at a concentration in excess of those used. To fibroblast cultures were added, after incubation for 1 day and disintegration of cells, (*a*) medium alone, (*b*) supernatant medium from intact macrophages alone and from intact cells maintained in medium containing PNO, and (*c*) supernatant medium from initially intact macrophages incubated with quartz (0·1 mg/2 × $10^6$ cells/ml.) and from intact macrophages incubated with quartz in the same concentrations together with PNO. In one experiment, 1 mg/ml. PNO was added to the macrophage cultures 1 h before adding quartz. Controls (*a*) and (*b*) showed no rise of HOP concentration. A significant increase of HOP occurred at 2 and 4 days in quartz cultures whether treated with PNO or not, there being no significant difference between the increases in the two cases. In a second experiment PNO was applied to macrophage cultures at 2 mg/ml. for 1 day before adding quartz. The experiment lasted only 2 days, but HOP production was raised with quartz alone ($P < 0.001$) but not at all with quartz in the presence of PNO.

The proximate mechanism of fibrogenesis by silicon dioxide is still obscure but the present experiments provide, apparently for the first time, tissue culture evidence of a quantitative nature that implicates the reaction between silica nd macrophages as an essential initial step in the process. The evidence permits the conclusion that this is a vital phenomenon, which does not necessarily involve a direct effect of dissolved or particulate silicate on fibroblasts. Moreover, the comparative experiments with titanium dioxide and PNO (higher concentration, longer action) suggest that the silica-macrophage reaction is specific. The nature of the active factor has still to be elucidated, but another experiment suggests that the debris from quartz-treated, disintegrated macrophages lacks the factor. The idea that the fibrogenic agent is lipid in nature[1,14] now faces preliminary evidence to the contrary[14].

Dr. Hans Leiteritz kindly provided the Dörentrup quartz, Dr. W. Twist the titanium dioxide and Professor Dr. H.-W. Schlipköter the PNO. This work was supported by a grant from the National Coal Board.

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## Haemolytic Activity of Asbestos and other Mineral Dusts

Silica in the particulate form has been shown to have a lytic action on intracellular structures[1–3] and on erythrocytes[3,4]. The present investigation has shown that chrysotile asbestos is as potent a haemolytic agent as silica powder. Other forms of asbestos lysed erythrocytes only after prolonged incubation with them, and a wide range of mineral dusts was inactive. The haemolytic action of chrysotile was completely prevented by disodium versenate and simple phosphates but only partially by polyvinylpyridine-*N*-oxide and aluminium, both of which protect cells against lysis by silica[1].

The following finely ground materials were used: crystalline and amorphous silica, silicic acid powder, silica gel, chrysotile, amosite, anthophyllite and crocidolite asbestos, serpentine, aluminium oxide, 'Sephadex *G*-25', high and low rank coal dust, mine dust, colloidal carbon, talc, kaolin, glass powder, carborundum and diamond dust.

For the majority of tests a standardized 2 per cent suspension of washed sheep erythrocytes in a veronal buffered isotonic saline buffer (*p*H 7·4) was used. Four millilitres of this suspension were completely lysed by 50 mg of chrysotile when this was incubated at 37° C with a fixed volume (4 ml.) of suspension for a period of 50 min. This was taken as the standard time for all subsequent quantitative tests. The effects of various agents in preventing haemolysis by silica and chrysotile were investigated after the minerals had been coated with suitable concentrations of the protective materials, followed by repeated washing with saline.

Significant haemolytic activity was found for chrysotile, serpentine and all forms of silica tested. (The particle size ranges of the active dusts were the following: chrysotile, 11 per cent < 0·5μ; 37 per cent 0·5–2μ; 33 per cent 2–5μ; 15 per cent 5–10μ; 4 per cent 10–50μ length; serpentine, very fibrous, no size distribution obtained; crystalline silica, 15 per cent < 2·5μ; 49 per cent 2·5–5μ; 30 per cent 5–7·5μ; 5 per cent 7·5–10μ; amorphous silica, 250–400 Å; silicic acid powder, not determined; silica gel, 10–40μ.) The remaining powders were either completely inactive or only weakly lytic. Quantitative values for the powders found to be lytic are shown in Table 1.

Table 1. HAEMOLYTIC ACTIVITY OF SALINE SUSPENSIONS OF CHRYSOTILE ASBESTOS AND SILICA DUSTS

| Mineral | Per cent haemoglobin in supernatant |
|---|---|
| Fully haemolysed erythrocyte suspension | 100 |
| Aerosil (colloidal silica) | 100 |
| Crystalline silica | 88† |
| Crystalline silica (acid-washed)* | 89† |
| Silicic acid powder | 68‡ |
| Silica gel | 64‡ |
| Chrysotile asbestos | 78‡ |
| Serpentine | 65‡ |

* Silica repeatedly washed with boiling hydrochloric acid to remove surface metals.
† No adsorption of haemoglobin—some erythrocytes left intact.
‡ Lower values caused by adsorption of haemoglobin on these dusts—erythrocytes completely haemolysed.



The rate of haemolysis by chrysotile was greater than that shown by silica, the apparent lower percentage haemolysis by the asbestos (Table 1) being the result of the adsorption of released haemoglobin during the early stages of lysis. Haemolysis by chrysotile took place rapidly, with the immediate adsorption of haemoglobin on the asbestos fibres. Because of this, the degree of haemolysis in the supernatant (as measured colorimetrically) never reached 100 per cent. This was also found in the case of the adsorbents, silicic acid and silica gel.

Several samples of amosite, anthophyllite and crocidolite asbestos gave consistently negative results when incubated for 50 min at 37° C. In the case of the first two, haemolysis started only after 5 h at 37° C and reached its maximum after 24 h. Crocidolite had a very mild haemolytic effect after 24 h.

Haemolysis by chrysotile was inhibited by serum and by low concentrations of disodium ethylenediamine tetraacetic acid (EDTA) and phosphate ions (35 mg $PO_4^{3-}$) in the standard test medium. In the case of EDTA it was found that 20 μmoles of chelating agent in 4 ml. of erythrocyte suspension at 37° C completely prevented the haemo-

lytic action of 50 mg chrysotile (Fig. 1). On the other hand, 2 per cent aluminium chloride, 1·25 per cent gelatine and 0·9 per cent polyvinylpyridine-*N*-oxide (all effective protective agents against haemolysis by silica) retarded lysis by chrysotile for short periods but failed to prevent it completely.

The potent haemolytic action of both chrysotile and silica has been demonstrated. The influence of any mechanical action of these materials on the erythrocyte surface can be largely excluded because other abrasive dusts, for example, carborundum, diamond dust and glass powder, gave little or no haemolysis under the same experimental conditions. Recent work[4] on silica suspensions and inert dusts confirms this observation.

The results of our investigations with protective agents suggest that silica and chrysotile differ in the nature of their haemolytic action. There is evidence that silicic acid interacts effectively with lipoprotein monolayers[5–7] and that hydrogen bonds are formed between keto-imino-acids and the hydroxyl groups of the silicic acid[6–7]. Recent investigations of the damage by silica to the phagosomal membrane of macrophages[1–3] have indicated that silica exerts its lytic effect by forming hydrogen bonds with the biological membrane of the phagosome. The preventive effect of polyvinylpyridine-*N*-oxide at the subcellular level was ascribed after experimental evidence[3] to the preferential formation of hydrogen bonds between the silica surface and the polymer. That the latter interacts more extensively with the silica surface than with the erythrocyte membrane is shown by the low degree of protection obtained when blood cells are preincubated with the polymer only[3].

The marked depression of silica solubility by aluminium (and, presumably, the protective effect of this ion against phagosomal membrane lysis by silica)[1] is caused by the formation of aluminium hydroxide by aluminium in the ionic form reacting with the hydroxyl groups of the silica surface.

In the case of chrysotile, a fibrous silicate containing 40 per cent magnesium (as oxide), the partial protection given by aluminium and polyvinylpyridine-*N*-oxide indicates that if hydrogen bond formation takes place, this must be relatively slight and may be accounted for by the molecular configuration of chrysotile which would offer not only hydroxyl groups but cations for surface interaction. Polyvinylpyridine-*N*-oxide is a neutral co-ordinating agent and would be expected to interact with cations such as magnesium (II) ions only when suitable anions are available to maintain electrical neutrality (T. Nash, personal communication).

That magnesium in the ionic form is the principal cationic reactant on the surface of chrysotile in haemolysis seems to be indicated by the effective inhibitory action of EDTA, a material which also prevents lysis by macromolecules containing ionized iron[8]. This belief is supported by the inhibitory action of simple phosphates in the medium. Nash has suggested (personal communication) that non-ionic compounds (for example, polyvinylpyridine-*N*-oxide) or betaine-like compounds would be expected to protect cells against haemolysis by silica because of the nonionic character of the latter. If magnesium ions are the lytic agents, however, as appears to be the case with chrysotile, polyacids would seem to be suitable inhibitors. Good co-ordinators like phosphate and polyphosphate should also be effective. The iron in chrysotile (up to 2 per cent) appears to play no part in lysis by this form of asbestos, because no inhibitory effect was obtained in the presence of α.α′-dipyridyl in the medium. On the other hand, the presence of magnesium and calcium ions had no enhancing influence on the weakly lytic effect of crocidolite and amosite.

The haemolytic effects of chrysotile are of interest in view of the relative inactivity of the amphibole asbestos forms, crocidolite and amosite, and the pronounced activity of certain polymerized silicates which, unlike monomeric forms, bind firmly to the erythrocyte membrane[9]. The active fibrogenic properties common to the types of asbestos investigated here cannot yet be associated with their haemolytic characteristics, a relationship suggested for different forms of silica[4]. The inactivity of crocidolite, amosite and anthophyllite after incubation for 50 min suggested that if haemolytic activity is to be related to fibrogenicity, contact of these forms of asbestos with erythrocytes would have to be prolonged; the weak haemolytic activity observed by us for crocidolite, amosite and anthophyllite after extended incubation with erythrocytes supports this possibility.

Finally, it also seems clear that the highly active lytic property of chrysotile is related to the adsorptive capacity of this form of asbestos. The present investigation has shown that under the same conditions, chrysotile adsorbs five times more protein from serum than silica. More protein is required to "cover" the haemolytic sites of chrysotile than of silica; this is not surprising in view of the chemical structure and morphology of the two minerals. Chrysotile also has the highest Mg/Si ratio of the three forms of asbestos tested and differs also in that iron is not formally included in the lattice. It has a very high zero point of charge (*p*H 10–12), that is, over most of the *p*H range it will carry a net positive charge[10].

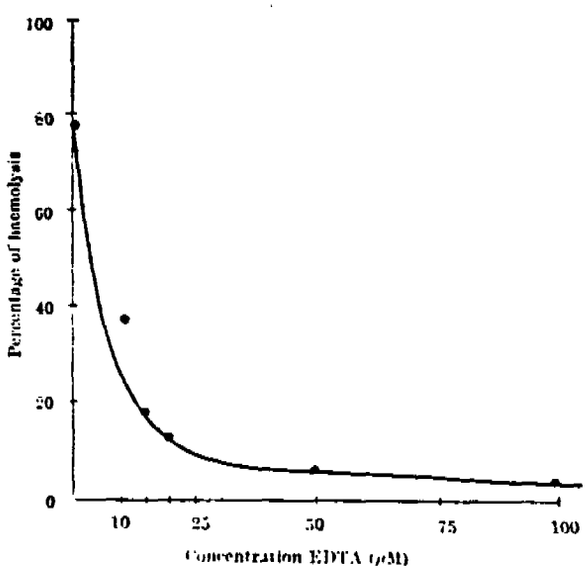


Fig. 1. Inhibitory effect of varying concentrations of EDTA on haemolysis by chrysotile (50 mg chrysotile, 2 ml. EDTA solution, 2 ml. erythrocyte suspension, 50 min incubation at 37° C; the highest value on the curve, 78 per cent, represents the effect of untreated chrysotile on erythrocytes).

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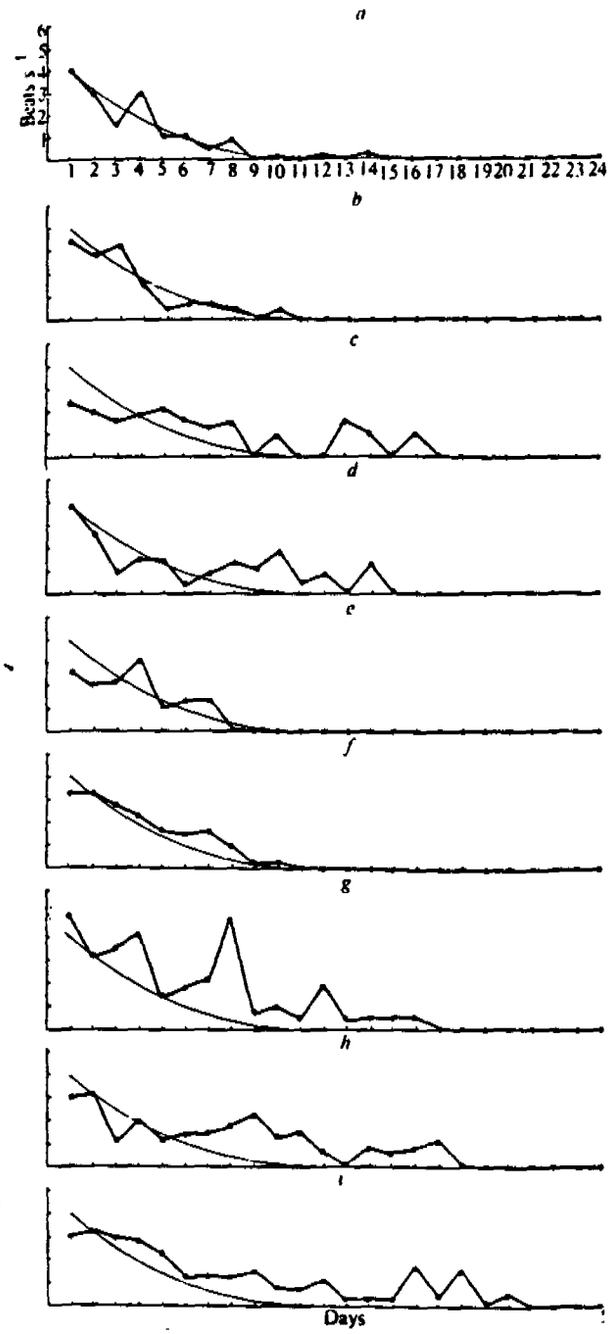


Fig. 2 Ventricular rates of the control hearts compared with the first 23 d for the test hearts.

seem reasonable to continue with further conventional clinical trials. If a new drug is depressant to the foetal myocardium above a certain concentration, it would be unwise to exceed this in a subsequent clinical trial. If the substance were to affect only young foetal hearts it might still be safe in late pregnancy.

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# Penetration of Asbestos through the Digestive Tract of Rats

MESOTHELIOMAS of the pleura and peritoneum[1-3] have been linked with the inhalation of asbestos fibres. We have shown[4] that asbestos fibres are present in drinking water, beer, wine and other beverages. Asbestos fibres can penetrate the mucosa of the stomach and intestine[5] and Telischi and Rubenstone[6] have found asbestos material in a gastric carcinoma. Godwin and Jagatic[7], reporting on mesotheliomas of the pleura caused by asbestos, noted that asbestos particles were widely distributed in various tissues. They suggested that particles may migrate from the lung through the blood and lymphatic system to all parts of the body. In the light of these reports, we surmised that asbestos consumed orally can pass through the gut into the blood stream and accumulate in various tissues.

To determine if this is the case we injected a suspension of asbestos fibres into the stomachs of rats. The suspension was prepared by shaking 2.5 g of chrysotile asbestos (Johns Manville No. 7RFO2) in 500 ml of water in a graduated cylinder. The suspension was allowed to settle for 30 min and the top 250 ml, which contained about $9.4 \times 10^9$ (1 mg) fibres $ml^{-1}$, was drawn off. Most of these fibres are 0.2 μm–2.0 μm long (Fig. 1). To ensure that fibres passed through the digestive tract without rats inhaling any of them, the asbestos was introduced into the gastrointestinal tract by opening the abdomen under anaesthesia and injecting the fibres directly into the stomach. Two to 4 d later the rats were killed and blood and tissues analysed for asbestos. To ensure that any fibres on the rat hair could not contaminate the organs removed during surgery, the rats were first thoroughly vacuumed and washed with double distilled water and methanol. During the removal of tissues wet towels were placed over the animals and around the incisions. Uncontaminated blood was drawn from the orbital sinus using a capillary tube.

To avoid formation of excess ash, which interferes with examination of specimens under the electron microscope, the tissues were first solubilized with soluene (Packard Instrument Company); the fibres were then centrifuged down, washed with methanol and ashed as previously described[4]. The ash was taken up in 1 ml of distilled water which had been filtered through a 0.2 μm filter, and 5 μl of this mixture was dropped on hydrophilic carbon-coated electron microscope grids supported on a ring peg. The grids were dried in a closed glass container (to avoid air contamination) under a heat lamp and were examined at magnifications of 20,000 and 80,000 with an electron microscope. The presence of chrysotile asbestos fibres was verified by electron diffraction. They were counted and the numbers corrected for dilution to determine the number in the original 1 ml sample. Results of recovery of asbestos fibres from rats treated in this way are shown in Table 1.

Rats in group 1 were given $9.4 \times 10^9$ fibres (1 ml) and killed 2 d later; those in group 2 received $94 \times 10^9$ fibres and were killed 4 d later. No asbestos fibres were detected in the blood of non-injected rats (controls) but $4.65 \times 10^6$ $g^{-1}$ (a statistically significant increase) were found in the first group.

This is the first time direct physical evidence for the presence of asbestos fibres in the blood has been obtained. Four days after treatment the amount of asbestos in the blood of rats in group 2 had dropped to $1.19 \times 10^6$ $g^{-1}$ even though these rats received ten times as much asbestos. This indicates that blood

Table 1 Numbers of Asbestos Fibres Recovered from Treated and Non-Treated Rats

| | Controls mean* | | Group 1† mean* | | Group 2‡ mean* | |
|---|---|---|---|---|---|---|
| Blood | 0.00 | — | 4.65¶ | 1.02 | 1.19 | 0.77 |
| Spleen | 2.33 | 0.67§ | 4.11 | 0.49 | 3.45 | 1.20 |
| Omentum | 2.46 | 0.46 | 2.74 | 0.87 | 18.25 | 7.79 |
| Heart | 2.09 | 0.41 | — | — | 2.28 | 0.40 |
| Brain | 0.05 | 0.02 | 0.31‖ | 0.19 | 0.29** | 0.11 |
| Lungs | 1.06 | 0.26 | 1.80 | 0.54 | 1.74 | 0.26 |

* Average of five rats in all cases except where noted. † Animals injected with $9.4 \times 10^9$ fibres and tissues examined 2 d later. ‡ Animals injected with $94 \times 10^9$ fibres and tissues examined 4 d later. § Standard error of the mean. ‖ Four rats only. ¶ $P < 0.01$; ** $P < 0.05$.
(The figures in the table are fibres $g^{-1} \times 10^{-6}$.)

can clear itself of the fibres. Tissues such as the spleen, heart and lung also seem to have, to a lesser extent, the ability to clear themselves of the asbestos fibres, as the figures obtained from rats in group 2 indicate. They are lower than those for group 1 rats even though they received a larger dose, but the tissues had longer to clear themselves. Indeed, recent work in our laboratory with radioactive asbestos indicated that some tissues, such as the lungs, can reduce their fibre content by half in 2 d. This clearing action could possibly have quite an effect on the numbers of fibres found in the liver and kidney of the rats but unfortunately, because of the considerable amount of ash present in these organs even after soluene treatment, we could not examine these specimens under the electron microscope.

Another factor may contribute to the higher fibre count obtained for the organs of rats in group 1 compared with animals in group 2, namely, that there were four times as many fibres in the blood of rats in group 1. The residual blood in the tissues on removal from the animal would of course contribute to the total fibre count of a particular tissue.

This factor could have created the anomaly in the fibre counts in brains. In the brains of treated rats this was about six times higher than in the controls, and the increase in fibre count is statistically significant for rats in group 2 but not, oddly, for rats in group 1 even though there were more fibres in these animals. The greater number of fibres per gram in the blood of rats in group 1 could be responsible for the larger standard error in the counts of rats in group 1 and therefore did not allow the values obtained to be significant.

The brain seems to have little ability to clear itself of asbestos fibres, but the omentum has even less. This tissue, which surrounds the small intestine, appeared to accumulate most asbestos. Although the asbestos fibre content of the omentums in rats in group 2 may seem rather high (one extreme count contributed to the high standard error) the omentum does not seem to respond as do the other tissues. As well as having little ability to clear itself, fibre accumulation in the omentum may take place much more slowly than it does in other tissues. As the omentum consists partly of peritoneum and partly adipose tissue, asbestos fibres may enter the peritoneum by direct penetration of the intestinal wall. Concerning the prolonged retention of asbestos fibres in the omentum in women asbestos workers, Keal[8] found a higher incidence of peritoneal cancer than of lung cancer.

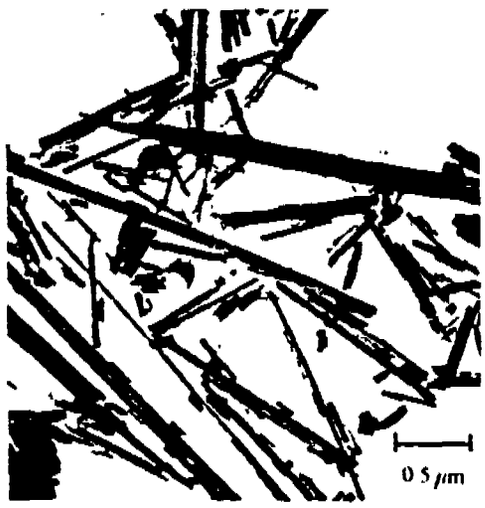


Fig. 1 Electron micrograph of the suspension of asbestos fibres injected into the rat stomach. Note the varying sizes of the bundles of fibres present.

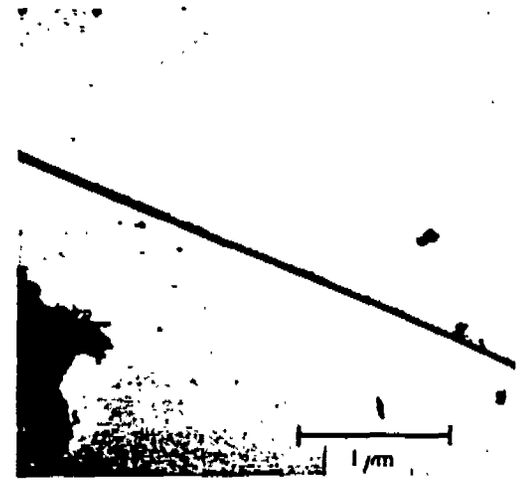


Fig. 2 Small bundle of asbestos fibres from the peritoneum of a rat. This bundle is at least 15 μm long.

The amount of asbestos found in the tissues of the controls is higher than we had anticipated, considering the age of the rats and the number of fibres they might have consumed in their drinking water[4]. But we do not know how much was in their feed or air. We have analysed the tissues of some people who died of natural causes and found somewhat similar levels. In one person there was twice as much asbestos in the brain as in the brains of our control rats and about one quarter as much in the omentum, but the omentum content was still higher than the brain.

Most of the fibres found were about 0.2–2.0 μm long but some measured 15 μm (Fig. 2) and one fibre in the blood of a rat was 23.55 μm long. We do not know how the fibres pass through the intestinal wall. Some long ones may pierce the gut like a needle, whereas pinocytosis may account for the absorption of the small ones.

We demonstrated earlier the presence of asbestos fibres in air, snow, city drinking water and a number of beverages[4]. The experiment described here shows that fibres of a similar size administered into the stomach of rats appear in the blood and accumulate in various tissues in the body.

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