Cholinesterase Variation as a Factor in Organophosphate Selectivity in Insects

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The suggestion that the selective toxicity of diisopropyl *p*-nitrophenyl phosphorothionate to bees as compared with houseflies is due to selective inhibition of the target enzyme, cholinesterase, has been investigated. The compound was found to be over 100 times more toxic to houseflies than to bees, and its phosphate derivative—presumed to be the actual toxicant—was 40 times more potent in vitro against housefly than against bee cholinesterase. However, four dialkyl *p*-nitrophenyl phosphates were made, and no over-all correlation was found between their selective toxicity for bees as compared with flies and selective inhibition in vitro of cholinesterases from these insects.

Many organophosphates are more toxic to insects than to mammals. This selective toxicity has been attributed to "target selectivity," i.e., differences in the susceptibilities of the target enzyme cholinesterase, in a few cases only. One case is that of DDVP or dichlorvos (dimethyl dichlorovinyl phosphate), which van Asperen and Dekhuijzen (*1*) showed was selectively toxic to insects and selectively inhibitory to insect cholinesterase. An important case is that of diisopropyl parathion (diisopropyl *p*-nitrophenyl phosphorothionate), which was stated by Metcalf and March in 1949 (*11*) to be a 1000 times better in vitro inhibitor of fly head than of bee head cholinesterase, and to be over 250 times more toxic to flies than bees. By contrast, parathion was only 2.2 times better as an inhibitor for fly brain, and only 4 times more toxic to flies. It was natural to conclude that the selective toxicity of diisopropyl parathion was due to differences in the cholinesterases. However, since 1949, it has been realized that carefully purified phosphorothionates of the parathion class are ineffective cholinesterase inhibitors in vitro, and any pronounced inhibition that such compounds may seem to show is usually a sign of the presence of impurities, usually isomers. The toxicity of the phosphorothionates is due to their conversion in vivo to their P(O) analogs.

The present authors have therefore undertaken to repeat and extend the study using the P(O) analogs of diisopropyl parathion and related compounds.

Methods and Materials

The dialkyl *p*-nitrophenyl phosphates (referred to below as dialkyl paraoxons) were synthesized by preparing the dialkyl phosphorochloridates (*10*) and reacting these with sodium *p*-nitrophenate (*4*). The compounds were purified by partition chromatography on a celite-isoöctane-methanol column (*2*). Purity of the compounds was determined by infrared spectroscopy using the Perkin-Elmer Infracord and by ascending paper chromatography on Whatman No. 1 paper utilizing Mitchell's nonaqueous solvent system *a* (*13*). The compounds were detected by spraying the chromatograms with two different chromogenic agents: phosphorus was detected by the method of Hanes and Isherwood (*6*) and the free *p*-nitrophenate ion by spraying with a 10% sodium carbonate solution. In all cases, the phosphorus and the *p*-nitrophenate ion had identical R_f's. The R_f's were paraoxon 0.047, diisopropyl paraoxon 0.37, di-*n*-propyl paraoxon 0.31, and dibutyl paraoxon 0.33.

The analyses of the new compounds (by Ilse Beetz, Nurnberg, Germany) were, in per cent: diisopropyl paraoxon, C calcd. 47.3, found 46.7; H calcd. 5.93, found 6.13. Di-*n*-propyl paraoxon, C calcd. 47.3, found 47.6; H calcd. 5.93, found 6.14. Di-*n*-butyl paraoxon, C calcd. 48.8, found 49.8; H calcd. 6.63, found 6.73.

Diisopropyl parathion was prepared by chlorinating diisopropyl phosphorodithioic acid (*5*), and reacting the chloridate in acetone with an equimolar amount of the sodium salt of *p*-nitrophenol. The salt was filtered off, the acetone removed under vacuum, and the residue dissolved in chloroform. The chloroform was washed with 5% $NaHCO_3$, followed by water. The chloroform was dried over sodium sulfate, removed under vacuum, and the oil induced to crystallize from ethanol-water mixture. The compound was obtained in 50% yield and had a m.p. of 56°–56.5° C. When Mitchell's dimethylformamide-isoöctane system (*13*) was used, the R_f of the compound was 0.49. A trace of *p*-nitrophenol was found at the origin. Calcd. C 44.9%, found 45.7%; calcd. H 5.62%, found 5.57%.

The fly heads and bee heads were collected according to the method of Moorefield (*14*). One gram of fly head was homogenized per 40 ml. of 0.1*M* phosphate buffer pH 8.0 in the cold, and the large particles were removed b filtering through glass wool, after whic the solution was centrifuged at 6100 × G for 10 minutes. Bee heads wer homogenized at 1 gram per 5 ml. c 0.1*M* phosphate buffer pH 8.0, and th pH was adjusted to 8.0 with 0.1*M* NaOH The procedure was then similar to tha described for the fly heads. The washe human erythrocyte ghosts were prepare as described previously (*15*).

The anticholinesterase activity of th dialkyl paraoxons was measured by th method of Hestrin (*7*). The final cor centrations were $8 \times 10^{-3}M$ acety choline and 0.05*M* phosphate buffer pl 8.0. The three enzyme sources wer diluted to give a hydrolysis of 2 μmol of acetylcholine in 30 minutes at 38° C This gave a linear rate of hydrolysi The cholinesterase was incubated fc 15 minutes with the inhibitor before th acetylcholine was added. Thirt minutes later, readings were taken an compared with that of a standard with out inhibitor. After log molar concentra tion was plotted against per cent inhib tion, the 50% inhibition values (th I_{50}) were determined by inspection. A I_{50} values were replicated at least thre times.

The mice used for toxicity studies wer female white mice (Rolfsmeyer Farm Madison, Wis.). The compounds wer dissolved in propylene glycol and in jected intraperitoneally. The housefli were a Wilson susceptible strain; 3- or 4-day-old adult females were in mobilized with CO_2 and treated topical on the dorsal thorax with 1 μl. each acetone in which the toxicants wer dissolved. The topical toxicity for th worker bees was determined in a simila manner, but chilling was used for in mobilization. Observations on the bee flies, and mice were made at 24 hours.

Attempts were made to purify th cholinesterase from bee heads. Th enzyme was extracted as described abov except that the supernatant liquid w

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centrifuged at 17,000 × G for 30 minutes at 2° C. The supernatant (phosphate extract) was then fractionated with ammonium sulfate at pH 7.4. The precipitate obtained between 25 to 65% saturation [calculated by the Kunitz formula (*8*)] was dissolved in 0.1*M* phosphate buffer pH 8.0. Because other enzymes might have been present, heat denaturation with protection by acetylcholine was used (*3*). The following day, the solution was fractionated again with ammonium sulfate at pH 6.4. The precipitate obtained in the 25 to 65% saturation fractionation was dissolved in 0.1*M* phosphate buffer pH 8.0. Protein was determined by spectrophotometric measurements at 260 and 280 mμ (*16*).

Results

Table I shows that the anticholinesterase activities were similar for the fly and erythrocyte cholinesterase with regard to all four phosphates assayed with the exception of dibutyl paraoxon, to which erythrocytes were 8 times more sensitive. The bee cholinesterase was 40 times less sensitive than the other enzymes to diisopropyl paraoxon, and 20 times less sensitive to propyl paraoxon; but it was equally sensitive to the other inhibitors.

Attempts were made to purify the bee head cholinesterase since evidence has been reported (*12*) of an aromatic esterase in bee head which can hydrolyze diethyl paraoxon, and this could possibly account for the lower I_{50} value for the bee with the diisopropyl paraoxon. With the initial ammonium sulfate fractionation, the I_{50} value decreased 2.5-fold (Table II). Heat denaturation with acetylcholine protection did not appreciably change the I_{50} value. With this procedure, a great deal of denaturation occurred, with a decrease in specific activity.

Finally, diisopropyl parathion itself was prepared and its toxicity determined. The LD_{50} values differ a little from those of Metcalf and March (*11*), but it remains true that the compound is highly selectively toxic to flies as compared to bees; this selectivity is more pronounced than in the case of diisopropyl paraoxon. Also diisopropyl parathion was substantially less toxic to mice than was diisopropyl paraoxon.

Discussion

The original claim (*11*) was that diisopropyl parathion was 250 times more toxic to flies than to bees, and this was attributed to its being 1000 times more potent against fly than against bee cholinesterase. The present finding is that it is 100 times more toxic to flies than to bees (Table III). Diisopropyl paraoxon is only 22 times more toxic to flies than to bees, and only 40 times better against fly than against bee cholinesterase. Furthermore, di-*n*-propyl paraoxon has greater selective toxicity than the diisopropyl (150-fold more toxic to flies than to bees) yet less selective anticholinesterase activity (20 times more effective against fly than against bee enzyme). The other two analogs show reverse selectivity, being actually more toxic to bees than to flies (10-fold for diethyl paraoxon, 1.7-fold for dibutyl paraoxon) in spite of being less potent against bee than against fly cholinesterase (1.6-fold and 2.0-fold, respectively).

Consequently, the hypothesis that the selective toxicity of diisopropyl parathion and diisopropyl paraoxon is due to selective anticholinesterase activity in vivo (i.e., target selectivity) must be considered as not proved, even though the data for the diisopropyl compound alone are compatible with the hypothesis.

In all cases, human and housefly cholinesterase preparations had almost identical sensitivities; bee cholinesterase preparation was, in two out of the four cases, markedly less sensitive. The cholinesterases of bee and fly might be different, or alternatively the crude bee preparation might contain enzymes that bind or degrade the inhibitor. Purification studies (Table II) indicated that the latter was true in part, for sensitivity to diisopropyl paraoxon increased slightly (2.5-fold) on ammonium sulfate fractionation. However, further purification had little effect, and therefore it seems that the bee cholinesterase is indeed different from the fly and human enzymes. Kunkee and Zweig (*9*) reached the same conclusion on the basis of substrate specificity of purified cholinesterase.

Table I. Effect of Alkyl Groups upon Anticholinesterase Activity

Inhibitor	pI_{50}[a] Fly head	Bee head	Erythrocytes
Diethyl paraoxon	7.7	7.5	7.5
Diisopropyl paraoxon	6.3	4.7	6.3
Di-*n*-propyl paraoxon	6.5	5.2	6.5
Dibutyl paraoxon	6.7	6.4	7.6

[a] pI_{50} = Negative logarithm of I_{50}, the molar concentration for 50% inhibition in 15 minutes.

Table II. Activity of Diisopropyl Paraoxon on Various Purified Fractions of Bee Cholinesterase

Fraction	pI_{50}	μmole Acetylcholine Hydrolyzed per 30 Minutes per Mg. Protein
Phosphate extract	4.7	0.18
First ammonium sulfate fraction	5.1	0.29
After heat denaturation and ammonium sulfate fractionation at pH 6.4	5.2	0.25

Table III. Toxicity of Paraoxon Analogs to Insects and Mice

Toxicant	LD_{50} (Mg. per Kg.) Housefly	Bee	Mouse
Diethyl paraoxon	1.6	0.16	1.5
Diisopropyl paraoxon	18	400	33
Di-*n*-propyl paraoxon	1.2	180	38
Dibutyl paraoxon	39	23	13
Diisopropyl parathion	5	522	>100

Acknowledgment

The authors are indebted to C. E. Dannelley and E. Schoenfeld for technical assistance, and to R. A. Morse for kindly donating bees. This investigation was supported by research grant GM 07804 from the National Institutes of Health, the U. S. Public Health Service.

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Received for review October 25, 1963. Accepted January 16, 1964.