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EFFECTS OF ALKYL PHOSPHOROTHIOATES ON THE HEPATIC MICROSOMAL MIXED-FUNCTION OXIDASE SYSTEM IN RATS

INHIBITION OF DRUG-METABOLIZING ENZYME ACTIVITY AND SELECTIVE INCREASE OF NADPH-CYTOCHROME *c* REDUCTASE ACTIVITY

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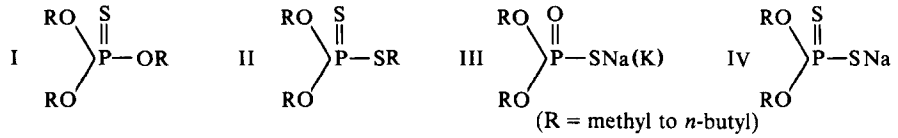
Abstract—Four series of alkyl phosphorothioates were administered to adult male rats by intraperitoneal injection, and their influences on the drug-metabolizing enzyme system in hepatic microsomes were examined. Among the alkyl phosphorothioates tested, *O,O,O*-trialkyl phosphorothioates (I) and *O,O,S*-trialkyl phosphorodithioates (II) significantly decreased hepatic microsomal cytochrome P-450 content and the metabolism of aniline and aminopyrine. Six hours after administration, triethyl compounds were the most effective of the trialkyl esters tested. In experiments on rats pretreated with phenobarbital or 3-methylcholanthrene, the inhibitory effects of triethyl esters were increased strongly by phenobarbital pretreatment and decreased by 3-methylcholanthrene. After the administration of I, a selective increase of NADPH-cytochrome *c* reductase activity was also observed. In the phenobarbital-pretreated rats, no further increase of NADPH-cytochrome *c* reductase activity was observed as a result of the administration of I. Except for the two dibutyl esters, *O,O*-dialkyl phosphorothioates (III) and *O,O*-dialkyl phosphorodithioates (IV) caused no significant inhibitory effect on the drug-metabolizing enzyme system under the same conditions.



(R = methyl to *n*-butyl)

A number of thiono-sulfur-containing compounds are used as pesticides, drugs, and industrial chemicals. Some of them, such as carbon disulfide, disulfiram, methimazole, and thiophosphate insecticides, are known to inhibit mammalian hepatic microsomal drug-metabolizing enzyme activities *in vivo* and/or *in vitro* [1–3], and the inhibitory effects of these compounds have been shown to be always due to a loss of cytochrome P-450 content.

With regard to the mechanism of inhibition by parathion, Neal [4] proposed that a highly reactive sulfur atom formed in the process of oxidative desulfuration mediated by mixed-function oxidases becomes covalently bound to the cytochrome P-450 molecule, predominantly to its apoprotein moiety, and that this sulfur atom causes the degradation of cytochrome P-450.

Other thiono-sulfur phosphorothioates, such as diethyl phenyl phosphorothionate, fenitrothion, diazinon and methyl parathion, have also been reported to inhibit hepatic microsomal drug-metabolizing enzymes [5, 6].

In this paper, we describe the relationship between the structures of simple alkyl phosphorothioates and their influences on hepatic microsomal drug-metabolizing enzymes when administered to rats. Alkyl phosphorothioates used in this study were *O,O,O*-trialkyl phosphorothioates (I), *O,O,S*-trialkyl phosphorodithioates (II), sodium (or potassium) *O,O*-dialkyl phosphorothioates (III) and sodium *O,O*-dialkyl phosphorodithioates (IV). In each of the four homologous compounds, the alkyl chains used were methyl, ethyl, *n*-propyl and *n*-butyl. These structures are shown above.

MATERIALS AND METHODS

Chemicals. *O,O,O*-Trialkyl phosphorothioates (I) were prepared from thiophosphoryl chloride and corresponding sodium alkoxides by the method of Mastin *et al.* [7]. *O,O,S*-Trialkyl phosphorodithioates (II) were prepared from sodium *O,O*-dialkyl phosphorodithioates (IV) and dialkyl sulfates (methyl and ethyl) or *n*-alkyl iodides (propyl and butyl) by the method of Yamazaki [8]. Their purities were checked using the TLC system of Trdlicka and Mostecky [9], mass spectroscopy, NMR spectroscopy and elemental analysis. Sodium (or

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potassium) *O,O*-dialkyl phosphorothioates (III) were prepared from corresponding hydrogen dialkyl phosphites and rhombic sulfur in the presence of sodium (or potassium) alkoxides by the method of Foss [10]. Hydrogen dialkyl phosphites were prepared from phosphorus trichloride and anhydrous alcohols by the method of Nylèn [11]. Sodium *O,O*-dialkyl phosphorodithioates (IV) were first prepared as their free acids from phosphorus pentasulfide and corresponding alcohols by the method of Yamazaki [12] and neutralized with anhydrous sodium carbonate in benzene. The purities of III and IV were checked as free acids using the TLC system of Stenersen [13, 14], mass spectroscopy and elemental analysis.

Animals and treatment. Male Wistar rats weighing 200–230 g were used. Trialkyl esters I, II and dialkyl esters III, IV dissolved in corn oil and 0.9% NaCl, respectively, were injected intraperitoneally. The concentration of each test compound was adjusted according to the volume of the injection (2 ml/kg body weight). As a control, the same volume of corn oil or 0.9% NaCl was injected. For the experiment involving phenobarbital pretreatment, each rat was given drinking water containing 0.1% phenobarbital sodium *ad lib.* for 2 days prior to treatment. 3-Methylcholanthrene was dissolved in corn oil and injected intraperitoneally at a dose of 20 mg/kg 2 days prior to treatment. In all experiments, except the time course studies, animals were fasted for 24 hr prior to being killed. After the injection of esters, rats were killed at intervals by decapitation, and blood was collected from the carotid artery.

Preparation of microsomes. Rat livers perfused *in situ* with ice-cold 0.9% NaCl solution were homogenized in a Potter–Elvehjem-type homogenizer with 3 vol. of 1.15% KCl solution. After centrifugation at 9000 *g* for 20 min, the supernatant fraction was centrifuged at 105,000 *g* for 60 min. The sedimented microsomal fraction was then resuspended in 1.15% KCl solution.

Biochemical assays. The constituents and enzymic activities of microsomes were determined by the following methods: cytochrome P-450 and cytochrome b_5 by the methods of Omura and Sato [15], aniline *p*-hydroxylase by the method of Imai *et al.* [16], aminopyrine *N*-demethylase by the method of Cochin and Axelrod [17], and NADPH-cytochrome *c* reductase by the method of Omura and Takesue [18]. Protein content was determined by the method of Lowry *et al.* [19] using bovine serum albumin as a standard, and serum cholinesterase activity was determined by the method of Ellman *et al.* [20].

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

RESULTS AND DISCUSSION

Effects of trialkyl esters on the hepatic microsomal mixed-function oxidase system. Table 1 shows the effects of a single intraperitoneal injection of triethyl esters (I-Et and II-Et) on hepatic microsomal cytochrome P-450 content at 4 hr after administration. Both compounds significantly decreased the cytochrome P-450 content. The effects were somewhat dose dependent up to a dose of 1.4 mmoles/kg.

Figures 1 and 2 show the time-dependent changes occurring in the hepatic microsomal mixed-function oxidase system after the injection of triethyl esters at a dose of 1.4 mmoles/kg (I-Et: 277 mg/kg; II-Et: 300 mg/kg). In the case of I-Et (Fig. 1), the activities of enzymes metabolizing aniline and aminopyrine were inhibited in parallel with the decrease of cytochrome P-450 content, maximum inhibition being observed between 6 and 8 hr after administration. On the other hand, NADPH-cytochrome *c* reductase activity increased directly after the injection, but cytochrome b_5 content was not affected by the treatment. In the case of II-Et (Fig. 2), cytochrome P-450 content, cytochrome b_5 content, and drug-metabolizing enzyme activities decreased quickly and were not restored within 24 hr after administration. In rats treated with II-Et, signs of severe toxicity were observed (described later).

Figures 3 and 4 show the dose-dependent changes occurring in the hepatic microsomal mixed-function oxidase system at 6 hr after the administration of triethyl esters. The inhibitory effects of both compounds were dose dependent up to a dose of about 1.0 mmol/kg. However, the dose-related inhibitory pattern of I-Et was different from that of II-Et. Aminopyrine N-demethylation was highly sensitive to II-Et inhibition. This observation suggests that each triethyl ester inhibits different forms of cytochrome P-450s.

We next examined the effects of homologous compounds of trialkyl esters under the same conditions. Figures 5 and 6 show the effects of *O,O,O*-trialkyl phosphorothioates (I) and *O,O,S*-trialkyl phosphorodithioates (II) at 6 hr after the administration of 1.4 mmoles/kg. Of the two types of trialkyl ester, triethyl esters had the stronger inhibitory effects on drug-metabolizing activity. The potency decreased in the order of ethyl $\geqq$ methyl $>$ propyl $>$ butyl. Aminopyrine N-demethylation was more sensitive than aniline p-hydroxylation to the administration of II. The administration of I esters increased NADPH-cytochrome *c* reductase activity.

The time-dependent effect of *O,O,S*-tributyl phosphorodithioate (II-Bu) was also examined. As shown

Table 1. Effects of I-Et and II-Et on hepatic microsomal cytochrome P-450 concentration at 4 hr after administration

Dose (mmoles/kg)	Cytochrome P-450 (nmoles/mg protein) I-Et	II-Et
0	0.636 ± 0.009 (100)	0.633 ± 0.054 (100)
0.35	0.478 ± 0.031* (75.2)	0.409 ± 0.015* (64.9)
0.70	0.407 ± 0.019* (63.9)	0.387 ± 0.009* (61.1)
1.40	0.371 ± 0.002* (58.3)	0.386 ± 0.032* (60.0)
2.80	0.424 ± 0.004* (66.6)	0.358 ± 0.040* (56.6)

Values are the means ± S.D. for three rats.
* Significantly different from control value ($P < 0.01$).

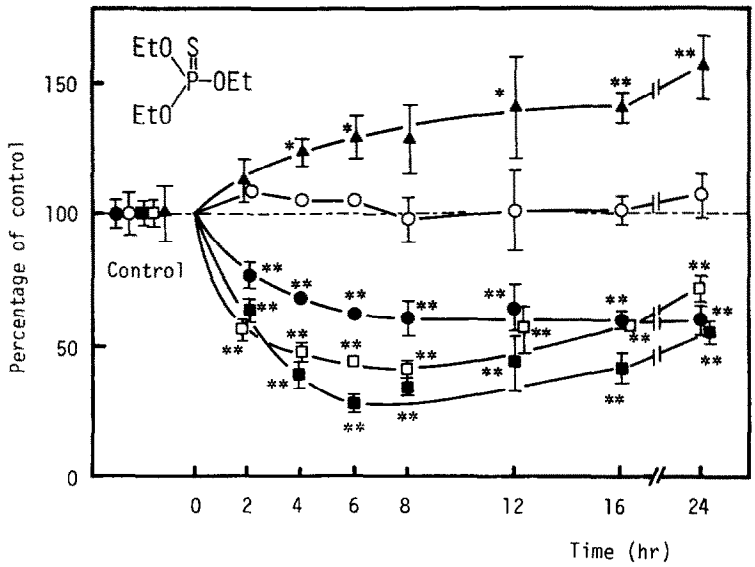

Fig. 1. Time course of the effect of *O,O,O*-triethyl phosphorothioate (I-Et) on the hepatic microsomal mixed-function oxidase system. I-Et: 1.4 mmoles/kg, i.p. Each point shows the mean ± S.D. (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. The control values were as follows: (●) cyt. P-450: 0.700 ± 0.032 nmoles/mg protein; (○) cyt. b_5: 0.371 ± 0.019 nmoles/mg protein; (■) aminopyrine *N*-demethylase: 6.40 ± 0.49 nmoles/mg/min; (□) aniline *p*-hydroxylase: 0.719 ± 0.061 nmoles/mg/min; and (▲) NADPH-cyt. *c* reductase: 60.2 ± 7.40 nmoles/mg/min.

in Fig. 7, its inhibitory effect was not as strong as that of I-Et at any time period. Aniline *p*-hydroxylation, cytochrome b_5 content and NADPH-cytochrome *c* reductase activity were no different from those of the control (data not shown), and no sign of toxicity was observed with this compound. Thus, the biological activities of these trialkyl esters probably decrease as the number of carbon atoms in the alkyl group increases.

The effects of drug-metabolizing enzyme inducers on the action of esters were next examined. Figures 8 and 9 show the effects of injected triethyl esters

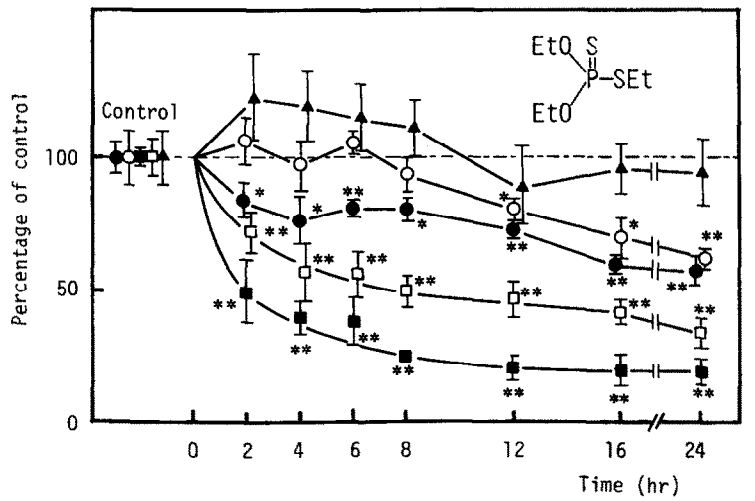

Fig. 2. Time course of the effect of *O,O,S*-triethyl phosphorodithioate (II-Et) on the hepatic microsomal mixed-function oxidase system. II-Et: 1.4 mmoles/kg, i.p. Each point shows the mean ± S.D. (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. The control values were as follows: (●) cyt. P-450: 0.708 ± 0.046 nmoles/mg protein); (○) cyt. b_5: 0.366 ± 0.040 nmoles/mg protein); (■) aminopyrine *N*-demethylase: 6.26 ± 0.19 nmoles/mg/min; (□) aniline *p*-hydroxylase: 0.683 ± 0.053 nmoles/mg/min; and (▲) NADPH-cyt. *c* reductase: 64.1 ± 8.68 nmoles/mg/min.

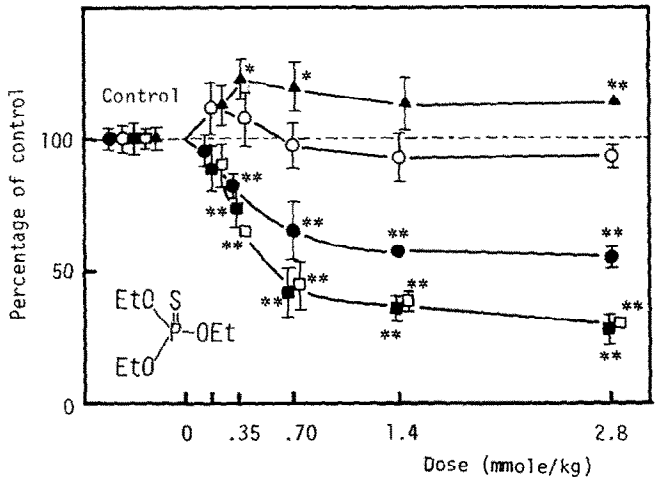

Fig. 3. Dose dependence of the effect of *O,O,O*-triethyl phosphorothioate (I-Et) on the hepatic microsomal mixed-function oxidase system at 6 hr after administration. Each point shows the mean ± S.D. (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. The control values were as follows: (●) cyt. P-450: 0.687 ± 0.025 nmoles/mg protein; (○) cyt. b_5: 0.348 ± 0.023 nmoles/mg protein; (■) aminopyrine *N*-demethylase: 7.59 ± 0.54 nmoles/mg/min; (□) aniline *p*-hydroxylase: 0.871 ± 0.030 nmoles/mg/min; and (▲) NADPH-cyt. *c* reductase: 85.7 ± 3.83 nmoles/mg/min.

(I-Et and II-Et) on the hepatic microsomal mixed-function oxidase system in rats pretreated with phenobarbital (PB) and 3-methylcholanthrene (3-MC). PB pretreatment significantly increased cytochrome P-450 content, NADPH-cytochrome *c* reductase activity, and the metabolism of aniline and aminopyrine. Subsequent treatment with triethyl esters equally decreased cytochrome P-450 content and drug-metabolizing enzyme activity. On the other hand, 3-MC pretreatment caused an increase of cytochrome P-450 content with a hypochromic shift in the difference spectrum and the metabolism of aniline only. Although triethyl esters also decreased the activity of drug-metabolizing enzymes, the extent of inhibition varied according to pretreatment with inducers. The inhibitory effects of both triethyl esters were strong in the case of PB and weak in the case of 3-MC pretreatment. Cytochrome P-450s induced

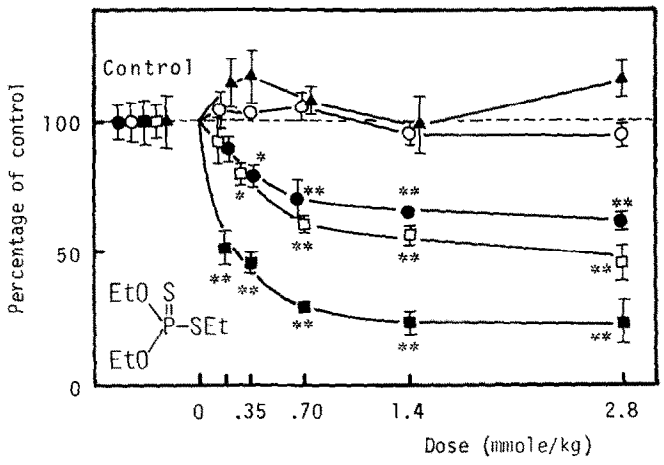

Fig. 4. Dose dependence of the effect of *O,O,S*-triethyl phosphorodithioate (II-Et) on the hepatic microsomal mixed-function oxidase system at 6 hr after administration. Each point shows the mean ± S.D. (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. The control values were as follows: (●) cyt. P-450: 0.616 ± 0.038 nmoles/mg protein; (○) cyt. b_5: 0.348 ± 0.026 nmoles/mg protein; (■) aminopyrine *N*-demethylase: 5.99 ± 0.44 nmoles/mg/min; (□) aniline *p*-hydroxylase: 0.576 ± 0.030 nmoles/mg/min; and (▲) NADPH-cyt. *c* reductase: 62.1 ± 6.77 nmoles/mg/min.

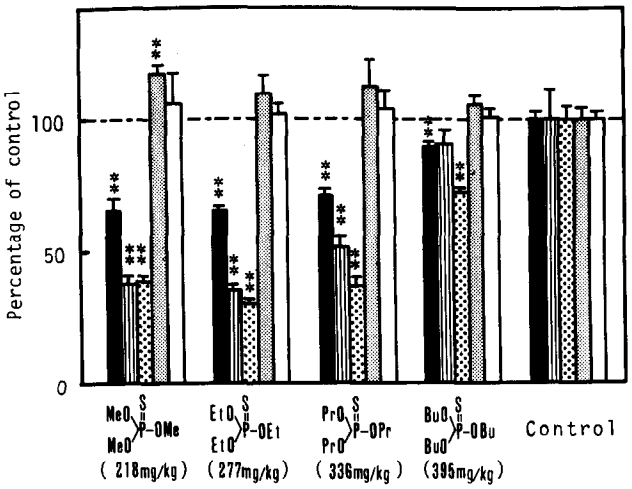

Fig. 5. Effects of *O,O,O*-trialkyl phosphorothioates (I) on the hepatic microsomal mixed-function oxidase system at 6 hr after administration of 1.4 mmoles/kg. Each column represents the mean ± S.D. (N = 3). Double asterisks indicate $P < 0.01$ vs control. The control values were as follows: (■) cyt. P-450: 0.593 ± 0.013 nmoles/mg protein; (▥) aniline *p*-hydroxylase: 0.766 ± 0.086 nmoles/mg/min; (▦) aminopyrine *N*-demethylase: 6.57 ± 0.29 nmoles/mg/min; (▨) NADPH-cyt. *c* reductase: 70.2 ± 2.57 nmoles/mg/min; and (□) cyt. b_5: 0.349 ± 0.008 nmoles/mg protein.

by 3-MC would be less sensitive to this type of thionosulfur compound. In addition, aniline *p*-hydroxylation was inhibited much more by the treatment of I-Et, whereas, in contrast, aminopyrine N-demethylation was more sensitive to II-Et.

An increase of NADPH-cytochrome *c* reductase activity as a result of I-Et administration was also observed in 3-MC-pretreated rats, but no further increase was observed in PB-pretreated animals. This result suggests that the increase in the activity of this enzyme may be controlled by a mechanism similar to PB-mediated enzyme induction. The induction of NADPH-cytochrome *c* reductase activity is a rather unusual effect for a drug-metabolizing enzyme inhibitor. Some kinds of inhibitors such as 2-diethylaminoethyl-2-2-diphenylvalerate (SKF-525A) are known to enhance the activity of the drug-metabolizing enzyme system as a rebound effect resulting from the inhibition [21, 22]. However, according to our unpublished data, no

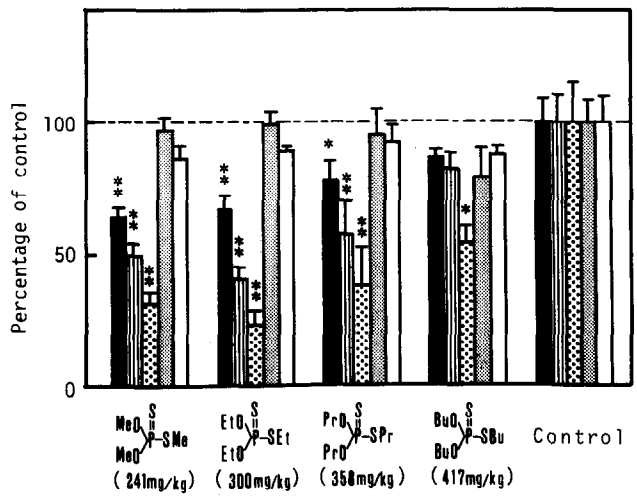

Fig. 6. Effects of *O,O,S*-trialkyl phosphorodithioates (II) on the hepatic microsomal mixed-function oxidase system at 6 hr after administration of 1.4 mmoles/kg. Each column represents the mean ± S.D. (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. The control values were as follows: (■) cyt. P-450: 0.624 ± 0.056 nmoles/mg protein; (▥) aniline *p*-hydroxylase: 0.640 ± 0.065 nmoles/mg/min; (▨) aminopyrine *N*-demethylase: 6.12 ± 0.89 nmoles/mg/min; (▦) NADPH-cyt. *c* reductase: 88.2 ± 7.29 nmoles/mg/min; and (□) cyt. b_5: 0.367 ± 0.034 nmoles/mg protein.

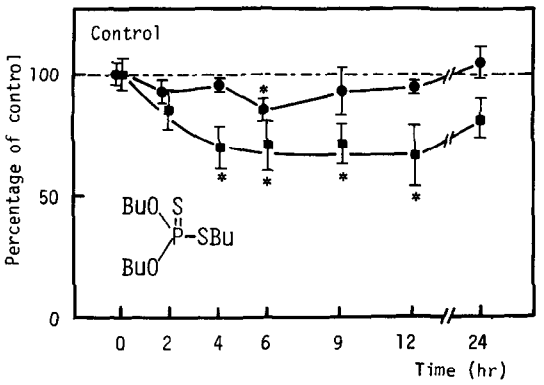

Fig. 7. Time course of the effect of *O,O,S*-tributyl phosphorodithioate (II-Bu) on hepatic microsomal cyt. P-450 content and aminopyrine *N*-demethylase activity (II-Bu: 1.4 mmoles/kg, i.p.). Each point represents the mean ± S.D. (N = 3). An asterisk indicates $P < 0.05$ vs control. The control values were as follows: (●) cyt. P-450: 0.670 ± 0.029 nmoles/mg protein; and (■) aminopyrine *N*-demethylase: 6.66 ± 0.51 nmoles/mg/min.

significant increase of the reductase was observed with SKF-525A treatment especially in its inhibition stage. Yoshida *et al.* [23] reported that treatment with the thiophosphate insecticide fenitrothion decreases the hepatic microsomal cytochrome P-450 content and drug-metabolizing enzyme activity. In their results, NADPH-cytochrome *c* reductase activity remained unchanged or was decreased slightly, unlike the results obtained in the present study.

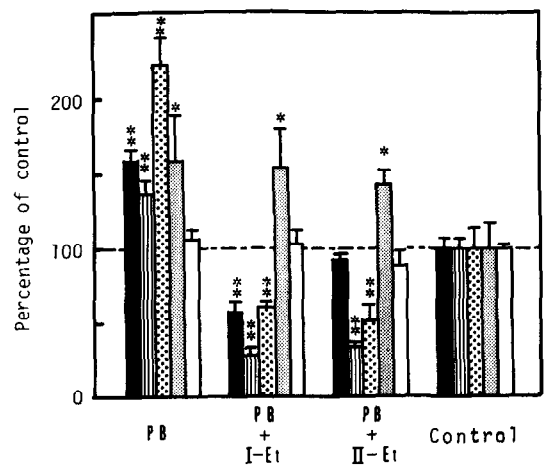

Fig. 8. Effects of I-Et and II-Et on the hepatic microsomal drug-metabolizing enzyme system of phenobarbital-pretreated rats at 6 hr after administration of 1.4 mmoles/kg of triethyl esters. Each column represents the mean ± S.D. (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. The control values were as follows: (■) cyt. P-450: 0.588 ± 0.032 nmoles/mg protein; (▥) aniline *p*-hydroxylase: 0.728 ± 0.051 nmoles/mg/min; (▩) aminopyrine *N*-demethylase: 3.40 ± 0.43 nmoles/mg/min; (▨) NADPH-cyt. *c* reductase: 38.0 ± 6.08 nmoles/mg/min; and (□) cyt. b_5: 0.283 ± 0.004 nmoles/mg protein.

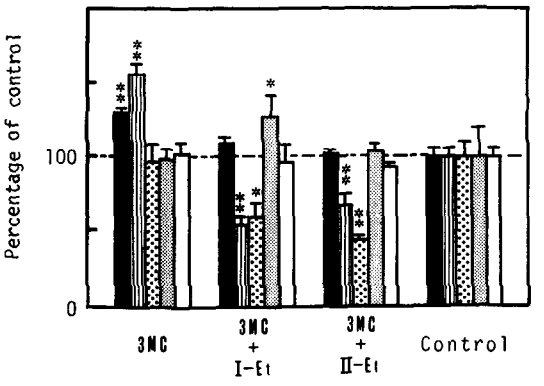

Fig. 9. Effects of I-Et and II-Et on the hepatic microsomal drug-metabolizing enzyme system of 3-methylcholanthrene-pretreated rats at 6 hr after administration of 1.4 mmoles/kg of triethyl esters. Each column represents the mean ± S.D. (N = 3). A single asterisk indicates $P < 0.05$ vs control; double asterisks indicate $P < 0.01$ vs control. The control values were as follows: (■) cyt. P-450: 0.533 ± 0.033 nmoles/mg protein; (▥) aniline *p*-hydroxylase: 0.515 ± 0.033 nmoles/mg/min; (▩) aminopyrine *N*-demethylase: 3.97 ± 0.38 nmoles/mg/min; (▨) NADPH-cyt. *c* reductase: 35.5 ± 6.99 nmoles/mg/min; and (□) cyt. b_5: 0.295 ± 0.019 nmoles/mg protein.

The effects of dialkyl esters (III and IV) on hepatic microsomal mixed-function oxidase system were also examined under the same conditions but, except for the two dibutyl esters, *O,O*-dialkyl phosphorothioates (III) and *O,O*-dialkyl phosphorodithioates (IV) caused no significant inhibitory effect (data not shown). In these experiments, strong signs of narcosis were observed just after the administrations of both dibutyl esters (III-Bu and IV-Bu), and some rats died as a result of deep anesthesia about 4 hr after the administrations. All of the constituents and enzymic activities in the hepatic microsomes of surviving rats were decreased to some extent, but these decreases seemed to be caused by general debility and were, therefore, different from the actions of trialkyl compounds. Accordingly, it seems unlikely that these hydrophilic dialkyl compounds are the substrates of the mixed-function oxidase system.

Other toxicological signs due to alkyl phosphorothioates. In addition to the inhibitory effects on the hepatic mixed-function oxidase system, various kinds of toxicological signs were observed following administration of alkyl phosphorothioates, as shown in Table 2. Narcosis was observed with the use of the lower trialkyl esters of I and II and the higher esters of III and IV, although the symptoms produced by the former compounds disappeared within 1 hr after administration. In contrast, the symptoms of narcosis resulting from the latter dibutyl esters lasted for more than 4 hr. Similar narcotic actions of other alkyl phosphates, alkyl phosphorothioates and phosphorothioate insecticides have been reported to be unrelated to cholinesterase inhibition [24, 25].

Serum cholinesterase was inhibited markedly by the lower homologs of II accompanied by characteristic symptoms such as lacrimation. The chol-

Table 2. Toxicities of alkyl phosphorothioates observed within 6 hr after administration of 1.4 mmoles/kg

	Compound	Narcosis	Cholinergic symptoms	Serum ChEase activity (% of control)	Other symptoms (a)	(b)
I	Me	++	−	95.3	−	−
	Et	+	−	104.0	−	−
	Pr	−	−	94.6	−	−
	Bu	−	−	96.9	−	−
	PB + Et	−	−	100.3	−	−
	3-MC + Et	−	−	102.9	−	−
II	Me	++	++	30.6	++	++
	Et	+	+	25.8	+	+
	Pr	−	−	22.1	+	−
	Bu	−	−	96.0	−	−
	PB + Et	−	++	26.5	++	−
	3-MC + Et	−	−	41.1	−	−
III	Me	−	−	92.2	−	−
	Et	−	−	97.7	−	−
	Pr	−	−	92.2	−	−
	Bu	+++	−	83.1	−	+(c)
IV	Me	−	−	N.D.	−	−
	Et	−	−	106.1	−	−
	Pr	−	−	N.D.	−	−
	Bu	++	−	92.2	−	+(c)

Toxicological signs are shown as follows: (+++, very serious and death occured; ++, serious; +, observed; −, not observed). Serum ChEase activity is shown as the mean value of two or three rats at 6 hr after administration. ND: not determined. (a) Diarrhea or urination. (b) Bleeding in the gastrointestinal tract. (c) Inflammatory hyperemia probably due to chemical stimulus.

inergic symptoms produced by II-Et were strengthened by PB pretreatment but weakened by 3-MC. These observations suggest that the oxygen analog of II-Et is a stronger inhibitor of cholinesterase than II-Et is itself, as known in the poisoning which occurs due to phosphorothionate insecticides. This fact is consistent with our experimental data, suggesting that 3-MC-inducible cytochrome P-450 is less sensitive to II-Et than the PB-inducible form.

Various other toxicological signs including diarrhea, urination, salivation, and bleeding from the nose, eyes and gastrointestinal tract were also observed in rats treated with the lower homologs of II. These signs were observed after 6 hr following II-Et administration, when the rats had become weakened with the course of time. Umetsu *et al.* reported the same type of toxicity in the form of delayed mortality in rats treated with impurities from the phosphorothioate insecticide malathion, i.e. *O,O,S*-trimethyl phosphorothioate, which is the oxygen analog of II-Me [26, 27]. From the fact that 3-MC pretreatment obviously decreased the toxicological signs of II-Et, the toxicity is most likely caused by the oxygen analogs of II. Imamura *et al.* [28] reported that pretreatment with PB decreased the toxicity induced by the oxygen analog of II-Me. In the present study, pretreatment with PB increased the urination and salivation, but decreased the bleeding, induced by II-Et. The PB-inducible form of cytochrome P-450 probably promotes both the formation of a toxic metabolite from II and its subsequent detoxification.

The decreasing effects on the hepatic microsomal mixed-function oxidase system, found especially in the lower homologs of II, may be closely related to some of these toxicities.

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