# Toxicity and Metabolism of Famphur in Insects and Mice

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Famphur [p-(dimethylsulfamoyl)phenyl dimethyl phosphorothionate] was essentially equitoxic by injection into mice, female and male cockroaches, and milkweed bugs, but was degraded at greatly differing rates in these organisms, decreasing in the above order. The level of the oxygen analog (famoxon) was examined in the whole organisms and roughly paralleled the famphur level. In the insects only, *N*-demethylation occurred. The slow degradation rate in the milkweed bug appeared to be compensated by a relative insensitivity of its cholinesterase to famoxon. The variations in degradation rate among mice and two sexes of cockroach were relatively small (1.9-fold) and were deemed not to require postulation of important differences in innate sensitivity of insects and mammals.

In examining a relatively new organophosphate, famphur [*p*-(dimethylsulfamoyl)phenyl dimethyl phosphorothionate], it was surprising to observe that on direct injection, its toxicity was astonishingly similar for several organisms, yet it was degraded extremely rapidly in some and very slowly in others. This situation questions the validity of attributing observed species varations in toxicity to parallel variations in degradation rate, an attribution frequently made in papers from this laboratory (see Discussion). This study presents the findings on famphur and considers their significance for the interpretation of differential toxicity.

Gatterdam and coworkers (*5*) have reported on the metabolism of famphur metabolites in sheep and a calf. The major metabolites found in urine were products of *O*- and *N*-demethylation and P-O-phenyl cleavage; the phenolic derivatives were excreted primarily as glucuronides. The solvent-extractable radioactivity present in blood was all either famphur or its P(O) analog, herein called famoxon.

## Materials and Methods

Radioactive famphur, ring - labeled with $H^3$ to give a specific activity of 78.5 mc. per mmole; its unlabeled P(O) analog, famoxon; and its unlabeled mono-*N*-demethylated derivative and ring - labeled *N* - dimethyl - *p*-sulfamoylphenol were kindly provided by R. J. Magee and Milon Bullock of American Cyanamid Co. Radioactive famoxon was prepared in 42% yield by $N_2O_4$ oxidation (*2*) of radioactive famphur and purified by chromatographing twice by the first thin-layer chromatography system given below.

White mice were obtained from the Rolfsmeyer Co., Madison, Wis. Only female mice weighing 20 to 30 grams were used. Separate experiments were done with adult male and female American cockroaches [*Periplaneta americana* (L.)] from our own culture. The milkweed bugs [*Oncopeltus fasciatus* (Dall.)] were obtained from our own culture; mixed sexes were used, 2 to 4 weeks old.

Radioactivity was counted in a Tri-Carb liquid scintillation counter, using 1 ml. of sample with 19 ml. of a toluene scintillation solution (*6*) where possible. For mouse homogenates, 1 ml. of homogenate was digested with 1 ml. of Hyamine hydroxide (1*M* in methanol) at room temperature for 2 hours, and counted with 18 ml. of a dioxane scintillation solution (*11*). For other aqueous preparations, 1 ml. was counted with 19 ml. of the dioxane counting solution.

Injections in the mice were made with a 1-ml. hypodermic syringe fitted with a No. 27 needle. Propylene glycol was used as a carrier solvent and a dose of 1 mg. per kg. was injected intraperitoneally. The American cockroach and milkweed bug were injected abdominally with 1 mg. per kg. of famphur in diethylene glycol, using an Agla microsyringe with a No. 27 needle for the cockroaches, and No. 30 for the milkweed bugs. All insects were cold-inactivated prior to injection. Treated insects were held at constant room temperature (25°) for 1, 7, and 24 hours.

After the holding period the mice were homogenized in a Waring Blendor in 0.05*M* tris(hydroxymethyl)aminomethane buffer, pH 8.0. The homogenate was filtered through six layers of cheesecloth, and the filter was rinsed with tris buffer. The final tissue concentration in the buffer was 10%. Two samples of 1 ml. of homogenate were taken and digested as above to determine the total activity in the mouse. When counts were made fresh, large blanks were encountered due to chemiluminescence (*4*): With 10% whole mouse tissue, 620 c.p.m. were found per ml. This decayed steadily, reducing to 80 c.p.m. in 24 hours and remaining constant thereafter. Therefore, samples were stored for 24 hours before counting.

At least 200 ml. of mouse homogenate was extracted with three volumes of toluene. The toluene phase was washed twice with tris buffer and filtered through anhydrous $Na_2SO_4$, and two samples of 1 ml. were taken to determine the toluene-soluble radioactivity.

Insects were blended in tris buffer, pH 8.0, in a Servall Omnimixer, to make a 10% homogenate. A 1-ml. sample was taken for the whole body count. The remainder of the mixture was strained through four layers of cheesecloth, and the precipitate was washed with buffer. A measured volume of the combined filtrate and washings was extracted with three volumes of toluene, which was then separated and washed twice with one volume of buffer, shaking for 3 minutes each time. The toluene phase was dried with anhydrous $Na_2SO_4$ and counted.

**Chromatography.** The toluene phases of the treated mice and insects were evaporated to dryness with a rotary evaporator. In the case of cockroach and milkweed bug, the residue was taken up directly in 2 ml. of chloroform. Because of the large amount of oils and lipids in the mouse residue, it was necessary to dissolve the residue in 150 ml. of hexane, and the radioactivity was extracted with 150 ml. of acetonitrile. The acetonitrile was back-extracted twice with equal portions of hexane. Weighing indicated that about 75% of the liquids went into the hexane phase, while 99 to 100% of the radioactivity went into the acetonitrile phase.

The acetonitrile was evaporated and the residue taken up in 2 ml. of chloroform. Famphur and famoxon were separated with thin-layer chromatography using silica gel G as adsorbent. Glass plates (20 × 20 cm.) were used, with a layer 0.25 mm. thick. Benzene with 10% methanol (v./v.) was used as developing solvent. Iodine vapor was used for detection (*1*). The $R_f$ values were 0.66 for famphur, 0.35 for famoxon, and 0.48 for *N*-desmethylfamphur.

The amount of extract that could be

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applied to the plate was limited by the amount of lipids; too much lipid led to interference, the compounds appearing as a single smear. From the 2 ml. of chloroform fraction, 30 to 50 μl. per spot could be applied in the case of the cockroach and milkweed bug, and in these cases eight spots were applied per plate. Of the mouse extract, 5 μl. could be applied; in this case 17 to 20 spots were applied per plate. Application was made with an Agla micrometer syringe. To each spot was then added 5 μl. of chloroform containing 50 μg. of nonradioactive reference compounds. After development and detection, the layer was scraped off the plate in strips and placed in counting vials, and 10 ml. of toluene scintillation solution was added. Care was taken that the iodine vapor was sublimed from the silica gel before the counting solution was added. Because of the quenching effect of the silica gel, the counting efficiency dropped down to 71% of the gel-free solution for a strip of gel 10 × 1 cm. and to 63% for a strip 20 × 1 cm. All data are corrected for this phenomenon. Little improvement in efficiency was obtained by using Cab-o-Sil gel in the scintillation solution, in order to suspend the silica gel.

**Anticholinesterase Activity.** Measurements were conducted at 38° C. with an incubation time of enzyme with inhibitor of 15 minutes. For housefly head and human red cells, the enzymes and procedures were as described by O'Brien (*13*). For cockroach nerve cord, 50 μl. of a 5% homogenate in phosphate buffer (0.067*M*, pH 7.4) were incubated with 50 μl. of inhibitor in phosphate buffer; inhibition was terminated by adding 100 μl. of 0.3% acetylcholine bromide in tris buffer [0.05*M* tris(hydroxymethyl)aminomethane, pH 8.0], with or without 1*M* NaCl as indicated. The reactions were carried out in 45- × 10-mm. tubes, using 0.25-ml. hypodermic syringes in place of pipets. After 15 minutes at 38° C., the residual acetylcholine was measured by the method of Hestrin (*7*) using microcuvettes with the Beckman DU spectrophotometer. Mouse brain was similarly treated but with 10× greater volumes throughout. For the milkweed bug, the enzyme preparation was 150 ganglia per ml. of phosphate buffer. Incubation with inhibitor was as for the cockroach, but 0.2% acetylcholine in tris buffer with added NaCl (1*M*) was used, and residual acetylcholine was measured after 30 minutes at 38° C.

## Results

**Toxicity and Anticholinesterase Activity.** The $LD_{50}$'s are shown in Table I. At or above the $LD_{50}$, all mice showed convulsions, starting 10 to 20 minutes after injection. If the mice did not die of respiratory failure, the convulsions could last as long as 45 minutes. For the surviving mice recovery was apparent 1 hour after injection.

The cockroaches showed tremors, starting approximately 15 to 50 minutes after injection; tremors in the milkweed bug did not start before 1 hour after injection. With both insects, the time of death was considerably later than for the mice.

**Table I. Toxicity of Famphur and Famoxon by Injection**

| | $LD_{50}$, Mg./Kg. at 24 Hr. | |
|---|---|---|
| | Famphur | Famoxon |
| Mouse | 11.6 | 5.8 |
| Female cockroach | 11.8 | 8.6 |
| Male cockroach | 9.0 | 4.6 |
| Milkweed bug (mixed sexes) | 8.0 | 3.0 |

**Table II. Anticholinesterase Activities of Famphur and Analogs**

| | $pI_{50}$[a] | |
|---|---|---|
| | Human red cell | Housefly head |
| Famphur | 3.7 | 5.7 |
| Famoxon | 5.5 | 7.6 |
| *N*-Desmethyl famphur | 3 | ca. 3 |
| *N*-Bisdesmethyl famphur | 3.2 | 4.6 |
| *N*-Bisdesmethyl famoxon | 4.0 | 5.9 |

[a] $pI_{50}$ is negative logarithm of concentration giving 50% inhibition at 38° C. for 15 minutes. Maximum final concentration obtainable is $10^{-3}M$; hence uncertainty of figure for *N*-desmethyl famphur with housefly.

Usually, in the absence of other activatable groups, phosphorothionates are poor anticholinesterases, and the corresponding phosphates are good anticholinesterases and are the actual toxicants (*15*). Table II shows that famphur is only about one hundredth as potent as famoxon against housefly head or human red cell cholinesterase. The *N*-demethylated analog of famphur was a much poorer anticholinesterase than famphur.

**Degradation.** Differential toxicity between species is often associated with differential degradation; one would therefore anticipate that because the toxicity of famphur was remarkably similar for the four organisms, its rate of degradation would be similar. This possibility was therefore examined. The most probable radioactive hydrolysis product was *N*-dimethyl *p* sulfamoylphenol, referred to below as "the phenol," and the first problem was to separate famphur from the phenol.

Because toluene is a convenient solvent for scintillation counting, its suitability as an extractant was explored. Partition studies showed that after one extraction of insect homogenates, 80% of added famphur and 53% of added phenol were removed in the toluene. After one wash of the toluene with tris buffer, 77% of the added famphur remained in the toluene but the phenol content dropped to 16%. After a second wash, 76% of the famphur was still in the toluene and only 5% of the phenol was present in the toluene. Such a separation was considered adequate. It gave an extraction of 72% of famoxon from a mouse homogenate.

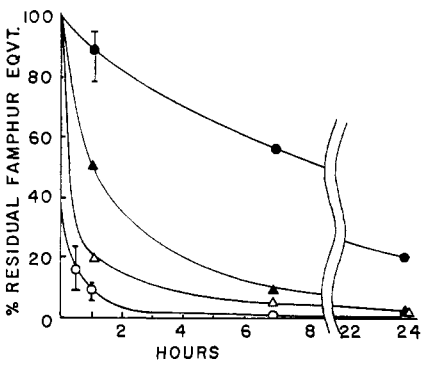


Figure 1. Hydrolysis of famphur

Values are for toluene-extractable radioactivity, expressed as famphur equivalents, and are means of three experiments. Vertical lines indicate range of data; where absent, range is less than size of symbol

● By milkweed bugs
▲ By male cockroaches
△ By female cockroaches
○ By mice

The data on metabolic degradation are shown in Figure 1. The results are expressed as percentages of equivalent amount of $H^3$-famphur added to freshly homogenized animals, then rapidly extracted and treated as usual. There is a remarkable variation in degradation rates, entirely unexpected in view of the close similarity in toxicities.

A possible explanation of this paradox would be if the organisms produced roughly equal amounts of famoxon (which the data given in Table II suggest to be the actual toxicant) in spite of the different degradation rates with respect to famphur. Since the species differences in degradation were most marked at 1 hour after injection, the nature of the toluene-extractable radioactivity at that time was determined by thin-layer chromatography. In addition to the radioactive spots of famphur and famoxon, a third radioactive spot was found in the case of the cockroach and the milkweed bug and to a very minor extent in the mouse (Table III). The metabolite was identified as *N*-desmethyl famphur on the basis of cochromatography with the unlabeled known compound on silica gel G as adsorbent, in two solvent systems. With a 1 to 1 mixture of hexane and ethyl acetate, each had an $R_f$ of 0.35, clearly separated from famphur at 0.44 and famoxon at 0.06. In the standard system (1 to 9 mixture of methanol and benzene), both had an $R_f$ of 0.48, clearly separated from famphur at 0.66 and famoxon at 0.35. It was clearly separated from the *N*-bisdesmethyl derivatives of famphur and and famoxon, whose $R_f$'s in the standard system were 0.29 and 0.09, respectively.

**Table III. Identity of Toluene-Extractable Radioactivity One Hour after Injection of Famphur, 1 Mg./Kg.**[a]

| | Famphur | Famoxon | N-Desmethyl Famphur |
|---|---|---|---|
| Female mouse | 8.11 (8.07–8.15) | 0.22 (0.14–0.29) | 0.01 (0.00–0.03) |
| Female cockroach | 16.84 (16.54–17.15) | 0.45 (0.40–0.51) | 1.20 (1.06–1.34) |
| Milkweed bug (mixed sexes) | 79.43 (75.93–82.93) | 2.15 (1.86–2.44) | 3.12 (2.98–3.27) |

[a] Values, expressed as percentages of original famphur, are means of three experiments for mouse and milkweed bug, two for cockroach; ranges are shown in parentheses.

It is apparent that the only anticholinesterase material present is famoxon. Clearly net famoxon production, like famphur persistence, is very small in the mouse and ten times greater in the milkweed bug, in spite of the great similarity in toxicities.

In a recent study on paraoxon selectivity (*17*), the 73-fold difference in toxicity between cockroach and frog was found to be due not to differential metabolism ("metabolic selectivity"), but to differing sensitivities of cholinesterase ("target selectivity"). The above paradox would be resolved if, in the various organisms, variations in degradation were compensated for by variations in cholinesterase sensitivity. The $pI_{50}$'s (negative logarithms of concentrations for 50% inhibition) for cholinesterase in nerve tissue homogenates were therefore determined. A standard procedure was first used, but when assays on milkweed bug enzyme were attempted, great difficulty in getting sufficient nerve tissue was encountered. However, by including 1*M* NaCl along with the substrate, and using 150 ganglia per ml., sufficient activity was obtained. Although there was little risk that this high NaCl concentrate would modify the results, the experiments with mouse, cockroach, and housefly were performed both with and without 1*M* NaCl. The findings for both these cases are reported below, with the values without NaCl in parentheses. The presence of the NaCl makes only a little difference. The $pI_{50}$'s obtained with an incubation time with inhibitor of 15 minutes for inhibition by famoxon of cholinesterase of nerve tissue were: mouse brain 6.5 (6.3); male cockroach nerve cord 6.0 (6.3); milkweed bug ganglia 5.0. These data may be compared with those in Table II (all performed without NaCl) for human red cell (5.5,) and housefly head (7.6). The housefly head value, when repeated with 1*M* NaCl, was 8.0. These variations in species sensitivity seem to resolve the above paradox very satisfactorily; the milkweed bug degrades famphur very slowly, but this is compensated by the insensitivity of its cholinesterase. The mouse degrades famphur most rapidly, but has the most sensitive cholinesterase. The male cockroach is intermediate in both respects.

## Discussion

In previous studies, the authors attempted to account for species selectivity of toxicants in biochemical and physiological terms. The rationale has been (*11*) that only five steps are involved in the development of a critical lesion: arrival in the body, metabolism, disposal (excretion and storage), and penetration to and attack on the target. Selective toxicity can be due only to variations in one or more of these steps, or to variations in the consequence of a successfully attacked target. By selecting cases of very clear-cut selectivity, we have in the past shown examples of three of the above five steps being involved: differential metabolism for malathion (*9*), diazinon, acethion (*10*), and in the case of mammalian variation, dimethoate (*18*); differential penetration to the target for certain basic organophosphates (*14, 16*); and differential cholinesterase sensitivity for paraoxon (*17*), and perhaps related compounds (*3*), and for dimethoate in insects (*19*).

Only very marked cases of selectivity were studied previously, in anticipation of a good deal of variation between species in their "handling" of any exotic compound: In cases of very marked selectivity, it is hoped that correspondingly marked variations in some determining factor will exist, and that our necessarily crude techniques will detect them. Even so, in the case of dimethoate's selectivity among insects, a polyfactorial situation has been indicated, with large variations in penetration, activation, and cholinesterase sensitivity all playing a role. The question therefore arises of how small a variation in toxicity is worth exploring if the aim is to analyze the factors responsible for selective toxicity. The authors have felt that a two- or threefold difference, for instance, is inadequate; the causes for such selectivity would probably be lost among the numerous variations expected for all of the above five steps.

What approaches are there to the more difficult task of examining variations in the consequences of a successfully attacked target? One would be to prove that in a case of very striking selectivity, between two species, all of the above five steps were identical. A rare case in favor of this argument is the claim by Hopf and Taylor (*8*) that one organophosphate eliminated cholinesterase activity in the ganglion of a poisoned locust, but that there were no harmful consequences. Another approach would be to take advantage of unusual circumstances such as this case of famphur, where its toxicity to several species is coincidentally similar, although one or more of the above five steps vary considerably. From Figure 1, the approximate ratios of degrading activity in the course of 1 hour for mouse: female cockroach: male cockroach: milkweed bug are 7.7:6.7:4.1:1.0. Since the milkweed bug's cholinesterase is 32 times more insensitive to famoxon than the mouse or cockroach, it seems reasonable to suggest that this compensates for the rather slow breakdown of famphur by this insect. Omitting the milkweed bug, one is left with a maximum of a 1.9-fold variation in breakdown rates among the three remaining organisms. This residual variation should probably be treated as negligible, and compatible with the essential equitoxicity of famphur for the three organisms.

## Conclusion

It seems unnecessary on the basis of the evidence herein to postulate any large difference—i.e., more than two- or threefold—in the innate sensitivity of insects and mammals to organophosphates. Instead, the interesting equitoxicity may be roughly accounted for on the basis of varying contributions of degradation rate and cholinesterase sensitivity.


## Acknowledgment

The authors are indebted to C. E. Dannelley for technical assistance.


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*Received for review November 30, 1964. Accepted May 13, 1965. Investigation supported in part by Public Health Service Research Grant No. GM 07804 from the National Institutes of Health.*

**FUNGAL METABOLISM OF HERBICIDES**

# Metabolites of Simazine by *Aspergillus fumigatus*

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**At least two $Cl^{36}$-labeled simazine metabolites were detected in culture solutions of the soil fungus *Aspergillus fumigatus* Fres. One of these was identified as 2-chloro-4-amino-6-ethylamino-*s*-triazine by spectral methods of analysis. The second $Cl^{36}$-labeled metabolite possessed an intact *s*-triazine ring but no alkyl substituents associated with the ring. A new pathway of metabolism, which does not involve the hydroxy analog reported to occur in higher plants, is proposed for degradation of the herbicide, simazine.**

THE isolation and identification of a soil fungus, *Aspergillus fumigatus* Fres., which degrades the herbicide 2 - chloro - 4,6 - bis(ethylamino) - *s*-triazine (simazine) have been reported (*9*). The organism reduced the amount of radioactivity in culture solutions containing $C^{14}$-chain-labeled simazine more rapidly than in culture solutions containing $C^{14}$-ring-labeled simazine. Subsequent investigations (*8*) revealed that $C^{14}O_2$ was evolved only from the chain-labeled simazine. This result suggested that the ring portion of the molecule was still intact.

Several new metabolites, unreported from simazine metabolism by higher plants (*5, 10, 15*), were detected in culture solutions of *A. fumigatus*. The purpose of the research reported in the present paper was to examine some of these metabolties and establish a metabolic pathway in *A. fumigatus*.

### Materials and Methods

**Source of Materials.** The relative mobility of several authentic substituted *s*-triazines was determined on paper and thin-layer chromatograms in order to identify simazine metabolites from *A. fumigatus* solution by cochromatography. The substituted *s*-triazines examined were

A. 2 - Hydroxy - 4,6 - bis(ethylamino)-*s*-triazine (hydroxysimazine)
B. 2 - Chloro - 4 - amino - 6 - ethylamino-*s*-triazine
C. 2 - Chloro - 4 - hydroxy - 6 - ethylamino-*s*-triazine
D. 2,4 - Dihydroxy - 6 - ethylamino-*s*-triazine
E. 2,4 - Dihydroxy - 6 - amino - *s*-triazine (ammelide)
F. 2,4,6 - Trihydroxy - *s* - triazine (cyanuric acid)

To facilitate discussion, these compounds are alphabetically designated. Compounds E and F were labeled with $C^{14}$ in the ring. Compound A was synthesized from simazine $C^{14}$ by a method similar to that of Castelfranco, Foy, and Deutsch (*2*). These suspected metabolites and $Cl^{36}$-labeled simazine (specific activity 0.324 μc. per mg.), $C^{14}$-ring-labeled simazine (specific activity 5.06 μc. per mg.), and $C^{14}$-chain-labeled simazine (methylene-labeled, specific activity 4.97 μc. per mg.) were generously supplied by the Geigy Chemical Corp., Ardsley, N. Y.

Silica gel G for thin-layer chromatography was purchased from the Research Specialties Co., Richmond, Calif. Florisil previously activated at 1200° F. was purchased from Floridan Co., Hancock, W. Va. Nuclear magnetic resonance and mass spectra and some of the infrared spectra were determined by the Stanford Research Institute, Menlo Park, Calif. Gas-liquid chromatography was performed with a flame ionization detector on a RSCo 600 series instrument.

**$C^{14}$-Labeled Simazine Experiments.** Culture conditions for *A. fumigatus* with respect to time, temperature, media composition, and inoculation have been described (*8, 9*). All solutions containing labeled simazine were prepared by first dissolving the compound in 5 ml. of chloroform and then adding the chloroform solution to the nutrient solution. Chloroform was removed by bubbling sterile air through the nutrient solution. The final concentration of simazine in each solution was 5 p.p.m. and contained the following amounts of radioactivity in 100 ml. of solution: $Cl^{36}$ simazine (0.162 μc.), $C^{14}$-ring-labeled simazine (2.53 μc.), and $C^{14}$-chain-labeled simazine (2.48 μc.). Aliquots of the culture solution were removed periodically after inoculation and extracted three times with chloroform, and the chloroform and aqueous phases were assayed for radioactivity. Chloroform extracts were reduced in volume and chromatographed on Whatman No. 1 paper and thin-layer plates (250 microns thick). Metabolites that appeared in the chloroform phase of $Cl^{36}$ simazine solutions were compared to metabolites recovered from ring- and chain-labeled simazine $C^{14}$ solutions.

**Large-Scale Incubation Experiments.** Mass culture experiments were conducted to obtain sufficient quantities of a