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Studies on *in Vitro* Chrysotile-Pleural Mesothelial Cell Interaction: Morphological Aspects and Metabolism of Benzo-3,4-pyrene

by M. C. Jaurand,* I. Bastie-Sigeac,* L. Magne,† M. Hubert-Habart‡ and J. Bignon*

Cultures of rat pleural mesothelial cells (PMC) were exposed to nonlethal doses of UICC chrysotile A. The morphology was studied by optical and electron microscopy. The consequences of chrysotile ingestion on the rate of pinocytosis of horseradish peroxidase (HPR) metabolism and benzo-3-4-pyrene (BP) were studied.

Nonlethal doses of chrysotile (5 μg/mL) induced a time-dependent vacuolation of PMC; a dose-dependent inhibition of the vacuolation was observed when PMC were pretreated with DMSO. The origin of the vacuoles is not clear, but some features of autophagy and lysosomal storage were observed.

Chrysotile fibers did not modify the rate of pinocytosis of HRP. Similarly, the metabolism of BP was unchanged when BP and chrysotile were both added to the culture medium or when PMC were preincubated with the fibers 24 hr prior to the addition of BP.

Introduction

In vitro studies performed with various cell lines have demonstrated that chrysotile fibers are cytotoxic (*1*). However, the effect is dose-dependent, and low concentrations do not kill the cells but induce some pathological features. The aim of this work was, first, to study the morphology of rat pleural mesothelial cells, (PMC) treated in culture with nonlethal concentrations of chrysotile fibers and, secondly, to examine the consequences of chrysotile ingestion on the metabolism of the PMC with regard to pinocytosis of exogenous macromolecules (horseradish peroxidase, HRP) or polycyclic hydrocarbons (benzo-3-4-pyrene, BP).

*Groupe de Recherches et d'Etudes sur les Affections Respiratories et l'Environnement (INSERM U 139 et ERA CNRS 845), Centre Hospitalier Intercommunal, 40, Ave de Verdun, 94010 Creteil Cedex, France.

†Laboratorie d'Etude des Particules Inhalées, DASS, Paris, France.

‡Chimie Cancérologique, Institut Curie, 75231 Paris Cedex 05, France.

Material and Methods

Cell Culture

PMC were cultured in NCTC 109 + 10% fetal bovine serum as described by Jaurand et al. (*2*). The cells were used at confluency between the 8th and 20th passage.

Chrysotile Fibers

UICC chrysotile A fibers were used. The fibers were dispersed by sonication in the culture medium (20 kHz, 20 W, 5 min).

Cell Viability

The cell viability was determined by trypan blue exclusion and also by measuring the lactate dehydrogenase (LDH) released in the culture medium as described elsewhere (*3*).

Phase Contrast Microscopy

The enumeration of vacuoles was carried out by using phase contrast microscopy (× 370). The fields

were randomly selected, and 100 cells crossing a line in the eye piece were counted. The treatment of PMC was unknown by the reader in order to avoid subjective results.

Electron Microscopy

Cells were prepared for electron microscopy as described elsewhere (2). The acid phosphatase staining was performed on cells fixed with 2.3% glutaraldehyde in 0.045 *M* cacodylate buffer and stained by the technique of Miller and Palade (4). The HRP was detected by the technique of Graham and Karnovsky (5).

Spectrophotometry for HRP

The HRP activity was measured by using the assay of Steiman and Cohn (6). No endogenous peroxidase was found in PMC.

Metabolism of BP

^{14}C-BP was added to the cells either untreated or treated with 2 μg/mL of chrysotile fibers. Both BP and chrysotile were added to the medium or PMC was preincubated with chrysotile for 24 hr prior to BP addition. After 24 hr of incubation with BP, the culture medium was extracted with ethyl acetate. The organosoluble metabolites were separated by HPLC and the proportion of conjugates, in the aqueous phase, was also determined.

Results

In order to incubate the cells with a nonlethal concentration of chrysotile fibers, a dose-effect relationship was established. The results are expressed in Table 1 and show that 5 μg/mL did not highly modify the viability of the PMC. The effect of BP was also studied, and no modification of the growth curves was observed with 1 μg/mL of BP.

Morphological Studies

Addition of 5 μg/mL of chrysotile fibers to confluent PMC resulted in the appearance of regular translucent vacuoles, often perinuclear and reaching approximately 2 μm diameter after 2 hr of incubation (Fig. 1). Their number was time-dependent and after 20 hr of incubation 80.2 ± 20% of PMC contained vacuoles (mean obtained with four different cell strains) (Fig. 2). The mean number of vacuoles per PMC was 3.0 ± 2.6 and 6.6 ± 3.7 after 8 hr and 20 hr of incubation with the fibers, respectively (Fig. 3).

Previous studies (7) showed that chrysotile fibers were ingested by PMC in culture and that degranulation of lysosomal enzymes occurred into the phagocytic vacuole. Following 2 hr of incubation with chrysotile fibers, electron microscopy revealed both the presence of phagocytic vacuoles containing chrysotile fibers and large regular vacuoles which were generally empty or contained traces of membrane whorls ressembling myelin figures (Fig. 4). Acid phosphatases were present not only in the phagosome (Fig. 5) but also in close contact with

Table 1. Viability of confluent PMC following 20 hr of incubation with UICC chrysotile A.

Concentration, μg/mL	n[a]	Unstained PMC, %	LDH released, %
0	3	100 ± 0	4.2 ± 4.2
5	2	99 ± 1	7.3 ± 4.5
10	3	97.7 ± 2.6	8.0 ± 10.9
20	2	87.3 ± 8	11.1 ± 8.9
50	3	73 ± 14	21.1 ± 16.9

[a]Number of cell strains.

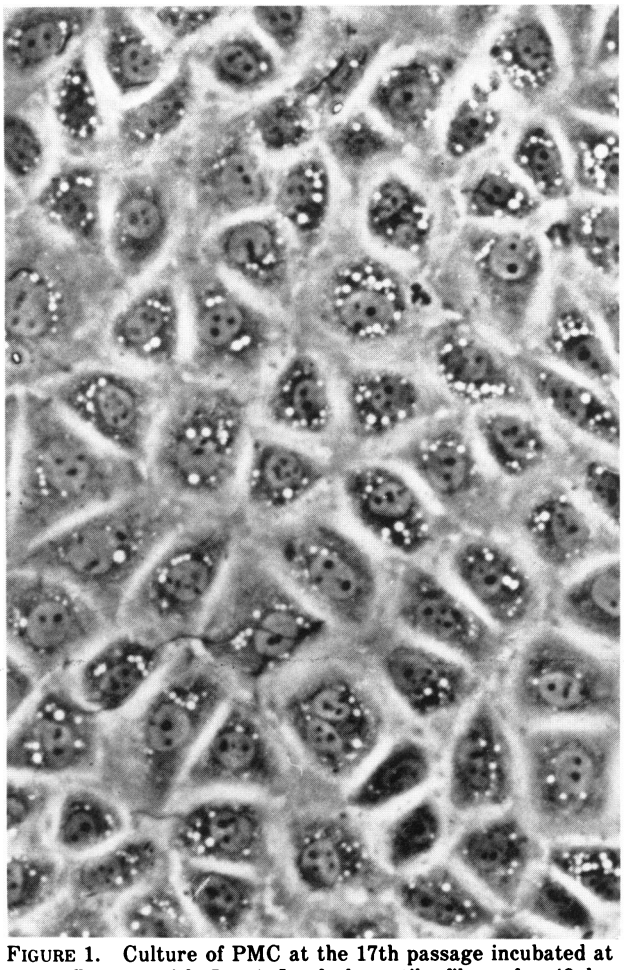

FIGURE 1. Culture of PMC at the 17th passage incubated at confluency with 5 μg/mL of chrysotile fibers for 48 hr. Phase contrast microscopy, × 310.

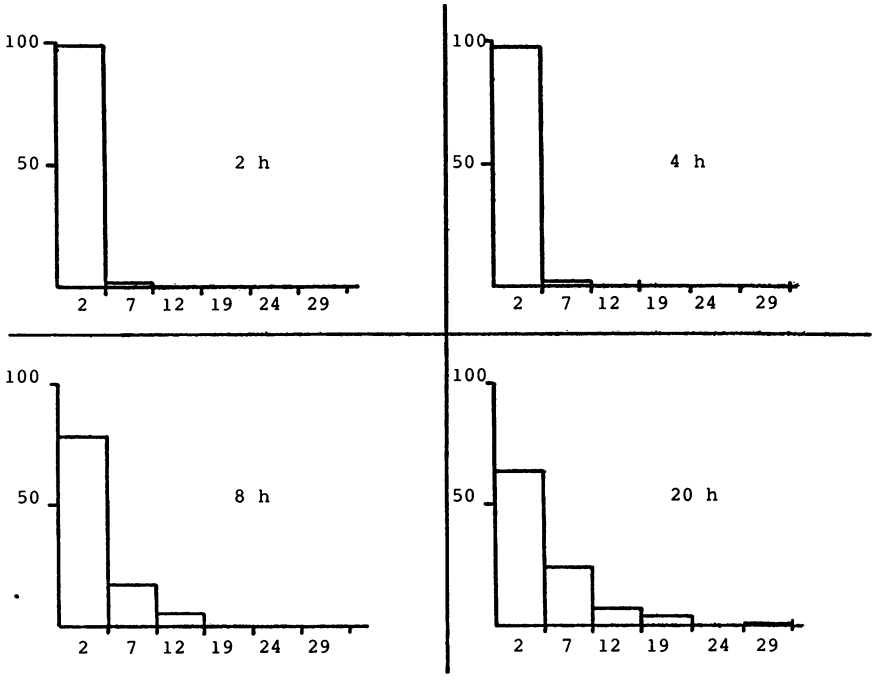

FIGURE 2. Kinetics of the formation of vacuoles by PMC incubated at confluency with 5 μg/mL of chysotile fibers. Histograms showing the distribution of the number of PMC with the number of vacuoles per cell, after various times of contact with the fibers. PMC were at the 8th passage.

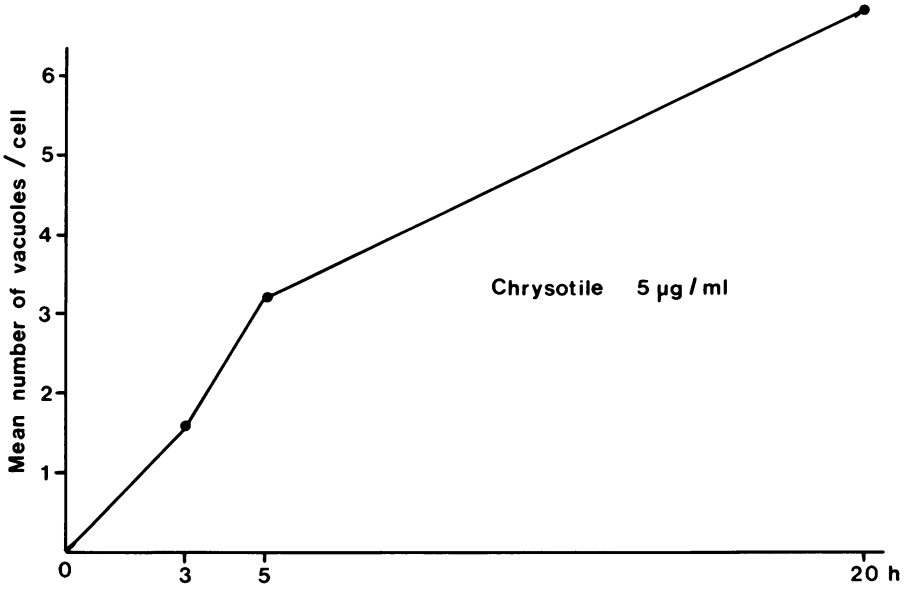

FIGURE 3. Variation of the mean number of vacuoles per cell, with the time of contact with the fibers. PMC were at 20th passage.

these structures, suggesting that they were secondary lysosomes.

In some cells cytoplasmic inclusions could be seen which had the appearance of lipid inclusions and were either highly ordered crystalloid or lamellated structures. These inclusions were contained by a single membrane.

Effect of DMSO

As BP was solubilized in DMSO (0.1%), the effect of DMSO was observed. DMSO was added to the culture medium 1 hr prior to the addition of chryso-

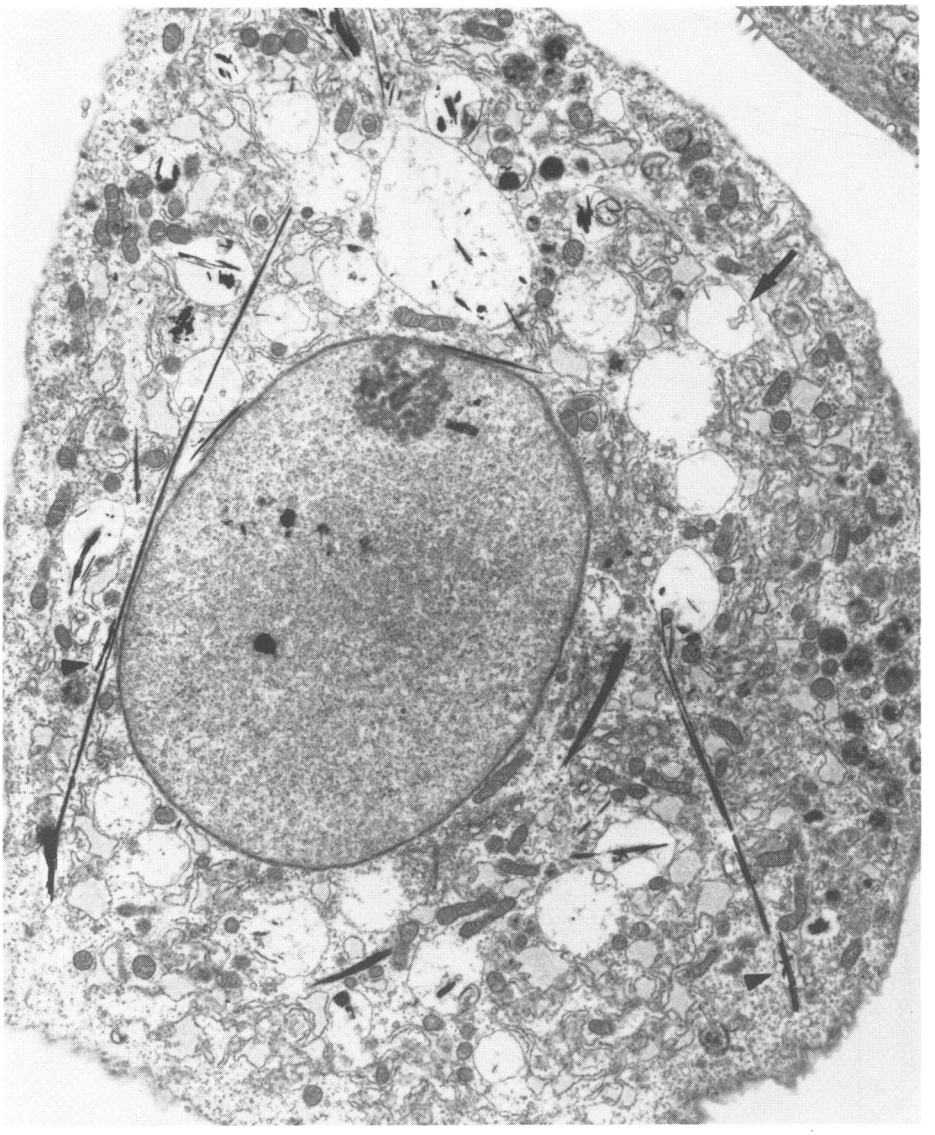

FIGURE 4. Electron microscopy of PMC treated as in Figure 2. Numerous vacuoles are seen, generally empty but sometimes with membrane whorls (arrow). The chrysotile fibers were generally in phagosomes (head of arrow) but were sometimes present inside the large regular vacuoles. × 5300.

tile. There was a dose-dependent inhibition of the vacuolation of PMC. In Figure 6, the variation of the mean number of vacuoles per PMC with the DMSO concentration is reported.

HRP Pinocytosis

The spectrophotometric assay revealed that HRP entered the cells when incubated with PMC. The amount of HRP ingested was time- and dose-dependent. The addition of 5 μg/mL of chrysotile fibers did not change the pinocytic rate (Table 2).

Pinocytic vesicles were also demonstrated by electron microscopy (Fig. 7). This revealed that HRP and chrysotile fibers were generally in separate vacuoles when both materials were incubated with PMC.

Metabolism of BP

BP was metabolized by PMC in culture. PMC were used at the 7th passage. After 24 hr of incubation 60% of the BP was metabolized. The propor-

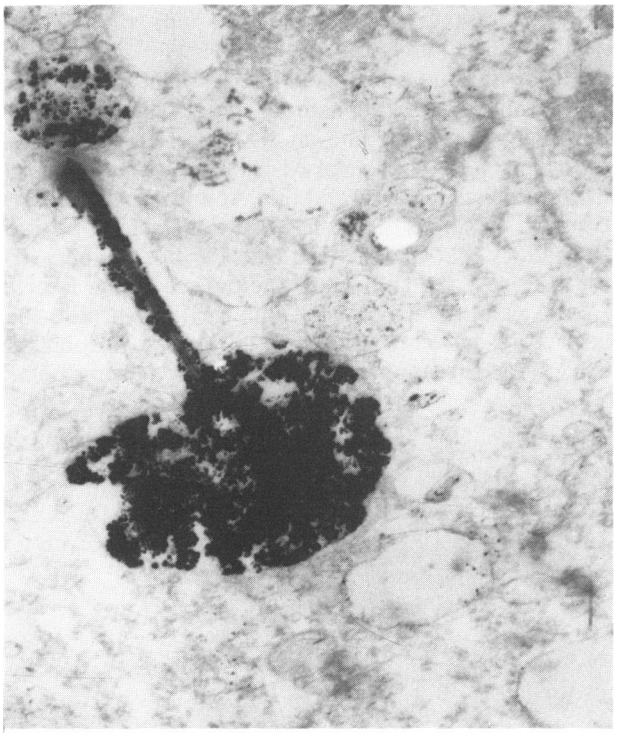

FIGURE 5. High magnification of a phagolysosome inside a PMC. The acid phosphatase reaction shows the presence of acid phosphatase inside the phagocytic vacuole. × 31,000.

tions of metabolites in the aqueous and organic phases were 60 and 40%, respectively. When PMC were incubated with both 2 μg/mL of chrysotile fibers and BP, the fraction of BP metabolized was not significantly different; similarly, the proportion of metabolites obtained in the aqueous or organic phases was unchanged. No modification of the proportion of water-soluble or organo-soluble radioactivity was found when PMC were pretreated with crystotile fibers (Table 3).

Discussion

The results reported here clearly demonstrate that nonlethal concentrations of UICC chrysotile A fibers induce morphological abnormalities in rat pleural mesothelial cells in culture. As far as the rate of pinocytosis of HRP and the metabolism of BP are concerned, the metabolism of PMC was unchanged.

This intense vacuolation of mammalian cells has been observed by others when cells were exposed to chloroquine (*8, 9*). The pictures shown by Wibo and Poole (*10*) of fibroblasts exposed to chloroquine are very similar to those observed with PMC. Some

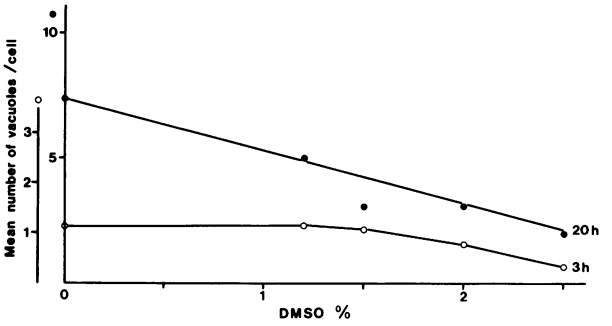

FIGURE 6. Effect of DMSO on the mean number of vacuoles per PMC. PMC were incubated for 1 hr with DMSO before the addition of 5 μg/mL of chrysotile fibers. The number of vacuoles was determined (○) 3 hr and (●) 20 hr following chrysotile exposure.

results reported here show that features of autophagy and lysosomal storage can be observed. However, large empty vacuoles are different from autophagic vacuoles; their significance is not clear, but it could be due to the storage of undigestible or undigested material conducive to an osmotic effect. The mechanism is not defined, and further studies must be performed in order to explain this effect. Such studies are of particular interest, since crocidolite fibers, in spite of being ingested, did not induce such an effect (*11*).

The inhibition of vacuolation observed after DMSO treatment is not clear; it could be due to a change in the physical state of the membranes since it is known that DMSO increases the transition temperature of some phospholipids (*12*). It is not due to an inhibition of phagocytosis, since numerous intracellular chrysotile fibers were identified by electron microscopy.

It is interesting to note that HRP pinocytosis is not increased during chrysotile exposure. This result indicates a distinction between the sites of at-

Table 2. Spectrophotometrical demonstration of pinocytosis of HRP.[a]

	Intracellular HRP activity after various incubation times, ng/flask		
HRP, mg/mL	60 min	105 min	180 min
HRP alone			
0.25	8.4	9.2	19.2
0.50	22.0	25.0	54.4
1.00	30.5	52.9	72.0
HRP + 5 μg/mL Ch			
0.25	8.5	12.1	17.3
0.50	24.3	33.8	35.7
1.00	34.2	39.3	72.0

[a]PMC at 13th passage were incubated with various amounts of HRP with or without UICC chrysotile A (Ch). PMC were then washed eight times with PBS, and the amount of HRP ingested was measured as described in Material and Methods.

Table 3 Proportion of radioactivity from ^{14}C-BP after extraction of the culture medium with ethyl acetate (EA).

PMC treatment	Water-soluble, %	EA-soluble, %
BP	37	63
BP + chrysotile	40	60
Chrysotile 24 hr, prior BP	37	63
Chrysotile 4 hr, prior BP	35	65

tachment of the chrysotile fibers on the plasma membrane and the sites of internalization of HRP. Pinocytosis of HRP follows an endocytic uptake of "dual carrier" type without adsorption. From the electron microscopic studies, and according to the previous results, chrysotile fibers may then be ingested on the single carrier model (*13*).

Other authors have studied the effect of asbestos fibers on the metabolism of polycyclic hydrocarbons. Our results do not show an increase in the metabolism of BP as observed by Mossman et al. (*14*) using methylcholanthrene (MCA) and crocidolite on tracheal organ cultures. The difference could be due to the fact that MCA was adsorbed on the fibers, to the origin of the cells or to the nature of the fibers. Daniel et al. (*15*) found a similar HPLC profile for untreated and chrysotile-treated human fibroblasts except for the appearance of an early eluting polar

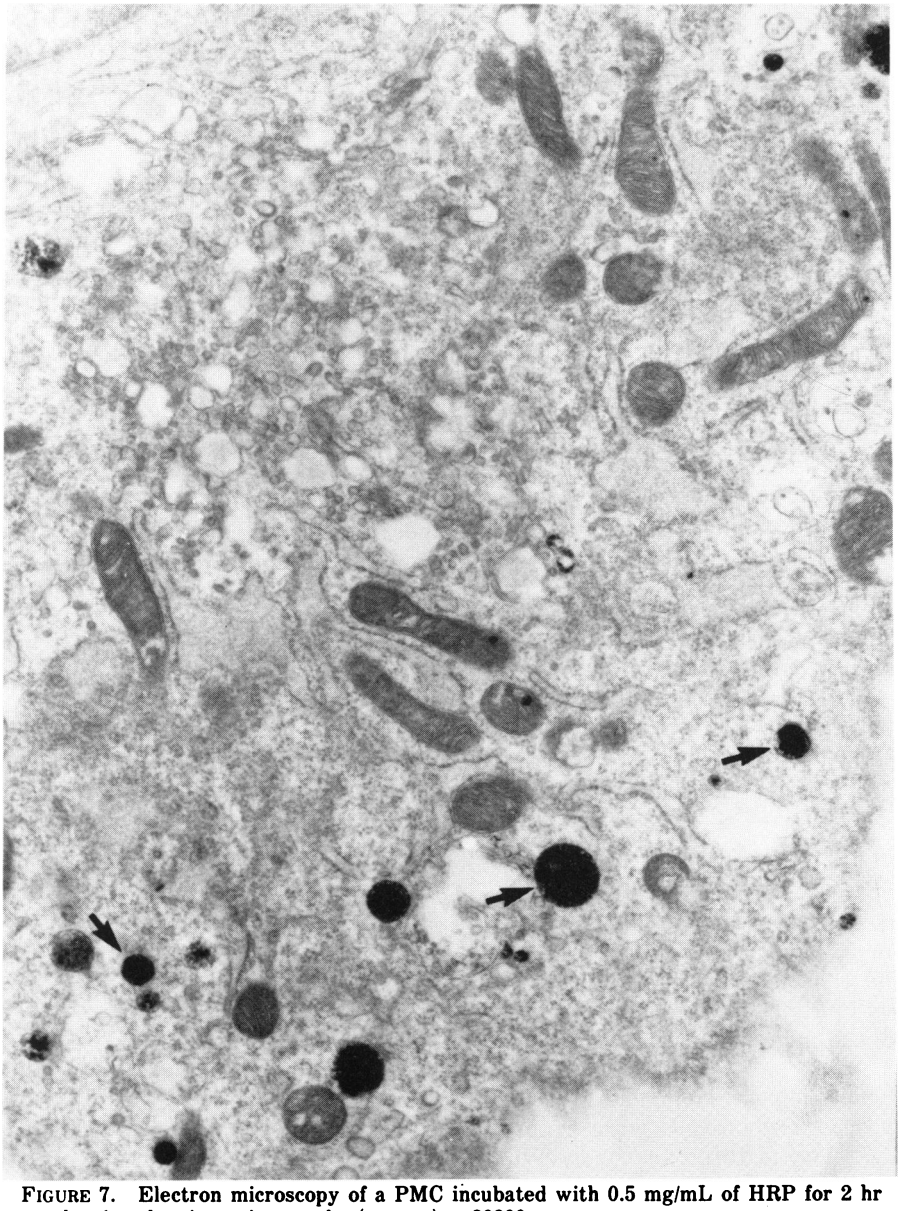

FIGURE 7. Electron microscopy of a PMC incubated with 0.5 mg/mL of HRP for 2 hr showing the pinocytic vacuoles (arrows). × 20200.

material observed when cells were treated with chrysotile 24 hr before the hydrocarbon was added. The stability in those aspects of metabolism described here confirmed that the viability of chrysotile-treated PMC was similar to the control cells.

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