DATA EVALUATION RECORD

(1) CHEMICAL: Trichlorfon

(2) TYPE OF FORMULATION: Unspecified

(3) CITATION: Hassan, A., Zayed, S.M.A.D., and Abdel-Hamid, F.M. 1965. Metabolism of organophosphorus insecticides: II. Metabolism of O,O-dimethyl-2,2,2-trichloro-1-hydroxy-ethyl phosphonate (Dipterex) in mammalian nervous tissue and kinetics involved in its reaction with acetylcholine esterase. Can. J. Biochem. 43:1263-1269

(4) REVIEWED BY:

Susan Rieth
Staff Scientist
Clement Associates
Washington, D.C.
(202) 333-7990

Signature: _____

Date: _____

Douglas Wilkening
Staff Scientist
Clement Associates
Washington, D.C.
(202) 333-7990

Signature: _____

Date: _____

(32B-0030)

(5) APPROVED BY:

Signature: _____

Date: _____

(6) TOPIC: This study has information pertinent to discipline toxicology, topic metabolism. It relates to none of the Proposed Guidelines data requirements.

(7) CONCLUSION: The metabolism of trichlorfon in an in vitro system by rat brain homogenates was investigated. Four metabolites, three acidic and one nonacidic, were demonstrated. The acidic metabolites were identified as monodemethylated trichlorfon (O-methyl-2,2,2-trichloro-1-hydroxyethylphosphonate), monomethylphosphate, and the third one was suggested to be 2,2,2-trichloro-1-hydroxyethyl phosphonic acid. The nonacidic compound was not identified. Preliminary evidence indicated that the reaction of trichlorfon and acetylcholine esterase was a first-order reaction. Addition of acetylcholine to the in vitro system before the addition of trichlorfon largely prevented inhibition of acetycholine esterase.

CORE CLASSIFICATION: Not applicable

(8) MATERIALS AND METHODS:

Test Substance: ^{32}P-trichlorfon of specific activity 0.6 mCi/g, and nonlabeled trichlorfon were used. Both compounds were 98% pure. Source was not specified.

Organism: Rats were used in the metabolism studies. No further information on the test animals was provided.

Experimental Procedure: Studies of both the metabolism of trichlorfon by rat brain homogenate and reaction kinetics

of trichlorfon and acetycholine esterase were carried out.

Metabolism Studies: Rat brain homogenate (20%) was incubated with ^{32}P-trichlorfon at 37° for 5 hours. In order to analyze the resulting metabolic products of trichlorfon, the reaction mixture was extracted with chloroform; the chloroform extracts were washed with water, and dried over anhydrous magnesium sulfate.

The aqueous layer (residue from the chloroform extraction and water washings) was applied on a 15x1-cm column of anion exchanges. Elution from the column was carried out following the procedure of Zayed and Hassan (1965. Can. J. Biochem. 43: pages not given). Acidic fractions were analyzed for radioactivity (by Geiger counter, with no allowance for self-absorption), and for total phosphorus content according to Casida et al. (1952. J. Econ. Entomol. 45:568). Radioactivity measurements were corrected for decay and background, with data expressed in counts per minute. Identification of the trichloroethyl grouping was by a modified Fujiwara test (1954. J. Agric. Food Chem. 2:1281).

The substances extracted with chloroform and those eluted with water or HCl from the anion exchanger were analyzed by ascending chromotography, using two solvent systems for each and developing for 16 hours. System A contained n-butanol-pyridine-water (12:8:6), and system

B contained 2-propanol-NH_4OH-water (75:24:1). The chromatograms were assayed radiometrically (by Geiger counter, as above), and the spots were made visible by spraying the chromatograms with Bandurski reagent (1951. J. Biol. Chem. 193:405), followed by irradiation for 10 minutes under a germicidal lamp.

Kinetic Studies: Rat brain homogenate was used as the source of acetylcholine esterase, and the reaction was assayed according to the method of Hestrin (1949. J. Biol. Chem. 180:249). The reaction mixture was composed of 0.2 M phosphate buffer, pH 7.2; 1.0 M magesium chloride solution; 1.0 M sodium chloride solution; 10% rat brain homogenate in isotonic KCl; trichlorfon in distilled water (final concentration 10^{-8}-10^{-3} M); and 48 mM acetylcholine chloride in 0.001 M sodium acetate, to be added after the preincubation period (final concentration 8 mM unless otherwise stated). The period of assay of the enzyme activity was 30 minutes.

Statistical Methods: No statistical analyses of the data were performed.

(9) REPORTED RESULTS:

Metabolism Studies: The results of analysis of the various fractions by paper chromatography are summarized in the following table.

Table 1

R_f Values of Radioactive Products Obtained from Metabolism of Trichlorfon by Rat Brain Homogenates

Material	R_f	
	System A	System B
Chloroform extract	0.49, 0.95	0.45, 0.80
Water washings	0, 0.48, 0.95	0, 0.45, 0.80
Acid eluate (pH 2.2-1.7)	0.07, 0.37	0, 0.48
Acid eluate (pH 1.6)	0.05	0.13
Monomethyl phosphate	0.05	0.13
Dimethyl phosphate	0.17	0.62
O-Methyl-2,2,2-trichloro-1-hydroxyethyl phosphonate	0.37	0.48
Trichlorfon	0.95	0.80

Chromatographic analysis of the chloroform layer showed the presence of trichlorfon and another substance with R_f 0.49 and 0.45 in systems A and B, respectively.

The aqueous layer was applied on an anion exchanger for separation of bands before chromatographic analysis. After washing the column with water, the three fractions with highest radioactivity were chromatographed in systems A and B, and R_f values of 0, 0.48, and 0.95 were obtained in System A. (See Table 1). The column was then eluted

with acid, and three peaks could be differentiated. Two bands were inadequately separated by eluting at pH 2.2-1.7, and a third peak of smaller magnitude was completely eluted at pH 1.6. The ratio of these three bands was 27:62:11, respectively, as shown by radiometry and total phosphorus determination.

The percentage of the metabolites characterized above by column and paper chromatographic analysis, in terms of percentage of total water-soluble metabolites recovered and as percentage of total radioactive material used was reported and appears in Table 2.

TABLE 2

Metabolite	Total Radioactivity (cpm)	% Total Water-soluble Metabolites	% Total Radioactive Material Used
Water-soluble metabolites	134,400		7.1
Substance with R_f 0 (in System A)	24,150	7	1.3
Substance with R_f 0.48 (in System A)	110,250	33	5.8
Acidic metabolites	198,000		10.4
Substance with R_f 0 (in System B)	52,800	16	2.8
Substance with R_f 0.13 (in System B)	23,070	7	1.2
Substance with R_f 0.48 (in System B)	122,000	37	6.4

The investigators identified or further characterized the metabolites of trichlorfon. One nonacetic water-soluble substance with an R_f of 0 was identified as probably protein bound by a positive biuret test. Two of the acidic compounds were identified as monodemethylated trichlorfon (O-methyl-2,2,2-trichloro-1-hydroxyethyl phosphonate) and monomethyl-phosphate. The third acidic metabolite was believed to be 2,2,2-trichloro-1-hydroxyethyl phosphonic acid.

Kinetic Studies: Based on a plot of the "reciprocal of inhibitor (trichlorfon) concentration" and "time in minutes for 50% inhibition (of acetycholine esterase)," the investigators found the type of reaction of trichlorfon and acetylcholine esterase to be bimolecular, with a velocity constant (k) for the reaction calculated to be 7.3×10^{-3} $mole^{-1}$ $minute^{-1}$.

When trichlorfon (10^{-6} M) was preincubated with acetylcholine esterase for 30, 60, or 120 minutes before addition of the substrate (acetycholine), the percent inhibition of acetycholine esterase was reported to be 20, 34, and 65%, respectively. When trichlorfon was added 30 seconds after addition of the substrate (4 mM acetylcholine), trichlorfon caused only slight enzyme inhibition after 60 minutes (5%) and 120 minutes (8%).

In their discussion, the investigators noted that the progressive nature of inhibition suggests an irrever-

sible inactivation of the enzyme. This irreversible inhibition by trichlorfon suggests that acetycholine esterase is not concerned with the detoxification of the insecticide to any significant extent.

In vitro studies with the substrate, acetycholine, indicated a competitive type of effect, suggesting that the organophosphorus compound binds itself to the substrate-binding group of the active center.

(10) DISCUSSION: Both the metabolism and kinetic studies reported in this paper contain some serious problems, which limit the usefulness of the results.

The results of the metabolism study are difficult to evaluate quantitatively because the authors provided no indication of the sensitivity of their radioactivity measurements. Although radioactivity determination using a Geiger counter with an end-window Tracerlab G-M tube was an acceptible method at the time of this study, more sensitive procedures are standardly used today (i.e., scintillation counting). Radiometric assay of the paper chromatograms was stated to be made with a "similar device," with no allowance made for self-absorption. The investigators also failed to account for the majority of the radioactivity used. Only the radioactivity measured in fractions from the aqueous layer was reported. Thus, it is impossible to state whether the remaining activity was associated

with unchanged trichlorfon or with other metabolic products that were not isolated. For this reason, the values reported for the contributions of metabolites to the total radioactivity used cannot be considered valid.

The R_f values of radioactive products from the metabolism of trichlorfon provide evidence for identification of two metabolic products, but their justification for suggesting 2,2,2-trichloro-1-hydroxyethyl phosphonic acid as a third acidic metabolite is unclear, since no R_f value for this compound was reported. It must be assumed that the standards, whose R_f values appear in Table 1, were determined in a similar manner to that of the experimental fractions, since no mention of their determination appeared in the Materials and Methods section. The protocol used and justification for choosing these particular standard compounds for chromotographic analysis should have been more explicit.

Problems associated with the kinetic studies are more serious. Because the investigators used whole brain homogenate as their source of acetylcholine esterase, their results are complicated by a highly impure incubation system. They were not simply measuring the reaction rates of trichlorfon and acetycholine esterase, as stated, but they were also dealing with an equilibrium between nonspecific binding of trichlofon and specific binding to the enzyme. The more lipid soluble a compound is, the greater the

role of nonspecific binding in the brain homogenate, and the longer it will take to achieve 50% inhibition of the enzyme.

Other problems with this paper arise because of ambiguity in the discussion of the results. It is unclear, for instance, whether the authors were characterizing the reaction of trichlorfon and acetylcholine esterase as a unimolecular or as a bimolecular reaction.

(11) TECHNICAL REVIEW TIME: 8.0 hours