# A BIOCHEMICAL STUDY OF PULMONARY FIBROSIS

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DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work here was carried out by the candidate himself. Due acknowledgement has been made of all assistance received.

Signed .....

(Candidate)

Signed .....

(Director of Studies)

## DEDICATION

In fulfilment of the wishes of all the members of my family, and as a sign of determination for my nephews and nieces so that they may strive harder and be more successful.

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## ABSTRACT

A biochemical study of pulmonary fibrosis

M Iqbal, 1984

The development of pulmonary fibrosis in the rat has been investigated following both the intratracheal and intrabronchial instillation of bleomycin. These studies showed that the intratracheal and intrabronchial instillation of an equivalent dose of bleomycin resulted in changes in the lung which were different when measured by both histological and biochemical techniques. Following the intrabronchial instillation of 0.5U of bleomycin (in 0.3ml saline) into the left lung, this lung showed extensive collagen deposition with eventual consolidation between 6 and 14 days. Comparison of the different models indicated that in each case the degree of fibrosis was dependent on both the dose of bleomycin and on the volume of saline in which it is instilled. In both models only those lung lobes which demonstrated histological evidence of extensive collagen deposition showed an increase in collagen concentration (μg hydroxyproline/mg lung dry weight). Biochemical and histological studies suggested that uninjured areas of the lung underwent compensatory lung growth following bleomycin instillation.

Studies undertaken to investigate the effect of hyperoxia (70% oxygen) on the development of bleomycin-induced pulmonary fibrosis showed that the fibrotic response could be enhanced. This enhancement was only observed when animals were exposed to oxygen immediately after the intrabronchial instillation of bleomycin.

Investigations into the biochemical mechanism of bleomycin toxicity indicated that its toxicity to lung cells differed from that of the herbicide paraquat. Bleomycin failed to stimulate both the pentose phosphate pathway and the oxidant defence enzymes. Furthermore, pre-exposure of rats to 85% oxygen failed to reduce bleomycin induced toxicity.

An assessment of different biochemical parameters that may be used as markers of bleomycin induced pulmonary fibrosis indicated that levels of lung transglutaminase activity and [$^{14}$C]-putrescine accumulation into lung slices may be of potential use in this context.

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# CHAPTER 1

## GENERAL INTRODUCTION


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## 1.1 FIBROTIC LUNG DISEASE AND ITS STUDY

"Fibrosis" is a classical morphological term used to describe the appearance of an increment in the connective tissue fibres as seen with the light microscope. In this regard, the "fibrotic lung diseases" are a group of heterogeneous, chronic disorders in which there is "fibrosis" of the alveolar structures. Pulmonary fibrosis has been classified into several histological groups each with a differing cellular response (Liebow, 1975; Fulmer and Crystal, 1979). Histologically, collagen appears to be increased in all of these groups. Several recent reviews discuss the clinical aspects of these disorders as well as the current concepts of their pathogenesis (Keogh and Crystal, 1981; Crystal et al, 1981).

One of the most studied human fibrotic diseases is idiopathic pulmonary fibrosis (IPF), a generally fatal disorder confined to the lung in which there is chronic inflammation and progressive interstitial fibrosis (Crystal et al, 1976). Idiopathic pulmonary fibrosis accounts for 30-40% of all clinical cases involving pulmonary fibrosis (Liebow, 1975).

Agents known to produce chronic interstitial pulmonary fibrosis in humans include, biologically active organic and inorganic dusts; ionizing radiations; gases such as ozone, oxygen and oxides of sulphur and nitrogen; metallic ions such as cadmium and mercury and drugs such as busufan, cytoxan and bleomycin (Carrington, 1968; Rennard et al, 1982). The natural history of pulmonary fibrosis is usually one of progresive deterioration of lung function with death due to cor-pulmonale and respiratory insufficiency (Crystal et al, 1976; Livingstone et al, 1964).

### 1.1.1 Components of the normal lung parenchyma

In order to understand the abnormal biochemical processes resulting in lung fibrosis, it is first necessary to understand normal pathways of maintenance of lung structure. Physiological and morphological data suggest that the initial events in lung fibrosis are parenchymal (tissue devoid of major airways and vasculature) and that airway abnormalities, although they do occur (Fulmer et al, 1976), are later manifestations of a primarily parenchymal process.

The normal adult lung contains $300 \times 10^6$ alveoli, which are grape-like structures that branch off terminal bronchioles (Figure 1). These are the functional units of the lung. Typical alveoli have an internal diameter of 200 to 300 μm and walls 5 to 10 μm in width (Crapo et al, 1982). The walls are lined with a single layer of epithelial cells resting on a basement membrane. Weaving through the interior of the alveolar walls, forming a branching network of tubes, are the pulmonary capillaries. The capillaries comprise a single layer of endothelial cells lying on a continuous basement membrane. Although the endothelial and epithelial basement membranes are distinct structures, they fuse at the locations where the capillaries come closet to the air spaces; these are the sites of gas exchange.

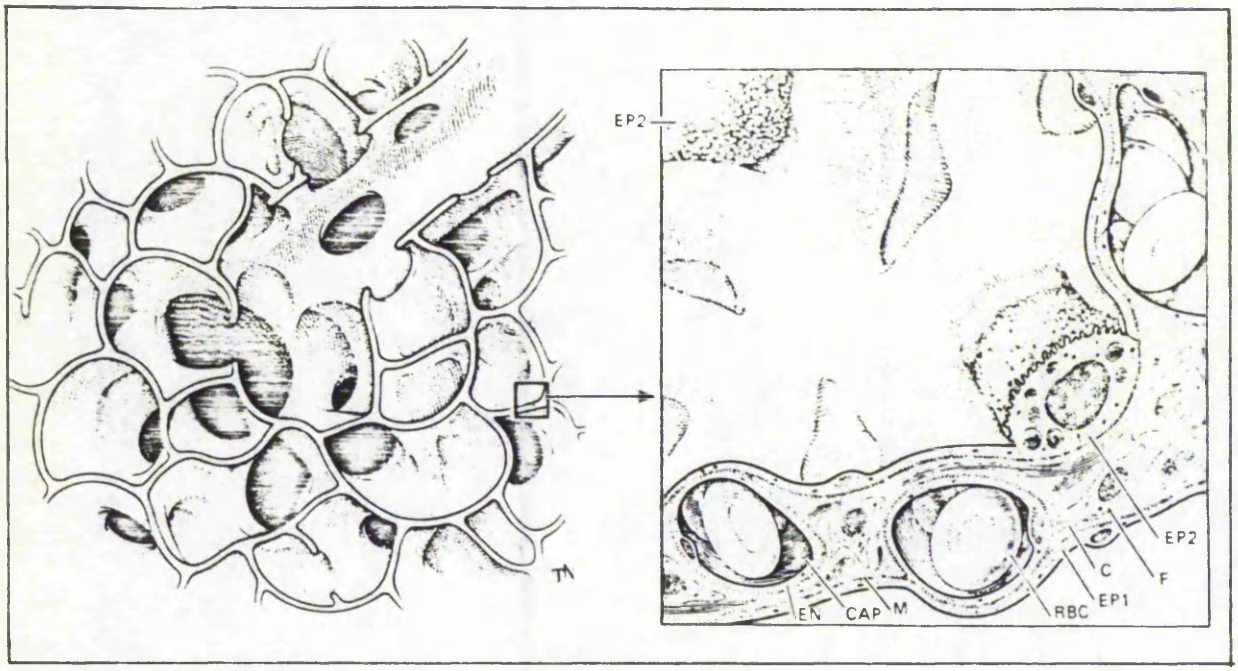



Figure 1: Structure of the Normal Lower Respiratory Tract

The left panel shows a low-power view of the distal lung; the terminal bronchiole is shown, but the pulmonary artery and vein are omitted. The right panel shows a high-power view of an alveolus; the cut surface demonstrates the type I (EP1) and type II (EP2) epithelial cells, endothelial cells (EN), basement membrane (M), red blood cells (RBC) in the capillaries (CAP), fibroblasts (F), and connective tissue (C). Inflammatory cells are not shown. (Reprinted by permission of the New England Journal of Medicine Vol 310, P154, 1983).

The epithelial and endothelial basement membranes define the boundaries of the alveolar interstium, a region made up of fibroblasts and a connective tissue matrix that together represents almost 50 percent of the tissue volume of the alveolar wall (Wiebel and Gil, 1977; Crapo et al, 1982).

The lung parenchyma represents about 80% of the total lung mass (Fulmer and Crystal, 1976). The major constituents of the lung parenchyma are listed in Table 1 and Figure 2. Twenty percent of the parenchymal mass is extracellular, composed primarily of three connective tissue components, collagen, elastin and proteoglycans. Collagen is the most abundant, representing 60-70% of the parenchymal connective tissue. There are four distinct collagen types in the lung parenchyma: The interstitial collagens (types I and III), basement membrane collagen (types IV) and the so-called "pericellular" collagen (type V). Of these collagens the type I is the most abundant, and is produced by a variety of cells, including fibroblasts, smooth muscle, epithelial and endothelial cells (Rennard et al, 1982).

Table 1

Classes of connective tissue macromolecules present in lung parenchyma.

| | Number of Known Types | % of Total Connective Tissue | General Chemical Nature | |
|---|---|---|---|---|
| | | | Protein | Carbohydrate |
| Collagen | 4 | 60-70% | 95% | < 5% |
| Elastic Fibre | 2 | 30-35% | 100 | ? |
| Proteoglycans | 2[a] | 1 | 10-30 | 70-90 |

a. Intact proteoglycans have not been studied in detail in the lung. However all seven types of glycosaminoglycans known are thought to be present in the lung (Rennard et al, 1982).

Much less is known about lung parenchymal elastic fibres although crude estimates suggest they make up to 30-35% of total parenchymal connective tissue (Horwitz et al, 1976). Elastic fibres have long been recognised as prominent components of lung connective tissue. They are known to be composed of at least two components, elastin and microfibrils (Rucker and Tinker, 1977; Gray and Franzblau, 1977).

The proteoglycans are macromolecules that make up the bulk of "ground substance". These macromolecules are comprised of a protein core to which a number of carbohydrate side chains are covalently attached. The carbohydrate side chains are repeating disaccharide subunits known as glycosaminoglycans, each of which contains an amino sugar and an acidic sugar. There are seven known types of glycosaminoglycans, each defined by the type of sugars comprising the disaccharide subunit. In general approximately 70 to 90% of the proteoglycan mass is carbohydrate and 10 to 30% protein (Hascall, 1977; Hascall and Heinegard, 1979). The total number of lung proteoglycans is presently unknown.

Eighty percent of the parenchymal mass is cellular. Although there are over 40 different cell types in the lung (Sorokin, 1970), the vast majority of these are of five types (Figure 2). These include the epithelial type I and epithelial type II cells, the endothelial cells, the interstitial cells (mainly fibroblasts) and the alveolar macrophage.

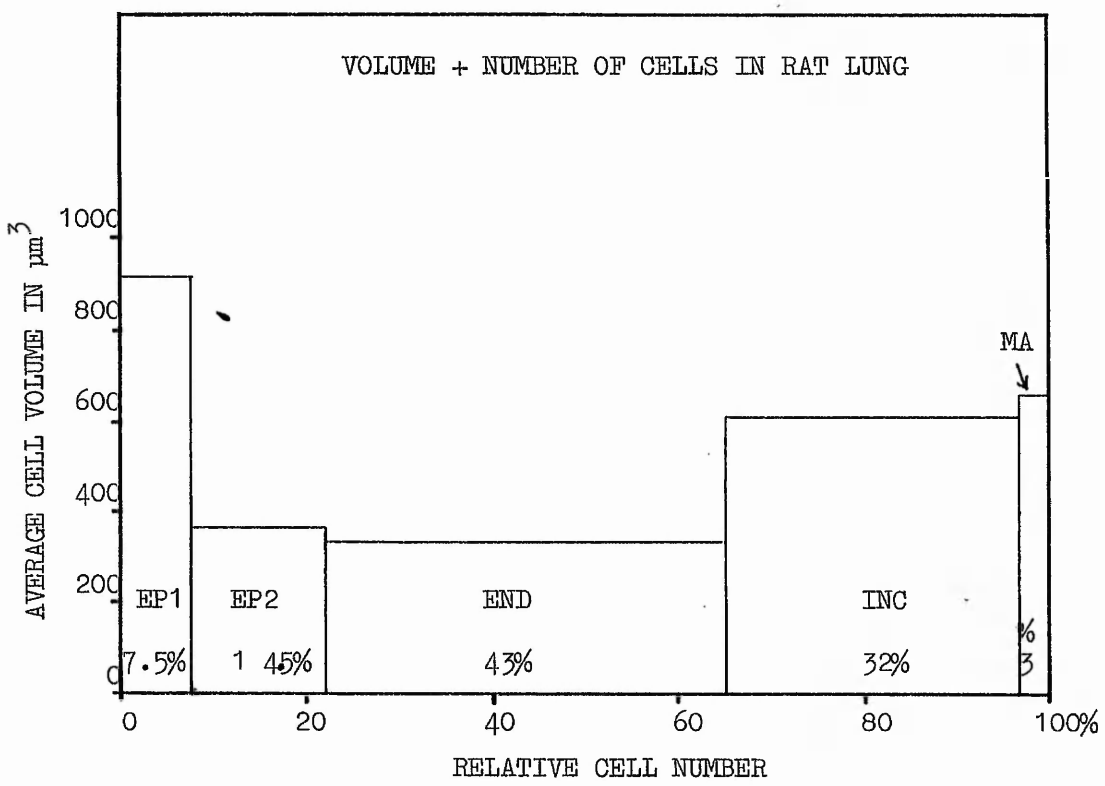



Figure 2: Comparison of average cell volumes and fraction of total cell number in the alveolar structures. EP1 = type 1 alveolar epithelium; EP2 = type II alveolar epithelium; END = endothelium; INC = interstitial cells; MA = macrophages. (Data for rat lung from Haies et al, 1981).

## 1.1.2 Maintenance of the alveolus

The parenchymal components are arranged in an ordered structure where gas exchange function is closely regulated by a multitude of physical, neural and endocrine processes. Four classes of cells (Figure 3) preserve this ordered structure and ensure that it is capable of responding to varied regulatory processes:

1) The parenchymal cells themselves; 2) blood cells (polymorphonuclear leucocytes, lymphocytes, monocytes); 3) cells of the alveolar lumen (pulmonary alveolar macrophages, lymphocytes); and 4) cells outside of lung (eg. endocrine cells, liver cells).

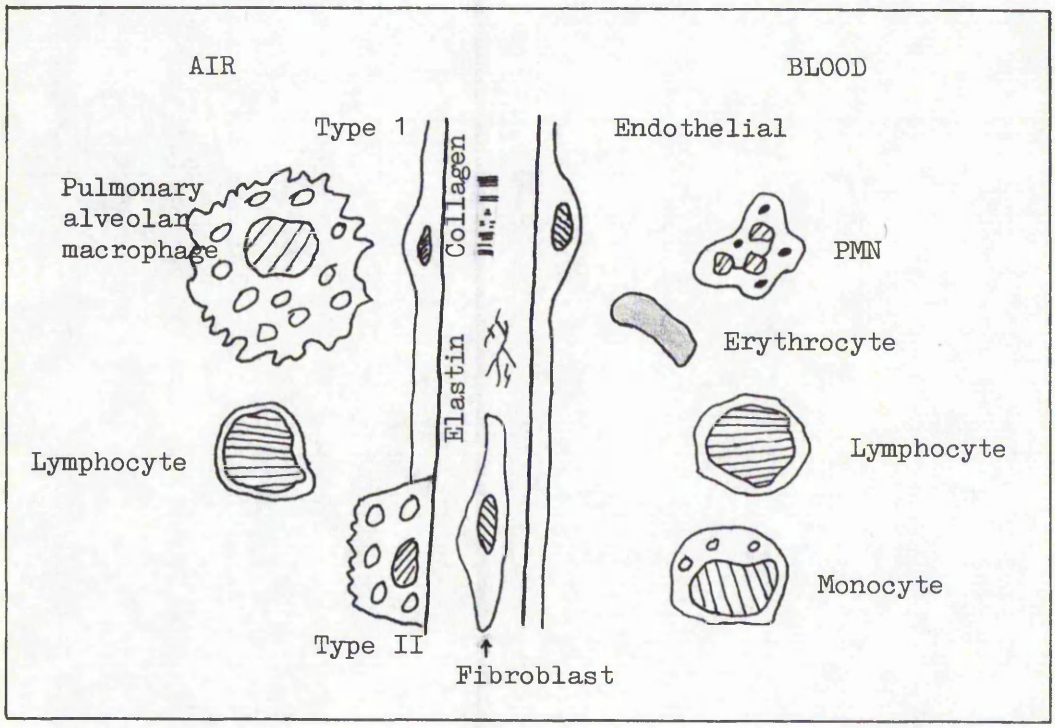



Figure 3

The cells involved in the maintenance of normal alveolar structure. A fourth class of cells, not part of lung structure, are also critical for modulating the inherent properties of the cells shown (e.g., cells of the adrenal cortex and liver). (From Crystal, 1976).

Two major mechanisms of alveolar maintenance have been described: A) Cell turnover (replication); B) Connective tissue turnover (Synthesis and degradation).

## A. Cell turnover

The study of cellular kinetics in the normal lung is prerequisite to an understanding of the pulmonary responses to injury and of the processes that initiate and control cellular regeneration and repair. Cell renewal as a mechanism can be described as a homeostatic means for replacement of ageing or injured cells (Bullough and Laurence, 1965).

### (i) Alveolar epithelium

The epithelium lining the walls of the alveoli is composed primarily of large, squamous type I cells and smaller, cuboidal type II cells. Type I cells cover over 90% of the alveolar surface, and type II cells are dispersed throughout the alveoli between type I cells (Figure 1). Both cell types lie on a common basement membrane. The type I cell does not have the capacity to replicate in situ, perhaps because of its topographical features (Weibel, 1974), but the type I cell population is maintained by its parent, the type II cell. The latter turns over at a rate of approximately 1% per day, maintaining its own, as well as the type I cell population (Fulmer and Crystal, 1976). More recent studies suggest that the rate of cell turnover may be much faster, with the entire epithelial cell population being renewed over a period of 30 days (Bowden, 1983).

Under normal conditions, the amount of epithelial cell proliferation following loss of cells is controlled in part by a negative feed back mechanism (Bullough and Laurence, 1965; Hennings and Elgjo, 1970). According to this theory, a decrease in the number of differentiated cells is followed by increased proliferation of progenitor cells until the differentiated cells are replaced.

(ii) Pulmonary endothelium

The most numerous cells in the lung are the endothelial cells lining the blood vessels (Weibel et al, 1976). Replicative mechanisms ensure that the endothelial cells are constantly replenished but at a rate of less than 1% of the total endothelial cell population daily (Fulmer and Crystal, 1976). The mechanism for renewal of the pulmonary endothelium has not been studied in detail. Most studies on endothelial cell renewal have been carried out on large vessels outside of the lung (Evans, 1982). Basically, the endothelium is different from the epithelium in that there are no specific progenitor cells in the former. Endothelial cells lost or damaged are replaced through division of other endothelial cells. Several studies have shown that after injury to vascular endothelium in the lung, the number of dividing endothelial cells increases greatly (Evans, 1982). To date, however, no studies have been performed to determine the process of division or the fate of the sister cells after division.

(iii) Pulmonary macrophages

Macrophages in the lung may be separated into two groups (Sorokin and Brain, 1975). The first group, alveolar macrophages, lie in the

surface-active film covering epithelial cells lining the alveoli. They move through this film over the alveolar surface and phagocytoze inhaled particles and other extraneous material. When they reach the regions of the terminal bronchioles, the majority either are carried upward in the airways in a mucous layer or enter the lymphatic system. In both cases, they are lost from the lung. The second group of macrophages reside in the connective tissue. They are equivalent to the fixed macrophages or histiocytes of other connective tissues in the body (Sorokin and Brain, 1975). Very few are found in the interstitial regions of the alveoli. Most are found in the connective tissues surrounding airways and in blood vessels.

Investigation so far has demonstrated two distinct means for supplying alveolar macrophages to the lung. The first is through migration of marrow-derived mononuclear cells through the vascular system into the alveoli (Evans, 1982); the second is division of alveolar macrophages in situ (Evans, 1982). Control of macrophage renewal is different from control of epithelial and endothelial cell renewal in that a demand for more macrophages does not appear to be dictated by the loss of macrophages. Instead, it appears to be associated with an increased need for a specific macrophage function.

(iv) Alveolar fibroblasts

The majority of the interstitial cells are fibroblasts. Normally these cells turnover slowly, probably less than 1% per day (Fulmer and Crystal, 1976). The number of fibroblasts within the interstitium has a profound effect on the alveolar structures, as this cell is the

primary cell responsible for the production of connective tissue elements. Although it is generally recognised that these fibroblasts have a limited life span, the precise signals within the interstitial microenvironment that control alveolar fibroblast replication, and thus the fibroblast population size, have not been elucidated.

Current concepts of the regulation of cell division suggest that besides controls intrinsic to the cell, cellular replication is modulated by exogenous growth factors, a class of molecules that instruct cells to synthesize DNA and divide (Temin, 1967; Van Wyk and Hintz, 1979; Ross and Sato, 1979). In this context, regulation of alveolar fibroblast replication largely depends upon the presence of growth factors within the local milieu.

B. Connective tissue turnover

In most species, the total amount of each lung connective tissue component remains constant once adulthood is reached (Home and Crystal, 1976). This requires that synthesis must be balanced by degradation.

Although pulmonary fibrosis involves a change in all the connective tissue components, it is the collagenous component of the matrix that characterises fibrosis (Crystal et al, 1976; Madri and Furthmayr, 1980; Rennard et al, 1982). Thus a deep understanding of the maintenance of collagen in the normal lung is of extreme importance.

(i) Structure of collagen

Although there are several types of collagen in the lung, the major types have several common properties. Each collagen molecule

is a rod-like unit 300nm long and 1.5 nm wide composed of three polypeptide chains coiled about each other in a triple helix. These polypeptide chains are known as $\alpha$-chains and each consists of about 1000 amino acid residues (Bornstein and Traub, 1979). The collagen molecules tend to aggregate to form fibrils and sometimes larger fibres. The structure of these fibrils is stabilized by covalent cross-links within and between the component collagen molecules (Bornstein and Traub, 1979). (Figure 4).

In general, all collagen polypeptide chains contain characteristic amino acids. For more than 90% of the length of each polypeptide chain, every third amino acid is glycine. Of the remaining amino acids, approximately 20% are proline, about half of which are modified to hydroxyproline after the polypeptide chain has been assembled. In addition, 3 to 5% of the amino acids are lysine. Like the hydroxylation of proline, modifications to lysine residues in the collagen chain occur after the polypeptide has been assembled (Bornstein and Traub, 1979).

The fact that a large proportion of proline residues are hydroxylated has been used by investigators desiring to quantitate the amounts of collagen present in various tissues (Green and Goldberg, 1964). With minor exceptions, this amino acid is unique for collagen and thus is used as a biochemical marker for collagen. In lung, hydroxyproline is also found in elastin (Rucker and Tusker, 1977, Gray and Franzblau, 1977), the C1q component of complement (Reid and Porter, 1976), two proteins present in epithelial surface fluid (Bhattacharyya et al, 1975, 1976) and possibly acetylcholinesterase (Rosenberry and Richardson, 1977). It is estimated, however, that these sources of hydroxyproline

constitute less than 5% of all lung hydroxyproline, and the error introduced by considering hydroxyproline unique for collagen is negligible (Bradley et al, 1974).

The various types of collagens differ in the sequence of amino acids of the component polypeptide chains, the relative degree of prolyl hydroxylations, lysyl hydroxylation, glycosylation of the hydroxy lysine residues, the presence of disulphide crosslinks, and lysine-derived covalent crosslinks. As a result, the ability and propensity of the various collagen types to aggregate into fibrils differs, giving each type a different macromolecular structure.

(ii) Production of collagen

In most species, the lung parenchyma of the adult utilizes 2 to 5% of its total protein synthesizing machinery to produce collagen (Bradley et al, 1974). The control of lung collagen production is complex and involves modulating factors both within and external to the lung cells (Figure 4). All collagens are synthesized and secreted as a high molecular weight precursor, procollagen. Procollagen contains the three pro $\alpha$- chains with globular extensions of 10-40,000 daltons at both the amino and carboxyl termini (Figure 4).

Specific steps in the biosynthesis of collagen include: assembly of the pre-pro $\alpha$-chains on membrane-bound ribosomes; cleavage of the pre-pro leader sequence; glycosylation by lipid-dependent glycosyl transferases of the C-terminal extensions of the pro $\alpha$-chains with an N-glycosidically linked carbohydrate side-chain containing N-acetylglucosamine and mannose residues; the hydroxylation of certain proline and lysine residues by specific iron-containing and vitamin C dependent oxygenases; the addition of galactose or glucosyl-galactose to some of the hydroxy lysine

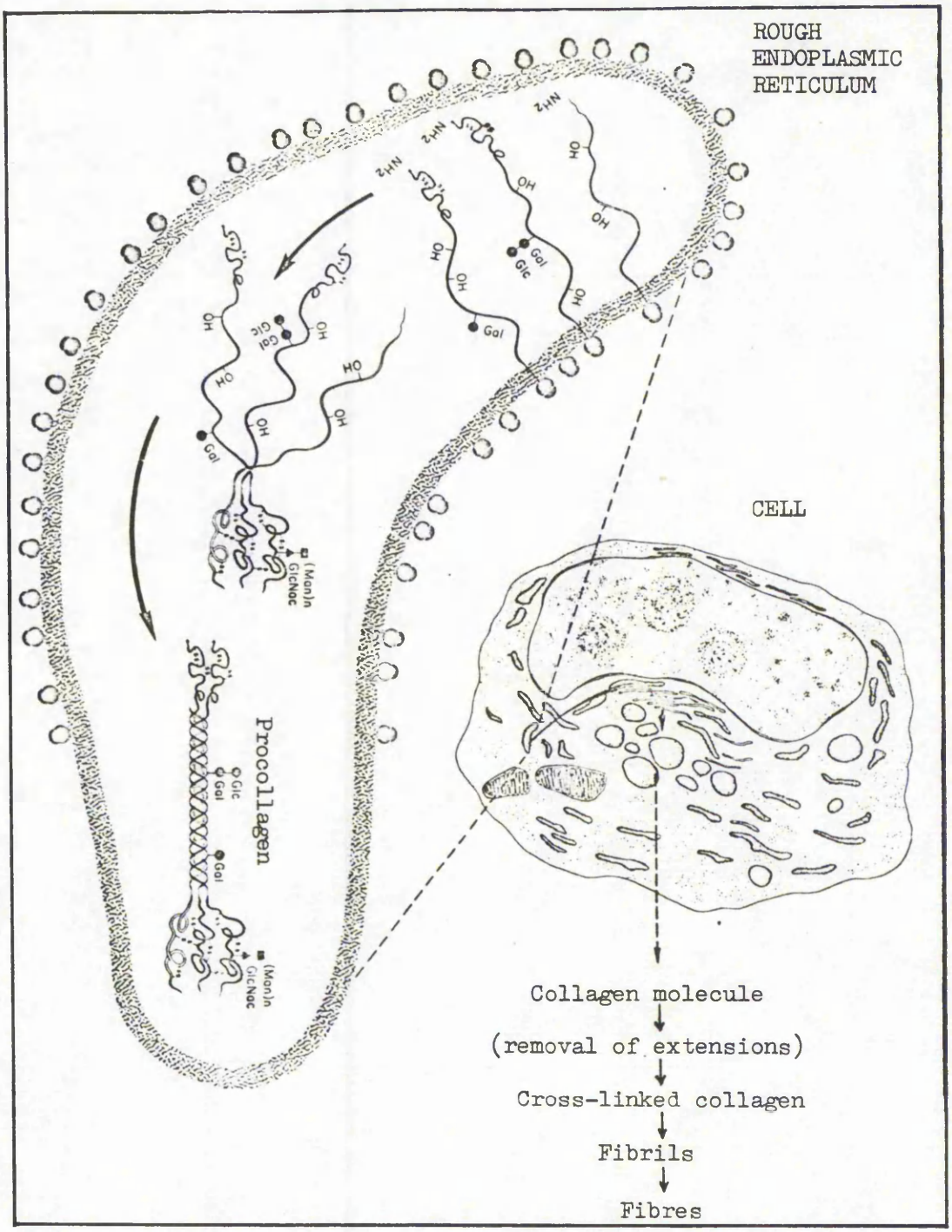



Figure 4: The steps in collagen biosynthesis

The diagram illustrates the various modifications during synthesis of the collagen pro$\alpha$-chains, followed by assembly to a pro-collagen molecule, secretion and conversion to collagen and deposition and cross-linking of individual collagen molecules to form the characteristic banded fibres observed by electron microscopy in connective tissues. Glc = glucose; Gal = galactose; Man = mannose; Glc Nac = N-acetyl glucosamine; S-S = disulphide cross-links (Reprinted by permission of the New England Journal of Medicine, Vol 301, P18, 1979).

residues, in an O-glycosidically linked bond; formation of interchain S-S bonds; and assembly of three pro$\alpha$-chains to form the procollagen molecules (Bornstein and Traub, 1979; Minor, 1980; Smith and Francis, 1982). The procollagen molecules are packed in the Golgi apparatus and secreted from the cell.

The extracellular cleavage of the non-helical terminal amino and carboxyl extensions by specific proteases prepares the collagen molecules for cross-linking. The cross-linking is performed by a copper containing enzyme, lysyl oxidase, which initiates the oxidative deamination of the $\epsilon$-amino groups in certain of the collagen lysine and hydroxylysine residues. The resultant lysine aldehyde then reacts with unreacted $\epsilon$-amino groups of other lysines (or hydroxylysines) in adjacent collagen molecules in the fibre. These cross-links give collagen fibres their tensile strength (Bradley et al, 1974; Fleishmajer et al, 1981).

(iii) Destruction of collagen

The normal, triple helical collagen molecule is remarkably resistant to proteolysis. However, specific enzymes, called collagenases, are capable of functioning under certain conditions in the extracellular milieu and of cleaving the collagen triple helix (Harris and Cartwright, 1977). These enzymes attack the collagen molecules at a specific site approximately three quarters of the distance from the N-terminal end of the molecule. Once the collagen molecule has been cleaved, the resulting pieces denature, the triple helical structure is lost and the remaining polypeptides can be degraded by non-specific neutral proteases.

Several different collagenases have been described (Harris and Cartwright, 1977), many of which are present in the lung. Alveolar macrophages from several species produce collagenase which attacks collagen types I, II and III (Wooley et al, 1976), a property which is also shared by lung fibroblasts (Kelman et al, 1977). The neutrophil, a cell present in a variety of lung diseases, contains a collagenase which has been shown to be more specific; this enzyme readily attacks type I, but not type III collagen (Horwitz et al, 1977). The activity of collagenase may, however, be regulated by various anti-proteases ($\alpha$-macroglobulin, $\alpha$-antitrypsin and $\beta_1$-globulin) (Jones et al, 1972; Eisen et al, 1970; Wooley et al, 1976) although the mechanisms are not clear.

Another degradative process controlling the quantity of lung collagen involves the intracellular degradation of newly synthesised molecules before they are secreted from the cell (Beinkowski et al, 1978). All of the mechanisms involved in this process have not been elucidated but it appears to be a way in which cells "monitor" the quality of newly synthesized collagen molecules and degrade those that are "defective".

### 1.1.3 Interruption of alveolar maintenance by fibrotic disease

Following severe injury, the lung responds by rapidly accumulating inflammatory and immune effector cells in the injured region as observed in "scar formation" in the skin (Madden, 1977). However, while the inflammation following limited skin wounding is almost always followed by orderly formation of scar tissue, inflammation in the lung either subsides without producing any recognisable derangement of the alveolar structures or more commonly, becomes chronic and associated with "fibrosis" (Crystal *et al*, 1976; Rennard *et al*, 1982). The mechanisms involved in the development of "fibrosis", however, are not clearly understood.

In an effort to gain a better understanding of what lung "fibrosis" is and how it occurs, a number of animal models of pulmonary fibrosis have been developed. The most widely used experimental animal models are induced by three main injurious agents, (i) bleomycin (an anti-neoplastic agent in common clinical use), (ii) paraquat (a powerful oxidant used as a herbicide), and (iii) radiation (usually external X-rays) (Rennard *et al*, 1982). Other experimental animal models used include those induced by N-nitroso-N-methylurethan, inhaled inorganic dusts (silica, asbestos and beryllium); toxic gases (ozone, high concentrations of oxygen and nitrogen dioxide); cigarette smoke and a variety of immunological insults (Rennard *et al*, 1982). The results obtained from these studies and those obtained from *in vitro* studies have led to the following general conclusions about the development of pulmonary fibrosis:

1. The disease is primarily a parenchymal disease. Owing to injury and desquamation, the relative percentage of cell types and the number of cells is probably changing.

2. Collagen is not the only component of connective tissue that changes following lung injury eg. elastin and glycosaminoglycan content may change as well.

3. Following an insult to the lung that results in pulmonary fibrosis, there seems to be an increase in collagen destruction as well as production.

4. Although some fibrosis-producing agents are directly toxic to lung cells (eg. bleomycin), almost all forms of pulmonary fibrosis are biologically complex and are mediated through effector cells of the inflammatory and immune systems.

5. Once fibrosis occurs within a group of alveoli, it is very unlikely that these alveoli will ever return to normal, ie. while the progression of fibrosis might be halted, the actual removal of the "fibrosis" is highly improbable.

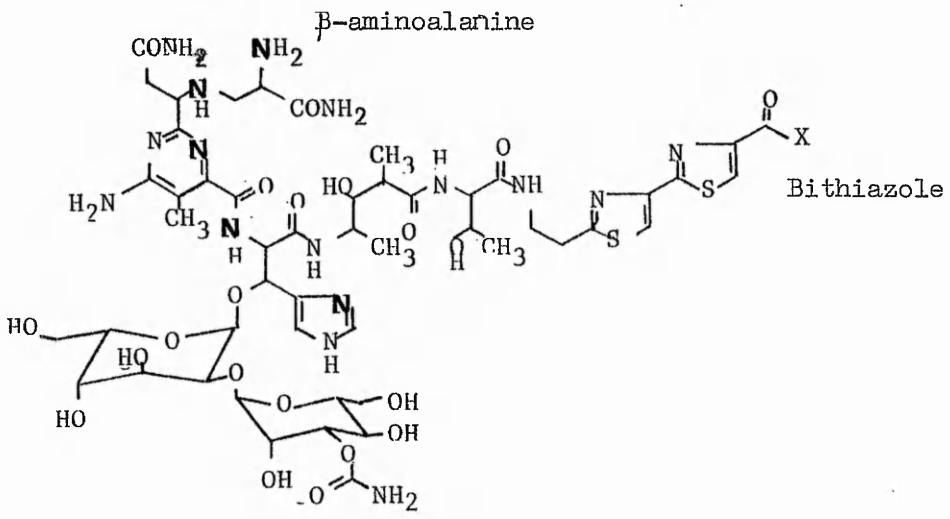
## 1.2 BLEOMYCIN

### 1.2.1 Chemistry and clinical activities

Bleomycin (BLM) is a secondary metabolite of a strain of Streptomyces verticillus (Actinomyces) obtained from soil. The bleomycins which were originally isolated as a copper (Cu (II)) complex by Umezawa et al, (1966a) are a family of histidine - containing glycopeptide antibiotics, and give rise to approximately 12 components after purification by ion-exchange chromatography (Umezawa et al, 1966a, b).

The proposed structure of bleomycin is shown in Figure 5. It is composed of the bleomycinic acid nucleus, shown in detail, and a terminal amine structure (X). It has a molecular weight of approximately 1400 which varies depending on the size of the terminal amine structure (Nakamura et al, 1974; Umezawa, 1974). Bleomycin A2 and B2, the structures of which are shown in Figure 5 account for 55-70% and 25-32% respectively of the material employed clinically. The other natural analogues account for less than 5% of the total. The clinically employed mixture is copper free (Crooke, 1981).

It is now clearly established that bleomycin can chelate a variety of divalent cations and that metal chelation has pronounced effects on the conformation and activities of bleomycin. Bleomycin and its analogues bind avidly to Co (II), Cu (II), Zn (II), Fe (II and III). They do not bind to Mg (II) or Mn (II) (Dabrowiak et al, 1978a, b). The metal binding sites (Figure 5) are thought to be the same for all the metals.




| BLEOMYCIN | TERMINAL SUBSTITUENT (X) | DERIVED TERMINAL AMINE (HX) |
|---|---|---|
| $A_1$ | $-NH-(CH_2)_3-SO-CH_3$ | 3-methylsulfinylpropylamine |
| $A_2$ | $-NH-(CH_2)_3-S^+-(CH_3)_2X^-$ | 3-aminopropyldimethylsulfonium salt |
| $A_2$-a | $-NH-(CH_2)_4-NH_2$ | 1,4-diaminobutane |
| $A_5$ | $-NH-(CH_2)_3-NH-(CH_2)_4-NH_2$ | spermidine |
| $A_6$ | $-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$ | spermine |
| $B_2$ | $-NH-(CH_2)_4-NH-\overset{\overset{NH}{\|\|}}{C}-NH_2$ | agmatine |

Figure 5: Structure of bleomycins. All analogs share a common glycopeptide backbone. Bleomycin analogs differ by variation of the terminal substituent X. For bleomycinic acid, X = OH. Examples of other substituents are shown below figure. Metal binding sites, characterised by study of a Cu II complex (Iitaka et al, 1978), are indicated by heavy lettering, and are belived to be the same for all metals (Takita et al, 1978; Dabrowiak, 1980). (From Raisfield et al, 1982).

Over the past 9 years, sufficient clinical evidence has accumulated to confirm the effectiveness of bleomycin as an anti-cancer drug against certain squamous cell carcinomas, lymphomas (Hodgkin's disease), and testicular cancer. The absence of haematopoietic toxicity or immuno-suppressive activity have been cited as the advantages of this therapeutic agent (Umezawa et al, 1967). Reviews concerning the use of bleomycin as a single agent are available (Blum et al, 1973; Carter and Blum, 1974), although more recent use of bleomycin involves its combination with various other anti-cancer agents (Crooke, 1981).

Bleomycinic acid is pharmacologically inactive and the presence of a positively charged terminal substituent is essential for ensuring anti-tumour activity, although the absolute structure of this moiety can be varied (Tanaka, 1977). Furthermore it has been shown that the structure of the terminal amine substituent is an important determinant of bleomycin induced pulmonary toxicity (Raisfield, 1979, 1980, 1981).

### 1.2.2 Clinical toxicities

The most significant dose-limiting toxicity induced by bleomycin is pulmonary fibrosis. It is estimated that approximately 10% of patients treated with bleomycin show significant clinical symptoms of pulmonary toxicity and approximately 1% show fatal pulmonary toxicity (Comis, 1978; Delena et al, 1972).

The development of bleomycin-induced pulmonary toxicity has been shown to be dependent on several factors and these include: the total dose of bleomycin, the age of the patient, and prior or concommitant exposure of the chest to radiotherapy. In addition, exposure to other potential toxins may contribute to the development of pulmonary toxicity.

The histopathological manifestations of bleomycin lung toxicity in humans are comparable to those noted in animals (Delena et al, 1972, 1973) and do not differ significantly from interstitial pneumonitis and fibrosis associated with many other lung toxins. The lesions consist of a fibrinous exudate, atypical proliferation of alveolar cells, hyaline membranes, interstitial and intra-alveolar fibrosis, and squamous metaplasia of the distal air spaces. Studies using electronmicroscopy have demonstrated type I alveolar cell destruction followed by type II cellular proliferation (Iacovino et al, 1976). In addition, nucleolar fibrillar centres and granular nuclear bodies have been shown in type I and type II alveolar epithelial cells and in fibroblasts.

Mucocutaneous toxicity induced by bleomycin is also frequent, occurring in approximately 47% of treated patients (Blum et al, 1973; Crooke and Bradner, 1976) and occasionally is severe.

Although data is not available on the tissue distribution of bleomycin in humans, studies in mice and rabbits have shown bleomycin to be widely distributed. Tissues tending to accumulate the highest concentrations were skin and spleen, but levels in the kidney, lung and heart were also high (Fujita, 1971). It has been suggested that the susceptibility of a tissue to bleomycin toxicity and anti-tumour

activity is inversely proportional to the level of an intracellular enzyme that metabolizes bleomycin (Yoshioka et al, 1978). This enzyme, known as bleomycin hydrolase, is an aminopeptidase that cleaves the amide group of the β-aminoalanine moiety of bleomycin (Umezawa et al, 1974). The cellular content of this cytosolic enzyme varies, depending on the cell type and tissue studied (Yoshioka et al, 1978). The product from bleomycin metabolism, desamidobleomycin has been reported to be one-twentieth as active as its parent compound with regard to DNA breakage and antibacterial activity (Umezawa, 1976).

### 1.2.3 The mechanism of action of bleomycin

#### 1.2.3.1 The effect of bleomycin on cells

The predominant morphological lesions in cells have been shown to be alteration in nucleolar morphology, including segregation and extrusion of fibrillar components, and induction of microspherules (Daskal et al, 1975; Madreiter et al, 1976; Yasuzumi et al, 1976; Daskal and Gyorkey, 1978). These effects have been observed in cultured cells, and also in cells of animal and human tissue susceptible to bleomycin toxicity. Other effects include the appearance of "bizarre" enlarged multiple nuclei (Krishan, 1973; Daskal et al, 1975) and chromosomal aberrations (Paika and Krischan, 1973; Hittleman and Rao, 1974; Daskal and Gyorkey, 1978).

While the mechanism that is responsible for the anti-tumour activity and pulmonary toxicity of bleomycin is unknown, its principal biochemical activity appears to be its ability to bind and break

DNA (Suzuki et al, 1969; Takeshita et al, 1974; Iqbal et al, 1976; Kohn and Ewing, 1976; Sausville et al, 1978a, b). Bleomycin induced DNA degradation has been shown in a variety of cell types including hepatoma cells, L cells and fibroblasts (Cox et al, 1975; Miyaki et al, 1973, 1975; Saito and Andoh, 1973).

### 1.2.3.2 The mechanism of DNA degradation by bleomycin

The bleomycin-induced DNA degradation reaction has been studied in cell-free defined reaction mixtures, but its mechanism is still not fully understood. However, using these in vitro systems, sufficient evidence has accumulated to support an updated hypothesis (Ekimoto et al, 1980) in which it is proposed that the DNA strand scission by bleomycin involves a two stage mechanism. In the first stage bleomycin binds to DNA by ionic interactions mediated through the terminal amine and intercalation by its bithiazole moiety (Figure 6). This is followed by the second stage during which free bases are released from DNA and both single and double strand breaks are produced by bleomycin.

Efficient breakage of DNA by bleomycin only appears to occur in the presence of oxygen, ferrous iron, and reducing agents such as mercaptoethanol, ascorbate, and dithiothreitol (Sausville et al, 1978 a, b; Lown and Sim, 1977, Suzuki et al, 1969; Onishi et al, 1975). $I_t$ is thought that the ferrous iron and bleomycin form a complex which is able to reduce molecular oxygen to the superoxide radical, hydrogen peroxide, and the hydroxyl radical (Figure 6 and 7). The byproduct is a ferric iron-bleomycin complex. The increase in efficiency of DNA strand breakage following the addition of reducing agents is thought to be due to their ability to reduce the ferric ion back to the ferrous state (Sausville et al, 1978 a, b; Caspary et al, 1979, 1981) thus resulting in a cyclic mechanism.

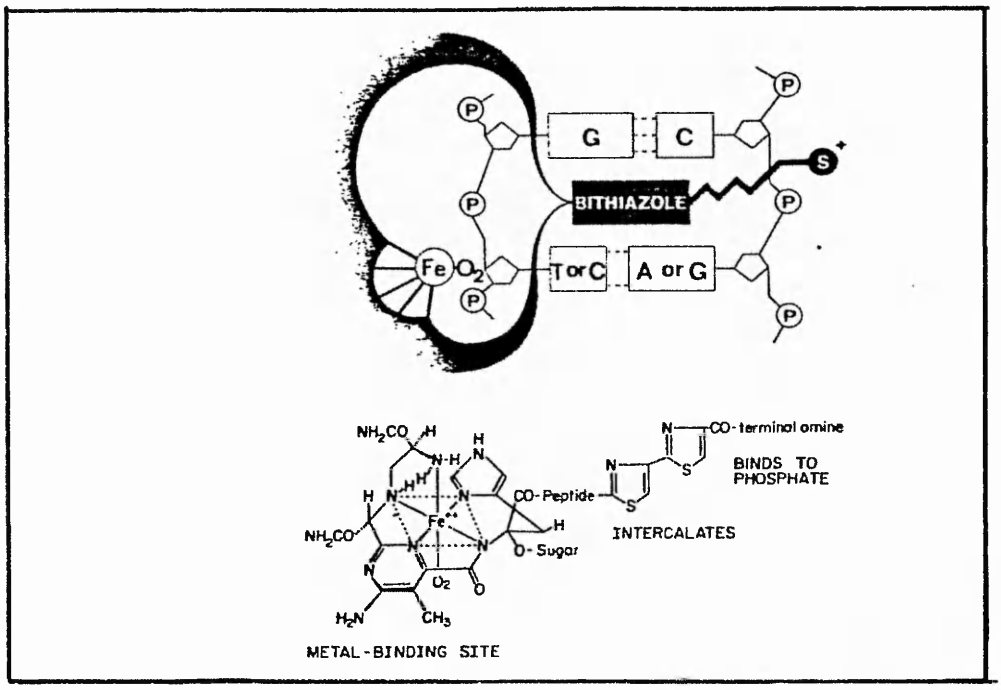



Figure 6: Schematic representation of the bleomycin Fe (II)-$O_2$-DNA complex (Reprinted by permission of Advances in Enz. Reg. Vol 18, P67, 1979)

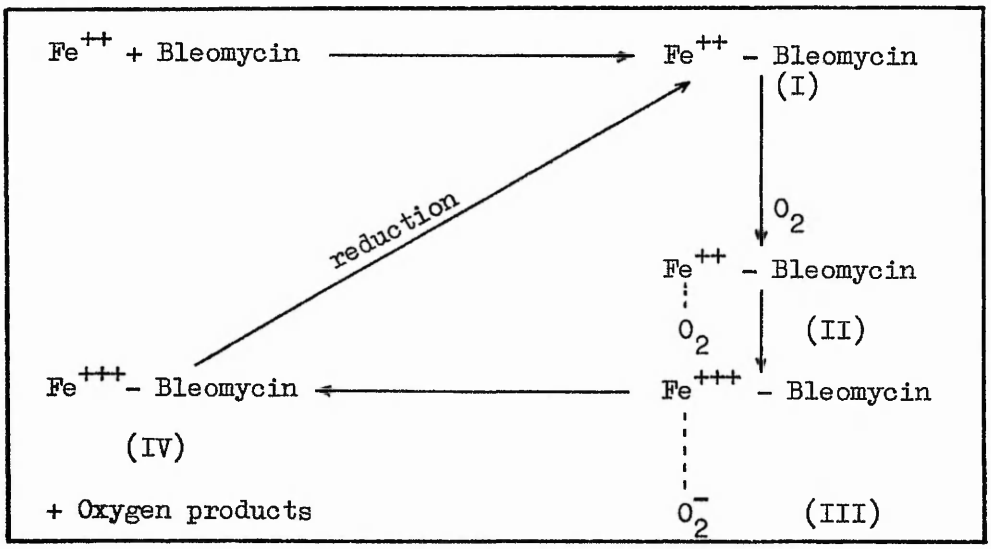



Figure 7: Proposed mechanism for the reaction of bleomycin, iron and oxygen. Structures II and III are included to demonstrate a dynamic process whereby an electron is transferred from the ferrous iron to oxygen. The actual structure and binding characterisitcs of these species have not been determined (Caspary et al, 1981).

## 1.3 AIMS

There were four principal aims of this study.

(i) To develop and characterise, using both histological and biochemical techniques, a suitable rat model of pulmonary fibrosis using bleomycin as the injurious agent.

(ii) To investigate the effect of hyperoxia on bleomycin induced pulmonary fibrosis.

(iii) To investigate and develop early biochemical markers of bleomycin induced pulmonary toxicity.

(iv) To investigate the biochemical mechanism of bleomycin induced pulmonary toxicity.

CHAPTER 2

# THE DEVELOPMENT AND CHARACTERISATION OF A FIBROTIC MODEL IN THE RAT LUNG FOLLOWING THE INSTILLATION OF BLEOMYCIN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## 2.1 INTRODUCTION

Bleomycin-induced pulmonary fibrosis is of interest as an animal model of human pulmonary fibrosis and as a clinical entity in humans that severely limits the usefulness of bleomycin as a chemotherapeutic agent. The pulmonary effects of bleomycin have been studied physiologically, morphologically, histologically and biochemically in various animals models in an attempt to define more precisely the genesis and evolution of fibrosis.

Most of the early animal model studies used repeated intravenous and intraperitoneal administration of bleomycin in laboratory animals. Fleischman et al (1971) and Schaeppi et al, (1974) studied beagle dogs, and Schaeppi et al (1973) studied rhesus monkeys all treated with repeated intravenous injections of the drug. Adamson and Bowden (1974) and Aso et al (1976) studied the effects of repeated intraperitoneal injections of bleomycin in mice. Bedrossian et al (1977) demonstrated the development of pulmonary fibrosis in pheasants after repeated intravenous injections of bleomycin. McCullough et al (1978) produced pulmonary fibrosis in baboons following repeated intramuscular injections of bleomycin. In all these investigations repeated injections of bleomycin had to be given over a period of 2 weeks to 14 months and pulmonary fibrosis was only observed in the late stages. For example, in the study with mice Adamson and Bowden (1974) observed fibroblastic hyperplasia around 8 to 12 weeks after the first injection of bleomycin.

Recently, intratracheal instillation of bleomycin directly into the lung has become a more widely used method of administration of this drug in laboratory animals. Animal models developed by this route

of administration include hamsters, rats, mice and rabbits (Snider et al, 1978; Starcher et al, 1978; Goldstein et al, 1979; Thrall et al, 1979; Phan et al, 1980; Clark et al, 1980; Giri et al, 1980; Marom et al, 1980; Raisfield, 1979; Hesterberg et al, 1981; Laurent et al, 1981). Administration of bleomycin by this route has several advantages over administration by the parenteral routes (eg subcutaneous). These include, the requirement of only a single injection of bleomycin, the use of very small quantities of the drug, and the rapid development of fibrotic lesions (within 1 to 2 weeks) (Snider et al, 1978).

The first intratracheal animal model was developed by Snider et al (1978) after the instillation of 0.5 units (U) of bleomycin in hamsters (IU = 1mg). These investigators described the histological changes in detail and reported that the lesions which developed in the hamster lung were similar to those seen in humans and other animal models following the parenteral administration of bleomycin. The histological changes included an early phase of oedema and polymorphonuclear cell infiltration, followed by marked alveolar and bronchilar epithelial cell prolieration, lymphocyte infiltration and interstitial fibrosis. The latter processes were maximal at 30 days declined thereafter and stabilized at 90 days, with foci of chronic inflammation and interstitial fibrosis still evident at 180 days (Snider et al, 1978). Similar histological changes have been reported in other animals following the intratracheal instillation of bleomycin (Starcher et al, 1978; Thrall et al, 1979; Marom et al 1980).

A common fibrotic model using the rat has employed a single intra-tracheal injection of 1.5U of bleomycin in 0.3ml of sterile isotonic

saline. In this model a 50% increase in biochemically detectable total lung collagen (measured by hydroxyproline determination) was seen by 2 weeks in treated rats. Apart from measurements of alterations in lung collagen , little attempt has been made to examine any other biochemical changes in this animal model. This lack of biochemical data is perhaps unfortunate when considering that Snider et al (1978) reported a large variability in the amount of diseased lung in the original intratracheal model, especially over the first 30 days after bleomycin instillation. This observation was further confirmed by Tryka et al (1982) who reported that only 29.2 ± 22.6% of the lung in the hamster was diseased by 30 days after the intratracheal instillation of 0.5U of bleomycin.

In an effort to reduce the variability in the amount of diseased lung the present study has investigated the use of the direct instillation of bleomycin into the left lung by the intrabronchial route. This method of instillation, which has previously been described by Wyatt et al (1981) using paraquat, has several advantages over the intratracheal method. Firstly it allows a more reliable and widespread distribution of a drug in the left lung, and secondly depending upon the dose and the dose volume it allows the study of a relatively uninjured area in the corresponding right lung.

Markers which have been used for the analysis of the resulting fibrosis include: lung morphology and histology, lung wet and dry weights and water content (markers of inflammation and oedema), DNA and protein content (a measure of the size of the cell population), DNA synthesis (a measure of the reparative/proliferative phase) and finally hydroxyproline (a measure of collagen content and hence fibrosis).

## 2.2 AIMS

(i) To investigate the dose-response relationship of pulmonary fibrosis following the instillation of bleomycin by the intrabronchial route.

(ii) To investigate the relevance of the route of bleomycin instillation in relation to the development of pulmonary fibrosis.

(iii) To develop and characterise, both histologically and biochemically, a fibrotic model in the rat lung following the intrabronchial instillation of a chosen dose of bleomycin.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Materials

Freeze dried bleomycin sulphate (copper free) was obtained from Lundbeck Ltd, Luton, UK. Bleomycin $A_2$ (copper form) (5 - methyl - $^3H$) (44.4 Ci/mmol) was obtained from New England Nuclear, Southampton, UK.

Halothane was obtained from Pharmaceutical Division, ICI Ltd, Cheshire. Oxygen: $CO_2$ (19:1) was obtained from Air Products Ltd, Lancs, UK.

(6 $^3H$-) Thymidine (30 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, UK. Soluene 350 and Dimilume 30 were obtained from Packard Instruments Ltd., Caversham, Berks, UK.

General chemicals used for buffers and physiological media were of Analar grade, while those used in chemical determinations also included the use of reagent grade chemicals. Chemicals were obtained from either BDH Chemicals Ltd, Warwickshire, UK or Sigma Chem. Co Ltd, Dorset, UK.

Alderley Park Wistar - derived SPF male rats (body weight of 180 - 200g) were used for all these studies.

### 2.3.2 Administration of bleomycin to animals

When animals were transported from Central Toxicology Laboratories ICI, Alderly Park, Cheshire, to Trent Polytechnic, Nottingham, Studies were performed on these animals after they had rested for 2 to 3 days.

2.3.2.1 Anaesthesia and exposure of trachea

After lightly anaesthetising with ether, the rats were kept under anaesthesia throughout the operation using halothane (3% in oxygen: $CO_2$ (19:1). The anaesthetic concentration was controlled by a fluotec (Cyprane Ltd) incorporated with a device for administering anaesthetic to small rodents. An incision was made in the neck of the rat and the trachea was exposed.

2.3.2.2 Intrabronchial instillation of bleomycin

After making a small incision between two cartilage segments of the trachea a plastic cannula (length 30mm and diameter 2mm) attached to a 1ml plastic syringe (sterile) was inserted into the trachea and passed into the left bronchus. Previous experience with this technique, using a vital dye, had shown that the cannula does enter the left bronchus. The contents of the syringe (in sterile isotonic saline) were instilled directly into the left bronchus quickly during inspiration, the cannula withdrawn, and the incision in the trachea sutured (one stitch only). The incision in the skin was also sutured and the rat allowed access to food and water after recovery.

2.3.2.3 Intratracheal instillation of bleomycin

Two methods of intratracheal administration of bleomycin were used and in both cases the rats were kept on an inclined surface (approx $45^{\circ}$) with the head upwards.

(A) After exposing the trachea as described in section 2.3.2.1, 1.5U of bleomycin was injected directly through the trachea in 0.3ml of saline over a period of 30 to 60 seconds.

(B) An incision was made in the trachea and a plastic cannula (length 10mm, diameter 2mm) was inserted into the trachea. The bleomycin (1.5U in 0.3ml saline) was then injected through the cannula as in (A). The incision was then sutured as described in section 2.3.2.2

### 2.3.3 Lung histology

After killing rats by exposure to halothane vapour the dorsal aorta was severed. The thoracic cavity was then opened to expose the lungs and trachea. Following the perfusion of the lungs through the pulmonary artery with isotonic saline, a cannula was inserted into the trachea and tied in place with a surgical thread. Fixative, formal saline (4% ($^{v}$/v) formaldehyde in phosphate buffered (0.05M, pH7) saline (0.9%, $^{v}$/v) was instilled into the lungs from the attached syringe, until the inflated lungs just filled the thoracic cavity. The trachea was ligated, and the lungs were submerged in formal saline and left for several days. When fixed, the left lung lobe and the right anterior, mid and posterior lung lobes were dehydrated and embedded separately in paraffin blocks by standard histological procedures using a Shandon 24 processor (Shandon Southern Ltd, Cheshire, UK).

Procedure for processing and embedding lungs prior to sectioning

| WASH | TIME(h) |
|---|---|
| 70% Methanol | 2 |
| 90% Methanol | 2 |
| 90% Methanol | 2 |
| 100% Methanol | 2 |
| 100% Methanol | 2 |
| 100% Methanol | 2 |
| Toluene | 1½ |
| Toluene | 1½ |
| Wax 1 | 1½ |
| Wax 2 | 1 |
| Wax 3 (under vacuum) | 1-2 |

Serial 5 µm thick paraffin sections were prepared and tissue sections were then stained with haemotoxylin and eosin in a Varistan 24-2 (Shandon Southern, Cheshire, UK) using the following procedure:

Haematoxylin and eosin schedules for the Varistan 24-2

| Position | Time |
|---|---|
| 1. Xylene | 2min |
| 2. Xylene | 1min |
| 3. Meths 74 | 2min |
| 4. Meths 74 | 1min |
| 5. Water | 1min |
| 6. Haematoxylin | 6min |
| 7. Water | 30s |
| 8. Water | 30s |
| 9. Acid alcohol | 10s |
| 10. Water | 30s |
| 11. Running water | 1min |
| 12. Scotts tap water | 1min |
| 13. Running water | 1min |
| 14. Eosin | 3min |
| 15. Eosin | 3min |
| 16. Meths 74 | 2min |
| 17. Absolute alcohol | 2min |
| 18. Absolute alcohol | 1min |
| 19. Xylene | 1min |
| 20. Xylene | 2min |
| 21. Xylene | 2min |

Suitably stained lung sections should show the nuclei as blue/black, the cytoplasm as varying shades of pink, the collagen as pale pinky red and the red blood cells as orange/red.

Separate lung sections were also stained with Martius Scarlet Blue (MSB), a trichrome collagen sensitive stain, by the following procedure:

(i) Solutions required

A. Celestin Blue

Iron Alum 5g

Celestin Blue 0.5g

Glycerine 14ml

Make up to 100ml with distilled water

B. Haemalum

Alcoholic alum

Haematoxylin (10% $^{w}/_{v}$) 10ml

Sodium iodate 0.2g

Potassium alum 50g

Chlorohydrate 50g

Citric acid 1g

Make up to 1000ml with distilled water

C. Martius Yellow

Martius Yellow 0.5g

95% Ethanol 100ml

Phosphotungstic acid 2g

D. Brilliant Cresyl Scarlet

Brilliant Cresyl Scarlet 1g

Distilled water 100ml

Acetic acid 2.5ml

E. Phosphotungstic Acid

Phosphotungstic acid 1g

Distilled water 100ml

F. Soluble Blue

Cotton Blue 0.5g

(Aniline Blue W.S)

Distilled water 100ml

Acetic acid 1ml

G. Mercuric chloride

3% ($^{W}/v$) Mercuric chloride in saturated alcoholic picric acid

(ii) Procedure for Martius Scarlet Blue staining

| | | |
|---|---|---|
| 1 | Remove wax from sections in Xylene | (3min) |
| 2 | Rinse in absolute alcohol | (2min) |
| 3 | Place sections in solution G | (20min) |
| 4 | Wash sections consecutively in 90% ethanol, 70% ethanol, iodine, sodium thiosulphate (5% $^{W}/v$) and water for 1min | |
| 5 | Stain with solution A | (5min) |
| 6 | Stain with solution B | (5min) |
| 7 | Wash sections in saturated lithium carbonate | (3min) |
| 8 | Wash well in water | (10min) |
| 9 | Rinse in 95% ethanol | (1min) |
| 10 | Stain in solution C | (2min) |
| 11 | Rinse in water | (1min) |
| 12 | Stain in solution D | (10min) |
| 13 | Rinse in water | (1min) |
| 14 | Stain in solution E | (5min) |
| 15 | Rinse in water | (1min) |

16 Stain in solution F (10 min)

17 Rinse in water and dehydrate sections in absolute alcohol

Suitably stained sections showed collagen as blue, fibrin as bright red, nuclei as blue black, cytoplasm as shades of mauve and blue and erythrocytes as yellow.

Procedure for the examination of lung sections under the electron microscope

Lung tissue which required examination under the electron microscope was fixed with modified Karnovskys fixative (formaldehyde (4%) - glutaraldehyde (1%), McDowell and Trump, 1976) instead of formal-saline. After 24h the Karnovskys medium was replaced with 0.1ml sodium phosphate buffer (pH 7.4). Lung tissue was diced into 1mm cubes and post-fixed with Millonigs 1% ($^{w}/v$) buffered osmium tetroxide (pH 7.3) and dehydrated using graded alcohols and epoxypropane before embedding in epoxy resin. Sections 1$\mu$m thick were cut and stained with lead citrate and uranyl acetate before being examined under the transmission electron microscope.

### 2.3.4 Lung dry weight and water content

The left lung and right lung lobes were removed from the thorax without perfusion and placed separately into preweighed glass vials. After weighing, the vials containing the lung lobes were placed in an oven at 106 $^{o}$C and dried to a constant weight over 2 days. The vial plus the dried lung were reweighed and the lung dry weight calculated. The water content was calculated as follows:-

$$\text{water content (ml water/g dry weight)} = \frac{\text{Wet weight - dry weight}}{\text{dry weight}}$$

In some experiments the dry weight of lung lobes was determined by weighing freeze-dried lung homogenates (0.5ml)

### 2.3.5 Biochemical determinations on lung homogenates

All animals were killed using halothane vapour as described in section 2.3.3

#### 2.3.5.1 Preparation of lung homogenates

Following the perfusion of lungs with isotonic saline through the pulmonary artery, the left and right lungs were homogenized separately in cold distilled water (1:10 $^{w}$/v), using the Ultraturrax (Janke and Kundel, KG) for 45 seconds. It is important to bear in mind that in this thesis the right lung refers to all three of the lobes, the anterior, mid and posterior, whereas the left lung refers to the large left lung lobe.

2.3.5.2 Lung hydroxyproline

Lung homogenates (0.5ml) were hydrolysed in 6M HCl for 18 hours at 100 °C in an oven in screw-cap pyrex tubes. The samples were then decolourised by addition of 50mg of "activated" charcoal. Following filtration the samples were neutralized with NaOH in graduated test tubes and the volume then adjusted to 4ml by addition of assay buffer (citric acid/sodium acetate pH 6.0). Samples (50 µl or 100 µl) were taken, depending on the degree of fibrosis, and assayed for hydroxyproline as described by Woessner (1961). A standard curve using crystalline L-hydroxyproline (Sigma Co Ltd) was prepared and the quantity of hydroxyproline in lungs determined from the standard curve.

2.3.5.3 Lung protein

Homogenised samples of lung were further diluted in distilled water (1:10 $^{v}/v$). The amount of protein in 20µl of this diluted sample was then determined using a modification of the method of Peterson (1977). Stock solutions for the assay comprised of (a) sodium dodecyl sulphate (SDS) (10% $^{w}/v$), (b) NaOH (3.2% $^{w}/v$), (c) $CuSO_4$ (0.1% $^{w}/v$), $Na_2$ tartrate, $2H_2O$ (0.23. $^{w}/v$), $Na_2CO_3$ (10% $^{w}/v$) and (d) distilled water. These solutions were mixed in equal proportions and 1ml of this reagent added to the sample to start the reaction. The samples were vortexed and after 15 min at room temperature 0.1ml of Folin-Ciocalteau reagent (BDH) (freshly diluted 1:1 with distilled water) was added. The samples were vortexed and left a further 20 min at room temperature before the measurement of absorbance at 750 nm. Standards of bovine serum albumin (BSA) in the range of 10 - 100µg were treated similarly.

2.3.5.4 Lung DNA

The DNA in 50µl of the homogenate was precipitated by the addition of 250µl of 10% ($^{v}$/v) ice-cold trichloracetic acid (TCA) in a 1.5ml plastic microfuge tube (Beckman Instruments Ltd, Bucks, UK). The samples were shaken and left on ice for approximately 5 min before being centrifuged for 4 min in a Beckman microcentrifuge (8749 xg). The pellet was then washed and re-pelleted several times as follows:

(i) twice in 5% ($^{w}$/v) TCA, (ii) once in ethanol (95% ($^{v}$/v)), (iii) once in diethyl ether: ethanol (1:1 ($^{v}$/v)); (iv) once in diethyl ether.

The pellet was then hydrolysed for 20 min in 0.1ml of perchloric acid (0.5M) at 70 °C. The hydrolysed DNA was cooled and 0.4ml of 1.75M perchloric acid was added to give a final concentration of 1.5M. The DNA present in each tube was then estimated by the diphenylamine method of Richards (1974). Standards were treated in a similar manner using calf thymus DNA (Boehringer). A standard curve ranging from 25µg - 100µg of DNA was constructed and unknown samples calibrated from the standard curve.

2.3.5.5 (6-$^{3}$H)-Thymidine incorporation into DNA

At various times after the instillation of bleomycin, rats were injected subcutaneously with (6-$^{3}$H)-Thymidine in isotonic saline (50µCi per 200g body weight). Animals were killed 1h later and their lungs removed and homogenised in cold distilled water (1:10 $^{w}$/v) for 30 s using a polytron vortex homogenizer (Northern Media Ltd). The DNA in 2ml of homogenate was precipitated by addition of 2ml of ice-cold 1.0M perchloric acid (PCA) and the precipitate then left to

floculate for 30 min at 4 °C before centrifugation at 2,000 xg for 10 min also at 4 °C (Beckman model TS-6). The supernatant was discarded and the pellet washed four times (by resuspending and recentrifuging) once with ice-cold 0.5M PCA, twice with absolute ethanol and finally with diethyl ether:ethanol (1:1 $^{v}/v$). The DNA in the pellet was hydrolysed using 2.5ml 5% ($^{w}/v$) TCA at 90 °C for 10 min and centrifuged at 2,000 xg for 10 min. The supernatant was saved and the pellet rehydrolysed with a further 2.5ml 5% ($^{w}/v$) TCA. This was again centrifuged and the supernatants pooled. Thymidine incorporation into the pooled hydrolysate was measured by mixing 0.5ml hydrolysate with 0.5ml distilled water and 10ml Instagel. These samples were measured for radioactivity using a Packard Tri-Carb 300 liquid scintillation spectrometer. The efficiency of counting was determined by the addition of an internal standard ($^{3}$H-hexadecane). DNA analysis on 0.2ml hydrolysate was performed by the method of Burton (1956). A standard curve was prepared from calf thymus DNA for each experiment.

### 2.3.6 Distribution of ($^{3}$H) - bleomycin in rat lung

The distribution of ($^{3}$H)-bleomycin (0.129μCi) in lungs was determined 1h after the instillation in appropriate doses of "cold" bleomycin. The "cold" doses of bleomycin which were used were 1.5U instilled in 0.3ml by the intratracheal route, 0.5U instilled by the intrabronchial route and 1.0U instilled in 0.1ml by the intrabronchial route. The instillation procedures were similar to those described in sections 2.3.2 and 2.3.3. The rats were killed and the left lung, right anterior, mid and posterior lung lobes

removed and placed in separate scintillation vials. The lung lobes were then dissolved by adding 2ml of Soluene 350 and heating to 50 $^{o}$C over a water bath. The contents of the vial were mixed following the addition of 20ml of Dimilume and stored in the dark at 4 $^{o}$C for four days to reduce chemiluminescence before measurement of radioactivity by scintillation counting. The efficiency of counting was determined by the addition of an internal standard ($^{3}$H-hexadecane). The results were then expressed as d.p.m. per lung lobe. The amount of bleomycin in the lung was expressed as a percentage of the initial amount instilled.

### 2.3.7 Statistical analysis

Throughout the thesis the control and treated means were compared using a Student's t test. The level of significance was chosen as $P < 0.05$.

## 2.4 RESULTS

### 2.4.1 Dose reponse of bleomycin induced fibrosis

The results of the dose-response experiments following the intrabronchial instillation of bleomycin into the left lung are shown in Figures 8, 10 and Table 2. The various doses of bleomycin were instilled in two different volumes of isotonic saline (for reasons that will be explained). Changes in lung collagen content and other components were determined 14 days following bleomycin administration. This time period was chosen on the basis of the results of Thrall et al (1979) who demonstrated a significant elevation in the total collagen content of the lung at this time after the intratracheal instillation of 1.5U of bleomycin.

#### A). Instillation of 0.25U-1.5U of bleomycin in 0.1ml saline into the left lung

In this study bleomycin (1.5U) was also instilled as an intratracheal injection in the same dose volume for comparison purposes. Following the intrabronchial instillation of bleomycin of doses ranging from 0.25U-1.5U, the left lung failed to show a significant increase in the total collagen content (as measured by hydroxyproline) above saline instilled control lungs by 14 days (Figure 8). However, significant increases in left lung collagen concentration (expressed as collagen per mg dry weight) were seen at this time following the instillation of 0.5U-1.5U of bleomycin. It was interesting to note that the doses which led to an increase in collagen concentration in the left lung also resulted in a reduction in the dry weight of the lungs although both of these changes only became

FIGURE 8: CHANGES IN LUNG COMPOSITION FOLLOWING THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN

Left and right lung dry weight, total collagen content (total hydroxyproline HP) and collagen concentration were determined at 14 days following the intrabronchial instillation of various doses of bleomycin in 0.1 ml isotonic saline. These changes were also measured in the whole lung ( ▨ ) following the intratracheal instillation of 1.5U of bleomycin in 0.1 ml isotonic saline. Lung dry weight was determined from lung homogenates (section 2.3.4.). The results show the mean determinations ± S.E.M. for 4 rats expressed as per cent of control values.

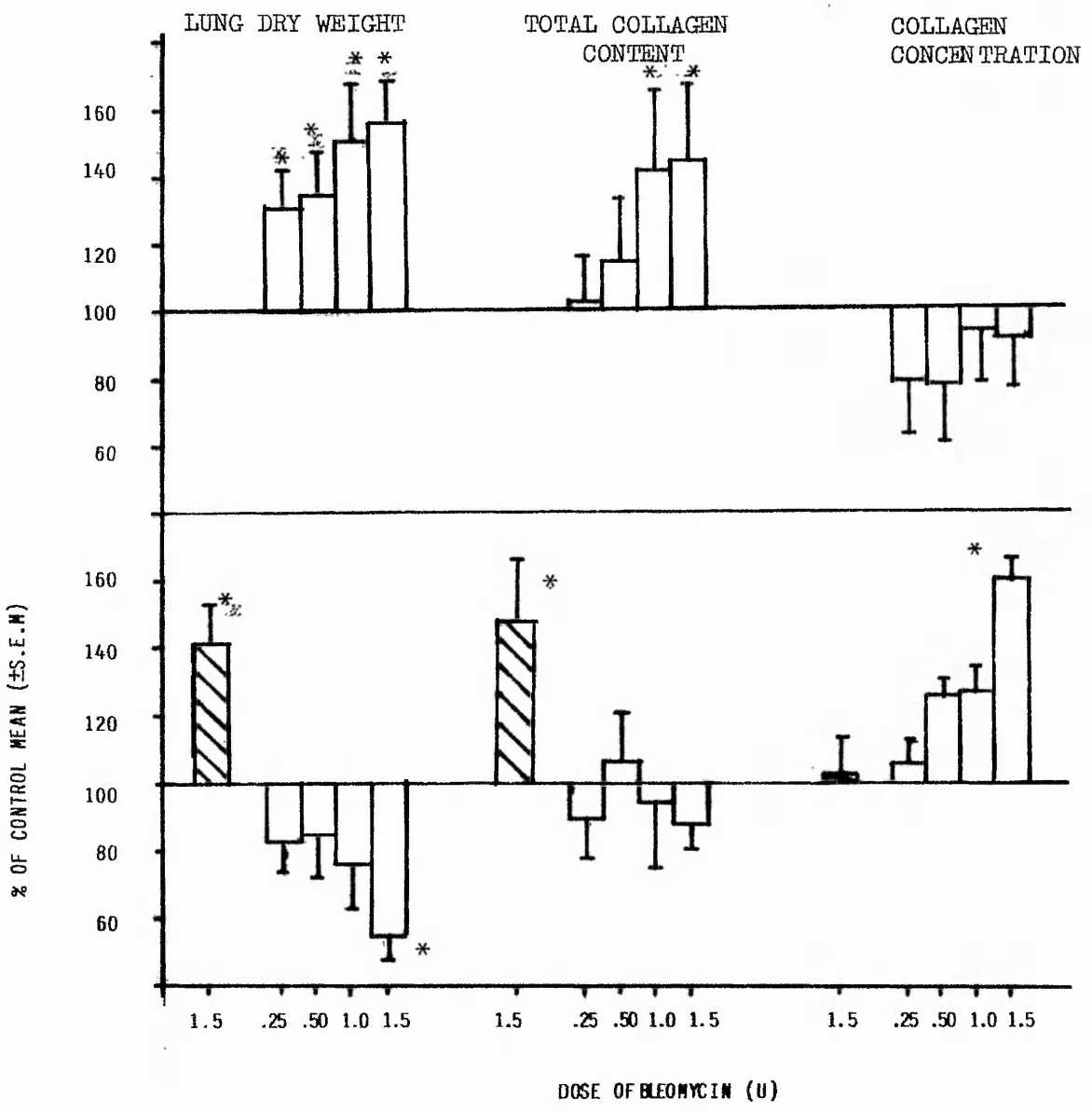
Control values obtained from rats (n = 4) instilled with isotonic saline were:

a) dry weight (mg), whole lung = 170 ± 8, left lung = 66 ± 6, right lung = 101 ± 5;

b) total hydroxyproline content (μg), whole lung = 2520 ± 70, left lung 1012 ± 43, right lung = 1493 ± 39;

c) hydroxyproline concentration (μg/mg of lung dry weight), whole lung = 14.87 ± 0.2, left lung = 15.30 ± 0.30, right lung = 14.72 ± 0.38.

* Significantly different from controls ($P < 0.05$)


LUNG DRY WEIGHT
TOTAL COLLAGEN CONTENT
COLLAGEN CONCENTRATION
RIGHT LUNG
LEFT LUNG
% OF CONTROL MEAN (±S.E.M)
160
140
120
100
80
60
1.5
.25
.50
1.0
1.5
DOSE OF BLEOMYCIN (U)

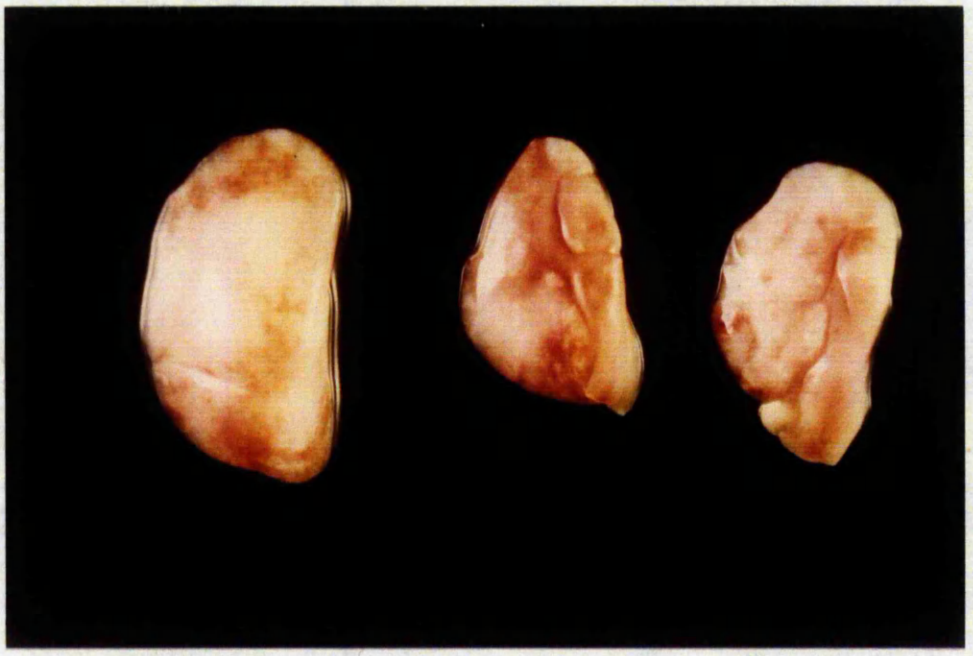
statistically different from controls ($P < 0.05$) at the bleomycin dose of 1.5U.

The changes observed in the corresponding right lung following the intrabronchial instillation of 0.25U-1.5U of bleomycin were quite different from those seen in the left lung. An increase in the dry weight of the right lung was seen for all the doses employed (Figure 8). Total collagen content was also elevated above controls, although this became statistically significant ($P < 0.05$) only after the instillation of 1.0 and 1.5U of bleomycin. In contrast to the left lung, no increase in right lung collagen concentration was observed for any of the doses instilled. It was interesting to note that the changes seen in the right lung following the intrabronchial instillation of 1.0U and 1.5U of bleomycin were similar to those seen in the whole lung following the intratracheal instillation of 1.5U of bleomycin in 0.1ml saline.

At the time of these biochemical studies (14 days), morphologically, the left lung appeared to have undergone shrinkage and consolidation of large areas. Following perfusion the consolidated areas appeared glossy and resisted inflation of the left lung through the trachea (Figure 9). The greatest degree of consolidation occurred with the highest dose of bleomycin.

One possible explanation for the failure to demonstrate an increase in the total collagen content of the left lung may be that the fibrosis occurs in a small localised area of the lung. This was suggested by the observation that the consolidation in the left lung occurred mainly along the mainstem of the bronchial tree (Figure 9). This in turn may have resulted from a high

FIGURE 9: MORPHOLOGICAL CHANGES IN THE LEFT LUNG AT 14 DAYS FOLLOWING THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN



Lungs were removed at 14 days following the intrabronchial instillation of 1.0U of bleomycin in 0.1 ml saline and perfused with cold isotonic saline. Extreme left shows a control left lung which received saline only. The other two left lungs show the two extremes of consolidation following bleomycin instillation.

localised concentration of bleomycin following the instillation of bleomycin doses in a small volume (0.1ml) of saline.

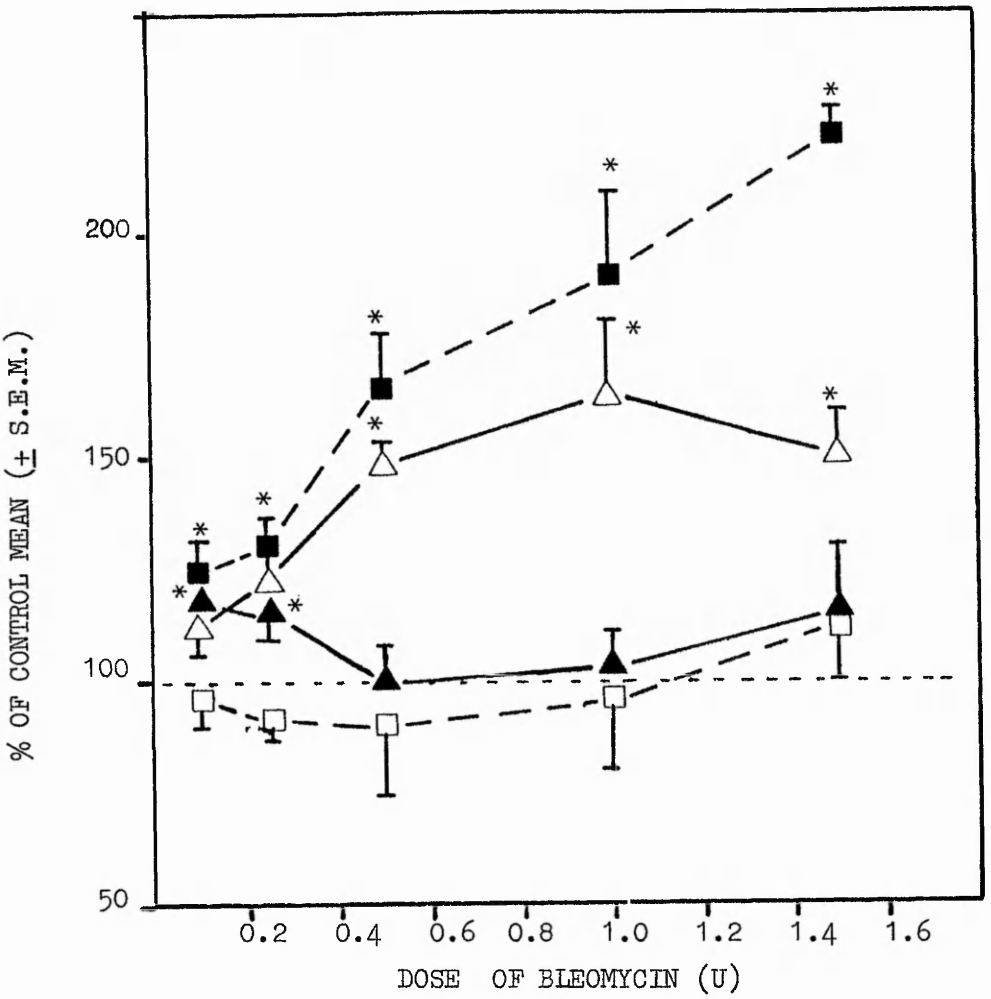
B) Instillation of 0.1U-1.5U of bleomycin in 0.3ml saline

In order to facilitate a more widespread distribution of bleomycin in the left lung, doses of bleomycin ranging from 0.1U -1.5U were instilled in 0.3ml saline by the intrabronchial route. The left and right lung were analysed after a 14 day period. The results obtained are shown in Figure 10 and Table 2.

A small but significant ($p < 0.05$) increase in the collagen content of the left lung was observed at this time period, but only after the instillation of 0.1U and 0.25U of bleomycin. In contrast the concentration of collagen in the left lung was significantly elevated above controls for all the doses ranging from 0.25U-1.5U of bleomycin (Figure 10). A significant drop in the dry weight, DNA and protein content of the left lung was also observed following the instillation of these doses (Table 2). Morphological examination of the left lungs indicated marked consolidation which was found to be more extensive than that seen after the instillation of the same dose of bleomycin in the smaller volume of 0.1ml saline (Figure 11B).

In the corresponding right lung, with the exception of collagen concentration, all the parameters measured showed increases for all the doses employed. However, the increase in these parameters was of variable magnitudes. For example the right lung collagen content increased in a dose related manner,

FIGURE 10: CHANGES IN TOTAL LUNG COLLAGEN AND COLLAGEN CONCENTRATION FOLLOWING THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN




Left and right lung total collagen (total HP) and collagen concentration were determined at 14 days following the intrabronchial instillation of various doses of bleomycin in 0.3ml isotonic saline.

Results show the mean determinations ± S.E.M. for 4 to 12 rats, expressed as per cent of control values. Total hydroxyproline content for the left lung is shown as ▲ and for the right lung as ■ and the control values were 1034±31µg for the left lung and 1577±28µg for the right lung. Hydroxyproline concentration for the left lung is shown as △ and for the right lung as □ and control values were 14.24±0.34µg for the left lung and 13.99±0.22µg for the right lung. Control values were obtained from 12 rats.

*Significantly different from controls ($P < 0.05$).

TABLE 2: CHANGES IN LUNG DRY WEIGHT, DNA AND PROTEIN CONTENT AFTER THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN

| Units of bleomycin instilled into the left lung. | LEFT LUNG | | | RIGHT LUNG | | |
|---|---|---|---|---|---|---|
| | % of Control | | | % of Control | | |
| | Dry Weight | DNA Content | Protein Content | Dry Weight | DNA Content | Protein Content |
| 0.10 | 105 ± 9 | 110 ± 12 | 140 ± 11* | 130 ± 4* | 141 ± 8* | 177 ± 10* |
| 0.25 | 94 ± 9 | 118 ± 16 | 96 ± 11 | 145 ± 8* | 149 ± 12* | 166 ± 17* |
| 0.50 | 70 ± 6* | 70 ± 6* | 78 ± 7* | 173 ± 9* | 148 ± 9* | 200 ± 13* |
| 1.00 | 60 ± 5* | 58 ± 12* | 72 ± 13* | 208 ± 22* | 176 ± 19* | 198 ± 13* |
| 1.50 | 78 ± 11* | 60 ± 12* | 82 ± 14 | 200 ± 22* | 147 ± 7* | 212 ± 18* |

Left and right lung dry weight, DNA and protein content were determined at 14 days following the intrabronchial instillation of various doses of bleomycin in 0.3ml isotonic saline. Lung dry weight was determined from lung homogenates (section 2.3.4). The results show mean determinations ± S.E.M. for 4 to 12 rats, expressed as per cent of control values.

Control values obtained from rats (n = 12) instilled with isotonic saline were:

a) dry weight (mg), left lung = 72.7 ± 1.7, right lung = 113.0 ± 2.4;

b) DNA content (mg), left lung = 2.50 ± 0.09, right lung = 3.82 ± 0.12;

c) protein content (mg), left lung = 55.11 ± 1.48, right lung = 79.80 ± 2.40.

* Significantly different from controls ($P < 0.05$).

whereas the total DNA content appeared to show a similar increase for all the doses employed (Figure 10 Table 2).

### 2.4.2 Changes in the composition of the left and right lung lobes following the intratracheal instillation of bleomycin

This study was undertaken to determine the changes occurring in the composition of the left and right lung lobes following the intratracheal instillation of 1.5U of bleomycin so that comparisons could be made with those results obtained from intrabronchial instillation. Bleomycin was instilled by two different techniques involving a direct injection or instillation through a cannula as described in section 2.3.2.3. Biochemical analysis on the lung lobes was performed after a period of 14 days, and the results shown in Table 3.

When bleomycin was administered as a direct injection the left lung failed to show any increase in either collagen concentration or DNA content. However, both total collagen content and dry weight of the left lung showed a significant increase ($P < 0.05$) above left lungs of control animals. All these parameters, with the exception of collagen concentrations, were found to be significantly elevated in the right lung when compared to the right lung values obtained from control animals.

Following the instillation of bleomycin through a cannula the left lung demonstrated increases in both total collagen and collagen concentration but the dry weight and DNA content of the left lung did not show any significant changes when compared to control animals. All the measured parameters, with the exception of

TABLE 3: CHANGES IN LUNG COMPOSITION FOLLOWING THE INTRATRACHEAL (IT) INSTILLATION OF BLEOMYCIN

| Lung parameter | Change in lung composition (% of control) | | | |
|---|---|---|---|---|
| | Direct (IT) injection | | Instillation through cannula | |
| | Left | Right | Left | Right |
| Total collagen (Total HP) | 138±19 * | 160±11 * | 143±22 * | 248 ±17 * |
| Collagen concentration | 100±12 | 74±16 * | 137±7 * | 92±12 |
| Dry weight | 137±17 * | 187±15 * | 110±14 | 267±22 * |
| DNA | 104±12 | 135±9 * | 93±15 | 161±10 * |

Data were obtained at 14 days following the intratracheal instillation of 1.5U of bleomycin in 0.3ml isotonic saline by the two methods described in section 2.3.2.3. Lung dry weight was determined from lung homogenates (section 2.3.4). The results show the mean determinations ± S.E.M. for 4 rats, expressed as per cent of control values. Control total hydroxyproline content (μg) for left lung = 802±70; for right lung = 1248±17. Control hydroxyproline concentration (μg/mg dry weight) for left lung = 11.50±0.41; for right lung = 12.70±0.76. Control dry weight (mg) for left lung = 68.9±4.7 for right lung = 108·5±6.8. Control DNA content (mg) for left lung = 2.38±0.21; for right lung = 3.68±0.32. Control values were obtained from 4 rats.

* Significantly different from controls ($P < 0.05$)

collagen concentration, showed significant increases above control values in the corresponding right lung.

Morphological examination of the left lung following the instillation of bleomycin through a cannula showed several lines of scar tissue which led to gross derangement of the lung lobe (Figure 11D). In contrast only one or two lines of scar tissue were observed after a direct injection, with large areas of the left lung appearing normal. Histological examination of these lungs at 21 days indicated that the scarring was due to collagen deposition, which led to the obliteration of the alveolar structure in the affected areas (Figure 11C, D).

### 2.4.3 Distribution of [$^3$H]-bleomycin in the lung

This study was undertaken to determine if the changes in the composition of the left and right lung were related to the amount of bleomycin encountered by the lung lobes.

Following the intrabronchial instillation of [$^3$H]-bleomycin both in 0.1ml and 0.3ml isotonic saline, the total amount of bleomycin recovered in the lungs after 1h was approximately 50% of the originally instilled dose (Table 4). Approximately 97% of the recoverable bleomycin was found in the left lung, while the remaining 3% was mainly distributed between the anterior and posterior lobes of the right lung.

When bleomycin (1.5U) was introduced into the lungs through the trachea as a direct injection in a volume of 0.3ml saline, only

FIGURE 11: DEVELOPMENT OF FIBROSIS IN THE LEFT LUNG FOLLOWING THE INTRATRACHEAL AND INTRABRONCHIAL INSTILLATION OF BLEOMYCIN

A) Section of a saline instilled control left lung (Martius Scarlet Blue stain, magnification x 4).

B) Left lung section at 21 days following the intrabronchial instillation of 0.5U of bleomycin (in 0.3ml isotonic saline) into the left lung showing extensive collagen deposition (blue stain) (Martius Scarlet Blue stain, magnification x 4).

C) Left lung section at 21 days after receiving 1.5U of bleomycin (in 0.3ml isotonic saline) as a direct injection through the trachea (Martius Scarlet Blue stain, magnification x 4).

D) Left lung section at 21 days after receiving 1.5U of bleomycin (in 0.3ml isotonic saline) as an injection through a cannula. Note the focal nature of the fibrotic lesions (Martius Scarlet Blue stain, magnification x 4).

FIGURE 11:

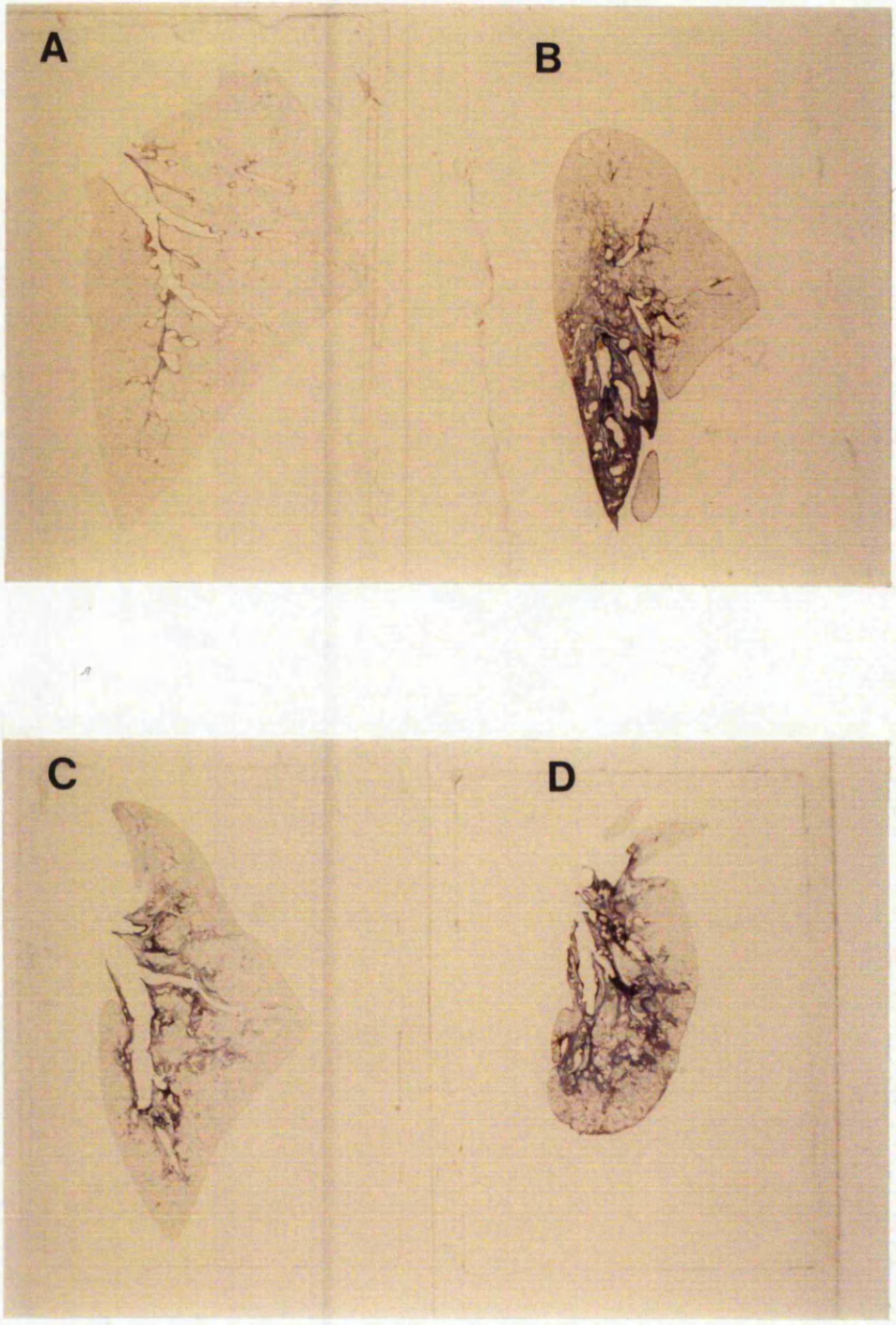


TABLE 4: DISTRIBUTION OF [$^3$H]-BLEOMYCIN IN THE LUNG FOLLOWING INSTILLATION BY THE INTRATRACHEAL (IT) AND INTRABRONCHIAL (IB) ROUTE

| Route and dose of bleomycin originally instilled | Per cent recovery of originally instilled radiolabel in | | | | |
|---|---|---|---|---|---|
| | Left Lung | Right Anterior | Right Mid | Right Posterior | Total (Left plus Right) |
| 0.5 U/0.3ml (IB) | 56.65±6.19 (0.28$\bar{U}$) | 0.40±0.20 | 0.02±0.01 | 0.74±0.32 | 57.80 |
| 1.0 U/0.1ml (IB) | 50.25±5.26 (0.50$\bar{U}$) | 0.57±0.23 | 0.15±0.06 | 0.77±0.35 | 51.73 |
| 1.5 U/0.3ml (IT direct) | 13.51±3.48 (0.20$\bar{U}$) | 5.73±2.28 | 0.65±0.37 | 6.72±2.07 | 26.61 |
| 1.5U/0.3ml (IT cannula) | 30.86±4.69 (0.46$\bar{U}$) | 4.19±1.10 | 0.61±0.13 | 17.14±3.32 | 52.80 |

[$^3$H]-bleomycin (0.129uCi) contained in the appropriate dose of "cold" bleomycin was instilled into rat lungs in isotonic saline. After 1h the rats were killed and the amount of radiolabel recovered in lungs was determined. The values in parenthesis show the amount of bleomycin present in the left lung at 1h after instillation. The results show the mean determinations ± S.E.M. for 4 rats.

26% of the total instilled dose was recovered (Table 4). In contrast 52% of the instilled dose was recovered in the lungs when the same amount of bleomycin was injected through a cannula. These results confirm earlier observations that following the instillation of bleomycin as a direct injection a substantial amount of bleomycin was lost through the mouth and nasal cavities during expiration. For each method of intratracheal instillation, the majority of the bleomycin recovered in the lungs was found in the left lung. The right lung lobes received considerably more bleomycin by the intratracheal route than they did by the intrabronchial route. For example, the anterior right lung lobe received approximately 10 times more bleomycin by both intratracheal methods of instillation than by intrabronchial instillation (Table 4 ).

2.4.4 The biochemical and histological changes occurring in the lung at various times following the intrabronchial instillation of bleomycin

This study was designed to give an insight into some of the events leading to pulmonary fibrosis resulting from the intrabronchial instillation of bleomycin. Sequential biochemical and histological changes occurring in the lungs after the instillation of 0.5U of bleomycin instilled in 0.3ml saline and 1.0U of bleomycin instilled in 0.1ml saline were therefore investigated.

These two doses were chosen because the amount of bleomycin entering the left lung following their instillation by the intrabronchial route was approximately equivalent to that entering the left lung after the intratracheal instillation of1.5U of bleomycin

in 0.3ml saline as a direct injection and instillation through a cannula, respectively (Table 4).

2.4.4.1 Biochemical changes occurring in the lung after the intrabronchial instillation of 0.5U of bleomycin in 0.3ml saline

A) Lung collagen content and collagen concentration

Following the intrabronchial instillation of bleomycin left lung collagen content failed to show any significant increase at anytime up to 14 days (Figure 12). However, when collagen concentration (collagen per mg dry weight) was measured in the left lung a significant reduction ($P < 0.05$) was found to occur at 2 days (50% of control) followed by an increase (40% above control values) at 14 days. In the corresponding right lung the collagen content started to increase around day 4, and then plateaued between day 8 and 14, being 60% above controls. The right lung collagen concentration did not show an increase above control values at anytime and was in fact significantly reduced ($P < 0.05$) at both day 2 and day 4 (Figure 12).

B) Lung DNA, dry weight and water content

The degree of oedema in the lung following bleomycin damage was determined from the water content (ml/g dry weight). The degree of inflammation resulting from the influx of cells was estimated from the DNA content and the dry weight of the lung. Reference to Figure 12 and 13 indicates that maximum oedema and influx of cells in the left lung as measured by the above parameters, occurred around 2 days after bleomycin instillation into the left lung. This was confirmed by histological examination of the left lung.

FIGURE 12: CHANGES IN LUNG COMPOSITION AT VARIOUS TIME INTERVALS AFTER THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN

Left and right lung total collagen (total HP), collagen concentration and DNA content were determined at various time intervals up to 14 days following the intrabronchial instillation of 0.5U of bleomycin in 0.3ml isotonic saline. The results show the mean determinations ± S.E.M. for 4 rats, expressed as per cent of control values. Total hydroxyproline content is shown as ■ and control values were 950 ± 55µg for the left lung and 1385 ± 33µg for the right lung. Hydroxyproline concentration (µg/mg of dry weight) is shown as □ and control values were 13.57 ± 0.43 for the left lung and 13.35 ± 0.28 for the right lung. †DNA content is shown as ○ and control values were 3.21 ± 0.28ug for the left lung and 5.19 ± 0.37ug for the right lung. Control values for 2, 4, 8 and 14 days were obtained from 3 to 4 rats at each time interval. Since the results in these groups did not differ significantly up to 14 days the data was pooled to obtain a mean value from 12 rats.

† DNA was extracted and estimated as described in section 2.3.5.5.

*Significantly different from controls ($P < 0.05$).

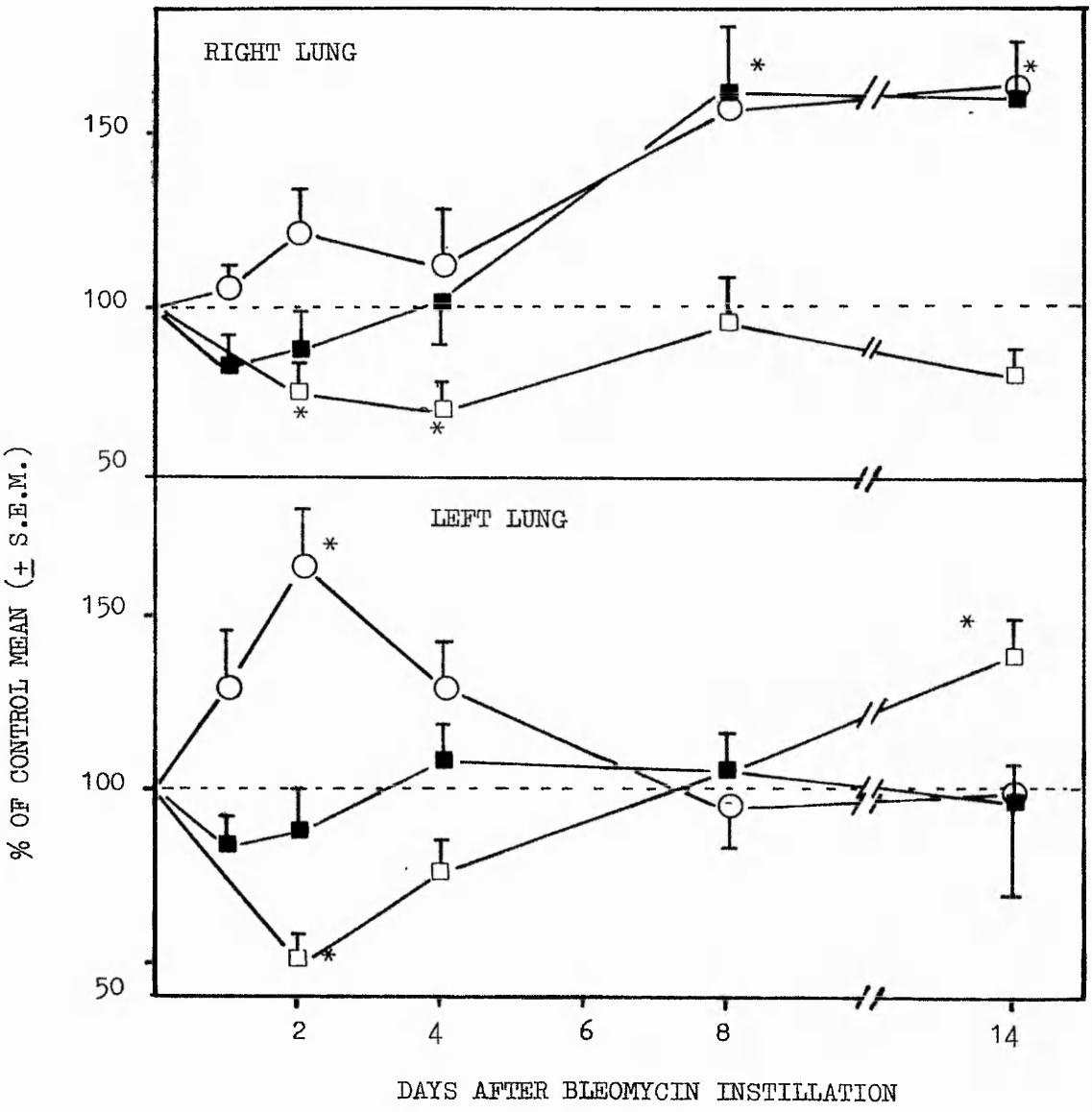
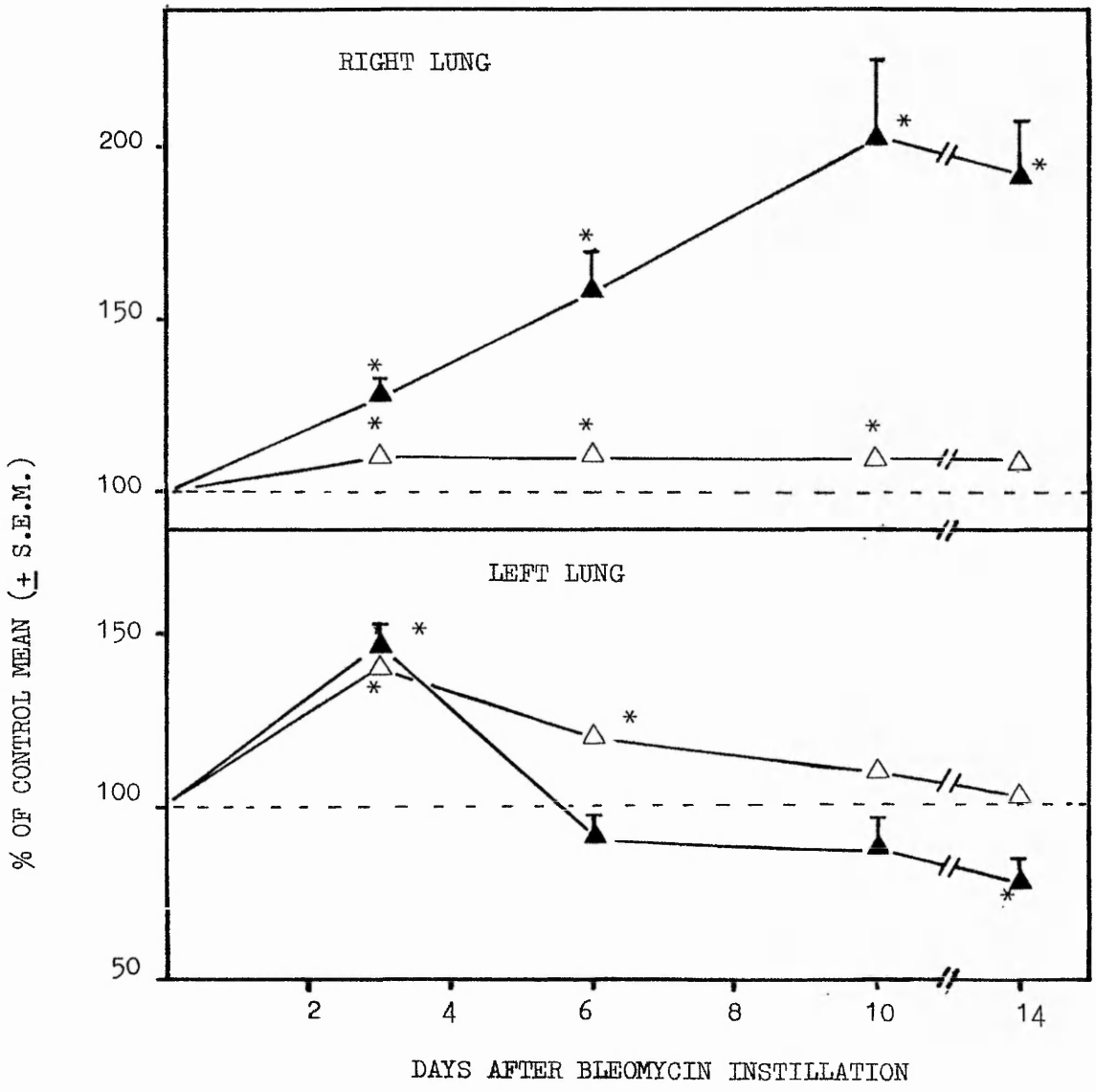

RIGHT LUNG
LEFT LUNG
150
100
50
150
100
50
2
4
6
8
14
% OF CONTROL MEAN (± S.E.M.)
DAYS AFTER BLEOMYCIN INSTILLATION

FIGURE 13: CHANGES IN LUNG DRY WEIGHT AND WATER CONTENT AT VARIOUS TIMES AFTER THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN

Left and right lung dry weight ( ▲ ) and water content ( Δ ) were determined at various time intervals up to 14 days following the intrabronchial instillation of 0.5U of bleomycin in 0.3ml isotonic saline. The results show the mean determinations ± S.E.M. for 4 rats, expressed as per cent of control values. Left lung dry weights (mg) obtained from saline instilled control rats ranged from 75.3 ± 2.1 at day 3 to 82.5 ± 2.3 at day 14 and that of the right lung ranged from 115.7 ± 4.1 at day 3 to 1225 ± 2.1 at day 14. Values obtained for the water content of control lung were (ml/g dry weight) for the left lung, 4.01 ± 0.07 and for the right lung, 3.93 ± 0.11. The water content of both the left and right lung did not differ significantly between 3 and 14 days (n = 3 to 4 rats per determination) and so control values were pooled.

*Significantly different from controls ($P < 0.05$).


RIGHT LUNG
LEFT LUNG
200
150
100
150
100
50
2
4
6
8
10
14
% OF CONTROL MEAN (± S.E.M.)
DAYS AFTER BLEOMYCIN INSTILLATION

In the corresponding right lung the changes in these parameters were quite different. DNA content showed a significant increase at 8 and 14 days, although no increase was seen up to day 4 after bleomycin instillation (Figure 12). Dry weight of the right lung showed a significant increase at 3 days and like the lung DNA content, reached a plateau between 8 and 14 days. A small but significant degree of oedema was seen by 3 days and this remained unchanged up to 14 days. Histological examination of the lungs showed very few focal lesions in the right lung, which compares favourably with the very minor changes in DNA content and water content in these lungs during early stages following bleomycin dosing.

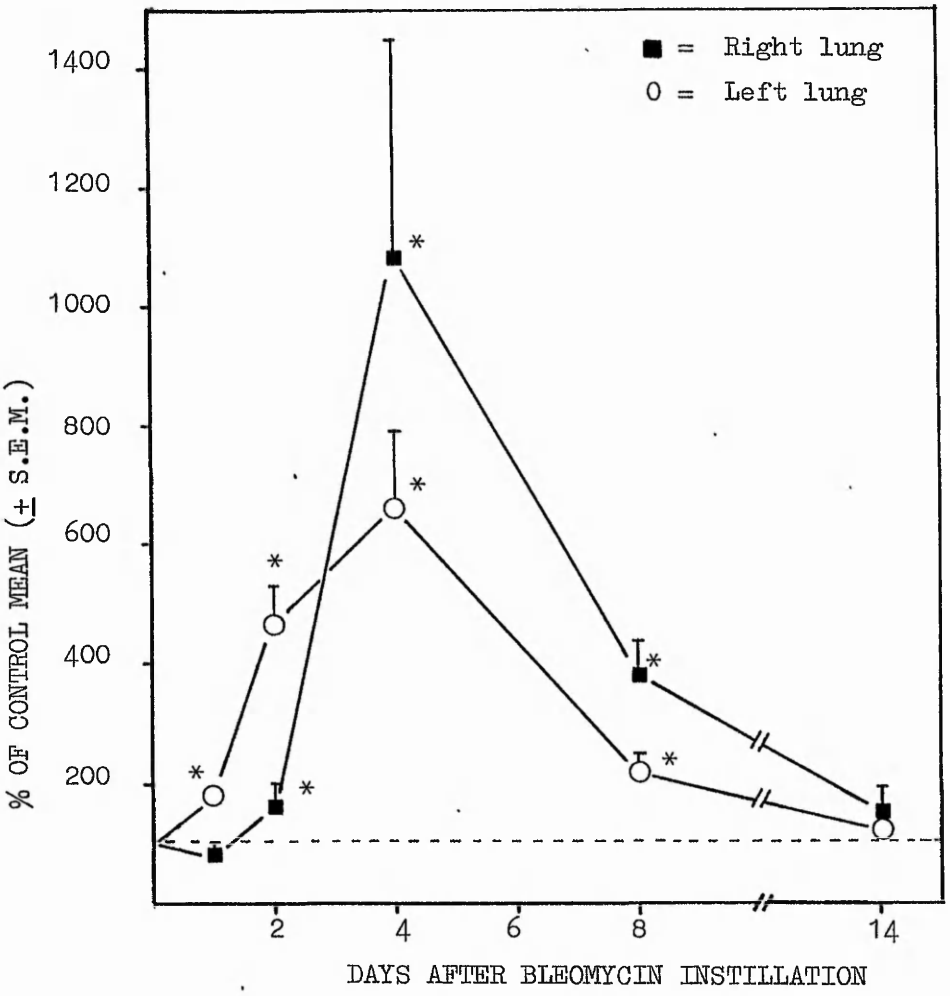
C) $(^3H)$- Thymidine incorporation into lung DNA

$(^3H)$- Thymidine incorporation into pulmonary DNA following administration of bleomycin was used as an indicator of DNA synthesis and cellular proliferation. In the left lung a significant increase in DNA synthesis occurred by day 1, reaching a peak at day 4 (6 times greater than control), and returning back to control values by day 14 (Figure 14). In the corresponding right lung DNA synthesis followed a similar pattern to that seen in the left lung although it was not significantly increased above controls until 2 days after bleomycin instillation.

2.4.4.2 Biochemical changes occurring in the lung after the intrabronchial instillation of 1.0U of bleomycin in 0.1ml saline into the left lung

Following the instillation of this dose only those changes in the left

FIGURE 14: THE INCORPORATION OF [$^3$H]-THYMIDINE INTO LUNG DNA AT VARIOUS TIME INTERVALS AFTER THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN




Rats received 0.5U of bleomycin (instilled in 0.3ml of isotonic saline) by the intrabronchial route. A subcutaneous injection of [$^3$H]-thymidine (section 2.3.5.5) was given to the animals 1h before killing and the incorporation of [$^3$H-] thymidine into lung DNA determined. The results show the mean determination ± S.E.M. for 4 rats, expressed as per cent of control values. Control values (dpm/mg DNA) for the left lung were 6406 ± 830 and for the right lung 6169 ± 714. The control values at each time interval were obtained from 3 to 4 rats. Since control values up to 14 days did not differ significantly from each other, data was pooled (n = 12) to obtain the overall mean values.

*Significantly different from controls $P < 0.05$.

lung were investigated. The results indicate that, apart from differences in magnitude, the biochemical changes were similar to those observed after the instillation of 0.5U of bleomycin in 0.3ml saline (Table 5). For example after the instillation of 1.0U of bleomycin in 0.1ml saline the increase in DNA synthesis in the left lung at day 2 and 4 was significantly less ($P< 0.05$) than that after the instillation of 0.5U bleomycin in 0.3ml saline. Furthermore a significant increase in DNA content (172% of control $P<0.05$) at day 1 was only seen after the instillation of 1.0U of bleomycin in 0.1ml of saline.

2.4.4.3 Histological changes occurring in the lung following the intrabronchial instillation of bleomycin

Histological examination of the lung was undertaken after the instillation of both 0.5U of bleomycin in 0.3ml and1.0U of bleomycin in 0.1ml. The development of pulmonary fibrosis was followed in detail up to 2 weeks and extended up to 10 weeks after the instillation of bleomycin. Histological changes were similar for both doses although the fibrotic changes were more extensive after the instillation of 0.5U of bleomycin.

Following the instillation of 0.5U of bleomycin in 0.3ml saline the left lung showed signs of congestion between day 1 and 2, as it was difficult to inflate and appeared plum red. This congestion was caused by a marked inflammatory response, which occurred throughout the left lung, although being most severe around the centre of the left lung. An increase in the influx of cells was seen from day 1 to day 2. The lung showed a marked increase in the number of

TABLE 5: CHANGES IN LEFT LUNG COMPOSITION FOLLOWING THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN

| Lung Parameter | Change in lung composition (% of control) | | | | | |
|---|---|---|---|---|---|---|
| | Days after bleomycin instillation | | | | | |
| | 1 | 2 | 4 | 6 | 8 | 14 |
| DNA content | *172±23 | *153±14 | 115±14 | 92±18 | 103±19 | 85±14 |
| Protein content | *151±20 | *177±21 | 115±17 | 91±18 | 101±19 | *75±5 |
| Water content | 106±4 | *113±7 | *109±5 | - | - | 100±5 |
| Dry weight | *118±11 | *134±14 | 94±4 | - | *67±4 | *69±6 |
| DNA synthesis | - | *241±35 | *356±56 | *263±48 | *232±24 | - |
| Total collagen (Total HP) | - | - | 77±10 | 87±14 | 103±5 | 101±8 |
| Collagen concentration | - | - | *65±9 | 100±19 | 119±19 | *127±13 |

Data were obtained at various time points following the intrabronchial instillation of 1.0U of bleomycin in 0.1ml isotonic saline into the left lung. The results show the mean determinations ± S.E.M. for 4 rats, expressed as per cent of control values. Control values obtained for the left lung were as follows: DNA content = 2.41 ± 0.12 mg (pooled results n = 6); protein content at day 4 was 50.30 ± 1.22 mg (n = 4) and at day 14 was 56.51 ± 3.3 mg (n = 3); water content = 3.98 ± 0.13 ml/g dry weight (pooled results n = 6); dry weight at day 4 was 75.3 ± 3.1 mg (n = 3) and at day 14 was 83.7 ± 2.4 mg (n = 3); DNA synthesis = 5583 ± 914 dpm/mg DNA (pooled results n = 6); total hydroxyproline content = 850 µg (n = 5); hydroxyproline concentration = 12.75 µg/mg dry weight (n = 5).

*Significantly different from controls ($P < 0.05$)

neutrophils by 2 days, which were present in both the interstitial and intra-alveolar areas (Figure 15B). There was a much smaller increase in the number of macrophages and lymphocytes, the former occasionally seen to be undergoing erythrophagocytosis. Perivascular oedema and both perivascular and intra-alveolar haemorrhage could be seen at this stage. Between 3 and 4 days the numbers of neutrophils became greatly reduced and an increase in the numbers of macrophages and lymphocytes was seen (Figure 16A). A large increase in the numbers of fibroblasts was also observed, which were present both in the interstitial and intra-alveolar regions. The alveolar septa became greatly thickened and extensive re-epithelialisation as well as early signs of collagen depositon were seen after collagen staining (Figure 17A, B). The bronchioles also appeared to be involved by day 4, with areas showing total loss of epithelium or flattened epithelium. Tissue examined by electron microscopy at this stage showed extensive damage to the epithelial Type I cells, which showed blebbing in some areas (Figure 16B) and in other areas a complete loss of cells of the alveolar lining was observed. Accumulation of macrophages, cell debris and some neutrophils as well as other mononuclear cells were seen in the alveoli. Although hypercellularity could be seen at 3 to 4 days, this became more prominent around day 6 (Figure 18A). By this time there was clear evidence of progressive fibrosis, with large areas of the left lung lobe being grossly distorted because of parenchymal collapse (Figure 19) due to hypercellularity and collagen deposition. At higher magnification the consolidated area was associated with collections of fibroblasts, mononuclear cells and increased collagen deposition between cells (Figure 18B).

The consolidated area was restricted mainly to the areas which showed the most intense inflammation by 2 days. At this stage the consolidated area was separated into areas in which the alveolar structure was totally obliterated and areas in which alveolar structure with greatly thickened septa could be seen. The latter merged with areas of normal alveolar structure, in which the inflammatory response had disappeared. Between 6 and 14 days the consolidated area covered over half of the left lung (Figure 19). No further increase in left lung consolidation was observed up to 10 weeks after bleomycin instillation.

The lesions in the right lung after the intrabronchial instillation of bleomycin, although of similar nature were focal and very few (Figure 20). Most of the right lung appeared to be normal.

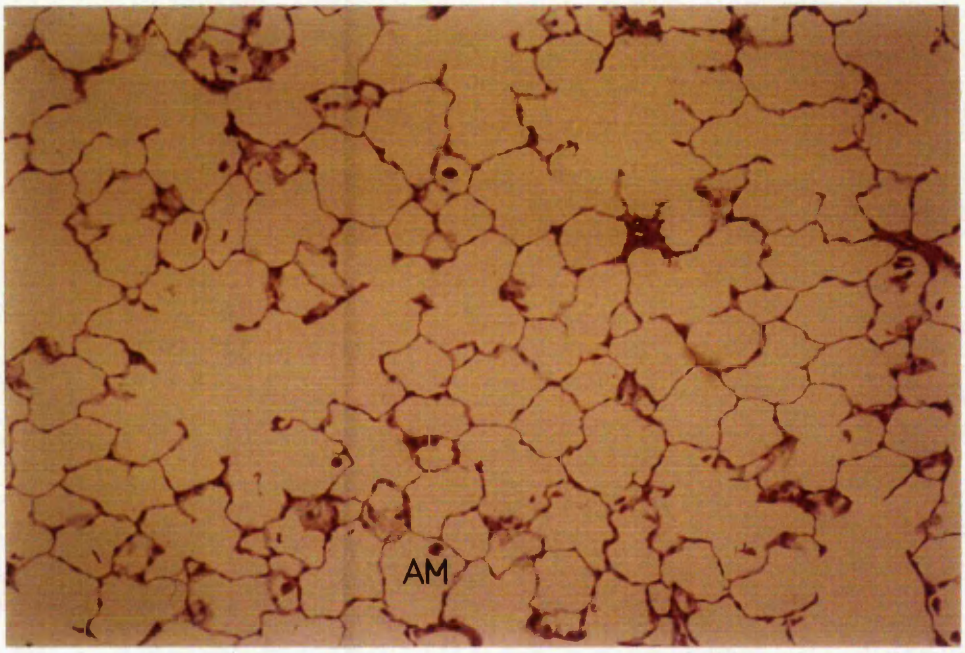
FIGURE 15: HISTOLOGICAL CHANGES IN THE LEFT LUNG AT DAY 2 FOLLOWING THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN

A) Normal lung architecture in saline instilled rats, showing an occasional alveolar macrophage (AM) in the alveolar space. Saline instilled lungs did not show any significant changes from those shown here throughout the study when examined up to 10 weeks (Haematoxylin and eosin stain, magnification x 490).

B) Influx of inflammatory cells both in interstitial and intra-alveolar regions of the left lung following the intrabronchial instillation of 0.5U of bleomycin in 0.3ml isotonic saline. Although an increase in the numbers of macrophages and lymphocytes can be observed, the majority of the inflammatory cells were polymorphonuclear leucocytes (PMN, neutrophils) (Haematoxylin and eosin stain, magnification x 196).

FIGURE 15:

A




B

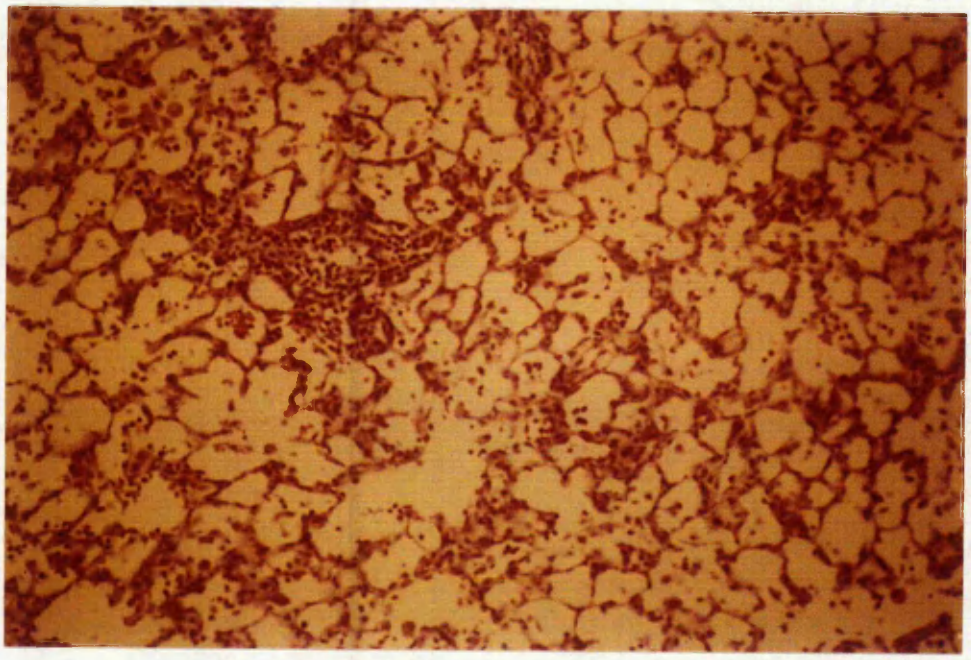


FIGURE 16 & 17: HISTOLOGICAL CHANGES IN THE LEFT LUNG AT DAY 4 FOLLOWING THE INTRABRONCHIAL INSTILLATION OF 0.5U OF BLEOMYCIN IN 0.3ml SALINE

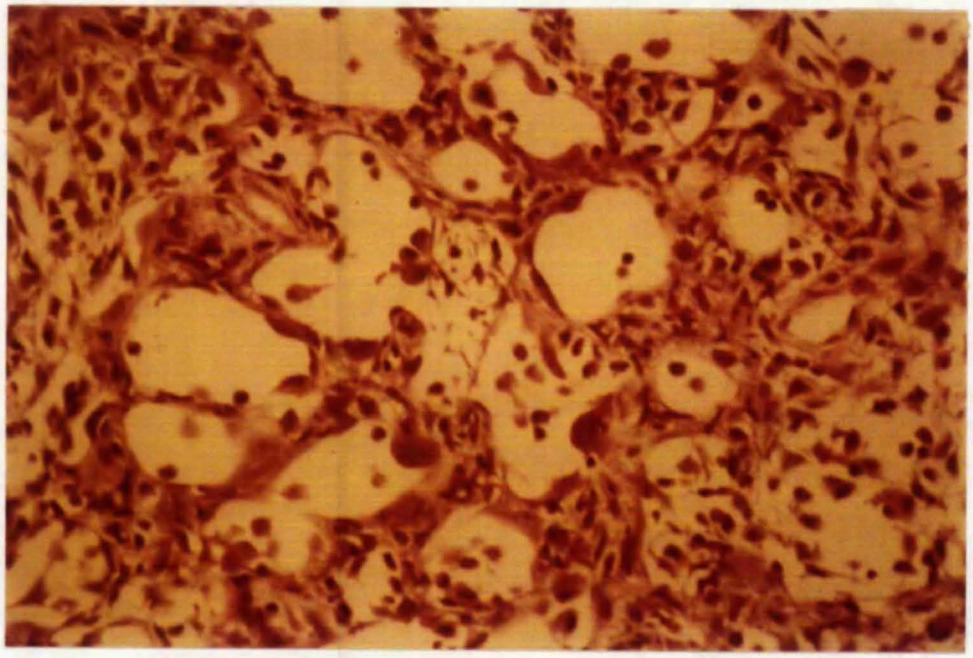
16A) Lung section examined under the light microscope showing a large increase in the number of macrophages, fibroblasts and lymphocytes. Thickening of the alveolar septa resulting from hyperplasia of the alveolar epithelium as well as fibroblastic hyperplasia (spindle shaped cells) can also be seen (Haematoxylin and eosin stain, magnification x 490).

16B) Lung section examined under the transmission electron microscope showing two macrophages (AM) and a polymorphonuclear leucocyte (PMN) in the alveolar space. Damage to epithelial type I cells ($E_1$) is indicated by their blebbing (magnification x 6,000).

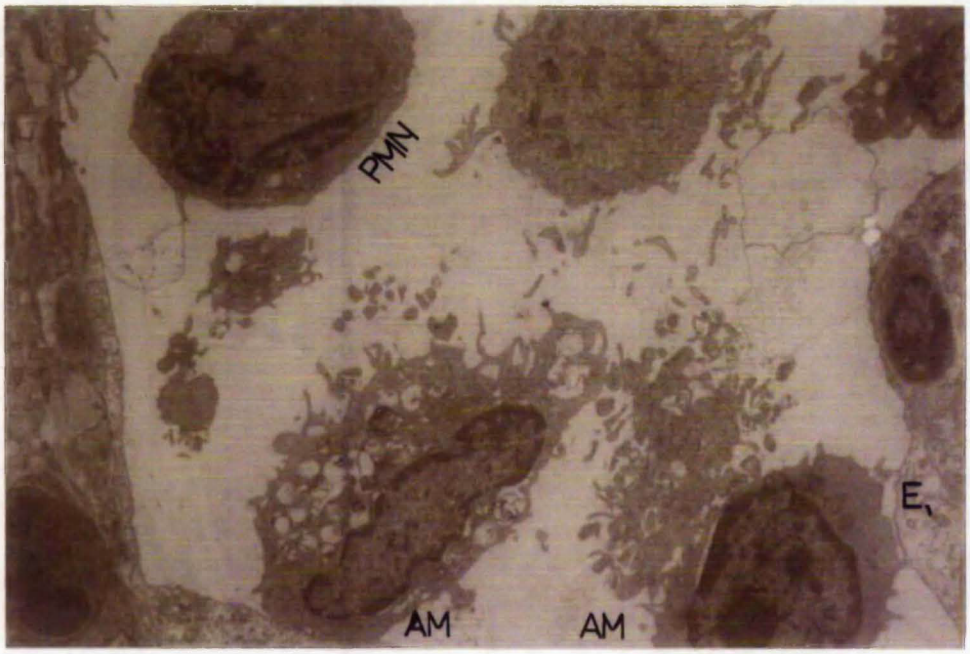
17A) Lung section examined under the light microscope showing extensive re-epithelialisation and collagen deposition (stained blue). (Martius Scarlet Blue stain, magnification x 196).

17B) Lung section examined under the electron microscope showing proliferating epithelial type II cells, characterised by numerous fat containing inclusions. The region of the alveolus shown here contained 5 type II cells (magnification x 7,800).

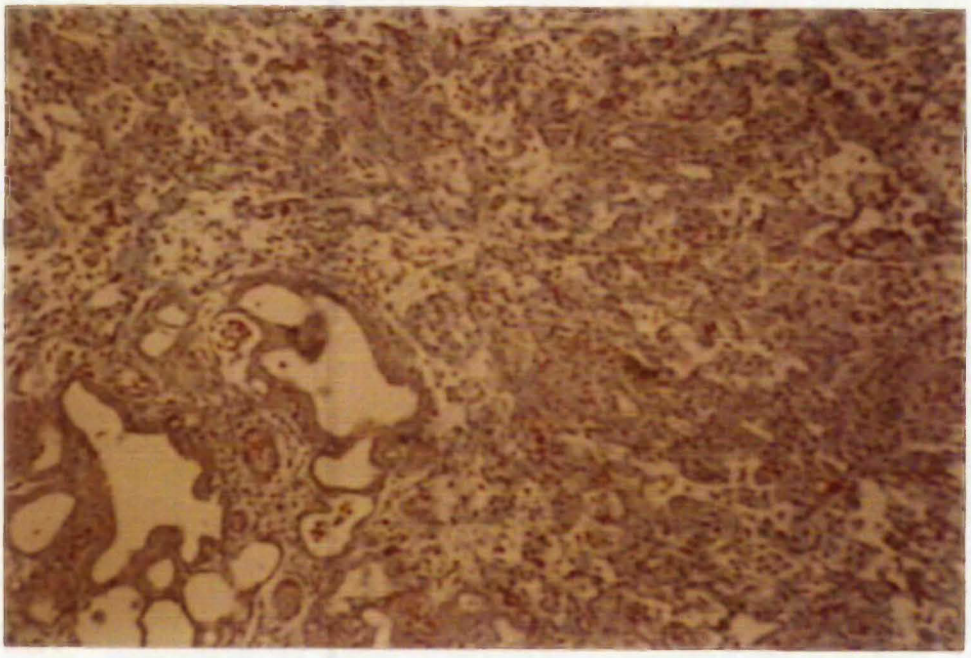
16A



16B



17A



17B

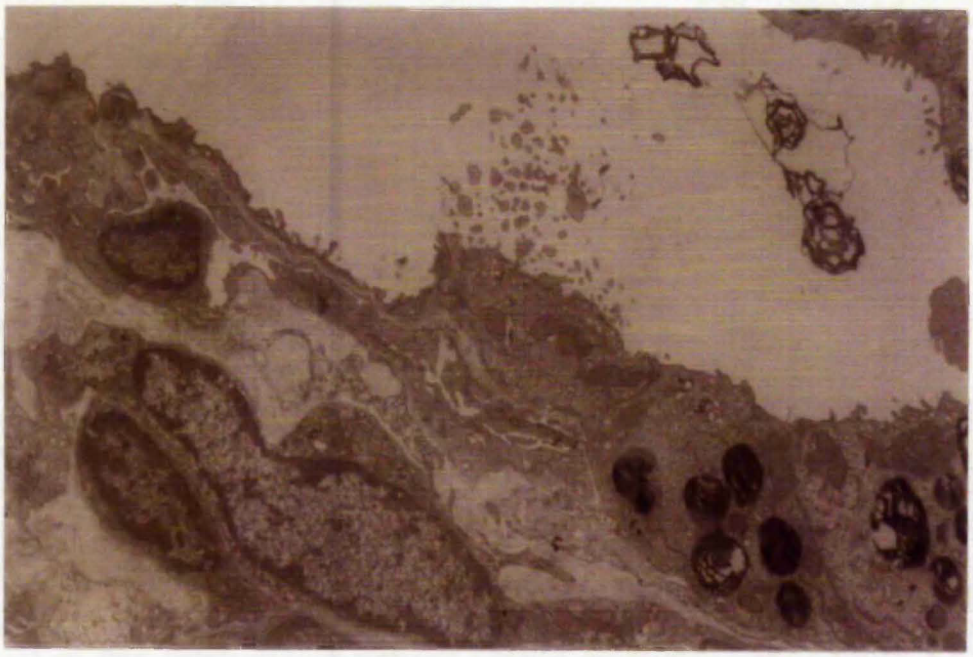


FIGURE 18 & 19: HISTOLOGICAL CHANGES IN THE LEFT LUNG AT DAY 6 FOLLOWING THE INTRABRONCHIAL INSTILLATION OF 0.5U OF BLEOMYCIN IN 0.3ml SALINE

18A) Lung section examined under the light microscope shows extensive deposition of collagen, hypercellularity and consolidation of large areas of the lung (Haematoxylin and eosin, magnification x 196).

18B) Lung section examined under the electron microscope shows the presence of fibroblasts and the deposition of collagen fibres and loss of normal alveolar structure (magnification x 7,800).

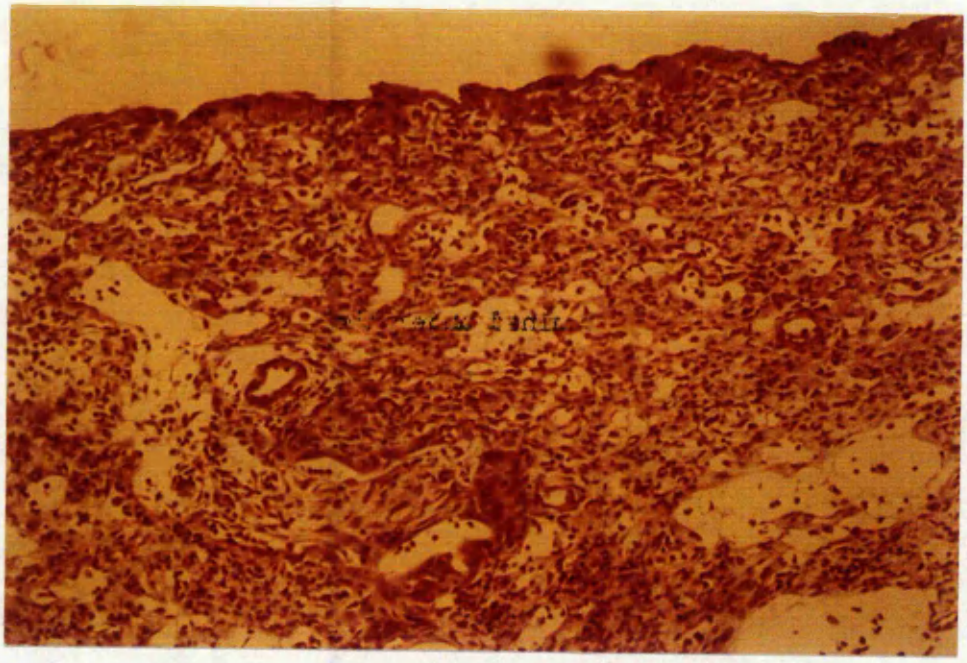
19) Left lung section showing extensive collagen deposition (blue stain). Areas which did not become consolidated returned towards normal between 3 and 10 weeks were completely normal. The consolidated area failed to show any recovery during this time (Martius Scarlet Blue stain, magnification x 4).

FIGURE 20: HISTOLOGICAL CHANGES IN THE RIGHT ANTERIOR LOBE AT DAY 6 FOLLOWING THE INTRABRONCHIAL INSTILLATION OF 0.5U OF BLEOMYCIN IN 0.3ml SALINE INTO THE LEFT LUNG

Fibrotic changes, although very few and patchy, were similar to those seen in the left lung (Haematoxylin and eosin stain, magnification x 77).

FIGURE 18:

18A



18B

FIGURE 19:

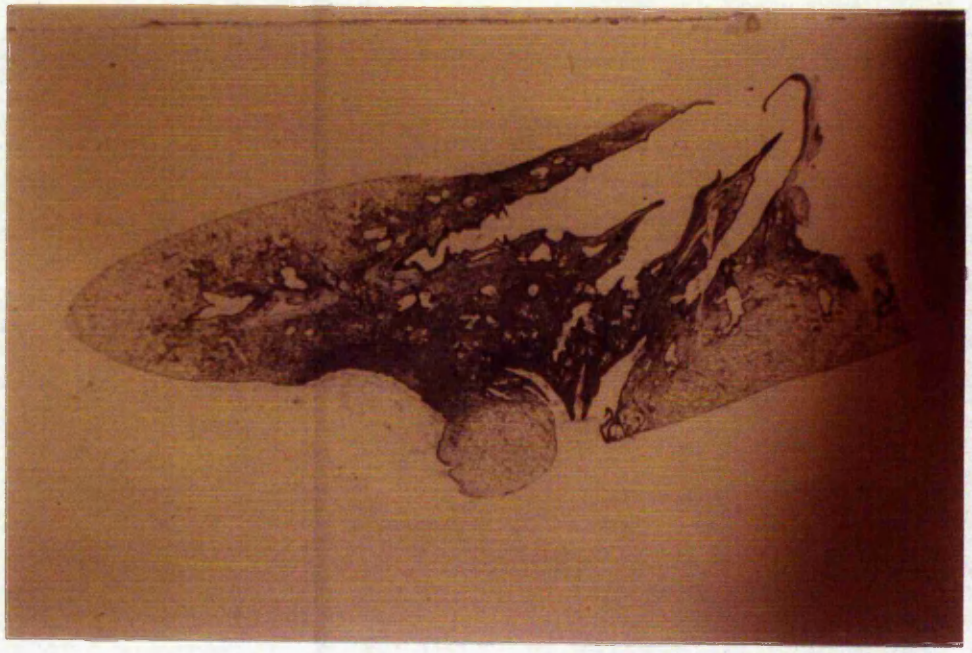
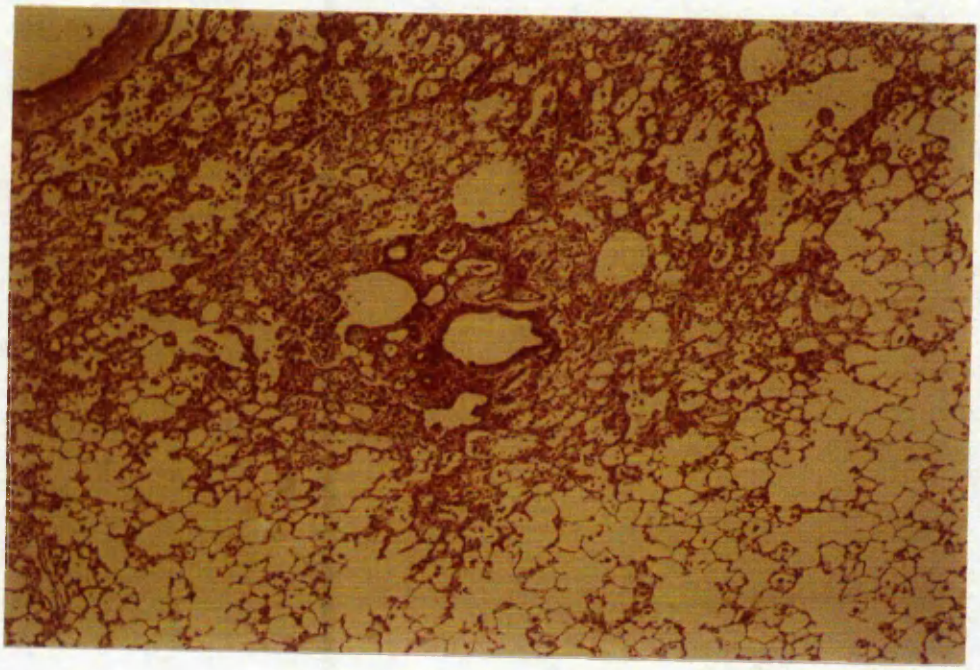


FIGURE 20:



## 2.5 DISCUSSION

Most investigators when assessing pulmonary "fibrosis" biochemically have tended to express the collagen, seen histologically, as total collagen content of the lung or lung lobes. This method of expression has been adopted for two reasons. Firstly because lung biopsy specimens obtained from patients with fibrosis have not shown the increased collagen concentration predicted from histological features of these lesions (Fulmer et al, 1976). Secondly because in several early animal studies a significant increase in total collagen content was observed although no increase in collagen concentration was seen (Göthe et al, 1968; Dale, 1973; McCullough et al, 1978). These investigators suggested that as parallel increases in other lung components were taking place during the fibrotic process (ie both numerator and denominator were changing) no increase in collagen concentration could be seen when expressed per mg of lung dry weight.

### 2.5.1 Dose response of bleomycin-induced fibrosis

It was therefore interesting to note that in the present study a significant increase in left lung collagen concentration above controls, was observed in histologically confirmed fibrotic lobes when assessed at 14 days after the intrabronchial instillation of 0.25-1.5U of bleomycin (instilled in 0.3ml of saline ) (Figure 10). Moreover, this increase in collagen concentration appeared to be dose related. Total collagen content of the left lung on the other hand was only found to be significantly elevated above controls by 14 days after the intrabronchial instillation of the smaller doses of bleomycin (0.1U and 0.25U of bleomycin instilled in 0.3ml saline).

With the exception of the dose of 0.25U of bleomycin, when a significant increase in collagen concentration in the left lung was observed, the dry weight, DNA and protein contents were all significantly lower than control values (Figure 10, Table 2). Although this type of pattern has not previously been reported after the intratracheal instillation of bleomycin, a similar phenomenon has been reported in several animal models of radiation induced pulmonary fibrosis in which specific lung lobes were irradiated (Law et al, 1976; Collins et al, 1978).

As expected, the corresponding right lung, following intrabronchial instillation of 0.1U-1.5U of bleomycin into the left lung (instilled in 0.3ml saline) did not show an increase in collagen concentration above control values at 14 days (Figure 10). However, a dose related increase in the total collagen content of the right lung was observed for these doses. Since histological examination of the right lungs showed very few focal fibrotic lesions, the large increases in total collagen content, as well as lung dry weight, protein and DNA content of the right lung are difficult to explain solely in terms of fibrotic changes.

The involvement of compensatory lung growth in uninjured areas of the right lung may possibly explain the large increases in the biochemical composition of the right lung. This phenomenon has been reported to occur in the uninjured lung lobes of baboons following the controlled exposure of lung lobes to radiation (Collins et al, 1978). Evidence for the role of compensatory lung growth in the present study is well illustrated by comparison of the data shown in Table 6.

TABLE 6: EVIDENCE FOR COMPENSATORY LUNG GROWTH IN RIGHT LUNG LOBES FOLLOWING THE INSTILLATION OF BLEOMYCIN. (Summarised data from results section)

| Route and dose of bleomycin originally instilled (a) | Dose of bleomycin encountered by right lung 1h after instillation (b) | Right lung collagen content (% of control) (c) |
|---|---|---|
| Direct intra-tracheal injection 1.5U | 0.197U | 160 ± 11 |
| Intratracheal instillation through cannula, 1.5U | 0.329U | 248 ± 17 * |
| Intrabronchial instillation, 0.5U | 0.006U | 167 ± 11 * |
| Intrabronchial instillation, 1.5U | 0.017U | 222 ± 6 * |

(a) All the doses were instilled in 0.3ml saline

(b) Determined from the distribution of [$^{3}$H]-bleomycin (Table 4)

(c) Determined at 14 days after bleomycin instillation

*Significantly different from controls $P < 0.05$

For example, the right lung collagen content at 14 days after intra-tracheal injection and intrabronchial instillation of 1.5U of bleomycin (instilled in 0.3ml of saline) showed increases, above control values of 60% and 122% respectively. Considering that histological studies showed a good correlation between fibrotic changes and the amount of bleomycin encountered by the lung lobes, the geater increase in right lung collagen content after the instillation of bleomycin

by the intrabronchial route cannot be accounted for by greater fibrotic change as it only received one tenth of the amount of bleomycin compared to that received by the right lung after the intratracheal injection of bleomycin.

The relative contributions of lung growth and the fibrotic process in the right lung cannot be determined with certainty from the biochemical data, and furthermore the nature of this lung growth is not entirely clear. Several animal studies have suggested that following pneumonectomy, the remaining lung undergoes compensatory growth and that the stretching of the remaining lung may provide the stimulus for protein synthesis and lung growth (Brody, 1975; Cowan and Crystal, 1975; Langston et al, 1977). Since the left lung in the fibrotic model induced by intrabronchial instillation of 0.5U-1.5U of bleomycin (instilled in 0.3ml) was very fibrotic and thus reduced in size the corresponding right lung may have been stimulated to grow.

Although other workers have examined bleomycin-induced lung damage in rodents in terms of dose-response, most of these studies have been restricted to describing the histological changes occurring in chronic models (Adamson and Bowden, 1974; Szapiel et al, 1979; Raisfield, 1980). Despite the fact that bleomycin was administered by different routes in these studies they all demonstrated a dose-related severity and incidence of fibrosis when assessed histologically. In the few studies in which collagen has been quantitated biochemically (expressed as total collagen per lung) a dose related increase has not been observed during the development of fibrosis (Starcher et al, 1978; Sikic et al, 1978) although a dose related increase in the synthesis of collagen has been demonstrated by

Hesterberg et al, (1981) following the intratracheal instillation of bleomycin in rats.

### 2.5.2 Characterisation of the fibrotic model following the intrabronchial instillation of bleomycin

Following the instillation of 0.5U of bleomycin in 0.3ml of saline and 1.0U of bleomycin in 0.1ml of saline by the intrabronchial route, the biochemical and histological changes were essentially complete within 1 to 2 weeks.

The left lung showed histological evidence of diffuse alveolar damage characterised by oedema, haemorrhage and inflammation, which was followed by a hypercellular "proliferative" phase, followed in turn by a loss of alveolar structure and the appearance of fibrosis. Although the histological changes in this model were similar to those reported by Snider et al (1978) after the intratracheal instillation of bleomycin, they occurred much more rapidly (ie 1-2 weeks as opposed to 4-8 weeks).

The influx of inflammatory and immune cells was found to be maximal at day 2 and consisted mainly of neutrophils and some macrophages. By day 4 much fewer neutrophils were seen and the number of macrophages, lymphocytes and fibroblasts had greatly increased with the greatest increase observed in the number of fibroblasts. Early signs of collagen deposition were present at this stage. The influx of cells and the deposition of collagen occurred both in the interstitial and intraalveolar regions, and between day 4 and 6 the alveolar structure in a large area of the left lung was totally obliterated. There was good correlation

between histological and biochemical changes. Although collagen concentration was significantly reduced at the time of maximum inflammation (day 2), it was seen to return towards normal by day 8 and was significantly elevated above controls by day 14. At no time was an increase in total lung collagen content seen in the left lung (Figure 12).

A few focal fibrotic lesions were also seen in the anterior and posterior lobes of the right lung and occasionally in the mid lobe. The cellular changes in these lesions were similar to those seen in the left lung. The focal nature of the lesions may partially account for the small increase in the water content (per g of dry weight) in the right lung (Figure 13). The biochemical changes occurring in the right lung were, however, much greater than those predicted from histology. Increase in right lung composition started occurring between 4 and 6 days after bleomycin instillation when the left lung started to consolidate. Thus the large increase in total collagen content seen at day 8 and day 14 was probably due to the occurrence of compensatory lung growth in the right lung and a small degree of fibrosis as previously discussed. The role of compensatory lung growth in the right lung is further supported by the large increase in DNA synthesis in the right lung by day 4.

### 2.5.3 Variables in the assessment and development of pulmonary fibrosis

a) Volume of instillation

The results of this study indicate that the volume of saline in which the bleomycin is instilled is important in determining the

magnitude of the fibrotic changes in the lung whether assessed biochemically or histologically. For example, the fibrotic changes in the left lung following the intrabronchial instillation of doses of bleomycin ranging from 0.25U-1.0U were always greater when these doses were instilled in 0.3ml of saline than 0.1ml of saline (Figures 8, 10 and Table 2). Similarly, changes seen in the biochemical composition of the left lung following the intrabronchial instillation of 0.5U of bleomycin instilled in 0.3ml saline were generally greater than those after the instillation of 1.0U of bleomycin in 0.1ml of saline when measured at different time intervals over a period of 14 days (Figures 12,13,14 and Table 5).

Although other investigators have produced a large number of animal models after the intratracheal instillation of bleomycin in different volumes of saline little discussion has centered on the importance of the dose volume. Clark et al (1980) using the intratracheal instillation of 1.0U of bleomycin in 0.1ml saline in hamsters reported that the histological changes were similar to those described by Snider et al (1978) and Goldstein et al (1979) who instilled 0.5U of bleomycin in 0.5ml of saline in hamsters. In the model developed by Clark et al (1980) lung dry weight, DNA and protein content were only significantly increased at 8,11 and 14 days after dosing, with an increase in collagen content occurring by 11 days. In the hamster model developed by Goldstein et al (1979) however, lung dry weight, DNA and protein content peaked at 30 days and remained elevated by 60 days. Collagen content in this model was only significantly

elevated by 30 days and reached a peak by 60 days. A good correlation between histological and biochemical changes was reported in this animal model. Surprisingly, no comment has been made to account for the differences observed in lung composition in these two hamster models.

b) Route of instillation

Both biochemical and histological studies indicate that the lung may show different fibrotic changes following the intratracheal and intrabronchial instillation of the same dose of bleomycin as illustrated by comparison of the data in Table 7. Histological examination of lungs showed that following the intrabronchial instillation of 0.25U-1.5U of bleomycin (in both 0.1ml and 0.3ml of saline) a continuous fibrotic lesion resulted in the left lung which extended along the mainstem of the bronchial tree (See also Figure 11B). This consolidated area covered over half of the left lung between 6 and 21 days when 0.5U of bleomycin was instilled by the intrabronchial route in 0.3ml of saline. In contrast, the fibrotic lesions in the left lung following the intratracheal instillation of 1.5U of bleomycin were focal in nature, with normal parenchymal tissue surrounding consolidated areas (Figure 11C,D), as previously described for the hamster model (Snider et al, 1978).

The extensiveness and distribution of the fibrotic lesions in the left lung, following both the intratracheal and intrabronchial instillation of bleomycin appeared to be related to the amount of bleomycin entering the lung lobe, as well as the volume in

Table 7 FIBROTIC CHANGES IN THE LEFT LUNG FOLLOWING THE INSTILLATION OF BLEOMYCIN (summarised data from results section)

| Changes in left lung | Route and dose of bleomycin originally instilled | | | |
|---|---|---|---|---|
| | Intratracheal/$^{1.5U/0.3ml}_{saline}$ | | Intrabronchial | |
| | Direct injection | Through cannula | 0.5U/0.3ml saline | 1.0U/0.1ml saline |
| Dose of bleomycin encountered by left lung 1h after instillation (Units) (a) | 0.20 | 0.46 | 0.28 | 0.50 |
| Volume of saline in which bleomycin is distributed (a) (ml) | 0.04 | 0.09 | 0.17 | 0.05 |
| Left lung collagen content (% of control) (b) | 138±19 * | 143±22 * | 101±8 | 101±8 |
| Left lung collagen conc'n (% of control) (b) | 100±12 | 127±7 * | 147±7 * | 127±13 * |
| Type of fibrotic lesions in the left lung |  |  |  |  |
| | Focal lesions | | Continuous lesions | |

(a) Determined from the distribution of [$^{3}$H]-bleomycin (Table 4)

(b) Determined at 14 days after bleomycin instillation

*Significantly different from controls $P < 0.05$

which it was instilled. Those lobes which showed extensive collagen deposition histologically also showed an increase in collagen concentration biochemically.

c) Number of lung lobes used for biochemical assays

When several lung lobes were analysed together, as in the case of the right lung, the occurence of compensatory lung growth and the presence of inflammatory cells in the lung may interfere with the biochemical assessment of fibrosis. The following examples may be used to illustrate this point. The collagen content of the left lung at 14 days after the intratracheal instillation of 1.5U bleomycin, both as a direct injection and as an injection through a cannula, showed approximately a 40% increase above controls, even though the lung lobe in the latter case encountered over double the amount of bleomycin and appeared much more fibrotic when assessed histologically. Although the right lung also received nearly double the amount of bleomycin when instilled through a cannula compared to a direct injection, the increase in right lung collagen content was much greater after the instillation of bleomycin through a cannula (248% of controls) than after a direct injection (160% of controls). The greater changes in the right lung after the instillation of bleomycin through a cannula probably resulted from both fibrosis and compensatory lung growth, since the left lung in this case was reduced in size and prevented from expanding by extensive fibrotic lesions.

In conclusion, at this stage it is difficult to say with certainty why the lung develops different types of fibrotic lesions following

the intratracheal and intrabronchial instillation of the same dose of bleomycin. It is possible that a slow intratracheal injection to the lung may lead to a patchy distribution of bleomycin in the form of droplets, thus resulting in focally high concentrations of bleomycin, which in turn may lead to the development of fical fibrotic lesions. In contrast, a rapid intrabronchial instillation in the left lung leads to an even distribution of bleomycin along the lung architecture, thus leading to the development of a continuous fibrotic lesion.

## 2.6 SUMMARY

These results have demonstrated that:

(i) an increase in collagen concentration was only observed in lung lobes which demonstrated histological evidence of extensive collagen deposition,

(ii) when several lung lobes were analysed together compensatory lung growth and inflammatory changes may dilute out the increase in collagen concentration,

(iii) intratracheal and intrabronchial instillation of an equivalent dose of bleomycin resulted in changes in the lung which were different when measured by both histological and biochemical techniques,

(iv) following the intrabronchial instillation of 0.5U of bleomycin in 0.3ml saline the left lung showed widespread fibrosis which resulted in the consolidation of the left lung within 2 weeks,

(v) uninjured areas of the lung appeared to undergo compensatory lung growth following bleomycin instillation by both routes,

(vi) the degree of fibrosis was not only dependent upon the dose of bleomycin but also upon the volume of instillation.

CHAPTER 3

# THE EFFECT OF HYPEROXIA ON THE DEVELOPMENT OF PULMONARY FIBROSIS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## 3.1 INTRODUCTION

The possibility of deleterious interactions between diffuse pulmonary injury and the breathing of oxygen is of great clinical importance since many patients with diffuse acute lung damage (such as occurs in the adult respiratory distress syndrome (ARDS)), have severe abnormalities of gas exchange that necessitate oxygen therapy for prolonged periods. From studies with animal models it is known that prolonged exposure to oxygen at partial pressures greater than that in ambient air results in toxic effects which become progressively more severe as the $pO_2$ and duration of exposure are increased (Haugaard, 1968; Clark and Lambertson, 1971; Fisher, 1980).

The finding that animals with experimental forms of lung injury survive prolonged periods in high concentrations of oxygen has led some investigators to conclude that the underlying lung injury might be protective against oxygen toxicity (Ohlsson, 1947; Smith et al, 1973). Alternatively it has also been suggested that oxygen may exacerbate the tendency to develop pulmonary fibrosis (Pratt et al, 1979; Witschi et al, 1981).

Clinical studies with patients receiving bleomycin therapy have suggested the possibility of increased susceptibility to pulmonary oxygen toxicity by these patients (Goldiner et al, 1978; Nygaard et al, 1978). More recently, investigations with animal models have suggested that oxygen may aggravate bleomycin-induced lung damage (Rinaldo et al, 1980; Toledo et al, 1982), although the evidence from these studies was not conclusive.

Considering the controversy on the effect of oxygen on the acutely damaged lung, the following section of work was designed to investigate the effect of hyperoxia on the development and progression of pulmonary fibrosis induced by the intrabronchial instillation of bleomycin.

## 3.2 AIMS

The experiments performed in the present section were undertaken to answer two basic questions.

(i) Does a diffusely damaged lung, resulting from bleomycin instillation show greater damage when exposed to 70% oxygen?

(ii) If exposure to hyperoxia leads to a greater extent of damage and fibrosis, what is the underlying mechanism for this interaction between bleomycin and oxygen?

Both histological and biochemical studies were undertaken to answer these questions.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Materials

Oxygen (99.9% pure) was obtained from Air Products Ltd, Lancs, UK Alderley Park, Wistar derived male SPF rats weighing 180-200g were used throughout. For other relevant materials see section 2.3.1

### 3.3.2 Exposure of animals to oxygen

Following the intrabronchial instillation of either sterile saline or 0.5U of bleomycin in 0.3ml saline, (as described in sections 2.3.2.2), the rats were divided into two main groups. One group was exposed to 70% oxygen for different time periods and the other group to air using special 60l perspex exposure chambers. Exposure times for animals in these chambers were: 0-3 day, 0-6 day and 6-12 day following instillation. The flow rate of air or 70% oxygen always exceeded 0.5l/min/rat and was controlled using Flowstat rate control valves and Flowmeters (Flowbits, Basingstoke, UK). The oxygen concentration and temperature were recorded continuously using a Beckman Fieldlab oxygen analyser, with daily spot checks for correct calibration using a Beckman $D_2S$ oxygen analyser (Beckman RIIC Ltd, Manchester, UK). Food and water were available ad libitum. At the end of the specified exposure times half of the rats were killed immediately for histological examination (section 2.2.3) and the other half were kept in room air for biochemical assessment of pulmonary fibrosis. All animals used in biochemical studies were killed at 14 days following instillation as described in section 2.3.5 Determination of hydroxyproline content, protein and DNA were performed

as described in sections 2.3.5.2, 2.3.5.3 and 2.3.5.4 respectively. Lung dry weight was determined by freeze drying 0.5ml of homogenate (section 2.3.4).

### 3.3.3 Retention of [$^{3}$H]-bleomycin in the lung

Bleomycin (0.5U) containing [$^{3}$H]-bleomycin (0.645uCi) was instilled in 0.3ml saline by the intrabronchial route as described in section 2.3.2.2. Animals were killed using halothane at 1h, 6h, 24h and 73h following instillation and the left lung processed for measurement of radioactivity as described in section 2.3.6. A semi-log plot of the amount of bleomycin retained in the left lung against time was used to determine the half-life of bleomycin in the lung.

## 3.4 RESULTS

### 3.4.1 The retention of bleomycin in the rat lung

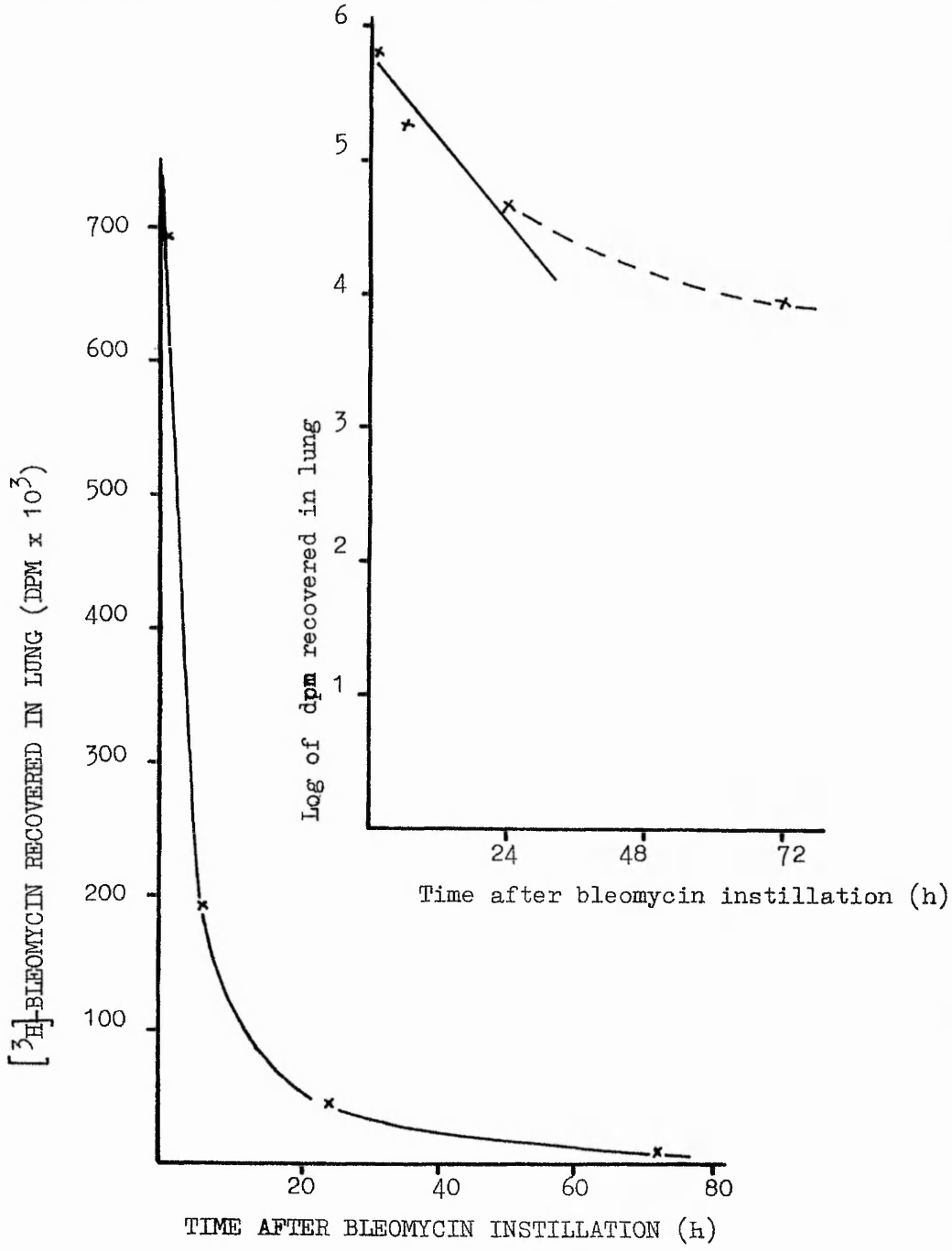
The retention of [$^{3}$H]-bleomycin in the left lung was measured over 3 days following the intrabronchial instillation of 0.5U of bleomycin in 0.3ml isotonic saline (Figure 21). The half life of bleomycin in the lung was determined by plotting a semi-log plot (Figure 21), and was estimated to be less than 13h over the first 24h after bleomycin instillation, Since the semi-log plot failed to follow a straight line after 24h this suggests that the normal functioning of the clearance mechanism in the lung may have been altered by this time due to bleomycin damage.

### 3.4.2 Histological and biochemical changes in the lung following exposure of bleomycin treated animals to 70% oxygen

In these studies rats were exposed to 70% oxygen at 3 different time intervals after bleomycin instillation. These periods were chosen to encompass the times when bleomycin was still present in the lung and when the initial inflammatory response had reached a peak (0-3 days). A time period which included the initial inflammatory response, maximum cellular proliferation and early signs of collagen deposition (0-6 days). Finally a time period when the fibrotic response was well underway (6-12 days).

Rats which received bleomycin by the intrabronchial route and subsequently exposed to air in the perspex chambers at the three different time periods were used as bleomycin controls. When analysed

FIGURE 21: THE RETENTION OF [$^3$H]-BLEOMYCIN IN THE LUNG FOLLOWING THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN




[$^3$H]-bleomycin (0.645 µCi) contained in 0.5U of "cold" bleomycin was instilled into the left lung in 0.3ml isotonic saline. Rats were killed at different times and the amount of radiolabel recovered in the left lung was determined and the half-life of bleomycin in the left lung calculated as described in section 2.3.6. The results show mean values for 5 rats per determination.

for fibrotic changes in the lung at 14 days after dosing these animals showed similar changes in the left lung total collagen (approximately 100% of saline instilled controls), collagen concentration (approximately 140% of saline instilled controls) and dry weight (approximately 70% of saline instilled controls) in all three treated groups of animals (Figure 22). Similar exposure of saline instilled rats to 70% oxygen did not lead to any significant changes in lung composition when compared to air exposed saline instilled controls. Histological examination of the lungs in these animals also showed that they were similar to those of saline instilled animals exposed to air.

The exposure of bleomycin treated animals to 70% oxygen after a delay of 6 days from the instillation time failed to show any significant changes in left lung collagen content, collagen concentration, and dry weight at 14 days after dosing when compared to bleomycin treated animals exposed to air (Figure 22). However, when animals were exposed to 70% oxygen for either 3 or 6 days immediately after bleomycin instillation a 30% drop in total collagen was observed ($P < 0.05$) when compared to both saline instilled control animals exposed to 70% oxygen and bleomycin treated control animals exposed to air. In the case of animals exposed to oxygen for 6 days after bleomycin instillation, left lung dry weight was also found to be significantly less than in animals treated with bleomycin but exposed to air for 6 days ($P < 0.05$).

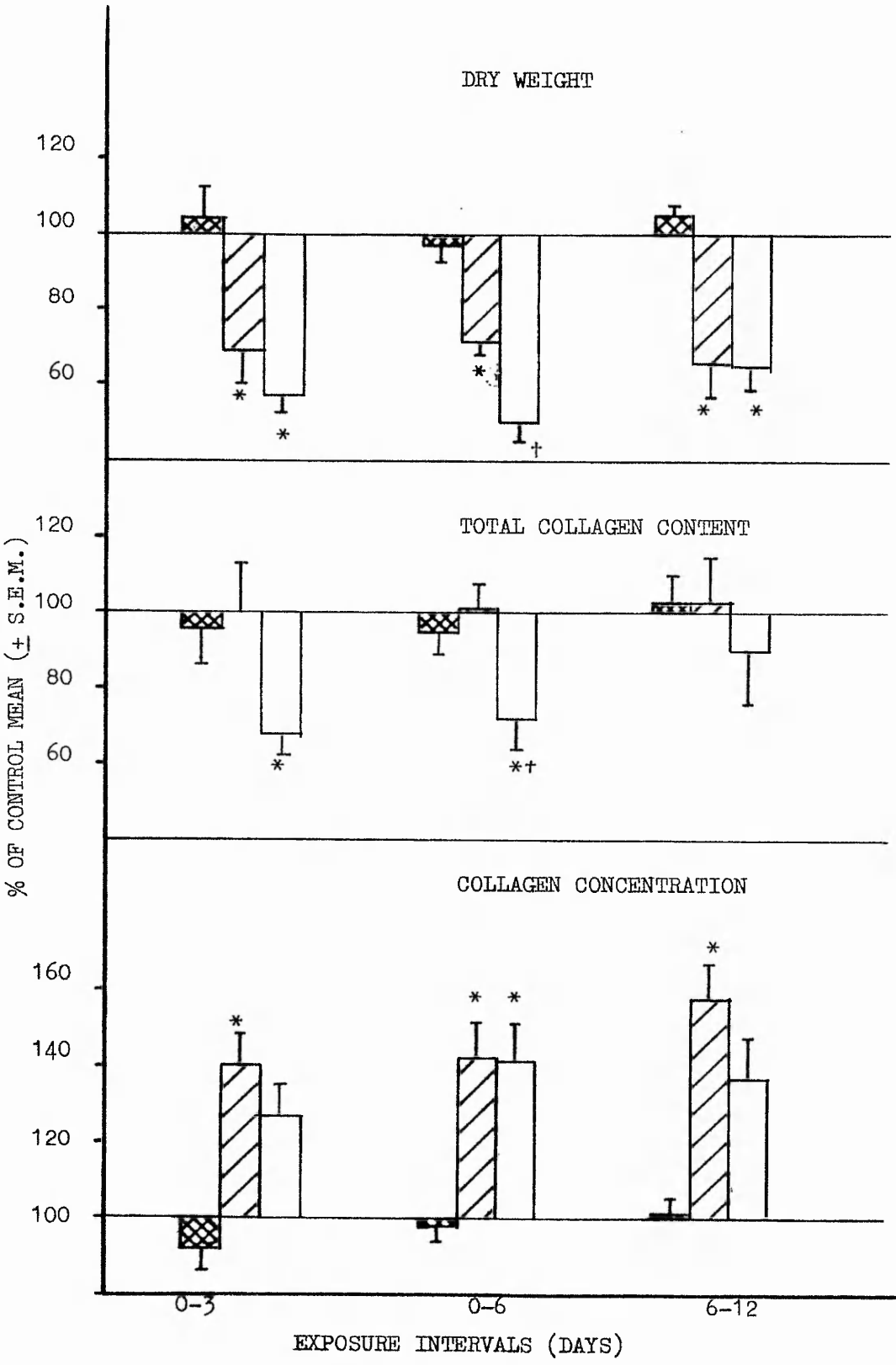
Morphological and histological examination of the left lung showed that there was no obvious difference in the lungs of bleomycin instilled animals exposed to air and the lungs of animals which underwent a delayed oxygen exposure 6 days after bleomycin instillation. On

FIGURE 22: CHANGES IN LUNG COMPOSITION FOLLOWING THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN AND SUBSEQUENT EXPOSURE OF RATS TO 70% OXYGEN

Left lung dry weight, total collagen content (total HP) and collagen concentration were determined at 14 days following the intrabronchial instillation of 0.5 U of bleomycin in 0.3 ml isotonic saline and subsequent exposure to 70% oxygen or air (section 3.3.2) for 3 different time periods. Bleomycin treated rats exposed to 70% oxygen are shown as ☐ (n = 3 to 4 rats); bleomycin treated rats exposed to air are shown as ▨ (n = 4 rats); saline treated rats exposed to 70% oxygen are shown as ▩ (n = 4 rats). The results show the mean determinations ± S.E.M. expressed as per cent of saline treated control rats exposed to air. The data from these control animals were pooled (n = 12), since the various parameters did not differ significantly ($P<0.05$) over the 3 different exposure times. Control left lung values were, dry weight = 72.7 ± 1.7 mg; total hydroxyproline content = 1034 ± 31 µg; hydroxyproline concentration = 14.24 ± 0.34 µg/mg dry weight.

* Significantly different from air exposed saline controls ($P<0.05$).

† Significantly different from bleomycin treated animals exposed to air ($P<0.05$).


DRY WEIGHT
120
100
80
60
TOTAL COLLAGEN CONTENT
120
100
80
60
COLLAGEN CONCENTRATION
160
140
120
100
% OF CONTROL MEAN (± S.E.M.)
0-3
0-6
6-12
EXPOSURE INTERVALS (DAYS)

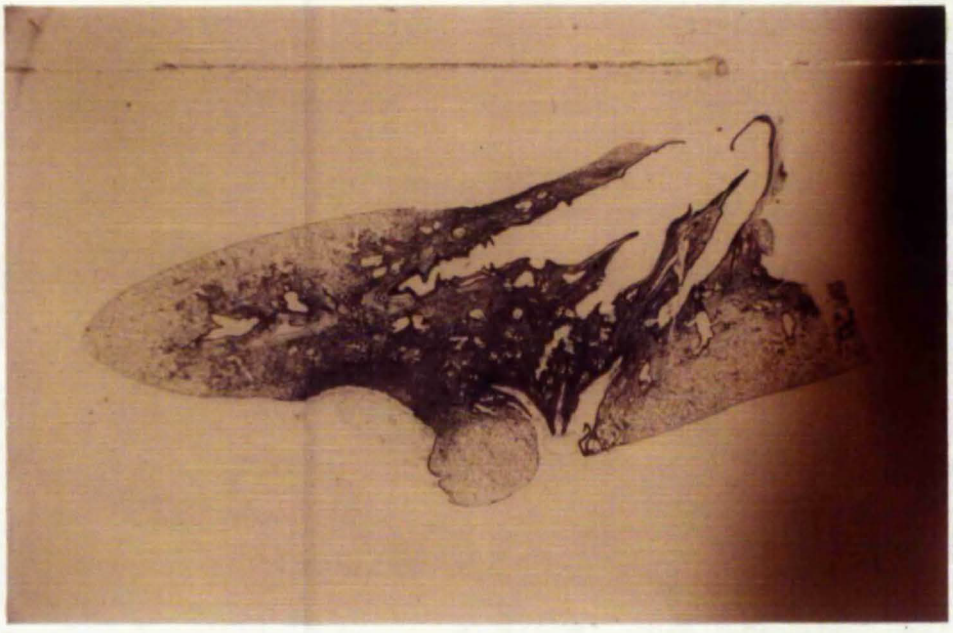
the other hand the lungs of animals exposed to oxygen for either 3 or 6 days immediately after bleomycin instillations were much more fibrotic than those which received bleomycin, but were exposed to air (Figure 23). Immediate exposure of rats to 70% oxygen for 3 days after bleomycin led to both more severe and more widespread inflammatory and proliferative response when compared to bleomycin treated controls exposed to air (Figure 24). However, biochemical asessment of fibrosis in the left lungs of the bleomycin plus oxygen treated animals failed to show the expected increase in collagen concentration above the bleomycin treated control animals exposed to air (Figure 22).

FIGURE 23: THE EFFECT OF HYPEROXIA ON THE DEVELOPMENT OF PULMONARY FIBROSIS

A) Left lung section at day 6 following the intrabronchial instillation of 0.5 U of bleomycin in 0.3 ml isotonic saline, showing extensive collagen deposition (blue stain) (Martius Scarlet Blue stain, magnification x 4).

B) Left lung section at day 6 following the intrabronchial instillation of 0.5 U of bleomycin in 0.3 ml saline and immediate exposure to 70% oxygen for 6 days. A much greater degree of consolidation in the left lung was observed when compared to the left lung of animals which received bleomycin and were exposed to air (A). (Martius Scarlet Blue stain, magnification x 4).

A



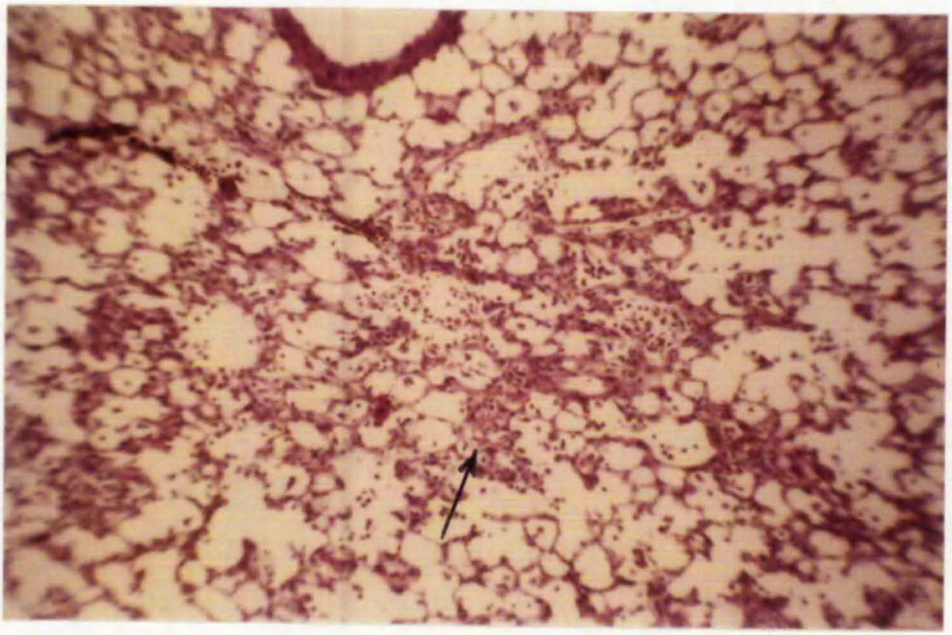
B

FIGURE 24: THE EFFECT OF HYPEROXIA ON THE DEVELOPMENT OF PLUMONARY FIBROSIS

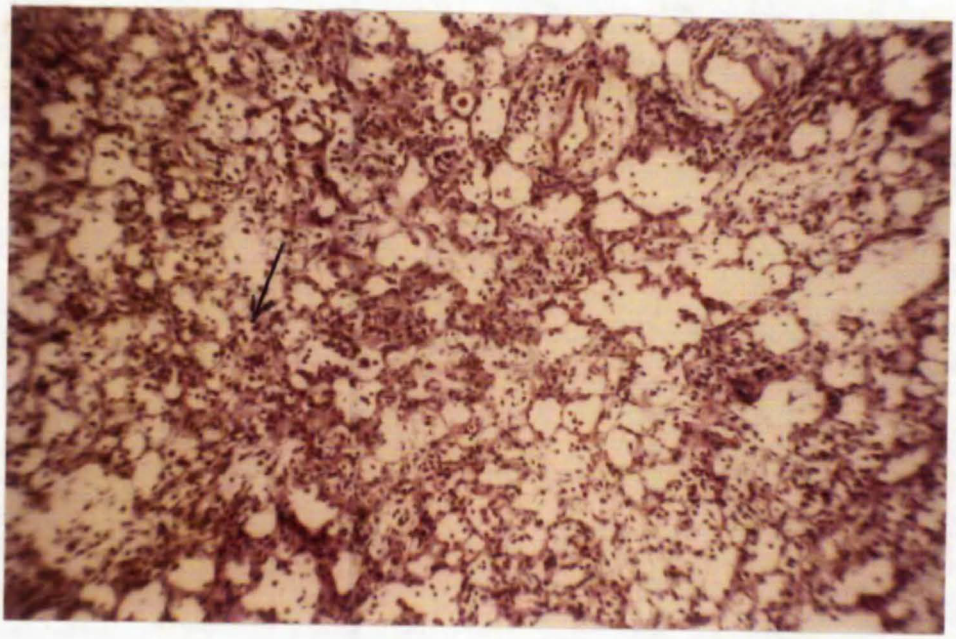
A) Left lung section at day 3 following the intrabronchial instillation of 0.5 U of bleomycin in 0.3 ml saline, showing an increase of inflammatory cells in both the interstitial and intra-alveolar regions, with some areas showing considerable epithelial and fibroblastic hyperplasia (arrow) (Haematoxylin and eosin stain, magnification x 123 ).

B) Left lung section at day 3 following the intrabronchial instillation of 0.5 U of bleomycin in 0.3 ml saline and immediate exposure to 70% oxygen for 3 days. A much greater degree of inflammation and fibroblastic hyperplasia (arrow) was observed when compared to the left lung of animals which received bleomycin and were exposed to air (A). (Haematoxylin and eosin stain, magnification x 123 ).

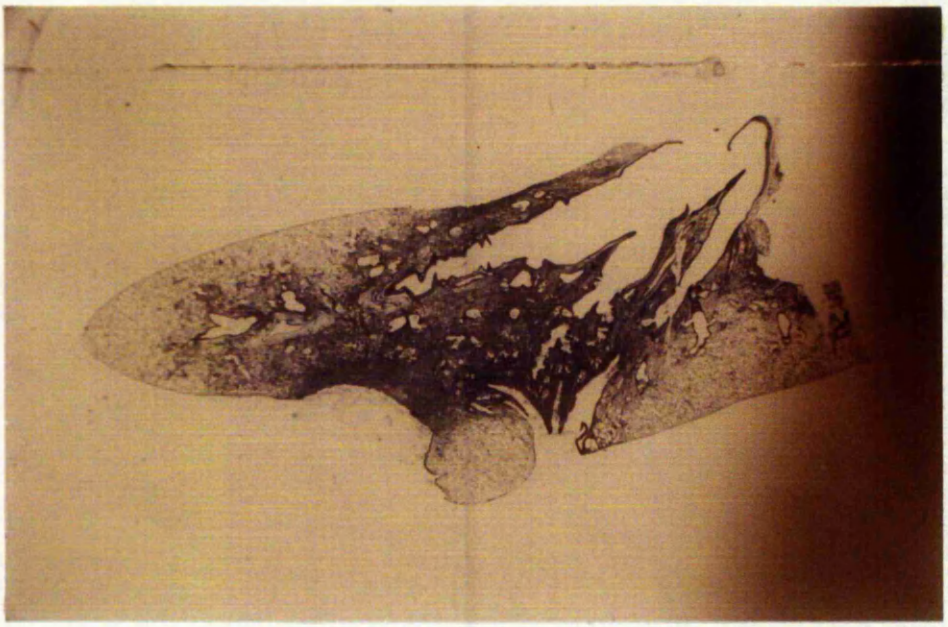
A



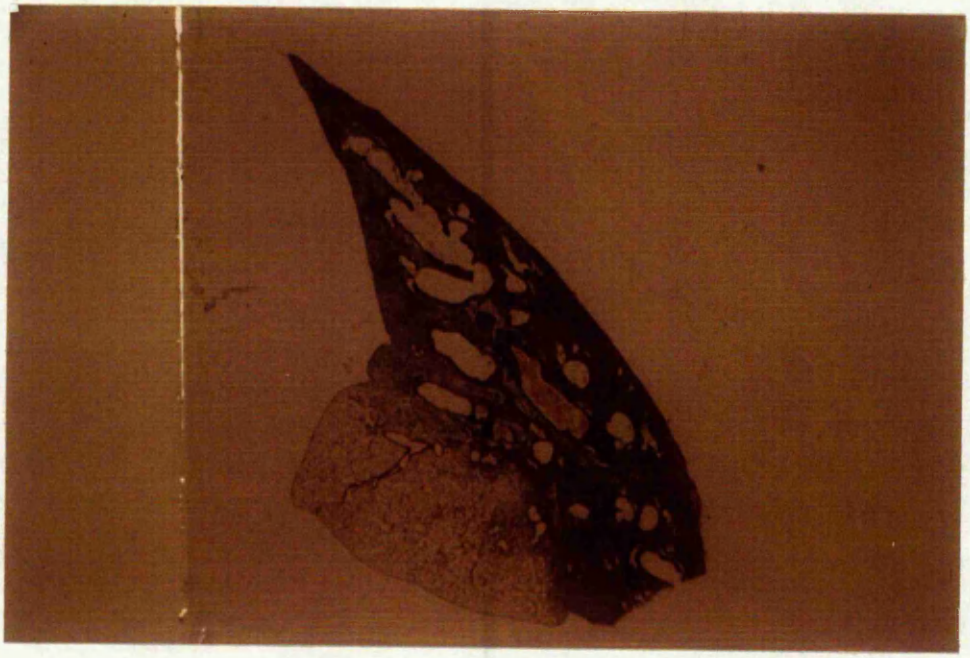
B



A



B



## 3.5 DISCUSSION

Prior to the investigations undertaken in this study two recent reports were published which attempted to evaluate the effects of hyperoxia on a bleomycin damaged lung (Rinaldo et al, 1980; Toledo et al, 1982). Both reports however proved to be inconclusive. Rinaldo et al (1980) indicated that the interaction between oxygen and bleomycin pulmonary toxicity in hamsters was bimodal; bleomycin induced lung damage resulted in early death from oxygen toxicity in some animals, while inducing oxygen tolerance in others. Toledo et al, (1982) demonstrated that the continuous exposure of mice to 40% oxygen, following repeated injections of bleomycin, shortened their median survival. On the basis of the morphological data obtained, the investigators could not however be certain that the site of the interaction between the two agents was the lung tissue.

Subsequently, in addition to the study undertaken in this section, several other workers have investigated the effect of hyperoxia on bleomycin induced lung damage, and a much clearer picture has emerged.

In hamsters treated with bleomycin and exposed to 70% oxygen for 3 days, more severe lung lesions developed than in animals treated with bleomycin alone (Tryka et al, 1982a). From morphological studies of the lung it was suggested by these investigators that the interaction between bleomycin and oxygen may be synergistic (Tryka et al, 1982b).

In the present study both histological and biochemical data demonstrated that the exposure of a damaged lung to hyperoxia may be detrimental. It has to be emphasized however that the increase in lung damage was only seen when the animals were exposed to 70% oxygen immediately after bleomycin instillation for either 3 or 6 days.

The correlation between the biochemical and histological changes in the lungs of animals exposed to oxygen for 3 or 6 days following bleomycin instillation was not as clear as that following the instillation of bleomycin alone. For example, the rats treated with bleomycin plus oxygen failed to show an increase in left lung collagen concentration above that seen in the lungs of animals treated with bleomycin and exposed to air as predicted from their histology (Figure 22 and 23). In these animals the best correlation of left lung histology was seen with changes in left lung dry weight and total collagen content. Since both of these parameters were significantly lower when compared to the left lungs of bleomycin treated animals exposed to air, there seems little doubt that the interaction of bleomycin with oxygen led to a greater degree of lung damage and development of fibrosis. These results further indicate the precaution required in the biochemical assessment of fibrosis.

In agreement with the observations of Tryka et al, (1982b), the histological changes seen in the lungs of rats treated with bleomycin plus 70% oxygen (for 3 and 6 days) were similar to those described after bleomycin alone, although more severe (Figure 23 and 24).

The ways in which oxygen and the underlying lung injury interact are obviously complex. Several investigators have suggested that the regeneration of the epithelium in the lung after injury normally suppresses fibroblast function and that if oxygen interferes with this regenerative process, fibrosis is enhanced (Haschek and Witschi, 1979; Witschi et al, 1981). However the results obtained for the interaction of bleomycin and oxygen in the model presented in this study, as well as those obtained by Hakkinen et al (1982) suggest that this mechanism may not be involved or that it may play a limited role. Hakkinen et al (1982) found that in mice given bleomycin by the intraperitoneal route, exposure to 70% oxygen during the period of maximum DNA synthesis (time of maximum epithelial type II cell regeneration) failed to show an increase in the severity of fibrosis. An increase in fibrosis was however observed when the animals were exposed to oxygen during the early period (1 to 8 days), before the occurence of maximum DNA synthesis.

Maximum DNA synthesis in the left lung of the animal model presented in this study occurred around day 4 after bleomycin instillation (Figure 14). Thus exposure of rats to 70% oxygen for 6 days immediately after bleomycin instillation was expected to produce a greater fibrotic response in the lung in comparison to rats exposed for 3 days only. However, this was not observed, and it appears that the event which leads to a greater fibrotic response probably occurs within 3 days after bleomycin instillation. This is supported by the observation that the overall loss in total collagen content at 14 days (which as previously stated correlated well with histological evidence of increased fibrosis)

was similar in both the 3 and 6 day oxygen exposed animals. Furthermore, histological examination of the left lung in these rats showed an enhancement of the inflammatory and proliferative process after 3 days of oxygen exposure compared to bleomycin alone (Figure 24). The enhancement of the inflammatory and proliferative process may be in response to the increased pulmonary toxicity resulting from an interaction between bleomycin and oxygen. Since bleomycin is rapidly lost from the lung following instillation by the intrabronchial route (with a half life of less than 13 hours over the first 24 hours) its interaction may occur in the early periods following instillation.

What mechanisms might account for the interaction between hyperoxia and bleomycin? Oxygen toxicity, at the molecular level, is thought to be due to the generation of reactive oxygen species (See section 5.1.1). The primary target in the lung appears to be the pulmonary endothelial cell (Deneke and Fanburg, 1980). Since reactive oxygen species have also been implicated in the injury to cells caused by bleomycin, it is possible that these two agents may cause cell injury by an increased generation of the reactive oxygen species. Despite the attractiveness of this hypothesis other interactions with oxygen might also be involved which may lead to an enhancement of fibrosis. These include: the inactivation of anti-proteases by oxygen (Bruce et al, 1981) and the hyperoxic injury to alveolar macrophages causing the release of chemotoxins and subsequent recruitment of neutrophils to the lung (Fox et al, 1981).

In this study a delay in the exposure of rats to oxygen (6-12 days) after bleomycin instillation did not result in any significant

changes from bleomycin treated animals exposed to air. However, this must not be taken to mean that in the clinical situations patients suffering from acute respiratory distress syndrome may be administered oxygen for long periods. Recently it has been demonstrated that the exposure of hamsters to 60% oxygen over a period of 21 days adds to the severity of the lung injury previously induced by bleomycin (Rinaldo et al, 1982).

CHAPTER 4

# EARLY MARKERS OF BLEOMYCIN INDUCED PULMONARY FIBROSIS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## 4.1 INTRODUCTION

Histological studies in man and various animal models suggest that bleomycin induced pulmonary toxicity may occur in four stages. Firstly there is endothelial cell damage characterised by endothelial cell blebbing and necrosis. Secondly there is injury to the type I epithelial cells. Damage to these two cell types results in the influx of inflammatory and immune cells, which in the third stage is followed by type II epithelial cell proliferation, appearance of cuboidal and/or ciliated epithelial cells and alveolar debris. Finally there is pronounced collagen deposition that characterises the onset of fibrosis (Delena et al, 1972; Iacovino et al, 1976; Adamson and Bowden, 1974, 1979; Bedrossian et al, 1977; McCullough et al, 1978; Sikic et al, 1978; Snider et al, 1978; Vates et al, 1979; Tom and Montgomery, 1980, Newman et al, 1980). In these animal studies bleomycin was administered by the intravenous subcutaneous, intramuscular, intraperitoneal or intratracheal route as a single dose or in repeated injections with treatments lasting from 2 days to 10 weeks. In most cases evaluation of the toxicity was assessed histologically. Little is known, however, about the changes in the biochemical processes of the lung, which accompany the morphological changes prior to collagen deposition.

A clearer understanding of some of the biochemical processes occurring in the lung following bleomycin administration would serve in developing useful biochemical markers for predicting the onset of fibrosis. In this regard the release of membrane bound

angiotension-converting enzyme (ACE) and the impairment of cellular clearance of certain biogenic amines, both of which are associated with the pulmonary endothelial cells, have been suggested as possible biochemical markers of bleomycin induced toxicity in animal models (Vats et al, 1979; Tom and Montgomery, 1978; Newman et al, 1980, 1981; Lazo, 1980; Lazo et al, 1981; Catravas et al, 1981). The importance or relation of these changes to the initiation or enhancement of the pulmonary fibrotic response is, however, not clearly understood. Recent studies of Newman et al, (1980, 1981) and Lazo et al (1981, 1983) suggests that these biochemical measurements of endothelial cell injury may be useful in providing an indication of acute pulmonary toxicity, but not the onset of pulmonary fibrosis.

### 4.1.1 The use of putrescine accumulation into the lung as a marker of pulmonary cell damage

One of the earliest events that occurs during cell growth, replication and differentiation is an increase in the levels of the polyamines, putrescine, spermidine and spermine, and the enzyme ornithine, decarboxylase (ODC), which is the rate regulating enzyme in the synthesis of these polyamines (Jänne et al, 1978; Williams-Ashman and Canellakis, 1979; Heby, 1981). Surprisingly there is a paucity of information concerning the role of and changes in polyamine levels in the lung during pulmonary fibrosis.

The biosynthesis of polyamines in mammalian cells and their structure is shown in Figure 25. The diamine putrescine is synthesised from ornithine through the activity of ornithine decarboxylase. Putrescine may then be converted to the other polyamines by the addision of aminopropyl groups catalysed by specific enzymes.

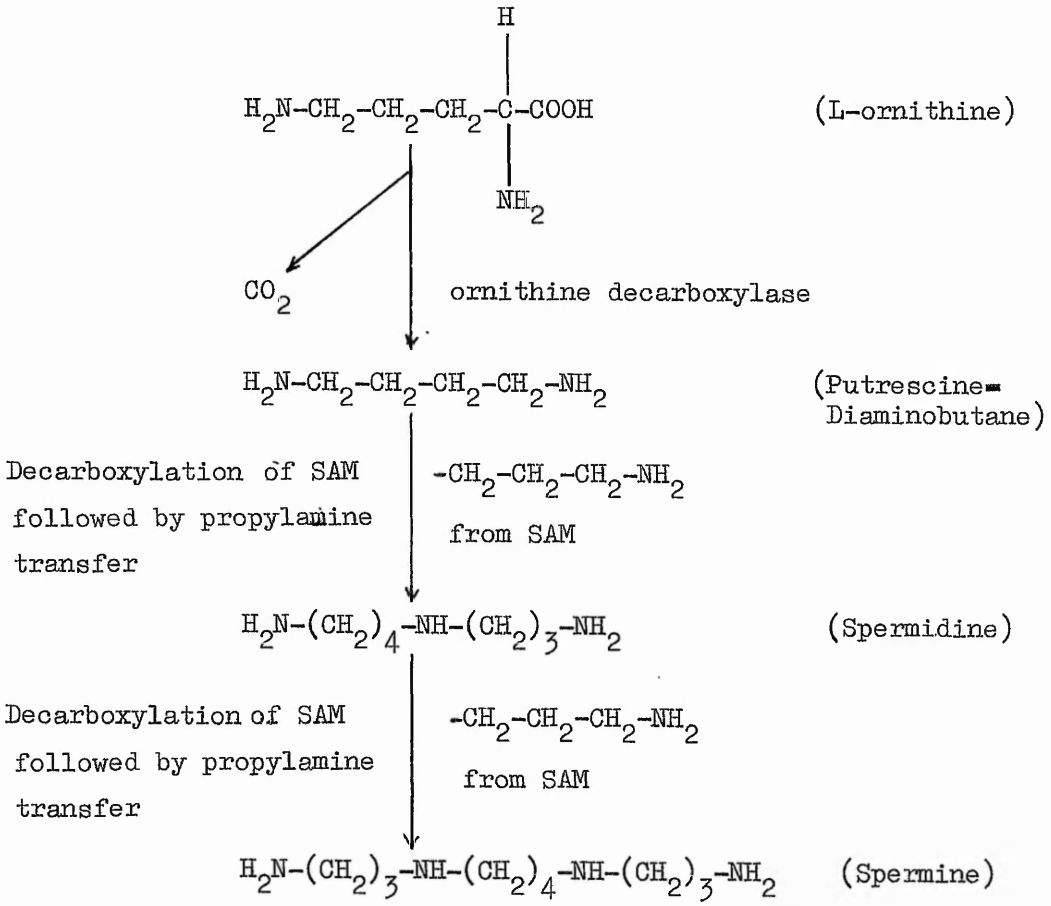



Figure 25: Polyamine biosynthesis in mammals

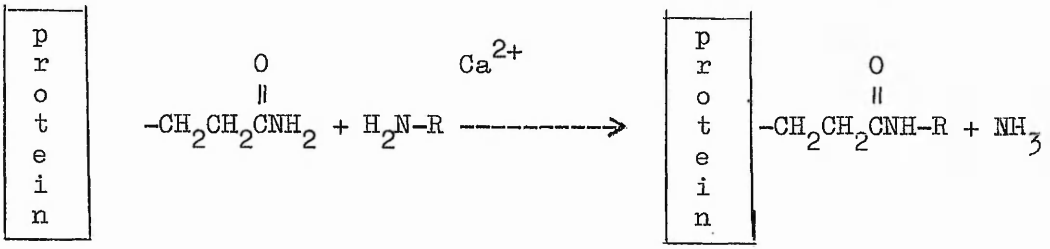
SAM= S-adenosyl-L-methionine

Recently, it has been demonstrated that lung tissue slices are capable of active accumulation of putrescine (Smith and Wyatt, 1981; Smith et al, 1982). It is believed that this uptake mechanism is probably responsible for the selective accumulation of the herbicide paraquat (Smith and Wyatt, 1981). The cells responsible for putrescine and paraquat accumulation are thought to be the epithelial cells, most probably the type II cells (Smith et al, 1976; Sykes et al, 1977; Keeling et al, 1981). The uptake process has been shown to obey saturation kinetics, and the structural requirement for selective accumulation into lung cells have been investigated (Smith et al, 1983a).

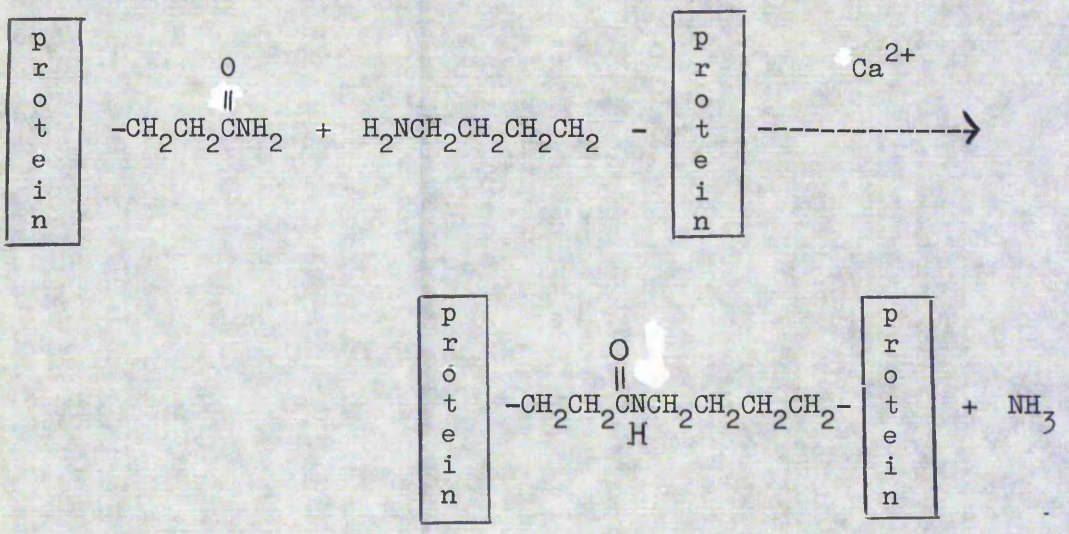
By following the changes in the accumulation of putrescine into lung slices after toxic lung injury it may therefore be possible to determine the time and severity of epithelial cell damage. This in turn may provide an insight into whether proliferating type II cells accumulate putrescine as efficiently as normal type II cells.

### 4.1.2 The use of transglutaminase activity in the lung as a potential marker of pulmonary fibrosis

Reactions catalysed by the $Ca^{2+}$-dependent enzyme transglutaminase (glutaminyl-peptide:amine $\gamma$-glutamyl transferase) lead to the post-translational modification of a protein either through the specific incorporation of amines into the $\gamma$-carboxamide groups of glutamine



or by the cross-linking of polypeptide chains by ε -(γ -glutamyl) lysine bridges



(For reviews see Lorand and Stenberg, 1976 and Folk and Finlayson, 1977). Although transglutaminase enzymes occur widely in Nature (Folk and Finlayson, 1977), the majority of the literature related to the physiological function of these enzymes has been directed to their ability to catalyse cross-links in polypeptide chains occurring in extracellular fluids (Ching, 1972; Murtagh et al, 1973; Mosher et al, 1979, 1980; Williams-Ashman et al, 1980; Williams-Ashman and Canellakis, 1980; Buxman and Wuepper, 1975; Buxman et al, 1980; Buxman, 1981).

The function of the ubiqitous so called "tissue transglutaminase" (Chung, 1972), however, is still poorly understood, although evidence suggests it may play an important role in the mediation of events at the cell membrane (Birckbichler et al, 1973; Birckbichler and Patterson, 1978; Siefring et al, 1978; Bjerrum et al, 1981; Fesüs et al, 1981).

The ability of transglutaminase enzymes to cause post-translational modifications of native protein has also led to investigations of their involvement in wound healing and in diseases such as atherosclerosis and pulmonary fibrosis (Lorand, 1969; Laki et al, 1972; Griffin et al, 1979). Griffin et al (1979) using an animal model of paraquat induced pulmonary fibrosis demonstrated a rise in lung transglutaminase activity, which was found to parallel the rise in the activity of prolyl hydroxylase. This activity was predominently associated with the "nuclear pellet" fraction obtained from lung homogenates. These authors suggested the possible importance of transglutaminase in the establishment of the connective tissue matrix during pulmonary fibrosis.

More recent studies have indicated the importance of transglutaminase in the activation of inflammatory and immune cells such as macrophages and lymphocytes (Novogrodsky et al, 1978; Folk et al, 1980; Schroff et al, 1981; Julian et al, 1983; Murtaugh et al, 1983). Since both these cell types are found in large numbers during bleomycin induced pulmonary fibrosis it is possible that transglutaminase activity may therefore serve as an indicator of the inflammatory stage during the development of pulmonary fibrosis.

## 4.2 AIMS

1 To investigate any changes in [$^{14}$C]-putrescine accumulation by lung slices following bleomycin instillation into the lung.

2 To investigate any changes in lung transglutaminase activity following bleomycin instillation into the lung.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Materials

(1,4-$^{14}$C-) putrescine dihydrochloride (113 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, UK. Scintillation cocktails (Instagel, Dimilume 30 and Scintillation cocktail T) and Soluene 350 were obtained from Packard Instrument Company Ltd, Caversham, Berkshire, UK. Tris-chloride and dithiothreitol were obtained from Sigma Chemical Co Ltd, Saint Louis, Missouri, USA. N,N$^{1}$-dimethylcasein was obtained from BDH chemicals Ltd, Warwickshire, UK.

Krebs Ringer Phosphate (KRP) buffer (pH 7.4) contained NaCl (130mM), KCl (5.2mM), $CaCl_2$ (1.9mM), $MgSO_4$ (1.29mM), $Na_2HPO_4$ (10µM) and glucose (10µM). All of these chemicals were obtained from BDH chemicals Ltd, Warwickshire, or Dorset, UK, and were of the highest possible grade.

### 4.3.2 Measurement of [$^{14}$C]-putrescine accumulation by lung slices

At various times after treatment with bleomycin rats were killed as described in Section 2.3.3 and the lungs removed without prior perfusion. Half of the left lung was then chopped so as to give 0.5mm thick transverse sections using a McIlwain automatic tissue chopper (Mickle Laboratory Engineering Co Ltd, Guildford, Surrey, UK). Lung slices weighing between 20-40mg were placed in pre-incubated 25ml flasks at 37 $^{o}$C containing 3ml of KRP and 10 uM putrescine (containing 0.1µCi of $^{14}$C-putrescine). The slices were incubated at 37 $^{o}$C in a shaking water bath shaking at 100 spm for

15, 30 and 60 min. At the end of each incubation 100µl aliquots of medium were taken and diluted to 1ml with water and the radioactivity measured after the addition of 10ml Instagel. The lung slices were removed, washed with fresh KRP medium, and dissolved in 1ml Soluene 350. Radioactivity was measured after the addition of 10ml Dimilume 30. Counting efficiency was determined by internal standardisation following the addition of [$^{14}$C]-hexadecane. The accumulation of putrescine into the lung slices was then determined by calculation of the slice to medium ratios (1mg of tissue slice was taken to represent a 1µl volume).

The unchopped half of the left lung was used to determine the water content of the lung as described in section (2.3.4). Slice to medium ratios were then corrected for oedema.

### 4.3.3 Preparation of lung homogenates for transglutaminase assay

Following the perfusion of rat lungs with ice cold 0.15M NaCl the left lung was removed and homogenized in 3 volumes of 0.25M sucrose/ 1mM EDTA/1mM Tris-chloride (pH 7.4) with an Ultraturrax homogeniser for 15 s. The homogenate was centrifuged at 600 xg (r av. 28cm) for 10 min and the resulting supernatant saved. The pellet was then rehomogenized in an equal volume of sucrose medium and combined with the first supernatant to produce the final homogenate. For some studies a sample of the homogenate was recentrifuged at 600 xg (r av. 28cm) for 10 min to provide a "cytoplasmic fraction" and a "nuclear pellet" which was resuspended in an equal volume of sucrose medium for further analysis.

4.3.4 Transglutaminase assay

Enzyme activity was measured by the method of ($^{14}$C)- putrescine incorporation into N-N$^{1}$-dimethylcasein using the filter paper assay of Lorand et al (1972).

The reaction mixture at 37 °C and pH 7.4 contained in a final volume of 100µl:Tris- chloride (28mM), dithiothreitol (3.85mM), $CaCl_2$ (2.5mM), ($^{14}$C)-putrescine (1.2mM, 3.97 mCi/mmol), N,N$^{1}$-dimethylcasein (5mg/ml).

Control incubations were also performed in which EDTA (5mM) replaced $CaCl_2$. The incubations were carried out in 1.0ml plastic microcentrifuge tubes in a water bath at 37 °C. The reaction was started by the addition of 45µl of enzyme sample; thereafter, samples (10µl) were taken at time intervals and deposited on pencil-labelled squares (1cm$^{2}$) of Whatman 3MM filter paper which were immediately placed in stirred ice-cold 10% (w/v) TCA (approximately 300ml) for at least 10 min. The filter paper samples were then freed of unbound radioactivity by transfer through a washing procedure that was comprised of:

3 x 10 min ice-cold 5% (w/v) TCA;

1 x 5 min acetone/ehtanol (1:1 (v/v));

1 x 5 min acetone.

The filter paper samples were then air-dried overnight and counted for radioactivity following the addition of 2ml Fisofluor 2 (Fisons) in a Packard Tri-Carb 300 Scintillation spectrometer.

Background was determined with filter paper squares which were taken through the washing procedure with the radioactive samples. At the end of the incubation period for each vial 10 µl of sample was deposited onto a filter square and allowed to dry overnight. The counts obtained from these filters were then used to calculate counting efficiency. The rates of $Ca^{2+}$- dependent incorporation of putrescine were then calculated after the subtraction of control values obtained from EDTA control vials. Units of enzyme activity were expressed as nmol putrescine incorporated/h under the conditions of assay.

## 4.4 RESULTS

### 4.4.1 Putrescine accumulation by lung slices following the instillation of bleomycin

The accumulation of putrescine by lung slices in all these studies was determined from the slice to medium ratios of ($^{14}$C)-putrescine at 15, 30 and 60 min. Since putrescine accumulation was found to be linear over 60 min, the results show the values at 60 min expressed as percent of the control values.

The change in putrescine accumulation at various time intervals following the intrabronchial instillation of 1.0U of bleomycin (instilled in 0.1ml saline) and 0.5U of bleomycin (instilled in 0.3ml saline) is shown in Figure 26. These two doses were used because the fibrotic changes occurring in the lung following their administration had been well characterised (Chapter 2).

The instillation of both of these doses failed to produce any significant reduction in putrescine accumulation in lung slices prior to the occurence of the inflammatory phase in the lung. Only by day 8 was a significant reduction in putrescine accumulation seen following bleomycin (1.0U) instillation. However, this represented the final stage of the fibrotic process when extensive collagen deposition was observed. The most interesting observation in these studies was the considerable increase in the accumulation of putrescine by lung slices during the inflammatory and proliferative phases (1-3 days) when compared to control lung slices from saline instilled animals.

In an effort to gain a better understanding of how bleomycin treatment changes putrescine accumulation in lung slices, the effect

FIGURE 26: PUTRESCINE ACCUMULATION IN LUNG SLICES FOLLOWING THE INTRABRONCHIAL INSTILLATION OF BLEOMYCIN

Putrescine accumulation into left lung slices following the intrabronchial instillation of 1.0U of bleomycin in 0.1ml isotonic saline (X) and a 0.5U of bleomycin in 0.3ml isotonic saline (O) was determined at various time intervals after instillation as described in section 4.3.2. For the measurement of putrescine accumulation into lung slices taken at day 1 and 3 after the instillation of 1.0U of bleomycin in 0.1ml saline the method was slightly modified and involved the homogenization of slices in 3ml of KRP using a polytron vortex homogenizer for 30s and the radioactivity counted in 0.1ml of homogenate after adding 0.9ml of water and 10ml of instagel. Control lungs were treated in the same manner. The results show the mean determinations $\pm$ S.E.M. for slice/medium ratio (S/M) for 4 rats expressed as per cent of control values.

During each measurement 3 to 4 control rats were always included. Control values (S/M ratio) for slices treated by solubilization in soluene were $37.04 \pm 3.73$ ($n = 6$) whereas control values treated by homogenization were $19.35 \pm 1.58$ ($n = 6$).

*Significantly different from controls ($P < 0.05$)

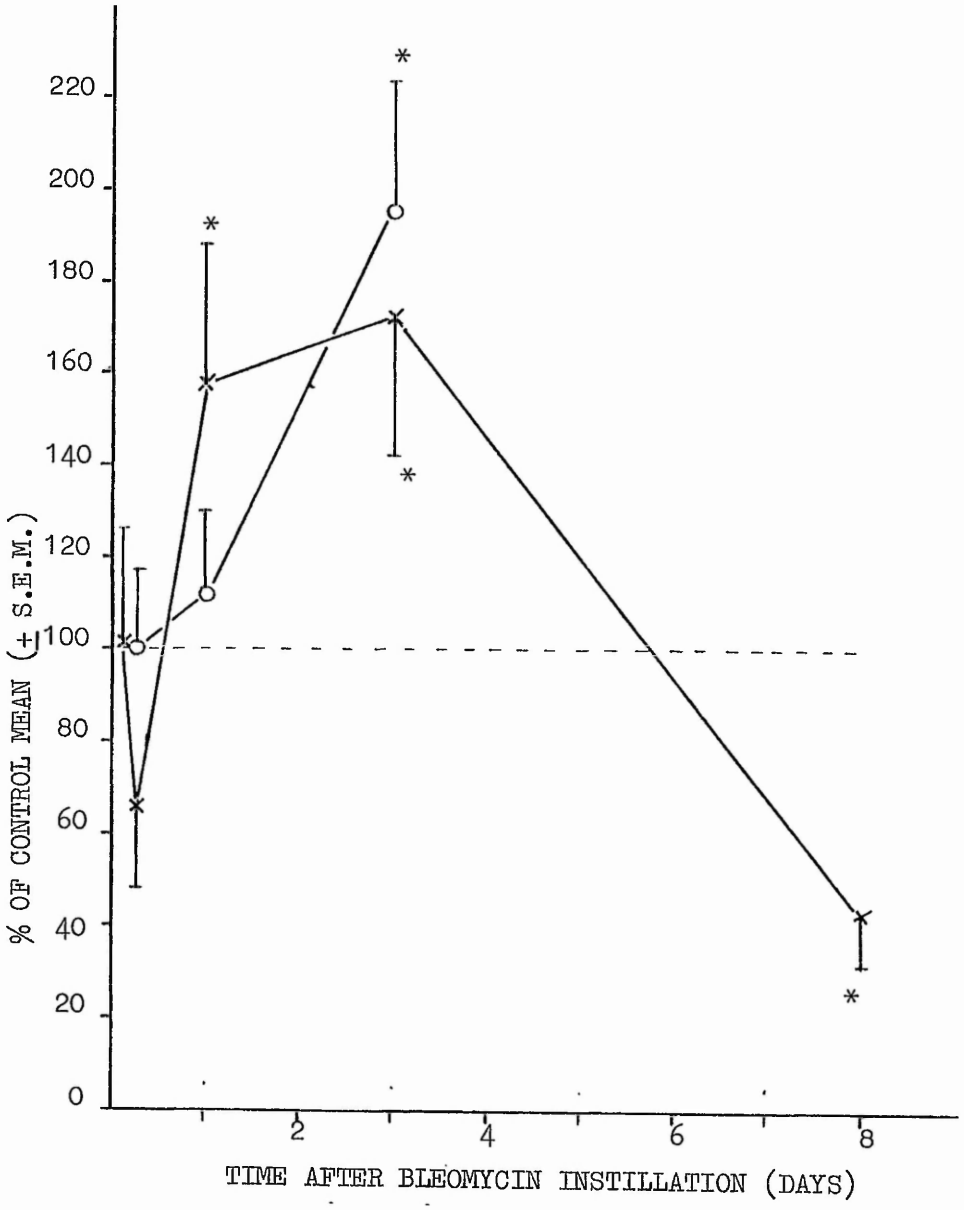
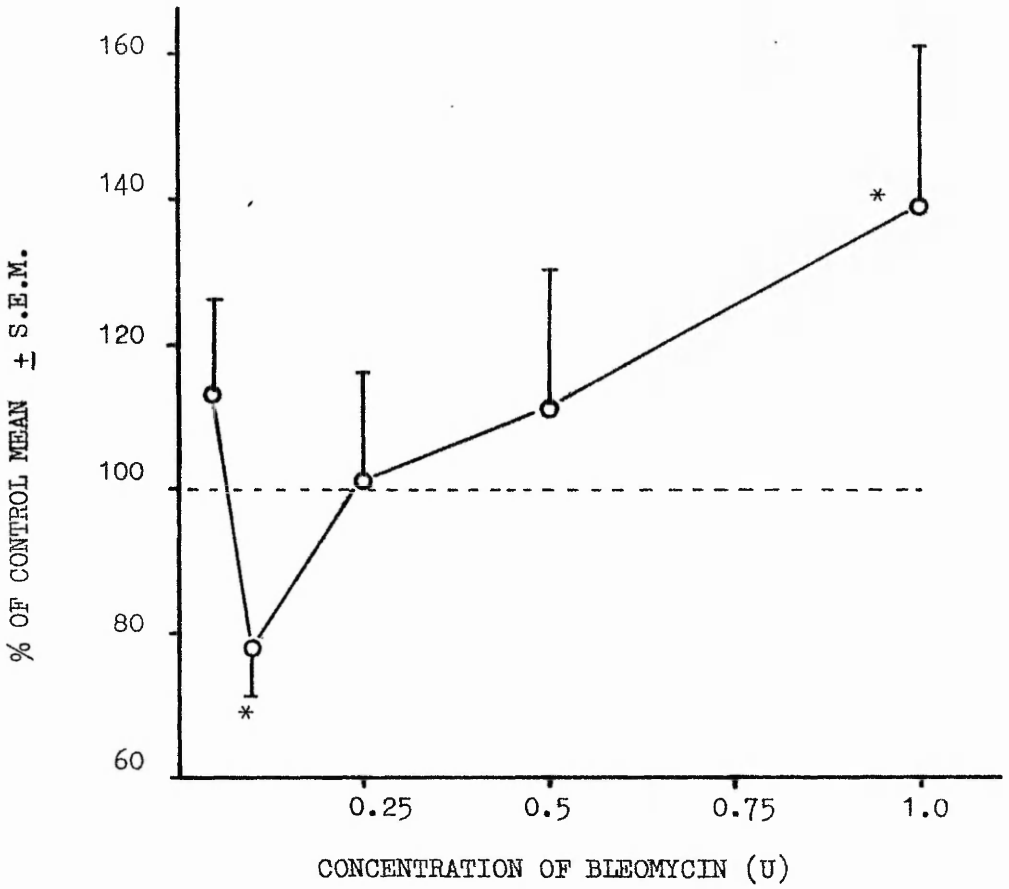

220
200
180
160
140
120
100
80
60
40
20
0
% OF CONTROL MEAN (± S.E.M.)
2
4
6
8
TIME AFTER BLEOMYCIN INSTILLATION (DAYS)

FIGURE 27: PUTRESCINE ACCUMULATION IN LUNG SLICES FOLLOWING THE INTRABRONCHIAL INSTILLATION OF VARIOUS DOSES OF BLEOMYCIN




Bleomycin was instilled into the left lung in 0.3ml saline and 1 day after instillation putrescine accumulation into left lung slices was then determined by the methodology described in section 4.3.2. The results are expressed as those documented in Figure 18. For each dose 4 rats were used. The slice to medium ratio calculated for putrescine accumulation into lung slices in control animals was $29.75 \pm 2.25$ ($n = 4$).

* Significantly different from controls ($P < 0.05$).

of the intrabronchial instillation of various doses of bleomycin (0.1 - 1.0U instilled in 0.3ml saline) on putrescine accumulation at day 1 was studied (Figure 27). Significant changes in putrescine accumulation were only observed following the instillation of 0.1U and 1.0U of bleomycin, although these changes proved to be opposite of one another. Following the instillation of 0.1U of bleomycin a significant reduction in the accumulation of putrescine was observed when compared to controls. On the other hand a significant increase was observed following the instillation of 1.0U of bleomycin.

### 4.4.2 Kinetics of putrescine accumulation by lung slices following bleomycin induced lung injury

Using a range of putrescine concentrations in the incubation medium the rate of putrescine uptake into lung slices taken from left lungs of rats 3 days after the intrabronchial instillation of 1.0U of bleomcyin in 0.1ml saline was determined (Table 8). This time period was chosen since it represents the time during the inflammatory and proliferative phase when an increase in putrescine accumulation was observed (Figure 26). Using this data a Lineweaver-Burke plot was used to determine the Km and Vmax for the uptake of putrescine. An apparent Km of 9.17 μM and Vmax of 457 nmoles/g wet weight/h (Corr. coeff = 0.998) were calculated for saline instilled rat lung slices. These values are in close agreement with those found by Smith and Wyatt (1981) for normal rat lung slices. Following the instillation of bleomycin the apparent Km was changed to 24.4 μM and Vmax was found to be 425 nmoles/g wet weight/h (Corr. coeff = 0.999).

TABLE 8: THE ACCUMULATION OF PUTRESCINE INTO RAT LUNG SLICES AT DAY 3 FOLLOWING THE INTRABRONCHIAL INSTILLATION OF 1.0U OF BLEOMYCIN IN 0.1ml SALINE

| Concentration of putrescine in medium (μM) | Rate of putrescine uptake (nmol/g wet weight of lung/h) | |
|---|---|---|
| | Saline instilled animals | Bleomycin instilled animals |
| 0.5 | 24 | 9 |
| 2.0 | 88 | 35 |
| 5.0 | 129 | 59 |
| 10.0 | 264 | 139 |
| 50.0 | 502 | 410 |
| Km (μM) | 9.17 | 24.40 |
| Vmax (nmoles $g^{-1}h^{-1}$) | 457 | 425 |
| Corr. Coeff. | 0.998 | 0.999 |

Lung slices were incubated in various concentrations of putrescine for 30 min only and the uptake of [$^{14}$C]-putrescine accumulated determined as described in section 4.3.2. Vmax is expressed as nmoles of putrescine accumulated per g of wet weight of lung per h.

### 4.4.3 Transglutaminase activity in lungs of bleomycin treated animals

Transglutaminase activity expressed in terms of total left lung activity was significantly increased over that of control left lung values between 2 and 6 days following bleomycin instillation. This increase in translutaminase activity was also seen when expressed per mg of DNA (an approximate measure of activity per cell) and per mg of protein. However the activity per cell was only significantly increased at day 4 and day 6 and the activity of transglutaminase expressed per mg of protein was only significantly increased at day 6 (Table 9 ).

In control lungs approximately 90% of the transglutaminase activity was associated with the 600g pellet ("Nuclear fraction") and a similar distribution pattern was found in lungs treated with bleomycin up to 6 days after instillation (Table 9 ). These results were at variance with those of Griffin *et al* (1979) although the control values were similar to those reported by Griffin *et al* (1978 ) for normal rat lung.

TABLE 9: TRANSGLUTAMINASE ACTIVITY IN LUNGS OF BLEOMYCIN TREATED ANIMALS

| TRANSGLUTAMINASE ACTIVITY (% OF CONTROL) | DAYS AFTER BLEOMYCIN INSTILLATION | | |
|---|---|---|---|
| | 2 | 4 | 6 |
| Units/left lung | 148 ± 18 * | 141 ± 12 * | 138 ± 12 * |
| Units/mg DNA | 101 ± 13 | 130 ± 12 * | 135 ± 14 * |
| Units/mg Protein | 98 ± 13 | 121 ± 15 | 132 ± 12 * |
| Activity in "Nuclear fraction" / "Cytoplasmic fraction" | 110± 28 | 76 ± 17 | 93 ± 14 |

Data for the left lung were obtained at various time intervals following the intrabronchial instillation of 0.5U of bleomycin in 0.3ml saline into the left lung. The results show the mean determinations ± S.E.M. for 4 rats expressed as percent of control values. Control values (n = 5) for the left lung were as follows: units/left lung = 1893 ± 170; units/mg DNA = 1035 ± 95; units/mg protein = 51.3 ± 4.7; enzymic activity in "Nuclear fraction" / "cytoplasmic fraction" = 9.45 ± 1.46

* Significantly different from controls $P < 0.05$.

## 4.5 DISCUSSION

### 4.5.1 The accumulation of putrescine into lung slices following bleomycin instillation

The results of this study suggest that measurements of putrescine accumulation into lung slices following bleomycin induced toxicity may not be a very reliable indicator of epithelial cell damage. A significant reduction in putrescine accumulation (78% of controls; $P < 0.05$) was only observed after the instillation of a dose of bleomycin which did not result in a large degree of inflammation (0.1U of bleomycin) by day 1 when putrescine accumulation was determined (Figure 27). The instillation of higher doses of bleomycin (0.25U - 1.0U) which led to extensive inflammation and subsequent fibrosis, showed either increased accumulation of putrescine or no significant change at day 1 after instillation. Furthermore the instillation of 0.5U of bleomycin instilled in 0.3ml saline and 1.0U of bleomycin instilled in 0.1ml saline failed to show any significant reduction in putrescine accumulation between 3h and 3 days after instillation (Figure 26). Although a significant reduction in putrescine accumulation was observed at day 8 (44% of controls; $P < 0.05$) following the instillation of the latter dose, this probably reflects a severely fibrotic lung and therefore dose not serve as an early indicator of cell damage.

The increased accumulation of putrescine during the inflammatory and hypercellular phase (3 days after bleomycin instillation) may be accounted for by changes in either the number of available carrier sites

(Vmax) or a change in the affinity of these sites (Km) which in turn may reflect a change in cell type. The two fold increase in the Km for putrescine in these lung slices compared to control values certainly suggests a change in the cell types present. However, since the Vmax failed to show a significant increase, it is difficult at this stage to explain the increased accumulation of putrescine.

The role of polyamines in normal lung function and in diseased states is not well understood. In a very recent study Thet et al (1984) have demonstrated an increase in the activity of ornithine decarboxylase (ODC) and the levels of polyamines in rat lungs undergoing repair and proliferation following the exposure of adult rats to 100% oxygen for 60 hours. Since pulmonary fibrosis is always preceded by a hypercellular phase it is possible that a suppression of this phase may lead to a reduction in fibrosis. Thus, the reduction or inhibition of ODC activity which is associated with proliferation of cells would appear to be a good target. However, Giri et al (1982) found that the administration of α-difluoromethylornithine (the irreversible inhibitor of ODC activity) to hamsters failed to reduce bleomycin induced pulmonary fibrosis.

It is possible that the two main cell types, the epithelial type II cells and the fibroblasts, which undergo rapid proliferation during fibrosis may be able to accumulate polyamines when the activity of ODC is low or is suppressed. Both of these cells have been shown to possess mechanisms for the active accumulation

of putrescine (Smith and Wyatt, 1981; Pohjanpelto, 1976). In this context, however, a recent study of Smith et al (1983b) suggested that proliferating epithelial type II cells cannot accumulate putrescine. They suggested that regenerating type II cells may be physiologically and biochemically similar to the type II epithelial cells of immature animals and thus fail to accumulate putrescine. This would be consistent with the observation that lung slices taken from young rats do not accumulate paraquat as readily as those taken from mature animals (Smith and Rose, 1977). Physiological and biochemical differences between mature and regenerating immature alveolar type II cells have also been demonstrated by other workers (Stevens and Autor, 1977). In view of these observations the changes in putrescine accumulation seen in this study may be related to the influx of the extra pulmonary cells. Nevertheless it should also be borne in mind that bleomycin is capable of causing epithelial cell metaplasia (Adamson and Bowden, 1979), and it is possible that the accumulation of putrescine may be affected in these morphologically distinct cells.

Transglutaminase activity in lungs of bleomycin treated animals

The increase in transglutaminase activity following bleomycin induced lung injury indicates that this enzyme may serve as a potential marker for the onset and progression of fibrosis as suggested by Griffin et al (1979) in studies using paraquat. The biochemical and cellular processes which lead to this increase however cannot be clearly identified from the data of this limited study.

The influx of plasma proteins during the oedematous phase between day 1 and 4 could conceivably lead to activation of the plasma transglutaminase (Factor X IIIa) which may contribute to the increase in transglutaminase activity. However, since approximately 90% of this transglutaminase activity was associated with the "nuclear pellet" fraction after bleomycin instillation, the role of the tissue transglutaminase, which is predominently a particulate associated enzyme, cannot be ignored.

It is possible that the increase in transglutaminase activity may be associated with the activation of inflammatory and immune cells such as macrophages and lymphocytes, which greatly increased in number between 2 and 6 days following bleomycin instillation. Recent studies using isolated lymphocytes and macrophages have shown that transglutaminase activity is increased during their activation (Novogrodsky et al, 1978; Folk et al, 1980; Schroff et al, 1981; Julian et al, 1983; Murtagh et al, 1983). The potential of this enzyme as a marker of the inflammatory phase cannot therefore be ignored. The functional significance of this increase, however, is unknown. Several lines of evidence have implicated tissue transglutaminase as a participant in the processing of ligand-receptor complexes by receptor-mediated endocytosis (Davies et al,

1980; Schroff et al, 1981; Fesus et al, 1981; Chuang, 1981; Leu et al, 1982). More recent evidence has indicated that the tissue transglutaminase may also play a role in the exocytic events occurring during cell secretion (Bungay et al, 1984). Therefore transglutaminase may not only be important in the activation of effector cells such as macrophages and lymphocytes but may be required for the subsequent secretory events performed by these cells.

Several investigators have also provided evidence for the involvement of the tissue transglutaminase in catalysing the covalent conjugation of polyamines into cellular proteins (See review Russell and Womble, 1982). It has therefore been suggested that the enzyme may be an important modulator of the effects of endogenous amines as well as of amine or alcohol containing pharmacological agents (Russell and Womble, 1982).

## 4.6 SUMMARY

(i) The measurement of putrescine accumulation into lung slices is not a reliable marker for epithelial cell damage following the instillation of doses of bleomycin which lead to extensive collagen deposition.

(ii) During the inflammatory and hypercellular phase after bleomycin instillation, the rate of putrescine accumulation into lung slices shows an increase above controls.

(iii) During the oedematous, inflammatory and hypercellular phase transglutaminase activity in the lung shows an increase above controls.

CHAPTER 5

# BIOCHEMICAL MECHANISM OF BLEOMYCIN INDUCED PULMONARY TOXICITY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## 5.1 INTRODUCTION

Many chemicals exhibit specific toxicity to particular organs. Specificity may be determined by the distribution, metabolism and excretion of the compound, while toxicity may be due to the parent compound itself, a chemically reactive metabolite or a chemically stable metabolite.

Numerous types of xenobiotic substances including industrial chemicals, naturally-occurring toxicants, and drugs are now recognised or suspected of causing chemical-induced toxicities through "metabolic activation". This is the *in vivo* biotransformation of relatively inert chemicals to highly reactive metabolites. Such metabolites may then interact with cells in numerous detrimental ways. These interactions include their covalent binding to essential cellular constituents, and of relevance to this thesis, the stimulation of the peroxidative decomposition of cellular lipids as well as damage to other cellular components (Boyd, 1980).

Based upon the toxicities of various agents to the lung Figure 28 schematically represents three important potential mechanisms of lung toxicity in which metabolic activation plays a crucial role. However all three of the mechanisms illustrated are highly simplified representations of early events involved in a complex series of events leading to lung damage.

The relatively high oxygen tensions in pulmonary tissue conceivably

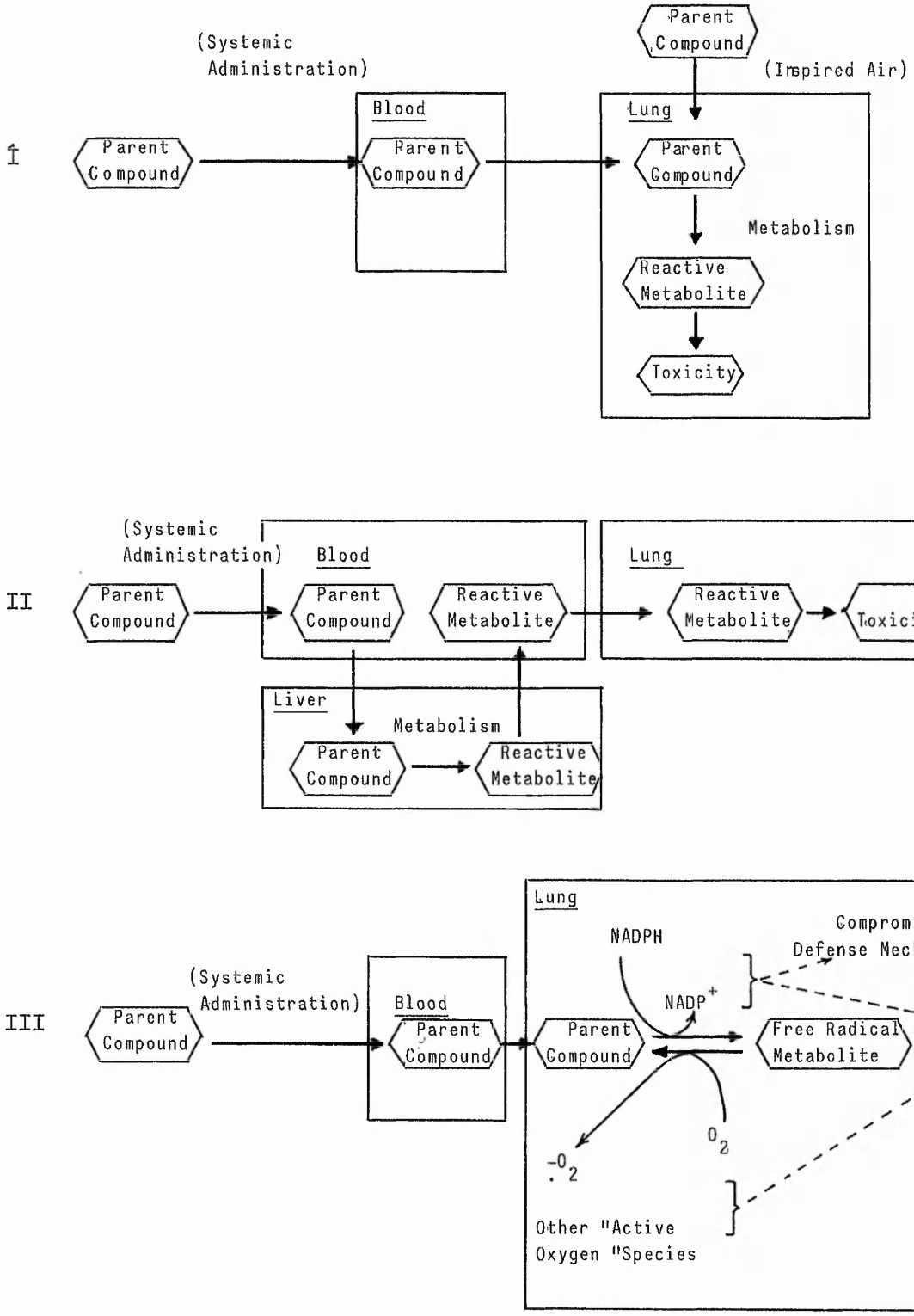



FIGURE 28: Schematic representation of three potential mechanisms of chemical-induced lung toxicity involving metabolic activation. (Boyd, 1981).

could contribute to preferential damage of the lung by agents acting through mechanism III. This mechanism is largely derived from the studies on lung toxicity induced by the herbicide paraquat. Available evidence suggests that the redox cycling of paraquat in certain lung cells leads to the consumption of cellular NADPH (reduced nicotinamide adenine dinucleotide phosphate) and the "activation" of molecular oxygen. The specific toxicological consequences of these effects are still not certain, but there is considerable evidence to suggest that the intracellular generation of superoxide anion ($O_2^-$) and its derivatives (such as singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($^{\cdot}OH$) leads to cell damage by stimulating the peroxidative decomposition of cellular lipids, while the depletion of NADPH contributes to lung cell damage by disruption of essential cellular metabolic pathways (Boyd, 1980).

The biochemical and molecular reactions by which bleomycin initially damages lung cells, thereby setting into motion the sequel of cellular events which ultimately lead to fibrosis is not yet known. However, numerous in vitro studies have suggested that bleomycin toxicity, like that of paraquat may be mediated by reactive oxygen species (Oberley and Buettner, 1979; Sugiura, 1979; Lown and Joshua, 1979; Ekimoto et al, 1980).

### 5.1.1 The biochemistry of oxygen toxicity

Oxygen entering the lungs dissolves in the lipid and aqueous phases of lung tissues, diffuses across alveolar membranes into the capillaries, and is picked up by haemoglobin in erythrocytes or

dissolves in the blood (Lambertson, 1978) for distribution to other tissues. Oxygen is used by the tissue in a variety of biochemical processes with different end products under normoxic conditions. Most of the molecular oxygen is reduced by mitochondrial cytochrome oxidase in a four electron reduction with water as the end product ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$ (Blum et al, 1978)). Enzymatic reactions in the cell also produce hydrogen peroxide ($H_2O_2$) by a two electron reduction ($O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$) or superoxide radical ($O_2^{\cdot-}$) by a one electron reduction step ($O_2 + e^- \rightarrow O_2^{\cdot-}$). Superoxide radical is also thought to be produced in the autoxidation of such cellular components as thiols, haemoglobin, and epinephrine (Misra and Fridovich, 1972; Misra, 1974; McCord and Fridovich, 1978), and by granulocytes during phagocytosis (Babior et al, 1973; Reis and Roos, 1978).

In addition to its importance in paraquat toxicity the superoxide radical has been suggested to be responsible for the toxicity of other exogenous agents including ionizing radiation (Petkau et al, 1975), haemolytic drugs (Goldberg et al, 1976), ozone (Mustafa, 1975) and hyperoxia (Crapo and Tierney, 1974) and the neurotoxin 6-hydroxydopamine (Cohen and Heikkila, 1974).

The currently held theory for the chemical mechanism of oxygen toxicity is that specific cell damage is caused primarily by the increased intracellular production of oxygen radicals (Figure 29; McCord and Fridovich, 1978; Fridovich, 1978; Halliwell, 1982). Oxygen radicals, in turn, participate in radical chain reactions causing lipid peroxidation, protein sulphydryl oxidation and cross-linking (leading to enzyme inactivation), and nucleic acid damage.

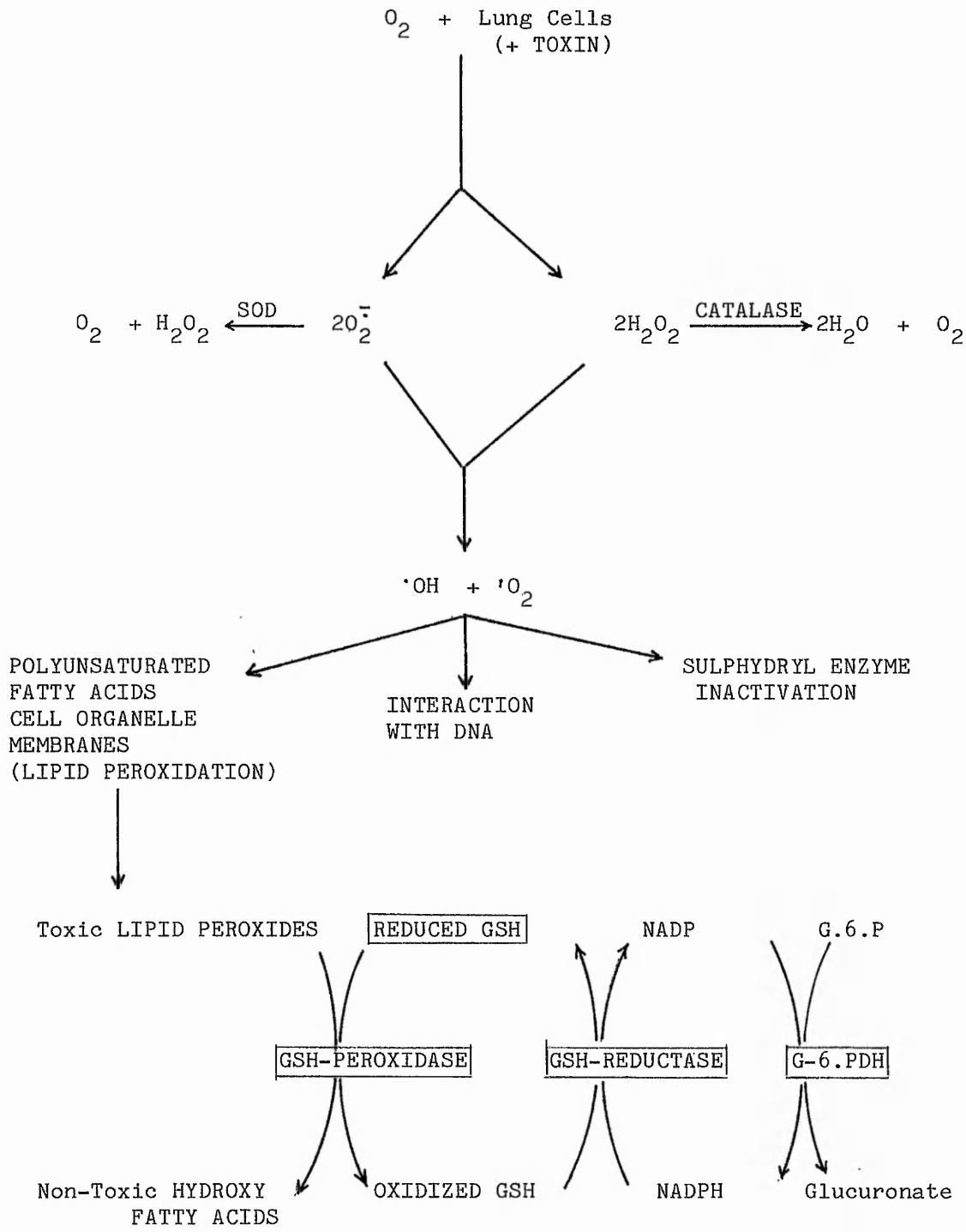



FIGURE 29: CHEMICAL MECHANISMS OF OXYGEN TOXICITY AND OXIDANT DEFENCE SYSTEMS.

The reactivity of the superoxide radical with cellular components is controversial and has recently received a great deal of discussion (Fee 1982; Halliwell, 1982). Superoxide radical generated in biological systems will react with itself as follows: $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ (Fridovich 1975). Alternatively, in the presence of iron chelates, superoxide radical may react with hydrogen peroxide to form the hydroxyl radical (Halliwell, 1978; McCord and Day 1978; Haber and Weiss, 1934) by the Haber-Weiss reaction as follows:

$$O_2^{\cdot -} + \text{Fe (chelate)}^{3+} \rightleftharpoons O_2 + \text{Fe (chelate)}^{2+}$$

$$H_2O_2 + \text{Fe (chelate)}^{2+} \rightleftharpoons {}^{\cdot}OH + OH^- + \text{Fe (chelate)}^{3+}$$

---

sum: $O_2^{\cdot -} + H_2O_2 \longrightarrow {}^{\cdot}OH + OH^- + O_2$ (Haber-Weiss reaction)

The hydroxyl radical produced is highly reactive and reacts with most biological materials. Chemical interactions between various oxygen and peroxy radicals can result in singlet excited oxygen ($^1O_2$) formation. Singlet excited oxygen is a highly reactive, electronically excited state of molecular oxygen that is toxic to many biological systems (Singh, 1978; Khan, 1978). At present hydrogen peroxide, superoxide radical, hydroxyl radical, and singlet excited oxygen are all considered possible agents which cause cellular damage and all fit into the loosely used term "activated oxygen" or "oxygen radical". The general consensus, however, indicates that the hydroxyl radical is the likely reactive species involved in producing cellular damage (Halliwell, 1982).

### 5.1.2 Defence mechanisms against oxygen induced damage

Natural defence mechanisms against oxygen induced damage include superoxide dismutase (SOD), which eliminates superoxide radical (Figure 29); catalase and peroxidases, which eliminate hydrogen

peroxide and lipid peroxides; and general radical quenchers and antioxidants such as reduced glutathione, $\alpha$- tocopherol (Vitamin E), and ascorbate (Vitamin C).

Glutathione in its reduced form (GSH) protects against an oxidizing environment by means of two major mechanisms. GSH protects against protein sulphydryl oxidation, since it is a preferred substrate for oxidants, and it can reverse the oxidation of protein sulphydryls. Glutathione is also used in its reduced form as a substrate for the enzyme glutathione peroxidase, which catalyses the inactivation of both hydrogen peroxide and lipid peroxides thus preventing the propagation of radical chain reactions and the deleterious reactions of hydrogen peroxide. The necessary GSH-GSSG ratios are maintained by the enzymes glutathione reductase and glucose - 6-phosphate dehydrogenase (G-6PDH). The series of related reactions are shown in Figure 29. The production of NADPH by G-6PDH is not only important for the maintenance of normal levels of GSH but it is also required for the biosynthetic processes necessary for the repair of damaged cellular components.

The likely involvement of oxygen in bleomycin toxicity led Matsuda et al (1980) to propose that alterations in cellular lipids or nucleic acids may be responsible for the pulmonary toxicity induced by this drug. To date, however, no attempt has been made to show DNA damage in lung tissue. Furthermore, several investigators have failed to show the involvement of lipid peroxidation in lung tissue following bleomycin administration (Tom and Montgomery, 1980; Muliawan et al, 1982). This finding, however, is not unique since the demonstration of lipid peroxidation for the herbicide paraquat

has also not been confirmed as a mechanism of toxicity (Shu et al, 1979; Steffen and Netter, 1979). In the case of paraquat this has led to an alternative hypothesis in which paraquat toxicity may occur in addition to or without causing lipid peroxidation. In this mechanism the involvement of NADPH and sulphydryls during the redox cycling of the paraquat radical (Figure 28) and the operation of the "oxidant defence system" (Figure 29) leads to their oxidation and eventual depletion (Fisher et al, 1975; Illet et al, 1974; Witschi et al, Keeling et al, 1982; Keeling and Smith, 1982).

## 5.2 AIMS

There were three principal aims of this study.

(i) To determine if increased NADPH consumption leading to its depletion was involved in bleomycin induced pulmonary toxicity.

(ii) To determine if the oxidant defence enzymes were stimulated following the instillation of bleomycin.

(iii) To determine if pre-exposure to a sub-lethal level of oxygen (85%) for seven days led to a reduction in bleomycin induced pulmonary fibrosis.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Materials

($1^{14}C$) and ($6^{14}C$) glucose (specific activity, 3mCi/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks, UK. Krebs Ringer Phosphate (KRP) was prepared as in section 4.3.1

Wistar derived SPF male rats weighing 180-200g were used throughout these studies.

### 5.3.2 Measurement of $^{14}CO_2$ production from ($1^{14}C$) and ($6^{14}C$) glucose.

Rats were killed by exposure to halothane vapour and the lungs perfused with cold KRP. The left lung was removed and transverse lung slices (0.5mm) were obtained using a McIlwain automatic tissue chopper (Mickle Laboratory Engineering Co Ltd., Guildford, Surrey, UK). Random slices weighing 20-40mg were selected and placed in 25ml respirometer flasks containing 3ml KRP at 37 $^{o}C$ in a shaking water bath. After allowing the slices to equilibrate for 15min 1µCi of either ($1^{14}C$) or ($6^{14}C$) glucose was added to each sample in duplicate and incubated for a further 60 min. $^{14}CO_2$ was collected in 0.2ml KOH (20% W/V) placed in the centre well of the flask together with a 2cm x 1½cm rectangle of hard filter paper (Whatman 452) to facilitate absorption. After incubation the filter paper and KOH were transferred to a glass scintillation vial , and the radioactivity measured after the addition of 0.8ml of distilled water and 10ml Instagel. Counting efficiency was

determined by the addition of internal standard ([$^{14}$C]-hexadecane).

### 5.3.3 Enzyme assays

#### 5.3.3.1 Preparation of lung samples

After killing the animals with halothane vapour the lungs were perfused with cold isotonic saline. The left lung was homogenised in 5mM phosphate buffer, pH7.8 (1:10W/V) for 1min using an Ultra-turrax homogeniser. Approximately half of the homogenate was then centrifuged at 40,000g (r.av 5.84cm) for 6 min at 4 $^{o}$C (MSE 50 centrifuge) to obtain a clear supernatant which was used in the assay for catalase.

#### 5.3.3.2 Superoxide dismutase assay

The activity of superoxide dismutase in lung homogenates was measured by the method of Beauchamp and Fridovich (1971).

The assay mixture in a final volume of 3ml of phosphate buffer (0.05M, pH 7.8) contained; 10mM methionine, 56 µM nitroblue tetrazolium, 20µM NaCN, 100µM EDTA, and 2.34 µM riboflavin. After the addition of 30µl of homogenate or 30µl of distilled water to controls, the reaction was started by irradiating the samples using tungsten lamps (90W) for 6 min inside an incubator maintained at 30 $^{o}$ C. Absorbance was then measured at 560nm using a PYE-Unicam SP 500 spectrophotometer. One unit of enzyme activitiy is defined as the amount which produced a 50% inhibition in absorbance at 560nm.

5.3.3.3 Glucose-6-phosphate dehydrogenase assay

Glucose-6-phosphate dehydrogenase activity was measured by following the substrate induced reduction of $NADP^+$ at 340nm (Lohr and Waller, 1974) using a Unicam SP 1800 spectrophotometer. The assay mixture in a final volume of 3ml at pH 7.5 contained; 46.7mM triethanolamine buffer, 0.5mM $NADP^+$, 100µl of enzyme sample and 0.67mM glucose-6-phosphate (G6P). The reaction was started by the addition of G6P and the temperature was maintained at 25 °C. One unit of enzyme activity was defined as the amount of enzyme which reduced 1µmole of $NADP^+$ per min under the conditions of the assay.

5.3.3.4 Catalase assay

Catalase activity in lung 40,000g supernatants (section 5.3.3.1) was measured by following the decomposition of $H_2O_2$ at 240nm (Lück, 1974) using a Unicam SP 1800 spectrophotometer. The assay mixture in a final volume of 3ml at pH 7.0 contained; 66.7mM phosphate buffer, 12.5mM $H_2O_2$ and 50µl of enzyme sample. The reaction was started by the addition of the enzyme sample and the temperature was maintained at 25 °C. One unit of enzyme activity was defined as the amount of enzyme that decomposed 1µmole of $H_2O_2$ per min.

## 5.4 RESULTS

### 5.4.1 The production of $^{14}CO_2$ from the oxidation of (1-$^{14}$C) and (6-$^{14}$C)glucose.

Most of the evidence for the increased consumption of NADPH during paraquat toxicity has come indirectly from *in vitro* studies which have shown a stimulation of the pentose phosphate pathway activity (Fisher *et al*, 1975; Rose *et al*, 1976; Keeling *et al*, 1982). Since the pentose phosphate pathway in the lung is a major source of NADPH (Holzer, 1959), then any increase in the cellular consumption of NADPH may be measured by comparing the amount of $^{14}CO_2$ evolved from lung slices incubated in the presence of (1-$^{14}$C) or (6-$^{14}$C)glucose.

The incubation of normal rat lung slices in the presence of bleomycin ($10^{-4}$M and $10^{-3}$M) as well as copper-bleomycin ($10^{-4}$M) did not lead to any significant increase in the oxidation of either (1-$^{14}$C) or (6-$^{14}$C)glucose (Table 10). In contrast incubation of lung slices in the presence of the lung toxin paraquat ($10^{-5}$M) resulted in a considerable increase in the activity of the pentose phosphate pathway such that the 1C:6C ratio for glucose oxidations was over 4 fold greater than that of control slices. Similarly the incubation of normal lung slices with diquat ($10^{-4}$M), which unlike paraquat is not actively accumulated by lung slices (Rose *et al*, 1976), also resulted in a significant increase in the activity of the pentose phosphate pathway, the 1C:6C ratio for glucose oxidation being over 5 fold greater than that of control slices.

TABLE 10: THE OXIDATION OF [1-$^{14}$C] and [6-$^{14}$C] GLUCOSE BY NORMAL RAT LUNG SLICES INCUBATED IN THE PRESENCE OF BLEOMYCIN AND THE HERBICIDES PARAQUAT AND DIQUAT.

| | DPM of $^{14}CO_2$/100mg slice weight | | $^{1C}/_{6C}$ |
|---|---|---|---|
| | [1-$^{14}$C] GLUCOSE | [6-$^{14}$C] GLUCOSE | |
| Control | 5535 ± 354 (7) | 2962 ± 135 (4) | 1.9 |
| Paraquat ($10^{-5}$M) | 29742 ± 2013(4) | 3524 ± 303(4) | 8.4 |
| Diquat ($10^{-4}$M) | 31704 ± 1305(7) | 2982 ± 203(8) | 10.6 |
| Bleomycin ($10^{-4}$M) | 4935 ± 195(4) | 2358 ± 148(4) | 2.1 |
| Bleomycin ($10^{-3}$M) | 5037 ± 217(4) | 2614 ± 131(4) | 1.9 |
| Copper-BLM ($10^{-4}$) | 5243 ± 203(4) | 2546 ± 171(4) | 2.1 |

Slices of rat lung (20 - 40mg) were incubated with the indicated concentration of bleomycin, paraquat or diquat and $^{14}CO_2$ production from either [1-$^{14}$C] glucose or [6-$^{14}$C] glucose measured for 1 hour as described in section 5.3.2. The values given are mean ± S.E.M. with the number of determinations in parenthesis.

Copper bleomycin was prepared by dissolving the required amount of bleomycin in an appropriate volume of $Cu(NO_3)_2.H_2O$ solution so that the molar ratio of 4:3 was given for bleomycin to Cu.

Following the intrabronchial instillation of a fibrogenic dose of bleomycin (IU instilled into the left lung in 0.1ml saline) the left lung failed to show any increase in either (1-$^{14}$C) or (6-$^{14}$C) glucose at anytime up to 72h after bleomycin administration (Table 11). In fact glucose oxidation by both the pentose phosphate pathway and the glycolytic pathway was significantly reduced between 24h and 72h. This is not surprising since a considerable degree of inflammation resulting from lung damage has been shown to occur during this time in earlier studies (Chapter 2). In contrast the intrabronchial instillation of a fibrogenic dose of paraquat ($10^{-3}$g) (in 0.1ml saline) into the left lung produced a substantial increase in (1-$^{14}$C) glucose oxidation, such that the 1C:6C ratio of glucose oxidation was over 6 fold greater than that of saline instilled control lung slices.

### 5.4.2 The activity of oxidant defence enzymes in the lung following bleomycin instillation

It is generally recognised that before an oxidant can cause lipid peroxidation in a tissue the oxidant defence system must be overcome. Several investigators have therefore suggested that the changes in the activities of these enzymes may give an indication of the involvement of reactive oxygen species (Crapo and Tierney, 1974; Kimball et al, 1976; Frank et al, 1978; Hoffman et al, 1980).

In this study changes in the activities of superoxide dismutase (SOD), catalase and glucose-6-phosphate dehydrogenase (G-6-PDH) have

TABLE 11: THE OXIDATION OF $(1-^{14}C)$ and $(6-^{14}C)$ GLUCOSE BY LEFT LUNG SLICES AT VARIOUS TIMES AFTER THE INSTILLATION OF BLEOMYCIN INTO THE LEFT LUNG

| Time after bleomycin instillation (h) | DPM/100mg slice weight | | 1C |
|---|---|---|---|
| | $[1-^{14}C]$ Glucose | $[6-^{14}C]$ Glucose | 6C |
| Control (9) | 4232 ± 78 | 1864 ± 100 | 2.3 |
| 3 (4) | 3948 ± 160 | 1692 ± 25 | 2.3 |
| 6 (4) | 3569 ± 224 | 1595 ± 76 | 2.2 |
| 12 (4) | 3847 ± 188 | *1310 ± 136 | 2.9 |
| 24 (4) | *3223 ± 175 | *1160 ± 74 | 2.8 |
| 48 (4) | 3639 ± 218 | *1103 ± 150 | 3.3 |
| 72 (4) | *3083 ± 208 | *1203 ± 56 | 2.6 |
| †Paraquat (after 3h)(4) | *33490 ± 2887 | *2592 ± 132 | 12.9 |

Bleomycin (1.0U) was instilled in 0.1ml isotonic sterile saline. Glucose oxidation was measured as described in section (5.3.2.). The values given are mean ± S.E.M. with the number of determinations in parenthesis. Control values at 6h, 24h and 72h did not differ significantly, and so the data was pooled.

†Paraquat ($10^{-3}$g) was instilled by the intrabronchial route in 0.1ml isotonic saline and glucose oxidation in left lung slices measured after 3h.

*Significantly different from controls ($P < 0.05$)

been determined following the intrabronchial instillation of three doses of bleomycin (1.0U instilled in 0.1ml saline, 0.5U and 0.1U both instilled in 0.3ml saline). This is the first study in which more than a single dose of bleomycin has been used to study the biochemical mechanism(s) involved in bleomycin induced pulmonary toxicity. These doses were chosen because they produced different degrees of fibrosis when instilled into the left lung (Chapter 2).

5.4.2.1 Superoxide dismutase activity

The total activity of SOD in the left lung, following the intrabronchial instillation of 1.0U of bleomycin (instilled in a 0.1ml saline), failed to show any increase and was in fact significantly reduced below control values at 24h (66% of control $P < 0.05$) and 48h (72% of control $P < 0.05$) (Figure 30). A similar pattern was observed when SOD activity was expressed per mg of lung DNA or per mg of lung protein (Figure 31).

Following the intrabronchial instillation of 0.5U of bleomycin (instilled in 0.3ml saline) the total lung SOD activity showed a time related increase, although only becoming significantly different from controls at 48h after instillation ($P < 0.05$) (Figure 32). In contrast the intrabronchial instillation of 0.1U of bleomycin (instilled in 0.3ml saline) produced a small but significant increase ($P < 0.05$) in total SOD activity above controls at 24h after instillation, although in this instance the increase was not time related. However, the activity of SOD failed to show any significant increase when expressed per mg lung DNA or per mg of lung protein (Figure 33 and 34) for both of these doses.

5.4.2.2 Catalase activity

The activity of catalase, whether expressed as total, activity per mg of lung DNA or lung protein, failed to show any significant changes from control values up to 48h following the intrabronchial instillation of 1.0U of bleomycin (in 0.1ml of saline) (Figure 30 and 31). This was also the case after the instillation of 0.1U of bleomycin (in 0.3ml of saline) (Figure 32 and 34). Following the intrabronchial instillation of 0.5U of bleomycin, (in 0.3ml of saline) however, a significant reduction in catalase activity was seen between 12h and 48h when expressed per mg of lung DNA or mg of lung protein. This reduction in catalase activity was not observed when the activity was expressed as total activity in the left lung (Figure 32 and 33).

5.4.2.3 Glucose-6-phosphate dehydrogenase activity

The total activity of G6PDH in the left lung following the intrabronchial instillation of 1.0U (in 0.1ml of saline) of bleomycin showed a 50% increase above controls ($P<0.05$) at 24h and a 106% increase above controls ($P<0.001$) at 48h after instillation (Figure 30). However, when the activity was expressed per mg of lung DNA or per mg lung protein a significant increase ($P<0.05$) was only observed at 48h (Figure 31).

Following the intrabronchial instillation of 0.5U of bleomycin (in 0.3ml of saline) total G6PDH activity showed a time related increase but only becoming significantly different from controls ($P < 0.05$) at 48h after instillation (Figure 32). However no

significant increase in the activity of this enzyme was observed when expressed per mg of lung DNA or per mg of lung protein (Figure 33). Surprisingly the instillation of 0.1U of bleomycin (in 0.3ml of saline) produced a 42% increase above controls ($P < 0.05$) in the activity of G6PDH at 12h when it was expressed per mg of lung protein (Figure 34), although it should be emphasised that this dose failed to show a significant increase in the activity of this enzyme at any other time regardless of how it was expressed.

### 5.4.3 Changes in lung composition following the intrabronchial instillation of bleomycin to rats pre-exposed to 85% oxygen

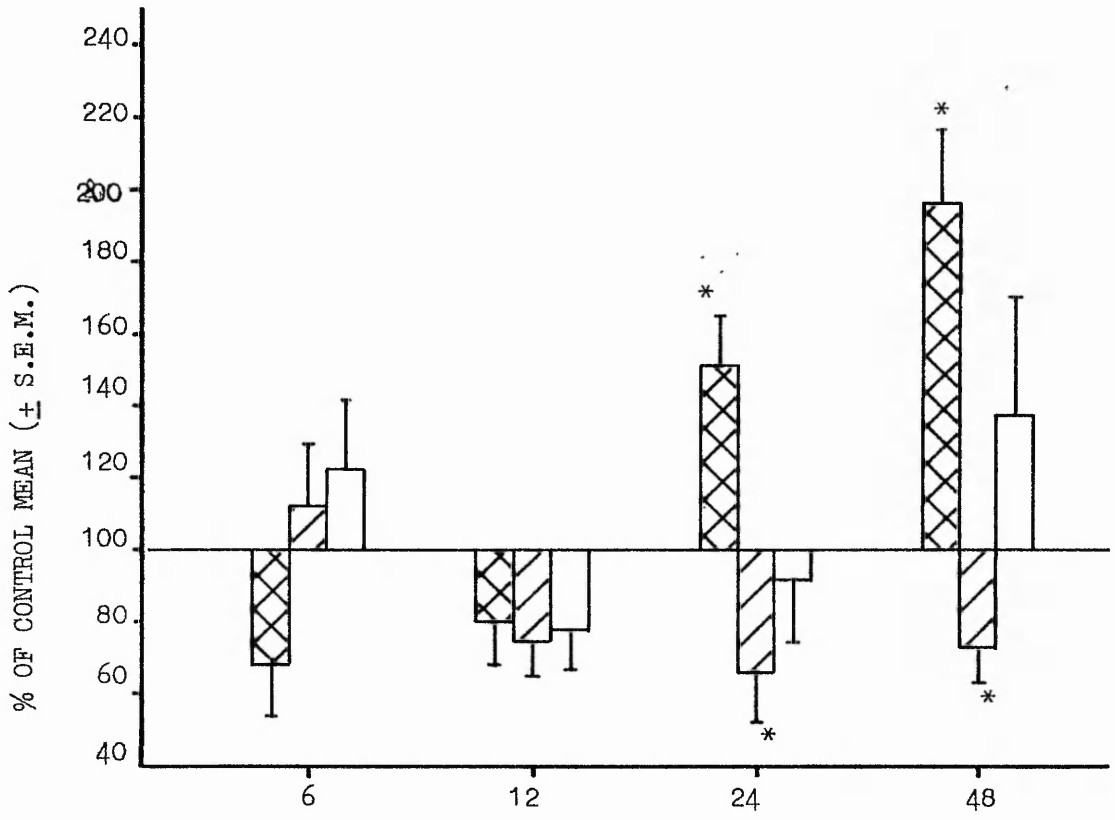
Most oxidants are presumed to cause damage by increasing the intracellular concentration of oxygen radicals and by overwhelming the natural defences of the cell. Several investigators, however, have demonstrated the inducibility of oxidant defence enzymes in prokaryotes and eukaryotes by exposure to sublethal levels of oxygen and the subsequent development of tolerance to oxidants such as 100% oxygen and paraquat (Fridovich et al, 1975; Crapo and Tierney, 1974; Bus et al, 1976). Pre-exposure of rats to 85% oxygen for 7 days has been shown to lead to increased activities of all the oxidant defence enzymes, and subsequent tolerance to 100% oxygen and paraquat (Crapo and Tierney, 1974; Kimball et al, 1976; Bus et al, 1976).

The changes in left lung dry weight, collagen content and collagen concentration at 14 days after the intrabronchial instillation of 0.5U of bleomycin (instilled in 0.3ml saline) to rats pre-exposed

to 85% oxygen for 7 days are shown in Figure 35.

Rats which received sterile isotonic saline following pre-exposure to 85% oxygen showed significant increases in both left lung dry weight (36% above control animals kept in air $P < 0.05$) and total collagen content (24% above controls $P < 0.05$). Following the intrabronchial instillation of bleomycin (0.5U) to rats pre-exposed to 85% oxygen a 35% reduction in left lung dry weight was observed when comparison was made to saline instilled control lungs of animals which had also been pre-exposed to oxygen. Animals which had been maintained in air and then treated with bleomycin (0.5U) showed a similar reduction (30%) in left lung dry weight when compared to saline instilled control left lungs of animals maintained in air. Therefore the reduction in dry weight observed due to bleomycin treatment was similar in animals pre-exposed to 85% oxygen or pre-exposed to air, when compared to their respective controls. A similar pattern of change was also observed for left lung total collagen content and collagen concentration.

FIGURE 30: CHANGES IN THE TOTAL ACTIVITIES OF LUNG SOD, CATALASE AND G-6-PDH FOLLOWING THE INTRABRONCHIAL INSTILLATION OF 1.0U OF BLEOMYCIN in 0.1ml SALINE.




Enzyme activities in the left lung were determined at various time intervals following the intrabronchial instillation of 1.0U of bleomycin in 0.1ml isotonic saline (section 5.3.3). Each point represents the mean ± SEM for 4 rats. The enzyme activities in saline-treated control rats did not differ significantly at various times after treatment and therefore these values were pooled together. Total left lung SOD activity is shown as ▨ and in controls was 503 ± 37 units (n = 8). Total left lung catalase activity is shown as □ and in controls was 630 ± 59 units (n = 8). Total left lung G 6 PDH activity is shown as ▩ and in controls was 1.58 ± 0.14 units (n = 7).

*Significantly different from controls ($P < 0.05$).

FIGURE 31: CHANGES IN THE SPECIFIC ACTIVITIES OF LUNG SOD, CATALASE AND G-6-PDH FOLLOWING THE INTRABRONCHIAL INSTILLATION OF 1.0U OF BLEOMYCIN

Enzyme activities were determined as described in Figure 20 earlier and the results expressed as units of activity per mg of left lung DNA or per mg of left lung protein. SOD activity is shown as ▨, catalase activity is shown as □ and G-6-PDH activity is shown as ▩ . Pooled data for the specific activities of the enzymes in saline instilled control rats were as follows: Left lung SOD activity was 250 ± 25 units/mg of DNA and 11.6 ± 1.5 units/mg of protein; left lung catalase activity was 274 ± 15 units/mg DNA and 32.99 ± 1.70 units/mg of protein; left lung G-6-PDH activity was 0.727 ± 0.071 units/mg DNA and 0.0280 ± 0.0027 units/mg of protein. Control values were obtained from 8 rats.

*Significantly different from controls ($P < 0.05$)

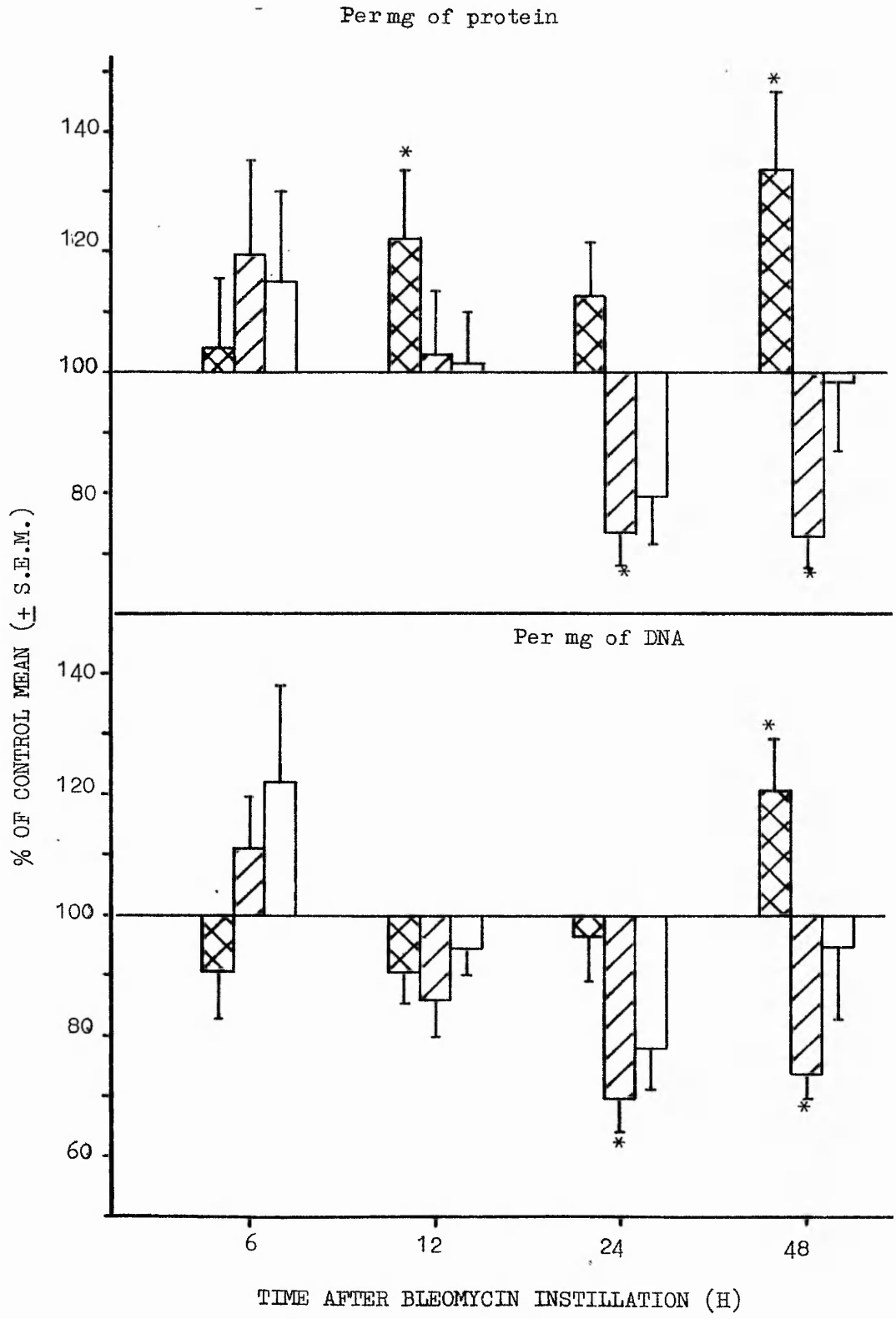

Per mg of protein
140
120
100
80
Per mg of DNA
140
120
100
80
60
% OF CONTROL MEAN (± S.E.M.)
6
12
24
48
TIME AFTER BLEOMYCIN INSTILLATION (H)

FIGURE 32: CHANGES IN THE TOTAL ACTIVITIES OF SOD, CATALASE AND G-6-PDH FOLLOWING THE INTRABRONCHIAL INSTILLATION OF 0.5U and 0.1U OF BLEOMYCIN IN 0.3ml SALINE

Enzyme activities in the left lung were determined at various time points following the intrabronchial instillation of the two doses of bleomycin. Each point represents the mean ± S.E.M. for 4 rats. SOD activity is shown as ▨, catalase activity is shown as □, and G-6-PDH activity is shown as ▩. The enzyme activities in saline treated control rats did not differ significantly at various times after treatment and therefore these values were pooled. Total left lung SOD activity in controls was 485 ± 41 units. Total left lung catalase activity was 703 ± 87 units. Total left lung G-6-PDH activity was 1.46 ± 0.17 units. Control values were obtained from 6 rats.

*Significantly different from controls ($P < 0.05$)

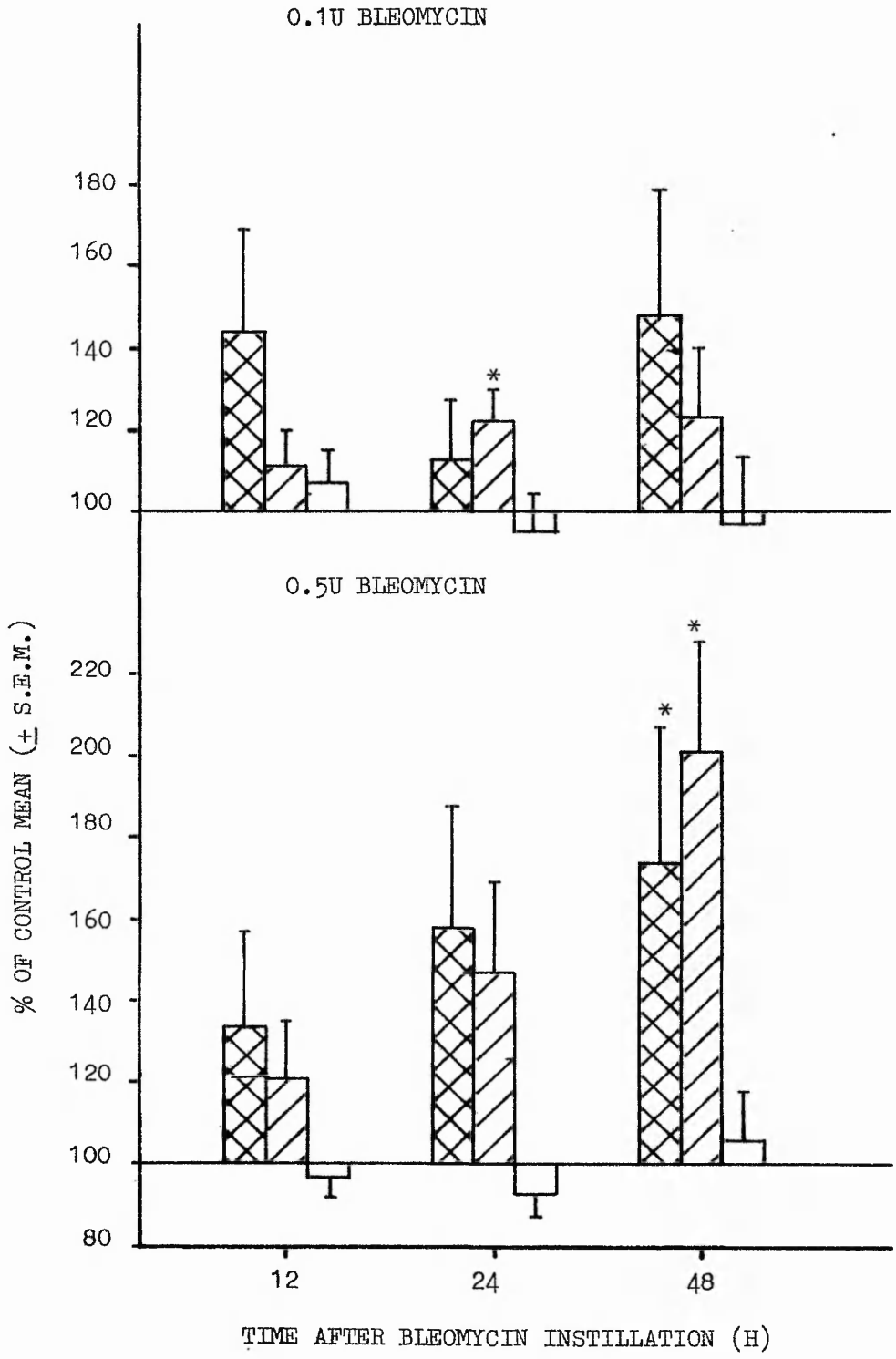

0.1U BLEOMYCIN
180
160
140
120
100
*
0.5U BLEOMYCIN
220
200
180
160
140
120
100
80
*
*
% OF CONTROL MEAN (± S.E.M.)
12
24
48
TIME AFTER BLEOMYCIN INSTILLATION (H)

FIGURE 33 & 34: CHANGES IN THE SPECIFIC ACTIVITIES OF LUNG SOD, CATALASE AND G-6-PDH FOLLOWING THE INTRA-BRONCHIAL INSTILLATION OF 0.5U and 0.1U OF BLEOMYCIN IN 0.3ml SALINE

Enzyme activities were determined as described in Figure 22 earlier and the results are expressed as units of activity per mg of left lung DNA or per mg of left lung protein. SOD activity is shown as ▨, catalase activity is shown as □, and G-6-PDH activity is shown as ⊠. Pooled data for the specific activities of the enzymes in saline instilled control rats were as follows: Left lung SOD activity was 209 $\pm$ 18 units/mg of DNA and 8.3 $\pm$ 1.4 units/mg of protein; left lung catalase activity was 296 $\pm$ 27 units/mg DNA and 36.81 $\pm$ 3.00 units/mg of protein, left lung G-6-PDH activity was 0.583 $\pm$ 0.060 units/mg of DNA and 0.0239 $\pm$ 0.0027 units/mg of protein. Control values were obtained from 6 rats.

*Significantly different from controls ($P < 0.05$)

Figure 33: Enzyme activities following the instillation of 0.5U of bleomycin

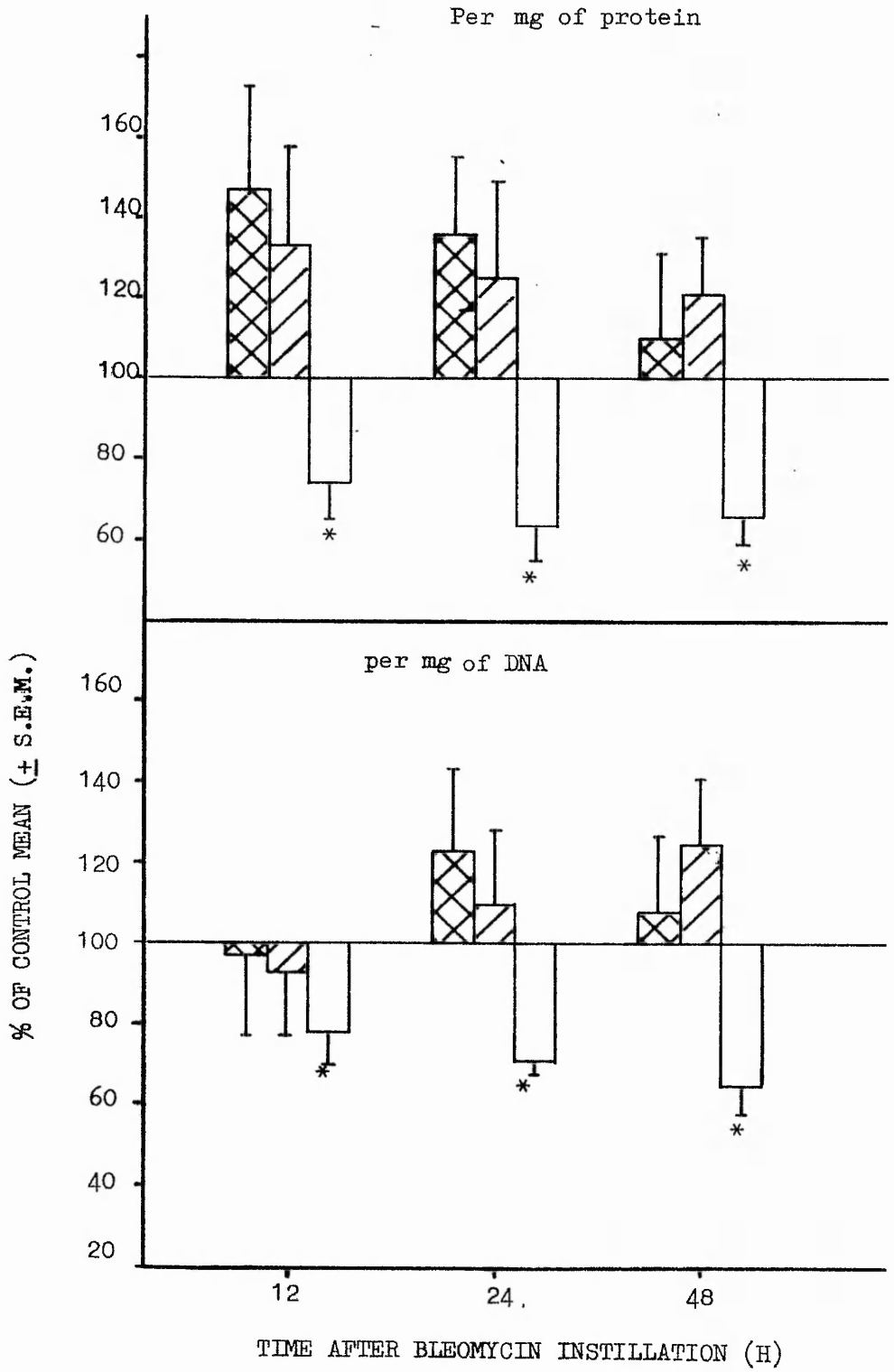


Figure 34: Enzyme activities following the instillation of 0.1U of bleomycin

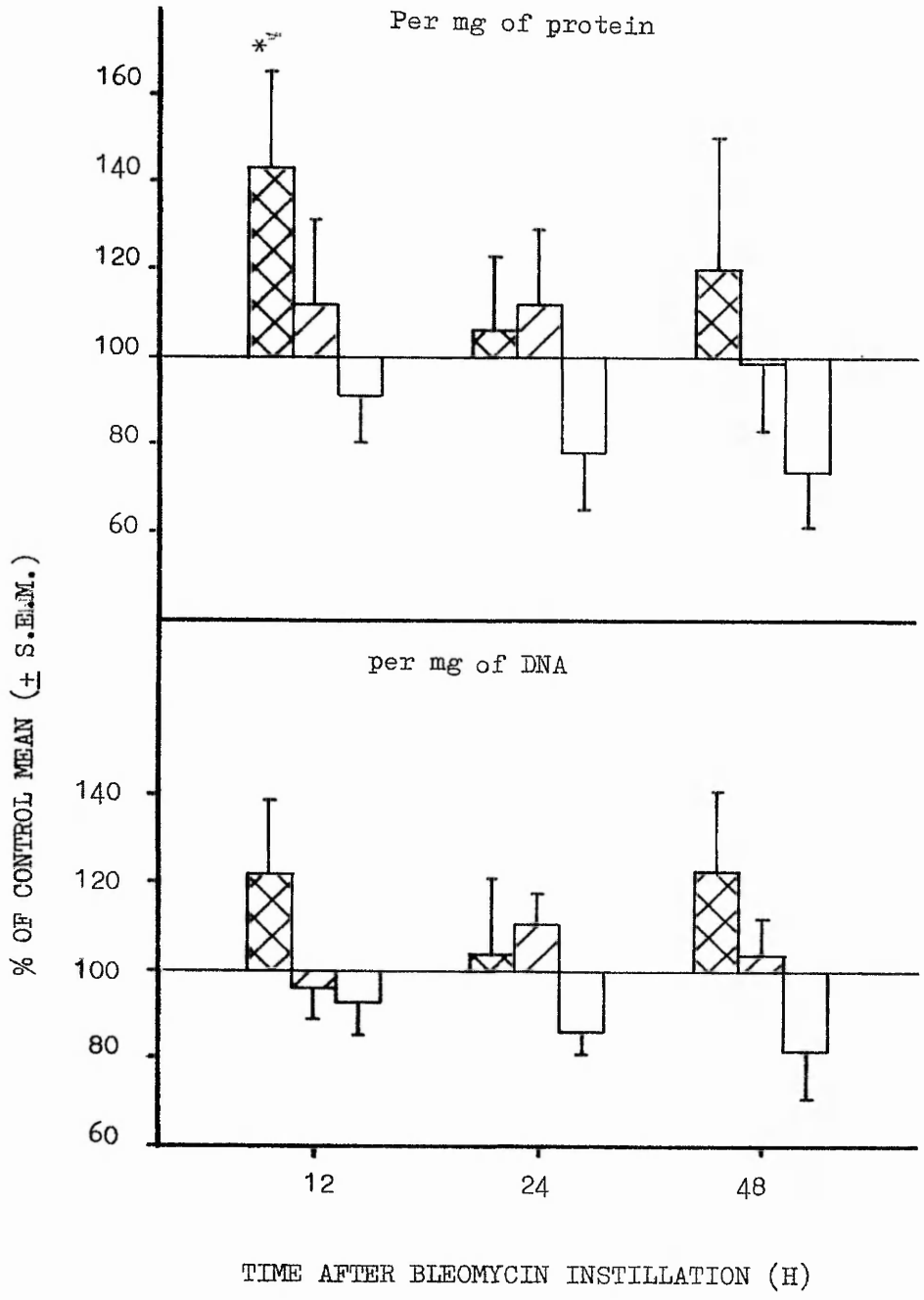
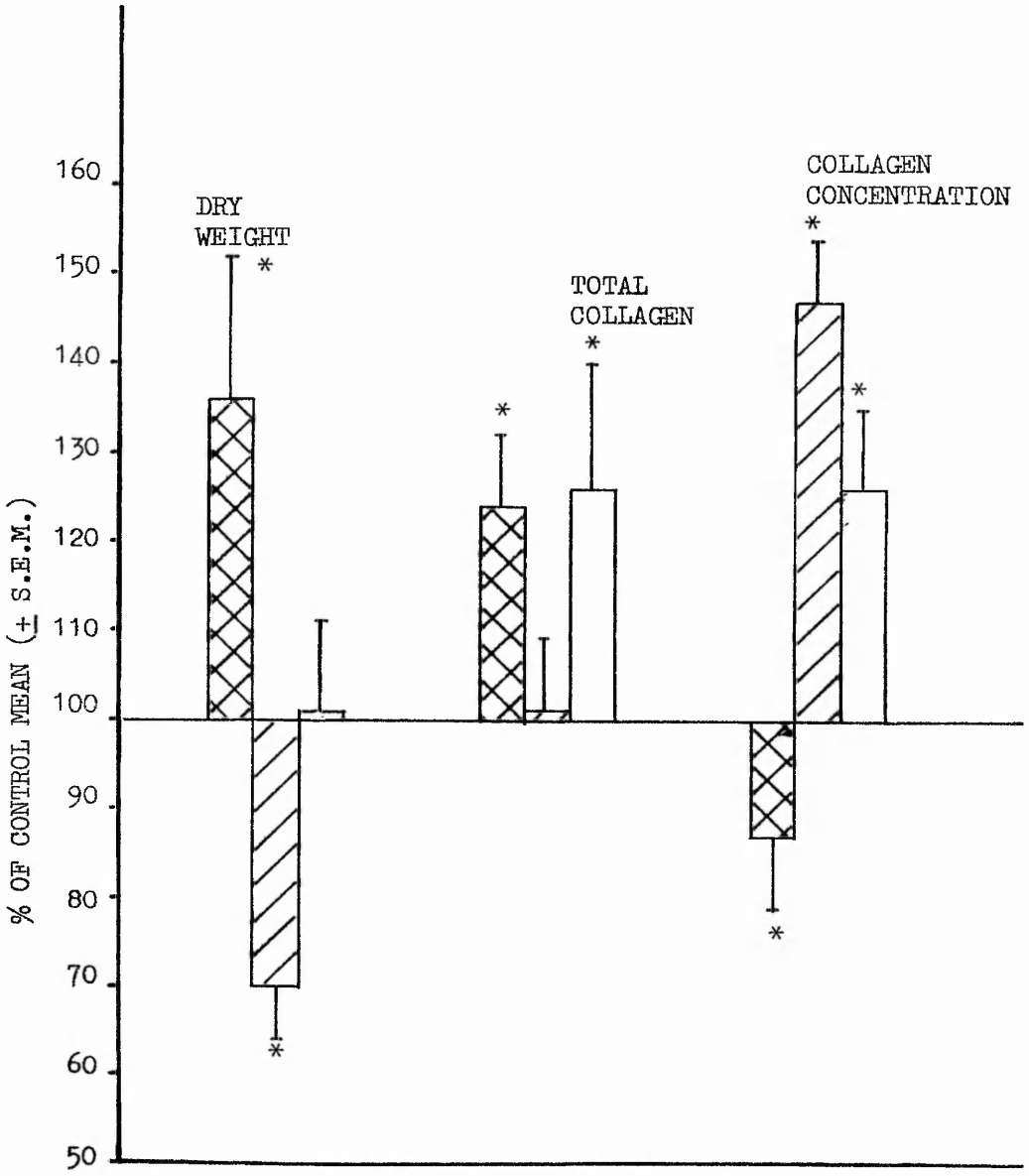


FIGURE 35: CHANGES IN LEFT LUNG COMPOSITION FOLLOWING THE INSTILLATION OF BLEOMYCIN IN RATS PRE-EXPOSED TO 85% OXYGEN

Data were obtained at 14 days following the intrabronchial instillation of 0.5U of bleomycin (in 0.3ml isotonic saline) into the left lung of rats pre-exposed to 85% oxygen for 7 days. Exposure of rats to oxygen was carried out as described in section 3.3.2. Lung dry weights were determined from lung homogenates (section 2.3.4). Test animals which received bleomycin (0.5U) following pre-exposure to 85% oxygen are shown as (□). Saline treated control animals pre-exposed to 85% oxygen for 7 days are shown as (▩). Bleomycin treated control animals pre-exposed to air for 7 days are shown as (▨). The results show the mean determinations ± S.E.M. (n = 4 or 5) expressed as a per cent of control values obtained from saline treated animals pre-exposed to air for 7 days (n = 4). Left lung control values for these animals were: dry weight = 71 ± 3mg; total hydroxyproline content = 1060 ± 38 µg; hydroxyproline concentration = 14.77 ± 0.34 µg/mg of lung dry weight.

*Significantly different from saline treated controls pre-exposed to air ($P < 0.05$).


160
150
140
130
120
110
100
90
80
70
60
50
% OF CONTROL MEAN (± S.E.M.)
DRY
WEIGHT
TOTAL
COLLAGEN
COLLAGEN
CONCENTRATION
*

## 5.5 DISCUSSION

### 5.5.1 Possible role of NADPH depletion in bleomycin toxicity

The possible role of NADPH depletion in bleomycin induced lung toxicity was investigated for several reasons. Firstly because the biochemical quantification of lipid peroxidation is difficult and this is further complicated in bleomycin toxicity because malondialdehyde (a by product of lipid peroxidation used to measure the process) is also released as a result of DNA cleavage by this drug (Gutteridge, 1979). Secondly because Tom and Montgomery (1980) failed to demonstrate any significant stimulation of lipid peroxidation in lung microsomal fractions following bleomycin administration to rats and finally, because it has been demonstrated that the action of bleomycin is enhanced in the presence of microsomes and NADPH (Yamanaka et al, 1978).

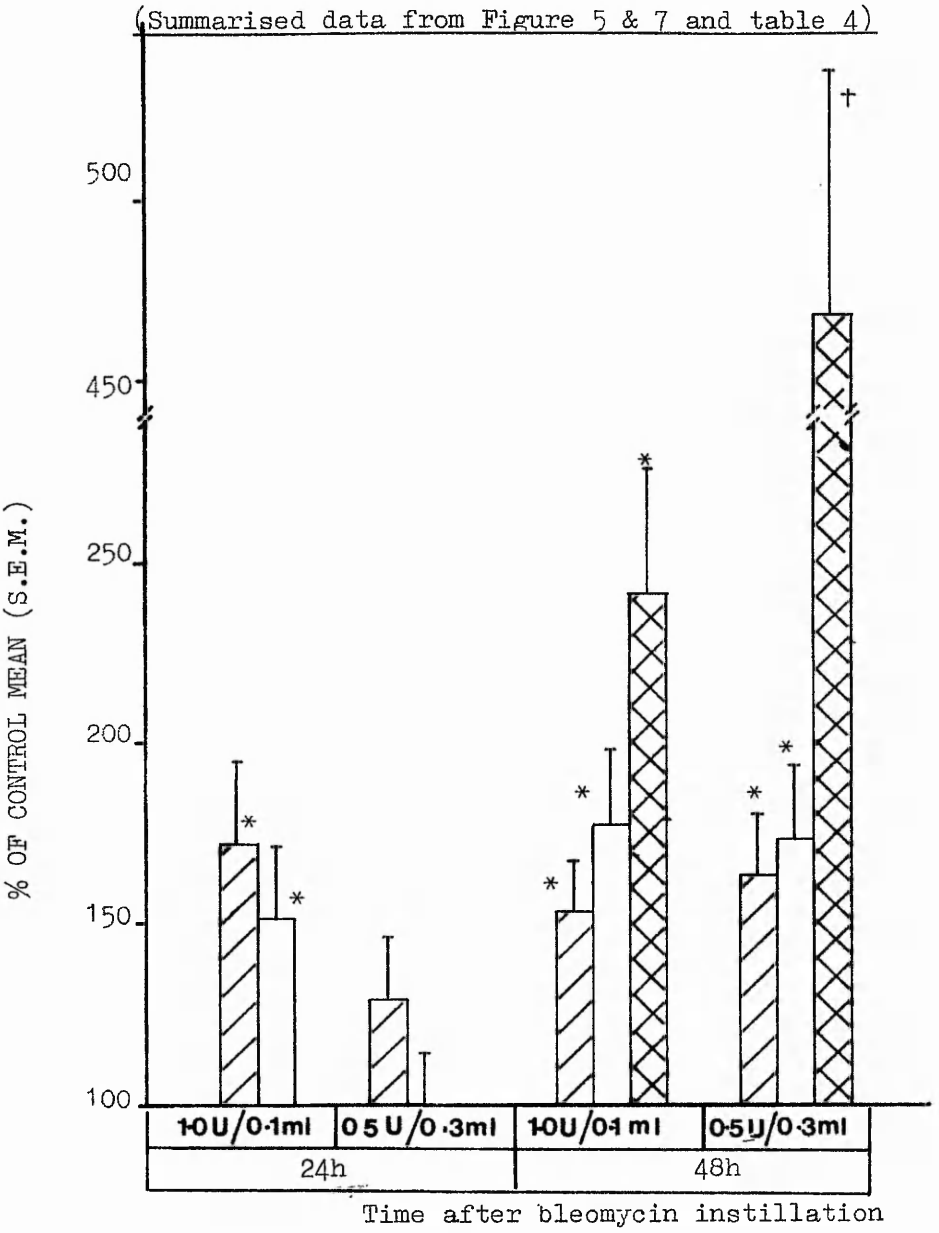
The results of this study provide little evidence for NADPH depletion as a mechanism involved in bleomycin induced pulmonary toxicity. This is supported by the observation that the pentose phosphate pathway activity failed to show any significant elevation above controls in both normal lung slices incubated in the presence of bleomycin and in lung slices taken from rats which had received a fibrogenic dose of bleomycin. In contrast normal lung slices incubated in the presence of paraquat or diquat, as well as lung slices taken from animals 3hr after the intrabronchial instillation of a fibrogenic dose of paraquat, showed greatly elevated pentose phosphate pathway activity.

### 5.5.2 Activities of lung oxidant defence enzymes

The total activities of both SOD and G6PDH following the intra-bronchial instillation of 1.0U of bleomycin showed similar changes to those reported in adult rats following either the intratracheal instillation of 1.5U of bleomycin (Fantone and Phan, 1979) or exposure to lethal levels of oxygen (100%) (Yam *et al*, 1978). These investigators suggested that a reduction in the activity of SOD may reflect the importance of this enzyme in the protective mechanism against the toxicity of bleomycin and oxygen. The results of this study, however, do not lend support to this conclusion. The data shows that the total activities of the oxidant defence enzymes in the lung vary according to the dose of bleomycin used. This variability may however be a reflection of the degree of cellular damage, inflammation and repair/proliferation occurring in the lung, rather than specific changes in the activities of these enzymes in response to bleomycin toxicity (Figure 36). This is supported by the following observations. The instillation of 0.5U of bleomycin into the lung failed to show an increase in the specific activity of SOD between 12 and 48h after instillation when expressed as units of activity per mg of DNA, an approximate measure of the activity of the enzyme per cell. This suggests that the influx of inflammatory cells is more likely to be responsible for the increase in SOD activity observed when expressed as total activity per lung (Figures 32 and 33). On the other hand the instillation of a higher dose of bleomycin (1.0U) resulted in a significant reduction in SOD activity between 24 and 48h after instillation, when expressed both as total activity and as specific activity per mg of DNA (Figures 30 and 31). This

FIGURE 36: MEASUREMENT OF THE DEGREE OF INFLAMMATION AND CELLULAR PROLIFERATION AFTER THE INSTILLATION OF 1.0U and 0.5U OF BLEOMYCIN IN 0.1ml and 0.3ml SALINE RESPECTIVELY

(Summarised data from Figure 5 & 7 and table 4)




Data shows changes in left lung DNA content (▨), Protein content (□) and DNA synthesis (⊠) following the intrabronchial instillation of bleomycin.

*Significantly different from saline controls ($P<0.05$)

†Significantly different from lungs receiving 1.0U of bleomycin in 0.1ml saline ($P<0.05$)

probably reflects more severe damage to lung cells than that after the instillation of 0.5U of bleomycin.

It is possible that the reduction in SOD activity following exposure to either 100% oxygen or bleomycin may reflect a similarity in the types of cells damaged by these two agents. The reduction in the total activity of SOD following the exposure of adult rats to 100% oxygen correlates well with the rapid decrease in the number of endothelial cells; the latter event being the primary cause of death in these animals (Yam et al, 1978; Crapo et al, 1980). Since the endothelial cells are the earliest site of damage following bleomycin administration (Adamson and Bowden, 1974, 1979) the reduction in the activity of SOD following the instillation of 1.0U of bleomycin may similarly reflect extensive damage to endothelial cells.

Although an increase in the activity of G-6-PDH, expressed both as total activity per lung and as specific activity per mg of DNA, was observed following the instillation of 1.0U of bleomycin, this may not necessarily be related to oxidant defence. In this regard, it has been suggested by Kimball et al (1976) that in order for G-6-PDH activity in the lung to double the infiltrating cells would have to make up approximately one third of the total lung weight. Since this condition appeared to have been fulfilled in the present study it provides a likely explanation for the increase in the total activity of G-6-PDH following the instillation of both 0.5U and1.0U of bleomycin. Furthermore, it is also recognised that

reparative, proliferative and growing processes in a number of tissues are characterised by increased activity of the pentose phosphate pathway (Warshaw and Rosenthall, 1972; Schwartz et al, 1974) which prowides ribose-5-phosphate for nucleic acid repair and synthesis and NADPH for reductive biosynthesis.

It may be argued that following the instillation of bleomycin doses which lead to the excessive deposition of collagen (1.0U and 0.5U) the oxidant defence enzymes are rapidly overcome and thus an increase in their activity may not be observed. Therefore, it was anticipated that the instillation of 0.1U of bleomycin may stimulate the oxidant defence enzymes, since this dose produced very little fibrosis in the left lung in earlier studies (Chapter 2). The results, however, showed that there was no clear and sustained stimulation of SOD, catalase or G6PDH between 12 and 48h after the instillation of this dose.

### 5.5.3 Possible protection against bleomycin toxicity following stimulation of the oxidant defence enzymes

Pre-exposure of adult rats to 85% oxygen for 7 days failed to reduce the toxicity of bleomycin to lung tissue, as determined from changes in lung dry weight, hydroxyproline content and hydroxyproline concentration when compared with respective saline instilled controls (Figure 35).

The increase observed in the left lung dry weight following the pre-exposure of rats to 85% oxygen was consistent with other

reports (Crapo et al, 1980). This increase signifies an increase in cell number as well as cell mass, particularly of epithelial type II cells.

## 5.6 SUMMARY

(i) NADPH depletion does not appear to be involved in bleomycin induced lung toxicity, and therefore its mechanism of toxicity differs from that of paraquat and diquat.

(ii) Following the instillation of bleomycin a significant increase in the activity of SOD, catalase and G-6-PDH (expressed per cell) is not observed.

(iii) Pre-exposure of adult rats to 85% oxygen for 7 days fails to protect against bleomycin induced lung toxicity.

# CHAPTER 6

## GENERAL DISCUSSION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| 6.3 | MECHANISMS LEADING TO INCREASED COLLAGEN DEPOSITION | 163 |

## 6.1 BIOCHEMICAL MECHANISM OF BLEOMYCIN INDUCED PULMONARY TOXICITY

The possibility of pulmonary cytotoxicity being initiated by bleomycin-mediated alterations in cellular lipids or nucleic acids was suggested by Matsuda et al (1980). However the inability of bleomycin to stimulate significant lipid peroxidation in lungs or in lung microsomes suggests that membrane lipids may not be target molecules for bleomycin (Tom and Montgomery, 1980; Muliawan, 1982; Trush et al, 1982a, b). An alternative mechanism of bleomycin toxicity involving the depletion of cellular NADPH levels may also be ruled out since in the present study bleomycin unlike paraquat, failed to stimulate the pentose phosphate pathway when tested both in vitro and in vivo. The most likely site of bleomycin interaction in pulmonary cells is therefore probably that of DNA. This is suggested by most of the in vitro studies undertaken by various investigators (see section 1.2.3).

DNA degradation by bleomycin is thought to involve the action of reactive oxygen metabolites, generated from the oxidation/reduction of the bound iron (Figure 7). Several investigators have shown that ferrous iron by itself is capable of producing random, non-specific strand breaks in DNA (Takeshita et al, 1976; Lown and Sim, 1977; Povirk et al, 1977) presumably by site - non specific autoxidation of ferrous iron and subsequent random hydroxyl radical attack on DNA. However, in the case of bleomycin it has been reported that DNA strand scission occurs at specific sites on the DNA (D'Andrea and Haseltine, 1978; Lloyd et al, 1978, 1979; Takeshita et al, 1978; Lown and Joshua, 1979; Mirabelli et al, 1979) and that the cleavage

results from the oxidation of the C-3 -C-4 bond of deoxyribose (Burger et al, 1980; Takeshita and Grollman, 1980). This suggests that the reactive oxygen metabolites are "site specific" and may be generated close to the deoxyribose on the DNA. Thus, the bleomycin may simply serve to transport and selectively position iron in the DNA chain. The reactive oxygen metabolite (eg $^{\bullet}OH$) which is then generated may interact with the nearest molecule, which is the deoxyribose of the DNA chain. This is further supported by the observation that in the absence of DNA bleomycin inactivates itself and subsequently is unable to bind iron or break DNA (Burger et al, 1981; Trush et al, 1982a, b).

The generation of "site specific" reactive oxygen metabolites may partially explain the failure to demonstrate a reduction in bleomycin induced lung toxicity in rats pre-exposed to 85% oxygen in the present study. It is possible that, even though the oxidant defence enzymes, such as SOD, in the lungs of these animals may be elevated after treatment with bleomycin, these enzymes are unable to get close to the reactive oxygen species being generated near the DNA. In this context, several in vitro studies have been undertaken by other workers to determine whether oxidant defence enzymes protect against bleomycin-mediated DNA damage, although the results have been contradictory. Studies of Onishi et al (1975) and Lown and Sim (1977) showed a reduction in bleomycin mediated DNA chain breakage by SOD, although this has been disputed by others (Ishida and Takahashi, 1975; Sausville et al, 1978b). More recently it has been suggested by Galvan et al (1981) that the inhibition of bleomycin-induced DNA degradation by SOD may be due to binding of the protein to DNA which then prevents the binding of bleomycin and the subsequent degradation of DNA.

The results of these studies must therefore be interpreted with care.

While the mechanism of bleomycin-mediated DNA degradation has been extensively studied in cell free systems it has only relatively recently been extended to biological systems. Trush et al, (1982a, b) investigated the interaction of bleomycin with rat lung microsomes and demonstrated significant bleomycin-mediated DNA chain breakage in the presence of oxygen, NADPH or NADH. Interestingly these investigators also found that in the absence of DNA, bleomycin led to a significant inhibition of microsomal lipid peroxidation. These results suggest that the chelation of iron by bleomycin may lead to both the inhibition of lipid peroxidation and also the bleomycin-microsome interaction leading to DNA damage. The iron in the bleomycin complex may be enzymatically maintained in the ferrous state by the microsomal mixed function oxidase while DNA damage is being produced (Kornbrust and Mavis, 1980; Trush et al, 1982a, b). Since mixed function oxidase activity is also associated with the nuclear membrane (Fahl et al, 1978), this form of interaction may also be responsible for the bleomycin induced damage in the nuclear region in cells.

An observation which is of interest to the present study is that the superoxide anion radical is capable of reducing $Fe^{3+}$ to $Fe^{2+}$ (Fong et al, 1976). Since it is commonly believed that oxygen toxicity is mediated by the superoxide anion radical (see section 5.1.1) it is conceivable that the observation in the present study of an increase in lung toxicity in rats treated with bleomycin plus 70% oxygen may also be related to an increased degree of

DNA damage by the interaction of these two agents. In support of this suggestion is the finding that superoxide anion radical produced by the redox cycling compounds such as paraquat, nitrofurantoin and mitomycin C increases bleomycin mediated DNA degradation (Trush et al, 1982b).

As an alternative to the reactive oxygen theory for bleomycin toxicity, studies on the fibrogenic structure-activity relationship of the bleomycin molecule have suggested that the terminal amine substituents may be of prime importance in the induction of fibrosis (Raisfield 1981; Raisfield et al, 1982). In these studies a difference in the fibrogenic potential of the different polyamine substituents was observed. For example, the fibrogenic potential of spermidine (the terminal substituent of bleomycin $A_5$) greatly exceeded that of agmatine (the terminal substituent of bleomycin $B_2$). However, more recently it has been suggested that the mechanism of toxicity of bleomycin analogs may be quite different from that of polyamines such as spermine and spermidine (Newman et al, 1983). These investigators demonstrated that in lung tissue both spermine and spermidine may be converted to the toxin acrolein, by the enzyme amine oxidase. Since intact bleomycin analogs, which contain terminal substituents such as spermine, spermidine, putrescine, agmatine and 3-aminopropyldimethyl-sulfonium salt, are poor substrates for amine oxidase, they are unlikely to induce toxicity by the production of acrolein.

Although most of the in vitro evidence points towards the interaction of bleomycin with cellular DNA, it may be concluded that the mechanism of bleomycin-induced pulmonary toxicity is complex. A significant contribution towards the understanding of bleomycin

toxicity would be the demonstration of DNA degradation in pulmonary cells following bleomycin administration. Undoubtedly, bleomycin-induced damage to DNA would be detrimental to a cell during replication. It is also possible that DNA damage may cause a disruption of important transcriptional processes in the cell and this would in turn lead to toxic cellular effects prior to replication.

## 6.2 Initial site of bleomycin pulmonary toxicity

Although endothelial cell damage may be the earliest site of damage following bleomycin administration (Adamson and Bowden, 1974, 1979) it is possible that this damage may not be directly induced by bleomycin. For example the observation that endothelial cells rapidly regenerate in the presence of bleomycin suggests that the bleomycin is not bound to the DNA of these cells. This observation is in complete contrast to that seen in epithelial cells (Adamson and Bowden, 1979). Considering that the most likely site of action of bleomycin in the cell is the DNA (see previous section) it is possible that the early influx of neutrophils into the lung may partially be responsible for endothelial cell damage. There is now considerable evidence which suggests that in many types of lung injury the aggregation and stasis of neutrophils in pulmonary capillaries may result in endothelial cell damage through the release of reactive oxygen species by these cells (Craddock et al, 1979; Hosea et al, 1980; Jacob et al, 1980; Fox et al, 1981; Hyers 1981).

Recent studies of Newman et al (1981) have shown that bleomycin may produce fibrotic changes in the lung in the absence of

endothelial cell injury suggesting that the initial site of bleomycin damage may be the epithelial cells. In this regard bleomycin induced histological changes in epithelial cells have been described in detail (Adamson and Bowden, 1974, 1979; Gyorkey, 1980). To date, however, no attempt has been made to quantitate epithelial cell damage biochemically. Therefore the possibility of using changes in putrescine accumulation by lung tissue as a monitor of epithelial cell damage was investigated in the present study following the treatment of lungs with bleomycin. Unfortunately the results failed to give a clear indication of the usefulness of this method in the assessment of epithelial cell damage. Although the measurement of changes in putrescine accumulation by lung tissue following treatment with low doses (eg 0.1U) of bleomcyin (which result in a small degree of inflammation and collagen deposition) was found to be useful in indicating epithelial cell damage, the use of higher doses (eg 1.0U) of bleomycin indicated this method to be unsuitable.

In order to elucidate the initial site of lung damage by a drug it may therefore be more appropriate to use acute animal models which do not show extensive fibrosis, thereby reducing the likelihood of gross secondary changes (eg. inflammatory cells) interfering with analysis. In this context it is known that acute endothelial or epithelial damage may be rapidly repaired without the onset of pulmonary fibrosis (Bowden and Adamson, 1974; Witschi, 1976), although severe injury to these cells with delayed or modified regeneration results in loss of fibroblastic control and eventual fibrosis (Adamson and Bowden, 1976, 1979, 1983; Brody et al, 1981). Therefore the assessment of acute endothelial and epithelial

cell damage during the early stages after drug administration may conveniently and rapidly be made by determining 5-hydroxytryptamine and putrescine accumulation respectively by lung slices (Junod, 1972; Smith and Wyatt, 1981). In order to relate these changes more closely to the situation in humans, bleomycin would of course have to be administered by the parenteral route.

A very significant contribution to understanding and elucidating the initial site of bleomycin injury would be the determination of the levels of the enzyme bleomycin hydrolase present in endothelial and epithelial cells of the lung. With regards to this, the studies of Adamson and Bowden (1979) are of considerable interest. These investigators showed that despite the fact that [$^3$H]-bleomycin was present in both the epithelial and endothelial cells only the epithelial cells showed abnormal regeneration, presumably due to the interaction of bleomycin with the DNA of these cells. It may therefore be speculated that the endothelial cells contain higher levels of the inactivating enzyme than the epithelial cells thereby preventing DNA binding and subsequent damage.

## 6.3 Mechanisms leading to the increased deposition of collagen

It is apparent from the discussions so far that bleomycin-induced pulmonary fibrosis is associated with a disorganisation of both cells and the connective tissue matrix. It is the collagenous component of the matrix, however, that characterises fibrosis (Basset *et al*, 1975; Crystal *et al*, 1976; Madri and Furthmayr, 1980; Rennard *et al*, 1982; Crystal *et al*, 1984). Furthermore, although it is generally appreciated that fibrosis likely includes changes in all types of collagens, it is the type I collagen that forms the thick, disorganised bands of fibres that characterise the fibrotic state (Rennard *et al*, 1982; Crystal *et al*, 1984). The

important contribution of type I collagen is the major cause of the stiff noncompliant functional properties of a fibrotic organ (Bornstein and Traub, 1979). Thus, in simplest terms, the most important matrix abnormality in the fibrotic state is an accumulation of type I collagen (Hance and Crystal, 1975; Seyer et al, 1976; Bateman et al, 1981). Analogous to this hypothesis is the finding that tissues that are relatively compliant (eg fetal skin, lung, intestine, blood vessels) have a low type I/type III ratio (Epstein and Munderloh, 1975), tissues with less compliance (eg adult skin, atherosclerotic blood vessel) have a higher ratio of type I to type III (Epstein, 1974; Epstein and Munderloh, 1975; McCullough and Balian, 1975); and tissues that are very rigid (bone, tendon) have only type I and no type III (Epstein and Munderloh, 1975). To understand the mechanisms leading to fibrosis, therefore, it is useful to focus on those processes that lead to the accumulation of type I collagen.

The accumulation of type I collagen within the alveolar wall probably results from a shift in the relative proportions of the parenchymal cells in the alveolar structures. The key change is the marked increase in the numbers of fibroblasts (Crystal et al, 1976; Crapo et al, 1978; Bitterman et al, 1981; Coalson, 1982; Rennard et al, 1982; Crystal et al, 1984). Since fibroblasts are the major producer of type I collagen in the alveolus (80 to 90 per cent of the collagen produced by fibroblasts is type I) (Hance and Crystal, 1975; Hance et al, 1976; Bradley et al, 1980; Rennard et al, 1982), an increase in the proportion of fibroblasts leads to an increase in the relative amount of type I collagen in the

interstitial matrix (Hance et al, 1976; Seyer et al, 1976; Bateman et al, 1981). The normal human fibroblast under conditions of defined extracellular milieu produces 150,000-300,000 type I molecules per cell per hour (Breul et al, 1980). These cells are thought to produce the same amount of collagen over most of their life span (Breul et al, 1980). In this context, a central aspect of the pathogenesis of fibrosis is the recruitment of fibroblasts and expansion of their numbers in areas where there has been significant damage.

An understanding of how this occurs can be attained by recognising that the reconstruction processes associated with fibrosis are preceded by the presence of activated inflammatory and immune effector cells in the area of injury (Rennard et al, 1982; Crystal et al, 1982, 1984) (the "alveolitis"). There is now ample evidence that several effector cells are capable of recruiting fibroblasts (Crystal et al, 1982). The two cell types which have received the greatest attention are the alveolar macrophages and lymphocytes.

The alveolar macrophage is thought to direct this process by virtue of its ability, when appropriately activated, to modulate both the local accumulation and the replication of fibroblasts. Alveolar macrophages during interstitial lung disorders release increased amounts of fibronectin, an adhesive glycoprotein that is chemotactic for fibroblasts, attaches them to matrix components, and provides them with one of the signals necessary for replication (Rennard et al, 1981; Ruoslathi et al, 1981; Bitterman et al, 1983). Alveolar macrophages also spontaneously release alveolar macrophage-derived growth factor (AMDGF),

an 18,000 dalton protein that induces fibronectin-primed fibroblasts to release an insulin-like growth factor, which in turn signals the same or nearby fibroblasts to replicate (Bitterman et al, 1982).

The exact role of lymphocytes in pulmonary fibrosis is not known. Their importance is suggested by the fact that treatment of rats with anti-lymphocyte globulin reduces the fibrotic response in rats (Thrall et al, 1979). Several in vitro studies have demonstrated that lymphokines from lymphocytes can influence fibroblast activity and collagen synthesis (Johnson and Ziff, 1976; Wahl et al, 1978). In addition lymphocytes can release factors that are chemotactic for fibroblasts and macrophages (Postlethwaite et al, 1976; Wahl and Wahl, 1981).

The alveolitis which invariably precedes fibrosis is a critical determinant not only of the reconstruction processes associated with fibrosis but also of the generalised derangement of the parenchyma and the extracellular matrix that accompanies this state. Of all the inflammatory cells, the neutrophil has the greatest capacity to inflict damage (Haverman and Janoff, 1978). The activated neutrophil is cytotoxic for most lung parenchymal cells, probably because it releases highly reactive oxygen metabolites (Till et al, 1982). Furthermore, the neutrophil carries potent connective-tissue proteases, including a collagenase that can degrade type I collagen and an elastase that can also degrade connective tissue components of the alveolar wall (Horwitz et al, 1977; Janoff et al, 1979; Rennard et al, 1982). It is understandable, therefore, that a chronic alveolitis involving large numbers of neutrophils is very damaging to the alveolar structures.

Consistent with this concept of the pathogenesis of fibrosis, in patients with idiopathic pulmonary fibrosis (IPF), the lung contains an active collagenase associated with increased numbers of neutrophils (Gadek et al, 1979). Thus, some of the connective tissue abnormalities in this disease are likely mediated by destructive processes followed by resynthesis of collagens in the incorrect proportions and locations, resulting in derangements in connective tissue that are characteristic of the disease. The fact that biochemical analysis of lung biopsies from these patients failed to show an increase in collagen concentration predicted from histological features suggests that a "shift" in the relative concentrations of the types of collagens may have occured (Crystal et al, 1976; Fulmer et al, 1976). Consistent with this concept, transmission electron micrographs of IPF lung show collagen fibres that are randomly arrayed, twisted, frayed and in abnormal locations (Basset et al, 1975). In addition, there is a difference in the types of collagen present in the IPF lung compared to the normal lung; in IPF, the normal 2:1 ratio of type I and III is increased to 4:1 (Seyer et al, 1976).

Animal models developed following the intratracheal instillation of bleomycin have produced results which are in conflict with some of the current concepts of pathogenesis of pulmonary fibrosis discussed above. For example, using rabbits Laurent et al (1981), failed to demonstrate an increase in the ratio of type I to type III collagen when measured over several weeks. In the rat model, however, an increase in this ratio has been observed at 3 weeks (Reisar and Last 1983), and an increase in ratio of newly synthesised

type I collagen to type III observed as early as 1 week (Reisar and Last, 1981). Nevertheless, in this model the time of maximal collagen synthesis did not correlate well with the time of maximal numbers of fibroblasts (Phan et al, 1980). This observation has also been reported in the hamster model (Clarke et al, 1980). In an effort to explain this discrepancy it has been suggested that the bleomycin may directly increase the rate of collagen synthesis per fibroblast in the lungs, since it has been demonstrated that bleomycin is capable of doing so in in vitro studies (Clarke et al, 1980).

Some of the intratracheal models have also yielded surprising results on the involvement of neutrophils and collagen degradation in the development of pulmonary fibrosis. In the rat model neutrophil depletion prior to bleomycin instillation leads to a greater increase in the total collagen content (Thrall et al, 1981). Similarly in the hamster model a greater increase in collagen synthesis has been reported, following neutrophil depletion (Clarke and Kuhn, 1982). These studies seem to suggest that the neutrophil may in fact be attempting to reduce the fibrotic response. With regard to collagen degradation, there is both direct and indirect evidence which suggests that collagen degradation may in fact be reduced following bleomycin instillation (Marom et al, 1980; Phan et al, 1981; Laurent et al, 1983).

It is possible that some of the discrepancies observed in the intratracheal models may be explained by the fact that in some of these models true "fibrosis" in lung lobes is not being assessed biochemically. It is apparent from the various studies presented in

this thesis that following the instillation of bleomycin into the lung two distinct types of change in lung collagen occur. These are, an increase in collagen (probably type I) in injured areas representing "fibrosis", and a general increase in collagen in uninjured areas representing compensatory lung growth. It is therefore likely that some of the discrepancies observed in the intratracheal models may be due to interference by compensatory lung growth, since biochemical assays of lung tissue would measure both of these processes together. Evidence for this was provided in the present study by the data obtained from the lungs of animals treated with bleomycin by the intratracheal route (see section 2.5.3C).

The changes occuring in the intrabronchial model described in this study differ in several respects from those reported for the intratracheal models. However, the sequence of events occurring in the left lung appear to be in agreement with the current concepts of the pathogenesis of pulmonary fibrosis discussed earlier. Both histological and biochemical studies showed a good correlation between the deposition of collagen (type I) and an increase in the numbers of fibroblasts, both being preceded by substantial numbers of inflammatory and immune effector cells.

The observations of an increase in collagen concentration in this model may have possibly resulted from a lesser degradation of collagen and/or a faster synthesis of collagen relative to that of other tissue components following bleomycin damage. There is evidence that collagen turnover is slower than

other proteins in a variety of tissues (Newberger et al, 1951; Laurent et al, 1978). Although no direct attempt was made to investigate collagen degradation in the intrabronchial model, both histological and biochemical studies suggested that it may be involved. The loss of collagen content in the left lung following treatment with bleomycin plus 70% oxygen clearly shows that collagen degradation certainly occurs when the damage to the lung is severe. It would therefore indeed be interesting to verify both collagen degradation and the increase in type I collagen by biochemical means in this model.

From both the studies presented in this thesis and those of other investigators it is apparent that fibrosis of the lung may manifest itself in two forms. For instance fibrosis of the interstitium is usually associated with diffuse septal thickening, whereas fibrosis of both the interstitial and intra-alveolar regions is associated with scar formation. The distribution and density of these scars may depend upon the route of administration of a drug and its concentration. For example, following the intratracheal instillation of bleomycin into the lung the scars are more localised and may have areas of normal alveolar tissue interspersed between them, whereas intrabronchial instillation of bleomycin leads to the formation of a continuous scar (Figure 11, Chapter 2). There is now considerable evidence which suggests that the type of fibrosis which develops in the lung may be related to the extent of the injury (Witschi et al, 1981; Crystal et al, 1982; Pickrell et al, 1983). Alveoli in which the injury to the parenchyma is so extensive that the basement membrane is disrupted probably develop dense

fibrous scars, which cannot be repaired (Vracko, 1974; Crystal et al, 1982; Pickrell et al, 1982; Chapter 2 present study). Conversely limited injury to the parenchyma resulting in diffuse interstitial pulmonary fibrosis may eventually be repaired, since it does not excessively interupt the pulmonary architecture (Pickrell et al, 1983). The irreversibility generally associated with scar formation is characteristic of fibrosis produced by a variety of etiologic agents in several species (Fulmer and Crystal, 1976).

Recent evidence suggests that the irreversible nature of fibrosis may be related to the formation of intermolecular cross-links between connective tissue proteins, thus contributing to a fibrotic matrix which is resistent to degradation (Richards and Curtis, 1984). Although the fibrotic lung tissue may contain several types of cross-links, those involving $\gamma$-glutamyl-$\epsilon$-lysine bridges catalysed by calcium dependent transglutaminase enzymes may be important (Griffin et al, 1978 ; Richards and Cur tis, 1984). If this is the case, then the current view of fibrosis as the over production, deposition and retention of collagen may be over simplified.

In conclusion it could be argued that the intrabronchial model developed in this study may be a more useful animal model than the intratracheal model, since it allows the study of "true fibrosis" (ie irreversible depositon of fibrous proteins) in the left lung and compensatory lung growth in the right lung, both at the same time. The data obtained from both the intrabronchial and intra-tracheal models in the present study suggests that the biochemical assessment of fibrosis is indeed a formidable task. Although both

collagen content and collagen concentration of the lung may be useful markers of fibrosis, data of this kind must be interpreted with care. Such biochemical parameters may show an increase, a decrease or may even be normal depending upon the extent of injury, the amount of compensatory lung growth and the extent of alveolitis. Perhaps the most accurate determinant of pulmonary fibrosis is the concentration of type I collagen as proposed by Crystal et al (1984) and which is further suggested by the results of the present study.

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# BIOCHEMICAL SOCIETY TRANSACTIONS

# A study of the pulmonary toxicity induced by the anti-tumour compound bleomycin

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Bleomycin is a mixture of glycopeptides used in cancer chemotherapy (Umezawa, 1974). Unfortunately a major side effect of bleomycin therapy is the development of pulmonary fibrosis (Delena *et al.*, 1972). The pulmonary toxicity of bleomycin, although limiting the clinical use of the drug, has led to its use as an injurious agent in the development of animal models of pulmonary fibrosis (Snider *et al.*, 1978). The mechanism of toxicity, however, is not fully understood.

Several studies have suggested that bleomycin exerts its anti-tumour activity by binding to DNA and causing strand scission (Suzuki *et al.*, 1969). The mechanism, as suggested by studies *in vitro* (Oberley & Buettner, 1979), involves the formation of a Fe(II)–bleomycin complex which in the presence of oxygen and a suitable reducing agent is thought to lead to the cyclic generation of reactive oxygen species such as $O_2^{-\cdot}$ and $OH^{\cdot}$.

The present study was designed to give information on the question of whether activated oxygen species play a significant role in bleomycin-induced pulmonary toxicity. The work includes a study of the effect of bleomycin on the lungs' oxidant-defence systems after the instillation of bleomycin into rat lungs, and also an examination of the effects of hyperoxia on bleomycin-induced pulmonary fibrosis.

The effects of bleomycin on the depletion of cellular NADPH either directly in the cyclic generation of reactive oxygen species or indirectly through depletion of glutathione in the lungs' oxidant-defence systems was tested by measuring stimulation of the pentose phosphate pathway. This was done by measuring any change in the rates of the oxidation of [1-$^{14}$C]glucose and [6-$^{14}$C]glucose in lung slices (Keeling *et al.*, 1982) from the left lungs of rats previously dosed intrabronchially with a fibrogenic dose of bleomycin (1.0 unit of bleomycin in 0.1 ml of iso-osmotic saline). The results (Fig. 1*a*) indicated that no great changes in the oxidation rates of [1-$^{14}$C]-glucose and [6-$^{14}$C]glucose could be detected that would be indicative of stimulation of the pentose phosphate pathway when measured in lung slices between 3 and 48 h after treatment. A similar study with the herbicide paraquat did, however, result in a 6-fold change in the C-1/C-6 ratio when lungs were examined 3 h after treatment.

When the oxidant-defence enzymes superoxide dismutase and catalase were measured (Beauchamp & Fridovich, 1971; Lück, 1965) in extracts of similarly treated lungs equivalent times after bleomycin instillation, no increase enzyme activities could be detected. A significant decre in superoxide dismutase occurred at both 24 and 48 h a dosing (Fig. 1*b*). Measurement of glucose-6-phosphate hydrogenase activities (Lohr & Waller, 1974), howe

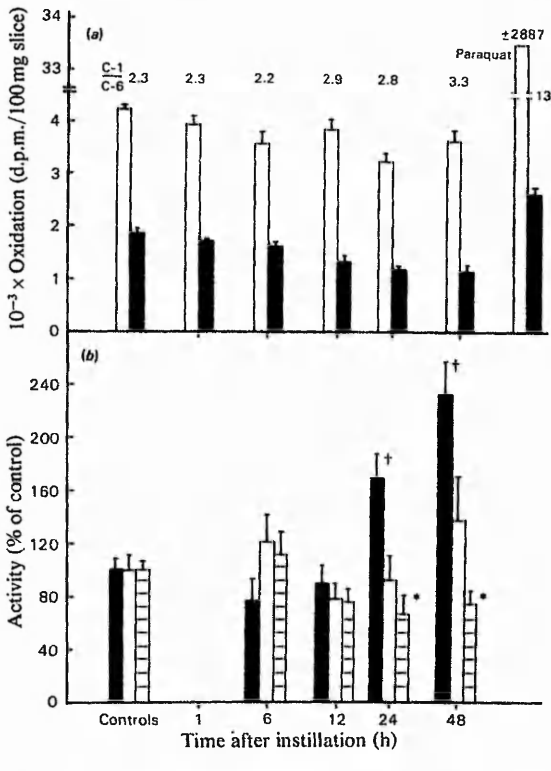


Fig. 1. *Effect of intrabronchial instillation of bleomycin on oxidation of* [1-$^{14}$C]*glucose* (□) *and* [6-$^{14}$C]*glucose* (■) *by lung slices and* (*b*) *lung activities of superoxide dismutase* ( catalase (□) *and glucose-6-phosphate dehydrogenase* (

Rats (180–200 g) received 1.0 unit of bleomycin in 0.1 m isotonic saline into the left lung, except for controls, wh received saline alone. Rats instilled with paraquat instea bleomycin received 3 mg of paraquat in 0.1 ml of sali Results show the mean values ± S.E.M. ($n = 4$). *$P \leq 0$ and †$P \leq 0.02$ for significance of differences from contr

indicated a significant increase in this enzyme at both 24 and 48 h after treatment.

Examination of the effects of non-lethal (70%) oxygen on the development of pulmonary fibrosis after intrabronchial instillation of bleomycin (0.5 unit in 0.3 ml of iso-osmotic saline) into rats indicated that animals exposed to both oxygen and bleomycin had increased pulmonary fibrosis as measured both histologically and biochemically (lung contents of DNA, protein, hydroxyproline, and dry weights) compared with animals treated with only bleomycin. No indications of fibrosis was detected in those animals exposed to oxygen alone.

Our results show no evidence for increased consumption of NADPH in bleomycin-induced pulmonary toxicity as measured by stimulation of the pentose phosphate pathway in lung slices. This is in direct contrast with studies with the herbicide paraquat, where consumption of NADPH (Keeling & Smith, 1982) and the generation of reactive oxygen species (Bus *et al.*, 1976) are well documented. The participation of reactive oxygen species in bleomycin-induced pulmonary toxicity is, however, unclear from our present data. Although no increase in catalase and superoxides dismutase activities could be detected in bleomycin-treated lungs, the decrease in superoxide dismutase and corresponding increase glucose-6-phosphate dehydrogenase have been described in lungs of rats exposed to lethal (96–98%) doses of oxygen (Yam *et al.*, 1976). Furthermore, increased pulmonary fibrosis was detected in bleomycin-treated animals exposed to non-lethal (70%) doses of oxygen, suggesting a synergistic effect of oxygen in bleomycin-induced pulmonary toxicity.

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# 3rd international colloquium on pulmonary fibrosis

October 17-19, 1984

PROGRAM AND ABSTRACTS

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CLINIQUE DE PNEUMO-PHTISIOLO
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# 3e colloque international sur la fibrose pulmonaire

17-19 octobre 1984

PROGRAMME ET RÉSUMÉS

# COMPARISONS BETWEEN THE DEVELOPMENT OF PULMONARY FIBROSIS FOLLOWING THE INTRATRACHEAL AND INTRABRONCHIAL INSTILLATION OF BLEOMYCIN. EVIDENCE FOR AN INCREASE IN COLLAGEN CONCENTRATION.

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Numerous animal models have been developed to study the mechanisms involved in bleomycin-induced pulmonary fibrosis. In this regard bleomycin administered by the intratracheal route has become of wide use. The fibrotic lesions in this model have been reported to develop focally and are thought to be non-progressive in nature. In the present study the development of pulmonary fibrosis in the rat has been investigated following both the intratracheal and intrabronchial instillation of bleomycin. These studies showed that the intratracheal and intrabronchial instillation of an equivalent dose of bleomycin resulted in changes in the lung which were different when measured by both histological and biochemical techniques. Following the intrabronchial instillation of 0.5U of bleomycin (in 0.3ml saline) into the left lung, this lung showed extensive collagen deposition with eventual consolidation between 14 and 21 days. Comparison of the different models indicated that the degree of fibrosis was dependent on both the dose of bleomycin and on the volume of saline in which it is instilled. In both models only those lung lobes which demonstrated histological evidence of extensive collagen deposition showed an increase in collagen concentration ( µg hydroxyproline/mg lung dry wright). Biochemical and histological studies suggested that uninjured areas of the lung underwent compensatory lung growth following the instillation of bleomycin by either the intratracheal or intrabronchial route. These studies demonstrate that the magnitude of the biochemical determinants used to assess pulmonary fibrosis may vary from one animal model to another depending upon the dose of bleomycin, the route of instillation and the volume of saline in which the bleomycin is instilled. Furthermore the results indicate that the method used to express biochemical data is of crucial importance when assessing fibrosis.

†This work was supported by an SERC Case Award.

## ABSTRACT

A biochemical study of pulmonary fibrosis

M Iqbal, 1984

The development of pulmonary fibrosis in the rat has been investigated following both the intratracheal and intrabronchial instillation of bleomycin. These studies showed that the intratracheal and intrabronchial instillation of an equivalent dose of bleomycin resulted in changes in the lung which were different when measured by both histological and biochemical techniques. Following the intrabronchial instillation of 0.5U of bleomycin (in 0.3ml saline) into the left lung, this lung showed extensive collagen deposition with eventual consolidation between 6 and 14 days. Comparison of the different models indicated that in each case the degree of fibrosis was dependent on both the dose of bleomycin and on the volume of saline in which it is instilled. In both models only those lung lobes which demonstrated histological evidence of extensive collagen deposition showed an increase in collagen concentration (μg hydroxyproline/mg lung dry weight). Biochemical and histological studies suggested that uninjured areas of the lung underwent compensatory lung growth following bleomycin instillation.

Studies undertaken to investigate the effect of hyperoxia (70% oxygen) on the development of bleomycin-induced pulmonary fibrosis showed that the fibrotic response could be enhanced. This enhancement was only observed when animals were exposed to oxygen immediately after the intrabronchial instillation of bleomycin.

Investigations into the biochemical mechanism of bleomycin toxicity indicated that its toxicity to lung cells differed from that of the herbicide paraquat. Bleomycin failed to stimulate both the pentose phosphate pathway and the oxidant defence enzymes. Furthermore, pre-exposure of rats to 85% oxygen failed to reduce bleomycin induced toxicity.

An assessment of different biochemical parameters that may be used as markers of bleomycin induced pulmonary fibrosis indicated that levels of lung transglutaminase activity and [$^{14}$C]-putrescine accumulation into lung slices may be of potential use in this context.