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INDUCTION OF THE HEPATIC MICROSOMAL CYTOCHROME P-450 SYSTEM BY TRIALKYL PHOSPHOROTHIOATES IN RATS

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Abstract—Single i.p. doses of *O,O,O*-triethyl phosphorothioate [OOO-Et(S)], one of the suicide substrates for cytochrome P-450, caused a rapid increase of NADPH–cytochrome *c* reductase activity in rat liver microsomes. The increase was dose dependent but did not coincide with the recovery from the inhibition of drug-metabolizing activities. There was no change of K_m value of the reductase in the induced state. The co-administration of cycloheximide repressed the stimulatory effect of OOO-Et(S), suggesting that a *de novo* synthesis of enzyme protein may be responsible for the increase in activity. Of four homologous tri-*n*-alkyl esters tested, the triethyl compound was the most effective at 24 and 48 hr after administration. Triethyl phosphate, the oxygen analog of OOO-Et(S), also caused an increase of the reductase activity, but carbon disulfide had no influence on this activity. Although *O,O,S*-triethyl phosphorodithioate [OOS-Et(S)] and its *n*-alkyl homologs also caused the inhibition of drug-metabolizing activities and the increase of the reductase activity, the recovery and the stimulation of enzyme activity were different from that of *O,O,O*-tri-*n*-aklyl phosphorothioates.

Some thiono-sulfur-containing compounds, such as carbon disulfide, disulfiram, methimazole and thiophosphate insecticides, constitute a class of inhibitors that decrease hepatic microsomal drug-metabolizing enzyme activities with a loss of cytochrome P-450 content [1–5]. *O,O,O*-Triethyl phosphorothioate [OOO-Et(S)] bears structural resemblance to thiophosphate insecticides, and we previously reported that the administration of this compound to rats significantly decreases hepatic microsomal cytochrome P-450 content and the metabolism of aniline and aminopyrine [6]. Furthermore, we observed a rapid increase of microsomal NADPH–cytochrome *c* reductase activity in the acute phase.

Although many inhibitors of the cytochrome P-450 system also act as inducers of the same enzyme system, the induction generally occurs in a subchronic or chronic phase [7]. Thus, the stimulatory effect of OOO-Et(S) on microsomal NADPH–cytochrome *c* reductase activity is a rather unusual effect of an inhibitor of the cytochrome P-450 system. With regard to thiono-sulfur-containing compounds, such as carbon disulfide and phenitrothion, no stimulatory effect was reported on the reductase activity especially in their actue phases [8, 9]. This paper reports further investigations on the effects of OOO-Et(S) and some structurally related compounds on the hepatic microsomal cytochrome P-450 system in rats.

MATERIALS AND METHODS

Chemicals. *O,O,O*-Tri-*n*-alkyl phosphorothioates and *O,O,S*-tri-*n*-alkyl phosphorodithioates were synthesized and purified as described previously [6]. Each homologous compound was abbreviated as OOO-R(S) and OOS-R(S) (R = methyl to *n*-butyl). Triethyl phosphate was obtained from Nakarai Chemicals Ltd. (Tokyo, Japan) and purified by fractional distillation under reduced pressure. Cycloheximide was obtained from Wako Pure Chemical Ind. Ltd. (Tokyo, Japan) and phenobarbital sodium from the Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All of the other chemicals were of the highest purity commercially available.

Animals and treatments. Male Wistar rats weighing around 200 g were used in all experiments. All trialkyl esters and carbon disulfide were dissolved in corn oil and administered intraperitoneally. The concentration of each test compound was adjusted so that its volume of administration was 2.0 ml/kg body weight. Phenobarbital sodium was dissolved in saline solution and administered i.p. at doses of 80 mg/kg body weight per day for 3 consecutive days. For the experiment involving cycloheximide co-administration, cycloheximide was dissolved in saline solution (1.0 ml/kg) and was administered i.p. in four doses of 0.5 mg/kg body weight at 3-hr intervals immediately after the treatment with 1.0 mmoles/kg of OOO-Et(S). This treatment schedule was adopted to ensure a long-lasting inhibition of protein synthesis. To avoid a rebound from the inhibition of protein synthesis, rats were killed 12 hr after the first treatment. As a control, the same volume of corn oil or saline solution was administered. In all experiments except for the time–course studies, animals were fasted for 24 hr prior to being killed. After the administration of compounds, rats were killed at various times by decapitation, and blood was collected from the carotid artery.

Preparation of microsomes. Hepatic microsomes were prepared as described previously [6].

Biochemical assays. Microsomal cytochrome P-

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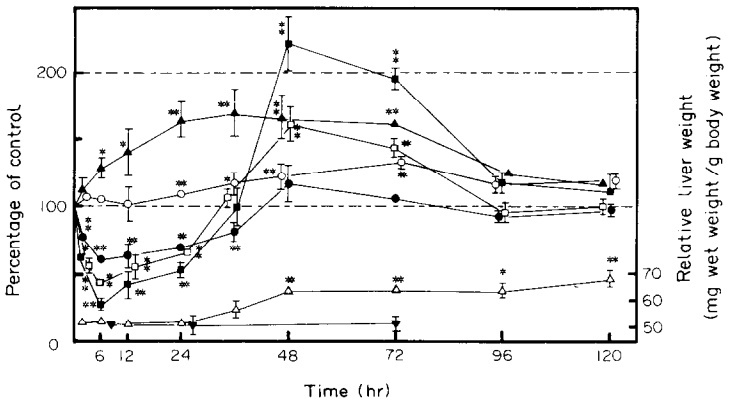

Fig. 1. Time–course of the effect of OOO-Et(S) on the hepatic microsomal cytochrome P-450 system and liver weight. OOO-Et(S): 1.4 mmoles/kg, i.p. Enzyme activities were expressed as percentage of the control in three separate experiments. The control experiments were run at 6, 24 and 72 hr after administration. Each point shows the mean ± SD (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. Key: (●) cyt. P-450; (○) cyt. b_5; (■) aminopyrine *N*-demethylase; (□) aniline *p*-hydroxylase; (▲) NADPH–cyt. *c* reductase; (▼) liver weight of control group; and (△) liver weight of OOO-Et(S)-treated group.

450 and cytochrome b_5 contents were measured by the methods of Omura and Sato [10]. Aniline *p*-hydroxylase was measured by the method of Imai *et al.* [11], aminopyrine *N*-demethylase by the method of Cochin and Axelrod [12], and NADPH–cytochrome *c* reductase by the method of Omura and Takesue [13]. NADH–cytochrome b_5 reductase was measured by the method of Takesue and Omura [14] using potassium ferricyanide as a substrate. Protein content was determined by the method of Lowry *et al.* [15] using bovine serum albumin as a standard. Serum glutamate-pyruvic transaminase (GPT) activity was determined according to the method of Karmen [16] using a Toshiba TBA-360 automatic analyzer.

Statistical analysis. The significance of the difference between two mean values was determined by Student's *t*-test.

RESULTS

Time–course of the effects of OOO-Et(S) on the hepatic microsomal cytochrome P-450 system. Rats were treated with OOO-Et(S) at an i.p. dose of 1.4 mmoles/kg (277 mg/kg), and time-related changes of hepatic microsomal enzymes were examined for 5 days. The dose of 1.4 mmoles/kg was selected to cause an effective inhibition of drug-metabolizing enzyme activities [6]. As shown in Fig. 1, the hepatic microsomal cytochrome P-450 content and the metabolism of aniline and aminopyrine decreased immediately and reached a minimum level 6 hr after administration. Thereafter, they recovered and increased beyond the control level and reached a maximum 48 hr after administration. During this recovery period, 20–30% increases were also observed in liver weight. These data suggest that a marked enzyme induction was occurring at this stage. In contrast, microsomal NADPH–cytochrome *c* reductase activity increased immediately and reached nearly a maximum at 24 hr after administration. Cytochrome b_5 content slowly increased and reached a maximum at 72 hr.

Dose-dependence of the effects of OOO-Et(S) on the hepatic microsomal cytochrome P-450 system. As shown in Fig. 2A, dose-related effects were observed

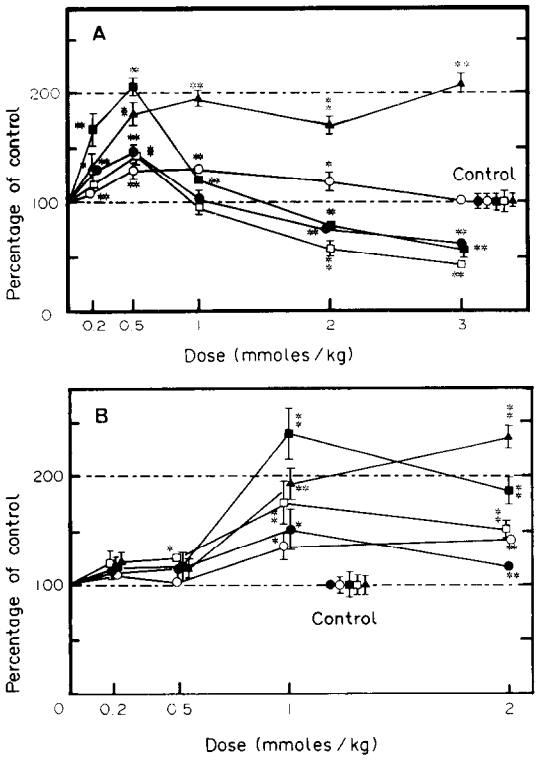

Fig. 2. Dose-dependence of the effect of OOO-Et(S) on the hepatic microsomal cytochrome P-450 system at 24 hr (A) and 48 hr (B) after administration. Each point shows the mean ± SD (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. Key: (●) cyt. P-450; (○) cyt. b_5; (■) aminopyrine *N*-demethylase; (□) aniline *p*-hydroxylase; and (▲) NADPH–cyt. *c* reductase.

on microsomal cytochrome P-450 content and drug-metabolizing enzyme activities at 24 hr after administration. As shown in Fig. 2B, decreased cytochrome P-450 and drug-metabolizing activities were recovered at 48 hr. These results indicate that the periods of inhibition and recovery were dependent on the dose. On the other hand, the increase of NADPH–cytochrome *c* reductase activity was directly dose dependent and reached a plateau at a dose of about 1.0 mmoles/kg. This result indicates that the increase of NADPH–cytochrome *c* reductase activity begins before the recovery from the inhibition of drug-metabolizing activity.

Kinetic parameters of NADPH–cytochrome c *reductase activity.* Kinetic parameters of the reductase were compared using microsomes from control, OOO-Et(S)-treated and phenobarbital-treated rat liver. Figure 3 shows the double-reciprocal plots of the reductase activity and cytochrome *c* concentration. Single doses of OOO-Et(S) increase the V_{max} values in a dose-dependent manner to a level as high as that observed after three consecutive doses of phenobarbital. However, no significant change was observed in apparent K_m values for cytochrome *c* by the treatments. This result suggests that the increase in the specific activity of the reductase may result from the increase of enzyme itself similar to phenobarbital-mediated induction.

Effect of cycloheximide on the induction of NADPH–cytochrome c *reductase by OOO-Et(S).* As shown in Table 1, the co-administration of cycloheximide significantly repressed the increase of the reductase activity. These data also indicate that a *de novo* synthesis of enzyme protein may be responsible for the increase of the reductase activity.

Effects of O,O,O-*tri*-n-*alkyl phosphorothioates on the hepatic microsomal cytochrome P-450 system.* Figure 4 shows the effects of four alkyl homologs of OOO-Et(S) on hepatic microsomal enzymes at 24 and 48 hr after administration at a dose of

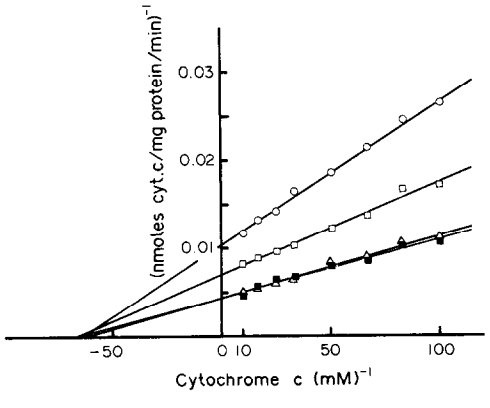

Fig. 3. Double-reciprocal plot of hepatic microsomal NADPH–cytochrome *c* reductase activity and cytochrome *c* concentration. Each point represents the mean of two or three determinations of a microsomal pool obtained from three rats each. Treatments and the apparent K_m values were as follows: (○) control: K_m = 15.5 μM; (□) OOO-Et(S), 0.2 mmole/kg (24 hr): K_m = 15.0 μM; (■) OOO-Et(S), 1.0 mmoles/kg (24 hr): K_m = 15.3 μM; and (△) phenobarbital sodium, 80 mg/kg/day for 3 days: K_m = 15.9 μM.

Table 1. Effect of cycloheximide co-administration on NADPH–cytochrome *c* reductase activity in liver microsomes of OOO-Et(S)-treated rats

Treatment	NADPH–cytochrome *c* reductase (nmoles/mg/min)
Control	70.5 ± 1.65 (100)
OOO-Et(S) alone	115.4 ± 6.22* (164)
OOO-Et(S) + cycloheximide	95.2 ± 6.51*† (135)
Cycloheximide alone	80.3 ± 11.73 (114)

Values are the mean ± SD for three rats. For experimental details, see Materials and Methods.
* $P < 0.01$ vs control.
† $P < 0.01$ vs OOO-Et(S) alone.

1.0 mmoles/kg. All compounds markedly induced microsomal NADPH–cytochrome *c* reductase, and the potency decreased in the order of ethyl > propyl > methyl > butyl. Although not shown, when rats were treated with these compounds at a dose of 2.0 mmoles/kg, OOO-Me(S) and OOO-Pr(S) increased the reductase activity to the induced level of OOO-Et(S) at 24 and 48 hr after administration. As shown in Fig. 4A, a slight increase was also observed in the activity of NADH–cytochrome b_5 reductase, a flavoprotein of the microsomal electron transport system. With regard to cytochrome P-450-mediated drug-metabolizing activities, early recovery from the inhibition was observed at 24 hr in the higher homologs, which have weaker inhibitory action at 6 hr after administration [6]. At 48 hr, the strongest stimulatory effect was observed with the triethyl compound and the magnitude of the increase was parallel to the inducibility of NADPH–cytochrome *c* reductase (Fig. 4B). The increase of aminopyrine N-demethylation was distinguished from that of cytochrome P-450 content and the other activities in the most stimulated states (Figs. 1, 2B and 4B).

Figure 5 shows the time-course of the effects of triethyl phosphate, an oxygen analog of OOO-Et(S), on hepatic microsomal enzymes at a dose of 1.0 mmoles/kg. At 6 hr after administration, a significant decrease was observed in cytochrome P-450 and aminopyrine *N*-demethylase activity but not in aniline *p*-hydroxylase activity. NADPH–cytochrome *c* reductase activity was also increased after some delay in comparison with the induction of OOO-Et(S). Cytochrome b_5 content did not change throughout the experiments.

Although not shown, the effects of carbon disulfide, the simplest thiono-sulfur compound, on hepatic microsomal enzymes were also examined at a dose of 1.0 mmoles/kg. At 6 hr after administration, microsomal cytochrome P-450 content and the metabolism of aniline and aminopyrine decreased to levels of 75.5, 50.7 and 69.5% of control respectively. These activities recovered to the levels of control rats at 48 hr after administration. However, no significant increase was observed in NADPH–cytochrome *c*

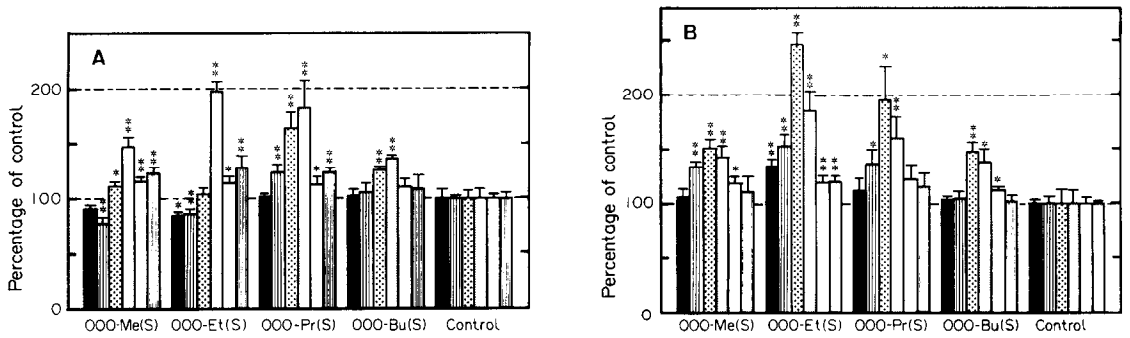

Fig. 4. Effects of *O,O,O*-tri-*n*-alkyl phosphorothioates on the hepatic microsomal cytochrome P-450 system at 24 hr (A) and 48 hr (B) after administration of 1.0 mmoles/kg. Each column represents the mean ± SD (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. Key: (■)cyt. P-450; (▥)aniline *p*-hydroxylase; (▩) aminopyrine *N*-demethylase; (▨) NADPH–cyt. *c* reductase; (□) cyt. b_5; and (▤) NADH–cyt. b_5 reductase.

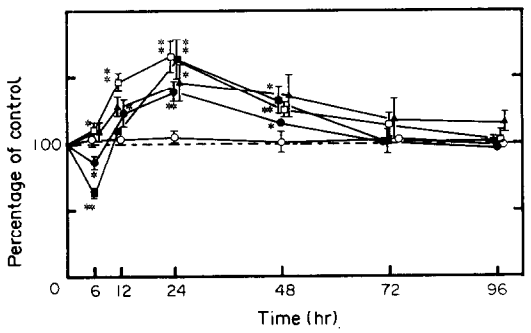

Fig. 5. Time–course of the effect of triethyl phosphate on the hepatic microsomal cytochrome P-450 system. Triethyl phosphate: 1.0 mmoles/kg, i.p. The data are expressed as percentage of control activities in two separate experiments. The control experiments were run at 6 and 48 hr after administration. Each points shows the mean ± SD (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. Key: (●) cyt. P-450; (○) cyt. b_5; (■) aminopyrine *N*-demethylase; (□) aniline *p*-hydroxylase; and (▲) NADPH–cyt. *c* reductase.

reductase activity or cytochrome b_5 content at any time during the recovery.

Effects of O,O,S-*tri*-n-*alkyl phosphorodithioates on the hepatic microsomal cytochrome P-450 system.* In a previous study, we reported that the four homologous *O,O,S*-tri-*n*-alkyl phosphorodithioates also decrease the hepatic microsomal cytochrome P-450 content and drug-metabolizing enzyme activities at 6 hr after administration. However, during this acute phase, no significant increase is observed in NADPH–cytochrome *c* reductase activity [6]. In the present study, we examined a more extended period of recovery from the inhibition caused by these dithioate esters. As shown in Fig. 6A, all the homologous compounds increased microsomal NADPH–cytochrome *c* reductase activity at 24 hr after administration, although the pattern of increase was different from that of each corresponding *O,O,O*-tri-*n*-alkyl phosphorothioate (Fig. 4). OOS-Et(S) was less effective in increasing reductase activity, and no recovery of drug-metabolizing enzyme activities was observed. In general, the recovery from the inhibition was slower than that of *O,O,O*-tri-*n*-alkyl phosphorothioate-treated rats. Moreover, no rebound activity was observed in those homologs even at 48 hr after administration (Fig. 6B).

In the rats treated with the lower homologs, serious toxicological signs including diarrhea, urination, salivation and bleeding from the nose, eyes, and gastrointestinal tract were observed, and all rats treated with OOS-Et(S) died with respiratory distress between 24 and 48 hr after administration. In our previous study, OOS-Me(S) seemed to be the most toxic compound among the alkyl homologs tested when observed within 6 hr after administration

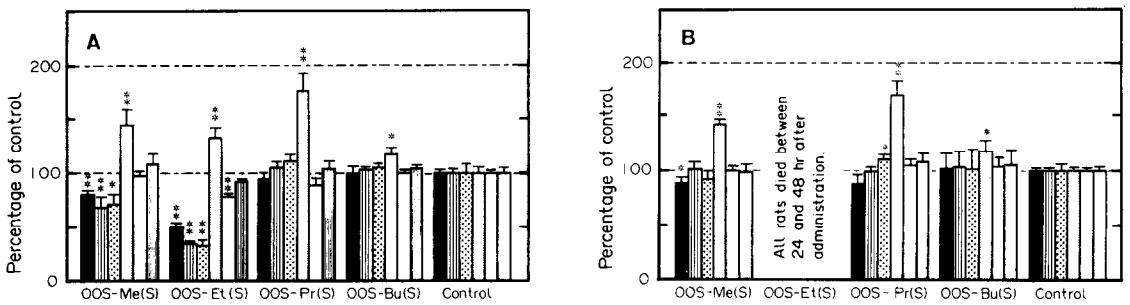

Fig. 6. Effects of *O,O,S*-tri-*n*-alkyl phosphorodithioates on the hepatic microsomal cytochrome P-450 system at 24 hr (A) and 48 hr (B) after administration of 1.0 mmoles/kg. Each column represents the mean ± SD (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. Key: (■)cyt. P-450; (▥)aniline *p*-hydroxylase; (▩) aminopyrine *N*-demethylase; (▨) NADPH–cyt. *c* reductase; (□) cyt. b_5; (▤) NADH–cyt. b_5 reductase.

Table 2. Effects of *O,O,O*-tri-*n*-alkyl phosphorothioates and *O,O,S*-tri-*n*-alkyl phosphorodithioates on serum GPT activity at 24 hr after administration of 1.0 mmoles/kg

Compound	GPT (I.U./l)
Expt. 1	
Control	34.7 ± 1.5
OOO-Me(S)	27.3 ± 7.8
OOO-Et(S)	27.0 ± 4.4*
OOO-Pr(S)	34.0 ± 6.1
OOO-Bu(S)	32.3 ± 3.1
Expt. 2	
Control	23.7 ± 3.8
OOS-Me(S)	52.0 ± 8.7†
OOS-Et(S)	141.0 ± 30.3†
OOS-Pr(S)	40.3 ± 9.3*
OOS-Bu(S)	35.3 ± 4.0*

Values are the means ± SD for three rats.
* $P < 0.05$ vs control.
† $P < 0.01$ vs control.

of a dose of 1.4 mmoles/kg. However, the present subacute study showed that OOS-Et(S) is the most toxic and OOS-Me(S) is next. No death occurred in rats treated with less than 4.0 mmoles/kg of OOS-Me(S), and rats recovered from the intoxication (data not shown).

Hepatic toxicity of trialkyl phosphorothioates. To examine the possibility of liver injury, serum GPT activity was determined in the rats treated with trialkyl phosphorothioates and trialkyl phosphorodithioates. As shown in Table 2, treatments with *O,O,O*-tri-*n*-alkyl phosphorothioates had no influence on serum GPT activity. On the other hand, treatments with *O,O,S*-tri-*n*-alkyl phosphorodithioates caused a significant increase of serum GPT activity. These data indicate that some hepatic damage occurred especially in the administration of OOS-Et(S).

DISCUSSION

Many xenobiotics, including drugs, pesticides and industrial chemicals, are known to inhibit the hepatic microsomal cytochrome P-450 system in mammals, whereas most of the compounds known to act as inhibitors, such as 2-diethylaminoethyl-2-2-diphenyl valerate (SKF-525A) and metyrapone, also act as inducers of the same enzyme system in a subchronic or chronic phase [17, 18]. In the present study, OOO-Et(S), a potent inhibitor of cytochrome P-450, also appears to act as an inducer in the subacute phase. The inducing effect of this compound was characterized by a rapid increase of NADPH–cytochrome *c* reductase activity. The data shown in Fig. 3 and Table 1 suggest that the increase of the reductase activity is not caused by activation but by *de novo* synthesis of this enzyme, as has been observed in the induction by phenobarbital.

The same induction of the reductase was also observed with the alkyl homologs and the oxygen analog (Figs. 4 and 5) but not with carbon disulfide. These results may indicate that the trialkyl ester structure of phosphate or the phosphorothioate is required to cause the rapid induction of the reductase and that the biosynthesis of the enzyme was not related to the loss of cytochrome P-450 caused by the thiono-sulfur moiety. Similar to the metabolism of parathion, a thiophosphate insecticide [4], OOO-Et(S) may be transformed partly to triethyl phosphate via oxidative desulfuration mediated by the cytochrome P-450 system. The inducing effects of OOO-Et(S) on the reductase cannot be explained by metabolic production of its oxygen analog, because the induction of OOO-Et(S) was more effective than triethyl phosphate. According to Madhukar and Matsumura [19], some thiophosphate insecticides, diazinon and malathion, act as potent inducers of NADPH–cytochrome *c* reductase when rats are treated with the pesticides consecutively at sublethal doses. Our results suggest that the characteristic induction pattern of these pesticides originates, in part, with phosphorothioate structures.

Similar to the reductase induction, subchronic induction of the other drug-metabolizing enzyme components cannot be explained by its oxygen metabolite. This is because the induction patterns of OOO-Et(S) are different from that of triethyl phosphate. For example, OOO-Et(S) caused a higher elevation of aminopyrine *N*-demethylase than of aniline *p*-hydroxylase and cytochrome P-450. This may depend in part on the induction of NADPH–cytochrome *c* reductase and cytochrome b_5, because cytochrome b_5-synergism is known in N-demethylation of aminopyrine but not in aniline hydroxylation [20, 21]. Another possibility is that the cytochrome P-450 species responsible for catalyzing demethylation was selectively induced. Although OOO-Et(S) was the most effective as an inhibitor and also as an inducer of the cytochrome P-450 system among homologous compounds, the potency of the two effects did not correlate with these compounds. For example, OOO-Me(S) was a strong inhibitor (Fig. 4A) but not an effective inducer (Fig. 4B). This observation indicates that the inhibition and the induction of enzymes of the cytochrome P-450 system may be controlled by some independent characteristics of the compounds.

The effects of *O,O,S*-tri-*n*-alkyl phosphorodithioates are more complicated. In the rats treated with these dithioate triesters, some severe toxic symptoms were seen and only NADPH–cytochrome *c* reductase was induced. In the recent decade, unique toxicities of some trimethyl phosphorothioates containing the thiolate-ester structure, such as *O,O,S*-trimethyl phosphorothioates, *O,S,S*-trimethyl phosphorodithioate and *O,O,S*-trimethyl phosphorodithioate [OOS-Me(S)] have been reported [22–26]. Although OOS-Me(S) is reported to be fairly low in toxicity among these trimethyl esters [22, 23, 25], our present study shows higher toxicity of the triethyl ester accompanied by some hepatic damage. Considering the strong induction ability of OOO-Et(S), OOS-Et(S) may also be expected to have strong inducing activity for NADPH–cytochrome *c* reductase. However, the induction of OOS-Et(S) was most likely repressed by its own toxic effects. In our previous report [6], no significant increase of NADPH–cytochrome *c*

reductase activity was observed in the rats treated with OOS-Et(S) or OOS-Bu(S) at a dose of 1.4 mmoles/kg. Our present result indicates that the induction of the reductase was repressed by some hepatic damage and/or indirect toxic effects such as loss of intake of food and water.

Konno *et al.* [26] reported that a single oral administration of *O,O,S*-trimethyl phosphorodithioate caused a dose-unrelated alteration in the activity of NADPH–cytochrome *c* reductase in liver and lung microsomes. It may also be the case that an inducing property of this compound was limited by physical conditions resulting from its toxicity.

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