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# Elastogenesis in the developing chick lung: a light and electron microscopical study

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## INTRODUCTION

The study of elastogenesis at an ultrastructural level has received some attention during the last decade. For this purpose tissues rich in elastin have been used; for example, the aorta of the chick embryo (Karrer, 1960; Tagaki & Kawase, 1967), the newborn rat aorta (Paule, 1963), the ligamentum nuchae of the developing calf, and rat tendon (Greenlee, Ross & Hartman, 1966). The ultrastructure of experimentally induced elastogenesis has been observed within regenerating tendon (Fernando & Movat, 1963), traumatic intimal thickening in the rat aorta (Kadar, Veress & Jellinek, 1969) and guinea-pig pleural wounds (Williams, 1970).

No ultrastructural study of developing pulmonary elastin has been recorded, although the light histology of elastogenesis in the human lung has been reported by Loosli & Potter (1959) and Hodel (1968). In comparison with mammals, only very small quantities of elastin are present in the air exchange area of the avian lung. Fischer (1905) described the distribution of elastic fibres in the lungs of six species of birds and found that elastin was situated predominantly in the walls of the primary, secondary and tertiary bronchi but was absent or sparse within the air capillaries. These observations have been confirmed recently by King (1966). The present study describes the development and fine structure of elastic fibres in the parenchyma of the embryonic chick lung.

## MATERIALS AND METHODS

*Electron microscopy.* Lungs from White Leghorn chick embryos incubated at 37 °C were examined at daily intervals from the thirteenth day until they hatched. Lungs from 3- and 7-day-old chicks and from mature cockerels were also examined. The embryonic lungs were removed separately and the left lung diced into small cubes and placed in 2·5 % buffered glutaraldehyde for periods varying from 30 min to 1, 2, 3 or 4 h, depending on the maturity of the lung. The lungs of the 3- and 7-day-old chicks and the cockerel were diced and placed in fixative and then degassed under vacuum. The fixed tissue was then washed three times in 24 h in buffer containing 3 mM-$CaCl_2$ and post-fixed in osmium tetroxide for 30 min (buffered at pH 7·4), prior to dehydration and embedding in Araldite. Thick sections (0·5–1·0 $\mu$m) were stained with alkaline toluidine blue. Thin silver or gold sections were stained with 1 % uranyl acetate and lead citrate and some sections were stained with 1 % aqueous phosphotungstic acid (P.T.A.). The sections were examined in an EM 6B electron microscope with an accelerating voltage of 60 kV.

*Light microscopy*. Tissue from the right lung was fixed in buffered 4 % formaldehyde, embedded in paraffin and sectioned. The sections were stained with haematoxylin and eosin and Masson's trichrome. Elastic fibres were stained with Victoria blue 4R and ethyl violet and counterstained with van Gieson's stain (Humberstone & Humberstone, 1969).

## OBSERVATIONS

The bronchial system of the mature chick lung is a complex system of anastomosing tubes connected to a number of air sacs. In each lung the primary bronchus gives off four groups of secondary bronchi. Some of the secondary bronchi anastomose

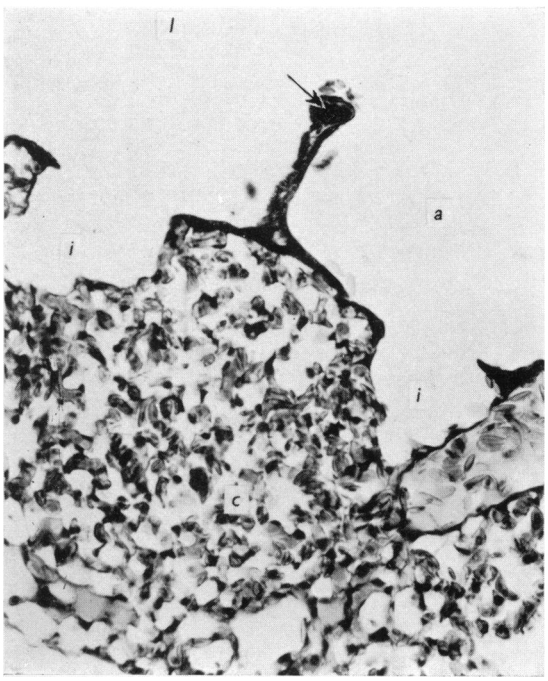


Fig. 1

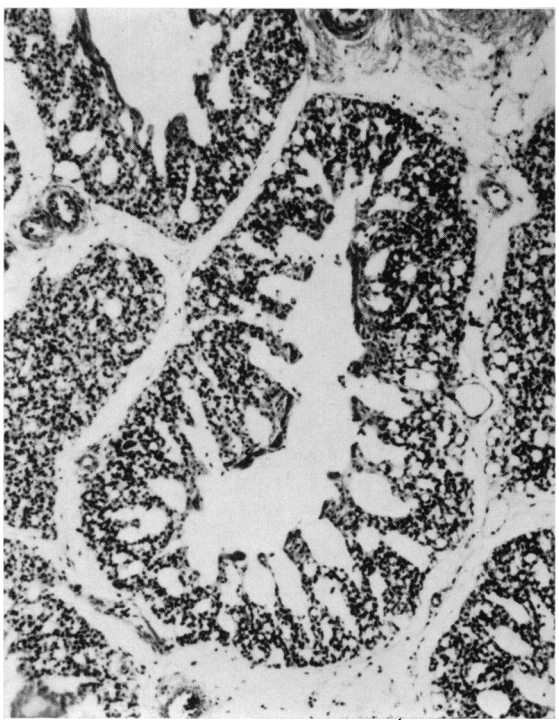

Fig. 2

Fig. 1. Cross-section of part of a tertiary bronchial unit of a mature cockerel showing the lumen (*l*) and the air capillary area (*c*). A smooth muscle bundle (arrow) cut in cross-section is supported by a connective tissue septum. The atrial area of the tertiary bronchus (*a*) lies between the smooth muscle and the air-exchange area. The infundibula (*i*) connect the air exchange area with the tertiary bronchial lumen through the atria. Masson trichrome, × 750.

Fig. 2. Cross-section of a tertiary bronchial unit in a 3-day-old chick lung. Masson trichrome, × 300.

directly with each other, but the main connections between the secondary bronchial systems are by means of straight or curved tertiary bronchi. Each tertiary bronchial lumen is the central axis of a respiratory unit which consists of a wall containing a network of smooth muscle bundles and a surrounding cylinder of air capillaries.

The air capillaries are connected with the tertiary bronchi through the atria, which are situated between the smooth muscle bundles (Figs. 1, 2).

In the chick lung, elastin is found in the walls of the primary and secondary bronchi. It is also found in the more peripheral parts of the lung in four situations: the tertiary bronchial walls, the connective tissue septa, the pulmonary vasculature and the pleura. The bulk of the parenchymal elastin is found in the tertiary bronchial wall in close association with the smooth muscle bundles.

At the fourteenth day of incubation the lung parenchyma is at a primitive glandular stage of development. It consists of mesenchyme which contains the endodermally derived simple tertiary bronchi with no elastin in their walls (Fig. 3). In contrast, elastic fibres in considerable number are present in the walls of the primary and

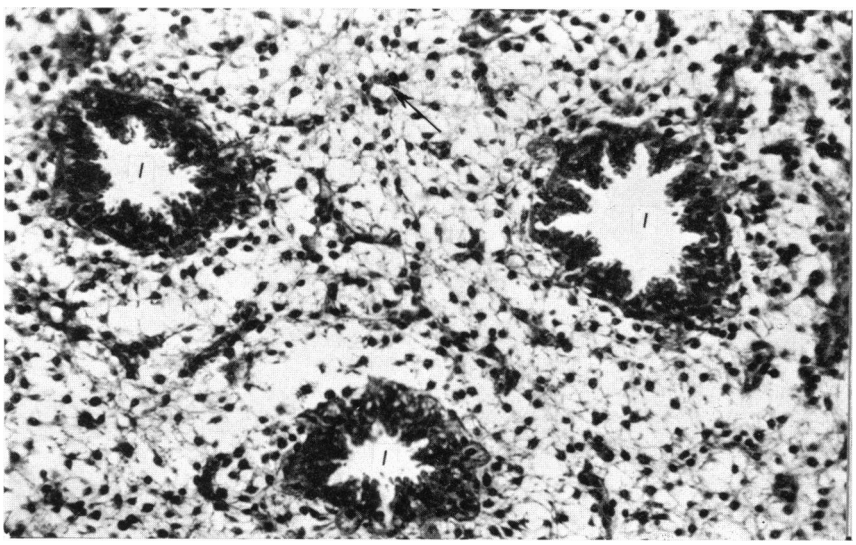

Fig. 3. The immature lung of a 14-day chick embryo, showing the primitive tertiary bronchi (*l*) lined by columnar cells. The bronchi are embedded in undifferentiated mesenchyme which contains capillaries (arrow). Haematoxylin and eosin, × 750.

secondary bronchi and the main pulmonary artery by the fourteenth day of incubation. As this account is concerned with elastogenesis in the more peripheral structures in the lung, the timing of elastic fibre formation in the central, better developed structures will not be further described.

### *Elastic fibres of the tertiary bronchus*

The wall of the tertiary bronchus is the area richest in elastin in the avian lung parenchyma. Elastic fibres intimately surround the smooth muscle bundles and are seen in the thin septa that support the smooth muscle and form the lateral walls of the atria. Elastic fibres form a network in the boundary zone between the atria and the air capillaries, and are continuous with those in the connective tissue septa that project into the lumen of the tertiary bronchus. No elastic fibres penetrate the walls of the air capillaries (Fig. 4).

In cross-section of the mature tertiary bronchus the smooth muscle and its supporting septum have the appearance of a 'club' (Figs. 5, 6). Elastic fibres occupy the centre of the stalk and surround the smooth muscle in the head of the club. The elastic fibres are situated in the interstitial space between the smooth muscle bundles and the attenuated pneumocyte covering the club. At the base of the club, the elastic fibres splay out into a loose irregular anastomosing network in the junctional area between the air capillaries and the atria.

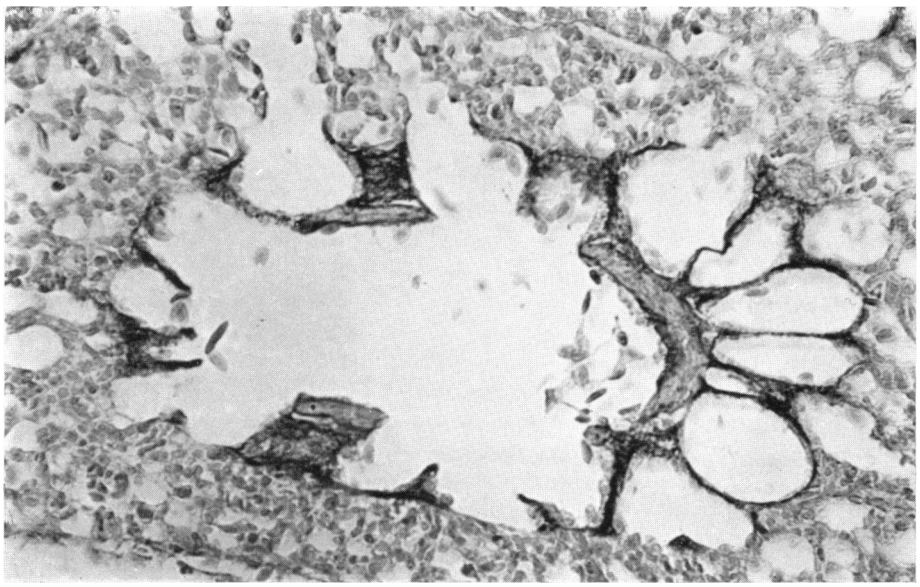

Fig. 4. The elastic fibre network of the tertiary bronchus of a 3-day-old chick lung. The elastic tissue is confined to the walls of the tertiary bronchus, where it occurs within the connective tissue septa and around smooth muscle bundles. The air-exchange area is devoid of elastic tissue. Victoria blue van Gieson, × 1000.

Elastogenesis commences at day 14, when small sparse bundles of microfibrils, the first stage in the development of elastic fibres, can be detected with the electron microscope around the periphery of the primitive cylindrical tertiary bronchi (Fig. 7). However, elastogenesis is more evident from day 16 onwards, when microfibrillary bundles are seen amongst the smooth muscle cells which partially surround the circular primitive tertiary bronchi. The bronchial lumen at this stage is lined by undifferentiated cuboidal cells. With increasing maturity, the smooth muscle migrates from the periphery of the bronchus into the lumen, taking with it an epithelium-covered septum containing elastin, collagen and fibroblasts. By 18 days incubation, the cuboidal lining cells have changed into attenuated membranous cells or into large cuboidal cells containing laminated osmiophilic inclusion bodies. These latter cells, which are analogous to the mammalian granular pneumocyte (Type II cell), are situated near the base of the stalk of the club (Fig. 8). Immature elastin clumps consisting of an amorphous central core with a peripheral mantle of microfibrils are found in the stalk and the head at day 18. The diameter of the smallest elastin

clumps showing this two-component system varies from 80 to 180 nm. (Figs. 9, 10). In the head of the club the elastin clumps are seen in the narrow interstitial space between the covering pneumocyte and smooth muscle cells. Often the small two-component clumps are found in the bays formed by the smooth muscle cell membrane as well as in contiguity with fibroblastic cell processes. Electron-optically it is rare to see fibroblastic nuclei in the interstitial space in the head of the club. However, active fibroblasts are seen in the stalk in close proximity to the small elastin clumps.

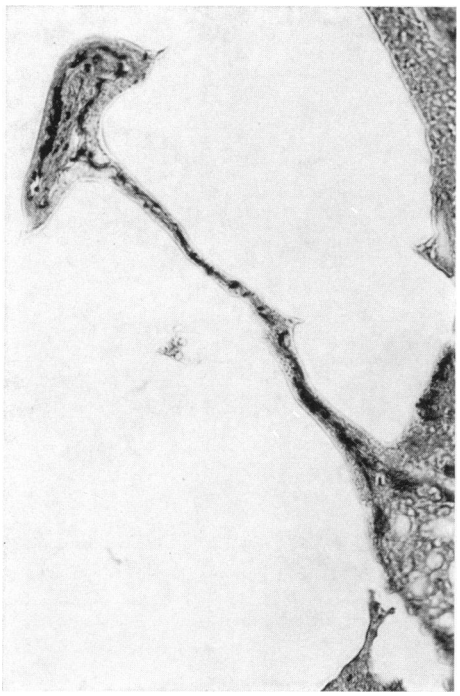

Fig. 5

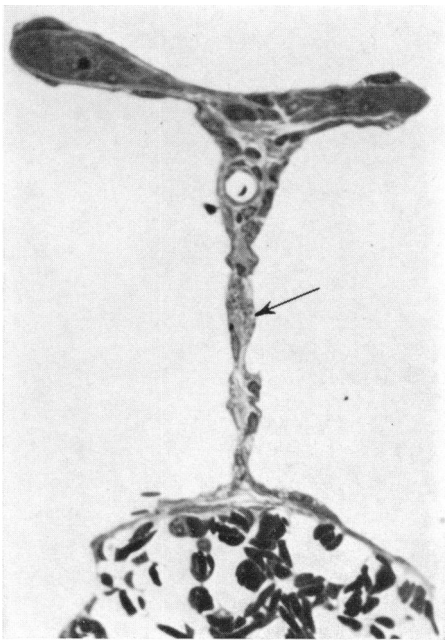

Fig. 6

Fig. 5. Transverse section of a tertiary bronchial 'club'. The elastic fibres occupy the centre of the supporting septum and surround the smooth muscle fibres in the head of the club. Victoria blue · van Gieson, × 1200.

Fig. 6. A 1 $\mu$m thick Araldite-embedded section stained with toluidine blue, showing a tertiary bronchial 'club' in a mature cockerel. The 'club' contains smooth muscle cells. The connective tissue septum is partly covered by granular pneumocytes, some of which contain inclusion bodies (arrow). × 1200.

By day 20 the elastic fibre network in the wall of the tertiary bronchus is approaching its fully developed state as seen by the light microscope (Fig. 11). Elastin synthesis is still active, as immature elastin clumps are still plentiful. But simultaneously more mature elastic fibres are beginning to appear. In these the central amorphous core is the major component and the microfibrillary portion is reduced. The development of elastic fibres in the wall of the tertiary bronchus is asynchronous, and elastin of all stages of maturity can be recognized up to the 7th day after hatching. Large immature two-component clumps are also present in the cockerel.

The two components of the developing elastic fibre show differences in staining. With uranyl and lead the microfibrils give a constant dense staining. By contrast the staining of the central core shows variation. In the smallest elastin clumps (diameter 80 nm) the amorphous central core has a light grey granular appearance (Fig. 9). As the material in the central core increases in amount the innermost area becomes completely non-staining or white, whilst the external rim adjacent to the microfibrils retains the light-grey granular stain (Fig. 14).

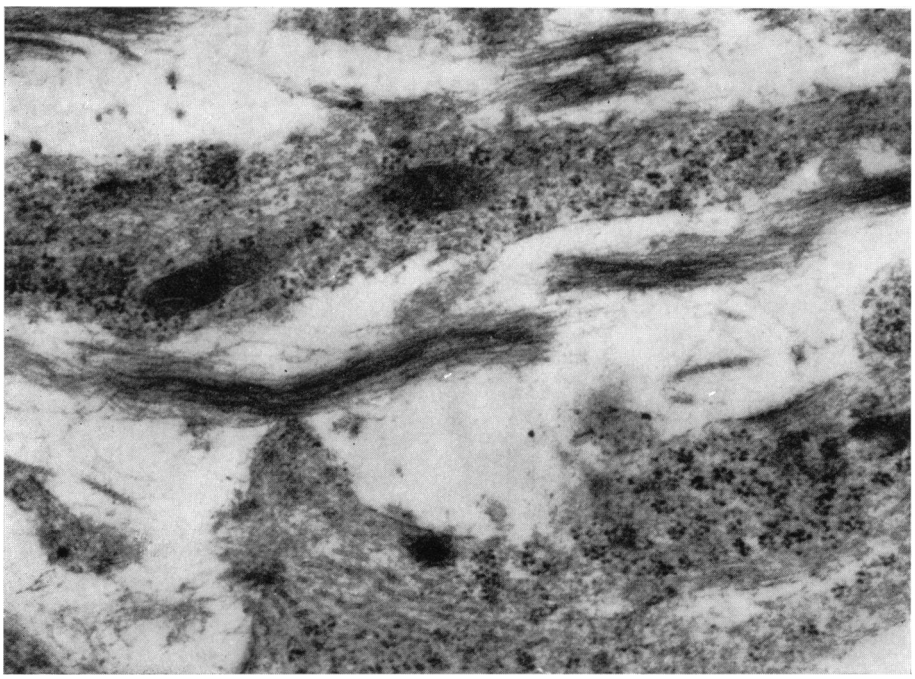

Fig. 7. Clumps of parallel elastin microfibrils at the base of a primitive tertiary bronchus. Day 14 of incubation. ×18750.

The amorphous core takes up phosphotungstic acid but in a somewhat unequal manner, the peripheral area tending to stain more densely than the centre of the material (Fig. 13).

Higher-resolution micrographs of cross-sections of individual microfibrils demonstrate that they have a light staining central area and a more dense staining circumferential rim indicative of a tubular structure (Fig. 14).

*Elastic fibre development in the small pulmonary vessels*

The blood supply to the lung parenchyma prior to day 14 consists of capillaries in the loose mesenchyme. At day 15 small arteries appear. These have a tall endothelial lining and the larger vessels have a thin muscular media. Between the 16th and 17th day the vessels increase in diameter and become more prominent, but no elastic fibres can be recognized by light microscopy until day 18. From this time onwards elastogenesis in the pulmonary vessels is exceedingly active: the fibres increase in

number and stain more intensely with Victoria blue. By day 19, muscularization of the small peripheral vessels is well advanced and in the larger arteries separate internal and external elastic laminae are recognizable. By day 19 the pattern of elastic fibres in the vessel walls is sufficiently distinctive to enable veins and arteries to be recognized.

The structure of the immature elastic lamina in a small pulmonary artery at day 19 is seen in Fig. 15. The lamina, which is bounded internally by tall endothelial cells

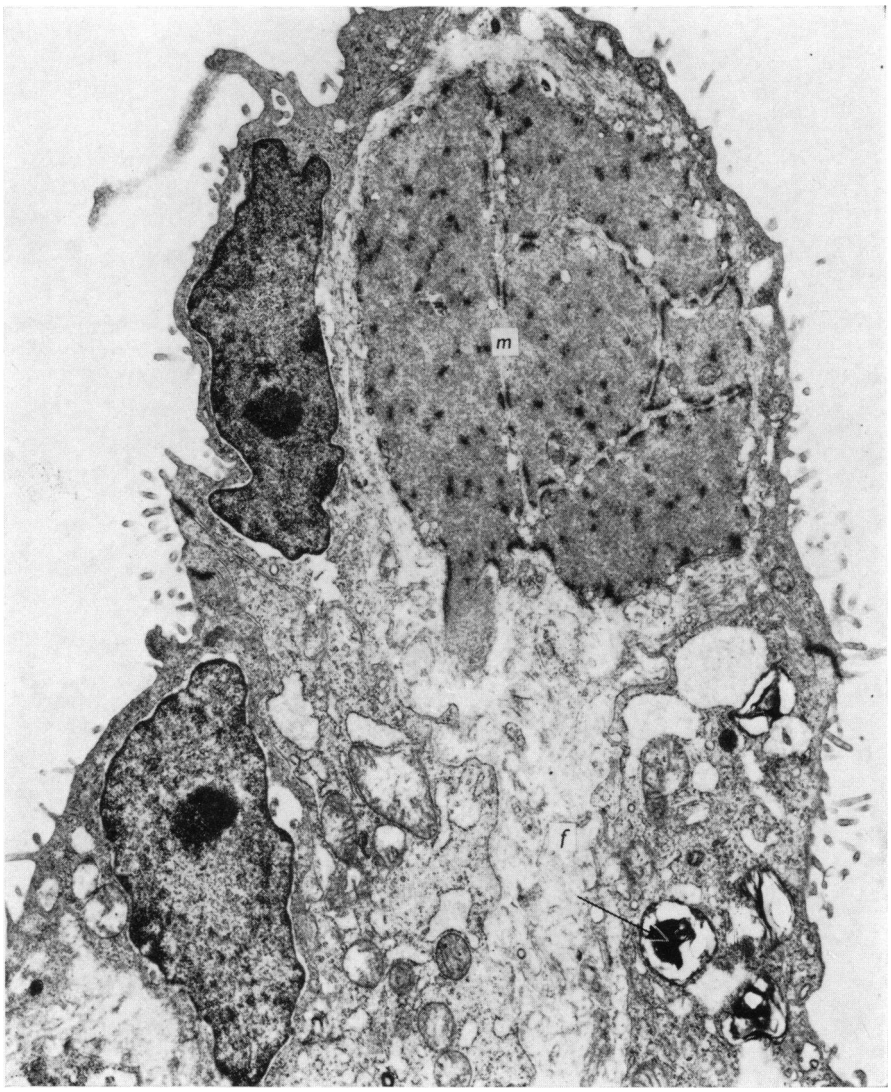


Fig. 8. An immature tertiary bronchial 'club' at 21 days incubation. The tip of the club is occupied by smooth muscle cells (*m*). The apex is covered by an attenuated cell which is devoid of inclusion bodies. The cell near the base of the club contains osmiophilic inclusion bodies (arrow). In the centre of the stalk there are processes of fibroblasts (*f*) and small elastin clumps. × 10000.

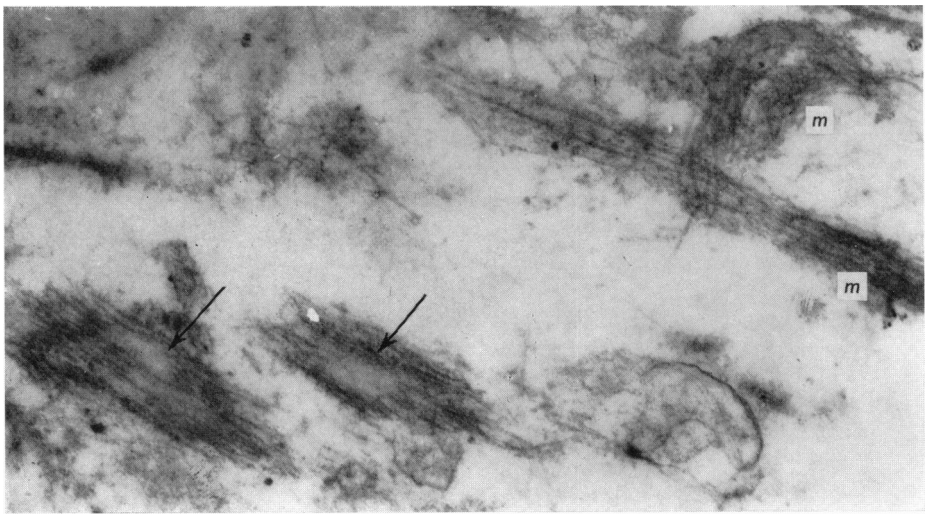


Fig. 9. Two small clumps of elastin (arrows) are cut obliquely to display a pale granular central core surrounded by microfibrils. Two bundles of microfibrils (*m*) devoid of central core materials are also seen. × 50000.

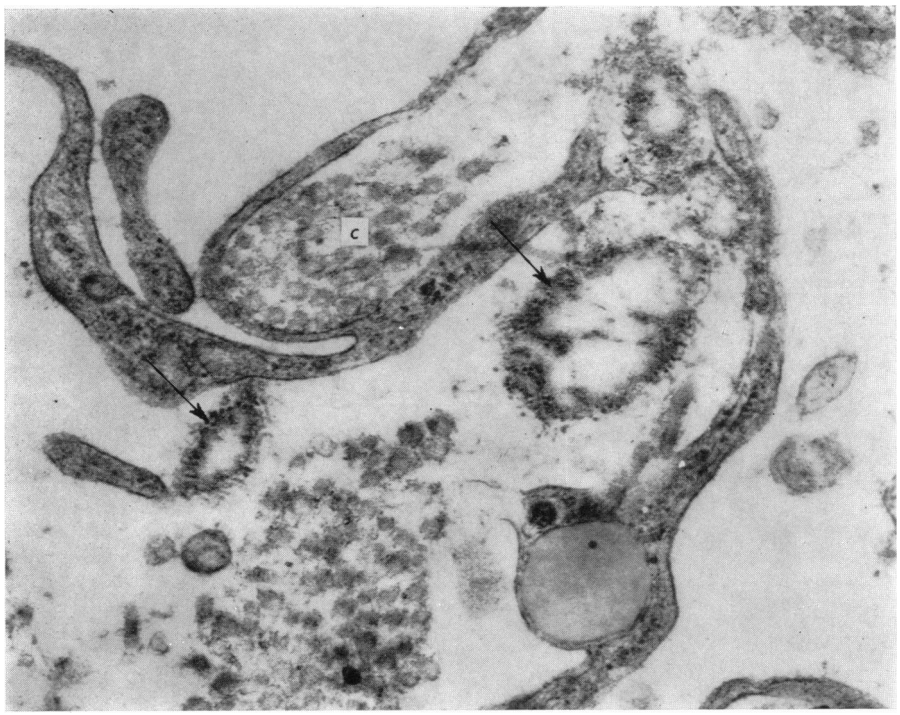


Fig. 10. Clumps of elastin (arrows) showing a two-component system of peripheral microfibrils and central amorphous core. The clumps are in contiguity with attenuated fibroblastic processes. Collagen fibres (*c*). × 40000.

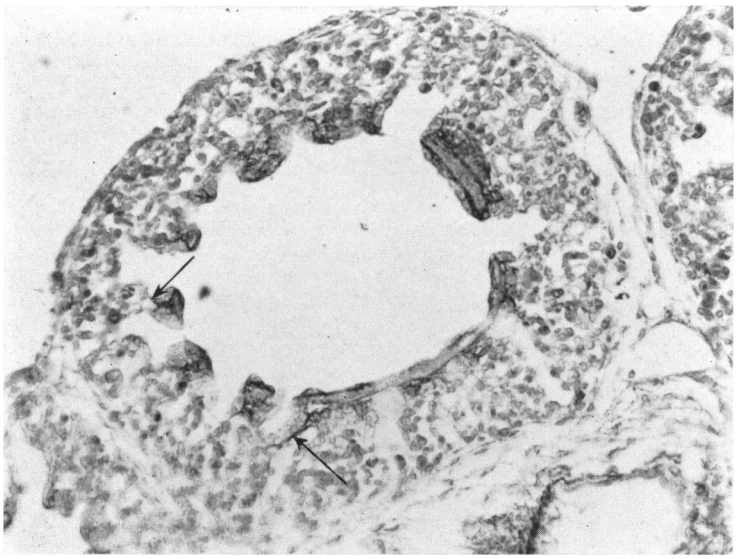

Fig. 11. Cross-section of a tertiary bronchial unit of a 20-day embryo. The arrangement of the elastic fibre network is discernible. The extremely fine elastic fibres are arranged around the smooth muscle of the tertiary bronchial wall and a few fibres are seen in the short, developing connective tissue septa (arrows). The surrounding air-exchange area is devoid of elastic fibres. Victoria blue van Gieson, × 750.

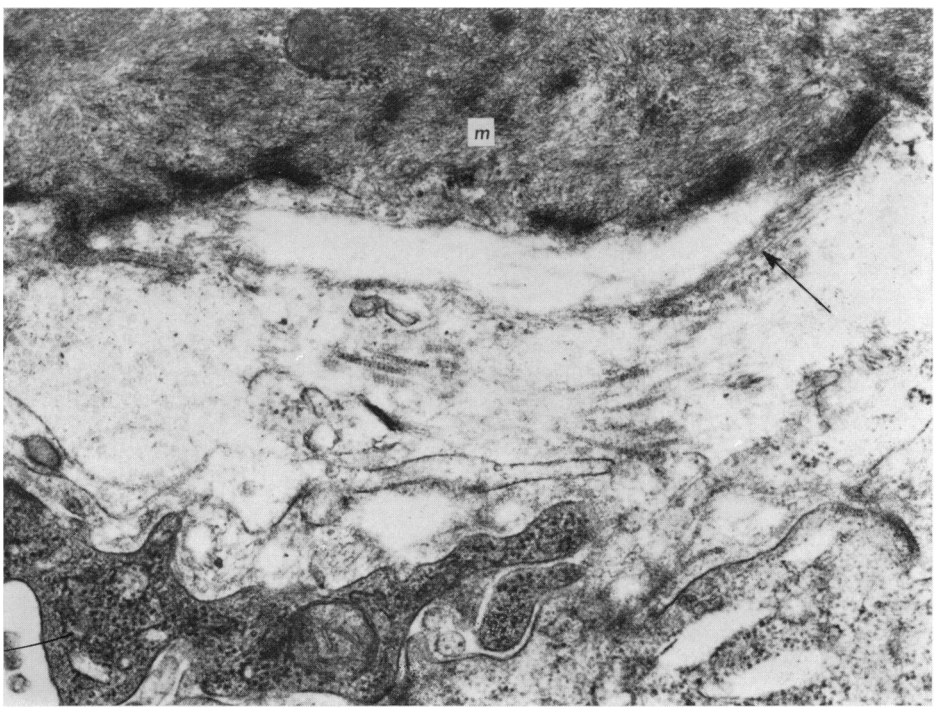


Fig. 12. A developing elastic fibre cut longitudinally from a chick lung on the third day after hatching. The fibre is situated adjacent to a smooth muscle cell (*m*) in the head of a tertiary bronchial 'club'. The greater part of the fibre consists of central core material which is non-staining and non-granular. The central core is partially surrounded by a thin mantle of microfibrils (arrow). × 30000.

and externally by smooth muscle cells, has a crenated appearance due to interdigitation of the cell processes of the boundary cells. Three components can be recognized in the lamina. There are groups of microfibrils cut in transverse section, the average diameter of which is 14 nm. Some of the microfibrils surround an electron-translucent central core. However, the greater part of the lamina at this stage is formed of intercellular matrix, which in this situation has the same staining characteristic with lead

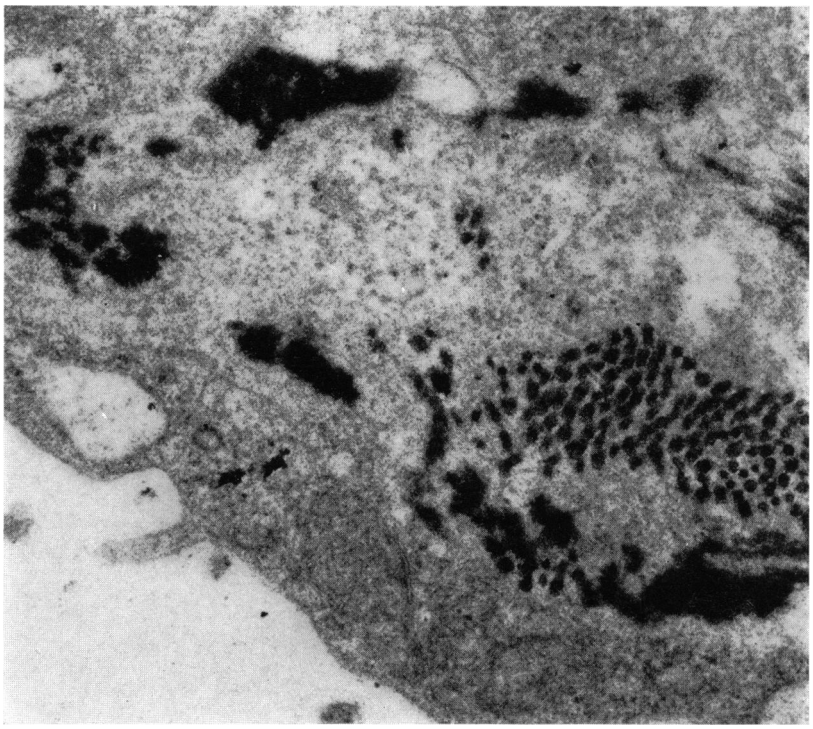

Fig. 13. Elastin clumps and collagen in a tertiary bronchial 'club'. Phosphotungstic acid stains the central core material of the elastin clumps and the collagen fibres but it does not stain their peripheral microfibrils. The elastin clumps have an irregular outline. × 50000.

and uranyl as the elastin central core material. The distinction between the two can be demonstrated with phosphotungstic acid, which stains the core material black but not the intercellular material.

Both the endothelial and the smooth muscle cell cytoplasm contain numerous vesicles, some of which have progressed to lacunar indentations of the cell membrane.

At 21 days the elastic lamina is composed predominantly of mature central core material, but it is still surrounded by a thin mantle of microfibrils. In cross-section the lamina shows the characteristic crenated appearance seen in undistended arteries. Although discontinuous in cross-section it is sufficiently organized to be recognized as laminated.

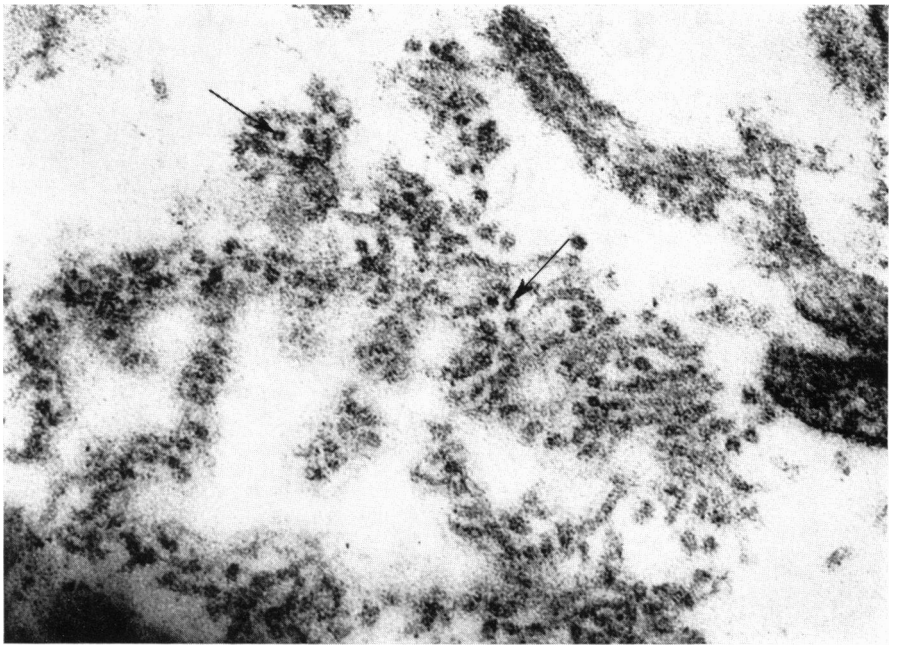

Fig. 14. A two-component elastin clump in cross-section. Some of the microfibrils have a tubular appearance in cross-section (arrows). The innermost part of the central core is relatively non-granular and electron-translucent, whilst the more peripheral part of the central core adjacent to the microfibrils has a granular appearance. × 120000.

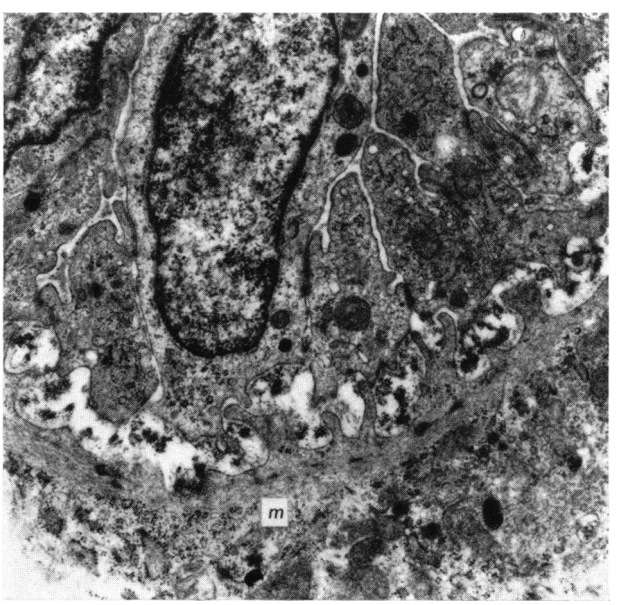


Fig. 15. Part of the wall of a small pulmonary artery in a 19-day embryo. The lumen is lined by columnar endothelial cells. The crenated elastic lamina is situated between the endothelial cells and a smooth-muscle cell (*m*). The potential elastic lamina consists of clumps of microfibrils, cut in cross-section, and ground substance. No central core material is seen. × 15000.

*The connective tissue septa and pleura*

The ultrastructure of developing elastic fibres in these two structures is similar to that seen elsewhere in the lung. However, the morphology of elastin-forming cells can be visualized best in the septa. The width of the mesenchyme between the tertiary bronchi diminishes as the tertiary bronchial units increase in diameter with age. With increasing maturity the cells in the connective tissue align themselves in loose, approximately parallel, rows. The cells have long and extremely attenuated cytoplasmic processes practically devoid of cell organelles. The rough endoplasmic reticulum in these cells is sparse but is often dilated. Elastin microfibrils can be recognized in the condensed mesenchyme at day 16. By the 18th day the connective tissue septum is an organized structure which can now be clearly recognised by light microscopy and which contains a few fine elastic fibres. Between days 18 and 21 the connective tissue septum becomes well delineated and clearly demarcates the edge of the air capillaries surrounding the lumen of the tertiary bronchus. During this period the elastic fibres increase in thickness and become more darkly stained.

## DISCUSSION

The ultrastructure of developing elastin in the chick lung is similar to that described in the more recent reports of developing elastin in other tissue and species (Greenlee, Ross & Hartman, 1966; Takagi & Kawase, 1967; Williams, 1970). The formation of elastic fibres takes place in three phases. The initial stage consists in the formation of approximately parallel groups of microfibrils, which are extracellular and show no periodicity. This is followed by the development of an amorphous core in the centre of the microfibrillary groups, so forming small elastin clumps. These clumps grow by enlarging the central core material, and the elastic fibre then forms as the result of aggregation of individual elastin clumps. At the same time the microfibrils diminish in number, and are probably incorporated into the central core material. The staining characteristics of the two components in the present study are similar to those described elsewhere. However, the microfibrils in the parenchyma of the chick lung did not take up phosphotungstic acid as they have been reported to do in the guinea-pig pleura (Williams, 1970).

There was a variation in the staining of the central core with lead and uranyl which appeared to depend on the size and maturity of the core material. In the smaller elastin clumps there was a light-grey granular staining of the scanty central core material which was present. In the larger clumps the central area of the amorphous core was non-staining and relatively electron-translucent, whilst a peripheral rim of varying width adjacent to the microfibrils retained the granular appearance of the earlier stage. Phosphotungstic acid, like lead and uranyl, also stained the periphery of the amorphous core material more densely.

Previous authors have maintained that there is no staining of the central cores with lead and uranyl. However, in a minority of the micrographs of Greenlee & Ross (1967) a light-grey staining of the central core is seen in the smaller elastin clumps. They state that this is due to primary fixation with glutaraldehyde and that it does not occur if fixation is primarily in osmium. Glutaraldehyde was the primary

fixative used throughout this study and yet a consistent variation in staining of the central core material was observed, suggesting the phenomenon was unconnected with the mode of fixation. It is more likely that the variation in staining of the central core is dependent on the age of the material, and is a reflexion of differences in chemical structure during maturation of the amorphous core.

The microfibrils show an affinity for anionic stains and the central core has an affinity for cationic stains. These different affinities of the two components of the elastic fibre indicate a difference in chemical structure. The amino acid analyses of separated central core material carried out by Ross & Bornstein (1969) indicate that it is of an amino acid composition similar to that previously described for elastin that had not been separated into its two components (Partridge, 1962). Ross & Bornstein have also found that the central core contains desmosine and isodesmosine. Amino acid analysis of the separated microfibrils revealed increased quantities of polar amino acids, but less proline, glycine and valine than the central core material.

Apart from the pulmonary vasculature, elastin appears to be formed in proximity to fibroblasts. The characteristic features of these cells are best seen in the connective tissue septa separating the tertiary bronchi and in the stalk of the tertiary bronchial 'clubs'. The fibroblastic cells have long attenuated cytoplasmic processes which are practically devoid of cytoplasmic organelles. Around the nuclei the cytoplasm contains a little rough endoplasmic reticulum which is usually widely dilated. Cytoplasmic vesicles, which have been described as prominent features of these cells (Greenlee & Ross, 1967; Williams, 1970), were not prominent in this material.

The cytoplasm of the fibroblasts formed bays or recesses in which were found groups of microfibrils or small clumps of elastin showing two components. Although in the tertiary bronchial clubs small elastin clumps were found in bays formed by the cell membranes of the smooth muscle cells, fibroblastic cell processes were always seen nearby and it was not thought that the smooth muscle cells played any part in elastogenesis in this situation. However, in the small muscular pulmonary vessels elastin synthesis took place alongside smooth muscle cells and in the apparent absence of cells with the characteristics of fibroblasts. The remoteness of the elastin clumps from all but smooth muscle cells suggests that in this situation elastin formation is a function of these cells. This is in agreement with observations of elastogenesis in the aorta by Karrer (1960), Takagi & Kawase (1967) and Kadar, Veress & Jellinek (1969).

The proximity of the moderately complex elastin network of the wall of the tertiary bronchus and the spiral smooth muscle bands indicates the functional relationships between the two. The appearance of the muscle network in the longitudinal and cross-sections of the bronchi, taken together with the functional studies of King & Cowie (1969), suggest that the bronchial musculature controls the diameter and consequently the airflow through the tertiary bronchi. Contraction of the smooth muscle will, besides reducing the overall diameter of the bronchi, diminish the width of the atria. Consequently the passage between the atria and air capillaries will be reduced. Gas exchange between the lumen and the air capillaries takes place predominantly by diffusion (Salt & Zeuthen, 1960) and this will be decreased.

The function of elastin in the tertiary bronchial units is probably to provide tension against which the spiral of smooth muscle may contract. Elastic tension in a vessel

is defined as the force tending to prevent stretching of the vessel wall to greater than the normal circumference by the intra-luminal pressure (Burton, 1954). Elastic fibres in the tertiary bronchi act in a similar way except that, as the elastic fibres are arranged predominantly in a radial direction along the projecting connective tissue septa, elastic tissue prevents undue narrowing of the bronchial lumen by contraction of the smooth muscle network. In the tertiary bronchi, therefore, the primary function of elastic fibres is to maintain the patency of the lumen rather than to prevent rupture, as in blood vessels.

## SUMMARY

Elastic fibres are found in four situations in the periphery of the chick lung: in the wall of the tertiary bronchi, in the pulmonary vasculature, in the connective tissue septa separating the tertiary bronchial units, and in the pleural covering. Elastin microfibrils first appear at day 14 of incubation but elastogenesis is most active between day 18 and 21. Elastic fibre formation can be considered to take place in three stages. The first stage consists in the formation of bundles of roughly parallel microfibrils, the average diameter of which is 14 nm. Secondly, small elastin clumps are formed by the development of an amorphous central core in the centre of the microfibrillary groups. Finally, elastic fibres are formed by the agglomeration of the small two-component clumps. Simultaneously the microfibrils diminish in number and are probably incorporated into the central core material.

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