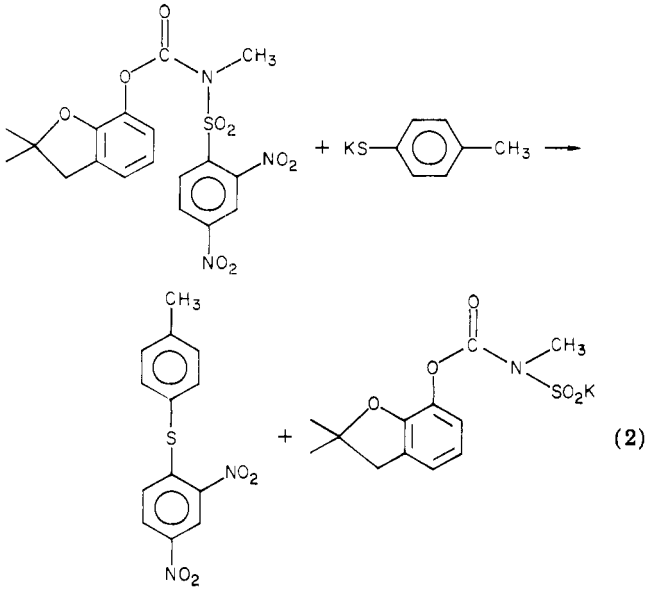
for 70 h at room temperature. However, when 14 was allowed to stand in a dichloromethane–ethanol mixture containing potassium *p*-thiocresylate, the major product identified was *p*-tolyl 2,4-dinitrophenyl sulfide and an unidentified water-soluble salt containing the carbofuran moiety. The reaction probably occurs as in eq 2. This



(2)

is in contrast to the reaction between the highly insecticidal *N*-toluenesulfenyl carbofuran and an arylthiolate anion where the major product isolated was carbofuran (Chiu et al., 1975).

When 14 was allowed to stand in the presence of excess triethylamine in dichloromethane solvent, the products which were isolated and identified were carbofuran phenol and 2,3-dihydro-2,2-dimethyl-7-benzofuranyl 2,4-dinitrophenyl ether. Thus, in neither case, i.e., in the presence of thiolate anion or triethylamine, was the toxic methylcarbamate carbofuran generated from 14. These findings are consistent with the poor insecticidal activity of the sulfonyl derivatives listed in the table.

Compounds 8 and 14 were examined for their toxicity to the white mouse. Neither compound produced any symptoms of intoxication in mice treated at 400 mg/kg. As a reference point, the LD_{50} value of carbofuran to mice is 2 mg/kg (Black et al., 1973a). Therefore, substituted benzenesulfonyl derivatives of toxic methylcarbamates are toxic neither to insects nor mammals.

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Yoshiro Kinoshita[1]
T. Roy Fukuto[2]

[1]Department of Agricultural Chemistry
Faculty of Agriculture
Ehime University
Matsuyama, 790, Japan
[2]Division of Toxicology and Physiology
Department of Entomology
University of California
Riverside, California 92521

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Anticholinesterase Effects of Carbamate Insecticide Thiofanox and Its Metabolites in Rats

The anticholinesterase activity of the carbamate insecticide thiofanox (P), 3,3-dimethyl-1-(methylthio)-2-butanone *O*-[(methylamino)carbonyl]oxime, and its three metabolites 3,3-dimethyl-1-(methylsulfinyl)-2-butanone *O*-[(methylamino)carbonyl]oxime (P_1), 3,3-dimethyl-1-(methylsulfonyl)-2-butanone *O*-[(methylamino)carbonyl]oxime (P_2), and 3,3-dimethyl-1-(methylsulfonyl)-2-butanone *O*-[(hydroxymethylamino)carbonyl]oxime (P_2OH) was investigated in the rat. The relative order of potency was $P_2 > P_1 > P > P_2OH$ for both the plasma ChE and RBC ChE. Time-dependent studies with P showed that maximum observed inhibition of cholinesterase (ChE) activity of plasma and red blood cells (RBC) of the rat was attained at 30 min after the peroral administration of 1 mg/kg P in aqueous solution. The maximum depression of brain ChE activity was observed 1–2 h postdose. In all three tissues, complete recovery of ChE activity was attained at 24 h after administration.

Thiofanox (P), 3,3,dimethyl-1-(methylthio)-2-butanone *O*-[(methylamino)carbonyl]oxime, is a potent systemic and contact insecticide developed by Diamond Shamrock Corp. Metabolic studies in soils (Duane, 1974) and plants (Whitten and Bull, 1974) showed rapid oxidation of P to its metabolities 3,3-dimethyl-1-(methylsulfinyl)-2-butanone *O*-[(methylamino)carbonyl]oxime (P_1) and 3,3-dimethyl-1-(methylsulfonyl)-2-butanone *O*-[(methylamino)-

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Table I. Effect of Thiofanox on Erythrocyte, Plasma, and Brain Cholinesterase Activities in Rats[a]

time after dose, h[b]	RBC			plasma			brain		
	no. of rats	ChE act., %[c]	statistical significance	no. of rats	ChE act., %	statistical significance	no. of rats	ChE act., %	statistical significance
0.25	5	22 ± 4	*d*	5	49 ± 11	*d*	5	76 ± 8	*e*
0.5	5	21 ± 4	*d*	5	42 ± 10	*d*	5	72 ± 5	*d*
1	10	23 ± 3	*f*	5	71 ± 18	*d*	5	68 ± 4	*d*
2	5	28 ± 9	*e*	5	55 ± 8	*d*	5	70 ± 5	*d*
4	10	48 ± 19	NS[g]	5	91 ± 34	*e*	5	89 ± 11	NS
6	10	70 ± 18	*e*	5	75 ± 11	*f*	5	85 ± 4	*e*
8	5	87 ± 20	*f*	5	83 ± 9	NS	5	81 ± 6	*e*
24	5	113 ± 9	NS	5	99 ± 21	NS	5	103 ± 7	NS

[a] Calculation based on 17 untreated female rats. Normal ChE activities in 1 mL of RBC, plasma, and brain supernatant were 7.25, 5.32, and 7.51 μmol of SH/5-min incubation, respectively. [b] Female rats were dosed perorally at 1 mg of thiofanox/kg of body weight. [c] Mean and standard deviation of cholinesterase activity as percentage of control values. [d] $P < 0.001$ as determined by Cochran's *t* test (Steel and Torrie, 1960). [e] $P < 0.01$ as determined by Cochran's *t* test. [f] $P < 0.05$ as determined by Cochran's *t* test. [g] NS means not significant.

carbonyl]oxime (P_2). Metabolic study of P in rats (Chin et al., 1980) showed that P_2 was hydroxylated further to form 3,3-dimethyl-1-(methylsulfonyl)-2-butanone *O*-[(hydroxymethylamino)carbonyl]oxime (P_2OH).

Cholinesterase (ChE) assays are gaining acceptance as a biological indicator of exposure to ChE-inhibiting insecticides (Wills, 1972). This study reports the extent of ChE inhibition by thiofanox and its metabolities P_1, P_2, and P_2OH using the rat as an animal model.

EXPERIMENTAL SECTION

Materials. P, P_1, P_2, and P_2OH were furnished by the T. R. Evans Research Center of Diamond Shamrock Corp., Painesville, OH. These chemicals were stored in the refrigerator. Reduced glutathione standard was purchased from Nutritional Biochemicals Corp., Cleveland, OH. Acetylthiocholine iodide (ATChI) was procured from Sigma Chemical Co., St. Louis, MO. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI.

A commercial synthetic rat diet (Knaak et al., 1965) containing 10% alphacel, 4% vegetable oil, 27% vitamin-free casein, 40% sucrose, 4% salt mixture, and vitamins was obtained from Nutritional Biochemicals Corp., Cleveland, OH. The diet was emulsified in 0.25% agar solution at a 3:5 ratio.

Methods. The instrumentation employed in this ChE assay was an Auto-Analyzer (Technicon Instrument Corp.). The Auto-Analyzer included a Sampler II, a proportioning pump, a dialyzer with type C membrane, a 37 °C heating bath (Model I), a colorimeter, and a recorder.

The flow diagram for this system is the same as that given by Levine et al. (1966), with the exception that a 0.02-in. internal diameter (i.d.) tubing was used for sampling tubes instead of a 0.03-in. i.d. tubing. Also, 0.9% NaCl replaced water in the wash reservoir. Samples were analyzed by using a timing cam marked "60/hour".

Reagents used were identical with those of Levine et al. (1966), except that an ATChI concentration for plasma, RBC, and brain supernatant was 7.6 mM (219 mg/100 mL of distilled water). Upon dilution in the manifold, the substrate concentration for these samples was 1.0 mM. Rats were killed by decapitation and the blood samples were collected in a 10-mL crucible with 2 drops of heparin. Heparinized blood samples were transferred to a 2-mL tube and centrifuged for 15 min at 600*g*. Plasma was removed by means of a pipet which left a trace amount of plasma remaining on the surface of the packed RBC layer. This trace amount of plasma was removed with a wick made of absorbent paper. The packed RBC were used without dilution. The brain was homogenized for 1 min with a Teflon–Pyrex homogenizer in ice-cold 0.25 M sucrose, and the final concentration of the homogenate was 10% w/v. Homogenates were centrifuged at 600*g* and the supernatant (Chin and Sullivan, 1968) was used for ChE assay.

The ChE activity is expressed in sulfhydryl (SH) units equivalent to micromoles of SH groups liberated in 5 min from 1 mL of either plasma, packed RBC, or brain supernatant.

Rats for this study were of the Harlan–Wistar strain from our own breeding colony, and they had been allowed free access to food and water throughout the study. The sex of rats for time-dependent and dose-dependent studies were female (150–200 g) and male (130–190 g), respectively.

Time-Dependent Study. The time-dependent studies on plasma, RBC, and brain ChE depression were conducted following a peroral dose of P at 1 mg/kg of body weight. The dosing solution of P was prepared fresh by dissolving 30 mg of P in 0.15 mL of ethanol, and the solution was then diluted to 30 mL with water. A single peroral dose of 1 mg/kg of body weight of this solution was administered by stomach intubation using a syringe fitted with a straight feeding needle to rats. Rats were killed by decapitation, and blood and brain were sampled for the ChE determination at 15, 30, and 60 min and at 2, 4, 6, 8, and 24 h. Five to ten treated rats and two to three control rats were used for each time interval.

Dose-Dependent Study. Relative anticholinesterase properties of P, P_1, P_2, and P_2OH were studied by comparing IC_{20} values (defined as amount of test chemical required to inhibit 20% of the control ChE activity). The test chemicals were dissolved in water or in the emulsified diet previously referred to (Knaak et al., 1965), and each solution was administered to male rats according to the following procedures.

(*A*) *IC_{20} Determinations of P and Its Metabolites Prepared in Water.* Plasma and RBC ChE activity was determined 30 min after a single oral dose of each test chemical by using water as a vehicle at the following concentrations. P, 0.25, 0.5, 0.75, and 1 mg 32 mL^{-1} (kg of body weight)$^{-1}$; P_1, 0.125, 0.25, and 0.5 mg 32 mL^{-1} (kg of body weight)$^{-1}$; P_2, 0.05, 0.1, and 0.15 mg 32 mL^{-1} (kg of body weight)$^{-1}$; P_2OH, 1.0, 2.5, and 4.0 mg 32 mL^{-1} (kg of body weight)$^{-1}$. Three rats were administered the test chemical orally at each dose and a total of 11 control rats were given the water at 32 mL/kg of body weight.

(*B*) *IC_{20} Determinations of P and Its Metabolites Prepared in Emulsified Diet.* Addition of the test chemicals was made to the emulsion diet to prepare various concentrations of P and its metabolites as with the water solution. Thirty-two milliliters of the emulsions was dosed

Table II. Effect of P_2, P_1, P, and P_2OH on RBC and Plasma Cholinesterase (Mean ± Standard Deviation)[a]

	ChE act., %																	
	P_2				P_1				P				P_2OH					
	RBC ChE		plasma ChE		RBC ChE		plasma ChE		RBC ChE		plasma ChE		RBC ChE		plasma ChE			
dosage, mg/kg	W[b]	ED[c]	W	ED	W	ED	W	ED	W	ED	W	ED	W	ED	W	ED		
0.05	62 ± 9	67 ± 6	88 ± 23	93 ± 6														
0.1	42 ± 4	57 ± 3	36 ± 0	76 ± 19														
0.125					44 ± 4	72 ± 11	60 ± 27	90 ± 5										
0.15	25 ± 4	50 ± 2	44 ± 17	71 ± 40														
0.25					25 ± 2	53 ± 7	67 ± 22	70 ± 31	44 ± 11	56 ± 9	75 ± 19	60 ± 23						
0.5					21 ± 2	43 ± 2	43 ± 22	35 ± 13	25 ± 5	53 ± 7	35 ± 9	50 ± 31						
0.75									23 ± 6	45 ± 5	44 ± 31	51 ± 33						
1.0									18 ± 2	27 ± 2	51 ± 9	39 ± 18	64 ± 3	74 ± 19	61 ± 27	82 ± 16		
2.5													53 ± 4	74 ± 5	49 ± 11	69 ± 34		
4.0													54 ± 14	60 ± 8	51 ± 21	64 ± 40		

[a] ChE activities are expressed as percent of 11 untreated male rats. Three male rats were used for each dose and they were killed 30 min after dosing. [b] W = chemical in water. [c] ED = chemical in emulsified diet.

Table III. IC_{20} Values of P_2, P_1, P, and P_2OH in Rats[a]

	IC_{20} values, mg/kg					
	RBC ChE			plasma ChE		
	dosing vehicle		ratio of	dosing vehicle		ratio of
chemical	ED[b]	W[c]	ED/W	ED	W	ED/W
P_2	0.026	0.018	1.4	0.09	0.03	3.0
P_1	0.06	0.02	3.0	0.14	0.06	2.3
P	0.12	0.04	3.0	0.12	0.18	0.7
P_2OH	0.8	0.4	2.0	1.2	0.35	3.4

[a] IC_{20} values are defined as milligrams of test chemical required per kilogram of body weight to inhibit 20% of the control ChE values. [b] ED = emulsified diet. [c] W = water.

per kg of body weight. Control rats were given the emulsion diets without added chemical at 32 mL/kg of body weight.

RESULTS AND DISCUSSION

The time-dependent studies on inhibition and recovery of ChE activities in RBC, plasma, and brain of female rats were conducted by peroral dosage of P at 1 mg/kg of body weight. Inhibition of ChE activity in RBC and plasma occurred very rapidly as compared to that of brain ChE with a maximum observed inhibition occurring 30 min after administration of P as shown in Table I. Inhibition of brain ChE occurred within 15 min, and the maximum inhibition was found between 0.5 and 2 h postdose. Recovery of ChE inhibition in all tissues tested was found after 2 h and was determined to be 80–90% of control values at 8 h. Complete recovery of ChE activities was attained at 24 h after administration. Because brain ChE had the least sensitivity to the inhibition by P, brain ChE was not measured in the subsequent dose-dependent studies.

Table II shows the effect of P, P_2, P_1, and P_2OH on male rat RBC and plasma ChE for the two different vehicles, water or emulsified diet. The relative inhibitory activity of each test chemical on ChE activity is easily recognized from this table. However, a direct quantitative comparison of anticholinesterase propeties of each chemical was difficult from the results in Table II. Therefore, to provide a convenient means of comparing the ChE inhibitory activity of P and P metabolites, we constructed plots using the percentage inhibition vs. the chemical concentration data shown in Table II. From these plots, the concentration of the chemical which inhibited 20% of the corresponding control ChE values (IC_{20}) was extrapolated.

The resulting extrapolated IC_{20} values of P, P_2, P_1, and P_2OH in rat RBC and plasma ChE for two different vehicles are shown in Table III. As shown in Table III, when water was used as the dosing vehicle the inhibitory activity of P_2 toward RBC ChE was 1-, 2-, and 20-fold more potent than that of P_1, P, and P_2OH, respectively. The inhibitory activity of P_2 toward plasma ChE was 2-, 6-, and 12-fold more potent than that of P_1, P, and P_2OH, respectively. Table III also shows that in either RBC or plasma, the anticholinesterase activity of P or P metabolites given in water was up to threefold more than that of these chemicals in the emulsified synthetic diet. Most insecticides inhibit pseudo (plasma) ChE more than true (RBC) ChE. For example, parathion (Serrone et al., 1969) and aldicarb [2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)-oxime] (Chin, 1968) inhibit plasma ChE activity more than RBC ChE activity. However, in this study, P and its metabolites inhibited RBC ChE activity more than plasma ChE activity. The most accessible sites being the blood, the effect of blood ChE can be used as an indictor of

exposure (Wills, 1972) to P and its metabolites.

There are discrepancies in in vivo ChE inhibition values among different laboratories for measuring blood ChE in carbamate poisoning. These discrepancies are due to sample dilution, substrate concentration, long reaction time, and washing of packed RBC (Witter, 1963; Winteringham and Fowler, 1966; Disney, 1966; Chin and Sullivan, 1968). All these factors influence the dissociation rate of the carbamate–enzyme complex and therefore result in underestimation of the degree of carbamate inhibition. For minimization of these factors, particular attention was given to the Auto-Analyze methodology used for this study. This methodology requires no predilution of plasma or RBC, low substrate concentration (1.0 mM), short reaction time (5 min), and no washing of packed RBC.

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Byong Han Chin*[1]
Marilyn J. Tallant[2]
Warren C. Duane[1]
Lloyd J. Sullivan[2]

[1]Diamond Shamrock Corporation
Department of Safety Assessment
T. R. Evans Research Center
Painesville, Ohio 44077
[2]Carnegie-Mellon Institute of Research
Carnegie-Mellon University
Pittsburgh, Pennsylvania 15213

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Isolation and Redefinition of the Toxic Agent from Cocklebur (*Xanthium strumarium*)

The highly toxic agent responsible for the poisonous properties of cocklebur, a common weed, has been isolated and identified as carboxyatractyloside. The toxin was identified by spectroscopic and chemical comparisons with authentic carboxyatractyloside.

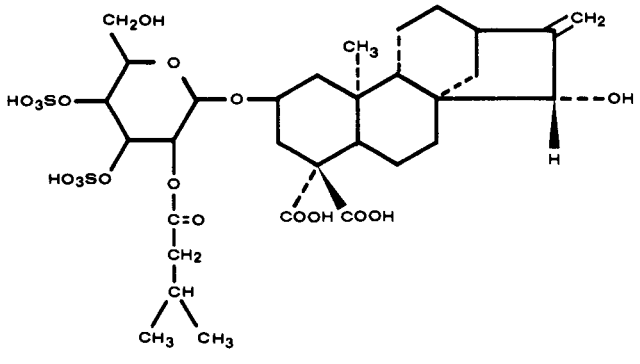
Cocklebur plants, *Xanthium spp.*, are widely distributed in the United States and are found in nearly all parts of the world. The plants have become especially noxious in South Africa and Australia. The poisonous properties of cocklebur are found only in seed and very young seedlings that still contain the cotyledon (cotyledon stage) (Marsh and Roe, 1924). Very young cocklebur seedlings, growing in pastures or fields, are particularly dangerous for swine in southern Georgia especially in the early spring. It was thought that the poisonous effects of cocklebur were largely due to the mechanical action of the burs (Hansen, 1920). More recently, Kuzel and Miller (1950) reported that the toxicity of the various species of *Xanthium* was due to hydroquinone present in the kernel and bur. We reexamined the etiologic agent in cocklebur as a result of an outbreak of cocklebur poisoning in South Georgia in the early spring of 1978. We were neither able to detect hydroquinone in cocklebur nor able to reproduce with it the characteristic lesions associated with cocklebur toxicity.

EXPERIMENTAL SECTION

Plant Material. Burs of *Xanthium spp.* were collected in fields in southern Georgia. Some burs were ground in a Wiley mill; others were germinated, and the very young seedlings (two-leaf stage) and older seedlings (four-leaf stage) were collected, lyophilized, and ground in a Wiley mill.

Biological Assay. The toxicity of crude extracts and other preparations was monitored with 30–40-lb shoats. The pigs were maintained on a complete 16% protein commercial swine ration and treated with an antihelmintic (Tramisol, Shell Chemical Co.). Initial crude preparations were given orally; purified toxin was administrated intravenously in sterile distilled water.

Isolation Procedure. Semiquantitative isolation of the potassium salt of carboxyatractyloside was performed by



first grinding either 100 g of whole burs or 33 g of freeze-dried seedlings in 1 L of acetone at high speed for 5 min. The slurry was filtered over two layers of cheesecloth, and the residue was returned to the blender and