

Enhanced lipid peroxidation and lysosomal enzyme activity in the lungs of rats with prolonged pulmonary deposition of crocidolite asbestos

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ABSTRACT The interaction of UICC crocidolite asbestos with biological membranes *in vivo* was studied in rats after a single intratracheal dose of a suspension of 20 mg of fibres per rat. Development of lung fibrosis (increased level of hydroxyproline, a collagen index together with corresponding pathomorphological alteration) confirmed the penetration of crocidolite fibres into the lungs in the course of seven months exposure. The pulmonary deposition of crocidolite affected the lysosomal membranes of lung cells as manifested by (1) enhanced lipid peroxidation with (2) stimulation (release) of activity of β -glucuronidase and cathepsin D. Enhanced lipid peroxidation and activity of β -glucuronidase may contribute to the delayed, carcinogenic effects of crocidolite asbestos.

The health hazard of exposure to asbestos in industry arises from its fibrogenic and carcinogenic activity.¹ The basic mechanism of the diverse biochemical events caused by asbestos at the molecular and sub-cellular level—that is, fibrosis, cytotoxicity, and carcinogenesis^{2,3}—may be linked with the interaction of asbestos or its organic components with cellular and subcellular membranes, or both.

A selective release of lysosomal enzymes⁴ with the stimulation of lipid peroxidation^{3,5} has been found after the addition of asbestos to cell culture which correlates with the alteration of permeability of cellular membranes and cell death.^{6,7} *In vitro* experiments with red blood cells⁵ and microsomes isolated from liver and lungs⁸ showed that asbestos stimulates lipid peroxidation. Since the rate of lipid peroxidation was dependent on the amount of asbestos bound to microsomes⁸ it is highly probable that the long term intimate contact of asbestos with biological membrane permits the reaction of short lived radicals with unsaturated fatty acids thus affecting the integrity and selective physicochemical characteristics of the membrane. It is suggested that free oxygen radicals may be linked with carcinogenesis: malondialdehyde, a prod-

uct of lipid peroxidation, has been found to be mutagenic and carcinogenic.⁹

The asbestos provoked peroxidative attack may possibly lead to an alteration in the fluidity of biomembranes thus affecting the activity of some membrane bound enzymes, especially in lysosomes, a target cellular subfraction for asbestos fibres. The release of lysosomal enzymes from pulmonary macrophages and inflammatory cells may influence the cleavage of endogenous components (protein and nucleic acids) and conjugated derivatives of xenobiotics (polycyclic aromatic hydrocarbons, adsorbed on asbestos and readily transferred to membranes).¹⁰

This investigation was undertaken to determine whether, *in vivo*, a prolonged pulmonary deposit of crocidolite UICC standard asbestos affects the activity of lysosomal enzymes and lipid peroxidation in this critical organ.

Materials and methods

ANIMALS AND TREATMENT

Experiments were carried out with male Wistar rats (200–250 g) fed with a laboratory diet—Murigran (Bioveterinary Industries, Gorzów Wielkopolski, Poland) and tap water as wanted.

The exposure of rats to crocidolite, the UICC reference asbestos standard (cf Timbrell¹¹), was achieved,

under anaesthesia with a drop of 4% xylocain sprinkled directly on the glottis, by a single intratracheal instillation of a suspension of 20 mg of asbestos fibres in 0.2 ml of physiological saline per animal. Control animals received only saline. The animals were killed at one, three, and seven months after dosing and their lungs were used for the tests.

BIOCHEMICAL ASSAYS

Rats were killed by decapitation under light ether anaesthesia and the lungs were excised, washed with physiological saline, blotted on a filter paper, and immediately processed.

Fresh lungs were weighed directly and dry weight was determined after an initial five hour desiccation at 50°C and then, to a constant weight, over phosphorus pentoxide.

Hydroxyproline in the collagen fraction of dry lungs was determined colorimetrically at 550 nm, after extraction, according to Stegemann and Stalder,¹² and converted to collagen by using a factor of 7.46 according to Schaub.¹³

For the isolation of the lysosomal fraction (cf Sawant *et al.*¹⁴), 10% (w/v) homogenate of fresh lungs in 0.25 M sucrose—0.01 M Tris-HCl, pH 7.4 obtained with a teflon glass Potter-Elvehjem homogeniser and squeezed through a bolting cloth (pores of 200 μ in diameter) was successively centrifuged at 4°C at 900 g for 10 minutes, 7000 g for 15 minutes, and 20 400 g for 35 minutes to sediment nuclei, mitochondria, and lysosomes, respectively. After washing with 1.15% KCl and recentrifugation at 20 400 g for 35 minutes, the lysosomal fraction was suspended by rehomogenisation in 1.15% KCl. Protein was determined according to Lowry *et al.*¹⁵

Activity of β -glucuronidase (EC 3.2.1.31) was assayed by the method of Nimmo-Smith¹⁶ with p-nitrophenol glucuronide as substrate; the p-nitrophenol formed was determined colorimetrically at 400 nm.

Activity of cathepsin D (EC 3.4.23.5) was assayed by the method of Anson¹⁷ with denaturated haemoglobin as substrate; acid soluble products of hydrolysis of the protein were determined with Folin phenol reagent.¹⁵

Lipid peroxidation in the homogenates and lysosomal membranes was evaluated on the basis of the detection of thiobarbituric acid reactive substances according to Mihara *et al.*¹⁸ An extinction coefficient of 156 000/M/cm according to Wills¹⁹ was used for malondialdehyde formation.

Results were statistically evaluated using Student's *t* test.

PATHOMORPHOLOGICAL EXAMINATION OF THE LUNGS

Lungs were fixed in situ at full inflation by the infusion of 10% neutral buffered formalin through the larynx and trachea. After ligation of the trachea, all the chest organs were excised and placed in 10% buffered formalin solution for continued fixation. The sections were gradually dehydrated in isopropanol (50–96%), cleared in xylene, and impregnated with paraffin. Haematoxylin and eosin stained paraffin sections were examined with a light microscope.

Results

Table 1 gives the data showing the effects of long term action of crocidolite fibres on the lungs. After three months the dry weight of the lungs of the experimental animals increased due to the development of fibrosis as inferred from the increase in their collagen content. Continued exposure to crocidolite for the next four months (seven months from the instillation of asbestos) resulted in a tendency for the fibrotic reaction in the lungs to diminish.

The biochemical findings were confirmed by the pathomorphological examination of the lungs (cf figs 1–4). Figure 1 shows a fragment of lung tissue from a

Table 1 Course of the increase in weight of the lungs and pulmonary content of collagen and lysosomal proteins in rats after intratracheal instillation of crocidolite UICC asbestos

Groups	Time after asbestos instillation (months)	Body weight (g)	Lungs, dry weight, (g)	Collagen† (mg per lungs dry weight)	Lysosomal proteins‡ (mg per lungs fresh weight)
Control (0.9% NaCl)	1	259 \pm 6	0.26 \pm 0.02	28.87 \pm 1.30	6.23 \pm 0.30
Crocidolite UICC		270 \pm 9	0.39 \pm 0.09	62.29 \pm 3.78**	11.56 \pm 0.71**
Control (0.9% NaCl)	3	324 \pm 9	0.26 \pm 0.01	30.81 \pm 2.27	8.08 \pm 0.38
Crocidolite UICC		342 \pm 15	0.46 \pm 0.04**	75.87 \pm 4.45**	12.36 \pm 0.79**
Control (0.9% NaCl)	7	351 \pm 13	0.32 \pm 0.01	38.64 \pm 1.75	8.27 \pm 0.39
Crocidolite UICC		341 \pm 6	0.35 \pm 0.01*	52.22 \pm 1.36**	13.95 \pm 0.84**

*, **Significantly different from concurrent controls at $p = 0.05$ and 0.001 , respectively.

†Hydroxyproline was converted to collagen using a factor of 7.46 according to Schaub.¹³

‡Determined according to Lowry *et al.*¹⁵

Results are the mean \pm SE for 16 rats.

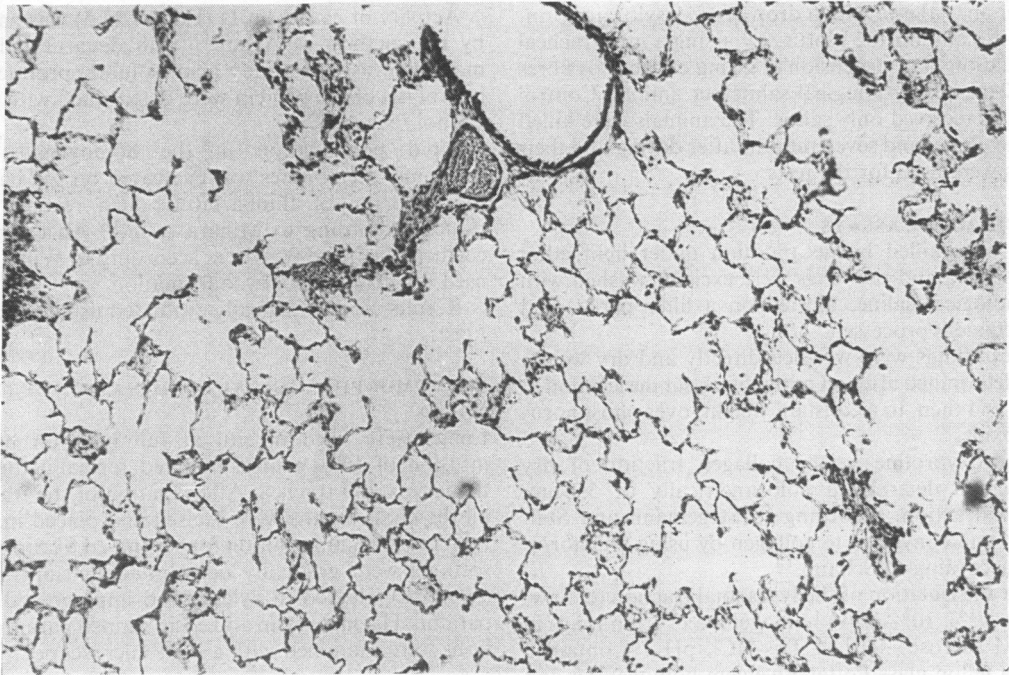


Fig 1 Lung of control rat, three months after intratracheal instillation of 0.2 ml physiological saline. Normal picture of pulmonary parenchyma with regular aerial space of alveoli and smooth contour of bronchi is seen. (H and E, $\times 40$.)

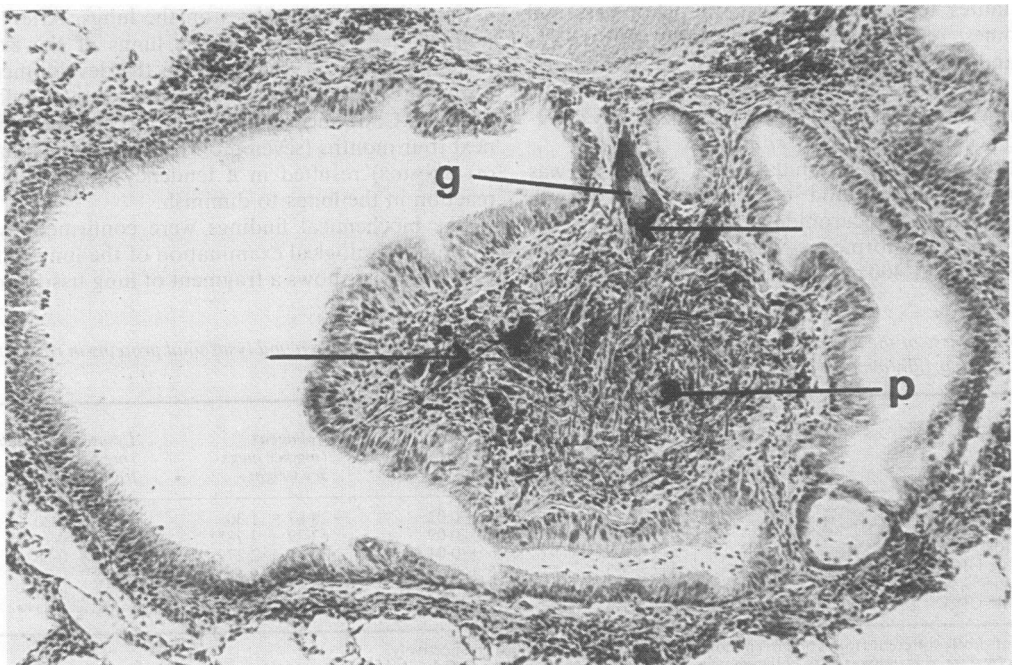


Fig 2 Lung of rat, three months after intratracheal instillation of suspension of 20 mg crocidolite UICC asbestos. Granuloma protruding into the lumen of bronchi with numerous crocidolite fibres (arrows), phagocytes (p), and a giant cell (g) are seen. (H and E, $\times 95$.)

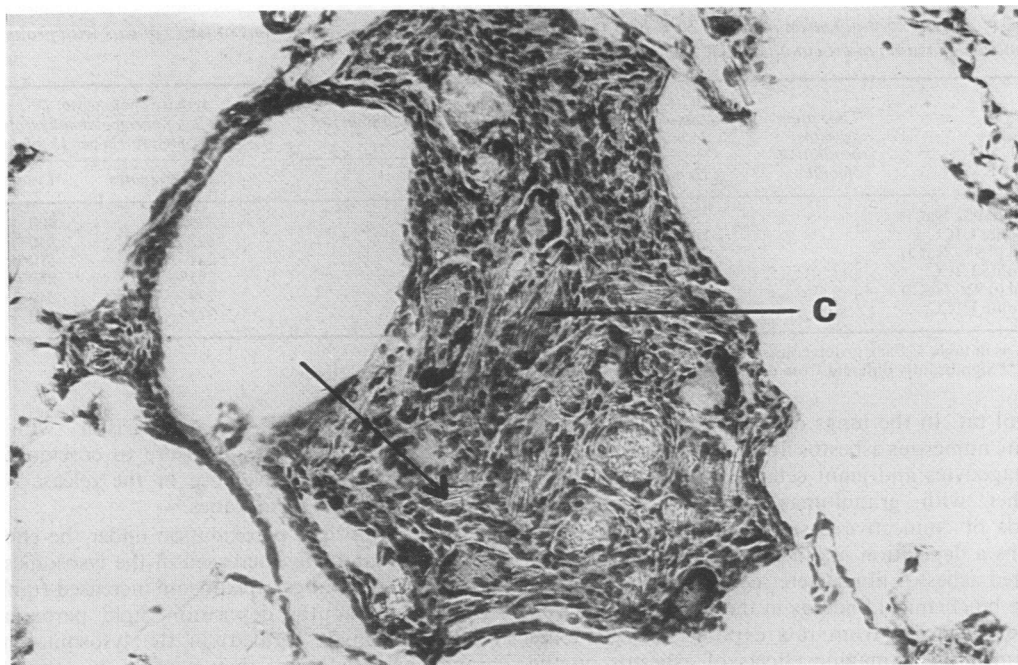


Fig 3 Lung of rat, seven months after intratracheal instillation of suspension of 20 mg crocidolite UICC asbestos. Mature, well organised granuloma containing asbestos fibres (arrow) and strands of connective tissue (c) are seen. (H and E, $\times 95$.)



Fig 4 Lung of rat, seven months after intratracheal instillation of suspension of 20 mg crocidolite UICC asbestos. A fragment of atelectotic pulmonary lobe. Typical, unremarked granuloma in vicinity of blood vessels with asbestos fibres (arrows) and giant cells (g). At periphery of granuloma, strands of connective tissue (c) and fibroblasts (f) are seen. (H and E, $\times 237$.)

Table 2 Activity of β -glucuronidase and cathepsin D in homogenates and lysosomal fractions of lungs of rats with prolonged pulmonary deposition of crocidolite UICC asbestos

Groups	Time after asbestos instillation (months)	Activity of β -glucuronidase (nmoles of p-nitrophenol formed per mg protein per 15 minutes)		Activity of cathepsin D (μ g of haemoglobin hydrolysed per mg protein per 15 minutes)	
		Homogenates	Lysosomes	Homogenates	Lysosomes
Control (0.9% NaCl)	1	52 \pm 5	60 \pm 10	273 \pm 26	400 \pm 50
Crocidolite UICC		180 \pm 20***	110 \pm 10**	623 \pm 41***	550 \pm 50*
Control (0.9% NaCl)	3	68 \pm 3	70 \pm 10	261 \pm 18	510 \pm 90
Crocidolite UICC		105 \pm 8***	100 \pm 10*	391 \pm 23**	850 \pm 100*
Control (0.9% NaCl)	7	66 \pm 12	70 \pm 10	279 \pm 16	340 \pm 40
Crocidolite UICC		91 \pm 11	90 \pm 10	372 \pm 36*	480 \pm 40*

Details as in table 1. Each group consisted of 10 rats.

*, **, ***Significantly different from concurrent controls at $p = 0.05, 0.01, \text{ and } 0.001$, respectively.

control rat. In the lungs of the rats exposed to crocidolite numerous asbestos fibres, locked on the inside of phagocytes and giant cells, were observed (fig 2) together with granulomas, asbestos fibres, and strands of connective tissue (figs 3–4). After seven months a deposition in atelectotic regions and accumulated asbestos fibres were seen (fig 4).

The biochemical findings in the lysosomal fraction of the lung cells from rats exposed to crocidolite confirmed the damaging effects of asbestos on the membranes; as exposure continued, the content of lysosomal protein in the lung cells increased (table 1) with a concomitant increase of activity of β -glucuronidase and cathepsin D (table 2). An almost parallel increase in the activity of the enzymes in the lysosomal subfraction and whole homogenates may suggest a crocidolite provoked release of the enzymes from the lysosomal membrane rather than a mere enhancement of their activities.

The highest increase in the activity of β -glucuronidase and cathepsin D in the lysosomes and whole homogenates was observed between one to three months after the asbestos instillation and this tended to diminish after seven months deposition, although the activity was still greater than the control value. The higher activity of lysosomal enzymes in the

whole homogenates by comparison with the lysosomal fraction after exposure to crocidolite for one month may be evidence of the release of the enzymes from the membranes.

The level of lipid peroxidation under the effect of crocidolite gradually increased in the lysosomes and homogenate of lungs as exposure increased (table 3). After seven months deposition, lipid peroxidation (malondialdehyde formed) in the lysosomal membranes increased more than twofold by comparison with the non-exposed control. In the lung homogenate the level of lipid peroxidation per whole protein basis, although lower than in the lysosomes, was still significantly enhanced under the long lasting influence of the crocidolite fibres.

Discussion

The present investigation found that UICC crocidolite asbestos administered to rats intratracheally in a single dose of 20 mg provoked, during the course of seven months, specific pathomorphological and biochemical alterations in the lungs. These changes included the development of fibrosis (cf figs 1–4, table 1) and an increase in the content of lysosomal protein, a concomitant increase in the activity of the lysosomal

Table 3 Lipid peroxidation in homogenates and lysosomal fractions of lungs of rats with prolonged pulmonary deposition of crocidolite UICC asbestos

Groups	Time after asbestos instillation (months)	Lipid peroxidation (nmoles of malondialdehyde per mg protein)	
		Homogenates	Lysosomes
Control (0.9% NaCl)	1	0.77 \pm 0.05	3.44 \pm 0.42
Crocidolite UICC		1.01 \pm 0.07**	3.73 \pm 0.34
Control (0.9% NaCl)	3	0.68 \pm 0.01	4.03 \pm 0.24
Crocidolite UICC		0.94 \pm 0.07**	5.03 \pm 0.24*
Control (0.9% NaCl)	7	0.63 \pm 0.07	3.06 \pm 0.30
Crocidolite UICC		1.17 \pm 0.08***	7.53 \pm 0.38***

Details as in table 1. Each group consisted of eight rats.

*, **, ***Significantly different from concurrent controls at $p = 0.05, 0.01, \text{ and } 0.001$, respectively.

enzymes β -glucuronidase and cathepsin D (cf table 2), and enhancement of lipid peroxidation (cf table 3).

The results of this *in vivo* exposure of rats to crocidolite via the tracheobronchial tract have confirmed previously reported *in vitro* effects of asbestos fibres on lipid peroxidation in red blood cells,⁵ cultured tracheal epithelial cells,³ and microsomal membranes of lungs and liver⁸ and on the activity of lysosomal enzymes in cultured macrophages⁴ and pulmonary lavage.²

The derangement of the integrity of biomembranes through the release of hydrolytic lysosomal enzymes and the stimulation of lipid peroxidation may be of importance for the delayed biological consequences of exposure to asbestos fibres.^{2,3} Stimulation of the activity of cathepsin D may contribute to the degradation of protein and probably collagen also, leading to a fibrotic reaction in the lung which diminishes in the course of prolonged exposure (cf tables 1 and 2). Asbestos mediated stimulation of the activity of β -glucuronidase may be linked with the carcinogenic potential of the fibres owing to the capability of β -glucuronidase to hydrolyse (reactivate) the inert, transport form of aromatic hydrocarbon glucuronide conjugates to their active derivatives.²⁰ *In vitro* experiments have shown an increased transfer of aromatic hydrocarbons to microsomal membranes when adsorbed to asbestos,¹⁰ resulting in an increase in the activity of aromatic hydrocarbon hydroxylases³ via cytochrome P-448 induction and, in effect, increased formation of ultimate carcinogens. Asbestos fibres might thus serve as a physical carrier mediating the transfer of carcinogens into the target tissue. This assumption may be supported by epidemiological evidence indicating that asbestos is cocarcinogenic—that is, a promoter—with cigarette smoke, generally known to contain volatile tar compounds.³

Brown *et al* on the basis of direct determinations suggest that asbestos can mediate two phenomena²¹: (1) the increased uptake of xenobiotics, aromatic hydrocarbons, and (2) the inhibition of the accumulation of glucuronides, conjugated derivatives of hydrocarbons. These phenomena may lead to the predominance of the processes of metabolic bioactivation of hydrocarbons and their binding to DNA over their detoxication. The stimulation of the activity of β -glucuronidase *in vivo* by prolonged deposition of crocidolite, as shown in the present paper (cf table 2) reinforces the second assumption of Brown *et al*.²¹

The cytotoxic and carcinogenic effects of crocidolite may also result from enhanced lipid peroxidation in biological membranes^{7,8,19} as confirmed *in vivo* in lysosomes from lungs with prolonged asbestos deposition (table 3). The enhanced formation of malondialdehyde, a product of lipid peroxidation

with mutagenic and carcinogenic properties^{9,22} most probably aggravates the pathological sequelae of exposure to asbestos. Iron, present in the structure of crocidolite fibres, is thought to be responsible for catalysing the peroxidation in a non-enzymatic way.⁸ As the chemically initiated (Fe^{2+} and ascorbate) lipid peroxidation of polyunsaturated fatty acids is able to mediate the oxidation of benz(a)pyrene to epoxide derivatives,²² it is possible that a similar process mediated by the Fe^{2+} in crocidolite may play a part in the activation of precarcinogens and finally in carcinogenesis.

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