Biochemical Pharmacology, Vol. 32, No. 21, pp. 3191–3195, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

SELECTIVE INHIBITION OF RAT PULMONARY MONOOXYGENASE BY *O,O,S*-TRIMETHYL PHOSPHOROTHIOATE TREATMENT

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(*Received* 22 *October* 1982; *accepted* 17 *February* 1983)

Abstract—The effects of oral administration of *O,O,S*-trimethyl phosphorothioate (OOS), an impurity present in widely used organophosphorus insecticides, were studied using pulmonary and hepatic microsomal enzymes of rats. The animals were treated with OOS at 10, 20 and 40 mg/kg, and were killed on day 3 after treatment. Their relative lung weights increased markedly at 20 and 40 mg/kg, increasing 94% at the highest dose, whereas the weight of liver decreased. At 20 mg/kg OOS, the cytochrome P-450 content of the lung and liver decreased to 83 and 80% of the control levels respectively. Pulmonary microsomal 7-ethoxycoumarin (7-Ec) *O*-deethylase decreased in a dose-dependent manner; activities were less than 10% of control at the 40 mg/kg dose. The activity of pulmonary coumarin hydroxylase also decreased following OOS treatment, but the decrease was not dose-dependent since no activity was detectable at doses over 10 mg/kg. In contrast, the effect of OOS treatment on hepatic monooxygenase activity was moderate. 7-Ec deethylase activity was not affected by OOS treatment at any dose level, while *p*-nitroanisole (*p*-NA) demethylase activity was decreased only at the 40 mg/kg dose of OOS. Pulmonary malathion carboxylesterase activity was not affected by OOS treatment. In contrast, a dose-dependent decrease was observed in the liver carboxylesterase. Time course effects of OOS treatment on these parameters were examined by treating rats at 20 mg/kg. The animals were killed 0.5, 1, 3 and 7 days after the treatment. The 7-Ec deethylase activity of pulmonary microsomes was decreased on days 0.5, 1 and 3 after treatment, the maximum decrease being observed on day 1. Significant decreases were not observed in hepatic microsomal activities of 7-Ec deethylase or *p*-nitroanisole demethylase throughout the experimental period; rather, these activities were higher on day 7. Hepatic microsomal malathion carboxylesterase was lower on days 0.5, 1 and 3 after OOS treatment.

O,O,S-Trimethyl phosphorothioate (OOS) is an impurity present in a number of widely used organophosphorus insecticides. For example, it is present in various amounts in technical grades of malathion [1–3], phenthoate [1], acephate [2] and fenitrothion [4]. All of these organophosphorus insecticides are generally regarded as safe, having rat LD_{50} values of 500 mg/kg or greater [5]. OOS has been shown to potentiate the acute toxicity of malathion and phenthoate [1]. In addition, at low doses, OOS causes an unusual delayed toxicity in rats without producing typical cholinergic signs [6–8]. The signs of delayed toxicity include weight loss and red staining around the nose, mouth and eyes.

Although the mechanism of delayed toxic action is not known, recent studies [9, 10] showed that a histopathological lung lesion was produced by OOS treatment. However, published data are not available on the changes in xenobiotic metabolism or lung biochemistry following OOS treatment. Electron microscopic observations [11] have indicated that OOS treatment (20 mg/kg, 3 days) of rats decreased the number and increased the diameter of Clara cells (nonciliated bronchiolar epithelial cells). Since Clara cells have been shown to be a locus of pulmonary monooxygenase activity [12], the present study was undertaken to examine whether this morphological alteration [11] could be characterized and localized biochemically by studying pulmonary xenobiotic metabolism.

Devereux and Fouts [13], using isolated pulmonary cells from rabbits, suggested that coumarin hydroxylase activity is selectively enriched in the Clara cells and 7-ethoxycoumarin (7-Ec) deethylase activity is enriched in type II cells. Therefore, the rate of hydroxylation of these two substrates was measured to determine the effect of OOS treatment on pulmonary microsomal xenobiotic metabolizing enzyme activity.

Malathion carboxylesterase activity has been found in different organs [14]. The liver contains the greatest amount of carboxylesterase activity, with 75% being attributable to the microsomal fraction. To determine whether enzymes which function independently of microsomal P-450 were affected, the activity of malathion carboxylesterase in lung and liver microsomes was measured following OOS treatment.

MATERIALS AND METHODS

Animals and treatment. Male Sprague–Dawley rats (140–160 g) were purchased from Simonsen Labora-

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tories, Gilroy, CA, or bred in our own animal facility from animals obtained from them.

The animals were housed in metal cages in an air-conditioned room and were maintained under an alternating 12 hr light/dark cycle. Food and water were available *ad lib.*, except for 16 hr prior to treatment with OOS when food was withdrawn.

OOS was dissolved in corn oil and administered orally at selected doses (10, 20 and 40 mg/kg). Control animals received corn oil alone at 2.0 ml/kg. The rats were killed by decapitation at the indicated times after OOS treatment.

Microsomal preparation. After sacrifice, rat lungs were perfused via the pulmonary artery with 10–15 ml of heparinized 0.9% NaCl solution. Lungs were removed and trimmed free of trachea and main bronchi, and the lobules from four rats were pooled [15]. They were minced quickly in 4 vol. of buffer (1.15% KCl, 0.02 M Tris, 75 mM dithiothreitol, 75 mM EDTA and 15% glycerol), pH 7.4, in a Waring blender at high speed for two periods of 10 sec each, and then further homogenized using six passes in a Potter–Elvehjem homogenizing tube. The homogenate was centrifuged at 18,000 *g* for 20 min, and the supernatant fraction was decanted and recentrifuged for 1 hr at 105,000 *g*. The microsomal pellet was resuspended in 0.02 M Tris–KCl, pH 7.4, and resedimented. The washed microsomal pellet was resuspended in 0.1 M phosphate buffer, pH 7.4, before use in incubation mixtures. Liver microsomes were prepared similarly, except that homogenization was done in 0.02 M Tris–1.15% KCl buffer, the Waring blender step was deleted, and the initial centrifugation was at 9000 *g* instead of 18,000 *g*.

The protein concentrations were determined by the method of Sedmak and Grossberg [16].

Cytochrome P-450 determination and enzyme assay. Cytochrome P-450 was quantified from the CO-difference spectra of dithionite-reduced microsomes between the peak wavelength near 450 and 490 nm [17]. An extinction coefficient of 91 cm^{-1} mM^{-1} for cytochrome P-450 was used.

7-Ec *O*-deethylase and coumarin hydroxylase activities were assayed by fluorimetric measurement of umbelliferone production by the method of Ullrich and Weber [18], at an excitation wavelength of 380 nm and an emission wavelength of 420 nm at room temperature. *p*-NA *O*-demethylase [19] and malathion carboxylesterase [14] activities were determined at 37° by methods described in the reference cited. The determinations of all the enzyme activities were carried out in the linear range. The means and the S.E.M. were calculated and the levels of significance between treated and control groups were determined using Student's *t*-test [20].

RESULTS

Effect of OOS on cytochrome P-450 content in rat lung and liver. The oral 28-day LD_{50} of OOS under the conditions of this study was 60 mg/kg with 95% confidence limits of 41–87 mg/kg [21]. An oral dose of 20 mg/kg was selected as a treatment dose since this dose resulted in the typical signs of delayed toxicity without causing a significant number of deaths during the 28-day observation period. At this dose the signs of delayed toxicity were most severe, and the morphological alterations of Clara cells were most distinctive on day 3 [11].

Cytochrome P-450 difference spectra of lung microsomes from control and OOS-treated rats on day 3 had a similar absorption maximum near 452 nm, although the peak was much broader (between 430 and 480 nm) and asymmetrical in OOS-treated lung microsomes (Fig. 1). Cytochrome P-450 content in lung microsomes from OOS-treated animals was about 83% of the control, and the reduction was statistically significant (Table 1).

In comparison, liver microsomes of OOS and control rats had similarly shaped peaks with absorption maxima at 450 nm, although the microsomal cytochrome P-450 content was reduced to 80% of the control (Table 1). The microsomal protein concentrations per gram wet weight tissue of both organs were not altered by the OOS treatment.

Dose-related effects of OOS treatment on microsomal enzymes in rat lung and liver. To characterize the difference found in the pulmonary P-450 after OOS treatment, we examined the ability of the pulmonary microsomes to metabolize 7-Ec and coumarin. The doses of OOS were 10, 20 and 40 mg/kg, and rats were killed on day 3 after the treatment. As shown in Table 2, body weights of the treated animals decreased significantly at doses of 20 and

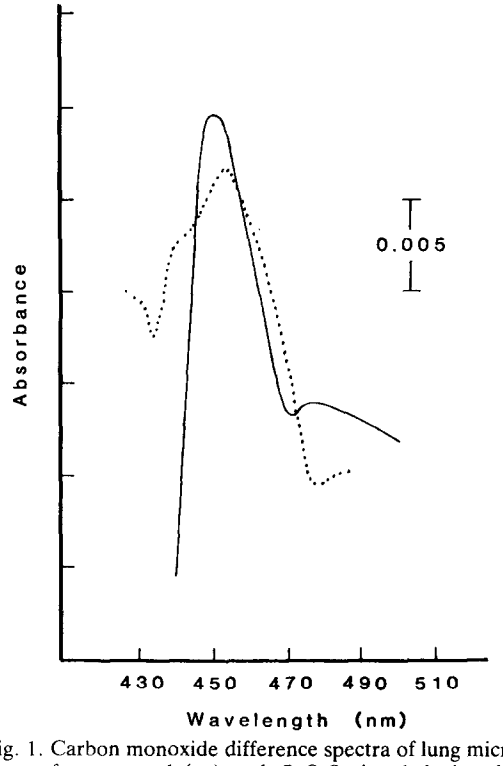

Fig. 1. Carbon monoxide difference spectra of lung microsomes from control (—) and *O,O,S*-trimethyl phosphorothioate (20 mg/kg, p.o., 3 days) treated rats (. . . . .). Both the sample and reference cells contained a microsomal suspension (1.65 to 1.55 mg of protein/ml, 0.1 M phosphate, pH 7.4) that had been treated with dithionite. The content of the sample cell was then saturated with CO, and the difference spectrum was measured after a few minutes.

Table 1. Cytochrome P-450 in lung and liver microsomes from control rats and from rats pretreated with O,O,S-trimethyl phosphorothioate (OOS)

Group	Microsomal protein* (mg/g wet wt)		Cytochrome P-450* (nmoles/mg microsomal protein)	
	Lung	Liver	Lung	Liver
Control (corn oil, 2.0 ml/kg, 3 days)	1.65 ± 0.15	8.12 ± 1.32	0.082 ± 0.007	1.04 ± 0.03
OOS (20 mg/kg, 3 days)	1.55 ± 0.16	7.72 ± 0.59	0.068 ± 0.006†	0.83 ± 0.11

* Values are the means ± S. E. of five separate pools of four organs each.
† Significantly different from control value ($P < 0.01$).

40 mg/kg. At these two doses, the relative weight of the lungs (mg wet wt/g body wt) of treated animals increased whereas that of liver decreased.

The activity of microsomal 7-Ec O-deethylase decreased markedly by OOS treatment in a dose-dependent manner, reaching 90% reduction at the highest dose (Table 3). In comparison, coumarin hydroxylase activity was abolished in OOS-treated lung microsomes at doses of 20 or 40 mg/kg, while the activity of malathion carboxylesterase was not affected even at the highest dose of OOS (40 mg/kg).

The data were compared to liver microsomal enzyme activities (Table 3). There was no change in p-NA demethylase or 7-Ec deethylase in the liver microsomes following OOS treatment. Malathion carboxylesterase activity in liver was reduced significantly at the 20 and 40 mg/kg doses.

Effects of OOS treatment on time courses of microsomal enzyme activities in lung and liver. The rats were treated with OOS at an oral dose of 20 mg/kg and were killed 0.5, 1, 3 and 7 days after treatment. No changes were observed in the wet weights of the lungs or in the microsomal protein per wet weight of lung throughout the time course (data not shown). As shown in Fig. 2, the activity of 7-Ec deethylase was reduced on days 1 and 3. The reduction reached a maximum on day 1 and remained significantly reduced until day 3. In contrast, malathion carboxylesterase activity was not reduced throughout the time course, but rather increased on the first day after treatment.

Table 2. Dose-related effects of O,O,S-trimethyl phosphorothioate (OOS) on body weight and relative organ weight

OOS treatment (mg/kg)	Body wt* (g)		Relative wet wt (mg/g)	
	Initial	Final	Lung	Liver
0	144 ± 1.2	168 ± 1.3	5.49	47.2
10	126 ± 2.0	125 ± 1.2	6.14	—
20	140 ± 1.6	106 ± 1.9	6.29	41.0
40	130 ± 3.5	98 ± 2.7†	10.64	40.0

* Values are means ± S.E. from eight animals. All the animals were killed on the third day after treatment.
† Significantly different from control value ($P < 0.05$).

The wet weight of the liver was significantly lower than in control rats throughout the time course; however, microsomal protein concentration per gram wet weight remained the same (data not shown). In contrast to that of the lung, the liver 7-Ec deethylase activity was not reduced at any time. Similarly, the activity of p-NA demethylase was not reduced by OOS treatment at this dose level but rather increased on the seventh day after treatment. As for malathion carboxylesterase, a significant reduction in activity was observed throughout the time course.

DISCUSSION

OOS is an impurity present in widely used organophosphorus insecticides and is capable of producing delayed toxicity in rats. Lung abnormalities in rats have been found after treatments with OOS and another impurity, O,S,S-trimethyl phosphorodithioate [10]. Although the liver is quantitatively the most important organ in the metabolism of most xenobiotics, the ability of the lung to metabolize foreign compounds may be important in chemically induced toxicities in this tissue [11]. Since the lung serves as the primary mode of entry for airborne environmental agents, including insecticides, and it is frequently the target organ for lesions produced by these agents, it is quite possible that the fate of these compounds is controlled by local metabolism by the pulmonary monooxygenase system.

OOS treatment produced a dose-related increase in the weight of the lung. This increase might be a result of the acute inflammatory edema accompanied, or followed, by the reparative-proliferative process as was found with another impurity, O,S,S-trimethyl phosphorodithioate [10].

Electron microscopic studies [11] have indicated that 3 days after oral OOS treatment at 20 mg/kg, there is a decrease in number (*ca.* 50%) and an increase in size of Clara cells. Since these cells are considered to be one of the major sites of P-450 activity in the lung [12, 22] a reduction in their number may result in a decrease in the monooxygenase activity of this organ. Such alterations should be characterized by the alteration of enzyme activities used in this study.

If the Clara cells are one of the major loci of P-450 in the lung and the morphological abundance of

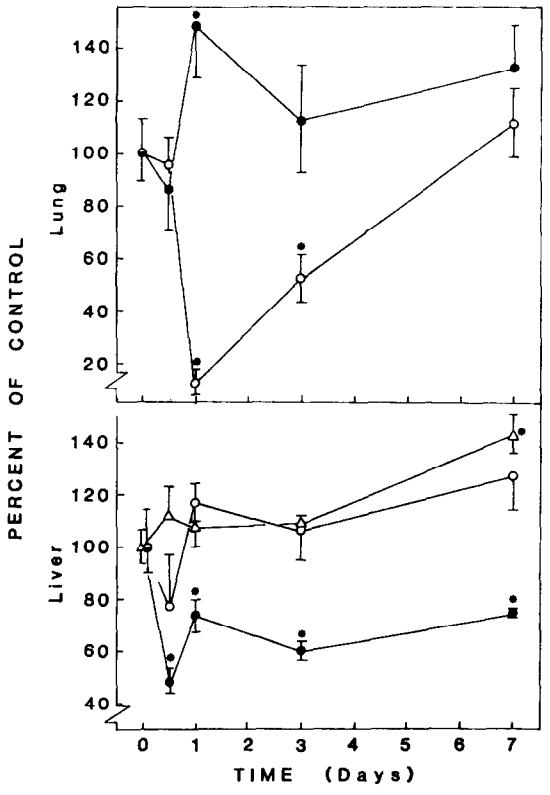

Fig. 2. Effects of *O,O,S*-trimethyl phosphorothioate (OOS) on the time course of the activities of 7-ethoxycoumarin *O*-deethylase (○) and malathion carboxylesterase (●) in lung and liver microsomes and *p*-nitroanisole demethylase (△) in liver microsomes. Male rats received OOS (20 mg/kg) orally and were killed at various times after the treatment. Values are means ± S.E.M. of determinations of four separate pools of four organs each. Values significantly different from controls are marked with an asterisk.

the cells directly correlates with the level of P-450, we expected a significant decrease in P-450 concentration after OOS treatment. Although there was a significant decrease in the P-450 content in lung microsomes prepared from OOS-treated rats, the decrease was small (about 17%) and was not as much as that expected from the extensive loss of Clara cells. It is possible that the increase in size of surviving Clara cells (as seen morphologically) is accompanied by an increase in P-450 content, or that sources of P-450 other than Clara cells (such as type II cells) make up a significant proportion of total rat lung P-450 content [13]. Qualitatively, there was a consistent observation that the P-450 CO-binding spectra (Fig. 1) of lung microsomes prepared from OOS-treated rats were much broader than those of controls. This suggests that the characteristics of the cytochrome P-450 might have been altered by OOS treatment, which may have occurred in Clara cells and/or other cell types in the lung.

In the present study, OOS treatment revealed a dose-related inhibitory effect on 7-Ec *O*-deethylase of pulmonary microsomes. The inhibition of the activity of coumarin hydroxylase was found to be more marked and not dose-dependent; the activity slightly increased at 10 mg/kg and was completely abolished at doses of 20 and 40 mg/kg. Assuming that the substrate-cell specificities found in rabbits by Devereux and Fouts [13] could be extrapolated to the present study with rats, our observation suggests that the OOS-induced decrease in the number of Clara cells resulted in a loss of the bronchiolar epithelial coumarin hydroxylase activity.

The decrease of 7-Ec *O*-deethylase activity may result from an effect of OOS on another cell type(s) of the lung, including type II cells. This also indicates that the cytochrome P-450-dependent monooxygenase enzymes are located in lung cells other than Clara cells, as shown by the data following treatment at 20 mg/kg. 7-Ec deethylase activity is known to be associated with both cytochrome P-450 and P-448 [23], whereas coumarin hydroxylase is associated only with cytochrome P-450 [24]. Therefore, the data may also indicate that OOS treatment selectively inhibited P-450-dependent monooxygenase at the 20 mg/kg dose. Pulmonary malathion carboxylester-

Table 3. Dose-related effects of *O,O,S*-trimethyl phosphorothioate (OOS) on lung and liver microsomal enzyme activity*

OOS treatment† (mg/kg)	7-Ec deethylase (pmole/min/mg)	Coumarin hydroxylase (pmole/min/mg)	*p*-Nitroanisole demethylase (nmole/min/mg)	Malathion carboxylesterase (nmole/min/mg)
Lung				
0	421 ± 55.1	85.0 ± 29.5		3.43 ± 0.36
10	219 ± 44.1‡	128 ± 7.3		6.48 ± 0.58‡
20	218 ± 38.3‡	ND§		3.81 ± 0.68
40	42.7 ± 24.8‡	ND		3.67 ± 0.75
Liver				
0	1720 ± 260		2.23 ± 0.14	151 ± 21.2
10	1995 ± 110		2.51 ± 0.32	136 ± 10.3
20	1830 ± 190		2.42 ± 0.07	116 ± 19.1‡
40	1532 ± 209		1.23 ± 0.18‡	85 ± 11.1‡

* Values are means ± S.E. from four to five separate pools of four organs each.
† All the animals were killed 3 days after oral treatment.
‡ Significantly different from control value ($P < 0.01$), two-tailed Student's *t*-test.
§ Not detectable.

ase, which does not require P-450, was not inhibited by OOS treatment. We have no explanation for the increase in lung microsomal malathion carboxylesterase activity following treatment with 10 mg/kg of OOS on day 3. The experiment was repeated three times, and the activity was elevated consistently.

In contrast to these observations in the lung, the effects of OOS treatment on hepatic monooxygenase measured by *p*-NA demethylase and 7-Ec deethylase were less severe and required the highest dose for inhibition of *p*-NA demethylase activity. Talcott *et al.* [25] demonstrated that very high doses (104 mg/kg) of OOS inhibited liver homogenate malathion carboxylesterase by 70%. The present study revealed that hepatic microsomal malathion carboxylesterase was inhibited in a dose-dependent fashion at much lower doses than previously reported. Since liver is the major source of malathion carboxylesterase activity, the decrease observed after OOS treatment may explain the potentiation of the cholinergic effect of malathion by OOS. Creaven *et al.* [26] have shown that rat liver microsomes contained no coumarin hydroxylating enzyme. Being compatible with their finding, we observed no measurable activity of this enzyme from either control or OOS-treated rat liver microsomes; therefore, the data were not listed in Table 3.

We have shown that the OOS-induced morphological alteration of rat lung bronchiolar epithelium correlates with the xenobiotic metabolizing capacity of the lung. Loss of the Clara cells resulted in the decrease in cytochrome P-450 content and P-450-mediated xenobiotic metabolism in the lung. Although the mechanism(s) of delayed death produced by OOS remains unknown, the ability of OOS to inhibit pulmonary microsomal enzymes may represent a primary site of action of OOS in the lung.

Acknowledgements—The authors are indebted to Dr Alan R. Buckpitt, College of Medicine, University of California, Irvine, for his assistance in performing the cytochrome P-450 measurements, for invaluable discussions throughout the project, and for his review of the manuscript. The authors also wish to thank Leslie Hasegawa for technical assistance and Linda McCloud for the care and breeding of the experimental animals. The project was supported in part by NIH Grant ES 02225.

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