, puffy pulmonary tissue which was difficult to cut. Small collapsed area in right lung. Mesenteric thrombosis.

Microscopic findings

Excess mucus in bronchial tree but no cellular evidence of bronchitis. Bronchiolitis with large numbers of goblet cells in the bronchioles. All stained for sulphomucins with alcian blue at pH 2.6 and at pH 1.0. Some peri-bronchiolar cellular infiltration but this was not extensive. A moderate eosinophilia was present in all the tissues.

Number	Origin	Age	Sex	Type
BW14	Edinburgh, necropsy at Glasgow	11	CM	Pony

Gross findings

Lungs diffusely overinflated, deflated soon after removal from carcase. Liver had multiple white roundish fibrous scars on its peritoneal surface and many large fibrous tags.

Microscopic findings

Widespread bronchiolitis with broad sleeves of lymphocytes around most. Small amounts of pus present in the lumen of some. Goblet cells in most bronchioles containing sulphomucins. A few isolated areas of eosinophilia.

Number	Origin	Age	Sex	Type
BW15	Glasgow	20	CM	Show-jumper

Gross findings

Lungs grossly overinflated and puffy especially anteriorly.

Microscopic findings

Widespread bronchiolitis with accumulations of lymphocytes around most bronchioles. Marked epithelial hypertrophy with many goblet cells. No intra-luminal exudate although goblet cells contained sulphomucins. Focal areas of mast cell hyperplasia.

Number	Origin	Age	Sex	Type
BW20	Glasgow files	11	F	Pony

Gross findings

Pale overinflated puffy lungs. Widespread speckling of the lungs and many greyish foci visible within the lung substance which were prominent bronchioles.

Microscopic findings

Widespread bronchiolitis with many goblet cells in the hyper-plastic epithelium. Marked lymphoid cellular infiltration of peribronchiolar tissue. Some excess mucus in the smaller airways which stained mainly blue with alcian blue at pH 1.0 and was therefore sulphomucin. Diffuse pulmonary eosinophilia and overinflated "stretched" looking alveoli.

Number	Origin	Age	Sex	Type
BW23	Edinburgh files	16	F	Pony.

Gross findings

Died overnight and not bled out so difficult to assess. Greenish inspissated material in smaller airways.

Microscopic findings

Some smaller bronchi contained excess mucus. Widespread areas of peribronchiolar fibrosis, bronchiolitis with a moderate eosinophilic reaction. Widespread post-mortem change and congestion obscured much of the detail.

Number	Origin	Age	Sex	Type
BW24	Edinburgh files	13	F	Clydesdale X

Gross findings

No report available.

Microscopic findings

Some lobules very overinflated in contrast to the remainder of the lung. In these areas the bronchioles were dilated but did not have a markedly hyperplastic epithelium. In the other areas there was bronchiolar epithelial hyperplasia with infiltration of fibrous tissue in the surrounding tissue. All the bronchiolar goblet cells were secreting sulphomucin but very few had excess mucus in the lumina. A moderate eosinophilia was present and most of these were concentrated in the alveolar walls.

Number	Origin	Age	Sex	Type
BW25	Edinburgh files	6	CM	Hunter

Gross findings

No report available.

Microscopic findings

Widespread bronchiolitis with infiltration of peribronchiolar tissue by lymphoid cells. Many goblet cells in the hypertrophied bronchiolar epithelium some of which are secreting the unusual type of sulphomucin which stains with alcian blue only at a pH of 1.0. Some bronchioles are surrounded by a substantial amount of fibrous tissue. There was a mild generalised pulmonary eosinophilia.

Number	Origin	Age	Sex	Type
BW 26	Edinburgh files	22	F	Pony

Gross findings

The pony died of torsion of the ileum . An associated extensive verminous arteritis was found at necropsy and a vertebral exostotic rupture into the abdomen with attendant haemorrhage. There were no apparent gross pulmonary lesions.

Microscopic findings

Widespread extensive bronchiolitis with fairly marked infiltration of the peribronchiolar tissue with lymphoid cells. Accumulations of polymorpho-nuclear leucocytes were found in the lumina of many small airways. Many of the goblet cells found in the hypertrophied bronchiolar epithelium looked empty or vacuolated. The mucin in them was sulphomucin sometimes of the type that

stained with alcian blue only at a pH of 1.0. The material was congested and there was a degree of post-mortem change present.

Number	Origin	Age	Sex	Type
BW29	Glasgow	16	CM	Pony

Gross findings

No generalised overinflation. There were several raised hemispherical areas on the lung, 5-10cm. diameter. When cut into these had a faint greenish appearance. No lungworms could be isolated. Several large white fibrous scars on liver and one calcified granuloma was found in the ventral lobe.

Microscopic findings

Mild bronchitis of the smallest bronchi and there was slight excess mucus in the airways. There did not seem to be an increase in the number of goblet cells present. Only a mild bronchiolitis was found and not all the bronchioles were affected. Goblet cells were seldom found in the bronchiolar epithelium. There was no obvious peribronchiolar cellular infiltration. A widespread intense eosinophilia was found. In places the bronchioles were completely surrounded by a mass of eosinophils. In contrast mast cells were very infrequent.

Number	Origin	Age	Sex	Type
BW31	Edinburgh, necropsy at Glasgow.	7	CM	Pony

Gross findings

Diffusely overinflated pale lungs which remained overinflated for several hours after removal from the carcass.

Microscopic findings

Widespread fairly marked bronchiolitis with extensive lymphoid infiltration of the peribronchiolar tissue. Goblet cells were frequently found in the bronchiolar epithelium and there were moderate amounts of mucus in the lumina of some of the airways. A mild pulmonary eosinophilia was present mainly in the alveoli and around the bronchioles. Some of the alveoli had an irregular disrupted appearance.

Number	Origin	Age	Sex	Type
BW33	Glasgow	10	F	Hunter

Gross findings

Diffusely overinflated pale, puffy lungs especially cranially. Some consolidation was found in the extreme caudal areas. One rib fractured on the right side and the mid-right caudal lung was adhered to this giving an extensive area of fibrinous pleurisy.

Microscopic findings

Extensive marked bronchiolitis with numerous goblet cells in the hypertrophied bronchiolar epithelium. Marked peribronchiolar infiltration by lymphoid cells. Most of the bronchioles did not have exudate in the lumina. Eosinophils were prominent and found mainly around the bronchioles.

In the caudal consolidated areas a very characteristic reaction was present. Each hyperplastic bronchiole was surrounded by several alveoli that had mucus accumulations in the air-spaces. Epithelial metaplasia had occurred in these alveoli so that they were lined by a cuboidal epithelium. Some fibrosis had occurred around these alveoli and there was a mild fibrotic vasculitis. No eosinophils were associated with this particular reaction

Number	Origin	Age	Sex	Type
BW34	Glasgow	11	F	Hunter

Gross findings

Extremely pale overinflated lungs. The cranial areas seemed to have gross emphysema as they were very puffy and pitted on digital pressure. No mucus was seen on lung section.

Microscopic findings

Widespread bronchiolitis but very little inflammatory reaction around the bronchioles. A few accumulations of polymorphonuclear leucocytes in the lumina but not enough to constitute plugging. One area found where mucus had accumulated in the alveoli. Moderate numbers of mast cells and eosinophils were found scattered amongst the pulmonary tissues.

Number	Origin	Age	Sex	Type
BW35	Glasgow	9	F	Hunter

Gross findings

Lungs had a grossly normal appearance.

Microscopic findings

Widespread mild bronchiolitis with fairly marked goblet cell infiltration into the bronchiolar epithelium in some but not in others. Mild cellular infiltration of peribronchiolar tissue. A few airways had mucus or polymorphonuclear leucocytes in the lumina but not enough to constitute obstruction. A few focal areas of alveolar collapse and inflammation were present. Large numbers of eosinophils were found particularly around the airways and blood vessels.

Number	Origin	Age	Sex	Type
BW36	Edinburgh, necropsy at Glasgow.	7	M	Clydesdale X

Gross findings

Overinflated pale lungs especially cranially and round the periphery of the caudal lobe. Several white fibrous scars on the ventricles of the heart which penetrated the thickness of the muscle.

Microscopic findings

Only a mild bronchiolitis was found and goblet cells were not always present in the hyperplastic epithelium. Mild infiltration of the peribronchiolar tissue with lymphoid cells. A few bronchioles contained small amounts of mucus. Quite marked alveolar disruption particularly in sections from the cranial lobe were seen.

The scars in the heart were also associated with a localised fatty degeneration of the muscle. No eosinophils were present around this lesion.

Number	Origin	Age	Sex	Type
BW37	Glasgow	12	CM	Pony

Gross findings

Cranial areas only overinflated, remainder normally collapsed, a few patches of pneumonia randomly distributed. Trachea semi-collapsed

along whole length except for a small portion in the mid-cervical area. There was no stenosis of the lumen and it had seemed normal on endoscopic examination the previous day.

Microscopic findings

Bronchiolitis with hyperplastic goblet cell containing epithelium. Excess mucus in the bronchioles sometimes forming plugs. Invasion of peribronchiolar tissue by lymphoid cells but large cuffs of eosinophils were found around many of the bronchioles far outnumbering the lymphoid cells. Extensive eosinophilia was found around many of the bronchi also. There was also a small increase in the numbers of mast cells around some of the airways. Some of the alveoli were mildly overinflated.

Number	Origin	Age	Sex	Type
BW38	Glasgow	12	CM	Pony

Gross findings

Overinflated diffusely pale lungs. There was some pus in the smaller airways which could be expressed by squeezing the cut surface of the lung.

Microscopic findings

Widespread bronchiolitis with goblet cells in the hyperplastic epithelium and excess mucus in some of the lumina. Fairly extensive lymphoid accumulation in the peribronchiolar tissue and polymorphonuclear leucocytic plugs in the lumina of many bronchioles and smaller bronchi. Several areas of alveoli had a very overinflated appearance.

Number	Origin	Age	Sex	Type
BW39	Glasgow	10	M	Welsh cob

Gross findings

Sent to Glasgow for removal of large area of granulation tissue on fore-limb but died under anaesthetic. At necropsy was found to have pale very bulky lungs. The cranial lobe and the periphery of the caudal lobe were particularly badly affected and were probably emphysematous.

Microscopic findings

Marked widespread bronchiolitis. Some plugged with polymorphonuclear leucocytic exudates and all surrounded by accumulations of inflammatory cells. Most bronchioles were affected with the typical bronchiolitis of C B but others were dilated and filled with large macrophages and mucus. All the mucus secreted was sulphomucin and stained with alcian blue at pH 2.6 and at pH 1.0. Large numbers of apparently discharging mast cells were found chiefly around the small airways and blood vessels. There were some very small focal areas of alveolar oedema associated with mast cell accumulations.

Number	Origin	Age	Sex	Type
BW40	Edinburgh, blocks sent to Glasgow.	13	CM	Hunter

Gross findings

No report available.

Microscopic findings

Marked widespread bronchiolitis with goblet cell-containing hyperplastic epithelium. Polymorphonuclear leucocytic plugs in many of the bronchioles and accumulations of lymphoid cells in the peribronchiolar tissue. Some bronchioles were dilated and filled with macrophages, a more advanced stage of this was that some bronchioles were completely obliterated leaving a granuloma-type reaction. However giant cells were not found.

Number	Origin	Age	Sex	Type
BW41	Glasgow	15	M	Pony

Gross findings

Pale evenly overinflated lungs. True emphysema visible in cranial lobes. Numerous subpleural parasitic nodules and some fibrous tags ventrally on the caudal lobe. Massive anterior mesenteric verminous arteritis. Liver extremely heavy, distorted and fibrosed. Whole surface pitted with parasitic nodules and scars.

Microscopic findings

Severe widespread bronchiolitis. Most are plugged with PMN's or mucus. In many areas the mucus hypersecretion affects the alveolar ducts and adjacent alveoli so that there are foci of mucus containing alveoli. Macrophages are also involved in this reaction. Cellular and fibrous reactions around most small airways. Mild bronchitis of smaller bronchi and PMN's are commonly found in their lumina. Mild eosinophilia and slight increase in numbers of mast cells. In the lower diaphragmatic area there is some alveolar consolidation with alveolar epithelial hyperplasia.