TOXICITY OF ASBESTOS IN LABORATORY ANIMALS, WITH SPECIAL REFERENCE TO THE CARCINOGENICITY OF INGESTED AND DISSEMINATED FIBRE

ROBERT EDWARD BOLTON, B.Sc.(Hons.)

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ABSTRACT

Some aspects of the toxicity of ingested and transported asbestos fibre are investigated in laboratory animals.
An introductory literature review of the effects of asbestos on both humans and experimental animals is followed by an account of several long-term toxicity studies on rats. Experiments on the effects, and consequences of, prolonged ingestion, subcutaneous injection and inhalation of asbestos are described, and a section giving details of a cytokinetic analysis of gastrointestinal tissues from animals exposed to ingested asbestos fibre is included. Some studies on the chemical reactivity of asbestos are described where they are of relevance to the reliable recovery and identification of fibre from tissues, and the results of searches for disseminated fibre are reported for each of the long-term experiments.

No adverse effects of prolonged ingestion of asbestos were found, with no signs of widespread tissue penetration or damage, and no gastrointestinal tumours or alterations to the proliferative status of exposed tissues. The subcutaneous injection of asbestos in rats was associated with a small degree of non-specific transport of asbestos to a variety of tissues, a strong carcinogenic response at the site of amphibole injection, and the production of several unusual tumours remote from the injection site. There was no direct association between the low levels of disseminated fibre and the tumours produced. The inhalation of asbestos was associated with a widespread dissemination of fibre via the lymphatic and

blood vascular systems to a range of tissues but there was no selective deposition in mesothelial tissues, and no adverse carcinogenic consequences of the disseminated fibre.

The experimental findings suggest that there is no risk of malignancy associated with the prolonged incidental ingestion of low levels of asbestos fibre in healthy humans, although it remains a possibility that individuals with abnormalities of the gastrointestine may be at risk from the consequences of asbestos fibre penetration. Any increases in gastrointestinal neoplasia amongst individuals occupationally exposed to high levels of inhaled asbestos fibre are therefore considered to arise as a result of the pulmonary lymphatic clearance and consequent widespread dissemination of the fibre.

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The experiments described herein were undertaken as an integral part of a long term research programme covering many aspects of the biological effects of the asbestos minerals, and involving many individuals. However, within this broad framework, the specific studies were conceived, devised, and undertaken by myself, unless expressly stated otherwise. Accordingly, I accept full responsibility for any errors and/or omissions in the holograph.

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UNITS and ABBREVIATIONS

A°	Angstrom 1×10^{-10} m
β	beta particle
°C	Centigrade
DNA	Deoxyribonucleic acid
d.p.m.	disintegrations per minute
EDAX	Energy dispersive X-ray analysis
°F	Fahrenheit
FLM	Fraction of labelled mitosis
G_0	The decycled, but potentially active, phase of the cell cycle
G_1	The phase of protein and RNA synthesis in the cell cycle
G_2	The short phase prior to mitosis in the cell cycle
G.F.	Growth fraction
G.I.	Gastrointestine (intestinal)
gm	gram
mg	1×10^{-3} gm
μg	1×10^{-6} gm
kg	1×10^{3} gm
H. + E.	Haematoxylin and eosin
^{3}HTdr	tritiated thymidine
Im	mitotic index
I_L	labelling index
I_p	proliferative index
I_{met}	metaphase index
K_B	cell birth rate
LM	light microscope (microscopy)
l	litre
ml	1×10^{-3} l
μl	1×10^{-6} l
M	Mitosis
m	metre
cm	1×10^{-2} m
μm	1×10^{-6} m
met	metaphase
P.C.A.	Perchloracetic acid

RNA	Ribonucleic acid
r.p.m.	revolutions per minute
r_m	rate at which cells enter mitosis
r_s	rate at which cells enter phase of DNA synthesis
S	DNA synthesis phase of cell cycle
SEM	Scanning electron microscope (microscopy)
s.e.m.	standard error of mean
TCA	Trichloracetic acid
TEM	Transmission electron microscope (microscopy)
t_A	the duration of metaphase arrest
t_c	true cell cycle time
$t_{c(a)}$	apparent cell cycle time
t_{G_1}	the duration of the G_1 phase of the cell cycle (q.v.)
t_{G_2}	the duration of the G_2 phase of the cell cycle (q.v.)
t_{met}	the duration of metaphase
t_s	the duration of the S phase of the cell cycle (q.v.)
UICC	Union Internationale Contra Cancer
z	atomic number

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction - the asbestos minerals

1.2 Review of epidemiology of asbestos-related disorders

1.3 Review of *in vitro* and *in vivo* experiments on asbestos bioeffects.

1.4 Summary and aims of research

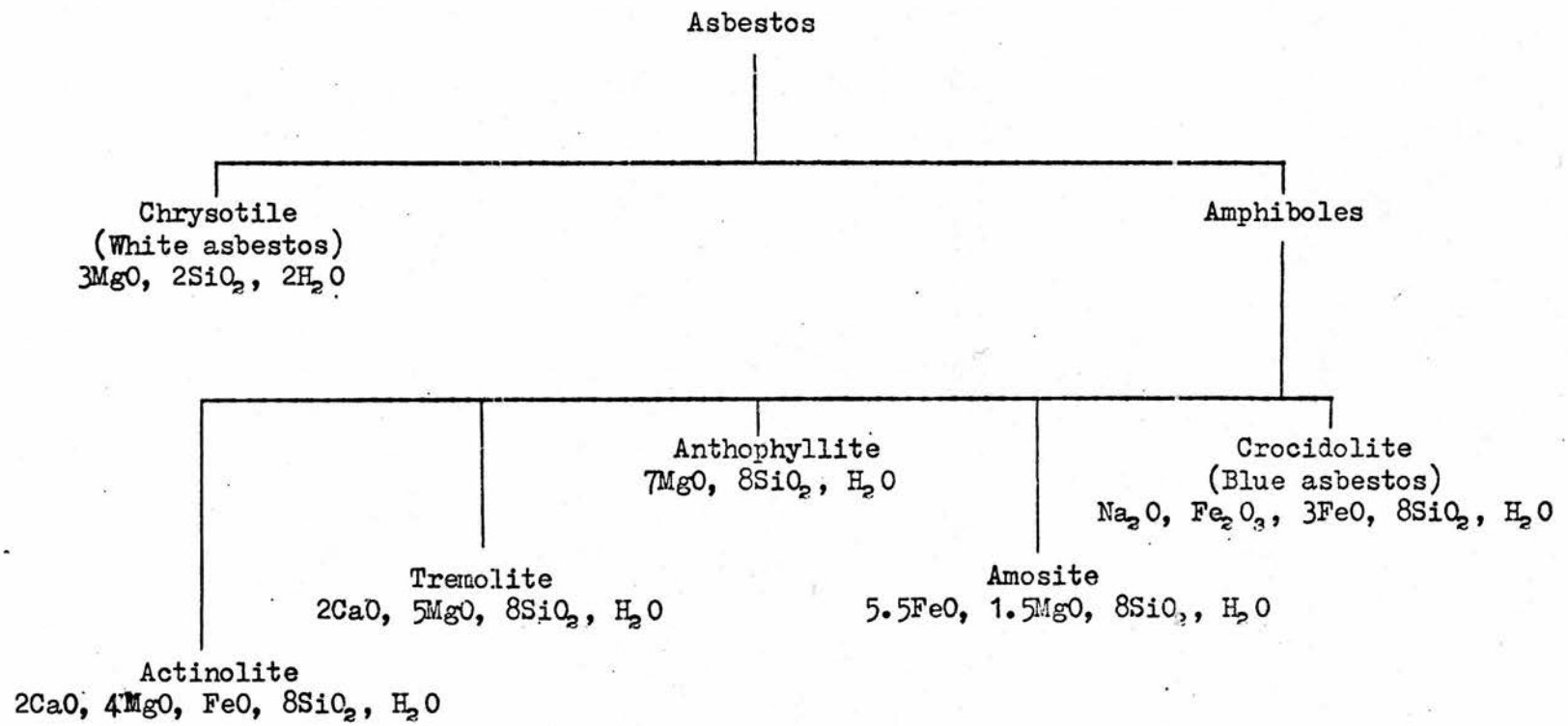
1.1 INTRODUCTION - THE ASBESTOS MINERALS

1.1.1 Definition, Historical Aspects and Occurrence

Asbestos is the generic name given to a class of natural fibrous silicates which vary considerably in their physical and chemical properties. They owe most of their useful and unusual physical and chemical properties to the fact that their individual fibres possess a truly crystalline structure. There are six species of asbestos known, and they can be classified on the basis of origin into two groups, the serpentines and the amphiboles (Fig. 1.1). Chrysotile, or white asbestos, is the only species derived from the serpentine rocks, but it is the most abundant and commercially the most important, accounting for over 90% of world production (Gaze, 1965). The other five species of asbestos are classified in the amphibole group (derived from Greek amphiboles meaning "ambiguous") and are, in descending order of their commercial importance - crocidolite, amosite, anthophyllite, tremolite and actinolite.

The useful qualities of asbestos have been recognised since the third millennium B.C. There is strong archaeological evidence that Stone Age man used the anthophyllite deposits in Finland to produce a primitive form of fireproof pottery (Kiviluoto, 1965). Plutarch refers to asbestos and its uses in his writings in 438 B.C., and the ancient Romans are known to have worked the chrysotile deposits in Italy to make asbestos cremation cloths and lamp wicks. In addition, Marco Polo describes asbestos in Russia during the thirteenth century, although he believed it was made from salamander skin.

FIGURE 1.1 Classification table of main asbestos types.



The discovery of vast deposits of chrysotile asbestos in Canada in the latter half of the nineteenth century, as a result of a forest fire, coincided with the increasing demands of the industrial revolution of that era to provide an efficient means of insulating the steam engine. As a result, the modern phase of asbestos exploitation can be traced to the development of commercial mining concerns in Eastern Quebec in the 1870's and some years later in the Bjanova area of the Ural mountains in Russia (Hodgson, 1965).

Crocidolite was discovered by a German geologist named Lichtenstein, some time between 1803 and 1806, whilst he was travelling near Prieska in the Orange River Valley area of South Africa. However, it was not until 1893 that a subsidiary of the great diamond mining concern De Beers Consolidated Mines was formed to exploit these deposits, and this region remains the major world source. The only known deposits of amosite asbestos were found in 1907 in the Eastern Transvaal (Hodgson, 1965). Mining of these deposits was initiated soon after this by Asbestos Mines of South Africa, and indeed amosite owes its name to the initials of this concern (Deer et al., 1962). Although the anthophyllite deposits in Finland have been worked for many centuries, the first real commercial mining can be considered to have started in 1918 (Hodgson, 1965). The other amphiboles are of little economic importance, but some mining of tremolite occurs in Italy and Pakistan, and a seam of actinolite has been found in the crocidolite mining area of Koegas in Cape Province.

Asbestos probably occurs in nearly every country in the world (Hendry, 1965), including Scotland, and in

addition to the principal areas of production outlined above, there is considerable mining in many other parts of the world (Fig. 1.2).

Finally, as a result of their continuous and interrelated geological nature, asbestiform minerals are found as "contaminants" in the bearer rocks of mines developed primarily for the exploitation of other minerals such as talc and mica (Speil and Leineweber, 1969), iron ore (Nicholson, 1974) and nickel (Wicks, 1971). In fact, Cralley (1968) has noted the presence of fibrous components in over 100 different natural minerals, and a large proportion of the fibres could be identified as asbestos.

1.1.2 Structure and Composition

The structure of asbestiform minerals has been the subject of considerable research over the years. Suffice it to say here that confirmation of the ultrastructure of asbestos fibres had to await the development of refined electron optical techniques. It is now recognised that the basic structural unit is a silicon atom in the centre of a tetrahedral arrangement with oxygen atoms at each of the four corners. These very stable tetrahedra can be linked in different ways by sharing the oxygens at the corners, edges or faces.

Warren and Hering (1941) first showed that the serpentine mineral chrysotile was essentially a layered structure based on a network of silica (SiO_4) units closely joined to a layer of brucite ($Mg(OH)_2$). Later, it was seen that there was a degree of asymmetry in the above structure such that the brucite layer was of slightly larger dimensions than the silica, with this potential source of tension relieved in chrysotile by the adoption of a curved

FIGURE 1.2 Countries with commercially exploited deposits of asbestos.

S. Africa

Rhodesia

Canada

Russia

U.S.A.

China

Australia

Finland

Bolivia

Bulgaria

Italy

Pakistan

Cyprus

morphology. There was some controversy over the extent of the curvature until 1960 when Maser, Rice and Klug were able to show from electron micrographs of transversely sectioned chrysotile fibrils that there was a definite concentric and spiral layering. Chrysotile is thus considered to be of "hollow" cylindrical fibres, with the centre partially occupied by a magnesium silicate gel and with an ultimate fibril diameter of approximately 240 A^{o} (Yada, 1967).

The basic crystal form of the amphibole minerals was first described by Warren in 1929 and his model description still stands today. Thus the amphiboles are described as consisting of pairs of double silica chains separated by a layer of hydrated cations and stacked in an ordered array. The cations must be capable of satisfying the negative charges of the silica chains, and as long as they do this the fibrillar structure of the amphibole asbestos minerals is maintained. The various amphiboles are characterised by the cations which occur in their structure, the principal being Mg^{2+}, Fe^{2+}, Fe^{3+}, Na^{+}, Ca^{2+} but some substitution of other cations such as K^{+} and AL^{3+} can and does occur. The asbestiform amphiboles can thus be regarded as being comprised of silicate fibres with varying amounts of accessory ions adding stability to the structure.

Research into the chemical composition of asbestos has shown that there is considerable variation in the composition of any asbestos type depending upon its geological source. As Hodgson (1965) points out, this picture is further complicated by the rather misleading analyses of amphiboles published before the 1950's due to the fact that they were invariably performed on what is

now known to be weathered and thus atypical deposits. However, by 1961 (Cilliers et al.), analyses of fresh samples of all types of asbestos were available, and since then many workers have published corroborative analyses.

Although bulk asbestos samples can usually be adequately described by optical microscopy methods (Hodgson, 1965; Julian and McCrone, 1970; Champness et al., 1976), the identification of submicrogram quantities of asbestos require the use of sophisticated techniques of electron diffraction (Clark and Ruud, 1974; Langer et al., 1974), X-ray spectrometry (Rickards, 1972; Timbrell, 1970), and analytical electron microscopy (Langer et al., 1974; Rubin and Maggiore, 1974; Champness et al., 1976). The techniques are expensive, time-consuming, and require skilled operation and interpretation of results, but they are the only reliable means of identification. The whole field of instrumental analysis in general has advanced considerably within the past ten years, and, in particular, analytical electron microscopy has benefited from developments in data processing and manipulation. The great advantage of analytical electron microscopy is that individual particles can be isolated and examined in detail, and such detailed analyses are often necessary to unambiguously identify fibres within the asbestiform range of minerals. Precise measurements of this nature are subject to considerable potential errors in interpretation caused, for instance, by alterations in structure and composition as a result of vigorous methods of sample preparation (Chatfield and Glass, 1977). Unequivocal identification of small and chemically labile particles such as asbestos fibres is thus difficult,

particularly where complicated sample preparation is necessary.

As research into the biological effects of asbestos developed it was realised that the compositional variations made it impossible to predict and compare the effects of differences in mineral composition, size distribution, associated organic matter and trace metals. Consequently it was decided at the First International Conference on Asbestos and its Effects held in New York in 1964 that standard reference samples should be prepared under the auspices of the Union Internationale Contre Cancer (UICC) and that these would be available for research workers. These samples, known world-wide as the UICC samples, were duly prepared and described by Timbrell and his co-workers (Timbrell et al., 1968; Timbrell, 1970; Rendall, 1970; Timbrell and Rendall, 1972), and it is these samples that have been used throughout this study, unless otherwise stated. Fig. 1.3 shows the published composition of the UICC samples. A more detailed description of the chemical composition of asbestos is available in Appendix I.

It will be seen later in this review that considerable research effort has been directed towards the significance of the presence of various minor constituents associated with the asbestos minerals to their biological effects. As a result of this, any description of the composition of asbestos must include some mention of the trace metals, organic residues, and sources of contamination. Trace elements of biological significance have been found associated with all types of asbestos, although their levels depend partly on the diligence of the searchers, and partly on the source of the asbestos. Organic residues have also been found, and, in particular, the known potent carcinogen benzo(a)pyrene has

FIGURE 1.3 Compositional analyses of UICC asbestos types.

Component	Percentage composition		
	Amosite	Crocidolite	Chrysotile A
SiO_2	50.8	51.16	39.8
FeO	33.6	21.2	0.4
Fe_2O_3	1.9	18.3	1.5
CaO	0.24	0.17	0.15
MgO	5.0	3.6	26.1
Na_2O	0.1	5.8	0.04

attracted a great deal of interest. Appendix I provides details of the presence of most of these contaminants, and at this point it should merely be said that the level of contamination increases with the degree of handling/processing of asbestos, as might be expected (Pylev and Shabad, 1973).

1.1.3 Properties

A detailed description of the physical and chemical properties of the asbestiform minerals is outwith the scope of this review. This section will restrict itself to a summary of those major properties that earn asbestos the description of "the mineral of unparalleled properties" (Badollet, 1961). Further information pertinent to their properties may be found in Appendix I and in Chapter 3.

Perhaps the most often quoted physical properties of the asbestos minerals are those of heat resistance and high tensile strength. Examination of the effects of heat upon asbestos shows that detectable chemical decomposition of all types of asbestos occurs at temperatures in excess of 500°C, and that a reduction in tensile strength and other properties is found at temperatures as low as 300°C. At first glance this would seem to be at variance with the widely held belief that asbestos is a highly heat-resistant mineral. It is therefore necessary here to emphasise that the heat resistance and insulating properties of asbestos depend more upon the particulate fibrous nature and the poor thermal conductivity of their decomposition products than on the stability of asbestos *per se*.

Over the years, there has been a good

deal of discrepancy between the published values for the tensile strengths of asbestos and the theoretical values computed for stable silicon oxygen bonds. For example Orowan (1948) suggested a theoretical tensile strength of the order of 100,000 kG/cm^2, whereas an acceptable experimental value has been found to be 35,000 (Hodgson, 1965). Hodgson attributes these discrepancies to the fact that there are many randomly positioned flaws in the linear silicon-oxygen structures of geologically occurring fibres, and that these flaws weaken the fibres. The development of techniques for measuring tensile strengths of very small fibres, thereby reducing the number of flaws, has meant that values approaching the theoretical tensile strength have been obtained. The significance of high tensile strengths, together with high modulus of elasticity lies in the fact that the asbestos minerals make perfect reinforcement matrices with a wide range of applications.

An extremely important property of the asbestos minerals is that of their surface reactivity. As might be expected from their differences in structure, the surface characteristics of chrysotile differ markedly from those of the amphiboles. The surface layer of hydroxyl groups on chrysotile confers upon it a very strong hydrophilic surface activity. As a result, it forms strong bonds either by partial solution or by attraction of opposite charges, with a very wide range of substances. This will explain the widespread use of chrysotile as a filter medium, as a stabiliser in many industrial processes, and its importance in providing an intractable reinforcement component in resins. One problem imposed by the hydrophilic nature of chrysotile in its use in

the cement industry is that it tends to drastically reduce the drainage properties, thus adversely affecting the setting time of the product. However, the amphiboles have a surface layer of hydrophobic silica sites renowned for their insolubility and thus their incorporation in a blend of asbestos cement products ensures the porosity and commercial acceptibility. The hydrophobic nature of the surface of the amphiboles can also be considered to contribute to their relative chemical inactivity.

The reactivity of asbestos minerals with acids and alkalis is well documented and has been described in detail by Badollet (1963). Once again there is a gradation in properties across the types of asbestos, with chrysotile being the most sensitive, and crocidolite the most resistant. This property of high acid resistance and generally low chemical reactivity of the amphibole minerals has made them eminently suitable for use in the marine environment and they are used widely in this context.

Finally, any review of the properties of asbestos must mention the fibrous nature of this group of minerals, since it is primarily this characteristic that both identifies them and distinguishes between them. The fact that all the properties outlined above are combined with a fibrous texture means that the asbestos can truly earn the title of "the Twentieth Century Mineral" (Gilson, 1965).

1.1.4 Uses

Fig. 1.4 shows the uses of asbestos in summary form. The major use of asbestos is in the manufacture of asbestos-cement products where it acts primarily as a support

FIGURE 1.4 Major uses of asbestos.

Asbestos cement products.

Asbestos paper, gaskets, linings, seals etc.

Friction lining - brakes and clutches.

Asbestos textile products.

Thermal insulation products.

Filtration materials.

reinforcement for the cement. Chrysotile is the most commonly used, making up about 10% of the finished product, but sometimes up to 40% of the amphibole is incorporated (Hodgson, 1965). In addition, the insulating properties of asbestos play a significant part in its use with cement in the manufacture of a wide range of insulating boards and panels.

Largely as a result of their large bond strengths, the next most important use of asbestos is as a reinforcement matrix in conjunction with organic resins in products such as vinyl floor tiles, prefabricated panels, etc. The additional frictional properties of chrysotile have meant that it can be considered an indispensable constituent of automotive brake and clutch linings.

The larger grades of asbestos are extremely useful in the manufacture of fireproof textiles such as clothing, safety curtains and conveyor belting. Amphiboles have a further important application in this area since their chemical resistance makes them ideal for chemical filters, etc. Resistance to attack by sea-water and a low thermal conductivity, were the main reasons why crocidolite asbestos was used very extensively for the fireproofing of naval vessels during and after World War II.

The suitability of asbestos as a filter material could be considered to be combined result of the fibrous particle nature, the chemical resistance, and the strong non-polar adhesive forces of the minerals. Until recently (1973) both the pharmaceutical and food industries used asbestos filtration extensively in the manufacture of many parenteral drugs and a wide variety of beverages.

It can be seen from the many diverse applications of asbestos minerals that they have assumed a role of considerable importance in modern technology. Were it not for the fact that they have also assumed an important role as a source of human disease, their widespread use would undoubtedly have continued unabated. It will be seen later in this review that one of the main reasons why asbestos can be a health risk is due directly to its ability to gain access to the body in a particulate form. It is therefore unfortunate that the very factors that endow the asbestos minerals with their many indispensable properties (Rosato, 1959) also result in their marked ability to fragment and produce a dust cloud. Further, the fact that the dust produced often contains a large proportion of particles within the respirable size range, and that these particles are relatively recalcitrant once they have gained access to the body, has meant that the asbestos minerals have become one of the major documented causes of occupational disease. It is only within the past few years that estimates of the populations at risk have revealed the true extent of the problem. One of the reasons for this has been a failure to appreciate the diversity of occupations incidentally exposed to asbestos fibre (Becklake, 1976). Once incorporated into manufactured products, asbestos fibre is relatively well bound for the duration of the workable life of the product (with some exceptions) and is therefore less likely to prove a health hazard. However, asbestos fibre may well be released as soon as the product is altered, removed, replaced, or destroyed. Fig. 1.5 shows a list of the populations of the workers now known to be at risk from asbestos, and it can be seen that this list includes

FIGURE 1.5 Populations of workers at risk from occupational asbestos exposure.

Miners

Millers

Textile workers

Dockyard workers

Insulators

Fitters

Welders

Plumbers

Electricians

Decorators

Mechanics

Demolition workers

Casual labourers

such people as welders, electricians, demolition workers and others who have received incidental but significant exposures. There is a lack of reliable data on the actual numbers of workers at risk in the U.K., or indeed the world, but an estimate for the United States in 1972 was 250,000 (D.H.E.W., 1972) and this is in a country where little actual mining of asbestos occurs.

1.2 REVIEW OF THE EPIDEMIOLOGY OF ASBESTOS-RELATED DISORDERS

1.2.1 Introduction

As production and use of asbestos increased the exposure of large numbers of workers to asbestos dust also occurred, and it was not until comparatively recent times that regulations were introduced to control the levels of dust in the working environment. In addition, there has been an increasing concern in recent years over the levels of asbestos contamination in the general urban environment, and the possible effects of this on the health of the population as a whole. However, it is important to keep the potential hazards associated with asbestos in perspective, and to this end, a review of the epidemiological evidence seems appropriate. Fig. 1.6 lists the range of pathological effects frequently associated with human exposure to asbestos. As indicated, the inclusion of some of the effects in a table of this type can only be tentative with the present state of knowledge. A fuller discussion of the position will be found under the relevant sections below.

It is not surprising that the dominant human response to an airborne particulate pollutant such as asbestos should be associated with the respiratory surfaces and it is with these that the review starts.

FIGURE 1.6 Pathological effects of asbestos exposure in humans.

Site	Effect	Association with exposure
Skin	Asbestos corns	Established
Larynx	Carcinoma	Possible
Lungs	Ferruginous bodies	Established
	Asbestosis	Established
	Carcinoma	Established
Pleura	Plaques and calcification	Established
	Effusion	Possible
	Malignant mesothelioma	Established*
Peritoneum	Malignant mesothelioma	Established*
Gastrointestinal tract	Neoplasia	Established*
Ovary	Carcinoma	Unlikely
Breast	Carcinoma	Unlikely
R.E. system	Neoplasia	Unlikely

*Association, but not cause, established.

(After Becklake, 1976)

1.2.2 Asbestosis

Asbestosis can be defined as a progressive diffuse interstitial pulmonary fibrosis with or without fibrosis of the visceral and parietal pleura. The first cases of diffuse pulmonary fibrosis after asbestos exposure were described by Murray in 1907, but it was not until 1927 that Cooke introduced the term asbestosis to describe this form of pneumoconiosis. Cooke showed that asbestosis was the major occupational hazard in the asbestos textile industry (the major asbestos industry of the time) and pointed out that as an occupational disease, it could be prevented by the appropriate controls. As recently as 1965, however, a report showed that the incidence of asbestosis in Britain was on the increase, this increase probably reflecting the increased utilisation of asbestos over the preceding 20 years (Harington et al., 1975).

It has been shown in the many epidemiological reports published since the first international conference on asbestos in 1964 that there is a strong dose response associated with asbestosis, with the disease being confined to those industries in which asbestos is extensively used. It would appear that all forms of asbestos fibre are capable of inducing pulmonary fibrosis to some extent, but the degree of involvement of the specific types has been the subject of a great deal of research. Documentation of the type and severity of exposure has been poor in most cases. Smither and Lewinsohn (1973) showed that of 71 papers on asbestos epidemiology reviewed by them between 1964 and 1970, one half were specific about occupation, the other half referring to their cohorts as "asbestos workers". Type of fibre was specified in

one third of the papers, and actual dust dosage in only 14%. Appendix II contains a summary of some of the reports of human fibrosis, together with details of geographical location and type of fibre, where possible. The position has been complicated by the fact that most workers have been exposed to a mixture of dusts in their occupational lifetime, and in this situation no clear indication of pathogenicity can be ascribed to any specific fibre type. However, using strictly defined parameters for describing populations it has been possible to specifically implicate an asbestos type. For example, McDonald (1973) found asbestosis amongst chrysotile miners, and similarly Meurman and Kiviluoto (1968) showed that anthophyllite can produce asbestosis.

There is strong evidence that cigarette smoking increases the risk of death from asbestosis and its complications (Hammond and Selikoff, 1972) although the synergistic effect is not as dramatic as that with asbestosis and bronchogenic cancer (q.v.). A strong dose response relationship has been shown to exist between asbestosis and dust exposure (Bader et al., 1970; Meurman et al., 1974; McDonald et al., 1974), although the fact that asbestosis may progress even after removal from dust exposure (Becklake, 1976) complicates attempts to establish a safe threshold value for asbestos dusts.

The regulations governing the use of asbestos in the working environment were specifically set to reduce the incidence of asbestosis to an "acceptable" level (less than 1% of exposed persons), and no regulations as yet in force in factories take account of the carcinogenic risks of asbestos. This came about

as a result of the fact that, historically, asbestosis has been the most disabling disease associated with asbestos exposure, and the first to appear, and impetus for its control came from the needs of compensation.

Despite the introduction of asbestos regulations in 1931, the incidence of asbestosis continued to rise in the U.K., and the disease was also found in occupational groups not covered by the regulations. As a consequence of this, new regulations governing the handling and processing of asbestos were introduced in 1969 using the rather scanty data of the British Occupational Hygiene Society (B.O.H.S., 1968, Annals Occup. Hyg. 11, pp. 47 - 69) to set the threshold limit values. The legislation relies upon assaying the fibre levels under specified conditions and expressing these in terms of fibres per cc of air sampled (not more than 0.2 fibres/cc for crocidolite, and not more than 2.0 fibres/cc for the other asbestos types). The definition of a fibre was restricted to cover "particles of length between 5 and 100 microns and having a length to breadth (aspect) ratio of at least 3:1" so that they were within the detection limits of the optical microscope. Although the optical microscope is of limited value in the measurement of the absolute number of asbestos fibres in a given sample (since a large proportion by number are of submicroscopic dimensions), it has been widely accepted as the only feasible method of assay currently available. The high costs of alternatives are likely to ensure the continued use of the light microscope for routine measurements of dustiness, but criticism in recent years has led to the adoption by some workers of the more accurate electron microscopy techniques for assays of environmental levels of asbestos pollution.

1.2.3 Asbestos bodies

Marchand (1906) was the first to describe "peculiar pigment crystals" in human lungs. Fahr and Feigel (1914) also described strange crystals in the lungs of an asbestosis case. In 1924 Cooke described"curious bodies" in an asbestos worker, and he thought them to be of fungal or vegetable origin (Cooke, 1924). He was unaware of the earlier description of Marchand. Stewart (1928) introduced the term "asbestosis bodies" when he noticed that the brown bodies were always associated with asbestosis. Soon after this, Gloyne (1929) found that they could be found in lungs without asbestosis, and he proprosed that the name be shortened to "asbestos bodies". He followed this with the observation that each body contained a central fibre (Gloyne, 1932). Many years later Schepers and Durken (1955) found these bodies in talc workers, and Gough (1965) preferred to call them "mineral fibre bodies" because he had found them in lungs with no known asbestos exposure. Finally, Gross (1968) proposed that the bodies be known as "ferruginous bodies", in line with the finding that they contained iron.

The interest and concern over the effects of asbestos exposure was reaching a peak in the late 1960's, and there were several published reports of the finding of ferruginous bodies amongst members of the general population (Thomson et al., 1963; Utidjian et al., 1968; Bignon et al., 1970). In a critical review, Wright (1969) points out that the presence of ferruginous bodies does not necessarily imply a history of asbestos exposure unless the core fibres are expressly identified as asbestos,

and he further criticises the "sweeping inferences" made in some papers about the presence of bodies in the general population.

Despite this, it is reasonable to use the presence of ferruginous bodies as an indication of exposure to mineral fibres. There is both a proven rural-urban gradient (Oldham, 1972) and a distinct age relationship (Doniach et al., 1975) in their incidence, even though the ratio of uncoated to coated fibres may be considerably higher than 10:1. Nowadays it is widely believed that the biological reaction leading to ferruginous body formation is aside from the main pathological effects of asbestos.

1.2.4 Pleural plaques and effusion

Some authors choose to describe these pleural reactions as different manifestations of the same syndrome, but an examination of the course and status of the lesions suggests there are basic differences (Becklake, 1976). Pleural plaques occur as discrete raised grey-white lesions on the parietal pleura of the rib-cage and diaphragm. In advanced cases, they may also be found on the pericardial surface (Meurman, 1966). Histologically, the plaques consist of collagenous connective tissue with few cells amongst masses of undulating collagen fibres. Dystrophic calcium deposition in the form of granules is a common feature particularly in relation to the anterolateral portion of the upper ribs (Meurman, 1966). Cuboidal mesothelial cells may occur at the edges of the plaques, and they are occasionally metaplastic (Lewinsohn, 1974). Meurman (1966) suggests that the plaques arise sub-pleurally between the mesothelial covering layer and the normal connective tissue. The pathogenesis of pleural plaques remains obscure, largely as a result of several contradictory findings. Fibres, but not bodies, have only recently been found in

association with the lesions, and then only with the aid of the electron microscope (Le Bouffant, 1972). Interestingly, they seem to be commoner amongst zones of calcification. Plaques are associated with little or no respiratory symptoms and indeed are usually present as an incidental radiological finding. There would appear to be some dose responsiveness, in that they are commoner with the higher estimated doses of asbestos, but they may be present in association with lungs which show little or no reaction to inhaled dust (Becklake, 1976).

"Idiopathic" pleural effusion has recently been described in association with asbestos (Chahinian, 1973) as a benign lesion to distinguish it from the effusions due to malignancy. Nyiredy (1975) describes the recent literature. The situation is complicated by the fact that recurrent pleural effusions may precede by months or even years the appearance of malignant mesothelioma of the pleura. Diagnosis of benign pleural effusion should therefore only be made by exclusion, and Gaensler and Kaplan (1971) further point out that the suspected association with asbestos need not have been recent or prolonged.

1.2.5 Bronchogenic cancer

In 1933 Gloyne and later Lynch and Smith (1935) independently reported a suspicion that there was an association with asbestosis and lung cancer. Merewether (1949) reported 31 cases of carcinoma in 235 asbestosis deaths between 1924-46. Epidemiological proof was to come in 1955 from Doll, and again from Knox in 1968, when it was shown that there was a tenfold risk of developing lung cancer amongst men employed in the industry before 1930.

So, by the 1950's, the association between lung cancer and asbestosis was shown to be very strong, with 50% of asbestotics dying of lung cancer, according to some estimates (Buchanan, 1965, and Selikoff, 1976). It has since been found that cigarette smoking is an important co-factor. Selikoff (1968, 1972) has shown that asbestos workers who were regular smokers had eight times the risk of dying of lung cancer than men who smoked but did not work with asbestos. This synergistic effect of cigarette smoking has been found to be multiplicative rather than additive, suggesting that there is a complicated etiology.

It is accepted nowadays that there is a strong dose response relationship between asbestos exposure and lung cancer (Enterline et al., 1972; Wright, 1969). This belief is supported by the finding by some authors that severely exposed persons who, also, by implication, have asbestosis, have a very high risk of dying of lung cancer, whereas low-to-modest exposured persons showed no significant increase in lung cancer (Berry et al., 1972). It follows from the dose response thesis that the problems associated with both lung cancer and asbestosis should diminish as the control measures for ventilation and worker protection take effect. The epidemiological literature shows that deaths from lung cancer and asbestosis reflect the conditions prevalent approximately 20 years previously. In theory the U.K. factory regulations have been operative for over 40 years (see Smither and Lewinsohn, 1972) but it is widely accepted that their meaningful enforcement has only occurred within the last 20 years. The need for control of dust levels in peripheral occupations (see Figure 1.5), where the peak dosage may be

many orders of magnitude higher than the permissible factory values (Harries, 1968), has only been appreciated comparatively recently (McEwen et al., 1970, and McDonald et al., 1970). In conclusion, it is reasonable to assume that the steady decline in both asbestosis and complicating lung cancer in British asbestos workers over the past few years will continue as the full hygiene standards take effect.

1.2.6 Mesothelioma

The fact that the definition of diffuse malignant mesothelial tumours has been the source of considerable controversy over many years emphasises their diversity. It is not proposed to review the history of the controversy here, but merely to point out some of the salient features of mesothelial tumours (see Davis, 1974, for further details). Mesotheliomas characteristically show great variation in structure, and may range histologically between dull connective tissue and full epithelial tissue.

Conventional pathology textbooks (e.g. Surgical Pathology, Ackerman and Rosai, 1974) tend to allocate mesothelial tumours into four clinico-pathologically distinct varieties: benign and malignant fibrous mesotheliomas, and benign and malignant epithelial mesotheliomas. This rather inflexible classification of mesothelial tumours is inadequate since it does not allow sufficiently for the co-existence of both fibrous and epithelial elements within the same tumour. Such mixed mesothelial tumours are widely acknowledged to occur. The only safe generalisation concerning the histological classification of mesothelial tumours is that

epithelial cell types predominate in peritoneal mesotheliomas.

Experimental studies show that the structural diversity is likely to be a result of the fact that mesotheliomas originate from the undifferentiated pluripotent primitive mesenchymal cells (Davis, 1974). The failure of early tumour classification systems to adequately categorise mesothelial tumours led some authorities to discount their very existence. Willis (1953) considered that all tumours of the body cavities were actually metastases arising from hitherto undiscovered primary cancers. It is worth emphasising here that any reports of the incidence of mesothelial tumours in humans based upon the results of needle biopsies should be interpreted with caution.

Pleural and peritoneal tumours associated with asbestos were described briefly in case reports (Wedler, 1943a, b, and Wyers, 1946) but it is generally considered that the association between mesotheliomas and asbestos was as a result of a publication by Wagner and his colleagues in 1960 (Wagner et al., 1960). They described 33 cases of mesothelioma arising in a crocidolite mining area of South Africa, in some of which the exposure could be considered to be incidental or environmental. By 1963 Wagner had collected 120 confirmed cases of mesothelioma of the pleura and also found primary peritoneal cases. Once again a large proportion of the cases had been exposed to crocidolite in the Cape Province area of South Africa, and more than half had no occupational exposure. By 1973, 360 pleural mesotheliomas had been found in South Africa (Webster, 1973).

Following the reports of Wagner et al.

in 1963, other studies confirmed that there was a greater risk associated with Cape crocidolite when compared with nearby Transvaal crocidolite (Oettlé, 1964) and that the differences were not attributable to differences in production or processing (Harington, 1967; Sluis-Cremer and Du Toit, 1973). Timbrell developed the concept of the importance of fibre geometry in a series of papers (1965; et al., 1968; et al., 1970a, b; et al., 1971; 1972) in which he described the parameters necessary for deep penetration and retention within the lung, and the characteristics of many types of asbestos. In 1971 he and his colleagues showed that differences in fibre geometry could explain the clear differences between the pathogenicity of Cape and Transvaal amphiboles, with the more active Cape crocidolite producing approximately 30 times more fibres per mass than Transvaal crocidolite. Despite the fact that the Transvaal amphiboles seem to be less carcinogenic in South Africa, there is ample evidence that exposure to amosite, which is mined exclusively in Transvaal, can lead to malignancy in other countries. Selikoff et al. (1973) have found both bronchogenic cancer and mesothelioma in considerable excess in a group of workmen exposed to amosite in the United States.

Chrysotile is also implicated in the production of mesotheliomas. Enticknap and Smither (1964); Elwood and Cochrane (1964); Selikoff et al. (1965) and Mancuso and El Attar (1968) all found mesotheliomas in chrysotile-exposed workers. Only anthophyllite would seem to be innocent of mesothelioma production in humans, despite intensive searches (Kiviluoto and Meurman, 1970).

Two important features have emerged

from the epidemiology of mesothelioma: one is that there would appear to be a long latent period between first exposure and malignancy (commonly regarded as 30 years or more); and the other is that the evidence suggests that the exposure may have been very low in some cases. In particular, the implication that small doses of asbestos may lead to the development of mesothelioma resulted in speculation that a large number of people may be at risk, and that the meteoric rise in the widespread use of asbestos may be mimicked by a similar meteoric rise in the incidence of mesothelioma 30 years later (Selikoff, 1975). Some support for this thesis was presented in the British Medical Journal (Anon, 1976) when the annual incidence of mesotheliomas reported to the U.K. Pneumoconiosis Panel was shown to be rising steadily. Taken out of perspective, it has been suggested that the minute traces of asbestos in the urban environment, in food and beverages, and in drugs, may lead to the development of cancer (Thomson et al., 1963). However, detailed epidemiological and pathological investigations are needed before this can be confirmed or denied (Lewinsohn, 1974).

It is worth noting here that peritoneal mesotheliomas are not as frequent as pleural, accounting for 24 out of 474 in one major study (Webster, 1974), and that there would seem to be greater potential for diagnostic confusion with the peritoneal tumours. The problem of accurate identification of the histologically diverse mesothelioma has been mentioned previously, and some authors have expressed the view that the annual increase in the incidence of mesotheliomas could in part be explained by the recent focussing of the attention of pathologists. It may well be that

misdiagnosis has been the main reason for the paucity of mesotheliomas in humans in the past, but it is unreasonable to explain the recent inexorable rises in these terms.

Another controversial aspect of the mesothelioma problem rests upon reports that there are cases with no known significant association with asbestos. Some of the more sensational interpretations regard this situation as a direct result of incomplete or inadequate occupational and environmental history. Persistent questioning will usually reveal some evidence of slight incidental exposure to asbestos amongst urban dwellers (Anon, 1976). However, it is surely illogical to implicate asbestos without unequivocal proof. Finally, there is some circumstantial evidence accumulating that there may be a dose response effect between asbestos exposure and the development of mesothelioma in humans (Newhouse, 1973).

1.2.7 Gastrointestinal cancers

Konig (1960) was the first to report an increase in the incidence of gastrointestinal (G.I.) cancer amongst asbestos workers. Selikoff et al. (1964) reported a threefold increase in G.I. cancers in a cohort of 1522 asbestos workers, and this was followed by accounts of similar findings from other epidemiological surveys including those undertaken by Elmes and Simpson (1971); Enterline et al. (1972); Vigliani et al. (1973); McDonald (1973); Selikoff et al. (1973), and Mancuso and El Attar (1973).

The significance of the mucociliary pulmonary clearance mechanism as the major source of asbestos within the G.I. tract has been appreciated for some time, although estimates

of the extent of the bronchial clearance component vary from 73% (Evans et al., 1973) to 98-99% (Gross et al., 1974) of all deposited particles, depending upon the exposure level. It follows that any observed increase in G.I. cancer amongst exposed persons could be attributable to penetration of the gut mucosa by ingested asbestos.

It is not yet widely accepted that asbestos exposure results in an increased incidence of G.I. cancers, and indeed some epidemiological studies have reported no significant increase in these neoplasms (Newhouse, 1973; Kleinfeld et al., 1967; Harries, 1971). The evidence that is available would suggest that the risk of gut cancers is only significant in the moderately and severely exposed occupational groups, although the absolute numbers are small (Schneiderman, 1974). In parallel with the development of public interest in the problems of mesothelioma, there has been considerable interest in the potential hazards associated with asbestos ingestion. This subject will be dealt with more fully in the Discussion (see Chapter 9), but uninformed sources have claimed that the noted world-wide increased incidence of gut cancer with increasing urbanisation and civilisation could be explained in terms of exposure to what might be termed "environmental" asbestos (i.e. that arising from a generalised low level of pollution associated with modern society's use of asbestos). Further, other workers would seek to explain the recorded very high incidence of stomach cancer in Japan in terms of the incidental ingestion of traces of tremolite asbestos present as a minor contaminant in the small quantities of talc used to "polish" rice (Merliss, 1971).

The concern over the potential effects

of ingested asbestos has grown out of proportion over the past few years, largely as a result of the discovery of large-scale amphibole contamination of Lake Superior in Canada in early 1973. The contamination arose as a result of the practice of dumping the waste from a taconite iron ore mining complex into the lake. These wastes consisted of rocks of the cummingtonite and grunerite series, and they have been shown to contain large amounts of mineral fibres, including some amosite asbestos. Several conurbations rely on Lake Superior for their water supplies, and fairly high levels of asbestos contamination have been found in the potable water supplies of the city of Duluth, Minnesota (Nicholson, 1974). A number of studies have been commissioned recently to investigate the many aspects of this problem of the effects of ingestion of asbestos. One of these studies suggests that there is no excess of G.I. cancer amongst the residents of Duluth when compared with those of Minneapolis, a city of similar size but with no known environmental asbestos hazard (Levy et al., 1974).

The question of the relevance of an environmental asbestos hazard has received a good deal of attention in recent years, and characterisation of the extent of any such hazard is a vital prerequisite for an answer. Lee (1974) considers that the finding by Spiel (1974) of large numbers of tiny chrysotile fibres in almost all the water supplies that he analysed from many parts of the United States emphasises that there is an inherent background level of naturally occurring asbestos. Of interest in this respect is the surprising finding that water from a river running through serpentine rocks contained no more chrysotile than water from a source not so

exposed (Spiel, 1974). A paper by Webster (1974) suggests that the possible level of exposure from the erosion of asbestos cement supply pipes is likely to be vanishingly small, although he did find some evidence that acidic sewage caused some mild pipe damage. Selikoff (1974) has suggested that crocidolite used in the asbestos cement pipes might be used as a marker for damage, since this does not occur naturally in U.S. aquifers.

Other reports detail the levels of fibre contamination of beverages (Biles and Emerson, 1968; Cunningham and Pontefract, 1971), foods (Fed. Register 38 (188) p.27076, 1973), parenteral drugs (Nicholson et al., 1972) and in the general urban environment (Thomson et al., 1963). An indication that background levels of ingested asbestos are not responsible for G.I. cancer in the general population might be inferred from the fact that the geographical incidence of colonic cancer in the United States does not correspond with the areas of apparent exposure of the general population.

Studies of the effects of ingestion of asbestos in animals form a large part of this thesis, and the literature will be reviewed in Section 1.3.3e.

1.2.8 Other cancers

Asbestos has been implicated in association with several other forms of cancer, although in most cases the association is considered tenuous or unproven. The tumours include carcinoma of the larynx (Stell, 1973; Newhouse, 1973; Libshitz, 1974; Selikoff, 1974), of the ovary (Graham, 1967) and of the breast (Doniach et al., 1975), lymphomas and leukaemias (Parkes, 1973;

Selikoff, 1974), and kidney, pancreas and brain (Selikoff, 1974). Of these, carcinoma of the larynx would seem to be most strongly implicated.

It is worth mentioning here that clarification of the role of asbestos in many of the above neoplasms must await the results of very large epidemiological investigations. The fact that they remain the centre of controversy despite the many epidemiological studies of heavily exposed persons suggests to me that their role within the spectrum of the pathological effects of asbestos is likely to be a very minor one. A closer look at some of the reports may reveal sources of misdiagnosis: Selikoff (1974) mentions that "pancreatic carcinoma" was often found to be peritoneal mesothelioma or G.I. cancer in their studies; and McCaughey (1958) pointed to the histological similarity between ovarian tumours and peritoneal mesotheliomas.

Obviously, any potential carcinogen, given access to vulnerable tissues, represents a threat, and asbestos is strongly indicated as a carcinogen in several sites in man. The presence of particles of asbestos in any tissue must be taken as a serious indication of potential malignancy. There is some evidence of widespread dissemination of asbestos within the tissues of heavily exposed humans with and without tumours (Godwin and Jagatic, 1970). Animal experiments investigating the question of the transport of asbestos are reviewed in the section on the <u>in vivo</u> effects of asbestos, and receive a good deal of attention in this thesis (see Section 1.3.3d).

1.3 REVIEW OF IN VIVO AND IN VITRO EFFECTS OF ASBESTOS

1.3.1 Introduction

There has been an enormous amount of experimental work into the biological properties of asbestiform minerals over the past 35 years, probably more than any other environmental and industrial contaminant. It is not surprising, therefore, that the copious literature on asbestos bioeffects contains many contradictions and controversies. It should be remembered that variables including type of animal, character of dose, route of administration, and the need for long-term survival of animals must be fully appreciated before any generalisations can be made in toxicological studies. It is not proposed to deal with the minutae of all animal experimental work associated with asbestos in this review, but to outline the trends that are emerging, mentioning in detail those aspects specifically relevant to the effects of transport and ingestion of asbestos, that were available up until the early 1970's.

The significance of both transport and ingestion in asbestos pathogenicity has only been considered in depth comparatively recently, since the majority of work has been naturally concentrated on investigations into the mechanisms of the major effects noted in humans. As a result, evidence for transport of asbestos is often circumstantial, the findings being an aside from the main purpose of study, frequently not reported in detail, but significant when considered in perspective with other studies. A general review of asbestos bioeffects therefore seems to be appropriate.

The literature can be broadly

classified into those papers dealing with investigations into the character of the fibrogenic and associated responses, and those dealing with carcinogenicity. Nowadays, the trend is towards investigations into the carcinogenicity of asbestos, this being of major concern now that control of asbestosis by legislation of dust levels is becoming a reality.

1.3.2 Fibrogenic and associated responses

Asbestosis is essentially a fibrogenic response of the lung to retained asbestos dust. There are proven large differences in physical and chemical properties of asbestos minerals and it is naturally important to find out if the different types of asbestos possess different degrees of fibrogenicity. Epidemiological evidence has shown that it is often difficult to unequivocally implicate one form of asbestos since most workers have been exposed to a mixture of dusts. As a result of this, both *in vitro* and *in vivo* experiments have been directed towards examining the fibrogenic response in detail.

1.3.2 a In vitro studies

Two main *in vitro* models have been used for a number of years to examine the biological reactions of asbestos: haemolysis and cytoxicity. The basic assumption behind the use of these models as bioassays has been that the haemolytic and cytotoxic activity of a given dust can be used as a guide to their fibrogenicity. Marks (1957), Allison (1971) and many others, have found a parallelism between cytotoxicity and fibrogenicity in animals, and Koshi *et al.* (1968) and Harington *et al.* (1971) have demonstrated correlations between the haemolytic and cytotoxic activity of a wide range of

mineral fibres. Some also suggest that there is direct correlation between *in vitro* haemolysis and lung fibrosis *in vivo* (Hefner and Gehring, 1975). However, this has been challenged (see Harington *et al.*, 1975), and it would seem that considerable caution is necessary in any direct extrapolations from the cellular to the organism level. Although all cytotoxic agents would seem to be fibrogenic, not all fibrogenic agents are cytotoxic. Despite these limitations, haemolysis provides a simple and rapid way of studying the effects of particles on biological membranes, and cytotoxicity a useful way of examining the sequels to any membrane effects (Harington *et al.*, 1975).

Haemolysis. The haemolytic activity of asbestos was first reported by MacNab and Harington (1967) and it was soon confirmed by many workers that the activity of some types of asbestos was similar in type to that of silica, with at least two mechanisms of action (Harington *et al.*, 1971). First, there is what has come to be regarded as the direct haemolysis caused by contact of particulates with erythrocyte membranes, and then there is indirect haemolysis in which silicates sensitise erythrocyte membranes to lysis by complement.

There are large differences in the extent and the rate of the haemolytic activity within the asbestos mineral groups: chrysotile has a marked haemolytic capability whereas crocidolite, amosite and anthophyllite have been shown to be less haemolytic (Secchi *et al.*, 1968; Schlipköter, 1968; Schnitzer and Pundsack, 1970). Further, it would seem that chrysotile haemolysis involves both direct and indirect mechanisms, and that

amphibole haemolysis relies more upon the indirect mode of action. Some authors would explain these differences in terms of the nature of the surface of chrysotile and its more immediate influence on membrane integrity (Harington, 1976). However, there are other theories to explain the differences, notably, variations in the solubility of constituents, magnesium ion concentration, iron concentration, surface area and particle number. Further work is necessary to resolve these problems and, in particular, a rigidly standardised technique is vital: contradictions may arise as a result of inadvertent residual serum contamination in washed erythrocyte preparations, or as a result of using different sources of erythrocytes, etc. (Harington, 1972).

Cytotoxicity. The main reaction of fibres *in vivo* is with macrophages (Harington *et al.*, 1975) and so most *in vitro* studies have utilised macrophage cultures maintained in appropriate media, usually with the addition of decomplemented serum. Toxicity can be measured as a loss of viability, either by using one of the dye exclusion techniques or by the assay of certain biochemical parameters. Despite differences in technique, source and type of cells, and dusts used (Robock and Klosterkötter, 1971; Harington, 1971), most workers are in agreement that chrysotile asbestos is more cytotoxic than the amphiboles (Koshi *et al.*, 1968; Beck *et al.*, 1971; Bey and Harington, 1971; Allison, 1972; Miller and Harington, 1972, and Robock and Klosterkötter, 1973). However, some authors (Parazzi *et al.*, 1968) found crocidolite to be the most cytotoxic, although the same group later considered that none of the asbestos dusts were toxic (Pernis and Castano, 1971).

It would seem that two types of cytotoxicity can be distinguished, an early form (within 1 hour) resulting from plasma membrane damage, and a delayed form apparently related to phagolysosome damage and its consequences (Allison, 1973; Harington et al., 1975). It is now widely accepted that chrysotile cytotoxicity exhibits both early and late forms, whereas any activity of the amphiboles would seem to be due to delayed cytotoxicity.

Since the ability of a given dust to damage or kill cells in vitro does not provide an insight into the mechanism of fibrogenicity, a number of workers have investigated the consequences of cells/particle interactions in vitro. The finding that macrophages seldom, if ever, differentiate into the collagen differentiating fibroblasts (Ross et al., 1970) resulted in speculation that fibrogenesis takes place by a two-stage mechanism. Support for this thesis was already available in the work of Heppleston and Styles (1967) who showed that mouse macrophages exposed to silica released a factor into the surrounding medium that was capable of stimulating collagen synthesis in chicken fibroblasts. Although other workers have had difficulty in confirming this phenomenon (Harington et al., 1973), Heppleston has studied the release of the stimulating factor in some detail (1969, 1970). It would appear that the factor depends upon the stimulation of dividing fibroblasts (Nourse et al., 1973), and that inhibition occurs if non-dividing fibroblasts are used.

In recent years some workers have investigated the rather longer term effects of asbestos on cells in vitro, and some of this work would indicate that asbestos has mutagenic

properties (Sincock and Seabright, 1975; Lavappa et al., 1975) or the ability to alter nucleic acid synthesis (Richards and Morris, 1973; Richards et al., 1977). These findings require corroboration, but they may ultimately provide an insight into the role of asbestos as a carcinogen.

There remains an urgent need for comprehensive studies into the relationship of fibre geometry to the differential cytotoxicities of the asbestos minerals. To date, these have not been forthcoming owing to the practical difficulties of producing clearly delineated fibre size fractions. However, the fact that cytotoxicity assays require only very small amounts of fibre will prove to be a great asset should such samples become available.

1.3.2 b In vivo studies

Asbestos has been administered by inhalation, and by intratracheal, intrapleural, and intraperitoneal injection into rabbits, rats, mice and guinea pigs and its fibrogenicity recorded. These many differences in technique have resulted in a certain amount of confusion about the fibrogenicity of mineral fibres over the past 50 years' experimentation. Despite this, some trends have emerged in recent years.

The first reported experiments used the intratracheal route, presumably since this was both technically simpler and required less fibre. Although the technique has obvious criticisms, it nevertheless remains a simple and effective bioassay that has since been used by many workers. Gardner (1931) reports the results of the first intratracheal experiments as part of a larger

study. The whole research programme spanning 25 years was described by Vorwald and his colleagues in 1951. They reported that when chrysotile fibres cut on a special microtome at lengths of 15 μ and 2.5 μ were injected intratracheally into rabbits, the larger sample produced significantly more fibrosis. King et al. (1946) administered long and short samples of chrysotile by intratracheal injections into groups of rabbits, and reported a generalised interstitial fibrosis with the short fibre group, and "silicotic-like" nodular lesions with long fibres. However, they soon reported that they could not produce the "silicotic"lesions in rats (Smith et al., 1951). Many other workers have since described similar studies where all the main types of asbestos produce a greater tissue reaction with increasing fibre length in a variety of animals (Scymczykiewicz and Wiecek, 1960; Klosterkötter, 1968; Hilscher et al., 1970; Davis, 1970, 1972 and Wright, 1977). In addition, the results of intrapleural and intraperitoneal injections would all tend to support the findings of the importance of fibre length to fibrogenicity.

Regarding differences between the asbestos minerals, there have been many contradictory reports of the relative fibrogenicities of the different asbestos types following injection. It will be seen later that it is likely that these contradictions have arisen as a result of the fact that precise characterisation of the doses used has seldom been obtained. The only confirmed finding is that most mineral fibres and all asbestos fibres will produce some fibrogenic response in animals following injection (Vigliani, 1968; Davis, 1972). There is an additional

finding that most workers report that chrysotile produces the most fibrosis following injection of equal masses of the different fibre types.

If anything, the results of inhalation experiments have served to further confuse the situation. The first known asbestos inhalation experiments were described briefly in 1928 by Simpson. He mentions the earlier work of Mavrogordato who exposed guinea pigs to chrysotile dust 2 hrs per day for 50 days, and found a slight generalised fibrosis with large numbers of asbestos bodies. Vorwald et al. (1951) exposed various species of animals to chrysotile clouds of either long-fibre (20 - 50 μ) or short-fibre and found peribronchiolar fibrosis only with the long-fibre cloud. These earlier studies using only chrysotile were followed some years later by a series of investigations into the differential fibrogenic properties of inhaled fibrous dusts. Wagner et al. (1963, 1965) and Morris et al. (1965) suggested that chrysotile produced less fibrosis than crocidolite or amosite for the same dose. On the other hand, Holt et al. (1965) found no differences in fibrogenicity between chrysotile, crocidolite, amosite and anthophyllite, and further deduced that their long-fibre dust clouds produced no more fibrosis than their shorter fibre clouds. Wagner et al. (1974) in a later study showed that Canadian chrysotile gave the most fibrosis and amosite the least. Reeves et al. (1974) reported that crocidolite produced the most fibrosis, chrysotile the least, with amosite in an intermediate position.

The question of whether or not experimental asbestotic lesions are progressive has been examined by

following the course of the lesions after the cessation of dusting. Vorwald et al. (1951) reported no progression of lesions in various animal species after chrysotile inhalation. Wagner (1963) found progression of lesions in guinea pigs, rabbits and velvet monkeys after inhalation of long-fibre clouds, but not with the short fibre. He later reported (Wagner et al., 1974) considerable progression in rats after exposure to all main types of asbestos. In 1965 Holt found progression occurred in guinea pigs, and Gross has variously reported progression in guinea pigs and hamsters (Gross and De Treville, 1967) but not in rats (Gross et al., 1967).

Information about the penetration, deposition and subsequent clearance of experimentally inhaled asbestos particles is of obvious importance in assessing the relative toxicities, since in the final analysis it is the dust that is deposited and retained that is pathogenic. Until very recently, the only information concerning the behaviour of airborne asbestos fibres was that of Wagner and his colleagues (Wagner and Skidmore, 1965; Morris et al., 1965; Wagner et al., 1974). They showed that exposure to similar airborne masses of chrysotile and amosite resulted in far more (up to six times) amosite dust being found in the lungs, and that there was a more rapid exponential clearance of chrysotile. The work of Timbrell is worth reviewing in this context.

In 1965 Timbrell drew attention to the fact that fibre diameter is the most important parameter affecting the free falling speed, and that it is the free falling speed that determines the deposition of most dust particles. From theoretical considerations of the sizes of pulmonary airspaces, it follows that

fibres of a diameter less than 3.5 microns and a length up to 200 microns can penetrate deeply into the lung. Timbrell and his colleagues further showed that differences in fibre diameter and hence respirability might explain the differences in pathogenicity of asbestos dusts. Thus, in humans, the more dangerous N.W. Cape crocidolite produces 30 times as many fibres as do the amphiboles from the Transvaal, and the former are also thinner. The Cape fibres are therefore inherently more efficient at deep penetration to the periphery of the lung. It is likely that differences in physical characteristics would also explain most of the rather contradictory experimental inhalation studies. When he found more amosite in rat lungs after exposure to equal mass doses of amosite and chrysotile, Wagner (Wagner and Skidmore, 1965) concluded that this was due to the fact that chrysotile was cleared much faster. The experiments reported by Timbrell (1970) would suggest that the curly nature of chrysotile is an important factor in determining its poor penetration and its deposition in the nasal turbinates and upper respiratory tract where there is a fast mucociliary clearance mechanism. This implies that Wagner's findings are explicable in terms of differential penetration and deposition rather than a clearance mechanism specifically stimulated by chrysotile.

Regarding the cellular clearance of deposited asbestos, there have been several studies supporting the view that longer fibres are more fibrogenic because they are less well phagocytosed and transported away (Hilscher et al., 1970; Beck, 1971; Friedrichs et al., 1971; Hilscher, 1972; Timbrell, 1972). Many authors have reported that short fibres are almost always seen intracellulary in tissues (Davis, 1972; Timbrell and Skidmore, 1968)

whereas longer ones are seldom incorporated into cells (Friedrichs et al., 1971). Harington (1975) takes this further and considers that transport of fibres begins with those measuring 20 μ in length and increases with decreasing length. The actual fate of the transported fibres is a separate question that will be discussed later. It is more appropriate at this point to examine the fate of those fibres longer than 20 μ that are, by virtue of their length, trapped in tissue. Most are destined to remain in the tissue and some of these become coated.

Although it is now known that only a small proportion of resident fibres become coated to form "asbestos" or "ferruginous" bodies, their frequent occurrence in asbestotic lungs and sputum prompted considerable research in the past. It has been suggested that the coating in some way protects the surrounding tissue from any toxic effects of the fibre, although this has yet to be proven. However, stimulated by the earlier work of Beger (1934), a series of reports describing the actual formation of the ferruginous body were published in the 1960's. In particular, Davis investigated their formation in a larger series of experiments (Davis, 1964; Davis et al., 1967, 1970) and by 1970 he and others (Davis, 1970) considered that the process involved the following features: (1) the process is intracellular, usually involving giant cells; (2) only relatively long fibres over 10 μ become coated to form typical bodies; (3) bodies are often found in cells with large numbers of uncoated fibres; (4) the ratio of uncoated to coated fibres may be 1000:1 or higher; (5) the first coating of the fibre is a mucopolysaccharide; (6) next, a layer of dense granules about 60 A^{o}

in diameter is laid down; (7) the granules are of iron containing protein, probably ferritin or haemosiderin; (8) there is usually a single layer, but where a number of layers occur, it is likely to be the result of repeated reingestion by giant cells; (9) an outer layer of calcium in the form of 60 A^{o} apatite crystals arranged radially may be present (Davis, 1971, reported that there is an association between the process of body coating and tissue calcification).

Ferruginous bodies have been produced experimentally in a number of animals and the mechanism of formation described above would appear to be independent of the species, route of administration or target tissue. There are, however, large quantitative species differences in their occurrence with the guinea pig and hamster mimicking the human condition of frequent formation, and the rat being particularly reluctant to produce them. Gross et al. (1969) examined the inconsistencies arising from the assumption that all ferruginous bodies contained asbestos in their core and showed that their formation could be considered as a relatively non-specific biological reaction to "inert" fibrous material. Most workers now consider the ferruginous body to be a curious and rather unimportant consequence of asbestos-tissue reactions.

The fate of ferruginous bodies is unknown. The fact that they can be found in sputum suggests that some of them gain access to the mucociliary escalator system and are subsequently cleared from the lung and ingested or expectorated. Analysis of the core of bodies found in human lungs showed that the majority of those identified as asbestiform were in fact amphiboles,

irrespective of exposure history (Fondimare and Desbordes, 1974). This led to speculation that amphibole asbestos types were particularly prone to ferruginous body formation and were generally more biologically active. However, close examination of ferruginous bodies revealed that fragmentation and dissolution of the core could occasionally be seen. Since chrysotile is chemically more labile, most authors now think that the finding of a preponderance of amphibole ferruginous bodies is a result of the preferential dissolution of the chrysotile bodies (Becklake, 1976). This agrees well with experiments showing that ferruginous body formation is dependent upon particle size rather than chemical composition (Gaensler and Addington, 1969).

1.3.3 Carcinogenic responses

There was very little animal experimentation into asbestos carcinogenicity carried out before the 1960's, largely as a result of the fact that it was not until then that there was a widely accepted association between asbestos and carcinogenicity in humans (Gilson, 1966). In 1962 Wagner published the results of animal experiments showing that tumours could be simply and consistently produced in rats, and since then there has been a great deal of research into the carcinogenicity of asbestiform minerals.

The work may be conveniently described in terms of the mode of administration of asbestos, since in most cases this determines the site and the characteristics of the tumours produced. Inhalation experiments most closely resemble the principal human exposure situation, but they are both technically difficult and

expensive to perform, and the findings can be difficult to interpret. The comparative simplicity of some injection experiments has been mentioned previously in connection with fibrogenicity (Section 1.3.2b), and injection has proved to be the most adaptable exposure method for carcinogenicity studies.

The review will commence with the results of inhalation experiments, since these were the earliest documented, then deal with intratracheal injections, injections in the body cavities, subcutaneous injections, and ingestion experiments.

1.3.3 a Inhalation

Apart from the practical difficulties associated with generating and characterising asbestos clouds of consistent known parameters over a long period of time, inhalation experiments have been hampered by the dual problems of combining the needs for the exposure period to be sufficiently long enough for the animal to acquire a meaningful dust burden, and yet be capable of surviving long enough for tumours to develop. Several species of laboratory animal have been used over the years, and it would now seem that the s.p.f. rat is the most suitable laboratory model to use in such studies: many strains of mice are known to spontaneously produce pulmonary tumours; hamsters tend to succumb to the effects of severe pulmonary fibrosis before sufficient time has elapsed for tumour development; guinea pigs are known to be reluctant to produce tumours, and rabbits and primates are expensive to maintain.

Nordmann and Sorge (1941) were the first to report the successful production of tumours following inhalation of asbestos. They exposed mice to chrysotile for periods

up to 13 weeks, and produced some bronchial carcinomas and many other abnormalities. Lynch et al. (1957) reported similar abnormalities some years later but did not produce tumours. In 1967 Gross et al. reported that exposing rats to chrysotile for periods up to 16 months produced many pulmonary carcinomas and one pleural mesothelioma. Reeves and his associates described the results of a very large study in which rats, rabbits, guinea pigs, gerbils and mice were exposed to asbestos clouds for 2 years (Reeves et al., 1971, 1974). They used amosite, crocidolite and chrysotile at high doses (50 mg/m^3), and produced many abnormalities, with the rats giving both lung and mesothelial tumours. The most detailed inhalation carcinogenicity study to date has been that of Wagner and his associates (Wagner et al., 1974). They exposed rats to all types of UICC asbestos for periods ranging from one day to 2 years, and found both lung cancers and mesotheliomas. Surprisingly, of the three mesotheliomas produced, one was of peritoneal origin, and one was produced after only one day's exposure to amphibole asbestos. In contrast to the above, although Shabad et al. (1974) exposed rats to chrysotile clouds of 230 mg/m^3 for up to 13.5 months, they produced no tumours. They did, however, report the development of a large number of lesions that they chose to describe as "precancerous", and they concluded that their study produced no tumours only because the animals did not survive long enough.

1.3.3 b Intratracheal injections

There have been relatively few intratracheal injection carcinogenicity studies associated with asbestos, the most prolific workers in this field being the Russians.

Following their finding that the carcinogenicity of benzopyrene was greatly enhanced by its injection in association with carbon particles (Shabad et al., 1964), Shabad and his co-workers went on to investigate the effects of asbestos and benzopyrene mixtures on animal lungs. They found that the clearance of benzopyrene from the lung is markedly decreased in the presence of asbestos, and concluded that this was because of the adsorption of the benzopyrene onto the surface of the particles (Shabad et al., 1974). Saffiotti et al. (1965) came to similar conclusions using particulate iron oxide and benzopyrene. The concept that carcinogen adsorption results in its prolonged retention and activity would explain the results of Miller et al. (1965) who showed that chrysotile, the most strongly surface active asbestos, produced the greatest carcinogenic response with benzopyrene. The work of both Vosamae (1971) and Pylev (1972) would tend to support these conclusions. Gross and De Treville (1967) used the intratracheal injection as a comparative bioassay when they injected rats with samples of the same dusts that they used in their inhalation studies. This showed that asbestos was capable of producing some pulmonary tumours without the aid of specifically introduced carcinogens, a finding that has since been confirmed by others. Interestingly, some authors have reported the occasional production of pleural mesotheliomas after repeated intratracheal injections (Pylev, 1972).

1.3.3 c Intrapleural and intraperitoneal injections

The pioneer work ~~of~~ was by Wagner (1962) who produced the first experimental mesotheliomas in laboratory animals by intrapleural injections of asbestos and established a cause and

effect relationship between asbestos exposure and mesotheliomas. It was later shown that injections of large amounts of asbestos into the main body cavities could produce a tumour incidence of up to 60% in groups of rats (Wagner, 1968; Wagner et al., 1970), and this established the technique as a useful bioassay for the study of the histogenesis of these unusual tumours, and the importance of certain co-factors in their production. As a consequence of this, most of the major hypotheses concerning the mode of asbestos as a carcinogen have been tested using the intrapleural and intraperitoneal injection techniques. The literature is understandably huge, and only a summary of the main findings are necessary here.

There is clear evidence that a dose response exists with all forms of asbestos (Smith et al., 1965, 1968; Wagner et al., 1973), although there is as yet no information about the existence of a threshold since the lowest intrapleural dose used (0.5 mg) produced tumours in rats (Wagner et al., 1973). The time between injection of asbestos and tumour production depends to some extent upon the dose used, with the first pleural tumours in rats usually developing after 15 months (Wagner et al., 1970) and the peritoneal after 9 months (Davis, 1974).

Harington's original suggestion that hydrocarbon contamination is responsible for carcinogenicity (Harington, 1962) has been refuted by the work of Wagner and his associates (Wagner and Berry, 1969; Wagner et al., 1973) who showed that exhaustive extraction of asbestos did not appreciably reduce the tumour yields. Although some workers have shown that the presence of excess carcinogenic hydrocarbon contamination does increase the

tumour yield, it is now generally accepted that the normal levels of contamination encountered are unlikely to be a major factor in the carcinogenicity of asbestos.

The apparent finding that mesotheliomas in humans were more frequently associated with crocidolite exposure added weight to the thesis that the carcinogenesis was associated with chemical effects. Amongst the many components variously considered responsible have been the silicon content. iron content, magnesium content, and various trace element contents and contamination. It has, however, been shown that fibres of diverse chemical structure are all capable of producing mesotheliomas under certain conditions (Wagner, 1970; Stanton and Wrench, 1972). Harington (1975) retains the belief that the chemical composition of asbestos does have a role to play in their carcinogenicity, and he calls on the results of haemolysis and cytotoxicity experiments to support his contention that supposedly inert fibres like glass do possess activity (Szentei, 1970; Beck et al., 1972).

The work of Timbrell has already been mentioned in connection with experimental fibrogenicity (Section 1.3.2b). He showed that physical size and shape of fibres has a profound effect upon their biological activity by virtue of the fact that it determines the site of deposition and retention within a tissue. Using the intrapleural injection model Wagner et al. (1973) developed this concept further and showed in a very large study that the carcinogenicity of asbestos was closely related to fibre geometry, with the finer and longer fibres being the most active. Stanton and Wrench (1972) amply confirmed that long, thin fibres were the most

dangerous in a study using the somewhat unusual technique of implantation of fibre impregnated discs into the pleural cavity. They proved that fibres of similar geometry but of diverse chemical composition produced similar tumour incidences, and that the fibrous form of a dust was active when the powdered was not. Most authors would draw a distinction between the effect of size and shape in asbestos carcinogenicity and the so-called Oppenheimer effect of inert foreign body carcinogenesis (Oppenheimer et al., 1961) on the grounds that the size discrepancies between the two mechanisms are incompatible.

The mesothelial tumours have a recognised variable histological structure, and it is often difficult to distinguish between them and secondary tumour deposits in humans. This has presented considerable ambiguity in the diagnosis of mesothelioma (McCaughey and Oldham, 1972) and as a result there have been many studies describing the fine structure of mesotheliomas (see Suzuki et al., 1973, and Davis, 1974). Their similarity with hemongiomas and synoviomas has been emphasized by Davis (1974), who suggested that they should best be simply considered as of mesenchymal origin (the totipotency of mesenchymal tissues is well established). There is some evidence to suggest that mesotheliomas arise from the relatively undifferentiated sub-mesothelial tissues rather than the specialised mesothelial layer itself (Davis, 1974).

1.3.3 d Subcutaneous injection and transport experiments

The subcutaneous injection technique has been used to investigate the consequences of asbestos transport through tissues, since it represents a simple method of introducing

large doses with the minimum trauma to the vascular system. The first of these experiments was reported by Roe et al. in 1965. By 1967 the same group had produced pleural and peritoneal mesotheliomas in mice after subcutaneous injection of crocidolite into the flanks, and they also found evidence of widescale dissemination of asbestos fibres throughout the body with a selective transport to the mesothelial tissues. In a later study, however, Kanazawa, Roe and others (Kanazawa et al., 1969) failed to produce either mesothelial tumours or evidence of selective transport to the mesothelial tissues, and the possibility remains that the 1967 findings were the result of mis-injections into the body cavities. Despite their negative tumour findings, Kanazawa and his colleagues did show that asbestos was transported from the injection site by the lymphatic system, and that fibres could be found in many other tissues. The authors observed that fibres trapped in the pleural lymphatic areas called "milky spots" might be significant for the future development of mesothelial tumours.

In 1970 Morgan and Holmes reported the results of subcutaneous and intrapleural injection experiments using labelled asbestos and radioactive tracer techniques. They found evidence of activity in the major body organs but considered the activity as an index of the leaching of labelled components from fibres rather than transport of fibres per se. This was followed, in 1971, by a report that chrysotile fibres found in the liver 73 days after intrapleural injection were apparently derived either from direct extension or migration from adjacent diaphragmatic lesions. In this and a subsequent paper (Evans et al., 1973) they confirmed their impression that the relatively high solubility of activation

productions of irradiated asbestos precludes their use for following the migration of small amounts of asbestos through tissues.

To date, there have been no other experiments reported that were specifically designed to look at the existence and/or consequences of the transport of asbestos fibres through tissues. Some circumstantial evidence exists in the descriptions of experimentally produced lesions and pathology of human asbestos related disease, but this will be considered later (see Chapter 9).

1.3.3 e Ingestion experiments

Until recently, little attention has been focussed on the effects of ingestion of asbestos, and there have been very few papers published on animal experiments. Westlake et al. (1965) were the first to report some evidence that ingested asbestos was capable of penetrating the gastrointestinal mucosa when they described finding some small chrysotile fibres in the colon of rats fed a diet of 6% chrysotile. Although Smith et al. (1965) did find small ulcerous lesions in the stomachs of hamsters fed asbestos, they also describe reports of similar findings in control animals. They did not specifically look for the presence of fibres in tissues, so there was no indication of penetration. There were other early ingestion studies that found no evidence of the adverse effects of asbestos and these either remained unpublished (Swinburne) or were reported very briefly (Bonser and Clayson, 1967).

The various reports of low levels of fibre contamination of foods and beverages (Biles and Emerson, 1968;

Cunningham and Pontefract, 1971; Eisenberg, 1974), and the work of Evans et al. (1973) clearly showing the levels of asbestos that may ultimately reach the digestive tract after inhalation, both indicate that most humans ingest some asbestos during their lives. The discovery in 1973 that Lake Superior in Canada was polluted with mining wastes containing asbestiform minerals has provided the impetus for a good deal of further research. This prompted Gross et al. (1974) to summarise not only work in progress but also some previously umpublished information, most of which concluded that there was no evidence of penetration of the lining of the gastrointestinal tract.

Despite the early report by Telischi and Rubenstone (1961) of the finding of asbestos fibres in a gastric carcinoma of an asbestos worker, there remains no direct proof that ingested asbestos gives rise to malignancies. The remaining published papers deal with the question of whether or not penetration occurs. In 1973 Cunningham and Pontefract reported the results of some very crude experiments in which they injected small-fibred chrysotile asbestos directly into the stomachs of rats after laparotomy, and subsequently found fibres in most tissues examined. They followed this by describing placental transfer of injected asbestos (Cunningham and Pontefract, 1974) and a brief mention that fibres could be found in the blood of rats on a 1% chrysotile diet (Pontefract, 1974). Further, Webster (1974) and Westlake (1974) have reported penetration of the gastrointestinal mucosa following ingestion of massive doses of asbestos over long periods. It is interesting to note that all those experiments describing penetration and translocation of fibres have used massive doses and unrealistic

methods of administration.

The only other ingestion work reported up until 1974 was that of Amacher and his colleagues (Amacher et al., 1974). In some short-term experiments using chrysotile, they have shown that pre-starved rats given very large doses of asbestos by gavage display evidence of a proliferative response in several gastrointestinal tissues.

There is some indirect evidence that asbestos fibres should be capable, by virtue of their size, of transmigration of the mucosal barrier: Clark (1959) showed that suckling rats and mice were capable of ingesting both whole proteins and "particulates" such as colloidal gold and Indian ink, but that this ability was lost within 18 days of birth. More significantly, Sanders and Ashworth (1961) clearly demonstrated the uptake and translocation of latex particles 2200 A^{o} in diameter in female rats, as part of a study on the adsorption of fats from the small intestine. In 1965 Evans and Mason reported a very high incidence of gastro-intestinal tumours in rats fed a diet including dried bracken although they did not know whether this was due to a bracken extract or to the irritability of the vegetable fibre component.

In 1968 Volkheimer introduced the term "persorption" to describe the phenomenon of passage of particulates between enterocytes in the gut wall. Volkheimer and his colleagues have since described the "persorption" of many particulates up to 90 μ on a surprisingly large scale (Volkheimer, 1974; Shreiber, 1974). However, corroboration of these findings by other workers is required before the phenomenon of persorption can be accepted as a normal physiological process.

1.4 SUMMARY AND AIMS OF RESEARCH

The asbestos minerals are united by their fibrous habit into a group of diverse structure, composition, properties, and uses. Their widespread use in modern technology has resulted in industrial and environmental exposures of large populations to asbestos fibres. The asbestos-related diseases would all appear to be dose-related, with the most severe disabilities amongst occupationally exposed groups. All the major types of asbestos would seem to be pathogenic, and the evidence to date suggests that fibre geometry is the most important pathogenic parameter.

Asbestos is an established carcinogen in man and animals: there is a proven and widely accepted association between lung cancer, mesothelioma and asbestos exposure. The available epidemiological data also shows an established increased incidence of gastrointestinal tumours, and there has been concern expressed in recent years over the etiology of these and some more obscure tumours. The question arises as to whether the increased incidence of gastrointestinal malignancy in heavily exposed workers is a result of the ingestion of asbestos, or the result of the inhalation, deposition and subsequent transport of asbestos fibre throughout the body to vulnerable tissues.

There is remarkably little conclusive information about the potential hazards of ingested and/or transported asbestos, and it is the aim of this thesis to investigate some aspects of the effects of ingested and transported asbestos in experimental animals using ingestion, injection and inhalation.

CHAPTER 2 METHODS

2.1 Animal husbandry

2.2 Histology

2.3 Electron microscopy

2.4 Photography

2.5 Cytokinetics

2.6 Development of techniques

2.1 ANIMAL HUSBANDRY

2.1.1 Source and type of animals

All the rats, mice and guinea pigs used in the various experiments reported in this thesis were supplied by the Edinburgh University Centre for Laboratory Animals and were maintained in the Institute of Occupational Medicine Animal Unit at Bush House.

Rats. A total of 507 Wistar AF/HAN rats were used. The strain was established from a small breeding colony using stock supplied by the Wistar Institute in Hanover, Germany, in 1971, and there have been no additions to the stock since. The animals were specific-pathogen-free (s.p.f.) derived and barrier maintained in the Institute of Occupational Medicine Animal Unit. Most animals were ten weeks of age at the start of the experiments reported. There was comparatively little information about the morbidity and mortality of this strain of rat at the start of this work in late 1973, and consequently a necessary addition to the long-term toxicological studies was an investigation into some basic parameters of the biology of the HAN Wistar laboratory rat.

Mice. A total of 15 CBA/ca s.p.f. mice were used. The inbred strain was established using breeding stock obtained from the Medical Research Council Laboratory Animal Centre, Carshalton, Surrey, and was maintained by close sibling mating of monogamous pairs.

Guinea pigs. A total of 20 Dunkin-Hartley s.p.f. guinea pigs were used. The strain was established from breeding stock obtained from the Animal Research Institute,

Moredun, Edinburgh, in 1971.

2.1.2 Caging and diet

The Institute of Occupational Medicine Animal Unit consists of an inhalation chamber area, five animal holding rooms, and appropriate laboratory space. All rooms were supplied with a flow of filtered air maintained at approximately 65 - 70°F, and no deliberate attempt was made to control the light regime - the animals received the normal seasonal and diurnal fluctuations.

Unless otherwise stated, the rats in the holding rooms were housed in polypropylene cages with stainless steel wire tops and bottoms (North Kent Plastics, Dartford, Kent). All rats were supplied ad libitum with Spratts LAD.1 Expanded Diet up to the age of six weeks, and thereafter with McGregors Special Rat Cake.

All the mice were housed in polypropylene boxes with stainless steel tops (North Kent Plastics) using a peat/wood shaving bedding material. They were supplied ad libitum with acidified water and Spratts LAD.1 Expanded Diet.

The guinea pigs were also housed in polypropylene cages, and were supplied ad libitum with B.P. Guinea Pig Diet.

2.1.3 Inoculation Methods

2.1.3 a Injection

All animals were injected under light ether anaesthesia. All asbestos preparations were dry heat sterilised before making up to the appropriate concentration with sterile phosphate buffered saline (PBS), to avoid possible compositional alterations from any temperature-sensitive solubility of asbestos constituents. The dose of asbestos used depended partly upon the aim of the experiment and partly upon the physical nature of the asbestos type, but was normally between 25 - 33 mg/ml. Subcutaneous injections were usually placed above the right scapula, intrapleural into the right thoracic cavity at the level of the fourth rib, intraperitoneal to the right of the mid line, and intravenous into the right lateral tail vein.

Injections of substances other than asbestos included: 10% nigrosin in PBS, administered via all routes, and designed to delineate specific regions of the lymphatic system; and the intraperitoneal injections associated with the cytokinetic studies; 1.0 mg/Kg vincristine sulphate (Oncovin Eli Lilly & Company, Basingstoke) made up to 2.0 ml with sterile PBS, or 0.5 mCi/Kg tritiated thymidine (6^{3}H thymidine specific activity 89 mCi/mg, Radiochemical Centre, Amersham) made up to 2.0 ml with sterile PBS.

2.1.3 b Ingestion

The method of administration depended upon the aim of the experiment. A prime consideration of the long-term asbestos ingestion experiments in rats was the prevention of a concomitant dust hazard, and accordingly the asbestos was prepared

as a margarine formulation. It was found that, when given free access to margarine as a dietary supplement, adult male rats would consume approximately 60 gms per week. The dose was accordingly set at 5 mg asbestos per gram of margarine, giving a total weekly consumption of 250 - 300 mg per rat, or approximately 50,000 times the human occupational ingestion maximum (see Schneiderman, 1974). Appendix IV shows that a weekly consumption of 60 gms of margarine was associated with a 30% reduction in the consumption of pelleted diet.

Attempts were also made to produce a similar dietary formulation for the guinea pig ingestion experiments. Guinea pigs are strictly herbivorous and consequently the margarine formulation described above was unacceptable. Extensive trials with alternative "inert" carriers such as gelatine and agar were unsuccessful, and it was finally decided to incorporate the asbestos into the guinea pig pelleted diet. Accordingly, with the cooperation of the diet manufacturer (Cooper Nutrional Products Ltd., Witham), asbestos was added at the level of 5 mg/gm to batches of the diet prior to pelleting.

Some of the short-term experiments on rats required the administration of relatively large amounts of intraluminal asbestos, and a gavage technique was consequently employed in which an asbestos slurry was introduced into the stomach via the oesophagus using a specially adapted trocar needle.

In addition, some experiments were designed to see if relatively large particles were capable of crossing the gastrointestinal mucosa under normal ingestion

conditions. Nigrosin was used in these short-term experiments, added to the drinking water (10% by volume) of both rats and mice.

2.1.3 c Inhalation

The primary aim of these inhalation experiments was to investigate the effects of prolonged exposure of rats to airborne asbestos, with particular emphasis on the pulmonary pathology produced. The results are reported in a paper by Davis et al. (1978). However, the experiments provided the opportunity to examine the extrapulmonary tissues of animals exposed to asbestos dust clouds and are consequently included in this thesis. Full details of the inhalation procedures are provided in the papers by Timbrell et al. (1970) and Beckett (1975). Essentially, rats were housed 12 per group, 4 groups per treatment, in specially designed inhalation chambers for the one year of dust dosing, and they were then maintained 2 per cage within the holding rooms for their full lifespan. Dust dosages were set at Amosite 10 mg, Crocidolite 5 and 10 mg, and Chrysotile 2 and 10 mg, all measurements being in milligrams of respirable fibre per cubic metre of air.

2.1.4 Autopsy Schedule

A full autopsy was carried out on all animals where possible. Moribund animals were killed, the method of despatch depending upon the aim of the experiment: in general, animals expected to yield tissue for electron microscopy were killed by cervical dislocation, and others were killed by administering deep ether anaesthesia followed by cervical dislocation. A carbon dioxide killing chamber was used when large batches of animals were killed for routine examination. Autopsy weight and details of

general body condition were recorded. Where possible, animals were exsanguinated via the right subclavian artery using the residual post mortem cardiac activity before the body cavities were opened. This was found to facilitate macroscopic examination of the peritoneal and pleural contents by reducing the amount of extravasation and haemorrhage. Fig. 2.1 gives details of the tissues routinely taken for histological examination. The lungs were normally inflation fixed with isotonic 10% formal saline. The gut was opened along its full length and the mucosal surface examined for abnormalities with a hand lens before selected areas were fixed. The external condition of all organ surfaces and cut faces were examined for abnormalities, and large organs such as the liver and kidney were sliced.

2.2 HISTOLOGY

2.2.1 Fixation

The methods of fixation and subsequent processing varied, depending upon the purpose of the histological preparation. Whilst it was recognised that perfusion fixation with a Karnovsky preparation was probably the best overall method of preserving tissue, it was seldom possible because of the time involved. Further vascular perfusion fixation of the gastro-intestinal tract was found to be incapable of preventing a certain degree of mucosal autolysis, and secondary immersion fixation was necessary. The vast majority of tissue examined histologically in connection with the investigations reported here were immersion fixed in isotonic 10% formal saline, since this general fixation method was considered adequate for most purposes. The exception

FIGURE 2.1 Tissues routinely fixed at autopsy.

Lungs

Heart

Liver

Spleen

Pancreas

Kidney

Adrenal

Omentum

Mesentery

Selected levels of gut

Any abnormalities

to this rule was the tissue prepared for the cytokinetic assays in which it was of primary importance to use an efficient nuclear fixative. Carnoys fix was therefore used (60% absolute alcohol, 30% chloroform and 10% glacial acetic acid), the tissue being transferred to cellosolve (2 Ethoxyethanol) after six hours to prevent hardening.

2.2.2 Dehydration, Embedding and Staining

Tissue sections were prepared in the usual manner. Following dehydration through the ascending alcohol series into xylene, the tissue was embedded in wax (melting point 57°C) in a vacuum oven, and sections cut at approximately 5 microns as routine. In the case of the tissue prepared for the cytokinetic analysis, it was important to avoid overestimates associated with nuclear overlap and so sections were cut at 3 microns. In addition, most photomicrographs were prepared from specifically recut and stained 3-micron sections.

All tissues were stained with haematoxylin and eosin (H and E) as routine. Other specialised stains frequently used were Weigert's elastic stain, Van Gieson's collagen stain, and Gordon and Sweet's reticulin stain. Full details of these standard procedures are available in Carleton's Histological Technique, 4th Edition, 1967, Oxford Univ. Press. Some slight modifications of existing techniques were required for the tissue prepared for the cytokinetic analysis: autoradiographic preparations required longer solution times for staining, differentiating and counterstaining as a consequence of the gelatine-based photographic emulsion covering, whilst stathmokinetic

preparations were counterstained with a diluted eosin (0.5% aqueous) solution since the resulting lighter background was found to aid nuclear identification when assessing the sections.

2.3 ELECTRON MICROSCOPY

2.3.1 Instruments

Several electron microscopes were used in the course of this study:

Transmission electron microscopes:

AEI EM6	Inst. of Occupational Medicine
EM6B	Dept. of Anatomy, University of Edinburgh
CORA TEM	Inst. of Occupational Medicine

Scanning electron microscopes:

Cambridge 2A	Dept. of Electrical Engineering, University of Edinburgh
S600 + Link EDA	Inst. of Occupational Medicine
S180 + Link EDA	MRC Unit, Western General Hospital, Edinburgh

2.3.2 Preparation of Biological Specimens

The interpretation of ultrastructural variations requires caution since it is known that artefacts can be introduced by alterations in preparation and presentation of specimens for electron microscopy (Ruska, 1960, 1961; Trier, 1964). Due cognisance was taken of this in the fixation of tissue and a rigidly standardised technique was used for each section of this study. Tissue for transmission electron microscopy was usually fixed in cold 4% osmium tetroxide for not more than two hours. In some (specified) cases, however, the primary fixative was 2% gluteraldehyde followed by a postfix in

osmium tetroxide. After fixation the tissue was dehydrated through the ascending alcohol series to propylene oxide, impregnated with araldite at room temperature overnight, and embedded in fresh araldite at 60°C for 48 hours. Sections were prepared using a Cambridge Huxley Ultramicrotome. Thick sections were stained with Toluidine Blue, and the thin sections (600 - 900 A°) double stained with lead citrate and uranyl acetate. Full details are available in Fixation Dehydration and Embedding of Biological Specimens, Vol. 3, Part I, Edited by A.M. Glauert; Part II edited by N. Reid, 1974, North Holland.

Tissue for scanning electron microscopy was fixed in filtered cold buffered 2% gluteraldehyde. Where the purpose of the preparation was to examine the in vivo surface of the tissue with the minimum of interference, great care was taken to fix the tissue without excessive disturbance. However, examination of the detailed cellular organisation of tissue surfaces such as gut mucosa required prior removal of mucus and associated debris with cold physiological saline before fixation. Tissue was subsequently dehydrated through acetone and liquid carbon dioxide in Polaron E3000 critical point drying apparatus according to a technique described by Boyde and Vesely (1972). Specimens were mounted upon aluminium stubs and coated with carbon and/or gold for examination. In the earlier stages of the studies reported here some specimens were treated with the ligand diphenylhydrazine to assist the relatively inefficient rotary evaporation coating methods (see footnote* for details). The AEI Metrovac Coating Unit Type 12 with the appropriate accessories

*Malick, L.E. and Wilson, R.B. (1975). Proc. Symp. SEM, 1975.

was used throughout, following the procedures detailed in the accompanying instruction manuals.

2.3.3 Preparation of inorganic specimens

The precise details of preparation depended largely upon the physical and chemical characteristics of the specimen and the aim of the investigation (see Section 2.6.1). Both the TEM and SEM were used, but the majority of examinations of inorganic specimens described in this thesis were performed using the S600 scanning electron microscope.

Samples for TEM were prepared on copper grids (size 200 or 400) using amyl acetate support films strengthened with carbon. Full details of techniques used are available in Techniques for electron microscopy, ed. D. Kay* 1967.

Samples for SEM were generally prepared by filtering down aqueous suspensions of the particulates onto 0.4 micron pore size Nuclepore membrane filters. This method was not always appropriate where, for instance, elemental analysis of the soluble constituents of samples were required. In this case samples were added directly onto double-sided cellotape mounted on SEM stubs. Definitive analyses of specimens required the use of carbon stubs to reduce the background "noise". Specimen coating was by evaporation with carbon and/or gold in the earlier preparations, and latterly with gold sputter coating.

2.4 PHOTOGRAPHY

2.4.1 Macrophotography

The macrophotographs included in

*Chapter 3, pp58-74 by D.E. Bradley.

this thesis were taken with a Nikon F camera using the standard lens and a bellows attachment. Ilford FP4 film was used for monochrome, and either Vericolor S, or Ektachrome 64 with commercial transprints, were used for the colour photography.

2.4.2 Photomicrography

All photomicrographs were taken using a Nikon SKE microscope adapted for photography using a 35 mm light box. Ilford Pan F film and a green filter were used for monochrome, and Ektachrome 64 with a blue filter and commercial transprints was used for colour photography.

2.4.3 Electron photomicrography

A variety of films were used, depending upon the instrument (see Section 2.3.1). For transmission electron micrography Ilford Special Lantern Contrasty Unbacked 3¼" x 3¼" glass plates, Ilford FP4 3¼" x 3¼" cut film, or 70 mm Ilford SP353 Electron Microscope Roll film was used. For scanning electron micrography 35 mm Ilford FP4, or 70 mm Ilford SP353 were used routinely. Towards the end of the study a 5" x 4" sheet film attachment to the S600 microscope was used with Ilford FP4 sheet film.

2.5 CYTOKINETICS

This section will be restricted to details of those procedures leading up to the production of what may be termed the "raw data", and the first stages of the data handling. The manipulation and interpretation of the data is described in Chapter 6. In addition, a brief introductory review of the subject of cytokinetics of the gastrointestinal tract may be found

in Appendix III.

2.5.1 Preliminary procedures

A total of 22 rats was used, distributed experimentally as shown in Fig. 2.2 (see Section 2.1.3a for details of the injection procedures). All injections were performed at 9.00 a.m. Animals injected with tritiated thymidine were killed alternately - one treated, one control - exactly one hour later. Animals injected with oncovin were killed in pairs 30, 60, 90, 120 and 150 minutes after injection. Since it was considered important to record the precise site of origin of the tissue samples analysed in case there were large local cytokinetic variations, the following procedure was adopted: immediately after cervical dislocation the animal was exsanguinated via the right subclavian artery and the entire peritoneal contents removed. The spleen and liver were dissected free, and the mesentery carefully cut away from the gastrointestinal tract at its points of attachment. The whole tract was then gently laid upon a piece of glass moistened with cold isotonic saline and allowed to adopt a "natural" length dictated by the smooth muscle tonicity. Tissue was removed as shown in Fig. 2.3. Samples destined for subsequent DNA estimations were immediately immersed in liquid nitrogen and stored at -70°C. Other tissue was cut into 3 cm lengths and fixed in Carnoys fixative (see Section 2.2.1). The individual sampling zones in the small and large intestines were expressed as percentages of the regional lengths to allow for any differences in the relative sizes of the intestine in the different animals.

FIGURE 2.2 Experimental layout for cytokinetics assay.

Number of animals used in :	Treatment		
	Amosite	Negative Control*	Positive Control
Autoradiographic and DNA assays (^{3}HTdr injected)	3	3	-
Stathmokinetic assays (oncovin injected)	5	5	6

*Negative Control animals were given normal laboratory diet. Positive Control were given diet with a margarine supplement (see Chapter 6).

FIGURE 2.3 Details of tissues samples for cytokinetics studies.

Tissue	Site of origin
Squamous forestomach	Taken from greater curvature
Glandular stomach	Taken from greater curvature
Upper small intestine	Taken from proximal 10 cms
Mid small intestine	Taken between 35 - 45 cms
Lower small intestine	Taken from distal 10 cms
Caecum	Taken from main body
Ascending colon	Taken from proximal 5 cms
Descending colon	Taken between 15 - 20 cms
Liver	Taken from left lobe
Spleen	Taken from anterior half

FIGURE 2.4 Details of tissues examined for each assay.

Tissue	Assay				
	DNA estimations	Autoradiography	Stathmokinetics		
			Amosite	Negative Control	Positive Control
Squamous forestomach		X	X	X	
Glandular stomach	X				
Upper small intestine	X	X	X	X	
Mid small intestine	X	X	X	X	
Lower small intestine	X	X	X	X	
Caecum	X				
Ascending colon	X				
Descending colon	X	X	X	X	X
Liver	X				
Spleen	X				

Figure 2.4 shows the tissues examined in detail for each individual assay and is self-explanatory.

2.5.2 DNA extraction procedures

The procedures used for the extraction of DNA are presented below. Further details on the development of this method and its validity are available in Section 2.6.2.

2.5.2 a Sample preparation

(i) Tissue samples of approximately 100 mg wet weight were used. Care was taken to remove any macroscopically viable mesenteric fat. Peyers patches were also avoided.

(ii) Tissue was homogenised in 3.0 ml cold 10% trichloracetic acid (TCA) over ice using a mechanical teflon pestle and glass tubes.

(iii) 7.0 ml cold 10% TCA was added with mixing, and allowed to stand for 20 minutes over ice.

(iv) Sample was centrifuged at 3000 r.p.m. for 20 minutes in a refrigerated centrifuge and the precipitate was kept.

(v) The precipitate was resuspended in 10 ml cold 95% ethanol.

(vi) Sample centrifuged as before (see item (iv)).

(vii) Resuspended precipitate in ethanol as before (see item (v)).

(viii) Centrifuged as above.

(ix) Resuspended precipitate in 10 ml cold ethanol/ether mix (3 parts ether : 1 part ethanol).

(x) Centrifuged as above.

(xi) Resuspended carefully in 5.5 ml cold 6% TCA

(0.5 + 5.0 ml) using a hand homogeniser to break up the precipitate.

(xii) The suspension divided into two aliquots of 2.5 ml for further treatment. One aliquot was used for liquid scintillation assessment of the β particle activity, and the other for estimations of the total DNA content.

2.5.2 b Radioactivity assay

(i) The aliquot from item (xii) above was centrifuged at 10,000 r.p.m. for 20 minutes at $0^{o}C$, and the precipitate carefully separated and kept.

(ii) 2.0 ml in NaOH added to the precipitate.

(iii) Precipitate dissolved by heating at $90^{o}C$ for 30 minutes in a water bath.

(iv) 500 µl duplicate aliquots were added to liquid scintillation counter vials.

(v) 200 µl of 100 vol. hydrogen peroxide (H_2O_2) was added to each vial.

(vi) Samples capped and left overnight to oxidise.

(vii) 400 µl 3N HCl was added to each vial with shaking.

(viii) 10 ml NE260 scintillant added to each vial with shaking.

(ix) Samples left to stabilise for 4 hours at $4^{o}C$.

(x) Each vial counted twice for 20 minutes at $4^{o}C$ using a Beckman L.E.350 liquid scintillation counter.

(xi) Appropriate background and quench samples also counted.

(xii) Activity expressed as "disintegrations per

minute" (d.p.m.).

2.5.2 c Total DNA estimations

(i) The 2.5 ml aliquot was made up to 4.0 ml with cold 6% perchloric acid (PCA).

(ii) Resulting suspension was extracted for 20 minutes at 100°C in a water bath.

(iii) Sample centrifuged as before. Supernatant removed and kept aside.

(iv) Precipitate re-extracted with further 2.0 ml of 6% PCA.

(v) Sample centrifuged as before. Supernatants pooled and mixed, and precipitate discarded.

(vi) Triplicate 1.0 ml aliquots of supernatant were added to test tubes.

(vii) 2.0 ml Burton's diphenylamine reagent added to each tube.

(viii) Tubes incubated overnight at 37°C.

(ix) Colour development measured at 600 nm using spectrophotometer.

(x) Appropriate DNA standards prepared and assayed using calf thymus DNA.

(xi) DNA content of samples expressed as micrograms. (The activity of the samples therefore expressed as d.p.m. per µg DNA.)

2.5.3 Autoradiographic and stathmokinetic procedures

All assessments were performed upon 3 micron tissue sections prepared as described in Sections 2.2 and

2.3. Subbed slides (1% chrome alum gelatine) were used for mounting the sections and autoradiographs were prepared using the dipping emulsion technique of Joftes and Warren (1955). Slides were dipped in Ilford K dipping emulsion, exposed for two weeks at 4°C, and developed with Kodak developer. Cells were assessed as labelled if they had 5 or more grains over them. The counting techniques were basically those of Cairnie et al. (1965). Full randomisation of all slides was used to remove observer bias, and 100 crypts were counted for each tissue. Only complete axial crypt sections, in which the base, middle, and top of the crypt were in the crypt section, were analysed. Care was taken to ensure that each crypt was only assessed once, and only the left-hand column of cells of each crypt were assessed. The cells were numbered counting from the bottom upwards, and this described as the "crypt column". During the counting procedure the position of mitotic and/or labelled nuclei were recorded, with mitoses situated midway between right and left crypt columns being scored as half. The criteria of Clarke (1970) were used to identify the stages of mitosis, and only prophasic and metaphasic nuclei were scored: prophase was considered to be from the appearance of chromosomes or threads of chromatin until the disappearance of the nuclear membrane; metaphase until the start of chromatin separation; anaphase until chromatin masses were fully separated, and telophase until the reappearance of the nuclear membrane. The number of cell columns per crypt was found by counting the number of cells in crypt cross-sections, using only the approximately circular crypts. Tannocks factor (see Appendix III) was also measured for each tissue from

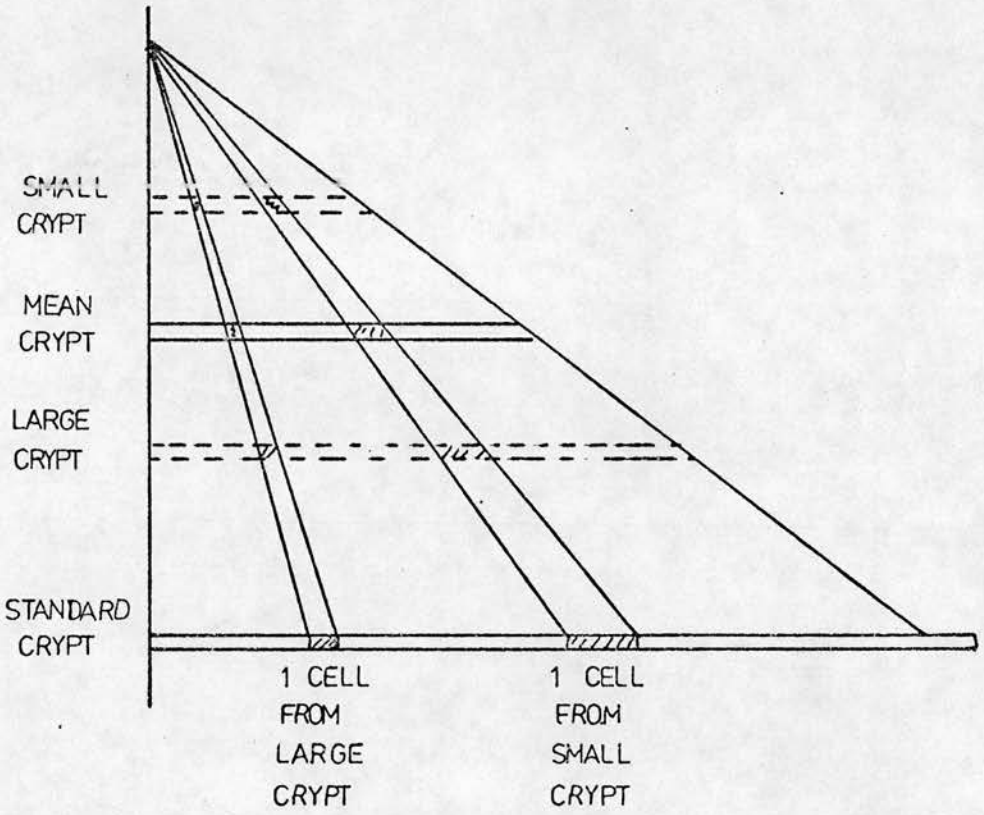
FIGURE 2.5 Format of data record sheets.

Animal No.: Slide No.:

Tissue: Sheet No.:

Cell Position	Crypt Section No.									
	1	2	3	4	5	6	7	8	9	10
1										
2										
3										
4										
•										
•										
•										
•										
•										
•										
•										
45										
TOTALS										

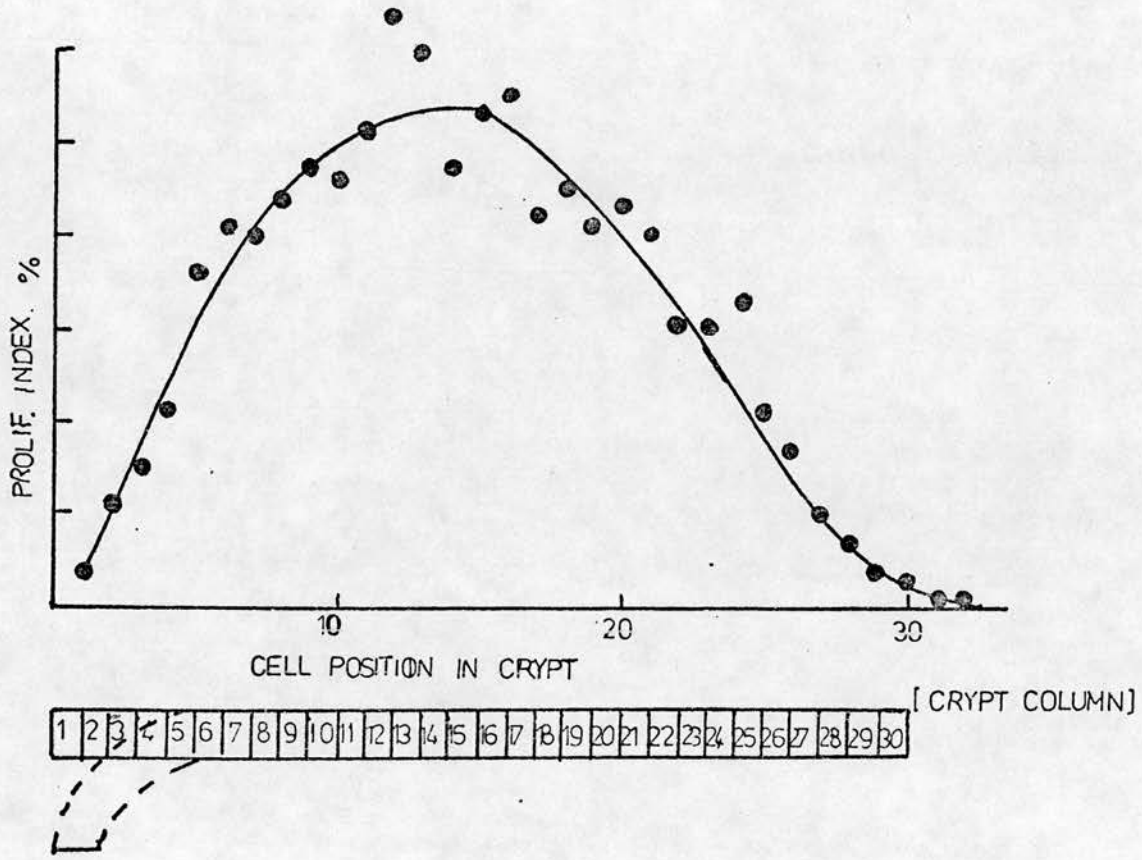
FIGURE 2.6 Procedure for crypt standardisation.



Diagrammatic representation of the crypt standardisation procedure used to compensate for the variation in crypt length when constructing proliferative index distribution curves.

(Redrawn from Wright, 1973)

FIGURE 2.7 Diagram of a specimen proliferative distribution curve and an associated crypt column.



(After Wright, 1973)

these cross-sections.

The data was recorded on sheets designed to facilitate the subsequent transposition directly onto punched cards (see Fig. 2.5). After card punching, the data was subjected to an ALGOL program run on an IBM 360/67 computer at Newcastle University, and a graphical output was produced for each tissue. The program "normalises" the data for each crypt by projecting the information onto a hypothetical crypt of 1.000 cells and then reducing this to a crypt of the tissue mean size for the expression of results. Fig. 2.6 shows the outline, and the full details of the procedure are given in Wright (1973). A specimen proliferative distribution curve is shown in Fig. 2.7.

Distribution curves were thus produced for each tissue examined in detail (see Fig. 2.4), and they formed the basis of the subsequent calculations. To avoid unnecessary duplication, the methods of calculation of various cytokinetic parameters and their interpretation are described in Chapter 6.

2.6 DEVELOPMENT OF TECHNIQUES

2.6.1 Preparation of tissue residues for electron microscopy

2.6.1 a Introduction

It can be seen that the results reported in Chapters 4, 5, 7 and 8 of this thesis rely heavily upon the electron microscopical examination of tissues for evidence of asbestos fibres, and consequently efficient methods of preparation were required. It is generally accepted in the literature that tissue ashing techniques are the simplest and quickest method of

reducing the bulk of the samples to manageable proportions. As a consequence of this, their suitability for the present investigation was examined. This exercise confirmed the efficiency of ashing techniques but a high level of contamination of samples with extraneous asbestos fibres was found. The presence of fibres in control tissue samples (i.e. tissue from animals known to have had no previous exposure to asbestos, taken under the strictest dust-free conditions) showed that the fibre contamination occurred during sample preparation. Exhaustive efforts were made to remove the sources of contamination by isolating the samples where possible, but asbestos could still be detected in all samples. The main problem was that the Institute facilities employed for the extraction procedures were simultaneously being used specifically for the treatment of large numbers of asbestos preparations and as such cross-contamination was inevitable. Although small, the levels of contamination were considered to be unacceptable, since it was the aim of the experiments to see if <u>any significant</u> asbestos penetration or transport had occurred. The experimental tissue samples were thus stored in a deep freeze until appropriate extraction methods could be developed. The simplest solution was to acquire duplicate ashing and associated apparatus and to use this apparatus in strict dust-free conditions. However, such a solution required a level of expenditure that necessitated a full investigation of the unsuitability of alternative methods. Consequently, the feasibility of the use of "wet" methods of tissue extraction was examined.

2.6.1 b Wet extraction methods

The primary aim of the extraction procedure was to reduce the bulk of the tissue requiring electron microscopical examination, but an equally important consideration was that the methods should not substantially alter any contained asbestos so rendering it unidentifiable. On this basis, prolonged chemical degradation of tissue with reagents such as concentrated hydrochloric and hydrafluoric acids could not be used (see Chapter 3). Many different reagents were, however, examined: trichloracetic acid, perchloric acid, ammonium acetate and acetic acid, formemide, strong caustic, sodium hypochlorite, soluene.

All reagents were used in excess, with two changes of each at three different temperatures (20°, 50°, 80°C) for varying times up to three days. Combinations of several of the reagents were also tested. The relative success of the reagents was assessed as the percentage of the original dry weight remaining after treatment. The absolute effectiveness of the various methods was limited by lack of equipment such as homogenisers, mechanical shakers, reflux apparatus, centrifuges, and the findings must be viewed with this in mind. The test tissues chosen were lung and liver since they are mainly parenchymal tissues that would be least affected by the lack of homogenising facilities. In general, the best results were obtained with tissue that had been defatted with xylene, dried and "powdered". The most successful procedure was found to be that using soluene: defatted dried tissue, three separate treatments with soluene for 24 hrs at 20°C, and one treatment with hydrogen peroxide with added octanol antifoam

reagent. About 4% of the original dry weight was left after this procedure. The procedure took seven days involving five separate solution changes, each requiring centrifugation, and the results must be compared with the simpler ashing process: 4% of original remaining after "wet" methods as compared with 0.1% after ashing.

2.6.1 c Ashing methods

On the basis of the above results the appropriate facilities for tissue ashing were acquired, and it proved possible to process samples without contamination. However, samples of control tissues were processed in parallel with each batch of experimental tissues to screen for any possible contamination. Batches in which controls were found to contain fibres were discarded. The treatment of ashed samples and their subsequent preparation for electron microscopy is an art that depends to a large extent on experience. The method offered muffle ashing and/or cold ashing, together with acid washing of residues.

Muffle ashing involved heating the samples to 380°C in a restricted oxygen environment that prevented excessively vigorous combustion. The temperature was set at 380°C to allow full oxidation of carbon whilst keeping the potentially damaging effects of heat upon asbestos to a minimum. "Cold ashing" may be defined as the process whereby low temperature gaseous plasmas are used to gently ash samples. In the present study, the plasma, a partially ionised oxygen gas, was produced by high frequency radio-wave excitation in a Nanotech Plasmaprep unit, and the operating temperature was kept below 100°C.

In practice, it was frequently found

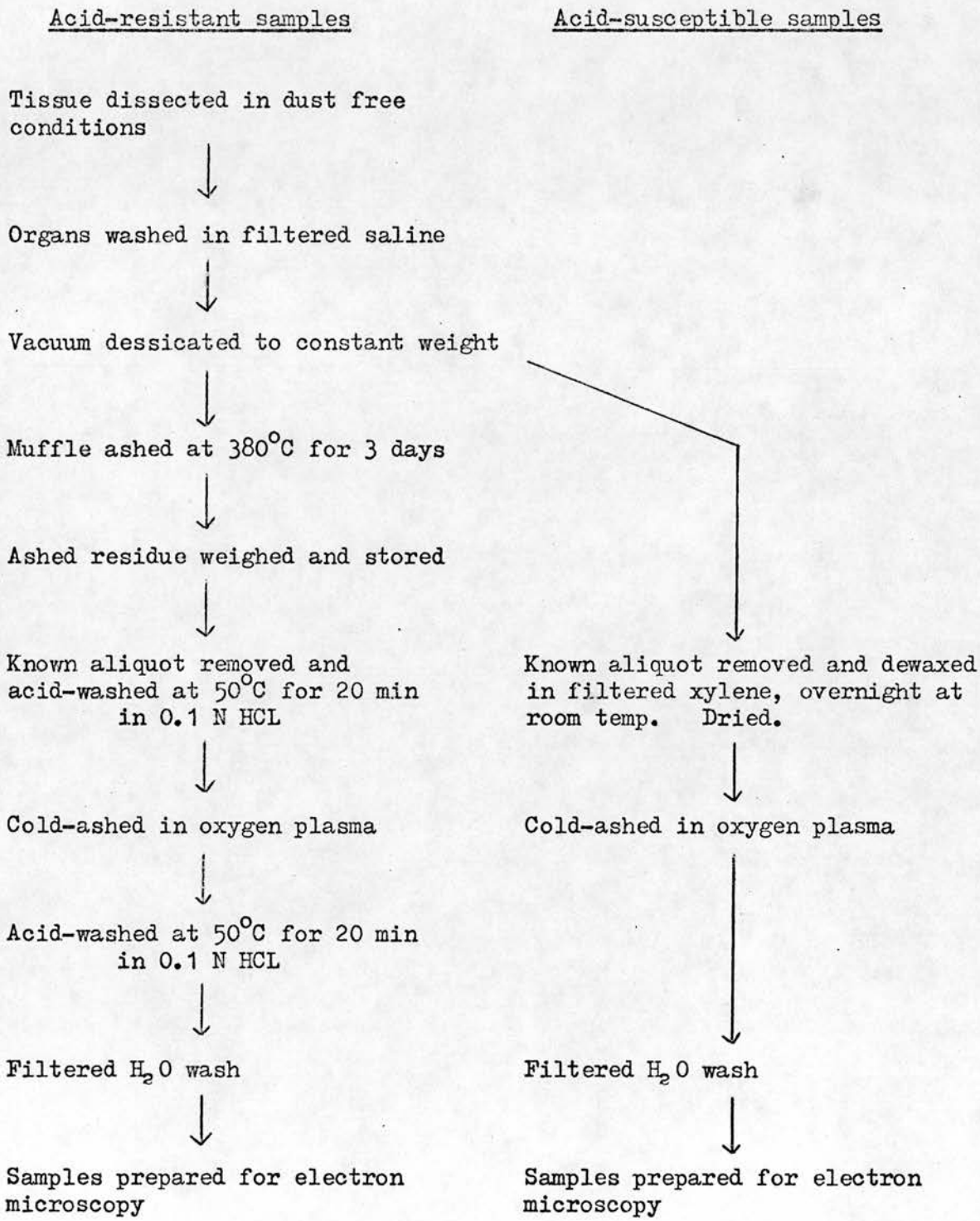
that even with long periods (longer than five days) of muffle ashing, there remained sufficient amounts of refractory carbon to require cold ashing. It was also found that cold ashing was unsuitable for the treatment of large samples, presumably since the oxidation is a surface-dependent etching phenomenon. The muffle ashing procedure was therefore used, where necessary, to reduce the bulk of the residue, and cold ashing for a secondary and final treatment. However, some tissue samples were considered to require full cold ashing treatment due to the potential heat susceptibility of any contained asbestos: for example, samples from chrysotile-treated animals could have contained chrysotile subjected to the damaging effects of long periods of leaching, and hence muffle ashing and some of the acid washing steps had to be omitted. Examples of typical ashing preparation procedures are given in Fig. 2.8.

2.6.2 Statistical considerations of electron microscopical fibre searches

2.6.2 a Introduction

Any discussion of the relevance of the results of electron microscopical searches for fibres requires some quantitative estimate of their significance. It is a straightforward statistical exercise to compute the total number of fibres in a given sample when some are found in a portion of the sample, but a more involved calculation is necessary if no fibres are found. The following accounts have been designed to cope with both eventualities. The scanning and transmission electron microscope search calculations are described separately since they require different approaches.

FIGURE 2.8 Examples of typical ashing preparation procedures.



2.6.2 b The scanning electron microscope searches

Tissue from animals exposed to asbestos had to be reduced in volume to reduce the examination time, and this was achieved by various ashing and washing treatments (see Section 2.6.1). The final residue was subsampled and the subsamples examined under the SEM for the presence of fibres. If no fibres were found in the subsamples, a statistical expression must be derived that represents the number of fibres that could exist in the sample and escape the subsampling methods. It was assumed, for the purposes of calculation, that no ties would occur in subsampling (i.e. no subsample would have two fibres in it). This assumption would be reasonable where the total number of fibres in a sample is considerably less than the number of possible subsamples, and the sample is mixed thoroughly. A further assumption was that the subsamples were very small in relation to the total sample, so that they could be discarded after examination without appreciably altering size of the sample. This was found to hold in practice. The following expression was thus derived:

Assume x is the total sample weight.

y is the total weight of all subsamples examined.

z is an estimated number of fibres in the whole sample x.

n is the number of subsamples examined.

Therefore, if no ties occur in subsampling:

Number of possible subsamples with 1 fibre = z

and " " " " " no " = $\frac{nx}{y} - z$

Assuming that the subsamples were randomly chosen:

Approx. probability of no fibre in n subsamples is -

$$\left[\frac{\frac{nx - z}{y}}{\frac{nx}{y}}\right]^n = \left[\frac{nx - zy}{nx}\right]^n = \left[1 - \frac{zy}{nx}\right]^n$$

and,

Approx. probability of exactly one fibre in y subsample is -

$$n\left[\frac{nx - zy}{nx}\right]^{n-1} = \left[\frac{zy}{nx}\right]$$

A probability table was constructed for each experiment by substituting different values for z (estimated number of fibres in whole sample) in the above formulae. Accepting 0.9 as a practical probability of an event occurring, the maximum number of fibres that could have been present in the original samples could be taken from the tables. An example of the use of the probability table is given in Fig. 2.9 , using data taken from the crocidolite ingestion experiment reported in Chapter 4 .

2.6.2 c The transmission electron microscope searches

The preparation of tissue for transmission electron microscopy is described by Glauert (1973). Essentially it involves mounting thin sections < 0.1 μ cut from small blocks of tissue ($\simeq$ 1 mm^3) subsampled from the whole organs. The sections are subsequently examined microscopically and it is assumed that if a fibre or part of a fibre is present in the section it is almost certain to be identified. In order to quantify the probability of detecting fibres for any given density of fibres per

FIGURE 2.9 Specimen probability table (using crocidolite ingestion experiment results - see Section 2.6.2b).

	No. of fibres in whole sample, x				
	100	200	500	1000	2000
Probability of having missed the fibres	0.64	0.41	0.1	<0.01	<0.0001

Accepting a probability level of 0.9, it can be seen that there were probably less than 500 fibres in the residue from crocidolite exposed animals.

unit volume of tissue, the following assumptions are necessary: the block of tissue subsampled is approximately spherical and of a volume V μm^3. Sections are defined as thin slices of tissue $\simeq$ 0.1 μm thick taken from the block in strips, and each section represents a thin disc of tissue from within the block. Any asbestos fibres present are all assumed to be needle-like, and to be randomly distributed with uniform density throughout the whole tissue at a rate of λ per μm^3.

The following details are concerned with the chance of detecting fibres within a single disc of tissue: it is clear that any fibre whose centre lies within the disc of tissue will certainly be detected whatever its orientation. It is also obvious that certain fibres whose centres lie outside the disc but which are close enough and have the correct alignment, will pass through or enter part of the disc and hence be detected. An evaluation of the exact probability that fibres would be detected requires consideration of all those fibres whose centres lie anywhere in the proximity of the disc and it is therefore very complicated calculation. An approximation has been used which should give results accurate to within 5% - an easily acceptable practical level. The approximate method considers only the disc of tissue and the disc-shaped volumes of tissue of thickness μm which lie immediately on each side of the disc. The chance that a fibre whose centre lies in one of these adjacent volumes of tissue cuts into part of the disc is approximately 0.5. No fibres (or parts of fibres) will be seen in the disc if: (i) there is no fibre whose centre lies within the disc, and (ii) there is no fibre whose centre lies within the adjacent

disc-shaped volumes of tissue, and (iii) there are fibres whose centres lie in the adjacent volumes of tissue but their alignments are in the wrong direction.

If the number of fibres per μm^3 is λ on average, the chance that n will have their centres in a given volume, V μm^3 is:

$$e^{-\lambda V}(\lambda V)^n/n^1 \qquad \text{(assuming Poisson distribution)}$$

Thus the chance that no fibres have their centres in volume V is $e^{-\lambda V}$. The chance that no fibres will be seen in the disc can now be expressed as:

$$e^{-\lambda V_1} \times \left[e^{-\lambda V_2} + \sum_{n=1}^{\infty} (e^{-\lambda V_2} (\lambda V_2{}^n/n^1) \ (\tfrac{1}{2})^n \right]$$

where V_1 is the volume of the disc

V_2 " " " " " adjacent disc-shaped pieces of tissue.

The expression simplifies to:

$$e^{-\lambda V_1} \times e^{-\lambda V_2/z}$$

or

$$e^{-\lambda(V_1 + \frac{1}{2}V_2)}$$

The chance that one or more fibres or parts of fibre will be seen in the disc is:

$$1 - e^{-\lambda(V_1 + \frac{1}{2}V_2)}$$

If d discs are cut per block and k blocks sampled from each tissue, then, assuming λ constant, the chance that asbestos fibres will be detected is:

$$1 - \left[e^{-\lambda(V_1 + \frac{1}{2}V_2)} \right] dk$$

or

$$1 - \exp\left[-\lambda dk(V_1 + \tfrac{1}{2}V_2) \right]$$

Substituting for $V_1 = \pi(\frac{4}{5}r)^2 w$ and $V_2 = 2\pi(\frac{4}{5}r)^2 \iota$ gives:

$$1 - \exp\left[-\lambda dk\pi(\tfrac{4}{5}r)^2(w + \iota) \right]$$

where λ is the density of fibres per μm^3.

d " " number of discs per block.

k " " " " blocks cut overall.

π " " constant 3.14159.

r " " radius of the spherical block in μm.

w " " width of the disc.

ι " " half length of the fibres in μm.

m " " number of sections (0.1 μm) forming a disc.

Three figures are given (Figs. 2.10–2.12) which show the chance of detecting asbestos fibres for various values of the factors of interest. The value of r is taken as 620 μm, this being the radius of a block of tissue of volume 1 mm^3. The fibre lengths considered run from 1 to 100 μm and a range of values of $k\lambda$ from 0.2 to 2000 is given. An estimate of λ as given from:

$$\lambda = \frac{\text{estimated number of fibres in whole gut}}{\text{number of possible blocks per gut}}$$

Fig. 2.10 shows the probabilities for the case when only one disc of 20 sections is taken per block (i.e. m = 20; w = 2; d = 1). Fig. 2.11 is concerned with two discs of 10

FIGURE 2·10 CHANCE OF DETECTING ASBESTOS IN K BLOCKS (if one discs of 20 sections used)

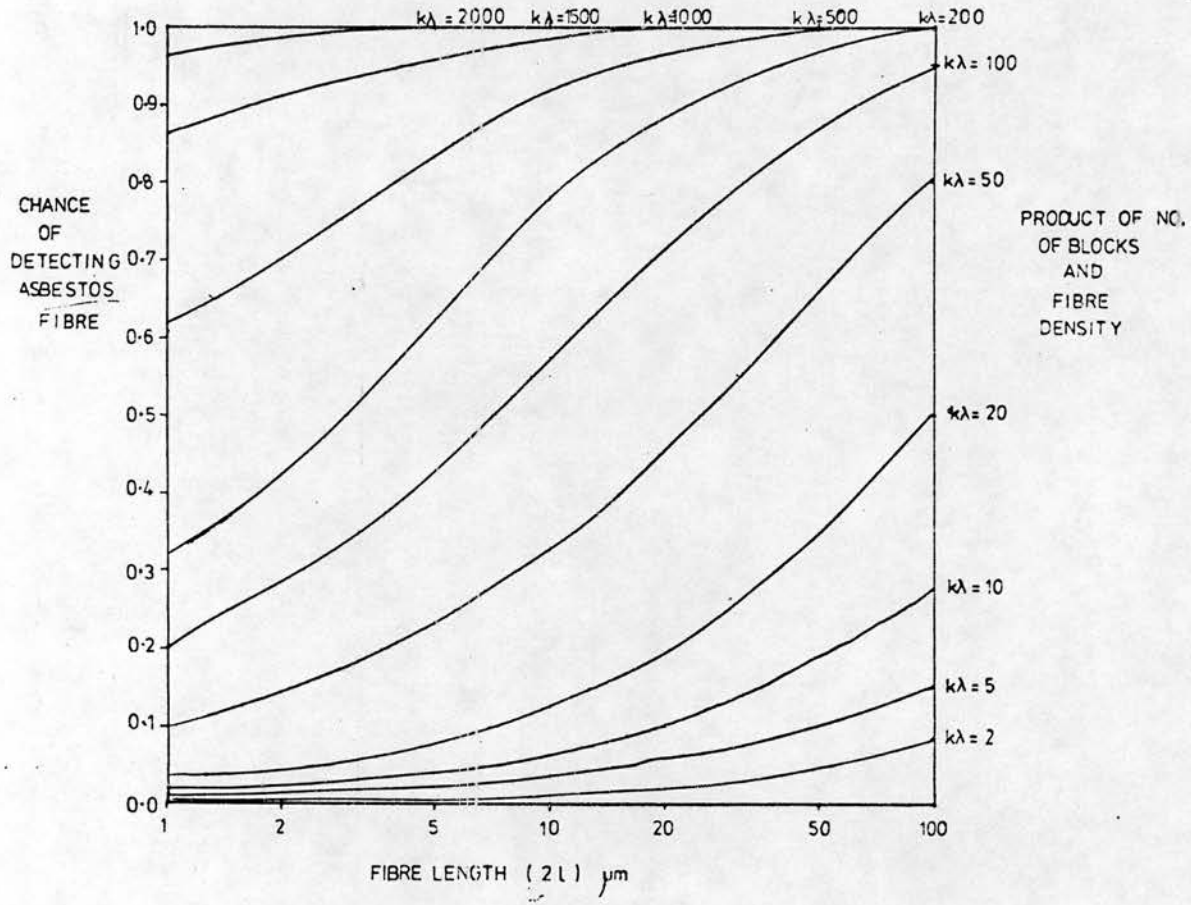


FIGURE 2·11 CHANCE OF DETECTING ASBESTOS IN K BLOCKS. (if two discs of 10 sections used)

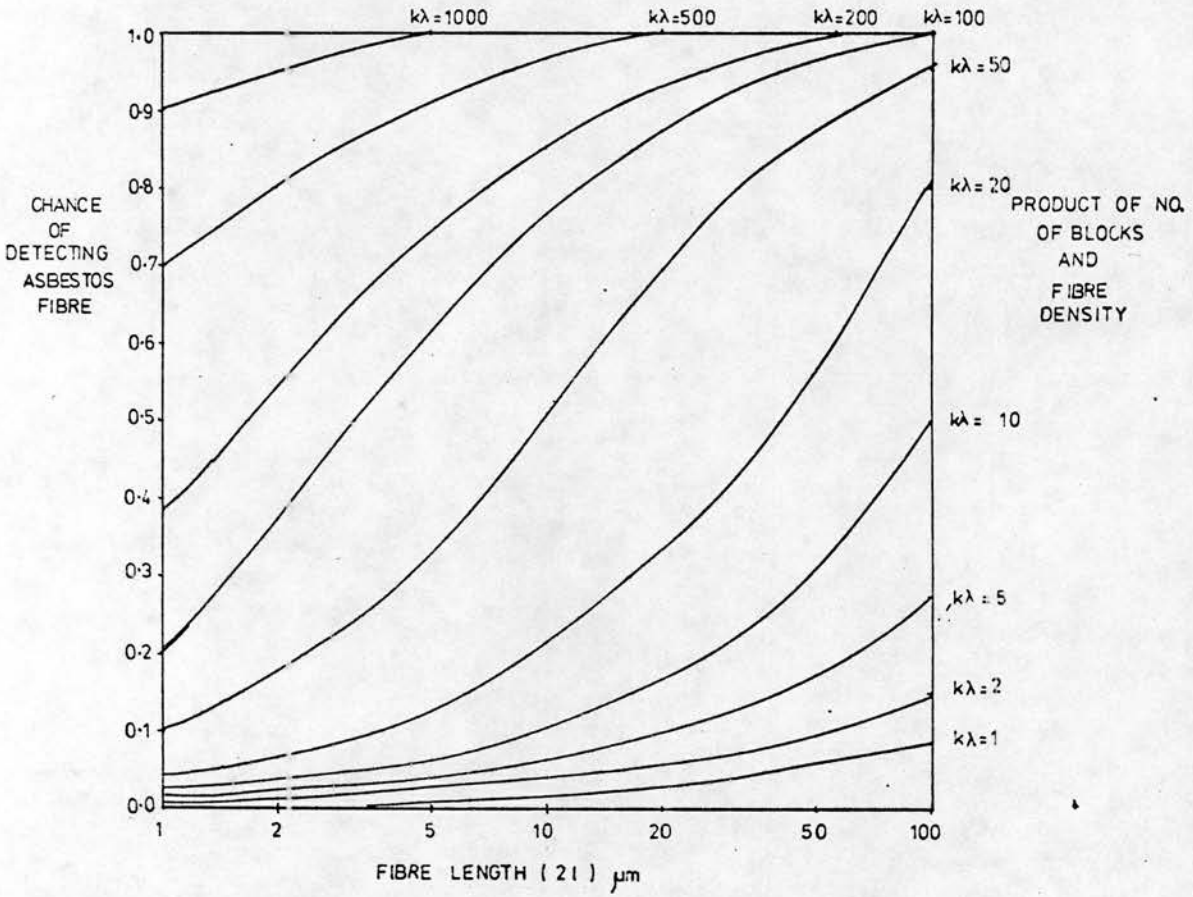
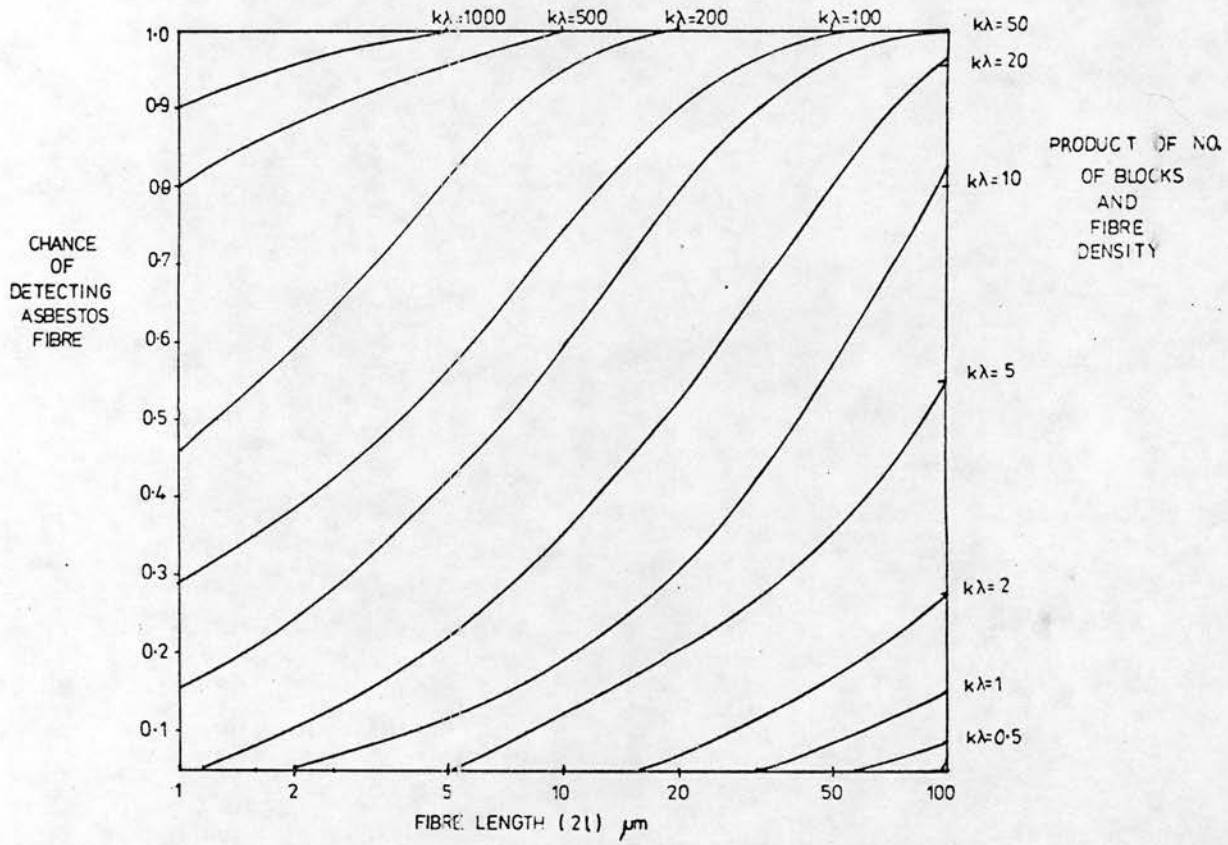


FIGURE 2·12 CHANCE OF DETECTING ASBESTOS IN K BLOCKS (if four discs of 5 sections used)



sections per block (m = 10; w = 1; d = 2), and Fig. 2.12 has four discs of five sections per block (m = 5; w = 0.5; d = 4). Note that for all three cases the total number of sections examined per block was 20 - a reasonable practical estimate of the microscope examination work undertaken for each sample.

Given the number of discs per block as one, two, or four, and given also λ and fibre length, the chance of detecting fibres can be set and the corresponding value of k (the total number of blocks to be cut) found. Alternatively the chance of fibres being detected after the examination of k blocks of tissue can be found.

For example, if λ is assumed to be 5 fibres per block, and the fibre length taken as 10 μm, the chance of detecting asbestos fibres in 10 blocks is 0.24, 0.37 or 0.57, depending upon whether 1, 2 or 4 discs are cut per block. Thus the chance of fibre detection increases if the sections are cut from several different levels of each block.

2.6.3 Development of DNA extraction techniques

2.6.3 a Introduction

The work reported in Chapter 6 of this thesis constitutes an attempt to investigate the proliferative responses of the gastrointestinal tract during asbestos ingestion. Although the emphasis has been placed upon the results of cytokinetic analysis, it was considered useful to include an assay on similar lines to that of Amacher and his colleagues (1974, 1975). Amacher's papers reported the results of asbestos ingestion experiments assayed by measuring the rate of incorporation of tritiated thymidine label

into DNA, using acid precipitation of DNA (see Section 1.3.3e and Appendix III). Their technique was itself a modification of that used by Shibko et al. (1967), and used perchloric acid for the DNA precipitation. An essential prerequisite of any method of quantitative extraction of DNA from tissues is naturally that the method should be both reliable and repeatable. Previous personal experience with acid precipitation methods of DNA extraction indicated that considerable caution was necessary to achieve an acceptable degree of repeatability, and so an investigation of the relative efficiencies of various extraction methods was warranted. The excellent reviews of Hutchison and Munro (1961) and Munro and Fleck (1966) clearly show that although acid precipitation techniques are beset with problems, they remain the most efficient available. The account that follows describes the modifications of Amacher's techniques used in Chapter 6, together with the reasons.

2.6.3 b Techniques

Amacher homogenised his tissue samples in cold sucrose buffer to minimise the enzymatic autolysis of DNA. The present work showed that successful homogenisation (as assessed by the absence of nuclei in Giemsa-stained smears) required a vigorous homogenisation that caused localised heating of the slurries despite the use of crushed ice or dry ice cooling. Up to 40% of the total DNA was lost as a result of this heating, and so an alternative homogenisation procedure was developed. This simply involved ultrasonic fractionation directly into chilled acid using a high speed motor driven teflon pestle and glass mortar. It was found that this method greatly reduced DNA loss, and there was no

acid damage of the apparatus.

The acid chosen for the homogenisation and precipitation was 10% trichloracetic acid (TCA) and not 2% perchloric acid (PCA) as used by Amacher. Two washings with 10% TCA were found to give a better recovery of precipitate than repeated PCA use, confirming the experience of many other workers (Moulé, 1953; Paul, 1958; Smillie and Kratkov, 1960). It was further found that, provided an excess of cold 10% TCA was used, efficient recovery occurred if the acid homogenate slurry was allowed to stand over ice for 20 minutes before centrifugation. Thus one wash with TCA was sufficient. Ten per cent TCA was chosen as the optimum concentration partly on the evidence of previous workers and partly in consideration for the potentially damaging effects of stronger acids on both the samples and the apparatus.

Two washes of cold 95% ethanol and one of cold ethanol-ether mix (3:1) were found to be necessary for the removal of lipid, phospholipid and lecithin components. This procedure was not found to damage the acid precipitate provided cold reagents were used throughout.

Development trials showed that less than 1% of the radioactivity of the tissue samples could be attributable to RNA activity and so the alkali RNA digestion step employed by Amacher et al. (1974) was omitted. This was a particularly useful omission since it avoided the real risk of simultaneous DNA degradation and loss associated with the use of even mild alkalis.

A potentially large source of error

was found to exist at the point where the washed and "purified" DNA-containing extract was divided into two equal aliquots for separate radioactivity and total DNA estimations. The major reason for this was that the precipitate was sticky and difficult to handle after TCA use, a factor considered by other workers to preclude the use of TCA. However, a secondary homogenisation in cold acid using a hand-held ground glass homogeniser was found to produce a fine slurry that could be easily sampled.

A 6% PCA extraction at 90°C was used to obtain a protein-free deoxypentose solution for DNA assay since the resulting solution produced a more uniform colour change (than TCA extraction) when assayed. Three separate 90°C extractions were found to be necessary for the effective solubilisation of DNA from the residues, and the supernatants of each extraction were pooled for assay. Burton's modification of the Dische diphenylamine assay for deoxypentose sugars was used to assess the total DNA per sample (Burton, 1956; Dische, 1930). A temperature of 37°C was used (instead of 30°C) owing to the availability of the appropriate incubator, and tests showed that incubation at 37°C produced a stable colour change between 16 - 34 hours. The use of fresh diphenylamine reagent and standard calf thymus DNA solution for each experiment avoided problems arising as a result of any alterations in colour development specificity.

One further modification adopted for the reported work concerned the radioactivity assay. Amacher used the PCA extracts prepared as above for the β particle counting in a liquid scintillant. Trials showed that PCA extracts produced

an unacceptably high degree of chemiluminescence and quenching, despite prior neutralisation of the acid. A more reliable assay was developed using hot alkali (IN NaOH at 90°C for 20 minutes) solubilisation of the acid precipitate, followed by overnight bleaching with hydrogen peroxide. Tritium activity was assessed using NE_{260} scintillant cocktail, counted at 4°C in duplicate. Separate tests showed that the radioactivity of the whole acid precipitate could reasonably be attributed to the tritium label located specifically within the DNA component.

Full details of the extraction procedures are given in Section 2.5.2. Three separate samples of each tissue were processed to give the results presented in Chapter 6.

CHAPTER 3 SOME ASPECTS OF THE STABILITY AND IDENTIFICATION OF ASBESTOS FIBRES

SOME ASPECTS OF THE STABILITY OF ASBESTOS FIBRES

3.1 INTRODUCTION

A description of certain aspects of the chemical stability and reactivity of asbestos formed a necessary prelude to the studies reported elsewhere. The results of the various experiments described in Chapters 5, 7 and 8 depend heavily on the ability to isolate and identify asbestos fibres in tissues, and relate the levels of any fibres found to pathological abnormalities. It was therefore vital to study the effects of the various tissue extraction methods on any asbestos fibres present, since there was little point in reducing the bulk of tissue to be examined at the electron microscopical level if, in so doing, any contained fibres were changed unrecognisably. It was similarly important to study the effects of prolonged periods of tissue residence on the stability of asbestos fibres in case this made them particularly susceptible to breakdown.

A large number of individual tests were performed in the course of this work. Most are described in general terms since they frequently duplicated or corroborated other published accounts of the chemical reactivity of asbestos. The descriptions have been further limited to cover the three main types of UICC asbestos: amosite, crocidolite, and chrysotile. The principal instrumental analytical system that became available for the characterisation of alterations to asbestos fibres was transmission and scanning electron microscopy with a microanalytical attachment to the scanning electron microscope only. A brief description of the advantages and limitations of energy dispersive analysis is included in a separate section, since some caution is necessary in the interpretation of spectra so produced.

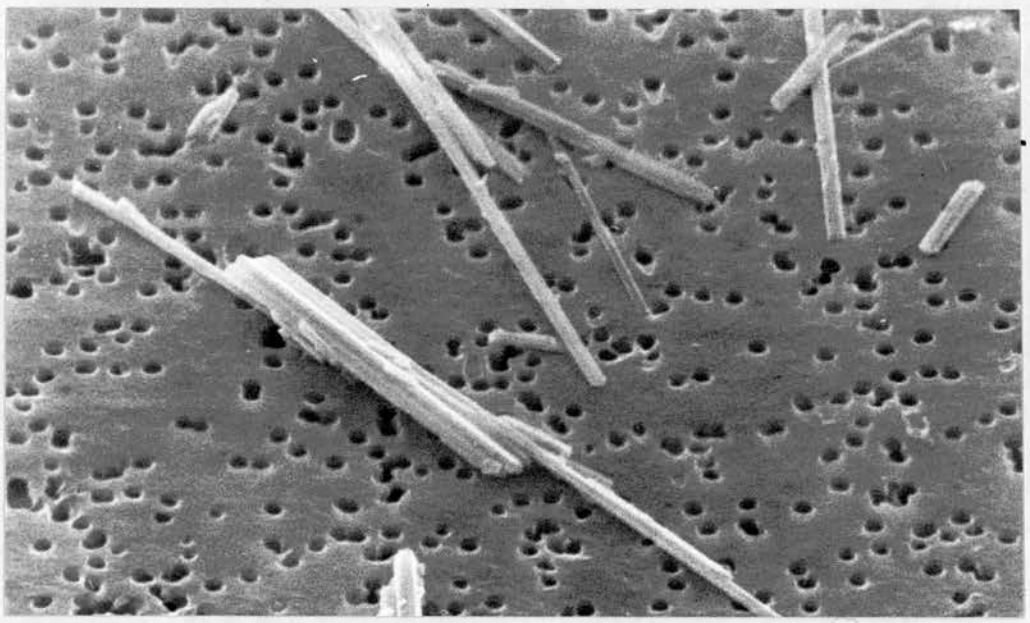


Figure 3.1 Scanning electron micrograph of UICC amosite asbestos. ⊢——⊣ 4 μ

Figure 3.1b Transmission electron micrograph of UICC amosite asbestos. ⊢——⊣ 4 μ

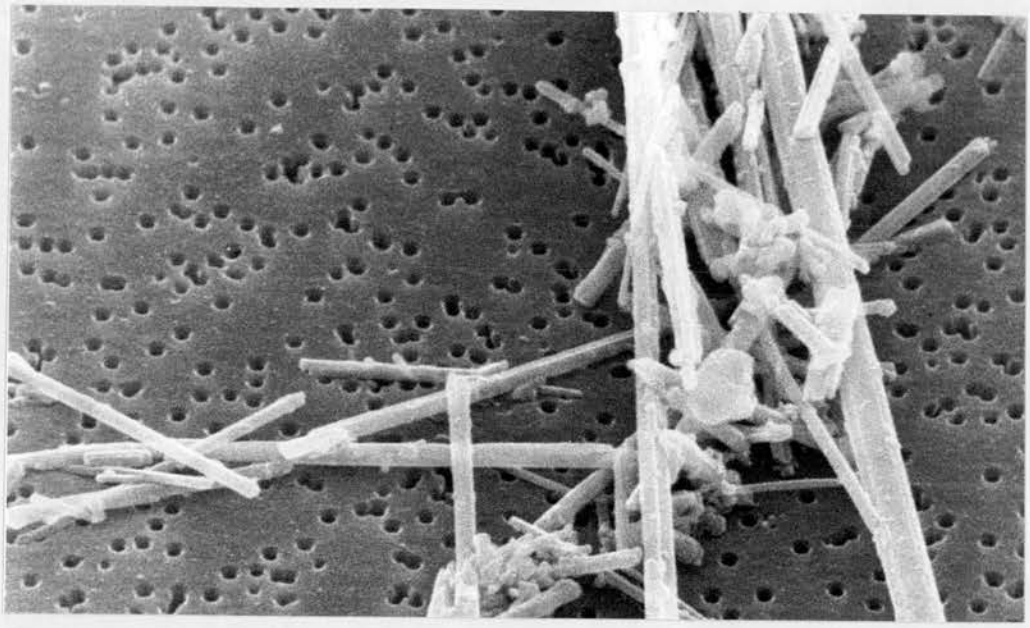


Figure 3.2a Scanning electron micrograph of UICC crocidolite asbestos. ⟵⟶ 4μ

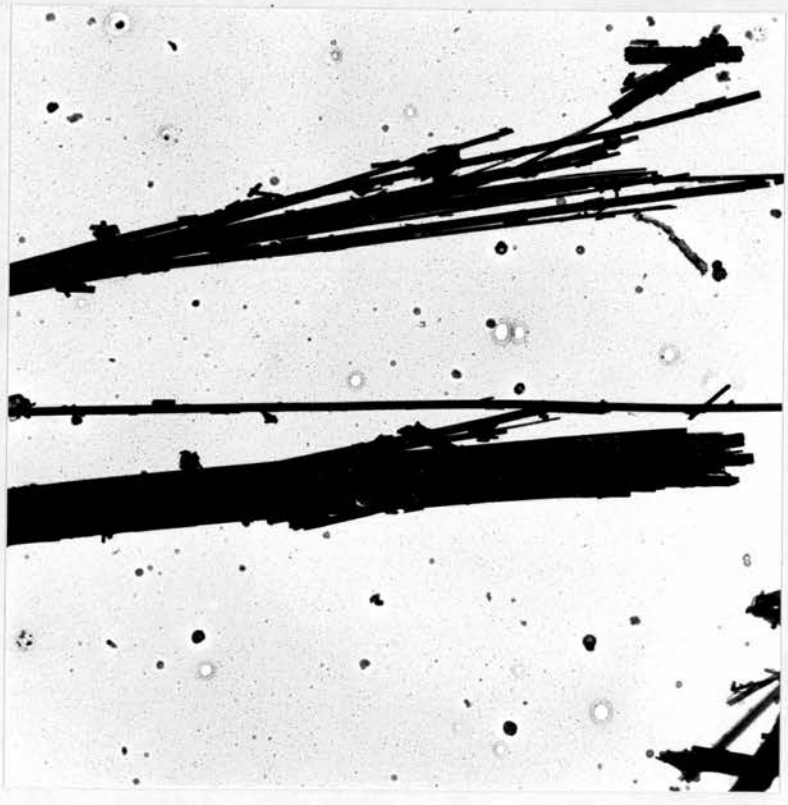


Figure 3.2b Transmission electron micrograph of UICC crocidolite asbestos. ⟵⟶ 4μ

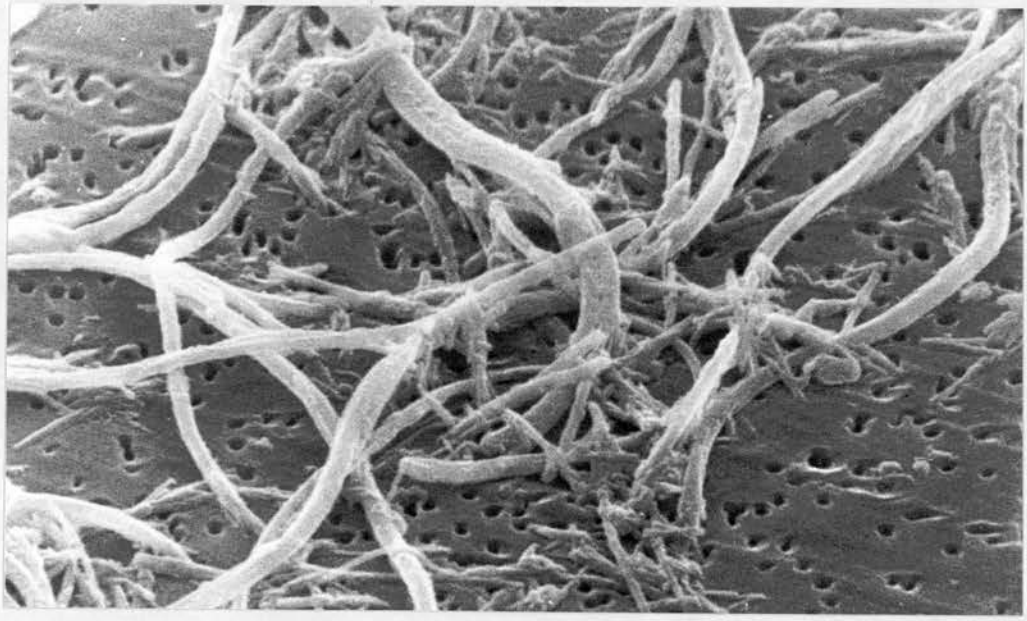


Figure 3.3a Scanning electron micrograph of UICC chrysotile asbestos. ⟵⟶ 4μ

Figure 3.3b Transmission electron micrograph of UICC chrysotile asbestos. ⟵⟶ 4μ (⟵⟶ 1μ inset)

3.2 ENERGY DISPERSIVE ANALYSIS

The energy dispersive analytical attachment to the S600 scanning electron microscope depends for its operating principles on the excitation and subsequent recovery of the electron configurations of individual atoms. The excitation is produced by means of a high kilovoltage electron source (in this case 25 Kv) which is focussed upon the specimen situated *in vacuo* on specimen stubs in the electron microscope, and the characteristic X-rays emitted by the individual constituent atoms are collected and analysed by means of a detector situated a small distance away. A more detailed description of the process is available in the work by CHANDLER (1977). The sensitive solid state detector is isolated from any potentially damaging contamination within the microscope column by a thin window of beryllium through which any X-rays emitted by the specimen has to travel before it can be amplified and identified. This beryllium window is thus a potential source of loss of sensitivity since it prevents some of the X-ray emissions from reaching the detector. In practice, the 'cut off' attributed to the window precludes the identification of such elements as oxygen, nitrogen, carbon, etc., but it also has some effects on identification of elements such as sodium, magnesium and aluminium.

Another source of a loss of sensitivity arises from the need to coat non-conducting specimens with a layer of conducting material prior to their irradiation. Many different conducting materials can be used, but in practice a layer of carbon, or gold, or a combination of these, were used routinely. Technical difficulties encountered with the everyday use of carbon coating techniques made the gold

sputter coating the method of choice, since this was both simple and efficient. The problems with the addition of a layer of gold (approx. 200 A^{o} thick) to the specimen are: firstly, the elemental gold itself produces strong emission spectra that can give rise to difficulties of interpretation of some closely adjoining spectra, and secondly, both the primary electron beam and the secondary emissions are required to penetrate the layer of artificially applied heavy metal before reaching the detector. These factors result in some loss of sensitivity, as can be seen from Figures 3.5 to 3.7 in which asbestos fibres have been analysed before and after coating, and there is a small but distinct decrease in the relative heights of some of the minor constituent elements' peaks.

One of the most serious limitations on the use of the energy dispersive analysis attachment to a scanning electron microscope is that quantitative analysis is only theoretically possible on infinitely flat and smooth specimens in which the constituent elements are randomly and evenly distributed. Since this seldom occurs in practice, certainly not in the case of asbestiform fibres, there are real problems in attempting to quantitatively analyse particles. As an approximation, it is only possible to relate the heights of the peaks produced by two elements to their abundance if they produce peaks that are of similar X-ray energy value. Thus referring to Figure 3.5a, it is possible to conclude that amosite contains a lot less magnesium than silicon, but it does not necessarily follow that the iron content is proportionally higher than magnesium. It will be appreciated that there can therefore be very real problems over the accurate identification of any fibres found in samples. It is

particularly difficult to discriminate between amosite and crocidolite fibres, where the main difference is in the relative levels of minor constituents such as sodium and magnesium.

3.3 ELECTRON OPTICAL EXAMINATION OF UNTREATED UICC ASBESTOS

Although phase contrast microscopy is frequently advocated for measuring the numbers of asbestos fibres in the working environment, it is widely accepted that the majority of asbestos fibres are below the resolution limits of optical microscopy, and that the electron microscope is necessary for any detailed morphological examination of individual asbestos fibres. Accordingly, Figures 3.1 to 3.3 show both transmission and scanning electron micrographs (TEM and SEM) of UICC samples of the three main types of asbestos.

The TEM micrographs of amphibole fibres show them to be electron dense, parallel sided and generally straight. The different fibres types tend to range characteristically in thickness and electron translucency, but the individual amphibole fibres cannot be accurately identified from TEM micrographs alone, and micro-analytical techniques are necessary. The TEM micrograph of chrysotile fibres shows them to be of unique fibrillar morphology in which an internal central capillary is surrounded by electron dense walls formed from rolled-up sheet lattices. An electron translucent amorphous material has been described both within and between chrysotile fibrils, particularly where the fibrils occur in large bundles. A feature of the larger chrysotile fibres is that the individual component fibrils can frequently be seen separating from the fibre ends. The morphology and the characteristic dimensions of chrysotile fibrils

enables the TEM to be used for a positive identification without the necessity to resort to microanalytical methods.

The SEM micrographs of amphibole fibres show them to be straight and needle-like, with a lathe-like arrangement discernible in some of the larger fibres, particularly of amosite. Most of the thinner amphibole fibres can be adequately identified and examined with the SEM. The SEM micrograph of chrysotile fibres clearly shows their curly nature. Although it is possible to see the splayed-out condition of the ends of many fibres, it is not possible to accurately resolve individual chrysotile fibrils within the bundles, and their characteristic tubular structure is not visible.

Selected area electron diffraction (SAED) has been used for the accurate identification of crystal structures, and the methods are applicable for the identification of individual asbestos fibres in a high voltage transmission electron microscope. The diffraction (SAED) pattern is produced by the interference of incident parallel monochromatic radiation (electron beam) with the radiation-scattered atoms in a crystal lattice array. The configurations that arise from the reflection of the incident radiation from the atomic planes in a crystal structures are in the form of an array of spots. The spacing of the spots is inversely related to the spacing of the planes in the crystal lattice, and the angle between the rows of spots is identical to the angle between corresponding crystal planes. Fig. 3.4 shows an example of an SAED pattern for UICC amosite asbestos. One of the major problems with the routine use of SAED is that the patterns produced are highly dependent upon the orientation of the crystals in the electron beam, and only certain orientations are suitable for

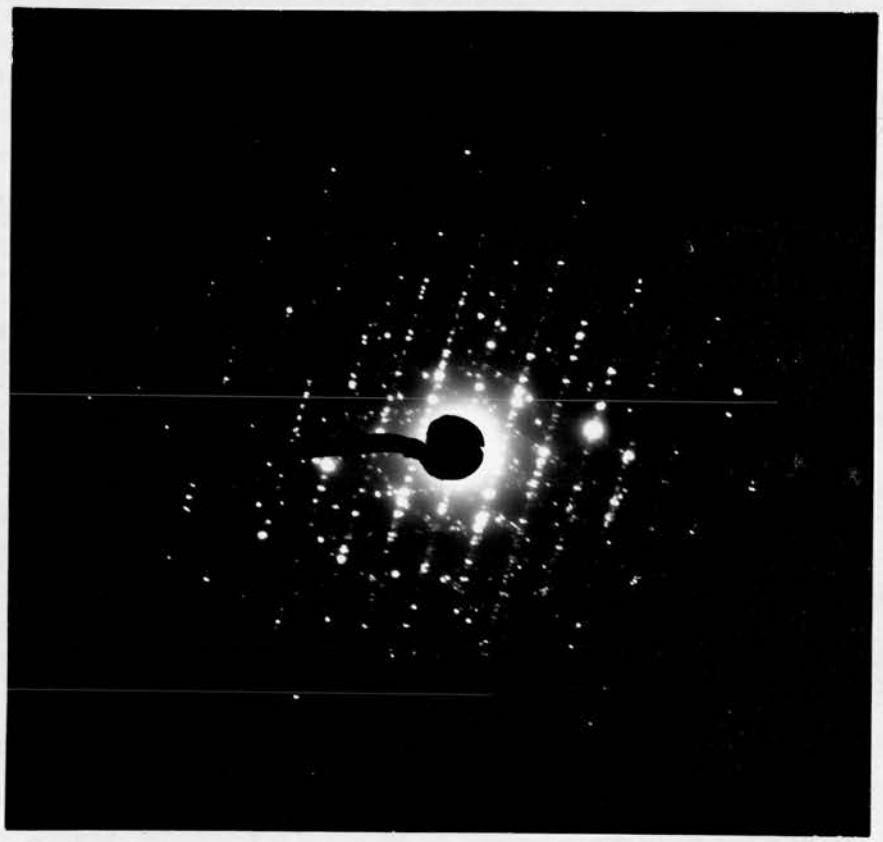


Figure 3.4 Typical selected area electron diffraction (SAED) pattern of amosite asbestos.

FIGURE 3·5

EDAX spectra of untreated amosite asbestos (UICC)

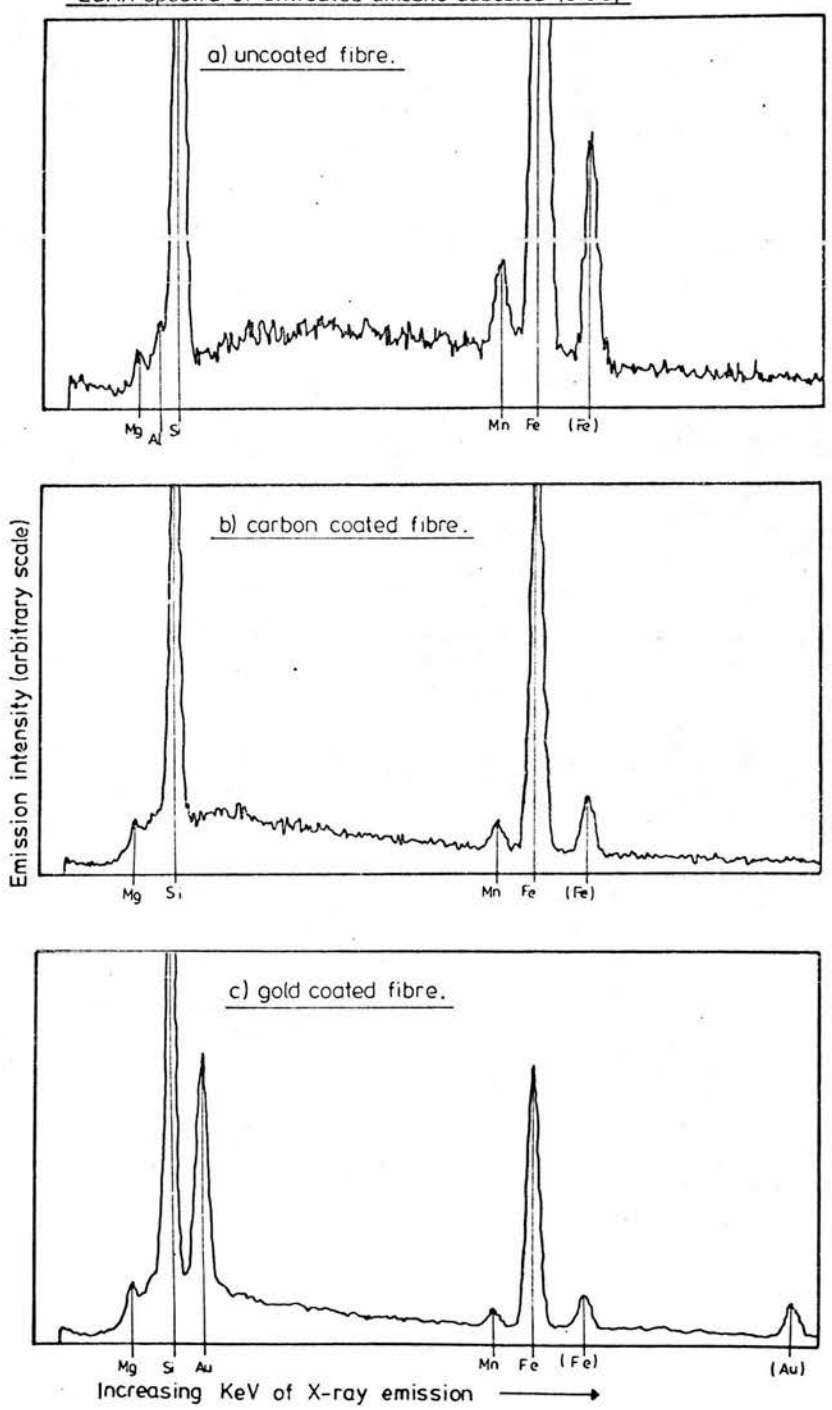


FIGURE 3·6

EDAX spectra of untreated UICC crocidolite asbestos.

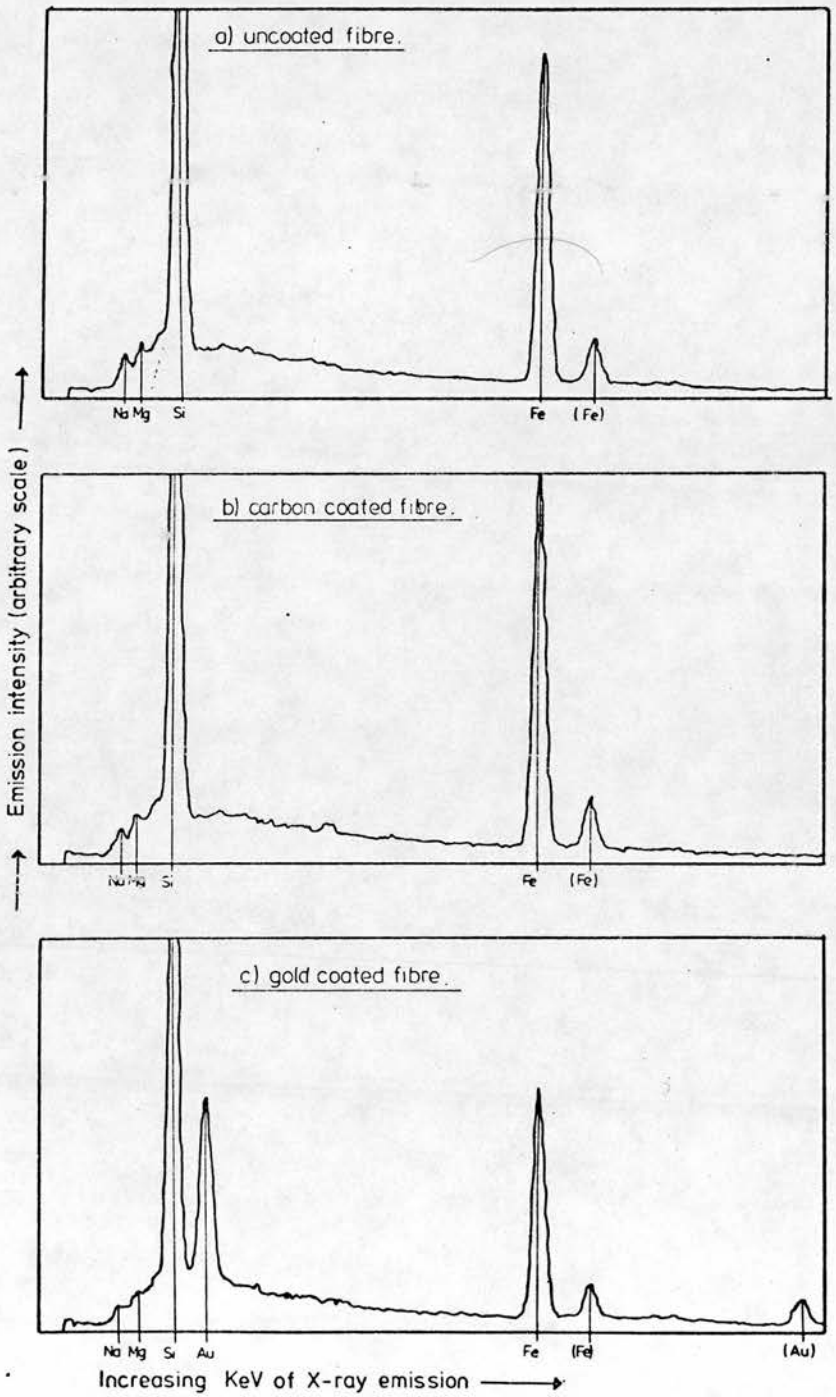
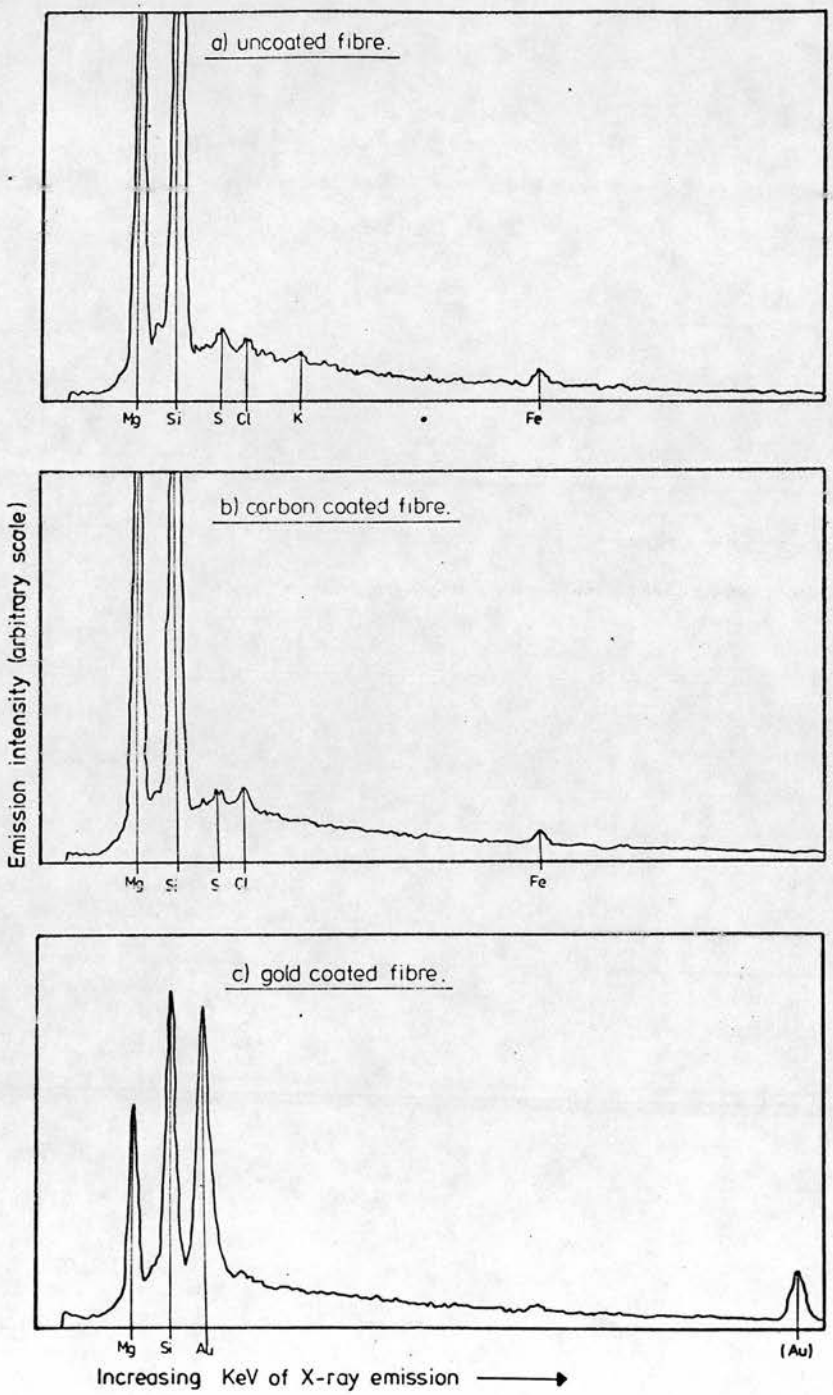


FIGURE 3·7

EDAX spectra of untreated chrysotile asbestos.



identification. This, combined with difficulties associated with the interpretation of the patterns, has meant that SAED has been largely superseded by EDAX analytical methods in recent years.

Figures 3.5 to 3.7 show the EDAX spectra of untreated samples of the three main UICC asbestos samples prepared for analysis under a variety of conditions and analysed in a scanning electron microscope. It can be seen that microanalysis of uncoated fibre samples provided the best spectra, in which even the minor constituent elements gave rise to distinct peaks. The addition of a surface coating of electron conducting material tends to reduce the discriminatory ability of the routine EDAX analyses to identify some of the minor constituents, and the effect can be particularly noticeable when the fibres are covered with a layer of gold (see Figures 3.5 to 3.7).

The EDAX spectra for UICC amosite asbestos in Figure 3.5 show magnesium, silicon, manganese and iron to be the principal constituents. Figure 3.6 shows that sodium magnesium silicon and iron are the principal constituents of UICC crocidolite. It can be seen therefore that the ability to discriminate between these two amphibole asbestos types depends on the fact that crocidolite contains some sodium but no detectable manganese, and amosite contains some manganese but no detectable sodium. Since these elements are amongst the minor constituents of the fibres that are near the lower end of the resolution limits of the EDAX detector system as fitted to the SEM, they could easily be overlooked in routine samples. Figures 3.5 to 3.7 show that the addition of a coating of gold can further reduce the resolution of the detector system. The EDAX

spectra for UICC chrysotile (Figure 3.7) show that magnesium and silicon are the principal constituents, and that a small peak of iron is also present. It is therefore relatively simple to discriminate between chrysotile asbestos and the amphiboles using the EDAX detector, provided the individual fibres can be recognised.

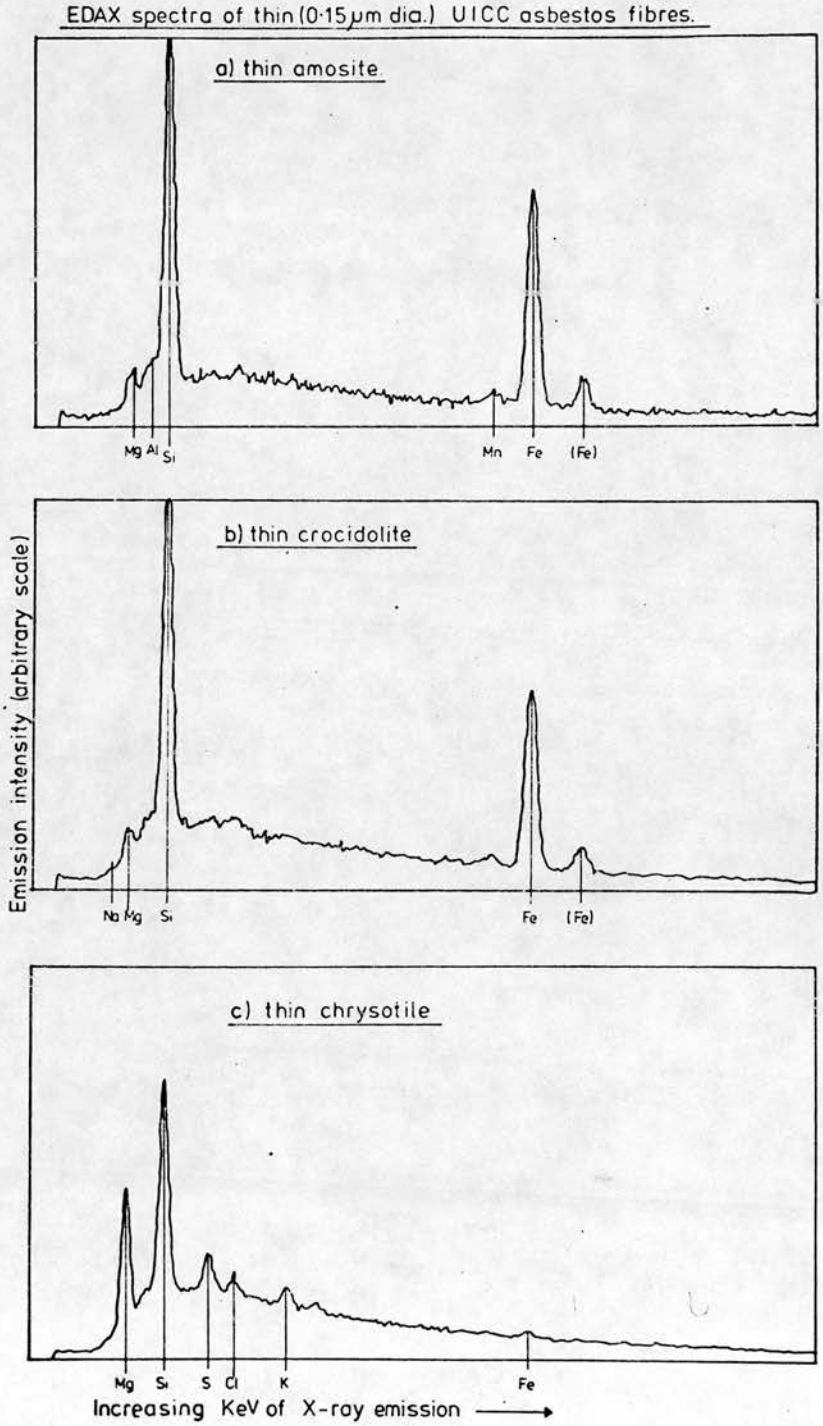
Figure 3.8 shows the effect of fibre size on the discriminatory ability of the EDAX analysis. The spectra were obtained by analysis of small fibres of less than 0.15μ diameter, and when compared with Figures 3.5 to 3.7, they illustrate the loss of sensitivity resulting from difficulties in accurately positioning the microprobe on very small objects. The loss of sensitivity can be even more pronounced if the very small fibres are coated with a layer of gold.

3.4 CHEMICAL REACTIVITY OF UICC ASBESTOS

Identification of asbestos fibres depended firstly on a morphological selection of those particles amongst ashed residues (see section 2.6.1c) that were of the correct dimensions to be asbestiform, and secondly on an EDAX analysis of those particles. This account is therefore restricted to describe the influence of various chemical treatments upon those aspects of the morphology and composition of asbestos fibres that are likely to affect their identification. The treatments may be separated into those of a biological nature such as effects of serum incubation, gut transit, and periods of tissue residence, on asbestos minerals; and those of a chemical nature such as effects of acids, alkalis, prolonged heating, and prolonged liquid storage on fibres.

FIGURE 3·8

EDAX spectra of thin (0·15 μm dia.) UICC asbestos fibres.



3.4.1 Biological treatments

A short term study in which small samples of asbestos were incubated with sterile serum at 37°C for periods up to 4 weeks and then examined by electron optical methods, failed to find any obvious morphological effects. Subjectively, there was some tendency for the larger chrysotile fibre bundles to be slightly more open ended after 4 weeks incubation with serum, but EDAX analysis showed no alterations in chemical composition.

The effects of transit along the gastrointestinal tract upon the UICC asbestos samples was examined using rats. The animals were prestarved for 48 hours, given 25 mg samples of asbestos by intraoesophageal injection (gavage), and killed serially thereafter over the next 24 hours. Apart from some remnants in the caecum and colon, the gut lumen was devoid of food debris as a result of the starvation regime. Electron microscopical preparations of fibre from different anatomical regions of the guts of those animals killed after gavage could thus be simply made from aqueous suspensions of the lumenal contents. This experimental system avoided the need for extraction procedures (as described in section 2.6.1) to isolate the fibres from food debris. Although gavage of prestarved animals could not be considered to reflect a normal physiological state, it was interesting to note that the pH of lumenal contents was found to be the same as that of animals maintained under normal dietary conditions.

Examination of the chrysotile fibre samples from the gut preparations strongly suggested that chrysotile fibrils

were more common, and the larger fibre bundles were less compact after a period of residence in the gut lumen. It appeared that this separation of the fibrils occurred in response to the gastric secretions, and no further alterations occurred as the fibres travelled along the gut. No sign of similar changes were found in the amphibole samples. EDAX analyses showed that the transit of asbestos was associated with small but definite increases in the sizes of the sodium, chlorine, and calcium peaks in all fibre types, the effect being most noticeable with chrysotile.

The effects of long periods of tissue residence on asbestos fibres was restricted to an examination of those animals in the subcutaneous injection experiments reported in Chapter 7. There was a substantial alteration in the morphology of chrysotile samples prepared from the injection site debris in that larger fibre bundles were not as common as they were in untreated UICC samples, and distinct fibrils were more difficult to find in the SEM preparations. Amphibole fibres were morphologically unaffected by prolonged tissue residence. The EDAX analysis showed that the composition of individual fibres tended to vary widely in any given sample, the most obvious differences being in the sizes of the iron peaks. It was noticeable that those asbestos fibres with indistinct ill-defined edges had a tendency to have larger iron-derived peaks, with the amphiboles also having larger magnesium-derived peaks. It is tempting to suggest that these findings were the result of some fibres being coated with biological material containing iron and magnesium, but this was not possible to prove quantitatively with the EDAX

attachment to the SEM. (A TEM attachment and full analytical facilities are necessary for detailed compositional analyses - see section 3.2.) One further consistent finding amongst all the asbestos samples was that some fibres tended to have quite large calcium-derived peaks.

3.4.2 Chemical treatments

The effects of various acids and alkalis of different temperatures and strengths upon asbestos fibres were studied both before and after heating. The effects of heat on asbestos were only considered in relation to alterations in the chemical reactivity of asbestos since preliminary trials with all fibre types showed no obvious morphological changes occurred at temperatures up to 400°C. Consequently samples of asbestos were heated at 100 - 150°C (to mimic cold ashing conditions) or 380 - 400°C (to mimic muffle ashing conditions) and thereafter they were subjected to chemical treatments.

In brief, all three types of asbestos were not obviously affected by alkali treatments, but acid treatments did produce profound alterations. Predictably, the most extensive alterations occurred when asbestos was treated with hot (100°C) concentrated hydrochloric acid. Macroscopically, all samples turned reddish-brown, and a yellowish supernatant remained after centrifugation or filtration. At the electron microscopical level, the amphiboles were morphologically unchanged, but extensive destruction of the fibrous morphology of chrysotile was observed. The chrysotile samples contained large amounts of irregularly shaped particles

and short fibrous remnants. There were few large fibre bundles, but those that could be found did not have the distinct split fibrillar ends characteristic of untreated chrysotile. Further examination of the effects of acids on chrysotile showed that even cold 0.1N hydrochloric acid resulted in some morphological alterations.

EDAX analyses of fibres after hydrochloric acid treatment showed that several hitherto undetectable elements could be found, apart from the ubiquitous chlorine from the acid. Aluminium, potassium and calcium were all found in acid treated amosite fibres, magnesium aluminium sulphur and calcium were found in crocidolite fibres, and some calcium was found in the chrysotile debris. Analyses of the residues formed by evaporation of the supernatant from acid treatments produced some interesting points. As suggested by the previously noted yellow-red colouration, there was a particularly strong emission spectrum for elemental iron in both the amphibole and the chrysotile supernatant samples. In addition, the amosite-derived supernatant was found to contain magnesium, aluminium, silicon, calcium and manganese, the crocidolite sample contained magnesium, aluminium, silicon and calcium, and the chrysotile sample showed a particularly strong spectrum for magnesium.

The effect of periods of prolonged heating at either "cold ashing" or "muffle ashing" temperatures was simply to accentuate any of the alterations arising after the acid treatment of samples described above. Chrysotile was

particularly susceptible to acid elutriation after periods of heating at 380 - 400°C, with an almost total loss of fibrous morphology occurring after agitation with 1N hydrochloric acid at room temperature. There was also strong evidence that prolonged heating at 380 - 400°C increased the fragility of amosite to such an extent that few large fibres could be found even after mild treatment with distilled water. The morphology of crocidolite asbestos remained unaffected by either the heat or chemical treatments.

One further study of the behaviour of asbestos fibres concerned the consequences of prolonged storage of asbestos in liquids of different.pHs. The principal aim of these experiments was to exaggerate some of the possible effects of the prolonged immersion of fibres in tissue fluids. Accordingly small samples of the three main types of UICC asbestos were stored in excesses of acids and alkalis of different strengths, and distilled water was also included. Aliquots of both fibre and supernatant were removed at regular periods for up to 2 years, and examined using electron optical methods. Once again the most striking alterations occurred with the acid treated samples in which there was a pronounced elutriation of sodium magnesium and iron from all asbestos types. Morphologically, chrysotile was the most affected, with gross degradation of fibrous morphology even at low acid concentrations (0.1N hydrochloric acid). These alterations to chrysotile were evident within an hour of the addition of acid, and within 3 days they reached a level after which no obvious further morphological degradation could

FIGURE 3.9

EDAX spectra of acid-treated(6N HCl) UICC amosite asbestos.

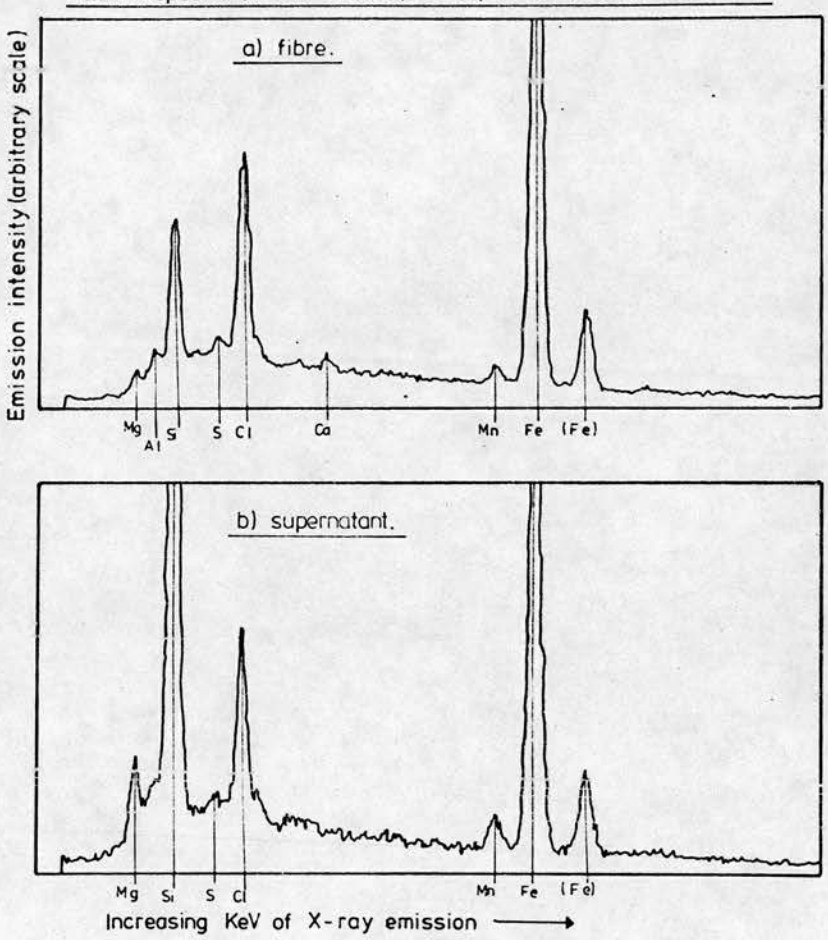


FIGURE 3·10

EDAX spectra of acid-treated(6N HCl) UICC crocidolite asbestos.

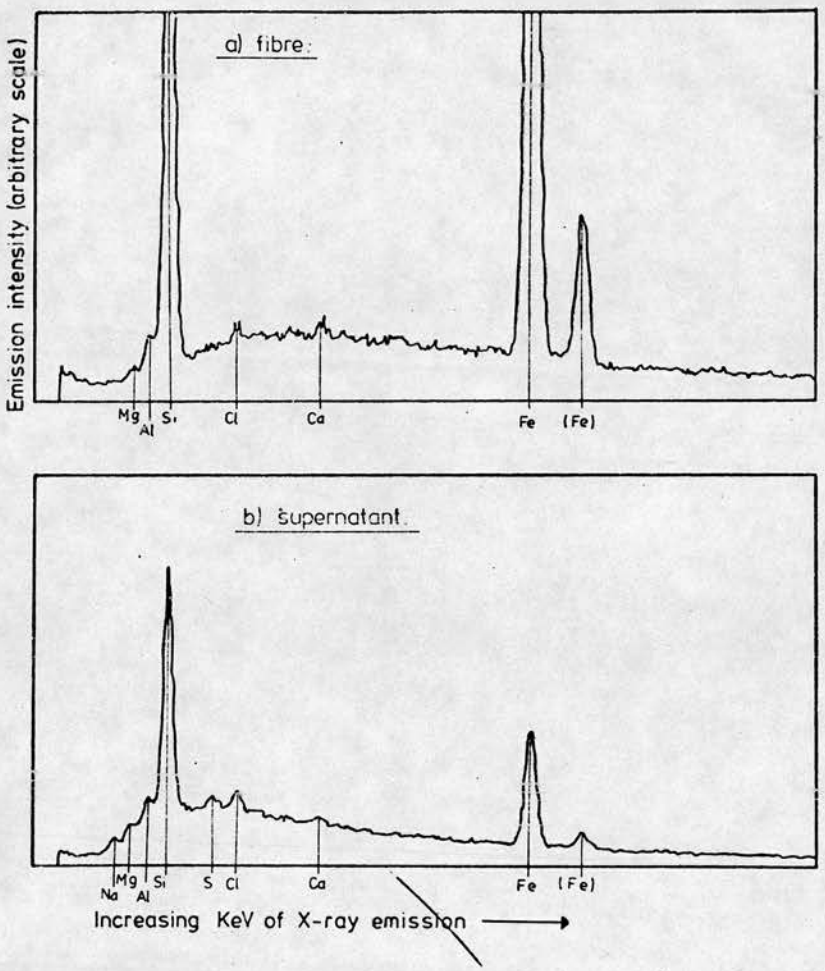
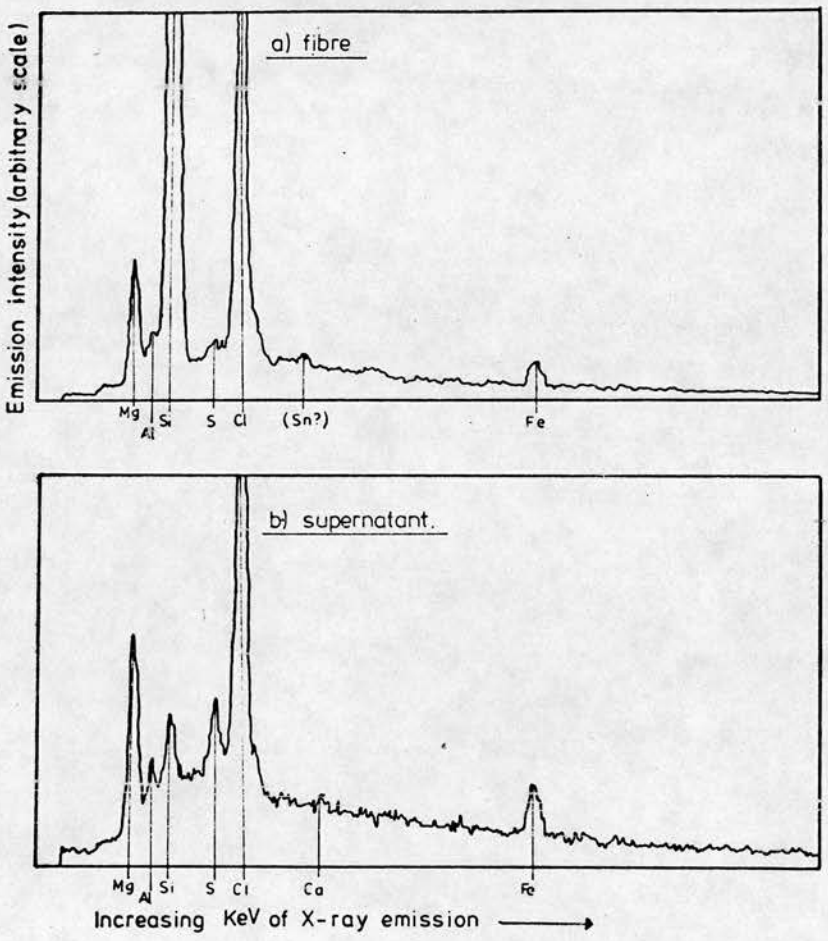


FIGURE 3-11

/ EDAX spectra of acid-treated(6N HCl) UICC chrysotile asbestos.



be seen.

The EDAX analyses of the prolonged storage samples were interesting in that they did show a gradual and consistent increase in the extent of elutriation of some of the constituent elements from the fibres, after an initial rapid reaction phase. Support for this finding came from the observation that there was a small but distinct increase in the yellowish colouration of the supernatant of the acid suspensions for periods up to one year after the start of the experiment. Some of the EDAX peaks produced from minor constituent elements became more pronounced over the same period. Figures 3.9 to 3.11 show some results of these analyses, with a representative analytical spectrum for both fibrous and supernatant components of the 6N hydrochloric acid storage test. It can be seen that all those elements found associated with the fibres are also found in the supernatant-derived residues implying that some leaching of all components occurred. It is interesting to note that the peaks for some elements become accentuated by the leaching process. Thus, for example, the calcium-derived peak for acid-treated amosite fibre shown in Figure 3.9 was much more prominent than shown in untreated UICC amosite (see Figure 3.5), suggesting that some "opening" of the molecular lattices may have occurred. It is also interesting to note that no sodium could be detected in the leached crocidolite fibre although there was a distinct peak in the corresponding supernatant, implying that the majority of the sodium associated with the fibre could be removed by the acid treatment. One unforeseen problem arose during the interpretation

of the effects of leaching in that it was not appreciated at the start of the experiment that storage of the samples in glass vials might result in leaching of constituents from the glass. Analysis of residues derived from pure 6N hydrochloric acid stored in the glass vials for two years did in fact suggest that some leaching of aluminium, magnesium progression and some lead had occurred.

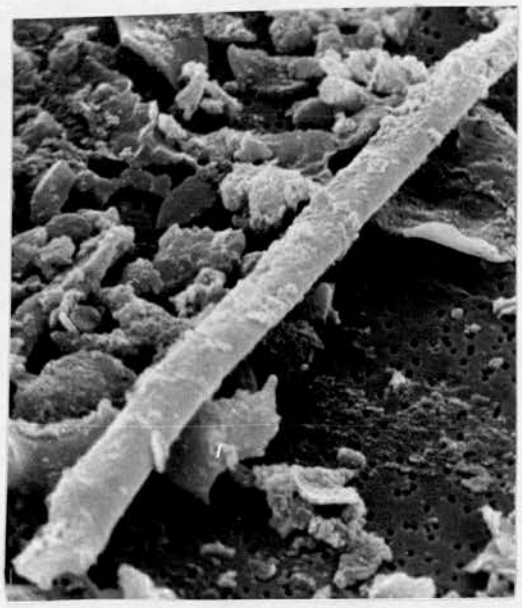
3.5 ANOMALOUS FIBRES

The primary screening of tissue-derived residues for the presence of asbestos fibres was based upon a morphological identification of fibres using mainly scanning electron microscopy as described in section 3.3. Those particles of the correct dimensions conforming to the 3 x 1 aspect ratio definition of "fibrous" (ARC Tech.Note 1*) were therefore categorised provisionally as putative asbestos until analysed further with the EDAX attachment. Previous sections (3.2, 3.3 and 3.4) give some indications of the difficulties associated with the accurate identification of the occasional asbestos fibres found amongst the non-specific debris of ashed residues. In particular, the potential influences of tissue residence and/or extraction procedures upon the composition and morphology of asbestos have been outlined. It is clear that fibrous shapes which contained substantial amounts of silicon but which failed to produce distinct asbestiform EDAX spectro could therefore present as an identification problem.

A selection of some of these anomalous fibres is shown in Figure 3.12. Most could be distinguished on morphological grounds as unlikely to be of asbestiform origin, but in some cases

* Asbestosis Research Council, London.

5 μ



5 μ

5 μ

2 μ

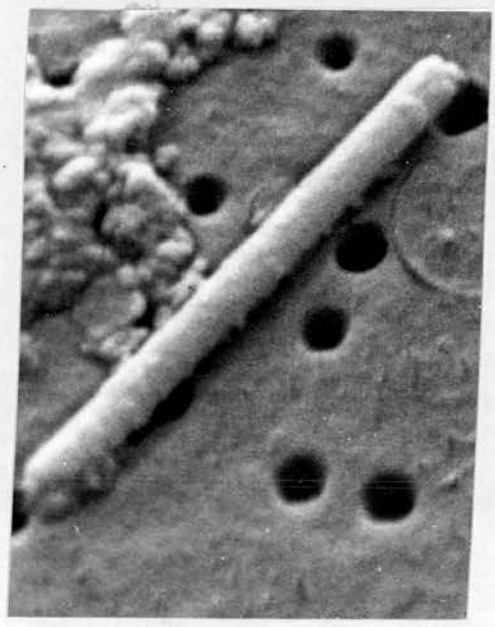


Figure 3.12 Scanning electron micrographs of four of the non-asbestiform "anomalous" fibrous shapes frequently found in the ashed tissue residues.

it was more difficult to provide accurate identification, particularly where small thin fibres were involved. Any fibres that could not be definitely excluded from an asbestiform classification were recorded as positive finds using a "fail-safe" maxim.

One particular variety of suspicious fibrous shapes was frequently found in some tissue residues, mainly of kidney origin, in the experiments described in Chapter 5. These differed from the fibrous shapes shown in Figure 3.12 in that morphologically they were very similar to amphibole asbestos, as can be seen from Figure 5.14. However, EDAX analyses suggested that they contained relatively little elemental silicon, and it was concluded that they were artefacts of the recovery process. (Refer to section 5.4 for a detailed account of these fibres.)

3.6 CONCLUSIONS

1. Energy dispersive X-ray analysis was the most practical method of routine analysis of fibres found in particulate residues, but there are considerable limitations in the discriminatory ability and resolution of the EDAX system when it is fitted to a scanning electron microscope. These limitations become particularly important when thin fibres coated with gold require analysis.

2. From a morphological point of view, the amphibole asbestos types have been shown to be remarkably stable in body fluids and after prolonged chemical treatments, but the converse is true of chrysotile asbestos. The dissolution occurring during tissue residence, and most forms of acidic treatment, may result

in a substantial disruption of the larger fibrous bundles that in extensive cases leads to a destruction of the fibrous character of chrysotile. Although no detailed fibre measurements were made, it seems reasonable to suggest that the small thin fibrils were particularly vulnerable to the effects of extensive leaching.

3. Prolonged heating of asbestos to temperatures approaching 400°C had no obvious effect on crocidolite asbestos morphology, apart from a brown colouration replacing the characteristic blue appearance of bulk samples. However, both amosite and chrysotile became much more brittle, and chrysotile was markedly more susceptible to chemical attack after prolonged heating.

4. Prolonged periods of tissue residence appeared not to alter the chemical resistivity of the amphibole asbestos types, but chrysotile did seem to be leached with, possibly, some disintegration of the smaller fibrils. There was strong evidence that some amphibole fibres became coated with a thin iron-containing layer of biological material, although no classical ferruginous bodies were found during the electron microscopical examination of the rat injection site residues. Prolonged tissue residence also produced some asbestos fibres with relatively large calcium-derived EDAX peaks. This may have been the result of either the deposition of ectopic calcium on to the fibres, or it might be the result of leaching of the fibres. The latter suggestion could be supported by the chemical leaching experiments described in section 3.4.2 in which the calcium-derived peak from EDAX analysis of amosite fibre was more prominent after prolonged storage in acid.

5. The amphibole asbestos types proved to be resistant to chemical degradation and alteration, even after prolonged heating. However, those changes that did occur were of importance since the elements that were affected were the ones usually used to discriminate between amosite and crocidolite. It was, therefore, not always possible to routinely identify a specific amphibole type in the ashed residues. Chrysotile was found to be chemically reactive, with even relatively mild treatments having a profound effect on the fibres. This meant that tissue residues from chrysotile-treated animals could only be processed using mild reagents (see section 2.6.1c) that were not capable of removing the majority of the non-asbestos debris. Fibre searches of residues derived from chrysotile-treated animals were consequently more difficult and time consuming.

6. There were some problems arising from the precise identification of certain fibrous particles in residues. In general, provided the fibres were of the appropriate dimensions, they were identified as asbestiform if they were found by EDAX analysis to contain significant proportions of silicon. Use of this rather broad classification would be likely to overestimate the actual number of true asbestos fibres present, but it must be remembered that a widely used definition of asbestos minerals groups them simply as "fibrous silicates" (see section 1.1.1).

CHAPTER 4 SHORT TERM INGESTION EXPERIMENTS

4.1 INTRODUCTION

This chapter contains the results of all those ingestion experiments of up to one year's duration. For convenience they have been grouped under three broad headings:

(i) Investigations into the methods of asbestos administration and their immediate effects.

(ii) Investigations into the rate of clearance of asbestos from the guts of healthy animals, and examination of the excreted asbestos for signs of alteration.

(iii) Detailed electron microscopical studies of the gastrointestinal tissues and residues for any evidence of fibre penetration and/or damage.

A total of 115 rats and 15 mice was used in these studies, and Figure 4.1 summarises the numbers used for each experiment.

4.2 FEEDING TRIALS

4.2.1 Administration

The first phase of experiments presented are the preliminary investigations into the methods of asbestos administration available for ingestion in laboratory animals, and the immediate effects of these methods. Administration methods included: incorporation of fibre into drinking water; into standard pelleted diets; into dietary supplements; the gavage of suspensions of asbestos by intraoesophageal injections, and the secondary ingestion of asbestos associated with the inhalation of asbestos fibre clouds. The last of these methods will be dealt with in more detail in Chapter 8. Of the remainder, gavage proved to be impractical for chronic ingestion dosing regimes, although it did

FIGURE 4.1 Short-term ingestion experiments : experimental layout.

Nature of experiment	No. of animals used per treatment				
	Anthophyllite	Amosite	Crocidolite	Chrysotile	Untreated
(i) Feeding trials:					
methods of administration	-	-	-	-	12
food consumption and defaecation studies	-	-	-	-	36
acute tests	-	-	-	-	16 + (15)
(ii) Clearance tests:	-	4	4	4	3
(iii) Detailed electron-microscope studies:					
intraoesophageal injection	-	-	4	4	2
clearance after one year of ingestion	2	2	2	2	4
microscopy of G.I. tract tissues	2	2	4	4	4
Totals	4	8	14	14	77 + (15)

FIGURE 4.2 Short-term ingestion experiments : effect of dietary asbestos on food uptake and excretion*.

Treatment	Food uptake consumption in gms dry wt. per rat per day		Excretion (per rat per day)	
	Diet	Margarine	Dry wt. faecal matter	No. of faecal pellets
Normal pelleted diet	21.40	-	4.09	37.2
Normal pelleted diet + margarine supplement	14.75	8.12	3.79	35.0
Normal pelleted diet + margarine supplement + chrysotile asbestos	15.03	7.98	3.82	35.3

* each figure represents the mean of observations on 12 rats of approx. 120 days old.

permit the application of very large doses to the gastrointestinal mucosal over short periods. Incorporation of fibre into the standard pelleted diet was rejected as labour intensive, impractical, and associated with an unacceptably high risk of airborne asbestos generation. The incorporation of asbestos into either drinking water or dietary supplements both appeared feasible. The addition of the fibre to drinking water supplies was finally rejected after trials with 8 rats supplied with a suspension of amosite in their drinking water in which the surrounding air was monitored for aerosol asbestos overnight (i.e. during periods of highest activity). Only relatively small numbers of fibres were subsequently detected, but an examination of the residues of the lungs of these animals after ashing (see Section 2.6.1c) clearly showed that the administration of asbestos by this method was associated with a significant burden of inhaled fibre. Problems of dosing caused by the sedimentation within, and the blocking of, the drinking water valves, and the possibility of dissolution of some of the constituents of chrysotile, (see Chapter 3) further supported the rejection of drinking water administration methods. Tests with the use of a margarine formulation confirmed the work of others that a dietary supplement constituted a practical method of supplying large doses of asbestos over prolonged periods with minimal contamination. The additional fortuitous finding that our strain of male rats would regularly consume 50-60 gms of margarine per week throughout the greater part of their life, greatly facilitated the administration of constant doses of asbestos. Details of the procedures may be found in Section 2.1.3b.

4.2.2 Effects of Asbestos Administration

A series of investigations into the effects of a relatively high dietary fat intake upon the normal ingestion assimilation and defaecation of the laboratory rat became a necessary part of the adoption of the asbestos-impregnated margarine dosing regime. Groups of male rats of various ages were given ad libitum access to known amounts of standard pelleted laboratory diet and/or margarine, and their food uptake rates were measured. Simultaneously, defaecation was assayed gravimetrically and numerically. A summary of the findings is presented in Figures 4.2 to 4.4. It can be seen that access to a regular supply of margarine was associated with a large (31%) reduction in the uptake and utilisation of pelleted diet, and the incorporation of asbestos into the margarine had no obvious effect on this reduction (Figure 4.2). Margarine ingestion was also associated with a slight (7%) reduction in the dry weight of faecal matter excreted per day, and a similar reduction in the number of faecal pellets passed (Figure 4.2). Figure 4.3 shows the effect of animal age on food uptake in which there is a gradual but consistent decrease in the total weight of food consumed with increasing age up to 750 days. Figure 4.4 shows that whilst the number of faecal pellets produced per day also decreased with increasing age, the actual weight of excreted matter remained fairly constant. This implies that, for both types of diet, there was a gradual increase in the size of the individual faecal pellets with increasing age, and further, that there was less utilisation of the ingested food.

4.2.3 Acute tests

The next phase of experiments was

FIGURE 4.3 Short-term ingestion experiments : effect of animal age on food uptake.

	Animal Age (days)				
	120	200	250	500	750
Mean Body Weight:-	358	406	430	461	472
Treatment					
Normal pellet diet (gms)	21.4	21.7	21.0	19.3	17.8
Normal diet (diet gms)	14.8	14.6	14.4	14.1	12.8
+					
Margarine (margarine gms)	9.3	8.5	8.2	8.1	7.6
Mean body wt.increase (gms/day)	5.1	1.3	< 1	< 1	-

FIGURE 4.4 Short-term ingestion experiments : effect of animal age on faecal excretion rates.

		Animal Age (days)				
		120	200	250	500	750
Mean Body Weight:-		358	406	430	461	472
Treatment						
Normal pelleted diet (12 rats)	(faecal dry weight gms)	4.09	4.11	4.10	4.05	4.01
	(No. of faecal pellets)	37.2	34.7	31.9	30.1	26.8
Normal pelleted diet + Margarine supplement (12 rats)	(faecal dry weight gms)	3.79	3.74	3.81	3.77	3.73
	(No. of faecal pellets)	35.3	31.9	30.0	28.9	23.6

designed to identify any acute effect of the ingestion of asbestos in the margarine formulation. It was assumed for these purposes that any acute effects were likely to be expressed as a loss of patency of the gastrointestinal mucosa, and thus attempts were made to identify any such areas of damage. Nigrosin was supplied in the drinking water and in the margarine of 16 rats, eight of which were also given amosite asbestos, and the guts were examined for periods up to two months for any signs of blackening of the mucosal surfaces and/or the local lymphatic tissues. No such blackening was seen. In addition, following a brief report in the literature of transmigration of nigrosin particles across the gastrointestinal mucosa of mice under similar conditions (Joel, D.D.* 1977). a group of 15 male CBA mice was supplied with nigrosin in their drinking water for periods up to three months. Examination of all gastrointestinal and lymphatic tissues at autopsy failed to confirm transmigration of nigrosin particles.

4.3 CLEARANCE TESTS

These tests were undertaken to establish the transit time of asbestos along the gastrointestinal tract and to test for possible retention in pockets within the gut lumen, as suggested by POOLEY (1974). Four rats were used with each asbestos type, together with controls, making a total of 15 animals. The experimental animals were given asbestos in margarine ad libitum for one month to establish constant ingestion conditions. At time zero the asbestos supply was removed and half the animals were killed. Twenty-four-hour faecal pellet samples were taken from the surviving animals at regular intervals for 28 days. On the twenty-eighth day the remaining animals including the controls were killed. Both gut tissues and

* Reported in Science News III, p 266.

faecal pellets were ashed and prepared for examination (see Section 2.6.1c) or analysis. Initially both TEM and SEM were used for the examination of residues. However, SEM proved to be the most useful technique since it allowed relatively large aliquots of sample to be prepared and examined without the obscuring effects produced by grid bars.

Figure 4.5 shows the mean number of faecal pellets produced daily per animal over the duration of the clearance tests, and Figure 4.6 the weights of the ashed residues. It can be seen that the cessation of asbestos and margarine ingestion was associated with a slight increase in the number of faecal pellets produced, and a more significant increase in the amount of excreted residue. Both these findings are to be expected from the results reported in Section 4.2.2. Figure 4.6 also includes the mean post ashed weights of the entire gastrointestinal tracts (with food contents) of several animals, and it shows this to be approximately the same as the weight of ashed faecal material excreted in any one 24-hour period.

Mass estimates of the amount of asbestos in cold ashed residues were made using an infra-red spectrophotometric technique described by BECKETT et al. (1975), and the results are summarised in Figure 4.7. The results of this technique in the present context were subject to large errors associated with the presence of a relatively high level of mineral impurity in normal rat diet residues. However, Figure 4.7 shows firstly that the constant ingestion conditions (i.e. those pertaining at "Time 0") resulted in approximately 35 mgs of asbestos within the gastrointestinal tract at any instant, secondly that the bulk of the asbestos was excreted during the first 24 hours after the cessation of administration, and thirdly that

FIGURE 4.5 Short-term ingestion experiments : faecal pellet production (per 24 hr period) following cessation of asbestos and margarine administration.

Collection Period	Mean number of pellets produced over 24 hr period			
	Treatment			
	Amosite	Crocidolite	Chrysotile	Control
0 - 24 hrs	49.0	45.0	45.0	47.5
24 - 48 hrs	47.0	43.5	52.0	50.0
48 - 72 hrs	54.0	46.0	55.0	52.0
6 - 7 days	62.5	48.5	47.5	55.5
1 - 14 days	55.5	53.0	50.0	48.0
19 - 20 days	50.0	61.0	54.5	57.0
27 - 28 days	52.5	44.5	45.5	48.0

FIGURE 4.6 Short-term ingestion experiments : post ashed weights of clearance test samples.

Sample Details	Mean sample weights (gms)			
	Treatment			
	Amosite	Crocidolite	Chrysotile	Control
G.I. tract + food Time 0	0.8071	1.3305	0.8304	-
Faecal pellet sample:				
0 - 24 hrs	0.8371	1.0327	0.8766	0.9512
24 - 48 hrs	1.1252	1.2087	1.2827	1.1867
48 - 72 hrs	0.9976	1.2989	1.2874	1.0899
6 - 7 days	1.2657	1.6295	1.3767	1.3795
13 - 14 days	1.3230	1.5723	1.5375	1.4591
27 - 28 days	1.2829	1.5379	1.5041	1.5500

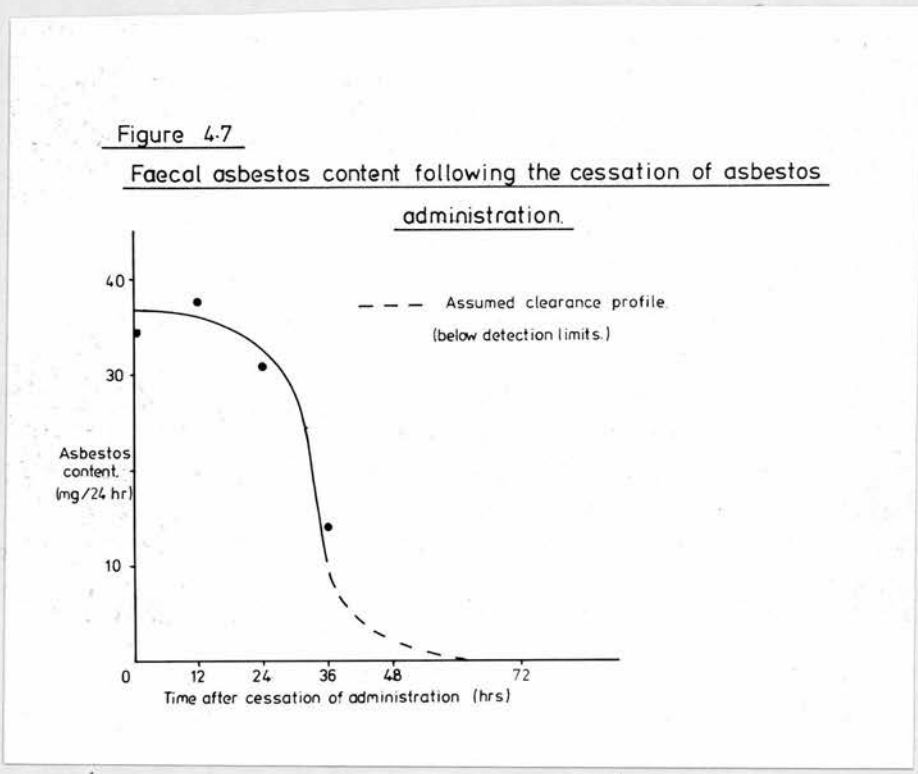


FIGURE 4.7 Faecal asbestos content following the cessation of asbestos administration.

there was no difference between the faecal clearance rates of the three main types of asbestos. The clearance was such that within 48 hours the levels of asbestos were below the resolution of the infra-red assay, and thereafter electron microscopical examination became necessary to detect the fibres (see Figures 4.8 to 4.11). Figure 4.8 shows a typical preparation from an amosite exposed animal taken during the feeding period and it can be seen that asbestos fibres comprise a very high percentage of the non-combustible material present in the gut at this time. Figure 4.9 shows considerably less asbestos is present in faecal remains up to 48 hours after the cessation of asbestos ingestion, Figure 4.10 that by 72 hours even single fibres were difficult to find, and Figure 4.11 is a typical view of all subsequent samples, showing no asbestos amongst the residues.

The morphology and qualitative compositional analyses of asbestos fibres in the faecal residues were examined for any signs of alterations arising from their transit along the rat intestine. Absolutely no sign of alteration of the amphibole types was found. However, chrysotile fibre bundles tended to be more fragmented than usual, even after taking into account the effects of cold ashing and the SEM sample preparation. Further, distinctly less magnesium was detected during EDAX analyses of chrysotile fibres from residues than would have been expected from untreated UICC chrysotile samples (Figure 4.12).

4.4 ELECTRON MICROSCOPICAL STUDIES

4.4.1 Tissue Examinations

This part of the study was undertaken

Figure 4.8 Scanning electron micrograph of the ashed lumenal contents taken from an animal ingesting asbestos, showing massive numbers of fibres amongst the debris.

⊢——⊣ 4 μ

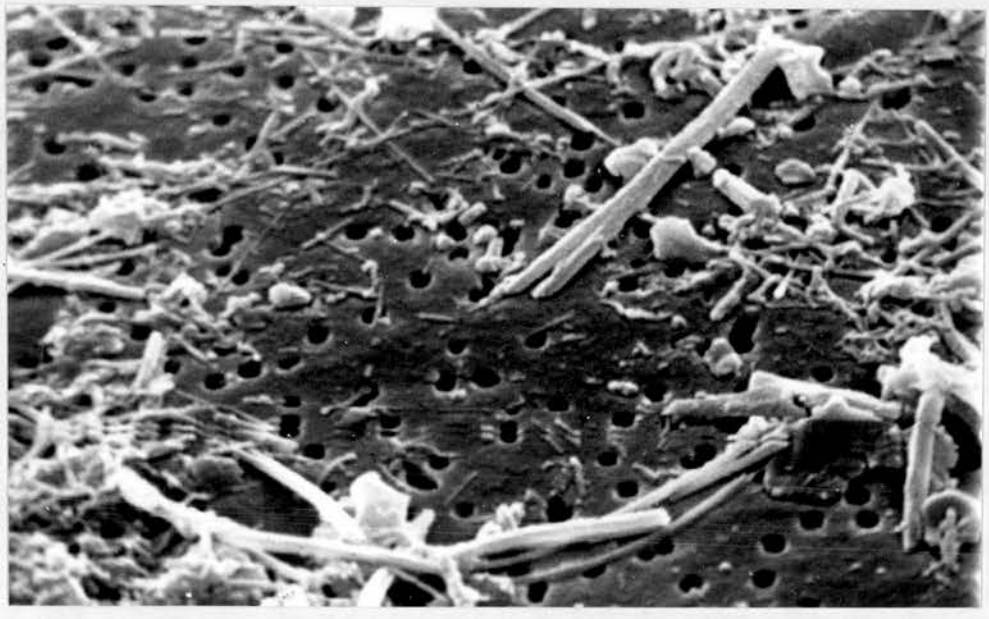


Figure 4.9 Scanning electron micrograph of the ashed GI. lumenal contents taken 48hrs after the cessation of asbestos ingestion, showing some asbestos fibre.

⊢——⊣ 3 μ

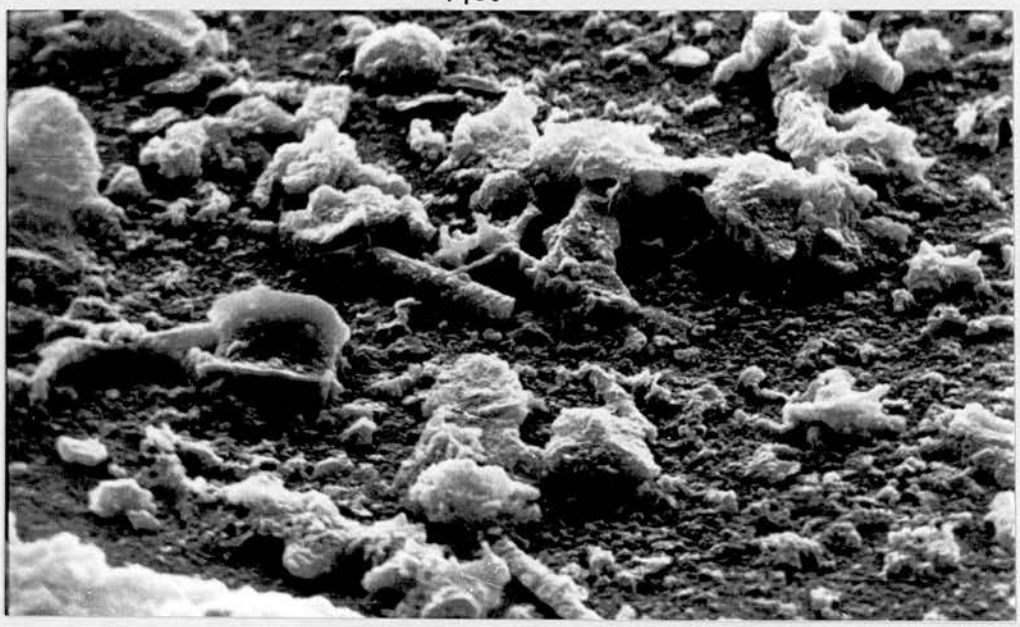


Figure 4.10 Scanning electron micrograph of the ashed GI. lumenal contents taken 72hrs after the cessation of asbestos ingestion, showing occasional fibres.

⟵⟶ 4 μ

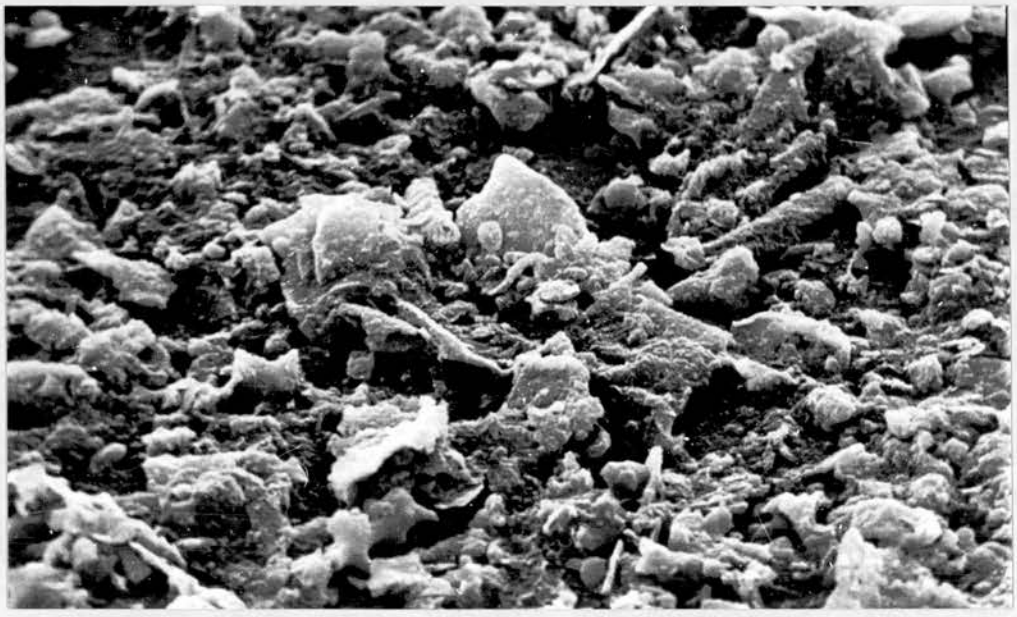


Figure 4.11 Scanning electron micrograph of the ashed GI. lumenal contents taken several days after the cessation of asbestos ingestion, with no asbestos fibre visible.

⟵⟶ 4 μ

FIGURE 4·12

EDAX spectrum of chrysotile fibre extracted from tissue.

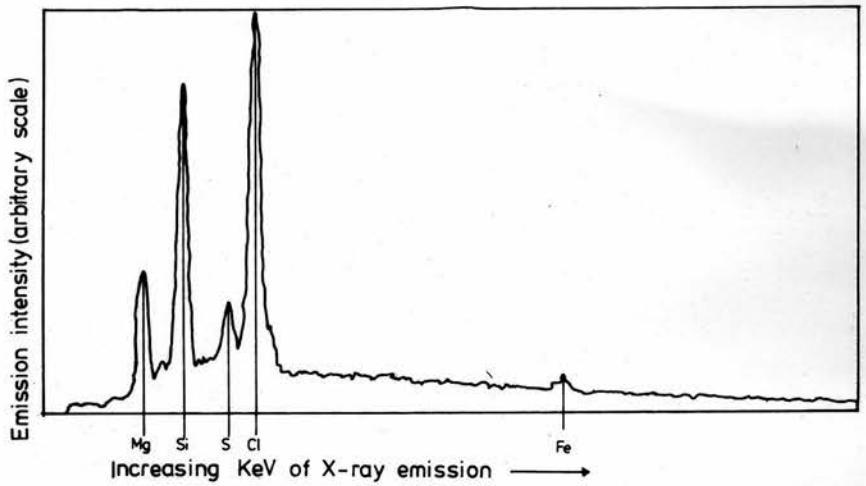


FIGURE 4.12 Typical EDAX spectrum of a chrysotile fibre extracted from rat tissue, showing the relative reduction of the intensity of the magnesium-derived peak.

to examine the gastrointestinal tissues of rats for any evidence of fibre penetration or cell damage which could be attributable to asbestos ingestion. Four animals were used for each type of asbestos and they were killed 2 weeks, 3 months, 6 months and 1 year after the start of asbestos ingestion. Randomly selected areas of all regions of all levels of the gut of each animal were fixed in osmium tetroxide for electron microscopical examination (see Section 2.3.2). The interpretation of ultrastructural variations requires caution since it is known that artefacts can be introduced by even small alterations in preparation and presentation of gastrointestinal specimens for electron microscopy (RUSKA, 1960; 1961; TRIER, 1964). A rigidly standardised technique was therefore used throughout this study. Representative areas of the remainder of the gastrointestinal tissues were taken for histological examination. Control animals were also examined at each time period in order to confirm the ultrastructural patterns of gastrointestinal tissues in the HAN s.p.f. rat.

Figure 4.13 summarises the details of the tissues sampled, and the total number of samples examined for each treatment. The complete absence of any damage in the tissues examined after 2 weeks and 3 months of asbestos ingestion suggested that it would be expedient to concentrate the major effort into examination of the samples taken at one year, since these would be the most likely to be affected. Consequently the bulk of the tissues inspected were from the later sampling time. There was some further selection of samples at these later killing times in that the sections examined tended to be either from the mucosal areas considered most likely to come into contact with the asbestos fibres,

FIGURE 4.13 Total number of tissue preparations examined using the TEM.

Treatment	Time after start of ingestion			
	2 weeks	3 months	6 months	1 year
Amosite	10	25	11	80
Crocidolite	10	29	-	74
Anthophyllite	10	10	-	25
Chrysotile	10	31	-	81

or similarly from the mesenteric lymph nodes.

Dissection of the gut followed by examination of the surface of the lumen with the aid of a stereoscopic microscope revealed no sign of abnormality or lesions. Histological examination of tissues likewise showed no pathological changes, and there was no evidence of the penetration of asbestos fibres into the tissues. When lengths of the gut were fixed with contents in situ large numbers of asbestos fibres could be seen amongst the digesting food (Figure 4.14). However, as this illustration shows, there was often a complete demarcation between the food and the mucosa with no evidence of solid food particles penetrating between the villi. The space usually seen between the surface of the gut contents and the tissue is probably due to contraction during fixation, but this does not explain the absence of food material between the villi.

The TEM examinations showed no sign of cell damage or penetration of the gut lining by asbestos fibres. Occasionally, cells from the tips of the villi in the small intestine showed disruptive changes in the uniformity of their microvilli with their normally cylindrical structures breaking down into irregularly sized droplets. These changes were, however, also found in the normal epithelial cell extrusion zones of control animals. Examination of the mesenteric lymph nodes with both light and TEM revealed a progressive increase with age in the number of reticulin fibres and macrophages containing cytosegresomes (Figures 4.15 and 4.16). However, none of the cytosegresomes examined contained any inclusions remotely similar to asbestos fibres, and no cytosegresomes

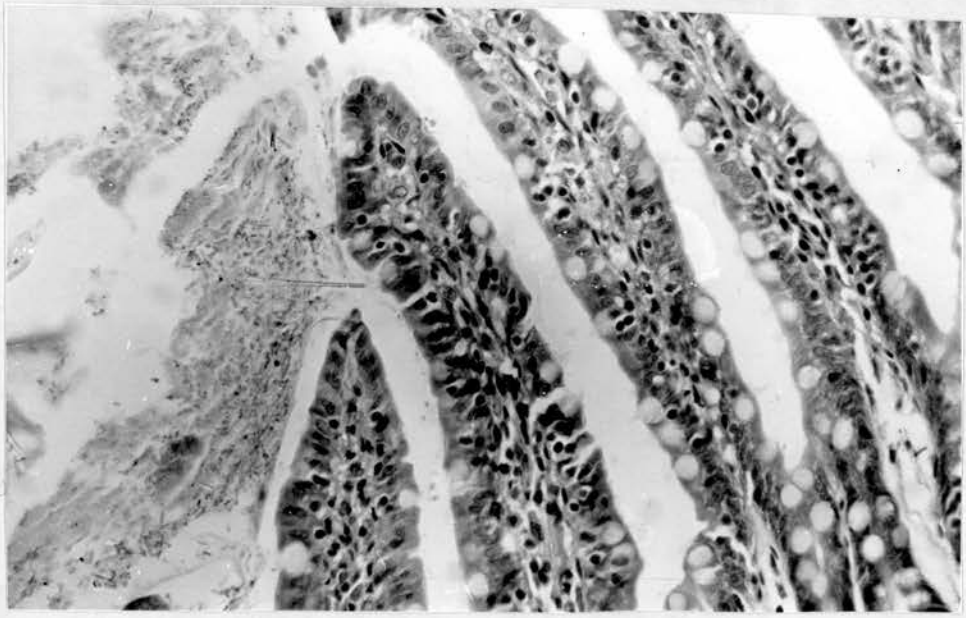


Figure 4.14 Section through rat intestine with lumenal contents *in situ*., showing close apposition of asbestos fibre and the mucosal surface.(focussed preferentially on the fibres.)

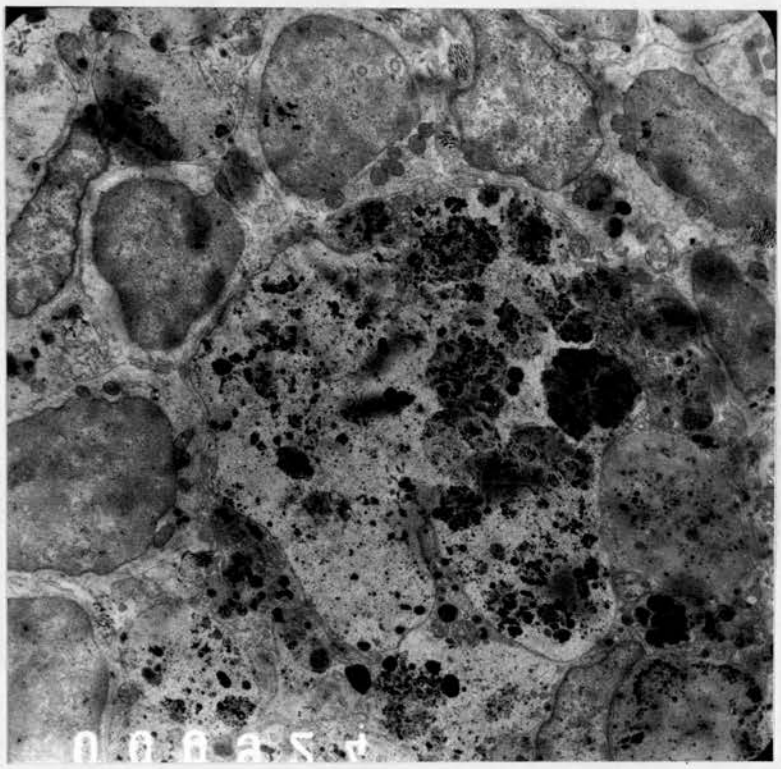


Figure 4.15 Transmission electron micrograph of an area of a mesenteric lymph node taken from a young animal (three months old).

⟵ 2 μ

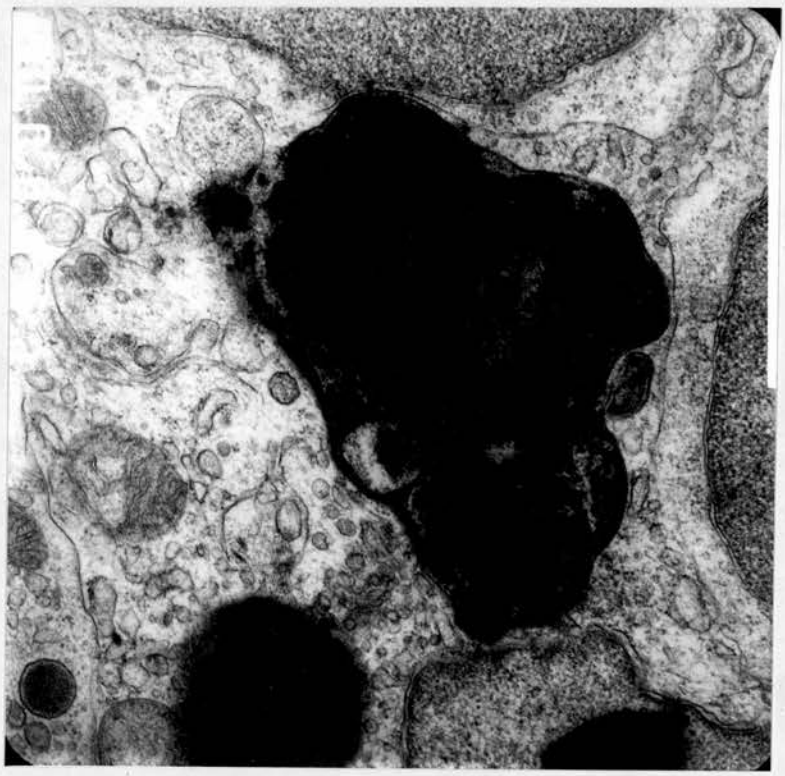


Figure 4.16 Transmission electron micrograph of an area of a mesenteric lymph node taken from an animal aged 15 months, showing some of the many cytosegrasomes containing electron dense material but no asbestos fibres. (compare with Fig. 4.15)

0·4μ

FIGURE 4.17 Number of tissue blocks to be examined (using the TEM) for any given chance of detecting fibres at a stated density of fibres per block of tissue sampled (assuming a fibre density of 1 per block for the examples below).

Fig. 4.17a Illustrating the advantage of adequate sampling, using the chances of detecting 5 micron fibres as an example.

Chance of detecting fibre	No. of blocks to be examined if:		
	20 consecutive sections	2x10 consecutive sections	4x5 consecutive sections
0.95	870	690	410
0.90	690	410	275
0.75	410	275	150
0.50	275	150	55
0.25	150	55	25
0.10	55	25	12

(Taken from Figs. 2.10 to 2.12)
(Refer to Section 2.6.2c)

Fig. 4.17b. Showing that the larger the fibre, the greater the chance of finding it in any given number of blocks.

Chance of detecting fibre	No. of blocks to be examined if fibres of stated length present		
	5 micron	10 micron	20 micron
0.95	370	240	160
0.90	240	160	80
0.75	160	80	37
0.50	80	37	15
0.25	37	15	8
0.10	15	8	3

(Taken from Fig. 2.12)
(Refer to Sections 2.6.2c and 4.4.1)

were found in any other situation within the nodes. This alteration of the mesenteric lymphatic tissue with age was also found in control animals.

Section 2.6.2c describes details of the development of a statistical approach to the analysis of the results of the fibre searches. Figure 4.17 shows the numbers of tissue sections that would need to be examined to be reasonably likely (for instance, at the 90% probability level) of detecting a fibre, given any assumed fibre density in the tissues. It is clear that a huge number of sections would need to be examined for even a relatively high assumed level of fibre penetration. Thus if it was assumed that the tissue density of asbestos fibres was 1 per cubic mm of tissue, and one ribbon of 10 sections was examined for each 1 cubic mm of tissue, to be reasonably sure (at the 90% probability level) of detecting a fibre of 5 μm in length, a total of 650 blocks would have to be examined. If it was assumed that there were ten 5 μm fibres in every mm of tissue blocked, then 65 blocks would have to be examined (c/f Section 2.6.2c).

Since the volume occupied by a damaged cell is much greater than that occupied by an individual asbestos fibre, transmission electron microscopical examination of tissues is more likely to produce results from tissue damage searches. Thus the TEM was used to look for any signs of damage, and the ashing techniques with SEM examination of the residues were used to look for long-term penetration and retention of fibres.

4.4.2 Ashing Analyses

The animals used for ashing analyses of

tissue had been given asbestos margarine mixtures for one year. Then, after one month on a diet free from asbestos, the animals were killed and the gastrointestinal tracts carefully removed. These, together with samples from control animals to check for possible fibre contamination during processing, were ashed and processed (see Section 2.6.1). The gastrointestinal tracts were carefully opened and washed in filtered saline to remove the bulky food contents thereby reducing the amount of residue requiring examination. The possibility of introducing fibre contaminants during dissection was carefully considered, but it was assumed that examination of the guts from control animals would detect any fibres from this source.

Scanning electron microscope examination of ashed residues of the entire guts of animals fed for 1 year on either crocidolite, amosite or chrysotile asbestos revealed that fibrous shapes were relatively common in this material. However, almost all of these structures were quite different from asbestos, and were present in the ashed gut residues of control animals as well as those from the experimental series (see Figure 4.18). It is considered that most of these fibres represent silicaceous plant skeletons included in the animal food pellets and not susceptible to complete degradation by the ashing preparatory procedures. This view was later supported by the finding that, although the fibrous shapes frequently contained significant amounts of silicon as shown by EDAX analysis (Figure 4.18), the analytical spectra differed from those of asbestos fibres (see Chapter 3). From the animals under consideration, only one possible asbestos fibre was found, although a total of 210 preparations of gut residues were examined in detail.

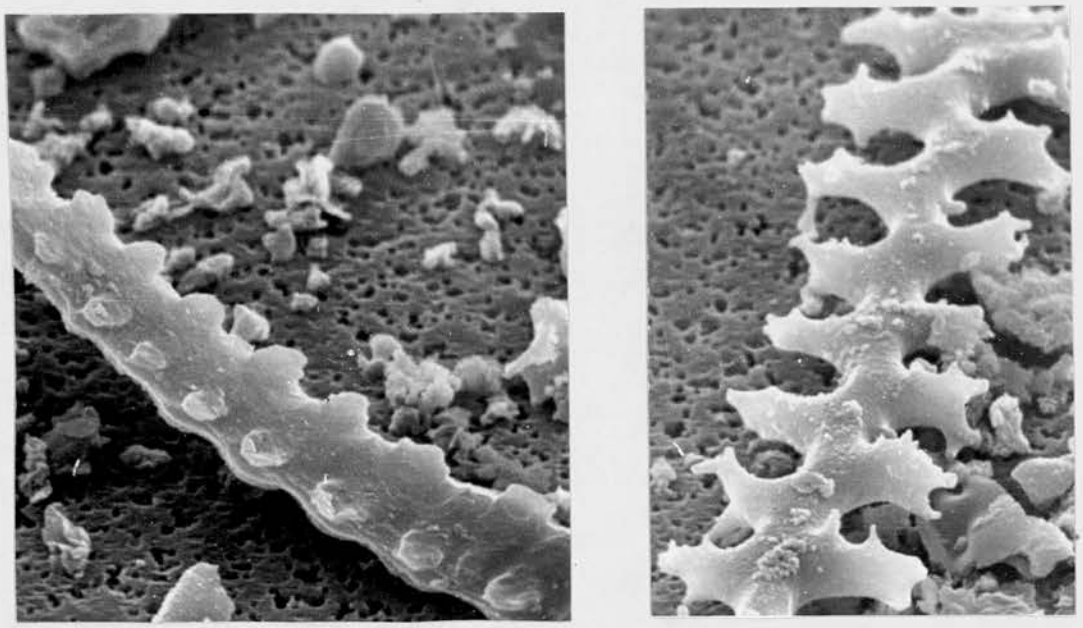
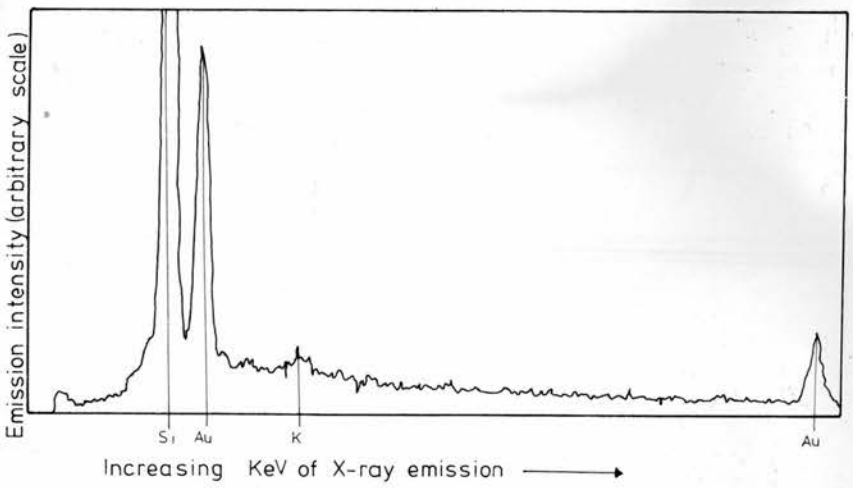


Figure 4.18 a and b. Scanning electron micrographs of two of the fibrous shapes of vegetable origin frequently found amongst the ashed residues of gut tissues.

4 μ

FIGURE 4·18c

EDAX spectrum of putative vegetable fibre.



In the case of the amphibole asbestos types, about 130 preparations were examined. For crocidolite these comprised approximately 0.4% of the total ashed residues. For amosite and anthophyllite, however, the gut tissues had been ashed after the removal of the contained food material, and the residue was therefore greatly reduced. This meant that approximately 4% of the total residues were examined in each case. With the chrysotile animals, it was not possible to use an acid wash to reduce the bulk of the final ashed sample (see Section 2.6.1) and therefore the 80 preparations examined comprised only 0.15% of the total. Figure 4.19 summarises the details of the amounts of residues available, and the numbers of samples examined.

In those animals fed anthophyllite, crocidolite or chrysotile, no fibres were discovered at all similar to any of the asbestos types. In one animal fed with amosite, however, a single fibre 11.6 μm x 0.5 μm was discovered (Figure 4.20). This fibre showed a very similar structure to known amosite (see Figure 3.1), and was accepted as being of this material. Note that it was not possible to confirm this since no EDAX analytical system was available on the scanning electron microscope facilities used for the fibre searches at the time of the examinations (1974 and early 1975).

Without the expenditure of an extremely large amount of time it was obviously impossible to examine all the ashed residue from each animal. It was, therefore, impossible to prove that there was absolutely no gut penetration in any of the animals examined in this study, but a statistical estimate

FIGURE 4.19a Amounts of ashed residue sampled and the number of SEM preparations examined.

Treatment	Mean wt. of ashed residue per animal (gm)	Total subsample wt. examined (mg)	Total no. of SEM preparations examined
Amosite	0.1320	5.2	16
Crocidolite	1.6780	6.9	74
Anthophyllite	0.1704	9.3	42
Chrysotile	1.0161	1.4	81

FIGURE 4.19b Probability Table. (The probability of having missed any fibres that were present in the original sample, given an assumed density of fibres per tissue.)

	Probability of having missed fibres				
Fibre density/sample:	100	200	500	1000	2000
Treatment:					
Amosite	0.01	<0.001	-	-	-
Crocidolite	0.64	0.41	0.1	<0.01	<0.001
Anthophyllite	0.004	<0.001	-	-	-
Chrysotile	0.87	0.77	0.5	0.25	0.06

(Refer to Section 2.6.2b)

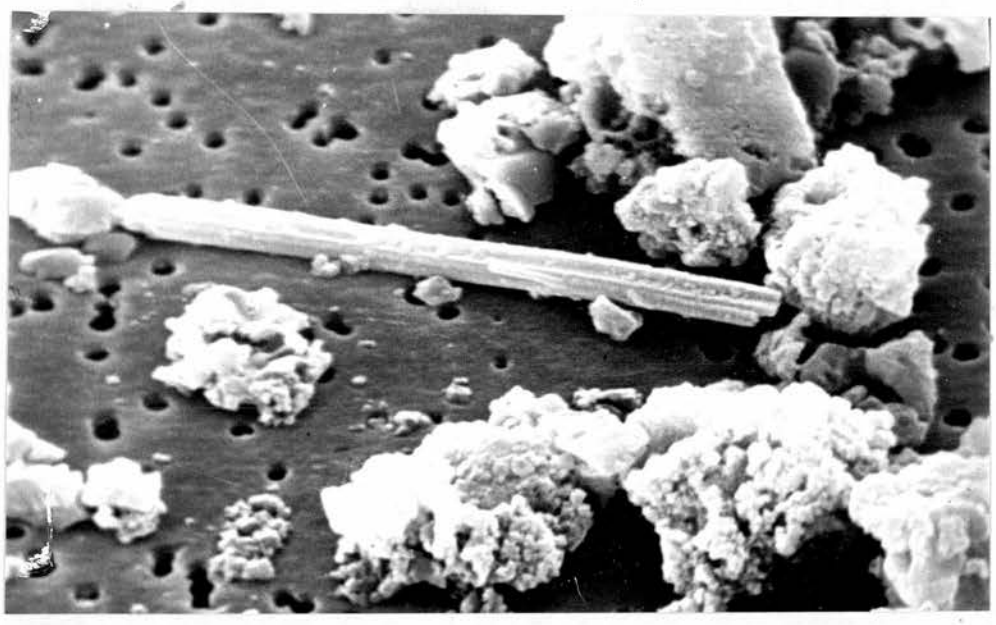


Figure 4.20 Scanning electron micrograph of the only asbestos fibre found amongst the gastrointestinal tissues of a rat that had been ingesting amosite asbestos.

Figure 4.21

Table showing the likelihood (probability) of having missed fibres amongst the ashed residues, given certain assumed total numbers of fibres per gastro-intestinal tract (using data from the crocidolite ingestion experiment).

Assumed no. of fibres per entire gut	Probability of having failed to find evidence of fibres
50	0.81
100	0.64
150	0.53
200	0.41
250	0.36
500	0.12
1000	0.01

of the probabilities can be made. Full details of the development of a statistical approach can be found in Section 2.6.2b, and it is assumed that if a Poisson distribution of fibres exists throughout the ashed residues, it is possible to calculate the maximum fibre content of these specimens that would be consistent with the observed data. Probability tables were constructed for each experiment as described in Chapter 2 and an example (for the crocidolite experiment) is shown in Figure 4.21. The calculations indicate a 90% probability that after 1 year's feeding, the maximum fibre penetration of an entire rat intestine would be less than 100 for amosite, 550 for crocidolite, 650 for anthophyllite, and 1500 for chrysotile. Similarly the figures for an even chance in each experiment are 40 for amosite, 170 for crocidolite, 240 for anthophyllite, and 500 for chrysotile. These figures do not reflect the relative risks of the four asbestos types but merely the difference in sample size. They do, however, take account of the fact that one amosite fibre was found, and that the examination of residues from animals treated with anthophyllite, crocidolite or chrysotile proved completely negative.

4.5 CONCLUSIONS

(1) The incorporation of asbestos into pelleted diet or into drinking water supply at projected experimental doses was associated with an unacceptably high risk of concomitant airborne fibre generation. This does not imply that the relatively low levels of fibre found in some human drinking water supplies may give rise to an inhalation hazard, but merely that exposure to fibre by any means other than ingestion had to be specifically avoided for the

ingestion studies to remain valid. This would have been particularly important had some evidence of tissue fibre burdens been found after chronic asbestos ingestion. Gavage was shown to be impractical for chronic studies but it did provide a technique for the administration of massive amounts of asbestos for acute studies. The incorporation of asbestos into margarine was shown to be a practical method of administration over long periods, with no risks of the generation of airborne asbestos.

(2) Ad libitum access to margarine resulted in a 31% reduction in the mass of pelleted food ingested, but only a 7% reduction in the mass of faeces excreted. This implies that margarine ingestion is associated with a reduction in the degree of breakdown and utilisation of the diet that was ingested. The fact that the 7% reduction in dry weight of excreted matter was also associated with a 7% reduction in the number of faecal pellets passed suggests that the pellets were the same size both with and without margarine in the diet. The addition of asbestos to the margarine had no detectable effect on any of these parameters.

(3) Increasing animal age was associated with a slight decrease in the amount of food consumed whilst the weight of excreted matter remained fairly constant. This implies that there was less utilisation of food with increasing age. The finding that the number of faecal pellets decreased with age whilst the total mass excreted did not alter suggests that the individual faecal pellets increased in size, possibly due to a reduction in bowel motility?

(4) The nigrosin ingestion tests demonstrated that there was no obvious transmigration of particles across the gut mucosa of

rats or mice, and the results of the acute tests showed no evidence of loss of this patency of the mucosal barrier associated with the ingestion of asbestos.

(5) The fact that the weights of entire ashed rat intestines including contents was approximately the same as the ashed weight of faecal pellets collected over a 24-hour period showed that the food takes approximately 24 hours to pass along the rat gut. This was supported by the clearance tests with asbestos, where the bulk of asbestos was cleared in the first 24 hours after the cessation of administration. The finding of occasional fibres 48-72 hours after cessation of administration is probably the result of delays to transit caused by mixing of the gut contents during digestion and absorption, particularly in the caecum.

(6) The amphibole asbestos types were not visibly affected by their passage along the gut, but there was a suggestion that chrysotile tended to lose some of its magnesium and, possibly, that the larger chrysotile fibre bundles tended to open up and disintegrate.

(7) Transmission electron microscopy showed absolutely no sign of penetration or damage arising from chronic asbestos ingestion. Even potentially weaker areas such as the epithelial villus extrusion zones remained unaffected by the presence of very large numbers of fibres. The complete absence of fibres within the mesenteric lymph nodes provided further strong evidence that no penetration took place, since it is here that fibres could be expected to accumulate following penetration.

(8) By virtue of the fact that it was possible to

examine greater proportions of tissues, the ashing and scanning electron microscopical examination of residues provided convincing corroboration of the TEM searches, and supported the suggestion that no large-scale penetration took place. The finding of a single amosite fibre cannot be ignored, however, and it remains a possibility that penetration can occur in some circumstances. The possibility that up to 100 amosite fibres might have penetrated the gastrointestinal tissues must be placed in perspective: the experimental doses of asbestos used in these studies (0.27% by weight of the diet) is equivalent to a projected human exposure approximately 50,000 times the ingestion maxima for severely occupationally exposed persons (SCHNEIDERMAN, 1974) and at least 1.6 million times that for environmental exposure (CUNNINGHAM and PONTEFRACT , 1971; 1973).

CHAPTER 5 LONG TERM INGESTION EXPERIMENTS

5.1 INTRODUCTION

Included under this heading are all those experiments in which animals were kept for their full lifespan to study the potential pathological effects of ingested asbestos. The bulk of the data concerns the effects of asbestos upon groups of male s.p.f. rats, but one experiment using guinea pigs is also included and described in a separate section at the end of the chapter.

Groups of rats were supplied *ad libitum* with asbestos in a margarine formulation (see Section 2.1.3b) for a period of 25 months and monitored for the remainder of their natural lifespan for the development of abnormalities. Fig. 5.1 gives details of the numbers of animals used.

All animals were regularly examined and weighed, and they were killed when found to be in obvious distress or moribund. A full autopsy was performed on all animals (see Section 2.1.4 for details of the autopsy procedure) and tissues were taken for histological examination. In addition, selected tissues (refer to Figs. 5.14 to 5.16) were carefully removed from a number of animals and processed for examination for the presence of fibres (see Section 2.6.1c for details of the ashing procedures).

5.2 MORBIDITY AND MORTALITY

Figure 5.3 gives details of the mean animal weights by treatment group throughout the duration of the experiment. It can be clearly seen from the graph that the three asbestos-treated groups and the positive (margarine-treated only) control group were noticeably heavier than the normal untreated control group. However, there was no significant difference in the mean body weights

FIGURE 5.1 Long-term asbestos ingestion in male rats : experimental layout.

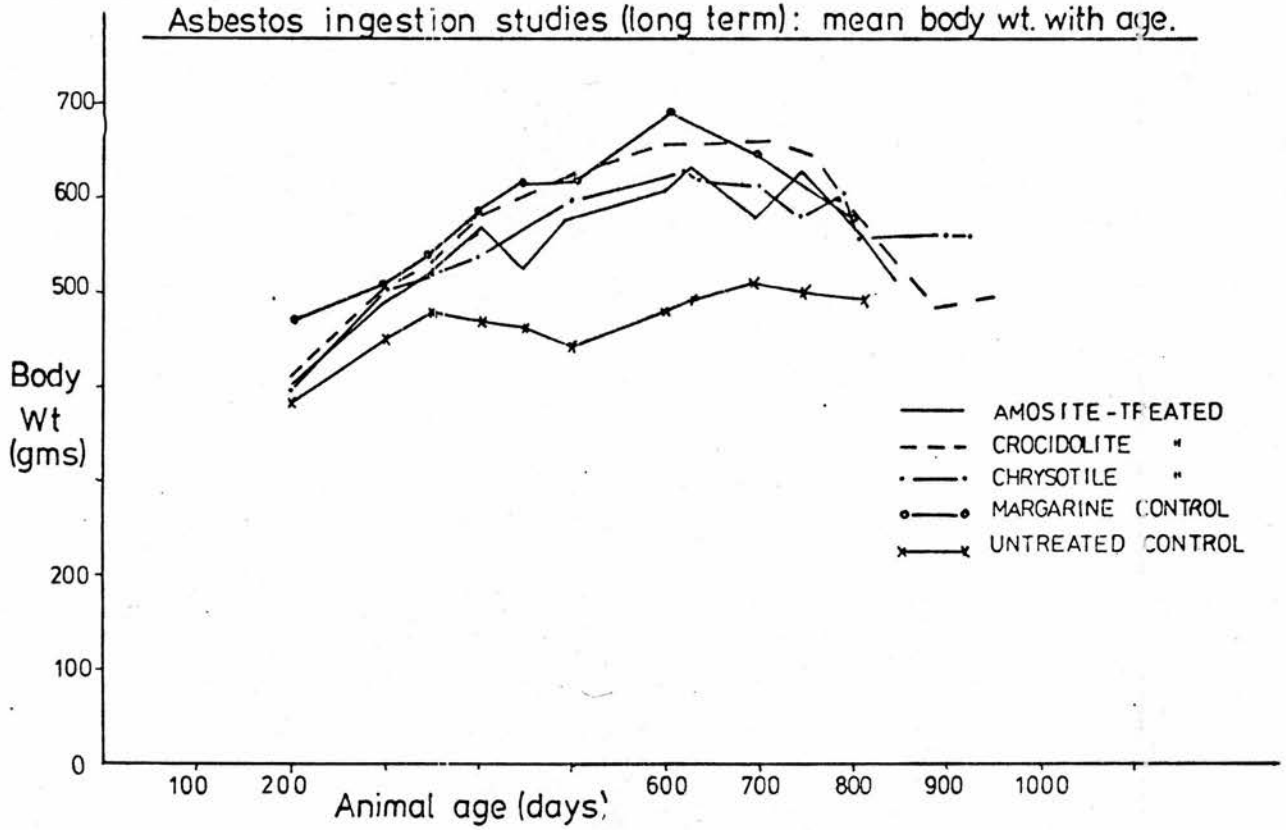
Treatment	No. of animals	Dosage Regime
UICC Amosite	24	Normal diet + 5 mg/gm asbestos/margarine
UICC Crocidolite	22	Normal diet + 5 mg/gm asbestos/margarine
UICC Chrysotile A	22	Normal diet + 5 mg/gm asbestos/margarine
Margarine control	24	Normal diet + ad libitum margarine
Untreated control	22	Normal diet only

FIGURE 5.2 Survival table.

	Number of animals surviving at specified age (days)													
Treatment	350	400	450	500	550	600	650	700	750	800	850	900	950	1000
UICC Chrysotile A	22	22	22	22	22	22	20	20	15	13	11	10	3	-
UICC Crocidolite	22	22	22	22	22	22	21	18	18	18	14	8	-	-
UICC Amosite	24	23	23	21	20	19	19	17	16	14	* 5	-	-	-
Margarine control	24	24	24	24	24	24	24	22	22	22	* 11	9	2	-
Untreated control	23	23	23	23	23	23	21	21	17	*16	8	-	-	-

* see section 5.2.

Figure 5·3


Asbestos ingestion studies (long term): mean body wt. with age.
Body Wt (gms)
700
600
500
200
100
0
100
200
600
700
800
900
1000
Animal age (days)
AMOSITE-TREATED
CROCIDOLITE "
CHRYSOTILE "
MARGARINE CONTROL
UNTREATED CONTROL

between these four treated groups, and it is reasonable to attribute the body weight increase to the margarine dietary supplement.

Figure 5.2 presents the survival of the groups in a tabular form. It is appropriate at this point to draw attention to the survival figures for the amosite and both control experiments: where indicated by the asterisks, a total of 22 animals were removed from the study and killed in connection with a cytokinetic analysis of the gastrointestinal mucosal as described in Section 2.5 and in chapter 6. They have been included in survival table to show that the majority of exposed animals were still alive at 800 days of age. The influence of this cull on the survival parameters can be seen in Fig. 5.4 where the mean survival times are compared with the mean body weight at autopsy for the different asbestos-treated groups. A total of eight animals were removed from the amosite-treated group and killed at 840 days of age. This had the effect of reducing the mean survival time for that group and, since the animals were on average younger and healthier at the time of death, this is reflected in the relatively heavier mean autopsy body weight of 583 gms.

The majority of animals were weighed at autopsy and the results are included in Appendix IV, where Figs. 1 - 5 show the relationship of the autopsy body weight to the survivors for each experimental group. (Animals killed as part of the cytokinetic assays are distinguished from those killed or dying as a result of infirmity by the symbol K.) A consistent feature of Figs. 1 - 5 in Appendix IV is that those animals dying in the course of the

FIGURE 5.4 Long-term asbestos ingestion in male rats : mean survival times and mean autopsy weights.

Treatment	Mean survival time (days)	Mean autopsy wt. (gms)
UICC Amosite	751	583
UICC Crocidolite	848	507
UICC Chrysotile A	833	492

FIGURE 5.5 Long-term asbestos ingestion in male rats : neoplastic lesions.

	Treatment				
	UICC Amosite	UICC Crocidolite	UICC Chrysotile	Margarine Control	Untreated Control
No. of animals per group	24	21	22	24	23
Malignant Tumours	1	1	5	4	2
Benign Tumours	1	5	11	2	1
Dysplasia	2	-	1	2	2

ingestion experiments were of lower body weight than the survivors. Loss of body weight is therefore shown to be an important indicator of morbidity.

5.3 PATHOLOGY

The pathological findings associated with the asbestos ingestion study are itemised in Figs. 6 - 10 of Appendix IV in which the principal lesions are described for each animal. The current problem of asbestos pathogenicity is now such that attention is normally focussed upon the neoplastic risks of exposure, and consequently Fig. 5.5 summarises the "neoplastic lesions" found in each treatment group. "Other lesions" have also been included and are presented in Fig. 5.6 to give an indication of any general toxicity/adverse effects of the exposure regimes.

Figure 5.6 shows that the most common lesion amongst all five groups of animals was the renal hypertensive damage, associated in the more severe cases with polyarteritis nodosa. This has proved to be a major source of morbidity within the HAN strain of laboratory rat used throughout this work, and Figs. 6 - 10 of Appendix IV show that hypertension, with its associated complications, was frequently found to be an important cause of death. A degree of hepatic fatty change was also common amongst all those animals supplied with the margarine-supplemented diet with or without added asbestos fibre. This is in accord with the previously noted higher body weight of the treated animals (see Section 5.2) and, together with an observed excess of peritoneal and subcutaneous fat deposition at autopsy, may be considered to be a result of a generalised obesity.

The neoplastic lesions summarised in Fig. 5.5 require some

FIGURE 5.6 Long-term asbestos ingestion in male rats : other lesions.

	Treatment				
	UICC Amosite	UICC Crocidolite	UICC Chrysotile	Margarine Control	Untreated Control
No. of animals per group	24	21	22	24	23
Pulmonary congestion	4	-	2	2	1
Renal hypertensive damage - Total No.	15	14	12	10	16
ext.	4	6	3	3	4
mod.	5	3	5	6	3
slight	6	5	4	1	9
Polyarteritis nodosa	3	2	5	1	2
Omental infarct.	1	-	2	-	-
Hepatic fatty change	9	4	5	9	2
Footpad granuloma	-	4	2	1	1
Miscellaneous	3	2	3	-	-

FIGURE 5.7 Long-term asbestos ingestion in male rats : details of malignant tumours.

	Treatment				
	UICC Amosite	UICC Crocidolite	UICC Chrysotile	Margarine Control	Untreated Control
No. of animals per group	24	21	22	24	23
Subcutaneous fibrosarcoma	-	-	1	-	1
Pleural histiocytic tumour	-	-	1	-	-
Adrenal medullary carcinoma	-	1	-	2	-
Adrenal cortical carcinoma	-	-	2	-	-
Plasma cell tumour	-	-	1	-	-
Lymphoma	-	-	-	-	1
Gastric leiomyosarcoma	1	-	-	-	-
Peritoneal sarcoma	-	-	-	1	-
Bladder adenocarcinoma	-	-	-	1	-
TOTALS	1	1	5	4	2

FIGURE 5.8 Long-term asbestos ingestion in male rats : details of benign tumours.

	Treatment				
	UICC Amosite	UICC Crocidolite	UICC Chrysotile	Margarine Control	Untreated Control
No. of animals per group	24	21	22	24	23
Subcutaneous fibroma	1	1	1	-	-
Peritoneal lipoma	-	-	1	-	-
Pancreatic endocrine adenoma	-	2	2	1	-
Pancreatic exocrine adenoma	-	2	3	1	1
Mesenteric haemangioma	-	-	4	-	-
TOTALS	1	5	11	2	1

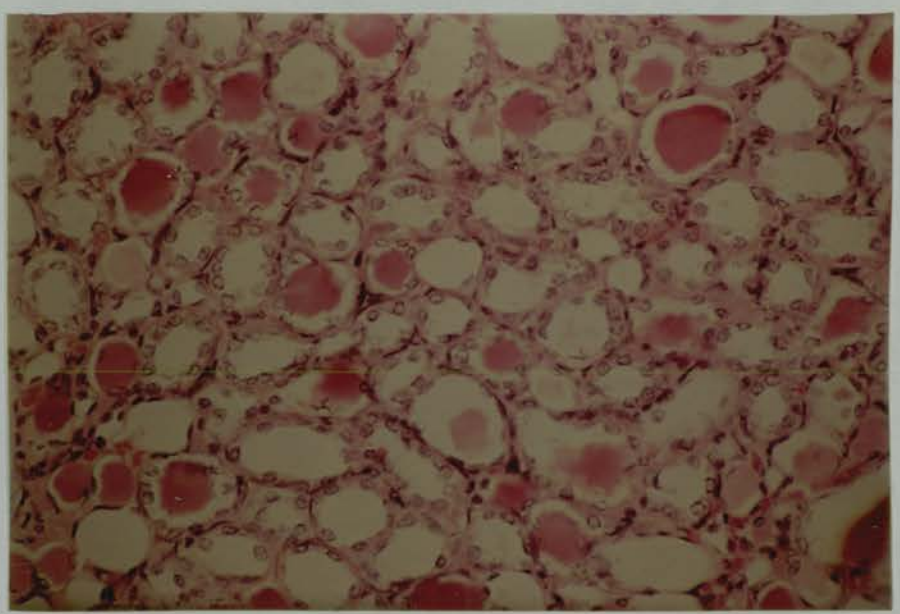


Figure 5.9 Typical section of kidney from ageing rat, showing the tubular distension and protein casts associated with renal hypertensive disease. (H.+ E.)

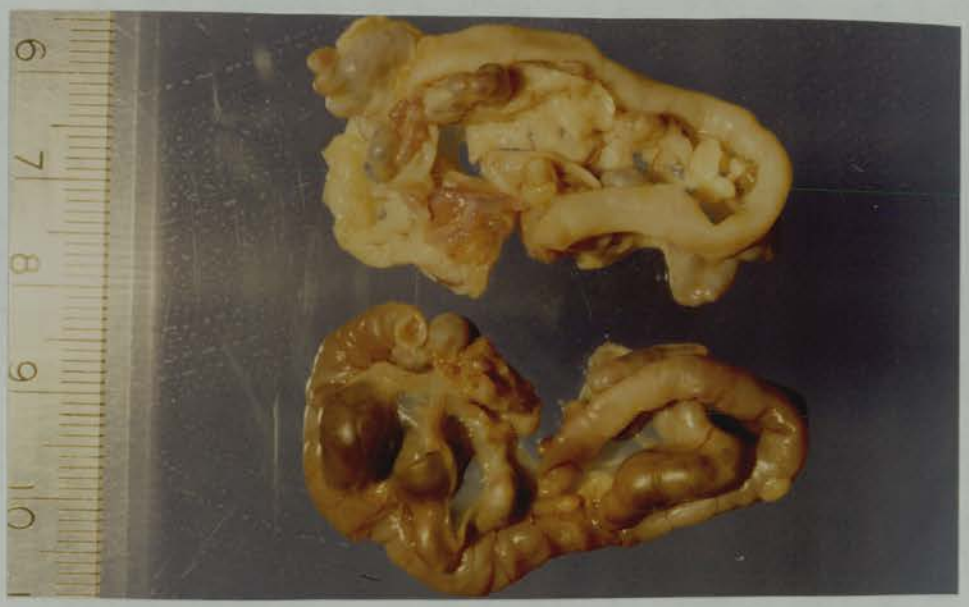


Figure 5.10a Macro photograph of advanced mesenteric polyarteritis nodosa, showing the characteristic nodular appearance.

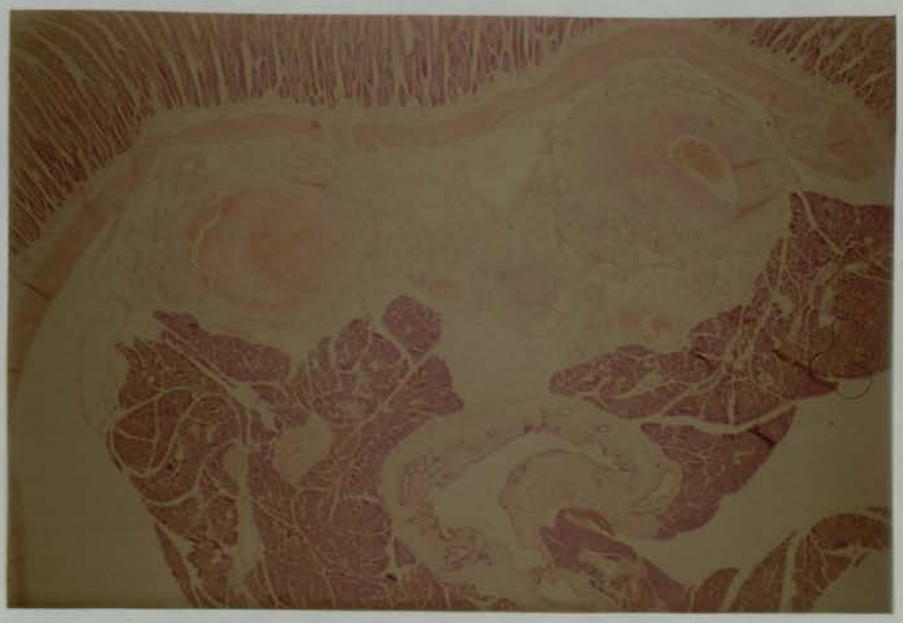


Figure 5.10b Polyarteritis. Section through a mesenteric blood vessel showing the reactive granulomatous proliferation. (H.+ E.)

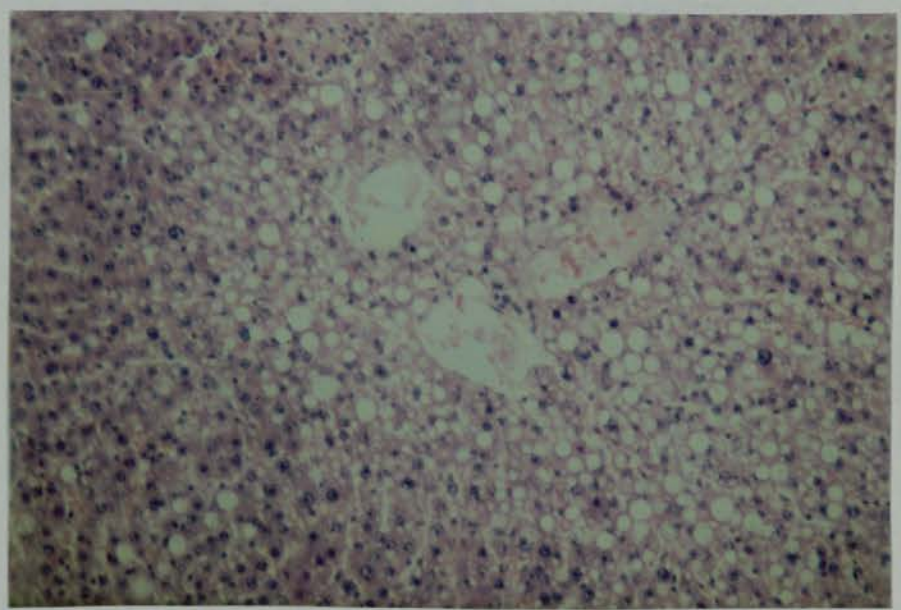


Figure 5.11 Hepatic periportal fatty change and fat lakes commonly found in the obese rats. (H.+ E.)

comment. In particular, the chrysotile-treated group have a relatively high incidence of malignant tumours, and a significant excess of benign tumours. Figures 5.7 and 5.8 present the information in more detail, and it can be seen that there were no primary neoplastic lesions of the gastrointestinal mucosa in any of the treatment groups. There is no clear pattern of malignant tumour incidence (Fig. 5.7), except that five out of 13 malignancies were associated with the adrenal. Of the other tumours, taken individually, there would appear to be no obvious relationship between treatment and the various sites of tumour origin. Taken collectively, however, the finding of a malignant tumour arising in the gastric muscle layers of an amosite-treated animal, and a pleural histiocytic tumour with several similarities to a conventional mesothelioma (Fig. 5.12), arising in a chrysotile-treated animal, suggests the need for caution in interpretation.

The excess of benign tumours in the chrysotile-treated group appear to be largely a result of the finding of mesenteric haemangiomas in four of the animals. The diagnosis of 'haemangiomas' has been used in this account to describe the benign vasoformative lesions (see Fig. 5.13) occasionally encountered in our strain of laboratory rat. However, an incidence of 4 out of 22 animals so affected is considerably higher than expected, and requires examination for any evidence of asbestos fibre penetration and/or residence. All of the other benign tumours are found amongst normal rats, and those occurring in this study are not considered to be associated with treatment.

Figure 5.12a

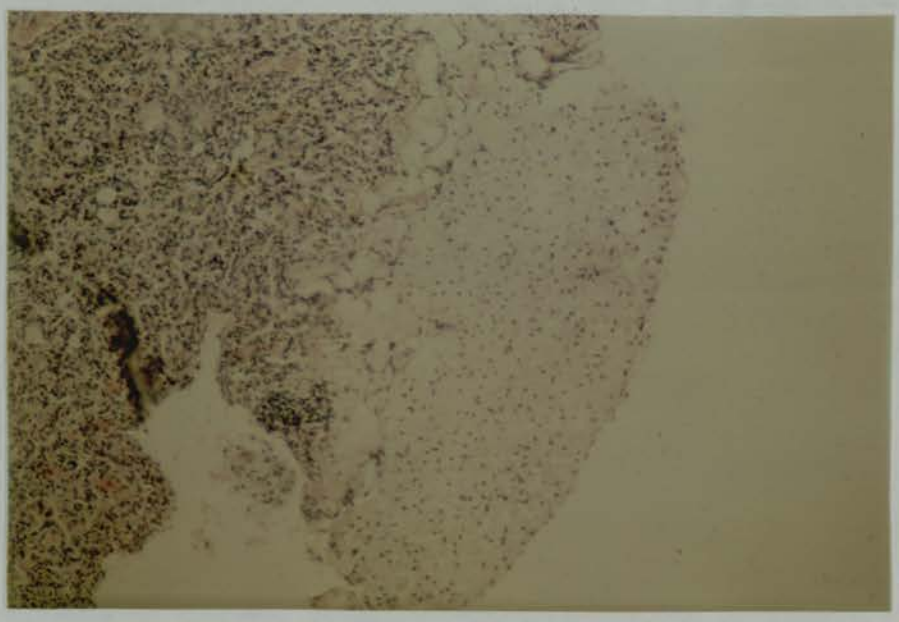


Figure 5.12b

Figures 5.12a and b. Two views of the pleural histiocytic tumour found in a chrysotile-treated animal aged 645 days. (H. + E.)

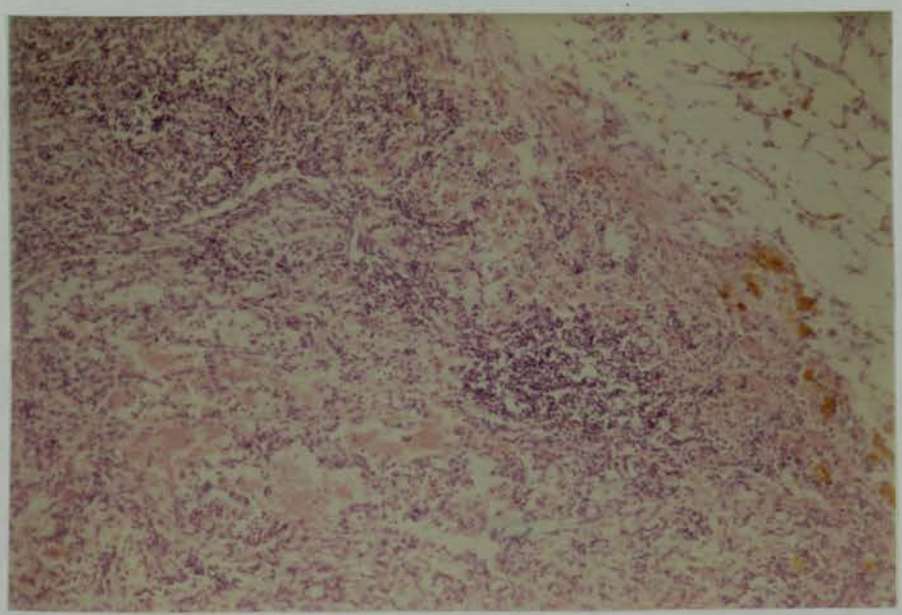


Figure 5.13 Typical view of a benign vasoformative haemangioma occasionally found in the HAN/AF strain of laboratory rat. (H.+ E.)

5.4 FIBRE SEARCHES

A wide range of tissues (see Section 2.1.4) were taken from the majority of those animals dying during the latter half of the study and stored at -20°C until appropriate facilities were available to enable a search for evidence of asbestos fibres within the tissues. A detailed description of the procedures developed can be found in Section 2.6.1.

Figures 5.14 to 5.16 summarise the results of these fibre searches, expressed in terms of the actual number of fibres found per tissue, and not the calculated total tissue burden. This method of presentation has been chosen to show the real density of asbestos fibres within the tissue residues in an attempt to emphasize their scarcity. However, appropriate 'sample conversion factors' are included in each table to allow a total tissue burden to be estimated. This factor varies with the tissue according to the amount of insoluble residue remaining after ashing and preparation. Thus a large and bulky organ such as liver or gut, with a relatively large amount of foreign matter associated with them, produced more residue when ashed. The residue then had to be appropriately diluted and subsampled to produce the optimum particle density for electron-microscopical searches. Conversely, ashing of tissues such as omentum or mesentery produced so little residue that the majority could be examined directly in detail for the presence of fibres. A more accurate statistical treatment of the likely number of fibres per tissue could be obtained using the expression derived in Section 2.6.2 b.

It can be seen from Figs. 5.14 to 5.16 that, for the

FIGURE 5.14 Long-term asbestos ingestion in male rats : amosite ingestion fibre search results.

		No. of fibres per sample per rat											
Animal age (days)		839	839	846	846	846	846	846	853	884	884	884	884
Tissue	Conversion Factor												
Lungs	351								3		-		
Liver	5200	-	-	-	1	-	-	1	-		-		1
Spleen	120	-	-	-	-	-	-	-	-		-	-	-
Kidney	176	1	-	1	-	-	-	-	-	-	-		4
Gut	1164	-		3	-	-	-	6	3	-		-	-
Mesentery	141	-	-	-	-	-	1	-	-	-	2	-	-
Omentum	30											-	-
Thoracic body wall	12640	-	-		-		-	-	-	-	-	-	-
Peritoneal body wall	6000	-	-		-		-	-	-	-	-	-	-

FIGURE 5.15 Long-term asbestos ingestion in male rats : crocidolite ingestion fibre search results.

		No. of fibres per sample per rat												
Animal age (days)		604	870	871	871	877	924	927	927	933	933	940	940	940
Tissue	Conversion Factor													
Lungs	351	1		1	-	-	-	-	-	-	2	-	-	-
Liver	5200	-	1	-	-	-	-	-	-	-	-	1		1
Spleen	120	-	-	-	-	-	-	-	-	-	-	-		-
Kidney	176	3	-	-	-	-	-	3	1	-	-		5	-
Gut	1164							-	4	2	-	-	-	2
Mesentery	141	-	1	-	-		-	-	-	-	-	1	-	-
Omentum	30	-	-	-	-	-	-	-	1	-	1	5	-	-
Thoracic body wall	12640	-	-		-		-			-	-	-	-	-
Peritoneal body wall	6000	-	-		-	-	-			-	-	-	-	-

FIGURE 5.16 Long-term asbestos ingestion in male rats : chrysotile ingestion fibre search results.

		No. of fibres per sample per rat												
Animal age (days)		645	757	833	909	918	937	939	939	948	948	960	960	961
Tissue	Conversion Factor													
Lungs	4212		1		3			3		2	2	1	1	
Liver	62400	-	-	1	-	-	-	-	-	-	-	-		2
Spleen	1440	-	-	-	1	-	-	4	-	-	-	-	-	
Kidney	2112		1	-		-	4							
Gut	13968			-		-	-	6	-	-		-	1	3
Mesentery	1692			-	-	1	-		-	-	-	-	1	-
Omentum	360	-	-	-	-	-	-		-	-	-	4	1	-
Thoracic body wall	151680			-		-	-	-	-	-		-	-	
Peritoneal body wall	72000	-		-		-	-	-	1	-		-	-	

purposes of these assays, no gastrointestinal tissues were sampled from animals that were actually exposed to ingested asbestos at the time of death. This apparent anomaly was necessary because (a) the finding of fibres under these circumstances could not differentiate between fibres resident within the tissues, and those from the gut lumen adhering in the mucous at the mucosal surface, and (b) the sensitivity of the preparation techniques was such that these tissues, together with their high asbestos fibre load, provided an unacceptably high risk of cross-contamination of other samples. This latter point was amply demonstrated in pilot trials in which attempts were made to remove all surface asbestos fibre by vigorous washing of the mucosal surface at autopsy. Subsequent scanning electron-microscopy of mucosal surfaces so treated showed that fibres frequently remained adhering, but not penetrating the surface. Further vigorous rinsing simply caused localised sloughing of the epithelium.

One further comment is necessary at this stage. Considerable difficulty was experienced with the identification of the analytical spectra produced from the energy dispersive analysis of suspicious fibres in some residue samples. In particular, kidney and some spleen residues were frequently found to contain numerous small fibres of similar dimensions and morphology to amphibole asbestos (Fig. 5.17), and they were initially identified as such. However, their consistent occurrence in both the chrysotile-treated animal residues and some control residues suggested the need for detailed examination. EDAX analysis showed that the fibres contained relatively little silicon, but high levels of iron and potassium, and

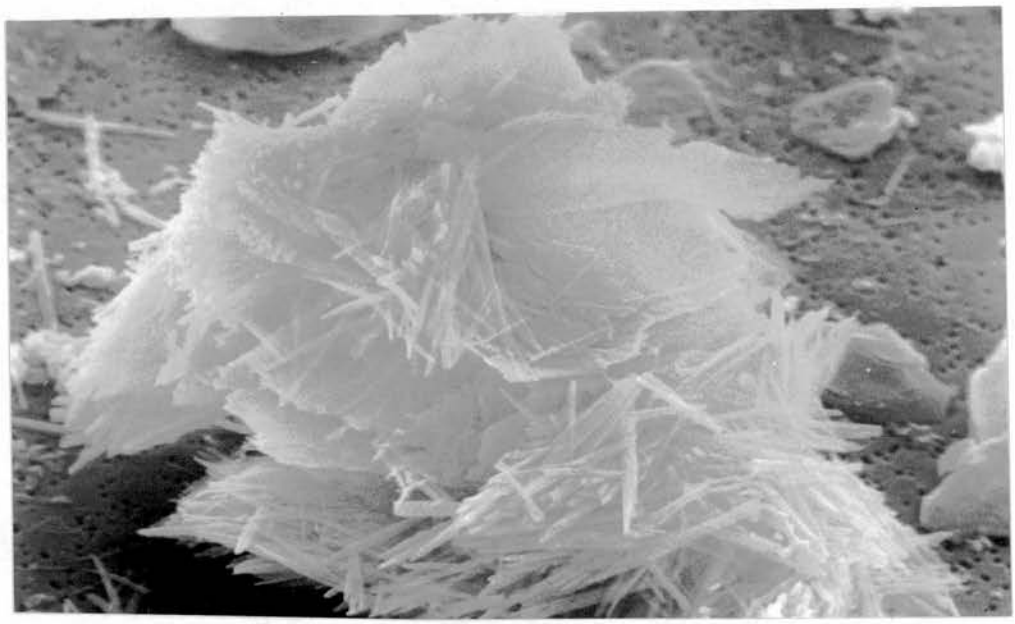
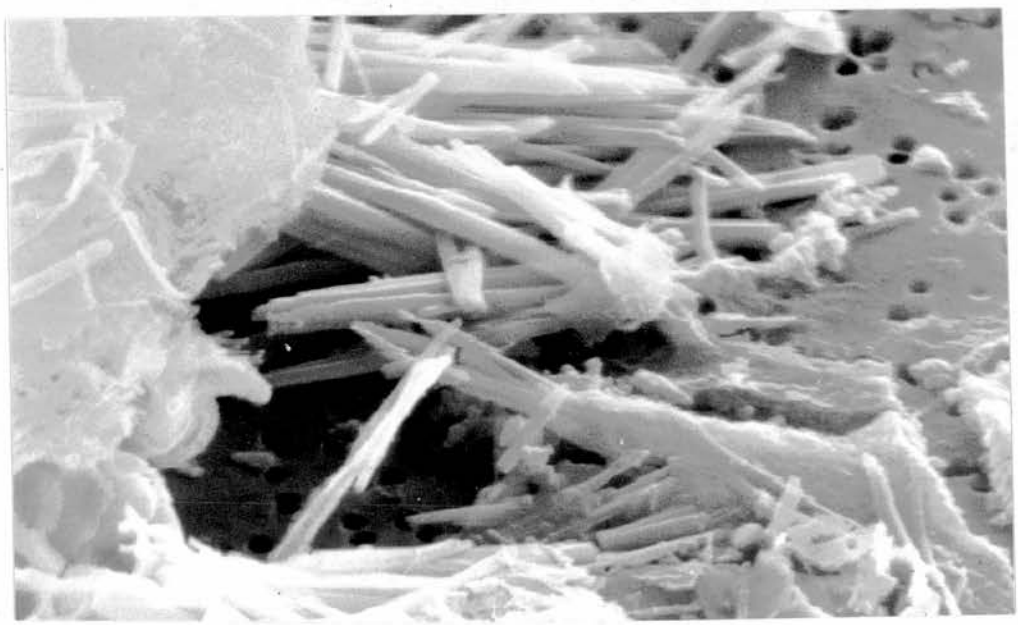


Figure 5.17a



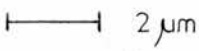


Figure 5.17b

Figures 5.17a and b. Two views of the anomalous fibres found in some ashed kidney and spleen tissue residue. (scanning electron micrograph)

FIGURE 5·17c

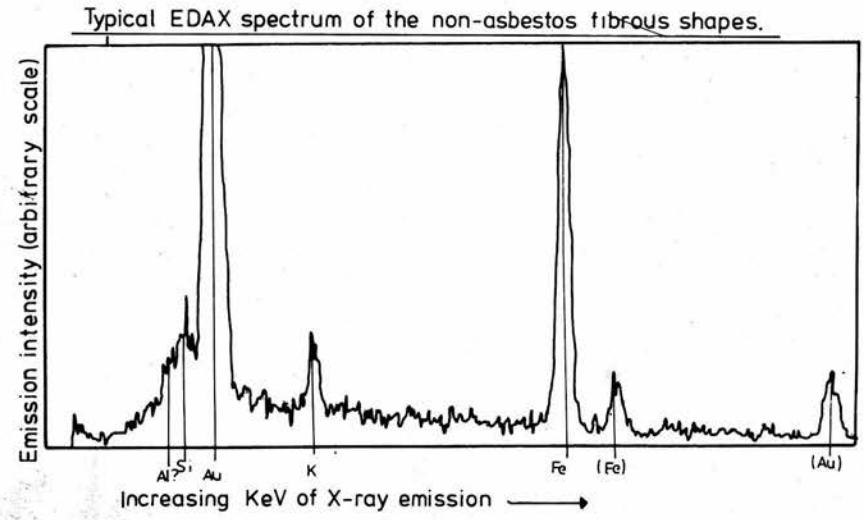


Figure 5.17c EDAX spectrum of one of the anomalous fibres shown in Figures 5.17a and b.

the spectra so produced (Fig. 5.17c)were distinct from those of known asbestos standards subjected to similar extraction procedures (see chapter 3). It was noticed that the fibrous shapes were much more numerous in those kidney and spleen samples that had been subjected to a period of muffle ashing to reduce their bulk (see Section 2.6.1 c). Early attempts to mimic their production by processing untreated rat kidney tissue resulted in the production of large numbers of these fibres. However, subsequent attempts totally failed to produce the fibrous shapes, despite identical conditions. It is difficult to account for the presence of the fibrous shapes other than to assume that they were a transitory artefact of the muffle ash process, possibly due to excessive local heating in the presence of some residual water vapour. Indeed, there is strong presumptive evidence that conditions prevailing during muffle ashing could favour the formation of iron containing fibrous crystals such as Goethite (A.P. Middleton, personal communication). It is clear that the fibrous shapes found were unlikely to be of asbestos origin: it is inconceivable that a removal of silicon from asbestos fibres on the scale indicated by the EDAX analyses could occur without the total disintegration of the fibres. Further, the silicon lattice of the amphibole is proven to be very stable, and it can withstand conditions such as prolonged reflux in concentrated acids (see Section 1.1.3).

Returning to Figs. 5.14 to 5.16, the most obvious result of the fibre searches is that comparatively very few asbestos fibres were found amongst the residues. Further, there is no noticeable

trend in the distribution of those fibres found, and no preferential finding of fibres in specific tissue sites. Finally, an attempt to relate the occasional presence of fibres with those animals exhibiting pathological lesions showed no correlation. If asbestos penetration and/or damage to the gut had occurred, it would have been reasonable to expect (a) relatively high levels of fibres within the tissues, (b) preferential occurrence of fibres within those tissues closely associated with the mode of entry, (c) possibly, highest fibre densities in those animals with pathological lesions. None of these conditions was fulfilled in the present study.

5.5 GUINEA PIG INGESTION EXPERIMENT

Twenty male guinea pigs were used in an asbestos ingestion experiment in which the asbestos was administered in a pelleted diet formulation (see Section 2.1.3 b for details of preparation). The principal aim of this experiment was to investigate the effect of asbestos upon an alternative gastrointestinal mucosal surface to the rat. The herbivorous habit of the guinea pig contrasts with the omnivorous rat, and there are other factors such as: the guinea pig's more vigorous turbulence as a result of the strongly developed taeniae coli, and the longer digestion times associated with cellulose breakdown, that suggested a possible increased likelihood of mucosal penetration and/or damage.

The experiment was initially designed to be of short duration, with the emphasis on histological and ashing searches for presence of asbestos fibre damage. However, no adverse effects were found after a short exposure period, and so the study was extended to one of a fifteen-month ingestion period, with a full lifespan

follow-up. Limited resources necessitated that only one asbestos type could be tested, and crocidolite was chosen on the grounds that its morphology was considered to be the most likely to penetrate and damage the gut mucosa, and its chemical resistance such that ashing recovery could be simplified by using the stronger acid washes.

The animals were housed two per cage, and the asbestos-impregnated diet was mixed with normal diet and supplied ad libitum to give the final dose of 250 mg per guinea pig per week. Attempts were made to detect any airborne asbestos generated during the normal feeding behaviour of the animals, but despite very long sampling times, no airborne asbestos fibre was found in the course of the study. Animals were regularly checked, killed when moribund, and a full autopsy performed (see Section 2.1.4). In addition to the histological tissue, samples were taken for ashing analysis for the presence of fibre from most of the animals. Figure 11 of Appendix IV lists the pathological findings for each animal, and the information is summarised in Fig. 5.18.

These figures show clearly that there were no neoplastic lesions found and that the most common lesion was a low-grade calcification of certain tissues. The calcium deposition was most frequently found around the kidney tubules, although a common site was also in the gastric glands. In some animals there was a rather irregular pattern of deposition within the gastrointestinal musculature. One animal was found with calcium deposits in the left myocardium. This degree of metastatic calcification is considered a normal feature of the strain of guinea pig as used in this thesis, and the pattern of deposition characteristic of the condition of hypervitaminosis D commonly found in laboratory rodents (Fig. 5.19).

FIGURE 5.18 Long-term crocidolite asbestos ingestion in twenty guinea pigs : pathological lesions found.

Lesion	No. of animals affected
Pneumonia	3
Pulmonary congestion	5
Coronary thrombosis	2
Calcification :	
renal	8
gastric	5
intestinal	2
myocardial	1
Hepatic fatty change	4
Miscellaneous	2

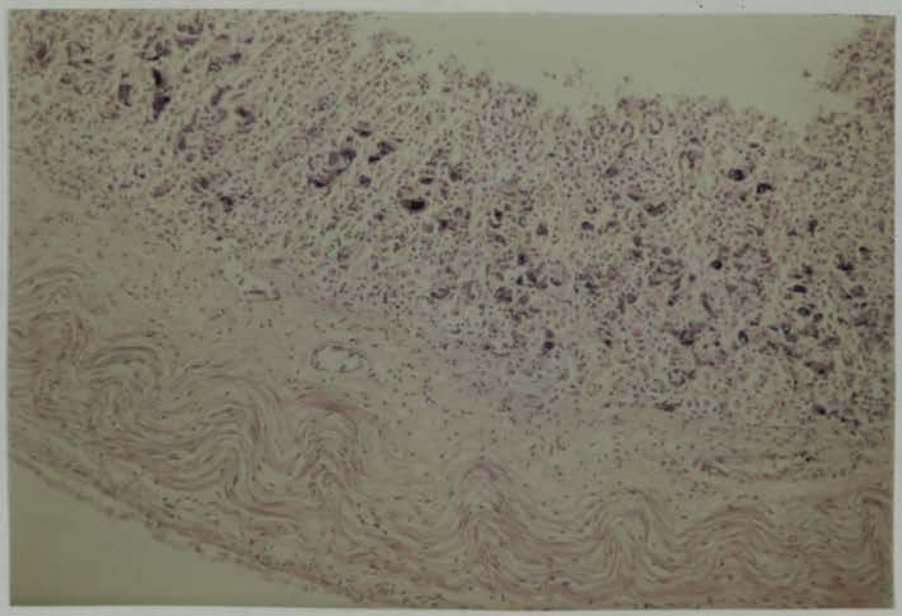


Figure 5.19a Glandular stomach.

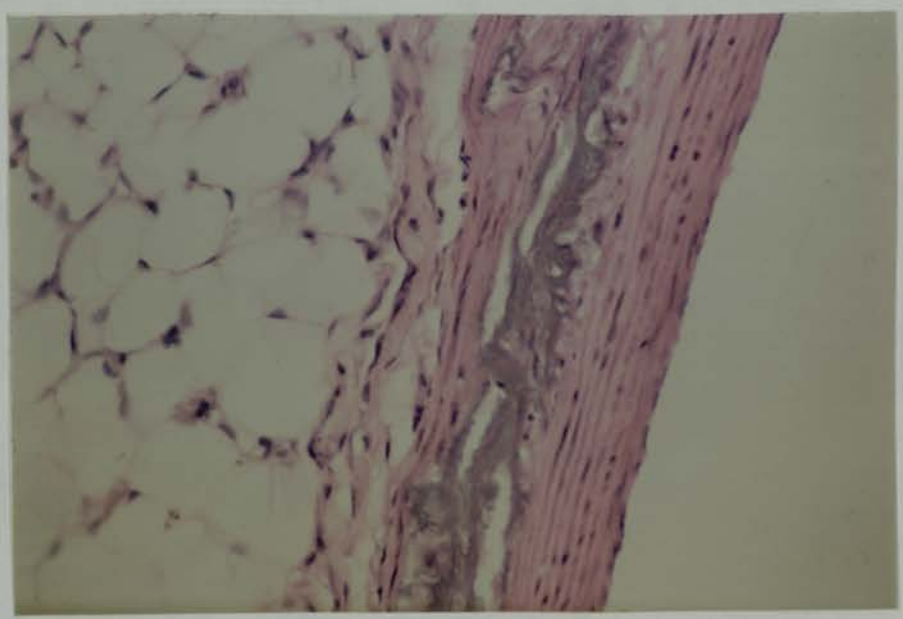


Figure 5.19b Aorta.

Figures 5.19a and b. Two views of the metastatic calcium deposition frequently found in guinea pig tissues.

Figure 5.20 summarises the results of the electron-microscopical searches of ashed and acid-washed tissue residues. (Note that, once again, the results have been expressed in terms of the numbers of fibres found per sample.) It can be seen that fibres were consistently found in lung tissue residues, although at a relatively low level, considering that the bulk of the pulmonary tissue was ashed. The method of administering asbestos in a pelletised formulation made it inevitable that some inadvertant inhalation of fibres would occur during ingestion and mastication. However, the airborne sampling, and these low levels of pulmonary asbestos fibre showed this route of entry to be unimportant. Amongst the other tissues examined, Fig. 5.20 shows that there was no widespread or consistent evidence of asbestos fibre penetration or deposition.

5.6 CONCLUSIONS

1. Rats ingesting approximately 50 - 60 gms of margarine per week were 25% heavier than controls. This increase in weight could be mainly accounted for by an increase in the size and extent of the peritoneal and subcutaneous fat depots. The pathological effects of the obesity were an increase in the degree of hepatic fatty change in older animals, and, occasionally, some fatty infiltration of the colonic musculature. There was no obvious difference in body weight, morbidity, or mortality, between the different asbestos treatments.

2. There was no obvious evidence of any carcinogenic effect of asbestos ingestion. No primary malignancies of the gastrointestinal mucosal tissues were found in any of the rats chronically ingesting large doses of asbestos for periods of up to 25 months. Taken collectively, however, it is worth noting that five of the 13

FIGURE 5.20 Long-term asbestos ingestion in male guinea pigs : crocidolite ingestion fibre search results.

		No. of fibres per sample per animal																
Animal age (days)		401	430	431	437	457	479	536	543	556	646	695	723	723	746	746	810	810
Tissue	Conversion Factor																	
Lungs	446	8	10	16				5	9	2							11	5
Liver	8170	-		-	-	-	-	-	1	-	1	-	-	-	-	-	-	-
Spleen	409			-	-		-	-	-	-	-	1	1			-	-	-
Kidney	182	-		-	1	-	-	-	2	-	3	-	-	-	-	-	-	-
Gut	1420	1	-	-	1	-	-		1	-	-	-	4	1	2	-	-	-
Mesentery	130	-	-	-	-		-		-	1	-	-	-			-	-	-
Omentum	30	-	-	-	-	-	-		-	-	-	-	-	-		-	-	3

malignant tumours were found in the chrysotile-treated animals, whereas there was only one in each of the amphibole asbestos types. Further, it is interesting that the only malignant tumour arising amongst the amosite-treated animals was a gastric leiomyosarcoma, and that a malignant pleural histiocytic tumour was found in a chrysotile-treated animal.

3. There was an excess of benign tumours in the chrysotile-treated rats, largely as a result of the finding of mesenteric haemangiomas in four of the animals. All the remaining benign tumours found are occasionally encountered in untreated HAN ♂ s.p.f. rats and were not considered to be associated with asbestos exposure.

4. There was no obvious association of any of the other lesions with the asbestos treatment, except that hepatic fatty change was more common amongst the margarine-treated rats, and there was slightly more mesenteric polyarteritis nodosa amongst those animals exposed to asbestos.

5. Very few fibres were found amongst the tissue residues of animals from the ingestion experiments, indicating that there was no widescale penetration of asbestos through the gut wall despite the large doses to which the rats were exposed.

6. No sign of fibre penetration or pathological damage was found amongst a group of 20 guinea pigs ingesting crocidolite asbestos.

CHAPTER 6 A CYTOKINETIC ANALYSIS OF THE GASTROINTESTINAL MUCOSAE OF RATS INGESTING ASBESTOS

A CYTOKINETIC ANALYSIS OF THE GASTROINTESTINAL MUCOSAL OF RATS CHRONICALLY EXPOSED TO INGESTED ASBESTOS

6.1 INTRODUCTION

The cytokinetic analysis of the gastrointestinal mucosal of rats involved in a long-term asbestos ingestion study was undertaken to examine the effects of asbestos on the mucosal proliferation. Three groups of animals were used: one exposed to ingested asbestos, one exposed to margarine as a control, and one untreated control group.

A dual approach was adopted for the analysis. The first procedure involved quantitative estimations of the amounts of DNA precursor taken up by the gastrointestinal tissues using DNA extraction and liquid scintillation counting methods. A full account of the development of these methods is available in Section 2.6.3. The second, more sophisticated approach involved a detailed assay of the rate of entry of cells into mitosis, and the extent of uptake of DNA precursor by a number of specialised cell types within the gastrointestinal mucosa. Details of the methods used may be found in Section 2.5.3. A brief introductory literature review is included in Appendix III. It is considered that the manipulation of the raw data, and the subsequent interpretation of the analyses so produced, require a certain amount of detailed explanation. The format of Chapter 6 has thus been arranged to provide scope for explanation where necessary.

At the time that resources became available to pursue a full cytokinetic analysis of the gut tissues using radioactive DNA precursor techniques, there was a small group of 8 animals that had been exposed to amosite asbestos ingestion for a period of 25 months. The asbestos dose had been set at approximately 50 000 times the estimated occupational maxima for asbestos workers (see Chapter 5),

and it was felt that any effects of asbestos on gastrointestinal cytokinetics should be expressed within this experimental regime. In view of the limited number of animals available (8 treated, 8 untreated age-matched controls and 6 margarine-treated positive controls), particular attention was paid to the design of the experiment, and it was decided to use the animals as shown in Figure 6.1. Within each group the animals were randomly allocated to the assays. The 6 positive, or blank margarine-fed, controls were kept aside until they were used only for a stathmokinetic analysis. Figure 6.1 shows that the other animals were used for autoradiographic and stathmokinetic assays. This combination of assays allowed the major effort to be concentrated upon the treated animals and their age-matched controls. In the event of a significant difference between the cytokinetic parameters of these two groups appearing, the material from the positive control stathmokinetic assay could be examined for evidence of the effects of margarine ingestion alone.

Section 2.5 provides full details of the methods used for the preparation of the tissues used in the various analyses. All animals were weighed at autopsy and the results are shown in Figure 6.2. It can be seen from this that those animals fed either margarine alone, or margarine and amosite asbestos, were considerably heavier than those not given access to margarine. This finding is in accord with the results described in Section 4.2.2. None of the animals used in these analyses had any pathological lesions that could be considered to have had any major effect on the proliferative status of their tissues. The principal findings were that the usual degree of renal hypertensive damage was found in most animals (see Chapter 5), and those on a

FIGURE 6.1 Cytokinetic analysis: experimental layout.

Assay	No. of animals per treatment		
	Amosite-fed	Negative control	Positive control
Autoradiographic and DNA assays	3	3	-
Stathmokinetic assays	5	5	6

FIGURE 6.2 Autopsy body weights (all animals aged 28 months at death).

	Treatment				
	Tritiated thymidine uptake		Stathmokinetic		
	Amosite	Control	Amosite	Control	Margarine Control
	676	482	616	528	780
	894	528	574	450	730
	802	568	692	510	740
			582	480	708
			678	540	743
					728
Means	790.7	526.0	628.4	501.6	738.2

margarine-supplemented diet had some periportal hepatic fatty changes. Further details may be found in Appendix IV, Figures 6 and 10.

6.2 ESTIMATIONS OF DNA SYNTHESIS IN GASTROINTESTINAL TISSUES

The extent of DNA synthesis was estimated using the rate of uptake of the DNA-specific precursor thymidine. Full details of the methods used to estimate the rate of uptake of tritiated thymidine are given in Section 2.5.2. Details of the development of these methods are available in Section 2.6.3. The assays produce results for the radioactivity of a given tissue, expressed in terms of the number of tritium disintegrations per minute per microgram of recovered DNA (dpm/μg DNA). Three tissue samples from each anatomical site were processed, and triplicate estimations were made from each sample. There were thus 9 values produced for each tissue site for each treatment, and examples of the data produced are given in Figure 6.3, using the values obtained for the upper small intestine and descending colon assays. It can be seen that there was considerable variation in the values found for the activity of the extracted DNA between the three samples. There was also considerable variation in the values measured for the triplicate aliquots of each sample, despite the fact that great care was taken over the standardisation of the extraction and purification procedures. This variability showed that only large differences between the rates of incorporation of tritiated thymidine may be detected by the extraction assays.

Figure 6.4 summarises the results of the DNA activity estimations for all the tissues examined. Each value represents the arithmetic mean, and the standard error of the mean is included to give some idea of the variability of the assays. It can be seen that

FIGURE 6.3 Typical results from DNA estimations, using upper small intestine and descending colon as examples.

Tissue	Tissue sample No.	Activity of extracted DNA (d.p.m. per μg)	
		Amosite-treated	Control
Upper small intestine	1	227.4	199.1
		242.0	290.3
		205.7	270.6
	2	337.4	626.4
		381.8	510.0
		293.9	432.3
	3	578.6	272.6
		380.6	384.1
		670.0	331.4
Descending colon	1	174.6	259.2
		132.4	184.7
		98.4	263.0
	2	157.4	178.3
		153.1	222.3
		177.1	214.4
	3	165.8	229.1
		152.8	195.8
		129.7	246.0

FIGURE 6.4 Cytokinetic analysis : summary of DNA estimations.

Tissue	Mean D.p.m./microgram of DNA ± standard error of mean		t test	Significance (16 degrees of freedom)
	Amosite-treated	Control		
Glandular stomach	59.37 ± 7.20	79.67 ± 8.91	-1.77	N.S.
Upper small intestine	368.60 ± 53.27	368.53 ± 45.00	0.0009	N.S.
Mid small intestine	668.70 ± 126.84	589.11 ± 50.10	0.437	N.S.
Lower small intestine	200.33 ± 42.66	186.62 ± 31.39	0.259	N.S.
Caecum	611.87 ± 37.97	506.51 ± 40.85	1.889	N.S.
Descending colon	183.00 ± 12.87	175.82 ± 6.34	0.500	N.S.
Descending colon	149.03 ± 8.36	221.42 ± 10.36	-5.44	$P < 0.001$
Liver	37.13 ± 5.39	23.37 ± 2.62	2.298	$P < 0.05$
Spleen	182.34 ± 32.50	148.21 ± 19.70	0.898	N.S.

only two tissues were found to have statistically significant differences in their rates of incorporation of tritiated thymidine. The descending colon samples from amosite-treated animals were found to have incorporated significantly less labelled thymidine than their age-matched controls over the same period, suggesting that amosite ingestion results in a depression of the cytokinetic activity in the colonic mucosa. The liver assays also showed a significance difference in thymidine incorporation, but in this case it was the control samples that had taken up less thymidine. It may be that this was due to the possibility that there was less circulating thymidine available in the control animals for uptake by the liver owing to the higher rate of incorporation within the control colonic mucosa. However, it must be remembered that the amosite-treated animals were also ingesting large amounts of margarine whilst the particular group of age-matched controls available for the thymidine uptake assays were not given access to margarine (see Section 6.1). It has already been found (Sections 5.3 and 6.1) that prolonged margarine ingestion was associated with a certain degree of hepatic peri-portal fatty change, and it is therefore possible that differences in uptake of thymidine by the livers of these animals may be related to the high fat diet and obesity.

6.3 FULL CYTOKINETIC ANALYSES OF SELECTED TISSUES USING HISTOLOGICAL SECTIONS

6.3.1 Preliminary remarks

Although all levels of the gastrointestinal tract were sampled and prepared for histological analyses, only tissues from four anatomical sites were examined in detail. These were: three levels of the small intestine, and one of the descending colon for each

animal, and they were considered to be representative of the proliferative activity of the other gastrointestinal tissues. (Some estimations of proliferative activity of the squamous forestomach samples were also made.) Those tissues not examined in detail were stored in case any differences found between amosite treated and control animals required further investigation.

The assays of the four tissues from each of 8 treated and 14 control animals generated an enormous amount of raw data, with over 400,000 cells being counted over a period of six months. It is not possible, therefore, to include details of all the individual calculations and assumptions associated with the cytokinetic analyses of each tissue. As a result, the methods of calculation of the various cytokinetic parameters are presented in detail for one typical tissue, with the results of the other similar calculations being presented in summary form only. The tissue 'selected' as a representative worked example was the upper small intestine of those animals ingesting amosite asbestos, and there thus follows an account of the generation and manipulation of the results of these assays.

6.3.2 A worked example

Section 2.5.3 describes the details of the cell counting procedures with the preparation of the standardised proliferative distribution curves for each tissue, and these formed the basis of subsequent calculations. Appendix III provides some background information on cytokinetics theory. The aim of the autoradiographic and stathmokinetic studies was to derive a reliable figure for the rate that cells in the proliferative zone of the

intestinal crypt enter into the mitotic phase of the cell cycle. Provided that the size (i.e. the number of cells) of the proliferation and maturation compartments of the crypt are also known, it is possible to calculate the proliferative rate of the whole tissue from the rate of entry of cells into mitosis (r_m). The r_m can also be used to calculate such additional parameters as the mean cell cycle time (t_c), the duration of mitosis (t_m), and the transit rate of cells up the crypt in cell positions per hour. The t_m calculated in this way refers to the mean duration of mitosis for all those cells dividing in the crypt. There is strong evidence (Wright et al., 1973) that the duration of mitosis varies with cell position in the crypt, and this can be proved from the mitotic accumulation rates plotted for each cell position.

The counts derived from the autoradiographic study were used to produce a figure for the labelling index (I_L) by dividing the number of labelled cells by the total number of cells counted. Since the presence of label within a cell nucleus indicates that the cell was in the synthesis (S) phase of the cell cycle in the hour immediately prior to death, the I_L provides an estimate of the number of cells synthesising DNA at any given time. I_L for the amosite-treated upper small intestine was found to be 28.29 (from 3,698 labelled cells divided by 9,372 total cells, x 100), or 28.29% of crypt cells were synthesising DNA at the time of assay. The autoradiographic counts can also be used to estimate the proliferative compartment size by finding the cell position at which the mitotic index (I_m) is at 50% of the maximum or peak value (see Cleaver, 1967). This proliferative index, or I_p, was found to be 0.56 for the amosite-treated upper small

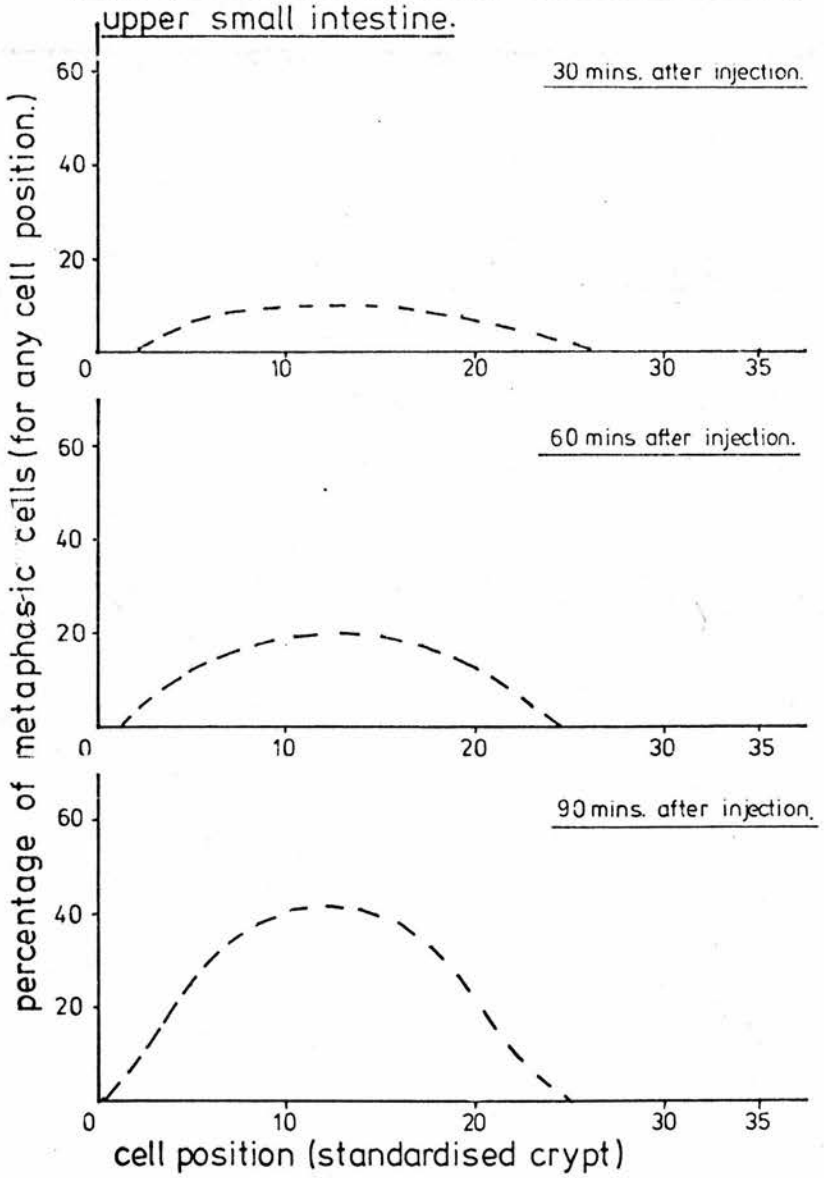
intestine, since the standardised proliferative distribution curve showed the mean column length was 32.67 cells, and the 50% peak occurred at cell position 18. I_p may also be obtained from the standardised proliferative distribution curves from the stathmokinetic data, but it is not as accurate when calculated in this way since the mitotic rate falls later than the labelling index as cells move up the crypt.

The counts derived from the stathmokinetic study were used to construct proliferative distribution curves for each of the five killing times after vincristine injection (30, 60, 90, 120 and 150 minutes - see Section 2.5.1) and r_m was calculated from the slope of the increase of I_m with time. Figure 6.5 shows examples of the graphical output of the proliferative distribution curves for the amosite-treated upper small intestine (see Section 2.5.3 for details of their preparation) in which the incidence of cells arrested in metaphase is given for each cell position in the crypt. It can be seen that there is a marked accumulation of arrested metaphasic nuclei with time. It is interesting to note from Figure 6.5 that the curve for 150 minutes of metaphase arrest shows that a plateau exists between cell positions approx. 9 - 18, and this is an indication that the stathmokinetic drug had caused a complete blockage of all those cells coming to metaphase over the 150 minute period. This could be confirmed from the linear nature of the plot of I_m against time of arrest as is shown in Figure 6.6.

Appendix IIIC provides some background details of the need for the Tannock's factor corrections to the raw data arising from over estimations of the mitotic index obtained from histological sections.

FIGURE 6·5

Cytokinetic analysis:- Demonstration of metaphase accumulation curves using amosite-treated upper small intestine.



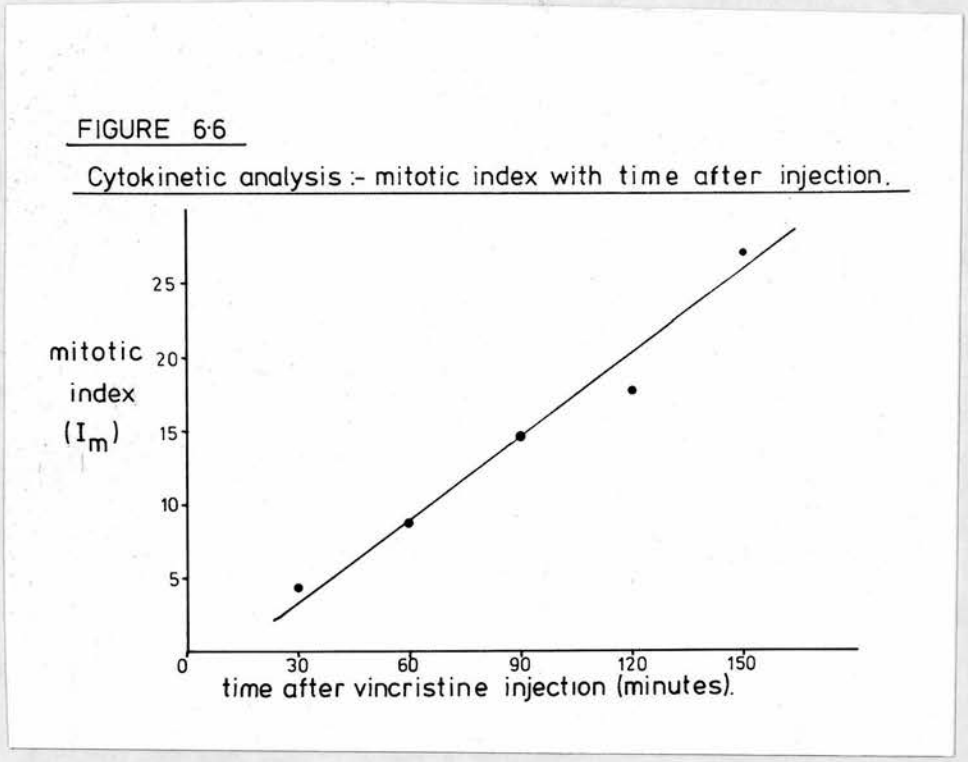


FIGURE 6.6 Graph showing the linear nature of metaphase accumulation following vincristine administration.

The Tannock's factor was found for each tissue in the course of the cytokinetic studies but since there was no difference between the three levels of the small intestine, a common factor of 0.52 was used for the amosite small intestine data. It is necessary to use the Tannock's factor correction to calculate the true I_m at each stathmokinetic arrest time-point before the r_m could be calculated. Figure 6.7 gives details of the calculation procedure. r_m is then the slope of the regression line from either the corrected I_m figures plotted against time of arrest if steady state conditions within the crypt population is assumed to occur, or the natural logarithm of the corrected I_m against time if exponential growth conditions are assumed. In the absence of any firm information to the contrary, the steady state or "rectangular" (see Appendix IIIB) growth conditions are considered to be more applicable in this study, although for comparison some parameters calculated for exponential conditions are included in the summaries reported in Section 6.3.3.

Whichever calculation method is used, the resultant r_m figure must be multiplied by 60 to convert it to the number of divisions per crypt cell per hour, and r_m was thus found to be 0.053 for amosite-treated upper small intestine. The production rate of a tissue in terms of the cells produced per crypt per hour can thus be simply calculated by multiplying r_m by the crypt size obtained from the product of the mean crypt column count and the mean column length. For the amosite-treated upper small intestine this was found to be 37.92 cells per crypt per hour, assuming steadystate conditions.

r_m may be used to derive a value for the apparent cell cycle time $t_{c(a)}$. For steady state growth conditions, the $t_{c(a)}$ can be

FIGURE 6.7 Cytokinetic analysis: table of calculations necessary for the determination of r_m for the amosite-treated upper small intestine.

Duration of Arrest	Measured I_m	Corrected I_m	$1 + I_m$ (corrected)	$\text{Log}_e \{1 + I_m\}$ (corrected)
30	4.68	2.43	1.0243	0.02401
60	8.84	4.60	1.0460	0.04497
90	14.74	7.66	1.0766	0.07381
120	17.73	9.22	1.0922	0.08819
150	27.25	14.17	1.1417	0.13252

found from the reciprocal of r_m, and the value for amosite-treated upper small intestine was thus 18.79 hours. If exponential growth conditions are assumed $t_{c(a)}$ is found to be somewhat different, since in this case $t_{c(a)}$ can be found from $\frac{0.693}{r_m}$, giving a value of 13.32 hours. The apparent cell cycle time is derived by assuming that all cells within the crypt are dividing but the measurement of the proliferative index (I_p) mentioned earlier shows that this is not the case (since I_p was less than unity). The true cell cycle, t_c, may be found by multiplying $t_{c(a)}$ by I_p. Since I_p of the amosite-treated upper small intestine was 0.56, t_c may be calculated as 10.52 hrs using steady state assumptions or 7.46 hrs using exponential assumptions.

The duration of mitosis (t_m) may be calculated from I_m/r_m (see Appendix III), provided that some adjustment is made for the units such that r_m is expressed as an index. This may be obtained by dividing the crypt production rate by the crypt size, and dividing the result by the Tannock's factor to give r_m as a percentage of cells per hour. A value of 11.29% per hour can thus be obtained for r_m of the amosite-treated tissue. The background or normal incidence of mitosis (I_m) may be found from the mitotic counts taken as part of the auto-radiographic determinations, in which there was no experimental interference with metaphase. I_m for the upper small intestine crypt of the amosite-treated animals was 4.77, and with r_m expressed as 11.29%, the mean duration of mitosis for the whole crypt is 0.42 hrs or 25.16 minutes. It has already been mentioned that the duration of mitosis varies with cell position within the crypt, but the mean value of t_m for the whole crypt will suffice for the purposes of the assays

described here.

6.3.3 The tissue summaries

This section will be restricted to summary listings of some of the calculated parameters of the cell population kinetics of those tissues examined together with interpretation of their significance. Appendix IV Figures 20-24 contain further details of the basic information from which the calculations were obtained as described in Section 6.3.2. Figure 6.8 lists some of the morphological findings. The Tannock's factor used for the calculations is also included for each tissue. A small difference was found between the factor determined for each tissue, and a comparison between amosite-treated and control tissues showed a marginally significant difference (using Student's t test). It was thought best to incorporate this difference into subsequent calculations, but to use a mean value for the three levels of the small intestine.

Figure 6.8 shows that the proliferative index, I_p, was similar for each level of the intestine sampled and there was therefore no large change in the growth fraction associated with treatment. This can be confirmed from the sizes of the proliferative compartment also shown in Figure 6.8. There was also a clearly defined tendency for the length of the crypt to decrease with progression along the small intestine and the decrease was found to be similar in both amosite-treated and control animals. The length of the descending colon crypt was shown to be considerably greater than the small intestine and once again there was no difference between treated and control. The information summarised in Figure 6.8 indicates that there was no detectable effect of the long-term chronic ingestion of amosite asbestos

FIGURE 6.8 Cytokinetics analysis: summary of some of the basic data.

	Mean Tannocks Factor	Proliferative Index (I_p)	Mean Column Length (cells)	Mean Column Count (cells)	Whole Crypt Size (cells)	Proliferative Compartment Size (cells)
Amosite						
Upper small intestine	0.52	0.56	35.69	19.97	712.73	399.1
Mid small intestine	0.52	0.53	32.62	20.83	679.47	360.1
Lower small intestine	0.52	0.51	31.45	20.38	640.95	326.9
Descending colon	0.62	0.43	41.13	20.20	830.83	357.3
Control (- ve)						
Upper small intestine	0.49	0.51	35.95	22.02	791.62	403.7
Mid small intestine	0.49	0.50	32.16	20.06	645.13	322.6
Lower small intestine	0.49	0.51	31.76	21.12	670.77	342.1
Descending colon	0.59	0.42	41.83	20.80	870.06	365.4
Control (+ ve)						
Descending colon	0.62	0.43	38.61	20.37	786.44	338.2

on the morphological parameters of those gastrointestinal tissues examined.

Figures 6.9 to 6.12 list some of the calculated parameters associated with the analyses. The values for the rate at which cells enter mitosis and the cell cycle time are shown with the standard errors to give some indication of the confidence limits of the data. Figure 6.9 gives the r_m and $t_{c(a)}$ values for the tissues, and both 'steady state' and 'exponential' assumptions have been included. It can be seen that the derivation of the calculation assumptions have had very little effect on the r_m values. The s.e.m* values included in Figure 6.9 show that the r_m estimations may be used with confidence in further calculations.

It must be remembered that the cytokinetics results were obtained from information derived from only one animal for each stathmokinetic time of kill, and yet the limits of errors were relatively small. There were, however, some fairly large differences in the rate of entry of cells into mitosis for the different tissues, and it is difficult to interpret the significance of these findings. Taken collectively though, there was a definite trend for r_m to decrease with progression along the intestinal tract such that r_m for the lower small intestine was always less than for the upper small intestine, and the descending colon r_m was always less than all levels of the small intestine.

There was no detectable difference between the r_m values obtained for the small intestine of amosite-treated and control animals. However, the information for the descending colon showed very large differences, with the r_m being much lower in the

* Standard error of mean.

FIGURE 6.9 Cytokinetic analysis: r_m and $t_{c(a)}$ values.

	Rate of entry of cells into mitosis* (r_m)		Apparent cell cycle time in hours $t_{c(a)}$	
	Steady state	Exponential	Steady state	Exponential
Amosite				
Upper small intestine	0.053 ± 0.006	0.052 ± 0.007	18.79 ± 2.08	13.32 ± 1.41
Mid small intestine	0.067 ± 0.009	0.061 ± 0.012	15.06 ± 2.61	11.32 ± 1.81
Lower small intestine	0.034 ± 0.005	0.033 ± 0.007	29.17 ± 5.19	21.30 ± 3.90
Descending colon	0.009 ± 0.002	0.015 ± 0.003	66.27 ± 11.67	47.15 ± 8.23
Control (- ve)				
Upper small intestine	0.050 ± 0.001	0.046 ± 0.001	20.14 ± 0.14	15.05 ± 0.04
Mid small intestine	0.041 ± 0.002	0.038 ± 0.003	24.66 ± 1.37	18.30 ± 0.89
Lower small intestine	0.041 ± 0.001	0.039 ± 0.002	24.28 ± 0.86	17.94 ± 0.54
Descending colon	0.028 ± 0.004	0.027 ± 0.005	36.17 ± 5.83	26.07 ± 3.98
Control (+ ve)				
Descending colon	0.014 ± 0.003	0.017 ± 0.004	71.43 ± 13.76	49.51 ± 7.04

* expressed in divisions per crypt cell per hour.

FIGURE 6.10 Cytokinetic analysis: cell cycle times.

	Calculated true cell cycle time (t_c)	
	Steady State Assumptions	Exponential Assumptions
Amosite		
Upper small intestine	10.52 ± 1.16	7.46 ± 0.79
Mid small intestine	7.95 ± 1.38	6.00 ± 0.96
Lower small intestine	15.25 ± 2.65	10.85 ± 1.99
Descending colon	28.50 ± 5.02	20.27 ± 3.54
Control (- ve)		
Upper small intestine	10.27 ± 0.07	7.68 ± 0.02
Mid small intestine	12.33 ± 0.69	9.15 ± 0.45
Lower small intestine	12.38 ± 0.44	9.15 ± 0.28
Descending colon	15.19 ± 2.45	10.94 ± 1.67
Control (+ ve)		
Descending colon	30.70 ± 6.11	21.28 ± 3.50

FIGURE 6.11 Cytokinetic analysis: proliferation rates.

	Proliferation rates (cells/crypt/hr)	
	Steady State Assumptions	Exponential Assumptions
Amosite		
Upper small intestine	37.92	37.06
Mid small intestine	45.25	41.58
Lower small intestine	21.98	20.88
Descending colon	7.54	12.13
Control (- ve)		
Upper small intestine	39.32	36.54
Mid small intestine	26.17	24.43
Lower small intestine	27.57	25.90
Descending colon	24.07	23.18
Control (+ ve)		
Descending colon	11.01	13.36

FIGURE 6.12 Cytokinetic analysis: duration of mitosis.*

	Mean duration of mitosis, t_m, (in minutes) for whole crypt
Amosite	
Upper small intestine	25.2
Mid small intestine	20.4
Lower small intestine	34.2
Descending colon	73.2
Control (- ve)	
Upper small intestine	24.6
Mid small intestine	30.0
Lower small intestine	29.4
Descending colon	46.8
Control (+ ve)	
Descending colon	75.6

* Steady state conditions assumed.

amosite treated tissue than in the control (see Figure 6.9). This implied a depressive effect of amosite asbestos treatment on the proliferative activity of the descending colon that required further examination. As described in Section 6.1, the experimental design permitted the cytokinetic analysis of a group of control animals that had been maintained on a margarine-supplemented diet. Accordingly, the descending colon tissues of these "positive" control animals were examined, and Figure 6.9 shows that the r_m value obtained was found to be closer to the amosite-treated tissue results. This was interpreted as demonstrating an effect of margarine alone on the descending colon cytokinetics. The apparent depressive effect of amosite ingestion was thus attributed to the "inert" carrier, and not to the asbestos per se. This conclusion is in accord with the findings reported in Chapter 4 where prolonged margarine ingestion was associated with a reduction in the food intake, and it may demonstrate an effect of lumenal contents on the gastrointestinal proliferative status.

The figures for $t_{c(a)}$ are included in Figure 6.9 and these show that there are large differences in the values calculated, depending upon the assumptions adopted. This is hardly surprising since it can be seen from Section 6.3.2 that each method manipulates the basic r_m information in a different way. Figure 6.10 lists the true cell cycle times, and a comparison with the $t_{c(a)}$ values in Figure 6.9 shows the profound influence of the growth fraction concept, with the true cell cycle time being nearly half the apparent. All three values for the control small intestinal tissues are remarkably close, and the amosite treated

upper small intestine figure is in good agreement with these. However, there are some problems with the interpretation of the results from the other two amosite-treated small intestinal zones. Although the absolute differences appeared large, they were not statistically significant, and it is possible that the high intake of dietary fats associated with the amosite treatment may have influenced the proliferative status of the small intestine. Such an effect might be due to an increased secretion of bile altering the proliferative status, and this might explain the apparently normal values obtained for the upper small intestine samples since they were taken from a site proximal to the bile duct opening. Bile has been shown to influence the intestinal flora and alterations in the intestinal flora are known to alter the proliferative activity of the intestinal tissues (Abrams et al., 1963). Further information on the influence of a high fat diet would be available from an examination of those tissues taken from the positive control animals. However, the cytokinetic study reported here concerned the effects of amosite in comparison with control tissues, and since the results of these analyses showed no statistical differences for the small intestine tissues, the matter was not pursued. Some support for the rather transient nature of any dietary effect may be found in Figure 6.10 in which it can be seen that the standard errors for the amosite treated tissues are proportionately much larger than the control samples.

Figure 6.10 emphasizes the significantly large differences in the response of the descending colon tissues to amosite treatment or control treatment mentioned earlier in connection with r_m values. Thus the cell cycle time of the amosite treated tissue is twice as long as the negative control. It is, however, similar to

that of the positive control tissue and there is therefore considered to be no effect of amosite treatment.

Figure 6.11 describes the proliferative activity of the tissues in terms of the number of cells produced per crypt per hour. Although the density and absolute number of crypts varied according to position along the gastrointestinal tract, there was no difference between treated and control animals, and the values shown in Figure 6.11 can be taken at face value. They show that the depression of the activity of the descending colon due to margarine ingestion could be expressed as a reduction in proliferative level to approximately one third of the untreated value. Since the lifespan of an individual cell on the intestinal surface is indirectly related to the proliferative rate within the crypts, it follows that the descending colon cells in the amosite treated animals were exposed to the lumenal contents (including large amounts of asbestos fibre) for considerably longer than the controls. When considered along with the absence of any significant fibre burden within the gut tissues as described in Chapter 5, this further supports the impression that ingested asbestos does not readily penetrate the gut mucosa.

Figure 6.12 shows the computed values for the mean duration of mitosis for the different tissues and provides some insight into the variations that can occur in the duration of one of the stages of the cell cycle. It must be pointed out that conventional cytokinetics theory considers the duration of mitosis to be relatively constant for mammalian tissues, with profound changes in t_c being mediated via increases in G_1 or G_2. The large differences in t_m shown in Figure 6.12 might reflect differences in the rate at

which the "slow cut off" model of Cairnie (Cairnie et al.,1965, a, b) operates for any given tissue. However, caution is necessary in the interpretation of such findings, and detailed information on the variation of the growth fraction with cell position would be necessary to resolve this matter. Such information is technically very difficult to obtain and is outwith the scope of the present work.

6.4 CONCLUSIONS

1. The animals used in the cytokinetic analyses showed no differences in the pathology arising between the different treatment groups, although those animals fed margarine were considerably heavier than those not so exposed.

2. It was found to be practical to estimate both DNA uptake and cytokinetic parameters from the same animal since different but adjacent pieces of tissue can be sampled.

3. The DNA extraction method had a large degree of variability associated with it, mainly due to problems with the quantitative extraction of DNA from the tissues. It may be concluded therefore that only large differences between treated and control may be detected using the DNA extraction techniques.

4. Only one gastrointestinal mucosal sample showed any consistent and statistically significant differences due to treatment, according to the DNA extraction techniques. This was the descending colon tissue in which the amosite treated samples were shown to have incorporated significantly less labelled DNA precursor than their age-matched negative controls. A small difference between liver samples was marginally significant but it was considered that this might be due to metabolic rather than proliferative differences.

5. Although time consuming, the stathmokinetic and autoradiographic analyses performed in this investigation did provide valuable information about the proliferative status of the various tissues. Many parameters were described and compared. Both steady state and exponential assumptions were used to calculate several of the parameters and it was shown that large differences in absolute values could be obtained using the different calculation methods. In the absence of information to the contrary, it was assumed that the steady state growth conditions more accurately described the proliferative compartment of the intestinal crypt.

6. The calculations showed that when the amosite-treated tissues were compared with their age-matched negative controls there were no detectable differences in the morphological parameters of the crypts.

7. When amosite asbestos treatment was compared with the negative control treatment, only the descending colon tissue showed any statistically significant proliferative differences, with amosite treatment being associated with a depression of proliferative activity. It is interesting to note that this is in accord with the results of the DNA extraction assays that showed a reduction in the amount of tritiated thymidine utilised by the descending colon tissue samples.

8. An examination of the proliferative activity of the positive margarine-treated control descending colon tissue indicated that the depression of proliferative activity noted above was associated with margarine ingestion and was not due to the ingestion of amosite asbestos. It is possible that differences in food transit time along the intestinal tract could account for the proliferative differences arising from a high level of margarine ingestion.

9. Of the various indices of proliferative activity, it appears that the rate of entry of cells into mitosis provides a useful indication of any differences found. The modification of r_m by taking into account differences in the growth fraction and cell populations make it possible to derive a figure for the actual number of cells produced per crypt, and this can be a useful figure in itself. It shows, for instance, that only one third of the number of cells are produced per hour in the descending colon of margarine exposed animals. Since there were no detectable differences in sizes of the cell populations, the above findings imply that the descending colon cells were exposed for longer periods to gut lumenal contents in the margarine ingesting animals. In animals ingesting asbestos, therefore, the commitment margarine ingestion increased any chances of the asbestos fibres inflicting damage to the cells. The fact that no such damage and no large scale penetration of the gut by asbestos fibres was found therefore provides further strong evidence that the prolonged asbestos ingestion was not harmful.

CHAPTER 7 ASBESTOS TRANSPORT FROM SUBCUTANEOUS INJECTION SITES IN RATS

ASBESTOS TRANSPORT FROM SUBCUTANEOUS INJECTION SITES IN RATS

7.1 INTRODUCTION

Asbestos fibres are known to cause both pleural and peritoneal mesotheliomas under certain circumstances. It would seem likely that a considerable degree of fibre penetration and transport is necessary for fibres to accumulate in the areas of susceptibility, particularly in the case of peritoneal mesotheliomas where the mesothelium is rather isolated from the normal routes of access of fibres to the body. Previous published work (see Section 1.3.3d) on the effects of the transport of asbestos using mice indicated that there was a specific preferential migration of fibres to submesothelial tissue sites, and that this was associated with the development of mesothelioma. However, the authors of this work later acknowledged that the injection of relatively large amounts of asbestos subcutaneously into the flanks of young mice was associated with a high risk of misinjection directly into the body cavities.

The present studies were therefore designed to determine the extent to which asbestos fibres are transported throughout the body from their initial site on entry and to assess the effects of any such transport. The subcutaneous site was chosen as the most suitable for the initial asbestos deposit, since there was likely to be comparatively little immediate vascular dissemination of fibre. A site over the right scapula was selected to minimise the chance of any misinjection into one of the body cavities, and rats were used instead of the mice of earlier workers. The animals were monitored for the development of abnormalities, and a wide range of tissues were sampled from a number of animals at autopsy and analysed for the presence of fibres (see Section 2.6.1c).

Figure 7.1 gives details of the numbers of rats used. All rats were injected at 12 weeks of age and housed two per cage thereafter. A full autopsy was performed on each animal (see section 2.14 for details), and selected tissues were processed for histological examination.

7.2 Morbidity and Mortality

Figure 7.2 gives details of the mean animal weights by treatment group throughout the duration of the experiment. It can be seen that there was a tendency for the control animals to be slightly heavier than the experimental groups from 300 days onwards, and that the chrysotile-treated group tended to be the lightest. However, these differences were not statistically significant.

Figure 7.3 presents the survival of the groups in tabular form, and it shows that by 900 days of age these were considerably more survivors in the chrysotile-treated group of animals than in those treated with either of the amphiboles or the control group. This difference is reflected in Figure 7.4 where it can be seen that the mean survival time of the chrysotile animals was at least 80 days longer than the other group. Note that there was no large difference in the mean autopsy weights between the different asbestos treated groups but that the control autopsy weights were slightly lower. The relationship of autopsy body weight to the body weights of the survivors is shown in Figures 12-15 of Appendix IV. The majority of animals killed or dying as a result of some infirmity during the course of the experiment can be seen to be of lower body weight than the survivors. This confirms the earlier finding (see Chapter 5) that loss of body weight is a useful indicator of morbidity.

FIGURE 7.1 Asbestos transport from subcutaneous sites in rats: experimental layout.

Treatment	No. of animals	Dosage regions
UICC Amosite	32	25 mg in 1.0 ml Dulbecco PBS
UICC Crocidolite	32	25 mg " " " "
UICC Chrysotile "A"	32	25 mg " " " "
Control	32	1.0 ml Dulbecco PBS only.

Figure 7·2

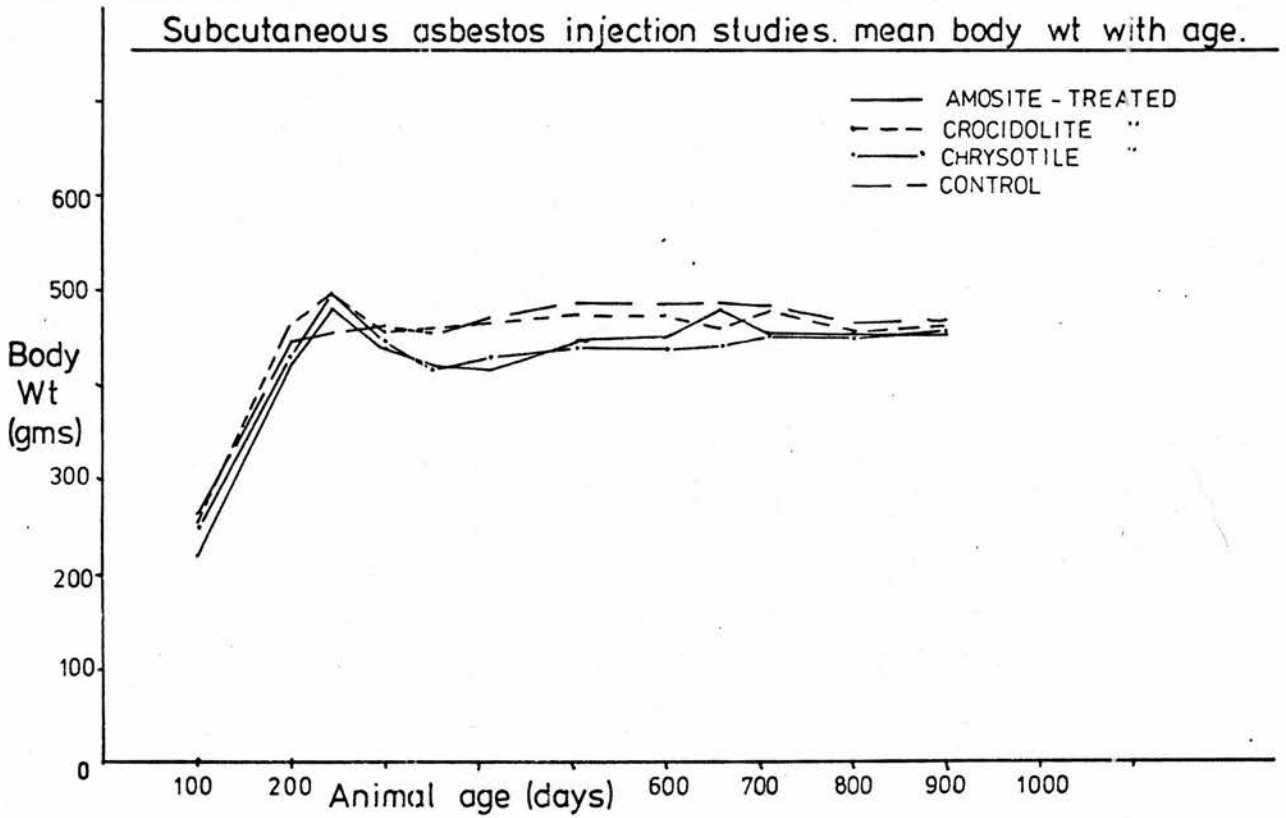

Subcutaneous asbestos injection studies. mean body wt with age.
AMOSITE - TREATED
CROCIDOLITE "
CHRYSOTILE "
CONTROL
Body Wt (gms)
600
500
300
200
100
0
100
200
Animal age (days)
600
700
800
900
1000

FIGURE 7.3 Asbestos transport from subcutaneous sites in rats: survival table.

	Numbers of survivors at specified age (days)															
	350	400	450	500	550	600	650	700	750	800	850	900	950	1000	1050	1100
Amosite	31	31	30	30	28	27	27	25	23	17	14	13	8	3	2	-
Crocidolite	32	32	32	32	32	32	32	28	25	24	18	11	9	1	1	-
Chrysotile	32	32	32	32	31	31	31	30	28	26	23	21	16	15	6	-
Control	32	32	32	31	31	31	30	27	25	22	18	13	10	6	4	-

FIGURE 7.4 Asbestos transport from subcutaneous sites in rats: mean survival times and mean autopsy wts.

Treatment	Mean Survival Time (days)	Mean Autopsy Wt (gms)
Amosite	809	398
Crocidolite	852	408
Chrysotile	931	401
Control	831	385

7.3 Pathology

The pathological findings associated with the subcutaneous asbestos injection experiment are itemised in Figures 13-16 of Appendix IV. The principal lesions found are described for each individual animal and both neoplastic and "other" lesions are listed. Although the most consistent autopsy findings were neoplastic some comments on the non-neoplastic lesions are included in the account given below. A summary of the neoplastic lesions is shown in Figure 7.5 where the number of malignant tumours arising at the injection site has been separated from those arising at other sites to avoid the misleading impression given by the simple totals. The total number of malignant tumours can be taken as an index of the overall malignancy of a given type of asbestos. It cannot, however, be taken as an index of the pathogenicity of transported asbestos since Fig. 7.5 clearly shows that many of the tumours arose at the site of application. Figure 7.5 summarises the incidence of benign tumours found amongst the treatment groups, and it can be seen that there was no difference between them.

It is necessary to examine the numbers of tumours arising at sites remote from the injection site to gain information on the carcinogenicity of transported asbestos, and Figure 7.5 shows that fewer tumours of other sites were found in the amphibole treated group of rats than in either the chrysotile or control groups. One explanation for this could be that there was a decrease in longevity in the amphibole animals as a result of the early mortality associated with the large numbers of injection site tumours. This is supported by the finding amongst chrysotile animals (Fig 7.6) that 8 of the total of 9 "other site" tumours arose in animals older than 851 days (the mean age at death of all animals with injection site tumours). Figure 7.6

FIGURE 7.5 Asbestos transport from subcutaneous sites in rats: neoplastic lesions.

	Treatment			
	Amosite	Crocidolite	Chrysotile	Control
No. of animals	32	32	32	32
Malignant Tumours				
Injection site	13	11	2	0
Other sites	5	6	9*	7
TOTAL	18	17	11	7
Benign Tumours	6	6	5	7

*One animal contained two primary tumours.

FIGURE 7.6 Asbestos transport from subcutaneous sites in rats: number of "other site" malignant tumours as a function of age.

	Treatment		
	Amosite	Crocidolite	Chrysotile "A"
No. of animals in group	32	32	32
Total no. of "other site" malignancies	5	6	9
Number found after 851 days of age	0	4	7
Mean age at death "other site" malignancies (days)	676	878	963
Mean age at death all malignancies (days)	803	863	936

also includes the mean age at death of all animals dying with malignant tumours, and it is clear that the differences in survival time shown in Figure 7.4 may now be explained in terms of differences in mortality associated with tumour development.

Figures 7.7 and 7.8 list details of the histological classification of the neoplastic lesions found during the subcutaneous injection experiments. There are some interesting points arising from the details of the types of malignant tumours listed in Figure 7.7. All the injection site tumours were fibrosarcomatous, usually with widespread local invasion of dorsal musculature, and occasionally with distant metastoses to lung, kidney etc. (Fig 7.9). Discrete fibres could be clearly seen associated with most amphibole derived injection site tumours, but it was extremely difficult to find chrysotile fibres in either of the two tumours arising following chrysotile injection, or in the connective tissues at the injection sites. The majority of injection site granulomata were sampled at autopsy and examined histologically for evidence of mis-injection etc. The asbestos deposits tended to have formed distinct well circumscribed granulomata by the time they were examined (most had been developing for at least 500 days before animals came to autopsy). The amphibole asbestos granulomata were usually fairly cellular, even in their centres, whilst the chrysotile granulomata tended to consist of a peripheral cellular zone and a denser tightly packed cellular fibrous core (Fig 7.10).

A malignant pericyte cell tumour, (Fig 7.11) was found in one rat nearly 650 days after chrysotile injection, and although this tumour was close to the injection site (approx. 2 cms) it did not appear to be associated with the scar tissue. It was considered to have arisen independently, and was accordingly classified amongst the tumours of "other sites".

FIGURE 7.7 Asbestos transport from subcutaneous sites in rats: details of malignant tumours.

	Treatment			
	Amosite	Crocidolite	Chrysotile	Control
No. of animals	32	32	32	32
Fibrosarcoma of injection site	13	11	2	
Anaplastic pleural tumour			1	
Pericardial tumour		1		
Peritoneal sarcoma	1		1	2
Osteosarcoma		1		
Lymphoma	3		1	2
Melanoma	1			
Seminoma			1	
Adrenal medullary tumour		1	1	
Adrenal cortical tumour		1		
Pericyte tumour			1	
Squamous carcinoma of skin		2	2	3
TOTALS	18	17	10	7

FIGURE 7.8 Asbestos transport form subcutaneous sites in rats: details of benign tumours.

	Treatment			
	Amosite	Crocidolite	Chrysotile	Control
No. of animals	32	32	32	32
Subcutaneous fibroma		2		1
Subcutaneous lipoma				1
Hepatic lipoma		1		
Pancreatic endocrine adenoma	2	2	1	1
Pancreatic exocrine adenoma	1	1	2	1
Mesenteric haemangioma	1		1	
Hepatic haemangioma				1
Thyroid "adenoma"			1	
Testicular fibroma				1
Mammary squamous papilloma	1			
Squamous papilloma of external auditory meatus	1			
TOTALS	6	6	5	6

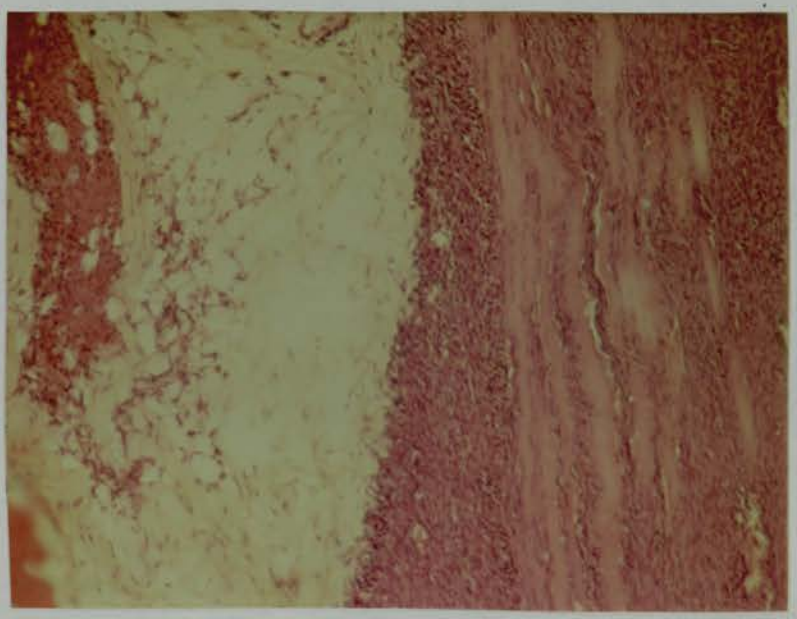


Figure 7.9a Section through the edge of a typical injection site fibrosarcoma, showing invasion of the dorsal musculature. (H.+ E.)

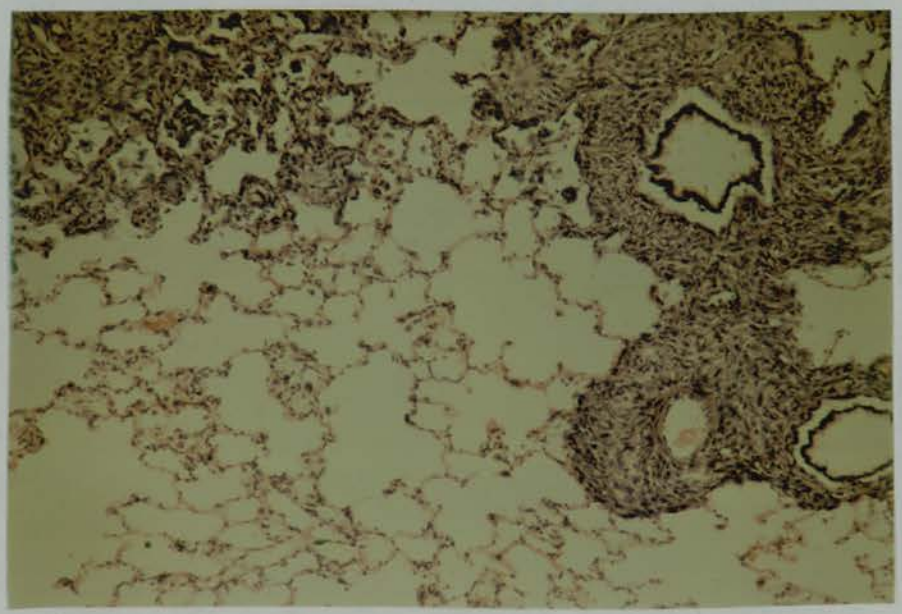


Figure 7.9b Section showing a pulmonary metastasis from an injection site fibrosarcoma. (H.+ E.)

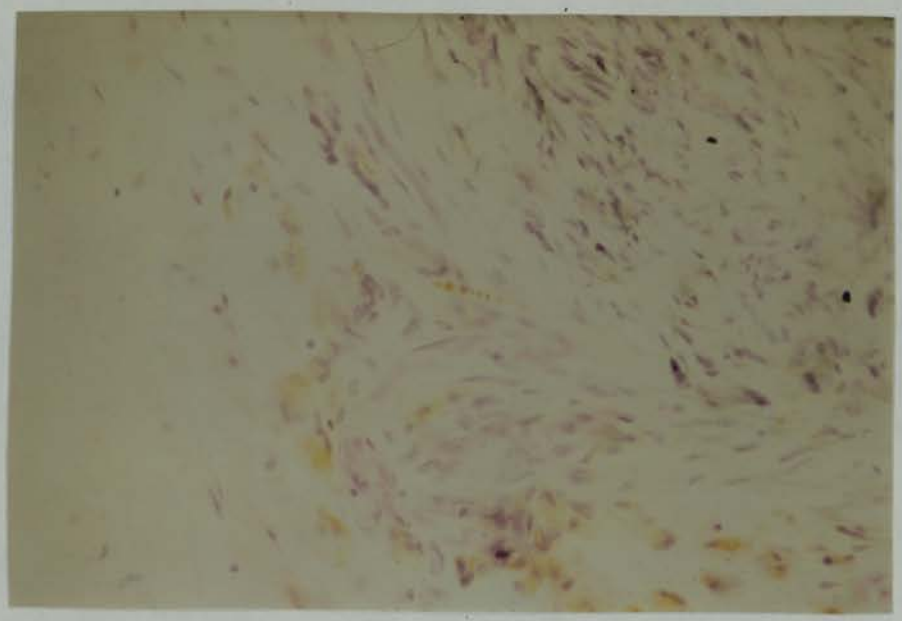


Figure 7.10a Section through an amphibole injection site granuloma, showing relatively cellular areas amidst the asbestos fibre deposits. (H.+ E.)

Figure 7.10b Section through a chrysotile injection site granuloma, showing the relatively acellular central area, with a distinct "capsular" peripheral fibrous tissue. (H.+ E.)

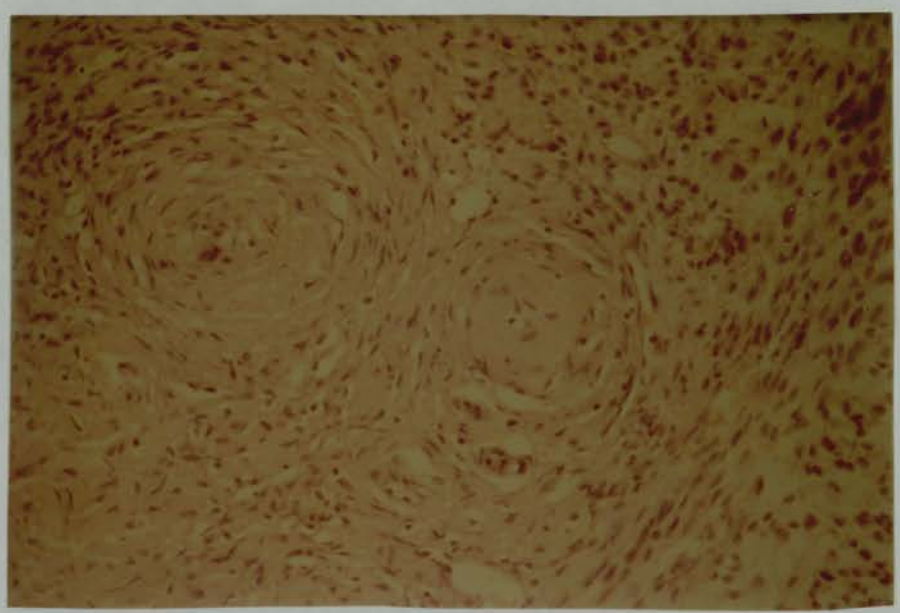


Figure 7.11 Section through a malignant pericyte cell tumour found in a chrysotile-injected animal aged 771 days. (H.+ E.)

A total of seven epidermal squamous carcinomas were found in the 128 rats involved in the experiments, but 3 of these occurred amongst the control group and the others were not considered to be related to asbestos exposure. All the tumours were well differentiated and locally invasive (Fig 7.12). No metastases were found.

A total of six generalised lymphomas were found: two in the control group, three in the amosite-treated group, and one in a chrysotile treated animal. There was a wide variation in the appearance of these tumours, as is shown by the examples in Figure 7.13, and some were difficult to categorise. Considerable difficulties were also found in the classification of 4 other tumours of diverse appearance found within the peritoneal cavity. All four were eventually loosely defined as peritoneal sarcomas. However, they differed from the other peritoneal sarcomas occasionally found in HAN male s.p.f. rats in that they did not appear to be strongly associated with the caecal mesentery as is usually the case, but tended to cover the surface of all the major peritoneal organs. Figure 7.14 shows one of these peritoneal tumours. This was found in a chrysotile treated animal of 1034 days of age, and it has the appearance of a round cell sarcoma. (There did not, however, appear to be the degree of invasion of underlying structures that might reasonably be expected of a widespread anaplastic round cell sarcoma.)

One malignant melanoma was found in a 595 days old amosite animal, with extensive involvement of the injection site region, and with widespread metastases (Figure 7.15). It was not possible to determine whether the injection site was the primary site of this tumour. A similar problem arose with the respect to the site of origin of a malignant pericyte tumour found in a 771 days old chrysotile-treated animal in which the extent of tissue destruction involving the clavicles and dorsal musculature made it impossible to accurately position the initial site.

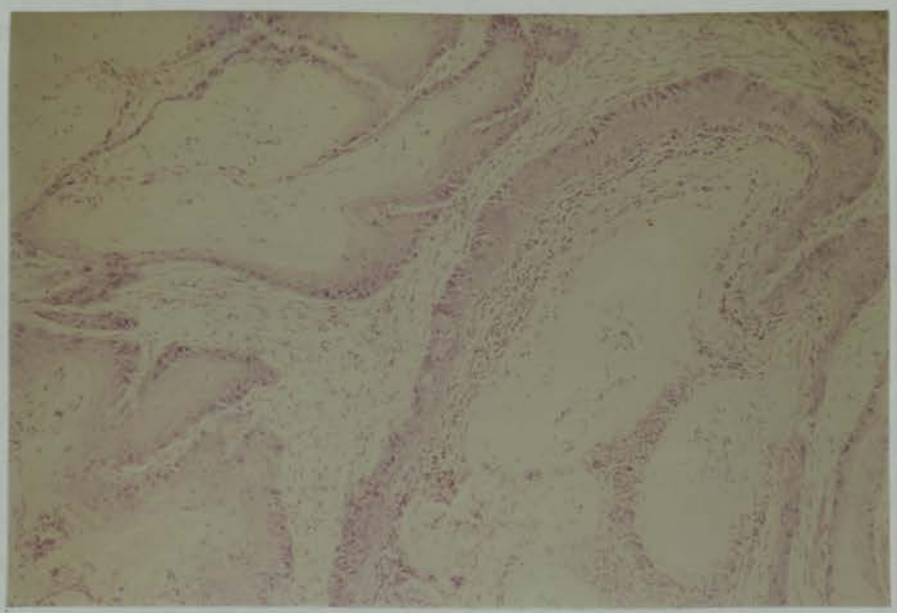


Figure 7.12 Section through a typical well differentiated squamous carcinoma. (H.+ E.)

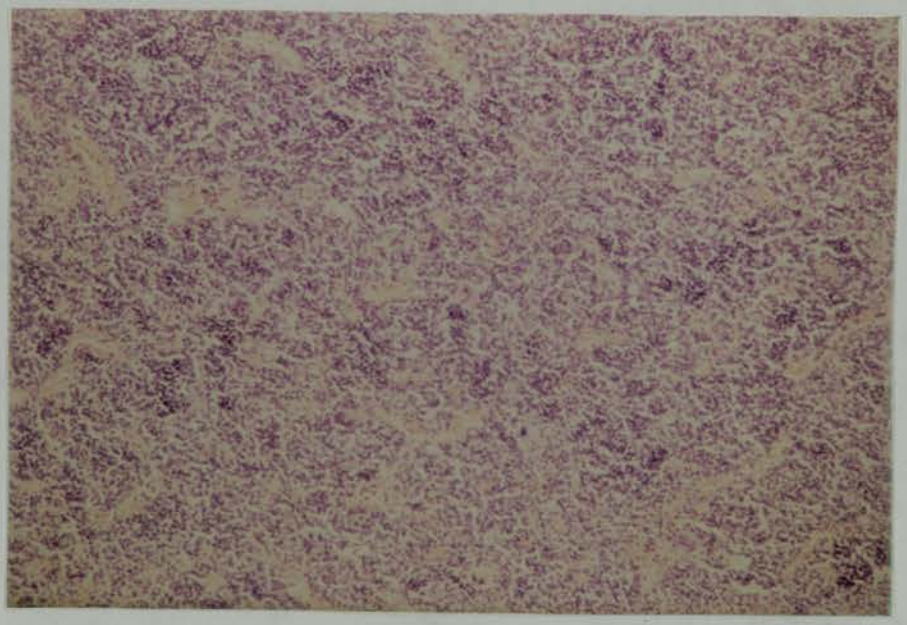


Figure 7.13a

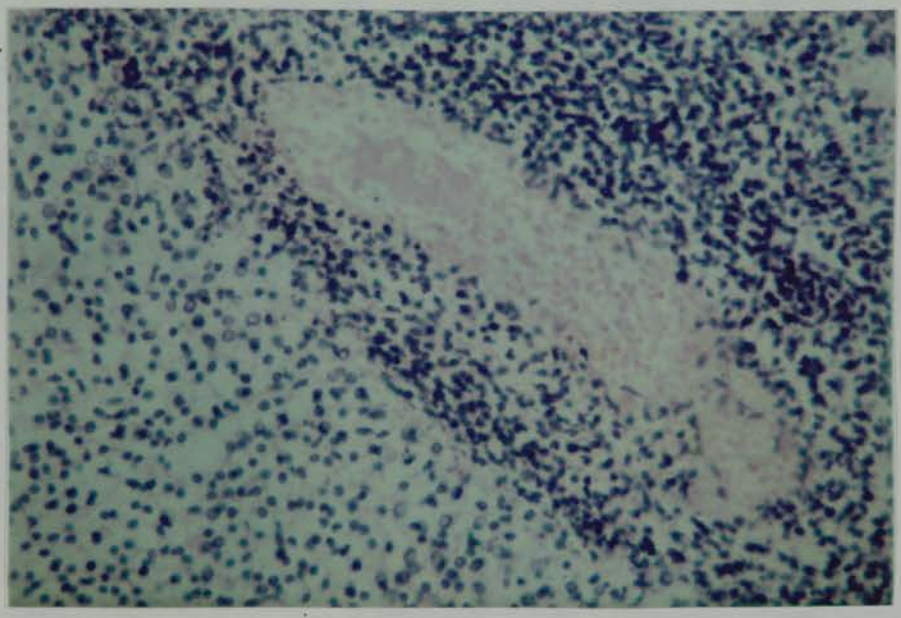


Figure 7.13b

Figures 7.13a and b. Typical views of two of the lymphomas found amongst the groups of rats, showing the wide variation in the histological appearance of different tumours. (H.+ E.)

Figure 7.14a

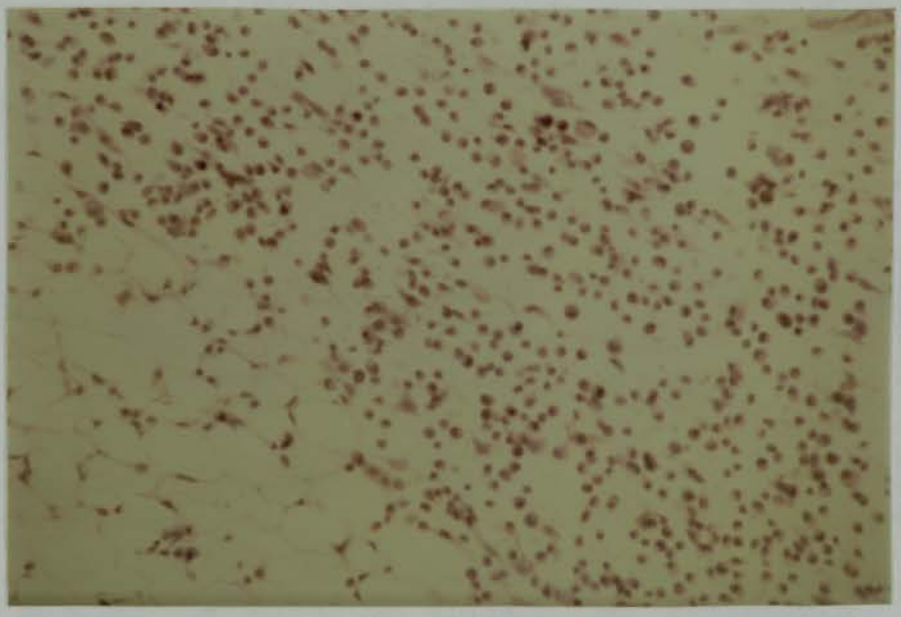


Figure 7.14b

Figures 7.14a and b. Two views of the peritoneal round cell sarcoma found in a chrysotile-injected animal aged 1034 days. (H.+ E.)

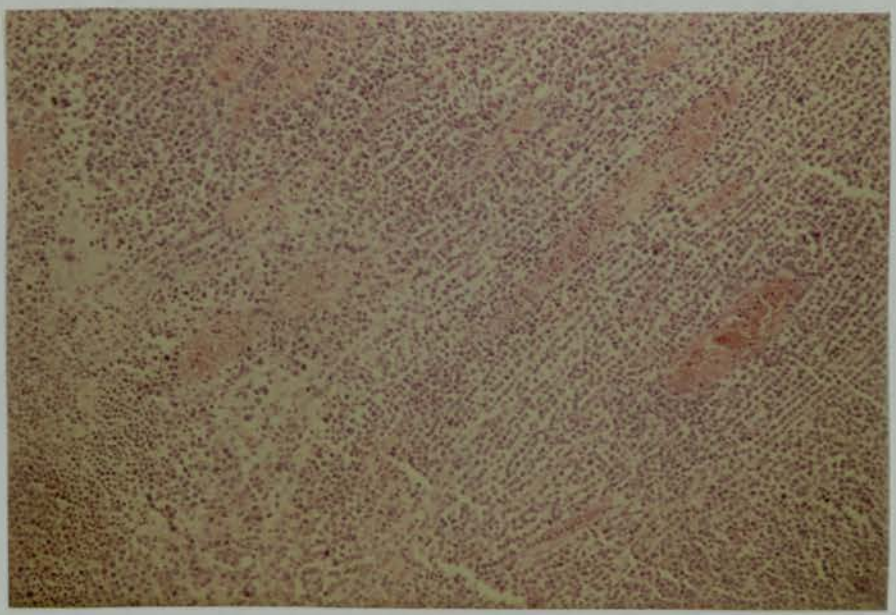


Figure 7.15a Section through a malignant melanoma found associated with an injection site area of an amosite-injected animal aged 595 days, showing the characteristic cords of cells. (H.+ E.)

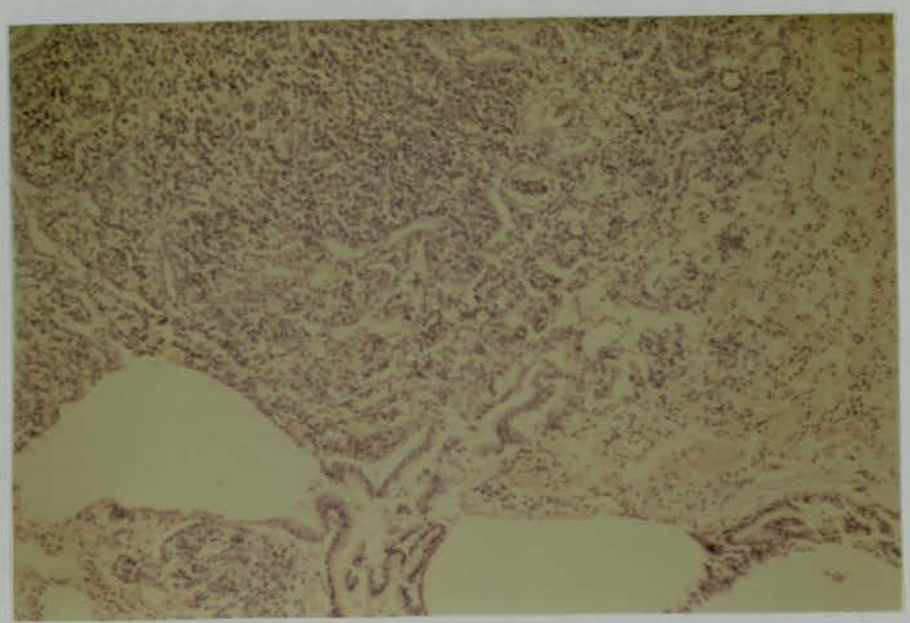


Figure 7.15b Section through a pulmonary metastasis of the melanoma shown above. (Fig. 7.15a). (H.+ E.)

Two other tumours, both involving the pleural cavity, require comment. The first was a malignant periodical tumour with extensive vascular invasion found in a 873 day old crocidolite-treated rat (Fig 7.16). The other was an extensive pleural tumour found in a 996 day old chrysotile-treated animal (Fig 7.17). Both these tumours had some areas similar in appearance to mesothelioma, but the associations were not strong enough to be considered typical of the tumours.

Taken collectively, although there is no clearcut link between malignant tumour incidence at "other sites" and asbestos treatment, there are one or teo aspects that require comment. The over-riding impression is that there was a wide diversity of unusual tumours amongst all the asbestos-treated groups, and that several of these tumours had some links with the injection site and surrounding tissues. Further, the finding of 2 pleural tumours out of a total of 27 tumours of "other sites" in 128 rats is a higher incidence than might be expected in untreated animals. Spontaneous pleural tumours are rare in our strain of laboratory rat, and have not been frequently reported in the literature. The significance of these findings must be viewed in the light of the results of fibre searches described in section 7.4.

Figure 7.8 shows clearly that there was absolutely no association between the incidence of benign tumours and asbestos treatment. Pancreatic adenomata were the most common (both endocrine and exocrine types), totalling 11 out of the 24 benign lesions found. Four haemangiomatous growths were identified, two arising in the mesentery, one in the kidney and one in the liver. The renal haemangiomas had some areas that were suggestive of a hamartoma. One neoplastic lesion of the thyroid was discovered as an incidental finding in a 747 day old chrysotile animal killed as a result of a large injection site sarcoma. This lesion (Fig 7.18) was identified as the benign thyroid adenoma using the complete

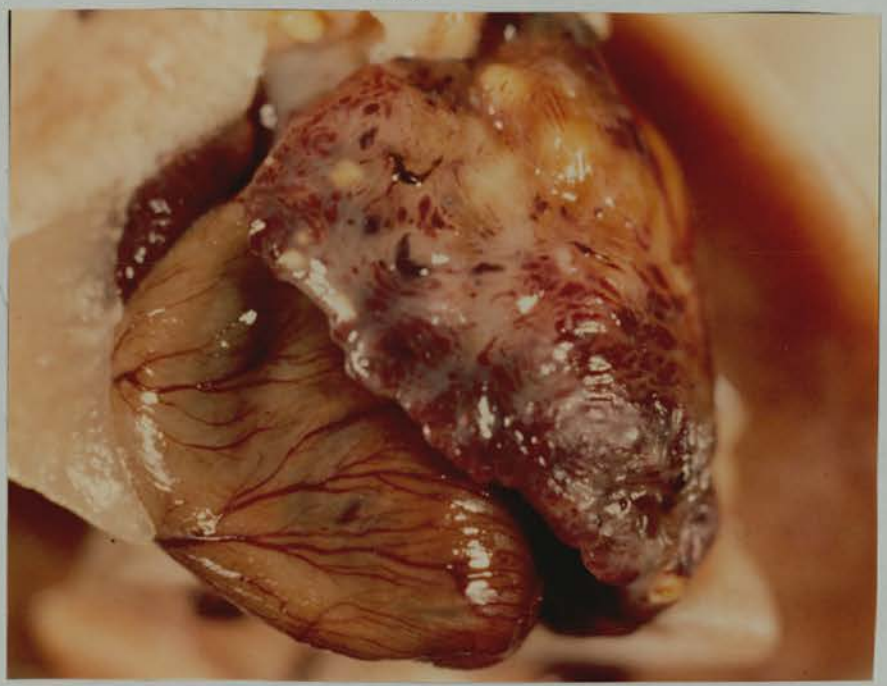


Figure 7.16a Macro photograph of the malignant pericardial cell tumour found in a crocidolite-injected animal aged 873 days.

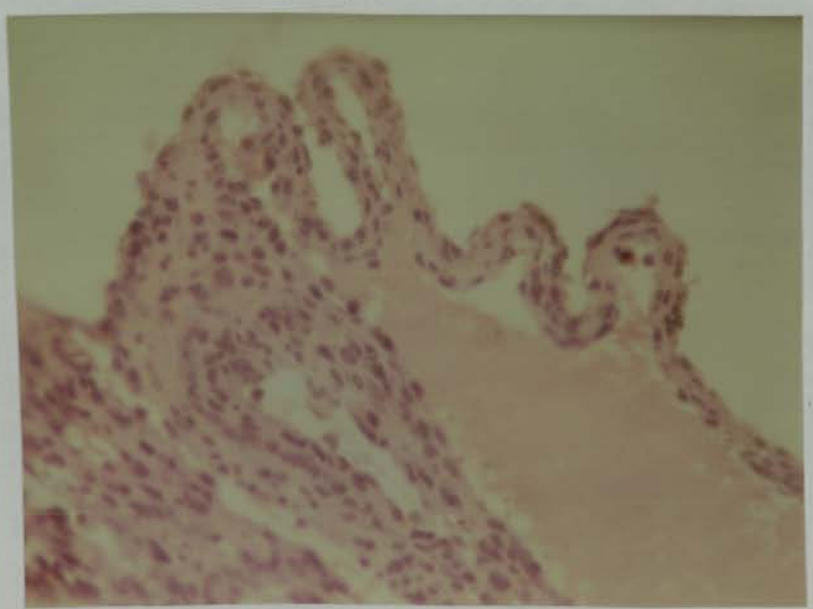


Figure 7.16b Histological section through the pericardial cell tumour shown in Figure 7.16a. (H.+ E.)

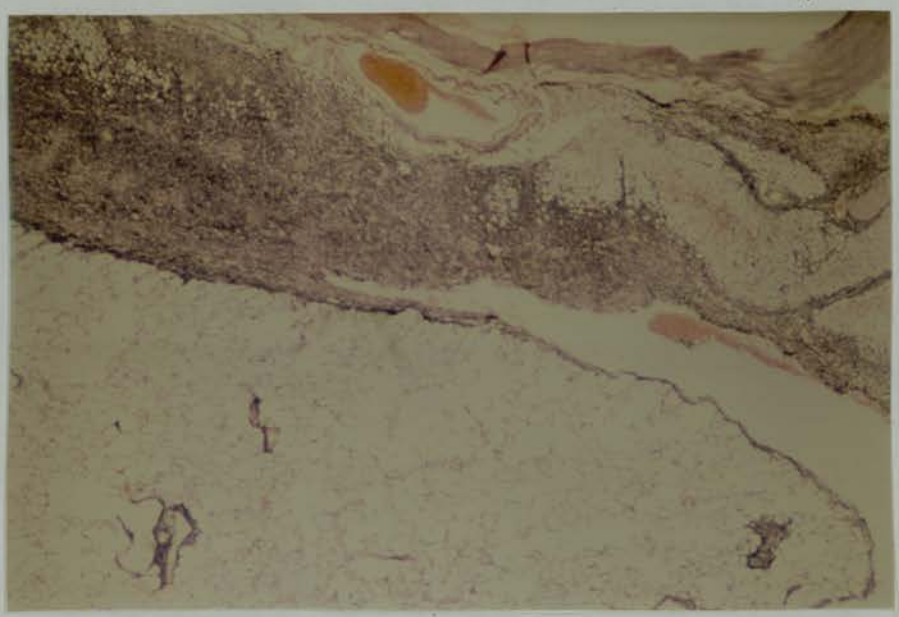


Figure 7.17a

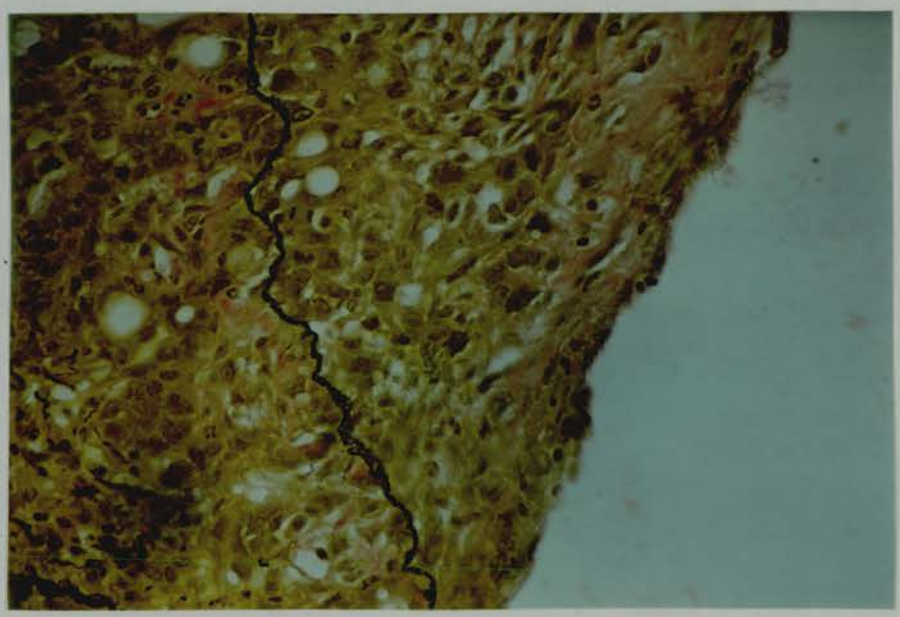


Figure 7.17b

Figures 7.17a and b. Two views of a malignant pleural tumour found in a chrysotile-injected animal aged 996 days, showing the extrapulmonary nature of the tumour.

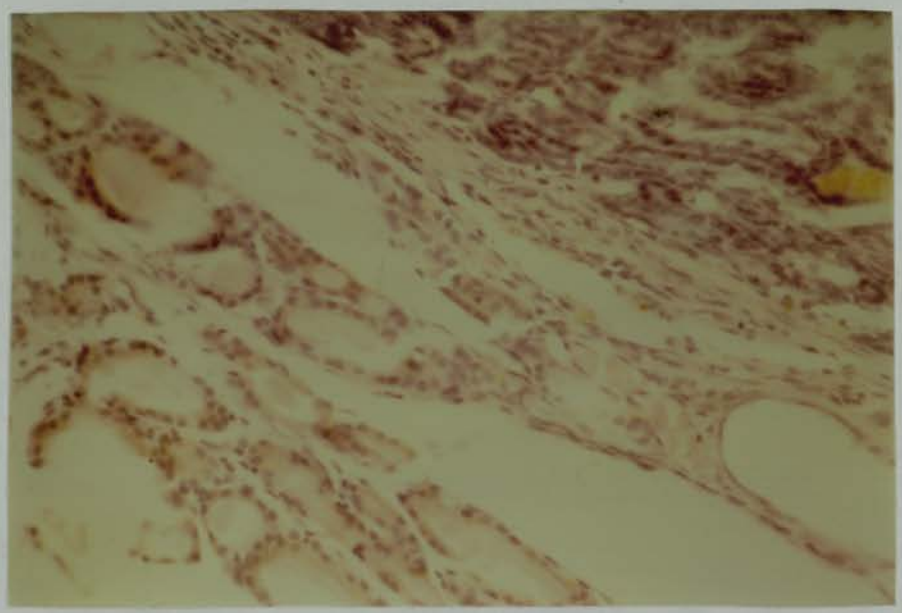


Figure 7.18 Histological section of a portion of a thyroid adenoma found in a 747 day old chrysotile injected animal.

lack of invasion as the main criterion and in full appreciation of the problems associated with accurate identification of slow growing neoplastic thyroid lesions.

Figure 7.19 presents the details of the incidence of non-neoplastic lesions encountered amongst the subcutaneous injection experiment animals, and it can be seen that once again (refer to section 5.3) renal hypertensive damage was the most common lesion. The association of mesenteric polyarteritis nodosa with renal hypertensive damage was also confirmed, and it was particularly prominent amongst the crocidolite-treated animals. In this group polyarteritis was twice as common in other groups, and seven out of the animals with extensive renal hypertensive damage also had mesenteric polyarteritis nodosa. (Appendix IV, Figure 17). Pulmonary venous congestion was also found in many animals at autopsy, particularly in the older animals. The higher incidence of pulmonary congestion amongst the chrysotile group is in agreement with the previously mentioned increased longevity in this group (section 7.2).

7.4 Fibre searches

A wide range of tissues (see section 2.1.4 and Figures 7.20 to 7.22) were taken from the majority of those animals coming to autopsy during the latter half of the experiment. The tissue samples were stored at -20°C until ashing facilities had been developed (section 2.6.1) as part of the procedure for an electron microscopical search for the presence of fibres.

Figures 7.20 to 7.22 summarise the results of these fibre searches, expressed in terms of the numbers of fibres actually found in each tissue. Sample conversion factors are also included to enable estimates of the total tissue burdens to be made as before (see section 5.4).

FIGURE 7.19 Asbestos transport from subcutaneous sites in rats: other lesions.

	Treatment			
	Amosite	Crocidolite	Chrysotile	Control
No. of animals	32	32	32	32
Pulmonary congestion	5	9	13	8
Renal hypertension:				
extensive	6	8	10	6
moderate	6	5	7	4
slight	5	8	7	6
TOTAL	17	21	24	16
Polyarteritis	2	8	4	3
Hepatic fatty change	1	0	4	2

FIGURE 7.20 Asbestos transport from subcutaneous sites in rats: amosite treated. Fibre search results.

		No. of fibres per sample per rat								
	Animal age (days)	415	698	752	823	902	916	939	959	1081
Tissue	Conversion Factor									
Lungs	340	1	1					2		1
Liver	5100	-	11	-	7	-	+	9	-	1
Spleen	125	-		2			-	-	-	
Kidney	164	3	-	4	-	-	12	3	-	2
Omentum	30	-	-	-	-	-	-	1	-	-
Mesenteric lymph nodes	150	-		-	1	-	1		-	-
Cervical lymph nodes	150	+	+		+	+	++	+		+
Axial lymph nodes	150	+	++	++	+	++	++	++	++	++
Thoracic body wall	16170	-	-	-		1	-	-	-	-
Peritoneal body wall	4300	-	-	1		-	-	1	-	-

FIGURE 7.21 Asbestos transport from subcutaneous sites in rats: crocidolite treated. Fibre search results.

		No. of fibres per sample per rat								
	Animal age (days)	659	742	793	830	868	884	906	963	1038
Tissue	Conversion Factor									
Lungs	340	1	2						1	3
Liver	5100	-	1	2	7	-	3	+	-	6
Spleen	125	-	-	2	-	1	-	1	-	-
Kidney	164	-	-	-	1	1	2	2	-	2
Omentum	30	-	1	-			-	-	-	-
Mesenteric lymph nodes	150	-	-	-	1	-	-	2	-	1
Cervical lymph nodes	150	+	+	+	-	+		++	+	+
Axial lymph nodes	150	++	++	++	++	++	++	++	++	++
Thoracic body wall	16170	-	-	-					-	-
Peritoneal body wall	4300	-	-	1					-	-

FIGURE 7.22 Asbestos transport from subcutaneous sites in rats: chrysotile treated. Fibre search results.

		No. of fibres per sample per rat								
	Animal age (days)	747	771	846	869	906	996	1005	1034	1075
Tissue	Conversion Factor									
Lungs	4000	-	-						1	
Liver	57000	4	-	3	-	-	1	-	-	1
Spleen	1320	-	1	-	-	-	-	1	-	-
Kidney	1950	-	4	-	4			-	-	-
Omentum	360	-	-	-	-	-	-	-		-
Mesenteric lymph nodes	1430	-	1	-	-	-	2	-	-	-
Cervical lymph nodes	1430	4	7	3	8	10		12	16	22
Axial lymph nodes	1430	++	++	+	29	12	17	-	7	13
Thoracic body wall	94700	-	-					-	-	
Peritoneal body wall	51500	-	-	-				-	-	

\+ = more than 10 fibres per SEM field at a magnification of x 2000.

++ = massive numbers of fibres per SEM field " " " " .

It was obvious from the macroscopic appearance that some lymph nodes around the injection site sampled at autopsy contained large amounts of asbestos. This was confirmed by preliminary examination, and the ashing procedures were modified slightly to minimise the risks of cross-contamination of other tissue samples. The modifications were simply that all local lymph nodes were processed separately from the other tissues and they were the last samples in the sequence of tissues to be processed. Most of the local lymph node preparations proved to contain far too many fibres to count, and their numbers were assessed visually as "more than 10 fibres per SEM field at a magnification of x 2000", or where the fibre density was very high, as "massive numbers per SEM field". Quantitative estimation by infra-red spectrometry of axial lymph nodes from 3 amosite injected animals suggested that they could contain up to 2mgs of asbestos within eighteen months of injection. Injection trials with subcutaneous deposits of nigrosin over the right scapula have shown that the axial, and in some cases the cervical, lymph nodes were frequently found to be heavily pigmented within one month from injection.

The indeterminate fibrous shapes described in section 5.4 that were found in some kidney and spleen sample residues from ingestion animals were also found occasionally in the subcutaneous injection experiment. Once again they tended to occur in kidney and some spleen residues, and although they were morphologically similar to amphibole asbestos, they contained very little silicon and were not classed as asbestiform.

It can be seen from Figures 7.20 and 7.22 that all axial and cervical lymph nodes examined from amphibole treated animals contained very large numbers of asbestos fibres, that there was no obvious change in the amounts of fibre with increasing animal age, and that there was no difference between the two types of amphibole. However, examination of

Figure 7.22 shows that the position was somewhat different with the chrysotile animals. Here there seems to be a distinct trend for the amounts of asbestos in the axial lymph nodes to decrease with animal age, with a possible very slight increase in the number of fibres found in the cervical lymph nodes. This alteration in the apparent sizes of the deposits within the lymph nodes is not taken as presumptive evidence for the widespread dissipation of fibres per se, but is considered more likely to be due to the dissolution of chrysotile in the body fluids. The histological appearance of the subcutaneous chrysotile injection deposits described in section 7.3 would support this concept of dissolution since it was difficult to find even large bundles of chrysotile fibres at the injection site by the time the animals come to autopsy.

It is interesting to note from Figures 7.20 to 7.22 that, whilst fibres were found regularly in several tissues from most animals, they were very much less frequent than in the local lymph nodes. (Note that the numbers in the columns represent the total number of fibres found in a search that usually involved at least 400 SEM fields examined for each sample, whereas the local lymph node results describe the appearance of a typical SEM field of view since a detailed search was unnecessary). Some tissues consistently showed a very low incidence of asbestos fibres, irrespective of which type was injected. These included the omentum, mesenteric lymph nodes, and thoracic and peritoneal body wall samples. There was thus absolutely no evidence for the preferential migration of asbestos fibres to the gastrointestinal lymphatic tissues, or to those areas of the parietal mesothelium that were included with the "body wall" samples.

Amongst the other tissues examined, there did appear to be a definite tendency for the liver, kidney, and to a lesser extent, the spleen,

to contain some fibres. This trend was most obvious with tissues from the amosite-treated animals, and least so with the chrysotile treated. (The residues from older chrysotile treated animals contained less fibres than the younger ones, and the relatively lower incidence amongst chrysotile treated animals might be due to the dissolution of chrysotile fibres mentioned earlier). Some asbestos fibres were also found in the lung tissue residues of most animals examined. The nature of the assays mean that it is not possible to make a detailed comparison of the relative levels of fibres in the different tissues, but there does seem to be some association between the frequency of fibre finds and the degree of vascularity of the tissue examined.

It can be seen from Figures 7.20 and 7.21 that two animals injected with amphibole asbestos were subsequently found to contain significant large hepatic deposits of asbestos fibre, suggesting that considerable dissemination of fibre had occurred. One of the animals, the one injected with crocidolite and dying aged 906 days, was found to have both an injection site fibrosarcoma and a malignant adrenal cortical tumour. The other animal, injected with amosite, had no tumour. It is possible that the malignancies found in the crocidolite animal would be related to the observed higher level of disseminated fibre. However, this is considered unlikely since a) a direct casual relationship with hepatic deposits of asbestos seems obscure; b) adrenal malignancies are fairly common in rats, irrespective of treatment; c) only two asbestos fibres were actually found after the examination of the kidney residues from the crocidolite animal, and the kidney had a similar blood supply to the adrenal from which the tumour arose; and d) the coincidental occurrence of two independent malignancies can be found elsewhere within the experiment, amongst animals without obvious excesses of disseminated fibre.

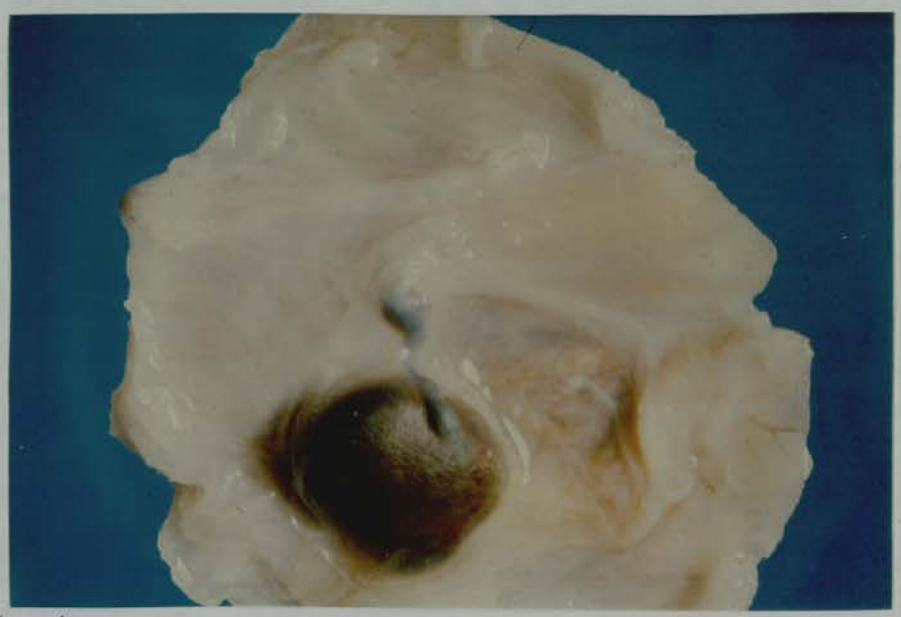


Figure 7.23 Macro photograph of a crocidolite asbestos injection site showing an afferent lymphatic vessel distended with asbestos fibre. Also shown is a portion of the brown multilocular fat of the "hibernating gland."

Figure 7.23 shows a local lymphatic vessel near the subcutaneous site of injection grossly distended with crocidolite asbestos. It would appear from this that large scale lymphatic and vascular dissemination of fibre did occur in some animals following the injection. Under these circumstances it is not surprising that fibres were regularly found in a wide range of tissues.

7.5 Conclusions

(1) There was no significant difference between the mean body weight of the different asbestos treatment groups and the control group of rats. The majority of animals were still alive at 800 days of age, and some survived to over 1050 days of age.

(2) The chrysotile injected group of animals lived longer than either of the amphibole treated groups, with 21 chrysotile animals alive at 900 days, only 13 in the amosite, and 11 in the crocidolite groups. The increased mortality in the amphibole treated animals was a direct result of the development of more injection site malignant tumours. The amphibole asbestos types were thus found to be more carcinogenic that the same mass of chrysotile. The mortality associated with this carcinogenicity at the injection site had the effect of reducing the number of malignancies at other sites.

(3) Several of the malignancies arising at "other sites" in the experimental animals proved difficult to classify. Amongst these were 2 tumours in the pleural cavity, 4 in the peritoneal cavity, and several associated with, but not at, the injection site. Four lymphomas were also found in the asbestos treated animals.

(4) There was no difference in the levels of benign tumours amongst any of the four groups of animals examined.

(5) There was more mesenteric polyarteritis nodosa amongst the crocidolite-treated animals than amongst any of the other groups. Although the link between mesenteric polyarteritis and extensive renal hypertensive damage was confirmed, with all treatment groups having a similar incidence of renal disease, more of those crocidolite animals with extensive renal disease developed polyarteritis.

(6) This study shows that there was some movement of asbestos fibre from the site of application, but that there was no selective transport to either the mesothelial or the gastrointestinal tissues. It is proposed that the main route for the asbestos fibre movement was from local lymphatic system and lymph nodes, into the thoracic duct, and from there into the blood vascular system for general dissemination. It is interesting in connection with this to recall that 4 lymphomas and 1 pericyte malignancy were found amongst the asbestos treated animals.

(7) Apart from the above, there was no obvious pattern in the incidence of malignancy in any of the groups examined, and those animals with malignant tumours did not, in general, have a greater degree of asbestos dissemination.

CHAPTER 8 SOME EFFECTS OF THE INHALATION OF ASBESTOS

Some effects of the inhalation of asbestos

8.1 INTRODUCTION

The principal aim of the inhalation experiment was to study the effects of fibre mass and fibre number of the three main types of asbestos on the respiratory system of laboratory rats. The results of these studies have been recently published (Davis et al 1978, 1979). Since pulmonary clearance involves both bronchial and lymphatic components, the inhalation experiment provided an excellent opportunity to investigate both the ingestion and the transport of asbestos fibres, and some results are presented below. The original study involved the exposure of groups of rats to two different doses of chrysotile, two of crocidolite, and one of amosite, over a long period. However, this account has been restricted to compare only one dose of each type of asbestos.

Full details of the complicated procedures developed for the generation and maintainance of exposure clouds of known dimensions over prolonged periods are available in the paper by Beckett (1975), and a detailed description is also included in the paper by Davis et al (1978). Basically, three types of UICC asbestos (amosite, crocidolite and chrysotile) were used to produce clouds containing 10mg/m^3 of respirable fibre. Groups of 48 HAN s.p.f. male rats were exposed for 7 hours a day, 5 days a week, for a total of 224 days during an elapsed time of nearly one year. No adequate comparable controls were available, but some information on the spontaneous disease incidence was obtained from control groups involved in other experiments simultaneously maintained in the animal unit. A total of 24 out of the 144 treated animals were killed at predetermined times during the course of the experiment to study the development of pulmonary fibrosis following asbestos inhalation: four animals were killed from each

treatment group at the end of the dusting period, and four 6 months later. A further eleven animals from each group were killed at the end of the experiment 17 months after the cessation of dusting. All of the other 87 animals were maintained and monitored for the development of disease. Animals were killed when moribund and a full autopsy performed as before (see Section 2.6) Figure 8.1 gives the details of the experiment.

The results of the investigations into the pathology arising in the treated groups of animals is presented in summary form only, but some details of particular relevance to the ingestion and/or transport of asbestos fibre are included. Further details, and the results of bulk estimations of the lung burdens of asbestos are available in the paper by Davis et al (1978). The results of the examinations of selected tissues for the presence of asbestos fibres are also presented (in Section 8.4). The tissues were taken at autopsy from a small number of those animals dying in the latter half of this study and were processed for an electron microscopical examination for the presence of fibres. Figure 8.2 lists the tissues sampled.

8.2 Morbidity and Mortality

Figure 8.3 presents the survival of the animals in the different treatment groups in tabular form, and it can be seen that there were no large differences between groups. (Those animals killed during the study as part of the interim pathology assays have been included in the table). The close similarity between the three asbestos treatments is further demonstrated in Figure 8.4. In particular, the mean autopsy weights of the terminal survivors are very close, suggesting that there was no excessive morbidity in any one of the treatment groups.

FIGURE 8.1 Inhalation of asbestos: experimental details.

Treatment	No. of animals at start	No. of animals followed to full term	Dose*	Duration
Amosite	48	29	10 mg/m³	One year
Crocidolite	48	29	10 mg/m³	One year
Chrysotile	48	29	10 mg/m³	One year

* 7 hrs/day, 5 days/week, for 224 days during an elapsed time of one year.

FIGURE 8.2 Inhalation of asbestos: tissues sampled for ashing.

Tissues sampled for electron microscopical examination for the presence of asbestos fibres
Mediastinal lymph nodes.
Cervical lymph nodes (includes nodes associated with salivary glands).
Mesenteric lymph nodes.
Renal lymph nodes.
Liver.
Spleen.
Kidney.
Selected gut.

FIGURE 8.3 Inhalation of asbestos: survival table.

	No. of animals alive at stated age (days)*											
Treatment	400	450	500	550	600	650	700	750	800	850	900	945
Amosite	48	45	42	42	40	36	32	28	23	19	14	12
Crocidolite	47	45	43	43	42	35	34	33	25	21	13	11
Chrysotile	45	43	41	40	39	35	31	27	23	17	15	11

*Planned kills at 452, 627, 946 days, see text for details.

FIGURE 8.4 Inhalation of asbestos: mean survival times and autopsy weights.

	Treatment		
Assay	Amosite	Crocidolite	Chrysotile
Mean survival time (days)	782.2 (48)	771.9 (48)	745.0 (48)
Mean autopsy wt. (gms)	416.6 (28)	389.1 (34)	399.5 (30)
Mean autopsy wt. of terminal survivors (see text 8.2)	385.5 (11)	390.0 (11)	378.9 (11)

(Figures in brackets refer to number of animals used for calculations.)

FIGURE 8.5 Inhalation of asbestos: pulmonary disease in rats killed at specific intervals.

	Treatment								
	Amosite			Crocidolite			Chrysotile		
The after start of exposure (months)	12	18	29	12	18	29	12	18	29
No. of rats per sample	4	4	6	4	4	6	4	4	6
Interstitial fibrosis	0.87	0.12	2.58	0	0.07	1.38	0.48	0.9	9.15
Peribronchiolar fibrosis	10.7	9.9	7.5	2.7	4.3	3.9	19.3	17.1	15.0
Bronchial epith. extension	2.27	3.90	3.05	0.85	2.45	1.96	2.68	2.40	1.43

8.3 Pathology

The degree of pulmonary pathology was estimated using a modified point counting method described in detail by Davis et al (1978) in which the areas of lung tissue involved in any specific type of lesion were expressed as a percentage of the total lung area from histological sections. Three types of lesion were found: interstitial and peribronchiolar fibrosis, and bronchial epithelial extension, and Figure 8.5 summarises the results of the estimations for each specific killing time. It can be seen that there was more pulmonary fibrosis in the chrysotile-treated animals than in either of the amphibole-treated groups at the end of dusting (12 months after the start of exposure). There was also a progressive increase in the extent of the interstitial alveolar wall fibrotic areas with time in all treatment groups. The peribronchiolar fibrosis did not show any increase in severity, and the slight apparent decrease in the size of the lesions was considered to be related to the larger areas of interstitial fibrosis that had developed by the later killing dates, obscuring the peribronchiolar lesions. There appeared to be no long term progression of the bronchial epithelial extensions in these studies, and no real differences in response between the three types of asbestos treatment. A full description of the of the development and progression of these pulmonary lesions is given in Davis et al (1978).

One further pulmonary lesion was noted in many animals exposed to the inhalation of asbestos (see Figure 8.6). Macroscopically the lungs were covered with prominent sub-pleural pale areas 2-3mm in diameter that tended to become much less prominent when the lungs were inflation fixed with a solution of formal saline. Histologically these lesions consisted of areas of sub-pleural accumulations of macrophages, some of which contained recognisable asbestos fibres. Similar accumulations of macrophages

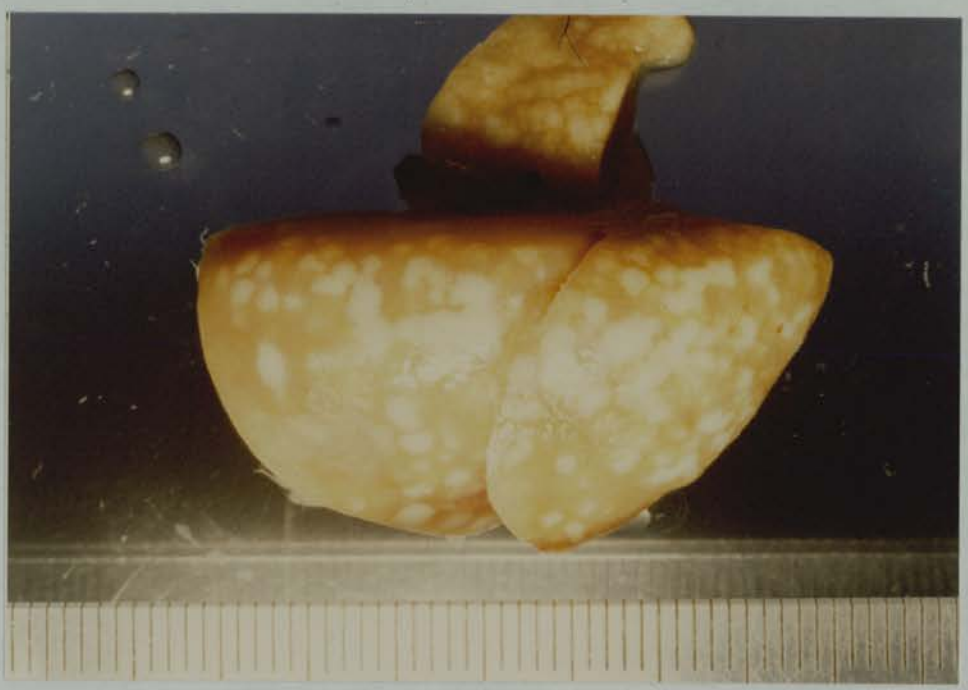


Figure 8.6 Macro photograph of excised lungs from an animal exposed by inhalation to chrysotile asbestos for one year, showing the subpleural "speckling".

may be occasionally found in untreated laboratory rats (Lamb, D. personal communication), and they may be associated with endemic viral infections. However, it was noticeable in the present inhalation studies that the sub pleural patches were more common in the chrysotile-treated group of animals killed 12 months after the start of dusting, and thereafter became a frequent finding in most older, more diseased animals, but particularly in the chrysotile-treated.

Figure 8.7 summarises the neoplastic lesions found in the inhalation studies and it can be seen that there were many more malignant tumours amongst the chrysotile-treated group, and that crocidolite appeared less carcinogenic than amosite. Figure 8.8 shows the numbers of pulmonary neoplastic lesions found, with the only malignant pulmonary tumours occuring amongst the chrysotile-treated animals. There were also more adenomatous lesions amongst the chrysotile-treated animals. It is apparent, therefore, that the difference in carcinogenicity of chrysotile from the amphibole asbestos types in Figure 8.7 could be accounted for mainly by the development of pulmonary tumours in the chrysotile group. This is further supported by the information in Figure 8.9 which shows that the chrysotile-treated produced only two more non-pulmonary malignant tumours than the amosite treatment. Figure 8.10 lists details of the benign non-pulmonary tumours, showing that in this case the highest number of lesions occurred in the amosite treated group, with crocidolite and chrysotile having a similar incidence.

It is interesting to examine the types of non-pulmonary malignant tumours found amongst the different groups of animals (see Figure 8.9). It can be seen that there was no distinctive trend in the histological classification of the tumours. There were the usual problems with the accurate identification. In particular, two malignant tumours found in the cervical

FIGURE 8.7 Inhalation of asbestos: summary of neoplastic lesions.

	Treatment		
	Amosite	Crocidolite	Chrysotile
No. of animals	43	40	40
Benign Tumours	13	7	13
Malignant Tumours	6	3	16

FIGURE 8.8 Inhalation of asbestos: pulmonary neoplastic lesions.

	Treatment		
	Amosite	Crocidolite	Chrysotile
No. of animals	43	40	40
Adenoma	2	1	7
Adenocarcinoma	0	0	6
Squamous carcinoma	0	0	6

FIGURE 8.9 Inhalation of asbestos: details of non-pulmonary malignant tumours.

		Treatment		
		Amosite	Crocidolite	Chrysotile
Lesion	No. of animals	43	40	40
Subcutaneous fibrosarcoma		2		
Adrenal angiosarcoma		2		
Cervical lymph node angiosarcoma		1		
Cervical anaplastic tumour				2
Thyroid carcinoma				1
Ileal leiomyosarcoma		1		
Peritoneal sarcoma			2	2
Epidermal squamous carcinoma			1	3
	TOTALS	6	3	8

FIGURE 8.10 Inhalation of asbestos: details of non-pulmonary benign tumours.

		Treatment		
		Amosite	Crocidolite	Chrysotile
Lesion	No. of animals	43	40	40
Subcutaneous fibroma		3	1	1
Pancreatic endocrine adenoma				1
" exocrine adenoma		1	1	1
Mesenteric haemangioma		2	1	
Hepatic adenoma				1
Thyroid/parathyroid adenoma		1	1	
Testicular seminoma		3	1	1
Epidermal squamous papilloma				1
" kerato acanthoma		1		
Sebaceous adenomatous cyst			1	
	TOTALS	11	6	6

region of the chrysotile exposed animals were classified as "anaplastic (sarcomatous) tumours" with some reservations. (Figure 8.11). There were strong resemblances between some areas of both tumours with the angiosarcomatous tumours found elsewhere in HAN rats. On this basis, it is possible that the tumours represent particularly anaplastic angiosarcomatous tumours, possibly arising in the large cervical lymph nodes. This view is supported by the finding of one clearly defined cervical lymph node angiosarcoma in an amosite exposed animal. The fact that most angiosarcomas and all the haemangiomas found in the Institute of Occupational Medicine Animal Experimental Unit were closely associated with lymphatic tissue further supports the view that the two anaplastic tumours found in the inhalation studies arose in the cervical lymph nodes.

The finding of four peritoneal sarcomas out of the total of 17 malignant non-pulmonary tumours requires comment. It was not possible to accurately define the site of origin of any of these tumours, except that the caecal mesenteric tissues were particularly heavily involved in all four cases. Peritoneal sarcomas have been noted amongst asbestos exposed rats in the other studies reported in Chapters 5 and 7 in much the same incidence. One leiomyosarcoma of the ileum was found in an amosite-treated animal (Figure 8.12) but there were no neoplastic lesions of the gastrointestinal mucosal tissues found in any of the 123 rats that survived for at least 18 months from the start of the dusting phase.

Figure 8.10 shows that there was no obvious link between the large number of non-pulmonary benign tumours in the amosite-treated group and any particular proliferative lesion. It is perhaps worth noting that three mesenteric haemangiomas were found, and they could be clearly seen to have arisen in the lymph node (Figures 8.13 and 8.14).

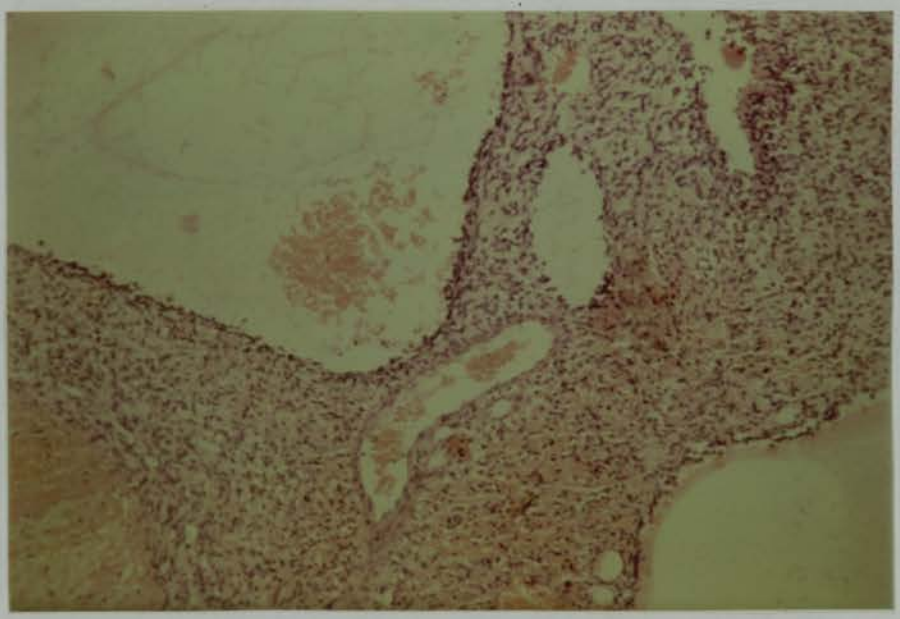


Figure 8.11 Section through an angiosarcomatous area of a malignant anaplastic tumour found in the cervical region of a chrysotile-exposed animal (near the left scapula.). (H.+ E.)

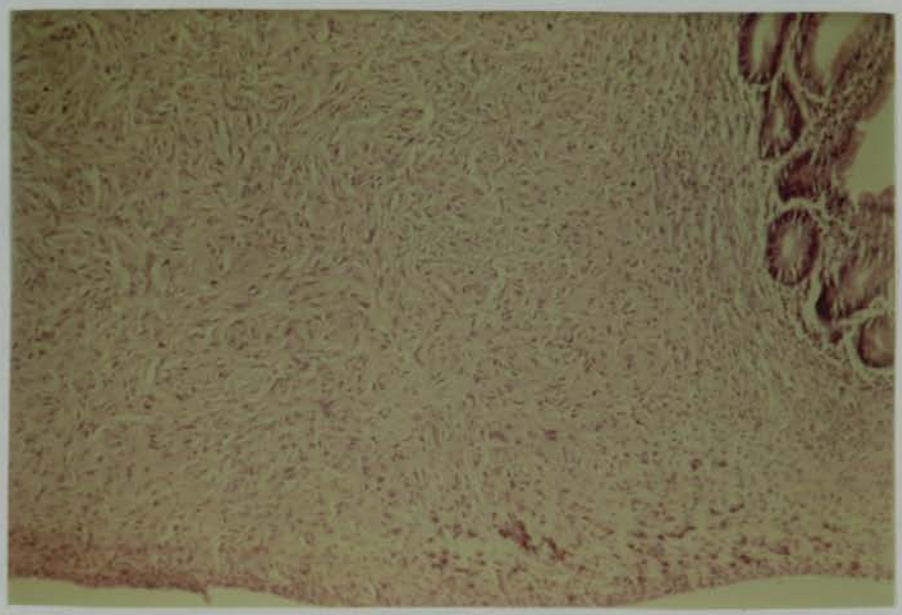


Figure 8.12 Section through a leiomyosarcoma of the ileum found in an amosite-exposed animal. (H.+ E.)

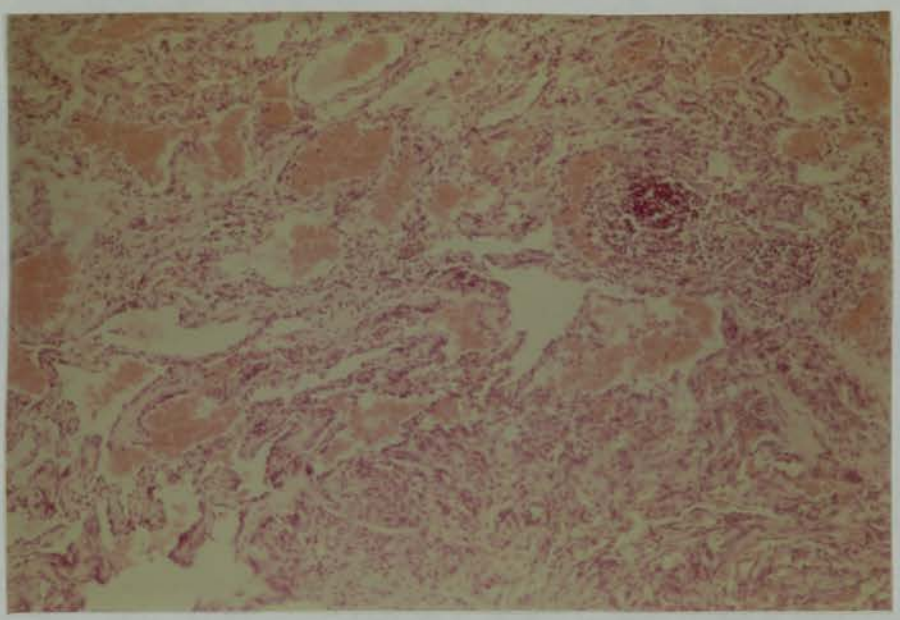


Figure 8.13 Low magnification photomicrograph of a section through an haemangioma, found in a lymph node, showing the typical appearance. (H.+ E.)

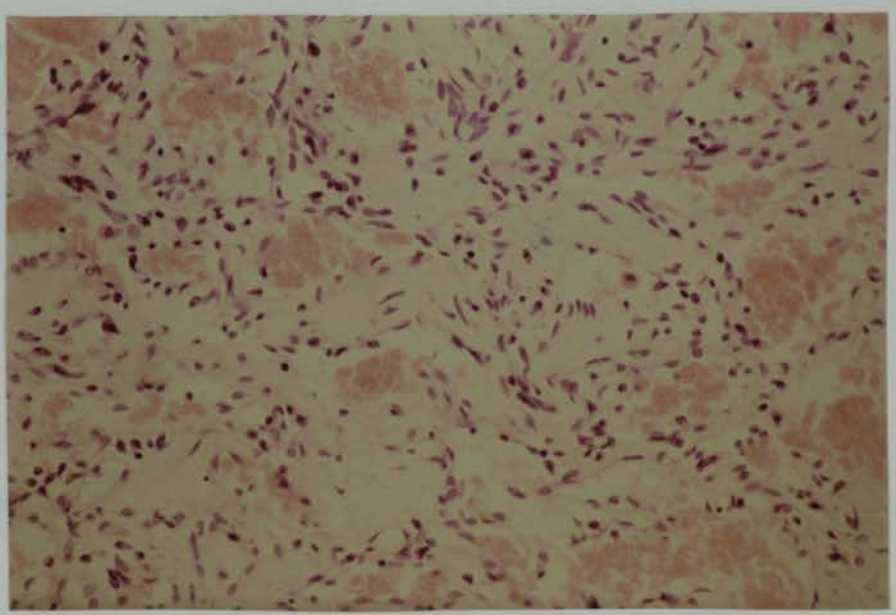


Figure 8.14 Higher magnification of a section through an haemangioma, showing the blood vessel formation. (H.+ E.)

Of the non-neoplastic pathological lesions, the most common was the usual renal hypertensive damage, frequently associated with mesenteric polyarteritis nodosa, found in aged male rats throughout these studies (see Section 5.3). There was no obvious link between the asbestos treatment and the renal pathology, but there were marginally more severe cases of mesenteric polyarteritis amongst the crocidolite treated animals (seven cases in the crocidolite group, two in the amosite, and four in the chrysotile). Other lesions occasionally found included some degree of hepatic fatty change, and some pulmonary congestion and oedema. However, of interest were two types of ulcerative gastrointestinal lesions: The first was the occasional finding of small erosive ulcerative areas of squamous forestomach (see Figure 8.15) in some exposed rats. These lesions have been occasionally found in untreated rats and are considered more likely to be associated with some dietary deficiency rather than the asbestos treatment. Histologically, the lesions were unremarkable with a low grade chronic inflammation and occasionally some small areas of focal haemorrhage. The second type of ulcerative lesion was one case of severe erosion, chronic inflammation and loss of mucosal architecture, of the caecum, found in a chrysotile exposed animal (Figure 8.16). Although it was the only caecal ulcer encountered in the HAN s.p.f. rat colony, it is not possible to describe it as having arisen because of the chrysotile treatment.

8.4 Fibre searches

8.4.1 Routes of access

Included under this heading are some estimates of the concomitant ingested fibre burden that is associated with any whole body inhalation exposure system. For practical reasons, it was not possible to produce direct quantitative figures for the extent

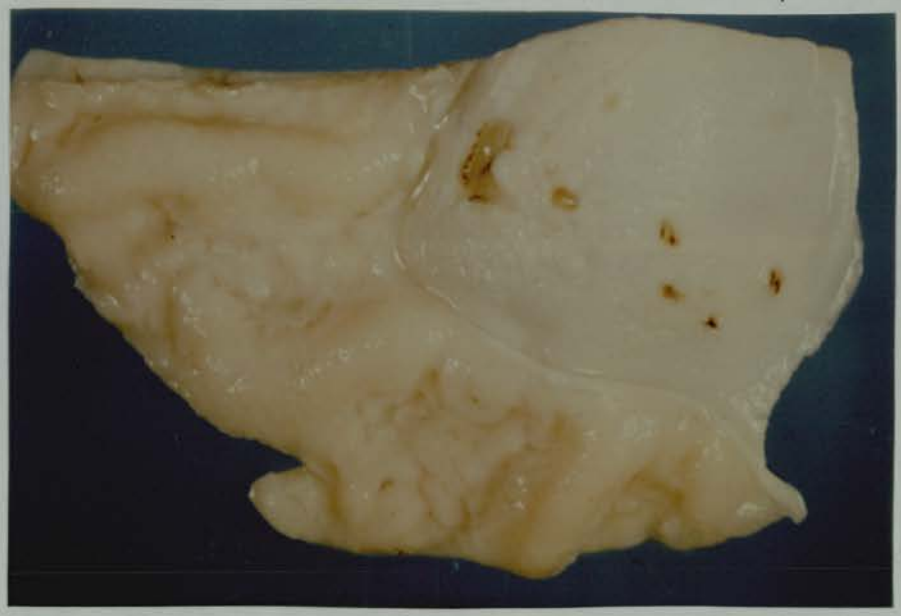


Figure 8.15 Macro photograph of the luminal surface of the squamous forestomach, showing the ulcerations occasionally found amongst the groups of animals.

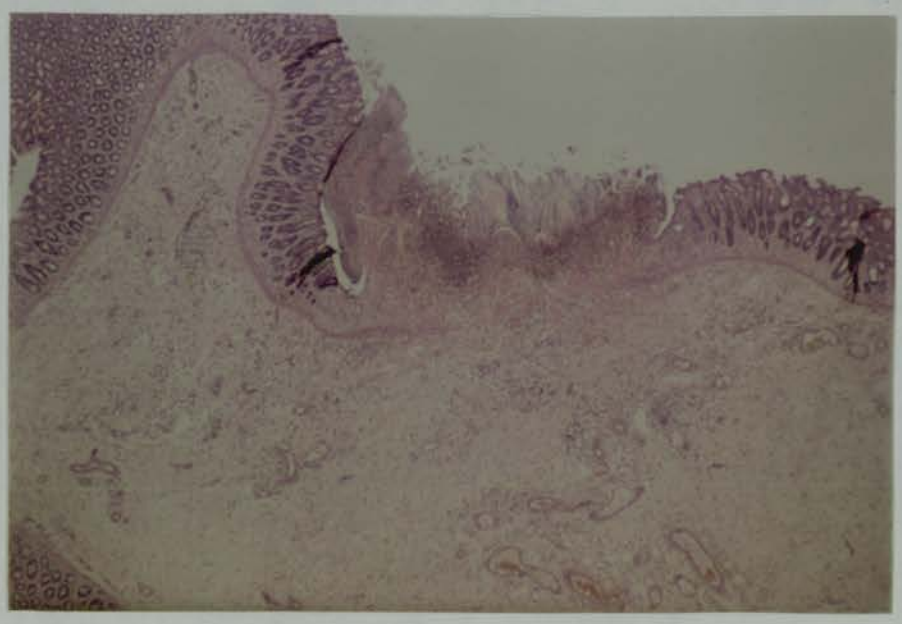


Figure 8.16 Section through the caecal ulcerative area found in a chrysotile-exposed animal. (H.+ E.)

of the ingested fibre burden, since the levels of fibre in the gut contents were below the resolution limit of the infra-red detection assays. However, in relative terms the burden of fibre amongst the ashed food residues was found to be considerable, with many fibres being visible at the SEM level (Figure 8.17).

The ingested fibre may be considered to have originated from three main sources. The first is that fibre directly ingested as surface contamination of the food pellets due to the deposition of dust upon the food. Chapter 4 shows that rats consume approximately 20gms of diet every 24 hours, and since feeding tends to be sporadic, with the rats gnawing at the surfaces of the pellets, it is likely that this is the major source of ingested fibre. Figure 8.18 shows an extreme example of the contamination of food with asbestos dust. The photograph shows the results of a heavy and albeit unusually high degree of fibre flocculation and deposition caused by a malfunction in the generation of chrysotile fibre clouds, and it has been chosen to illustrate food contamination. It should be stressed that Figure 8.18 was photographed following a breakdown in the normal dust generation and it is not intended to demonstrate the normal levels associated with the long term dosing of rats in the exposure chambers.

A second source of ingested fibre is that collected by the animals during their normal grooming of their fur. Grooming is an integral part of the behaviour of healthy rats, and many hairs are ingested as may be seen from a macroscopic examination of the contents of the rat forestomach. It is difficult to estimate the extent of the contribution of fibre ingested in this way to the total burden, but it is likely to be much less than the direct ingestion of fibre with food.

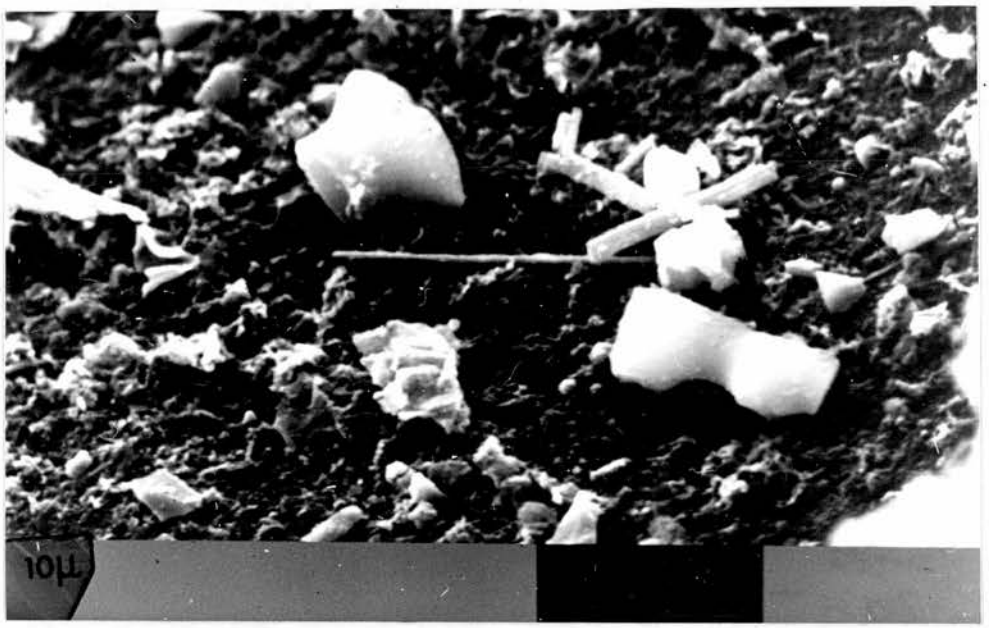


Figure 8.17 Scanning electron micrograph of ashed GI. lumenal contents taken from an animal exposed to inhaled asbestos, showing the associated ingested fibre burden.

Figure 8.18 Macro photograph of the inside of an inhalation chamber, showing a heavy contaminating layer of asbestos fibre covering the chamber contents, including the food pellets and the animals. (Photograph illustrates an extreme example - see section 8.4.1 .)

The third route of fibre ingestion following inhalation of asbestos is the ingestion of material cleared from the lungs by the mucociliary clearance mechanisms. Although only a minor component of the total burden during the dusting phase, this differs from the other two sources in that it persists after the actual period of inhalation dosing ceases. It is widely accepted that the major component of lung clearance is via the bronchial mucociliary escalator, and it follows, therefore, that the majority of the cleared asbestos is subsequently ingested. Figures for the pulmonary clearance of inhaled asbestos dust in rat exposed under the same conditions as described in this work, but for only 6 weeks, show that the rate of removal of dust may be up to 20 micrograms per rat per day in the earlier (faster) phase. (Middleton et al 1977). It is reasonable to assume that most of this asbestos passes along the gastronintestinal tract.

Although assumed to be of only minor importance, the lymphatic clearance mechanism is involved in some clearance of particles from the lung and this is clearly shown in Figure 8.19. Here the mediastinal lymph nodes of an animal killed after one year of crocidolite inhalation can be seen as prominant blue protrusions from the surrounding connective tissue. The natural blue colouration of crocidolite proved to be a useful indicator of the presence of large deposits of fibre, and blue mediastinal lymph nodes were observed in many of those animals autopsied after crocidolite inhalation. Ashing examination of the lymph nodes of rats exposed to either of the other dusts showed that very large numbers of fibres could also be found. Figure 8.20 shows an ashed residue sample of one of the crocidolite exposed mediastinal lymph nodes, and it is interesting to note that the majority of the fibres are relatively short.

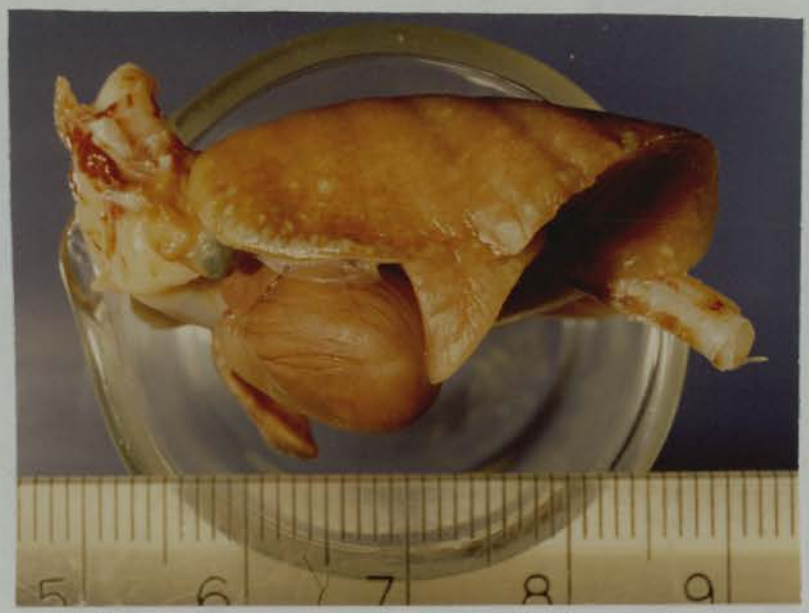


Figure 8.19 Macro photograph of the contents of the pleural cavity excised from an animal exposed by inhalation to crocidolite asbestos fibre, showing the mediastinal lymph node loaded with the blue asbestos fibre.

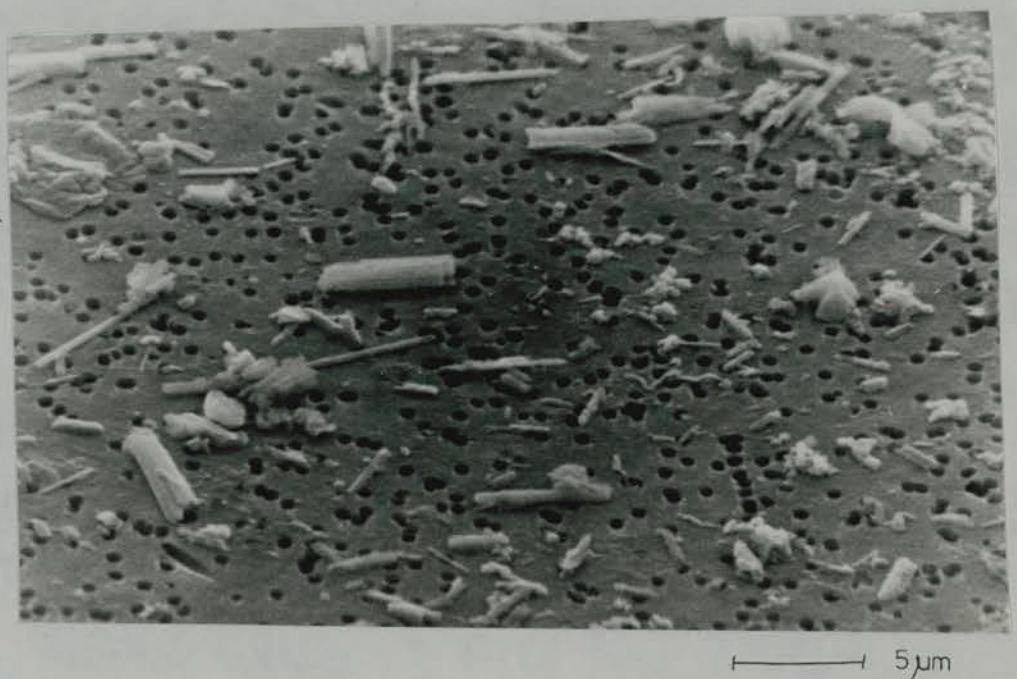


Figure 8.20. Scanning electron micrograph of ashed residue from a mediastinal lymph node taken from an animal exposed by inhalation to crocidolite asbestos, showing predominately short fibre.

This might be expected since it is possible that macrophages containing the smaller fibres would be more likely to penetrate into the lymphatic system than cells hindered by longer fibres. Figure 8.20 also demonstrates that asbestos fibres could be found in very large numbers in the mediastinal lymph nodes following the inhalation of asbestos. Further mention of this will be found in Section 8.4.2.

8.4.2 Details of findings

A selection of tissues (Figure 8.2) were taken from a small number of animals from each treatment group and processed by ashing (see Section 2.6.1) prior to an electron microscopical search for the presence of asbestos fibres. Figures 8.21 to 8.23 summarise the results of these fibre searches, expressed in terms of the numbers of fibres actually found in each tissue residue. Sample conversion factors are included to enable estimates of the total tissue burden to be made as before (see Section 5.4). Section 8.4.1 shows the large numbers of fibres that could be found in mediastinal lymph node residues. As a result this high density of fibres, precautions were taken to minimise the risks of cross contamination of other residue samples, and the actual numbers of fibres were assessed visually, (see Section 7.4 for further details).

It was shown in Chapter 3 that asbestos fibres tended to become brittle when heated and acid treated, and that this effect tended to be particularly pronounced following periods of tissue residence. Since some of the ashing procedures involved limited heating and acid washing steps, it was considered inappropriate to measure the dimensions of any fibres found amongst the residues. However, there was one exception to this rule. The mediastinal lymphatic tissues generally contained such large numbers

FIGURE 8.21 Asbestos transport following inhalation of fibre: amosite treatment. Fibre search results.

		No. of fibres found per sample per rat			
Animal age (days)		448	949	949	949
Tissue	Conversion Factor				
Liver	5450	4	12	2	7
Spleen	120	2	8		4
Kidney	170	7	10	6	12
Selected gut	1400	31	11	8	10
Mesenteric lymph nodes	140	4			5
Cervical lymph nodes	150	12		3	9
Mediastinal lymph nodes	50	++	++	+	++

FIGURE 8.22 Asbestos transport following inhalation of fibre: crocidolite treatment. Fibre search results.

		No. of fibres found per sample per rat			
Animal age (days)		448	950	950	950
Tissue	Conversion Factor				
Liver	5450	10		7	15
Spleen	120	-	6	3	
Kidney	170	19	8	9	11
Selected gut	1400	36	17	24	12
Mesenteric lymph nodes	140	12	16	9	22
Cervical lymph nodes	150	2	9	-	4
Mediastinal lymph nodes	50	++	++	++	++

FIGURE 8.23 Asbestos transport following inhalation of fibre: chrysotile treatment. Fibre search results.

		No. of fibres found per sample per rat			
Animal age (days)		448	950	950	950
Tissue	Conversion Factor				
Liver	60500	6	3	4	
Spleen	1600	5	-		5
Kidney	2100	-	3	8	4
Selected gut	15000	-	9	-	6
Mesenteric lymph nodes	1740	8	9	1	4
Cervical lymph nodes	1950	4		3	-
Mediastinal lymph nodes	100	+	+	++	+

+ = more than 10 fibres per SEM field at a magnification of x 2000.

++ = massive numbers of fibres " " " " " "

of fibres that it was not necessary to remove the bulk of the tissue residue in order to find fibres. As a result, the residues could be gently dispersed in distilled water and the preparations examined using the SEM. It was shown that such a mild treatment did not alter the size distributions of control amphibole samples, and it was therefore considered that some comments on the dimensions of fibres recovered from the mediastinal lymph nodes could be made. Accordingly, Figure 8.20 shows a typical view of crocidolite fibres recovered from the mediastinal lymph nodes of an animal that has been inhaling crocidolite for one year prior to death, and it can be seen that almost all the fibres are less than five microns in length.

It should be noted from Figures 8.21 to 8.23 that tissues from four animals were examined for each treatment. One of these was killed at the end of the dusting phase, and the other three were killed when the experiment was terminated 17 months later. An examination of the Figures shows that many more fibres were found in these tissues than in either the ingestion or subcutaneous injection experiments described in Chapters 5 and 7. In particular, the mediastinal lymph nodes of all animals examined contained large numbers of fibres. Both types of amphibole treated tissue samples appeared to have more fibres associated with them than the chrysotile, but this might reflect problems over the recovery and identification of chrysotile in residues.

It is interesting to note that the only tissue that showed a real variation in the numbers of fibres with animal age was the selected gut sample, in which the tissue from animals killed immediately after the dusting phase had more fibres. This is to be expected since the ingested burden of fibres is likely to be higher at this time then 17 months after the end of dusting (see Section 8.4.1). The finding of any fibres in the

gut tissue residues should be treated with caution, since although care was taken to remove the lumenal contents prior to ashing, experience with tissues from the ingestion experimental animals indicated that it was impossible to discount the possibility of fibres adhering to the gut mucosal surfaces. The presence of fibres within the mesenteric lymphatic tissues does however suggest that at least some of the fibre was within the gut tissues.

As regards the other tissues examined, Figures 8.21 to 8.23 show that almost all the amphibole-derived tissues examined contained some asbestos fibre. This is in marked contrast to the tissues from the ingestion and subcutaneous injection experiments, and it suggests that the inhalation of asbestos is associated with a certain degree of widespread fibre dissemination. It seems likely that this occurs as a result of lymphatic spread from the lung via the mediastinal lymph nodes rather than gastrointestinal penetration by ingested fibre.

8.5 Conclusions

(1) There was no difference in the morbidities of the three groups of rats exposed for one year to either amosite, crocidolite or chrysotile, with the mean survival times and the autopsy weights being similar.

(2) Of the three asbestos treatments, chrysotile was associated with the greatest degree of pulmonary damage when measured in terms of interstitial fibrosis, peribronchiolar fibrosis and bronchial epithelial extension. The interstitial fibrosis was shown to be progressive after the end of the dusting phase such that the lungs of chrysotile dosed animals were extensively fibrosed by the end of the study.

(3) The interesting finding of sub-pleural accumulations of macrophages that were particularly prominent as pale patches in the lungs of chrysotile exposed animals requires further investigation. Taken on face

value, it could indicate a specific movement of macrophages, some of which may contain asbestos fibres, out towards the pleural surface, and this has far reaching implications for the potential development of mesotheliomas. However, it must be remembered that the macrophage accumulations have been associated with viral lung disease and it is possible that the noted link with chrysotile treatment may simply be secondary one due to an increase in viral lung disease in animals with widespread pulmonary fibrosis.

(4) Chrysotile inhalation was the only treatment to produce malignant pulmonary tumours. There were also many more adenomatous lung tumours amongst the chrysotile treated animals. The link between benign and malignant lung tumours in this study was sufficiently strong to suggest that there might be a direct progression from benign proliferation to malignancy.

(5) There was no major difference in the numbers of non-pulmonary malignant tumours produced amongst the different treatment groups, although the chrysotile group did have two more than the others. It is interesting to note that the chrysotile group contained two anaplastic angiosarcomatous tumours in the cervical region, and it is possible that these tumours arose in the cervical lymph nodes. No gastrointestinal mucosal malignancies were found in any of the inhalation-exposed animals, but one leiomyosarcoma of the small intestine was found in an amosite treated animal.

(6) More non-pulmonary benign tumours were found amongst the amosite treated animals (11 in the amosite treated group, 6 in the crocidolite, and 6 in the chrysotile) but there was no clear pattern in the types of tumour found.

(7) The whole body inhalation exposure system was shown to have an associated level of ingestion. Although high in comparison to human environmental exposure, this level of "secondary" ingestion is still a lot lower than that used in the ingestion experiments reported in Chapters

4 and 5. Despite this, however, asbestos fibres were found in a variety of tissues including the gut, and to a greater extent than in either the ingestion or the subcutaneous transport studies. There was no strong evidence that the gastrointestinal mucosal barrier of animals exposed by inhalation to asbestos was any less potent than that of the animals used in the ingestion experiments, with only one inhalation animal being found with some abnormal caecal ulceration. This suggests that the high level of fibres in tissues following inhalation is not associated with the subsequent ingestion of inhaled material.

(8) The fibre search results show that there was considerable movement of the smaller asbestos fibres to the mediastinal lymph nodes, and it seems likely that further movement of fibres round the body could occur via the lymphatic and vascular systems. The finding of fibres in most of the tissues from inhalation exposed animals that were examined in detail supports the concept that transport of fibres throughout the body does occur following inhalation.

CHAPTER 9 DISCUSSION

DISCUSSION

9.1 INTRODUCTION

The introductory literature review given in Chapter 1 outlines the background information on asbestos and its effects that was available by the end of 1973 (when the majority of the studies reported in Chapters 2 - 8 were initiated). Each of the chapters describing specific studies includes a small section summarising the immediate conclusions of the experiments, but the wider implications of these findings will be explored below. This discussion will also include reviews of the recent relevant literature that has been published since the start of the studies, and the experimental findings will be discussed in connection with the published work. Finally some suggestions for further work will be included.

It has already been shown in Section 1.1.4 that the needs of modern industrialised society have resulted in the widespread and increasing use of asbestos, and Section 1.1.4 shows that there has been an associated increase in the number of persons exposed to asbestos. The major portal of entry of asbestos into the body is via the respiratory system and Sections 1.2.2 to 1.2.5 present in some detail the effects of prolonged and usually heavy exposures to airborne asbestos upon the human respiratory system. Historically, numerically and economically the most important of these effects has been pulmonary interstitial fibrosis, or asbestosis, a progressive and often debilitating disease affecting many severely exposed persons. However, current methods of dust control appear to be reducing the incidence of asbestosis, and that is reassuring since the statutory regulations were specifically

aimed at controlling the asbestosis (see Section 1.2.2).

Nowadays attention is focused upon the carcinogenic risks of asbestos exposure, and Sections 1.2.5 to 1.2.8 review some of the epidemiological evidence for the association between asbestos exposure history and the development of various types of malignant tumours. By far the most common malignant tumour amongst asbestos exposed individuals is lung cancer, and the incidence of death from bronchogenic cancer has been shown to be much higher amongst the heavy exposure groups than amongst comparable control groups. Section 1.2.5 shows that this carcinogenic effect is grossly accentuated if the individuals are also cigarette smokers. There would also appear to be a strong dose response relationship operative, since the bronchogenic tumours are normally only found amongst the heavily exposed individuals who also have asbestosis. It is possible, therefore, that the dust control measures mentioned above may also significantly reduce the incidence of bronchogenic cancer. Measures specifically taken to reduce the asbestos dust levels to below the "carcinogenic threshold" have only recently been suggested but they have not yet been widely accepted. The main cause of this delay concerns the establishment of appropriate absolute levels of airborne asbestos fibre.

The establishment of standards of occupational hygiene in terms of a minimum effective dose depends upon detailed studies of the precise relationship between asbestos exposure and the development of malignancies. Epidemiological evidence points to the existence of a number of non-pulmonary malignant tumours that appear to be linked with asbestos exposure, and it is in connection with

these that major regulatory problems emerge. With most of the non-pulmonary malignancies the increased incidence is not high enough to provide unequivocal support for a direct link with asbestos exposure, and is certainly not high enough to permit regulatory standards for their prevention to be derived (see, for example, Section 1.2.7, where the evidence for an increased risk of gastrointestinal cancers has not been universally accepted).

The non-pulmonary malignancies suspected of being associated with asbestos exposure may be broadly classified into two groups. The first, and possibly the most widely known of these, are the tumours of the serous body cavities known as pleural and peritoneal mesotheliomas, and they are described in some detail in Section 1.2.6. It can be seen that one of the worrying aspects of the etiology of the human mesothelioma is that this usually very rare tumour has been frequently linked with obviously very low and often transient asbestos exposure, although there is some evidence for a dose response effect (Newhouse, 1973). The implications of the development of mesotheliomas following low exposures for the regulatory authorities are immense, since it is likely that precise control of incidental asbestos exposure may prove impossible. The existence of peritoneal mesotheliomas amongst asbestos exposed groups provides a further dilemma, since it is necessary to account for their development remote from the supposed (pulmonary) portal of entry of asbestos into the body. Mechanisms of transport and selective deposition of fibres in the mesothelial tissues have been proposed to account for peritoneal mesothelioma, and part of the work reported in the studies in preceding chapters was concerned with an examination of the mechanisms of any such transport.

Of the other group, the epidemiological evidence suggests that gastrointestinal cancer may be numerically the most important of the non-pulmonary malignancies linked with asbestos exposure (Wright, 1978) although the link is considered by some to be tenuous (Miller, 1978). Section 1.2.7 reviews the relevant literature, showing that the association has been attributed to the ingestion of asbestos fibre. The discovery in early 1973 of widespread pollution of some Canadian drinking water supplies with an asbestos-like mineral provided additional impetus for research into the potential hazards of ingested asbestos, and an important part of the work reported in the preceding chapters concerns investigations into the effects of ingested asbestos fibre. The links between cancers at other sites and asbestos exposure are still tenuous (see Section 1.2.8) and require further detailed epidemiological studies before proof can be established. However, animal experimentation should provide some information on general asbestos carcinogenicity that may be of relevance.

The picture therefore emerges of an urgent need for an experimental examination of the toxicity of asbestos, with special reference to the carcinogenicity of ingested and disseminated fibre. The comparatively small elevation in the incidence of certain malignancies in asbestos workers that has led to disputes over the validity of any link with exposure has also led to difficulties over experimental design. It has been shown that an experiment with less than 59 animals in any dose group cannot detect a carcinogenic risk of less than 5%, assuming that the spontaneous tumour rate in the animals is zero (Ehrenberg and Holmberg, 1978). Since the

spontaneous tumour rate in the HAN strain of laboratory rat appears to be at least 20% (see tables in Appendix IV), the number of animals per treatment group must be increased accordingly (Zbingen, 1973).

Practical limitations on the resources available precluded a strict adherence to the above animal experimental group sizes in the present studies, and attention was concentrated on attempts to increase the effective asbestos fibre dose received by the animals, thus increasing the carcinogenic risk. For example, an experimental ingestion dose of 50,000 times the human occupational ingestion maximum might reasonably be expected to increase the risks of developing gastrointestinal cancer in rats accordingly. In the absence of any carcinogenic expression, the tissues subjected to such a regime might reasonably be expected to demonstrate the extent of any fibre penetration and/or movement. Considerable emphasis was therefore placed on detailed examinations of tissues from the experimental animals for the presence of asbestos fibre, since the presence of a tissue burden of fibre would indicate a potentially harmful outcome.

The extrapolation of the results of *in vivo* carcinogenicity tests to man requires considerable caution, not least for limitations arising from the choice of experimental animal as the vehicle for the bioassay. The laboratory rat was selected for the present long term carcinogenicity studies largely for convenience, but also for its proven longevity and susceptibility to various carcinogens. In retrospect it was perhaps unfortunate that the laboratory rat has been shown by several workers (Schardein *et al.*, 1968; Kihlstrum and Clements, 1969; Mackawa and Odashima, 1975; Sass *et al.*, 1975), to be relatively resistant to spontaneous gastrointestinal malignant

tumour development. Zaidi (1974) comments on the unsuitability of the rat as an experimental model for an investigation of human gastrointestinal disease, and the comparisons between rat and human intestinal function reported by Drasar et al., (1970) would certainly suggest that the rat is not the ideal animal. An assay depending upon the enhancement of a natural propensity of an animal to develop a specific lesion might be considered more likely to express evidence of toxicity than an assay using resistant animals. The question of the relevance of any subsequent increase in incidence of a lesion due to exposure to a toxic substance should then be the subject of further experimentation. In the human context gastrointestinal malignancies have been shown to be a major source of cancer mortality in Western society (Waterhouse et al., 1976), and any influence of asbestos exposure is superimposed upon this "background" incidence. The fact that a significant proportion (20%) of all human cancer deaths have been attributed to exposure to occupational pollutants (Bridband et al., 1978), with asbestos being considered the most important single agent, shows the potential extent of the problem. The possibility of the role of asbestos as a co-carcinogen could not be examined with the experimental designs of the ingestion experiments reported in the preceding chapters, which were limited by a lack of resources.

Similar criticisms regarding the relevance of the laboratory rat apply to a lesser extent to the subcutaneous injection and inhalation experiments reported in Chapters 7 and 8. However, of specific relevance to the subcutaneous injection experiments, it would appear that the choice of the injection site over the right scapula was particularly unfortunate: it was subsequently

discovered that the brown multilucolar fat of the interscapular "hibernating gland" is both well supplied with blood vessels and it has been shown to exhibit an atypical connective tissue neoplastic response to a variety of injected agents (Grasso and Goldberg, 1966). The site was originally chosen as one that minimised the risks of injection of asbestos fibre directly into the body cavities as found in mice by Kanazawa et al., 1970, and it was also thought to provide a suitable site for the local application of relatively very large doses of fibre with minimal vascular interference. Once again, therefore, emphasis must be placed on the results of searches for the presence of asbestos fibre remote from the site of application rather than the tumour incidence within the groups of animals.

It follows that the isolation of individual asbestos fibres from tissues and their subsequent accurate identification is thus a vital part of the ingestion and transport studies, and some comment on the methods of extraction are appropriate. Several methods of extraction of particulate material from tissues have been developed over the years, and most have been concerned with pulmonary tissues. The methods include use of one or more of the following reagents: hydrogen peroxide (Jaunarajs and Liebling, 1972), sulphuric and nitric acids (Gerstel, 1934), hydrochloric acid (Wright, 1957; Cartwright and Nagelschmidt, 1961), alkalis (King and Gilchrist, 1945), glacial acetic acid (Bergman, 1966), formamide (Thomas and Stagemann, 1954), and bleach (Utidjian et al., 1968). Other methods have used muffle ashing at 380°C (King et al., 1956), or cold ashing in nascent oxygen (Gleit, 1963). The applicability of any one particular technique depends to a large extent on the stability of the particulate material to be extracted: a vigorous

extraction technique involving for example hot concentrated hydrochloric acid could be used for the extraction of silica, but not asbestos. Asbestos fibres may be substantially damaged by such treatments (see Chapter 3) and a milder extraction regime must be used. The choice of method has been the subject of some debate, and a trial of the relative efficiencies of different techniques has been published (Gylseth et al., 1979). One criterion for assessing the absolute efficiency of any method has been a comparison of the length distributions of recovered fibre in which it has been assumed that the procedure producing the largest number of long fibres is the most suitable, on the grounds of minimal fibre breakage. However, this basic tenet is suspect: a size distribution containing a large number of long fibres could be obtained by removal, or loss of, ~~long~~ short fibres, during such procedures as filtration, centrifugation separation etc. that are inherent in methods of chemical degradation and extraction. Confirmation of the problems of recovery of finely divided minerals from liquid suspensions (Jaunarajs and Liebling, 1972) was obtained during the development of extraction procedures described briefly in Section 2.6.1. As a result, cold ashing of dried tissue samples has been used for all the studies reported in Chapters 4, 5, 7 and 8.

The effects of various chemical treatments on the integrity of asbestos types was examined in detail in Section 3.4.2, with particular reference to the effect of any alterations on the criteria of identification of fibres. The occurrence of non-asbestiform fibrous particulates in many residues necessitated analysis of all suspicious shapes where possible, and any modifications of the fibre analyses arising from the extraction procedures had to be

taken into account. The modifications to fibres were assessed using electron optical methods. The small size of the majority of fibres in UICC asbestos preparations has meant that the transmission electron microscope with attached microprobe analytical apparatus provides the only absolute method of examining fibres in tissues or residues, and the use of the scanning electron microscope (SEM) and energy dispersive analysis (EDAX) can at best only be considered a compromise between full transmission electron microscopy (TEM) and light microscopy. Some of the problems of the use of the SEM and EDAX facility available at the time of the fibre searches described in Chapters 4, 5, 7 and 8 are presented in Section 3.2, and it can be seen that there was considerable scope for error and misidentification. In view of this, any fibrous shapes were generally selected on morphological grounds as putative "asbestiform", and then analysed for the presence of silicon. A positive silicon assay was taken as confirmation that the fibre was asbestos, and the use of this rather broad classification scheme was therefore likely to overestimate the absolute number of asbestos fibres present in a tissue residue (see Section 3.6). However, it must be remembered that the working resolution of the SEM precludes the identification of any fibres appreciably less than 0.2μ diameter, and as such there was a real possibility that asbestos fibres could be overlooked (since many UICC fibres are thinner than 0.2μ). It must be concluded, therefore, that the techniques used in the fibre searches in Chapters 4, 5, 7 and 8 were likely to underestimate the absolute number of fibres present.

To return to the examination of the chemical reactivity of asbestos described in Section 3.4. In general, these studies confirmed the work of others (Hodgson, 1965; Harington, 1965;

Monkman, 1971; Choi and Smith, 1972), with chrysotile being the most reactive and the amphibole types relatively resistant to chemical attack. A further examination of the influence of varying periods of storage and tissue residence produced some interesting findings: the dissolution and leaching of deposite of chrysotile in tissues confirmed the findings of many workers (Morgan et al., 1971; Rahman et al., 1974, and Jaurand et al., 1976), whilst morphologically the amphiboles seemed unaffected. However, the results of analyses of recovered amphibole fibres strongly suggested that many were coated with a thin iron-containing layer of biological material.

Ferruginous bodies are only occasionally seen in the rat (although none ~~was~~ were seen at the electron microscope level), yet the implication remains that some amphibole fibres are coated with a layer containing iron. Working with human lung extractions, many workers have shown that ferruginous bodies make up a small proportion of the total fibre burden (Ashcroft and Heppleston, 1973; Le Bouffant et al., 1976). They tend to refer to "uncoated" and "coated" fibres, whereas it is perhaps more likely that all fibres are coated to some extent, but only a proportion are recognisable as ferruginous bodies. Desai and Richards (1978) have demonstrated the adsorption of a range of biological macromolecules on to mineral dusts, and asbestos minerals are known to have a highly adsorptive surface (see Section 1.1.3). Davis (1970) describes the process of ferruginous body formation, and the process of giant cell formation (Davis, 1963). Since giant cells tend to form round the larger fibres, and ferruginous body formation tends to involve the longer fibres, it is possible that the excessive coating of the longer fibres occurs predominantly in giant cells. Some support for the suggestion that fibre coating

may be simply a question of degree rather than an absolute phenomenon is available from comparative studies on body formation. It is unreasonable to account for the differences in extent of ferruginous body formation between rat, guinea pig and hamster (see Section 1.3.2b) in terms of different mechanisms. It would be interesting to examine the asbestos fibres recovered from guinea pig injection or inhalation experiments for evidence of more fibres with a thin coating of biological material.

9.2 THE INGESTION OF ASBESTOS FIBRE

The studies described in Chapters 4, 5 and 6 have shown no evidence of any widespread penetration of, or damage to, the gastrointestinal mucosae following prolonged ingestion of large amounts of asbestos fibre. The asbestos-margarine preparation was shown to be a practical method of administering asbestos in the diet over a long period, and there was no associated inhalation hazard. The high fat diet produced changes in the uptake and utilisation of pelleted food (Chapter 4), resulted in a generalised obesity with some hepatic periportal fatty change (Chapter 5), and caused some depression of the mucosal proliferation of the descending colon (Chapter 6). Examination of the intestinal lumenal contents of animals actively ingesting asbestos showed that the fibre had adequate access to the mucosal surface, and thus the negative findings could not be explained in terms of the unsuitability of margarine as the carrier medium.

It would appear that the intestinal mucosa of healthy rats presents an effective barrier against potentially harmful lumenal contents such as asbestos fibre. The results of the fibre searches of tissue residues presented in Chapter 5 imply that there is almost

no penetration of the gut mucosa after very long periods of asbestos ingestion. Despite the stringent precautions taken against contamination, it remains a distinct possibility that those fibres that were found could be the result of contamination during the sample preporation. It is clear that if penetration did occur, the amount of fibre in the tissues represents a minute proportion of the exposure dose. Since the dose was set at 50,000 times the human ingestion maximum, it is reasonable to conclude that the potential levels of fibre penetration were proportionately higher than would be expected after the ingestion of foods and beverages contaminated with asbestos fibre.

Chapter 5 contains details of the malignant tumours found in animals ingesting asbestos, and it can be seen that there were no primary gastrointestinal mucosal malignancies. This is not unexpected in view of the above findings of minimal fibre penetration in the animals.

The ingestion work therefore suggested that healthy individuals with a normal gastrointestinal function are not at risk from the normally encountered levels of ingested mineral fibre. The problem of the potential penetration in areas of mucosal abnormality such as intestinal ulceration etc. has not been investigated. It would appear that the mucosal barrier can maintain its integrity even under conditions of extreme loss of mucosal architecture and epithelial cell structure associated with Whipple's disease (Trier and Rubin, 1965), but a complete breakdown of organised mucosa might well provide a portal of entry for asbestos fibres. The effects of unbalanced and/or inappropriate diets and the use and abuse of tobacco and alcohol are all known to have an adverse effect

on normal intestinal function in modern man (Piper and Raine, 1959; Hunt, 1963; Davenport, 1972). One estimate of the intestinal disorders in modern man suggests that 6.5% of otherwise healthy individuals have some loss of integrity of intestinal mucosa arising from the pressures of Western society (Doll and Avery Jones, 1951). It is reasonable to suggest that such a breakdown of the mucosal barrier could be associated with an increased risk of penetration of particulate or other foreign matter. Inflammation and infection of ulcerated areas is not uncommon in certain regions of the gastrointestinal tract (Hunt, 1963). A clear correlation between diet and certain forms of gastrointestinal neoplasia has been shown in humans (Dunn and Buell; 1967; Burkitt, 1971; Spiller and Aman, 1975), although in general it has not been established whether the dietary changes lead directly to tumour formation as a result of contained carcinogens, or whether intestinal dysfunction provides expression for the normally occurring low levels of potentially carcinogenic materials such as biliary pigments (Hunt, 1963).

It would be interesting to examine the effects of prolonged ingestion of asbestos on animals with areas of damaged intestinal mucosa. It may be that the apparent excess of malignant gastro-intestinal tumours arise amongst those heavily exposed individuals who have also had a non-specific deficiency of the mucosal barrier. It is worth noting that the excesses of tumours so far reported tend to involve those sites known to be susceptible to ulceration, diverticula formation, etc. Pozharisski (1975) has shown a strong relationship between non-specific caecal injury and experimentally induced tumours in rats. The gastrointestinal tissues may be highly

susceptible to the carcinogenic influence of asbestos once the fibre has gained access to the vulnerable tissues.

The phenomenon of "persorption" of particulate material through the wall of the gastrointestinal tract has been reported by Volkheimer (1974) and by Schreiber (1974). The implications of frequent penetration of particles of up to 90 μm diameter into the lymphatic system and beyond, as reported by Volkheimer, are far-reaching and require comment. It is interesting to note that whilst Clark (1959), Sanders and Ashworth (1961) and others have reported absorption of very small latex spheres by pinocytosis in the small intestine, no mention was made of massive transmigration of material on a scale envisaged by Volkheimer. Persorption of particles as large as 90 μm could only occur following a breakdown in the intestinal mucosal barrier. Such breakdowns have not been reported in the copious literature of fine structure of the intestine (see Trier and Rubin, 1965). Some of the photomicrographs demonstrating "persorption" presented in the paper Volkheimer (1974) may be simply reproduced by careless handling of lengths of gut before full fixation. In addition, one would expect that if the phenomenon of persorption were the norm, examination of the mesenteric lymph nodes would reveal a heavy burden of particulate matter. Gross et al., (1974) emphasized that even in workers occupationally exposed to particulate matter such as coal and silica dusts, there is no involvement of the mesenteric lymph nodes, whilst alveolar penetration by the dusts can be confirmed by examination of the hilar lymph nodes. However, ~~In addition~~, pronounced black pigmentation of the liver is sometimes observed at autopsy of coal miners (Lamb; personal communication).

Some support for the concept of persorption is provided by the finding of particulates in human urine (Keal, 1960; Schreiber, 1974; Cook and Olson, 1979). Wyss first reported asbestos fibres in the urine of a person exposed to asbestos (Wyss, 1953), although his comments imply that he considered the pulmonary circulation the most likely source of the fibre. The recent work of Cook and Olson (1979) reports the results of electron microscope searches of urine collected from human volunteers, drinking water contaminated with asbestiform fibre in which direct penetration of the gut wall is implied. It is difficult to reconcile these observations with the complete lack of widescale penetration and movement of asbestos described in Chapters 4 and 5. In a letter to the Editor of Archives of Environmental Health, Gross (1974) presented some damning evidence against the published findings of Schreiber relating to the ingestion and excretion of particulate vegetable matter, that the author (Schreiber) completely failed to answer adequately. Some further comments on the movement of particulates will be found in Section 9.3.

Following the initial findings of asbestos contamination in Canadian drinking water supplies (see Section 1.2.7), there have been several detailed studies of the actual levels of fibre (Mudrock and Kramer, 1974; Cook et al., 1974; Brown et al., 1976; Durham and Pang, 1976). It has been established that 60,000 tons of taconite debris per day were dumped in to Lake Superior from the mining operations, and that this has resulted in levels of fibre up to 8.7×10^6 per litre in drinking water supplies. However, three epidemiological surveys of the populated areas at risk from this contamination have to date failed to find any excess of

gastrointestinal neoplasia (Mason et al., 1974; Levy et al., 1976; Wigle, 1977). It would appear, therefore, that there is no neoplastic risk from water supplies contaminated with asbestos fibre, although it would be wise to follow a cohort for at least 40 years after first exposure to exclude the possibility of the development of tumours with long latent periods.

Epidemiological surveys of individuals occupationally exposed to asbestos published in recent years have given conflicting views of the incidence of gastrointestinal tumours, but, in general, there is evidence that an increased risk exists, and that there is some dose response (Kuschner et al., 1974; Schneiderman, 1974). It remains possible that studies finding no significant excess in gastrointestinal cancer (e.g. Lumley, 1976) require a further period of follow up. Selikoff and Hammond (1978), for example, have found an increase in pulmonary X-ray changes in dockyard workers that they believe clearly indicates a significant previous asbestos exposure that may well lead to future increases in cancer at many sites, including gut. This reinforces earlier papers by Selikoff (1975) and by Newhouse and Berry (1976) who predict that the highest incidences of asbestos-related neoplasia will occur during the last two decades of the twentieth century. In a review of some of the literature concerning the health effects of ingested asbestos, Hallenbeck and Hesse (1977) suggest that further work is required before any firm conclusions may be drawn.

The fears of increased risks of gut cancer in humans exposed to products containing low levels of mineral fibre have not been confirmed. Hence a relationship between stomach cancer in Japan and mice treated with talc containing traces of asbestos

fibre (Merliss, 1971) is soundly disputed by Blejer and Arlon (1973) who point out that in a world-wide context no correlation exists. A study by Rubino et al., (1976) shows no excess of cancer amongst talc miners and millers, and to date there is no proven carcinogenic risk of occupational exposure to fibreglass (Gross, 1976) or cummingtonite-grunerite minerals (McDonald et al., 1978). However, in the latter paper McDonald et al believe that a high mortality from pneumoconioses may have obscured any carcinogenic influence.

It is necessary to look closely at experimental studies for corroboration of the epidemiological findings. The work described in Chapters 4 and 5 showed no sign of asbestos fibre penetration and no increase in pathology following prolonged ingestion of asbestos. These findings agree well with some recent publications. Thus Gross (1974), Gross et al., (1974), Smith (1973), Gibel et al., (1976) and Schepers (1976) all found no excess of gastrointestinal neoplasia in animals ingesting large amounts of various fibrous particulates over long periods. Chapter 8 describes the results of asbestos inhalation experiments with rats in which no excess of gastrointestinal tumours were found. In a similar inhalation study, but using larger numbers of animals, Wagner (1974) reported no adverse gastrointestinal effects. He described briefly the potential extent of the level of fibre ingested from such sources as the animal food, drinking water, and fur. Reeves et al., (1971, 1974) also make no mention of an increased incidence of gastro-intestinal disease in a large inhalation study involving several animal species. In an interesting article, Lee (1974) rightly comments on the problems over the widespread acceptance of negative

findings, and the above must be set against the many recent reports of the results of ingestion experiments in which pathogenicity of fibrous particulates are either expressly proven or implied.

It is interesting to note, however, that none of the studies actually found increases in the incidence of gastrointestinal tumours, but are restricted to descriptions of the extent of fibre penetration and some biochemical consequences of such penetration. Fibre penetration has been reported by Pooley (1974), Webster (1974), Westlake (1974), Cunningham et al., (1977), and Hallenbeck and Patel-Mandlik (1978) in a variety of experiments using rats, guinea pigs, or primates. In general, electron microscopy was used for tissue examinations, but few details of attempts to minimise fibre contamination of samples are given, and one of the studies reports regularly finding asbestos fibres in untreated control tissue samples (Cunningham et al., 1977). In a bizarre experiment involving laparotomy followed by ligation and inflation of a section of the small intestine with an asbestos suspension, Storeygard and Brown (1977) present some electron photomicrographs of "penetration" of the mucosa and then proceed to infer a risk of penetration under more realistic conditions. Zaidi et al., (1976) found evidence of dissolution of nickel from asbestos after catheter instillation of prestarved guinea pig stomachs with huge (500 mg) asbestos doses, and they suggest that solubility products of asbestos may cause cancer in the absence of any direct penetration. Using a somewhat similar technique in rats, Amacher et al., (1974, 1975) found an increase in DNA synthesis in several gastrointestinal tissues that persisted for up to two weeks. In summary, it would appear possible to produce indication of intestinal penetration by asbestos if either

unphysiological exposure regimes and/or insufficient precautions against fibre contamination of microscopy samples are employed.

However, the results of Jacobs et al., (1977, 1978) are more difficult to explain. Working with rats, they have found an increase in DNA within the lumen of those animals ingesting asbestos either with or without added cigarette smoke condensate, implying an increased attrition rate of intestinal villus cells. Their animals had been exposed to reasonable levels of chrysotile asbestos for long periods (of up to 15 months), and as such they were not subject to the criticisms of technique described above. Chapter 6 describes detailed analyses of the proliferative parameters of animals maintained for 25 months on an amosite-containing diet in which no alteration in proliferative rates and therefore, by implication, in attrition rates, was found. It is possible that the differences between these results and those of Jacobs and co-workers may be due to the fact that the latter used the more cytotoxic chrysotile (Bolton and Appleton. In press).

9.3 THE TRANSPORT OF ASBESTOS FIBRE

The interest and concern over the potential problems associated with the transport of asbestos fibres throughout the body has arisen over the years in an attempt to explain the development of a diverse range of human malignant tumours, many of which apparently arise remote from the site of the major asbestos deposits (see Sections 1.2.8, 7.1 and 9.1). There is some evidence that a widespread dissemination of fibre can occur in humans following its initial deposition. Thus Keal (1960) reviews the earliest findings of asbestos and ferruginous bodies in urine thyroid and spleen of asbestos workers: Hourihane (1965) describes finding asbestos fibres

in both pleural and peritoneal mesotheliomas, Levine and Rubnitz (1965), Levine (1968) describe finding asbestos bodies in the peritoneal cavity; Telischi and Rubenstone (1961) reported asbestos fibre in a gastric carcinoma, and Wagner (1965) found some in both gastric mucosa and spleen. In the discussion following a paper by Newhouse and Thompson (1965), Schepers reported finding fibre in both adrenal and renal carcinoma, and Selikoff described finding fibres in bone marrow, kidney, liver, spleen and gastrointestinal tract following ashing extraction techniques. Godwin and Jagatic (1970) found both fibres and bodies in a wide variety of tissues from six cases of human mesothelioma. Finally, Le Bouffant and his co-workers (Le Bouffant, 1974; Le Bouffant et al., 1976), report many small asbestos fibres in the pleural plaques, and this is interesting since plaques are almost always restricted to the parietal pleura (see Section 1.2.3).

The experimental evidence for the transport of asbestos fibres through tissues stems from the work of Roe et al., (1967) who reported transport of asbestos in mice from a subcutaneous injection site, selective deposition in submesothelial tissues, and the subsequent development of mesothelioma. However, a later paper by the same group of workers (Kanazawa et al., 1970) suggested that the "selective transport" to mesothelial tissues and the mesothelioma development was an artefact arising from the direct erosion of injected material into the peritoneal cavity. In the repeat experiment they did, however, find widespread dissemination of fibre to many tissues, and they suggested that both the lymphatic and the blood vascular systems were involved. Several of the experimental ingestion studies that apparently found evidence of gastrointestinal

penetration also found fibres in a variety of tissues (Cunningham et al., 1977; Hallenbeck and Patel-Mandlik, 1978), and the papers relating to persorption and excretion are also relevant (refer to Section 9.2). Working with a man-made silicate-containing fibre, Styles and Wilson (1976) reported finding fibre in the lungs and lymph nodes of rats after intraperitoneal injection.

However, it remains a possibility that the general but consistent low level of asbestos found amongst most of the animals injected with one of the three types of asbestos may have had some influence on the number and diversity of malignancies developing. A larger experiment involving more animals would be necessary to answer this point. The present experiment merely showed that there was no obvious adverse effect of disseminated asbestos. Having accepted that some fibre could be found remote from the original site of application, it is necessary to explain how the dissemination occurred. The subcutaneous asbestos injection experiment suggests that the most likely route was from injection site to local lymph nodes via the lymphatic system, then to the thoracic duct into the blood vascular system and a generalised dissemination.

It would appear from the above that the lymphatic and possibly the vascular dissemination of asbestos fibre from various sites is well established. The findings of very low but consistent levels of fibre in a variety of rat tissues following subcutaneous injection certainly confirms this view (see Chapter 7). Widespread particulate movement can easily be demonstrated by the injection of nigrosin suspensions into many sites, and indeed this technique was widely used by the early anatomists to delineate the lymphatic system (Job, 1915; Higgins, 1925). Work with nigrosin injections at the

Institute of Occupational Medicine has clearly established the role played by the lymphatic system in the movement of particulates. Of specific relevance to the finding of Styles and Wilson (1976) described above, direct communication between the peritoneal and pleural cavities can be demonstrated following intraperitoneal injection of particulate suspensions (see also Allen, 1936 and Wang, 1975). Retrograde flow from the pleural to the peritoneal cavity also seems to occur, although to a much lesser extent.

Using neutron activation of asbestos samples, Morgan and his co-workers (Morgan et al., 1971, 1975; Evans et al., 1973) found what they considered to be leaching of soluble constituents (particularly magnesium) from asbestos, and their subsequent movement to a variety of tissues. The dissolution was particularly noticeable with chrysotile, confirming the results of experiments described in Chapters 3 and 7, and the work of many other investigators (Rahman et al., 1974; Jaurand et al., 1976). Harington (1965) considers many metals to be carcinogenic and has suggested in several of his papers that asbestos toxicity is due directly to the levels of magnesium associated with the fibres (see Harington et al., 1976). However, the carcinogenicity of the dissolved magnesium that has been moved from the fibres is not expressly considered. Zaidi et al., (1976) have vaguely suggested such a possibility for nickel dissolved by gastric acids.

The carcinogenic consequences of any transported asbestos are more difficult to characterise. It is not sufficient to merely record the finding of asbestos fibre within tumour material since its presence does not establish on a priori relationship. Tumours might arise remote from a site of application as a result of direct

movement of the fibre, dissolution and movement of some of the constituents of the fibre, or by the transformation and subsequent movement of cells that have been in contact with a fibre. The work reported in the preceding chapters has been essentially concerned with the first of these mechanisms, and a good deal of effort has been put into the examination of tissue residues for evidence of fibre transport. Chapter 7 describes the tumours arising after subcutaneous injections of asbestos in which the only unusual finding was the variety of tumours produced rather than the absolute number. Commenting on a similar variety of tumours after the experimental ingestion of chrysotile-containing filters, Gibel et al., (1976) suggest that it is necessary to consider all the tumours arising in the course of an experiment, irrespective of their histological classification or site of origin. In this connection, it is interesting to note that many of the tumours found in the subcutaneously injected animals (see Chapter 7) were associated with the reticuloendothelial or haemotopoeitic systems, particularly since any dispersion of fibres, dissolved constituents, or cells, must occur via the lymphatic and/or vascular systems. However, any link with asbestos fibre transport must remain tenuous since animals with tumours were found to have similar levels of disseminated fibre as those without tumours.

The inhalation experiments described in Chapter 8 show that widespread and significantly higher levels of fibre dissemination are a consequence of the pulmonary deposition and clearance of asbestos. This supports the findings of Godwin and Jagatic (1970) in humans that asbestos reaches the hilar lymph nodes of occupationally exposed individuals. Blau (1978) demonstrates that the lymph nodes are not an

absolute filter of particulate material, and this supports the findings in Chapter 8 that fibres occur in significant numbers distal to the mediastinal and hilar lymph nodes. There was, however, no selective movement of fibre to either the mesothelial or the gastrointestinal tissues and no excess of non-pulmonary tumours associated with the fibre dissemination. It is possible, however, that under some circumstances certain tissues have an enhanced response to resident asbestos fibres. Some support for this suggestion is available in a paper by Szymczykiewicz (1965) describing details of a somewhat bizarre experiment in which small doses of asbestos fibre were injected directly into the lungs and the gut wall, with the small intestinal tissue response being much greater than that in the lung.

9.4 SUMMARY

The studies reported in the preceding chapters demonstrate that:

1. The effects of tissue residence on asbestos fibres include chrysotile dissolution with possibly an increased likelihood for dissemination, and coating of fibres with a biological iron-containing layer that appears to be particularly prominent with the amphibole fibre types.

2. The ingestion of asbestos in rats is associated with no obvious fibre penetration, no increases in pathology amongst the treated groups, and no evidence of interference with the normal gastrointestinal proliferative mechanisms.

3. The experiments involving the subcutaneous injection of asbestos show some evidence of a limited but non-selective dissemination of fibre, with some evidence of an increase in the

variety of tumours arising amongst the treated groups.

4. The inhalation of asbestos is associated with a widespread non-selective dissemination of asbestos, particularly the shorter fibres, throughout a variety of tissues. All the animals examined were so affected, but there was no obvious increase in the numbers of non-pulmonary tumours with this dissemination.

5. It would appear that there is no risk of malignancy connected with the prolonged incidental ingestion of low levels of asbestos fibres, ~~in healthy humans~~ in rats exposed with margarine-supplemented diets. However, it remains a possibility that individuals with abnormalities of gastrointestinal function may be at risk from the consequences of asbestos fibre penetration.

6. Any increases in gastrointestinal disease amongst individuals occupationally exposed to high levels of asbestos fibre dust are therefore considered likely to be as a result of the pulmonary lymphatic clearance mechanisms and subsequent widespread dissemination of fibre. It is possible that the gastrointestinal tissues might be particularly susceptible to asbestos fibre once they have gained access to the vulnerable tissues via the lymphatic and vascular systems.

9.5 SUGGESTIONS FOR FURTHER WORK

The preceding chapters have described a diverse range of experiments investigating some of the pathological effects of asbestos. Many techniques have been used, and there is therefore a correspondingly diverse range of follow-up experiments that might be undertaken. However, the suggestions for further work will be restricted to those of specific relevance to the consequences of ingested and/or transported asbestos, and thus will exclude certain aspects of

instrumental microanalysis of mineral fibres, gastrointestinal cytokinetics, etc.

Ingestion of asbestos. From an epidemiological point of view, there is an urgent need for an unequivocal appraisal of the risks of asbestos ingestion, with details of the specific gastrointestinal (G.I.) sites at risk of tumour development, and of the classification of any such tumours. The ingestion experiments have shown that healthy laboratory animals are capable of maintaining an effective G.I. mucosal barrier that prevents the penetration of asbestos. However, it is necessary to examine the consequences of a breakdown in the integrity of the mucosal barrier, since such a breakdown may lead to widespread penetration and tissue damage. It might be possible to induce chronic intestinal dysfunction using pharmacological, immunological, or parasitic infestation as the experimental protocol. In addition, it must be remembered that the asbestos samples used throughout these studies for the purposes of comparison were specifically prepared under the auspices of the UICC to give a high proportion of small and respirable fibres, and it might be of interest to look at the effects of the ingestion of asbestos fibres of different dimensions. It is possible that the larger fibres have a greater chance of being deposited in the upper airways, cleared to the G.I. tract, and subsequently penetrating the mucosa. Experiments to examine the susceptibility of gastrointestinal tissues of laboratory animals to asbestos fibre would also be useful using direct inoculation of fibre into the tissues, since they would help in the rational choice of a suitable experimental animal.

Subcutaneous injection of asbestos. The unusual variety of tumours produced following the subcutaneous injection requires

some further investigation, although it would appear that very large numbers of animals would be necessary using the subcutaneous route. It is likely that the vascular inoculation of asbestos would produce more information on the consequences of disseminated asbestos, and allow a reasoned judgement of the significance of any fibre movement under more normal circumstances. The subcutaneous injection route might provide a useful opportunity to study the effects of prolonged tissue residence of asbestos, particularly vis-a-vis dissolution and/or coating of fibres.

Inhalation of asbestos. Perhaps the most valuable finding of the present work has been the discovery that the inhalation of asbestos would appear to be associated with a degree of widespread lymphatic and vascular dissemination of fibre. It is now imperative to examine this finding in detail. Quantification of both the extent of the lymphatic pulmonary clearance component and the dimensions of the fibres involved would be a useful first step. Examination of the mechanisms of clearance and of the ultimate fate of the disseminated fibre might provide interesting information of the development of extrapulmonary abnormalities in both humans and animals exposed to asbestos.

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APPENDIX I COMPOSITION AND PROPERTIES OF ASBESTOS

APPENDIX I Structure and properties of asbestos.

APPENDIX I, FIGURE 1 Typical percentage composition of UICC samples of asbestos.

	Chrysotile		Crocidolite	Amosite
	A	B		
S_1O_2	39.8	39.7	51.16	50.8
FeO	0.4	0.8	21.2	33.6
Fe_2O_3	1.5	1.4	18.3	1.9
CaO	0.15	0.09	0.17	0.24
MgO	26.1	26.6	3.6	5.0
Na_2O	0.04	0.02	5.8	0.1

Modified from Rendall 1970
Timbrell 1970

APPENDIX I, FIGURE 2 Comparison of percentage composition of major components of amosite and cummingtonite, showing their similarities.

Constituents	Amosite	Cummingtonite
SiO_2	49.47	54.67
FeO	35.63	27.16
Fe_2O_3	4.15	0.73
Al_2O_3	0.63	0.27
CrO	0.52	0.99
MgO	6.57	13.66
MnO	0.61	0.80
Na_2O	0.02	0.05
K_2O	0.20	0.06
TiO_2	0.25	0.03
H_2O	2.40	1.27

APPENDIX I, FIGURE 1 Typical percentage composition of UICC samples of asbestos.

	Chrysotile		Crocidolite	Amosite
	A	B		
$S_1 0_2$	39.8	39.7	51.16	50.8
FeO	0.4	0.8	21.2	33.6
$Fe_2 0_3$	1.5	1.4	18.3	1.9
CaO	0.15	0.09	0.17	0.24
MgO	26.1	26.6	3.6	5.0
$Na_2 0$	0.04	0.02	5.8	0.1

Modified from Rendall 1970
Timbrell 1970

APPENDIX I, FIGURE 2 Comparison of percentage composition of major components of amosite and cummingtonite, showing their similarities.

Constituents	Amosite	Cummingtonite
SiO_2	49.47	54.67
FeO	35.63	27.16
$Fe_2 0_3$	4.15	0.73
$Al_2 0_3$	0.63	0.27
CrO	0.52	0.99
MgO	6.57	13.66
MnO	0.61	0.80
$Na_2 0$	0.02	0.05
$K_2 0$	0.20	0.06
TiO_2	0.25	0.03
$H_2 0$	2.40	1.27

APPENDIX I, FIGURE 3 Trace Metal Content of Samples of Asbestos.

Type	Source			Micrograms/gm			
				Ni	Cr	Co	Mn
Chrysotile	UICC Africa		bulk	1432	1378	54	393
	"A"		under 10μ	1290	1050	33	245
	UICC Canada		bulk	802	317	45	444
	"B"		under 10μ	665	288	23	227
	Africa		over 10μ	1588	1204	58	305
			under 10μ	2048	1649	76	684
	Canada		over 10μ	1356	591	46	452
			under 10μ	2006	1317	57	898
	Russia		over 10μ	1353	793	78	530
			under 10μ	1720	1400	73	718
Crocidolite	Africa	Cape	over 10μ	25	26	9	723
			under 10μ	170	239	7	1914
		Transvaal	over 10μ	15	15	13	410
			under 10μ	120	162	15	721
	UICC		bulk	8	20	10	833
			under 10μ	30	34	11	800
Amosite	Africa		over 10μ	55	27	22	7539
			under 10μ	193	273	6	5071
	UICC		bulk	33	31	11	13347
			under 10μ	79	65	8	12650
Anthophyllite	Finland		over 10μ	547	1075	72	538
			under 10μ	827	55	31	1448
	UICC		bulk	414	584	24	986
			under 10μ	397	407	18	752

(From Cralley and Lainhart 1973)

APPENDIX I, FIGURE 4 Percentage composition of some commercially available sources of asbestos (major constituents only).

Constituent	Chrysotile					Crocidolite						Amosite		Anthophyllite	Tremolite
	Thetford	Cassiar	Asbest	Shabani	Havelock	Koegas	Kuruman	Pomfret	Transvaal	Bolivia	Australia	Penge	Weltevreden	Finland	Pakistan
SiO_2	38.75	40.75	39.00	39.70	39.93	50.90	50.70	52.00	59.41	55.65	52.85	49.70	51.30	57.20	55.10
FeO	2.03	0.28	1.53	0.70	0.45	20.50	17.50	17.65	15.11	3.84	14.94	39.70	35.50	10.12	2.00
Fe_2O_3	1.59	0.44	0.54	0.27	0.10	16.85	18.30	16.05	14.03	13.01	18.55	0.03	0.90	0.13	0.32
CaO	0.89	0.35	2.03	1.08	1.02	1.45	1.30	1.20	0.49	1.45	1.07	1.04	0.95	1.02	11.45
MgO	39.78	41.28	38.22	40.30	40.25	1.06	3.05	4.28	3.53	13.09	4.64	6.44	6.90	29.21	25.65
MnO	0.08	0.03	0.11	0.26	0.05	0.05	0.06	tr.	tr.	tr.	tr.	0.22	1.76	-	0.10
Na_2O	0.10	0.07	0.07	0.04	0.09	6.20	5.30	6.21	4.63	6.91	5.97	0.09	0.05	-	0.14
K_2O	0.18	0.04	0.07	0.05	0.09	0.20	tr.	0.06	0.28	0.39	0.05	0.03	0.51	-	0.29
H_2O	12.82	13.64	12.14	12.81	13.28	2.59	2.82	2.69	2.21	1.78	2.99	1.91	2.36	2.46	3.68

(From Hodgson 1965)

APPENDIX I, FIGURE 5 Content of benzopyrene in chrysotile asbestos from two different sources at various stages of preparation and use.

	Bazhenovsk mine chrysotile (up to 10 cm long)	Dzhetygarinsk mine chrysotile (up to 1 mm long)
Crude ore	9.96	-
Pre-dried ore	3.10	2.54
Post-dried ore	3.80	2.39
Ore 5th stage concentration	5.70	-
Ore 6th stage concentration	5.40	4.79
Factory asbestos	36.97	16.00
Asbestos cloth	18.87	-
Dust from electro-filters	11.71	3.92

(Taken from Krivosheeva and Pylev 1974)

APPENDIX I, FIGURE 6 Organic contaminants and benzopyrene in geological sources of asbestos.

	No. of sources	Oil-Wax range mgm/100 gm fibre	Benzopyrene range mgm/100 gm fibre
Crocidolite	12	Trace - 200 mg	0.2 - 2.4
Amosite	15	Trace - 20	0.2 - 2.4
Chrysotile	19	Trace - 7.6	None detected

APPENDIX I, FIGURE 7 Comparison of tensile strengths of various materials.

Material	Tensile strength lbs/in^2 x 10^6
Ingot iron	45,000
Wrought iron	48,000
Carbon steel	155,000
Ni-Cr steel	243,000
Piano steel wire	300,000
Cotton fibre	73,000 - 89,000
Rock wool	60,000
Glass fibre	100,000 - 200,000
Chrysotile	450,000
Crocidolite	500,000
Amosite	350,000
Anthophyllite	240,000

APPENDIX I, FIGURE 8 Comparison of fibre diameters.

Material	Fibre diameter in microns
Human hair	40
Wool	20 - 28
Cotton	10
Rayon	7.5
Nylon	7.5
Glass	6.5
Rock wool	3.5 - 7.0
Asbestos	0.02

(Taken from Lindell 1973)

APPENDIX I, FIGURE 9 Solubility of asbestos*.

Per cent loss in weight a) after refluxing for 2 hrs in 25% acid or caustic					
	HCl	CH_3CO-OH	H_3PO_4	H_2SO_4	NaOH
Chrysotile	55.69	23.42	55.18	55.75	0.99
Crocidolite	4.38	0.91	4.37	3.69	1.35
Amosite	12.84	2.63	11.67	11.35	6.97
Anthophyllite	2.66	0.60	3.16	2.73	1.22
	b) after 528 hrs in 25% acid or caustic at room temperature (26°C)				
	HCl	CH_3CO-OH	H_3PO_4	H_2SO_4	NaOH
Chrysotile	56.00	24.02	56.45	56.00	1.03
Crocidolite	3.14	1.02	3.91	3.48	1.20
Amosite	12.00	3.08	11.83	11.71	6.82
Anthophyllite	2.13	1.04	3.29	2.90	1.77

*deals with commercial asbestos, not UICC samples.

(Taken from Lindell 1973)

APPENDIX I, FIGURE 10 Effect of heat on tensile strength of chrysotile.

Temperature	Tensile strength lbs/in²	Per cent of original tensile strength
Ambient	400,000	-
600°F	120,000	91.6
800°F	96,000	73.3
1000°F	78,000	59.5
1200°F	42,000	32.0

APPENDIX I, FIGURE 11 Summary of Thermal Decomposition Reactions of three main types of asbestos.

	Under oxidizing conditions °C			Under neutral conditions °C		
	Dehydrogenation	Dehydroxylation	Breakdown	Dehydroxylation	Dehydrogenation	Breakdown
Chrysotile	100 - 700	600 - 780	800 - 850	150 - 700	575 - 800	800 - 1000
Crocidolite	400 - 600	-	900	550 - 700	-	800
Amosite	600 - 800	800 - 900	600 - 900	600 - 800	600 - 800	600 - 900

(From Hodgson 1965)
(Berry 1971)

APPENDIX I, FIGURE 12 Effect of temperature on loss of weight of asbestos fibres.

Temperature for 2 hrs		Percentage loss in weight			
°F	°C	Chrysotile	Crocidolite	Amosite	Anthophyllite
400	204	0.30	0.08	0.23	0.05
800	426	2.17	0.73	0.98	0.38
1000	537	3.99	0.86	1.16	0.44
1200	649	12.75	1.04	1.39	0.54
1400	760	13.43	1.03	1.43	0.54
1800	982	13.77	0.77	1.53	2.30

(After Rosato 1959)

APPENDIX I, FIGURE 13 Properties of main types of asbestos.

	Chrysotile	Crocidolite	Amosite
Minim fibre diameter (μ)	0.02	0.08	0.1
Specific gravity	2.55	3.37	3.45
Modulus of electricity	1.65×10^6	1.9×10^6	1.65×10^6
Tensile strength	31,000	35,000	25,000
Flexibility	Good	Good	Fair
Hardness (mohs)	2.5 - 4.0	4.0	5.5 - 6.0
Colour	White	Blue	Fawn/Grey
Solubility-25% HCl 2 hrs reflux wt loss %	55.69	4.38	12.84
25% NaOH " " " " "	0.99	1.35	6.97
Surface change	Positive	Negative	Negative
Heat resistance	500°C	200°C	200°C

(Taken from Spiel and Lieneweber 1965)
Becklake 1976)
Gaze 1965)
Hendry 1965)

APPENDIX II EPIDEMIOLOGY OF ASSOCIATION WITH ASBESTOS EXPOSURE

APPENDIX II Epidemiology of association with asbestos exposure.

APPENDIX II, FIGURE 1 Evidence of asbestos fibre fibrogenicity in man.

Country	Fibre type	Reference
Australia	Crocidolite	McNulty 1970
Bulgaria	Anthoph. trem. chrys.	Zolov et al 1968
Canada	Chrysotile	McDonald 1973
	Unspecified	Cartier 1968
Cyprus	Chrysotile	McDonald 1973
E. Germany	Unspecified	Roitzsh 1968
Finland	Anthoph. trem.	Kiviluoto 1965
	Anthophyllite	Meurman et al 1973
France	Chrysotile	Avril and Champeix 1970
Italy	Chrysotile	Vigliani 1970
Rhodesia	Chrysotile	Gelfand and Morton 1970
S. Africa	Croc. Amos. Chrys.	Sluis-Cremer 1965
	" " "	Webster 1970
	Croc. and Amosite	Sluis-Cremer 1970
	" " "	Solomon et al 1971
	Mixed dusts	Goldstein and Webster 1971
U.S.A.	Insulation materials	Selikoff et al 1970
	" "	Cooper and Miedema 1973
	Chrys. and Croc.	Enterline and Weill 1973
	Unspecified	Enterline 1968
	"	Kleinfeld 1968
		Mancuso and El Altov 1968
U.K.	Chrys. Croc. Amos.	Smither 1965
	Chrys. Amos., little Croc.	Smither and Lewinsohn 1972
	Unspecified	McVittie 1965

APPENDIX II, FIGURE 2 Prevalence of ferruginous bodies in routine autopsy material.

Location	No. of individuals sampled	Prevalence (% age)	Examination Method	Year*	Reference
London, U.K.	127	0.0	Sections	1936	Um 1971
London, U.K.	100	3.0	"	1946	"
London, U.K.	100	14.0	"	1956	"
London, U.K.	394	36.8	"	1975	Doniach et al 1975
Newcastle, U.K.	311	20.3	Scrapings	1968	Ashcroft 1968
Glasgow, U.K.	100	23.0	Smears	1967	Roberts 1967
Belfast, U.K.	200	40.5	"	1965	Elmes et al 1965
Cape Town, S. Africa	500	26.4	"	1963	Thomson et al 1963
Johannesburg, S. Africa		47.0	"	1965	Anon. 1963
Perugia, Italy	109	1.0	"	1969	Peacock et al 1969
Milan, Italy	100	51.0	"	-	Ghezzi 1967
Schwerin, E. Germany	234	9.0	"	1971	In: Hagerstrand 1973
Dresden, E. Germany	250	43.2	"	1967	In: Hagerstrand 1973
Sarajero, Yugoslavia	100	38.0	"	1971	In: Hagerstrand 1973
Malmo, Sweden	97	48.4	"	1967	In: Hagerstrand 1973
New York, U.S.A.	100	53.0	Ashed sections	1934	"
New York, U.S.A.	100	60.0	" "	1967	"
New York, U.S.A.	1,975	47.7	Smears	1966	"
Ann Arbor, U.S.A.	100	18.0	-	1969	Dicke and Naylor 1969
Miami, U.S.A.	500	27.2	Smears	1966	Thomson and Graves 1966
Pittsburg, U.S.A. . .	100	41.0	"	1965	Cauna et al 1965
Montreal, Canada	100	48.0	Scrapings	1966	Anjilvel and Thurlbeck 1966
Melbourne, Australia	200	43.5	-	1969	Kipell and Bhathal 1969

*Year of publication usually within one or two years of survey.

(Modified from Becklake 1976)

APPENDIX II, FIGURE 3 Correlation between asbestos exposure and degree of respiratory disability.

Exposure (MPPCF x Yrs)*	Disability	Reference
Environmental	Cough: 8% incidence Dyspnoea: 7% incidence	Schall 1965
10 - 100	Bronchitis in non-smokers 19% incidence	
25	Chronic cough: 14% incidence Dyspnoea: 12% incidence	Schall 1965
40	Detection of pulmonary fibrosis	Schall 1965
50 - 60	Detection of asbestosis	Wells 1965
100	Incidence of terminal fibrosis	Kleinman and Cooper 1967 Wells 1965
200	Detection of mesothelioma	Thomson et al 1963 Sluis-Cremer 1970
200	Bronchitis, wheezing: 46% incidence	Schall 1965
400	Chronic cough: basal rales: finger clubbing: mesothelioma.	Kleinman and Cooper 1967
800	Highest risks of all forms of respiratory pathology	Schall 1965

*Exposure measured or estimated as "Millions of particles per cubic foot" x years of exposure.

APPENDIX II, FIGURE 4 Comparative cancer mortality rates of asbestos workers.

Cause of death	Observed	Expected	Ratio
Cancer, all sites	1008	309.3	3.26
Pulmonary carcinoma	475	89.4	5.31
Pleural mesothelioma	52	-	-
Peritoneal mesothelioma	101	-	-
G.I. cancer	140	67.9	2.06
Other cancer	240	152.0	1.58

(Taken from the papers of Selikoff et al)

APPENDIX II. FIGURE 5 Association between asbestos exposure and mesothelioma.

Location	Period of Study	Mesothelioma Subjects		Control Subjects		Exposure	Reference
Case-control Studies		No.	% exposed	No.	% exposed		
U.K.: London	1917-1967	76	53	76	12	Factory crocidolite	Newhouse and Thomson 1965
Newcastle	1948-1969	41	95	56	41	Shipyard general	Ashcroft 1973
Belfast	1950-1964	42	76	42	21	Shipyard and insulation	Elmes et al 1965
Scotland	1950-1967	80	67	80	32	Shipyard general	McEwen et al 1970
Italy: Piedmont	1960-1970	50	18	50	2	Manufacturing	Rubino et al 1972
Sweden: Malmo	1957-1966	34	53	34	12	Shipyard general	Hagerstrand et al 1968
Germany: Hamburg	1958-1968	150	71	104	24	Shipyard general	Wain et al 1974
Holland: Walcheren		67	72	67	18	Shipyard general	Zielhaus et al 1975
Canada	1960-1972	190	26	182	7	Mainly production	McDonald 1973
U.S.A.	1972	99	48	86	20	Production and manufacturing	McDonald 1973
Uncontrolled Studies							
U.K.: Liverpool	1955-1970	52	80			Shipyard general	Whitwell and Rawcliffe 1971
France: Rouen		14	86			Textile manufacture	Pondimare et al 1974
Germany: Hamburg	1958-1968	98	58			Shipyard general	Bohlig et al 1970
Holland: Walcheren	1962-1968	25	88			Shipyard general	Stumphuis 1971
U.S.A.: Harisburg	1958-1963	42	74			Textile manufacture	Lieben and Pistawka 1967
Chicago	1969	77	86			Manufacture general	Godwin and Jagatic 1970
Somerville	1948-1970	72	83			Mainly chrysotile	Borow et al 1973
Australia: Victoria	1962-1968	15	87			Crocidolite or mixed	Milne 1969
S. Africa: Cape area	1956-1960	33	97			Crocidolite mining	Wagner et al 1960
Cape area	1956-1971	252	82			Crocidolite mining	Webster 1972

(Modified from Becklake 1976)

APPENDIX II, FIGURE 6 Relative frequencies of pleural and peritoneal mesotheliomas.

Populations examined	Site of Tumour			References
	Pleural	Peritoneal	Unknown	
Asbestos exposed: both occupational and neighbourhood	20	Some	15	Anspach 1962
Asbestos:				
Factory workers	8	15	0	Newhouse and Thompson 1965
Laggers	6	2	0	
Relatives	7	2	0	
Neighbourhood	11	0	0	
No known exposure	18	7	0	
Asbestos: occupational	34	8	0	Lieben and Pistawka 1967
Asbestos: occupational and locality	44	10	0	Roitzch 1968
General population: Scotland 1950–1967	75	3	2	McEwen et al 1970
Asbestos: shipyards	54	1	0	Harries 1971
shipyards	35	6	0	Ashcroft 1973
Pneumoconiosis panel	246	30	0	Greenberg and Davies 1974
Asbestos: occupational	51	21	0	Borow 1973
Coastal cities	234	16	0	Knappmann 1972

APPENDIX III CYTOKINETICS - LITERATURE REVIEW AND BACKGROUND INFORMATION

APPENDIX III - CYTOKINETICS

This account will commence with an introduction justifying the inclusion of cytokinetic techniques in a study of the effects of ingested and transported asbestos, then progress to an introduction of the basic concepts of cytokinetics with some mathematical treatment. There then follows a very brief historical review with a summary of some of the experimental values obtained in the rat and their significance.

III A Introduction.

B Summary of pertinent cytokinetics theory.

C Historical review.

D Review of the experimental data pertaining to the rat intestine.

IIIA Introduction

It soon emerged in the course of this work that there was no obvious large-scale transmigration of fibres across the gut wall following the ingestion of asbestos. However, it was remotely possible that the increase in gastro-intestinal cancer noticed epidemiologically could be due to an effect upon the proliferative homeostasis of the mucosal tissues of the alimentary canal. Such an effect could be caused either by altering the turnover rate of epithelial cells to compensate for a direct toxic effect of intraluminal asbestos, or to some as yet unexplained regulatory influence of minute traces of fibre within the proliferative zones. A preliminary and rather crude attempt to examine this problem was published by Amacher and his colleagues in 1974, in which they reported an increase in DNA synthesis within various gut tissues of rats after gavage with massive amounts of chrysotile asbestos. Using the same radio-isotope labelling procedures, and several different doses of chrysotile, they later reported a dose response effect evident in several tissues three days after the gavage (Amacher et al., 1975). Apart from the use of an unrealistic experimental protocol, these reports are open to the criticisms that an assay measuring incorporation in whole organs does not permit any examination of the response of specific tissues to the asbestos, nor is it sufficiently sensitive to detect small changes in the proliferative status of tissues.

In view of the attention focussed upon the ingestion problem throughout the world, it was decided to

examine the possibility of an influence of asbestos ingestion upon gastrointestinal mucosal kinetics in some detail. Following Amacher's work (Amacher et al., 1974, 1975), it was felt that a more realistic approach to the problem would be to assess the effects of prolonged ingestion of lower, but still considerable, amounts of asbestos. The combination of methods chosen for this assay were selected to produce the maximum amount of information from a small group of animals using the conventional cytokinetics analysis approach, and simultaneously to compare these results with those produced by the whole organ DNA extraction techniques employed by Amacher and his colleagues.

It is not sufficient merely to describe an increase in the rate of incorporation of labelled thymidine, since this provides little useful information about the tissue response. There are several parameters of proliferative populations that can be simply described providing sufficient care is taken in their assessment, and these parameters can be used to provide an analysis of the functional state of the tissues. It would be useful at this point to introduce some of the concepts from the rapidly developing discipline of cytokinetics, although the account will be brief in the interests of clarity. A more comprehensive review is available in the thesis by Wright (1973) and in Aherne et al. (1977).

IIIB Cytokinetics theory

Cytokinetics is essentially a study of dynamic histology, and has historically depended upon the development of autoradiography as a technique in 1946 (Belanger and

Leblond, 1946). The full potential of autoradiography in this field had to await the discovery of a highly specific label for DNA in 1957 (Taylor et al., 1957), and since this date there has been an enormous number of publications involving cytokinetic techniques. Any quantitative description of a dynamic system must of necessity involve a mathematical definition of those kinetic parameters that can be measured experimentally. In practice, a full kinetic description of an in vivo cell population is extremely difficult to produce, but it is possible to arrive at a useful workable approximation. The calculations are simplified by the use of a series of assumptions about the behaviour of cells in populations.

In 1953 it was discovered by following the uptake of radioactively labelled precursors that DNA was only synthesised during a limited part of the cell cycle, and this led to the concept that the proliferative cell goes through a number of distinct phases between one division and the next. Howard and Pelc (1953) accordingly divided the cells in a population into four phases: Mitosis phase, M, where chromosome segregation occurs to form two daughter cells; Growth phase, G_1, where RNA activity and protein synthesis are at their peak; Synthesis phase, S, where DNA is synthesised and any incorporation of label occurs; and the Second Growth phase, G_2, usually a relatively short but vital period in which the spindle proteins are coordinated in preparation for mitosis. Later, a fifth phase, G_0, was added to describe those cells that have decycled and are deemed to be in a period of dormancy.

The situation becomes more complicated in vivo since most populations are a mixture of proliferating and non-

proliferating cells, and the terms growth fraction or proliferative index is used for the ratio of proliferating to total number of cells. Clearly, when all cells are actively proliferating, the growth fraction is unity and the time taken for the number of cells to double will equal the time taken for a cell to proceed through the four main phases of the cell cycle described above. Similarly, where a proportion of the cells in a population are out-with the proliferative compartment, the growth fraction is less than one, and the apparent cell cycle time will be longer than the true cell cycle time.

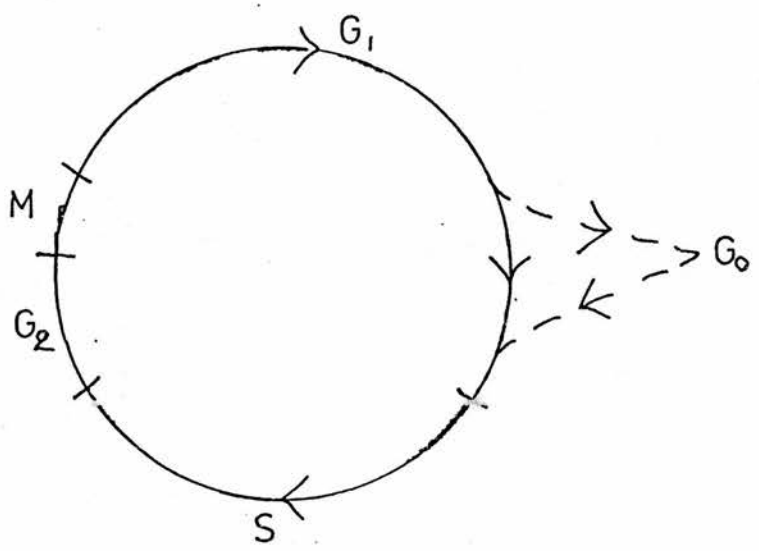
There are other definitions to be considered. The gastrointestinal mucosal tissues are widely accepted to fulfil a series of conditions that can best be described as conforming to a steady state, in which cell production equals cell loss. An extremely useful characteristic of steady state populations is that the number of cells in any given phase is proportional to the duration of that phase as a fraction of the total cell cycle time, providing the cells are dividing asynchronously (a common, and fairly safe, assumption for in vivo cell populations). This fact has been used as an entrée to measuring cell cycle times, as will be seen later.

On a practical level, it is assumed that those cells found by autoradiography to have incorporated the DNA-specific label tritiated thymidine after an appropriate time were in the S phase during that period. Hence the labelling index is taken to be the same as the synthetic index, or the proportion of cells in the S phase. Similarly, the fraction of cells found in

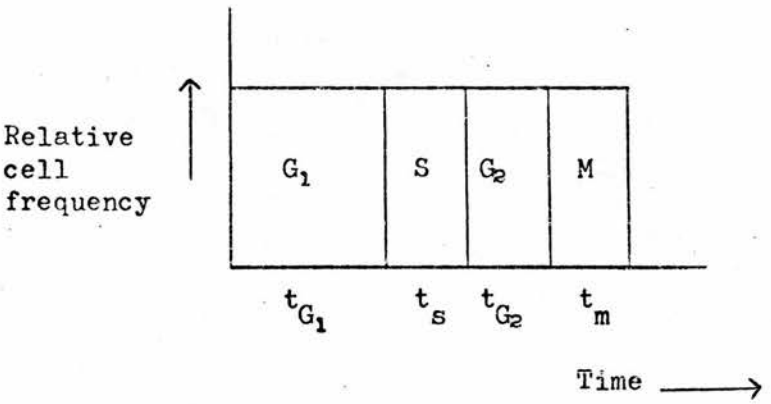
mitosis is taken as the mitotic index. The rate of entry of cells into mitosis can be deduced by administering a stathmokinetic drug such as vincristine that disrupts the spindle proteins and thus arrests all cells in the metaphase stage of mitosis. An assay of the increasing proportion of metaphase cells with time after drug administration thus yields an estimate of the birth rate of cells within a steady state population. The growth rate of a population is an essential parameter in cell dynamics: an increase in the number of cells produced by a given population may be brought about either by a shortening of the cell cycle time, by an increase in the number of cells actually proliferating, or by a combination of both. In the gastrointestinal mucosa the growth fraction is deduced from spatial maps of the positions of the proliferative cells. A proliferative zone is thus delineated and cells within this zone are considered to be proliferating, so making the growth fraction a simple proportion of number of zone occupants to total number of cells in the population.

So, it can be seen that labelling index, mitotic index, birth rate and growth fraction can all be measured experimentally. A brief mathematical description follows:-

The cell cycle:



For steady state conditions:



Thus $t_c = t_{G_1} + t_s + t_{G_2} + t_m$

and,

No. of cells in any phase = $\frac{\text{duration of that phase}}{\text{duration of total cell cycle}}$

For the synthetic (S) phase:

synthesis index $I_{(S)} = \frac{t_s}{t_c}$ [In labelling experiments, labelling index, $I_L = I_S$]

and, for the mitotic (M) phase:

mitotic index $I_{(m)} = \frac{t_m}{t_c}$

Growth Fraction or proliferative index $=$ $\frac{\text{number of proliferating cells}}{\text{total number of cells}}$

or $I_p = \frac{N_c}{N}$

The birth rate (K_B) depends upon the cell cycle time and the number of cells involved in the cell cycle:

$$\text{or } K_B = \frac{I_p}{t_c}$$

Since, for steady state conditions, the birth rate is the same as the rate of entry of cells into the mitosis phase (r_m), we have:

$$K_B = r_m = \frac{I_p}{t_c}$$

for use in stathmokinetic experiments, this can be rewritten as:

$$K_B = r_m = \frac{I_{met}}{t_A}$$

where I_{met} is the incidence of metaphase and t_A the period of drug-induced metaphase arrest.

The duration of mitosis (t_m) can be found from:

$$t_m = \frac{I_m}{r_m}$$

and the apparent cell cycle time ($t_{c(a)}$) from the reciprocal of the rate of entry into mitosis (r_m):

$$t_{c(a)} = \frac{1}{r_m}$$

By rearranging the above equations we can arrive at:

(i) $t_c = \frac{t_m}{I_m} = \frac{t_s}{I_s}$

(ii) $I_p = \frac{Nc}{N}$

(iii) $K_B = \frac{I_p}{t_c} = r_m = \frac{I_{met}}{t_A}$

(iv) $I_L = I_s = \frac{t_s}{t_c}$

(v) $t_{c_{(a)}} = \frac{1}{r_m}$

where I_m, I_{met}, r_m, I_L, I_p, N are all measurable.

IIIC Historical review of cell proliferation in the small intestinal mucosa

Patzelt (1882) first noticed that cells were produced in the crypts of Lieberkuhn, but Bizzozero (1888, 1892, 1893) is usually credited with the observation. It was some years before continuous cell loss from the villi was noticed (Ramond, 1964) and it was not until 1948 that a full steady state renewal system was proposed by Leblond (Leblond and Stevens, 1948) with the loss of live cells. Friedman (1945) showed that epithelial cells migrated from crypt to villus when he followed the progress of irradiated and swollen goblet cells, and this was confirmed by Leblond and his colleagues in 1948 with one of the first autoradiographic studies. The pioneer work of Leblond and his colleagues using successive refinements of the autoradiographic procedures has since contributed substantially to small intestine cell kinetics research.

Pernice (1889) first described large numbers of metaphases in the intestinal mucosa of a dog treated with an extract of the autumn crocus (colchicum autumnale) although colchicum was not specifically used as a stathmokinetic drug until the 1930's. Experimentally, Stevens Hooper (1961) produced the first definitive stathmokinetic analysis using a critical and logical approach that removed many of the anomalies of previous workers in the field.

In a comprehensive paper Quastler and Sherman (1959) introduced many of the basic concepts of steady state proliferative systems that now form the basis of cytokinetics theory, and in particular their deduction of a "critical decision phase" (point at which a cell may decycle) influenced many future workers.

The "modern" phase of research owes much to the experimental work of Cairnie, Lamerton and Steel (1965) and the theoretical approach of Cleaver (1967). Cairnie et al. (1965) used morphometric methods to look at proliferation at different levels of the crypt and delineate the proliferation and maturation compartments. They were able to develop the concept of the critical decision phase of Quastler into that of the "slow cut-off model" in which the point at which cells leave the proliferative cycle should best be thought of as a region, and a region capable of a certain degree of flexibility. Thus a movement of the "cut-off" area will alter the growth fraction within the crypt and this represents a potentially powerful and simple mechanism by which the proliferative capabilities of the crypt can be altered. The paper of Cairnie et al. (1965) made use of an established technique that has come to be known

as the FLM technique, or "fraction of labelled mitoses", and it is probably true to say that the FLM technique remains one of the most powerful tools available to the experimental worker to date. In essence it consists of following a labelled cohort of cells for a period in excess of one cell cycle time and mapping variations in the proportion of labelled mitoses observed with time. From a practical point of view the refinements of the FLM technique must be weighed against the considerable disadvantage that it is expensive in terms of effort and animals, and it is for this latter reason that the FLM technique could not be used in the present study.

Cleaver (1967) used the work of Cairnie et al. (1965) as a model for his theoretical analysis and he showed that the growth fraction could be simply and accurately determined from labelling index distribution maps. Cairnie and Bentley (1967) further formalised the analysis of cytokinetic data by developing a computerised method of deriving a "standard" crypt, and a modification of this method was used in the present study. Basically it relies upon the now proven assumption that a long crypt ($\geq$ 36 cells) can be considered to be a stretched short crypt ($\leq$ 30 cells), so a specific tissue assay consisting of labelling or mitosis distribution maps for 100 crypt columns can be readily combined to produce an index distribution curve. The procedure is described briefly in Section 2.5.3, and a fuller treatment is available in the original paper of Cairnie and Bentley (1967) or the M.D. thesis of Wright (1973).

In 1967 Tannock pointed out the need for an important modification to current theory based upon the

geometrical relationships of the cells of a sectioned crypt. Since a mitotic nucleus tends to adopt a central rather than a basal intracellular position, they tend to be found nearer the crypt axis. The counting methods detailed in Section 2.5.3, and widely used throughout the literature, require selection of axially sectioned crypts, and there is thus a greater likelihood of a nucleus in mitosis being sectioned. This leads to an overestimate of the metaphase or mitotic index and the Tannock factor compensates for this by multiplying the apparent index by $\frac{a}{b}$, where "a" represents the distance of the mitotic nucleus from crypt axis, and "b" the crypt radius. The use of the factor $\frac{a}{b}$ ignores any variations in the overall sizes of mitotic as compared with interphasic nuclei.

IIID Review of the experimental data pertaining to the rat intestine

There now follows a brief general account of some of the values obtained experimentally for the various kinetic parameters of the rat intestine. The account will be restricted in the main to the small intestine since there is very little published information on the detailed kinetics of the rat large intestine. To a large extent, the lack of agreement between some estimates of the many parameters has historically depended upon the experimental techniques used and on the subsequent interpretation of the results, so it is intended to outline the broad trends only. The lack of agreement can best be illustrated using the example of estimates of the duration of mitosis (t_m) in the rat small intestine: Leblond and Stevens (1948) produced a value of 68 minutes for t_m

using long periods of metaphase blocking, and Stevens Hooper (1961) produced a value of 61 minutes using similar techniques. However, following the critical approach of Tannock (1967), Clarke modified the stathmokinetic procedure and produced an estimate for t_m of 23 minutes. This has been supported by Wright et al. (1972) who found that t_m for the whole rat crypt was 26 minutes, and that t_m varied with position in the crypt. It is now accepted that t_m is of the order of 30 minutes in the mammalian small intestinal crypt.

In general, cell cycle parameters have been found to be uniform in a wide variety of experimental animals such that the cell cycle time (t_c) is 10 - 14 hours, t_m is $\frac{1}{2}$ hour, t_s is 6 - 8 hours, t_{G_2} is about 1 hour, and t_{G_1} is highly variable (Cairnie et al., 1965). The mitotic index for the whole crypt has been found to be 4 - 5%, the pulse labelling index 30 - 35% (Cairnie et al., 1965), and estimates of the proliferative index (I_p) or growth fraction are of the order of 0.5 - 0.6 in normal adult rat small intestine. Apart from high values for the proliferative parameters in the lactating female, there would appear to be no significant sexual dimorphism. The migration rate of cells up the crypt column is approximately 1.3 - 1.4 cell positions per hour, and the total cell production rate per crypt 33 - 38 cells per hour, with no significant change with anatomical site (Altmann and Enesco, 1967; Clarke, 1970). The total epithelial turnover time, or time taken for all cells in a population to be replaced, is a parameter of rather limited value, but it was found to vary with anatomical position, getting larger distally. This phenomenon is largely a function of the fact that the relative density of crypts is

reduced from duodenum to ileum, so the crypts are effectively supplying more villi and the cells have a longer functional life, given that there is no real variation in the rates of production of cells. Clarke (1970) concluded that cell production rate has a linear relationship with villus surface area, or, in kinetic terms: the size of the functional compartment may govern the activity of the proliferative compartment. For this to hold true, the linear relationship must vary with anatomical site, a hypothesis supported by the work of Altmann and Leblond (1970) on the effect of certain intraluminal factors on functional homeostasis.

There is evidence available to show that both t_c and t_s increase with age (Lesher, Fry and Kohn, 1961), although Thrasher and Greulich (1965) found no alteration of t_s with age between 10 and 638 days. A general slowing of small intestinal proliferative activity seems to occur in ageing animals, due largely to reductions in the growth fraction (Thrasher and Greulich, 1965). Forrester (1972) and Clarke (1972) have shown that the overall number of villi in the rat small intestine (128,000) does not change with body weight within the range 60 - 600 gms, and Clarke (1972) has further shown that it is the crypt density that increases with age - possibly by a process of longitudinal division.

Despite the long-standing contradictory findings of no detectable diurnal variation (Pilgrim et al., 1963; Leblond and Stevens, 1948; Bertalanffy, 1960) or a detectable variation (Klein and Geizel, 1947; Bullough, 1948; Alov, 1963), it is now accepted that such a diurnal variation exists (largely as a result of the elegant studies of Sigdestad et al., 1969,

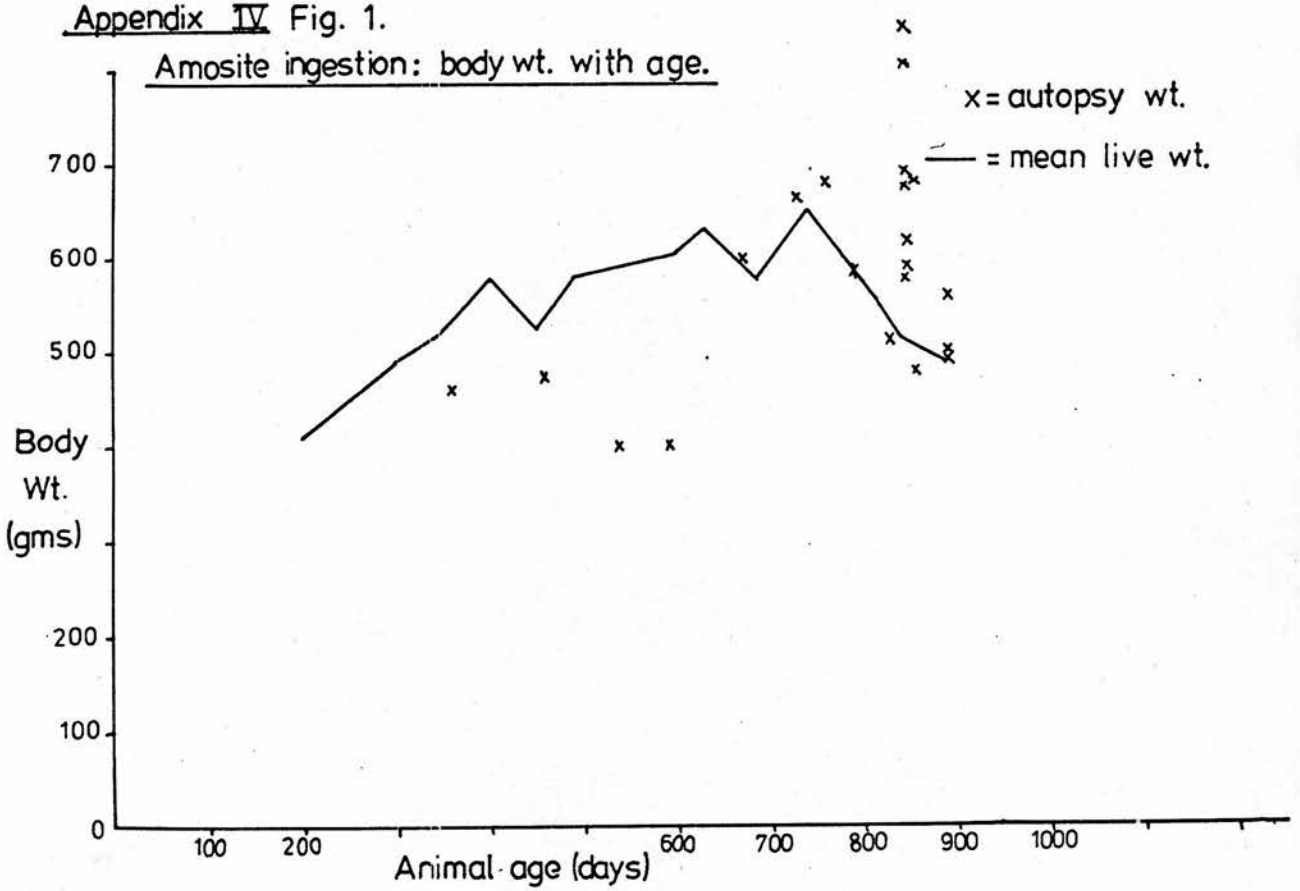
1970, and 1971). The peak of proliferative activity is found at 0300 h and the nadir at 1500 h, suggesting that attrition of lumenal contents may play a part in the variations (Alov , 1963). A comparison of germ-free and standard rats shows that the presence of normal lumenal gut flora contributes to a stimulation of proliferation (Abrams et al., 1963; Lesher, Walberg and Sacher, 1964).

Amongst the responses of the small bowel renewal system to experimental regimes, the effects of starvation and/or refeeding are pertinent to the work reported in Chapter 6 of this thesis: several workers have found that prolonged starvation in rats - up to 10 days - results in an overall reduction in the proliferative activity of the gut (Stevens Hooper and Blair, 1958; Hopper et al., 1968; Clarke, 1972) although Klein and Geizel (1947) have shown that at least some evidence of diurnal variation persists. Refeeding has been found to initiate a rapid and significant compensatory increase (Cameron and Cleftman, 1964; Altmann, 1972; Al-Dewachi, 1975).

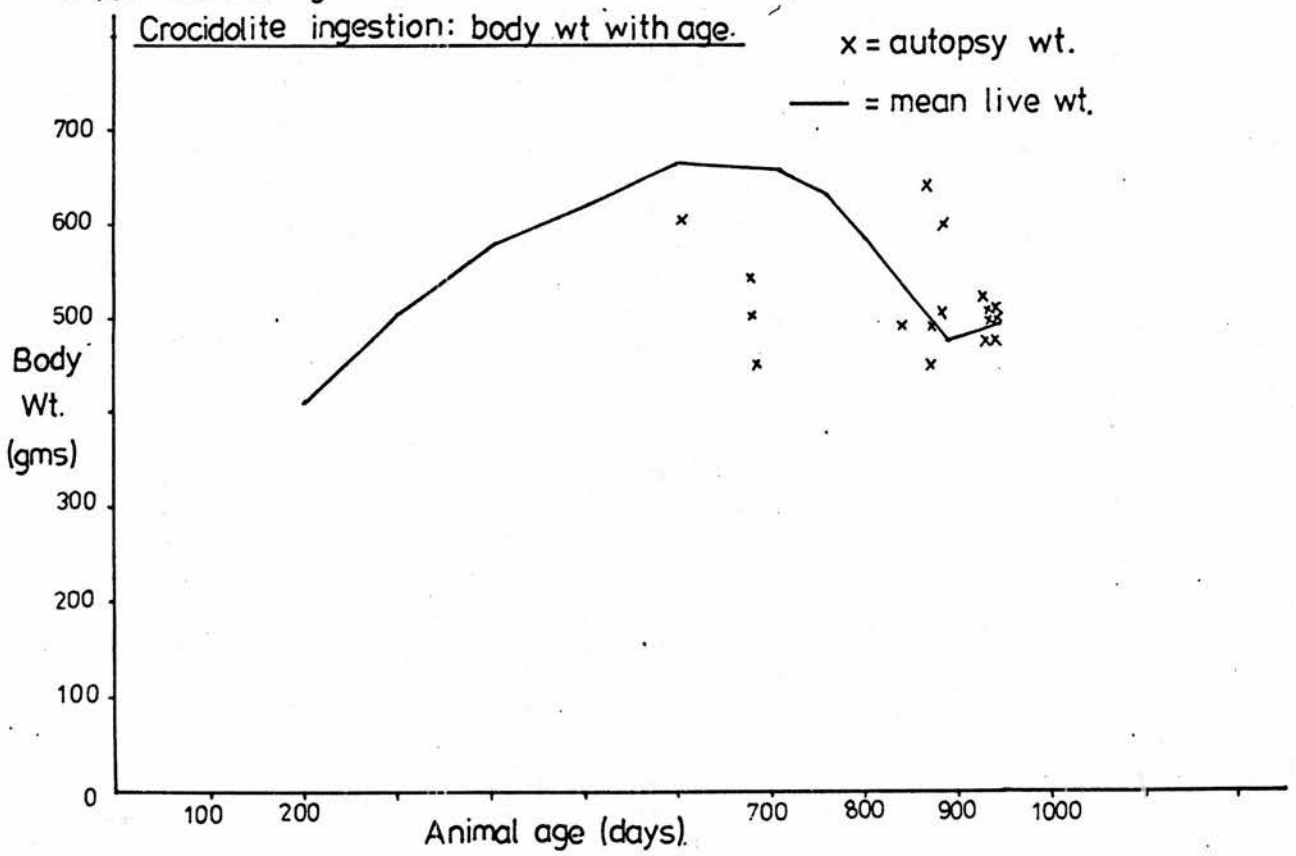
The evidence available suggests that proliferation within the large intestine is somewhat slower than the small intestine, and that there is some variation depending upon anatomical site (Knudtson et al., 1963).

APPENDIX IV BIOLOGY AND PATHOLOGY OF INDIVIDUAL EXPERIMENTAL ANIMALS

Appendix IV Fig. 1.

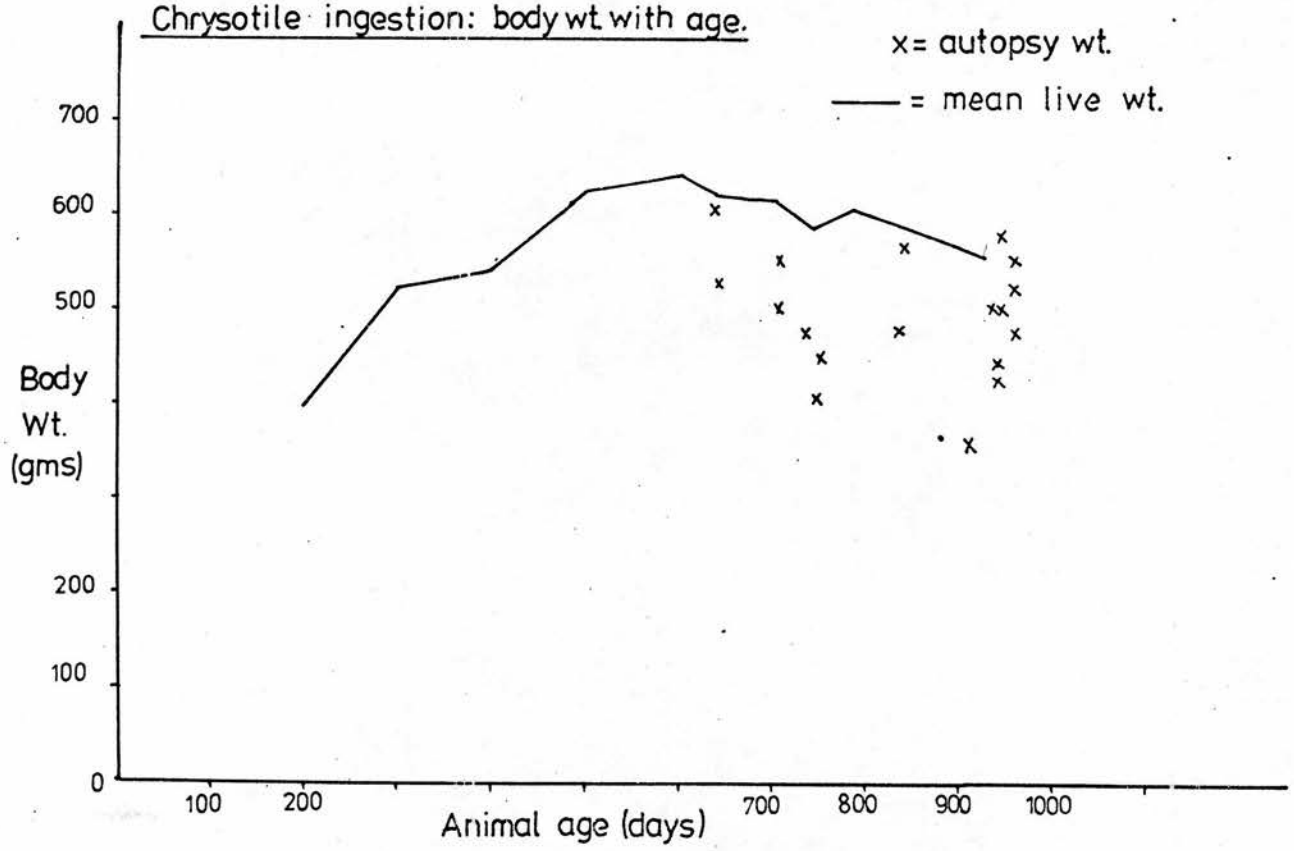


Appendix IV. Fig. 2.

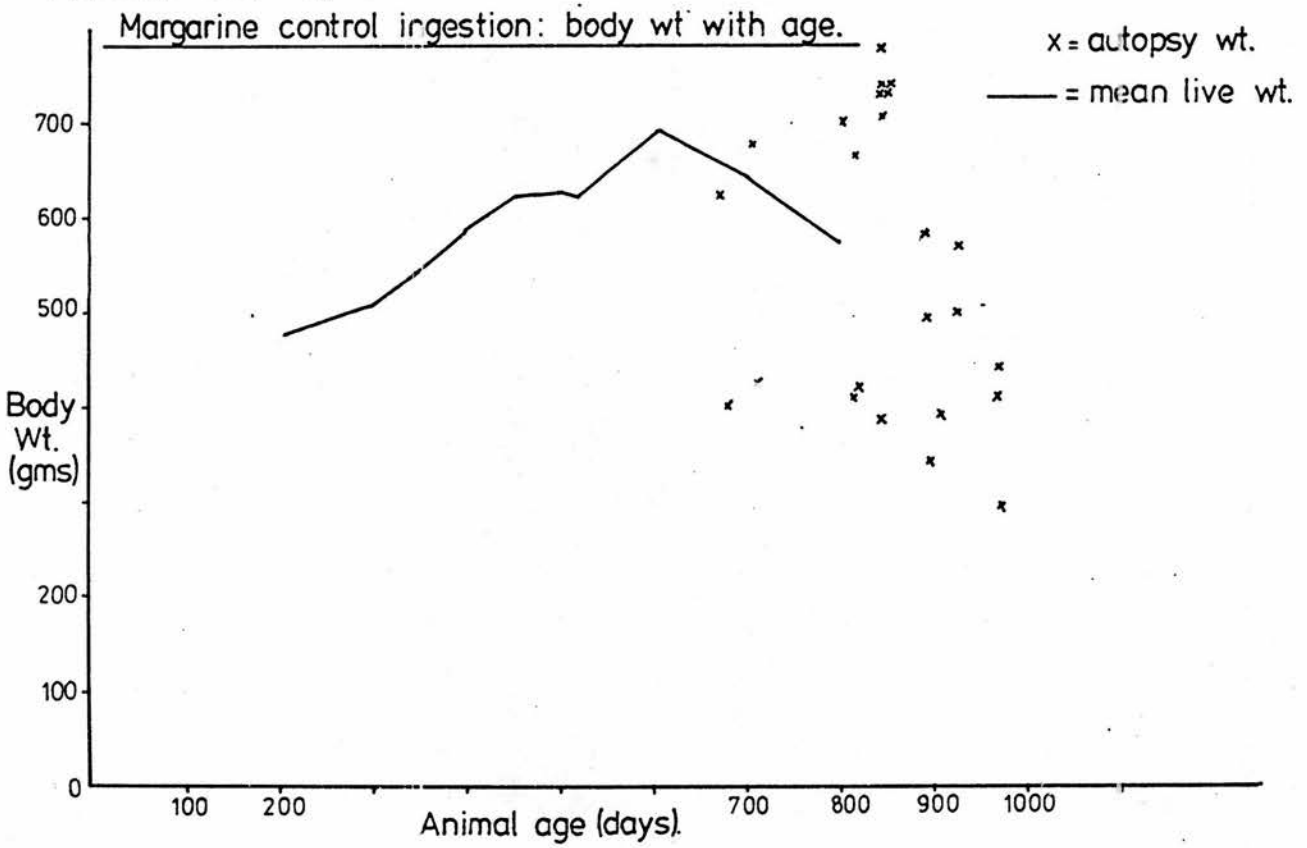


Appendix IV Fig. 3.

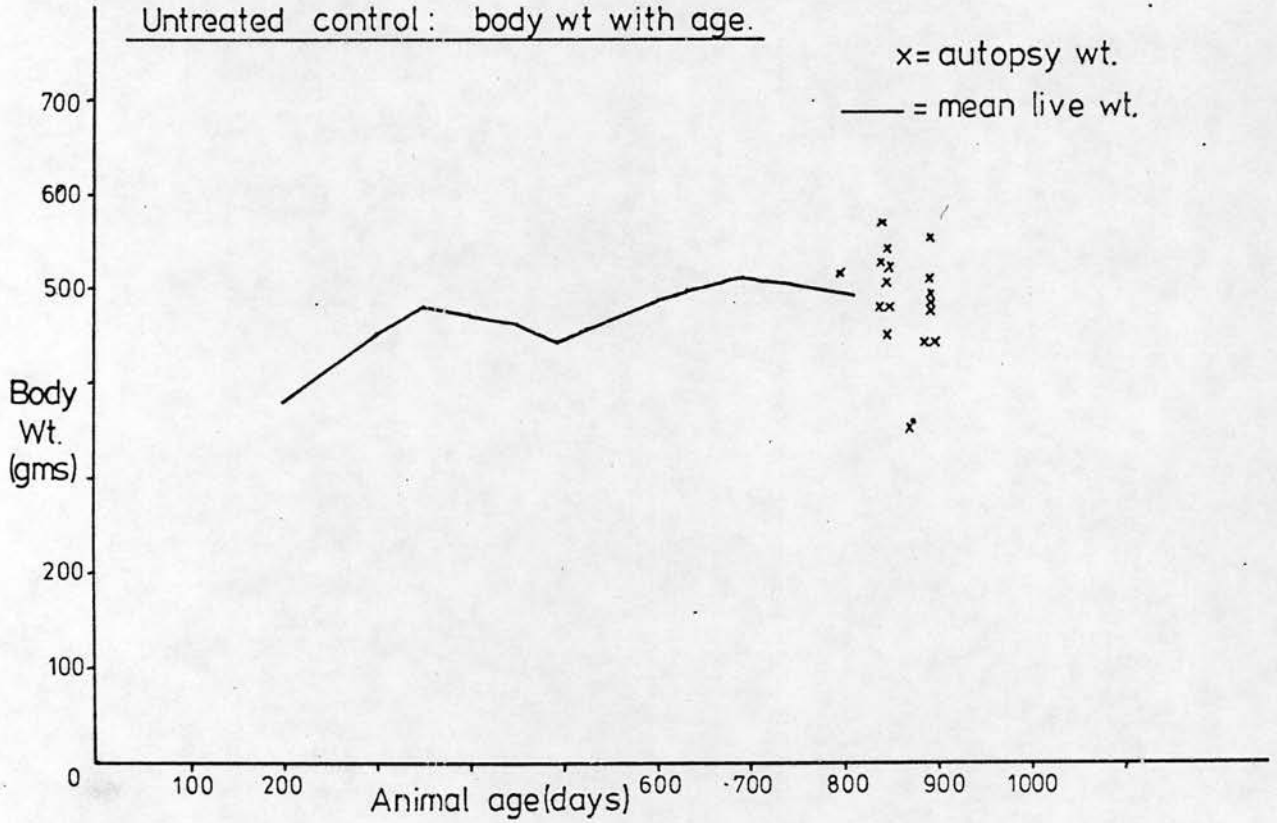
Chrysotile ingestion: body wt. with age.



Appendix IV Fig. 4



Appendix IV Fig. 5.



APPENDIX IV, FIGURE 6 Pathological findings in rats following long-term amosite asbestos ingestion.

(animals arranged chronologically, lesions in order of importance)

Age at Death (days)	Autopsy Wt. (gms)	Details of Pathology
365	464	Pulmonary venous congestion. Chylous pleural effusion.
463	475	Decomposed - no obvious abnormality.
482	-	Pulmonary venous congestion. Peritonitis.
539	400	Malignant leiomyosarcoma of greater curvature of glandular stomach. Pulmonary oedema.
588	400	Moderate renal hypertensive damage. Some hepatic fatty change (peri-portal).
673	600	Pulmonary congestion, oedema, and pleural effusion.
679	-	Decomposed - no obvious abnormality.
728	662	Subcutaneous fibroma. Some renal hypertensive damage. Some hepatic fatty change (peri-portal).
755	680	Extensive renal hypertensive damage and calcification. Moderate mesenteric polyarteritis nodosa.
787	590	No detectable histopathology.
833	508	Extensive renal hypertensive damage. Extensive mesenteric polyarteritis nodosa. Pulmonary venous congestion.
839	676	Moderate renal hypertensive damage. Some hepatic fatty change (peri-portal).
839	894	Extensive hepatic fatty change. Some renal hypertensive damage.
839	802	Moderate renal hypertensive damage. Some hepatic fatty change (peri-portal).
846	616	Extensive renal hypertensive damage. Some hepatic fatty change (peri-portal).
846	574	Some renal hypertensive damage. Some hepatic fatty change (peri-portal).
846	692	Some renal hypertensive damage. Some hepatic fatty change (peri-portal). Omental adipose infarct.
846	582	No detectable histopathology.
846	678	Some renal hypertensive damage. Some hepatic fatty change (peri-portal).
853	480	No detectable histopathology.
884	482	Extensive renal hypertensive damage. Hyperplastic seminal vesicle. Hypertrophic salivary gland.
884	566	Moderate renal hypertensive damage.
884	502	Some renal hypertensive damage.
884	495	Moderate renal hypertensive damage. Moderate mesenteric polyarteritis nodosa.

APPENDIX IV, FIGURE 7 Pathological findings in rats following long-term crocidolite asbestos ingestion (animals arranged chronologically, lesions in order of importance)

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
604	600	Pancreatic endocrine adenoma. Fatty infiltration of colonic musculature.
676	545	Extensive hepatic fatty change. Pancreatic exocrine adenoma.
676	500	Fatty infiltration of colonic musculature.
686	490	Moderate hepatic fatty change. Fatty infiltration of colonic musculature.
821	450	Extensive renal hypertensive damage.
830	384	Extensive renal hypertensive damage. ? ischaemic damage to gastric glandular mucosa.
837	-	Moderate renal hypertensive damage. Footpad granuloma.
841	490	Extensive renal hypertensive damage. Extensive mesenteric polyarteritis nodosa.
870	640	Moderate renal hypertensive damage and some calcification. Hepatic necrosis, infarction and congestion.
871	500	Footpad granuloma.
871	450	Extensive renal hypertensive damage. Moderate mesenteric polyarteritis nodosa.
877	600	Subcutaneous fibroma. Slight renal hypertensive damage.
880	505	Decomposed - no obvious abnormality.
924	-	Footpad granuloma.
927	525	Some renal hypertensive damage. Some hepatic fatty change. Footpad granuloma.
927	475	Some renal hypertensive damage.
933	500	Some renal hypertensive damage.
933	500	Some renal hypertensive damage. Pancreatic endocrine adenoma.
940	475	Malignant adrenal medullary carcinoma. Extensive renal hypertensive damage.
940	500	Extensive renal hypertensive damage. Pancreatic exocrine adenoma.
940	500	Moderate renal hypertensive damage. Some mesenteric polyarteritis nodosa.

APPENDIX IV, FIGURE 8 **Pathological findings in rats following long-term chrysotile asbestos ingestion (animals arranged chronologically, lesions in order of importance)**

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
637	605	Decomposed - no obvious abnormality.
645	525	Malignant pleural histiocytic tumour, with nodules on diaphragm, pleural septae, and parietal surfaces. Fatty infiltration of colonic musculature. Slight peri-portal hepatic fatty change. Slight renal hypertensive damage.
713	475	Extensive renal hypertensive damage. Pancreatic exocrine adenoma.
713	500	Pulmonary venous congestion. Pancreatic endocrine adenoma.
713	550	Malignant subcutaneous fibrosarcoma. Extensive hepatic fatty change. Eosinophilic gastric sub-mucosa, but no obvious parasites.
713	475	Small lipoma peritoneal body wall. Omental adipose infarct.
736	475	Extensive renal hypertensive damage. Haemangioma in duodenal mesentery. Pancreatic exocrine adenoma.
751	400	Decomposed - no obvious abnormality.
757	450	Small haemangioma in caecal mesentery.
833	475	Some renal hypertensive damage. Some generalised hypertensive change to arteries. Footpad granuloma. Some lymphocyte cuffing of bronchioles.
839	560	Moderate renal hypertensive damage.
863	-	Subcutaneous fibroma.
909	-	Malignant adrenal cortical cell tumour.
918	350	Malignant plasma cell tumour ventral neck region. Mesenteric polyarteritis.
937	440	Extensive renal hypertensive damage. Some generalised hypertensive change to arteries. Haemangioma in duodenal mesentery. Pancreatic exocrine adenoma.
939	425	Malignant adrenal cortical cell tumour with lymphatic and venous invasion. Pulmonary venous congestion. Moderate hepatic fatty change.
939	500	Moderate renal hypertensive damage. Some ? ischaemic damage to gastric mucosa.
948	500	Slight renal hypertensive damage. Footpad granuloma.
948	575	Slight renal hypertensive damage. Pancreatic endocrine adenoma.
960	550	Moderate renal hypertensive damage. Haemangiomas in caecal mesentery. Omental adipose infarct.
960	475	Moderate renal hypertensive damage. Generalised hypertensive blood vessel change. Pancreatic exocrine hyperplasia. Moderate hepatic fatty change.
961	525	Moderate renal hypertensive damage. Generalised hypertensive blood vessel change.

APPENDIX IV, FIGURE 9 Pathological firdings in rats following long-term margarine (control) ingestion (animals arranged chronologically, lesions in order of importance)

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
673	626	Pulmonary congestion. Some hepatic fatty change (peri-portal).
680	398	Extensive renal hypertensive damage. Some mesenteric polyarteritis nodosa.
708	676	Decomposed - no obvious abnormality.
713	425	Malignant peritoneal fibrosarcoma.
805	702	Pancreatic endocrine adenoma. Some hepatic fatty change (peri-portal). Some renal hypertensive damage. Footpad granuloma.
816	670	Decomposed - no obvious abnormality.
817	406	Moderate renal hypertensive damage. Some hepatic fatty change. Pancreatic exocrine hyperplasia.
824	420	No detectable histopathology.
847	384	Extensive renal hypertensive damage. Extensive hepatic fatty change with necrotic areas.
848	780	Some hepatic fatty change (peri-portal).
848	730	No detectable histopathology.
848	740	Malignant adrenal medullary carcinoma. Moderate renal hypertensive damage. Moderate hepatic fatty change (peri-portal).
848	708	No detectable histopathology.
848	743	Pancreatic exocrine adenoma. Some hepatic fatty damage (peri-portal).
848	728	Some hepatic fatty change (peri-portal).
890	578	Decomposed - no obvious abnormality.
893	340	Malignant. Adenocarcinoma of bladder, with renal inflammation.
893	490	No detectable histopathology.
911	390	Decomposed - no obvious abnormality.
924	500	Pulmonary congestion and oedema. Moderate renal hypertensive damage. Pancreatic exocrine hyperplasia.
926	568	Moderate renal hypertensive damage.
968	294	Malignant adrenal medullary carcinoma. Extensive renal hypertensive damage.
971	408	Moderate renal hypertensive damage.
971	436	Moderate renal hypertensive damage. Some hepatic fatty change (peri-portal).

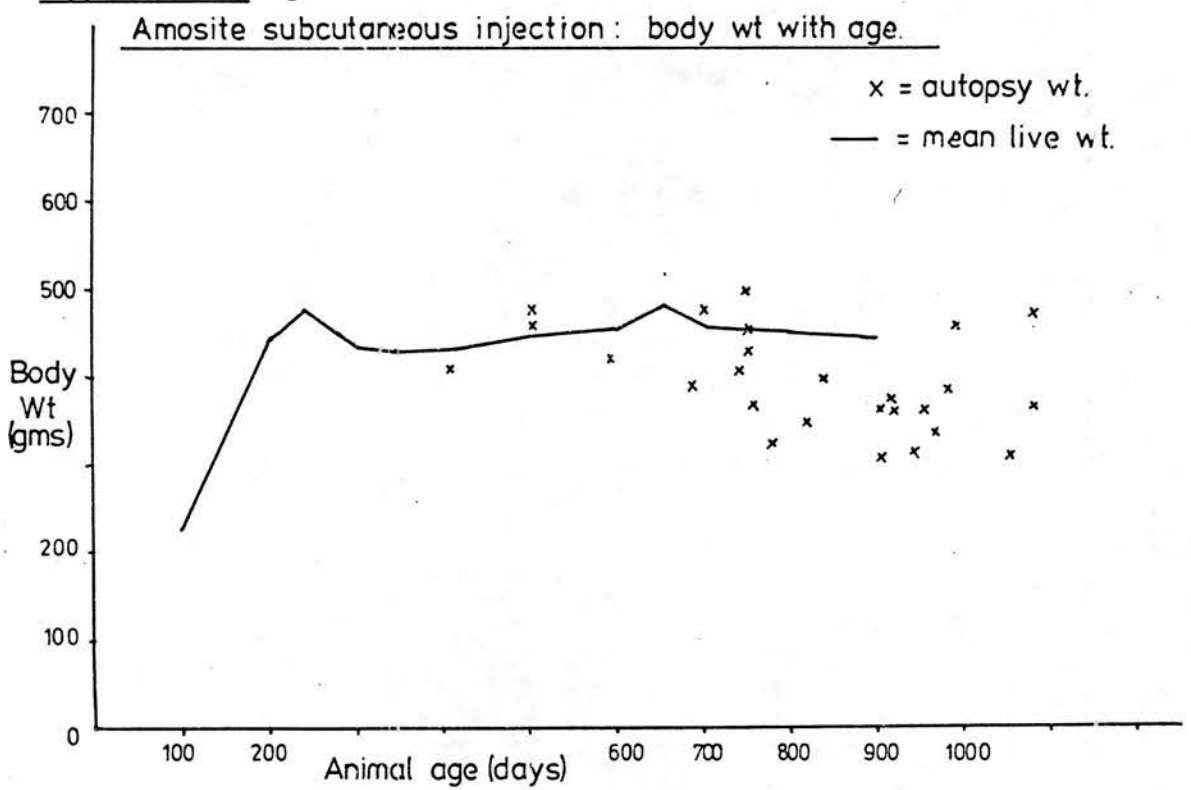
APPENDIX IV, FIGURE 10 Pathological findings in untreated control rats
(animals arranged chronologically, lesions in order of importance)

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
605	-	Malignant subcutaneous fibrosarcoma. Highly reactive spleen.
640	-	Pulmonary congestion.
724		Some renal hypertensive damage. Hepatic congestion. Pancreatic exocrine hyperplasia.
724	-	Some renal hypertensive damage. Some hepatic fatty change.
724	-	Extensive renal hypertensive damage.
724	-	Some renal hypertensive damage. Pancreatic exocrine hyperplasia.
797	518	Malignant. Lymphoma.
839	482	Some renal hypertensive damage.
839	528	Extensive renal hypertensive damage.
839	568	Some renal hypertensive damage.
846	528	Pancreatic exocrine adenoma. Some renal hypertensive damage.
846	450	Moderate renal hypertensive damage.
846	510	Extensive renal hypertensive damage. Extensive mesenteric polyarteritis nodosa.
846	480	Moderate renal hypertensive damage. Some hepatic fatty change (peri-portal).
846	540	No detectable histopathology.
873	354	Extensive renal hypertensive damage. Extensive mesenteric polyarteritis nodosa and haemorrhage.
884	446	No detectable histopathology.
884	486	Some renal hypertensive damage.
884	496	Some renal hypertensive damage.
884	440	Some renal hypertensive damage.
884	550	Footpad granuloma.
884	490	Moderate renal hypertensive damage.

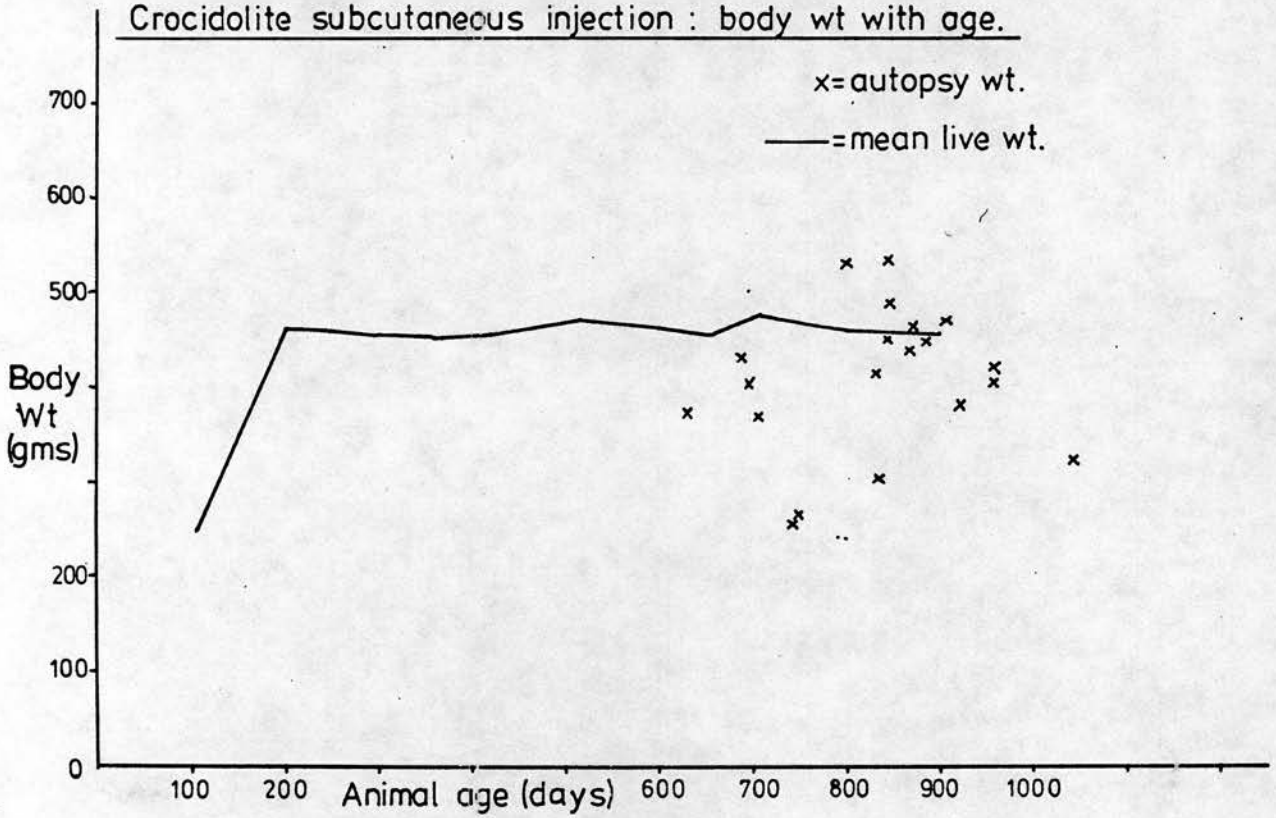
APPENDIX IV, FIGURE 11 Pathological findings in guinea pigs following long-term crocidolite asbestos ingestion (animals arranged chronologically, lesions in order of importance)

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
401	-	Early focal renal and gastric calcification.
430	500	Gastric calcification.
431	450	Intestinal blockage due to volvulus.
437	350	Pneumonia, congestion and oedema. Widespread calcification of kidney, stomach, and intestine.
457	377	Pyelonephritis, some renal calcification and cystitis. Pulmonary congestion and oedema.
479	650	Pulmonary congestion.
536	503	Hepatic fibrosis and necrosis. Congested pancreas. Hyperactive spleen.
543	750	Myocardial infarct, coronary thrombosis and cardiac aneurysm with pleural effusion.
556	-	No detectable histopathology.
580	460	Decomposed - no obvious abnormality.
646	-	Pneumonia. Some hepatic fatty degeneration.
695	-	Coronary thrombosis. Widespread calcification of kidney and myocardium.
723	448	Some pulmonary congestion. Renal calcification.
723	372	Pulmonary congestion. Renal hypertensive damage. Some hepatic fatty degeneration.
729	472	Decomposed - no obvious abnormality.
746	416	Pneumonia, congestion and pericardial effusion.
746	512	Widespread renal, gastric, intestinal calcification. Hepatic fatty degeneration.
799	654	Decomposed - no obvious abnormality.
810	854	Hepatic fatty degeneration. Renal calcification.
810	812	Renal and gastric calcification.

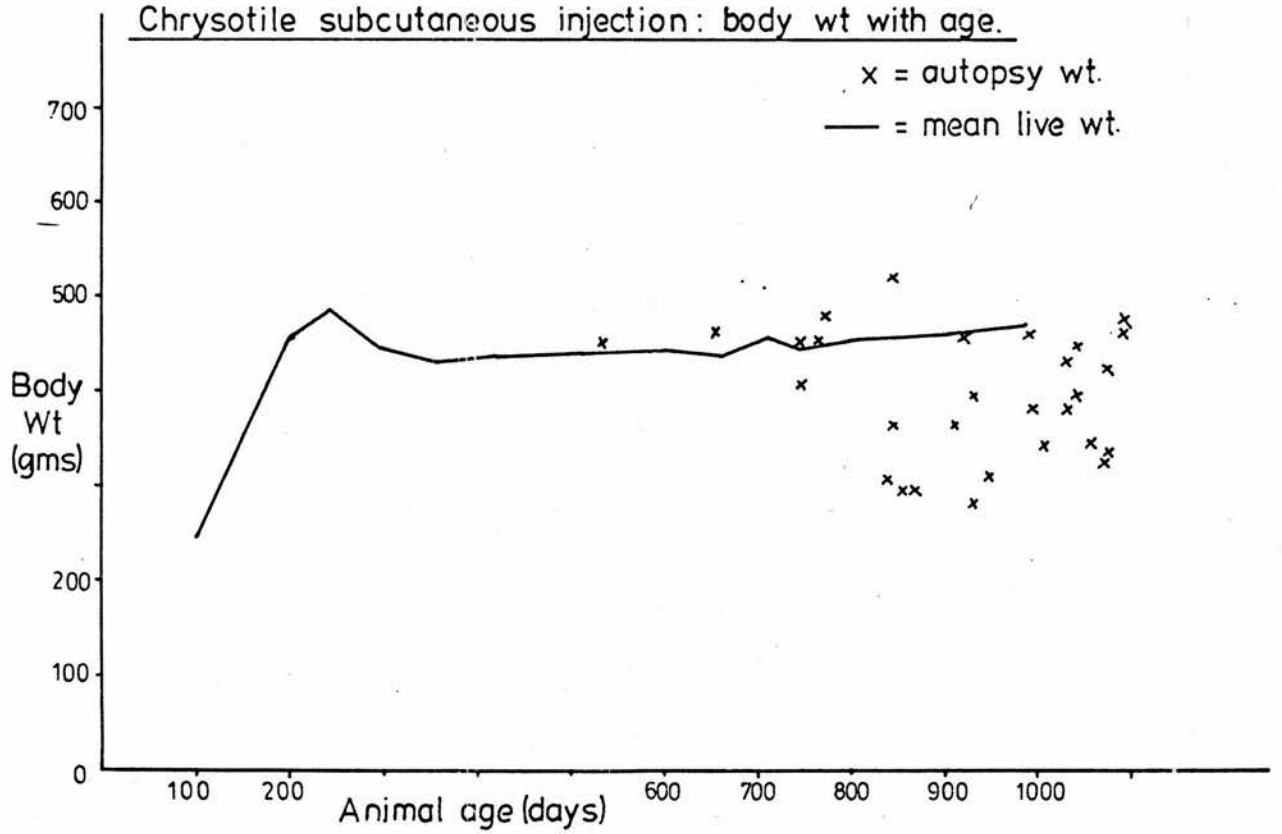
Appendix IV Fig. 12



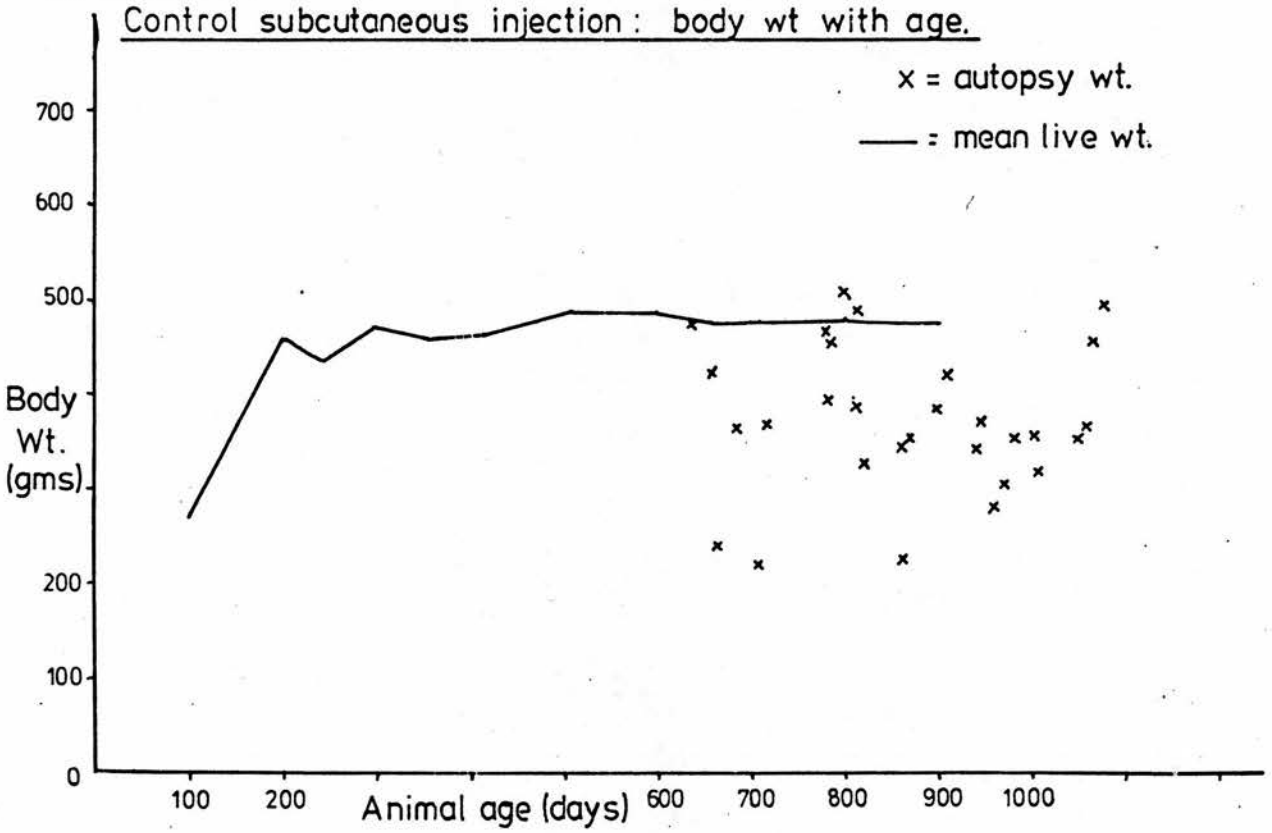
Appendix IV Fig. 13.



Appendix IV Fig. 14.



Appendix IV Fig. 15



APPENDIX IV, FIGURE 16 Pathological findings in rats following subcutaneous amosite injection (animals arranged chronologically, lesions in order of importance)

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
385	-	No detectable histopathology.
415	400	Eye ?
503	460	Extra-medullary erythrocytopoeisis - ? anaemia.
507	472	Lymphoma.
595	422	Melanoma with pulmonary metastases.
688	385	Injection site fibrosarcoma. Some renal hypertensive damage.
699	-	Injection site fibrosarcoma.
704	471	Peritoneal fibrosarcoma arising in coecal mesentery.
743	410	Peritonitis andascites. Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery. Infarction, ischaemic change, and fibrosis of liver and gut.
752	428	Lymphoma.
752	496	Injection site fibrosarcoma. Moderate renal hypertensive damage. Pulmonary venous congestion.
752	450	Pulmonary venous congestion. Pancreatic exocrine hyperplasia.
763	366	Injection site fibrosarcoma with subcutaneous metastases. Moderate renal hypertensive damage. Pancreatic endocrine adenoma.
781	320	Injection site fibrosarcoma. Bronchiolar lymphocyte cuffing.
789	472	Injection site fibrosarcoma.
822	482	Injection site fibrosarcoma. Some renal hypertensive damage.
823	344	Lymphoma.

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
837	394	Injection site fibrosarcoma.
891	-	Extensive renal hypertensive damage.
902	360	Moderate renal hypertensive damage. Pancreatic endocrine adenoma.
907	308	Extensive renal hypertensive damage. Squamous papilloma of external auditory meatus. Small benign adhesion of left lung to diaphragm.
916	370	Moderate renal hypertensive damage.
917	358	Injection site fibrosarcoma. Moderate renal hypertensive damage.
939	312	Some renal hypertensive damage.
959	361	Injection site fibrosarcoma. Pulmonary venous congestion. Some renal hypertensive damage.
966	336	Pulmonary venous congestion. Moderate renal hypertensive damage. Pancreatic exocrine adenoma.
984	384	Extensive renal hypertensive damage. Small mammary papilloma.
992	455	Injection site fibrosarcoma. Pulmonary venous congestion.
994	-	Injection site fibrosarcoma.
1054	304	Bronchopneumonia and pleural effusion. Haemangioma in mesentery. Extensive renal hypertensive damage. Moderate polyarteritis nodosa of mesentery.
1081	362	Extensive renal hypertensive damage. Hepatic peri-portal fatty degeneration.
1081	468	Injection site fibrosarcoma with widespread metastases. Some renal hypertensive damage.

APPENDIX IV, FIGURE 17 Pathological findings in rats following subcutaneous crocidolite injection (animals arranged chronologically, lesions in order of importance)

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
659	372	Pulmonary arterial hypertensive change.
669	-	Injection site fibrosarcoma.
687	430	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery.
699	402	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery.
706	368	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery.
736	-	Decomposed - no obvious abnormality.
742	260	Moderate renal hypertensive damage. Moderate polyarteritis nodosa of mesentery.
747	276	Extensive renal hypertensive damage. Moderate polyarteritis nodosa of mesentery. Gastric mucosal ischaemic damage.
793	240	Subcutaneous fibroma (ventral flank). Some renal hypertensive damage. Inflammation of submandibular salivary gland duct and surrounding tissues.
800	530	Injection site fibrosarcoma.
830	412	Injection site fibrosarcoma. Squamous carcinoma of skin. Pulmonary venous congestion.
833	302	Osteosarcoma of lower jaw.
841	453	Injection site fibrosarcoma.
844	530	Injection site fibrosarcoma. Some renal hypertensive damage. Some pulmonary venous congestion. Pancreatic endocrine adenoma.
847	486	Pulmonary venous congestion and oedema. Moderate renal hypertensive damage.
866	437	Injection site fibrosarcoma. Some renal hypertensive damage. Pancreatic exocrine adenoma.
868	390	Malignant APUD-oma associated with bile duct. Hepatic portal triad fibrosis, inflammation, and biliary retention. Extensive renal hypertensive damage, pyelonephritis and papillary necrosis.
868	451	Injection site fibrosarcoma.
873	467	Malignant pericardial tumour with vascular invasion. Pulmonary venous congestion and oedema. Some renal hypertensive damage.
879	449	Injection site fibrosarcoma. Squamous metaplasia of mammary gland duct. Some renal hypertensive damage.
884	412	Injection site fibrosarcoma. Hepatic lipoma.
897	-	No detectable histopathology.
906	472	Injection site fibrosarcoma. Malignant adrenal cortical tumour. Moderate renal hypertensive damage. Pulmonary venous congestion.
911	474	Moderate renal hypertensive damage. Pulmonary venous congestion.
953	409	Moderate renal hypertensive damage.
963	515	Subcutaneous fibroma. Some renal hypertensive damage.
963	419	Squamous carcinoma of skin. Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery. Pancreatic endocrine adenoma.
973	380	Extensive renal hypertensive damage. Moderate polyarteritis nodosa of mesentery. Pulmonary venous congestion.
986	362	Injection site fibrosarcoma with pulmonary metastases. Some renal hypertensive damage.
995	410	Pulmonary congestion and oedema. Some renal hypertensive damage.
997	-	Footpad granuloma.
1038	324	Extensive renal hypertensive damage. Some polyarteritis nodosa of mesentery. Pulmonary venous congestion. Pancreatic exocrine hyperplasia.

APPENDIX IV. FIGURE 18 Pathological findings in rats following subcutaneous chrysotile injection (animals arranged chronologically, lesions in order of importance)

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
532	450	No detectable histopathology.
655	460	Seminal vesiculitis.
747	408	Pulmonary venous congestion. Moderate renal hypertensive damage. Pancreatic exocrine adenoma.
747	446	Injection site fibrosarcoma. Epidermoid sebaceous cyst lower flank. Moderate renal hypertensive damage. Pulmonary venous congestion. Adenoma of thyroid.
764	451	Decomposed - no obvious abnormality.
771	478	Malignant pericyte tumour over clavicle. Moderate renal hypertensive damage. Bronchiolar lymphocyte cuffing.
843	304	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery. Pulmonary arterial hypertensive change.
844	520	Pulmonary venous congestion. Moderate renal hypertensive damage. Pancreatic exocrine hyperplasia.
846	362	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery. Pulmonary venous congestion. Hepatic fatty change.
856	292	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery.
869	296	Lymphoma. Squamous carcinoma of skin of lower jaw. Moderate renal tubular damage. Pulmonary venous congestion.
906	460	Subcutaneous fibroma with ? early malignant change. Pulmonary venous congestion. Some renal hypertensive damage.
911	360	Injection site fibrosarcoma. Pulmonary venous congestion.
931	280	Extensive renal hypertensive damage.
934	391	Buccal cavity abscess. Pulmonary venous congestion. Some renal hypertensive damage.
949	314	Seminoma of testis (malignant). Extensive renal hypertensive damage. Pulmonary venous congestion. Pancreatic exocrine hyperplasia.
996	379	Malignant.
1005	341	Squamous carcinoma of skin of lower jaw. Pulmonary venous congestion. Some renal hypertensive damage.
1005	498	Some renal hypertensive damage. Pancreatic endocrine odenoma. Footpad granuloma.
1013	432	Malignant Apudoma associated with adrenal medulla. Moderate renal hypertensive damage. Pulmonary venous congestion.
1032	-	Extensive renal hypertensive damage. Extensive hepatic fatty infiltration with excess peritoneal fat depots. Pancreatic exocrine adenoma.
1034	506	Malignant.
1034	374	Some renal hypertensive damage. Peritoneal adipose infarct.
1038	392	No detectable histopathology.
1038	424	Some renal hypertensive damage.
1039	444	Pneumonia. Extensive renal hypertensive damage. Hepatic fatty degeneration. Pancreatic exocrine hyperplasia.
1056	348	Extensive renal hypertensive damage. Some polyarteritis nodosa of mesentery. Pulmonary venous congestion.
1068	326	Malignant.
1075	330	Haemangioma in coecal mesentery. Extensive renal hypertensive damage.
1076	420	Extensive renal hypertensive damage. Pulmonary venous congestion. Pancreatic exocrine hyperplasia.
1090	458	Some renal hypertensive damage.
1090	472	Moderate renal hypertensive damage.

APPENDIX IV, FIGURE 19 Pathological findings in rats following subcutaneous saline control injections (animals arranged chronologically, lesions in order of importance)

Age at death (days)	Autopsy Wt. (gms)	Details of Pathology
452	-	Pyelonephritis and extensive renal hypertensive damage.
633	472	No detectable histopathology.
655	429	Squamous carcinoma of external auditory meatus.
664	240	Lymphoma ? Pulmonary interstitial pneumonitis.
686	366	Pulmonary oedema.
709	220	Squamous carcinoma with infection and inflammation of skin.
717	367	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery.
777	469	No detectable histopathology.
777	391	No detectable histopathology.
783	451	No detectable histopathology.
800	507	Peritoneal sarcoma associated with coecal mesentery.
811	388	No detectable histopathology.
814	325	Pulmonary venous congestion and bronchiolar lymphocyte cuffing. Moderate renal hypertensive damage. Infarct of adrenal. Pancreatic exocrine hyperplasia.
814	590	Subcutaneous fibroma. Pulmonary venous congestion. Some renal hypertensive damage.
862	338	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery. Hepatic fatty degeneration.
864	220	Squamous carcinoma of skin of lower jaw. Moderate renal hypertensive damage. Pulmonary venous congestion.
866	350	Fibroma of testis. Some renal hypertensive damage.
867	658	Pulmonary venous congestion. Moderate renal hypertensive damage. Subcutaneous lipoma.
899	383	Lymphoma.
907	422	Malignant ill-defined peritoneal sarcoma. Some renal hypertensive damage.
938	342	Extensive renal hypertensive damage. Extensive polyarteritis nodosa of mesentery.
942	368	Some renal hypertensive damage.
958	278	Renal haemangioma.
969	305	Decomposed - no obvious abnormality.
982	350	Pulmonary venous congestion. Some renal hypertensive damage.
995	-	No detectable histopathology.
1001	354	Pulmonary venous congestion. Benign angioma in liver. Pancreatic exocrine adenoma.
1004	314	Extensive renal hypertensive damage. Hepatic fatty degeneration.
1052	351	Pulmonary venous congestion and bronchiolar lymphocyte cuffing. Extensive renal hypertensive damage.
1059	364	Pulmonary venous congestion. Pancreatic endocrine adenoma.
1066	456	Moderate renal hypertensive damage.
1079	492	Some renal hypertensive damage.

APPENDIX IV, FIGURE 20 Cytokinetic analyser : column length estimations

	Column length estimations (No. of cells)*					
Amosite-treated:-	Mean values for 100 individual crypt counts per animal					Overall means
	1	2	3	4	5	
Upper small intestine	33.15	36.91	40.41	34.71	34.59	35.95
Mid small intestine	31.54	32.91	32.68	32.51	31.14	32.16
Lower small intestine	32.22	31.82	34.48	28.59	31.67	31.76
Descending colon	41.83	45.22	42.28	41.87	37.95	41.83
Control (-ve)	1	2	3	4	5	Overall means
Upper small intestine	32.46	34.49	42.94	33.05	35.49	35.69
Mid small intestine	30.87	31.84	36.08	31.26	33.05	32.62
Lower small intestine	30.50	29.52	33.98	30.98	32.25	31.45
Descending colon	40.08	40.22	45.64	40.78	38.91	41.13

* Taken from stathmokinetic determinations.

APPENDIX IV, FIGURE 21 Cytokinetic analyses : column length estimations

	Column count estimations			
Amosite-treated:-	Mean values for 100 column counts per animal			Overall means
	Animal 1	Animal 2	Animal 3	
Upper small intestine	18.00	21.63	20.30	19.97
Mid small intestine	19.03	22.27	21.20	20.83
Lower small intestine	17.00	22.87	21.27	20.38
Descending colon	18.73	21.23	20.63	20.20
Control (-ve)	1	2	3	Overall means
Upper small intestine	22.83	20.73	22.50	22.02
Mid small intestine	20.46	20.50	19.23	20.06
Lower small intestine	22.50	20.07	20.80	21.12
Descending colon	22.80	20.97	18.63	20.80

APPENDIX IV, FIGURE 22 Cytokinetic analyses : Tannock's factor

	Tannock's factor			
	Amosite		Control (-ve)	
	Mean*	Studied Deviations	Mean*	Studied Deviations
Upper small intestine	0.525	0.094	0.468	0.125
Mid small intestine	0.509	0.116	0.488	0.086
Lower small intestine	0.515	0.083	0.503	0.102
Descending colon	0.617	0.116	0.586	0.126

* Mean of 50 observations.

APPENDIX IV, FIGURE 23 Cytokinetic analyses : mitotic indices from autoradiographic assays

*Mitotic index, Im, (Estimated from autoradiographic measurements							
Amosite treated				Control (-ve)			
upper intestine	mid intestine	lower intestine	descending colon	upper intestine	mid intestine	lower intestine	descending colon
4.77	4.35	3.72	1.78	4.17	4.14	4.10	3.63

* Figures are raw data i.e. <u>not</u> corrected with Tannock's factor.

APPENDIX IV, FIGURE 24 Cytokinetic analyses : mitotic indices from stathmokinetic assays

*Stathmokinetic mitotic indices					
Amosite	Time of arrest				
	30	60	90	120	150
Upper small intestine	4.68	8.84	14.74	17.73	27.25
Mid small intestine	4.15	7.00	16.38	18.39	30.38
Lower small intestine	3.81	7.04	13.13	14.56	16.53
Descending colon	1.62	3.38	4.40	4.71	7.04
Control (-ve)					
Upper small intestine	6.03	11.00	17.35	21.06	26.34
Mid small intestine	6.25	10.09	14.81	17.59	23.25
Lower small intestine	5.40	8.71	14.07	17.49	22.07
Descending colon	2.03	4.14	6.65	7.48	12.12

*Figures are raw data i.e. not corrected with Tannock's factor.

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THE SHORT-TERM EFFECTS OF CHRONIC ASBESTOS INGESTION IN RATS

R. E. BOLTON and J. M. G. DAVIS
Institute of Occupational Medicine, Edinburgh

Abstract—The effects of ingestion of asbestos in laboratory rats were examined by feeding a diet supplemented with an asbestos/margarine formulation for periods up to 1 yr. UICC standard reference samples of chrysotile A, crocidolite and amosite were used throughout this study. Animals were examined for short-term clearance and evidence of penetration of, or damage to, the gut mucosa. There was no evidence of asbestos retention within the gut lumen, and no sign of cell penetration or damage to the intestinal mucosa in any of the animals under test. Results of scanning electron microscope investigations are presented, together with statistical analyses, to show that if penetration of the gastrointestinal tract did occur, it could only have done so to a minimal extent.

INTRODUCTION

UNTIL recently, research into the pathology of asbestos disease has been centred around the pathogenic effects after inhalation and little attention has been given to the concomitant effects after asbestos ingestion. KONIG (1960) was the first to report an increase in the incidence of gastro-intestinal cancer amongst asbestos workers. SELIKOFF *et al.* (1964) reported a threefold increase in gastro-intestinal cancer in a cohort of 1522 asbestos workers and this was followed by accounts of similar findings from other epidemiological surveys including those undertaken by ELMES and SIMPSON (1971), ENTERLINE *et al.* (1972), VIGLIANI *et al.* (1973), MCDONALD (1973), SELIKOFF *et al.* (1973) and MANCUSO and EL ATTAR (1973).

The significance of the mucociliary pulmonary clearance mechanism as the major source of asbestos within the gastro-intestinal tract has been appreciated for some time, although estimates of the extent of the bronchial clearance component vary from 73% (EVANS *et al.*, 1973) to 98–99% (GROSS *et al.*, 1974) of all deposited particles, depending upon the exposure level.

In addition to neoplasms of the gut lining it is known that peritoneal mesotheliomas as well as pleural mesotheliomas are associated with asbestos exposure and it has seemed possible that these are caused by asbestos particles penetrating through the gut wall into the peritoneal cavity.

Some studies on the effects of asbestos ingestion in experimental animals have been undertaken and WESTLAKE *et al.* in 1965 reported finding some small chrysotile fibres in the lining of the colon in rats fed a synthetic diet containing 6% of chrysotile asbestos. These fibres were seen by electron microscopy in both the glandular epithelial cells and in the tissues of the lamina propria. They did, however, appear to be free in the cell cytoplasm and not surrounded by cell membranes as is usually the case with intra cytoplasmic asbestos (DAVIS, 1967). Other studies using similar ingestion techniques proved negative and were either unpublished (Swinburne) or reported very

briefly (BONSER and CLAYSON, 1967). Recently, however, considerable concern has developed over the occurrence in drinking water supplies of both genuine asbestos, and also other mineral fibres of similar size and shape. This has been particularly true of fibres from cummingtonite and grunerite rocks that have been found in association with taconite iron ore. This potential hazard has resulted in a focusing of attention on the effects of asbestos ingestion, and prompted CROSS *et al.* (1974) to summarize not only work in progress but also some previously unpublished information. In general, these studies showed no evidence of penetration of asbestos fibres into the walls of the gastro-intestinal tract. SMITH *et al.* (1965) and SMITH (1973) had also reported negative findings from groups of hamsters fed asbestos for their full life span. Other studies recently published include those by PONTEFRACT and CUNNINGHAM (1973). These workers reported finding large numbers of asbestos fibres in rat tissue after the fibres had been initially administered via the gut. In this series of experiments, however, the asbestos had been injected directly through the stomach wall after laparotomy and there was a high risk of tissue contamination.

Most previous studies have relied on light microscope examination of gut tissues or the digestion of these tissues followed by electron microscope examination of the digest in order to obtain evidence of asbestos penetration of the gut wall. It was considered important, however, to use electron microscopy to examine the fine structure of the gut lining cells during periods of asbestos ingestion in order to determine whether or not the cells might become damaged in the absence of actual penetration. The present study was therefore undertaken and rat tissues were examined after periods of up to 1 yr of asbestos ingestion. In addition to transmission electron-microscopy of some tissue samples, entire gastro-intestinal tracts were ashed and the residues examined both by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

MATERIALS AND METHODS

Male SPF Han rats, initially 10 weeks old, were used throughout this study. They were housed two per cage and maintained on the standard pelleted laboratory diet. The asbestos was administered as an *ad libitum* supplement to this diet in a margarine formulation. It was found that rats consumed on average 50–60 g of margarine per week and the dose level was set at 5 mg of asbestos per 1 g of margarine so that the average weekly consumption of asbestos was 250–300 mg per rat. The effects of chrysotile, crocidolite and amosite asbestos were examined in this study, and the UICC standard reference samples were used throughout (TIMBRELL, 1970).

Three experiments are reported in this study: 1. short-term gut clearance tests, 2. ashing analysis of tissue after 1 yr of asbestos ingestion, and 3. detailed microscopical examination of gut tissues at intervals during 1-yr exposure period.

1. *Short-term gut clearance*

These tests were undertaken to establish the transit time of asbestos along the gastro-intestinal tract and to test for possible retention in pockets within the gut lumen, as suggested by POOLEY in 1974. Four rats were used with each asbestos type, together with two controls. The experimental animals were given asbestos in margarine *ad libitum* for 1 month to establish constant ingestion conditions. At time zero the

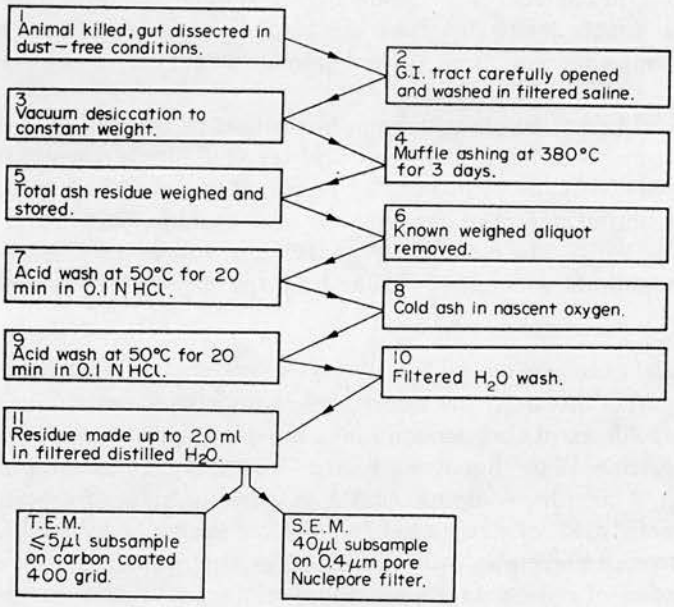

FIG. 1. Stages in preparation of samples for electron microscopy.

asbestos supply was removed and half the animals were killed. Twenty-four-hour faecal pellet samples were taken from the surviving animals at regular intervals for 28 d. On the 28th day the remaining animals including the controls were killed. Both gut tissues and faecal pellets were ashed and prepared for examination by the techniques shown in Fig. 1. Initially both TEM and SEM were used for the examination of residues. However, SEM proved to be the most useful technique since it allowed relatively large aliquots of sample to be prepared and examined without the obscuring effects produced by grid bars. In addition, SEM gave clear pictures of the characteristic morphology of amphibole fibres and greatly simplified the recognition of these asbestos types.

2. *Ashing analysis of tissue*

The second group of experiments was undertaken to test for deep penetration and long-term retention of asbestos fibres. Again chrysotile, crocidolite and amosite asbestos were all tested, using two animals for each asbestos type. The animals were given asbestos margarine mixtures *ad libitum* for 1 yr. Then, after 1 month on a diet free from asbestos, the animals were killed and the gastro-intestinal tracts carefully removed. These, together with samples from control animals to check for possible fibre contamination during processing, were muffle ashed and washed as before (Fig. 1). All samples were carefully examined for fibre content using both TEM and SEM. Stage 2 was introduced halfway through the study after it became apparent from the examination of the guts of the first animals that the bulk of the residue after muffle ashing was from the food contents of the gut. It was therefore decided to reduce this residue and hence increase the resolution of the technique by dissecting

and washing out the contents of the gastro-intestinal tract. The possibility of introducing fibre contaminants during dissection was carefully considered, but it was assumed that examination of the guts from control animals would detect any fibres from this source.

Stages 7 and 9 had to be omitted from the analysis of animals from the chrysotile ingestion experiments owing to the know acid-susceptibility of chrysotile (HODGSON, 1965). Tests with UICC asbestos samples suggested that there was a real possibility of altering the fibrous nature of chrysotile by acid washing, particularly after 3 d of muffle ashing at 380°C. The omission of stages 7 and 9 from the scheme resulted in a five-fold increase in the amount of residue from the chrysotile-fed animals requiring examination.

3. *Microscopical examination*

The third part of this study was undertaken to examine the gastro-intestinal tissues of rats for any evidence of fibre penetration or cell damage which could be attributable to asbestos ingestion. Four animals were used for each type of asbestos and they were killed 2 weeks, 3 months, 6 months and 1 yr after the start of asbestos ingestion. Randomly selected areas of all regions of all levels of the gut of each animal were fixed in osmium tetroxide and embedded in Araldite for electron microscope examination. The interpretation of ultrastructural variations requires caution since it is known that artefacts can be introduced by alterations in preparation and presentation of specimens for electron microscopy (RUSKA, 1960, 1961; TRIER, 1964). A rigidly standardized technique was therefore used throughout this study. The remainder of all the gastro-intestinal tissue was taken for light microscopy. Control animals were also examined at each time period in order to confirm the normal ultrastructural patterns of gastro-intestinal tissues in the strain of rat used in this study.

RESULTS

1. *Short term gut clearance*

The results of the gut clearance experiment showed that for all asbestos types, transit time for the vast majority of fibres is less than 48 h, that at 72 h only trace levels of asbestos are detectable, and that from 7 to 28 d no asbestos was detected in the faeces. Analysis of the whole gut and contents for asbestos 28 d after the cessation of asbestos ingestion similarly proved negative, indicating that all the asbestos had been cleared and that no tissue penetration had occurred. During the period of asbestos feeding, asbestos fibres made up a very high proportion of the non-combustible gut contents (Fig. 2), but this proportion dropped rapidly until at 72 h even single asbestos fibres were difficult to find. These findings agree with those of EVANS *et al.* (1973) and confirm that in rats asbestos fibres are not retained at any point in pockets within the gut lumen. This means that any asbestos fibres found by ashing the entire gut with its food content more than 14 d after the cessation of asbestos feeding, must have penetrated the tissues of the gut lining, if atmospheric contamination of the specimens can be excluded.

2. *Ashing analysis of gut tissues following asbestos feeding for* 1 yr

Scanning electron microscope examination of ashed residues of the entire guts of animals fed for 1 yr on either crocidolite, amosite or chrysotile asbestos revealed that

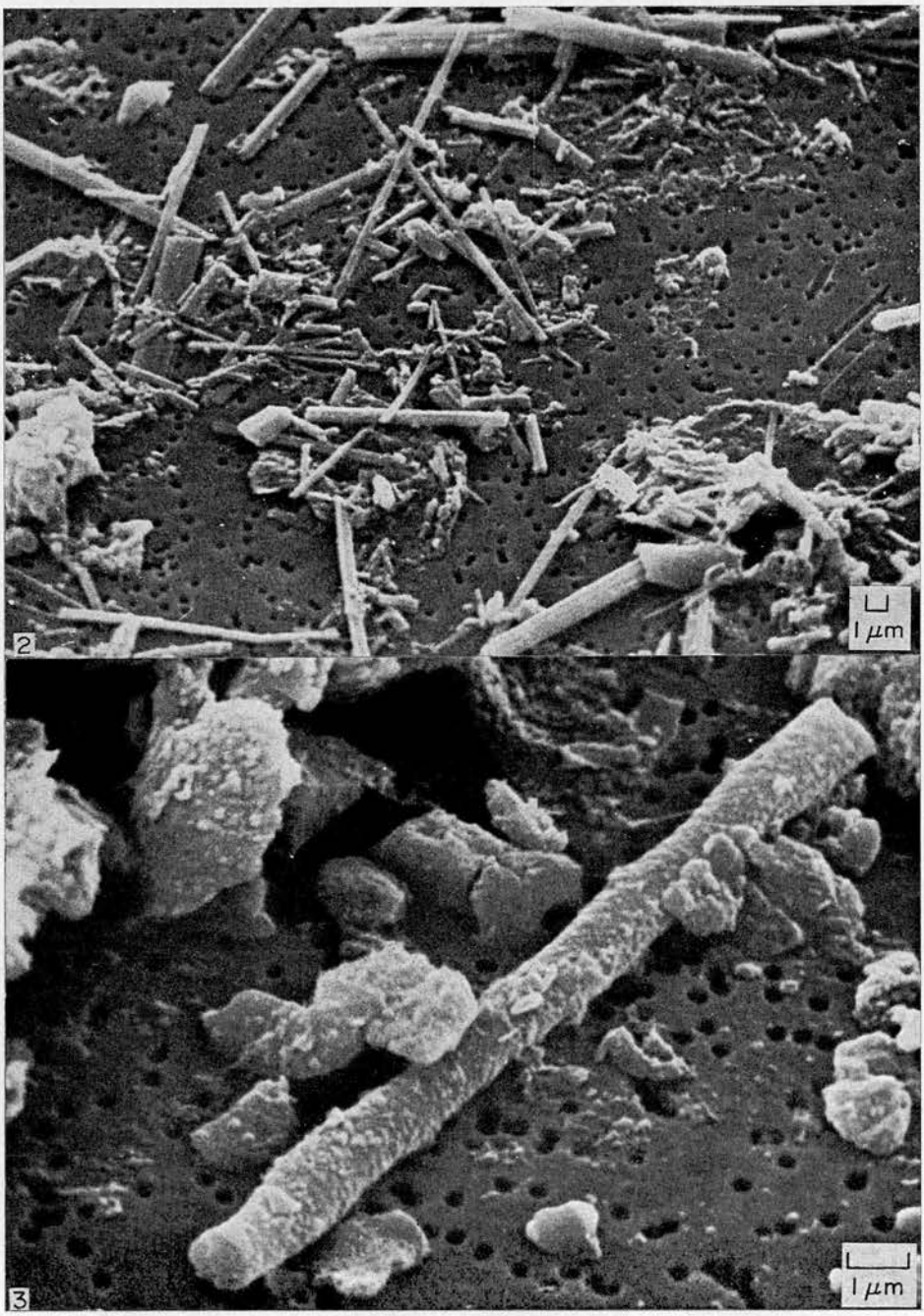

FIG. 2. A sample of ashed gut residue taken from the amosite gut clearance experiment. This sample was taken during the feeding period and shows that the asbestos fibres comprise a very high percentage of the non-combustible material present in the gut at this time. S.E.M. preparation.

FIG. 3. Example of a typical fibrous shape from residue of amphibole fed animals. This fibre is in the correct size range for amphibole fibres, but appears to be cylindrical rather than foliate as shown in Fig. 2. Similar fibres were found in all control animals. S.E.M. preparation.

(f.p. 124)

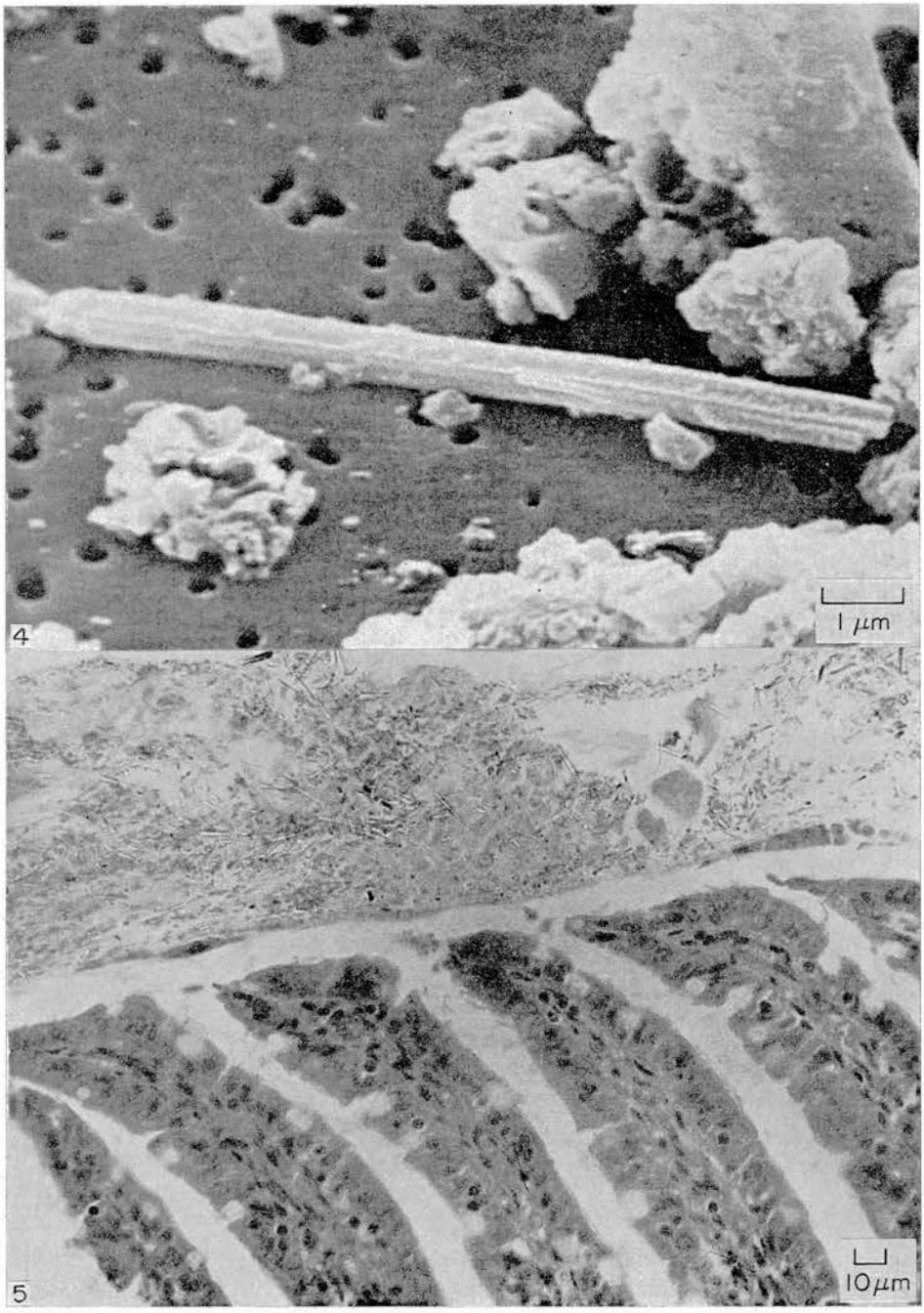

FIG. 4. A single fibre found in ashed gut samples from an animal after 1 yr of amosite ingestion followed by a 1-month period without asbestos in the diet. The similarity between this fibre and known amosite can be seen from a comparison with Fig. 2. S.E.M. preparation.

FIG. 5. Light photomicrograph of rat small intestine with food containing asbestos fixed *in situ*. Large numbers of asbestos fibres can be seen amongst the food, but the main food mass appears to be within reach of the tips of the villi only. The intervillous spaces are devoid of particulate matter.

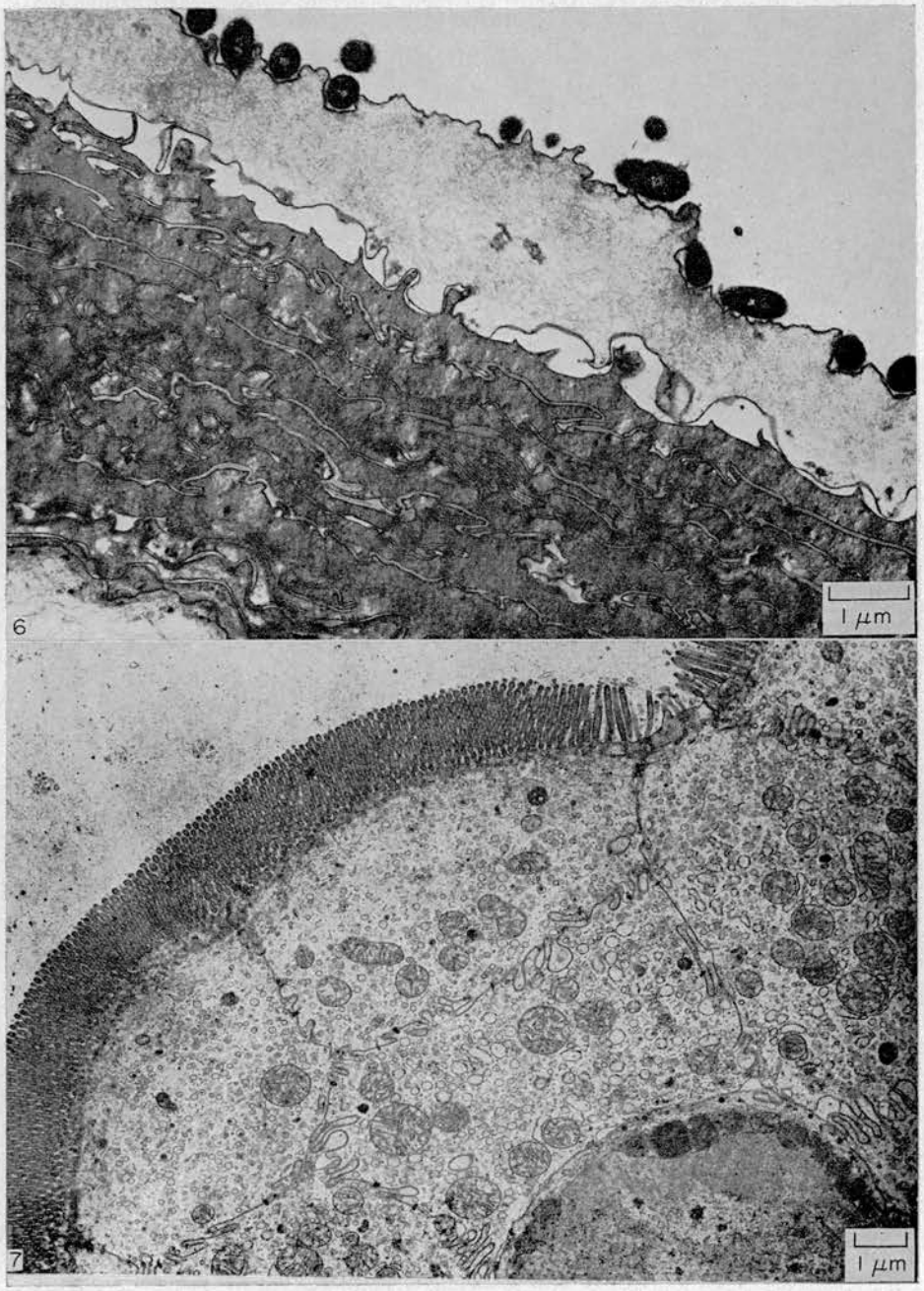

FIG. 6. Section of the keratinizing squamous epithelium from the oesophagus. It can be seen from the presence of bacteria on the outer hydrated layer only that the multilayered stratum granulosum represents a formidable barrier against potential penetration. T.E.M. preparation.

FIG. 7. Photomicrograph showing a section through several intestinal epithelial cells from the apical region of a villus. The microvilli that make up the 'brush border' have been sectioned mostly in the tangential plane. The cell structure appears normal and there is no sign of asbestos penetration. T.E.M. preparation.

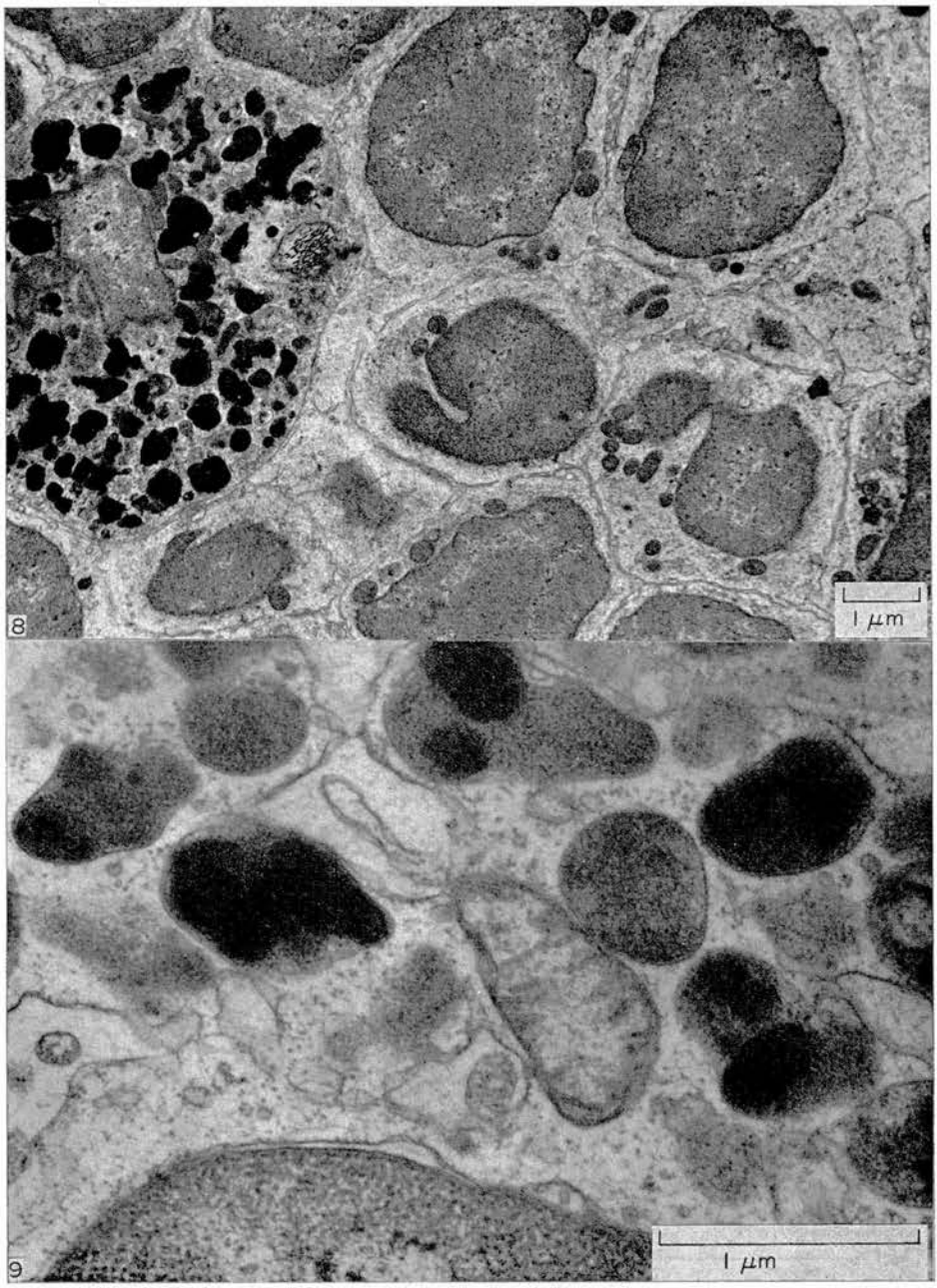

FIG. 8. A typical view of rat mesenteric lymph node showing several normal lymphocytes and part of a macrophage containing large numbers of cytosegresomes. This type of macrophage was found to occur more frequently in older animals. T.E.M. preparation.

FIG. 9. High power view of part of a macrophage from rat mesenteric lymph node showing that the cytosegresomes contain an amorphous material bound by a distinct membrane. There was no evidence of fibrous inclusions in the cytosegresomes of any of the animals examined. T.E.M. preparation.

fibrous shapes were relatively common in this material. However, almost all of these structures were quite distinct from asbestos (Fig. 3), and were present in the ashed gut residues of control animals as well as those from the experimental series. It is considered that most of these fibres represent fragments of silicaceous plant skeletons included in the animal food pellets and not susceptible to complete degradation by the ashing procedures used in this study. From the animals under consideration, only one possible asbestos fibre was found, although a total of 180 preparations of gut residue were examined.

In the case of the two amphibole asbestos types, about 100 preparations were examined. For crocidolite these comprised approximately 0.4% of the total ashed residues. For amosite, however, the gut tissues had been ashed after the removal of the contained food material, and the residue was, therefore, greatly reduced. This meant that 4% of the total residue was examined. With the chrysotile animals, it was not possible to use an acid wash to reduce the bulk of the final ashed sample and therefore the 80 preparations examined comprised only 0.15% of the total.

In those animals fed either crocidolite or chrysotile, no fibres were discovered at all similar to either of these asbestos types. In one animal fed with amosite, however, a single fibre 11.6 μm $\times$ 0.5 μm was discovered (Fig. 4). This fibre showed a very similar structure to known amosite (Fig. 2) and was accepted as being of this material. No X-ray analysis equipment was available on the scanning microscope used in the present investigation.

Without the expenditure of an extremely large amount of time it was obviously impossible to examine all the ashed residue from each animal. It was, therefore, impossible to prove that there was absolutely no gut penetration in any of the animals examined in this study, but a statistical estimate of the probabilities can be made. If a Poisson distribution of fibres throughout the ashed residues is assumed, then it is possible to calculate the maximum fibre content of these specimens that would be consistent with the observed data. These calculations indicate a 90% probability that after 1 yr's feeding, the maximum fibre penetration of an entire rat intestine would be less than 100 for amosite, 550 for crocidolite, and 1500 for chrysotile. Similarly the figures for an even chance in each experiment are 40 for amosite, 170 for crocidolite and 500 for chrysotile. These figures do not reflect the relative risks of the three asbestos fibres but merely the difference in sample size. They do, however, take account of the fact that one amosite fibre was found, and that the examination of residues from animals treated with either chrysotile or crocidolite proved completely negative.

3. *Microscopical examination of gastro-intestinal tracts*

Gut tissues from animals fed on either chrysotile, crocidolite or amosite were examined at intervals from 2 weeks to 1 yr after the start of the experiment. Dissection of the gut followed by examination of the surface of the lumen with the aid of a stereoscopic microscope revealed no sign of abnormality or any pathological lesions. Histological examination of tissue sections likewise showed no pathological changes and there was no evidence of the penetration of asbestos fibres into the tissues. When lengths of the gut were fixed with contents *in situ* large numbers of asbestos fibres could be seen among the digesting food (Fig. 5). However, as this illustration shows, there was often a complete demarcation between the mucus-bound food package

and the gut lining cells, and there was no evidence of solid food particles penetrating between the villi. The space usually seen between the surface of the gut contents and the tissue is probably due to contraction during fixation. However, the lack of food between the villi suggested that in the rat at least the particulate fold material passes through the gut as a mucinous package and, at most, only touches the tips of the villi. In these circumstances the areas of mutual contact between particulate food and the intestinal mucosa must be small, and the chances of tissue penetration in the rat must also be small.

In addition to the light microscope studies, detailed ultrastructural examination by TEM showed no signs of cell damage or penetration of the gut lining by asbestos fibres (Figs. 6–9). Occasionally, cells from the tissue of small intestine villi showed disruptive changes in the uniformity of their microvilli, the normally cylindrical structures breaking down into irregularly sized droplets. These changes were, however, also found in the normal epithelial cell extrusions of control animals.

Examination of mesenteric lymph nodes from all animals with both light microscopy and TEM revealed progressive increase with age in reticulin fibres and the number of macrophages containing cytosegresomes (Figs. 8 and 9). However, none of the cytosegresomes examined contained any inclusions remotely similar to asbestos fibres and no cytosegresomes were found in any other situation within the nodes. The alteration of the mesenteric lymphatic tissue with age was also found in control animals.

DISCUSSION

The present study has shown that in rats at least, prolonged asbestos feeding does not result in any widespread penetration of the gut lining by asbestos fibres in any size range. Without unlimited manpower and greatly prolonged use of the scanning electron microscope it was not possible to prove that no fibres at all had penetrated the gut tissues in these animals but the maximum figure for fibre penetration was reduced to below 550 for amphibole fibres. These, because of their stiff, spiky nature, appear the most likely to penetrate soft tissues. However, extensive searches of ashed gut residues produced only one fibre that could be accepted as asbestos. Since, in any laboratory using asbestos for experimental purposes, the risk of specimen contamination cannot be eliminated entirely, it is possible that even this fibre was not present in the original gut sample. The maximum possible penetration dose of 550 amphibole fibres after 1 yr of asbestos ingestion must be placed in perspective beside present day knowledge of the carcinogenicity of asbestos in other sites. The evidence is unfortunately sparse, but some studies have been undertaken on the dose of asbestos required to produce mesotheliomas in experimental animals following intrapleural injections. SMITH *et al.* (1973) found no tumours in two groups of hamsters injected with 1 mg doses of either harsh chrysotile or amosite. WAGNER (1973) on the other hand, although finding that tumour production was roughly dose-related, did produce occasional tumours after the injection of 0.5 mg of chrysotile and crocidolite into rats. It has been calculated that 550 amphibole fibres 5 μm in length would weigh approximately 1×10^{-6} mg so that the maximum penetration in the present study is five hundred thousand times lower than the smallest asbestos dose yet proved to be carcinogenic.

However, the possibility must remain that large numbers of asbestos fibres continually passing through the gut mixed with normal food material might be able to

exert a carcinogenic effect without any tissue penetration occurring. The contents of the gut have been shown to effect the proliferative activity of the mucosal layers (ABRAMS *et al.*, 1963; BULLOUGH, 1965; RIJKE *et al.*, 1974), and AMACHER and his colleagues (1974) have shown that ingestion of large doses of chrysotile, administered by lavage, did cause a temporary increase in the DNA synthesis of the rat gastro-intestinal tract. This type of response might conceivably occur in some severely-exposed asbestos workers continually swallowing relatively large amounts of asbestos, but does appear most unlikely in people who ingest minute quantities of asbestos in drinking water, or in beverages filtered through asbestos filters.

The feeding of asbestos mixed with margarine to rats as a supplement to a normal pelleted diet gives no evidence of asbestos penetration of the intestinal mucosa. Some workers using different experimental systems, however, have reported penetration of the gut wall by asbestos and other particulate matter.

WEBSTER (1974) and WESTLAKE (1974) reported finding evidence of asbestos penetration after ingestion of massive doses of asbestos fibres (6% of the diet in the case of Westlake's experiments). The *ad libitum* dose of 5 mg asbestos per gram of margarine used in the present study represents 0.27% of diet for rats. To keep this in perspective, a diet containing 0.27% asbestos is equivalent to a projected human exposure approximately 50 000 times the ingestion maximum for severely occupationally-exposed workers calculated from inhalation data (ENTERLINE *et al.*, 1972; MCDONALD and MCDONALD, 1973; KUSCHNER *et al.*, 1974; SCHNEIDERMAN, 1974) and at least 1.6 million times that for environmental exposure (CUNNINGHAM and PONTEFRACT, 1971; 1973).

Widespread penetration of gastric mucosa followed by asbestos transport to various regions of the body has been reported by CUNNINGHAM and PONTEFRACT (1973) following laparotomy and transmural gastric injection of chrysotile. GROSS *et al.* (1974) and others have pointed out that this technique has an inherently high risk of contamination artefacts which are difficult to evaluate. The phenomenon of 'persorption' of particulate material through the wall of the gastro-intestinal tract has been reported by VOLKHEIMER (1974) and by SCHREIBER (1974). The implications of frequent penetration of particles of up to 90 μm diameter into the lymphatic system and beyond as reported by Volkheimer are far reaching and require comment. It is interesting to note that whilst CLARK (1959), SANDERS and ASHWORTH (1961) and others have reported adsorption of very small latex spheres by pinocytosis in the small intestine, no mention was made of massive transmigration of material on a scale envisaged by Volkheimer. Persorption of particles as large as 90 μm could only occur following a breakdown in the intestinal mucosal barrier. Such breakdowns have not been reported in the copious literature of fine structure of the intestine (TRIER and RUBIN, 1965). In addition, one would expect that if the phenomenon of persorption were the norm, examination of the mesenteric lymph nodes would reveal a burden of particulate matter. GROSS *et al.* (1974) emphasized that even in workers occupationally exposed to particulate matter such as coal and silica dusts, there is no involvement of the mesenteric lymph nodes, whilst alveolar penetration by the dusts can be confirmed by examination of the hilar lymph nodes. TRIER and RUBIN (1965) have studied the ultrastructural abnormalities associated with the malabsorption syndrome of Whipple's disease. This is characterized by variable alterations in villous architecture and epithelial cell ultrastructure, but even in these extreme conditions the mucosal barrier retains its integrity.

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MASS AND NUMBER OF FIBRES IN THE PATHOGENESIS OF ASBESTOS-RELATED LUNG DISEASE IN RATS

J. M. G. DAVIS, S. T. BECKETT, R. E. BOLTON, P. COLLINGS AND A. P. MIDDLETON

From the Institute of Occupational Medicine, Roxburgh Place, Edinburgh EH8 9SU

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Summary.—Five groups of rats were treated by inhalation for 12 months with the U.I.C.C. preparations of the 3 main commercially used asbestos types, chrysotile, crocidolite and amosite. The experiment was designed so that the effects of both fibre mass and fibre number could be examined. The results indicated that chrysotile dust caused far more lung fibrosis than either amphibole type even when the fibre numbers in the dust clouds were similar. All malignant pulmonary neoplasms found during this study occurred in animals treated with chrysotile. The fibre-number calculations used for the generation of dust clouds were evaluated using the parameters recommended by the Health and Safety Executive in 1976, by which all fibres over 5 μm long are counted using a phase-contrast light microscope. When fibre-length distributions were calculated using a scanning electron microscope, however, it was found that the chrysotile clouds used in this study contained many more fibres over 20 μm long than either of the amphibole clouds. The results, therefore, support previous suggestions that long asbestos fibres are more dangerous than short. They also indicate that neither a single mass standard, nor the present fibre-number standards are satisfactory.

THE INHALATION of asbestos dust may cause both lung fibrosis and neoplasia in those involved in the industrial processing of this material and for this reason the maximum level of dust in asbestos factories is governed by strict standards in most countries. Although the major types of asbestos used commercially differ both physically and chemically, the legislation in many countries lays down one standard which is applied to several or all forms (Zielhuis, 1977). For coal dust, the work of Jacobsen *et al.* (1970) has shown that the mass of respirable airborne dust corresponds more closely with radiological change than does particle number. Unfortunately similar data do not exist for asbestos. This fact was noted by the I.A.R.C. Advisory Committee on Asbestos and Cancer in 1973. Previous work carried out in Edinburgh (Beckett, 1975) has shown that for each type of asbestos there is a different relationship between the airborne fibre number and mass concentrations. This means that if a gravimetric standard is adopted the permitted fibre number for chrysotile is much higher than for amosite, while if a fibre-number standard is operated the permissible mass for amosite is greater than that for chrysotile. This situation has no doubt arisen because reliable evidence relating to the relative pathogenicity of asbestos dust has been difficult to obtain from human epidemiological studies, since most factories have, in the past at least, used more than one asbestos type. In addition, the information from animal inhalation studies has often been conflicting. Holt, Mills and Young (1965) found no differences in the fibrogenic potential of chrysotile, crocidolite, amosite and anthophyllite, while Wagner (1963) and Wagner and Skidmore (1965) and Morris *et al.* (1967) suggested that chrysotile produced less fibrosis than amosite or crocidolite for the same mass

dose. In a later inhalation study, using the U.I.C.C. standard reference samples, Wagner *et al.* (1974) reported that amosite dust invariably gave the least fibrosis and Canadian chrysotile the most. Crocidolite and Rhodesian chrysotile were intermediate.

Knowledge of the relative importance of the different asbestos tyes in the production of neoplasia is no more precise and, although crocidolite has been specially linked with the production of mesotheliomas (Wagner, Sleggs and Marchand, 1960), subsequent epidemiological studies have indicated that at least some of the other asbestos types may also cause this type of tumour (McDonald, 1973; Selikoff, Hammond and Seidman, 1973). Similarly, since the report of Doll (1955) showing a greatly increased risk of bronchial carcinoma among asbestos workers, no reliable human epidemiological data have been produced indicating whether or not all industrially used asbestos types are equally potent in the production of these lung tumours. This is due to the fact that most workers in factories handling asbestos have been exposed to more than one type during their working lives.

Most early animal inhalation studies produced no lung tumours, and those later ones which did result in the production of bronchial carcinomas and mesotheliomas gave positive results with different asbestos types in each experiment (Gross and De Treville, 1967; Reeves *et al.*, 1971). However, both Wagner *et al.* (1974) and Reeves, Puro and Smith (1974) published the results of studies in which all the major asbestos types had been administered to rats. Wagner used amosite, anthophyllite, crocidolite and 2 varieties of chrysotile. He found the highest number of malignant tumours in animals treated with Rhodesian chrysotile, and the lowest number in those treated with amosite. Anthophyllite, crocidolite and Canadian chrysotile gave about the same number of tumours. Reeves used chrysotile, crocidolite and amosite, and obtained similar tumour incidences with all 3. Since both these authors used gravimetric dust estimations, the results could indicate that more fibres of chrysotile are required for tumour production than any of the amphibole types. However, it appeared desirable to reappraise this problem with a series of experiments in which the effects of both fibre mass and fibre number could be compared in the same study.

MATERIALS AND METHODS

For any given mass, the U.I.C.C. sample of amosite has the fewest fibres, and chrysotile the most, with crocidolite fibres being somewhere between the 2. It was decided therefore to use amosite as the reference dust, and to compare its pathological effects with those produced by both crocidolite and chrysotile clouds of equal fibre mass in one instance, and equal fibre number in the other.

The equal-mass concentration clouds had a target mean concentration of 10 mg/m^3, which was considered to be high enough to cause significant pathological change (Wagner *et al.*, 1974). This figure is more than 100 $\times$ the present British hygiene standard. Higher concentrations were avoided, because chrysotile asbestos tends to produce more "thistledown" flocs, and so form clouds which have a high proportion of non-respirable material. As this does not occur for amphibole asbestos it is very difficult to draw a direct comparison between the different types at higher concentrations. The chrysotile and crocidolite clouds calculated to have the equivalent number concentrations had 2 mg/m^3 and 5 mg/m^3 respectively (Beckett, 1975).

This study was undertaken using white SPF rats of the Han strain. The 5 groups each consisted of 48 animals aged 3 months at the start of the experiment. They were exposed to asbestos fibre for 7 h/day, 5 days a week, for a total of 224 days during an elapsed time of one year. Twenty rats of similar age were maintained in the same unit as controls. So that a comparison could be made with previous experiments, U.I.C.C. samples of amosite, chrysotile A and U.I.C.C. crocidolite asbestos dust samples (Timbrell, Hyett and Skidmore, 1968) were used. The clouds were generated with a modified Timbrell dust generator (Timbrell *et al.*, 1970) and the inhalation chambers were of design similar to Timbrell's but with dimensions modified

slightly to fit the available space. The dust was size-selected by a cyclone system (Beckett, 1975) before being added to the chamber airstream. This ensured a high proportion of respirable dust in the clouds. Gravimetric monitoring was carried out during dusting, and daily mass concentration measurements obtained for all the chambers. The N.C.B.-M.R.E. sampler (Casella Type 113A; Dunmore, Hamilton and Smith, 1964) was used to measure the concentrations in the crocidolite and amosite chambers. At 10 mg/m^3 with chrysotile this instrument had been found to undersample, and a vertical elutriator system (Beckett, 1975) was therefore used to monitor the chrysotile clouds. This had been shown to give similar results to the M.R.E. with both crocidolite and amosite. Measurements were also made of the total dust in the chamber.

For the chambers whose clouds were planned to be of equal fibre number, additional monitoring was undertaken, using the standard sampling method described by the Asbestosis Research Council (1971). Each membrane-filter sample was taken using an open Gelman filter holder facing downwards, at a flow rate and sampling time calculated to give an optimum density for the microscopical examination (1–3 fibres per graticule area). The filters were counted with a phase-contrast microscope containing a "Walton and Beckett" eyepiece graticule (Walton and Beckett, 1977) to define the area of the field of view being evaluated. At least 50 samples were taken in each chamber during the inhalation period, not more than one per day. The fibres counted were those with a length greater than 5 μm, a diameter less than 3 μm and an aspect ratio of more than 3 : 1. Fibre length and diameter distributions were obtained partly by phase-contrast microscopy and partly by scanning electron microscopy (Beckett, 1973).

Four animals from each inhalation chamber were killed one year after the start of dusting, and 4 more 6 months later. The remaining animals were left with the intention of allowing them to survive their full life-span, in order to study the frequency of lung-tumour development. However, the survival of the population was extremely good and 56 animals were still alive 860 days after the start of dusting. It was decided to terminate the experiment at this point, and all the remaining animals were killed.

Tissue used for histological examination was fixed with 10% formal saline solution and embedded in paraffin wax. Lungs were fixed by inflation. Sections were stained with either haematoxylin and eosin (H. and E.), Van Geison's method for collagen or Gordon Sweet's stain for reticulin.

For the quantitative estimation of fibrotic lesions produced in the rat lungs by the different asbestos clouds, the following method was adopted. Lung tissue was examined from all the animals killed at the first 2 intervals 12 and 18 months after the start of dusting. Of the animals that survived until the final killing date at 860 days, 6 were examined from each group. The remaining animals were examined only for the presence or absence of tumours. The quantitative estimations of the fibrous lesions produced in rat lungs by the different asbestos clouds were undertaken using the following procedure. The entire lung tree with the heart was embedded together, and sections were cut in the coronal plain to include parts of all lobes. Sections were cut at 4 different levels in each block, and were at least 1 mm apart, and groups of serial sections were mounted from each of these levels for use with the different staining techniques. For all lesions, the H. and E. sections from each animal were scanned with the light microscope using an eyepiece graticule consisting of a 1 cm square subdivided into 100 units of 1 mm^2. Viewing magnification was $\times$ 60. The area of large regions of interstitial fibrosis was estimated for each slide by counting the number of grid squares involved and presenting the results as a percentage of the total lung tissue in the section. An average figure for the animal was produced by combining the result from all 4 sections. The very early fibrotic lesions were usually much smaller than one grid square at the magnification involved and since they were associated with the respiratory bronchials they were also widely scattered. For this type of small lesion, the calculations were based on the number of squares that contained the small areas of fibrous tissue and the results from all 4 sections were again presented as a percentage.

Asbestos retained in the lungs of selected animals was recovered by a low-temperature ashing process. This was conducted in a stream of O_2 excited by a radio-frequency discharge (Gleit and Holland, 1962). Any residual lung salts were removed by washing

the samples in 3 ml of cold ($\sim 20°C$) 0·2M HCl before gravimetric estimations of the amounts of asbestos recovered were made using the infra-red spectrophotometric techniques described by Middleton, Beckett and Davis (1977). To determine the percentage retention of the different dust types it was assumed that the rats breathed at the rate of 100 cm^3/min during dusting. Calculations were made using this volume and the gravimetric levels of each dust cloud.

Dust retention estimations were undertaken on the left lungs of animals, the right lung being retained for histological study on each occasion. At the first killing date (12 months after the start of dusting) 2 left lungs were analysed from each group of animals, but on the second occasion 6 months later 4 left lungs were available from each group.

Because of the suggested association between laryngeal carcinomas and asbestos in humans (Stell and McGill, 1973) the larynxes were examined from all animals, both in the 5 experimental groups and in the controls. For histological examination the larynx was serially sectioned in the longitudinal plane and approximately 8 evenly spaced sections were mounted for examination from each specimen.

RESULTS

The dust parameters for the 5 chambers over the period are given in Table I. The mass concentrations were very close to the target set at the beginning of the experiment. More than 50% of the daily concentration measurements in the equal-mass chambers were within 3 mg/m^3 of the target concentration. The 3 equal-number chambers were dosed at gravimetric concentrations determined by a number *vs* mass correlation obtained during previous short-term experiments (Middleton *et al.*, 1977). This correlation was based on 30 membrane-filter samples for each type of asbestos and had a large uncertainty (coeff. of variation $\sim 70\%$). This was due to the fact that the mass concentrations were integrated measurements taken over 7 h. The counting samples, on the other hand, were limited to a few minutes, owing to the high dust concentrations giving deposits which were too dense to evaluate for the larger-volume samples. As fluctuations in concentration occur during the day, and membrane filter samples cannot be evaluated with a reliability better than $\pm 30\%$ (National Health and Medical Research Council, 1976), uncertainties of this order are inevitable.

In this present study, between 50 and 100 membrane-filter samples were evaluated during the 12-month inhalation period to check this correlation, and gave mean fibre concentrations of 550 fibres/ml for amosite, 390 fibres/ml for chrysotile and 430 fibres/ml for crocidolite. This meant that 0·1 mg of dust/m^3 of air was equivalent to 19·5, 8·6 and 5·5 fibres/ml for chrysotile, crocidolite and amosite respectively. The uncertainty in the measurements was of a similar order to that in the previous experiments. There was no significant difference between the fibre-number concentrations in the crocidolite and chrysotile chambers ($P = 0·4$), but the amosite chamber was significantly

TABLE I.—*The Mean Mass and Fibre-number Concentrations over the Exposure Period*

Asbestos Type	Chrysotile	Chrysotile	Crocidolite	Crocidolite	Amosite
Type of cloud	Equal mass	Equal fibre number	Equal mass	Equal fibre number	Equal mass and fibre number
Target concentration (mg/m^3)	10·0	2·0	10·0	5·0	10·0
Mean mass concentration (mg/m^3)	9·9	2·0	10·0	4·9	10·0
Mean ratio of total to respirable dust	1·4 : 1	1·3 : 1	1·2 : 1	1·1 : 1	1·15 : 1
Mean fibre-number concentration (fibres/ml >5 μm)	1950*	390	860*	430	550
Mean fibre-number concentration (fibres/ml >20 μm) (estimated from size-distribution data)	360	72	34	17	6

* Estimated figure

different from the other two ($P<0{\cdot}01$). The animals in this chamber were therefore probably dosed with a slightly higher average number of fibres. The difference between the fibre-number concentrations was, however, very much smaller than for the equal mass chambers.

Taking a series of short-period samples to monitor the fibre number exposure, although subject to this large uncertainty, does in fact correspond closely to the industrial situation, where 10 min samples are frequently taken to monitor a person's exposure (Department of Employment and Productivity, 1970).

A series of samples on Nuclepore filters were taken in addition to those on membrane filters. These were used to measure the size distribution of the fibres using a scanning electron microscope. No significant difference was found between the different samples from the same chambers. The length distribution of fibres longer than 0·6 μm and the diameter distribution of fibres broader than 0·2 μm are shown in Fig. 1 and Fig. 2 respectively.

The survival times from the animals from the five inhalation chambers are shown in Table II. These indicate that there were no significant differences in survival times between animals treated with the different asbestos clouds. When the average weight of animals in the different groups was considered, however, some differences were noticeable. At the end of the 12-month inhalation the rats from the 3 amphibole chambers averaged between 500 and 510 g each. Those from the high and low chrysotile chambers, however, averaged 465 and 467 g respectively. This differential was gradually reduced with time, until at 20 months after the start of dusting all groups averaged slightly over 500 g per animal, with the exception of the low-crocidolite group where the average was 494 g per animal. Subsequently, with advancing age, all animals gradually lost weight, but there were no significant differences between the different dust groups.

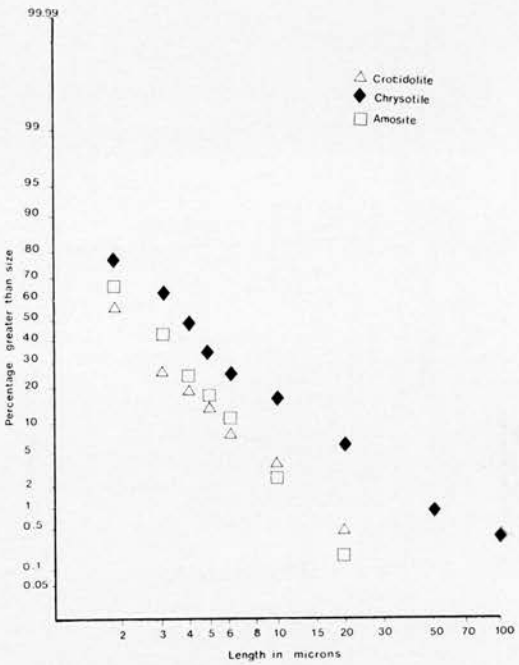

FIG. 1.—Length distributions of fibres longer than 0·6 μm. (Scanning electron microscope measurements.)

TABLE II.—*Survival patterns for the Animals in the Different Inhalation Groups. The Experiment was Terminated at* 29 *Months. Groups of* 4 *animals from Each Chamber were Killed at both* 12 *and* 18 *months*

	Months after start of exposure			
	12	18	24	29
10 mg/m³ Chrysotile	48	40	21	12
2 mg/m³ Chrysotile	48	40	26	7
10 mg/m³ Amosite	47	39	22	11
10 mg/m³ Crocidolite	47	41	25	11
5 mg/m³ Crocidolite	46	37	22	8

Light-microscope examination of lung tissue from animals in the 5 dust groups killed 12 months after the start of dusting showed 3 distinct types of lesion that could be associated with asbestos dust. None of these lesions were seen in control animals. The first type of lesion consisted of

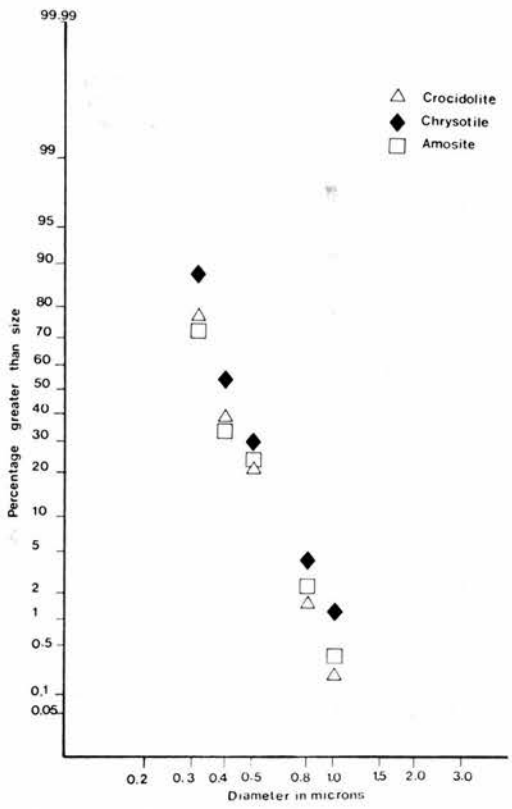

FIG. 2.—Diameter distribution of fibres broader than 0·2 μm. (Scanning electron microscope measurements.)

aggregates of dust-containing macrophages, giant cells and fibrous tissue in association with the respiratory bronchioles and alveolar ducts (Fig. 3). These areas stained strongly positive for reticulin and more weakly for collagen, although some collagen was always present at this stage. The second type of lesion consisted of the replacement of the epithelial lining of many respiratory bronchioles, alveolar ducts and associated alveoli by epithelium of bronchiolar type. It was not possible, however, to determine whether this was due to hyperplasia of the bronchiolar lining or metaplasia of the alveolar epithelial cells (Fig. 4). Both these types of lesion were frequently found together around any one respiratory bronchiole, but either could appear on its own. The third type of lesion consisted of the thickening of alveolar septa over quite large areas of lung tissue (Figs. 5 and 6). The alveoli involved were lined with rounded epithelial cells, probably Type 2 pneumocytes, and Gordon Sweet's stain showed an increase in the reticulin network in the septa walls although no collagen was present in the early stages. While most sections of alveoli in each animal contained only an occasional macrophage packed with asbestos fibres, those from areas of interstitial fibrosis were often filled with dust-containing cells. In these cases, however, it was noticeable that each cell contained relatively little dust. The areas of interstitial fibrosis could become quite large, often 4–5 mm in diameter, especially in the oldest animals, but early lesions were small, and appeared to be centred on one bronchiole. With the increasing age of the animal the depositions of fibrous tissue in the interstitial space was often greatly increased, so that the total alveolar wall thickness could become as much as 50–100 μm (Fig. 6). In these advanced cases, the thickened septa stained positive for both reticulin and collagen.

An alternative to advanced fibrosis, however, was the continued growth of the rounded epithelial cells, with the subsequent compression of the alveoli to produce an adenomatous appearance. In some cases, positive adenomas were found forming in these areas. In a few animals small areas of squamous metaplasia of the alveolar epithelium were also found.

Quantitative estimations of these 3 types of lesions are shown in Table III. It was found that both of the chrysotile clouds had produced much more of the early granulomatous deposits around terminal bronchioles and alveolar ducts than any of the amphibole dusts ($P<0{\cdot}001$). The 10 mg/m^3 chrysotile cloud had produced significantly more peribronchial fibrosis than the 2 mg/m^3 chrysotile cloud ($P<0{\cdot}001$). These lesions showed no further increase in numbers after the end of the inhalation period. Subsequent studies of tissues taken at either 6 or 17

months after the end of dusting in fact showed a slight decrease in the frequency of the lesions. However, this was due to the increased areas of interstitial fibrosis that had developed by these times, which reduced the area of tissue in which the peribronchial lesions could be recognized with certainty. The amphibole dusts

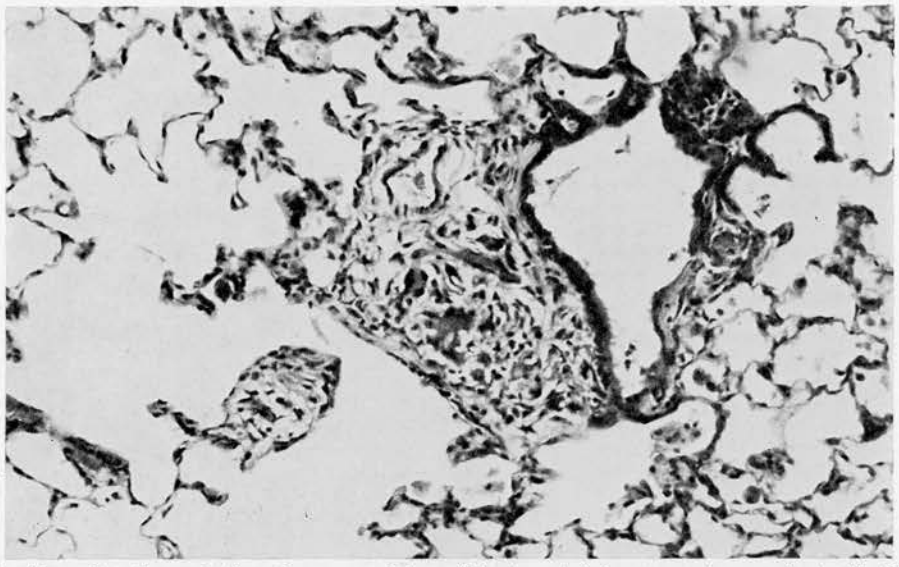

FIG. 3.—Deposits of granulation tissue, consisting of dust-containing macrophages, giant cells, fibroblasts and reticulin fibres, associated with a terminal bronchiole and several alveolar ducts. This lesion developed in a rat treated for 12 months with a cloud of chrysotile asbestos of 10 mg/m^3. $\times 250$.

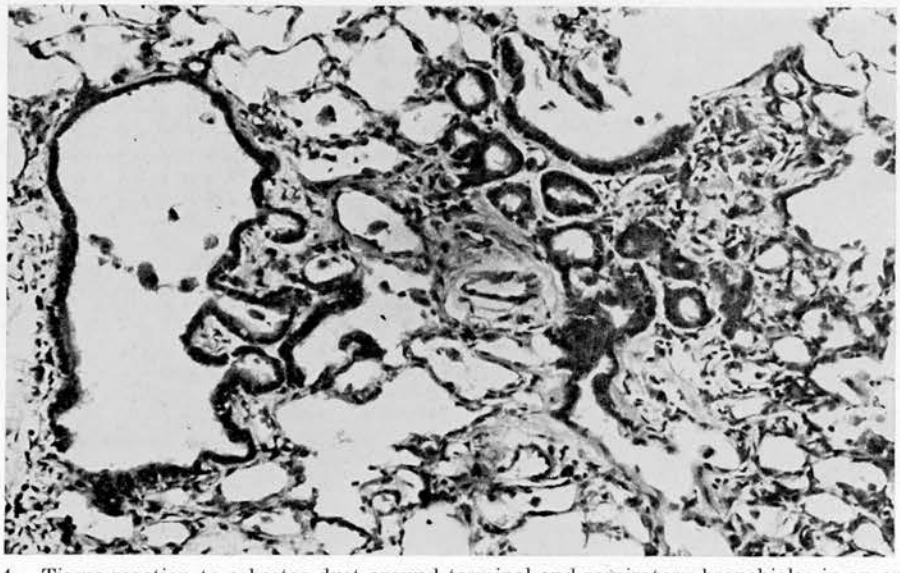

FIG. 4.—Tissue reaction to asbestos dust around terminal and respiratory bronchioles in an animal treated with chrysotile asbestos. Bronchial epithelial cells now line some alveolar spaces. $\times 250$.

TABLE III.—*Levels of Lung Fibrosis Produced by the Diffe*

	10 mg/m³ Chrysotile			2 mg/m³ Chrysotile		
Time after start of exposure (months)	12	18	29	12	18	29
Peribronchiolar fibrosis	19·3 (12·7–24·5)	17·1 (15·1–19·2)	15·0 (12·7–20·1)	10·7 (7·8–12·7)	9·9 (7·5–11·77)	7·5 (5·2–9
Extension of bronchial epithelium to alveolar ducts and alveoli	2·68 (1·28–4·4)	2·4 (2·3–2·6)	1·43 (0·7–1·9)	1·7 (1·1–2·5)	4·03 (2·8–6·6)	1·0 (0·5–
Interstitial fibrosis	0·48 (0–1·8)	0·9 (0·25–1·85)	9·15 (3·8–14·4)	0·35 (0–1·2)	0·83 (0–2·9)	3·8 (0–7
No. of rats in sample	4	4	6	4	4	6

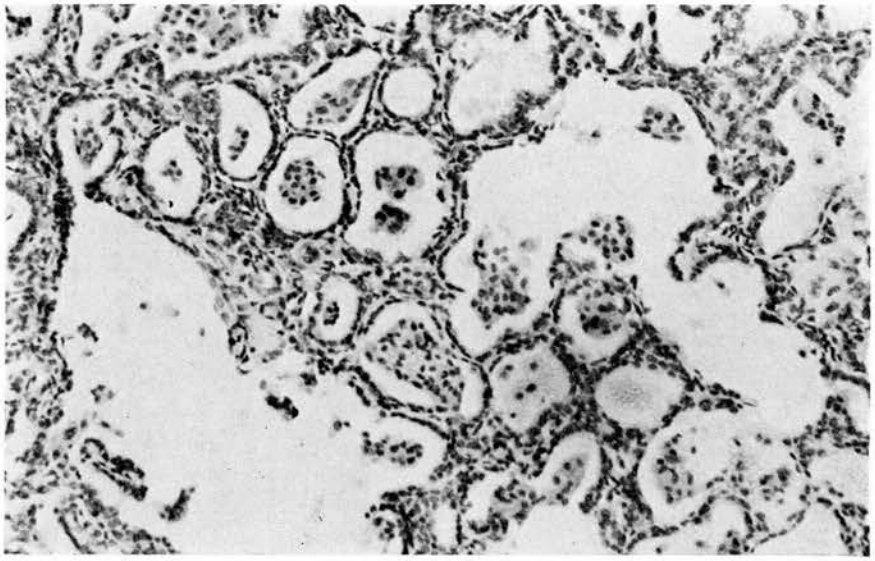

FIG. 5.—An area of interstitial fibrosis from an animal treated with chrysotile asbestos for 12 months. The alveolar septa are thickened and they are surfaced with rounded epithelial cells. Most alveolar spaces contain aggregates of cells, many of which are dust-containing macrophages. × 250.

produced relatively little early peribronchial fibrosis (Fig. 7) but dust-containing macrophages still aggregated around all the terminal bronchioles. For the most part they did not appear to be held in place by any reticulin network, and yet some aggregates were still present without associated fibrosis in the oldest animals examined (Fig. 8). The extension of bronchial epithelial cells in alveolar ducts and alveoli varied much less between the different dust clouds than the peribronchiolar fibrosis. In common with these fibrotic areas, however, there appeared to be no long-term progression of the lesions after 12 months from the start of dusting.

While areas of peribronchiolar fibrosis and peribronchiolar alveolar epithelialization appeared evenly distributed through the lungs of any animal examined, areas of widespread interstitial fibrosis were much more haphazardly arranged, and these areas were completely absent from some animals examined at between 12 and 29 months after the start of the experiment. Consequently the figures for interstitial fibrosis at the 12-month stage based on only 4 animals in each group are not considered to show significant differences between the asbestos types. Only 4 animals were included in the groups taken at 18 months, so that the same consideration might apply although by this time most animals treated with chrysotile had

)estos Clouds (Parameters as Described in Methods section)

10 mg/m³ Amosite			10 mg/m³ Crocidolite			5 mg/m³ Crociodolite		
12	18	29	12	18	29	12	18	29
4·12	5·1	4·2	2·68	4·25	3·9	2·8	2·3	2·47
)–5·5)	(3·8–5·9)	(2·5–5·5)	(1·25–4·05)	(2·6–6·4)	(2·5–6·0)	(2·2–3·8)	(2·1–2·6)	(1·25–4·1)
2·27	3·9	3·05	0·85	2·45	1·96	1·35	1·25	1·69
6–3·2)	(1·0–6·0)	(1·8–5·5)	(0·47–1·27)	(1·3–4·9)	(1·1–3·6)	(0·9–1·6)	(1·2–1·6)	(0·97–3·4)
)·87	0·12	2·58	0	0·07	1·38	0·42	0·04	0·76
-3·24)	(0–0·4)	(1·1–5·1)	(0)	(0–0·27)	(0–4·1)	(0–1·7)	(0–1·7)	(0–2·23)
4	4	6	4	4	6	4	4	6

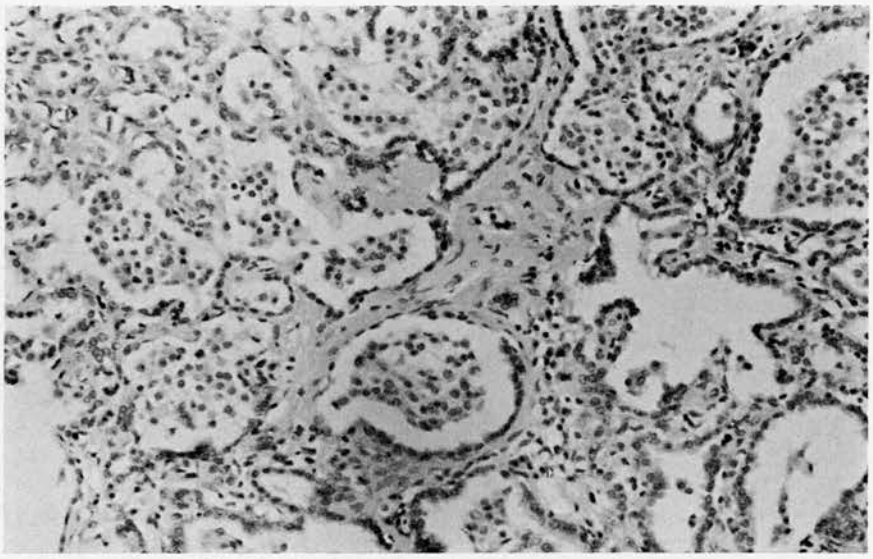

FIG. 6.—Advanced interstitial fibrosis in a 32-month-old rat after the inhalation of chrysotile dust. Some alveolar septa are $>100\ \mu m$ in thickness and stain strongly positive for collagen. $\times 250$.

noticeably more interstitial fibrosis than those treated with either amphibole sample. For the last sample, 17 months after the end of dusting, 6 animals were available from each group. The figures show that all groups had by this time developed significantly more interstitial fibrosis than had been present 11 months earlier. The high chrysotile cloud had produced large areas of fibrosis in all the animals examined, with average figures more than double those for any other group ($P<0{\cdot}001$). The differences between the other 4 asbestos clouds were less dramatic, but there appeared to be a definite gradation, with the low chrysotile cloud having produced more interstitial disease than any of the amphiboles ($P<0{\cdot}01$). At the same time, amosite appeared to have produced more damage than the 2 crocidolite clouds and the high crocidolite had produced more fibrosis than the lower cloud of the same material. The levels of significance of these latter observations is, however, low.

The incidence of neoplasms of the lung and mesotheliomas that were found in the different experimental groups is shown in Table IV. The incidence of lung tumours closely follows the level of lung fibrosis, and all the malignant lung tumours were found in animals that had inhaled chrysotile dust ($P<0{\cdot}001$). Even benign pulmonary adenomas were more frequent in these

TABLE IV.—*Lung Tumours and Mesotheliomas*

Tumour	10 mg/m³ Chrysotile 40 animals	2 mg/m³ Chrysotile 42 animals	10 mg/m³ Amosite 43 animals	10 mg/m³ Crocidolite 40 animals	5 mg/m³ Crocidolite 43 animals	Control 20 animals
Adenoma	7	6	2	1	2	0
Adenocarcinoma	6	1	0	0	0	0
Squamous carcinoma	2	1	0	0	0	0
Pleural mesothelioma	0	0	0	0	1	0
Peritoneal mesothelioma	0	1	0	0	0	0

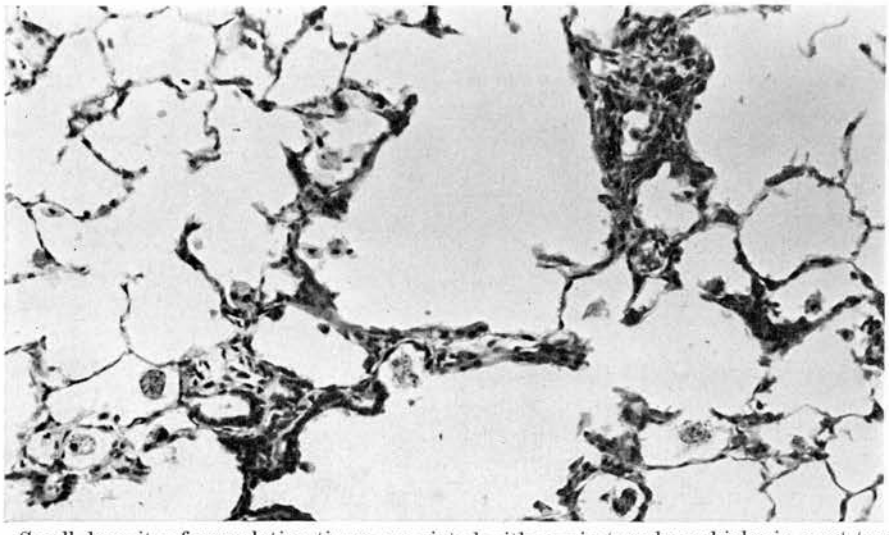

FIG. 7.—Small deposits of granulation tissue associated with respiratory bronchioles in a rat treated with amosite dust. These areas contained dust-laden macrophages, fibroblasts and reticulin fibres. Giant-cell formation was rare in the lesions caused by both amosite and crocidolite asbestos. ×250.

2 groups than in animals treated with either variety of amphibole ($P = 0{\cdot}006$). Four of the adenocarcinomas had metastasized to the pleural cavity (Fig. 9). The typical histological pattern of one of the squamous tumours is illustrated in Fig. 10. Neither had metastasized to the pleural cavity, although they had reached diameters of 5 and 11 mm respectively and had caused marked swelling of the lung lobes involved. Both showed evidence of direct invasion into the surrounding tissues. Only 2 mesotheliomas were found in this study, one solitary spindle-cell tumour in the pleural cavity of an animal treated with crocidolite, and an abdominal mesothelioma in an animal that had inhaled chrysotile. This latter tumour showed the histological pattern previously described (Davis, 1974). No pulmonary tumours were found in control animals.

The tumour incidence from sites other than the lung, and excluding mesotheliomas, is shown in Table V. If the tumour totals for each group are compared, the high chrysotile group and the amosite group appear to have more evidence of neoplasia than controls. However, with the relatively small groups of animals these differences are not significant. Of interest was the finding of relatively large numbers of peritoneal connective-tissue tumours. One was a leiomyofibroma that had developed on the wall of the small intestine. The remaining tumours, however, were malignant and multiple, and macroscopically were very similar to peritoneal mesotheliomas. Histological examination,

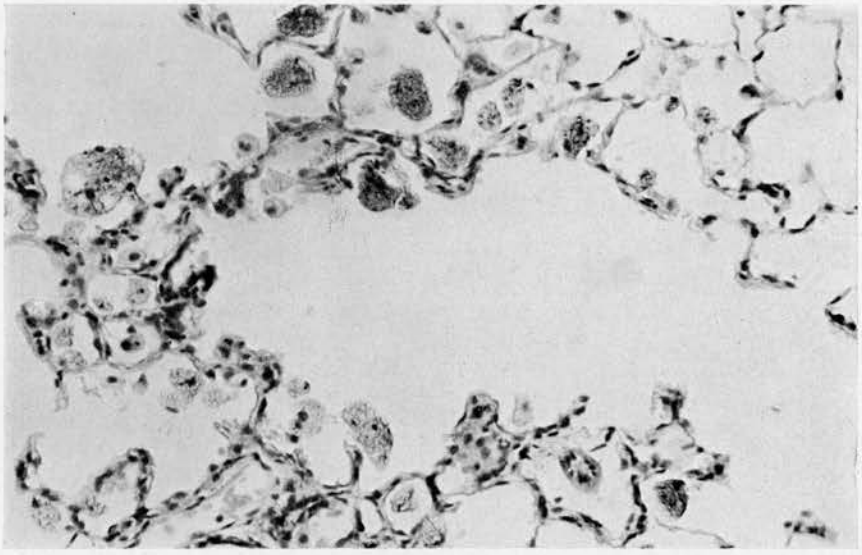

FIG. 8.—Aggregations of macrophages packed with amosite fibres around alveolar ducts in the lungs of a 32-month-old rat. ×250.

TABLE V.—*Sites of Tumours other than Lung* (*B, benign, M, malignant*)

	Chrysotile 10 mg/m³ 40 animals		Chrysotile 2 mg/m³ 42 animals		Amosite 10 mg/m³ 43 animals		Crocidolite 10 mg/m³ 40 animals		Crocidolite 5 mg/m³ 43 animals		Controls 19 animals	
Site of tumour type	B	M	B	M	B	M	B	M	B	M	B	M
Subcutaneous connective-tissue tumours	1			1	2	3		1		1		1
Peritoneal connective-tissue tumours		2		1	1	1		2		3		1
Osteosarcomas		1		1								
Testicular tumours			1		4		1					
Squamous tumours of the Epidermis	1	1			1			1				
Parotid tumours		2								1		
Adrenal tumours			1			1						
Thyroid tumours		2		1	1					1		1
Lymphoma/leukaemia				1								
Pancreatic tumours	2											
Totals	4	8	2	5	9	5	1	4	0	6	0	3

however, showed marked differences from the mesotheliomas normally found in rats. Some appeared to be poorly differentiated fibrosarcomas, others showed gross cellular and nuclear pleomorphism and 2, including one found in a control animal, contained large multinucleate cells mixed with small spindle cells.

Histological examination of larynxes from the animals in this study showed no tumours. In the oldest animals that had inhaled asbestos dust, some small areas of epithelial hyperplasia were found involving squamous cells, usually at the bases of the vocal cords. However, similar areas of hyperplasia were found in control animals, and it was assumed that these changes were associated with advanced age.

The weights of asbestos dust extracted from the lungs of animals in the different inhalation groups is summarized in Table

VI. Although on this occasion only left lungs were available for dust estimation, previous short-term inhalation studies had involved dust estimation from both lungs taken separately, and these had indicated that the asbestos content ratio between the left and right lung was 0·6 to 1. Figures in Table VI, therefore, indicate actual left lung content and the estimated total lung content calculated from the above ratio. These calculations indicate that, for a given dust cloud, far more amphibole asbestos is deposited and retained in the lung than is the case with

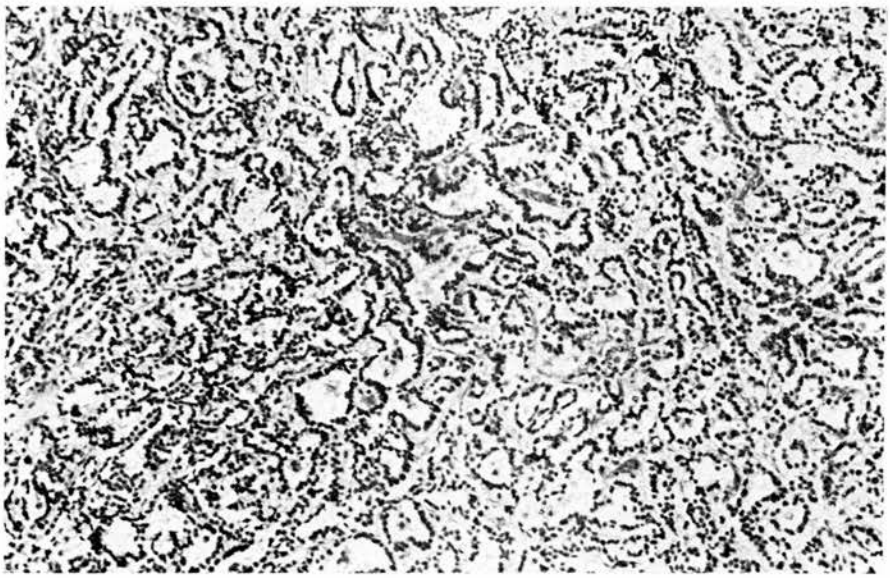

FIG. 9.

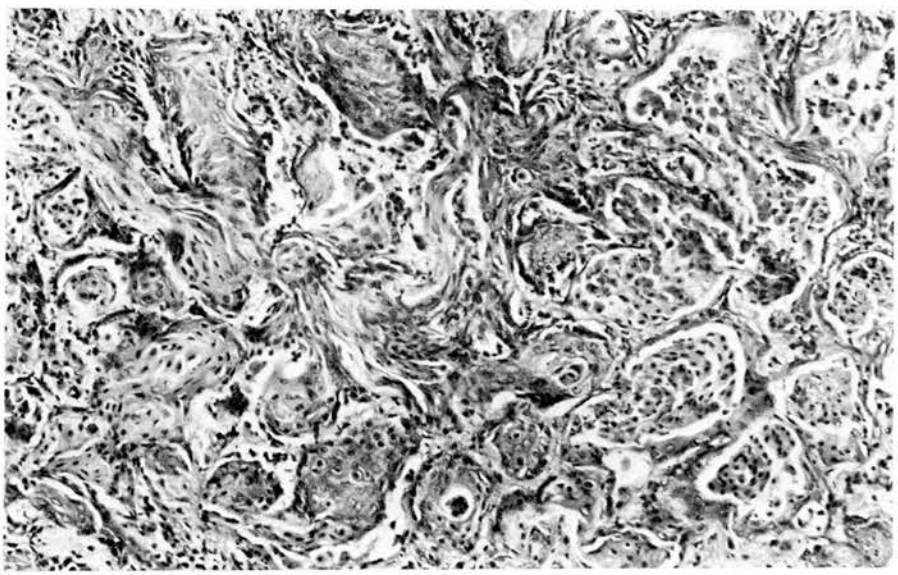

FIG. 10.

FIGS. 9 and 10.—Histological patterns of an adenocarcinoma and a squamous-cell carcinoma that developed in the lungs of rats treated with chrysotile asbestos. ×250.

TABLE VI.—*Levels of Asbestos Recovered from Lung Tissue*

Type of asbestos	Respirable concentration (mg/m³) Target	Actual	Days after exposure	Recovered asbestos μg/left lung (Actual)	μg/rat (Estimated)	Estimated % retention
Chrysotile	10	9·9	7	520	1417	1·5
			182	228	648	0·7
Chrysotile	2	2·0	7	193	526	2·8
			182	66	180	1·0
Crocidolite	10	10·0	7	3212	8750	9·3
			182	2731	7440	7·9
Crocidolite	5	4·9	7	1279	3484	7·6
			182	976	2659	5·8
Amosite	10	10·0	7	3366	9169	9·7
			182	2577	7020	7·5

chrysotile. For the clouds of 10 mg/m³, the lung content of both amosite and crocidolite at the end of the dusting period was very close, while the chrysotile content was only 15 to 16% of this figure. A comparison of the 2 chrysotile clouds indicated that the percentage retention after 12 months of dusting was twice as high for the 2 mg/m³ cloud as for 10 mg/m³. With crocidolite, however, the percentage retention for the 10 mg/m³ cloud was slightly higher than for the 5 mg/m³ cloud. The differences in lung asbestos content between 7 and 182 days after the end of dusting would indicate that chrysotile had been cleared from the lung much more quickly than the amphiboles. During this period both the chrysotile groups showed a reduction in lung dust content of 50 to 70% while the comparable figures for the amphibole clouds were only 15 to 25%.

DISCUSSION

In this study of the effects of fibre mass and fibre number on asbestos-related lung disease, it was clearly demonstrated that a given airborne mass of U.I.C.C. Rhodesian chrysotile produced far more lung fibrosis than the same airborne weight of U.I.C.C. samples of either amosite or crocidolite. This indicates that a single mass standard for all types of asbestos would be inappropriate. To some extent, a comparison of the 3 dust types on a fibre-number basis was spoilt by the high fibre count of the amosite cloud. However, the figures for the 2 mg chrysotile and the 5 mg crocidolite clouds were extremely close, 390 and 430 fibres/ml respectively Once again the animals treated with chrysotile had developed significantly more lung fibrosis than those treated with crocidolite. All these results could be taken to indicate that chrysotile is much more fibrogenic than either of the amphiboles, and they might be considered to agree with *in vitro* cytotoxicity studies which have reported that chrysotile causes greater cell damage than either amosite or crocidolite (Klosterkötter and Robock, 1975). From this it might be suggested that the standards for chrysotile should be more strict than for either amosite or crocidolite. In fact, however, consideration of the fibre length distribution of the various dust clouds given in Fig. 1 suggests another possibility. Fibre counting for monitoring the dust clouds supposed to have equal fibre numbers was undertaken using the procedure laid down for the present hygiene standards (Health and Safety Executive, 1976) which records all fibres over 5 μm in length but does not allow for fibre variations above this length. A complete fibre-length distribution produced by scanning electron microscopy showed that the chrysotile clouds used in the present study had many more fibres over 20 μm in length than either of the amphibole clouds (Fig. 1 and 2). No reliable estimates are available relating fibrogenic potential to fibre length, but a number of authors have suggested that for mesothelioma production at least, carcinogenicity depends on fibre lengths in excess of 10 to 20 μm (Maroudas, O'Neal and

Stanton, 1973; Stanton *et al.*, 1977). Since in the present study the only malignant lung tumours were produced by chrysotile and this was also by far the most fibrogenic, these results could support the long-fibre theory of carcinogenesis in general, and also indicate that the same parameters are involved in the fibrogenic response. This would indicate that the present protocol for fibre counting is inadequate, and that either the 5 μm limit for counting should be raised, or counts should be broken down into different fibre-length groups. Since, however, biological knowledge on the exact lengths of fibre that cause damage is still not definite, a suitable compromise might be to retain the present 5 μm lower limit, but to include an additional count of fibres >20 μm long. It might be found that this latter figure correlates better with epidemiological data for asbestosis and bronchial carcinomas than the 5 μm counts.

The finding that chrysotile asbestos produced far more lung fibrosis and pulmonary neoplasia than the amphibole asbestos types was not expected from previous animal inhalation experiments. Wagner *et al.*, in a large study published in 1974, had included groups of rats treated for 12 months with 10 mg/m^3 clouds of U.I.C.C. samples of Rhodesian chrysotile, amosite and crocidolite, so that the results should have been directly comparable with those of the present study. However, they reported similar levels of lung fibrosis for all groups, and the number of malignant lung tumours produced by the chrysotile and crocidolite clouds were closely comparable although the amosite cloud produced only one such tumour. The reason for this discrepancy between the 2 studies is difficult to determine, since the dust-retention figures in both studies are extremely close. It may be that the elutriation systems used in the 2 studies differed. Wagner did not give fibre-length distributions for the dust clouds used and it seems likely that the chrysotile clouds used in the present study had a higher proportion of long fibres.

Whether the increased fibrogenic and neoplastic effect of chrysotile found in this study was due to chrysotile itself, or to increased fibre lengths in the chrysotile clouds, it does not change the position regarding human hazards from chrysotile exposure, since the present British industrial asbestos dust standards were based on epidemiological data from chrysotile-exposed working populations. The human position regarding the types of chrysotile cloud met with in industry is, therefore, already known, but the new data indicate that some amphibole clouds may be less dangerous than previously expected.

At present crocidolite is considered in most countries to be the most dangerous asbestos type and its use is banned in some cases. However, this situation is largely due to the association of crocidolite with the production of mesotheliomas in humans. This connection is well documented, but there are no epidemiological data indicating that crocidolite is worse than the other asbestos types at producing lung fibrosis or bronchial carcinomas. The present study would indicate that as far as lung pathology is concerned crocidolite is the least dangerous of the absestos types examined, even though as much as 6$\times$ more crocidolite than chrysotile was retained after one year of dusting. Because mesothelioma production in response to asbestos inhalation is a very rare event in both animals and humans, animal studies so far undertaken have been unable to produce statistically significant numbers of these tumours for an accurate comparison between the various forms of asbestos. Wagner et al. (1974) reported 5 mesotheliomas from 76 animals with tumours in both the chrysotile and crocidolite groups. We found only 2 mesotheliomas in 123 animals but again both crocidolite and chrysotile were implicated. No mesotheliomas were produced by amosite in either study after 12 months, but Wagner did find one mesothelioma in an animal treated for only one day with amosite dust.

The use of asbestos clouds of differing density over a long inhalation period of 12

months has made it possible to continue the study of asbestos deposition and retention that was commenced using short-term administration of asbestos dust (Middleton *et al.*, 1977). It has been confirmed that the percentage lung retention of chrysotile is much lower than either amphibole types and also that retention is reduced when the density of the cloud is increased. With a 2 mg/m^3 cloud the percentage retention of chrysotile is almost double that for a 10 mg/m^3 cloud. The retention of crocidolite, however, shows the reverse, and retention is marginally higher with the denser dust cloud. The reasons for this have not been determined with certainty, but measurements of fibre-length distribution of retained lung dust in rats is in progress. These results may indicate the reasons for these differences.

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