原　著

Activation and Degradation of Sumithion, Methylparathion and their Oxygen Analogs by Mammalian Enzymes *in Vitro*.

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1. 肝臓細胞分劃による *in vitro* におけるスミチオン，メチルパラチオンおよびスミオキソン，メチルパラオキソンの活性化と分解.

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1. モルモット，白ネズミ，マウスの肝臓ホモジネートを用い，トリチウムでフェノール側を標識した上記4種の化合物の分解をしらべた．スミチオン，メチルパラチオンはいずれも，そのオキソン，フェノール，脱メチル体に変化した．モルモット，白ネズミでは，この両化合物はほぼ同程度に分解され，マウスでは，スミチオンの方が若干よく分解をうけ脱メチル体の生成がやや多かった．オキソン型はチオ型よりもよく分解をうけフェノール，脱メチル化合物を生成する．モルモットではフェノールの生成がいちじるしく，マウスでは脱メチル体が多かった．白ネズミではスミオキソンよりの構造不明の分解産物が多量に生成した．

2. これら3種の動物肝臓のミクロゾーム分劃を用い，$NADPH_2$を助酵素としてスミチオン，メチルパラチオンよりのスミオキソン，メチルパラオキソンの生成をしらべた．いずれのミクロゾームにおいても，スミオキソンの生成量はメチルパラオキソンに比してわずかに多く，同じ条件下でスミオキソンの分解がメチルパラオキソンのそれと同じか，やや多いことを考え合わせれば，スミオキソンの生成の方が容易にすすむと考えられる．

3. スミチオン，メチルパラチオンおよびそれらのオキソンの脱メチル反応を触媒する酵素は，細胞の上清分劃にありグルタチオンを必要とすることが知られている．1. でマウスのホモジネートがもっともよく脱メチル化合物を生成することが認められたので，マウス肝臓の上清を用い，上記4種の化合物の脱メチル化をしらべた．スミチオンはメチルパラチオンに比しやや，またスミオキソンはメチルパラオキソンに比し若干速く，脱メチル化をうけることがわかった．

4. 以上の結果よりすれば，スミチオン，メチルパラチオン（およびスミオキソン，メチルパラオキソン）の活性化および分解には若干の差が認められるものの，これらにもとづいてスミチオンの哺乳動物に対する低毒性を説明することは困難と考えられる．

Introduction

In one of the preceeding papers designed to elucidate the lower toxicity of Sumithion®, *1) to mammals[6], Sumithion was demonstrated to be activated to sumioxon*2) even more rapidly, and to be decomposed a little more slowly *in vivo* than methylparathion*3) which closely resembles Sumithion in chemical structure. From the results difference in the respects of both activation and degradation in mammals was presumed to play a role of rather minor importance in lowering the toxicity of Sumithion. In the experiments reported here, activation and degradation of Sumithion and sumioxon by mammalian liver preparations *in vitro* were compared with methylparathion and methylparaoxon*4), in order to lend further support for the above presumption.

Materials and Methods

Special reagents

Sumithion, methylparathion and their oxygen analogs, sumioxon, methylparaoxon, labelled with tritium in the phenol moiety of the molecule, with a specific activity of 11.0, 10.1, 26.6 and 25.5 mCi/mmole, respectively, were supplied by Takarazuka Radiation Laboratory, Sumitomo Atomic Energy Industries, Ltd.[1]. The chemical purity of the preparations was above 99% and no radioactive impurities were detected. These

*1) *O, O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate.

*2) *O, O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorate.

*3) *O, O*-dimethyl (*O*-4-nitrophenyl) phosphorothioate.

*4) *O, O*-dimethyl(*O*-4-nitrophenyl) phosphorate.

radioactive compounds were suitably diluted with the corresponding non-active compound and used.

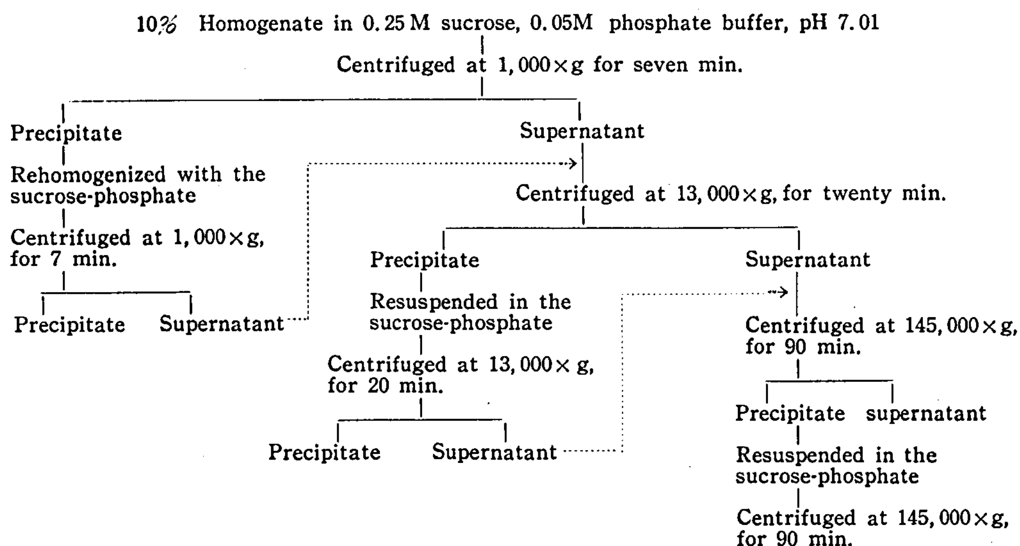
Desmethylparaoxon[*5)] was prepared by the reaction of methylparaoxon with equimolar sodium iodide in acetone at 60°C for four hours and recrystallized from acetone containing a minute amount of ethanol. The sodium salt was analyzed; calculated for $C_7H_7NO_6P$ Na, C, 32.94 ; H, 2.74 ; P, 12.15 ; N, 5.49% found, C, 32.94% ; H, 3.00 ; P, 12.44 ; N, 5.25%. Desmethylsumioxon[*6)] was prepared in the similar way. The sodium salt of desmethylparathion[*7)] was prepared by the action of sodium thiophenolate in absolute ethanol[3,5)]. The resultant sodium salt was recrystallized from a mixture of chloroform and *n*-hexane and analyzed; calculated for $C_7H_7NO_5SP$ Na·H_2O, C, 29.06 ; H, 2.42 ; P, 10.73 ; S, 11.07 ; N, 4.84%, found, C, 28.86 ; H, 3.05 ; P, 10.43 ; S, 11.96 ; N, 4.64%. Desmethylsumithion [*8)] was similarly obtained and recrystallized from acetone-chloroform. As these four compounds were more or less hygroscopic, the melting point was not accurately determined. Each of these authentic compounds gave single spot by thin layer chromatography and their IR and NMR spectra were consistent with the proposed chemical structure. Other authentic compounds such as Sumithion, sumioxon, methylparathion, methylparaoxon, 4-nitrophenol and 3-methyl-4-nitrophenol were synthesized in this laboratory.

NAD[*9)], $NADH_2$[*10)], NADP[*11)], $NADPH_2$[*12)] were purchased from Shigma Chemical Co.

Enzyme preparation and incubation

Three heads of male Wistar rats (body weight 170~200g), mice (15~18g) or Guinea pigs (200~230g) were sacrificed by decaptation and liver was dissected out and washed with the solution containing 0.25M of sucrose and 0.05M of phosphate buffer, pH 7.01. Liver was homogenized with nine times of the above sucrose and phosphate solution in a Potter-Elvehjem homogenizer with teflon pestle under cooling. The microsomal fraction of liver was obtained from animals abstained from food overnight as in the following scheme.

The precipitated microsomal fraction obtained



*5) *O*-methyl *O*-hydrogen *O*-(4-nitrophenyl) phosphorate.
*6) *O*-methyl *O*-hydrogen *O*-(3-methyl-4-nitrophenyl) phosphorate.
*7) *O*-methyl *O*-hydrogen *O*-(4-nitrophenyl) phosphorothioate.
*8) *O*-methyl *O*-hydrogen *O*-(3-methyl-4-nitrophenyl) phosphorothioate.
*9) nicotinamide adenine dinucleotide, oxidized.
*10) nicotinamide adenine dinucleotide, reduced.
*11) nicotinamide adenine dinucleotide phosphate, oxidized.
*12) nicotinamide adenine dinucleotide phosphate, reduced.

from 2.5g of liver was suspended in 1 ml of the sucrose-phosphate solution. Mouse liver supernatant fraction was prepared by centrifuging the 10% homogenate at 145,000×g, for ninety minutes and by discarding the precipitate.

By using the above enzyme preparations, decomposition of the phosphorothioates and their oxygen analogs was examined. Incubation was carried out at 37.5°C, vapor phase, air. The composition of the incubation mixture was:

Organophosphorus compound	(5×10^{-3}M)*	0.1 ml
Coenzyme	(5×10^{-3}M)**	0.2
Nicotinamide	(2×10^{-1}M)	0.05
Magnesium sulfate	(2×10^{-1}M)	0.05
Phosphate buffer	(1×10^{-1}M, pH7.01)	0.3
Enzyme solution		0.3
		1.00 ml

* Stock solution (1×10^{-1}M in ethanol) was diluted with 19 volumes of distilled water.

** NAD, $NADH_2$, NADP or $NADPH_2$.

Desmethylation of the organophosphorus compounds by mouse liver supernatant was tested under the conditions below:

Organophosphorus compound*		0.1 ml
Reduced glutathione	(1×10^{-2}M)	0.1
Phosphate buffer	(1×10^{-1}M, pH 7.01)	0.5
Enzyme solution**		0.3
		1.0 ml

* At the various concentration.

** When 0.1 ml of the enzyme solution was used, 0.2 ml of the sucrose phosphate solution was added.

The mixture was incubated at 37.5°C, vapor phase, air.

At specified intervals an aliquot of the incubation mixture (usually 1 ml) was pipetted into 9 ml of distilled water, and shaken with 40 ml, then additional 20 ml of chloroform. Aqueous layer was centrifuged to remove coagulated proteins. By this treatment the phosphorothioates, their oxygen analogs, their component phenols and some other unidentified compounds were extracted into chloroform, while desmethyl compounds remained in aqueous layer. As chloroform is a strong quencher in the liquid scintillation counting, especially when tritium is to be analyzed, an aliquot of chloroform layer was evaporated, and the residue was dissolved in toluene. This toluene solution as well as aqueous layer was analyzed for radioactivity with a liquid scintillation counter. Scintillator solution composed of 4.8g of 2,5-diphenyl oxazole (PPO), 0.12g of 2,2'-*p*-phenylene-*bis*-(5-phenyloxazole) (POPOP) in 1.2 l of toluene and 0.8 l of absolute ethanol was used. Results were expressed as disintegration per minute and compared.

Separation and identification of decomposition products

Separation and identification of chloroform- and water-soluble compounds were carried out as in the following. Chloroform layer was concentrated *in vacuo* below 40°C and the residue was co-chromatographed with the authentic samples on thin layer of silica gel (Merck, HF, thickness 0.5mm). The mixture of benzene: ethyl acetate (4 : 1, v/v) (solvent A) was used as a developing solvent. After development, silica gel was cut into 20 equal portions transversely, and radioactivity of each portion was counted. Identity was ascertained by extracting the radioactive spots separated with (A) into chloroform and by rechromatographing them on thin layer of silica gel with the second solvent, *n*-hexane:chloroform: acetone (40:15:3, v/v) (solvent B). Rf values of the authentic compounds were;

	solvent A	solvent B
Sumithion	0.95	0.75
Methylparathion	0.94	0.73
Sumioxon	0.29	0.41
Methylparaoxon	0.30	0.42
4-nitrophenol	0.58	0.38
3-methyl-4-nitrophenol	0.56	0.37

Aqueous layer obtained as above was concentrated *in vacuo* below 40°C and the residue was co-chromatographed with the authentic compounds on silica gel thin layer (Merck HF, thickness 0.5mm). The following two solvent systems were used.

(C) butanol:acetic acid : water (4:1:2, v/v).

(D) isopropanol : concentrated ammonium hydroxide : acetone (60:20:3, v/v).

The Rf values of the authentic samples were;

	solvent C	solvent D
Desmethylsumithion	0.75	0.94
Desmethylparathion	0.70	0.95
Desmethylsumioxon	0.53	0.89
Desmethylparaoxon	0.47	0.89

The amount of each radioactive compound was determined similarly to the chloroform-soluble compounds and identity was confirmed by re-chromatographing the developed spots of (C) with the solvent (D).

Results

1. Decomposition of phosphorothioates and their oxygen analogs by homogenate.

Decomposition of radioactive Sumithion, methylparathion and their oxygen analogs was tested by liver homogenate fortified with NADP. (Different from the case of microsomal fraction, general pattern of decomposition was not altered significantly either by NADP or $NADPH_2$) The radioactive compounds formed were separated and identified. The results are shown in Fig. 1 and Table 1. As shown in Fig. 1, Sumithion and methylparathion were evidently decomposed to the same degree by liver homogenate of Guinea pig or white rat, while mouse liver homogenate

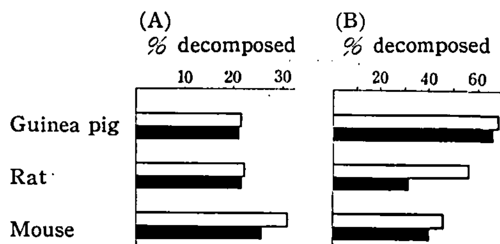


Fig. 1. Decomposition of phosphorothioates and their oxygen analogs by liver homogenate. 5×10^{-4}M of organophosphorus compound, 1×10^{-3}M of NADP were used. Incubated for one hour at 37.5°C. Organophosphorus compound added was taken as 100%. Mean of three replicated trials.
(A) phosphorothioate.
□ Sumithion ■ methylparathion
(B) phosphorate
□ sumioxon ■ methylparaoxon

Table 1. Identification of decomposition products from phosphorothioates and their oxygen analogs by liver homogenates.

	Compound added			
	Sumithion	Methylparathion	Sumioxon	Methylparaoxon
Guinea pig				
Oxygen analog	4.50%*)	3.55%*)	—*)	—*)
Desmethyl phosphorothioate	9.19	9.65	—	—
Desmethyl phosphorate	1.50	1.85	11.20%	12.77%
4-nitrophenol	—	2.94	—	52.11
3-methyl-4-nitrophenol	4.75	—	53.26	—
Unidentified**)	1.59	2.81	4.85	1.79
Rat				
Oxygen analog	4.85	4.47	—	—
Desmethyl phosphorothioate	11.58	12.19	—	—
Desmethyl phosphorate	1.81	0.84	24.07	19.94
4-nitrophenol	—	3.00	—	9.37
3-methyl-4-nitrophenol	3.39	—	5.51	—
Unidentified**)	0.27	0.76	26.28	2.20
Mouse				
Oxygen analog	1.96	2.43	—	—
Desmethyl phosphorothioate	21.58	18.25	—	—
Desmethyl phosphorate	3.74	2.40	40.59	34.36
4-nitrophenol	—	3.55	—	6.24
3-methyl-4-nitrophenol	3.54	—	3.62	—
Unidentified**)	0.07	0.26	2.30	0.30

*) Compound added was taken as 100%.
**) Chloroform soluble.
Incubation conditions, same as in Fig. 1.

degraded Sumithion slightly better than methylparathion. All of the three homogenates decomposed oxygen analogs much better than the phosphorothioates. Rat liver transformed sumioxon appreciably more easily than methylparaoxon, while in the homogenate of other two animal species difference in the decomposition rate between two oxygen analogs was not so greater. Table 1 demonstrates that an amount of oxygen analog and the component phenol was formed from the phosphorothioates and the major product was desmethyl compound. Although there founds no appreciable difference in the formation of these products between two phosphorothioates, the amount of desmethyl compound from both phosphorothioates was in the increasing order of Guinea pig, white rat and mouse, which reflected upon total decomposition rate of each substrate. Desmethyl compounds from the oxygen analogs added were formed in the same order among three animal species, mouse liver having the highest activity. Guinea pig liver was exceptional in producing much amount of the phenol from the respective phosphorate: approximately three quarters of the total decomposed were 4-nitrophenol or 3-methyl-4-nitrophenol. Rat liver transformed fairly large portions of sumioxon, but not methylparaoxon, into unidentified chloroform-soluble compound (Rf 0~0.2 with solvent A). This was solely an outstanding difference between two series of compounds, Sumithion, sumioxon and methylparathion, methylparaoxon through three animal species.

2. Phosphorothioate activation by microsomal fraction.

As there are ample evidences that the mammalian liver microsomal fraction catalyzes conversion of phosphorothioates into corresponding oxygen analogs, microsomal fraction was prepared from liver of Guinea pig, white rat and mouse and transformation of Sumithion or methylparathion into sumioxon or methylparaoxon, respectively, was examined. Degradation of the oxygen analogs was also tested. As from the preliminary experiments shown in Fig. 2, $NADPH_2$ was found the most effective coenzyme in Sumithion activation, washed liver microsome was fortified with $NADPH_2$. The results of transformation of the phosphorothioates into the phosphorates are reproduced in Table 2 and decomposition of the oxygen analogs by the

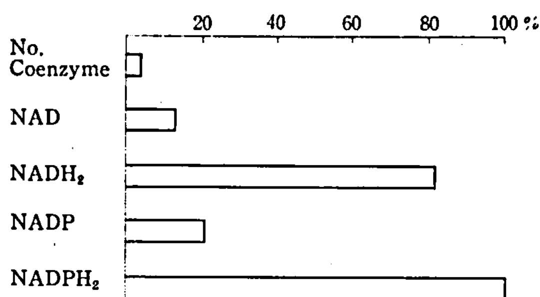


Fig. 2. Coenzyme requirement in Sumithion activation by rat liver microsome. 5×10^{-4}M of Sumithion, 1×10^{-3}M of coenzyme were incubated with washed rat liver microsome for one hour at 37.5°C. Sumioxon present by the addition of $NADPH_2$ was taken as 100%. Mean of three replicated trials.

Table 2. Oxygen analog formation by liver microsome.

	Phosphorothioate decomposed	Oxygen analog formed
Guinea pig		
Sumithion added	24.3%*)	9.8%*)
Methylparathion added	20.7	7.7
Rat		
Sumithion added	12.5	6.0
Methylparathion added	9.0	5.4
Mouse		
Sumithion added	15.3	6.4
Methylparathion added	12.6	5.0

*) Phosphorothioates added were taken as 100%.

5×10^{-4}M of Sumithion or methylparathion, 1×10^{-3}M of NADPH, were used. Incubated for one hour at 37.5°C. Mean of three replicated trials.

Table 3. Decomposition of oxygen analogs by liver microsome.

	Sumioxon added	Methylparaoxon added
Guinea pig	44.53%*)	32.57%*)
Rat	15.92	13.35
Mouse	16.48	17.86

*) Oxygen analogs added were taken as 100%.

Incubation conditions, same as in Table 2, except that 5×10^{-4}M of sumioxon or methylparaoxon was used as substrate. Mean of three replicated trials.

fraction, in Table 3. The content of the oxygen analogs formed in the incubation mixture was highest when Guinea pig microsome was used, amounting approximately to 40% of the phosphorothioate decomposed, followed by white rat or mouse. Products other than oxygen analog were component phenol and trace amount of desmethyl compound, as was the case of homogenate. Guinea pig microsome was found to degrade the phosphorates much more actively than the other, as shown in Table 3, and majorly to their component phenols. As is clear from Table 2, the amount of sumioxon formed was slightly larger than that of methylparaoxon through three animal species, and if taken into account of the decomposition rate of two oxygen analogs in Table 3, Sumithion was seemingly even more easily activated into sumioxon than methylparathion under the experimental conditions.

3. Desmethylation by mouse liver supernatant fraction.

As described above, mouse liver homogenate was found most active in catalyzing desmethylation reaction of phosphorothioates and phosphorates and difference in the formation rate was suspected between Sumithion and methylparathion, or sumioxon and methylparaoxon. The enzymes catalyzing desmethylation or transmethylation of such compounds were reported to be present in the supernatant fraction of mammalian liver, and glutathione is considered essential as an activator or methyl acceptor[2,4,8]. Therefore, by using mouse liver supernatant fortified with reduced glutathione, the rate of desmethylation of Sumithion and sumioxon into the corresponding desmethyl compounds was compared respectively with methylparathion and methylparaoxon. (Under the experimental conditions no radioactive products other than the corresponding desmethyl compounds were formed.) The results of time course studies of the reaction are shown in Fig. 3. The rate of conversion versus substrate concentration was also tested. Fig. 4 gives the results. In accordance with the results obtained with the homogenate, Sumithion was found to be converted rather rapidly into desmethyl compound than methylparathion, and sumioxon, more rapidly than methylparaoxon.

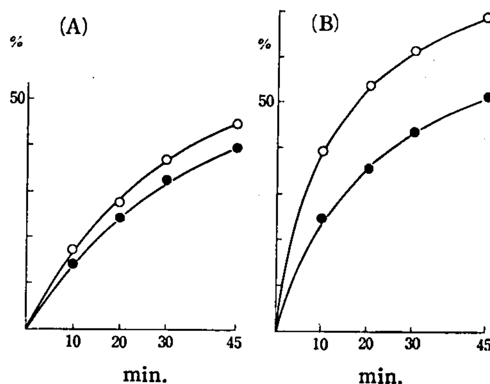


Fig. 3. Formation of desmethyl compound from phosphorothioates and their oxygen analogs by mouse liver supernatant.
5×10^{-4}M of organophosphorus compound, 1×10^{-3}M of reduced glutathione and 0.3ml of supernatant in 1ml were incubated at 37.5°C. Mean of duplicated trials.
(A) phosphorothioate.
○ Sumithion ● methylparathion
(B) phosphorate
○ sumioxon ● methylparaoxon

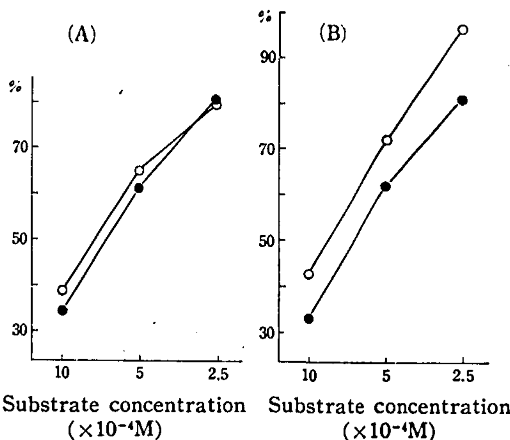


Fig. 4. Formation rate of desmethyl compounds from phosphorothioates and their oxygen analogs by mouse liver supernatant.
1×10^{-3}M of reduced glutathione was used. Mean of duplicated trials.
(A) phosphorothioate.
○ Sumithion ● methylparathion
0.3ml of supernatant in 1ml of incubation mixture. Incubated for thirty minutes at 37.5C.
(B) phosphorate.
○ sumioxon ● methylparaoxon
0.1ml of supernatant in 1ml of incubation mixture. Incubated for fifteen minutes at 37.5°C.

Discussions

As widely accepted, phosphorothioates such as Sumithion and methylparathion are oxidized by microsomal enzymes of animal tissues into physiologically active phosphorates and these phosphorates exert toxic effects upon the whole animal. On the other hand, these phosphorothioates and phosphorates are decomposed and detoxified. From the results of experiments aiming at explaining the selective toxicity of Sumithion to mammals, one of the present authors concluded that Sumithion was converted into sumioxon not more slowly in mammals *in vivo* than methylparathion and that the former compound was not always subject to easier decomposition than the latter[6]. He therefore presumed that the difference in the respects of both activation and degradation in mammals plays a role of rather minor importance in the lower toxicity of Sumithion than methylparathion, and attributed the selective toxicity to another mechanism[7]. In searching for an explanation of the selective toxicity of Sumithion, Vardanis, *et al.*[9] tested activation and degradation of Sumithion and methylparathion by mouse liver *in vitro* and surmised that the selective toxicity of the former compound resulted from the slower oxidizability and more rapid degradation than the latter. However, their assay methods were indirect and the results obtained were seemingly rather ambiguous. The results in Table 2 and Table 3 indicate that mouse liver microsomal enzymes as well as those of Guinea pig and white rat oxidized Sumithion even more rapidly than methylparathion, which is in accordance with the results already obtained *in vivo*[6]. As to the degradation of these phosphorothioates and their oxygen analogs, Hollingworth, *et al.*[3] stated in a recent publication dealing with the *in vivo* metabolism of the above two compounds in mice that the high selectivity level of Sumithion depends on the ability of cleaving P-O-alkyl bond thus forming desmethyl compounds to play an enhanced role in detoxication as the dosage is increased. Moreover, they examined the toxicity and metabolism of *O*-methyl phosphonate analogs of Sumithion and methylparathion to verify the above hypothesis. As the results they found that the toxicity ratio of these sumiphonothion and methyl paraphonothion was less than that of Sumithion and methylparathion, also that desmethylation of these phosphono analogs occurs only slightly. However, the present authors think that the results obtained by Hollingworth, *et al.* are not always so conclusive, as the enhanced desmethylation of Sumithion might be the results of lower toxicity of Sumithion, rather than the cause, and that their conclusions lack the evidences that the reduction of toxicity ratio observed in phosphono analogs comes solely from the inability of these compounds to be desmethylated. Although indeed mouse liver supernatant catalyzed desmethylation of Sumithion and sumioxon more rapidly than methylparathion and methylparaoxon, respectively, as shown in Fig. 3 and Fig. 4, the difference was not so large as to explain the lower toxicity of Sumithion.

Thus, the above *in vitro* results of activation and degradation of Sumithion and sumioxon compared with methylparathion and methylparaoxon are seemingly not incompatible with the presumption drawn from the previous *in vivo* experiments that the difference in activation and degradation of Sumithion and sumioxon did not result in the lower toxicity of Sumithion toward mammals.

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