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STUDIES ON THE METABOLISM OF THE PNEUMOTOXIN *O,S,S*-TRIMETHYL PHOSPHORODITHIOATE—II
LUNG AND LIVER SLICES

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Abstract—The metabolism of *O,S,S*-trimethyl phosphorodithioate (OSSMe), a pneumotoxic impurity in some organophosphorus insecticides, was investigated by incubating rat lung and liver slices with 1 mM OSSMe, labelled with ^{3}H or ^{14}C on one of its thiolo-methyl (CH_3S-) groups. Protein bound radioactivity was higher in lung slices than in liver slices. In lung slices the predominant diester produced was *O,S*-dimethyl phosphorothioate ($OSMeO^-$), whereas in liver slices it was *S,S*-dimethyl phosphorodithioate ($SSMeO^-$). Other studies had shown binding of radioactivity and $OSMeO^-$ production to be cytochrome P-450-dependent processes in microsomes and $SSMeO^-$ production to result from the action of cytosolic glutathione-*S*-transferase on OSSMe. Preincubation of slices with 10^{-5} M paraoxon did not influence the amount of protein-bound radioactivity, suggesting that binding of radioactivity did not simply result from protein phosphorylation. Pretreatments of the rats with *O,O,O*-trimethyl phosphorothioate [OOOMe(S) 0.5, 2.5 and 12.5 mg/kg p.o.], with *p*-xylene (1 g/kg, i.p.) or with bromophos (5.3 mg/kg, i.p.) which all protect against the lung toxicity of OSSMe probably by inhibiting pulmonary mixed-function oxidase, also led to significant decreases in both protein binding of radioactivity and $OSMeO^-$ production in lung slices, but not in liver slices. These results show that tissue slices are a convenient system for investigating xenobiotic metabolism in the lung and they suggest that the susceptibility of the lung to OSSMe probably results from a relatively high rate of activation, coupled with a relatively low rate of metabolism by non-toxic pathways and/or removal of reactive metabolites in some lung cells, possibly the alveolar type I cells.

In vitro studies on the metabolism of *O,S,S*-trimethyl phosphorodithioate (OSSMe), a pneumotoxic impurity of organophosphorus compounds [1], were conducted using microsomal preparations on the one hand, and tissue slices on the other. The experiments with microsomes, described in an accompanying paper [2] showed that the production of a metabolite capable of binding to protein was, at least in part, a cytochrome P-450-dependent process possibly involving sulphoxidation of OSSMe. The K_m for the metabolic activation of OSSMe was found to be considerably lower in lung microsomes than in liver microsomes, thus suggesting that the selective toxicity of OSSMe for the lung is due to a high rate of activation of OSSMe by pulmonary cytochrome P-450 isozyme(s) compared to hepatic isozymes.

In this paper we present the results obtained with tissue slices, used first to compare the overall metabolism of OSSMe by lung and liver, and later to understand the effects of various animal pretreatments known to modulate the lung toxicity of OSSMe.

The pretreatments given included *p*-xylene, *O,O,O*-trimethyl phosphorothioate [OOOMe(S)], and bromophos. *p*-Xylene, when given in large doses, results in a selective destruction of pulmonary cytochrome P-450 without decreasing hepatic cytochrome P-450 and without apparently causing cellular damage in the lungs [3, 4]. Pretreatment with *p*-xylene results in protection of rats against OSSMe-induced lung damage, without substantial alteration in the toxicokinetics of OSSMe [5]. Pretreatment or co-treatment with small amounts of OOOMe(S) has been shown to abolish the delayed toxicity of even large doses of *O,O,S*-trimethyl phosphorothioate (OOSMe) [6] or OSSMe [5]. Later it was found that many other thiono-phosphorus (P = S) compounds, such as bromophos, are protective, even at very low doses, against the pneumotoxic effects of OSSMe [7].

MATERIALS AND METHODS

Experimental animals. The same source and type of female rats were used as described in the accompanying paper [2]. In addition, some experiments were carried out in the Unit of Industrial Toxicology and Occupational Medicine of the U.C. Louvain in Brussels (Prof. R. Lauwerys) with Sprague–Dawley rats. This strain of rats is somewhat less susceptible to OSSMe-induced toxicity, but does otherwise exhibit similar lung damage to the Carshalton strain (unpublished).

Chemicals. The sources of the chemicals which were also used in the experiments using microsomes are described in the accompanying paper [2]. In addition, *O,O,O*-trimethyl phosphorothioate [OOOMe(S)] was obtained from Ventron (Karlsruhe, F.R.G.), *p*-xylene from Carlo Erba (Milano, Italy), and parathion, paraoxon and bromophos (*O*-4-bromo-2,5-dichlorophenyl *O,O*-

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dimethylphosphorothioate) were obtained from SBA Chemie (Belgium).

Preparation and incubation of lung and liver slices. Rats were killed by transection of abdominal vessels after deep anaesthesia with ether (first experiment) or by decapitation. The lungs, dissected free of major airways and vessels, and a piece of liver, cut to give slices of approximately the same section as lung slices, were sliced with a McIlwain tissue slicer [8] in 0.8-mm-thick slices.

The slices were immediately placed in 10 ml Krebs–Ringer–phosphate–glucose buffer (KRPG) (NaCl 129 mM, KCl 5.2 mM, $CaCl_2$ 1.8 mM, $MgSO_4$ 1.3 mM, Na_2HPO_4 10 mM, pH 7.4, glucose 11 mM) and preincubated at 37° in a shaking water bath (about 120 strokes/min). After 10–15 min preincubation, slices (200–700 mg) were transferred to 25 ml conical flasks or 20 ml scintillation vials containing 2–4 ml of the final incubation medium, i.e. 1 mM radiolabelled OSSMe in KRPG, and incubated for up to 120 min at 37° (or 0°). One or two slices and aliquots from the medium were removed at intervals during the incubation.

Determination of tissue bound radioactivity. The slices were immediately placed in 1 M perchloric acid and they were later washed twice in H_2O, twice or three times in ethanol/diethyl ether/water (7/10/3) and then again in H_2O. They were finally blotted, weighed, and solubilized in 1 ml BTS 450® (Beckman). Some slices from the longest incubation were solubilized in 1 M NaOH at 60° for the determination of protein content [9]. After the addition of 10 ml scintillant (Ready-Solv MP®, Beckman) containing 1% acetic acid, the radioactivity in the dissolved tissue was measured by scintillation counting as described in the accompanying paper [2].

In Brussels the scintillation cocktails used were 4 ml Picofluor® (using minivials) or 10 ml ES299® from Packard, and scintillation counting was done using a Betaszint BS5000 (Berthold) scintillation counter with calculation of dpm by the external standard method.

Separation and quantitation of diester metabolites. The same procedure and TLC system were used as for the experiments with microsomes [2].

Presentation of results. Results are presented as means with standard deviations, and statistical comparisons were made using Student's *t*-test or one-way analysis of variance, adopting a level of $P < 0.05$ as statistically significant.

RESULTS

Metabolism of OSSMe by tissue slices from control rats

Figure 1 shows that the amount of radioactivity bound to lung and liver tissue increased with time of incubation at 37°. The amount of [^{3}H]binding per mg of protein was significantly greater in lung than in liver slices, but the quantity of ethyl acetate insoluble radioactivity in the medium was lower with lung slices than with liver slices. Figure 1 also shows that the pattern of diesters produced by the liver and the lung differed considerably: in the lung the predominant diester was $OSMeO^-$, whereas in the liver the predominant diester was $SSMeO^-$. The ratio

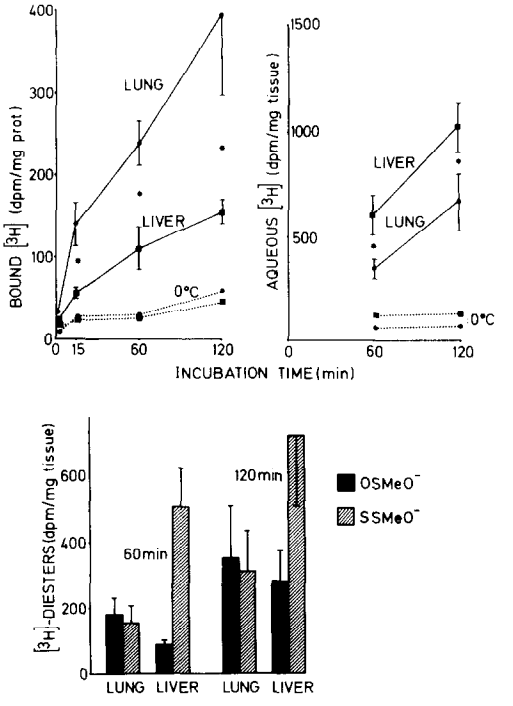

Fig. 1. Metabolism of *O,S,S*-trimethyl phosphorodithioate (OSSMe) by rat lung and liver slices. Lung and liver slices from 4 female LAC-P rats were incubated at 37° or 0° with 1 mM [^{3}H]-CH_3S]-OSSMe (specific activity 800 dpm/nmol). Bound radioactivity is the radioactivity remaining in the slices after extensive washing; aqueous radioactivity is the radioactivity remaining in the medium after three ethyl acetate extractions; *O,S*-dimethyl phosphorothioate ($OSMeO^-$) and *S,S*-dimethyl phosphorodithioate ($SSMeO^-$) were determined by TLC of 25 µl samples of these extracts followed by scintillation counting of the appropriate areas (data expressed assuming all aqueous radioactivity consisted of $OSMeO^-$ and $SSMeO^-$). Specific activity of $OSMeO^-$ is half that of $SSMeO^-$. Values are means ± SD (N = 4 or 3) for incubations at 37°, and means of 2 determinations for incubations at 0°. *$P < 0.05$ for lung vs liver.

(dpm) $OSMeO^-/SSMeO^-$ was 1.16 ± 0.18 at 60 min and 1.11 ± 0.28 at 120 min for lung slices and respectively 0.19 ± 0.06 ($P < 0.001$), and 0.40 ± 0.09 ($P < 0.05$) for liver slices. In other words, taking into account that the specific activity of $OSMeO^-$ will be half that of $SSMeO^-$, the lung slices produced about twice as much $OSMeO^-$ as $SSMeO^-$, whilst in liver slices the proportion of $OSMeO^-$ was about half that of $SSMeO^-$.

In this first experiment kidney slices were also prepared and similarly incubated. No significant binding of radioactivity occurred in these slices and the amounts of total ethylacetate insoluble radioactivity produced were lower than with lung slices; kidney slices produced about 50% more $SSMeO^-$ than lung slices and very little if any $OSMeO^-$ (not shown).

Figure 2 shows the effect of 30 min preincubation of slices with parathion (10^{-5} M) or paraoxon (10^{-5} M (both dissolved in methanol, 1% v/v final concentration). Binding of radioactivity and metab-

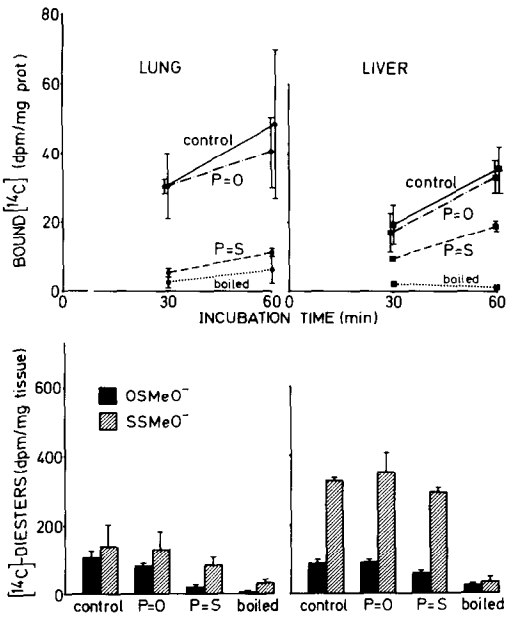

Fig. 2. Effect of preincubation with paraoxon or parathion on the metabolism of *O,S,S*-trimethyl phosphorodithioate (OSSMe) by rat lung and liver slices. After 30 min preincubation at 37° without (control) or with 10^{-5} M paraoxon (P = O) or 10^{-5} M parathion (P = S), or after boiling for 10 min, lung and liver slices from 2 female Sprague–Dawley rats were incubated at 37° or 0° with 1 mM [^{14}C-CH$_3$]-OSSMe (specific activity 369 dpm/nmol). Bound radioactivity, aqueous radioactivity and diesters were determined as indicated in the legend of Fig. 1, except that TLC was carried out on 50 μl samples containing 33 μl acetone + 17 μl aqueous extract of the medium after 60 min incubation. Values are means of 2 determinations with their range.

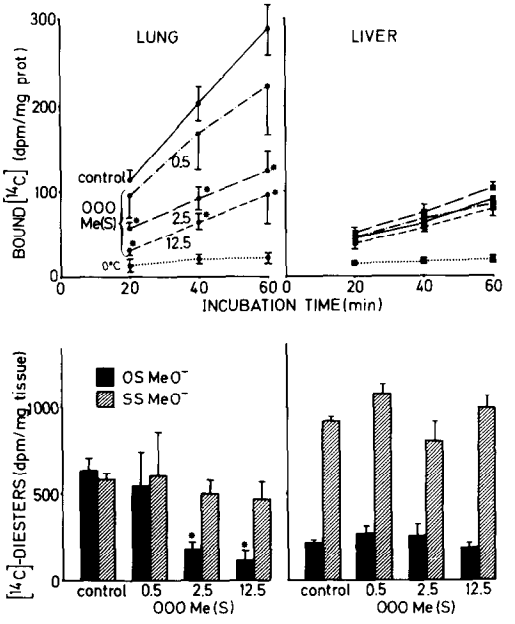

Fig. 3. Effect of pretreatment with *O,O,O*-trimethyl phosphorothioate [OOOMe(S)] on the metabolism of *O,S,S*-trimethyl phosphorodithioate (OSSMe) by rat lung and liver slices. Lung and liver slices from female LAC-P rats given an oral dose of OOOMe(S) (indicated in mg/kg), 2 hr previously, were incubated at 37° or 0° with 1 mM [^{14}C-CH$_3$]-OSSMe (specific activity 845 dpm/nmol). Bound radioactivity, aqueous radioactivity and diesters were determined as indicated in the legend of Fig. 1, except that TLC was carried out on 30 μl samples containing 20 μl acetone + 10 μl aqueous extract of the medium after 60 min incubation. Values are means ± SD (N = 3), *P < 0.05 compared to control. Line types correspond to similar conditions for lung and liver slices.

olite production were not affected by paraoxon in either lung or liver slices, whilst parathion reduced both the binding and the production of OSMeO$^-$, particularly in lung slices.

Effects of pretreatments on the metabolism of OSSMe by tissue slices

Figure 3 shows the effects of pretreatment with OOOMe(S) administered by oesophageal intubation (starved rats), 2 hr before killing, in doses of 0, 0.5, 2.5 or 12.5 mg/kg in glycerol formal (0.1 ml/kg). In lung slices there was a significant and dose-related effect of pretreatment with OOOMe(S) on the amount of covalent binding, whilst no such effect was observed in liver slices. Pretreatment with OOOMe(S) also led to a decrease in the production of ethyl acetate insoluble metabolites in lung slices only. This decrease in lung slices was attributable to a lowering of the amount of OSMeO$^-$, without change in the amount of SSMeO$^-$. The ratio (dpm) OSMeO$^-$/SSMeO$^-$ was 1.01 ± 0.09 in control lung slices and decreased to 0.80 ± 0.04 ($P < 0.05$), 0.53 ± 0.18 ($P < 0.05$) and 0.34 ± 0.10 ($P < 0.001$) in slices from rats pretreated with 0.5, 2.5 and 12.5 mg OOOMe(S) respectively. No significant alteration in this parameter occurred in liver slices (0.28 ± 0.06, N = 12).

Figure 4 shows the effects of pretreatment with *p*-xylene (1 g/kg, 5 hr before killing) and bromophos (5.3 mg/kg, 2 hr before killing), both substances being dissolved in olive oil and administered i.p. to rats that had been starved overnight. *p*-Xylene pretreatment markedly reduced the binding of radioactivity and the production of OSMeO$^-$ (and also that of SSMeO$^-$) in lung slices, but had no effect in liver slices. Bromophos pretreatment reduced the binding of radioactivity in both lung and liver slices, and also the production of OSMeO$^-$ in lung slices.

DISCUSSION

The initial experiments (Fig. 1) showed that lung and liver tissue were capable of metabolizing OSSMe, and suggested that part of this metabolism resulted in reactive metabolites becoming bound to tissue proteins. In addition, these experiments showed that binding of radioactivity was higher in lung slices than in non-target tissues and that the predominant diester in the lung was OSMeO$^-$, whilst in the liver it was SSMeO$^-$. The qualitative correlation between the production of OSMeO$^-$ and the binding of radioactivity naturally led to the hypothesis that it is metabolism of OSSMe to

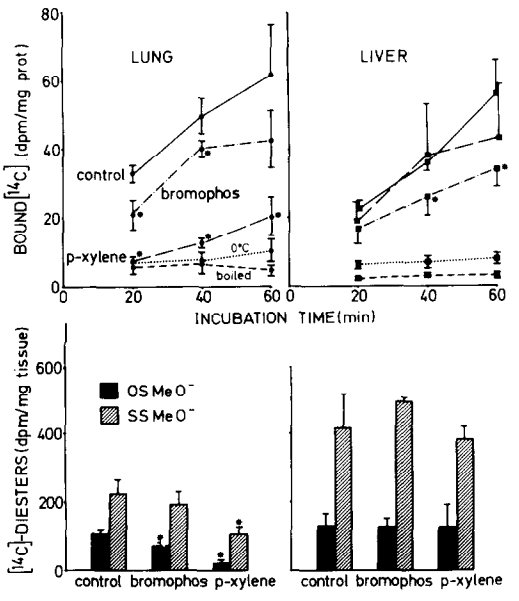

Fig. 4. Effect of pretreatment with *p*-xylene or bromophos on the metabolism of *O,S,S*-trimethyl phosphorodithioate (OSSMe) by rat lung and liver slices. Lung and liver slices from female Sprague–Dawley rats given an i.p. dose of *p*-xylene (1 g/kg), 5 hr previously, or of bromophos (5.3 mg/kg), 2 hr previously, were incubated at 37° or 0° with 1 mM [^{14}C-CH_3S]-OSSMe (specific activity 335 dpm/nmol). Bound radioactivity, aqueous radioactivity and diesters were determined as indicated in the legend of Fig. 1, except that TLC was carried out on 50 μl samples containing 33 μl acetone + 17 μl aqueous extract of the medium after 60 min incubation. Values are means ± SD (N = 3), *P < 0.05 compared to control. Line types correspond to similar treatments for lung and liver slices.

$OSMeO^-$ which is associated with the generation of reactive intermediates, capable of binding to macromolecules.

It is remarkable that this working hypothesis could be made from data obtained with such a simple experimental system as lung and liver slices. A strong argument in favour of the use of slices for metabolic studies involving extrahepatic organs is that slices allow several replicate measurements to be made from individual animals, whereas the organs of several animals often have to be pooled in order to obtain satisfactory microsomal preparations. It should be realized that yields of mixed-function oxidase in microsomal fractions are only 20–30% of the original activity in tissue homogenates, both in liver [10] and in lung [11]. Such losses probably do not occur in tissue slices, which have the added advantage that delays between killing and incubation are minimal, when compared to the several hours required for preparing microsomes. Moreover, microsomal preparations only inform about the intrinsic metabolic capability of a tissue, whereas data obtained from slices describe a more complex and compartmentalized system with different metabolic options. The utility of tissue slices is also apparent from work on bromobenzene [12].

The *O*-demethylation of OSSMe yielding $SSMeO^-$ probably results predominantly from the action of glutathione-*S*-transferases. An earlier study had shown that the latter reaction could occur in the liver cytosol [13], and further experiments showed that little, if any, $SSMeO^-$ is produced in microsomes [2]. The glutathione-*S*-transferase-mediated conversion of OSSMe of $SSMeO^-$ is probably essentially a detoxication reaction.

We have no conclusive evidence that the radioactivity retained in slices after extensive washing represented covalently-bound material resulting from the generation of reactive metabolites of OSSMe, but it is reasonable to assume that this was the case, since no significant radioactivity could be released from the washed slices after mechanical homogenization and further washing (not shown), and more convincingly on the basis of the low levels of radioactivity found in incubations carried out at 0° or after boiling the slices. Admittedly this does not exclude the possibility of metabolic incorporation of radioactivity, but does rule out simple trapping of metabolites or the parent chemical.

The experiment carried out with paraoxon and parathion (Fig. 2) was designed to check whether some of the bound radioactivity might not result from phosphorylation of proteins by OSSMe, since OSSMe had been shown to be capable, albeit poorly, of phosphorylating serine esterases [14].

However, blocking the available esteratic sites with the direct inhibitor paraoxon did not reduce the binding of radioactivity. When parathion was used, the binding was reduced, but this was presumably due to decreased metabolic activation of OSSMe as evidenced by a diminished production of $OSMeO^-$. This inhibitory effect of parathion is to be interpreted in the light of the effects of other thiono-compounds, such as OOOMe(S) or bromophos, discussed below.

It is also improbable that the binding of radioactivity resulted from the chemical methylation of proteins by OSSMe. Methylation of cysteine residues of haemoglobin has been shown to occur after *in vitro* incubation of OSSMe or after *in vivo* treatment (unpublished experiments with P. B. Farmer). However, such chemical methylation would probably involve the unlabelled O-methyl group rather than a thiolo-methyl group, since C–O cleavage is favoured over C–S cleavage in neutral conditions (see [15]). The latter is supported by the fact that no *S*-demethylation occurs in the (glutathione-*S*-transferase catalyzed) methylation of glutathione by OSSMe [13].

It may be concluded that the binding of radioactivity probably is not due to direct phosphorylation or methylation of proteins, but that it probably results from the generation of reactive metabolites during the metabolism of OSSMe, most likely its conversion to $OSMeO^-$. This hypothesis was further supported by the effects from pretreatments.

Thus *p*-xylene strongly inhibited both the covalent binding and the conversion of OSSMe to $OSMeO^-$ in the lung, without affecting metabolism in the liver. The mechanism for the destruction of pulmonary cytochrome P-450 by *p*-xylene is not clear, but it has been suggested to result from a high rate of oxidative metabolism of *p*-xylene in some lung cells, coupled with a relative deficiency in aldehyde dehydrogenase [16].

Pretreatment with OOOMe(S) was also associated in lung slices, and not in liver slices, with dose-related decreases both in covalent binding and in the amount of $OSMeO^-$ produced. It is unlikely that the effects found in lung slices were due to a reduced viability of these slices, since OOOMe(S) even in high doses does not cause any cellular lung damage (unpublished). As with OOOMe(S), pretreatment with bromophos also resulted in a reduced ability of lung slices to metabolize OSSMe to $OSMeO^-$.

The following mechanism, illustrated in Fig. 5, is proposed to explain the inhibition by phosphorothionates of the metabolic activation and lung toxicity of OSSMe: (i) OSSMe and the phosphorothionates undergo sulphoxidation by the same cytochrome P-450-dependent mixed function oxidase; (ii) in the case of OSSMe, sulphoxidation of the thiolo-sulphur (P—S—CH_3) leads to the production of $OSMeO^-$ and a putative reactive intermediate [CH_3S^+] capable of covalent binding to cellular macromolecules (cf. Fig. 4 in accompanying paper [2]); (iii) in the case of phosphorothionates, OOOMe(S), bromophos or parathion, sulphoxidation of the thiono-sulphur (P = S) leads to the oxon-form (P = O) with consequent release of highly reactive atomic sulphur and "suicidal destruction" of cytochrome P-450, as has been demonstrated for parathion and CS_2 [17, 18].

Verschoyle and Aldridge [7] have recently shown that pulmonary mixed-function oxidase is extremely sensitive to this effect of phosphorothionates, since low doses of several phosphorothionates were capable of rapidly and dramatically reducing the activity of pulmonary 7-ethoxycoumarin-*O*-deethylase, with very little loss of hepatic enzyme activity.

Figure 5 also indicates that, as for other chemicals [19], the relative efficiency of detoxication processes must also be important in determining the cellular toxicity of OSSMe. Reduced glutathione (GSH) has been variously implicated in the protection against chemically-induced injury, including in the lungs [19]. Thus our experiments in which the presence of GSH completely prevented the binding of radioactivity in microsomes incubated with labelled OSSMe [2] and the work of others [20] with *in vivo*-administered labelled *O,O,S*-trimethylphosphorothioate (OOSMe) strongly suggest that GSH plays a protective role "downstream" of the activation process probably by trapping reactive metabolite of OSSMe or OOSMe. In addition, GSH probably also plays a detoxifying role "upstream" of the activation process, in that GSH will, via the action of GSH-*S*-transferases [13], prevent OSSMe from being metabolized by mixed function oxidase. Our results suggest that liver slices have a greater ability than lung slices to thus detoxify OSSMe to $SSMeO^-$, a finding which

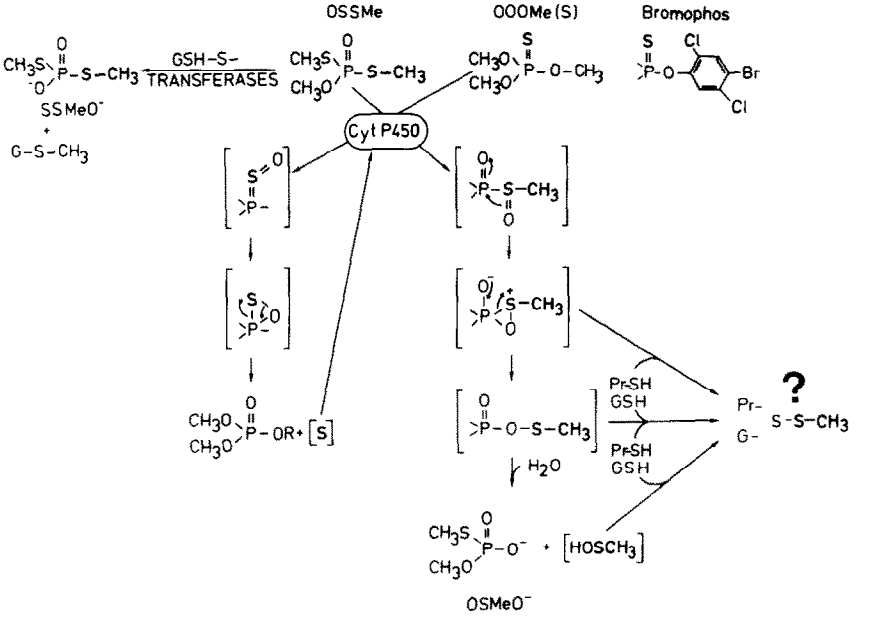

Fig. 5. Hypothetical mechanism for the metabolic activation and toxicity of *O,S,S*-trimethyl phosphorodithioate (OSSMe) and for the inhibitory effect of phosphorothionates. In the endoplasmic reticulum, sulphoxidation by cytochrome P-450-dependent mixed-function oxidase results in covalent binding of [CH_3S] to protein (Pr–SH) and production of *O,S*-dimethyl phosphorothioate ($OSMeO^-$) [2]; this metabolic activation occurs to a greater extent in the lung than in the liver. Phosphorothionates, such as *O,O,O*-trimethyl phosphorothioate [OOOMe(S)] or bromophos, also undergo sulphoxidation by cytochrome P-450-dependent mixed-function oxidase, but this results in the release of atomic sulphur (cf. [17, 18]) which destroys cytochrome P-450 and thus prevents activation of OSSMe; pulmonary mixed function oxidase seems to be particularly sensitive to this action of phosphorothionates [7]. In the cytosol, the *O*-methyl group of OSSMe is transferred to glutathione (GSH) by glutathione-*S*-transferase, giving methyl-*S*-glutathione and *S,S*-dimethyl phosphorodithioate ($SSMeO^-$) [13]; this detoxication reaction occurs to a greater extent in the liver than in the lung. In addition, GSH may be protective by trapping reactive metabolites, in which case Pr–SH = GSH [2].

is compatible with the higher activity of GSH-*S*-transferase in liver tissue than in lung tissue [21].

In conclusion, this study has shown that tissue slices are a convenient system for investigating xenobiotic metabolism in the lung and our data indicate that the susceptibility of the lung to OSSMe probably results from a relatively high rate of activation coupled with a relatively low rate of metabolism by non-toxic pathways and/or removal of reactive metabolites in lung tissue compared to liver tissue. Although it cannot be excluded that type I epithelial cells, which are the target-cells for the toxicity of OSSMe [22], are damaged by metabolites produced in other cells, e.g. type II cells, it seems reasonable to speculate that the type I cells are themselves capable of activating OSSMe.

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