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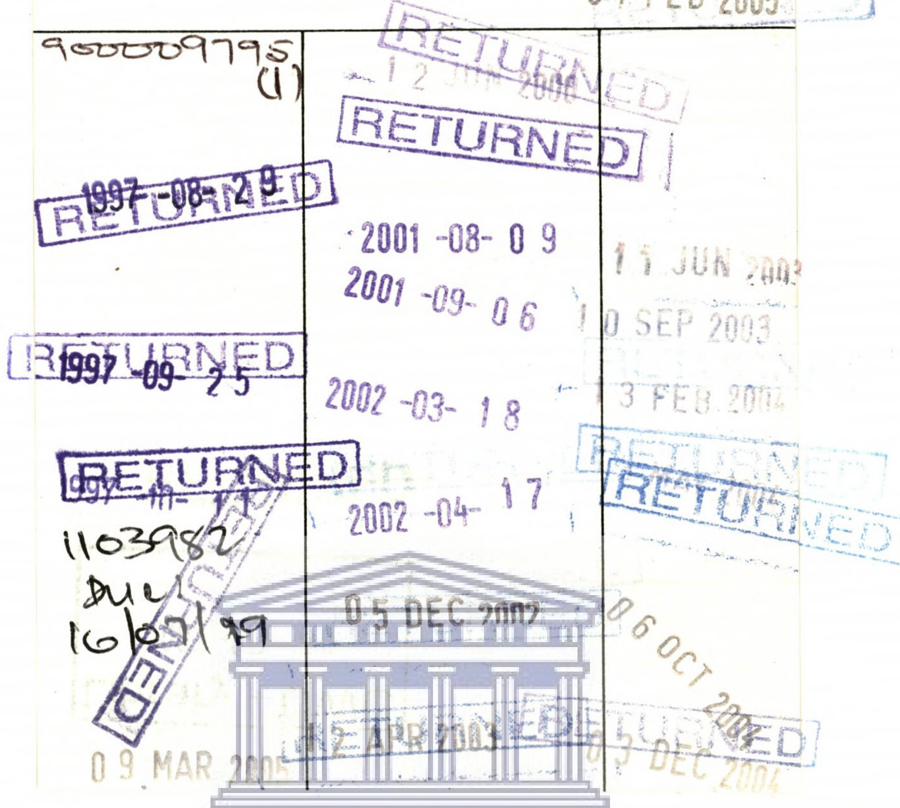

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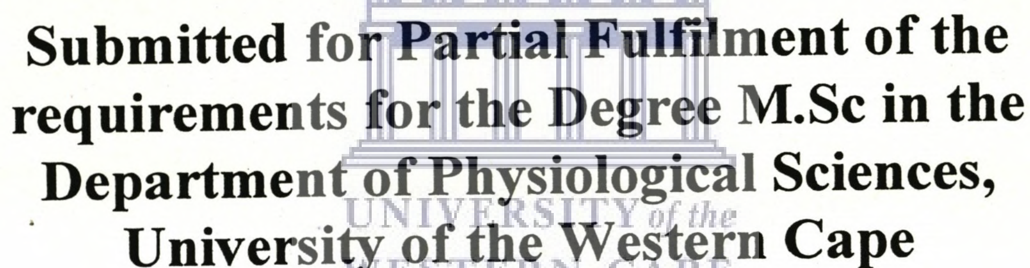
THE EFFECT OF MATERNAL NICOTINE EXPOSURE ON THE QUANTITY AND QUALITY OF NEONATAL RAT LUNG CONNECTIVE TISSUE

BY

LARRY DOLLEY



Submitted for Partial Fulfilment of the requirements for the Degree M.Sc in the Department of Physiological Sciences, University of the Western Cape

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DEDICATION

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TO ROBIN DOLLEY

WHO STIMULATED MY INTEREST IN LUNG DYSFUNCTION

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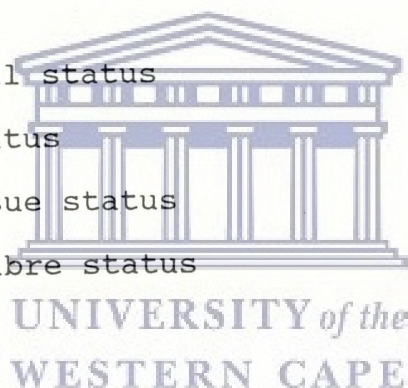

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Appendix 1: List of abbreviations.

Ala	-	Alanine
g	-	Gravitational acceleration (9,8 m/s^2)
g	-	Gram(s)
Gly	-	Glycine
H and E	-	Haematoxylin and eosin
Hyp	-	Hydroxyproline
kPa	-	Kilopascal
ℓ	-	Litre
Lys	-	Lysine
mRNA	-	Messenger RNA
mℓ	-	Millilitre(s)
PAS	-	Periodic acid-Schiff
Pro	-	Proline
RNA	-	Ribonucleic acid
UDP	-	Uridine diphosphate
Val	-	Valine

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Abstract:

The infants of smoking mothers (compared to non-smoking mothers) have been shown to have a lower birth mass, a lower brain mass, an increased perinatal mortality rate as well as a predisposition to respiratory abnormalities in later life. Evidence suggests that one of the reasons for the latter is abnormal lung structure due to changes in the connective tissue skeleton.

This study evaluated the _in vivo_ effects of maternal nicotine exposure (1mg/kg/day subcutaneously - designated the experimental group), which is equivalent to smoking 32 cigarettes per day, on the connective tissue status of the neonatal (7, 14 and 21 day old) Wistar rat lung. The control group received sterile saline as a placebo. The specific aspects investigated were:

(1) the morphological changes in lung structure and connective tissue (collagen, elastic tissue and reticulin) distribution by means of light microscopy.

(2) the quantities of collagen and elastic tissue in the lung.

(3) the ultrastructure of the lung connective tissue skeleton by means of scanning electron microscopy.

Emphysema-like morphological changes are present at all ages. The histochemical appearance of collagen is not affected while reticular fibres appear to be abnormal in structure. On day 7 there appears to be no elastic tissue in the nicotine-exposed lung compared to the control lung. This difference is not

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noticeable on days 14 and 21.

Biochemical quantitation indicated that, for the three age groups studied, there was no significant difference in collagen content between experimental and control animals. Elastic tissue was significantly higher in 7 day old experimental lungs than in the control group, contradictory tothe results of the histochemical studies. This difference was not significant for 14 and 21 day old lungs.

Ultrastructural studies of the lung connective tissue skeleton shoed abnormal fibres in the experimental group. Changes included fibre breaks, a beaded appearance of certain fibres and a deficiency in normal fibre arrangement due to the direct or indirect effects of nicotine.

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The effects of nicotine on neonatal rat lung after maternal nicotine exposure is described. The direct mechanisms for these events are still not known but speculation as to this are presented here. Further studies which could explain these mechanisms are also suggested.

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CHAPTER 1

INTRODUCTION

According to voluminous literature published over the last three decades, the respiratory system is underestimated as far as its function and sensitivity to internal and external modulating factors is concerned. Besides its important role in the exchange of respiratory gasses in the lungs, another major function includes conditioning of inspired air by the removal of coarse particles and gaseous impurities, warming and humidifying it simultaneously (Ganong, 1983). Also, because the whole cardiac output is exposed to the lung before reaching any other tissues, it has an important metabolic role as far as activation, deactivation, synthesis and release of hormonally active substances are concerned (Wijnands, 1987).

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To carry out these functions, the respiratory system has a basic structure common to man and a number of other animals, including the rat. Figure 1.1 shows this basic structure without showing any proportionality between the different elements.

The different portions of the respiratory system are sensitive to different stimuli, especially the respiratory portion, leading to defective or insufficient functionality. This in turn could lead to physical and/or chemical abnormalities in the individual concerned. One common diseased state of the lung is emphysema, a chronic obstructive pulmonary disease characterised by

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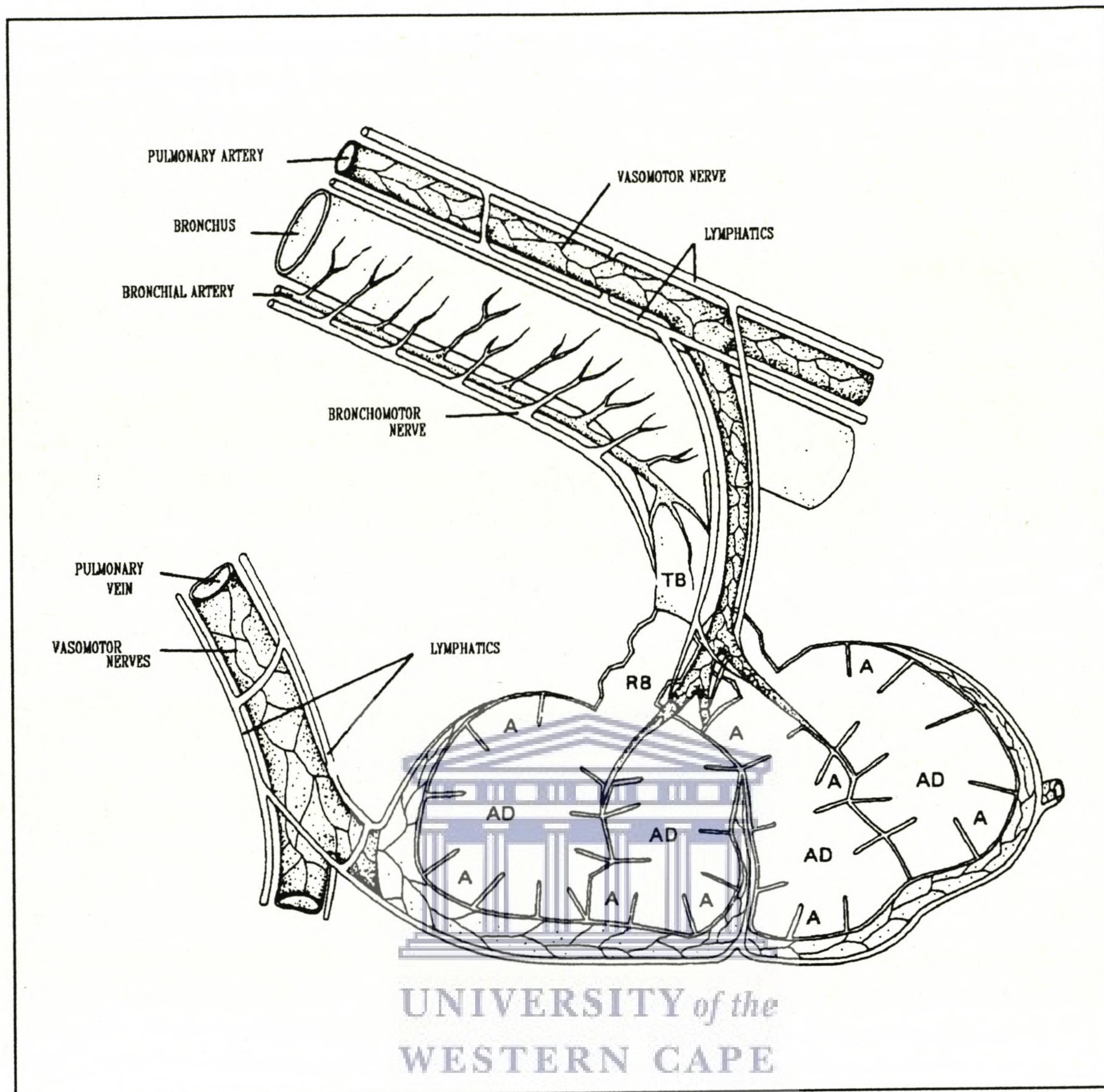

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Figure 1.1: Schematic representation of a part of lung structure at the level of the alveolus. RB = respiratory bronchiole; AD = alveolar duct; A = alveolus; TB = terminal bronchiole.

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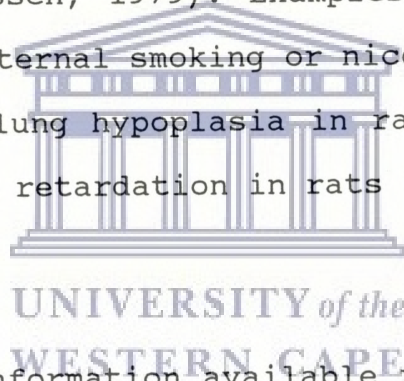
decreased lung distensibility and impaired gas exchange (Ganong, 1983). Exposure of the fetus to nicotine during gestation, has a number of deleterious effects on the prenatal and postnatal lung (Hamosh et al., 1979; Nasrat et al., 1986; Clark, 1987). In man, it has been known for over a century that tobacco smoke was associated with diseases of the respiratory tract (Pott, 1775 as cited in Pirani, 1978), substantiating the evidence of Cameron et al (1968). Examples of abnormalities in man are lowered birth mass (Cope et al., 1975), a higher rate of perinatal mortality (Pirani, 1978; Butler et al., 1972) and severe damage to the vessel walls of the umbilical artery, vein and vessels of the placental villi (Asmussen, 1979). Examples of disturbances in animal models where maternal smoking or nicotine administration is applied are fetal lung hypoplasia in rats (Collins et al., 1985) and fetal growth retardation in rats (Bassi et al., 1984; Maritz, 1988).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So far, most of the information available regarding normal and abnormal lung metabolism and function has been obtained using animal models, in particular the rat. To compare the human and rat lung with respect to metabolism and/or diseased states, a description of the development and morphology of both systems need to be addressed. For the purpose of clarity, this description will be based on the following broad steps:

1. fetal development
2. neonatal development

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3. diseased states with special reference to chronic obstructive pulmonary diseases and smoking, especially the effect on connective tissue.

The third section in particular will be further elucidated by this investigation. The first two aspects will be surveyed purely from a morphological point of view. Other aspects, such as biochemical development, cell types, connective tissue framework and function will be described in a later section.

1.1 Fetal lung development in man and animal models.

1.1.1 Fetal lung development in man.

Anatomically the lungs begin as an outpouching of the primitive gut (Wall *et al*., 1985), the latter which develops from the lower part of the laryngotracheal groove (Williams and Warwick, 1980). The start of this growth is characterised by the formation of stem bronchi and left and right lung buds covered by mesenchyme. This mesenchyme eventually gives rise to the connective tissue, cartilage, smooth muscle and vasculature of the bronchi and lungs (Williams and Warwick, 1980). This embryonic phase is sometimes termed stage one of development (Farrel, 1982).

Fetal lung development and differentiation, as described below, occurs in three phases (Wigglesworth, 1987; Williams and Warwick, 1980):

1. Psuedoglandular stage. This is also called the glandular stage (Williams and Warwick, 1980). It occurs from the 7th

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to the 17th week of gestation. This phase is characterised by low columnar epithelial tubules, the cells of which are rich in glycogen, surrounded by mesenchyme (Williams and Warwick, 1980). The lumina of these tubules are almost obliterated. The bronchial divisions are formed during this phase. The epithelial cells have scattered RNA and glycogen, a prominent Golgi apparatus and few organelles. Between the epithelial cells are mesenchymal cells and vasculature, the whole bud being similar to glandular tissue.

2. Canalicular stage. This occurs from the 17th week to the 24th week of gestation. The tubules dilate and the epithelium thins out to a cuboidal form. Some cells become squamous and are intimately associated with the expanding vascular system (capillaries) to form initial blood-air barriers. Elastic tissue also makes its first appearance within the airways (Wigglesworth, 1987; Williams and Warwick, 1980).

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3. Terminal sac stage. This phase runs from week 24 to week 40 i.e. full term (Kauffman, 1980). During this phase cellular differentiation results in the formation of type I and type II alveolar cells. At term up to 65% of the alveoli are formed (Dornan and Meban, 1985). The latter figure was debated for a number of years, some saying that a smaller percentage was already formed at birth (Thurlbeck, 1975). These alveoli are still in an immature form but fully capable of sustaining ventilation (Wall *et al*., 1985).

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During this time the alveoli become more mature and reach their full functional capabilities.

Farrel (1982) also describes a 5th stage (stage 1 to 4 being the embryonic stage and the above 3 stages) i.e. the alveolar phase, which is initiated in the perinatal period and runs up to about 8 years of age. Since much alveolarisation occurs after birth, the term "terminal sac stage" replaced the term "alveolar stage" (Kauffman,1980).

All these phases, when looked at as a continuous rather than segmented process, show the following developmental processes which occur in the lung (Farrel, 1982):

1. the early stages involve the formation of airways together with a slowly increasing vasculature and cartilaginous tissue.
2. arterial vasculature occurs along dividing airways while the veins remain surrounded by mesenchyme.
3. capillarisation of developing acini occur with vessel diameters remaining larger at the proximal ends.
4. saccules with thin septae develop and surfactant appears.
5. alveolarisation occurs mainly postnatally where alveolar numbers increase from 20 million at birth to 300 million at age 8 years (Thurlbeck, 1975).

The last period of gestation finds the potential air spaces of the lungs filled with "fetal pulmonary fluid", expulsion of which occurs with the onset of labour and vaginal delivery (Farrel,

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1982). Production of this so-called "tracheal fluid" ceases about 2 days before birth (Kitterman et al., 1979). There is evidence that this fluid is important in promoting lung growth (Alcorn et al., 1977; Kitterman, 1984).

During development it was noted that lower surfactant production occurs in the late gestational stage of the female fetus (Torday et al., 1981). These changes and differences are related to a higher incidence of respiratory distress syndrome in premature male infants (Adamson and King, 1984). During the development process in man, blood-air barrier epithelial thinning occurs mainly in the canalicular phase and fusion between epithelial and endothelial basement membranes also occur at about 19 to 20 weeks of gestation (DiMaio et al., 1989).

1.1.2 Fetal lung development in animal models:

Since the development of fetal lung in man has been conveniently divided into three phases (ignoring the 5 phases of Farrel, 1982), it may serve well to compare the time frames during which these occur in man with the rat as an animal model (see table 1.1 below).

Stage	Man	Rat
Pseudoglandular	7 - 17 weeks	0 - 13 days
Canalicular	17 - 24 weeks	13 - 21 days
Terminal sac	24 - 40 weeks	21 - 23 days

Table 1.1: Comparison of the time of occurrence of the three fetal lung phases during gestation in man and rat. Above figures obtained from Boyden (1976) and Meyrick and Reid (1977).

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From this it can be seen that profound changes which take place over a period of weeks in man, occur over a few days (and sometimes hours) in the rat and other animals. As much as this simplifies the study of these changes, it goes hand in hand with a possible loss of discernible detail due to the rapid changes occurring.

Regarding fetal lung growth, cell proliferation is at its highest (for epithelium and mesenchyme) during the pseudoglandular stage in the rat (O'Hare and Townes, 1970). In fetal sheep the lungs grow at a rate proportional to the fetal body and is mainly due to an increase in cell number (Kitterman, 1984). During this stage, loose mesenchymal tissue still surrounds buds of endodermal cells (Farrel,1982). Kauffman (1980) describes these buds as epithelial rosettes (peripheral buds) in which the cells are rich in glycogen and have a high mitotic activity.

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The canalicular period of the rat and mouse is characterised by the transformation of potential lumina of rosettes into canals. These canals of the endodermal buds are the future air sacs (Kauffman, 1980; Farrel, 1982). During this period other changes which occur are the thinning of the interstitium, rapid development of capillaries and the angulation of vessels between alveolar epithelial cells (Kauffman, 1980). This is also accompanied by the differentiation of type I and type II alveolar epithelial cells and the appearance of pulmonary surfactant (Campiche et al., 1963). The formation of new septa coincides

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with interstitial fibroblast proliferation (Burri,1974) and the appearance of elastic tissue at the tip of newly formed septa.

Finally, in the terminal sac stage, the proportion of dividing epithelial cells falls to low levels (Kauffman, 1980) and the resting cells remain quiescent until the early postnatal growth period. Surfactant production proceeds and further differentiation of the respiratory region occurs. Saccules with thin septae tend to become more prominent, leading to a marked increase in the internal surface are of the lungs (Farrel, 1982). At this stage, similar to the fetal lamb, the potential air spaces are filled with "fetal pulmonary fluid". Lobar differences in development are apparent in a number of animal models (Kikkawa et al., 1971) i.e. upper or cephalad lobes show faster maturation than lower or caudad lobes. The upper lobes also show faster radiographic clearing during recovery from hyaline membrane disease (Farrel, 1982).

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A study of mesenchymal versus epithelial proliferation, using [^{3}H]-thymidine indices, showed a higher mesenchymal activity in the terminal sac stage of fetal rat lung (O'Hare and Townes, 1970). This activity is carried over into the neonatal period where endothelial and fibroblast activity was 8 to 10 times higher than the alveolar epithelium.

There are two recognizable types of mesenchyme viz.

(a) tracheal mesenchyme consisting of highly oriented connective tissue and collagen

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(b) loose non-orientated mesenchyme from bronchial tips. A relationship was recognised between the quantity of mesenchyme in developing lung and the pattern of cytodifferentiation where greater amounts of mesenchyme were necessary for type II cell differentiation than for bronchiolar cell differentiation. This was attributed to a polypeptide called "fibroblast-pneumocyte factor" which is produced by fibroblasts (Smith, 1979). Fibroblasts are also thought to produce a chemotactic factor acting on bronchial epithelial cells (Shunsuke et al.,1989).

A comparison of rat littermates grouped according to sex was done by Adamson and King (1984). They ascertained that lung epithelial cell labelling increased, especially in the female, above that of the male (fetal days 17 - 20). The amount of surfactant was also significantly higher in the female. From this stage up to full term the male tended to "catch up". Essentially, lung structural differences in the male and female predates surfactant synthesis. This must be kept in mind when studying both rat and human lung development and function during the immediate prenatal period.

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1.2 Postnatal lung development:

1.2.1 Gross structural development:

One of the biggest debates regarding postnatal development was whether alveoli are formed before birth or whether new alveoli are formed postnatally. A number of researchers have attempted answering this question, mainly using light microscope techniques

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(Dunnill, 1962; Adamson and King, 1988; Burri, 1974). It has since been well documented that lung development is not yet complete at birth and new alveoli are still formed postnatally (Burri, 1974). In man, alveolarisation is only completed at about eight to ten years of age (Vaccaro and Brody, 1978). In the rat, the comparative age for cessation of alveolar multiplication is about postnatal day 15 (Burri, 1974). Angus and Thurlbeck (1972) report that the number of alveoli per human lung can vary greatly depending on a number of factors e.g. body height. Farrell (1982) reports a relatively constant figure of 300 X 10^6 alveoli per adult human lung.

The course of lung development in mouse is similar to that of other animals, including that of man. This has been shown by many authors up to now (Leeson and Leeson, 1964; Conen and Balis, 1969; Burri, 1974; Amy et al., 1977; Ten-have Opbroek, 1981; Farrell, 1982). Since the proposed study to be undertaken is in postnatal rat lung, further descriptions of lung development will deal primarily with the latter animal.

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At birth (day 0) the conducting airways occupy a relatively large percentage of lung volume when compared to mature lung. At this stage a number of different levels of conducting airway may be identified, viz.:

(a) bronchi - airways with cartilage and seromucous glands.

(b) bronchioles - those airways lacking the above structures.

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Epithelia of the peripheral airways flatten and form straight, smooth-walled channels. The septa are thick with a relatively large central layer of connective tissue having capillaries on both sides. Groups of fibroblasts are formed where several septa join (Burri, 1974).

Day 4 in the postnatal rat lung shows thin septa with small humps containing fibroblasts, collagen, a bundle of elastic tissue and sometimes a capillary loop. Distal parts of the conducting channels also show that cuboidal epithelial cells start thinning out, showing a blood-air barrier typical of the gas exchange region (Burri, 1974).

Between days 4 and 7 drastic changes in morphology are evident. The initially smooth septa show "buds" protruding into the air space. These buds (ridges or crests) form "secondary septa". The capillaries in this bud are usually wider at the base of the crest and may even be closed at the peak of the crest (Burri, 1974).

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From days 10 to 13, respiratory bronchioli become evident, lined by a flattened epithelium. Elastic fibres are well-developed and are usually found at the mouth of the developing alveoli. After day 13 an expansion and thinning out of the alveolar septa occurs. Total tissue and interstitium thickness as well as the septal thickness decreases. An increase of the gas exchange surface area is also an indication of "septal stretching" (Burri, 1974).

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During this development, formation of secondary septa seems to be due to growth of mesodermal derivatives (Burri, 1974). Elastic fibre bundles at the tip of ridges seem to play a role in this process (Loosli and Potter, 1959). Tension induced by the presence of these elastic fibres could stimulate fibroblasts,thereby influencing the rate and direction of fibrous tissue development (Burri,1974).

1.2.2 Cytology of the developing postnatal rat lung:

The morphological changes described by Burri (1974) have been confirmed and extended by Vaccaro and Brody (1978) and Farrell (1982). The adult rat lung is known to be an extremely complex organ and contains approximately forty different cell types. The parenchymal region only has five basic cell types as listed in table 1.2.

Parenchyma cell types	Cell number distribution (%)
Type 1 epithelial cells	10
Type 2 epithelial cells	12
Endothelial cells	40
Alveolar macrophages	5
Interstitial cells	33

Table 1.2: Lung parenchymal cell types. Data for this table is obtained from Weibel and Gil (1977).

A further description of the cell types in the developing alveolus requires an idea of the microscopic structure of the alveolus. The alveolar epithelium is continuous with that of the alveolar ducts and the bronchioles. Because alveoli lie adjacent to each other, connective tissue and its contained blood vessels

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are sandwiched between two epithelial layers. This forms the interalveolar septum. The epithelial lining of each alveolus consists of a number of different cells as described below.

1.2.2.1 <u>Type 1 pneumocytes</u>:

These cells are also known as type 1 alveolar epithelial cells. They are flattened cells which, together with its basement membrane and underlying capillaries, only measure about 0,05μm in thickness (Williams and Warwick, 1980; Ganong,1983). The main function of this cell type is to form a barrier of minimal thickness to allow for greater diffusibility of atmospheric and blood gasses. Type 1 pneumocytes are also incapable of repair when damaged and first have to be replaced by type 2 pneumocytes (see below).

1.2.2.2 <u>Type 2 pneumocytes</u>:

These cells are also known as granular alveolar pneumocytes (Spencer, 1978), great alveolar cells or septal cells (Ganong, 1983). They occur in much lower numbers than the type 1 pneumocytes. These rounded epithelial cells are attached to the alveolar epithelial basement membrane and have a secretory function. The major secretory product is surfactant which is derived from cellular inclusions called lamellar bodies. These lamellar bodies in turn consist of layered phospholipids in storage form (Gil and Reid, 1973). The type 2 cells respond to injury by rapid proliferation. Meban (1972) showed the presence of alkaline phosphatase in these cells, an enzyme which may be

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used as an indicator of type 2 cell rupture in the alveolar lavage.

1.2.2.3 Type 3 pneumocytes:

Also known as brush cells (Meyrick and Reid, 1968), they are very seldom encountered in a microscopic examination of human lung tissue. These cells have short microvilli on its free surfaces and fine filaments in the cytoplasm. It makes up about 10% of the pneumocyte population. Possible absorptive functions are associated with this cell type (Meyrick and Reid, 1968).

1.2.2.4 Pulmonary macrophages:

Also known as dust cells, they are of special interest because of their ability to synthesise and secrete collagenase and also elastase in thioglycollate-stimulated intraperitoneal macrophages (Werb and Gordon, 1975). The activity of these cells and their enzymes could have profound effects on lung structure and function, especially with respect to chronic obstructive pulmonary diseases such as emphysema (Werb and Gordon, 1975). These cells, although derived from blood monocytes, are found in the interstitium of the alveoli and the alveolar spaces (Leeson and Leeson, 1985). Because they have to migrate through the alveolar wall, they generally have an irregular shape. This is due to both its phagocytic role (folding of the membrane) and its movement and spreading (similar to diapedesis).

In addition to the role of the alveolar macrophage as a defence mechanism against inhaled dust particles, the macrophage also

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appears to play a role in the turnover of pulmonary surfactant (Naimark, 1973). Maritz and Woolward (1990) showed that this is a strong possibility due to the observation of these macrophages close to the sites of surfactant synthesis.

1.2.2.5 Interstitial space cells:

These are cells found between the alveoli and the larger blood vessels. They usually occur together with free fluid, collagen and elastic tissue. Weibel (1974) demonstrated pericytes (Rouget cells) in human lung, their exact location being in the basement of the capillaries. A second fixed cell type is the fibroblast which is usually associated with the connective tissue components of the interstitium. Vaccaro and Brody (1978) showed two types of fibroblasts, one of which occurs at the tip of new septa and is involved in elastogenesis.

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1.2.2.6 Pulmonary endothelial cells:

These are the cells of the capillaries associated with the alveolus. Besides forming a component of the blood-air barrier (see later), these cells have a number of other functions common to the endothelium of other vascular beds (Ryan and Ryan, 1977). Some of these additional functions are as follows:

(a) contribution of coagulation and fibrinolytic factors

(b) processing of lipids and chylomicrons

(c) final monitoring of the whole cardiac output before it is transported to the systemic circulatory system

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1.3 <u>The blood-air barrier and surfactant</u>:

This barrier (also called the alveolar-capillary membrane) is where the intracellular and extracellular matrix of the lung is closest to the atmosphere and all its components, both toxic and non-toxic. Because of its importance in maintaining optimal aeration of blood and indirectly maintaining blood pH, this barrier is of utmost importance to the well-being of man and animal.

The main layers (Atwal, 1988) making up this continuous barrier consists of (from the direction of the capillary lumen to the alveolar space):

(a) capillary endothelial layer

(b) two fused basement membranes

(c) squamous epithelial layer lining the alveolus.

The total thickness of the barrier is approximately 0,35μm (Leeson et al., 1985). The absolute thickness can vary depending on which part of the barrier the measurement is made. The two fused basement membranes measure about 50nm and in some cases can be much thinner (Weibel, 1964).

As well as allowing the movement of gases in both directions, the cells of the blood-air barrier have additional functions or capabilities. Pinocytic vesicles are present in both cell layers and they play a role in the uptake of alveolar fluid and serum proteins in surfactant (Bignon, 1975). This idea was furthered by Schneeberger (1978) who used horse radish peroxidase which was

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introduced into the trachea of newborn mice. This enzyme eventually appeared in the epithelial cell pinocytic vesicles.

The synthesis and secretion of surfactant increases in the immediate perinatal period (Torday et al., 1981). Disturbance of this process may lead to respiratory distress syndrome (Rooney, 1985; Mason, 1987), due to lung immaturity in the newborn rat and in man. Even at this early stage of life, sex differences are evident in lungs i.e. male fetal rat lungs are less developed than those of their female counterparts (Adamson and King, 1984).

The type 2 pneumocyte synthesises and secretes surfactant. It usually appears as cellular inclusions called lamellar bodies (Harwood, 1987). Lamellar bodies are the storage form of synthesised surfactant (Chevalier and Collet, 1972).

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1.4 Development of the mesenchyme:

According to Ten Have-Opbroek (1981), who surveyed the results of lung development in mammals (and mice in particular), conclusions drawn for the latter animal may hold for other mammals, including man. This is based on the fact that both prenatal and postnatal development is comparable in a number of species. Because much of the proposed study centres on postnatal lung connective tissue architecture and composition, the following section will attempt to compare the development of the mesenchyme (interstitium) of the lung in man and other animal models, including the rat.

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During gestation in the rat, the fetal mesenchyme has a 50% tritiated thymidine labelling index when compared with epithelial cells (Adamson and Bowden, 1975), indicating slow growth of the mesenchyme. This is purported to result in branching of the rapidly growing epithelial tubules. During the terminal sac period i.e. the last intrauterine phase prior to parturition, this labelling index changes. Day 22 in fetal rat lung shows an increase in the labelling index from 50% to 90% (Kauffman, 1980), which indicates a higher activity of endothelial cells and fibroblasts. In man (terminal sac stage at 24 to 40 weeks gestation) this is associated with thinning of interalveolar septa and an increased volume of the developing saccules (Farrell, 1982). At birth the rat has underdeveloped saccules with thick septa containing a central layer of connective tissue rich in fibroblasts. At regions where a number of septa join (septal junction) there is usually a concentration of fibroblasts (Burri, 1974). Elastic tissue is ill-developed at birth, only being found in the walls of the larger bronchi, but the stimulus provided by respiratory movements lead to its early appearance. At the third month of postnatal life it is readily observed in alveolar walls, pleura and vasculature (Spencer, 1978).

All the cell types viz. epithelial cells, endothelial cells and fibroblasts, grow in concert during these different phases but it is believed that the key to postnatal rat lung growth is the connective tissue component (Thurlbeck, 1975). This is evident when connective tissue development is interfered with using a proline analogue which blocks collagen triple helix formation by

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blocking the hydroxylation of procollagen (Carrington et al., 1985). Administration of this analogue during the fetal phase causes a permanent decrease in average postnatal rat lung size and affected normal airway branching patterns even though each structural unit appears normal at three weeks of age (Spooner and Faubion, 1980). This implies that inhibition of interstitial fibroblast function and interference with extracellular collagen synthesis significantly alters postnatal lung growth. In addition it is known that blocking elastic tissue cross-linking, which is an extracellular process, also retards future lung growth (Kida and Thurlbeck et al., 1980). Also, diminished fibroblastic activity due to the administration of analogues may reduce the production of the peptide fibroblast-pneumocyte factor which is involved in fetal type 2 cell differentiation.

In the rat, postnatal day 15 to 28 is also characterised by thinning of the lung septa (see previous page for similar findings in man). These septa usually consist of a single capillary and a sheet of connective tissue (Burri, 1974). This is similar to the situation in adult rat lung which has walls which are thin, less cellular and have few interstitial cells. Where interstitial cells are present, they tend to occur in the corners of alveoli (Vaccaro and Brody, 1978). At this stage the cell ultrastructure is indicative of a dormant cell which is not actively engaged in protein synthesis.

The fibroblasts noted in developing rat lung appear to be of two types, those occurring at the base of the developing septa and

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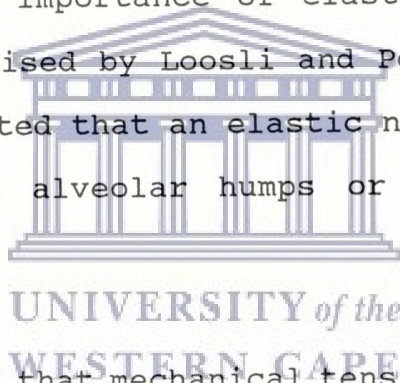
those at the tips of these septa. Those at the base (Burri, 1974) are designated corner fibroblasts by Vaccaro and Brody (1978). Their dormant appearance is characterised by few organelles and extensive accumulations of intracellular lipid. The role of this type of fibroblast is uncertain (Vaccaro and Brody, 1978). At the tips of the septa the fibroblasts are actively synthesising and secreting connective tissue. At an ultrastructural level elastic tissue predominates in the formation of new septa (Vaccaro and Brody, 1978). Sparse, randomly scattered collagen in the early postnatal period becomes thick, well-bonded groups after most alveoli have formed, with a concomitant increase in lung lysyl oxidase activity. The importance of elastic tissue to septal development was recognised by Loosli and Potter (1959) amongst others. It was also noted that an elastic network in developing septa indicates where alveolar humps or crests will appear (Burri, 1974).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It is also hypothesised that mechanical tension introduced in the developing lung tissue by the elastic tissue component would locally increase the rate of cell division. It would therefore follow that the tension of these elastic fibres could be responsible for the arrangement of the collagen skeleton forming the septal support system (Burri, 1974).

Considering the importance of the connective tissue skeleton and architecture as far as lung structure and function is concerned, it is of vital importance that the development of this system is not interfered with. As has been described, interference in the

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postnatal period leads to changes in size (smaller lungs) even though lung morphology is apparently normal. Changes other than size may also occur e.g. overall lung composition, total connective tissue content and individual connective tissue element content may be adversely affected. Changes may also be permanent, leading to susceptibility to a number of different disorders later in life.

1.5 Lung collagens:

Connective tissue fibres, collectively termed scleroproteins, are made up of collagen, elastin/elastic tissue and reticulin. These tissues are characterised by their insolubility, their relative permanence and also their high tensile strength (Pierce et al., 1961). Notwithstanding the harsh conditions needed to isolate and study these proteins, they can be obtained relatively easily by making use of cultured mesenchymal cells from different tissue (Peterofsky and Prather, 1974; Kao et al., 1977; Berman et al., 1978; De Clerck and Jones, 1980). In addition, culturing these cells allows the development of models for the biosynthesis, regulation and degradation of these proteins (De Clerck and Jones, 1980).

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Considering the importance of the connective tissue skeleton in lung structure and development, it follows that damage of this tissue and its numerous protein components can cause defects in lung structure and function in both prenatal and postnatal phases in man and animals. For the purpose of this study it is of extreme importance to survey the biosynthesis of the protein

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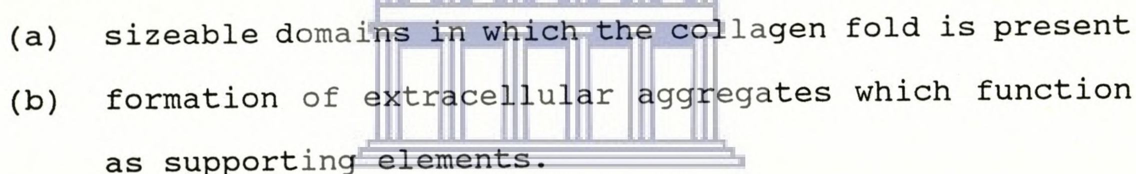
components as well as the degradative processes involved. In addition, classification of the different genetically distinct types of proteins as well as their specific functions and site of occurrence in an organism would also have relevance in this context.

Since the further discovery of more and more genetically distinct collagens, the amount of information regarding these molecules has proliferated at an ever-increasing pace. Gene cloning techniques have also added to this store of information (Miller and Gay, 1987). All collagen molecules found in the variety of collagens are slightly different only as far as primary structure is concerned, but all have the same basic characteristics:

(a) sizeable domains in which the collagen fold is present

(b) formation of extracellular aggregates which function as supporting elements.



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Currently, eleven different collagens are recognised, depending on the source from which they are obtained.Because of the complexity of the family of collagens, they are usually (but not definitively) classed into three groups as shown in table 1.3 (Miller and Gay,1987). The known collagens may also be classified on the broad basis of the tissue group to which they belong as indicated in table 1.4.

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Group 1	Group 2	Group 3
Type I	Type IV	Type IX
Type II	Type VI	Type X
Type III	Type VII	
Type V	Type VIII	
Type K		

Table 1.3: The different types of collagen classed into three groups.

Collagen	Non-cartilaginous	Hyaline cartilage
Group 1	I, III, V	K, II
Group 2	IV, VI, VII, VIII	
Group 3		IX, X

Table 1.4: Collagen types grouped according to the types of tissues they are derived from.

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Of the above groups, group 1 molecules undoubtedly constitute the major fibril-forming and fibre-forming molecular species of collagen in vertebrate organisms (Miller and Gay, 1987).

1.5.1 Collagen Synthesis:

The synthesis of collagen follows the generally accepted rules of protein synthesis. This process has been exhaustively described by Hance and Crystal (1975 and 1976). An abbreviated version of the process of collagen synthesis up to the point of triple helix formation is presented (figure 1.2). Similar to many

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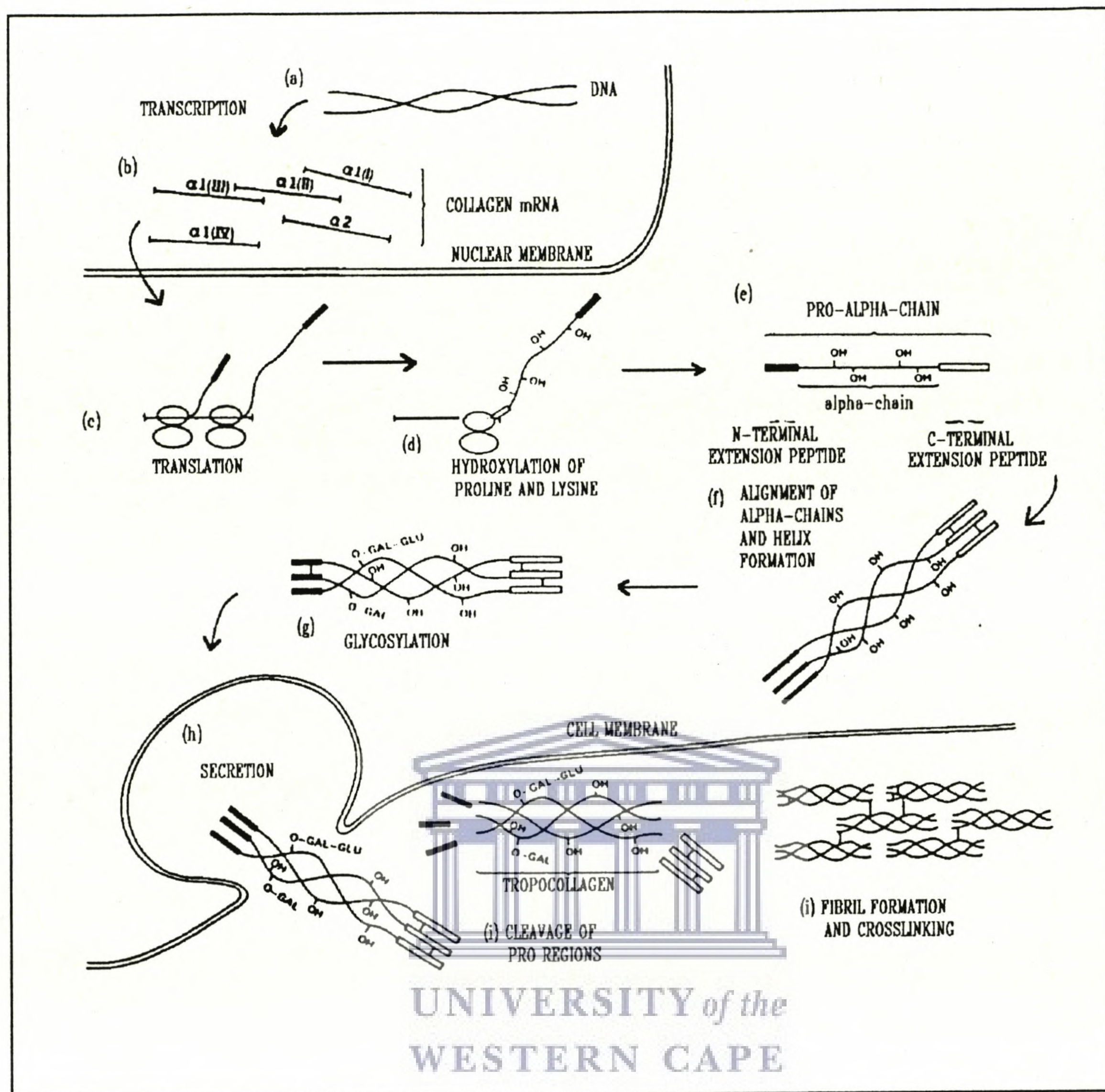

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<u>Figure 1.2</u>: Schematic illustration of collagen synthesis, via generally accepted mechanisms, with modifications introduced at various stages prior to secretion and ultimately fibril formation. The process starts with transcription (a) leading to the synthesis of mRNA (b) and subsequent translation (c), hydroxylation (d), pro-α-chain formation (e), helix formation (f), glycosylation (g), secretion (h), cleavage (i) and finally fibril formation (j). Modified from Hance and Crystal (1976).

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proteins with an extracellular function, collagen is synthesised in a precursor form termed tropocollagen.

1.5.1.1 **Translation of collagen MRNA:**

The collagen MRNA, after translocation to the cytoplasm, is translated on polysomes attached to the ribosomes, as expected for proteins which are to be secreted later on. In keeping with the large collagen MRNA, many ribosomes (up to 10) can be accommodated in the polysome (Hance and Crystal, 1975).

1.5.1.2 **Procollagen:**

Currently it is accepted that the product of translation is the pro-α-chain (molecular weight of about 150 000 daltons) with a translation time of 7 to 8 minutes. The intact pro-α-chain contains three regions (Hance and Crystal, 1976):

(1) N-terminal non-collagenous extension.

(2) middle α-region.

(3) C-terminal non-collagenous extension.

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The non-collagenous "pro" extensions (also known as teleopeptides) do not contain the Gly-X-Y triplets found in the α region. In addition, Try is present in the C-terminal extension (Hance and Crystal, 1976). Just before release, 3 pro-α-chains become aligned, most likely by "pro" region interaction, including disulphide bond formation. This is followed by coiling of the α-regions, forming protropocollagen, the form in which it is secreted. Besides the above function of the "pro" region of

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the pro-α-chain, the following is a list of postulated functions of the "pro" regions:

(1) enhances solubility during intracellular and extracellular transport.

(2) promotes proper orientation of tropocollagen in fibrilogenesis and intermolecular cross-linking between tropocollagen molecules.

(3) inhibits premature cross-link formation.

Protropocollagen (newly secreted) is converted to tropocollagen via a series of extracellular steps. Procollagen peptidase removes the N-terminal extension to form a p-collagen intermediate. The C-terminal extension is subsequently removed, probably by a separate protease. Dermatosparaxis, inherited skin fragility in cattle, is due to the absence of procollagen peptidase and thus a disordering of collagen fibrils. This enzyme is also absent in the lungs of these cattle. Prior to secretion, the new pro-α-collagen chains must undergo a number of post-translational modifications. These are described in the following subsections (Hance and Crystal, 1975 and 1976).

1.5.1.3 <u>Hydroxylation of proline in collagen</u>:

Mostly in the nascent procollagen chain, Pro residues are hydroxylated at position 4 by prolyl hydroxylase, an enzyme bound to the rough endoplasmic reticulum membrane. The enzyme requires ascorbic acid (or similar reducing agent) and other activators for activity. A separate enzyme is suspected to be responsible for hydroxylation at position 3. Hydroxylation occurs at position

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4 in mammals when occurring in the sequence Gly-X-Pro-Gly and at the 3 position in the sequence Gly-Pro-X-Gly. The degree of hydroxylation, indicated by the Hyp:Pro ratio, is different between the different types of collagen. Although the function of Hyp is not completely understood, it is known to increase stability of the triple helix of collagen. It has now been noted that inhibition of hydroxylation results in lowered collagen secretion (Hance and Crystal, 1975 and 1976).

1.5.1.4 <u>Hydroxylation of lysine</u>:

Lysyl hydroxylase has similar requirements to prolyl hydroxylase (Fe^{++}, α-ketoglutaric acid and vitamin C) and is specific for Gly-X-Lys-Gly. Activity has been demonstrated in chick lung. The degree of hydroxylation varies with age, collagen type, tissue type and animal species. Lysyl and hydroxylysyl residues are implicated in cross-link formation. The nature and strength of the cross-link depends on whether a lysyl or hydroxylysyl group is involved. Bonds involving hydroxylysyl residues are very stable and the degree of hydroxylation here may play a very important role in the nature of the final collagen network. Another characteristic of hydroxylysyl residues are that they are the only known sites for glycosylation (Hance and Crystal, 1975 and 1976).

1.5.1.5 <u>Glycosylation of collagen</u>:

This involves addition of a single galactose unit or disaccharide unit (glucosylgalactose) to certain hydroxylysine residues. The addition occurs in a stepwise process. In the first step a

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galactose unit (in the form of UDP-galactose) is added to the hydroxyl group of hydroxylysine by the enzyme UDP-galactose: galactosyl transferase. After this, some of these-hydroxylysine-linked galactose units are glycosylated by UDP-glucose: glucosyl transferase. Glycosylation takes place during the nascent phase but can also occur on the native triple helix conformation (Hance and Crystal, 1975).

The activity of the two glycosylating enzymes are high in young rat lung (only cartilage has a higher activity) and decreases with age, paralleling the decrease in the rate of collagen synthesis (Hance and Crystal, 1976). Glycosylation is considered a post-translational modification of collagen which occurs in the cytoplasm of fibroblasts (Hance and Crystal, 1975 and 1976). Glycosylating enzymes have also been reported in lung basement membrane (Hudson and Spiro, 1972). The higher basement membrane content of young rat lung tissue versus older tissue is thought to explain the higher glycosylating activity in the younger animals (Hance and Crystal, 1975 and 1976).

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It has been found that collagen with a more amorphous structure has a high degree of glycosylation, suggesting a very important role in determining collagen fibril organisation. This also relates to the fact that glycosylated hydroxylysine residues participate in major cross-links. In addition to this, other postulated functions of glycosylation are (Hance and Crystal, 1976):

(1) identification as a secretory protein.

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(2) induction of collagen-induced platelet aggregation.

(3) partial control of cross-link formation.

1.5.1.6 Triple helix formation and secretion:

Helix formation is thought to take place in the cisternae of the rough endoplasmic reticulum immediately after translation and hydroxylation. This process involves interaction of the non-collagen region of 3 pro-α-chains by means of disulphide bonds. Formation of this helix is essential for secretion of the final product. Secretion of protropocollagen is assumed to be a typically merocrine process involving movement from the rough endoplasmic reticulum to the Golgi apparatus from whence to Golgi-derived vacuoles and finally to the extracellular space. A modification of this process has also been proposed which involves movement straight from the rough endoplasmic reticulum to the cytoplasm in vesicle form and from here to the extracellular space (Hance and Crystal, 1975 and 1976).

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1.5.1.7 Collagen cross-links:

The ultimate ability of collagen to perform its function is its ability to form a framework, leading to a strong, inert matrix. Cross-linking is not necessary for collagen fibril formation but it does give the fibril a much higher tensile strength. Cross-linking requires conversion of protropocollagen to tropocollagen followed by intramolecular and intermolecular covalent bonds or cross-links. The cementing substance in the framework formed would be proteoglycans and other non-collagen connective tissue glycoproteins. Cross-linking occurs via lysine and hydroxylysine

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residues (figure 1.3). The first step is catalysed by lysyl oxidase (protein lysine 6-oxidase; EC 1.4.3.13), which oxidatively deaminates the ϵ-amino group of lysyl or hydroxylysyl residues to the corresponding δ-semialdehyde, forming allysine and hydroxyallysine. Aldol condensation of two allysine aldehydes, or reaction of allysine (or hydroxyallysine) with a lysyl (or hydroxylysyl) residue to form a Schiff base, gives rise to these cross-links (Hance and Crystal, 1975). Collectively, this is the only enzyme required for the cross-linking of both collagen and elastic tissue. In collagen, this enzyme is specific for highly conserved sequences (teleopeptide region) at helical cross-linking sites (Eyre et al., 1984). Lathyrogens (chemicals which prevent the crosslinking process), such as β-aminoproprionitrile, are irreversible inhibitors of the cross-linking enzyme (Kida and Thurlbeck, 1980).

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Where collagen forms fibrils, the individual tropocollagen elements overlap by 67nm (or multiples of this distance) with intermolecular cross-links being formed in this region. These links have been described in both the C-terminal and N-terminal ends of nearby tropocollagen molecules. The appearance of Cys in the α-chains of some collagen types raises the possibility of intermolecular and intramolecular disulphide bonds (Hance and Crystal, 1976).

The strong connective tissue framework formed by collagen is largely dependent on the formation of cross-links between collagen molecules. A similar situation holds for elastic tissue,

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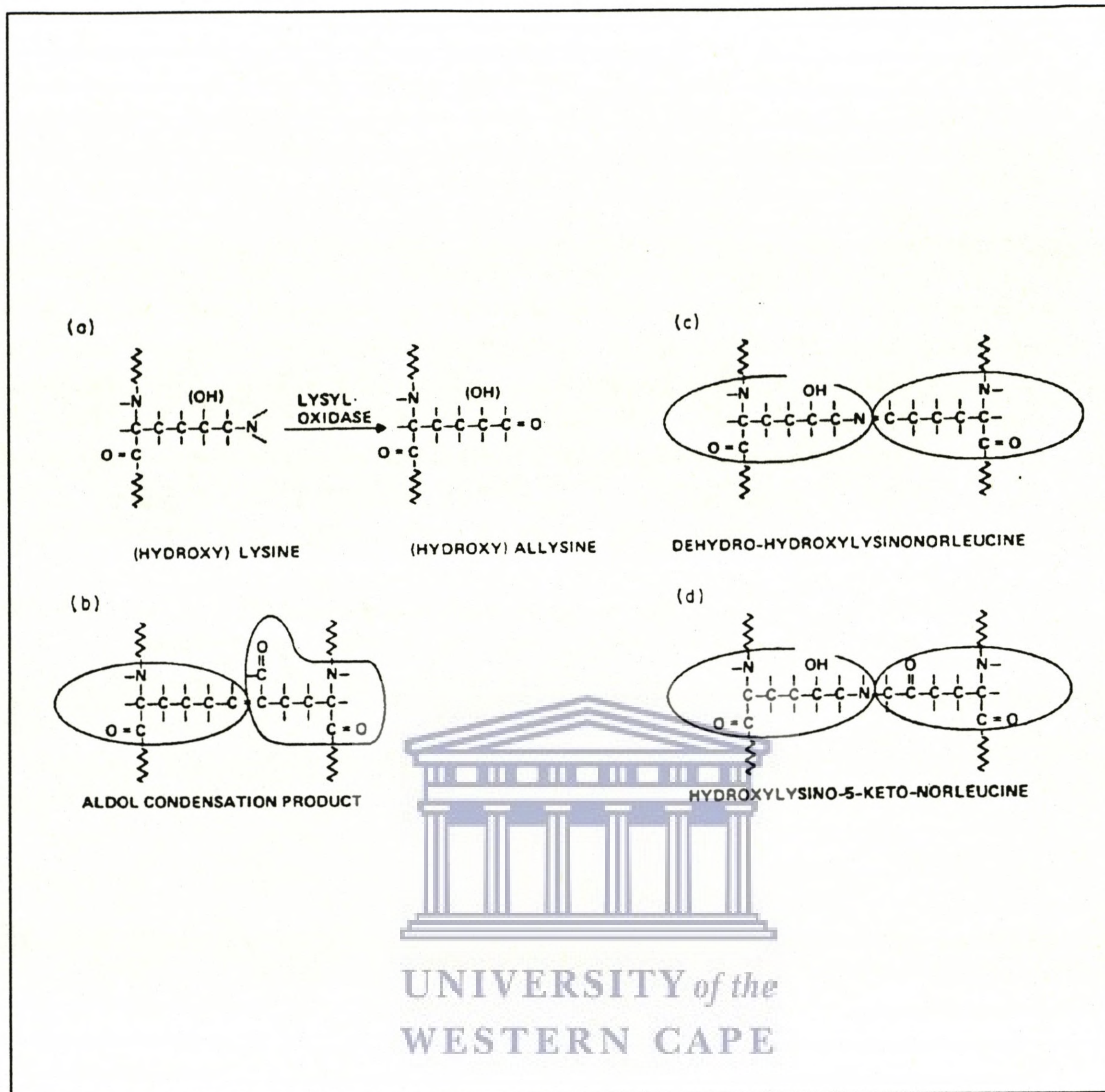

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Figure 1.3: Cross-linking molecules formed during collagen synthesis (Eyre et al., 1984).

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where cross-linking amino acids have also been identified. But, even after years of research into the exact nature of the main cross-linking residues of collagen, very little is known about it. Up to now an assortment of borohydride-reducible intermediates have been identified and these usually disappear as the collagen matures (Eyre et al., 1984).

Although there is still debate about the exact cross-linking residue(s) in collagen, a pathway for possible cross-link formation and tentative cross-link structure is available. According to Eyre (1987) collagen cross-linking is dependent on the formation of collagen aldehydes formed from a few specific lysine or hydroxylysine residues generated by the enzyme lysyl oxidase . The latter process is thought to be the committed step for cross-link synthesis (Eyre et al., 1984). An assortment of di-, tri- and tetrafunctional cross-linking amino acids can form spontaneously by the inter- and intramolecular reactions of these aldehydes within newly-formed collagen polymers. Two possible pathways have been suggested (see figure 1.3) for the development of cross-links in banded fibrillar collagens (67nm repeat), one based on lysine aldehydes and the other based on hydroxylysine aldehydes (Eyre, 1987; Eyre et al., 1984).

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The major forms of mature cross-linking in the hydroxylysine aldehyde pathway are the trivalent 3-hydroxypyridinium residues hydroxylysylpyridinoline and lysylpyridinoline (Eyre, 1987; Eyre et al., 1984). The cross-linkers for the allysine pathway are

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still unknown, although histidine may be a component in the process.

1.5.1.8 **Collagen degradation:**

Collagen turnover in lung is apparent from the fact that adult lung continually synthesises new collagen but the content remains constant (Hance and Crystal, 1975). In rabbit lung parenchyma, 30% of newly synthesised collagen, intracellularly or soon after secretion, is degraded. This may be a means of monitoring the biosynthetic process for abnormal proteins (Harper, 1980). Continued collagen proteolysis was shown in rat lung where collagen was labelled during a rapid growth phase and subsequently was lost over the period of a year. Since 30% of body protein is made up of collagen, it is of importance that the role of enzymes and other degradative procedures be understood (Traub and Piez, 1971). There are only three possible sources of enzymes (Hance and Crystal, 1975) responsible for collagen breakdown (collagenolytic activity):

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(1) collagenase originating from microorganisms.

(2) cathepsin B[1] (lysosomal origin).

(3) vertebrate collagenase (extracellular compartment).

Of these three, the first one is of interest only in infected lung while the second would only be of importance for phagocytosed collagen, where the correct condition for activity is maintained. Production of the third form has been noted in lung parenchyma explants (Hance and Crystal, 1975).

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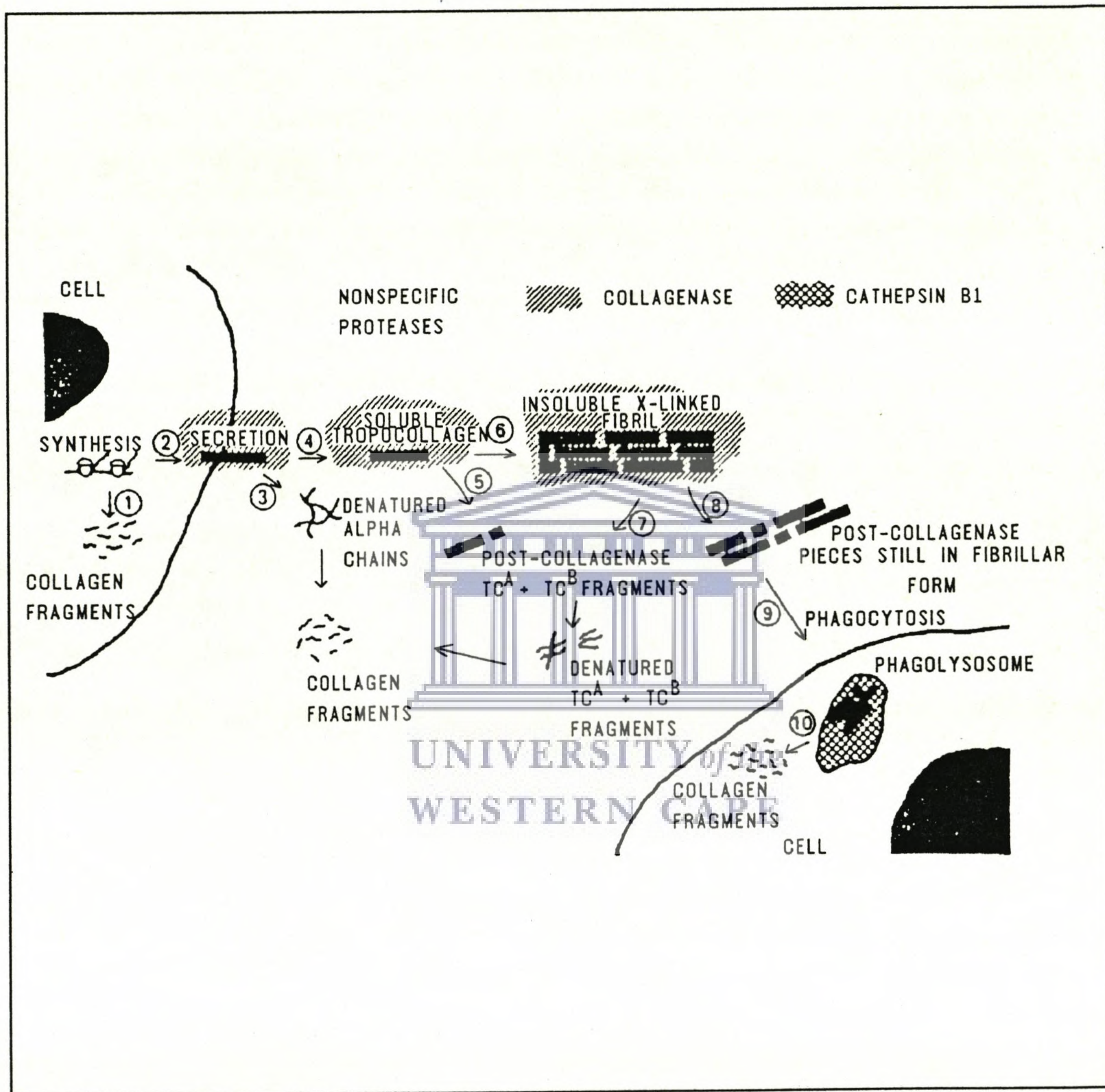

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Figure 1.4: Schematic representation of the mechanism for collagen degradation. After synthesis (1) some fragments of protein are produced while some denaturation occurs during secretion (2) from the fibroblast. The rest (4) exists as soluble tropocollagen which may be degraded by collagenase (5) or be converted to mature cross-linked collagen (6). Eventually the latter is also degraded by collagenase (7 and 8) absorbed (9) by other cells where further degradation occurs (10). Modified from Hance Crystal (1975).

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Most interest was initially centred on the prokaryotic form of collagenase synthesised by Clostridium histolyticum. Vertebrate collagenase is the only proteolytic enzyme which can attack collagen in the triple helix formation. This hydrolysis is specific in that it cleaves a bond about 300 residues from the C-terminus of the molecule (Hance and Crystal, 1976). The two helical peptides produced by this attack denature at 37°C (as opposed to the high heat stability of the triple helix) and in this form can be attacked by general proteases. The reaction products due to collagenase activity has been shown to induce chemotaxis of human monocytes and fibroblasts. This implies that the supply of monocytes to an area of inflammation and also tissue repair by fibroblasts will increase (Postlethwaite et al., 1978).

There are two known sources of vertebrate collagenase which can attack lung collagen, namely polymorphonuclear leucocytes and alveolar macrophages. It is not entirely sure which plays a major role in collagen turnover. If lung collagen is not degraded by non-collagenase mechanisms (non-helical collagen) it may therefore be degraded in the helical form by enzymes from either of the above two sources, following which there are two possible pathways for further degradation (Hance and Crystal, 1975):

(1) denaturation and attack by non-specific proteases in an extracellular medium.

(2) phagocytosis and intracellular degradation in phagolysosomes (analogous to cathepsin B[1]).

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1.6 Lung Elastic Tissue.

Together with collagen, elastic connective tissue makes up the basic framework for lung structure and function. As well as having a structure different to that of collagen, elastic tissue co-exists intimately and, in instances, is physically bonded to collagen. It is a highly insoluble protein rich in hydrophobic amino acids (Eyre et al., 1984).

With the initial description of elastic tissue, an element of confusion has crept in to the nomenclature of the different components of this tissue. For the purposes of this study I will use the terms as described in Hance and Crystal (1975) to describe elastic fibres. These include:

(1) a fibrillar component to be called microfibrils or microfibrillar component.

(2) an amorphous component called elastin.

The above two components constitute the elastic fibre. With age the amorphous (elastin) component increases with respect to microfibrils (Hance and Crystal, 1975).

The microfibrils are about 10 - 12nm in diameter with a denser periphery than the centre, therefore giving a tubular appearance. With maturation, almost the entire centre of the fibre is amorphous. Biochemical definition shows that these two components are different and do not have a precursor-product relationship. It has already been shown that the two components have differing amino acid compositions:

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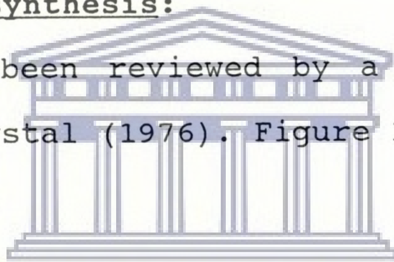
(a) The microfibrils are rich in polar amino acids such as aspartic acid and glutamic acid. It also contains methionine, cysteine and have hydroxyproline cross-links.

(b) elastin is rich in non-polar amino acids (alanine, valine, leucine, isoleucine), and has no cysteine and methionine. Very small amounts of hydroxyproline and cross-links occur.

Both components do not contain any hydroxylysine (Hance and Crystal, 1975).

1.6.1 Elastic tissue synthesis:

This tissue has also been reviewed by a number of authors, including Hance and Crystal (1976). Figure 1.5 is a synopsis of this synthetic process.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1.6.1.1 Translation of elastin MRNA:

A number of different cell types, including the fibroblast, are known to synthesise elastic tissue. In the lung this takes place on the rough endoplasmic reticulum of mesenchymal cells and follows the general rules of protein synthesis. The initial product of this process is a soluble elastin precursor viz. tropoelastin.

1.6.1.2 Tropoelastin:

Depending on the method used for its isolation, tropoelastin has a molecular mass of 40 000 to 70 000 daltons (the large difference in molecular mass can be ascribed to the presence of

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a possible super-precursor - protropoelastin). Because of its amino acid composition, tropoelastin is not readily soluble in water. There is also very little resemblance to the amino acid sequences in collagen. Some repeating sequences that do occur are Gly-Gly-Val-Pro, Pro-Gly-Val-Gly-Val and-Pro-Gly-Val-Gly-Val-Ala.

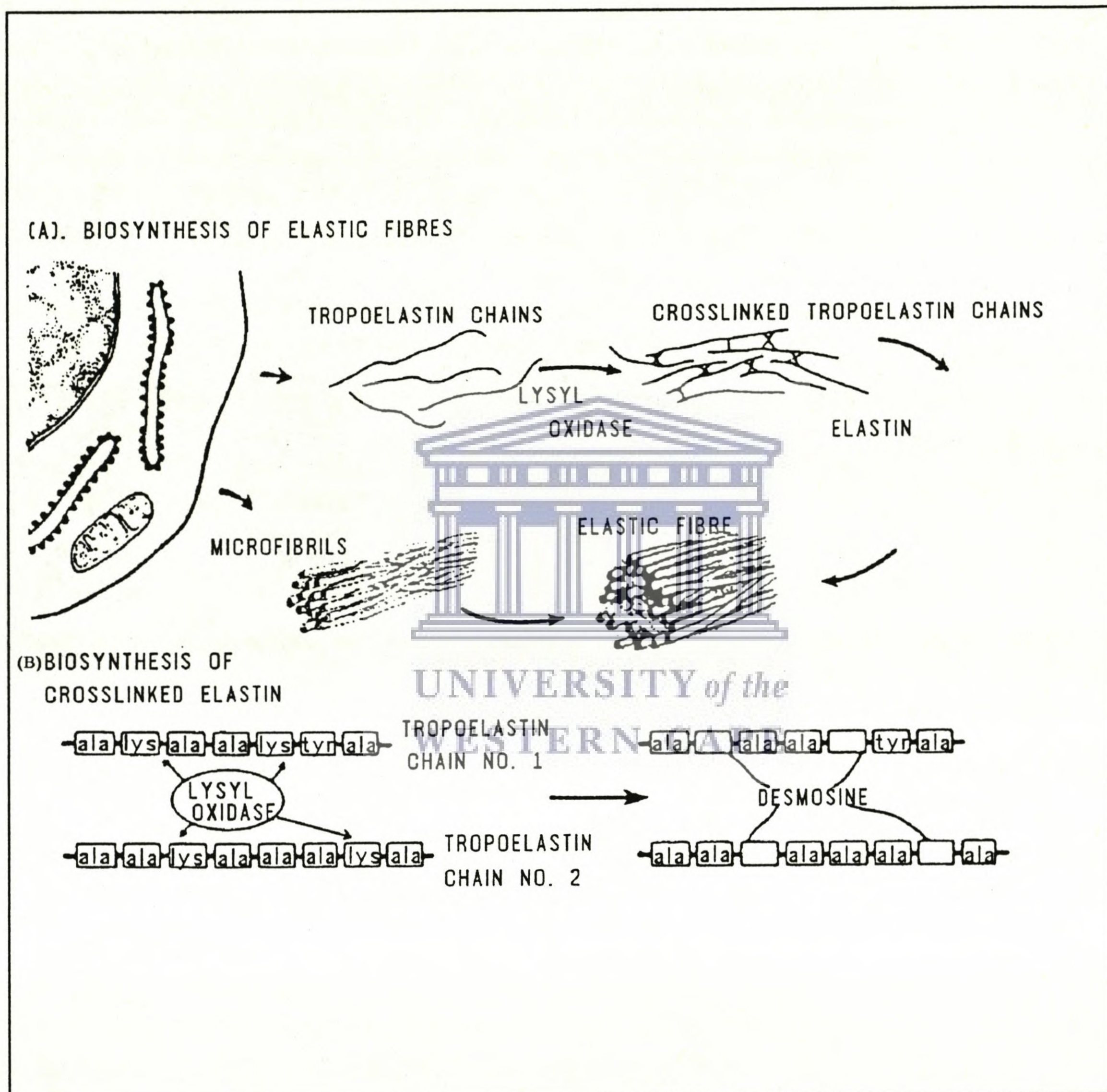

Figure 1.5: Schematic representation of elastic tissue synthesis via generally accepted mechanisms. (A). Synthesis of mature elastic fibres including cross-linking under the influence of lysyl oxidase. (B). Representation of the cross-linking mechanism and the amino acids involved. Modified from Hance and Crystal (1975).

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1.6.1.3 Hydroxylation of proline and lysine:

The hydroxylation of proline and lysine is considered to be a post-translational modification of tropoelastin. Only a few of the proline residues are hydroxylated. Similar to collagen, hydroxylated lysine forms cross-links in the tropoelastin molecule, which explains the absence of hyroxylysine in the final product (Hance and Crystal, 1975). Lysyl oxidase, as with collagen, catalyses the oxidative deamination of the ϵ-amino group of lysine to an aldehyde, the latter which can then link with the aldehyde forms of other lysyl residues. This formation of cross-links, such as desmosine, isodesmosine and lysinonorleucine (see figures 6 and 7), signals the conversion of tropoelastin to elastic tissue. Desmosine is derived from four lysine residues in two tropoelastin sequences and in some cases up to four tropoelastin sequences may be joined in this fashion. The relation between crosslink formation and association of elastin with the microfibrillar component is not known Hance and Crystal, 1975).

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1.6.1.4 Microfibril synthesis:

Much less is known about this component of elastic tissue. Their differences in structure and composition is characterised by microfibril susceptibility to proteolysis by trypsin and pepsin as well as the ability of dithiothreitol to solubilise the microfibrils away from elastin (reduction of disulphide bonds evidently play a role in this process). Microfibrils have a higher level of cysteine and methionine but do not contain hydroxyproline, proline, desmosine, isodesmosine or lysinonor-

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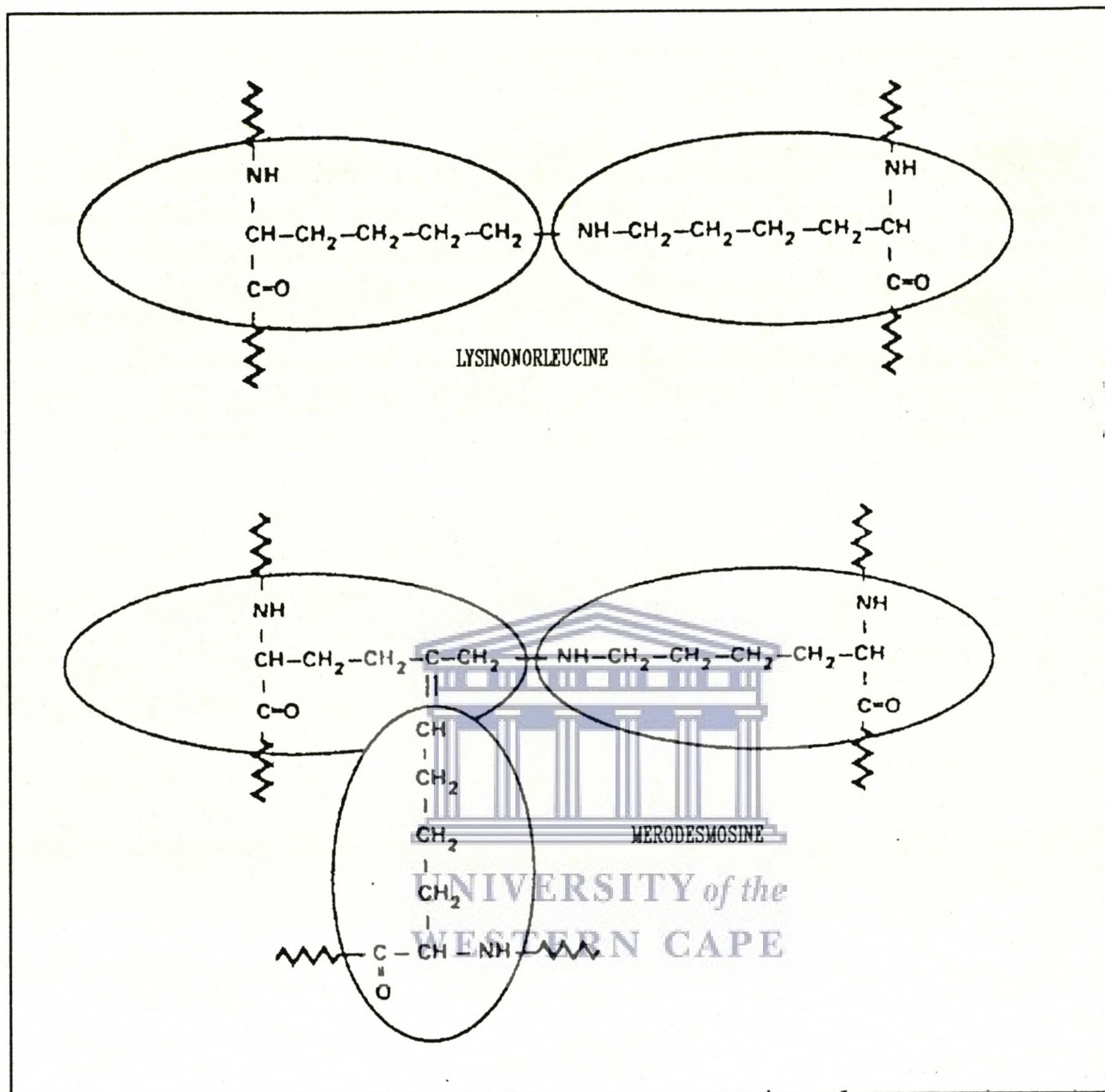

<u>Figure 1.6</u>: Lysinonorleucine and merodesmosine formed under the influence of lysyl oxidase for elastic fibre cross-linking (Eyre <u>et</u> <u>al</u>., 1984).

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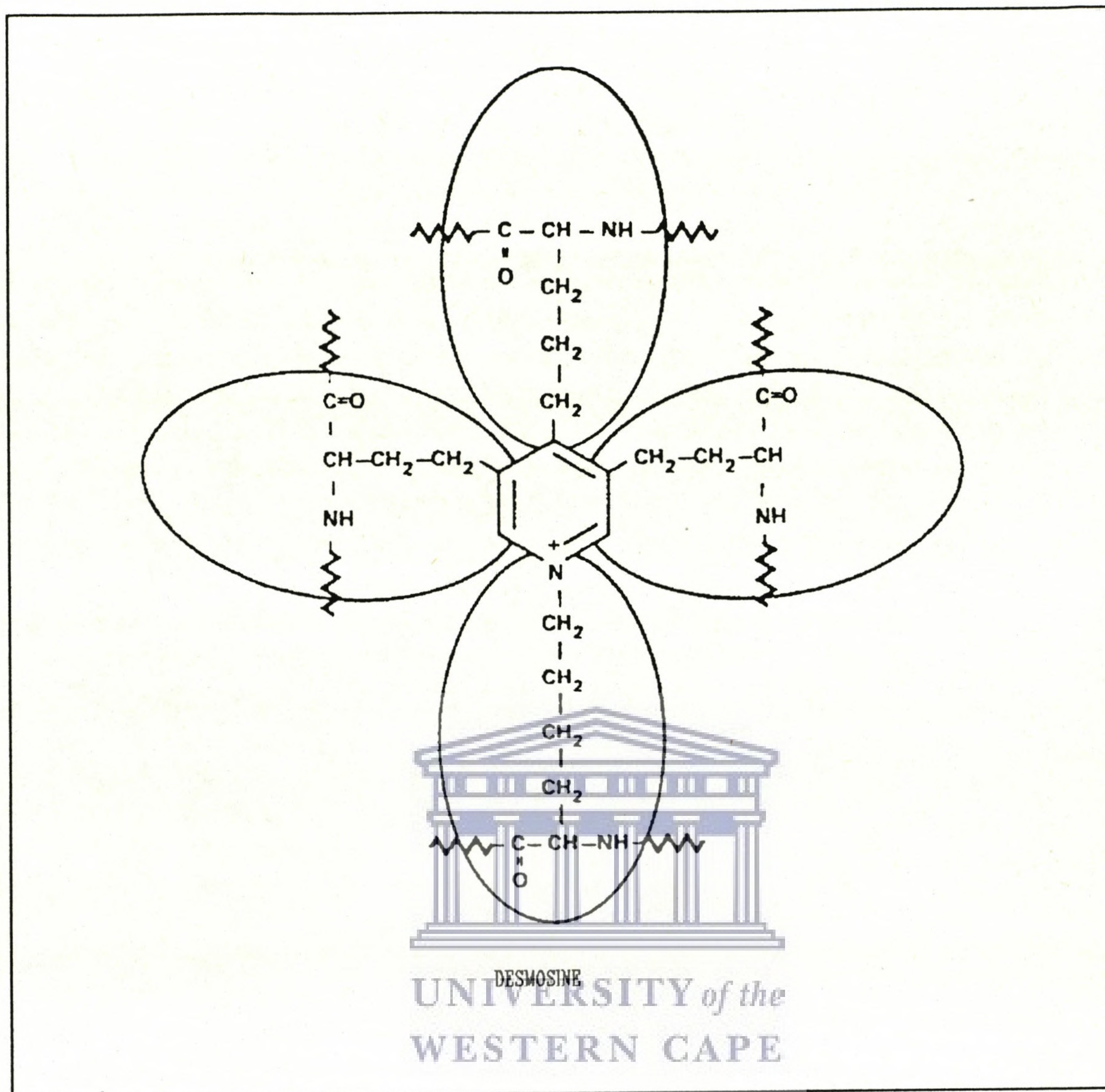

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Figure 1.7: Desmosine, another cross-linker of elastic fibres formed by the action of lysyl oxidase (Eyre et al., 1984).

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leucine. The absence of hydroxyproline assumes that no collagen is present in the microfibrillar component. Certain hexose and hexosamines are incorporated in microfibrillar structure (Hance and Crystal, 1975).

1.6.1.5 Elastic fibre degradation:

Radioactive tracer studies show a very low turnover of elastic tissue. It seems that new and immature elastic tissue is turned over more rapidly than mature elastic tissue. This is important when considering the process of lung elastolysis and its role in emphysematous conditions.

Elastolysis is centred solely on the enzyme elastase. It is possible that this enzyme is highly specific for mature elastic tissue fibres while the immature forms may be degraded by a variety of proteases, including trypsin. Pancreatic porcine elastase has a molecular mass of 25 000 daltons with a precursor (proelastase) activated by tryptic cleavage and an optimum pH of 8,80 (Hance and Crystal, 1975).

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Sodium chloride can act as an inhibitor of elastase while a serum α-globulin known as α_1-antitrypsin also has this effect. The structure, active site and mechanism of inhibitors would have possible future importance in controlling elastolysis in diseased states (Hance and Crystal, 1975).

Confusion regarding elastase in the literature has arisen due to a number of reasons (Hance and Crystal, 1975):

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(1) purified elastase is not specific for elastic tissue but can degrade other proteins e.g. ribonucleases.

(2) assaying for elastase activity using substrates other than pure elastic tissue give a possible source of error.

(3) elastases from various tissue and organs have not been proven to be identical.

(4) many "elastase" preparations have not been highly purified and may have other proteolytic activity.

These factors have to be kept in mind when analysing and interpreting data regarding these enzymes.

1.7 Lung reticulin.

These fibres are also called argyrophyllic reticular fibres (Hance and Crystal, 1976) because of their ability to stain black with silver methionine dyes (leaving collagen a light-brownish-yellow.

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1.7.1 Composition and structure:

Because reticular fibres stain differently to collagen it was assumed that they were of different composition and structure. Using electron microscopic techniques, it was noted that reticular fibres have the same periodicity as that of collagen (same degree of helicity). This only indicates a molecular structure similar to collagen, not necessarily the same composition (Leeson et al., 1985).

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It was also noted with the light microscope that, with sufficient reticular fibres present, PAS staining resulted in a positive result (Leeson _et_ _al_., 1985), indicating the presence of a glycoprotein. The latter test is negative with collagen. Chemical analyses also showed that the carbohydrate content of these reticular fibres was 4% (10 times more than collagen), explaining this difference. It now appears as if the carbohydrate acts as a coating on the fibre, explaining silver staining at the periphery of the fibres (Leeson _et_ _al_., 1985).

This has lead to the belief that the reticular fibre is actually collagen with a coating of glycoprotein and proteoglycan. The structure of this fibre is much finer than the collagen fibre (Leeson _et_ _al_., 1985).

1.7.2 Synthesis and degradation:

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In loose connective tissue the reticular fibres (also sometimes called reticulin according to Hance and Crystal, 1976) are formed by fibroblasts, while in blood-forming tissues they are formed by reticular cells (Leeson _et_ _al_., 1985). Reticulin is sensitive to a number of enzymes, including collagenase, but also resistant to trypsin, again indicating a collagenous nature (Hance and Crystal, 1976).

1.8 PROTEOGLYCANS

"Ground substance" is a general term used to describe a wide variety of components which make up the acellular, non-fibrillar component of the intercellular matrix of all tissues. The term

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"amorphous" is also used with the above term i.e. amorphous ground substance. This term encompasses the following cellular and non-cellular components (Horwitz et al., 1976):

(a) intrinsic and serum-derived proteins (glycoproteins, enzymes, etc.)

(b) cell metabolites

(c) electrolytes

(d) small neutral molecules e.g. glucose, urea, etc.

(e) metabolites of collagen and elastic tissue

(f) hormones

(g) proteoglycans.

The proteoglycans are the best-defined components of the amorphous ground substance. It is a combination of mucopolysaccharide (a disaccharide repeating unit termed glycosaminoglycans) covalently bonded to protein. An overview of the structure and function of different proteoglycans has been adequately dealt with by Heinergard and Sommarin (1987). A short summary of the main features of the proteoglycans regarding the study to be undertaken is presented below.

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1.8.1 Structural features:

(1) Carbohydrate portion: The glycosaminoglycans are composed of hexosamines and uronic acids or galactose. All the glycosaminoglycans, with the exception of keratin sulphate, contain uronic acids. There are different categories of glycosaminoglycans in different tissue types:

(a) those with glucosamine or galactosamine

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(b) those with glucuronic acid, iduronic acid or galactose.

Of the above two groups, some have either no sulphate, one sulphate or 2,5 sulphates per disaccharide.

(2) Protein portion: The proteoglycans may consist of a number of chains of glycosaminoglycans per protein. Attachment of the two components occur via the hydroxyl group of serine (sometimes threonine). More than one type of glycosaminoglycan may be attached to the protein. The protein component is not easy to separate in its native state for further study. The above two components which make up the native proteoglycan are different to the mucin glycoproteins of the respiratory tract. The latter have a shorter carbohydrate portion than the proteoglycans and are also different with respect to their susceptibility to enzyme digestion.

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1.8.2 Proteoglycan biosynthesis:

Because of the dearth of information regarding the protein component, it is assumed that they are synthesised on endoplasmic reticulum-bound polysomes, to allow for export to the extracellular space.

The three components of the carbohydrate portion, which are easily traced via radioactivity studies, are acetate, glucosamine or sulphate. Acetate enters by conversion to acetyl-CoA, glucose through phosphorylation and subsequent interconversions of the

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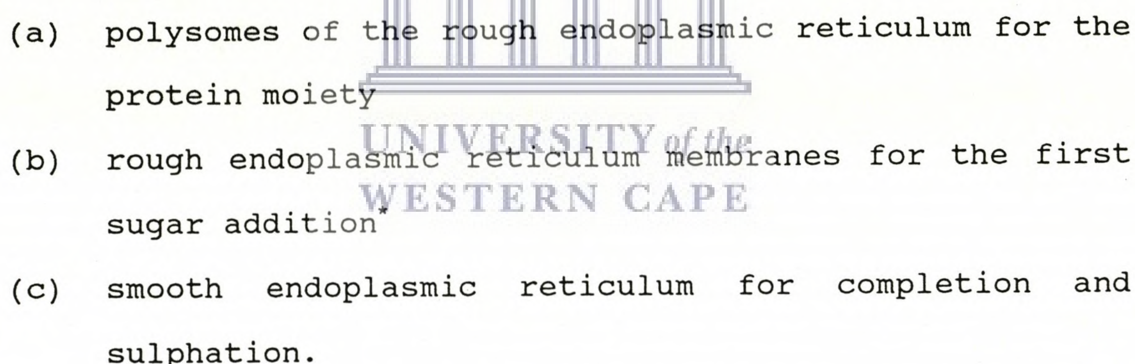
nucleotide sugar, and sulphate through formation of 3'-phosphoadenylsulphate before incorporation.

Synthesis of glycosaminoglycan is initiated by the sequential addition of xylose, galactose and glucuronic acid by separate enzyme activities. Enzymes for chain elongation have not been solubilised but are believed to be membrane-bound enzymes. Sulphation studies, using PAPS and polysaccharide acceptors, shows soluble enzymes are responsible for this process. It may be that sulphation enzymes form part of the complex used in elongation (Heinegård and Sommarin, 1987).

The subcellular sites of synthesis have been determined autoradiographically as: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(a) polysomes of the rough endoplasmic reticulum for the protein moiety

(b) rough endoplasmic reticulum membranes for the first sugar addition*

(c) smooth endoplasmic reticulum for completion and sulphation.

*The addition of sugars at this point is similar to the first addition of sugars to newly synthesised collagen. This link, though, seems to be an extremely weak one (this may still be important in the case of lung damage due to nicotine and/or smoke inhalation).

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1.8.3 Proteoglycan degradation:

As with many other cellular and tissue components, there is a balance struck between synthesis and degradation. The approximate half-lives of different glycosaminoglycans differ from one tissue to another. This ranges from 5 days (skin hyaluronic acid) to 2 weeks (skin dermatan sulphate). Hydrolytic enzymes for degradation may be cellular constituents (hyaluronidase in rat lung) or blood borne cell constituents brought to the tissue (alveolar macrophages have enzymes which can affect chondroitin sulphate).

During the study of genetic defects of these molecules, stepwise degradation by enzymes such as glucuronidases, hexosamidase, sulphatases and iduronidases have been elucidated. These enzymes are stored in lysosomes. Lysosomal stability, cellular fragility and phagocytic activity would all affect catabolism of the glycosaminoglycans.

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1.8.4 Lung glycosaminoglycan synthesis:

The major glycosaminoglycans of lung are heparin, heparin sulphate and dermatan sulphate. A higher content occurs in the trachea as compared to lung parenchyma itself. The rate of synthesis of the different types of glycosaminoglycans differ with age (Heinegård and Sommarin, 1987).

There are a number of cells which could possibly synthesise these glycosaminoglycans. It seems as if mast cells make heparin and hyaluronic acid while fibroblasts synthesise heparin sulphate,

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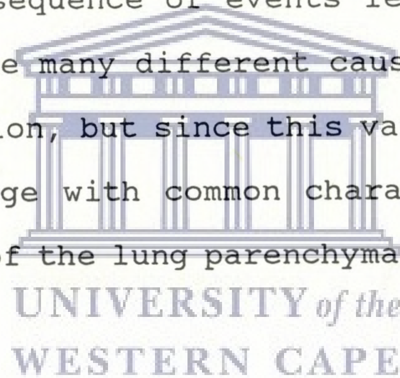
dermatan sulphate and chondroitin sulphate (Heinegård and Sommarin, 1987).

1.9 <u>Abnormal physiology of the lung</u>.

Pulmonary emphysema is characterised by the destruction of lung parenchyma, often involving walls of respiratory bronchioles, resulting in centrilobular lesions. It can also extend to true alveoli, resulting in panlobular lesions (Janoff, 1983). The final result is a distension of air spaces, leading to a lower gas exchange surface area and therefore inefficient aeration of blood. Emphysema itself is a final stage and it is therefore not easy to construct the sequence of events leading to this stage (Laros, 1972). There are many different causative factors which can lead to this situation, but since this variety of factors can lead to this final stage with common characteristics suggests that a very basic part of the lung parenchyma is involved (Laros, 1972).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When trying to establish the causes of generalised pulmonary emphysema, Laros and Kuyper (1976) divides them into two broad categories:

(a) extrinsic causes which include chronic bronchiolitis, chronic bronchitis, bronchial and bronchiolar obstructions, chronic irritation by inhalation, vascular changes, thromboembolic phenomena, changes in surface-active properties,enzyme deficiencies and congenital dysfunctions (Laros, 1972).

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(b) the molecular disintegration theory caters for those possible causes which do not fit into the extrinsic theory. These include a higher incidence of emphysema in menopausal females, a significantly higher incidence in males, a tendency to appear in families and the predisposition to emphysema even when exposed to similar factors such as air pollution and cigarette smoke (Laros and Kuyper, 1976).

It is evident that there is a large variety of causative factors involved in "generalised emphysema". From a histological viewpoint, pathological changes of connective tissue fibres or the ground substance, or both, may be at the basis of these emphysematous changes (Laros, 1972). Based on this, the present study deals with the possible changes in lung connective tissue quantity as well as the quality of the connective tissue framework. In particular, these factors will be looked at in the immediate postnatal period to establish the effects of maternal predisposing factors on lung integrity. For this reason, a survey of the effects of some predisposing factors on the development of emphysema and emphysematous-like changes in neonatal lung follows.

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1.9.1 Normal connective tissue architecture in lung.

(A) Functional anatomy of alveolar wall connective tissue:

As the main supporting tissue of lung, the connective tissue component consists of fibroblasts, macrophages, mast cells,-non-

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differentiated cells, fibres and a ground substance consisting of previously described macromolecular polysaccharide-protein complexes (Laros, 1972). This series of components can be divided into three different systems (Laros and Kuyper, 1976):

(i) A collagen-ground substance system maintains the shape and stability of the alveolar structure. When stretched, the non-distensible fibres limit the distension in the physiological range.

(ii) elastic tissue-collagen system allows the lung to function as a bellows. The elastic fibres can only be stretched when both ends are attached to the fibre network. The ground substance does not hamper the stretching process.

(iii) the non-fibrillar ground substance acts as a cementing substance for the interaction of the two fibre types. It also plays a role in determining the viscosity of the system i.e. the ease of uncoiling of collagen and "lubrication" of elastic fibres. It also plays a role in determining the osmotic pressure of the system.

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(B) Molecular anatomy of alveolar wall connective tissue:

(i) Collagen-ground substance system:

The collagen fibres are found predominantly in the basement membranes (Bradley et al., 1975) and are usually glycosylated. The latter may influence peptide packing and consequently porosity of the membrane.

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(ii) Elastin-collagen system:

The elastic fibres must be anchored to the total fibre framework and this is probably achieved by the cementing properties of the proteoglycans and glycoproteins (Pump, 1974). The elastic fibres are surrounded by an environment of acidic polysaccharides, allowing free movement of the fibres.

(iii) Non-fibrillar ground substance:

The acidic mucopolysaccharides (also called the glycosaminoglycans), chondroitin-6-sulphate, dermatan sulphate, heparin, heparin sulphate and hyaluronic acid, are fundamental for the properties of the connective tissue framework (Laros, 1972). Hyaluronic acid consists of coiled chains of high molecular mass which does not bond to, but entangles and anchors, elastic fibres without chemical interactions (Sharon, 1974). The main cementing substances are the sulphated proteoglycans with negative charges, which give shape and stability to the fabric of fibres in the alveolar wall (Sharon, 1974). Of the monosaccharides present in the ground substance, nine link with only five of the twenty amino acids, namely aspartic acid, hydroxylysine, hydroxyproline, serine and threonine. These five amino acids are present in collagen but not elastic tissue, indicating the anchoring of collagen, and not elastic tissue, to the ground substance.

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1.9.2 Emphysematous lung connective tissue status:

The study of human emphysema, as reported in the numerous papers, was done using human post-mortem tissue as well as biopsy and pneumonectomy material. Animals models have also been studied.

Laros and Kuyper (1976) state that the following general trend holds for the emphysematous lung.

(a) There is no significant difference between collagen content and composition between normal and affected specimens

(b) An inconsistent lowering of elastic tissue content and a change in its amino acids composition has been noted

(c) Total glycosamines were not significantly different but glucosamine:galactosamine ratio was increased for the emphysematous lung group. This ratio indicates the hyaluronic acid to chondroitin sulphate ratio. The change in this ratio points to the loss of cementing properties of the ground substance and also decreased fibrillogenesis from tropocollagen (Laros, 1972).

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According to Laros and Kuyper (1976), the human female is less susceptible to emphysema than males, and that this is due to female sex hormones. It is proposed that these hormones protect the connective tissue framework of the lung by stimulating fibroblastic activity (Ozzello and Bembry, 1964). It is also noted that the development of the fetal lung is affected by these hormones. This includes a greater synthesis of surfactant in late gestation in the rat as well as faster growth rate. This effect

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is eventually cancelled out in the early postnatal period due to the male "catching up" with female development (Adamson and King, 1984). The effect of cigarette smoking in humans on the development of chronic obstructive pulmonary diseases (COPD's) has been well- documented over the last thirty years. Cigarette smokers have been shown to be more susceptible to COPD's such as chronic bronchitis, emphysema and lung function abnormalities (Higgins, 1984). According to Chen et al. (1991), female smokers are more susceptible to COPD's than male smokers for reasons not yet known, while for non-smokers there is no significant difference in this respect. This could be related to fetal developmental aspects especially as far as the lung is concerned.

1.10 Smoking, nicotine and lung metabolism:

Smoking is an addiction primarily due to nicotine in tobacco smoke (Stepney, 1982). Considering the movement of nicotine across the uteroplacental barrier it is important to know the possible effects of this drug on the developing fetus in the prenatal and postnatal periods. A number of authors have studied various facets of maternal and fetal metabolism in this regard. The following is a brief list of some of some of these facets:

(a) fetal coronary artery development interfered with as seen in autopsied human babies (Lehtovirta et al., 1984).

(b) Smaller neonatal lungs in postnatal day 8 of the rat. This situation "catches up" to normal by day 21 (Maritz, 1988).

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(c) retarded fetal growth in human due to impairment of uteroplacental circulation via the vasoconstrictive effect of nicotine (Mochizuki et al., 1984; Bassi et al., 1984).

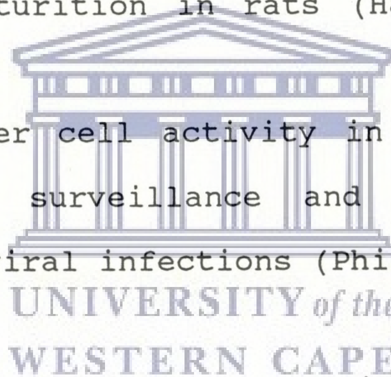
(d) increased alveolar permeability of humans (Jones et al., 1980) and rabbits (Witten et al., 1985).

(e) increased maternal rat lipolysis and increased fetal rat adiposity (Williams and Kanagasabai, 1984).

(f) lower milk production in rat dams interferes with normal offspring development (Hamosh et al., 1979).

(g) reduced embryo growth, delayed implantation and retarded parturition in rats (Hammer and Mitchell, 1979).

(h) natural killer cell activity in the lung decreased giving less surveillance and protection against tumours and viral infections (Phillips et al., 1985).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These are some changes and interferences with the development of the fetus as a result of smoking.

1.11 Effect of smoking and nicotine on lung connective tissue:

Cigarette smoking is probably the single most important etiologic cofactor in the production of human emphysema (Karlinsky and Snider, 1978). Since the architecture, anatomy and functionality of the lung are heavily dependent on the connective tissue status, any factor affecting this status could lead to defective functioning. Also, since maternal smoking during pregnancy results in respiratory disease in the postnatal period, it is

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feasible that changes effected in the fetus during this period predisposes the neonate to respiratory disease (Taylor and Wadsworth, 1987). Earlier studies concentrated mostly on parental smoking and its passive effects on children but a congenital effect is now also proven due to maternal smoking.

(A) Collagen status:

Since the role of connective tissue is crucial to lung mechanics, its quality and quantity in lung tissue has been investigated extensively and related to emphysematous changes. According to Laros and Kuyper (1976) in a review paper, there are no significant differences between the composition of collagen of emphysematous and normal lungs. When looking at the immediate postnatal rat lung, the first week is associated with a rapid growth phase, including alveolarisation. This is also associated with an increased rate of protein synthesis (both collagen and non-collagen proteins). This rate eventually returned to a base level at 21 days of age and remained constant (Garrett, 1978). The rate of *in vitro* collagen synthesis in the newborn rat lung is high and remains so up to about 14 days. Garret (1978) has also shown that when the lung is exposed to cigarette smoke under *in vitro* conditions (1 - 95 days of rat life), collagen and-non-collagen protein synthesis is severely depressed.

For human lung, protein synthesis reaches a peak in the late fetal stage (Garrett, 1978) and then returns to normal levels. According to Pierce *et al*. (1961) the contents of collagen and elastic tissue of adult human lung is not different when normal

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and emphysematous lung is compared. This implies that the quantities of collagen remain the same notwithstanding the effects of emphysema on the lung. Does this mean that a fetal lung (as in the rat), when exposed to nicotine or smoke, would also have depressed protein synthesis, but would eventually "catch up" at some stage?

Kida and Thurlbeck (1980) have done further studies using-β-aminoproprionitrile (BAPN) in rats. This molecule prevents correct cross-linking of the scleroproteins during synthesis of the scleroproteins. They suggest that damage to the fibrous proteins during the first four weeks of life may lead to irreversible damage. This may in turn result in structural and growth alterations in the lung, predisposing it to injury in later life. Since the lesions developed during general emphysema are similar to those when treated with BAPN, it is possible that fetal lung may be affected by maternal smoking resulting in an abnormal connective tissue framework in later life.

It must also be remembered that collagen structure changes with age. More stable cross-links are derived from older animals and this affects factors such as tensile strength, thermal shrinkage, solubility and susceptibility to collagenase digestion (Rickert and Forbes, 1972). These changes are in the main due to the formation of more stable cross-links (Hance and Crystal, 1975; Eyres, 1987; Eyres et al., 1984). The initial cross-links formed have been shown to be relatively unstable (borohydride-reducible) compared to the more mature cross-links as age increases. There

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is also a dose-related loss of lysine as well as a decreased solubility when collagen is exposed to tobacco smoke (Rickert and Forbes, 1972). This could result in premature aging of the lung of smokers and the lungs of the fetus after exposure to nicotine. If the initial synthesis of collagen is interfered with, it may result in the synthesis of relatively unstable cross-links. Since collagen plays an important role in lung mechanics and recoil, these changes could result in loss of lung function.

De Clerck and Jones (1980) have also investigated the role of ascorbic acid (vitamin C) in collagen synthesis in rat smooth muscle cells. They showed that hydroxylation of collagen in the presence of ascorbic acid was essential for the correct percentage of cross-links to be formed. The formation of more soluble collagen occurred in the presence of ascorbic acid. In this context, ascorbic acid could play a role in either preventing or reversing changes of this nature in collagen structure.

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Proposed mechanisms for the lowered synthesis of protein in ,lung exposed to smoke <u>in vitro</u> have been put forward by Garrett (1978):

(a) depressed energy levels in the cell via inhibition of cytochrome oxidase

(b) interference with transcription or translation

(c) a reversible process depending on the removal of the water-soluble toxic component on washing.

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An additional factor which can cause emphysematous changes in immediate postnatal rat lung is nutritional status (Kerr et al., 1985; Kalenga and Eeckhout, 1989). When the diet is severely protein-restricted the lungs show characteristic air-space enlargement and alveolar wall disruption (Kerr et al., 1985). The latter authors have determined this using adult rats while Kalenga and Eeckhout (1989) have used rats from postnatal days 8 to 49. The general findings by these authors indicates the following:

(a) Protein deprivation affects the elastic tissue concentration in lung, thereby affecting lung elasticity

(b) Collagen undergoes less severe changes

(c) Lung size was decreased

(d) No nett loss of structural protein occurs.

Kalenga and Eeckhout (1989) suggest that the different effects with respect to collagen and elastic tissue is most probably due to different mechanisms for the modulation of these fibrous proteins. Considering the effects of smoking on dietary intake, a side-effect leading to emphysematous changes may be mediated through this mechanism.

In their paper on the perinatal effects of nicotine on rat metabolism, Nasrat et al. (1986) suggests that the adverse effect of maternal nicotine administration on the fetus is possibly due to poor maternal nutrition and appetite. In addition, they also suggest unspecified direct effects of nicotine on metabolism. Hamosh et al. (1979) also show that less milk is available to rat

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pups born during maternal nicotine administration. In addition, studies using human placenta shows that nicotine also suppresses amino acid transfer across the uteroplacental barrier (Barnwell and Sastry et al., 1980).

Much emphasis in research into smoking, emphysema and its relation to lung development was placed on whole smoke or condensates (Cope et al., 1975; Low, 1974; Laurent et al., 1983), while later studies tended to concentrate on nicotine and its effects (Nasrat et al., 1986; Hamosh et al., 1979; Clarke, 1987). This water soluble molecule is implicated in a number of lung changes, especially with respect to the connective tissue of lung Maritz and Woolward, 1992). Maternal smoking slowed prenatal lung growth (Collins et al., 1985) after being absorbed across the uteroplacental barrier (Greenberg et al., 1984). This is aggravated by the fact that nicotine tends to accumulate in the respiratory tract after absorption (Szuts et al., 1978).

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(B) Elastic tissue status:

Much more information has been obtained regarding this connective tissue component and its changes during emphysema than for collagen. As stated previously, lung protein synthesis is depressed by tobacco smoke during in vitro exposure (Garrett, 1978) by unknown mechanisms during the first 95 days of life. Since nicotine interferes with lung growth (Maritz, 1988; Collins et al., 1985) it is probable that this is the active component which interferes with protein synthesis. Since emphysematous changes are associated with lower alveolarisation, the effects

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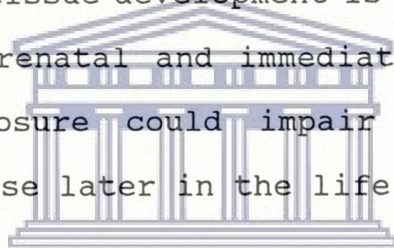
of this alkaloid on the interstitial fibroblast could be involved in these changes (Brody and Vaccaro, 1979).

Maritz and Woolward (1992) used light microscopic techniques to determine the effect of maternal nicotine exposure on the elastic tissue content of neonatal lung. They showed that the control rat pup lung had normal elastic tissue development in the alveoli and also the vasculature of the lung. The nicotine exposed rat pup at days 1 and 7 had virtually no elastic tissue in the alveolar portion but normal development in the vasculature. This was also accompanied by a high degree of air saccule collapse. Since elastic (and collagen) tissue development is essential for airway branching during the prenatal and immediate postnatal period, maternal nicotine exposure could impair lung function and susceptibility to disease later in the life of the offspring.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For rat lung, the most important phase for synthesis of elastic tissue is from postnatal days 4 to 20 (Powell and Whitney, 1980). As stated before, decreased elastic tissue synthesis during this period leads to loss of lung elasticity (Kalenga and Eeckhout, 1989). The role of cigarette smoke and also nicotine in the pathogenesis of emphysema has been widely investigated, especially its effects on elastic tissues (Auerbach et al., 1974; Chen et al., 1991; Huber, 1989; Idell and Garcia, 1989).

To explain the effects of smoking (or cigarette smoke) on the development of elastic tissue, a number of theories and studies have been proposed. Many of these postulates centre on one of two

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basic mechanisms resulting in the decreased level of elastic tissue in lungs of emphysematous patients:

(a) Lowered synthesis and deposition of this tissue

(b) Increased degradation of this tissue in lung.

For the early development phase of the lung, the lowered elastic tissue content is related to a lowered protein synthetic ability as proposed by Garrett (1978) who used an in vitro model. The findings of Maritz and Woolward (1992) are based on an in vivo model using the fetuses of dams exposed to nicotine during pregnancy. Their observations are based solely on histological evidence and conclusive biochemical quantification is necessary. No exact mechanism for this occurrence is given except that it is not likely related to an antiprotease deficiency, since the latter is unaffected by nicotine (Janoff and Carp, 1977).

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In a further study, Maritz et al. (1993) showed that maternal nicotine administration leads to impaired lung structure in the neonate. The lowered radial alveolar counts for the nicotine exposed group indicates a link between the lower elastic tissue content and formation of fewer alveoli. The controls show an even spread of elastic tissue with accumulations at the mouth of alveoli. These authors are of the opinion that differences in content and distribution of elastic tissue in nicotine samples leads to incorrect and inefficient alveolar formation.

By far the strongest evidence for a causative factor in emphysema is attributed to the protease-antiprotease hypothesis which was

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first described by Laurell and Eriksson (1963). This theory ascribes the destruction of elastic tissue (elastolysis) and the loss of air sacs to the development of emphysematous lesions. The increase in elastolysis is not necessarily due to an increased level of elastase (or other proteases) but rather a lowered level of protease inhibitors, in particular α-1-antitrypsin (Idell and Garcia, 1989).

A number of studies have been undertaken which have shown that proteolytic enzymes such as papain, when administered intratracheally or as an aerosol, caused destruction of elastic tissue to such an extent that emphysematous lesions appeared (Colombo and Steinetz, 1975). Johanson et al (1973), using a papain aerosol, showed that this protease leads to emphysematous lesions with loss of the elastin amorphous component but not the associated microfibrils. In this study collagen appeared normal. Others have shown the effect of smoking and nicotine on the levels of elastases, anti-proteases and consequently the development of emphysema (Idell and Garcia, 1989). Human subjects were used by Olsen et al. (1975) to show that there is no difference in serum AAT (α-1-antitrypsin) between smokers and non-smokers while lung lavage and alveolar macrophage levels of AAT in smokers showed much higher levels. These increased levels of AAT in the lung lavage is proposed to be due to the increased recruitment of macrophages and neutrophils to the lungs of smokers (Janoff et al., 1983).

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In a further in vitro study, Laurent et al. (1983) showed that a water-soluble component of the gas phase of cigarette smoke blocks elastic tissue repair mechanisms. This of course would mean a weakened repair mechanism for overcoming the simultaneous recruitment of elastase-secreting cells and functional capacity to inhibit elastase. An in vivo study, using hamsters, shows evidence for the suppression of elastic tissue cross-link formation (Osman et al., 1985).

1.12 Perspectives on smoking and emphysema:

Besides nicotine as an important and addictive component of cigarette smoke, there are many other components which are also potentially deleterious in nature. The effects of these many different components eventually result in one of a number of conditions, including pulmonary emphysema. In addition to this, the smoker is also potentially more susceptible to pulmonary aberrations later in life. Thus far, no one has yet elucidated that single initial reaction (or the exact chemical involved) which leads to the multifaceted conditions consistent with those seen in the habitual smoker. According to recent estimates, approximately 15% of smokers develop clinically significant emphysema or other chronic obstructive pulmonary diseases (Huber, 1989).

All research up to now has lead to formulation of a triad of biochemical effects due to smoking (Huber, 1989):

(A) The generation of chemotactic agents leading to inflammatory cell recruitment to the lung. An

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important cell in this regard is the polymorphonuclear leucocyte which, because of its high levels of elastase and other proteases, increases the proteolytic burden of the pulmonary parenchymal tissue.

(B) The inhibition of antiproteases. This can occur via free radicals in smoke as well as those free radicals produced by alveolar macrophages. The latter cells also accumulate in smaller airways under the effect of nicotine, lending strength to this line of reasoning.

(C) Suppression of repair mechanisms by tobacco smoke. The decreased activity of lysyl oxidase is associated with this loss of ability to repair damaged lung tissue.

This triad hypothesis has possibly caused a block in thinking and research into the exact causes of, and initial reaction causing emphysema (Huber, 1989). The present study to be undertaken will centre on the effect of nicotine intake of the gestating rat on the fetal and neonatal rat lung tissue. It is suspected that one or more of the above elements may play a role in perinatal rat lung metabolism and structure.

A final factor in the puzzle regarding the role of nicotine (and cigarette smoke by implication) in the pathogenesis of pulmonary emphysema is the effect of oxidising free radicals. Free radicals are known to be tumorigenic. The carcinogenic effect implies transformation of the genetic material and/or modulation of gene expression (Church and Pryor, 1985). The latter effect could have

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far-reaching consequences on overall lung structure and stability. Free radicals are also known to participate in lipid oxidation, including the lipid component of cell membranes (Church and Pryor, 1985). Any tissue damage is initiated by damage to single cells, such as free radical peroxidation of lipids. This line of thought is also used by Maritz et al. (1993) with respect to lung tissue damage due to the effect of maternal nicotine administration.

1.13 Motivation for this study:

Based on a survey of the research carried out over the years, it is obvious that much emphasis has been placed on the effects of smoking or whole cigarette smoke on both adult and fetal lung tissue. Many researchers have also utilised in vitro techniques which in itself introduces many discrepancies regarding its relation to actual smoking and exposure of pulmonary cells to nicotine and other smoke components via the blood. Since nicotine is supposedly the central molecule implicated in lung aberrations, it was decided to investigate the effect of this molecule on early neonatal lung structure and development, and attempt to link this with the later onset or susceptibility to respiratory disturbances.

The study to be undertaken will focus on the following aspects of lung structure and development:

(A) Light microscopic confirmation of neonatal lung morphological changes as a function of maternal nicotine exposure.

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(B) Light microscopic investigation of changes in neonatal lung connective tissue (collagen, elastic tissue and reticulin) as a function of maternal nicotine exposure.

(C) Biochemical quantitation of the soluble protein and insoluble protein (connective tissue) as a function of maternal nicotine exposure.

(D) Electron microscopic examination of the connective tissue architecture of the postnatal lung and the effect of maternal nicotine exposure.

The aim of the study is to gather evidence for the formulation of a hypothesis to explain the susceptibility of the adult lung to emphysema, and other respiratory indispositions, due to exposure to nicotine via the placenta (fetal phase) and via the lactating mothers' milk (during the neonatal phase). Most of the evidence to date deals with abnormalities of the respiratory system due to smoking or other pollutants. Much has been explained regarding the effects and very little regarding the cause of these abnormalities. Since the exact causative mechanisms which initiate emphysema and other similar respiratory conditions is not known, it is also hoped that more light would be shed on these processes. Ultimately, this knowledge gained may possibly be used to develop a regimen to either prevent, alleviate or reverse the effects of maternal nicotine exposure on the developing fetus.

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CHAPTER 2

THE EFFECT OF MATERNAL NICOTINE EXPOSURE ON NEONATAL RAT LUNG MORPHOLOGY AND CONNECTIVE TISSUE STATUS

2.1 Introduction:

For the lung to function efficiently, it must have undergone all the normal phases of development described in the previous chapter. Once this has been accomplished without interruption and interference, the correct structure and structural relationships would have been established. This includes the required quantities of connective tissues as well as the spatial and chemical integrity of elastic tissue and collagen. From the previous discussion it is also evident that connective tissue development, its quantity and also its quality play an important role in dictating alveolar space development and septal branching. The correct prenatal and neonatal development also dictates to a large extent the quality of lung function and susceptibility to disease in later life.

A number of authors have used light microscopic methods to study the changes occurring with respect to these tissues (Maritz and Woolward, 1993; Maritz and Woolward, 1992; Laros and Kuyper, 1976; Laros, 1972). The following section will attempt to confirm the result obtained regarding the histochemical studies of the connective tissues and will also be used to comment on the structural integrity of the lungs of the different samples used.

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2.2 Materials and Methods:

2.2.1 Mayer's Haematoxylin and Eosin Stain. (From Bancroft and Stevens, 1990)

a) Principle

Haematoxylin is oxidised to the natural dye haematin by sodium iodate. To increase its affinity for tissue, a mordant in the form of aluminium salt is added during the preparation of the stain. This stain (haemalin) combines with the acidic nuclei by means of covalent bonding and van der Waal's forces, the latter being the most important due to the molecular size of the mordant-dye complex. Staining is allowed to take place progressively, whereafter the material is counterstained with eosin to stain the cytoplasm. Blueing in Scott's water serves to enhance the staining of the nuclei.

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b) Reagents

1) Absolute alcohol (absolute ethanol). This can be diluted to appropriate concentrations using distilled water.

2) Xylene: use as supplied by the manufacturer.

3) DPX: use as described by the manufacturer.

4) Buffered neutral formaldehyde: Dissolve 4 grams sodium dihydrogen phosphate (NaH_2PO_4) and 6,5 grams disodium

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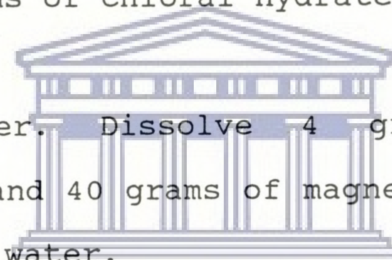
hydrogen phosphate (Na_2HPO_4) in a solution containing 100mℓ 40% formaldehyde and 800mℓ distilled water. Make this solution up to a final volume of 1ℓ in a volumetric flask.

5) Eosin. Dissolve 5,0 grams in 1ℓ of distilled water.

6) Mayer's haemalum. Dissolve 1 gram of haematoxylin in 1ℓ of distilled water. Add 50 grams of potassium ammonium alum and shake to dissolve fully. Then add 0,2 grams of sodium iodate, 1,0 gram of citric acid and 50,0 grams of chloral hydrate.



7) Scott's water. Dissolve 4 grams of potassium bicarbonate and 40 grams of magnesium sulphate in 2ℓ of distilled water.

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c) **Procedure**

1) Fix the sections to the glass slide by placing in a hot air oven at 80°C for 20 minutes.

2) Dewax in xylol for 2 minutes, then hydrate by placing the sections for 30 seconds in each of 100%, 80% and 70% alcohol, in that order.

3) Fully hydrate and remove all traces of alcohol by placing the sections in running tap water for 1 minute.

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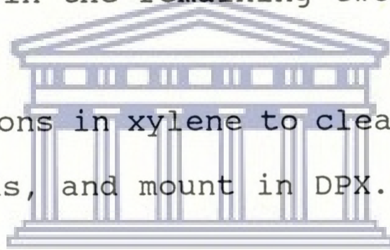
4) Stain in Haemalin for 10 minutes.

5) Blue in Scott's water for 1 minute and wash in tap water for 30 seconds.

6) Stain in Eosin for 1 minute.

7) Wash in tap water for 30 seconds.

8) Dehydrate through graded alcohols of 70%, 80% and 100% respectively for 10 seconds in the first and 30 seconds each in the remaining two alcohols.



9) Dip the sections in xylene to clear, using two changes for 30 seconds, and mount in DPX.

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d) Interpretation of Results

The nuclei of the cells stain blue whilst the cytoplasm stains red to pink. The red blood cells appear yellow to red in colour.

2.2.2 Verhoef's Elastic Fibre Stain: (from Bancroft and Stevens, 1990)

a) Principle:

When staining for elastic fibres, cognisance must be taken of the presence of collagen fibres in the tissue. After staining with Verhoef's reagent, all the tissue is stained black. Ferric

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chloride is used to differentiate by removing excess stain from the tissue, only leaving the elastic fibres black against a pale background. To further differentiate between elastic and collagen fibres, Van Gieson's reagent, an anionic dye, is used to stain the collagen red. The binding of stain to elastic fibres is due to hydrogen bonding, as evidenced by poor binding in the presence of a reducing agent such as urea (Goldstein, 1962).

b) <u>Reagents</u>:

(1) Absolute alcohol (absolute ethanol). As in section 2.2.1.

(2) Xylene: as in section 2.2.1.

(3) DPX: use as described by the manufacturer.

(4) Buffered neutral formaldehyde: As in section 2.2.1.

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(5) Aqueous 2% (or 10%) ferric chloride ($FeCl_3$): dissolve 2 grams (or 10 grams) $FeCl_3$ in 100mℓ distilled water.

(6) Alcoholic haematoxylin: dissolve 5 grams haematoxylin in 100mℓ absolute alcohol.

(7) Lugol's iodine: dissolve 1 gram of iodine and 2 grams of potassium iodide in 100mℓ distilled water.

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(8) Saturated picric acid: approximately 2% picric acid dissolved in distilled water.

(9) Van Gieson's stain: Add 10mℓ of aqueous 1% acid fuchsin to 100mℓ of saturated picric acid.

(10) Verhoef's stain: Add 5mℓ of a 5% alcoholic haematoxylin solution to 5mℓ absolute alcohol. Add 4mℓ of 10% $FeCl_3$ to 4mℓ Lugol's iodine. Mix these two solutions.

c) **Procedure:**

(1) Fix, dewax and hydrate as in section 2.2.1.

(2) Stain with Verhoef's stain for 20 minutes.

(3) Differentiate in 2% $FeCl_3$ for 1 minute and check whether elastic fibres can be observed against a pale background.

(4) Rinse with distilled water and differentiate further if necessary.

(5) Wash in running tap water for 5 minutes.

(6) Dip in alcohol for 5 minutes to remove all traces of iodine.

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(7) Rinse in tap water for 5 minutes.

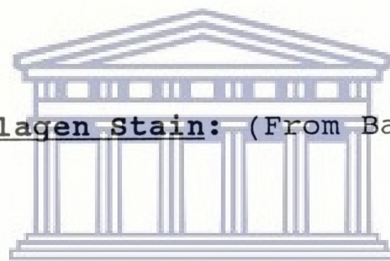
(8) Counterstain with Van Gieson's stain for 5 minutes.

(9) Wash in tap water for 1 minute.

(10) Dehydrate, clear and mount as in section 2.2.1.

d) Interpretation of results:

The cytoplasm and muscle tissues stain pale yellow (forming the background) while collagen contrasts red against the black elastic fibres.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2.2.3 Van Gieson's Collagen Stain: (From Bancroft and Stevens, 1990)

a) Principle:

In this procedure, iron haematoxylin is used in place of haemalum because the latter is removed by picric acid. Initial staining of the nuclei takes place with iron haematoxylin, after which differentiation with acid alcohol removes the stain. Because collagen fibres stain strongly with acid fuchsin/picric acid, this stain is used after the differentiation step.

b) Reagents:

(1) Alcohol, as described under section 2.2.1.

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(2) Acid alcohol: Add 10mℓ concentrated hydrochloric acid (HCl) to 990mℓ 70% ethanol.

(3) Van Gieson's stain: Add 10mℓ of 1% aqueous acid fuchsin to 100mℓ saturated picric acid in distilled water.

(5) Weigert's iron haematoxylin: Dissolve 1 gram of haematoxylin in 100mℓ absolute alcohol (solution A). Add 4mℓ 10% aqueous $FeCl_3$ and 1mℓ concentrated HCl to 100mℓ distilled water (solution B). Mix equal parts of solution A and B immediately prior to use.

c) **Procedure:**

(1) Fix, dewax and hydrate as described under section 2.2.1.

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(2) Stain the slides in an appropriate rack with Weigert's iron haematoxylin for 40 minutes.

(3) Wash in tap water for 30 seconds.

(4) Differentiate in acid alcohol for 1 minute.

(5) Wash in tap water for 30 seconds.

(6) Place in Van Gieson's stain for 3 minutes.

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(7) Wash in tap water for 30 seconds.

(8) Dehydrate, clear and mount as described under section 2.2.1.

d) Interpretation of results:

Collagen stains a bright red against blue-black nuclei and yellow cytoplasm of muscles and red corpuscles.

2.2.4 Gomori's Reticular Fibre Stain: (From Bancroft and Stevens, 1990)

a) Principle:

This metal impregnation technique makes use of silver in a form which is readily able to precipitate as metallic silver. Reticulin fibres have little natural affinity for silver solutions, thus a reducing agent such as formalin is required to produce sensitised sites on the fibres where silver deposition can be initiated. Excess silver in the tissue is removed by treatment with a sodium thiosulphate solution. Toning with a gold salt solution renders the preparation permanent and produces a neutral black colour of high intensity.

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b) Reagents

1) Formalin. A 10% aqueous solution is prepared by adding 300 mℓ of distilled water to 100 mℓ of a 40% formaldehyde solution.

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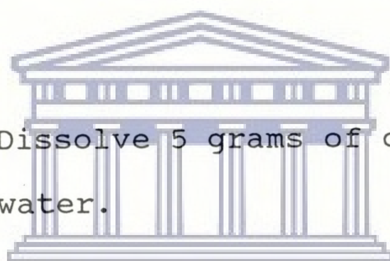
2) Gold chloride. Prepare a 0,2% solution by dissolving 200 mg of gold chloride in 100 mℓ of distilled water.

3) Hypo. Dissolve 5 grams of sodium thiosulphate in 100mℓ of distilled water to prepare a 5% solution.

4) Mordant. Dissolve 2 grams of ferric iron alum in 100mℓ of distilled water. Filter through Whatman No. 1 filter paper before use.

5) Neutral red. Dissolve 1 gram in 100 mℓ of distilled water.



6) Oxalic acid. Dissolve 5 grams of oxalic acid in 100mℓ of distilled water.

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7) Potassium permanganate. Dissolve 0,5 grams of potassium permanganate in 100 mℓ of distilled water.

8) Wilder's silver nitrate solution. Add 10mℓ of a 10% silver nitrate solution to 2mℓ of a 10% potassium hydroxide solution in a 100mℓ measuring cylinder. Allow the precipitate to settle for a few minutes and then pour off the supernatant until just before the precipitate comes out. Wash the precipitate three times with distilled water and pour off in the same fashion, except for the last rinse where at least 10mℓ of the mixture should be retained. Add concentrated

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ammonia solution dropwise until the precipitate has dissolved. Make up to 100mℓ with distilled water.

c) **Procedure**

1) Fix, dewax and hydrate as described under section 2.2.1.

2) Oxidise by placing in potassium permanganate for 5 minutes.

3) Wash well in distilled water for 2 minutes.

4) Bleach until white by placing the slides in oxalic acid for 3 minutes.

5) Wash thrice in distilled water, 1 minute per wash.

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6) Flood the slides with mordant and allow to react for 2 minutes.

7) Wash 3 times with distilled water, 1 minute per wash.

8) Impregnate by placing the slides in Wilder's silver nitrate solution for 3 minutes.

9) Rinse with distilled water for 1 minute.

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10) Reduce by placing the sections in formalin for 3 minutes.

11) Rinse with distilled water for 1 minute.

12) Tone by placing in gold chloride for 3 to 5 minutes.

13) Rinse in distilled water for 1 minute.

14) Rinse with hypo for 1 minute.

15) Rinse well in distilled water for 3 minutes.

16) Stain with neutral red for 40 seconds.

17) Rinse with distilled water for 1 minute.

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18) Dehydrate, clear and mount sections as described under section 2.2.1.

c) **Interpretation of Results**

The reticulin fibres stain black, collagen fibres red and the nuclei of the cells various shades of grey.

2.3 Experimental animals:

All subsequent experiments and treatments were approved by the Ethics Sub-committee of the Research Committee of the University of the Western Cape. The animals used in this study was white

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Wistar rats from the colony maintained at the Department of Physiological Sciences, University of the Western Cape. Animals were routinely examined for any obvious signs of ill-health before use in the experimental procedures. A standard diet of Epol rat cubes were fed as required. Water bottles filled with tap water were recharged on a daily basis. Cages and animal room conditions were maintained in a suitable state of cleanliness to ensure good animal hygiene.

Simulation of an alternating 12 hour day and night cycle was obtained using a timer switch. This prevents any experimental variation which may be due to circadian rhythm disturbances. Room temperature was regulated at a constant 25°C by means of an air conditioner.

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Litters for study were obtained by allowing mating to take place overnight (12 hours) and removing the sires on the following morning. Females were then randomly assigned to control or experimental groups without replacement (Matthews and Farewell, 1988; Daniel, 1984). The appearance of a mating plug or the presence of sperm in a vaginal smear was used to indicate whether mating had occurred. The day on which this occurred was designated day 0 of gestation. Rats were then weighed on a daily basis for the next 7 days. A significant weight increase indicated successful blastocyte implantation. Animals were then placed in separate cages from day 7 to the end of gestation.

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Once separated, appropriate treatment of the respective control and experimental groups was started. The nicotine-exposed group, hereinafter referred to as the experimental group, received subcutaneous injections of 1mg nicotine/kg body mass/day (diluted in sterile saline) between 09H00 and 10H00. According to Armitage et al. (1974), this is equivalent to the amount of nicotine inhaled by an average 70kg adult who smoked 32 cigarettes per day. Control animals were administered a placebo consisting of sterile saline (0,9% NaCl) using a volume of 1mℓ/kg body mass. After parturition, the dams received further administration of nicotine (or placebo) via intraperitoneal injection to ensure that the nicotine first entered the blood before appearing in the milk during suckling.

All injections were done with 23 gauge 1,0mℓ disposable plastic syringes. To prevent or minimise infection, only gamma-irradiated syringes were used. During injection and handling of the rats, care was taken not to inflict pain or to unduly excite the animals. The day of birth of the pups was designated day 0. At least three litters from each age group was used for this study and at least three pups randomly selected from these litters. For this study, pups were sampled on days 7, 14 and 21 after birth.

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2.4 Lung tissue excision and preparation:

Rat pups of the different age groups were sacrificed by decapitation and the lungs were rapidly removed from the thorax. Handling was minimised to prevent tissue damage. Samples of the inferior lobes of the lung were fixed in buffered formalin for

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24 hours, after which it was washed in running tap water for 30 minutes. Each sample was then placed in a suitably labelled plastic cassette. Dehydration and wax impregnation was done on a Shandon tissue processor using the following program:

1.	70% ethanol	1 hour
2.	90% ethanol	30 minutes
3.	95% ethanol	30 minutes
4.	100% ethanol	1 hour
5.	100% ethanol	1 hour
6.	Xylene	1 hour
7.	Wax bath	1 hour
8.	Wax bath	1 hour

On the same day the tissue was embedded in paraplast wax using a Tissue-Tek embedding system. These embedded samples were then refrigerated at 4°C for a minimum of 3 hours before cutting sections. Using a rotary microtome, thick sections of 10μm were first cut to get down to the correct tissue plane. After this, 5μm sections were cut in ribbons of three to four sections. Ribbons were floated out in a waterbath at 45°C and transferred to clean microscope slides and finally fixed overnight at 37°C in an incubator. The slides were then stored in appropriate slide boxes for later staining while the cassettes were stored under cool, dry conditions for later use. Prior to staining, slides were labelled on the frosted end with a graphite pencil and then fixed in a hot-air oven at 80°C for 20 minutes.

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2.5 Photography and printing:

All colour photography was done on a Polyvar microscope with camera attachment and automatic exposure setting. Fujifilm HG400 36 exposure film was used. Developing and printing of photographs was done by Media Graphics at Groote Schuur Hospital, University of Cape Town.

2.6 Results:

2.6.1 Morphological Status:

Haematoxylin and eosin sections of 7 day old control rat lung tissue (Figure 2.1) shows alveoli with a smooth-walled structure. Numerous well-defined buds destined to form new alveoli are also noted. The corresponding experimental lung tissue at day 7 (Figure 2.2) shows poorly developed alveoli which seem flattened or deformed. The alveolar buds in this case are also poorly developed. The alveolar septa seem thicker with more cells, but no conclusive statement regarding this can be made using the evidence available.

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At day 21 the control rat lung tissue (Figure 2.3) shows thin, complete interalveolar septa and alveolar ridges. In the case of the experimental animal (Figure 2.4) at day 21 the interalveolar septa appear incomplete or broken at different places. This lung damage is also seen in the alveolar ridges.

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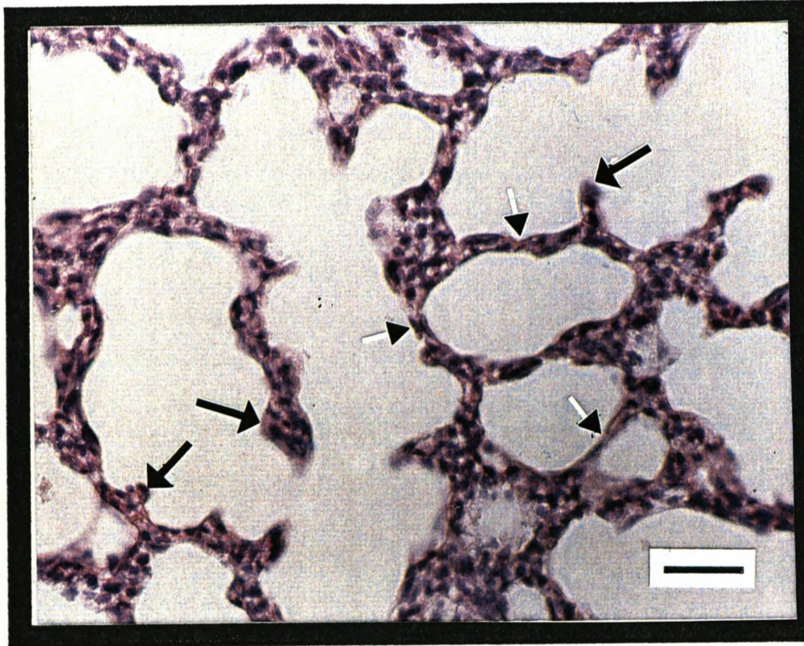

Figure 2.1: H and E stain of 7 day old control rat lung. Black arrows = alveolar buds; White-tailed arrows = thin, smooth alveolar walls. Bar = 30μm.

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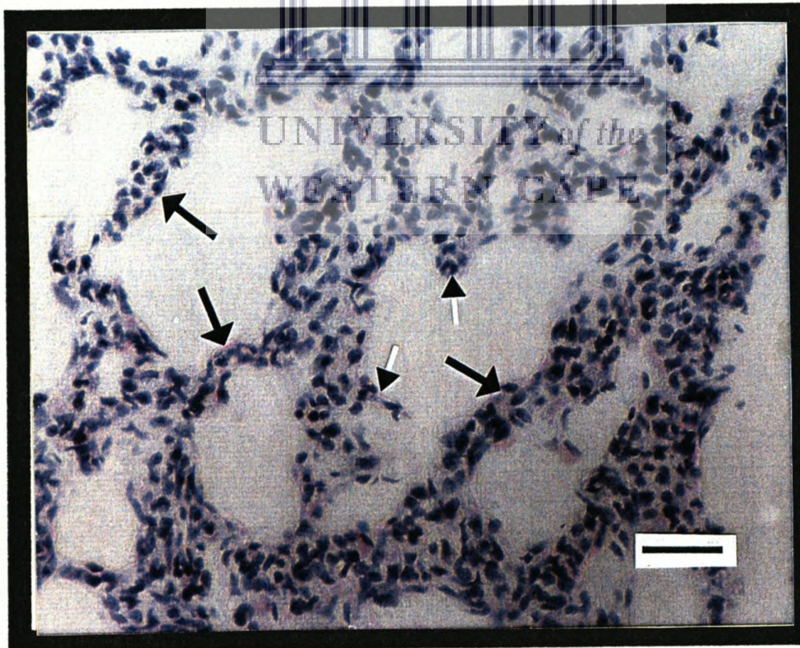

Figure 2.2: H and E stain of 7 day old experimental rat lung. Black arrows = alveolar buds; white-tailed arrows = thick, uneven alveolar walls. Bar = 30μm.

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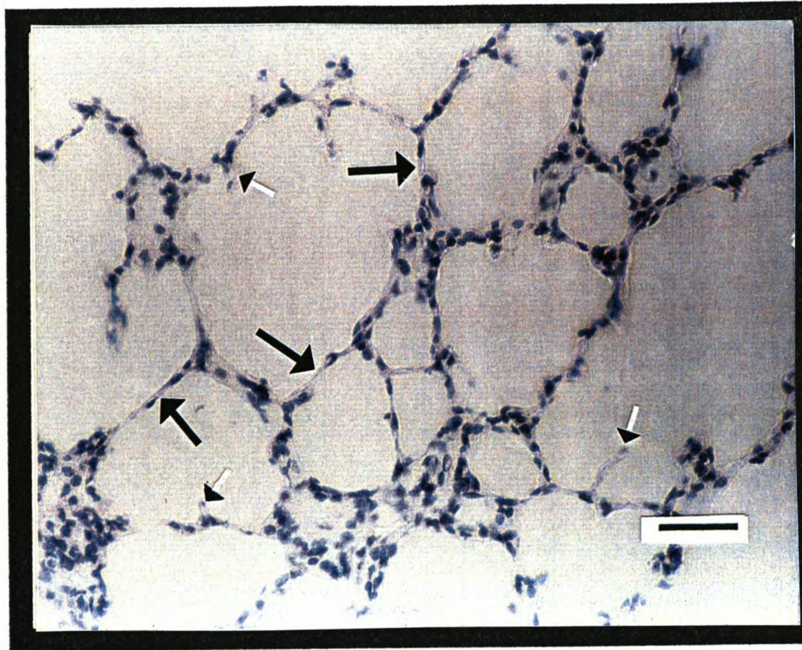

Figure 2.3: H and E stain of 21 day old control rat lung. Black arrows = thin, undamaged alveolar walls; White-tailed arrows = undamaged alveolar ridges. Bar = 30μm.

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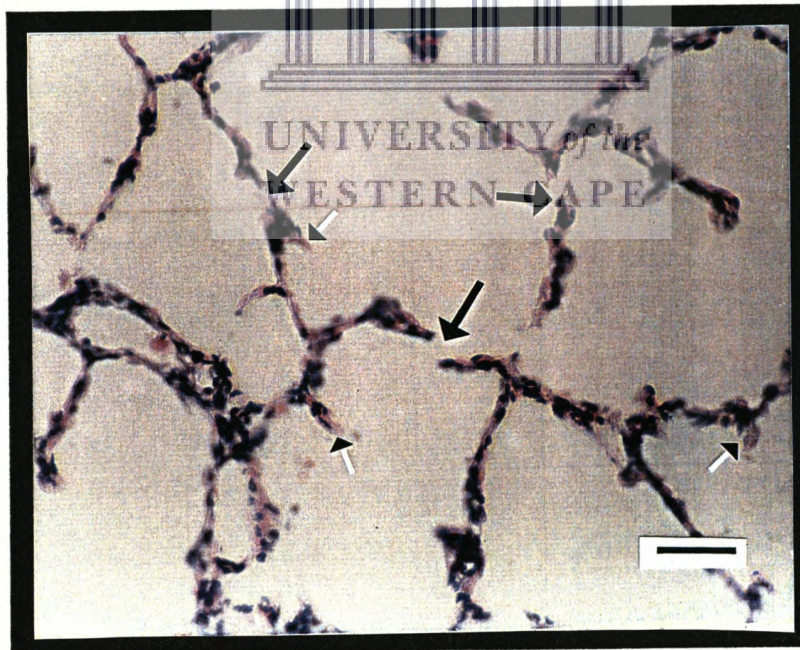

Figure 2.4: H and E stain of 21 day old experimental rat lung. Black arrows = torn alveolar walls; White-tailed arrows = damaged alveolar ridges. Bar = 30μm.

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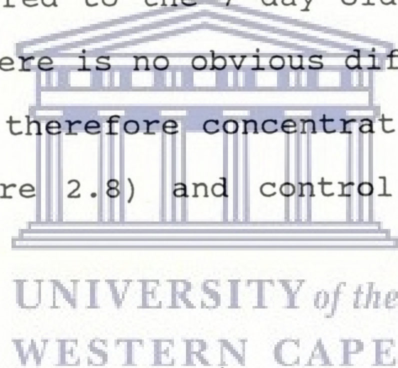
2.6.2 Collagen Status:

The lung of 7 day old control (Figure 2.5) and experimental (Figure 2.6) animals show traces of collagen in the already-formed alveolar septa at the lung periphery. It is not possible to see any quantitative differences between the control and experimental lungs.

The lungs of 14 day old control animals (Figure 2.7) stain more strongly for collagen than the 7 day old control lungs (Figure 2.5), implying a higher collagen concentration. Experimental rat lung at 14 days (Figure 2.8) also shows this increase in collagen staining capacity compared to the 7 day old experimental lung tissue (Figure 2.6). There is no obvious difference in collagen staining capacity (and therefore concentration) between 14 day old experimental (Figure 2.8) and control (Figure 2.7) lung tissue.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There is no visible difference in collagen content between control (Figure 2.9) and experimental (Figure 2.10) 21 day old animals. Besides morphological differences at this stage (described previously), one other marked difference between the two 21 day old samples becomes apparent at higher magnifications. 21 day old control lung (Figure 2.9) shows an area where the collagen in the alveolar walls is evenly distributed. In experimental animals (Figure 2.10) the collagen of the alveolar walls sometimes appears unevenly distributed compared to the control lung collagen (Figure 2.9). The molecular nature of these

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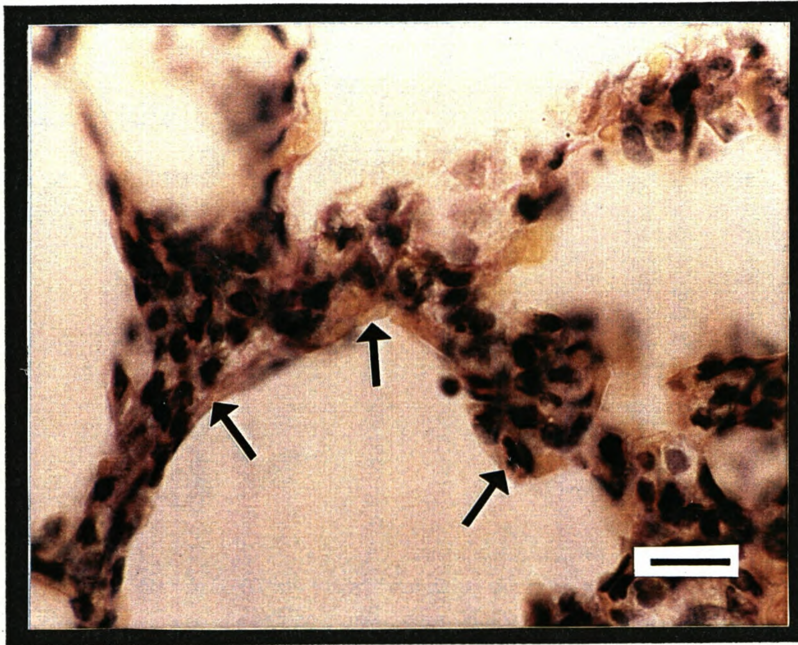

Figure 2.5: Van Giesons stain of 7 day old control rat lung. Arrows indicate red collagen in alveolar septa. Bar = 12μm.

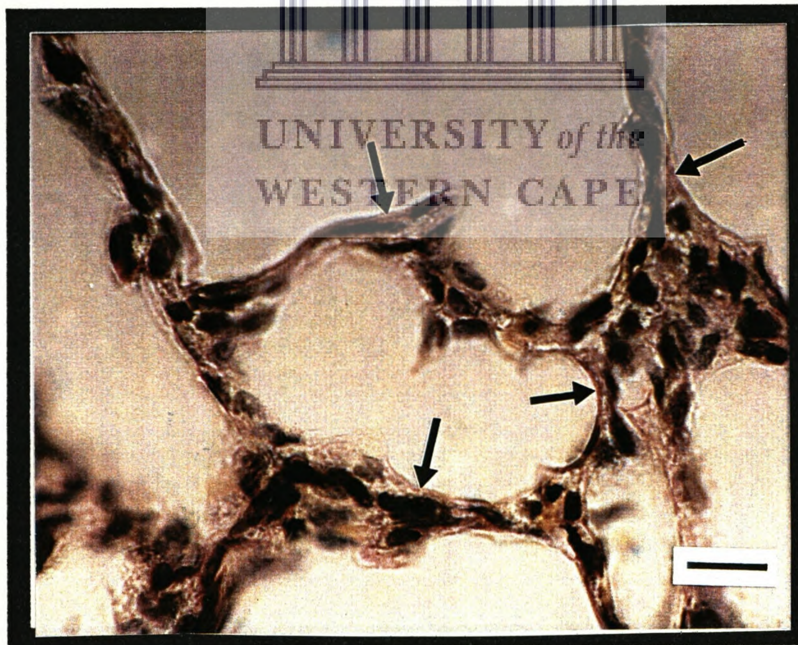

Figure 2.6: Van Giesons stain of 7 day old experimental rat lung. Arrows indicate red collagen in alveolar septa. Bar = 12μm.

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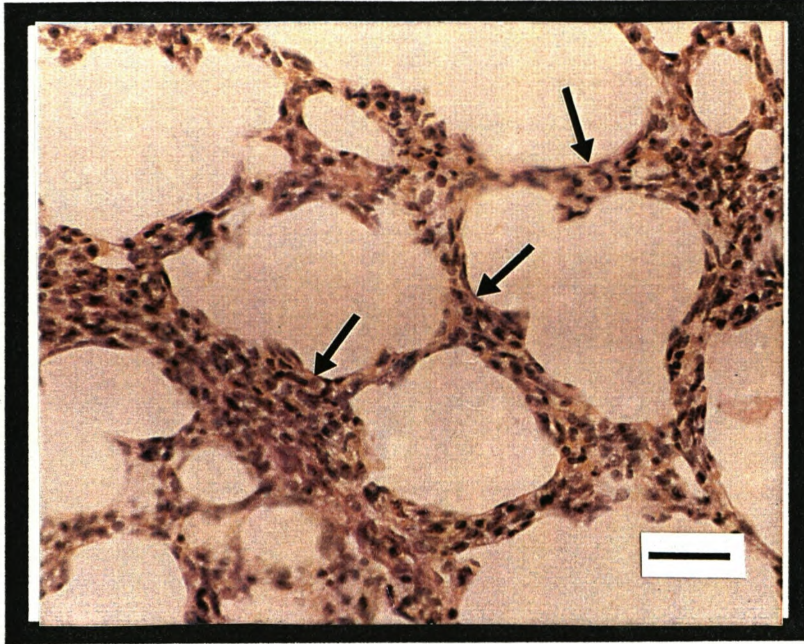

Figure 2.7: Van Giesons stain of 14 day old control rat lung. Arrows indicate red collagen in alveolar walls. Bar = 30μm.

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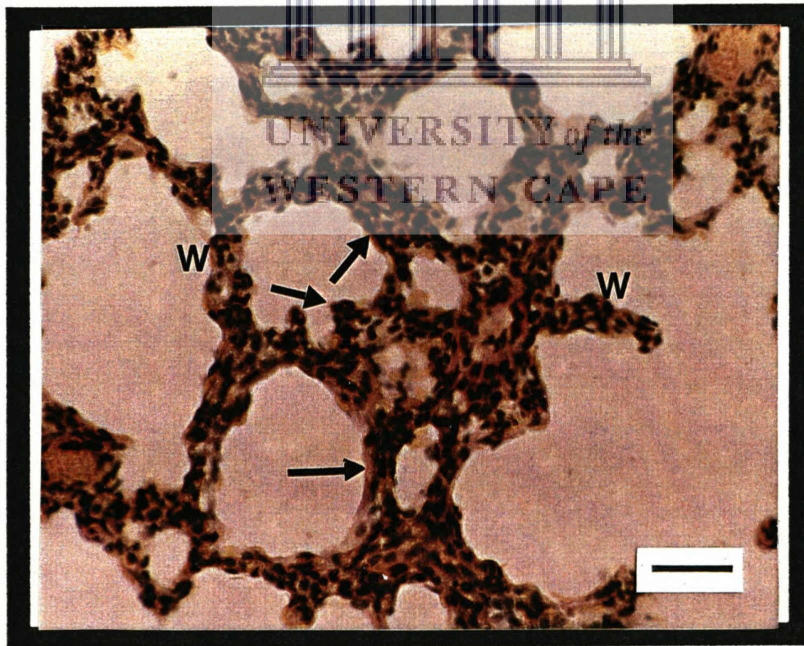

Figure 2.8: Van Giesons stain of 7 day old experimental rat lung. Arrows indicate red collagen in alveolar walls; W = unevenly thickened alveolar walls. Bar = 12μm.

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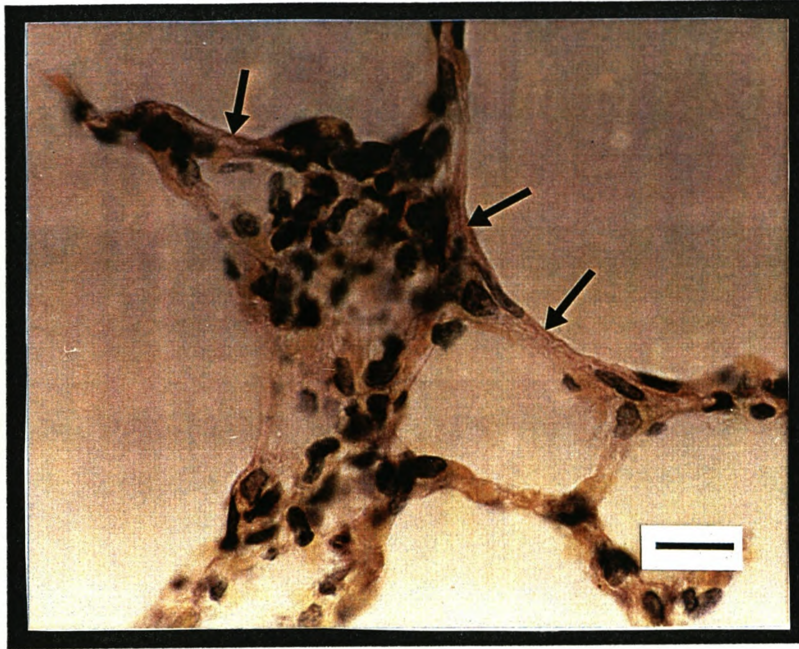

Figure 2.9: Van Giesons stain of 21 day old control rat lung. Arrows indicate red collagen in alveolar walls. Bar = 12μm.

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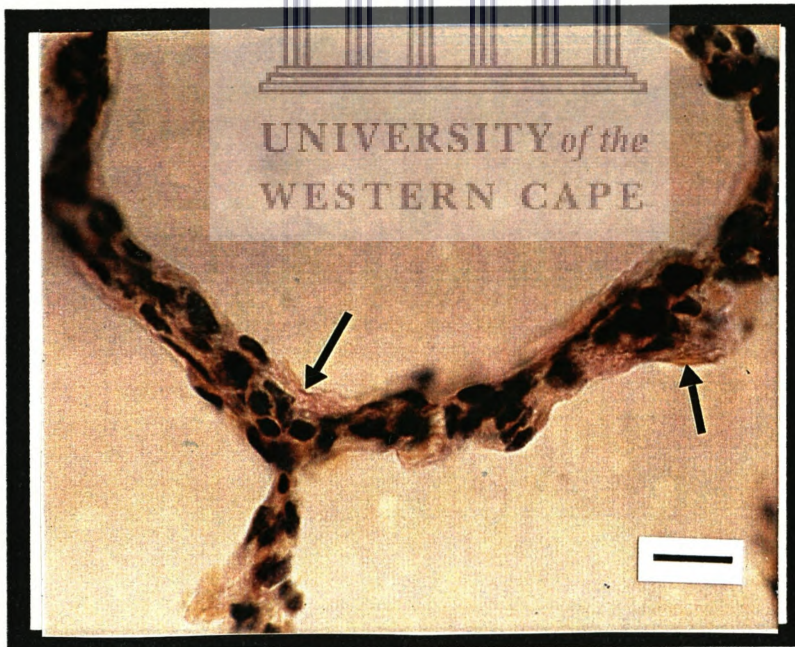

Figure 2.10: Van Giesons stain of 21 day old experimental rat lung. Arrows indicate red collagen fibres in alveolar walls. Bar = 12μm.

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differences is not apparent at this level. Again there is no indication of quantitative differences as regards collagen but more probably a difference in quality and three-dimensional structure.

2.6.3 Elastic Tissue Status:

At day 7 the experimental rat lung (Figure 2.11) shows very little staining for elastic tissue in the septal regions. The control animals of the same age group (Figure 2.12) showed abundant elastic fibres in the septal regions of air-tissue interfaces as well as in vascular elements.

In 14 day old control (Figure 2.13) and experimental animals (Figure 2.14) no appreciable differences in the histochemical quantity of elastic tissue is noted. Concentrations of elastic tissue is evident for these animals at the mouth of the alveoli i.e. at the tips of the septal buds. A similar situation holds for 21 day old animals as demonstrated by the 21 day old control rat lung (Figure 2.15) and experimental rat lung (Figure 2.16).

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2.6.4 Reticular fibre status:

Seven day old control rat lung (Figure 2.17) shows reticular fibres presenting strongly on the luminal side of the alveolar septum. The fibres are well-defined, complete and show a slightly wavy appearance. The amplitude of the wave pattern formed is small and in some cases hardly evident. Those fibres lining the alveolar septum appear to occur in groups. Figure 2.17 shows

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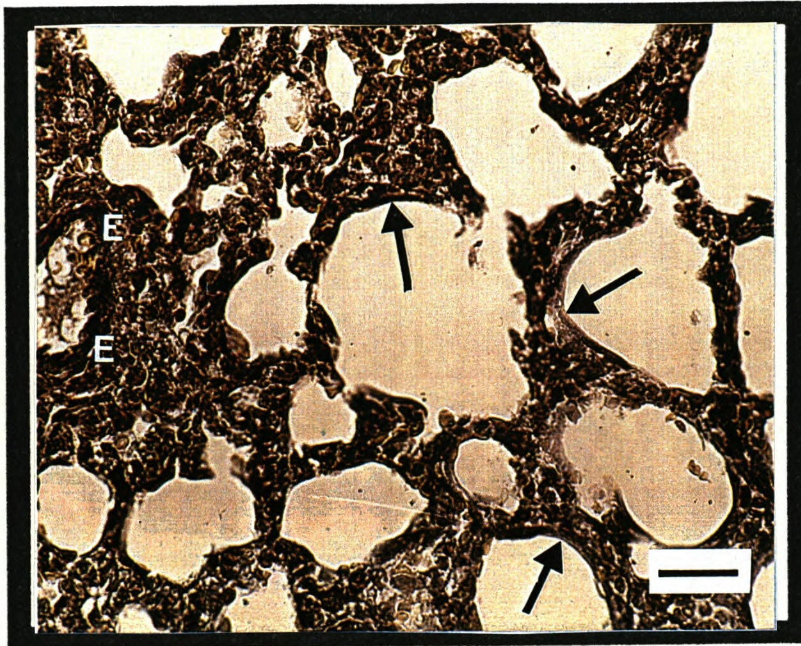

Figure 2.11: Verhoefs stain of 7 day old experimental rat lung. Few elastic fibres at alveolar surface (arrows) while evident in vascular elements (E). Bar = 30μm.

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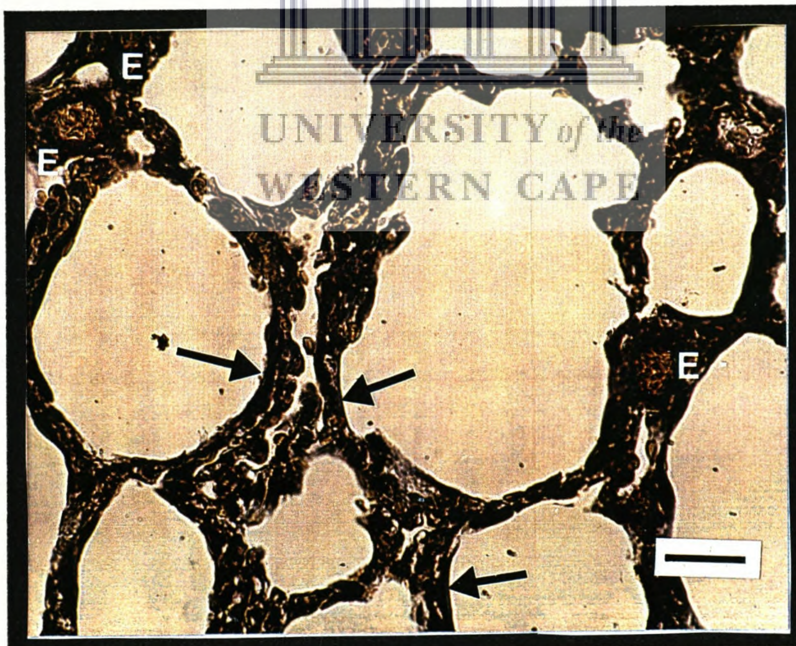

Figure 2.12: Verhoefs stain of 7 day old control rat lung. Elastic fibres seen at alveolar surface (arrows) and in vascular elements. Bar = 30μm.

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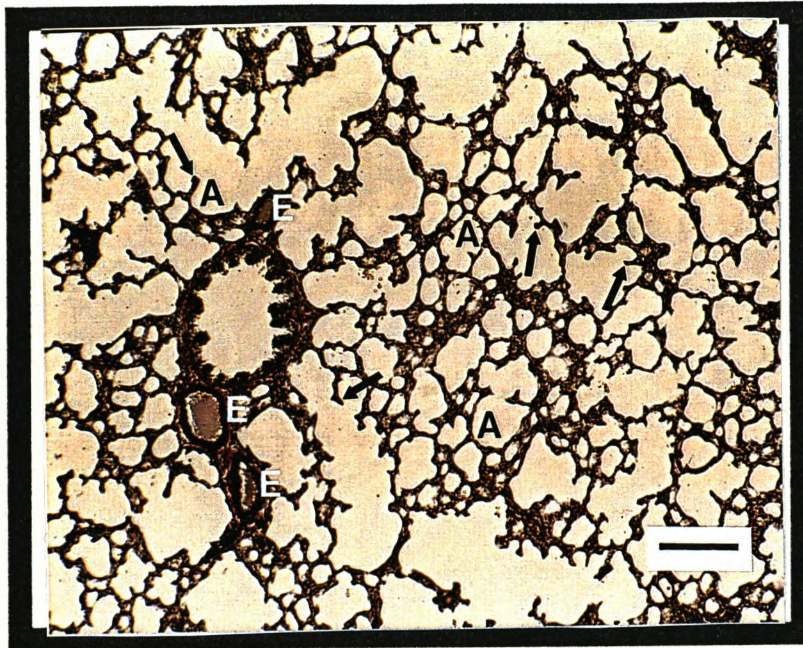

Figure 2.13: Verhoefs stain of 14 day old control rat lung. Thin-walled alveoli (A) with elastic fibres in buds (arrows) and vascular elements (E). Bar = 120μm.

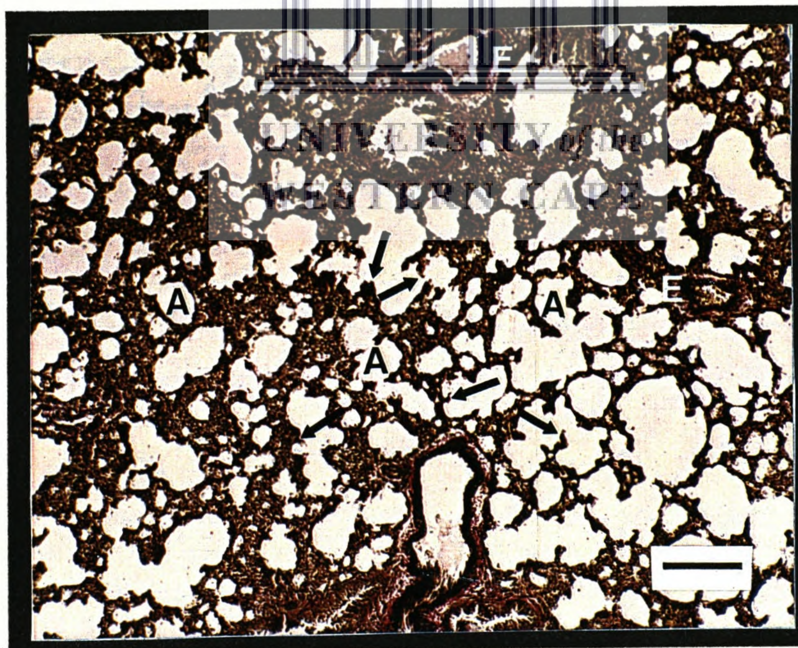

Figure 2.14: Verhoefs stain of 14 day old experimental rat lung. Alveoli (A) with elastic fibres in ragged buds (arrows) and vessel elements (E). Bar = 30μm.

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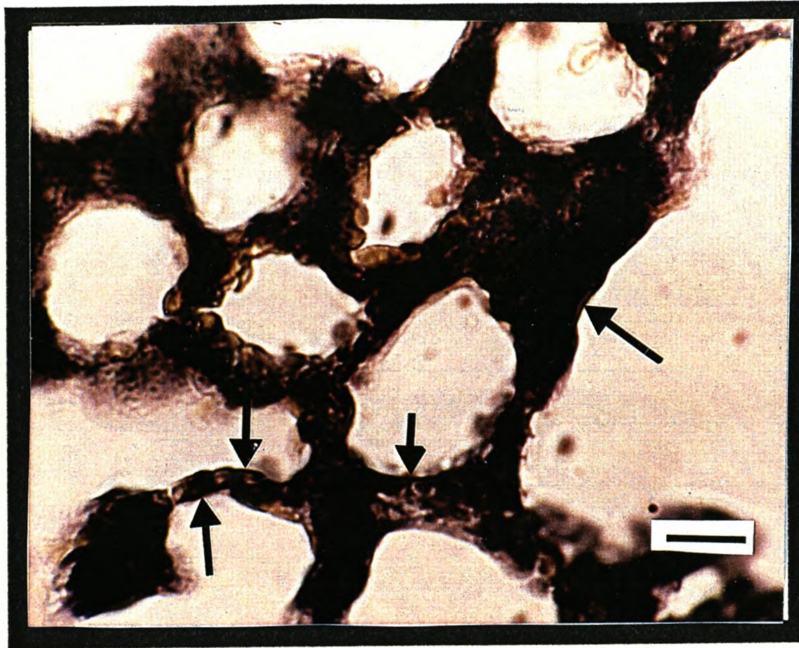

Figure 2.15: Verhoefs stain of 21 day old control rat lung. Elastic fibres (arrows) present at alveolar surface. Bar = 12μm.

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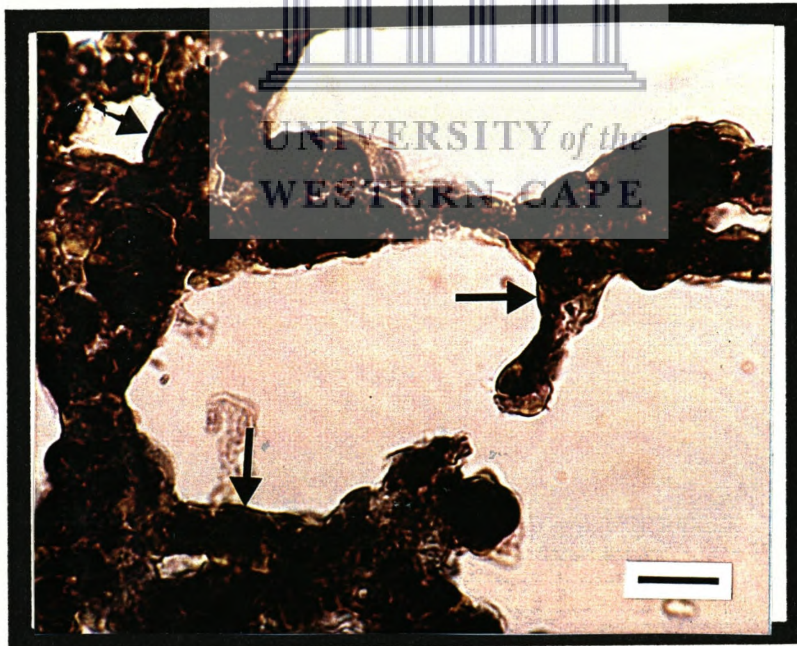

Figure 2.16: Verhoefs stain of 21 day old experimental rat lung. Elastic fibres lining alveoli (arrows) are similar to that of control lung. Bar = 12μm.

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fibres completely lining the alveolus. In the interstitial tissue the fibres appear well-defined and dispersed as thick fibres. The 7 day old experimental rat lung tissue (Figure 2.18) shows reticular fibres which appear much thicker and also having a wavy appearance (or maybe a helix if viewed three-dimensionally). The amplitude of the wavy structure in this case is also much larger than for the control rat lung tissue of the same age group. In addition, the reticular fibres of the experimental rat lung tissue is not intact where it lines the alveolar septum. Another difference is the occurrence of a loosely organised, diffuse fibre network in the interalveolar tissue mass of the experimental rat lungs.

At day 14, little has changed regarding the reticular fibres lining the alveolar septum in the experimental lung tissue (Figure 2.19). Breaks occur frequently in the fibre structure. As regards the amplitude of the wavy structure formed by the above fibres, the difference is not as noticeable at this stage. In the control rat lung tissue (Figure 2.20), the reticular fibres in the tips of the alveolar ridges occur as discrete, concentrated areas whereas, in the experimental lung tissue, the fibres in these alveolar buds end loosely, as if damage to this area has occurred. Fibres in the interalveolar areas of the control rat lung tissue appear to be evenly distributed in the form of discrete, aster-shaped fibres as well as separate fibres running between cells. In the experimental rat lung tissue the fibres appear as thick, wavy fibres that are not as well-

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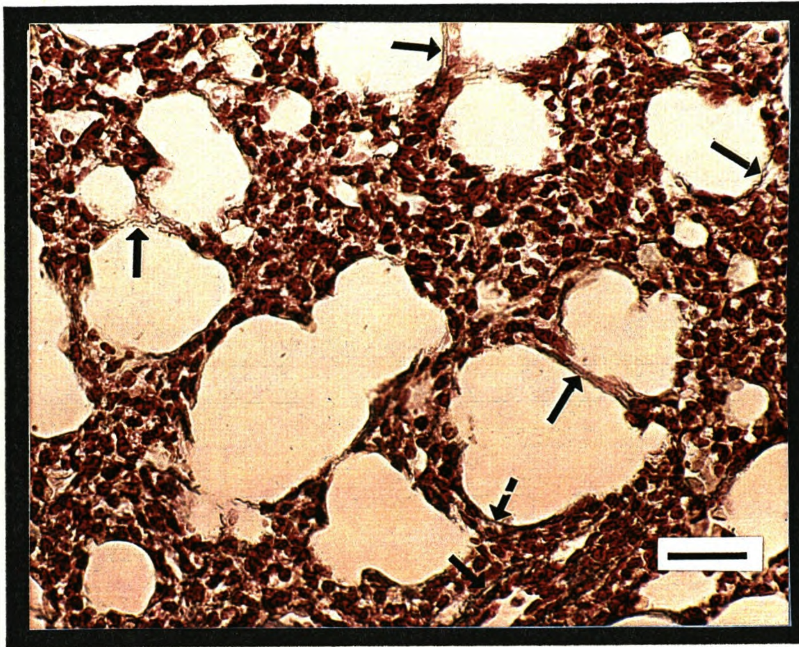

Figure 2.17: Gomori stain of 7 day old control rat lung. Fine reticular fibres (arrows) line alveoli and are weakly present in interstitial areas. Bar = 30μm.

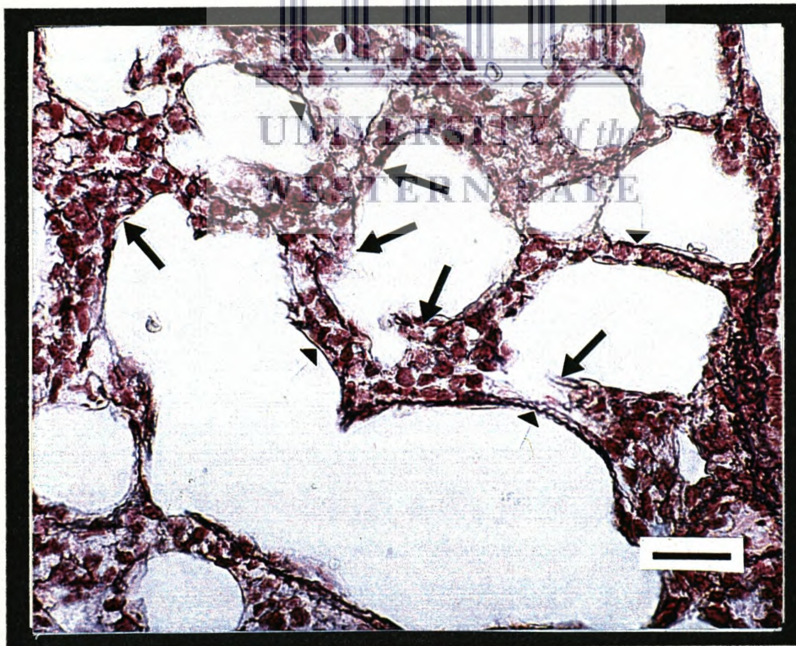

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Figure 2.18: Gomori stain of 7 day old experimental rat lung. Thicker reticular fibres (white-tailed arrows) and altered fibres (black arrows). Bar = 30μm.

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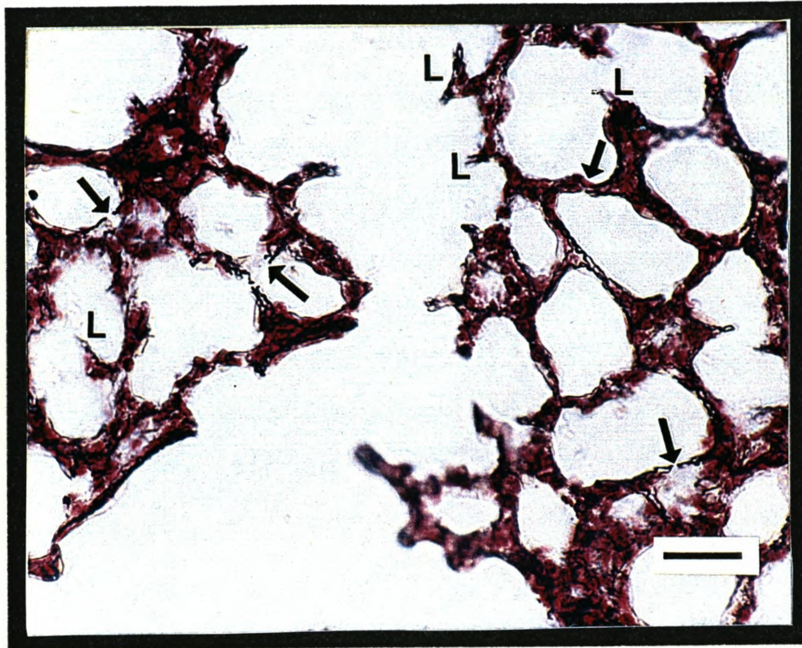

Figure 2.19: Gomori stain of 14 day old experimental rat lung. Arrows indicate breaks in reticular fibres and loose fibres in alveolar buds (L). Bar = 30μm.

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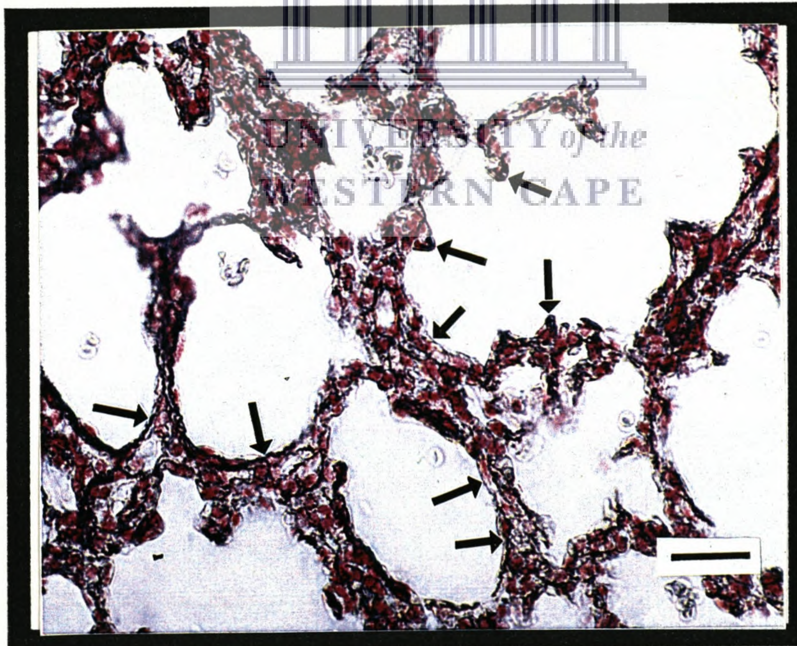

Figure 2.20: Gomori stain of 14 day old control rat lung. Fine fibres (arrows) line alveoli with discrete concentrations in alveolar buds. Bar = 30μm.

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dispersed as in the control lung tissue. In control lung tissue the reticular fibre improved between days 7 and 14 as indicated by the more intense staining characteristics on day 14 (Figure 2.20).

At day 21, further differences are noted between the two groups. The experimental group (Figure 2.21) exhibits fibres which are much thicker than those of the control rat lung tissue. As in the previous two age groups, the experimental lung reticular fibres lining the alveolar wall has numerous interruptions in its structure compared to the well-defined fibres of the control rat lung (Figure 2.22). The control lung tissue also exhibits fine fibres which are more evenly distributed in the interalveolar tissue areas. These fibres seem to form a meshwork in this area as opposed to thick, disjointed fibre bundles in the experimental rat lung. Again, as in the previous two age groups, the alveolar ridge tips (buds) of the control rat lung is much more well-defined with a bundle of fibres present, while the experimental lung shows degenerate tips with fibres seeming to be ending loosely and incompletely.

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2.7 <u>Discussion</u>:

The efficiency of the lung as a gas exchange organ is dependent on the development of a maximum number of physiologically developed and functional alveoli. Disruption of the ultrastructure and the structural and spatial relationships of the individual units and substructures would not only interfere with gas exchange, but ultimately put the animal at risk

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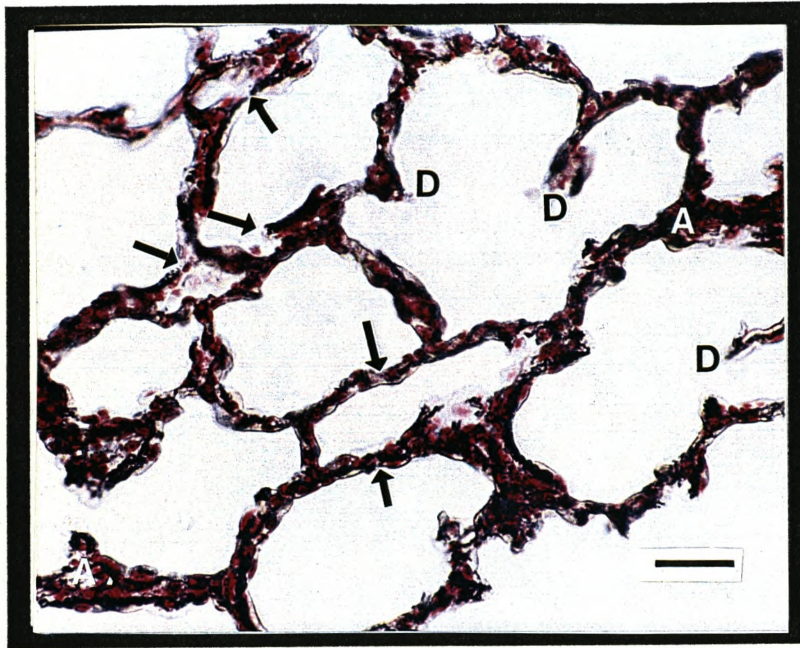

Figure 2.21: Gomori stain of 21 day old experimental rat lung. Thick broken fibres (arrows), degenerate buds (D) and abnormal fibre structure (A) noted. Bar = 30μm.

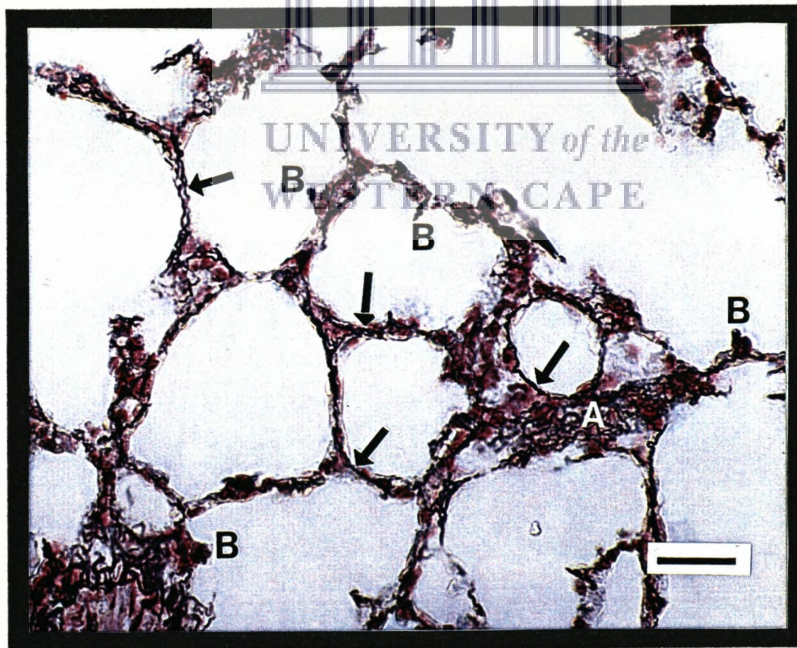

Figure 2.22: Gomori stain of 21 day old control rat lung. Fine alveolar fibres (arrows), interstitial network (A) and well-defined buds (B) noted. Bar = 30μm.

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regarding physiological maintenance of blood homeostasis. The final structure and efficiency of the lung is dependent on developments early in the fetal stage. It is during this stage that important structural developments occur which would dictate the final structure and functional capacities.

The connective tissue of lung is important to the maintenance of tissue integrity and growth. Defective collagen synthesised during the fetal stage would lead to decreased lung size and also interferes with normal airway branching patterns (Thurlbeck, 1975; Carrington et al., 1985; Spooner and Faubion, 1980). Interference with the elastic tissue component also retards lung growth (O'Dell et al., 1978; Kida and Thurlbeck, 1980). The development of these two components is linked by the fact that elastic tissue tension could influence collagen fibre arrangement (Burri, 1974). Production of these two connective tissue components occurs via two different populations of fibroblasts (Schellenberg et al., 1987). Synthesis of these two components by the relevant lung fibroblasts is also dependent on hormonal and protein factors which play a role in type 2 cell differentiation (Thurlbeck et al., 1980), indicating the interrelationship between various cells in the developing lung. By implication, any alteration in the quantity or quality of these components would affect lung structure and function.

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When comparing normal neonatal lung tissue with experimental samples, a clear pattern of morphological effects are seen. It is easily seen that the alveoli of the experimental animals of

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different age groups are not rounded or smooth-walled but take on a flattened appearance which could be construed as alveolar collapse as in atelectasis. The alveolar collapse is not as easy to see in 7 day old animals because of the thicker alveolar septa but does become more evident up to day 21. This appearance is similar to changes seen in man and animals suffering from chronic obstructive pulmonary diseases such as emphysema (Karlinsky and Snider, 1978). Janoff et al. (1983) states that these changes involve respiratory bronchioles (centrilobular lesions) and also extends to the alveoli (panlobular lesions). Figure 2.1 shows a 7 day old control lung which has neat, thin-walled alveoli with clearly defined buds or septa. The latter is indicative of developing alveoli. Figure 2.2 shows the 7 day old lung of experimental animals and in this photomicrograph it is evident that the buds are thicker and very often irregular. Some also seem to be torn or interrupted. This could be due to rupture of alveolar septa with structurally unstable connective tissue components. Auerbach et al. (1974) showed alveolar fragility in humans who smoked. Laros (1972) also describes similar changes and ascribes them to the result of both primary changes of composition of lung tissues and also changes in the magnitude and distribution of mechanical stress on the lung tissues. The changes described above greatly decreases the gas exchange capacity of the lung in the diseased state.

As the lung matures postnatally, the alveoli enlarge and a larger percentage of airspace becomes evident (alveoli and different levels of conducting tubes). In the case of the experimental lung

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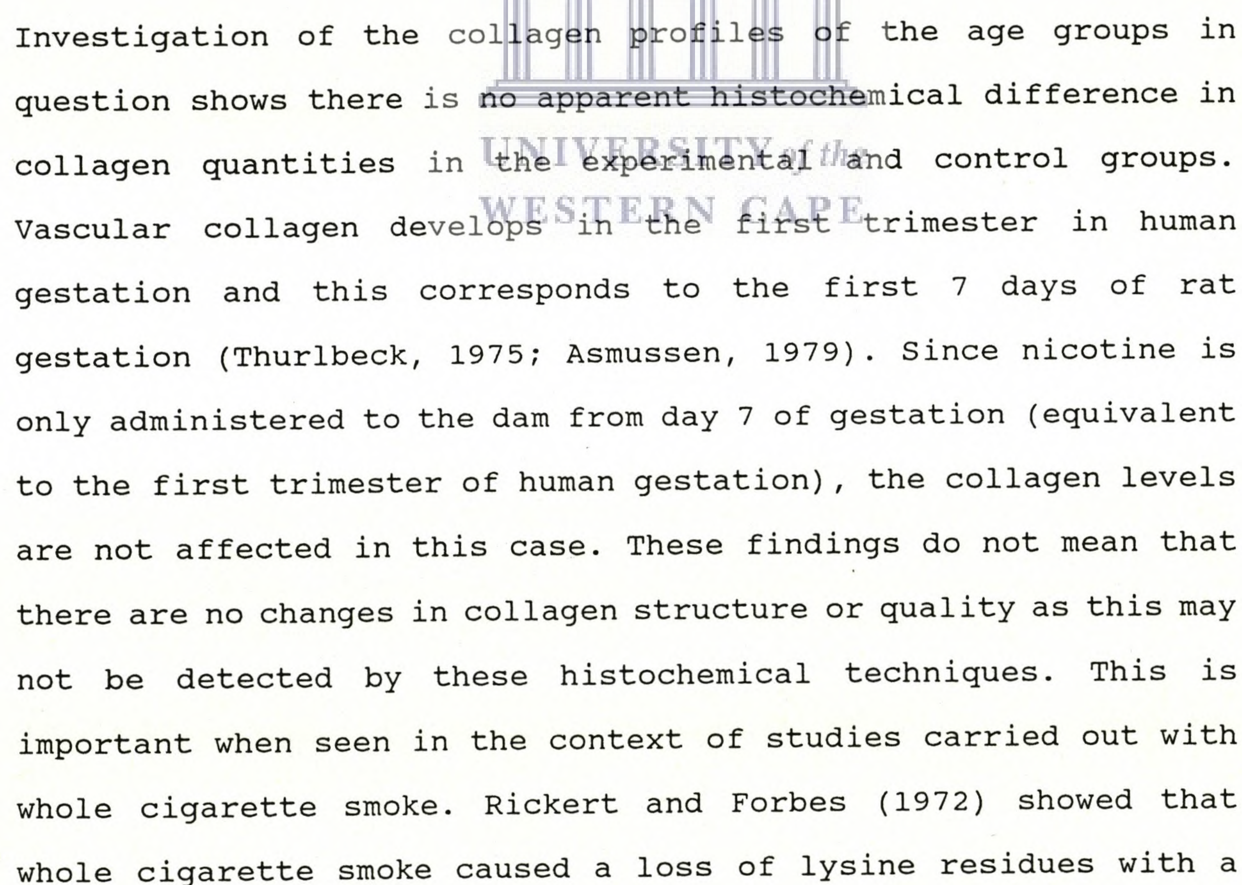
tissue it seems as if it matures faster because of an increase in percentage airspace earlier than that of control lung tissue. This is a false perception created by the disruption of interalveolar septa and "coalescence " of individual alveoli (Karlinsky and Snider, 1978). The septa of the experimental group appear thicker and is also an indication of immaturity as shown by Maritz and co-workers (1993). These findings regarding nicotine may be equated with the effect of cigarette smoking during pregnancy on the fetal and neonatal stages of the human where lowered lung mass (Butler et al., 1972; Hammer and Mitchell, 1979), susceptibility to lower respiratory tract infections and a higher incidence of perinatal mortality is experienced (Pirani, 1978).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Investigation of the collagen profiles of the age groups in question shows there is no apparent histochemical difference in collagen quantities in the experimental and control groups. Vascular collagen develops in the first trimester in human gestation and this corresponds to the first 7 days of rat gestation (Thurlbeck, 1975; Asmussen, 1979). Since nicotine is only administered to the dam from day 7 of gestation (equivalent to the first trimester of human gestation), the collagen levels are not affected in this case. These findings do not mean that there are no changes in collagen structure or quality as this may not be detected by these histochemical techniques. This is important when seen in the context of studies carried out with whole cigarette smoke. Rickert and Forbes (1972) showed that whole cigarette smoke caused a loss of lysine residues with a

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resultant decrease in solubility of the collagen and, by implication, a loss of the ability to form stable cross-links.

When considering elastic tissue levels (Figures 2.11 and 2.12), it is evident that up to day 7, nicotine exposure results in a drastically lowered level of elastic tissue as opposed to controls, especially in the region of the alveolar septa. Vascular elastic tissue shows up strongly. The difference in elastic tissue content between the experimental and control groups decreases up to day 14 and at day 21 there is no visible difference using our histochemical technique. Since it seems as if the experimental lung has "caught up" by day 21, it could be assumed that the lungs of both groups would function equally well.

Lung elastic tissue synthesis in the rat usually precedes alveolar formation and is essential in this process (O'Dell et al., 1978). This has also been verified in mouse lung (Amy et al., 1977). This effect manifests itself in the disruption of alveoli as seen at 14 and 21 days (Figure 2.4) in experimental rat lung. This disruption may be ascribed to the virtual histochemical absence of elastic tissue at day 7 in the experimental neonate (Figure 2.12). A study by Maritz et al. (1993) confirms a lowered total alveolar count in animals treated in this manner.

Reticular fibrous tissue (reticulin), the third fibrous component investigated, has also shown very definite changes due to

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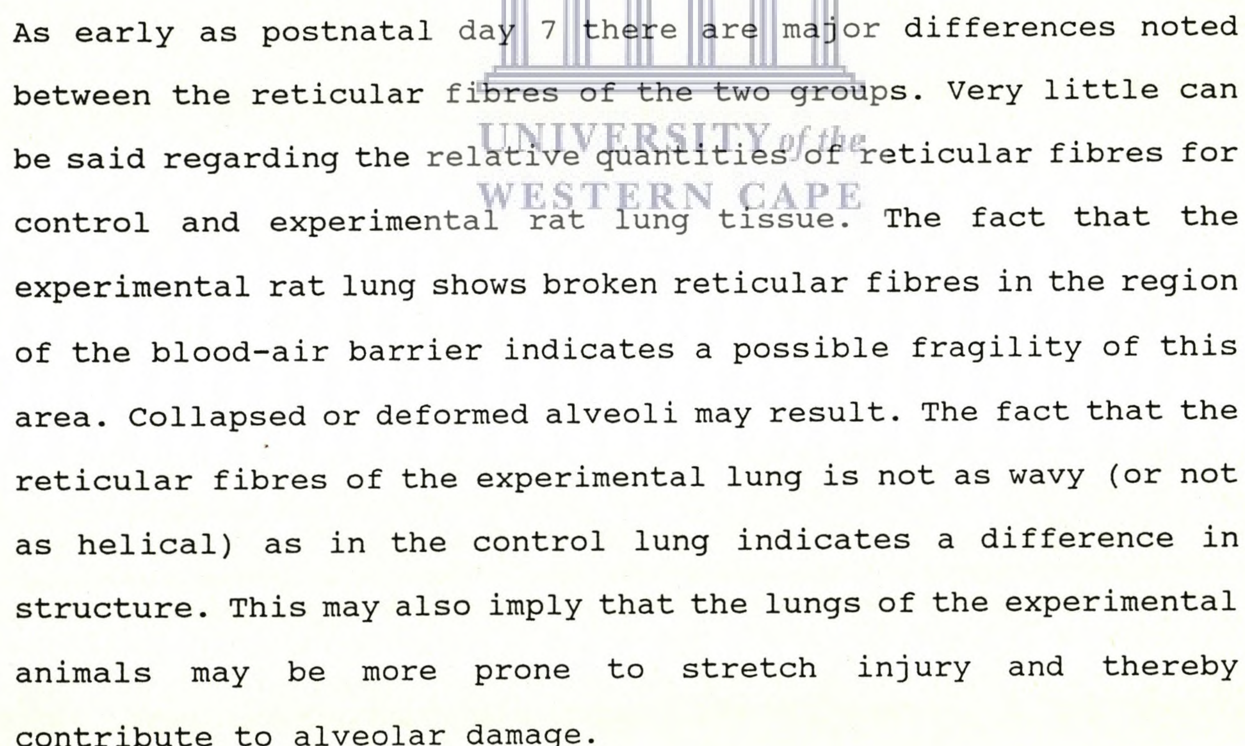
nicotine administration. These fibres, a glycosylated variant of collagen coated with 4% carbohydrate, is especially associated with ground substance as well as loose connective tissue associated with capillaries. In all ages of the control lung tissue studied, this fine wavy network is evident and consistent with functionally and structurally correct reticular fibres. Damage of this fibrous tissue, together with previous damage and connective tissue alterations described, also implies possible changes to the structural and functional characteristics of the ground substance. The importance of this component of the interstitial material in maintaining lung integrity could be one of a number of related changes leading to breakdown of lung integrity (Laors and Kuyper, 1976).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As early as postnatal day 7 there are major differences noted between the reticular fibres of the two groups. Very little can be said regarding the relative quantities of reticular fibres for control and experimental rat lung tissue. The fact that the experimental rat lung shows broken reticular fibres in the region of the blood-air barrier indicates a possible fragility of this area. Collapsed or deformed alveoli may result. The fact that the reticular fibres of the experimental lung is not as wavy (or not as helical) as in the control lung indicates a difference in structure. This may also imply that the lungs of the experimental animals may be more prone to stretch injury and thereby contribute to alveolar damage.

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At two weeks of age, the similarities between reticular fibres in the alveolar septal area of the control and experimental rat lung regarding their wavy appearance seems to indicate a repair mechanism or recovery process. This recovery process is not necessarily complete as evidenced by degenerate reticular fibres in the alveolar buds. This could further contribute to alveolar collapse.

At day 21 , defective reticular fibres in the experimental lung indicates a possible unstable connective tissue framework, as indicated by the morphological status of the lung. At this stage of development the control lung interalveolar reticular fibres exhibit a fine, hairy network which is reminiscent of mature fibres found in other tissues and organs, implying an immature fibre network in the experimental rat lung. Whether this situation is rectified later in life may only be determined by further longitudinal studies. The alveolar ridges are degenerate in previous morphological studies and this is paralleled by the conformation of abnormal reticular fibres in this area. It is not easy to say whether the fibre degeneration is a consequence of alveolar ridge degeneration or vice versa.

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The connective tissue fibres of the 21 day old experimental rat lung seems to be thicker than that of the 21 day old control animal as well as being more wavy (or helical). The fibres in the interstitium of the experimental lung in particular seem knotted in little bunches (Figure 2.21). When looking at the younger ages

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(7 and 14 day old) of the experimental groups this condition does not seem that well developed.

The overall picture presented by these findings can be summarised in the four categories below:

(1) Nicotine exposure results in changes in lung morphology, in particular destruction of alveolar walls, leading to a loss of gas exchange surface area and capacity.

(2) Nicotine exposure seems to lower lung elastogenesis in the early neonatal phase (up to day 7). This is based on the assumption that the elastic tissues of both the control and experimental groups is equivalent, since defective cross-linking is associated with poor staining properties (O'Dell et al., 1978). By day 14 (and later) the experimental lung seems to recover its normal elastic tissue status.

(3) Nicotine exposure does not seem to interfere with lung collagen synthesis at the three ages studied. This does not rule out qualitative changes in collagen structure.

(4) Nicotine exposure alters the structure of lung reticular fibres. The evidence available does not allow any conclusion to be drawn regarding relative quantities of these fibres.

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All the above changes are consistent with the reported effects of smoking as seen using different laboratory models. Hoidal and Niewoehner (1983) showed a change in lung elastic recoil in hamsters treated with cigarette smoke, a factor influenced by the connective tissue balance of the lung. Using in vitro studies, Laurent et al. (1983) showed that the gas phase of filtered cigarette smoke blocked cross-linking of elastic tissue. Rickert and Forbes (1972) used in vitro studies to show that the gaseous phase of cigarette smoke causes structural changes resulting in a decreased solubility of collagen. Garrett (1978) also showed that tobacco smoke, not necessarily nicotine, depressed protein synthesis in rat lung.

The findings of this study thus far confirms, and in some cases carries further, the findings of other researchers in this field (Maritz and Woolward, 1992; Maritz et al., 1993; Woolward, 1992). Although shedding light on the morphology and connective tissue status of the lung tissue under the conditions described, we can at this stage not explain the exact causative reactions leading to lung lesions due to the influence of nicotine. Biochemical quantitation of collagen and elastin could further elucidate the influence of maternal nicotine exposure on neonatal lung connective tissue.

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CHAPTER 3

QUANTITATION OF COLLAGEN AND ELASTIC TISSUE IN CONTROL AND EXPERIMENTAL NEONATAL RAT LUNGS

3.1 Introduction:

From the previous chapter it is evident that there does not seem to be any difference in collagen content of the neonatal rat lung when comparing control and experimental animals of each age group. This does not necessarily rule out qualitative changes to the collagen framework formed under the influence of nicotine. In the case of elastic tissue, histochemical evidence shows the virtual absence of elastic tissue in 7 day old experimental rat lung when compared to the appropriate control lung tissue. At days 14 and 21 this difference is not apparent. Reticular fibres show very obvious differences in quality as evidenced by numerous breaks and other abnormalities in structure in experimental lung tissue. On the basis of this evidence, and because the latter two components have been implicated in emphysematous changes (Laros, 1972) it was deemed necessary to use biochemical techniques to quantitate collagen and elastic tissue to verify any differences in content. Reticular fibres are not easily quantified because of their similarity with collagen and also because they have not been as extensively characterised and studied.

The importance of the presence and quantities of these connective tissues is evident from the studies of Carrington et al. (1985) and Spooner and Faubion (1980) who showed that interference with

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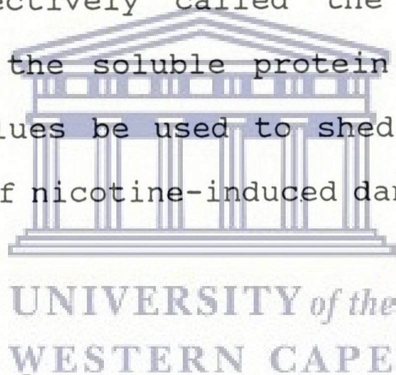
collagen triple-helix formation results in decreased lung size and abnormal branching of the airways. Similarly, preventing elastic tissue cross-linking also retards further lung growth and development. In both these cases, a decrease in mature connective tissue content causes these adverse changes. These alterations of connective tissue levels can be due to:

(i) changes in connective tissue content.

(ii) changes in effectiveness or maturity of the connective tissue.

The aim of this study was to determine the collagen and elastic tissue contents (collectively called the insoluble protein component) as well as the soluble protein component. It was intended that these values be used to shed more light on the pathology and etiology of nicotine-induced damage to the neonatal rat lung.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3.2 Materials and methods:

3.2.1 Lung tissue excision and storage:

Wistar rat neonates (as described under section 2.2 dealing with light microscopy) of appropriate ages were used. After decapitation of the neonate, the lungs were removed via an incision in the thorax. As much of the whole lung as possible was removed without including branches of the trachea.

The excised tissue was rapidly frozen in liquid nitrogen or on dry ice and stored in plastic bottles at -70°C in a Revco

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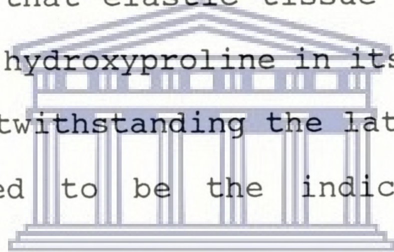
freezer. The tissue was kept cold until directly before use to prevent any deteriorative biochemical changes.

3.2.2 Quantitation of collagen:

(a) Principle:

Connective tissues, being poorly soluble, are normally difficult to quantitate directly. Since collagen is the only protein to contain appreciable quantities of hydroxyproline (Kivirikko et al., 1967), the basis of this determination is rather the quantitation of hydroxyproline (Kivirikko et al., 1967). It must be noted at this point that elastic tissue contains very small and variable amounts of hydroxyproline in its structure (Kalenga and Eeckhout, 1989). Notwithstanding the latter, hydroxyproline is generally considered to be the indicator of choice to quantitate collagen.



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The method used is based on that of Prockop and Udenfriend (1960). They initially used the method to determine collagen in urine samples, but it has since been successfully applied to,lung tissue (Kalenga and Eeckhout, 1989; Stanley et al., 1975). It involves the hydrolysis of lung tissue homogenate in 6M HCl, breaking down all proteins to their free amino acids, amongst which is hydroxyproline. The free hydroxyproline is then oxidised with 0,2M chloramine-T (N-chloro-p-toluenesulfonamide) solution. After boiling, the oxidation products are extracted into toluene and then allowed to react with Erlich's reagent (a preparation of para-dimethylaminobenzaldehyde). The stable chromophore formed

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is measured spectrophotometrically at 560nm using appropriate standards and a water blank. This method is suitable for quantities of hydroxyproline ranging from 5 to 12μg. For the hydrolysate produced during this study, 4ml contained a quantity of hydroxyproline falling in this range. As this only quantitates hydroxyproline, conversions to the equivalent mass of collagen is done by multiplying by a factor of 7,41 (Kalenga and Eeckhout, 1989).

(b) <u>Reagents and equipment</u>:

1. 0,15M NaCl.

2. 6M, 0,1M and 0,05M HCl.

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3. 1% Phenolphthalein in alcohol.

4. 12M, 1M, 0,1M and 0,05M KOH.

5. Standard hydroxyproline - 10mg/100mℓ distilled water. This can be stored in aliquots in a freezer for up to three months.

6. KCl crystals.

7. 10% Alanine: dissolve 10g in 90mℓ distilled water, adjust the pH to 8,7 with KOH and make up to 100mℓ. This can be stored in a refrigerator for a maximum of two weeks.

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9. Potassium borate buffer (pH 8,7): dissolve 61,84g boric acid and 225g KCl in about 800mℓ distilled water, adjust the pH to 8,7 with 10M and 1M KOH and make the volume up to 1ℓ.

10. 0,2M Chloramine-T: prepare a 0,2M solution in methyl cellosolve (2-methoxyethanol) on a daily basis (0,91g/20mℓ cellosolve).

11. 3,6M Sodium thiosulphate.

12. Toluene.

13. Erlich's reagent:

(a) slowly add 27,4mℓ concentrated H_2SO_4 to 200mℓ absolute alcohol in a beaker and cool.
(b) add 120g p-dimethylaminobenzaldehyde to 200mℓ absolute alcohol in a second beaker and cool.
(c) slowly stir the acid ethanol mixture into the second beaker in an ice bath.
(d) store for three weeks in a refrigerator. Any crystals formed during storage can be redissolved by warming.

14. Silicon oil bath at 100°C.

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15. 115 X 16mm Kimax culture tubes with teflon-lined screw caps.

16. Hitachi UV/Vis Spectrophotometer.

17. Boiling water bath.

18. Polytron homogeniser with PTA-10S aggregate.

19. Table model centrifuge/ high speed centrifuge.

20. Vortex mixer.

21. Pasteur pipettes.

22. Crushed ice.

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(c) Procedure:

1. Homogenise weighed tissue (10% W/V) in cold 0,15M NaCl. A mass of 0,25 - 0,45g of tissue is suitable. Homogenisation procedure involves use of the Polytron with PTA-10S aggregate at full speed for two 30 second bursts in Hitachi centrifuge tube. Keep cold by immersion in ice at all times.

2. Remove two 100$\mu\ell$ aliquots (for duplicate analyses) and place in a Teflon-lined Kimble screw-capped tube for hydroxyproline determination. Treat the rest of the

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homogenate as under the procedure for elastic tissue determination later in section 3.1.3 (c).

3. Add 1mℓ 6M HCl and heat on a silicon oil bath at 100°C overnight (12 hours).

4. Add 1 drop of a 1% phenolphthalein solution (made up in alcohol) and adjust to pink with 12M and 0,1M KOH.

5. Dilute to 15mℓ mark with distilled water, cap and mix by inversion. Filter each sample through Whatman no. 4 paper to remove any heavy pigments.

6. Pipette a 4mℓ aliquot of hydrolysed supernatant (equivalent to 5 - 12μg hydroxyproline) into another screw-capped tube (in duplicate) and dilute to 15mℓ exactly with distilled water.

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7. Simultaneously prepare the following tubes:

   water blank: 4mℓ

   5μg standard hydroxyproline: 50μℓ std. + 3,95mℓ water

   10μg standard hydroxyproline: 100μℓ std. + 3,90mℓ water

8. Add a drop of phenolphthalein (where necessary) as above and adjust to pink with 0,1 and 0,05M KOH or HCl as required.

9. Saturate with KCl by adding 3g solid crystals.

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10. Add 0,5ml 10% alanine and 1mℓ potassium borate buffer.

11. Stand at room temperature for 20 to 30 minutes with occasional stirring.

12. Oxidise by adding 1mℓ 0,2M chloramine-T solution and mix immediately and thoroughly.

13. Stand at room temperature for 25 minutes with occasional mixing and then add 3mℓ 3,6M $Na_2S_2O_4$.

14. Add 5mℓ toluene and cap tightly with the screw cap.

15. Shake vigorously (full speed on Gerhard shaker) for 5 minutes and centrifuge at 600*g* (Heraeus Christ table model centrifuge) for 2 minutes to separate the toluene layer.

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16. Carefully remove and discard the toluene with a pasteur pipette.

17. Cap the tube tightly and place it in a boiling water bath for 5 minutes.

18. Cool with running tap water and add 5mℓ toluene by means of a pipette.

19. Recap, shake for 5 minutes and centrifuge at (600*g*) as in step 15 above.

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20. Pipette 2,5mℓ of the toluene layer into a clean test tube and add 1mℓ Erlich's reagent.

21. Mix rapidly as the reagent is added to prevent layering.

22. Read the absorbance at 560nm after standing for 30 minutes at room temperature.

(d) Calculations:

The mass of hydroxyproline (in μg) was calculated for each sample duplicate tube using the following formula:

$$\frac{A_{560} \text{ of sample}}{A_{560} \text{ of standard}} \times \text{mass hydroxyproline per standard volume}$$

This mass of hydroxyproline is then converted to the equivalent mass of collagen, correcting for all dilution factors and finally multiplying by 7,41. The results have all been expressed as mg/g of wet tissue mass.

3.2.3 Quantitation of elastic tissue:

(a) Principle:

A number of methods are in use for determining elastic tissue quantities for the past few years, all based on the initial removal of collagen, proteins and protein-polysaccharide complexes (glycosaminoglycans and mucoproteins). These methods usually require a large amount of tissue. Naum and Morgan (1973)

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developed a semi-micro method involving extraction of a tissue homogenate with 5M guanidine to remove all soluble proteins. Subsequent autoclaving solubilises collagen. The remaining elastic tissue residue is made soluble with elastase digestion and the resulting peptides are estimated spectrophotometrically by the method of Lowry et al. (1951).

(b) Reagents and Equipment:

1. 5M Guanidine hydrochloride.

2. Elastase: Sigma grade porcine pancreas type III (product number E-0127) elastase (EC 3.4.21.36).

3. 0,02M $NaHCO_3$ buffer (pH 8,80). Add 46mℓ 0,1M Na_2CO_3 to 40mℓ 0,1M HCl. Mix and then add 50mℓ water. Adjust pH to 8,80 and make up 172mℓ with water.

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4. Elastase working solution: Suspend elastase in bicarbonate bufer solution at a concentration of 0,1mg/mℓ. Store frozen in aliquot form prior to use.

5. Hitachi HIMAC refrigerated centrifuge and RPR 20-2 rotor.

6. Techne shaking waterbath.

7. Parafilm.

8. Autoclave - Erma Optical Works (Tokyo).

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9. Polytron with PTA-10S aggregate.

10. Hitachi recording spectrophotometer.

(c) Procedure:

1. Centrifuge the remaining homogenate obtained from 3.1.2 (c) at 2 600*g*.

2. Decant and retain the supernatant on ice for subsequent protein determination.

3. Suspend the pellet in 1mℓ 5M guanidine hydrochloride (depending on the pellet size 2mℓ may be used).

4. Extract the pellet for 24 hours on a shaking waterbath at 25°C. Ensure that the tube is closed with Parafilm.

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5. Centrifuge for 45 minutes at 40 000*g* and <u>carefully</u> discard the supernatant. If a solid pellet is not formed, the supernatant may be aspirated using a pasteur pipette.

6. Re-extract the pellet for a further 24 hours as in step 4 above.

7. Repeat step 5.

8. Wash the pellet with 1mℓ distilled water and centrifuge for 20 minutes at 20 000*g*.

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9. Resuspend the pellet in 1mℓ distilled water and autoclave at 101,35 kPa (121°C) for 45 minutes.

10. Centrifuge as in step 8 above and decant the supernatant. At this stage the pellet is regarded as elastic tissue.

11. Resuspend the residue in 3mℓ distilled water with Polytron set at setting 5 for 15 seconds.

12. Transfer 1mℓ of the elastic tissue suspension (in duplicate) to new tubes and add 1mℓ elastase. Also prepare an elastase blank containing 1mℓ water and 1mℓ elastase. Incubate for 30 minutes on a shaking waterbath set at 37°C.

13. Remove tubes to ice and use 100μℓ aliquots for protein determination by the method of Lowry et al. (1951).

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(d) Calculations:

Use a serum albumin standard curve to calculate the amount of elastic tissue peptides present in the samples. Accounting for dilution factors and aliquot sizes, the mass of elastic tissue may be calculated and averaged for all samples. Results are expressed as mg elastic tissue per gram wet lung tissue mass.

3.2.4 Quantitation of soluble protein:

(a) Principle:

The method of Lowry et al. (1951) has been widely used for the

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detection and quantitation of peptide bonds (and consequently proteins) in solution. The method involves the reduction of divalent copper ions with the peptide bonds. The further reaction of this copper-protein complex with Folin-Ciocalteau's reagent results in the formation of a blue complex. The colour intensity can be measured spectrophotometrically at 540nm. This procedure shows a linear relationship between absorbance and concentrations of protein from 50 to 300μg per ml of homogenate/solution. The colour complex is stable for 30 minutes.

(b) Reagents:

1. Bovine serum albumin fraction 5 (Seravac) - 3mg/mℓ. Dissolve 300mg bovine serum albumin in 100mℓ distilled water. Store frozen in aliquot form for up to three months.

2. Lowry reagent A - 2% sodium carbonate in 0,1M sodium hydroxide. Prepare a 0,1M NaOH solution by dissolving 2g NaOH pellets in 500mℓ distilled water. In this solution dissolve 10g anhydrous Na_2CO_3. Store in the refrigerator at 4°C for up to two weeks.

3. Lowry reagent B_1 - 1% copper sulphate. Dissolve 1g $CuSO_4.5H_2O$ in 100mℓ distilled water. Store in the refrigerator at 4°C for up to two weeks.

4. Lowry reagent B_2 - 2% sodium potassium tartrate. Dissolve 2g sodium potassium tartrate in 100mℓ distilled water. Store in the refrigerator at 4°C for up to two weeks.

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5. Lowry reagent C must be prepared fresh on the day of the experiment. Add reagents to a 100mℓ conical flask in the following order:

500μℓ reagent B_2

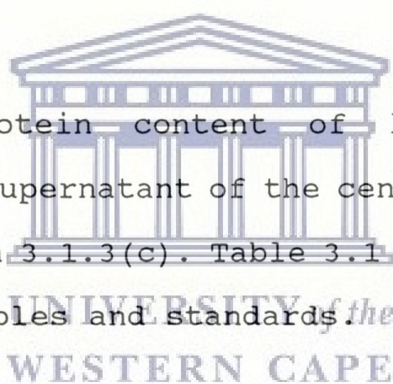
500μℓ reagent B_1

50mℓ reagent A

Stir and store on ice until used.

6. Lowry reagent D. Dilute commercial grade Folin reagent with equal parts of distilled water. Prepare fresh on the day of experiment. Store on ice prior to use.

(c) <u>Procedure</u>: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1. Total soluble protein content of lung tissues were determined on the supernatant of the centrifuged homogenate obtained in section 3.1.3(c). Table 3.1 below specifies the preparation of samples and standards.

Tube	1	2	3	4
Sample (μℓ)	30	-	-	-
Standard (μℓ)	-	50	75	-
Distilled water(μℓ)	470	450	425	500

<u>Table 3.1</u>: Lowry spectrophotometric determination of total soluble protein.

2. All samples and standards are prepared in duplicate where tube 4 is a blank. The 50μℓ standard is equivalent to 100μg

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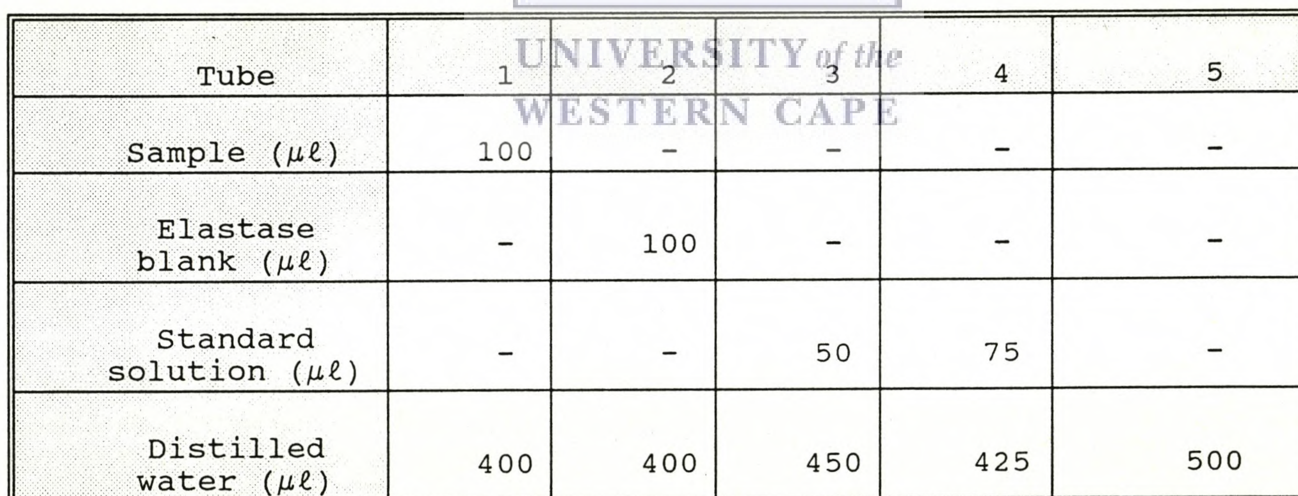
protein and the 75$\mu\ell$ standard is equivalent to 150μg protein.

3. Add 2,5mℓ reagent C to all tubes and mix immediately. Stand for 10 to 15 minutes at room temperature.

4. Add 250$\mu\ell$ reagent D and mix immediately. Allow tubes to stand for 30 minutes at room temperature.

5. The absorbance of all tubes were read at 540nm against the reagent blank in the spectrophotometer.

6. Table 3.2 below is used when determining elastic tissue peptides as prepared in step 13 of 3.1.3 (c):



Tube	1	2	3	4	5
Sample ($\mu\ell$)	100	-	-	-	-
Elastase blank ($\mu\ell$)	-	100	-	-	-
Standard solution ($\mu\ell$)	-	-	50	75	-
Distilled water ($\mu\ell$)	400	400	450	425	500

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<u>Table 3.2</u>: Lowry spectrophotometric determination of elastic tissue peptides after elastase hydrolysis.

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7. All samples and elastase blanks were prepared in duplicate. Tube 5 is the reagent blank. All tubes were subsequently treated as in steps 3 to 5 above.

(d) Calculations:

Since the standard concentrations used are in the linear portion of the curve relating absorbance to concentration, the ratio of the two readings should be 1:1,5. If so, the standard values are suitable for calculating sample concentrations.

For total soluble protein concentration, the following formula is used to arrive at a figure expressing the mass (mg) of protein per gram wet mass of sample:

$$\frac{A_{540} \text{ of sample}}{A_{540}(50\mu l \text{ standard volume})} \times 50$$

In the case of elastic tissue peptide determination the following equation may be used to arrive at the mass (mg) of elastic tissue per gram wet tissue mass:

$$\frac{A_{540}(\text{sample}) - A_{540}(\text{elastase blank})}{A_{540}(50\mu l \text{ standard}) \times 3{,}75} \times \frac{1}{\text{sample mass} - 0{,}001g}$$

3.2.5 Statistical treatment of data:

All data obtained for soluble protein contents, collagen contents as well as elastic tissue contents is presented as mean values ± the standard error of the mean (mg/g wet lung mass). These values were obtained by an unpaired t-test using Medcalc, a

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statistics package used in the Department of Physiological Sciences, U.W.C.

3.3 Results:

3.3.1 Soluble protein quantitation:

Quantitative studies of soluble protein (expressed per gram of wet tissue mass) of lung is presented in Table 3.3 for all three age groups of both the control as well as the experimental animals. For the lungs of control animals, the soluble protein content increases significantly from day 7 up to day 21 ($p<0,05$), although between days 7 and 14 as well as between 14 and 21 days, there is no significant difference ($p<0,05$). The same situation exists for experimental neonatal lung ($p<0,001$). When comparing the soluble protein content between the control and experimental groups, no significant differences are apparent for the three age groups studied ($p>0,1$ for all age groups).

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The protein contents at the different age groups can also be used to determine the rate of protein synthesis (Table 3.4) averaged over the time periods indicated. The rate of soluble protein synthesis in control lung between days 7 and 14 is, at 495,7μg/g/day, only 5,5% lower than that of the experimental group (524,7%). During the third week the rate of synthesis of soluble proteins was 708,6μg/g/day in control lung, this being 90,1% slower than the 1347,2μg/g/day for lung tissue of experimental rat pups. Due to this higher rate of protein synthesis, the soluble protein content of the lung tissue of

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AGE	PROTEIN CONCENTRATION (mg/g wet mass ± SEM)		
	CONTROL	EXPERIMENTAL	p
7 DAY	48,03 ± 2,67 (n=11)	47,22 ± 2,03 (n=21)	>0,5
14 DAY	51,50 ± 3,74 (+7,2%)	50,89 ± 2,85 (+7,8%)	>0,5
	(n=15) (p>0,1)	(n=17) (p>0,1)	
21 DAY	56,47 ± 2,26 (+9,7%)	60,32 ± 1,07 (+18,5%)	>0,1
	(n=13) (p>0,1)	(n=10) (p<0,05)	

Table 3.3: Soluble protein concentration of control and experimental neonatal rat lung expressed as milligrams protein per gram wet mass (mean ± SEM). Percentages and p values relate to change in content between the ages in each group. $p<0,05$ is considered significant.

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AGE	RATE OF PROTEIN SYNTHESIS (μg/g/day)		
	CONTROL	EXPERIMENTAL	% DIFFERENCE
7 - 14 DAYS	495,7	524,3	+5,5
14 - 21 DAYS	708,6	1347,2	+90,1
% DIFFERENCE	+42,5	+156,9	

Table 3.4: Rate of soluble protein synthesis of control and experimental neonatal rat lung expressed as microgram soluble protein per gram wet mass per day.

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experimental animals is at 60,32 ± 1,07 mg/g a total of 18,5% higher ($p<0,05$) than the 50,89 ± 2,85 mg/g of the two week old experimental animals. The slower rate of protein synthesis in the control animals results in a soluble protein content of 56,47 ± 2,26 mg/g at week 3 after birth. This is not significantly different ($p>0,05$) from the 51,50 ± 3,74mg/g at two weeks of age.

3.3.2 Collagen quantitation:

Table 3.5 presents the collagen contents of control and experimental animal lungs expressed as a function of wet mass. The collagen content for control neonatal lung shows a significant increase from day 7 to day 21 ($p<0,001$), the same is observed in experimental animals ($p<0,001$). Of interest is the fact that, for control animals, there is a significant increase of 70,5% in lung collagen content between days 7 and 14 ($p<0,01$). After day 14 the lung collagen content is unchanged. In the experimental animals the lung collagen content increases significantly by 65,3% between days 7 to 14 after birth ($p<0,001$). Between days 14 and 21 after birth no further increase is noted ($p>0,1$). When comparing animals of the same ages between groups, there is no significant difference in content at each stage ($p>0,1$).

The rate of collagen synthesis can be calculated from the data in Table 3.5. These calculated values are presented in Table 3.6. A comparison of the rate of synthesis for control animals shows that the rate of collagen synthesis during week 2 is 334,2 μg/g wet lung mass/day and in the experimental animals 344 μg/g wet

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AGE	COLLAGEN CONCENTRATION (mg/g wet mass ± SEM)		
	CONTROL	EXPERIMENTAL	p
7 DAY	3,32 ± 0,30 (n=11)	3,69 ± 0,25 (n=22)	>0,1
14 DAY	5,66 ± 0,31 (+ 70,5%)	6,10 ± 0,44 (+65,31%)	>0,1
	(n=11) (p<0,01)	(n=16) (p<0,001)	
21 DAY	6,66 ± 0,46 (+17,7%)	6,55 ± 0,48 (+7,38%)	>0,5
	(n=13) (p>0,1)	(n=11) (p>0,1)	

Table 3.5: Collagen content of control and experimental neonatal rat lung expressed as milligrams of collagen per gram wet mass (mean ± SEM). $p < 0,05$ is considered significant. Shaded p values indicate significance of differences between ages in the same group. Unshaded p values indicates the significance of differences between groups at a specific age. Percentage differences are indicated for different ages in each group.

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AGE	RATE OF COLLAGEN SYNTHESIS ($\mu g/g/day$)		
	CONTROL	EXPERIMENTAL	% DIFFERENCE
7 - 14 DAYS	334,2	344,3	+3
14 - 21 DAYS	142,9	64,29	-55

Table 3.6: Rate of collagen synthesis of control and experimental neonatal rat lung expressed as micrograms collagen per gram wet mass per day.

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lung mass/day. During the third week the rate of collagen synthesis in control animals and in the experimental animals declines to such an extent that the collagen content of the control and experimental lungs are not different to that seen two weeks after birth.

3.3.3 **Elastic tissue quantitation:**

Values for the determination of elastic tissue (expressed as mg per gram of wet lung mass) is presented in Table 3.7. When assessing the development of the neonatal control rat lung with respect to elastic tissue, there is a significant increase in elastic tissue content between day 7 and 14 ($p<0,05$). There is no significant difference in content between days 14 and 21 ($p>0,05$). The trend is the same for the experimental neonatal lungs over the same time-frames. Looking at a comparison between control lungs and experimental lungs, the only significant difference noted is at day 7 where the elastic tissue content of the experimental samples of 8,09 $\pm$ 0,31mg/g was higher than the 6,41 $\pm$ 0,67mg/g of the control group ($p<0,05$). These figures can also be used to determine the rate of synthesis of elastic tissue as presented in Table 3.8. Regarding the rate of elastic tissue synthesis in control lung, it is clear that synthesis peaked after day 7 but before day 14 after birth. It is however interesting to note that in experimental animals, the rate of synthesis peaked before day 7 after birth. This explains the higher elastic tissue levels of experimental lung samples on day 7 after birth as compared to control lungs.

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AGE	ELASTIN CONCENTRATION (mg/g wet mass ± SEM)		
	CONTROL	EXPERIMENTAL	p
7 DAY	6,41 ± 0,67 (n=7)	8,09 ± 0,31 (n=15)	<0,05
14 DAY	9,43 ± 0,92 (+47,11%)	9,49 ± 0,61 (+17,31%)	>0,5
	(n=12) (p<0,05)	(n=12) (p<0,05)	
21 DAY	7,17 ± 0,40 (-24,00%)	7,50 ± 0,65 (-21,00%)	>0,5
	(n=8) (p>0,05)	(n=6) (p>0,05)	

Table 3.7: Elastic tissue concentration of control and experimental neonatal rat lung expressed as mg per gram wet mass (mean ± SEM). p < 0,05 is considered significant. Shaded p values indicates the significance of differences between ages in the same group.

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AGE	RATE OF ELASTIN SYNTHESIS ($\mu g/g/day$)		
	CONTROL	EXPERIMENTAL	% DIFFERENCE
7 - 14 DAYS	431,4	200,0	-53,6
14 - 21 DAYS	-322,9	-284,3	-11,9

<u>Table 3.8</u>: Rate of elastic tissue synthesis of control and experimental neonatal rat lung expressed as micrograms per gram wet mass per day.

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An important factor in the actual quantitation process for elastic tissue could shed light on possible differences between experimental animal lungs and control lungs. Step 5 of 3.1.3(c) makes mention of the pellet being inconsistent. In fact, the lungs of the experimental animals rendered a jelly-like mass after centrifugation while the control lungs rendered a solid pellet which adhered strongly to the centrifuge tube wall. The supernatant was easily poured off in the latter case. This, more than any other evidence presented here yet, is an indication of altered quality of the elastic tissue pellet of experimental animals when comparing it to control lung elastic tissue.

3.4 Discussion:

3.4.1 Soluble protein content:

Analysis of the data presented for the control animal lung tissue shows a gradual increase of protein content between days 7 and 21 of neonatal life. The observed increase between days 7 and 14 as well as days 14 and 21 is insignificant ($p<0,05$) although the rate of synthesis in the third week is 42% faster than during the second week of life. It is sensible to argue that this increase in protein content is due to control of protein synthesis commensurate with general growth and maturation of the lung.

Protein synthesis in the lung tissue of the experimental rat pups shows that the rate of protein synthesis between days 7 and 14 is the same as for control rat pups of the same age. However, from day 14 to 21, the rate of protein synthesis exceeds that of

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the control rat pups by 90,1%. If the assumption is true that the increase between days 14 and 21 is due to control of soluble protein synthesis to assure optimal lung development, it implies that nicotine exposure of the fetus and neonate interfered with this control mechanism. The mechanism of interference and consequence thereof is unknown.

The change from a diet of mothers milk to solid foods in the third week of life implies a lowered exposure to nicotine via milk intake in the experimental rat pups. One could argue that, since the level of any cellular component is a function of its rate of synthesis and degradation, nicotine seems to have interfered with this process in some as yet unknown way, thereby enhancing the rate of soluble protein synthesis. On the other hand, considering that the weaning phase implies partial release from the amount and effect of ingested nicotine, it is possible that this results in higher levels of soluble protein synthesis. The mechanism could be either lowered inhibition of synthesis in the absence of nicotine or stimulation of degradation. The overall effect could also possibly lead to over-correction of the protein levels in response to lowered nicotine intake. The fact that the levels of anti-elastases are lowered in animals exposed to cigarette smoke (Hoidal and Niewhoner, 1983; Janoff and Carp, 1977), implying increased elastolytic activity, could argue against the lowering of the degradative effects.

The anomaly of this increased rate of soluble protein synthesis in experimental lung tissue at day 21 may be partially explained

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by the speculation of Goldspink (1987) who, after studying lung protein content and turnover from the fetal stage to old age, attributed developmental changes in protein synthesis to a possible alteration of the ratio of cell types in the total lung cell population. Lowered nicotine intake during weaning indeed alters this population ratio and if the argument of Goldspink (1987) is true, it is conceivable that the increase in the rate of soluble protein synthesis, in response to nicotine exposure, may indirectly affect protein synthesis by interfering with the normal ratio of the cell types in the experimental lung tissue.

3.4.2 Collagen content:

Agreeing with accepted developmental and growth trends for lung tissue, there is a steady increase in collagen content for the control and experimental lung tissue during the phase being studied (Laros and Kuyper, 1976). According to Burri (1974), at birth collagen occurs as a layer of connective tissue in alveolar septa, the content of which increases with age and formation of alveoli. The increase in content between days 7 and 14 is significant for both control lungs and experimental lungs which is in accordance with Burri (1974) and Bradley et al. (1975), the latter study relating to rabbit lung collagen synthesis. According to the latter authors, there is a redirection of total protein synthesis (soluble and insoluble) towards collagen synthesis during the third trimester before birth which then flattens off in early adulthood. The actual control mechanisms for this switch can only be speculated on at this stage. However, it appears that it is at the transcriptional level that control

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is exerted (Bradley et al., 1975). The increase in collagen content during the third week of neonatal life of both control and experimental rat pups was not significant (Table 3.6). This implies that, by day 14, the rate of collagen synthesis is slowed down as the lung completes its development process. It is interesting to note that the period of relatively rapid collagen formation (Table 3.7) corresponds with the phase of rapid alveolarisation which occurs between days 4 and 13 after birth (Burri, 1974). This is an indication that a relationship exists with the formation of the collagen component of the connective tissue framework. Studies by Spooner and Faubion (1980) indeed illustrate that collagen synthesis is associated with branching of airways and blood vessels. Histological validation of the increased collagen content with age has already been provided in the previous chapter.

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Histological studies (chapter 2) suggests that the collagen contents between control and experimental groups at the three age levels is not significantly different. The quantitative data given in this chapter confirms this observation. This can be taken as an indication that nicotine exposure made no impact on neonatal lung collagen content. This lack of influence is in concert with the effect seen on soluble protein content of the lung tissue at different ages. Since collagen is associated with branching of airways (Spooner and Faubion, 1980) during lung development, it is conceivable that nicotine will also not affect the process of lung growth and development to maturation via changes in collagen contents.

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Of interest is the fact that changes in the quality of protein synthesised is noted histologically in reticular fibres. These changes have been pointed out at 14 days of age in chapter 2. Since reticular fibres are closely related structurally to the collagens, it is reasonable to assume that the causative mechanism for reticular fibre changes may also affect collagen quality (and maybe soluble proteins too) in the experimental neonatal lungs.

The structural changes of fibres referred to above have an added implication since the connective tissue content and rate of synthesis are determinants of airway branching and alveolar development. This could have a detrimental effect on further lung development and stability of the connective tissue skeleton of the lung. Kida and Thurlbeck (1980) have shown that, by interfering with the process of collagen cross-linking, changes may be brought about in the growth and structure of the lungs in later life. Spooner and Faubion (1980) have also stated that normal airway branching (implying cell division, mesenchymal and airway cell synthesis as well as control of the direction of growth), is dependent on connective tissue deposition. In other words, there is an interplay between connective tissue, its distribution and content, as well as the optimal formation of functional and stable lungs. This implies that a balance exists between cells and fibres. The balance in this case is not static but rather dynamic due to the ratios of the components changing to suit the needs and design of the adult lung. This can only be achieved through proper control mechanisms. Further investigation

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of the qualitative aspects of rat lung collagen is necessary, as well as the effects of this on lung connective tissue architecture and functional ability. It would also be of interest to use longitudinal studies to investigate what happens as regards recovery from the detrimental effects of nicotine on lung development and functionality.

3.4.3 Elastic tissue content:

It is already known that the early neonatal period is essential for complete lung development, especially the period up to 14 days of age. Alveolarisation still occurs in rat lung up to day 15 in the neonate (Angus and Thurlbeck, 1972). This process entails the thickening of septa and the formation of buds or secondary septa (Burri, 1974). Connective tissue synthesis plays a role in dictating these events. The elastic tissue component in particular plays a vital role in the development of secondary septa (Burri, 1974). By day 14 the presence of elastic tissue, as well as the tension introduced by the latter in lung tissue (Loosli and Potter, 1959; Burri, 1974), is thought to stimulate fibroblasts, in this way influencing the rate and direction of fibrous tissue development.

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Initially in the control group there is a significant increase in lung elastic tissue content from day 7 to day 14. The situation in the experimental group shows that elastic tissue was formed earlier than this. The initial increase is due to synthesis of elastic tissue during the second week of life, after which there is a marked thinning of the alveolar septa together

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with a higher mitotic activity due to tension introduced by the elastic tissue (Loosli and Potter, 1959; Burrie 1974). This is illustrated by the studies of Kalenga and Eeckhout (1989) who have determined elastic tissue content of 49 day old rat lungs. Their value obtained is approximately 10mg/g wet lung mass and is only marginally higher than the values obtained for 14 day old rat lungs used in this study (about 9,4mg/g).

What is of extreme importance is the fact that at day 7 there was a significantly lower ($p<0,05$) elastic tissue content in the controls compared to experimental lungs of animals of the same age. The reason is not known. In addition to this is the fact that this difference is negated by day 14, after which no significant differences are noted up to day 21. This clearly illustrates that elastic tissue formation was completed before day 7 in the lungs of rat pups exposed to nicotine via the placenta and mothers' milk.

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From these results, it appears as if nicotine exposure has an effect on elastic tissue synthesis in the fetal and early neonatal (prior to day 7) stages of lung development. If the rate of synthesis in the experimental lungs were increased prior to day 7, it would be expected that the peak of synthetic activity would be reached earlier. This also suggests that the experimental lung slows down its synthesis earlier. This is borne out by a 53,6% lower rate of synthesis of elastic tissue between days 7 and 14 while this difference decreases 11,9% between days 14 and 21. From this it may also be assumed that the rates of

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synthesis in later life would be similar when comparing control and experimental animals.

From the figures presented though, it is interesting to note that the rate of elastic tissue synthesis between day 7 and 14 is higher in the control lung (approximately double) while the rate of synthesis decreases to a similar extent in the third week of life for these animals. The intriguing question of the events prior to day 7 still remains to be answered. Maritz and Woolward (1992) have investigated neonatal lung elastic tissue in these laboratories using histological techniques on 1 to 21 day old rat pups. Experimental rat lungs at day 1 have a lower staining intensity for elastic tissue compared to the control animal of the same age. I have corroborated the latter histological studies for days 7 to 21 and also have shown an "apparent" disagreement with the quantitative studies presented here. The histochemical results show that, at 7 days of age, there is a lowered staining intensity for experimental lung elastic tissue than for appropriate control lungs, indicating a lower elastic tissue concentration. The findings of the present quantitative studies contradicts these results. Again, as with collagen quantitation, care must be taken when interpreting the histochemical information because, if there is any change in structure or quality of experimental elastic tissue, this could manifest itself in a lowered staining capacity. This again raises the question of altered quality and architecture of this connective tissue component. O'Dell <u>et al</u>. (1978) have noted that copper-deficient rats have a lowered activity of the cross-linking

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enzyme lysyl oxidase, leading to decreased staining properties of elastic tissue. Also, since the elastic tissue contents are not significantly different by day 21, it could be assumed that both control and experimental lungs are fully developed and functional in this respect. This agrees with histochemical evidence presented in chapter 2 regarding the similarities in elastic tissue content at days 114 and 21 in both control and experimental rat lung tissue.

A crude indicator of possible qualitative differences between the two groups has already been mentioned in section 3.1.3(c). When centrifuging the insoluble elastic tissue during the quantitative determination, a solid pellet was formed for control animal elastic tissue. For the experimental lungs, the pellet formed was not solid but a jelly-like mass which did not adhere to the centrifuge tube. This necessitated the removal of the supernatant by aspiration.

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The importance of elastic tissue for optimal lung development and functionality has already been pointed out. Although the histochemical and quantitative studies yield seemingly opposing results (for whatever reason), the important point is that there is a difference indicated. In particular, there are major morphological differences between the lung structures i.e. the emphysema-like lesions of the experimental lungs. Lung elastic tissue is found predominantly at the region of the alveolar septa. Amy *et al*. (1977) and Spooner and Faubion (1980) have shown that the development of connective tissue in general, and

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elastic tissue in particular, is necessary to dictate airway branching and the correct structure of alveoli. Any interference with connective tissue development during this sensitive phase would have serious implications for alveolarisation. This seems to be the case in this study, even though there is sufficient elastic tissue in the experimental lungs, there is disruption or interference with alveolar development, leading to emphysema-like changes observed under the light microscope. This could be due to the interfering effect of nicotine on the quality of elastic tissue produced. This could affect alveolar formation during the critical period up to day 14. After this, a recovery of the ability to form mature elastic tissue may have taken place, leading to both quantitative and histochemical studies eventually showing no obvious differences.

Emphysema-like changes, as much as being due to interference with the synthesis of elastic tissue (and collagen) under the influence of nicotine (or cigarette smoke), could also be due to impaired degradation of these connective tissues. A number of reviews regarding the protease-antiprotease hypothesis have attempted to summarise this process. Laurel and Eriksson (1963) first put forward this hypothesis which blamed increased elastolysis on the deficiency of a specific protease inhibitor (α-1-antitrypsin). It is unlikely that a deficiency of the antiprotease could be implicated in possible increased elastolytic activity (Janoff and Carp, 1977). Maritz and Woolward (1992) showed a lowered radial alveolar count for experimental lungs at each age group studied. This lowered count is ascribed

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to alveolar rupture as illustrated morphologically in chapter 2. These studies infer that this alveolar fragility, which results in emphysema-like lesions, is due to a more uneven distribution of elastic tissue in the lung septa, even though there is no significant quantitative difference.

3.4.4 Conclusion:

There is a definite element of interference in the synthesis of elastic tissue and reticular fibres by exposure to nicotine in the neonatal rat lung. The level of interference seems to have two facets:

(1) alteration in terms of content in the case of elastic tissue where it is significantly higher in the 7 day old experimental group compared to the control group. As already pointed out, there may be an element of structural interference too.

(2) alteration in terms of quality with respect to deformed reticular fibres noted at all age groups in the experimental group.

The evidence available at this stage does not rule out structural alterations of collagen but does point out that there is no significant alteration of collagen content between the two groups.

All of these changes, since they occur early in life, together may have a profound influence on later lung stability, susceptibility to disease and any other factor which could impinge on lung morphology and function. To ascertain the extent

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to which nicotine interferes with lung connective tissue biochemistry and, in particular, lung connective tissue architecture, scanning electron microscopy of the lung at different age groups was decided upon. Since possible changes in scleroprotein structure is also anticipated by these results, either protein sequencing for both groups or analysis of genetic material (using molecular biological techniques) is indicated. The feasibility and applicability of the latter studies need to be investigated.

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CHAPTER 4

ELUCIDATION OF NEONATAL LUNG CONNECTIVE TISSUE ARCHITECTURE WITH THE AID OF A SCANNING ELECTRON MICROSCOPE

4.1 Introduction:

The picture of the lung gained from the previous two chapters show the following important characteristics for the experimental tissue:

(a) definite morphological changes to alveolar structure.

(b) changes to both the quality and quantity of the elastic tissue component at day 7.

(c) definite alteration of reticular fibre structure and arrangement at all three age groups studied.

The aim of this chapter was to translate these changes into a three-dimensional picture regarding the connective tissue framework of the experimental lung tissue.

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In addition to all the biochemical analyses (quantitation, characterisation of connective tissues) and physiological functionality tests (pressure-volume curves) as well as physico-chemical studies (elastic recoil), another important aspect to emphysema and those conditions related to it is the connective tissue quality-architecture relationship. Because emphysema is a final stage in itself, the actual initiation and propagation steps leading to this phase is not easily determined or studied (Laros, 1972). Because a wide variety of factors may induce emphysema, it stands to reason that all these factors must have

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some common effects, in all likelihood via a common component of the lung. It is not difficult to understand that the initiation of emphysema, or any other condition involving tissue or organ injury, is mediated via a first step involving cell injury (Cottran, 1989; Maritz et al., 1993). The term "injury" refers not only to physical insult to a cell but also chemical modulation leading to cell damage and ultimately a multifaceted condition such as the emphysematous lesions as noted in the light microscopic examination in chapter 2. Laros (1972) contends that either changes to the connective tissue component or the ground substance or both may be the basis of these changes. The previous chapter has concentrated on quantitation and also dealt in speculation regarding the connective tissue component. Bradley et al. (1975) states that collagen fibres are found predominantly in the ground substance. Sharon (1974) showed that hyaluronic acid (a component of the ground substance) also anchors elastic fibres. This means that, with respect to lung architecture, the connective tissues must be viewed as one of a pair of component roleplayers. The cementing role of the ground substance is also important in cell-cell adhesion due to its glue-like properties (Sharon, 1974).

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In the light of the above facts, a study of the three-dimensional ultrastructure of the developing alveoli, as well as the connective tissue framework, is appropriate. Laros (1972) has indicated a loss of cementing properties of the ground substance due to the alteration of the glucosamine:galactosamine ratio in emphysematous lungs. This could manifest itself in increased

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alveolar fragility leading to emphysematous lesions. Changes in collagen, and possibly elastic tissue, cross-linking due to cigarette smoke could also induce this fragility of the alveolus (Kida and Thurlbeck, 1980; Rickert and Forbes, 1972).

Since we are interested in the development of emphysematous lesions due to nicotine, or other pollutants, the further study of the lung ultrastructure may give some important clues as to the exact component which is first affected in this process.

This study was divided into two subsections:

(a) an ultrastructural study of sectioned lung of appropriate control and experimental animals before alkali digestion

(b) an ultrastructural study of lungs after alkali digestion. This will enable investigation of the connective tissue framework of the lung at a three-dimensional level.

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In order to study the three-dimensional connective tissue structure of the lung, it was necessary to first develop the technique of removing all cellular components in order to expose the connective tissue framework. Due to the higher fragility of neonatal lung at day 7 versus that of the 21 day old rats, adjustments were made to accommodate this. It is therefore necessary to report in detail on the technique followed.

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4.2 Materials and Methods:

4.2.1 Preparation for scanning ultramicroscopy:

(a) Principle:

The technique used to examine the different lung specimens is based on accepted procedures regarding tissue preparation and microscope operation as applied to scanning electron micrography. The basic principle involves dehydration of the respective tissue samples in graded acetone followed by critical point drying and then sputter coating with a 5nm gold layer. For studies of the connective tissue framework, lung samples were digested with NaOH for appropriate periods (see later) and prepared for scanning electron microscopy as for the undigested samples.

(b) Reagents and Equipment:

1. Hitachi HPC-2 critical point dryer.

2. Edwards S150B sputter coater.

3. Hitachi model X650 scanning electron microscope.

4. Acetone (analytical grade).

5. Xylene (analytical grade).

6. Sodium hydroxide pellets (analytical grade).

7. Pill bottles.

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(c) **Procedure:**

1. Methods for the operation of each instrument was according to manufacturers specifications.

2. Tissue samples used were remaining wax blocks from light microscopic examination as described in chapter 2. The respective tissue samples in the wax block was cut into pieces approximately $3mm^3$ with a sharp scalpel. Minimal tissue damage occurs due to the support provided by the wax blocks.

3. The tissue chips produced in this way were placed in a new labelled cassette and the wax removed in a Shandon tissue processor using the protocol in Table 4.1:

	SOLVENT	TIME
1.	xylene	1 hour
2.	xylene	30 minutes
3.	100% alcohol	30 minutes
4.	95% alcohol	15 minutes
5.	90% alcohol	15 minutes
6.	80% alcohol	15 minutes
7.	70% alcohol	15 minutes
8.	distilled water	30 minutes
9.	distilled water	30 minutes

Table 4.1: Protocol for dewaxing of embedded lung tissue.

4. For examination of alkali digested lung tissue, the tissue was treated according to the method in step 5 below. For examination of the undigested lung tissue, the relevant

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tissue pieces were taken from the distilled water to 100% acetone in 50ml pill bottles using the protocol in Table 4.2. During each phase the bottles were agitated gently. This dehydrated tissue was then dried by the critical point method, sputter coated and examined with the scanning electron microscope.

	SOLVENT	TIME
1.	70% acetone	30 minutes
2.	80% acetone	30 minutes
3.	90% acetone	15 minutes
4.	95% acetone	15 minutes
5.	100% acetone	15 minutes
6.	100% acetone	15 minutes

Table 4.2: Protocol for acetone dehydration of lung tissue prior to critical point drying and sputter coating.

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5. Tissue pieces from step 3 were transferred to 20ml pill bottles containing NaOH for an appropriate period of time (the concentration of NaOH and time of digestion differs for different types of tissue and size of sample pieces - see section 4.4.1 for determination of these parameters). Every second day half the NaOH was siphoned off and replaced with fresh NaOH solution.

6. The fragile digested tissue was then rinsed in distilled water and dehydrated according to the protocol in step 4 and prepared for viewing under the scanning electron microscope.

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4.2.2 Optimisation of lung digestion:

The method used by Wang and Ying (1977) for the digestion of lung tissue and the subsequent exposure of the connective tissue framework (in particular alveolar elastic tissue) was modified to suit the conditions and requirements of this phase of the study. The above authors digested both human and rabbit lung tissue for up to 25 days in 0,1M NaOH to elucidate their particular structural characteristics. The process is time-consuming as it was intended to show the effects and structures exposed by slow hydrolysis whereas the present study simply aims to expose the connective tissue framework. Therefore, to make this process more efficient for this study, a number of parallel digestions of both control and experimental 21 day old lung tissue was undertaken using two different concentrations of NaOH, namely 0,75M and 1,0M. The protocol employed for digestion is presented in Table 4.3.

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NaOH CONCENTRATION	DIGESTION TIME		
0,75M	64 HOURS	5,5 DAYS	9,5 DAYS
1,0M	64 HOURS	5,5 DAYS	9,5 DAYS

Table 4.3: Processing time and concentration for optimisation of digestion procedure using both 21 day old experimental and control animal lung tissue.

4.3 Results:

4.3.1 Undigested lung structure:

Photomicrographs are presented of both control and experimental neonatal rat lungs at various stages of development. The lungs

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of 7 day old control rat pups (Figure 4.1) have smooth-walled alveoli with just-perceptible gaps occurring between some component cells. The corresponding experimental lung (Figure 4.2) shows alveoli with an uneven wall surface with very clear demarcations or gaps between cells. In addition, pores or fenestrations are noted in some instances, as well as the odd erythrocyte in the alveolar cavity.

Experimental lung tissue at 14 days of age (Figure 4.3) shows a less organised structure than the control animal of the same age (Figure 4.4), especially regarding the more continuous and smoother outline of the alveolar ridges of the control group.

At 21 days of age, more profound differences are noted between control and experimental rat lung tissue. Figure 4.5 shows control rat lung tissue with relatively smooth alveolar walls surrounded by well-developed, intact alveolar ridges. The experimental rat lung tissue (Figure 4.6) by comparison has a much more uneven alveolar wall surface. Alveoli also appear to be more shallow than in control lung. The alveolar ridges in this case also appear fragmented and ragged.

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4.3.2 Optimisation of lung digestion:

When compared to Figures 4.1 and 4.2, it is obvious that digestion for 64 hours in 0,75M NaOH causes very little obvious changes to control (Figure 4.7) and experimental rat lung structure (Figure 4.8). In some cases, small areas of digestion can be seen, but this is too small to allow further study of the

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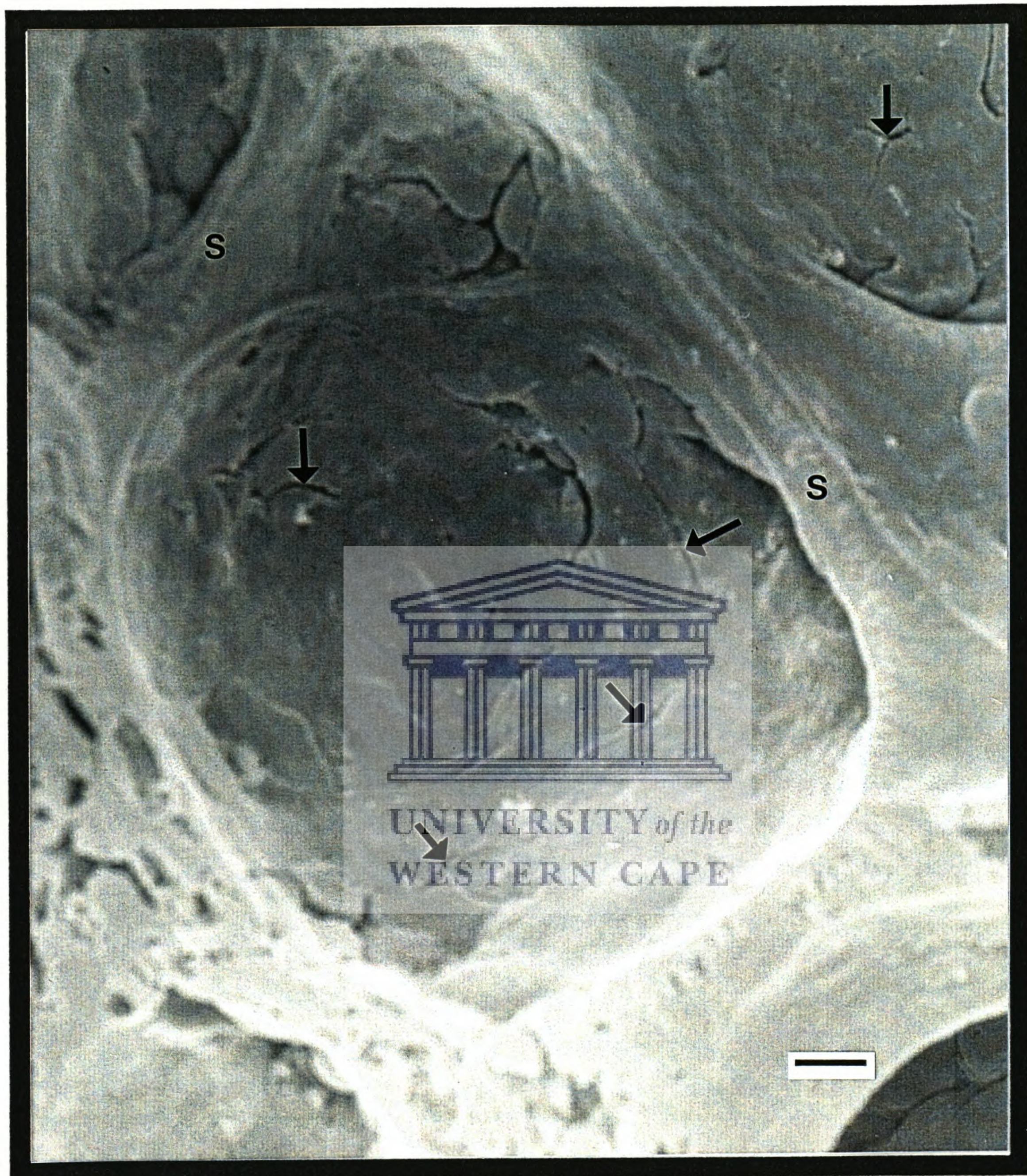

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Figure 4.1: 7 Day old control rat lung (undigested). Arrows indicate borders between alveolar cells. Smooth alveolar ridges (S) are also visible. Bar = 5μm.

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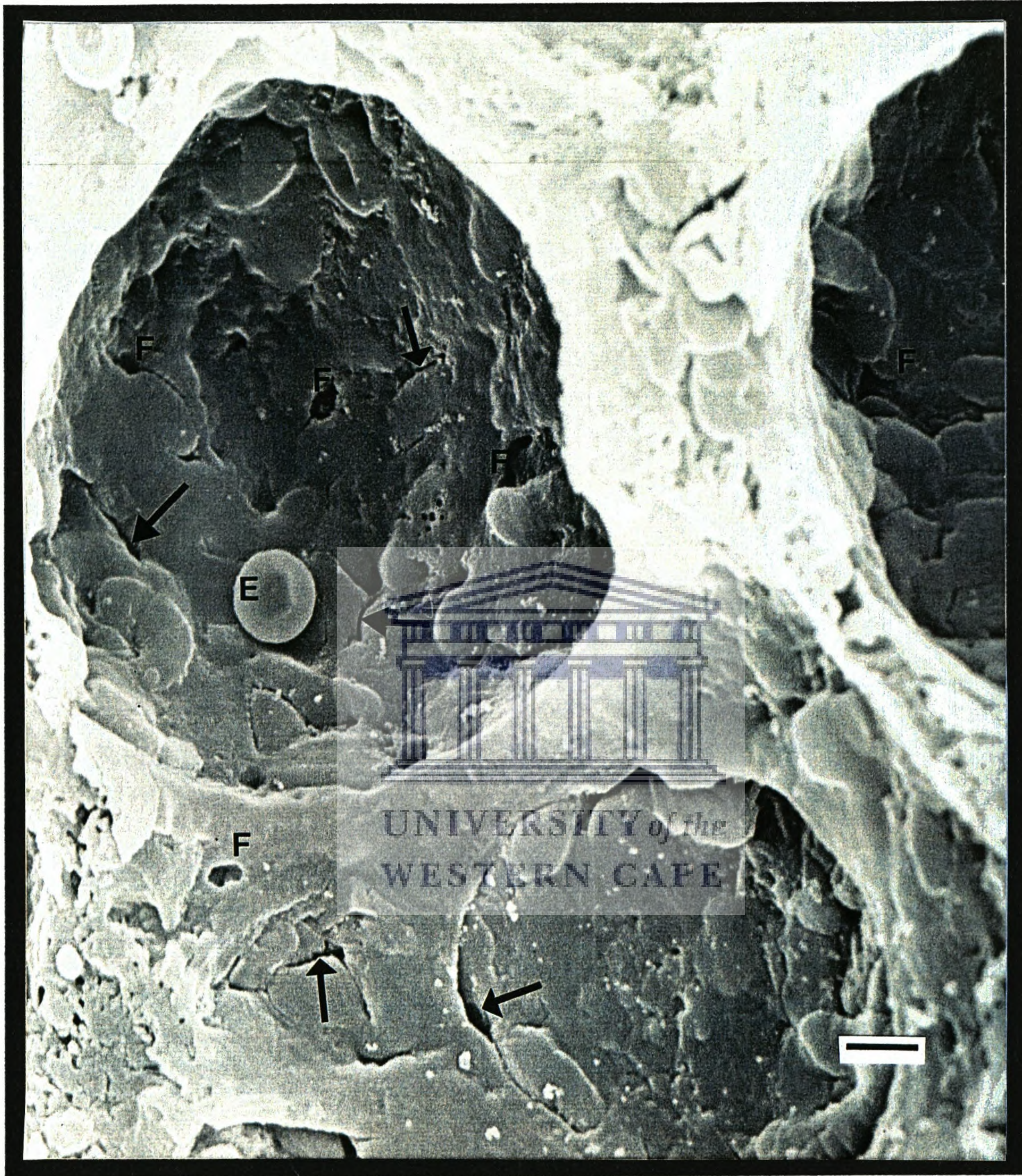

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Figure 4.2: 7 Day old experimental rat lung (undigested). Abnormal fenestrations (F), erythrocytes (E) in alveolus as well as large intercellular gaps (arrows) noted. Bar = 5μm.

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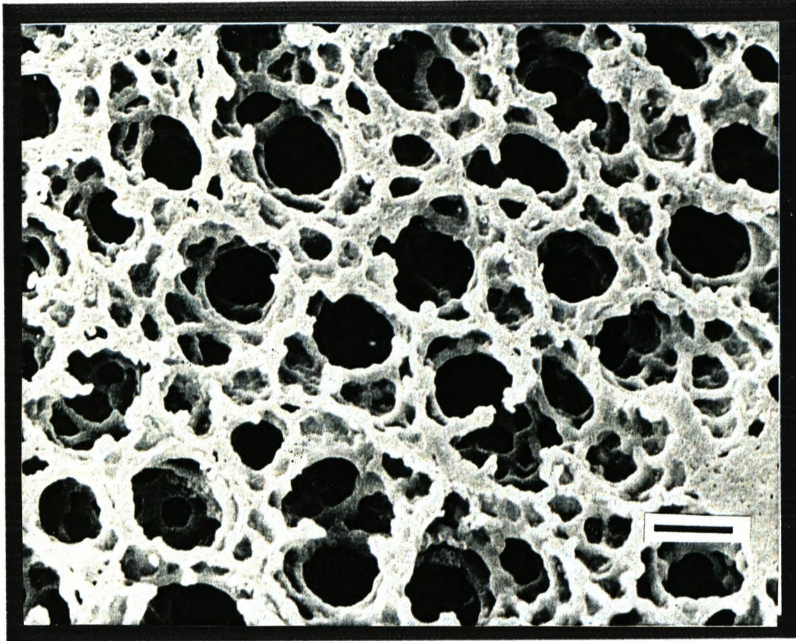

Figure 4.3: 14 Day old experimental rat lung (undigested). Irregular lung structure noted. Bar = 44μm.

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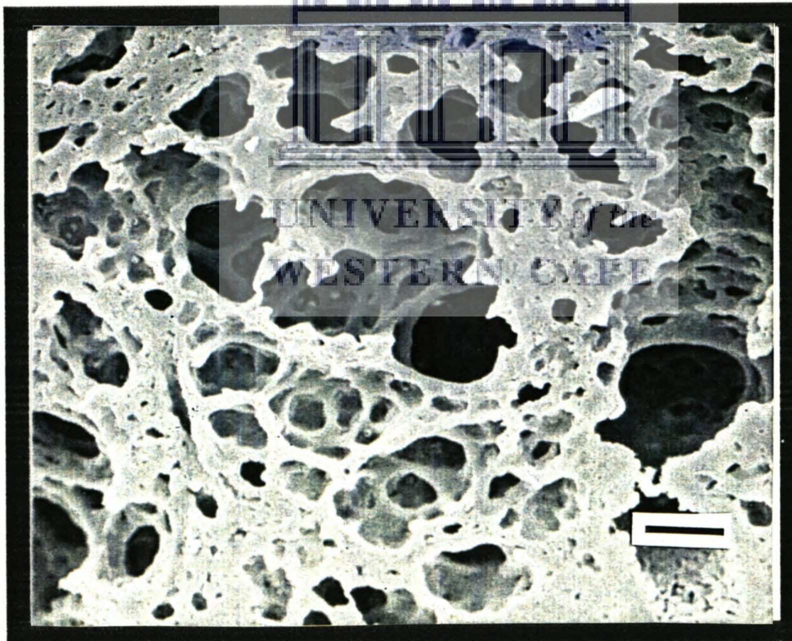

Figure 4.4: 14 Day old control rat lung (undigested). More regular and smooth lung structure compared to fig. 4.3. Bar = 44μm.

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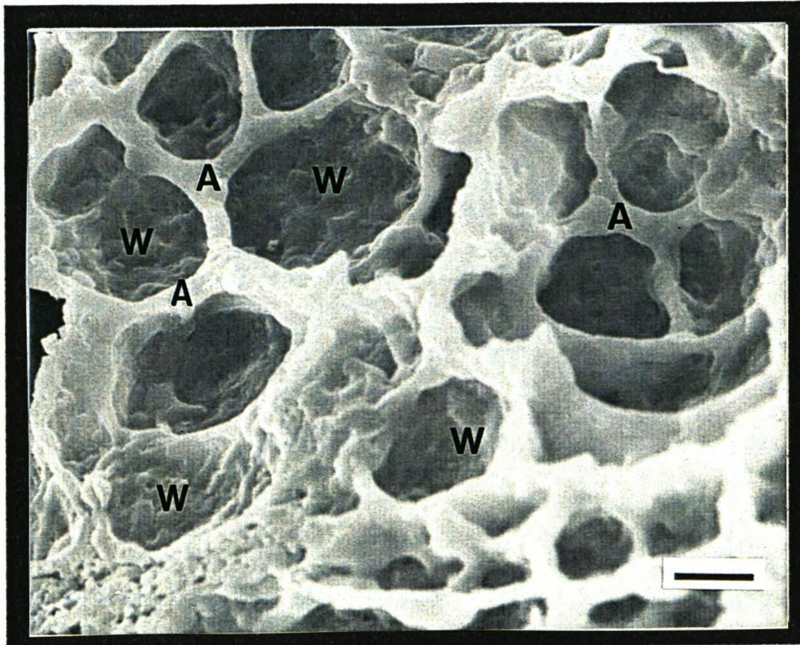

Figure 4.5: 21 Day old control rat lung (undigested). Smooth alveolar ridges (A) and walls (W) are evident. Bar = 14μm.

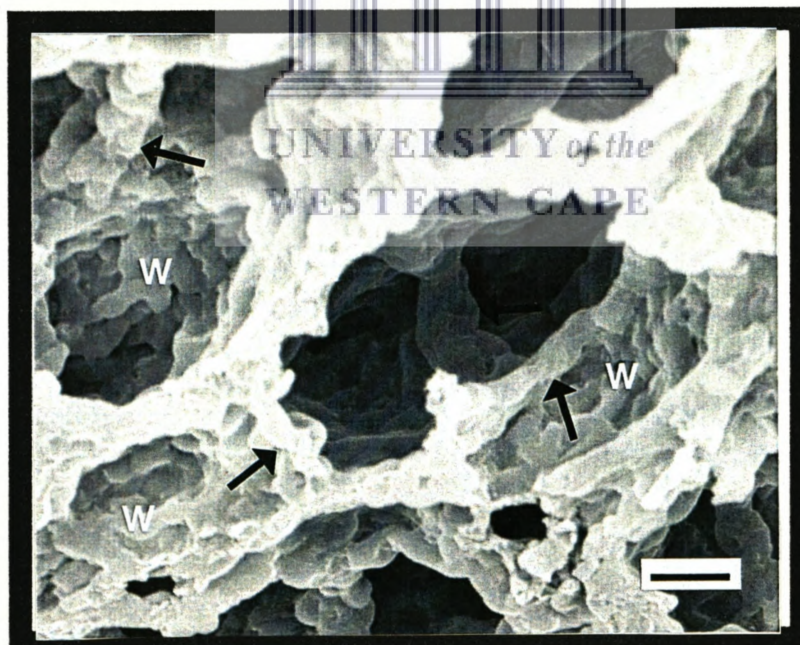

Figure 4.6: 21 Day old experimental rat lung (undigested). Unevenly thickened alveolar ridges (arrows) and walls (W) are evident. Bar = 14μm.

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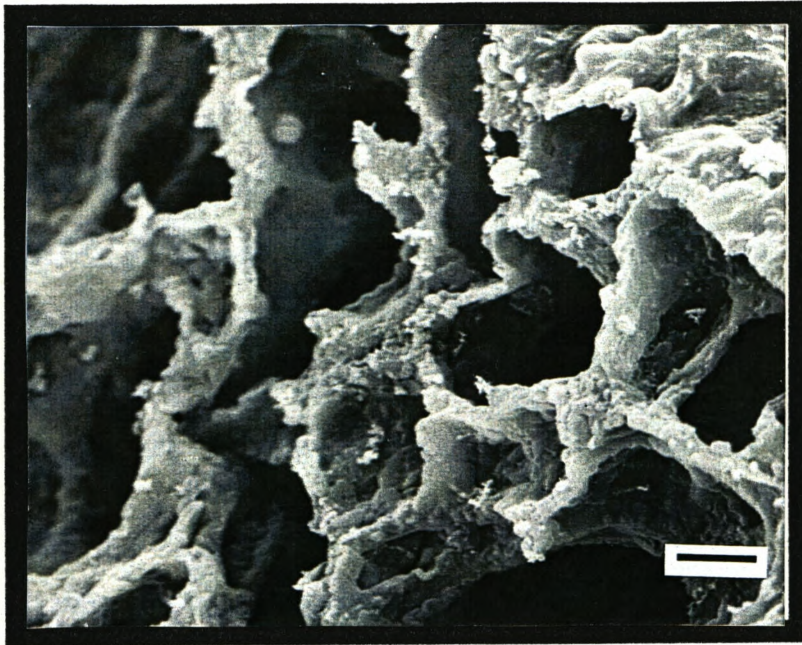

Figure 4.7: 21 Day old control rat lung after 64 hours in 0,75M NaOH. Bar = 16μm.

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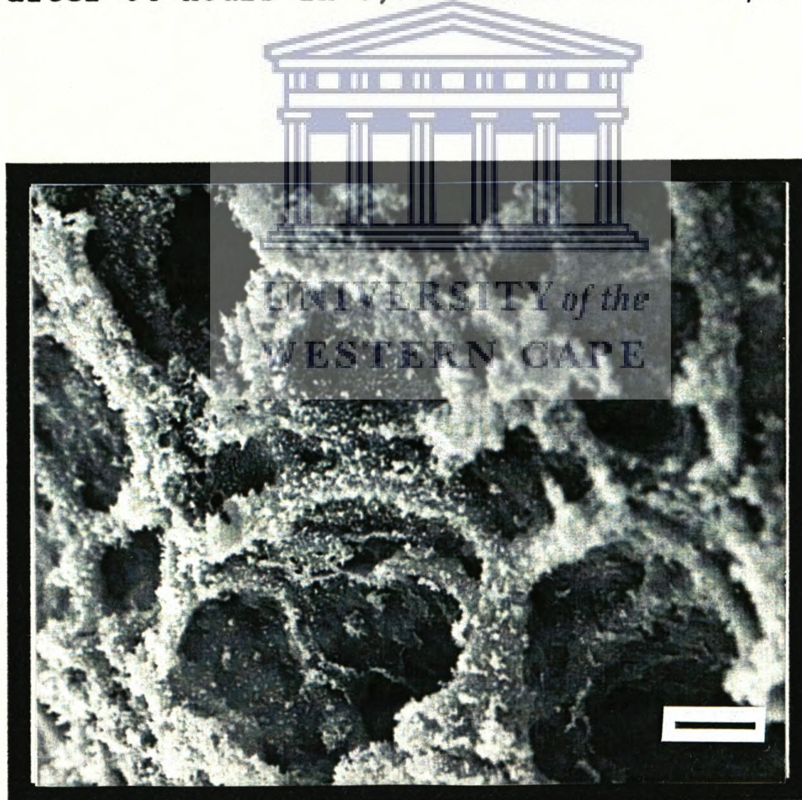

Figure 4.8: 21 Day old experimental rat lung after 64 hours in 0,75M NaOH.Bar = 16μm.

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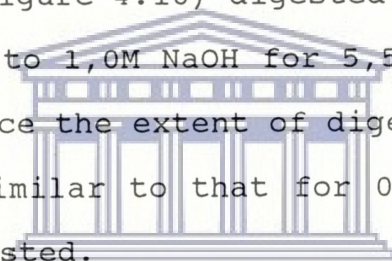
connective tissue architecture. In one particular case, the undigested alveoli of an experimental lung specimen showed a particularly ragged (Figure 4.8) or frizzy appearance but no fibrous tissue was evident. Digestion for the same period of time in 1,0M NaOH showed more, but not sufficient, signs of tissue removal (results not shown here). Fibres are exposed but still much tissue mass is evident for both control and experimental rat lungs.

Digestion for 5,5 days in 0,75M NaOH shows further signs of tissue removal in control animals (Figure 4.9), while in experimental animals (Figure 4.10) digested alveolar ridges are also evident. Exposure to 1,0M NaOH for 5,5 days shows similar signs of digestion. Since the extent of digestion was still not complete and appears similar to that for 0,75M NaOH, a longer digestion period was tested.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Digestion of 21 day old experimental lung tissue in 0,75M NaOH for 9,5 days showed adequate digestion, exposing fibres, ridges, vascular tissue and other alkali-resistant tissue elements (Figure 4.11). In particular, thick ridges and thin elastic tissue fibres were present in the same tissue preparation. Digestion in 1,0M NaOH for 9,5 days caused serious disintegration of tissue elements (not shown here), leading to an unmanageable tissue mass which proved too difficult to process further.

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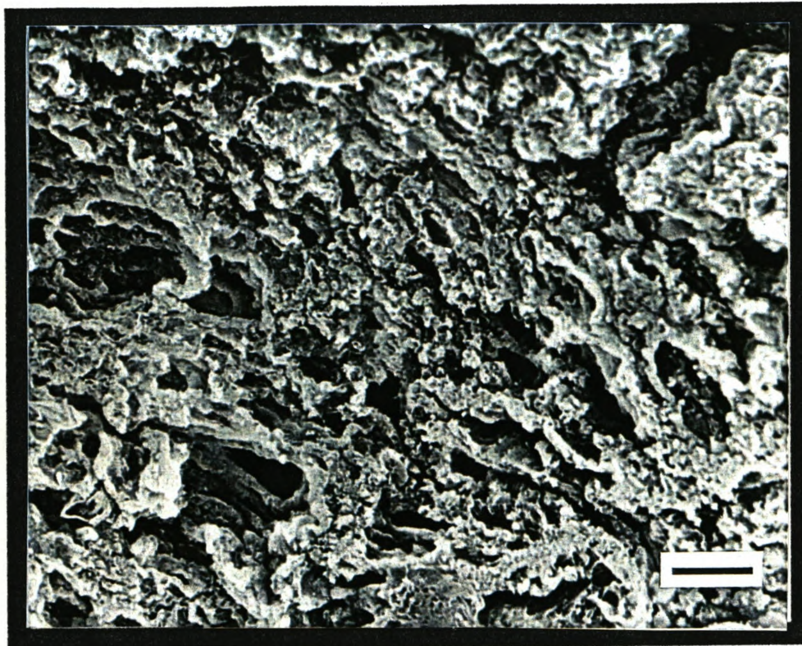

Figure 4.9: 21 Day old control rat lung after 5,5 days in 0,75M NaOH. Bar = 44μm.

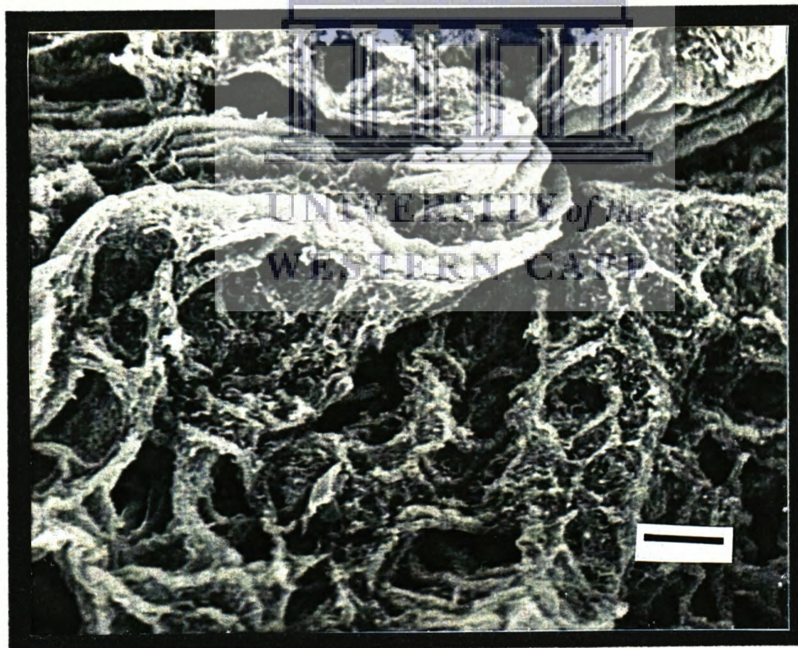

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Figure 4.10: 21 Day old experimental rat lung after 5,5 days in 0,75M NaOH. Bar = 44μm.

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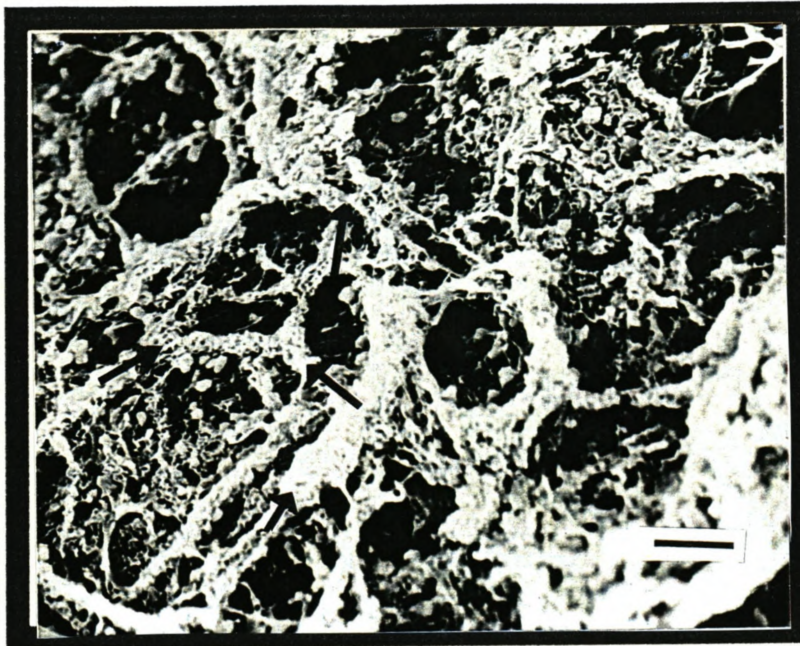

Figure 4.11: 21 Day old experimental rat lung after 9,5 days in 0,75M NaOH. Arrows indicate chains of connective tissue. Bar = 14μm.

4.3.3 Connective tissue framework:

Seven day old control lung shows a connective tissue skeleton consisting of fibres which clearly outline alveolar ridges (Figure 4.12). The thin fibres are complete and uninterrupted. Thick bands of fibres form whorls indicative of alveolar ridges (Figure 4.13). The connective tissue skeleton shows sheets of fibres presumably containing collagen. The connective tissue framework of 7 day old experimental lungs appear different to the control lung tissue in that a less smooth and more fuzzy appearance is apparent (Figure 4.14). Whorls of connective tissue indicating alveolar sacs and alveoli are also seen. At higher magnification the experimental lung connective tissue shows numerous breaks as well as presenting a "spiky" appearance

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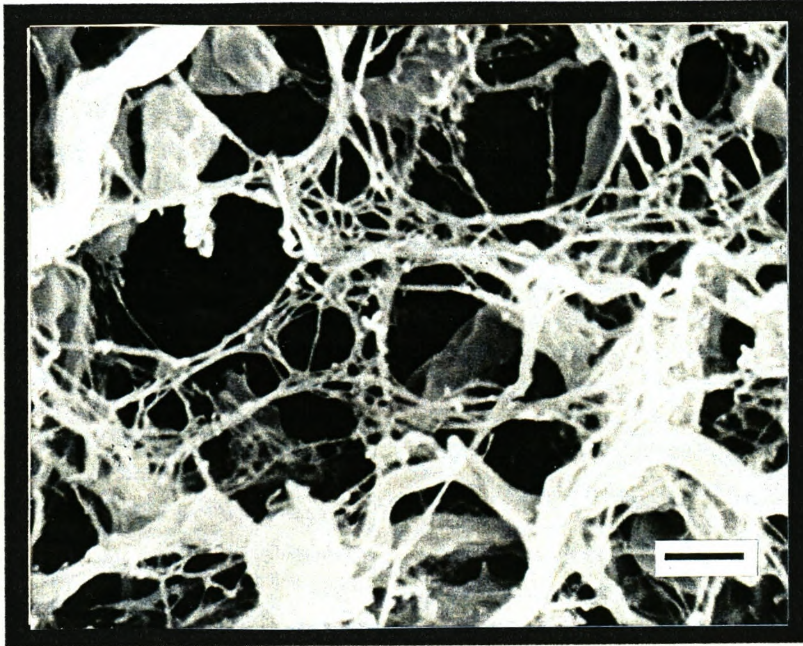

Figure 4.12: 7 Day old control rat lung (digested). Bar = 2μm.

Figure 4.13: 7 Day old control rat lung (digested). Thick fibre bands of alveolar ridges (arrows) are visible. Bar = 5μm.

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reminiscent of barbed wire (Figure 4.15). A beaded appearance of the fibres are also noted in experimental connective tissue. In both control and experimental rat lung tissue the fibres of connective tissue seem to intertwine around each other.

Fourteen day old rat lung tissue shows similar characteristics to those described above. Outlines of alveolar ridges are formed by the connective tissue fibres as well as associated blood vessel connective tissue (the latter is not seen individually but it is deemed to be present). The control lung tissue of 14 day old rats (Figure 4.16) shows intact fibres of the connective tissue framework. Experimental lung tissue (Figure 4.17) has fibres and other structures which show a hairy or spiky appearance. Many of the fibres appear to be broken or incomplete in the experimental lung tissue. Difficulty was experienced with the digestion process at this stage as evidenced by the well-digested experimental lung tissue as opposed to a less-digested control lung. At day 21, the control lung tissue (Figure 4.18) has a well-filled appearance with strongly demarcated whorls of connective tissue and vessel elements. Alveoli are easy to identify in this mass of digested remains while primary and secondary septa are easily identified by the chains and ringlets of connective tissue formed. At higher magnifications the primary ridges are clearly seen to consist of remains of vessel elements plus fibres (Figure 4.19), presumably collagenous in nature. A strong elastic tissue network forms the background to alveolar structure. The latter structure forms both thick and thin elastic fibres. It is difficult to identify any broken or damaged fibres

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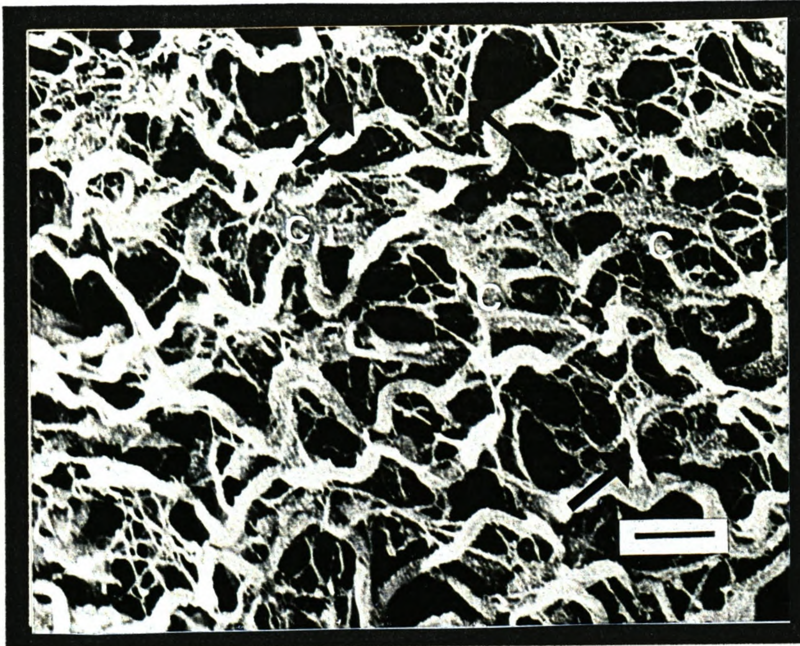

Figure 4.14: 7 Day old experimental rat lung (digested). Alveolar ridge fibres (arrows) and furry connective tissue patches (C) are visible. Bar = 5μm.

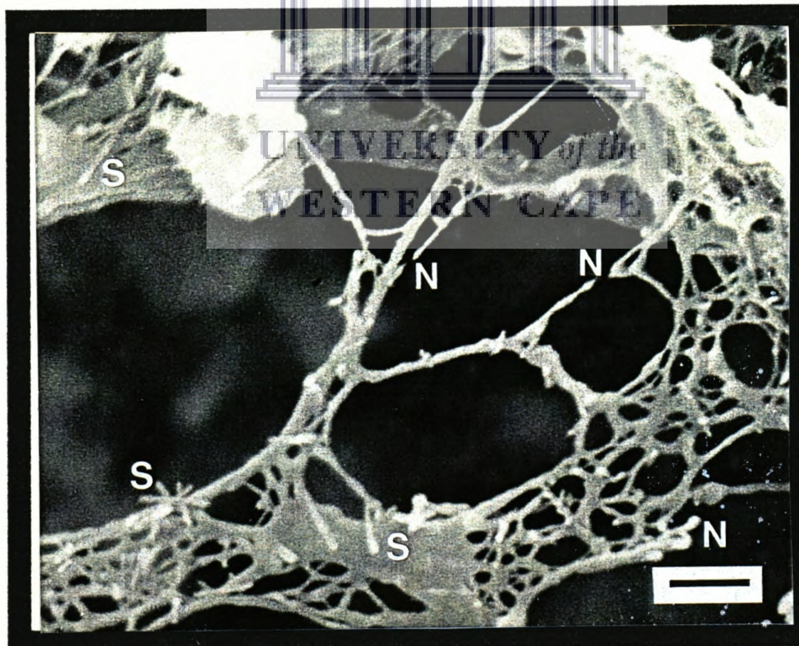

Figure 4.15: 7 Day old experimental rat lung (digested). Broken fibres (N) and spikes (S) are evident. Bar = 2μm.

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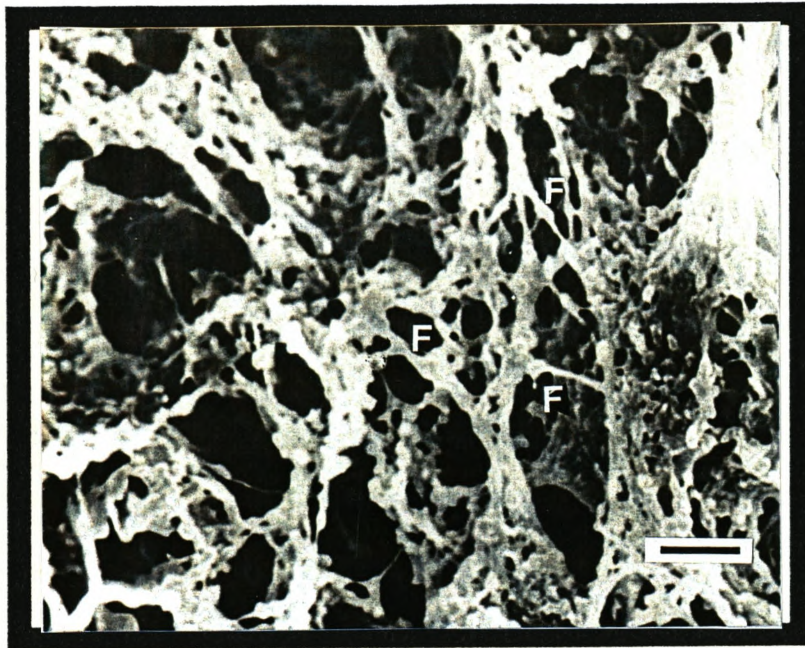

Figure 4.16: 14 Day old control rat lung (digested). Intact fibres (F) are evident. Bar = 20μm.

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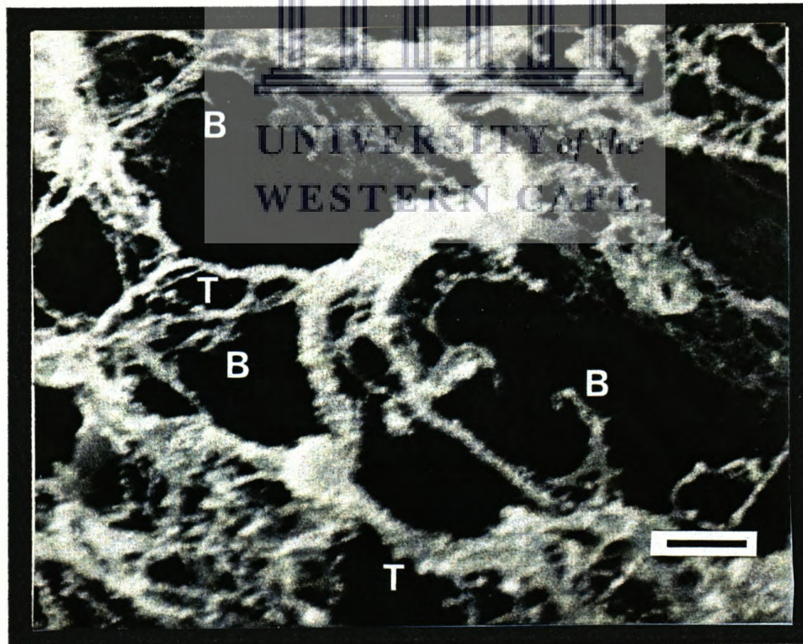

Figure 4.17: 14 Day old experimental rat lung (digested). Broken fibres (B) abound as well as fuzzy fibre bundles (T). Bar = 2μm.

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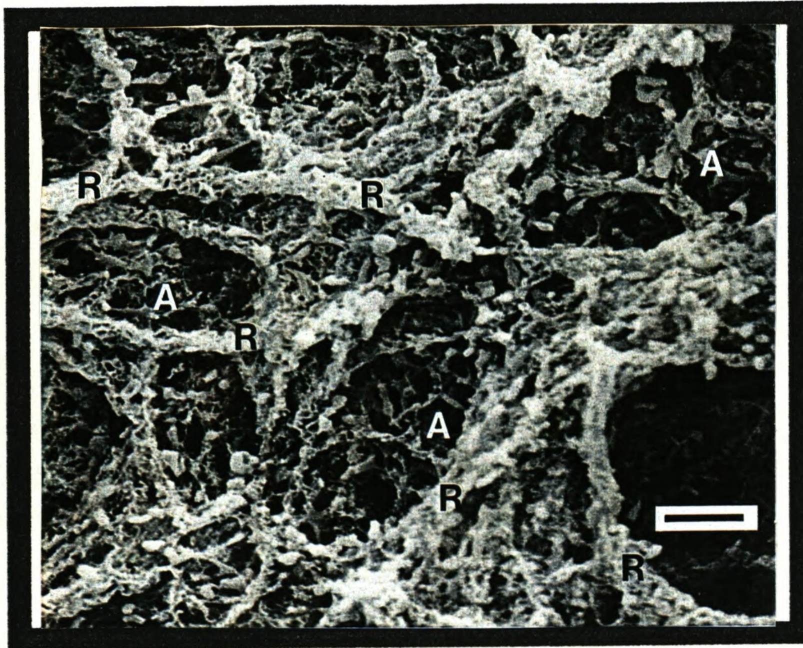

Figure 4.18: 21 Day old control rat lung (digested). Connective tissue bundles of major ridges (R) are visible as well as outlines of alveoli (A). Bar = 14μm.

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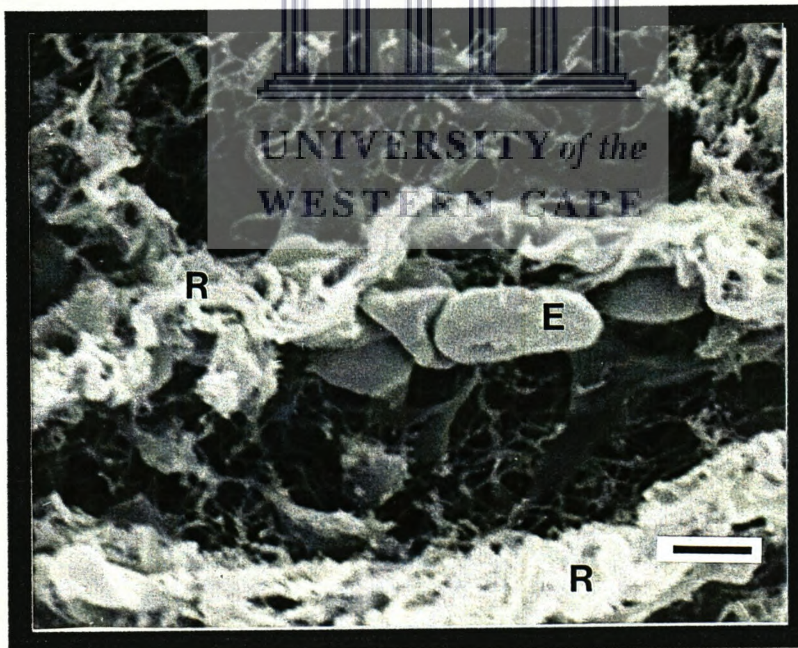

Figure 4.19: 21 Day old control rat lung (digested). Major alveolar ridge fibres (R) and remnants of erythrocytes (E) are present. Bar = 2μm.

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in the control lung tissue. The experimental 21 day old lung tissue, after digestion, also shows well-defined primary and secondary ridges (Figure 4.20). The background elastic tissue network is also well developed, but not as dense as that of the appropriate control lung i.e. it has a grainier or coarser appearance. This lower network density is more apparent (Figure 4.21) while the primary and secondary ridge structure appears similar to that of the control lung. The furriness or spikiness associated with experimental lung tissue of younger animals is not as marked here, but it is still noticeable in places. Many broken or incomplete elastic tissue fibres are noted in the experimental lung (Figure 4.21).

4.4 Discussion:

4.4.1 Undigested lung structure:

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Results for the 7 day old control and experimental rat lung tissue indicates that, even at this early stage of development, nicotine has a structural effect on the experimental rat lung, both for the alveolus as a whole and a substructural effect (uneven alveolar wall surface and loss of cell-cell adhesion). The oval-shaped appearance of the alveoli is in agreement with the histological findings reported in chapter 2. The uneven alveolar surface is indicative of some form of alveolar wall derangement. The fact that the cells in the alveolar wall of the experimental rat lung seems to be losing physical contact with each other may be due to abnormal cell adhesion. The cementing role of the ground substance in this regard has already been

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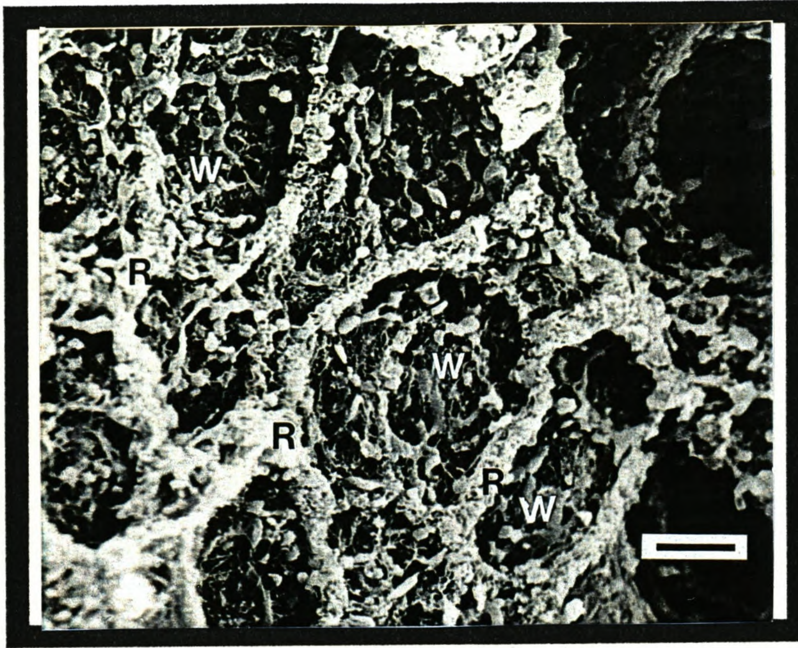

Figure 4.20: 21 Day old experimental rat lung (digested). Alveolar ridge (R) and alveolar wall connective tissue (W) are indicated. Bar = 14μm.

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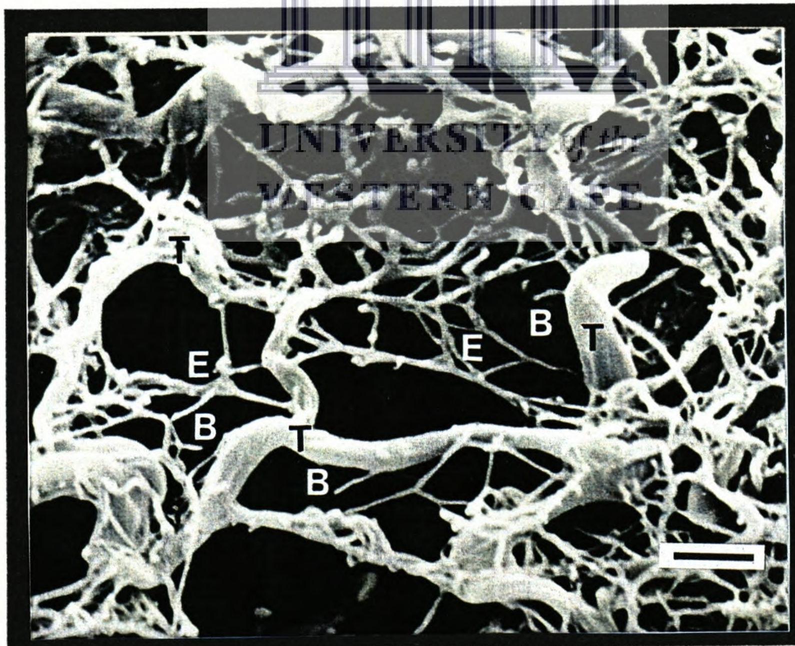

Figure 4.21: 21 Day old experimental rat lung (digested). Thick connective tissue fibres (T), thin elastic fibres (E) and broken fibres (B) are noted. Bar = 2μm.

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discussed in chapter 2. The possible change in composition and its relationship with the connective tissue component of lung tissue in the experimental rat lung could also account for this lack of cell-cell adhesion (Pump, 1974). Alternatively, a weakened alveolar wall, due to connective tissue alterations, could also rupture due to normal force distribution. Laros and Kuyper (1976) have already described the role of both the scleroproteins and the cementing ground substance in maintaining alveolar integrity. The appearance of erythrocytes in the alveolar cavity is also indicative of a form of tissue damage with subsequent leakage of blood into the alveolar cavity. Studies by Maritz et al. (1994) indeed indicated rupturing of blood-air barriers of nicotine-exposed neonatal lung. It is entirely plausible that alveolar damage is due, in part, to alteration of the connective tissue integrity, elastic tissue in particular (Maritz and Woolward, 1992). Alterations in elastic tissue quantity or quality could lead to alveolar rupture due to the loss of elasticity. This could also account for the appearance of alveolar pores or fenestrations of abnormal size reported by Maritz et al. (1994) in 2 to 3 week old animals.

The above findings in 7 day old rat pup lungs is carried through in 2 week old pups with similar differences noted between control and experimental animals. These abnormal conditions in the experimental neonatal rat lungs intensifies by day 21. This indicates an ongoing process of damage. This seems out of step with histological and biochemical studies regarding elastic tissue i.e. by day 21 the experimental rat lung has "caught up"

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with respect to elastic tissue. The three-dimensional morphology exhibits a more serious degree of damage. Maritz et al. (1994) have indicated the appearance of membrane blebs and alveolar wall rupture.

Many different reasons have been put forward for alveolar damage of this nature and for emphysematous lesions in rat neonatal lung. A central theory is the elastase-antielastase hypothesis balance (Laurell and Eriksson, 1963). Further supporting evidence obtained in subsequent phases of this study needs to be viewed together to obtain a more rounded picture of the possible processes involved in lung damage due to nicotine exposure. The underlying mechanism, involving the first step of cell damage, is not clearly indicated here. Loss of cellular adhesion could lead to abnormal force distribution over the lung tissue, this latter event promoting alveolar rupture. Intercellular communication may also be disturbed by this occurrence, introducing asynchrony in the functioning of the alveolar cell population.

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4.4.2 Optimisation of lung digestion:

In order to expose the connective tissue framework of the lung, it was necessary to digest all the cellular components and structures. Weak alkaline solutions (0,1 - 1,0M NaOH) are known to digest tissue components. Time of digestion as well as sample size affects the completeness of digestion but since relatively uniform pieces of tissue were cut from a lung sample, the latter factor is not significant in this instance. Since the aim of this

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phase of neonatal lung study was to determine the lung connective tissue structure in three dimensions, it was imperative that the digestion process/protocol be such that only connective tissue remains. Prolonged digestion of tissue will eventually lead to complete dissolution of even the connective tissue components. Prolonged digestion could also lead to artefactual changes of the connective tissue skeleton of the lung.

From the results presented it is obvious that digestion in 0,75M and 1M NaOH for up to 5,5 days did not result in complete exposure of the connective tissue skeleton. Too much interstitial tissue remains and would interfere with any investigation of the scleroprotein framework. Since digestion in 1M NaOH for 9,5 days affected the lung integrity and manageability so badly, it was also discarded as a method of choice. A digestion period of 9,5 days in 0,75M NaOH was decided upon. This period would also be more than sufficient to digest 14 day old and 7 day old rat lung tissue since these tissues are less developed and hence more easily digested. Since temperature could also play a role in the rate of digestion, the reaction should be allowed to proceed at a temperature between 18 to 22°C.

4.4.3 Connective tissue framework:

When comparing the connective tissue framework of 7 day old rat lung, the control and experimental lung specimens show very definite differences. At low magnification the whorls of connective tissue appear denser in the control than in the experimental lung tissue, probably due to a higher density of

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fibres in the control lung (Figure 4.12). This seems to suggest a defective rate of fibre synthesis or degradation in the experimental rat lung. This assumption is a sweeping one at this stage as there are a wide variety of possible causes for this situation, including post-translational modification of fibres as well as the quality of fibres synthesised. Since fibre deficiency seems to occur primarily between alveolar ridges, it is assumed to be mostly a deficiency of elastic tissue, since this area of the alveolus consists largely of elastic tissue. This finding appears to be contrary to the biochemical quantitation of elastic tissue where the content of the latter component is significantly higher in the experimental lung than in the control rat lung tissue. According to Kalenga and Eeckhout (1989), loss of elastic tissue implies a loss of lung elasticity. These scanning electron micrograph findings do agree with the light microscopic studies presented in chapter 2 regarding elastic tissue quantities. On the other hand, further analysis of photomicrographs of the control and experimental animal lung tissue show that the elastic fibres between alveolar ridges appear thicker in experimental rat lung than in the lungs of control animals. This could account for what appears to be a lower elastic tissue content in the experimental animal as determined by histochemical techniques in chapter 2. The fact that the elastic fibres of the experimental rat lungs appear thicker is indicative of possible qualitative differences between the elastic tissues of the two groups. The evaluation of elastic fibre thickness has not been done by actual measurement, but is

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based solely on the visual appearance of a number of photographs studied.

Another important difference between the two groups is that the thick, tortuous fibre bundles in the control animal have long, thin hair-like, wavy threads originating from them. It can easily be imagined that these hair-like threads are new and growing elastic tissue elements which still have to be cross-linked to form the elastic tissue network of the alveolus. The beaded appearance of some fibres is reminiscent of damage to elastic fibres as described by Johanson et al. (1973) and Karlinsky and Snider (1978). The latter changes could be due to a disturbance of lysyl hydroxylase activity, as documented by O'Dell et al. (1978). This is further supported by the existent fibre network being attached to the mainly collagen-containing connective tissue sheaths. The elastic and recoil function of the elastic tissue is also pertinently shown by the coiled appearance of some of the finer fibres. In contrast to this branching and cross-linking in the control rat lung tissue, the experimental lung connective tissue shows a developed architecture with a number of shortcomings or differences. These may be summarised as follows:

(a) spikes or short fibres branch off from main connective tissue fibres, reminiscent of incompletely developed structures due to either a delayed start to synthesis of the fibre or rapid breakdown of existing fibres.

(b) numerous breaks appear in existing or fully developed elastic tissue fibres of the experimental lung tissue.

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Again, this could be explained on the basis of defective synthesis or degradation or breakage due to mechanical stress.

Based on the functional anatomy of the alveolar wall connective tissue three different systems (see section 1.9.1) may be identified (Laros and Kuyper, 1976). The functions of the elastic tissue network can only be carried out properly if both ends of the fibres are attached to the collagen framework. Loss of attachment, or breakages, would lead to loss of elasticity and therefore also affect lung recoil (Kalenga and Eeckhout, 1989). Laurell and Eriksson (1963) and Idell and Garcia (1989) suggest that breakages in elastic tissue could be due either to increased levels of proteolytic enzymes or a lack of α-1-antiprotease. Proteolytic loss of elastic tissue has been demonstrated using a papain aerosol (Colombo and Steinetz, 1975).

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All of the above, taken in perspective at this stage of development, indicates a possible defect in elastic tissue metabolism. A quality difference is also evident during successive centrifugation steps during elastic tissue quantitation. An important clue to this defect could be the fact that the scanning electron micrographs and light microscopic studies seem to indicate a lowered quantity and quality of elastic tissue with no obvious changes in the collagen component. Biochemical studies have challenged this by showing a higher elastic tissue content in the experimental lung on day 7 after birth. The anomaly as regards elastic tissue quantities in

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control and experimental lung tissue may be ascribed to either of the following:

(a) a change or deterioration in the quality of the 7 day old experimental lung elastic tissue could lead to it not being stained or stainable under the conditions used in this instance.

(b) the biochemical estimation process may quantitate both elastic tissue and other protein components which increases the apparent elastic tissue quantities for the experimental lung tissue.

The bottom line to all this is that something happens to the elastic tissue of the experimental lung elastic tissue and/or its synthesis/degradation in the early neonatal period, rendering it either non-functional or poorly functional.

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In the 14 day old animal, the rat lung tissue shows characteristics similar to that described for 7 day old animals. Apart from the expected developmental differences due to the lung tissue being 7 days older, there are other similarities and differences which are of particular concern regarding the effects of nicotine. The 14 day old control rat lung tissue appears to be more resistant to digestion than its experimental counterpart, as witnessed by an intact framework with complete fibres. The 14 day old experimental lung tissue shows a much more fragile network of fibres. The numerous breaks in these fibres indicates a weakened structure or an incompletely synthesised fibre. Reasons for this have been previously proposed (Janof _et al_., 1983; Hoidal and Niewoehner, 1983). What is of particular

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interest is the increased number of spikes which, according to previous speculation, indicates development of elastic tissue which is either late or incomplete, for whatever reason. Because of the previously described difficulty associated with the digestion process at this age, comment cannot be made as regards the relative quantities of the connective tissue components. The above structural differences exist even though both the histological and biochemical data indicate that connective tissue components occur in similar quantities for both control and experimental rat lung tissue.

At 21 days of age, many of the major differences seen between the control and experimental lung tissue, at both days 7 and 14, virtually disappeared. A more dense structure seems to prevail in the control lung tissue than in the experimental lung tissue. At low magnification it is not possible to identify the exact components responsible for this difference. What is of extreme importance is the well-developed ridges and whorls which occur in the control lung tissue. The experimental lung tissue also shows similar ridges although here they appear incomplete or disjointed, almost as if the structural integrity of the ridge has been interfered with. This agrees with the histological findings described in chapter 2 and by Woolward (1992) where the collagen fibres or bundles in the experimental animal lung tissue shows a patchy appearance, whereas the control lung tissue staining indicates an even distribution and cross-linking of these fibres. The collagen tissue in the alveolar ridges plays an extremely important role in alveolar stability, being

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responsible for recoil and prevention of overdistension and collapse of the alveolus. Since morphologic studies (both light and electron microscopic studies of lung tissue) show collapsed or distorted alveoli in the experimental lung (see chapter 2 and this chapter), especially at 14 and 21 days of age, the disruption of the collagen framework in the alveolar ridges could be considered responsible for this process.

At higher magnification, a survey of the fibre organisation and structure shows significant differences between control and experimental lung tissue. Control lung fibres, similar to previous phases, are intact while those of the experimental lung tissue still shows signs of spikes or incomplete fibres projecting from thicker connective tissue bundles as well as finer fibres. This phenomenon is not as evident or strongly developed as in 7 and 14 day old lung tissue, leading one to believe that, at 21 days of age, the experimental lung seems to be recovering from the initial nicotine insult. This also falls in line with the evidence of lung recovery supplied by Maritz (1988), especially with respect to qualities of connective tissue, as well as the biochemical studies presented in this volume. The degree of fibre breakage, although still evident, appears much less, indicative of repair or recovery of damaged tissue.

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4.5 <u>Conclusion</u>:

The changes in connective tissue architecture of the lung may be summarised as follows:

(a) elastic tissue damage as manifested by growth and structural abnormalities.

(b) collagen abnormalities are noted, especially at days 14 and 21, leading to degenerate alveolar ridges and consequently collapsed alveoli.

(c) an apparent recovery with respect to elastic tissue by day 21.

The study presented here cannot differentiate between reticular fibres and collagen fibres after the digestion process. From histologic studies presented in chapter 2, the importance of reticular fibres as well as the ground substance in which it is embedded must not be forgotten. Any disturbance of this basic cementing substance (as seen with reticular fibres under the influence of nicotine) could lead to a number of changes, including loss of cell-cell integrity.

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CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE PROSPECTS

5.1 General conclusions:

A summary of the findings of this study as well as an evaluation of success in attaining the objectives as set out in chapter 1 is presented below.

(A) This study has confirmed the damage and morphological changes that maternal nicotine exposure causes to the neonatal rat lung. This may also be considered to be indirect evidence of the role nicotine plays in neonatal lung lesions developing after maternal smoking in humans.

(B) The histochemical study of the effects of nicotine on the connective tissues of the lung has confirmed the alteration of some components. Lung collagens are unaffected as far as content is concerned and is also apparently not altered structurally. Reticular fibres are altered, either by direct damage or via developmental effects. This has raised the question of possible alterations in the quality of collagen (a molecule which is closely related to reticulin) but which is not visible using histochemical techniques. Elastic tissue is shown to be depressed in 7 day old experimental animals after which this situation seems to return to normal when compared to controls.

(C) Biochemical quantitation yielded results which showed

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no significant differences in soluble protein and collagen contents between experimental and control animals. A significantly higher content of elastic tissue was noted at day 7 in experimental animals when compared to control rat lungs. This difference was negated by days 14 and 21. These results allowed reasonable assumptions to be made regarding the role of nicotine in changing the quality of elastic tissue, especially the possible changes in cross-linking characteristics of the tissue.

(D) Scanning electron microscopy of fixed lung tissue showed obvious changes in the integrity of the experimental rat lung alveolus, including abnormal fenestrations, loss of cellular adhesion and abnormal alveolar ridges. This again allowed reasonable assumptions to be made regarding the role of maternal nicotine exposure on alveolar integrity and the development of emphysematous lesions.

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(E) Scanning electron microscopy of the connective tissue framework showed obvious changes due to either abnormal development or direct damage under the influence of maternal nicotine exposure. The changes noted were consistent with alterations in the cross-linking of the elastic tissue component of the framework. This again allowed reasonable assumptions to be made regarding the possible role of cross-linking enzymes or proteolytic enzymes in causing this change in structure.

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The ultimate aim of the study was to describe the mechanism or mechanisms by which maternal nicotine exposure causes changes to neonatal lung structure. This study has isolated the elastic tissue component and its integrity as a central factor in this regard. The reticular fibre component is also structurally affected and is another facet in the mechanism of damage to nicotine exposed lung tissue. The exact mechanisms by which this occurs is still not explained by the results obtained here.

5.2 Future prospects:

Since the central theme uncovered in nicotine-induced alteration of lung structure involves individual fibre elements, further study in this regard is called for. To explain exactly when, where and how damage (or alterations) are induced, it may be necessary to investigate the following:

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(A) the prenatal status of lung connective tissue quality and quantity.

(B) the connective tissue cross-linking capacity of prenatal and neonatal lung.

(C) the degree of cross-linking of connective tissue elements in neonatal and prenatal lung.

(D) the primary structure of precursor elements of neonatal and prenatal connective tissue (pro-collagen and tropo-elastin).

(E) the base sequence of the relevant mRNA species coding for the precursor forms mention in (D) above.

(F) the genes coding for the different connective tissue

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elements as described above.

Working backwards in this fashion, right down to the level of the gene, it may be possibly to isolate the exact point of damage and when it occurs during prenatal and neonatal lung development,

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Chapter 6

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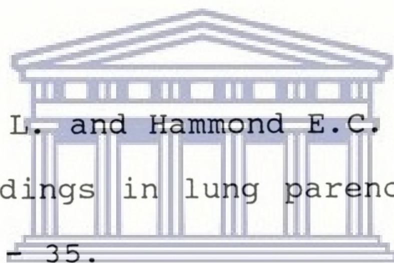

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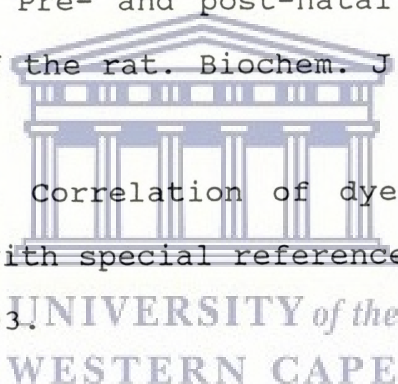
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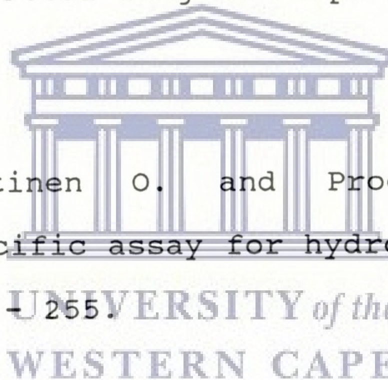

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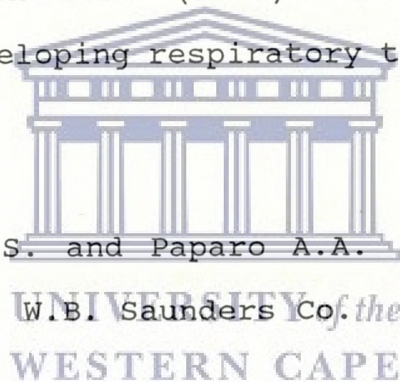

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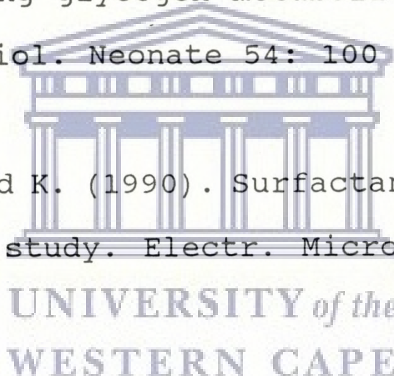
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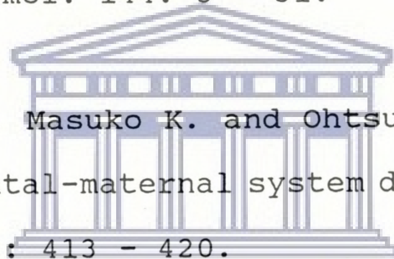

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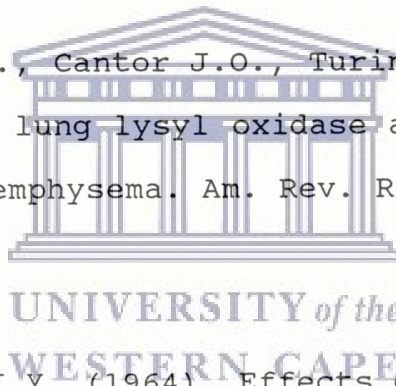
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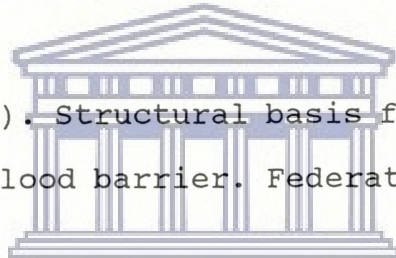

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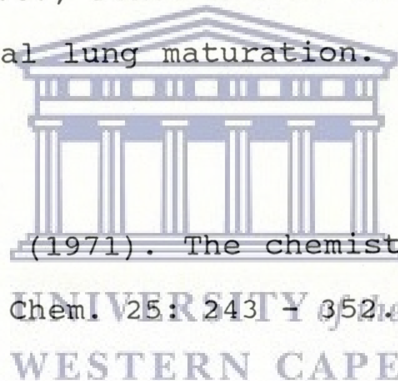

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