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HEMOLYSIS BY ASBESTOS

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ABSTRACT

Various experimental factors that affect hemolysis by crocidolite and chrysotile were studied. Microscopic observations of cell-fibre interactions were made using a video-scanning technique.

The same general pattern of hemolysis can be observed for the two asbestos materials: attachment of a cell to asbestos fibre is an essential requirement for hemolysis; hemolysis of a cell is preceded by a prolytic stage in which the optical density of the cell does not change; hemolysis is a comparatively sudden event in which hemoglobin is released over a period of about 30 s.

Nevertheless, significant differences can be observed in the interaction of the two materials with erythrocytes, namely:

(Continued)

(i) There are observable differences in the mode of attachment of cells to fibre. These presumably arise from the differing surface properties of the two minerals.

(ii) The duration of prolysis observed for crocidolite is very much greater than for chrysotile, so the rates of hemolysis are correspondingly less.

(iii) The duration of prolysis is, on average, reduced by increasing the extent of agitation of cell-crocidolite suspension.

These results suggest that boundary layer diffusion may be an important factor in determining rates of prolytic transport phenomena.

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HEMOLYSIS; ASBESTOS; ERYTHROCYTES

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1. INTRODUCTION

Hemolysis of plasma-free erythrocytes by asbestos has been studied frequently, and it has been concluded by many workers that the chrysotiles are powerful hemolysing agents, whereas the amphiboles are either inactive or weakly lytic [Macnab and Harington 1967; Harington et al. 1968, 1971; Secchi and Rezzonico 1968; Schlipköter 1968; Klösterkotter 1968; Schnitzer and Pundsack 1970]. No such distinction can be made for long-term toxic effects *in vivo*; both classes of asbestos represent an occupational hazard arising from the fibrogenic and carcinogenic properties of inhaled asbestos fibres [Selikoff et al. 1964, 1972; Harington, 1969; Wagner et al. 1973].

The contrast between short-term *in vitro* effects and long-term toxicity reported for the amphiboles, which has been considered as something of a paradox, has recently been discussed by Light and Wei [1977a,b]; they suggested that the slow leaching of asbestos fibres *in vivo* and continuous alteration of the fibre surface charge could explain why chrysotiles and amphiboles, which differ markedly in hemolytic properties, are not obviously different in their long-term toxicities. However, we have examined the assumption that amphiboles as a class are only weakly hemolytic or inactive. Three Union Internationale Centre le Cancer (UICC) asbestos reference samples were compared in order to evaluate the method used to assess hemolytic activity and study various experimental factors that determine the measured degree of hemolysis.

2. EXPERIMENTAL

2.1 Materials

Hemolysis was measured for three standard UICC asbestos reference samples (chrysotile A, chrysotile B and the amphibole crocidolite) provided by the Medical Research Council Pneumoconiosis Unit, Penarth, UK.

2.2 Measurement of Hemolysis

Samples of human blood were obtained by venepuncture during routine medical examinations. Standard measurements using EDTA as anticoagulant were made of hemoglobin content, red cell count and hematocrit. Hemoglobin was determined by the cyanohemoglobin method using Drabkins' solution, and

hemoglobin concentrations were measured photometrically at 540 nm by means of a Bausch and Lomb Spectronic 20 photometer.

For most hemolysis experiments we adopted a standard method of measurement as follows:

Cells, obtained from whole blood which had been stored at 4°C for 16 hours, were washed three times with isotonic saline and then mixed in saline to give a suspension containing about 4 vol. per cent of cells. A weighed amount of asbestos was shaken for one minute with 2 mL saline in a polystyrene container (60 mm long x 20 mm diameter). Two millilitre of cell suspension was added to the asbestos suspension at ambient temperature (18-20°C). The sealed vessel containing cell-asbestos mixture was then placed on a Mathum mixer and rotated at 20 rev min^{-1} for one hour at 37°C, and the mixture was subsequently centrifuged. One millilitre of lysate was then diluted with 1 mL of Drabkins' solution plus an appropriate amount of saline; hemoglobin concentration of the diluted lysate was determined photometrically.

Blank experiments were also carried out periodically, using cell suspensions alone or cell suspensions mixed with calcined gamma-alumina powder (BET surface area of $\sim 2\ m^2\ g^{-1}$). In each blank experiment carried out by the standard procedure, the hemolysis was either undetectable or less than 2 per cent.

Some experiments varied from the standard method; these are discussed in Section 3.

2.3 Microscopic Examination

A video camera, monitor and video recorder were used in conjunction with a microscope, to give magnifications up to 2000 X. Samples were placed between Perspex slides and cover slips, separated by a thin band of vacuum grease around the edges, or within the disc-shaped volume formed by 25 mm diameter Perspex cover slips held 2 mm apart by a stainless steel annulus. This permitted observation of erythrocytes floating free of any constraints arising from attachment to the cover slips.

3. RESULTS AND DISCUSSION

3.1 Crocidolite is Hemolytically Active

Figure 1 shows the percentage hemolysis, h_w, plotted as a function of dose, d_w (pg/cell), for both chrysotile and crocidolite. The magnitude of h_w measured by our standard method is, in general, less for crocidolite (curve Q) than for chrysotile (curve P). Hemolysis by crocidolite is nevertheless substantial, especially at the higher doses, and greater than previously reported; for example, curve Q indicates 70 per cent hemolysis for 150 pg crocidolite/cell, whereas Harington et al. [1971] found that $h_w \cong 15$ per cent for human erythrocytes exposed to UICC crocidolite at about 140-160 pg/cell. In calculating this dose range it was assumed that a 2 vol. per cent suspension of cells contains 3.0×10^8 - 3.5×10^8 cells/mL. It should be noted, however, that Morgan et al. [1977] obtained $h_w \sim 25$ per cent for a maximum dose of ~ 50 pg/cell for UICC crocidolite. This value coincides with curve Q of Figure 1.

Techniques used by previous workers for assessing hemolytic activity of asbestos differed from our standard method in several respects: reported values of h_w often referred only to a single dose of asbestos; suspensions of asbestos fibre and erythrocytes were usually preheated separately to 37°C before mixing, although sometimes mixtures were prepared and maintained at room temperature; cell-fibre mixtures were sometimes incubated in veronal-buffered solution; usually the mixtures were either not agitated during incubation or were mixed gently by inverting the incubation vessel periodically, although Morgan et al. [1977] employed a rotary mixer for continuous agitation.

Curve Q in Figure 1 shows that hemolysis by crocidolite is not measurably affected by preheating suspensions to 37°C, or by veronal buffer. On the other hand, the method of agitating a hemolysing mixture can affect lysis substantially. This can readily be seen from a comparison of the curves Q and R of Figure 1 and from the data of Table 1.

The extent of hemolysis by crocidolite is not influenced by heating the cell suspension to 37°C immediately before mixing, but if preheating is continued for 1 hour at 37°C, then some diminution in h_w can be observed. However, as is shown in Table 2, hemolysis is again determined predominantly by the method of agitation during incubation of the cell-fibre mixture.

3.2 Dispersal of Asbestos Fibre in Saline

Thorough dispersal of chrysotile fibres in saline before mixing with cells is essential for maximum hemolysis; otherwise cell-fibre interaction is confined in part to the exterior of poorly-dispersed fibre bundles. The effect of fibre dispersal can be seen in Figure 2. Curve X refers to hemolysis by chrysotile which had been well-dispersed in saline by vigorous shaking before the addition of cells, whereas the generally lower hemolysis indicated by curve Y was obtained by adding cell suspension to the dry fibre. Dispersion of fibre bundles may also occur during incubation with cells, but in this case we believe that interior fibres are partially deactivated by adsorbed hemoglobin before dispersion occurs. This view is confirmed by the results of separate experiments which indicate that, after exposure to hemoglobin solution, well-dispersed chrysotile is only slightly hemolytic, even in the conditions of our standard method. Deactivation of chrysotile by adsorbed hemoglobin has been noted and discussed by other workers [Harington et al. 1971; Desai et al. 1975; Morgan et al. 1977].

Crocidolite fibres disperse in saline more readily than chrysotile, so that efficient cell-fibre contact is more easily ensured. Cells adhere to the fibres and, almost immediately after mixing, the cell-coated fibres flocculate and then settle out at a rate faster than that observed for the suspension of crocidolite alone, and, at sufficiently high dose (e.g. 50 pg/cell), a cell-free solution remains above the flocculated sediment. Predispersal of crocidolite by vigorous shaking in saline is not therefore such a critical factor in determining subsequent hemolysis.

3.3 Microscopic Observation by Videoscan of Cell-Fibre Interaction

As shown by the photomicrographs of Figure 3, cells adhere to both chrysotile and crocidolite. Videoscan records indicate that cells adhere to fibres immediately after mixing, and that the attached cells cannot easily be dislodged from either form of asbestos by agitation *in situ*.

Agitation, although ensuring adequate dispersion of fibre before mixing with cells and maximising the number of cell-fibre contacts during initial mixing, will not thereafter significantly alter the number of cells adhering to fibres; nor will there be any significant degree of detachment and re-attachment of cells to fibre during agitation. Attachment to asbestos fibre appears to be an essential requirement for lysis of an erythrocyte; for

example, it can readily be shown that, when excess cells are mixed with chrysotile (e.g. 10 pg chrysotile/cell), the non-adhering cells can be separated from cell-coated fibres by washing with saline through filter paper. The separated cells are intact and hemolyse only very slowly after separation.

Cells adhering to chrysotile often show extensive areas of cell-fibre contact, with severe distortion of the attached cell, whereas the area of cell-fibre contact is much less for crocidolite, and cells are relatively undistorted. We believe that the observed differences in the mode of attachment of cells reflect the differing surface properties of the two classes of asbestos. The surface charge of UICC chrysotile is positive, as indicated by a zeta potential, Z, of about 45 mV at pH 7.4, whereas crocidolite fibres carry a negative charge with $Z \sim -44$ mV [Light and Wei 1977]. Because the net surface charge of the erythrocyte is negative, it follows that for cell-chrysotile interactions, the force of attraction involves a substantial electrostatic component; but cell adhesion to crocidolite has to be considered on the other hand as a balance between electrostatic repulsion and intermolecular forces of the van der Waals type.

The sequence of microphotographs in Figure 3(a-d), refers to events occurring some 5 min. after mixing cells and chrysotile together, but within the relatively short period of 80 s. These data show that hemolysis of a cell (see arrowed cell) is preceded by a prolytic stage in which the optical density of the cell does not change and no hemoglobin is released. Subsequent hemolysis of the cell is a relatively catastrophic event in which hemoglobin is released from the cell over a period of approximately 30 s.

Observation of crocidolite-cell mixtures by videoscan reveals the same sequence of microscopic events as described above for chrysotile, although the duration of prolysis is, on average, greater for crocidolite. However, following agitation of cell-crocidolite mixtures *in situ*, there can be observed an increase in the number of hemolysing cells; that is, agitation reduces the duration of prolysis, even though cells remain attached to fibres.

3.4 Pattern of Hemolysis

The pattern of hemolysis for both crocidolite and chrysotile is one in which lysis of a single cell leads to complete (or nearly complete) liberation of hemoglobin, so that within a population two types of cell can be observed; these are faintly visible ghost cells and optically opaque unhemolysed cells

which can be clearly distinguished microscopically (Figure 3). Percentage hemolysis is therefore a measure of the fraction of cells transformed into ghosts. Some events that precede and accompany hemolysis of single cells by asbestos can be readily observed microscopically with the aid of videoscanning. These are in the sequence:

(i) Adhesion of cells to asbestos fibre. This occurs immediately on first contact with asbestos.

(ii) Prolysis in which an adhering cell does not hemolyse, but remains optically opaque for some time t_h.

(iii) Hemolysis of the cell occurs after time t_h (the hemolysis time), as indicated by a rapid decrease in optical density, although size and shape of the cell do not change significantly during the release of hemoglobin. Transformation to a barely visible ghost occurs within about 30 s, so that if Δt is the time interval over which hemolysis of the single cell occurs, then $\Delta t < t_h$; that is, the prolytic stage is rate-determining in terms of hemolysis for the cell population.

Within the cell population there can be observed a variation in the magnitude of t_h for individual cells. Furthermore, the duration of the prolytic stage is, on average, much greater for cells exposed to crocidolite than for chrysotile-cell mixtures: this difference in behaviour of the two asbestos forms is reflected in the measured kinetics of hemolysis as illustrated in Figure 4; the curve h_w versus incubation time for chrysotile approaches a terminal value of h_w after about 10 min. incubation at 37°C, but hemolysis by crocidolite continues at a finite rate even after 60 min. incubation. In general, these kinetic data confirm the findings of Harington et al. [1971] who noted a similar difference in the behaviour of the two asbestos forms with a finite rate of hemolysis even after 4 hours' incubation with crocidolite.

Taking into account the pattern of hemolysis established by visual observation, we can now formally describe the hemolytic activity of both chrysotile and crocidolite, and define some differences in behaviour of the two materials. We postulate a probability density function (PDF) for t_h alone, $\phi(t_h)$, without specifying the exact form of $\phi(t_h)$.

If N_o = total number of cells in system, and

N_h = number of hemolysed cells at time t,

then the rate of hemolysis is given by

$$dN_h/dt = \phi(t_h) \quad . \tag{1}$$

If N_a = number of cells attached to fibre, and

t_a = time at which all N_a cells are hemolysed, then

(i) for $t < t_a$

$$h_w = 10^2/N_o \int_0^t \phi(t_h)dt, \text{ and } dh_w/dt > 0 \; ; \tag{2}$$

(ii) if $t \geqslant t_a$, then

$$h_w = 10^2 N_a/N_o = (10^2/N_o \int_0^{t_A} \phi(t_h)dt) \text{ , and} \tag{3}$$

$$dh_w/dt = 0 \text{ at time t .}$$

From the curve of h_w versus t given in Figure 4 for chrysotile and the kinetic data of Harington et al. [1971], we can assume that after incubation for 1 hour virtually all cells attached to chrysotile are hemolysed, so $N_h = N_a$ and $t_a < 1$ hour. The extent of hemolysis measured by our standard method is, therefore, given by Equation 3. Furthermore, from the standard curve X of Figure 2, it can be seen that for a dose > 8 pg/cell, all cells are attached to fibre and all are hemolysed after 1 hour; that is $N_w = N_a = N_o$. Thus for chrysotile, the degree of hemolysis measured by our standard method is determined by the geometrical factors that affect the proportion of cells attached to fibres.

Hemolysis by crocidolite continues even after incubation for 1 hour: therefore $t_a > t_h$, $dh_w/dt \neq 0$ for t = 1 hour, $N_a > N_h$, and h_w is given by Equation 2. That is, the degree of hemolysis by crocidolite (as measured by our standard method) is determined by kinetic factors and, in particular, by factors that affect prolysis and so also affect the form of $\phi(t_h)$. The exact form of $\phi(t_h)$ is not known. However, Stuart and Shying [1980], in studying hemolysis by uranyl compounds, have shown that an experimental PDF can be

derived from precise kinetic data obtained using automated analytical techniques. As we have noted, continuous agitation of cell-fibre mixtures is an important influence in determining kinetics of hemolysis by crocidolite. The microscopic evidence indicates that agitation does not initiate shear-induced hemolysis and cell fragmentation, but reduces the duration of prolysis t_h. These results suggest the hypothesis that following attachment of cells to crocidolite, prolytic events such as potassium release or diffusion of water into the cell are markedly affected by agitation; that is, boundary layer diffusion may be an important factor in determining rates of certain prolytic transport phenomena.

4. CONCLUSIONS

The results described point to the uncertainties encountered in comparing hemolytic properties of different materials and the difficulty in defining hemolytic activity. There has been much interest in the hemolytic technique as a possible means of assessing the toxic potential of airborne particulates, but a correlation has not yet been convincingly demonstrated between the hemolytic properties of mineral dusts or fibres and their pathogenic effects in humans or intact animals. It could be argued, therefore, that *in vitro* work should be directed towards other cell systems (for example, macrophages and related tumour cell lines) on the assumption that the properties of these cells are more likely to correlate with toxic effects *in vivo*. However, some experimental factors that affect hemolysis are likely to be important in other *in vitro* methods.

We believe that continuing investigation of erythrocyte-particulate interactions is necessary:

(a) as a useful complement to other *in vitro* methods, and

(b) as a means of defining damage to biological membrane in terms of changes in transport properties, permeability, rearrangement of membrane components and chemical change in molecular constituents of the membrane.

5. ACKNOWLEDGEMENT

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TABLE 1
HEMOLYSIS BY UICC CROCIDOLITE

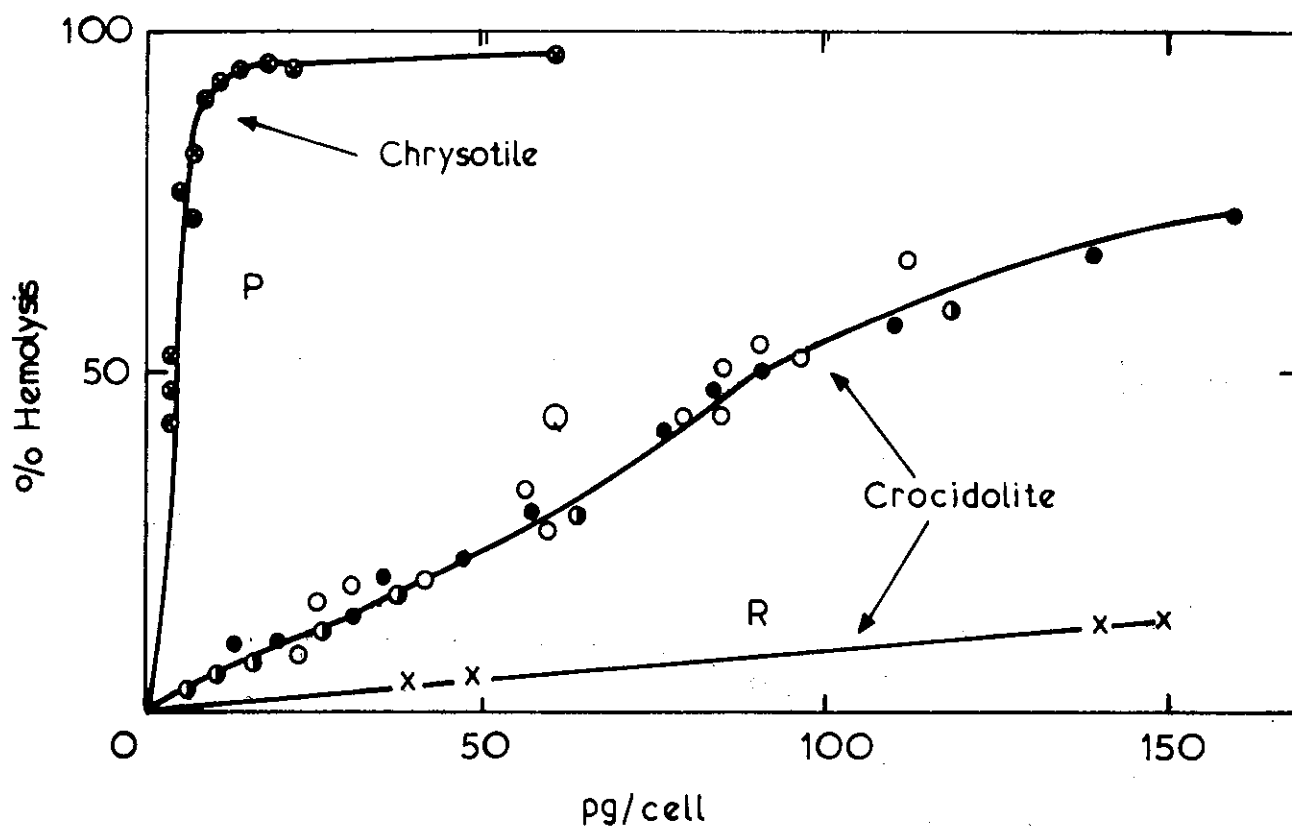
2 mL 4 per cent cell suspension mixed at 18°C with 2 mL asbestos suspension. Mixture incubated at 37°C for 1 hour. 30 pg/cell

Agitation	% hemolysis
Nil	1.8
Incubation tube inverted gently	5.1 5.5
Continuous on Mathum mixer at 20 rev min^{-1}	17.3 18.2

TABLE 2
HEMOLYSIS BY UICC CROCIDOLITE

Cell suspension heated for 1 hour at 37°C before mixing. Conditions otherwise as given in Table 1.

Agitation	% hemolysis
Nil	1.2
Incubation tube inverted gently	2.6 2.3
Continuous on Mathum mixer at 20 rev min^{-1}	11.9 13.2



Curve P: chrysotile B; ⊗ standard method

Curve Q: crocidolite; o standard method

◑ fibre suspension and cell suspension each heated to 37°C before mixing, veronal buffered;

● suspensions preheated to 37°C, no buffer.

Curve R: crocidolite; x no Mathum mixer, incubation tubes gently inverted at 10 min intervals.

FIGURE 1. HEMOLYSIS BY UICC STANDARD ASBESTOS REFERENCE SAMPLES; INCUBATION FOR 1 HOUR AT 37°C

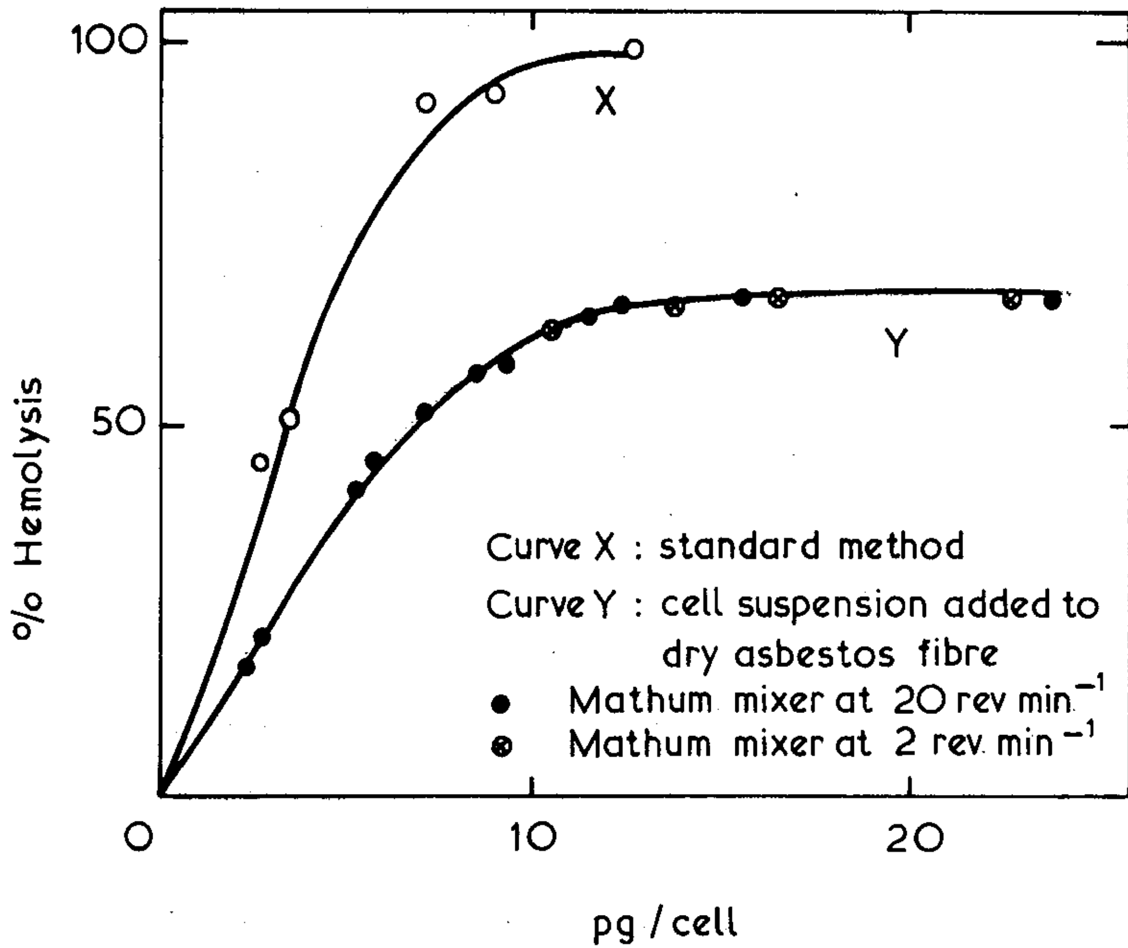
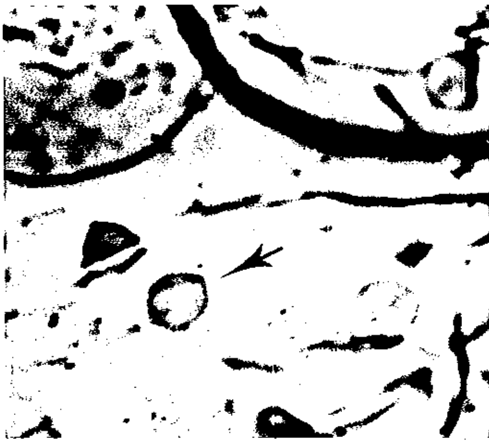


FIGURE 2. HEMOLYSIS BY UICC CHRYSOTILE A



(a) 5 min after mixing

(b) 10 s after (a)

(c) 20 s after (a)

(d) 80 s after (a)

(e) UICC crocidolite, 300 s after mixing

FIGURE 3. VIDEO-SCAN PHOTOMICROGRAPHS OF ASBESTOS FIBRE-CELL SUSPENSIONS, (a - d) SEQUENCE FOR UICC CHRYSOTILE A

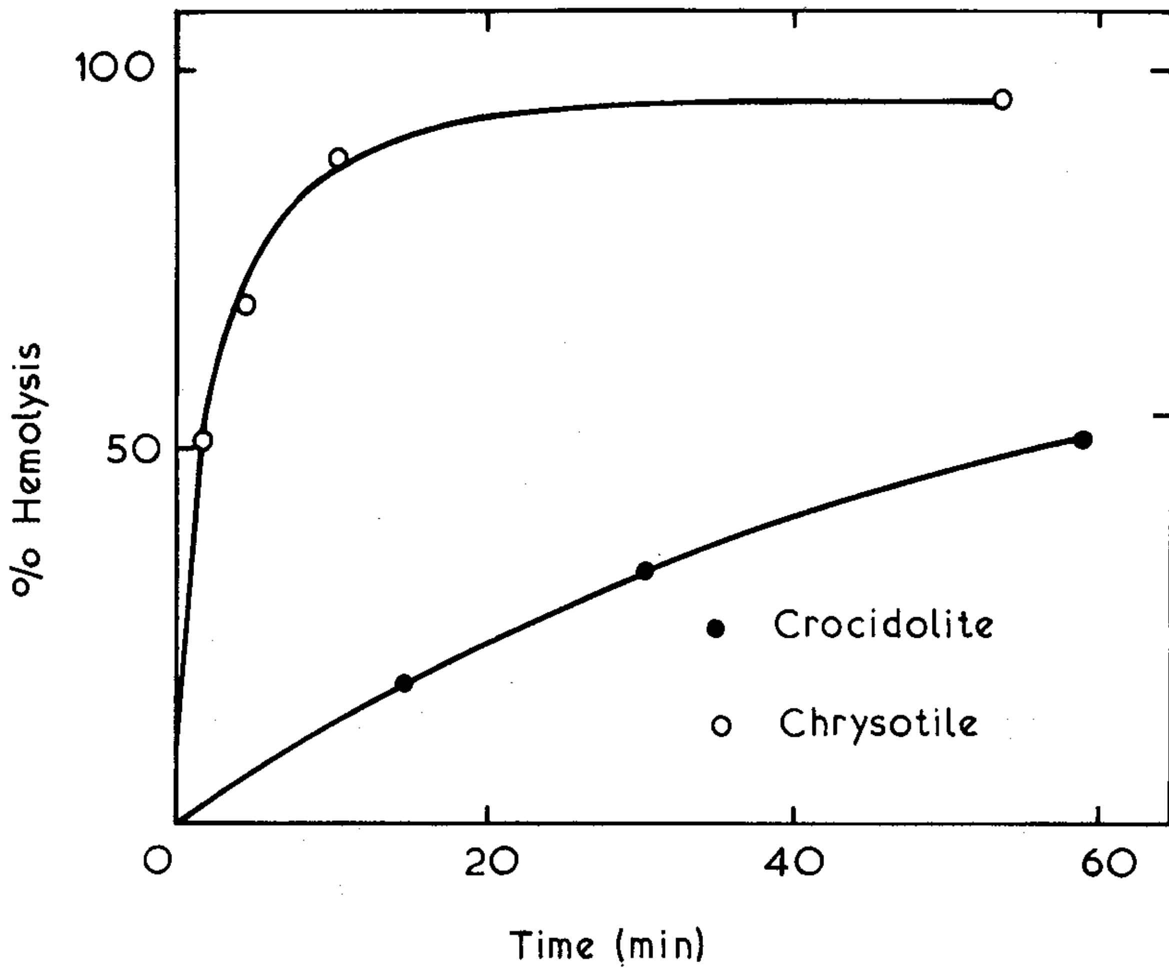


FIGURE 4. HEMOLYSIS BY ASBESTOS; EFFECT OF TIME OF INCUBATION FOR CHRYSOTILE B AND CROCIDOLITE