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A Comparative Study on the Hemolytic Action of Short Asbestos Fibers on Human, Rat, and Sheep Erythrocytes[1]

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The hemolytic activity of short asbestos fibers isolated by a sedimentation procedure was studied using human (HRBC), rat (RRBC), and sheep (SRBC) red blood cells. The initial velocity (V_i) of hemolysis is proportional to the concentration of fibers with HRBC and RRBC, but not with SRBC. Initial velocity (V_i) is 14.30 for HRBC, 5.75 for RRBC, and 3.60 for SRBC at a concentration of 1,000 μg of fibers/ml. Maximum velocity (V_m) is reached at 400 μg/ml with HRBC and its value is 16.7. With RRBC and SRBC, V_m is reached at 600 μg/ml and its value is 10 and 3.6, respectively. The 50% hemolytic concentration (HC_{50}) at 60 min of incubation is 75 μg/ml for HRBC, 240 μg/ml for RRBC, and 260 μg ml for SRBC. It appears that the sensitivity against the short asbestos fibers of the three types of RBC used is in the following order: HRBC > RRBC > SRBC.

INTRODUCTION

The hemolytic activity of chrysotile asbestos was reported by Macnab and Harington (1967). Since then, this *in vitro* model has been extensively used to study the biological activity of asbestos. Although many hypotheses have been put forward to explain the precise nature of the damages caused by the asbestos fibers to the red blood cell membrane, the mechanism by which these fibers cause the damages is still not completely elucidated.

Most studies on the hemolytic activity of asbestos fibers were done with erythrocytes of sheep (Macnab and Harington, 1967; Schnitzer and Pundsack, 1970; Harington *et al.*, 1971a, b; Schnitzer *et al.*, 1971; Light and Wei, 1977a, b) or of man (Secchi and Rezzonico, 1968; Harington *et al.*, 1971b; Schnitzer *et al.*, 1971; Morgan *et al.*, 1977; Jaurand *et al.*, 1979b, 1980). Horse blood cells were used in only a few studies (Schnitzer *et al.*, 1971; Harington *et al.*, 1971a). Rat blood cells (Hefner and Gehring, 1975; Harington *et al.*, 1971a) and rabbit erythrocytes (Harington *et al.*, 1971a; Desai *et al.*, 1975) were also used.

Because the chemical composition of the red blood cell membrane from different species is known to be different, it appeared pertinent to do a comparative study on the hemolytic effect of well-characterized asbestos fibers on erythrocytes from different sources. This paper describes the kinetics of the hemolytic action of short chrysotile fibers on human, sheep and rat erythrocytes.

[1] This work was supported by Grant G0179 from the CRSNG of Canada.

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MATERIALS AND METHODS

Preparation of erythrocyte suspensions. Human (HRBC), rat (RRBC), and sheep (SRBC) blood cells were prepared at a concentration of 4%. Human blood was collected from healthy donors by venipuncture; sheep blood was collected from the radial vein; and rat blood was collected from the inferior vena cava of Long Island rats weighing 250–300 g. Cells were washed three times with Veronal buffer, pH 7.28.

Asbestos fibers. Chrysotile fibers, isolated by a sedimentation procedure, from Quebec 4T30 chrysotile (Dunnigan *et al.*, 1980) were used in this study. The surface charge represented by the zeta potential (Light and Wei, 1977a), at pH 7.3, had a value of +52 mV. The length distribution of the fibers as determined by Jolicoeur *et al.* (1981) showed that more than 90% of the fibers were shorter than 2.0 μm and that the maximum fiber length was 8 μm. These same authors have also determined the specific area of the short sedimented fibers from nitrogen adsorption and water adsorption: the first method yielded a specific area of 20 m^2/g and the second 28 m^2/g.

Hemolysis. Weighed amounts of asbestos fibers (200, 400, 800, 1200, and 2000 μg/ml) were suspended in Veronal buffer using a Dounce tube. Veronal buffer was prepared as described by Harington *et al.* (1971a) and just before use, one part of the stock solution was diluted with four parts of distilled water. Exactly 1 ml of the 4% suspension of erythrocytes previously warmed at 37°C was added to 1 ml of the suspension of fibers at the desired concentration (final concentration of red blood cells was 2% and that of asbestos fibers was 100, 200, 400, 600, or 1000 μg/ml). The Falcon tubes were immediately placed into a Dubnoff metabolic shaking incubator set at 37°C and at 45 strokes/min. After 0.5, 1–10, 15, 30, and 60 min of incubation, 0.2 ml of a 2.5% solution of glutaraldehyde was added to each sample to stop hemolysis. The samples were then centrifuged at 900*g* for 5 min and the optical density of the clear supernatant was read at 541 nm in a Bausch and Lomb Spectronic 21 spectrophotometer.

Complete lysis was obtained by preparing a 2% erythrocyte suspension in distilled water with two drops of Triton X-100 and 0.2 ml of glutaraldehyde. The hemolytic activity of the fibers was calculated from the average optical density of three replicates and was expressed as a percentage of the totally lysed control (100%) optical density.

Electron microscopy. A drop of a diluted suspension of the short fibers was placed on a Formvar-coated grid and allowed to dry. A second Formvar-coated grid was deposited over the dried fibers and the "sandwich" was observed in a Philips EM 300 electron microscope.

RESULTS

The short asbestos fibers prepared by the sedimentation procedure measure less than 8 μm in length (Fig. 1). They are well dissociated and large aggregates are very infrequently observed.

The curves of hemolysis obtained with the erythrocytes of the three different species make it possible to identify different stages during the hemolytic process.

FIG. 1. Suspension of short chrysotile fibers dispersed with Dounce ball tube. ×5460.

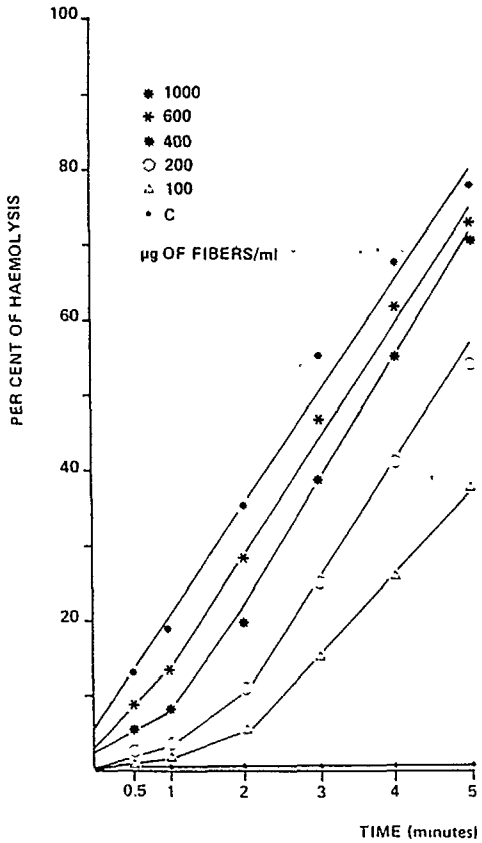

FIG. 2. Human RBC. Percentages of hemolysis during the 0- to 5-min period. Correlation coefficients: 1000 μg ml 0 to 5 min (r = 0.9950), 600 μg ml 1 to 5 min (r = 0.9965), 400 μg ml 2 to 5 min (r = 0.9988), 200 μg ml 2 to 5 min (r = 0.9991), 100 μg ml 2 to 5 min (r = 0.9990).

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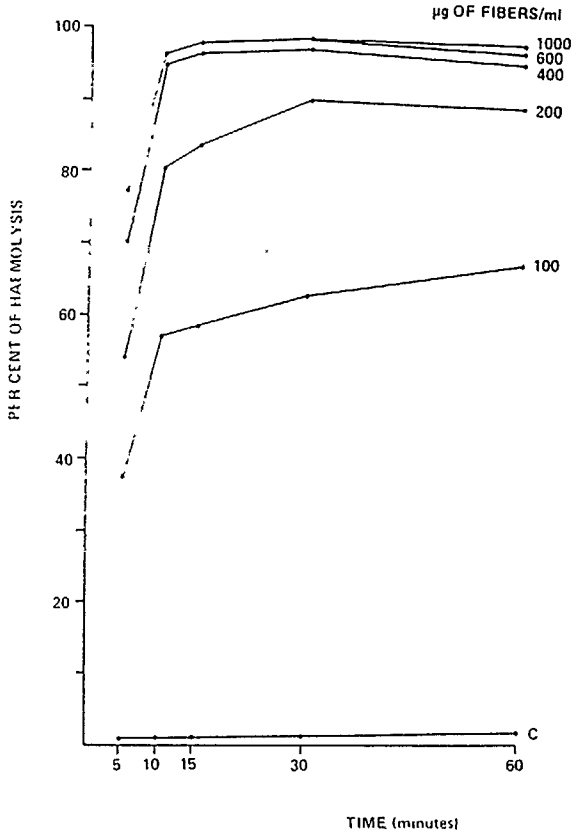

FIG. 3. Human RBC. Percentages of hemolysis during the 5- to 60-min period.

With HRBC, between 0 and 5 min, the kinetics is not linear when the concentrations of fibers are from 100 to 600 μg/ml (Fig. 2). The initial velocity (V_i) between 0 and 1 min and the maximum velocity (V_m) between 1 or 2 and 5 min were calculated. At a fiber concentration of 1000 μg/ml, the kinetics becomes linear and V_i is identical to V_m. Figure 3 shows the kinetics of hemolysis between 5 and 60 min of incubation. At higher doses (400–600 μg/ml), the curves reach a plateau at 10 min. At 200 μg/ml, a plateau is reached at 30 min only, but at 100 μg/ml a maximum is still not reached at 60 min.

With RRBC (Fig. 4), V_i was calculated as occurring between 0 and 2 min and V_m between 2 and 8 min. The kinetics of hemolysis with RRBC is not linear even at the highest concentrations of fibers. Later during incubation, a plateau is reached at 30 min for all the concentrations used (Fig. 5).

When SRBC are used, the kinetics of hemolysis is linear between 0 and 15 min (Fig. 6) and V_i is identical to V_m. Between 30 and 60 min (Fig. 7), hemolysis is not completed and a positive slope is still observed even for the highest concentrations of fibers.

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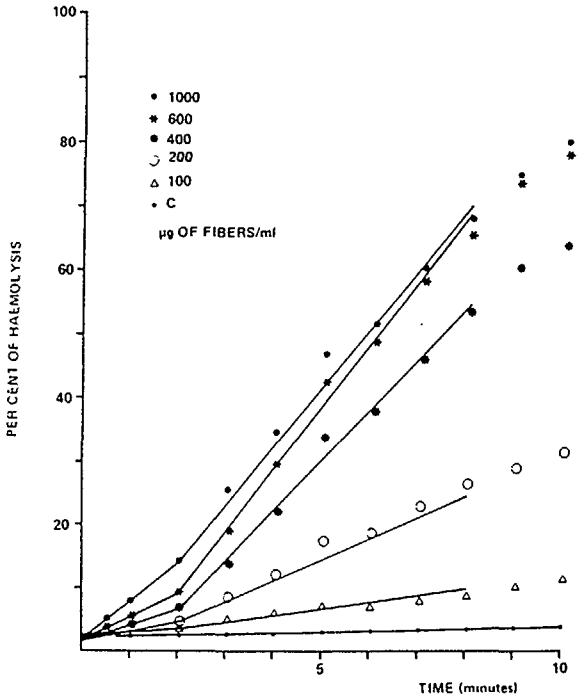

FIG. 4. Rat RBC. Percentages of hemolysis during the 0- to 10-min period. Correlation coefficients: 1000 μg/ml 2 to 5 min (r = 0.9920), 600 μg/ml 2 to 6 min (r = 0.9951), 400 μg/ml 2 to 8 min (r = 0.9958), 200 μg/ml 2 to 8 min (r = 0.9924), 100 μg/ml 2 to 8 min (r = 0.9729).

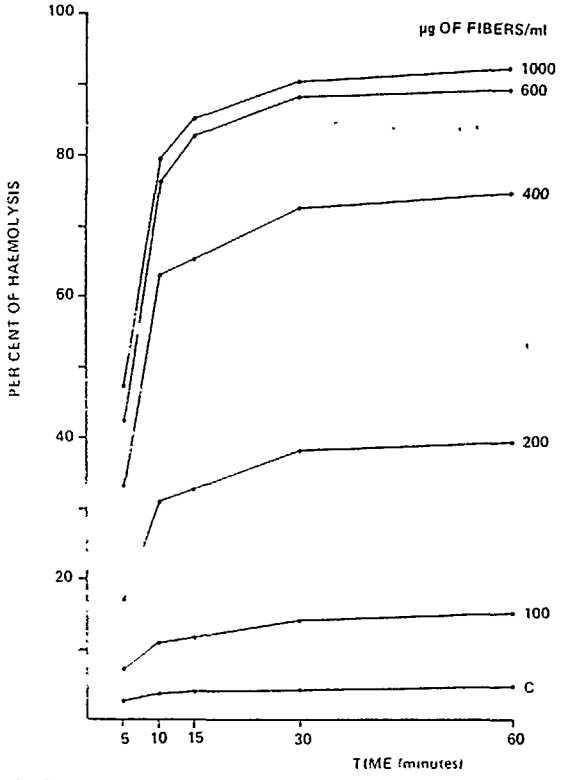

FIG. 5. Rat RBC. Percentages of hemolysis during the 5- to 60-min period.

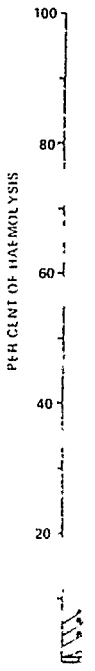

FIG. 6. Sheep RBC. Pe
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1000 μg/ml (r = 0.9976).

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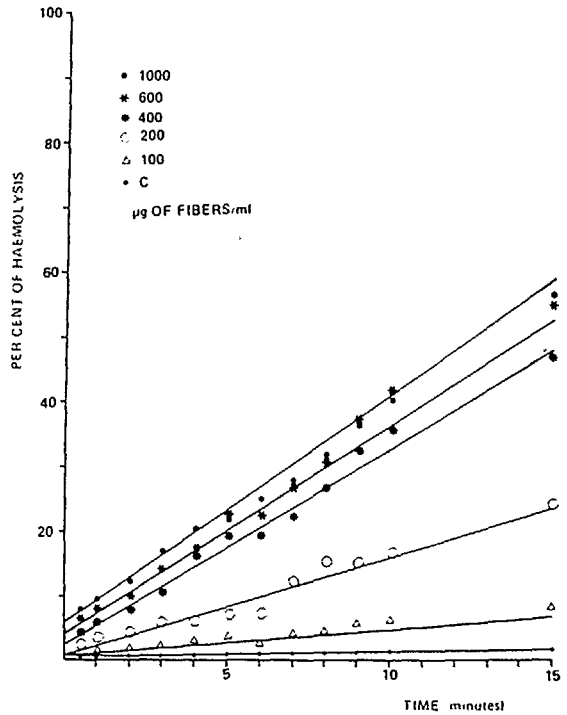

FIG. 6. Sheep RBC. Percentages of hemolysis during the 0- to 15-min period. Correlation coefficients: 100 μg/ml (r = 0.9905), 200 μg/ml (r = 0.9873), 400 μg/ml (r = 0.9900), 600 μg/ml (r = 0.9956), 1000 μg/ml (r = 0.9976).

If V_i for the three types of blood is plotted on the same graph, (Fig. 8), it is easily seen that velocity is proportional to the concentration of fibers when HRBC and RRBC are used, but it is not when SRBC are used. The value of V_i is 14.30 for human blood, 5.75 for rat blood, and 3.60 for sheep blood at a concentration of 1000 μg of fibers/ml; V_i is expressed as the percentage of hemolysis per minute. At concentrations between 0 and 400 μg/ml, V_i of hemolysis is comparable for RRBC and SRBC.

Figure 9 shows V_m for the three types of blood. V_m is reached at 400 μg/ml with HRBC and its value if 16.7; with RRBC and SRBC, V_m is reached at 600 μg/ml and their values are 10 and 3.6, respectively.

The percentages of hemolysis observed at 60 min of incubation (when the concentration is 1000 μg of asbestos/ml) are not very different for the three types of blood used. However, with lower concentrations, one can see that the sensitivity of the three types of blood are different. Between 30 and 60 min, the percentages of hemolysis reach a plateau; at 60 min, we can determine the HC_{50} (HC_{50} represents the 50% hemolytic concentration; see Schnitzer *et al.* (1971)). In the portion of the curves corresponding to 50% of hemolysis, the percentage is almost propor-

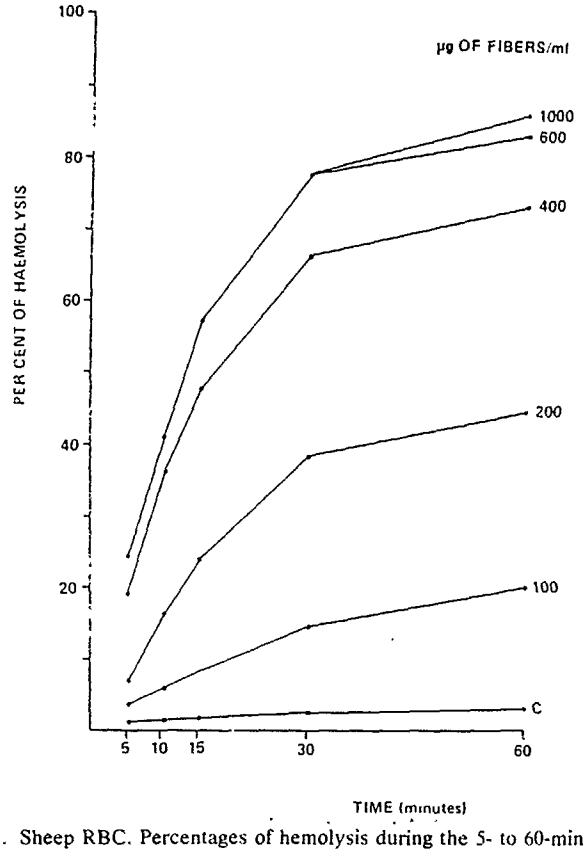

FIG. 7. Sheep RBC. Percentages of hemolysis during the 5- to 60-min period.

tional to the concentration of fibers. Therefore, this HC_{50} can be interpolated for the three types of blood: for HRBC it is 75 μg/ml, for RRBC it is 240 μg/ml, and for SRBC it is 260 μg/ml.

DISCUSSION

The results on the hemolytic activity of chrysotile asbestos fibers found in the literature are difficult to compare because of (1) the different types of blood used, (2) the utilization of fibers of diverse origin often poorly characterized, (3) the different protocols used to measure the hemolytic activity, (4) the important differences in the controls used, and (5) the temperature and the duration of the incubation. To illustrate this diversity we have summarized the results reported by different authors on the hemolytic activity of chrysotile fibers (Table 1).

In the present work, we have used well-characterized chrysotile fibers isolated by a procedure that does not modify the structure of the native fibers. Furthermore, these short fibers give stable and homogeneous suspensions. It is well known that ball-milling modifies considerably the physicochemical properties of

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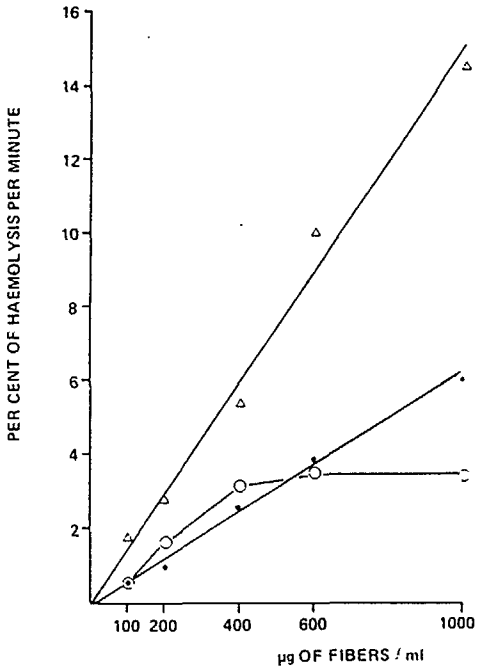

FIG. 8. Initial velocity (V_i). Percentage of hemolysis per minute. SRBC (○), RRBC (●) (r = 0.9970), and HRBC (△) (r = 0.9924).

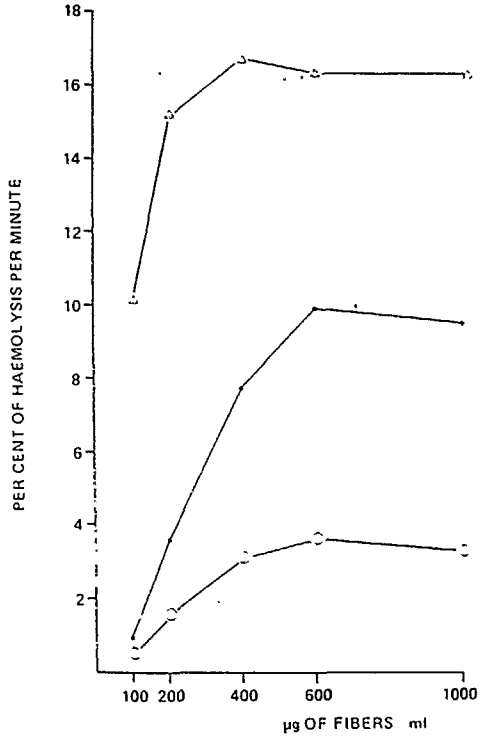

FIG. 9. Maximum velocity (V_m). Percentage of hemolysis per minute. SRBC (○), RRBC (●), and HRBC (△).

TABLE 1

SUMMARY OF THE PERCENTAGES OF HEMOLYSIS WITH CHRYSOTILE FIBERS FOUND IN THE LITERATURE

Source	Fiber	Species RBC-dilution[a] (%)	Fiber dose[a]	Incubation time temperature	Percentage of Hemolysis
Rahman *et al.* (1974)	Indian chrysotile	Sheep 0.1	250 μg/ml	2 hr 37°C	73.1
Morgan *et al.* (1977)	UICC Rodhesian chrysotile	Man ·0.2	400 μg/ml	10 min 25°C	19.8
Langer *et al.* (1978)	Calidria chrysotile RC144, fibrils 77% <0.5 μg/ml, fibers 95% <5 μm	Sheep 2	1000 μg/ml	2 hr 37°C	80.0
			400 μg/ml		50.0
Schnitzer and Bunescu (1970)	Chrysotile from Jeffrey mine, Quebec	Sheep 2	150 μg/ml	2 hr 37°C	50.0
Schnitzer and Pundsack (1970)	UICC chrysotile A and B	Sheep 2	3125 μg/ml		100.0
	Chrysotile from Jeffrey mine, Quebec, surface-area 63,200 cm²/g	Sheep 2	125 μg/ml	2 hr 37°C	7.5
			312.5 μg/ml		45.0
			625 μg/ml		100.0
Schnitzer *et al.* (1971)	Chrysotile B UICC, surface-area 44,000 cm²/g	Sheep 2	240 μg/ml	2 hr 37°C	50.0
	Chrysotile B UICC, surface-area 44,000 cm²/g	Man 2	230 μg/ml	2 hr 37°C	50.0
	Chrysotile B UICC surface-area 44,000 cm²/g	Horse 2	270 μg/ml	2 hr 37°C	50.0
	Chrysotile from Jeffrey mine, Quebec, surface-area 11,560 cm²/g	Sheep 2	800 μg/ml	2 hr 37°C	50.0
	Chrysotile from Jeffrey mine, Quebec, surface-area 63,200 cm²/g	Sheep 2	110 μg/ml	2 hr 37°C	50.0
	Chrysotile from Jeffrey mine, Quebec, surface-area 63,200 cm²/g	Man 2	155 μg/ml	2 hr 37°C	50
Desai *et al.* (1975)	UICC Canadian chrysotile B	Rabbit 0.25	2.5 mg/ml	50 min	·90.0

TABLE 1—*Continued*

Source	Fiber	Species RBC-dilution[a] (%)	Fiber dose[a]	Incubation time temperature	Percentage of Hemolysis
Koshi *et al.* (1968)	Chrysotile from Japan and South Africa	Rat	1 mg/ml	1 hr 37°C	—
Secchi and Rezzonico (1968)	UICC chrysotile A	Man 2	12.5 mg/ml	30 min 37°C	100.0
			12.5 mg/ml		
	UICC chrysotile B	2	12.5 mg/ml		96.0

	surface area 44,000 cm²/g	2		37°C	
	Chrysotile B UICC surface-area 44,000 cm²/g	Horse 2	270 μg/ml	2 hr 37°C	50.0
	Chrysotile from Jeffrey mine, Quebec, surface-area 11,560 cm²/g	Sheep 2	800 μg/ml	2 hr 37°C	50.0
	Chrysotile from Jeffrey mine, Quebec, surface-area 63,200 cm²/g	Sheep 2	110 μg/ml	2 hr 37°C	50.0
	Chrysotile from Jeffrey mine, Quebec, surface area 63,200 cm²/g	Man 2	155 μg/ml	2 hr 37°C	50.0
Desai *et al.* (1975)	UICC Canadian chrysotile B	Rabbit 0.25	2.5 mg/ml	50 min	~90.0

TABLE I—*Continued*

Source	Fiber	Species RBC-dilution[a] (%)	Fiber dose[a]	Incubation time temperature	Percentage of Hemolysis
Koshi *et al.* (1968)	Chrysotile from Japan and South Africa	Rat 1	1 mg/ml	1 hr 37°C	—
Secchi and Rezzonico (1968)	UICC chrysotile A	Man 2	12.5 mg/ml 12.5 mg/ml	30 min 37°C	100.0
	UICC chrysotile B,	2	12.5 mg/ml		96.0
	Canadian short fibers,	2	12.5 mg/ml		94.5
	Canadian medium fibers,	2	12.5 mg/ml		88.5
	Canadian long fibers	2	12.5 mg/ml		81.0
Jaurand *et al.* (1979a)	UICC chrysotile A	Man 0.5	500 μg/ml	60 min 37°C	100.0
Jaurand *et al.* (1979b)	UICC chrysotile B	Man 0.5	500 μg/ml	60 min 37°C	~95.0
	UICC chrysotile B	0.5	220 μg/ml		~82.0
	UICC chrysotile B	0.5	100 μg/ml		~60.0
	UICC chrysotile B	5	500 μg/ml		~35.0
Light and Wei (1977a)	UICC chrysotile A	Sheep 0.75	1.25 mg/ml	1 hr 23°C	81.9
	UICC chrysotile B	0.75	1.25 mg/ml	23°C	60.5
Harington *et al.* (1971b)	UICC chrysotile	Man OD lysat 0.7	800 μg/ml	50 min 37°C	70.0
Harington *et al.* (1971a)	UICC chrysotile, 80% < 50 μm surface area 22.8 m²/g	Sheep OD lysat 0.7 -1	12.5 mg/ml	50 min 37°C	73.0
	UICC chrysotile	Man	12.5 mg/ml	50 min 37°C	77.0
	UICC chrysotile	Rat	12.5 mg/ml	50 min 37°C	74.0
Macnab and Harington (1967)	Chrysotile 80% < 5 μm	Sheep	12.5 mg/ml	50 min 37°C	100.0

[a] Final concentration.

the fibers (Langer *et al.*, 1978). The hemolytic action of these short fibers was measured using human and sheep red blood cells which are reported in the literature to be the most frequently used on asbestos and also red blood cells of the rat which is the most frequently utilized animal in laboratories.

Our results show that the three types of blood used have a different sensitivity to the same chrysotile fibers. Human erythrocytes are the most vulnerable to the hemolytic action of these fibers. With human blood, hemolysis is the most rapid and the calculated V_i is 2.5 times that of rat erythrocytes and 4 times that of sheep erythrocytes; for human blood, V_m is the highest and its estimated HC_{50} is 3 times lower than that of the two other types of blood. When the results for rat and sheep blood are compared, it seems that the latter is slightly less sensitive than the former to the hemolytic action of the fibers used.

It is difficult to explain the differences in the responsiveness of the three types of blood to the same chrysotile fibers. Nevertheless, one can point to the different physicochemical properties of the red blood cells of the three species used. For example the mean diameters of human and sheep red blood cells are 8.5 and 5.2 μm, respectively, with the results that for HRBC and SRBC, the mean surfaces are 163 and 67 μm^2, respectively (Weinstein, 1974). The surface charges expressed as the electrophoretic mobility ($\mu m\ sec^{-1}\ V^{-1}\ cm$) at 25°C are 1.08, 1–1.28, and 1.14, respectively for HRBC, RRBC, and SRBC (Seaman, 1975). The permeability of the three types of blood toward glycerol or phosphate ions is different (Van Deenan and De Gier, 1974). Finally the phospholipid composition of the membrane of the three types of RBC as reported by Van Deenan and De Gier (1974) also underlines these differences with SP/PC ratios (SP: sphingomyelins, PC: phosphatidylcholine) of 1/4 for RRBC, 2/3 for HRBC, and 12/1 for SRBC. These different physicochemical properties probably play an important role in the sensitivity of the RBC to chrysotile fibers but it would be imprudent to venture which one is the most important: surface is probably involved but the chemical composition of the RBC membrane could be as determinant.

In conclusion, although there are some differences in the results obtained with the different types of blood used, it can be said that all three types are acceptable to perform these studies on hemolysis. At this time, it appears that the use of well-characterized fibers and the method used to obtain the suspension of fibers are matters of the utmost importance.

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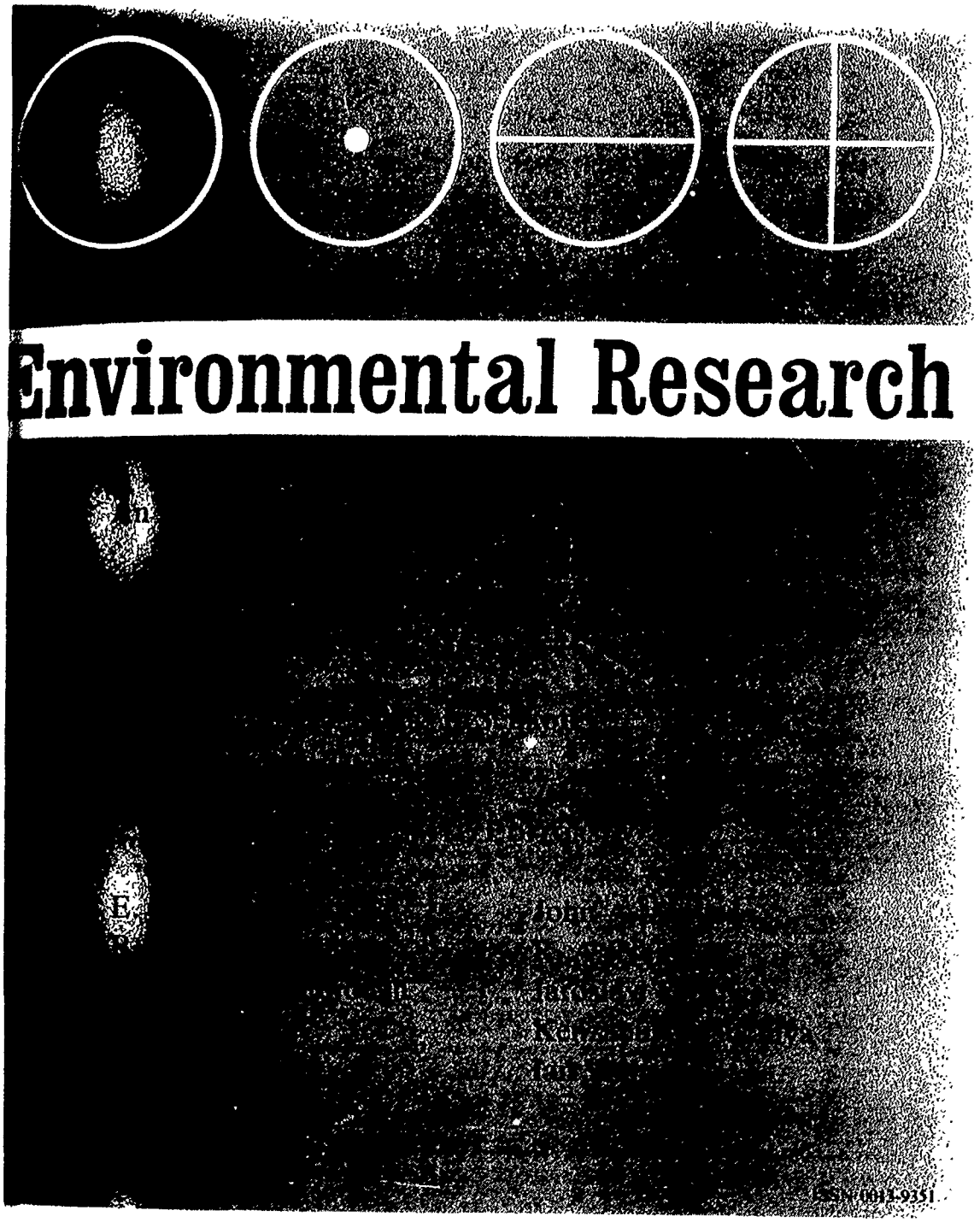

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