Ann. occup. Hyg., Vol. 26, Nos. 1–4, pp. 569–582, 1982.
Printed in Great Britain
Inhaled Particles V

0003-4878/010569-14$03.00/0
Pergamon Press Ltd.
© 1982 British Occupational Hygiene Society.

VARIATIONS IN THE CARCINOGENICITY OF MINERAL FIBRES

R. E. BOLTON, J. M. G. DAVIS, K. DONALDSON and ANNETTE WRIGHT

Institute of Occupational Medicine, Edinburgh, U.K.

Abstract—An intraperitoneal injection assay system in laboratory rats was used to examine the relative carcinogenicity of five asbestiform dusts of chrysotile origin and two of amosite origin. Twenty-five milligram suspensions of dust were injected into each of 32 rats for each treatment and the animals subsequently monitored for the development of tumours. The intraperitoneal route was shown to provide a useful assay of the tumour-producing potential of particulate materials, with an increased sensitivity when compared with intrapleural inoculation methods. The results showed that the chrysotile samples tended to be more carcinogenic than the amosite preparations and some of the possible reasons for this are discussed. It was concluded, from estimations of particle size using the scanning electron microscope, that relative fibre length did not provide a useful measure of the carcinogenicity of the seven dusts, owing in part to problems arising from the preparation of realistic, representative, samples for microscopy.

INTRODUCTION

THE ASSOCIATION between asbestos exposure and primary mesothelioma of the pleura and peritoneum was first demonstrated by WAGNER *et al.* (1960) amongst crocidolite miners and millers of South Africa. Several epidemiological studies have since shown that a history of exposure to asbestos is associated with an increased risk of development of mesothelial tumours many years later (SELIKOFF *et al.*, 1970; NEWHOUSE, 1973; MCDONALD and LIDDELL, 1979). All the three main commercial types of asbestos (chrysotile, crocidolite, amosite) have now been implicated and there is some evidence that crocidolite is particularly active.

The experimental production of mesothelioma in animals following asbestos inhalation has proved to be somewhat difficult with only occasional mesotheliomas developing after prolonged exposure (REEVES *et al.*, 1974; WAGNER *et al.*, 1974; DAVIS *et al.*, 1978), although, once again, all the three main types of asbestos have been implicated. WAGNER (1962) showed that mesothelial tumours could be simply produced in appreciable numbers in rats by intrapleural inoculation with suspensions of asbestos dust. Whilst this method may be criticized for failing to provide an adequate mimic of the biological protective clearance mechanisms associated with the normal (pulmonary) portal of entry of fibre into the body, it has proved to be a useful carcinogenicity bioassay of particulate pollutants. The work has since been extended by many workers using either intrapleural injection techniques (SMITH *et al.*, 1965; WAGNER and BERRY, 1969; WAGNER *et al.*, 1970) or a direct intrapleural implantation technique (STANTON and WRENCH, 1972), to provide comparisons of the biological activity of fibrous particulates.

In our laboratory industrial mineral dusts are tested for their harmful effects and we routinely undertake both inhalation studies to examine the dusts' potential to produce

EXHIBIT
48
labelbar

pulmonary fibrosis and tumours and injection studies to explore their potential for mesothelioma production. The present study is part of a programme designed to examine and compare the biological effects of the standard UICC asbestos samples with dusts collected from other sources, including experimental and industrial applications. We report here the results of the first of the injection assays, in which the carcinogenicity of seven different types of asbestiform material have been examined using laboratory rats. We have chosen to use the intraperitoneal injection route, since previous experience in our laboratory has indicated that this may be a more sensitive method of bioassay than intrapleural inoculation. Some comparison will be made with the results of *in vitro* tests using the same dusts (Wright *et al.*, 1980). The first of the parallel inhalation studies has been reported elsewhere: UICC dust samples (Davis *et al.*, 1978); factory dust samples (Davis *et al.*, 1980).

METHODS

A total of 232 male AF/HAN random-bred spf Wistar laboratory rats was used, 8–10 weeks old at the time of injection, housed four/cage with *ad libitum* access to standard pelleted laboratory diet (BP Nutrition Ltd) and tap water. They were stratified by age and then randomly allocated into seven treatment groups of approx. 32 animals each, as shown in Table 1. An additional four animals were added to two of the chrysotile-treated groups to replace those dying from acute peritonitis in the first few days following injection. The seven dusts are described in summary form in Table 1. All the samples are described as 'elutriated' to signify that they were collected from airborne asbestos clouds generated as part of the inhalation studies (Beckett, 1975) by using an absolute filter assembly in the ducting between the dust generator and the animal exposure chamber. This was undertaken in an attempt to provide a closer comparison of the results of inhalation and injection experiments by using dusts prepared in a similar manner. Representative samples of the various dusts were prepared on Nuclepore membrane filters (Beckett, 1973), both from the airborne clouds and from the liquid injection suspensions, for estimations of the particle size distributions using a scanning electron microscope (Cambridge Instruments S.600) and the criteria of Schneider (1979). Thus an aspect ratio of 3:1 was used and all particles that conformed to or exceeded this were assessed. For practical purposes, constraints imposed by the working resolution of the scanning electron microscope

Table 1. Experimental layout

Dust sample (25 mg injected i/p in 2.0 ml PBS)	No. of animals injected
Elutriated UICC amosite	32
Elutriated factory amosite	32
Elutriated UICC chrysotile 'A'	36
Elutriated factory chrysotile	32
Elutriated heated chrysotile	32
Elutriated parent chrysotile	36
Elutriated wet-dispersed chrysotile	32

meant that there was a minimum fibre diameter slightly less than 0.2 μm and therefore, by inference, a minimum fibre length of approximately 0.6 μm. There were no effective maximum limits to the fibre dimensions. The data is expressed graphically as cumulative fibre length distributions from which the relative proportion of fibres exceeding certain stated lengths may be obtained.

Table 1 shows that elutriated samples of UICC amosite and chrysotile 'A' have been used, together with two 'factory' samples that were originally obtained from the exhaust filter units at asbestos factories. These factory samples were found to contain other undefined non-asbestos, largely non-fibrous, impurities. The contamination was considerable: the chrysotile sample contained only 60% chrysotile by i.r. estimation, and the amosite sample was found to contain 90% amosite. The non-asbestos component was not further characterized, but analysis did confirm that there was no cross-contamination of each sample with other asbestos types. The other asbestiform samples tested for carcinogenicity were: a chrysotile preparation heated up to 850°C; an unheated 'parent' commercial Canadian chrysotile sample, and an experimental preparation of 'wet dispersed' chrysotile produced during the development of anionic surfactant textiles (HERON and HUGGETT, 1971).

After the elutriated samples of each dust had been collected, they were dry heat sterilized at 60°C for 30 min, mixed with sterile Dulbeccos PBS (Biocult) and injected intraperitoneally into rats, each animal receiving 25 mg of dust suspended without ultrasonication in 2.0 ml PBS under light ether anaesthesia. All animals were examined daily and were killed when distressed or moribund. Representative histological tissue samples were taken from the majority of mesothelial tumours and from any other abnormalities noted at autopsy.

The results were analysed using estimates of the survival function based on the product limit (KAPLAN and MEIER, 1958) method of calculation. This is similar to the actuarial life table methods used by other workers (BERRY and WAGNER, 1969), except that it is based on individual survival times, thus providing the maximum amount of information from the relatively small experimental groups. The method permits a survival function to be calculated for each individual death, based upon the proportion of individuals within the treatment group surviving at the time of each death. The mortality experience of each treatment group was divided into two components, one related to mesothelioma and one related to other causes of death, and the survival functions calculated for each death within each group. A BMD program was used for the computations (BMDP 1L, revised November 1979, originally developed at the Health Services Computing Facility, University of California, under NIH Grant RR3).

RESULTS

A total of 11 deaths from acute peritonitis occurred amongst the experimental groups in the first 10 days following injection. Of these, five occurred with the elutriated UICC chrysotile 'A' sample and three with the elutriated 'parent' chrysotile sample. An extra four animals were added to each of these two treatment groups, making a total of 221 animals available for analysis, distributed as shown in Table 2. The number of peritoneal tumours produced in each treatment group is also shown in Table 2, and it can be seen that the dose level of 25 mg of dust caused the production of mesothelial tumours in almost all animals in six treatments out of seven.

Table 2. Details of the numbers of mesotheliomas produced amongst the treated animals

Treatment	No. of animals injected	No. of animals after 14 days*	No. of peritoneal mesotheliomas	Percentage with peritoneal mesothelioma
Elutriated UICC amosite	32	32	30	94
Elutriated factory amosite	32	31	29	94
Elutriated UICC chrysotile 'A'	36	31	30	97
Elutriated factory chrysotile	32	30	29	97
Elutriated heated chrysotile	32	32	13	41
Elutriated parent chrysotile	36	33	33	100
Elutriated wet-dispersed chrysotile	32	32	32	100

* i.e. the number of animals available for long-term follow-up.

The structure of the peritoneal mesothelioma produced in experimental animals by the injection of asbestos has been described in detail by Davis (1974a). The majority of the peritoneal tumours produced in the present study were of the classical advanced multinodular type with copious straw-colour or blood-stained ascites, numerous free floating nodules, and widespread nodular growth over the visceral and parietal surfaces. A small proportion (11 out of a total of 196) of the tumours were macroscopically predominantly plaque-like, with sheets of thickened cellular tumour masses covering both parietal and visceral surfaces. The variable histological appearance of peritoneal mesothelioma previously reported for both humans and animals (Enticknap and Smither, 1964; Davis, 1974b) was confirmed in the present study, with both epithelial and connective tissue elements visible in the earlier stages and a more pronounced fibrosarcomatous form being more common in advanced tumours.

Table 3 records both the time taken for the first tumour to become apparent and the mean mesothelioma survival time (i.e. the mean time from injection to death for mesothelioma-bearing animals) for each treatment group. It can be seen that the first mesothelioma developed within 178 days of injection (with the wet-dispersed chrysotile preparation) and that there is a considerable range of values between the seven different treatments, with the heated chrysotile sample producing the first mesothelioma only after 621 days. Similarly there are large differences between the mean mesothelioma survival times of the seven treatments, with the wet-dispersed chrysotile treatment having the shortest time and the heated chrysotile the longest.

Figure 1 presents the information in graphical form, with the cumulative proportion surviving plotted against the time (in days) from injection. This method of presentation means that the survival curves for the most carcinogenic dusts are those closest to the ordinate. There is a clear separation between the curves for five out of the seven treatment groups, although there is little difference between the results for UICC

TABLE 3. COMPARISON OF THE TIME TAKEN FOR THE DEVELOPMENT OF THE FIRST TUMOUR AND OF THE MEAN MESOTHELIOMA SURVIVAL TIME FOR EACH TREATMENT

Treatment	Time taken for first mesothelioma (days)	Mean mesothelioma survival time ± S.E. (days)
Elutriated UICC amosite	292	505 ± 21
Elutriated factory amosite	377	566 ± 20
Elutriated UICC chrysotile 'A'	279	400 ± 24
Elutriated factory chrysotile	245	373 ± 18
Elutriated heated chrysotile	621	840 ± 31
Elutriated parent chrysotile	307	438 ± 13
Elutriated wet-dispersed chrysotile	178	312 ± 24

chrysotile and parent chrysotile. This separation of the curves provides an indication that there are differences in the carcinogenic potential of the dusts, even though there is little difference between the total numbers of tumours produced by all the dust samples except heated chrysotile.

Table 4 summarizes the information by listing the various treatments in descending order of the carcinogenicity (as described by their survival functions from Fig. 1) and it can be seen that the carcinogenicity of the various dust suspensions may be ranked in a similar descending order using either of the other indices of activity—mean mesothelioma survival time or the time to onset of the first tumour. The results of the *in vitro* cell viability assays of these same dust samples reported previously in the form of a cytotoxicity index (WRIGHT *et al.*, 1980) are also included for comparison. Information

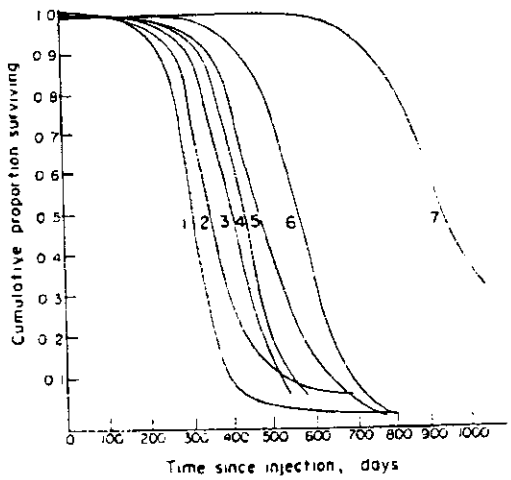

FIG. 1. Plot of cumulative survival of tumour bearing animals against time, showing the relative carcinogenicities of the seven elutriated dusts.
Key: 1 = Wet-dispersed chrysotile, 2 = Factory chrysotile, 3 = UICC chrysotile 'A', 4 = Parent chrysotile, 5 = UICC amosite, 6 = Factory amosite, 7 = Heated chrysotile.

TABLE 4 SUMMARY OF THE INDICES OF CARCINOGENICITY, WITH A COMPARISON WITH *IN VITRO* VIABILITY ASSAY

Treatment (Ranked by survival function)*	Time for first mesothelioma (days)	Mean mesothelioma survival time (days)	Percentage viability at 48 h ć 10 μg/ml
Elutriated wet-dispersed chrysotile	178	312	12.9
Elutriated factory chrysotile	245	373	71.7
Elutriated UICC chrysotile	279	400	58.2
Elutriated parent chrysotile	307	438	72.5
Elutriated UICC amosite	292	505	79.3
Elutriated factory amosite	377	566	80.4
Elutriated heated chrysotile	621	840	81.1

* i.e. in order of descending carcinogenicity (taken from Fig. 1).

from on
with th
cytotoxi
prepara
The
Figure
obtaine
became
samples
dispersi
estimat
dust sar
other t
destruc
the par
distribu
the cha
produc
measur
before
remem
fibrous
accoun
The
dust sa

FIG. 2.

from only one dose (10 μg/ml) after 48 h incubation is shown, this giving the best fit with the *in vivo* data. There is broad agreement between the injection and the cytotoxicity tests, with the most carcinogenic dust (the wet-dispersed chrysotile preparation) also proving to be the most toxic *in vitro*.

The information available on the fibre dimensions is shown in Figs. 2 and 3. Figure 2 consists of relative fibre length distributions of five of the dust preparations obtained by sampling the airborne clouds at the time of collection. It subsequently became possible to measure the relative fibre size distributions of the various dust samples following a liquid preparation procedure that more closely reproduced the dispersion state of the samples at the time of injection, and the results of these estimations are presented in Fig. 3. Figures 2 and 3 present results for only five of the dust samples, since satisfactory fibre length distributions could not be produced for the other two. The procedure of heating chrysotile to 850°C resulted in considerable destruction of the fibrous chrysotile morphology and, although a small proportion of the particulate material was fibrous when examined under the SEM and a length distribution could therefore be produced, it would not be a representative assessment of the character of the sample. At the other extreme, the wet dispersed chrysotile sample produced aggregations of very long thin fibres of such tangled complexity that measurement of length distributions were not possible. A further caveat is necessary before the relative fibre length distributions are examined closely: it must be remembered that both of the factory-derived samples contained a substantial non-fibrous component (40% in the case of the factory chrysotile sample) that is not accounted for in Figs. 2 and 3.

The fibre length distributions given in Fig. 3 represent the best available data on the dust samples actually injected into the rats. Since the samples of both chrysotile and

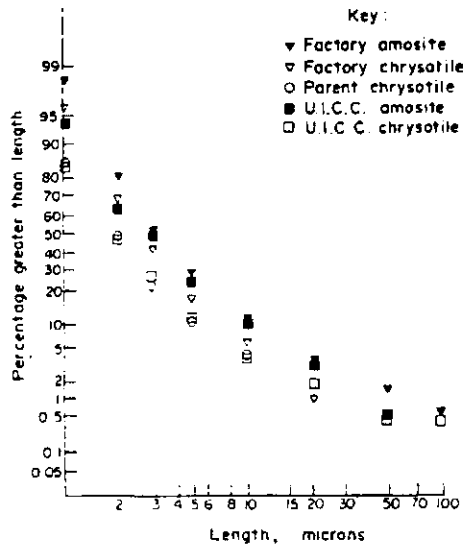

FIG. 2. Relative fibre length distributions of elutriated asbestos samples, prepared from airborne clouds, and estimated using a scanning electron microscope.

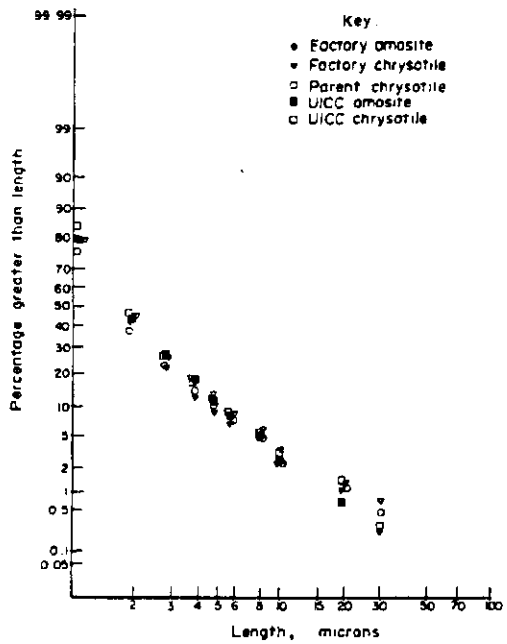

FIG. 3. Relative fibre length distributions of elutriated asbestos samples, prepared from liquid suspensions, and estimated using a scanning electron microscope.

amosite appeared to have a similar relative size distribution in these liquid suspensions and since the chrysotile samples were more carcinogenic than the amosite, the percentage of long fibres in a dust sample would not appear to be the most important factor in determining carcinogenicity.

DISCUSSION

The present work has confirmed that the peritoneal cavity provides a suitable site for the investigation of the development of mesothelioma. The issue of the relative isolation of the peritoneum from the effects of airborne dusts under normal circumstances requires some comment. Having accepted that inoculation methods of investigation of the response of mesothelial tissues to insult are useful (WAGNER *et al.*, 1973), despite their circumvention of pulmonary deposition and clearance mechanisms, the actual choice of the target mesothelium for the measurement of any response is mainly a technical consideration.

It would appear that the peritoneal injection method has several advantages over the pleural site of inoculation. Firstly, the technique is simple, quick, and with less chance of misinjection or inoculation fatality than the intrapleural method. Although LEWIS *et al.* (1966) found that 19.6% of animals inoculated by the intraperitoneal route were misinjected, we found no evidence of misinjection on this scale in the 232 animals

used in the present study. This compares favourably with the experience of various workers using the intrapleural technique.

A second advantage is that the peritoneal site appears to be more sensitive. In our experiments, mesothelial tumours developed in over 90% of animals injected intraperitoneally with 25 mg samples of six out of seven asbestos dusts, with the first tumour detected within 300 days of injection in most treatments. This may be compared with the results of intrapleural injection studies (WAGNER *et al.*, 1973) in which intrapleural injections of 20 mg samples of a range of asbestos types produced mesothelial tumours in up to 60% of treated animals, with the first appearing after about 500 days. One possible reason for this observed enhanced sensitivity of the peritoneal assay could be that at the (presumably) saturation dose level used in the present study, the surface area of mesothelium available for transformation becomes an important factor, and there is a greater mesothelial surface area within the peritoneal cavity than the pleural cavity. In the absence of information on the dose response of injected asbestos in the peritoneal cavity, the dose of 25 mg/rat was chosen for the present investigation, since previous experience has shown that this dose of UICC chrysotile could be expected to produce mesotheliomas. Full dose response studies using the UICC reference samples were initiated soon after, and these are still in progress. The near 100% response encountered in several of the treatment groups in this study provides some problems of interpretation, since it is not possible to discriminate between treatments on the basis of the number of tumours produced. However, the mean tumour survival time provides a useful indication of relative carcinogenicity where a common mechanism of induction may be assumed for several treatments; and analyses of survival have been used in this study to differentiate between different treatments.

A useful corollary of the higher level of response and decrease in the tumour latent period following intraperitoneal injection is that the influence of natural mortality from spontaneous disease is minimized. Experience with the AF/HAN strain of Wistar rat in our laboratory indicates that there is a spontaneous tumour incidence of the order of 30%, mainly occurring within the latter third of the animal's normal life-span. There was no evidence for the existence of a proportion of animals not susceptible to mesothelioma formation as suggested by BERRY and WAGNER (1969) after intrapleural injection. In the present study, the majority of animals had succumbed to mesothelioma within 550 days (i.e. approximately half their normal life-span). The product-limit method of analysis (KAPLAN and MEIER, 1958) used in this study calculates a cumulative survivial function for each treatment group and it therefore takes into account the number of animals surviving at the time of death of any individual animal. The method provides for the exclusion or censoring of those animals dying from causes other than mesothelioma, and this has been taken into account in the construction of Fig. 1.

This study showed that chrysotile tends to be more carcinogenic than amosite when equal masses of dust are injected, thus confirming the experience of other workers (SMITH *et al.*, 1965; WAGNER *et al.*, 1973). That the heated chrysotile proved to be the least carcinogenic in our assay is probably due to the radical effect of heating to 850°C, resulting in almost total destruction of the fibrous chrysotile component. It might therefore have been more appropriate to describe this sample as a forsterite preparation. Within the other chrysotile samples, there does appear to be a correlation between fibre

length and carcinogenicity. Thus, the wet-dispersed chrysotile (*probably* the longest) was the most carcinogenic, the parent chrysotile the least, with the other two samples in between.

Previous workers have used estimates of fibre size to account for differences in the carcinogenicity of asbestiform minerals, using either phase contrast or electron optical methods of estimation. The consensus now strongly favours the view that fibre length is the most important descriptor of carcinogenic potential of asbestos sample. Thus studies by STANTON and WRENCH (1972) and STANTON *et al.* (1977) indicated that the carcinogenicity of several types of mineral fibre correlated best with the number of fibres of length exceeding 8 μm and a diameter of less than 0.25 μm.

Assessments of fibre size were undertaken in the present study, but an association between increasing fibre size and biological activity is not obvious from Figs. 1, 2 and 3. Figures 2 and 3 summarize the information acquired in fibre length, expressed as the cumulative relative proportions of fibres over certain stated lengths, and based upon different preparations of the test materials. Fibre numbers were not estimated for the samples used in this study. Length distributions previously obtained from the original airborne samples taken as part of the inhalation studies showed the chrysotile clouds to contain a relatively higher proportion of longer fibres than the amosite clouds (DAVIS *et al.*, 1978). However, when the airborne samples taken during the collection of the 'elutriated' dusts for injection were assessed, the amosite samples were shown to have a greater relative proportion of longer fibres (Fig. 2). When these same elutriated samples were prepared for microscopy using a liquid dispersion procedure somewhat similar to the injection process, there were no statistical differences between the length distributions of the five samples examined (Fig. 3). Similarly, when the elutriated samples were subjected to sufficient ultrasonication in suspension to provide the degree of dispersal necessary for *in vitro* toxicity tests (WRIGHT *et al.*, 1980), the differences in length observed between the five samples were not statistically significant.

It is considered that these variations are mainly associated with the problems of preparing accurate reproducible samples for the electron microscopy, rather than with limitations of resolution associated with the use of the scanning electron microscope for size estimations. It is possible that the degree of dilution and dispersion of fibre samples necessary to provide preparations of sufficient technical quality for size estimation (particularly from the liquid slurries used for injection) may introduce a degree of disaggregation not representative of the sample *in vivo*.

However, Fig. 3 provides the best estimate available of the fibre size distributions of five of the samples injected in the present study and it can be seen that the samples had a similar relative length distribution. Despite this, there is some support for the general concept of STANTON and his co-workers (1972, 1978) that mineral fibre carcinogenicity is related to fibre length. It is known that experimental chrysotile preparations (including the UICC reference samples) tend to contain substantially more fibres per unit mass than amosite, using either phase contrast microscopy (DAVIS *et al.*, 1978, 1980) or electron optical techniques (unpublished observations). Although we were unable to obtain sufficient information on the number of fibres per unit mass in the present study, it is reasonable to assume a similar trend, with chrysotile samples containing more fibres per milligram than the amosite. Given the similar relative size distributions, it follows that the chrysotile samples contain more fibres of any specific length. It is not, however, possible to discriminate between an effect due to length, or to

some other parameter of particle size such as surface area. Whilst STANTON and LAYARD (1978) did not find a strong correlation between carcinogenicity and their estimate of the total surface area of 17 glass fibre preparations implanted intrapleurally, there was a link with estimated surface area amongst their longer fibre preparations, suggesting a possible role for the fibre surface area exposed as a result of incomplete phagocytosis.

Table 4 shows that both the time for development of the first mesothelioma, and the mean mesothelioma survival time, correlate well with the calculated cumulative survival function, confirming that preliminary information may be obtained on the carcinogenicity of a given particulate material by examination of the time taken for the first tumour to develop. With this in mind, it is possible to predict that several other dusts currently being studied at this Institute will finally be shown to be much less carcinogenic than asbestos. These include a ceramic (aluminium silicate) sample, and several forms of calcium silicate, none of which have yet produced mesothelial tumours, although the animals have survived for 650 days to date. The observed reasonable correlation of the injection experiments with *in vitro* cell viability assays is also noteworthy. Whilst it is accepted that not all cytotoxic dusts are carcinogenic, and some of the results of our viability assays are sufficiently close to be within the limits of experimental variability, there is broad agreement that the cytotoxic fibrous particles were also more carcinogenic. This is despite the fact that prolonged (2 min) ultrasonication of the *in vitro* dust samples was required to achieve adequate dispersion whilst the injected samples received no such treatment.

It is possible to conclude from this study that the relative carcinogenicity of several similar dust samples may be reasonably assessed from the mean tumour latent period rather than the total number of tumour-bearing animals produced. This does, however, presuppose that the mechanism of carcinogenicity is the same for each dust type under investigation, a reasonable assumption under the present circumstances. This study has produced relatively little information on the mechanisms of asbestos carcinogenesis, although there is some evidence that the more active samples contained more longer fibres. However, more information on the interrelationship of particle size, number, and surface area is required before length can be shown to be the principal determinant of carcinogenicity. Examination of these factors, together with investigations into the influence of particle dose, animal age and sex on the incidence of peritoneal mesotheliomas, is in progress.

Acknowledgements—The authors would like to thank Dr S. T. Beckett for the preparation of some of the fibre size distributions, and Mr B. Miller for his statistical help and advice

REFERENCES

BECKETT, S. T. (1973) The evaluation of airborne asbestos fibres using a scanning electron microscope. *Ann. occup. Hyg.* **16**, 405–408.

BECKETT, S. T. (1975) The generation and evaluation of UICC asbestos clouds in animal exposure chambers. *Ann. occup. Hyg.* **18**, 187–198.

BERRY, G. and WAGNER, J. C. (1969) The application of a mathematical model describing the times of occurrence of mesotheliomas in rats following inoculation with asbestos. *Br. J. Cancer* **23**, 582–586.

DAVIS, J. M. G. (1974a) Ultrastructure of human mesotheliomas. *J. natl Cancer Inst.* **52**, 1715–1725.

DAVIS, J. M. G. (1974b) Histogenesis and fine structure of peritoneal tumours produced in animals by injections of asbestos. *J. natl Cancer Inst.* **52**, 1823–1838.

DAVIS, J. M. G., BECKETT, S. T., BOLTON, R. E., COLLINGS, P. and MIDDLETON, A. P. (1978) Mass and number of fibres in the pathogenesis of asbestos-related lung disease in rats. *Br. J. Cancer* **37**, 673–688.

DAVIS, J. M. G., BECKETT, S. T., BOLTON, R. E. and DONALDSON, K. (1980) A comparison of the pathological effects in rats of the UICC reference samples of amosite and chrysotile with amosite and chrysotile from the factory environment. In *Biological Effects of Mineral Fibres* (Edited by WAGNER, J. C.) Lyon, IARC Publication No. 30, pp. 285–292.

ENTICKNAP, J. B. and SMITHER, W. J. (1964) Peritoneal tumours in asbestosis. *Br. J. Ind. Med.* **21** (1), 20–31.

HERON, G. F. and HUGGETT, R. (1971) Dispersion based textiles. Paper 42, *Int. Conf. Physics and Chemistry of Asbestos Minerals*, Louvain, Belgium.

KAPLAN, E. L. and MEIER, P. (1958) Non-parametric estimation from incomplete observations. *J. Am. statist. Ass.* **53**, 457–481.

LEWIS, R. E., KUNZ, A. L. and BELL, R. E. (1966) Error of intraperitoneal injections in rats. *Lab. Anim. Care* **16**, 505–509.

MCDONALD, J. C. and LIDDELL, F. D. K. (1979) Mortality in Canadian miners and millers exposed to chrysotile. *Ann. NY Acad. Sci.* **330**, 1–9.

NEWHOUSE, M. L. (1973) Asbestos in the work place and the community. *Ann. occup. Hyg.* **16**, 97–102.

REEVES, A. L., PURO, H. E. and SMITH, R. G. (1974) Inhalation carcinogenesis from various forms of asbestos. *Environ. Res.* **8**, 178–202.

SCHNEIDER, T. (1979) The influence of counting rules on the number and on the size distribution of fibres. *Ann. occup. Hyg.* **21**, 341–350.

SELIKOFF, I. J., HAMMOND, E. C. and CHURG, J. (1970) Mortality experience of asbestos insulation workers, 1943–1968. In *Pneumoconiosis: Proc. Int. Conf.*, Johannesburg, 1969. (Edited by SHAPIRO, H. A.) pp. 180–186, Oxford University Press, Capetown.

SMITH, W. E., MILLER, L., CHURG, J. and SELIKOFF, I. J. (1965) Mesotheliomas in hamsters following intrapleural injection of asbestos. *J. Mt Sinai Hosp.* **32** (1), 1.

STANTON, M. F. and LAYARD, M. (1978) The carcinogenicity of fibrous minerals. In *Definitions and Measurement Methods* (Edited by GRAVATT, C. C. *et al.*) National Bureau of Standards Special Publ. 506, pp. 143–151.

STANTON, M. F. and WRENCH, C. (1972) Mechanisms of mesothelioma induction with asbestos and fibrous glass. *J. natl Cancer Inst.* **48**, 797–821.

STANTON, M. F., LAYARD, M., TEGERIS, A., MILLER, E., MAY, M. and KENT, E. (1977) Carcinogenicity of fibrous glass. *J. natl Cancer Inst.* **58**, 587–603.

WAGNER, J. C. (1962) Experimental production of mesothelial tumours of the pleura by implantation of dusts in laboratory animals. *Nature, Lond.* **196**, 180–181.

WAGNER, J. C. and BERRY, G. (1969) Mesotheliomas in rats following inoculation with asbestos. *Br. J. Cancer* **23**, 567–581.

WAGNER, J. C., BERRY, G. and TIMBRELL, V. (1970) Mesotheliomas in rats following the i/pl. inoculation of asbestos. In *Pneumoconiosis: Proc. Int. Conf.*, Johannesburg, 1969. (Edited by SHAPIRO, H. A.) pp. 216–219, Oxford University Press, Capetown.

WAGNER, J. C., BERRY, G. and TIMBRELL, V. (1973) Mesotheliomata in rats after inoculation with asbestos and other minerals. *Br. J. Cancer* **28**, 173–185.

WAGNER, J. C., SLEGGS, C. A. and MARCHAND, P. (1960) Diffuse pleural mesothelioma and asbestos exposure in North Western Cape Province. *Br. J. Ind. Med.* **17**, 260–271.

WAGNER, J. C., BERRY, G., SKIDMORE, J. W. and TIMBRELL, V. (1974) The effects of inhalation of asbestos in rats. *Br. J. Cancer* **29**, 252–269.

WRIGHT, A., GORMLEY, I. P., COLLINGS, P. L. and DAVIS, J. M. G. (1980) The cytotoxicity of asbestos and other fibrous dusts. In *The* in vitro *Effects of Mineral Dusts* (Edited by BROWN, R. C., GORMLEY, I. P., CHAMBERLAIN, M. and DAVIES, R.) pp. 25–32, Academic Press, London.

DISCUSSION

G. BERRY: I should like to preface my comments by congratulating the authors on their experiment which points to the possibility of increasing the sensitivity of animal injection experiments. This is obviously worth attempting, as animal experiments are not cheap. Increased sensitivity allows a planned reduction in the number of animals and in the average length of time they have to be kept. The increased sensitivity has been achieved by increasing the mesothelioma rate so that most of the tumours occur before instead of concurrently with natural mortality. In all experiments of this type the most efficient analysis uses the times when the tumour, or death from other causes, occurred as well as the number of animals developing tumours. The method that you have used to produce Fig. 1 does have this desirable feature as also does the Weibel distribution (which we have used) and Cox's method. I think it is a little unfortunate, therefore, that you seem to suggest abandoning this efficient analysis in favour of cruder measures such as the mean tumour latent period or, even worse, the time to first mesothelioma. These measures are correlated with the efficient analysis but not completely, and it is this lack of complete correlation which indicates a loss of efficiency in using them. In addition, these measures would not be expected to relate to dose in a clearly interpretable manner. Any abandonment of an efficient analysis is equivalent to sacrificing some of the extra sensitivity of the experimental technique, and is therefore undesirable in economic terms, not just from a purely statistical methodological viewpoint.

Dr BOLTON: I did not intend to suggest we should abandon the use of statistical analysis. That certainly it is the better way of presenting the data, but it is also interesting to note that in our experiments both the other two simpler indices ranked the tumours in the same order. Nevertheless, I believe we should use the cumulative survival function, or some similar function, whenever we can.

K. MILLER: In spite of the rank order you have for the different dust samples, there is almost 100% tumour induction. Could this high rate be related to the large amount of sample injected (25 mg); could it have caused mechanical irritation? Did you include an irritant dust in your experiments and have you looked for dose–response relationships in the development of tumours?

Dr BOLTON: We have no positive irritant control of any other dust in this particular study. As regards the dose, this is the first time we tried to quantify the intraperitoneal injection route and it was surprising, if not embarrassing, to find that six out of seven of our treatments produced near 100% response. We have in progress dose response studies going down to 0.01 mg and we await the outcome. I agree that the TD 50 or the LD 50 test is quite a useful one although in the literature on asbestos to date there have been no reports of the use of the TD 50 to discriminate between treatments.

A. E. COCKCROFT: You point out the correlation between *in vitro* tests and animal experiments. Looking at Table 4 and Fig. 1 in your paper, although the fibre types are in the same order, in Table 4 (the *in vitro* tests) the major step seems to be between the wet-dispersed chrysotile and the rest, whereas in Fig. 1 (the animal tests) the major step seems to be at the other end of the spectrum, between heated chrysotile and the rest. Why do you think this is so?

Dr BOLTON: You will appreciate that there is some error in the percentage cell viability figures that we quote. Perhaps it was almost fortuitous that we managed to achieve the ranking that we did between some of the more closely ranked specimens. I find it very difficult to know why the order and spacing is as it is. There are lots of things different about the wet-dispersed chrysotile preparations and the other types of asbestos minerals that we used in this study and it could be any one of the many well known or documented effects such as particle number, size, surface area, diameter, length generally.

B. T. MOSSMAN: Did you examine the physico-chemical properties of the wet-dispersed chrysotile in detail? For example, were the surface-charge or surface constitution of the fibres different compared with factory or UICC chrysotile?

Dr BOLTON: I am sorry I cannot answer that in detail. I think the specific surface area would be definitely greater, although I do not, as yet, have any measurements.

J. C. WAGNER: I offer my congratulations on this elegant experiment, but make one major criticism, namely that all the materials you used were elutriated samples. This prevents comparison with previous pleural or intra-peritoneal cavity implanatation experiments. This could have been avoided by including the two untreated UICC standard samples of chrysotile and amosite.

Dr BOLTON: We originally set out to compare this test with the results of our inhalation experiments, and the elutriation was an attempt to provide a closer match between the dusts that we were using in the chambers and

in this particular assay. The elutriation was rather coarse and designed only to remove large irrespirable flocs from the cloud produced by the Timbrell dispenser. It is unlikely that the basic fibre size distribution would have been substantially altered, as the flocs also contained fine fibres. Experiments using the standard UICC samples are in progress and we hope to be able to provide the comparison you refer to.

G. H. Pigott: The dusts you have tested so far have all been fibrogenic. As a given amount of dust will produce more fibrosis in the peritoneal than in the pleural cavity, this may be significant. Dr Wagner recently reported an enhanced response to crocidolite when it was mixed with a fibrogenic agent (carageenin). In this context it will be interesting to examine, with particular reference to the induction period, the results from essentially non-fibrogenic products which are currently under test.

With regard to size–distribution, it has been our experience that the determinant of carcinogenicity in such systems is particle diameter as well as length. It is therefore not surprising that there is no correlation between carcinogenicity and a measurement of overall length. If the length distributions had been confined to fibres of diameter less than 0.5 μm the correlation may well have been evident.

Dr. Bolton: We did measure, by scanning electron microscopy, the diameter of these fibres, but we have not reported them because the results were very similar. However, the limited resolution of the scanning electron microscope does not allow us to say much about the diameters. I think it is fair to say that chrysotile samples are likely to contain, on average, thinner fibres and leave it at that.

T. L. Ogden: In earlier size-distributions reported by Beckett, he did not measure fibres less than 0.2 μm in diameter. Does this limitation apply equally to your results?

Dr Bolton: The limit of detection was slightly below 0.2 μm, but we measured all the fibres that were visible at a magnification of 10 000 × using SEM. Since we were not happy with the critical measurements of diameter, we did not report them.

V. Timbrell (written comment): I would like to comment on your fibre-size data. It is not possible to reach any conclusions on the relative number of fibres in materials from length distributions alone. Number of fibres per gram is inversely proportional to diameter-squared times length and, although, for instance, your wet-dispersed chrysotile had a lower proportion of long fibres than the amosite, the number in the 25 mg injected would be greater if the chrysotile was sufficiently thinner. Considering the ability of chrysotile but not amosite to split longitudinally into very thin fibrils, one suspects that long fibres are several times more abundant in the wet-dispersed chrysotile than in the amosite.

In our experience, to reach conclusions as to the importance of fibre length and diameter, it is necessary: (a) to know the number of fibres per gram of the material, (b) to determine, preferably, the joint distribution according to length and diameter and not only the two distributions separately, and (c) to use the TEM for these measurements.

Your experiment has demonstrated several useful features of the intraperitoneal injection system including the potential advantage of its high sensitivity. 'Potential', because this sensitivity must, in one way, be considered a disadvantage in that six of the materials all produced similar, high percentages of mesothelioma, suggesting that they were equally carcinogenic. I presume that in future experiments you will use this sensitivity to reduce the dose below saturation level and improve the discriminatory power of this test.

Dr Bolton: I agree that information on the number of fibres per unit weight and on the distribution of length and diameter would greatly assist the interpretation of the results of both inhalation and injection experiments. We attempted to examine these in this pilot study and we hope to obtain better information in the follow-up work. The size of chrysotile in liquid dispersions is affected by the method of preparation and is particularly difficult to define, as has been pointed out in your own publications. It is notable that the published characteristics of the UICC samples did not include diameter distributions. Moreover, very few users of these materials have reported measurements of the fibre dimensions in their preparations, which we think is a serious omission. Whilst we accept that the TEM provides the most accurate information, we believe that the SEM usefully extends the information obtainable by phase contrast microscopy.

As mentioned earlier, dose response studies are in progress using standard and elutriated forms of the UICC asbestos samples. However, in the absence of detailed information on the dose response, we believe that estimates of biological activity based upon the survival function are valid where the same carcinogenic mechanism can be assumed to operate, as in this study.