The Selectivity of Sumithion Compared with Methyl Parathion. Metabolism in Susceptible and Resistant Houseflies

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The metabolism and fate of methyl parathion and Sumithion has been studied in organophosphorus-susceptible and -resistant houseflies in an investigation of the nature of the resistance mechanism. The two compounds behaved almost identically with respect to penetration, activation, and degradation in the two strains. Resistance was attributed to the presence of enhanced phosphatase activity in the resistant strain which resulted in greater degradation of the activation products, methyl paraoxon and Sumioxon, and thus a three- to fourfold lower level of these active toxicants was present in the resistant strain. A secondary factor in resistance may be the slower penetration into the resistant flies. The high level of resistance could be at least partially attributed to saturation of the penetration and activation mechanisms at high dosage levels, which leads to a decreased rate of accumulation of the activation product. At the same time, the over-all rate of degradation of these insecticides by the resistant flies at the high dosage was still as great as with the susceptible flies at the low dosage.

Because of the great interest in the exceptionally low mammalian toxicity of the insecticide *O,O*-dimethyl *O*-(3-methyl 4-nitrophenyl) phosphorothioate (Sumithion or fenitrothion), there have been a number of detailed studies of its metabolism in higher animals (*3*). However, little attention has been paid to studies of Sumithion metabolism in insects, although these would be valuable both from the standpoint of comparative metabolism and in relation to investigations of insecticide resistance. The present investigation, therefore, sought to determine the metabolism and fate of Sumithion and methyl parathion after topical application to susceptible and organophosphate resistant houseflies.

Qualitatively, the metabolism of this type of organophosphate proceeds similarly in both insects and mammals, although the over-all rate of degradation is often lower in insects and there may be a different emphasis among the pathways followed (*14*). Activation of phosphorothionates by oxidation to the corresponding phosphate has been confirmed in insects by several workers (*1*, *9*, *10*), although Tomizawa *et al.* (*15*) presented evidence that isomerization of methyl parathion may occur also in some cases. Degradation of the P-O-aryl bond is found with both phosphates and phosphorothionates, and the enzyme carrying out this reaction with parathion in resistant houseflies has been purified partially by Matsumura and Hogendijk (*7*). Cleavage at the P-O-alkyl bond also is commonly observed in insects (*13*, *14*), and enhanced desalkylation has been implicated as the resistance mechanism of the rice stem borer to parathion by Kojima *et al.* (*4*).

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The comparative behavior of methyl parathion in susceptible and resistant houseflies has been investigated in detail by Mengle and Casida (*8*), who could find no difference in penetration, activation, or detoxication sufficient to explain the selectivity between the two strains. Plapp *et al.* (*12*), on the other hand, in similar studies with parathion discovered that the resistant flies detoxified both parathion and paraoxon by hydrolysis faster than susceptible flies.

Materials and Methods

P^{32}-labeled methyl parathion and Sumithion were prepared from high activity dimethyl phosphorochloridothionate—specific activity 26 mc. per mmole, The Radiochemical Centre, Amersham, England. The condensation with the requisite sodium *p*-nitrophenoxides and subsequent purification have been described (*3*). At the time of use, the compounds had specific activities of 18 to 21 mc. per mmole and gave 45,000 to 50,000 c.p.m. per μg. on a gas flow counter. They were at least 99% radiochemically pure as shown by paper chromatography (*3*).

Houseflies. Two strains of houseflies were used, the susceptible S_{NAIDM} with female topical LD_{50} values for methyl parathion 1.2, Sumithion 3.1, methyl paraoxon 2.5, and Sumioxon 4.3 μg. per gram, and the resistant R_{SC} strain with female topical LD_{50} values for the same compounds of 89, 126, 25, and 141 μg. per gram, respectively (*2*).

One hundred eighty 2-day-old female flies were each treated topically on the pronotum with a 1.0 μl. droplet of an acetone solution of the desired P^{32} compound. They were placed in a 750-ml. glass jar with a plastic gauze cover at 70° F. without food or water and kept for periods of 0.5, 1, 2, and 4 hours. The treatments took about 10 minutes and were always carried out at the same time of day. The dosages used for most of

the determinations were 0.032 μg. per fly for methyl parathion (1.8 μg. per gram for S_{NAIDM} and 2.2 μg. per gram for R_{SC}) and 0.034 μg. per fly with Sumithion. The R_{SC} flies also were treated with methyl parathion at 0.98 μg. per fly (64 μg. per gram) and Sumithion at 1.01 μg. per fly. All these dosages were below the mean 24-hour LD_{50} value for the particular compound and strain at the time of testing. The higher dosage level for the R_{SC} flies was chosen since here the flies showed mortality and rate of development of symptoms similar to those of the S-flies at the lower dosage level.

After the appropriate time interval, the flies were sacrificed by deep freezing the jar in dry ice for 15 minutes. Further treatment of these flies was carried out within 1 hour of freezing. A full analysis of metabolism was made on the flies sacrificed 2 hours after dosage. At other times, penetration, oxidation, and over-all degradation only were investigated.

Before extraction of metabolites, the flies were washed successively for 1 minute periods with 25 ml. of CH_3CN, 25 ml. of warm H_2O, and then with an additional 10 ml. of CH_3CN. The combined "washings" were assayed for radioactivity to determine penetration. The insects were then homogenized for 1 minute in 75 ml. of CH_3CN in a Waring Blendor, filtered, and washed with CH_3CN; the residue was rehomogenized with 75 ml. of distilled H_2O, filtered through Celite, and washed with 25 ml. of H_2O. The extraction step was repeated with 50-ml. portions of CH_3CN and H_2O in turn, followed by washings with 25 ml. of the same solvent and, finally, extraction was made with 50 ml. of acetone-methanol (1 to 1). All filtrates were collected together to form the "extract." The holding jars and covers were washed thoroughly with 25-ml. portions of CH_3CN, H_2O, CH_3CN, and H_2O to remove the excreta, and the washings were combined to form the "jar-wash" fraction. Finally, the residual material from the extraction was dried and the organic material completely digested by warming on the steam bath for 4 hours with 75 ml. of concentrated HNO_3 and filtered to give the "unextractable" fraction. All washes and extracts were kept in the freezing compartment until analyzed. Exhaustive efforts were made to extract the maximum amount of P^{32} from the flies and the procedure described was as effective as any tested, giving recoveries of the applied material which were almost always >90%. No method extracted all the internal P^{32} from the flies since some was firmly bound to insoluble body constituents.

Analysis of Metabolism. The total radioactivity in all washes, extracts, and residual fractions was assayed by evaporation of aliquots in planchets and counting on a gas flow counter. A sample was then taken from each wash or extract and partitioned against $CHCl_3$ to show the relative amounts of hydrolyzed and unhydrolyzed compounds present. To estimate the amounts of the phosphate and phosphorothionate in the fly extract, the $CHCl_3$ phase from partitioning of the extract was evaporated gently, the radioactive compounds were taken up in CH_3CN to remove most of the fatty material and applied to a thin-layer plate—Adsorbosil 1 silica gel, 150 μ thick. After development with a mixture of $CHCl_3$–ethyl acetate (9 to 1), the phosphate and phosphorothionate were located by the position of cochromatographed compounds, and a series of 2-cm. strips including these compounds was scraped from the plate into planchets and counted. The radioactivity in this $CHCl_3$ layer was composed almost entirely of the unhydrolyzed phosphate and phosphorothionate with only traces of hydrolysis products present.

A full analysis of the metabolites present was carried out on the fly extracts obtained 2 hours after dosage. The metabolites were separated, identified, and estimated by ion-exchange chromatography, as described previously (*3*), after removal of the organic solvent from the extract by a rotary evaporator. Considerable amounts of the unchanged phosphorothionates (metabolite 4) were present in the extracts which appeared as peaks just after, and often partially obscuring those of the corresponding phosphates (metabolite 5).

Discussion

The quantitative metabolism of methyl parathion and Sumithion in the S_{NAIDM} and R_{SC} houseflies, involving a number of duplicate runs, is presented in Table I and summarized in Figure 1. At the dosage levels used, these compounds caused little or no mortality during the 4-hour period of study and generally less than 50% mortality after 24 hours. In one assay at the 2.0 μg. per gram dosage, neither methyl parathion nor Sumithion caused any apparent change in the behavior of S_{NAIDM} flies for 2 hours, at 3 hours some hyperactivity occurred with both compounds, and with Sumithion, a few flies showed incoordination. At 6 to 7 hours, the methyl parathion–treated flies also exhibited some loss in coordination and 5 to 10% were knocked down, while the Sumithion-treated flies still displayed hyperactivity and incoordination, with 3 to 5% knocked down or dead. At 12 hours, the methyl parathion–treated flies showed 33% knockdown and 10 to 15% mortality, but Sumithion-treated flies behaved as at 7 hours. The final 24-hour mortality in this test was 55 to 60% for methyl parathion and 5 to 10% for Sumithion.

The R_{SC} strain at the 2.0 μg. per gram dosage showed no symptoms of intoxication, but at 65 μg. per gram some incoordination and 3 to 5% knockdown was noted with both compounds. At 4 hours, 3 to 5% mortality occurred with Sumithion and 5 to 10% with methyl parathion. After 17 hours, 35 to 40% of the methyl parathion–treated flies were dead or moribund as compared with 30% for Sumithion. The final 24-hour mortalitites were 40% for methyl parathion and 47% for Sumithion.

Penetration into Flies. The absorption of both compounds as measured by rate of surface loss was very rapid and generally 80 to 90% had penetrated after 30 minutes at lower dosage levels. The rate was markedly reduced in the 60 μg. per gram dosage where 50 to 65% penetrated after 2 hours. When log percentage of applied dose unpenetrated is plotted against time allowing for the small percentage of dose rubbed off on the jar (3 to 5%) (Figure 2), reasonably straight line plots were obtained over the first hour, until the surface

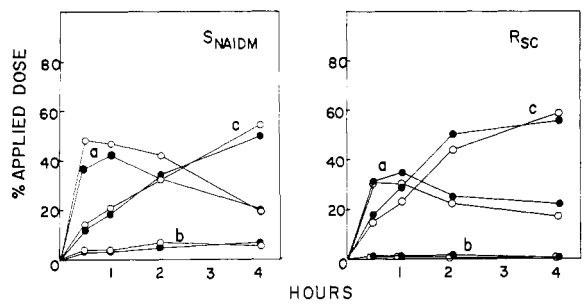

Figure 1. Summary of metabolism of methyl parathion, ●, and Sumithion, O, in susceptible and resistant houseflies

a Internal phosphorothionate, *b* internal phosphate, *c* hydrolysis products

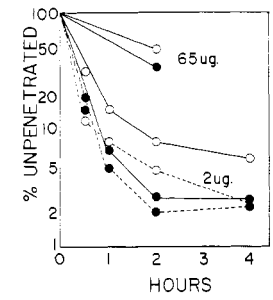

Figure 2. Rates of penetration of methyl parathion, ●, and Sumithion, O, into susceptible and resistant houseflies at indicated dosage levels

—— R flies; - - - S flies

dosage was largely exhausted. From such plots, t_{50} values for penetration of half of the applied dose were obtained (Table II). Little, if any, difference could be detected in rates of penetration of methyl parathion and Sumithion into S_{NAIDM} flies, but methyl parathion was absorbed considerably more rapidly than Sumithion into the R_{SC} flies at both low and high dosage levels. Both methyl parathion and Sumithion penetrated more rapidly into the S_{NAIDM} flies than into R_{SC} flies, but the differences seem too small to explain the level of resistance.

Metabolism in S- and R-Flies. Figure 1 shows that significant differences exist between the two strains in rate of detoxication, since for both Sumithion and

Table I. Metabolites Isolated from Susceptible and Resistant Houseflies Two Hours after Treatment with Methyl Parathion or Sumithion

		Dose, μg./G.					
		1.8	1.9	2.1	2.2	63	65
		Total Dose, μg.					
		5.73	6.05	5.73	6.05	177	182
		S_{NAIDM}		R_{SC}			
Metabolite		M.P.[a]	S.[b]	M.P.	S.	M.P.	S.
1	%[c]	2.1	1.3	0.8	0.6	0.2	0.1
Unknown	μg.	0.09	0.06	0.03	0.02	0.2	0.1
2	%	1.5	0.8	0.6	0.7	0.2	0.3
Phosphoric acid	μg.	0.07	0.04	0.02	0.03	0.2	0.2
3	%	0.7	0.6	1.9	0.6	0.3	0.4
Methyl phosphoric acid	μg.	0.04	0.03	0.07	0.02	0.3	0.3
4	%	5.5	3.6	17.2	16.1	7.7	6.7
Dimethyl phosphoric acid	μg.	0.24	0.16	0.68	0.66	6.5	3.7
5	%	11.0	15.5	3.8	4.1	1.9	2.0
Phosphate	μg.	0.48	0.67	0.15	0.17	1.6	1.1
6	%	41.6	46.3	31.6	43.5	51.1	59.0
Phosphorothionate	μg.	1.83	2.02	1.24	1.78	43.2	32.3
7	%	19.7	21.2	14.0	14.3	9.4	8.6
Dimethylphosphorothioic acid	μg.	0.87	0.92	0.54	0.59	7.9	4.7
8	%	2.8	2.4	3.6	3.9	2.4	1.6
Desmethyl phosphate	μg.	0.12	0.10	0.14	0.16	2.0	0.9
9	%	15.1	8.3	25.2	16.0	26.8	21.4
Desmethyl phosphorothionate	μg.	0.66	0.36	0.99	0.66	22.6	11.7

[a] Indicates methyl parathion.
[b] Indicates Sumithion.
[c] Per cent of total radioactivity in the extract.

Table II. Half-times (t_{50}) in Minutes for First Order Processes in the Metabolism of Phosphorothionates Topically Applied to Resistant and Susceptible Houseflies

	Dose, μg./G.					
	1.8	1.9	2.1	2.2	63	65
	S_{NAIDM}		R_{SC}			
	M.P.[a]	S.[b]	M.P.	S.	M.P.	S.
Penetration	12	12	15	20	85[c]	125[c]
Hydrolysis of the dose penetrated	180	180	105	105	150	220

[a] Indicates methyl parathion.
[b] Indicates Sumithion.
[c] Figures in these columns are approximate and derived from a single measurement.

methyl parathion there is a considerably larger production of hydrolytic products in the resistant strain at all times. Since the insecticides penetrate somewhat more slowly into the resistant strain, the levels of hydrolysis products shown represent an even larger fraction of the dose which has penetrated than is apparent from this figure. An over-all measure of the relative rates of detoxication in the S_{NAIDM} and R_{SC} flies has been made by determining the ratio of per cent of dosage penetrated/per cent of dosage hydrolyzed, including in the analysis the water soluble material both on the flies and the holding jars. The ratios (Table III) indicate the almost identical behavior of Sumithion and methyl parathion and that the R-strain hydrolyzes about 50% more of the internal dose than does the S-strain over the first 2 hours. The data show that by 2 to 4 hours after treatment, the R-strain has detoxified almost all the available internal dosage and the slower detoxifying S-strain is beginning to catch up.

Plots of log per cent of penetrated dosage not hydrolyzed against time give reasonably straight lines over the first 2 hours, and as with penetration, over-all detoxication is a first order process, the rate depending only on the concentration of unchanged material. The t_{50} values (Table III) emphasize the greater detoxifying capacity of the R_{SC} flies. The t_{50} values for the R_{SC} flies at 65 μg. per gram are tentative but are considerably higher than those for 2 μg. per gram, suggesting that like penetration, degradation is saturated at this elevated dosage.

Figure 1 and Table I establish more directly the effects of the hydrolytic detoxication systems on the internal levels of the activated phosphate analogs methyl paraoxon and Sumioxon, which are the actual anticholinesterase toxicants. Hollingworth, Fukuto, and Metcalf (*2*) have shown that the brain cholinesterases in the S_{NAIDM} and R_{SC} flies are almost identical in enzymic activity and in susceptibility to methyl paraoxon and Sumioxon. Thus, equal internal concentrations of these two compounds should produce equally toxic effects. Therefore, at all times and with both insecticides, the level of methyl paraoxon and Sumioxon was from 3.5 to 4.0 times higher in the susceptible than in the resistant strain, with a maximum difference of 6 to 8 times higher. In the R_{SC} strain, these compounds constituted 0.8 to 1.9% of the applied dose over the 4 hours studied, while in the S_{NAIDM} flies they represented from 2.7 to 7.5%.

The decreased levels of activated phosphates in the R-flies most likely would result from decreased oxidation of the thionates or enhanced hydrolysis of the phosphates. Since the data in Table IV show that oxidation, as judged by the total levels of all oxidized products in the extract, occurs slightly faster in the R-flies, the phosphates are produced at about the same rates in both strains of flies. This conclusion is supported by the observation that the R-flies are substantially more resistant to the phosphate analogs than the S-flies, which suggests that activation is not an important feature of the resistance mechanism. On the other hand, Tables I and IV show that the R-flies contain higher levels of P=O hydrolysis products, owing to the presence of from 3 to 4 times as much dimethyl phosphoric acid, as in the S-flies at equivalent dosages. Thus, despite the differences in the amounts of dimethyl phosphoric acid and intact phosphate in the two strains, the combined amount of these two metabolites represents a fairly constant proportion of the applied dosage in both strains—i.e., for S_{NAIDM} with methyl parathion and Sumithion, the combined amount is 11.5 and 13.7%, respectively, and for the R_{SC} strain the same compounds give 14.5 and 13.7%.

At the 2.0 μg. per gram dosage the selectivity between the S- and R-flies can be explained by the more rapid detoxication of the phosphate analog in the R-strain which leads to a lower rate of accumulation of this active toxicant. The two strains degrade the phosphorothionates themselves at a comparable rate, although as the data in Table I show, the S_{NAIDM} flies are more efficient in cleaving the P(S)-O-aryl bond, while the resistant strain produces greater degradation of the P(S)-O-alkyl bond. Since the R_{SC} strain was not derived directly from the S_{NAIDM} and is presumably of different genetic origin (*2*), such differences are to be

Table III. Fraction of Penetrated Dose Hydrolyzed by Resistant and Susceptible Houseflies at Various Times after Topical Application of Phosphorothionates

	S_{NAIDM}		R_{SC}			
Dose, μg./g.	M.P.[a] 1.8	S[b] 1.9	M.P. 2.1	S. 2.2	M.P. 63	S. 65
Hours						
0.5	0.17	0.18	0.24	0.25	...	...
1	0.21	0.23	0.33	0.30	...	...
2	0.37	0.35	0.54	0.51	0.42	0.31
4	0.54	0.58	0.62	0.67	...	...

[a] Indicates methyl parathion.
[b] Indicates Sumithion.

Table IV. The Per Cent of the Internal Dose in Various Forms Two Hours after Topical Application of Phosphorothionates to Resistant and Susceptible Houseflies

	Dose, μg./G.					
	1.8	1.9	2.1	2.2	63	65
	S_{NAIDM}		R_{SC}			
	M.P.[a]	S.[b]	M.P.	S.	M.P.	S.
Total P=O containing compounds	21.5	22.9	27.1	25.4	12.5	11.0
Hydrolysis products containing P=S	34.8	29.5	39.2	30.3	36.2	30.0
Hydrolysis products containing P=O	10.5	7.4	23.3	21.3	10.6	9.0

[a] Indicates methyl parathion.
[b] Indicates Sumithion.

expected. The above conclusion is supported by the observation of March (*5*) that the R_{SC} strain was more active than an S-strain in degrading malaoxon in vitro, and these results generally have a similarity to those of Plapp *et al.* (*12*) for a parathion-resistant strain of houseflies. However, the data presented here are not in agreement with the mutant aliesterase theory of van Asperen and Oppenoorth (*11*) since there is a relatively gross difference in the proportion of the phosphate present which is degraded by the two strains. In addition, March (*6*) discovered that the R_{SC} strain has an average aliesterase activity slightly higher than that of the S_{NAIDM} strain.

The Resistance Level of the R-Strain. Raising the dosage level of the R_{SC} from 2 to 65 μg. per gram radically changes the fate of the applied insecticide. These changes are indicated in Table I, which compares metabolism of Sumithion at the two dosages. Almost identical results were obtained with methyl parathion. At the 65 μg. per gram level, saturation of the oxidation system has occurred, as indicated by the much reduced level of oxidation products (Table IV) and the increased levels of methyl parathion and Sumithion internally (Table I). This, plus the saturation in absorption previously noted, favors low toxicity by decreasing the rate of phosphate production. Saturation of the P(S)-O-aryl hydrolysis has occurred also which explains the lower percentage of the penetrated dose hydrolyzed at the higher dosage. However, hydrolysis of P(S)-O-alkyl, P(O)-O-alkyl, and P(O)-O-aryl is unsaturated since the same percentage of the available dose is detoxified by these pathways at both 2.0 and 65 μg. per gram. Thus, over-all detoxication is still as effective with the R-flies at 65 μg. per gram as for the S_{NAIDM} at 2.0 μg. per gram (Table III). These relationships explain the ability of the R_{SC} flies to withstand dosages much higher than the relatively small difference in the level of P=O compounds between the two strains at the 2.0 μg. per gram dosage would suggest. An anomaly exists, however, in the difference in absolute levels of phosphate in the bodies of the S-flies at 2.0 and R-flies at 65 μg. per gram. Thus, after 2 hours the S-strain contained a mean value of 0.0030 μg. per fly of Sumioxon and 0.0021 μg. of methyl paraoxon, while the R-strain at 65 μg. per gram contained 0.0061 and 0.0089 μg. per fly, respectively. The two- to four fold higher phosphate concentration in the R-flies is not large in view of the 35-fold higher dosage of phosphorothionate applied and the greater severity of symptoms of the R-strain over the period of 2 to 4 hours after dosage. However, since comparable mortalities result in both strains, and equal internal concentrations of phosphate should have equal effects in the two strains judging from the response of the brain cholinesterase, the R_{SC} strain may have some further protective advantage such as more favorable distribution of the phosphate or a greater resistance to the train of physiological events leading to death after cholinesterase inhibition. This latter would explain why symptomatology is more severe, but mortality the same, in S_{NAIDM} at 2.0 and R_{SC} at 65 μg. per gram. That death results indirectly from cholinesterase inhibition is shown clearly by the fact that the maximum internal level of phosphate analog occurs in the first 2 to 4 hours after dosage, yet the greatest rate change in symptomatology and the increase in mortality occur considerably later than this.

Comparative Metabolism in Mouse and Fly. The metabolic data shown in Table I indicate the presence of nine metabolites in the housefly and a pattern qualitatively similar to that of the mouse (*3*). Metabolite 1 of unknown nature was found also in mouse urine on occasion, but in very small amounts and too diffuse to form a true peak. Desmethylation is an important detoxifying process in both strains of houseflies, and its relative importance increases slightly at higher dosages when the other detoxication systems become saturated (Table I). Apparently desmethylation is a system which is not easily saturated in either species, although this property is more marked in the mouse under test conditions.

The main difference in the way in which the fly and the mouse degrade relatively similar doses of these insecticides lies in the much greater emphasis on oxidized hydrolysis products in the mouse (60 to 70% of the hydrolysis products contain P=O at 3.0 μg. per gram) than in the housefly (15 to 35% of the hydrolysis products contain P=O at 2.0 μg. per gram). Since mammalian tissue seems to be considerably more active in degrading Sumithion and methyl parathion than insect tissues in vitro (*14*, *16*), the lower proportion of phosphate hydrolysis products in insects probably re-

sults from slower oxidation and deficiency of phosphate-degrading systems in this species rather than from faster detoxication of the thionate. This view is compatible with the higher toxicity of both methyl parathion and Sumithion to the housefly compared with the mouse.

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