Detection of Cholinesterase-Inhibiting Pesticides Following Separation on Thin-Layer Chromatograms

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A new thin-layer chromatographic procedure is described for separating and detecting 17 cholinesterase-inhibiting pesticides on aluminum oxide and silica gel. The detection of the anticholinesterase activity is based on the hydrolysis of indophenyl acetate by bee brain cholinesterase. The inhibiting pesticides show up on the chromatoplate as white spots on a blue background. Limits of detectable cholinesterase inhibitors were at the subnanogram level and the time required for detection is about 40 minutes. Semi- to quantitative determination of the inhibitors can be achieved by use of a Xerox-copied chromatogram.

The recent utilization of thin-layer chromatography (TLC) for the separation and detection of cholinesterase-inhibiting pesticides and metabolites has been enumerated by several investigators (Ackerman, 1966; Bunyan, 1964; Crosby *et al.*, 1965; El-Refai and Hopkins, 1965; Mendoza *et al.*, 1968; Menn and McBain, 1966; Ortloff and Franz, 1965). The most sensitive detection technique for a number of the oxidized thiophosphates has been reported by El-Refai and Hopkins (1965). This procedure employs cellulose layers on chromatoplates which are washed and dried prior to coating with polar and apolar stationary phases. The chromatoplates are then developed with immiscible mobile phase solvents. Because of the time required in preparing and handling these chromatoplates, such a system is not as advantageous as a simple TLC procedure using conventional adsorbants such as aluminum oxide and silica gel.

The present study for separating and detecting cholinesterase-inhibiting pesticides utilizes the more simple techniques in preparing TLC plates. The system is based on the study by Archer and Zweig (1959), who showed that bee brain cholinesterase is extremely sensitive for detecting the carbamate insecticide, Sevin, as well as many organophosphorus pesticides and their oxygen analogs or oxons. The employment of a combination of TLC with this cholinesterase detection method permits a very rapid and sensitive procedure for the separation and detection of 17 cholinesterase-inhibiting pesticides. Time required for detecting inhibitors following development of chromatoplates is less than 40 minutes and sensitivities are reported in the subnanogram levels for oxidized and unoxidized thiophosphates as well as other inhibiting pesticides.

Quantitation can be achieved by use of a Xerox-copied chromatogram by cutting out the representative inhibited spot from the Xerox copy, weighing on an analytical balance, and plotting on semilogarithmic paper.

Hollingworth *et al.* (1967) reported that the anticholinesterase activity of a particular pesticide and its analogs depends a great deal on the cholinesterase source. Therefore, the newly developed method using bee brei as the cholinesterase source was compared to a previously reported method (Crosby *et al.*, 1965) using human plasma. Such a difference in activity permits a qualitative determination for unknown pesticides in a biological system.

MATERIALS AND METHODS

Apparatus. Thin-layer chromatograms were prepared on 20 × 20 × 5 cm. crystal glass plates supported by a stainless steel mounting board and using a thin-layer applicator (Desaga-Brinkman) to give a 250-micron thick layer.

A Brinkman application box (Model DB) was used to spot all pesticides in a nitrogen environment. The spotting of pesticides without a nitrogen environment often resulted in artifacts which could not be distinguished from a

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pesticide response as well as the occurrence of occasional streaking due to possible hydrolysis of unstable pesticides.

Reagents. Aluminum oxide G and silica gel H (E. Merck, Darmstadt, Brinkman Instruments, Westbury, N. Y.).

Two solvent systems were used to give optimum separation. Acetone-toluene-hexane (1:1:5) was used to separate the less polar and acetone–toluene–hexane–ethyl acetate (2:1:2:1) was used to separate the more polar compounds.

Human plasma indicator solution was prepared by mixing 1 part (by volume) of human plasma with 3 parts of 0.1% cresol red indicator dissolved in 0.01*N* aqueous sodium hydroxide solution.

Honey bee brain cholinesterase was prepared by modifying the procedure reported by Archer and Zweig (1959). Honey bees (*Apis mellifera*) were stored in a −30° C. deep freeze until ready for use. The heads were separated from the bodies by shaking with dry ice (Moorefield, 1957) through two U. S. standard sieves, 6- and 8-meshes to the inch, respectively. Two thousand frozen heads were then ground in a prechilled mortar and pestle with 30 ml. of salt solution (8.12 grams of manganous chloride and 8.77 grams of sodium chloride per liter) and 5 to 10 grams of washed sand. The contents were then transferred to a 50-ml. centrifuge tube with two 5-ml. aliquots of the salt solution and centrifuged for 10 minutes at 10,000 r.p.m. in a Model SS-1 Sorvall superspeed centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The supernatant liquid was then decanted into a graduated cylinder and again the heads were mixed and centrifuged with 30 ml. of the salt solution. The extraction procedure was repeated once more and the combined supernatant solutions adjusted to 100 ml. with the salt solution so that each milliliter was equivalent to the extractives of 20 bee heads. The solution containing the bee brain cholinesterase was then transferred to cellophane dialysis tubing and placed in the Oxford multiple dialyzer (Oxford Lab., San Mateo, Calif.) containing distilled water. The entire apparatus was stored at 4° C. Every day for one week the water was replaced with fresh distilled water. At the completion of the dialysis period the enzyme solution was transferred in 10-ml. units to glass vials and stored in a −30° C. freezer until ready for use. The bee brain cholinesterase was stable for over a year. Working solution of bee brain cholinesterase was prepared by mixing one part of the stock bee brain brei with three parts of buffer solution (20 grams of H_3BO_3 per liter and 275 ml. of 1*N* NaOH, pH is adjusted if necessary to 9.7 ± 0.2 with NaOH).

A 0.2*M* solution of acetylcholine bromide was prepared for the human plasma cholinesterase. The substrate used for the bee brei cholinesterase was prepared according to the method of Archer and Zweig (1959). The reagents used in preparing the indophenyl acetate substrate must be used immediately as they decompose once exposed to atmospheric conditions. A working solution of indophenyl acetate was made with dimethyl sulfoxide at a concentration of 1 mg. per ml.

Standard Solutions. Stock solutions were prepared by weighing and mixing 100 mg. each of Betasan, Bidrin, diazinon, Dylox, ethion, Furadan, Gardona, Guthion, Ethyl Guthion, methyl parathion, parathion, paraoxon, Phosdrin, Sevin, Temik, Trithion, and Methyl Trithion with 100 ml. of redistilled chloroform. Working solutions of each standard were prepared by diluting each stock solution with chloroform to a concentration where 1 to 10 μl. of the pesticide could be applied to the plate. Each pesticide with its chemical name and manufacturer is listed in Table I.

PROCEDURE

Aluminum oxide plates are prepared according to the method of Kovacs (1963), followed by the thin-strip thin-layer chromatography technique of Beckman and Winterlin (1966). Each strip is 15 mm. wide, permitting 10 strips to a plate. The plates are then placed in the application box with a flow of nitrogen of 150 ml. per minute and spotted with the respective pesticide using a 10-μl. syringe. The chromatoplates are marked with a line 30 mm. from the top and placed in the presaturated developing chamber which was lined with Whatman No. 1 chromatography paper. As soon as the solvent reaches the

Table I. Organophosphorous Pesticides Separated by Thin-Layer Chromatography

Common or Trade Name	Chemical Name	Manufacturer
Betasan	*N*-(beta-*O*,*O*-diisopropyl dithiophosphoryl ethyl) benzene sulfonamide	Stauffer
Bidrin	3-hydroxy-*N*,*N*-dimethyl-cis-crotonamide dimethyl phosphate	Shell
Diazinon	*O*,*O*-diethyl *O*-(2-isopropyl-6-methyl-4-primidinyl)phosphorothioate	Geigy
Dylox	*O*,*O*-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphonate	Chemagro
Ethion	*O*,*O*,*O*′,*O*′-tetraethyl *S*,*S*′ methylene phosphorodithioate	FMC
Furadan	2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate	FMC
Gardona	2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate	Shell
Guthion	*O*,*O*-dimethyl *S*-4-oxo-1,2,3-benzotriazin-3(4*H*)-ylmethylphosphorodithioate	Chemagro
Ethyl Guthion	*O*,*O*-diethyl S-4-oxo-1,2,3-benzotriazin-3(4*H*)-ylmethylphosphorodithioate	Chemagro
Parathion	*O*,*O*-diethyl *O*-*p*-nitrophenyl phosphorothioate	American Cyanamid
Methyl parathion	*O*,*O*-dimethyl-*O*-*p*-nitrophenyl phosphorothioate	American Potash
Paraoxon	*O*,*O*-diethyl *O*-*p*-nitrophenyl phosphate	American Cyanamid
Phosdrin	3-hydroxy-alpha-methylcrotonate, dimethyl phosphate	Shell
Sevin	1-naphthyl *N*-methylcarbamate	Union Carbide
Temik	2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl) oxime	Union Carbide
Trithion	*O*,*O*-diethyl *S*-(*p*-chlorophenylthio) methyl phosphorodithioate	Stauffer
Methyl Trithion	*O*,*O*-dimethyl *S*-(*p*-chlorophenylthio) methyl phosphorodithioate	Stauffer

premarked line, the plates are removed and dried with a heat gun for 5 minutes. For the thiophosphate compounds the sulfur analogs are converted to their corresponding oxons by exposure to bromine vapor. The dried chromatoplates are inverted and placed with the adsorbant facing down with the plate resting on four No. 8 cork stoppers in a glass-covered borosilicate glass baking dish containing a 2.5% bromine in carbon tetrachloride solution. The chromatoplates are exposed for 30 seconds to the bromine solution at room temperature, 24 ± 1° C., and removed from the baking dish. The remaining bromine vapor must be completely removed by means of air for several minutes. The dried plates are placed in the baking dish in a horizontal position and sprayed with the enzyme-buffer solution until the plates appear glossy due to saturation. Care must be exercised not to spray beyond this point. The baking dish is covered and the plates are allowed to incubate for 20 minutes at room temperature. At the completion of the incubation period the plate is sprayed with the indophenyl acetate solution until the surface is slightly yellow. After 10 minutes, clear and well-defined white spots appear on a very intense blue background.

An alternate procedure for applying the substrate to the plate may be utilized by saturating an 8 × 8 inch sheet of Whatman No. 1 chromatography paper, freshly dipped in the indophenyl acetate solution, and placed on top of the developed chromatoplate. After the development time is completed, the white spots are marked with a water soluble pencil and the paper removed for permanent filing.

A detection system using human plasma as the cholinesterase source (Crosby *et al.*, 1965) was also utilized. The system involves spraying the developed plates with human plasma indicator solution. After 20 minutes an 8 × 8 inch sheet of Whatman No. 1 chromatography paper is dipped in a 0.2*M* aqueous solution of acetylcholine bromide and placed on the developed chromatoplate. Within 15 minutes the cholinesterase inhibited spots were visible as red spots on a yellow background.

RESULTS AND DISCUSSION

The detection limits of 17 cholinesterase-inhibiting pesticides and their oxidized analogs using two different enzyme systems are shown in Table II. The detection system using bee brei as the cholinesterase source was generally more sensitive to the anticholinesterase pesticides; however, difficulties usually encountered with thin-layer chromatography prevent significant conclusions when comparing the two systems, if the data falls within a twofold range. Of the 17 pesticides studied only Temik proved to be considerably more sensitive to the human plasma detection system.

Bee brei cholinesterase was generally very sensitive to the oxidized analogs of the thiophosphates as well as the carbamate Sevin with the range in detectable residue running from 1 to 500 picograms. The increase in sensitivity of the oxidized thiophosphates following the oxidation with 2.5% bromine increased the detectable amount of pesticide from 10 to 500 times. Time of exposure, temperature of the plate surface, concentration of bromine vapor, and distance of plate from bromine solution are all very critical factors when considering activity of a particular pesticide due to oxidation. Figure 1, *A* and *B*, best illustrates the effect of the bromine concentration on the oxidation of Trithion and Methyl Trithion to their respective oxygen analog or analogs. A series of thin-layer chromatograms, all of which were developed and handled simultaneously, were exposed to individual concentrations of bromine vapor ranging from 0.05 to 2.5%. All other factors were held constant, including the time required to remove all traces of bromine vapor.

Table II. Detectable Limits in Nanograms and R_f Values of Cholinesterase-Inhibiting Pesticides on Aluminum Oxide Thin-Layer Chromatograms

Compound	Human Plasma Cholinesterase		Bee Brain Cholinesterase		R_f	
	Unoxidized	Oxidized	Unoxidized	Oxidized	I[a]	II[b]
Bidrin	2		1		0.00	0.15
Dylox	2		1		0.00	0.03
Furadan	1		0.05		0.21	0.70
Gardona	10		0.05		0.32	0.76
Paraoxon	1		0.01		0.11	0.73
Phosdrin	1000		5		0.00, 0.03	0.57, 0.68
Sevin	25		0.001		0.28	0.67
Temik	0.05		10		0.31	0.65
Betasan	500	50	5	0.5	0.22	0.77
Diazinon	50	0.1	25	0.05	0.80	0.92
Ethion	20	1	2	0.2	0.77	0.90
Ethyl Guthion	20	0.1	10	0.05	0.43	0.87
Guthion	2000	20	25	0.1	0.35	0.83
Parathion	100	1	2	0.02	0.65	0.90
Methyl parathion	1000	20	50	0.05	0.51	0.86
Trithion	100	1	2	0.05	0.83	0.94
Methyl Trithion	5000	100	5	0.1	0.75	0.87

[a] Solvent system v. v. acetone-toluene-hexane, 1:1:5.
[b] Solvent system v. v. acetone–toluene–hexane–ethyl acetate, 2:1:2:1.

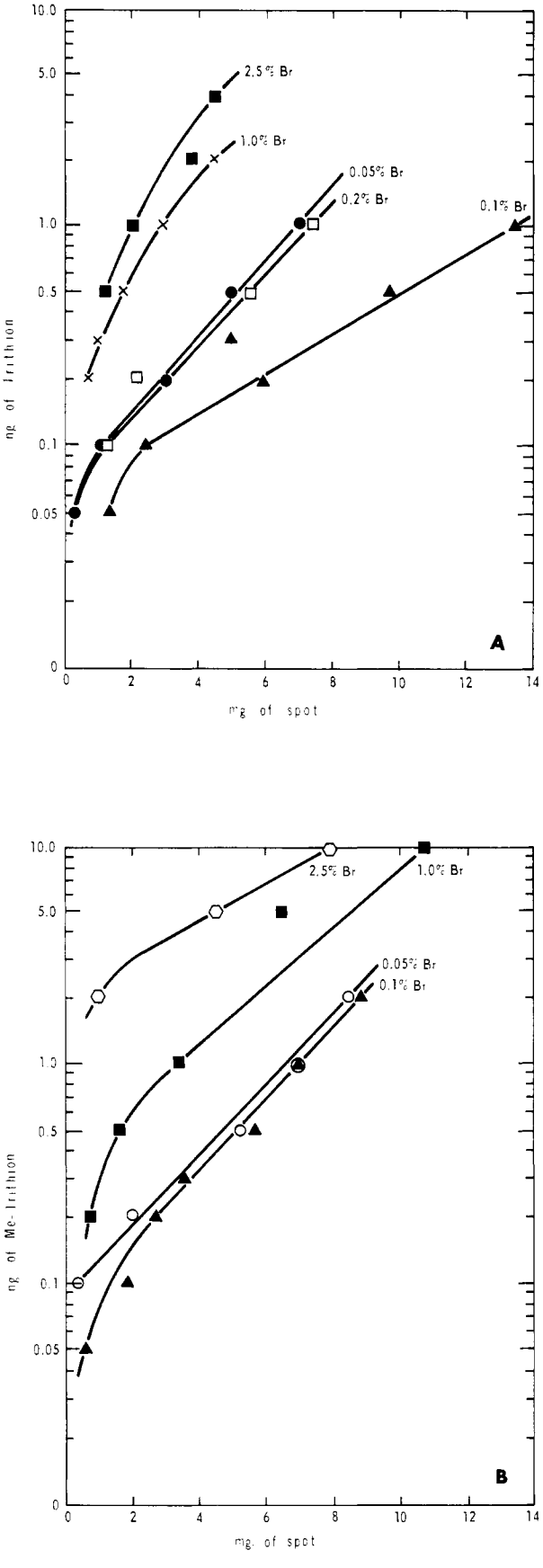

Figure 1. A. Effect of bromine concentration on the oxidation of Trithion B. Effect of bromine concentration on the oxidation of Methyl Trithion

The plates were then treated with the bee brei enzyme-buffer solution and indophenyl acetate substrate. At exactly 10 minutes following the application of the substrate, the area of the particular developed spots on the thin-layer chromatograms were marked with a sharp pointed object. The plates were then placed in a Xerox copying machine and a copy of the chromatogram indicating the marked spots was produced. The area of the marked spots was then cut out and weighed on an analytical balance.

When Trithion was exposed to concentrations of 0.05, 0.10, 0.20, 1.0, and 2.5% active bromine in carbon tetrachloride for 30 seconds the optimum response with maximum sensitivity was the 0.1% concentration. Response with 2.5% bromine was considerably lower. The optimum concentration of bromine vapor with Methyl Trithion was 0.05 or 0.10% and the difference between the optimum concentration and 2.5% was not as great as with Trithion. From this and other studies it was concluded that each thiophosphate pesticide has its own reaction rate; however, studies conducted by other investigators (Bunyan, 1964; Irudayasamy and Natarajan, 1965) as well as by our own laboratory have found a bromine concentration ranging from 2.5 to 10% for 20 to 30 seconds to be adequate for activation of most thiophosphate pesticides.

Utilization of the Xerox copy technique permitted semi- to quantitative information. The weight of the extracted paper representing the inhibited area on the chromatogram was plotted along the logarithmic scale (abscissa) on semi-logarithmic paper and the quantity of the pesticide expressed in nanograms was plotted along the linear scale (ordinate). Quantitation was generally limited to a 100-fold range or less for most pesticides studied. Quantitation depends considerably on standard conditions; therefore plates must be the same thickness and consistency and must be handled simultaneously. Environmental conditions such as moisture on the adsorbant, temperature, humidity, and timing are all extremely pertinent variables and must be considered when quantitation is desired.

The detection system using bee brei cholinesterase works very well on silica gel H (E. Merck, Darmstadt, West Germany, distributed by Brinkman Instruments, Inc.). The only appreciable difference from the aluminum oxide chromatoplates is in preparing the enzyme buffer solution. The buffer solution should be diluted 50% with distilled water before mixing with the bee brei solution. Lower limits of detectability were nearly equivalent using either adsorbant with the exception of Bidrin and Phosdrin. The detectable limit using these two pesticides was 10 and 500 times greater, respectively, using silica gel. The detection system using human plasma cholinesterase does not work on silica gel H and the peculiarities are presently being investigated.

The R_f values of the 17 cholinesterase-inhibiting pesticides using the two solvent systems are shown in Table II. The acetone-toluene-hexane solvent system separated most of the pesticides with the exception of the more polar compounds. Incorporating ethyl acetate into the solvent system improved separation of the more polar compounds but unsatisfactory separation resulted for the less polar compounds. However, by utilizing both solvent systems

a satisfactory separation of nearly all the pesticides was made possible with the exception of diazinon-Trithion and Methyl Trithion–ethion. One of the main problems characteristic of TLC is the change in R_f value due to overloading of a particular pesticide. Experience in this laboratory has shown that R_f values are relatively dependable providing other factors are constant; however, when a chemical has been spotted at a concentration exceeding its limit of detection by 100-fold, the R_f values may change significantly.

Since anticholinesterase activity of organophosphate and carbamate pesticides is dependent upon the cholinesterase source, the utilization of two or more enzyme sources permits a qualitative, sensitive, and rapid means of identification of an unknown pesticide. This is best illustrated using the carbamate, Temik, which is relatively nonsensitive to bee brei cholinesterase but highly sensitive to human plasma cholinesterase. The opposite is true with the carbamate, Sevin. It would be relatively simple to distinguish between these two pesticides if present in biological materials using this approach to their separation and detection.

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