Organophosphorus Insecticides

Paul A. Giang

Organophosphorus insecticides inhibit the cholinesterase of animals and insects. This accounts to a large extent both for their effectiveness in the control of harmful insects in agriculture and for their toxicity to warm-blooded animals. Many of these insecticides, and also some of their metabolic products, are such powerful inhibitors of cholinesterase that very sensitive enzymatic methods for their micro-determination have been developed utilizing this inhibitory property.

The most widely used method[1] in which the inhibition of an enzyme is used for the analysis of insecticides is described below. It is based on *Michel*'s[2] simplified method for the estimation of cholinesterase activity in human red blood cells and plasma. The cholinesterase used in this method must be carefully standardized to avoid any interference from other related esterases[3].

Principle

The sample is extracted with an organic solvent, the solvent is evaporated off and the residue is incubated for 30 min. with a known excess of cholinesterase (ChE) in a buffered solution. At the end of this "inhibition" period, a known excess of acetylcholine (ACh) is added to the reaction mixture. After 60 min. the acetic acid produced by the hydrolysis of acetylcholine is measured by the change in pH (with a pH-meter).

(1) Cholinesterase + inhibitor $\longrightarrow$ inhibited cholinesterase + free cholinesterase

(2) Acetylcholine $\xrightarrow{\text{free ChE}}$ acetic acid + choline + unhydrolysed acetylcholine

The less cholinesterase is inhibited in reaction (1), the more acetic acid will be formed in reaction (2) during the specified time of hydrolysis.

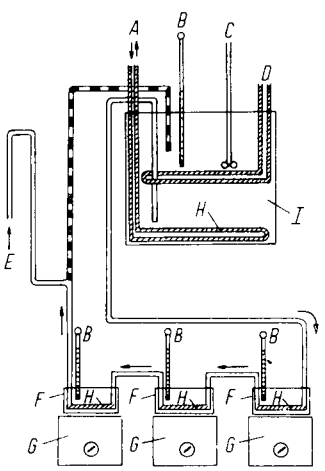

Fig. 1. Diagram of the constant temperature apparatus: A = cooling water; B = thermometer; C = stirrer; D = heating element with thermostat; E = tube for compressed air; F = small, insulated constant temperature baths (crystallization dishes); G = magnetic stirrers; H = copper coil; I = large constant temperature water bath.

[1] *P. A. Giang* and *S. A. Hall*, Analytic. Chem. *23*, 1830 [1951].
[2] *H. O. Michel*, J. Lab. clin. Med. *34*, 1564 [1949].
[3] *R. L. Metcalf:* Organic Insecticides. Interscience Publishers, New York 1955, p. 273.

Apparatus

The following are required: pH-meter; constant temperature bath; crystallization dishes (9 cm. diameter) as small constant temperature baths; magnetic stirrer (magnetic flea: iron wire sealed in glass; 1 cm. long, 2 mm. outside diameter); 10 ml. beakers, each enclosed by a lead-solder wire coil to hold the beaker in place in the water bath; syringes (2 and 25 ml.); stopwatch.

Fig. 1 shows the arrangement of the water baths, thermostat and magnetic stirrers.

Reagents

1. 5,5-Diethylbarbituric acid (veronal, barbital)
2. Sodium hydroxide, 1 N
3. Potassium chloride, KCl
4. Potassium dihydrogen phosphate, KH_2PO_4
5. Hydrochloric acid, 0.1 N
6. Sodium chloride, NaCl
7. Dichloromethane, redistilled
8. Acetylcholine chloride
9. Cholinesterase

The substrate and buffer concentrations described here are suitable for the purified bovine and human serum cholinesterase which can be obtained from Winthrop-Stearns, Inc., New York 18, N. Y., U.S.A. By suitable alteration of the substrate and buffer concentrations the following preparations can be used: "Lyovac" plasma (Sharpe & Dohme, Philadelphia, Pa., U.S.A.); normal horse serum (Pittman-Moore Co., Division of Allied Laboratories, Inc., Indianapolis, Ind., U.S.A.); human plasma (old blood from blood banks or hospitals)[4-6]. For other reagents, see "Experimental material".

Preparation of Solutions

I. Buffer (pH 8.1):
Add 6.647 g. 5,5-diethylbarbituric acid (veronal) to 800 ml. distilled water with stirring and add 36 ml. 1 N NaOH to dissolve the veronal. Add 89.9 g. KCl and 1.089 g. KH_2PO_4 to this solution. Adjust the pH of the solution to 8.1 with *ca.* 7 ml. 0.1 N HCl, and dilute to 1000 ml. with distilled water (volumetric flask). Add 2 drops of toluene.

II. Sodium chloride (0.9% w/v):
Dissolve 0.9 g. NaCl in 100 ml. distilled water, sterilize the solution (heat to boiling) and pour into sterile bottles.

III. Sodium chloride (10% w/v):
Dissolve 10 g. NaCl in 100 ml. distilled water.

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4) *J. W. Cook*, J. Assoc. off. agric. Chemists *37*, 561 [1954].
5) *J. Epstein, M. Demek* and *V. C. Wolff*, Analytic. Chem. *29*, 1050 [1957].
6) *J. Hensel, A. E. Hewitt, U. M. Sheets* and *R. C. Scott:* Procedure for the Determination of Residues of Cholinesterase-Inhibiting Insecticides and their Metabolites in Plants. American Cyanamid Co., Stamford, Conn., 1956.

IV. Sodium chloride (saturated):

Heat 40 g. NaCl in 100 ml. distilled water, allow to cool to room temperature and decant.

V. Acetylcholine standard solution (0.22 M):

Dissolve 4 g. acetylcholine chloride in 100 ml. distilled water and add 2 drops of toluene

VI. Cholinesterase

a) Stock solution (*ca.* 1000 units*)/ml.):

Introduce into a vial containing 22000 units of dried cholinesterase 22 ml. of ice-cold, sterile NaCl solution (II) with a 25 ml. sterile syringe (puncture the stopper of the vial) and mix by shaking. Immediately place the solution in a refrigerator (0 to 5° C). The activity of this stock solution must be determined (see "Determination of the activity of the cholinesterase stock solution").

b) Dilute solution (*ca.* 20 units*/ml.):

Add to 1.0 ml. of the stock solution the volume of 0.9% NaCl solution calculated from the results of the cholinesterase activity assay (p. 621). The dilute solution should reduce the pH of the control mixture C2 by 2 units under the conditions given under "Assay". Add 2 drops ot toluene to the dilute solution and store at 0 to 5° C. For other solutions, see "Oxidation of insecticides".

Stability of the solutions

Store the acetylcholine and buffer solutions in a refrigerator. The stock cholinesterase solution keeps for 6 months at 0 to 5° C without any appreciable loss of activity.

Procedure

Experimental material

Extract the sample with dichloromethane, concentrate the extract to about 50 ml. and pour into a separating funnel and wash with 10 ml. portions of 10% NaCl solution (III) until the washings are neutral to litmus. Filter the neutral extract through a plug of dry cotton wool into a 100 ml. volumetric flask. Dilute to approximately 100 ml. with dichloromethane (filtered through the same cotton wool plug). Place the volumetric flask in an ice bath for 20 min. and dilute to 100 ml. with ice-cold dichloromethane.

Determine the approximate insecticide content of the solution as follows: pipette into a 10 ml. beaker containing a magnetic flea

2 ml. ice-cold sample

and evaporate to dryness in a current of air. Add

3 ml. cholinesterase solution (VI b)
3 ml. buffer (solution I)

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*) A unit is the amount of enzyme which liberates 1 µl. CO_2/min. in the assay according to *R. Ammon*, Pflügers Arch. ges. Physiol. Menschen, Tiere *233*, 486 [1933]. For a description of the assay method, see p. 774.

and stir for 30 min. in a water bath at 25°C. Mix in

0.6 ml. acetylcholine solution (V),

continue to stir at 25°C and after exactly 10 min., determine the pH of the solution. Subtract the reading from pH 8.0 and multiply the difference by 6 (10 min. → 60 min.). With this value obtain the approximate insecticide content of the sample from a standard curve constructed for the particular insecticide. Dilute the solution of the sample so that it contains. about 0.015 μg. insecticide/ml.

Oxidation of insecticides

Practically all the organophosphorus insecticides are thio- or dithiophosphates. Normally these compounds are oxidized *in vivo* to their respective oxygen analogues or to their sulphoxides or sulphones, which are powerful inhibitors of cholinesterase. For the *in vitro* oxidation of insecticides there are four methods which are suitable with all the enzymatic procedures. The insecticides need not be oxidized before the enzymatic reaction if it is already in the oxidized form, for example, DDVP (2,2-dichlorovinyl-dimethyl phosphate), TEPP (tetra-ethylpyrophosphate) or Paraoxon (diethyl-*p*-nitrophenyl phosphate).

1. Oxidation with bromine or N-bromosuccinimide[7]

Add 0.4 ml. saturated bromine water to 100 ml. distilled water. Add 1 ml. of this solution to the dry residue from the dichloromethane extraction of the sample, immediately before the addition of buffer and enzyme (see under "Assay"). The bromine content of the bromine water is not critical, since ten-fold variations give the same results.

Bromine reacts with most thiophosphates immediately. Certain compounds, such as Demeton (*O,O*-diethyl-*O*-[2-(ethylthio)-ethyl] thiophosphate) or Sulfotepp (tetra-ethyl dithiopyrophosphate) are exceptions, as neither can be converted to cholinesterase inhibitors by bromine water or by *N*-bromosuccinimide. However, they are slowly oxidized by *N*-bromosuccinimide in chloroform, carbon tetrachloride or trichloroethane. In this case, add 1 ml. of a solution of 25 mg. *N*-bromosuccinimide in 100 ml. of one of the above-mentioned solvents to the dry residue of the dichloromethane extraction. Allow to stand for 5 min. at room temperature, add 1 ml. of a 0.02% solution of phenol in chloroform and evaporate off the solvents. Treat the residue with buffer and enzyme solution as described under "Assay".

2. Oxidation with H_2O_2-acetic acid[8,9]

Extract the sample with benzene. Add 3 ml. of a freshly prepared mixture of 30% H_2O_2 and glacial acetic acid (1:5 v/v) to 5 ml. of the extract in a test tube (with a ground-glass stopper) containing boiling chips. Stopper the tube, shake briefly, remove the stopper, heat for 20 min. at 75°C and cool in an ice bath. Add 5 ml distilled water, stopper and shake thoroughly. When the phases have separated, pipette a portion of the benzene layer into a 10 ml. beaker and add a drop of Nujol (paraffin oil). Mix thoroughly, evaporate off the benzene and use the residue for the assay.

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7) *H. O. Fallscheer* and *J. W. Cook*, J. Assoc. off. agric. Chemists *39*, 691 [1956]; *J. W. Cook*, *ibid.* *37*, 984, 987 [1954]; *38*, 150 [1955].

8) *R. Miskus*, *M. E. Tzanakakis* and *S. M. Smith*, J. econ. Entomol. *52*, 76 [1959].

9) *G. G. Patchett* and *G. H. Batchelder*, J. Agric. Food Chem. *8*, 54 [1960].

3. Oxidation with nitric acid[1)]

Evaporate 5 ml. dichloromethane extract to dryness in a 125 ml. round-bottomed flask, cool the flask in an ice bath and carefully add 10 ml. of a mixture of conc. HNO_3 and fuming HNO_3 (1:1 v/v). Wet the walls of the flask with the solution, remove the flask from the ice bath and allow to stand for 5 min. at room temperature. Carefully add 25 ml. cold distilled water and pour the solution into a 125 ml. separating funnel. Rinse the flask with two 25 ml. portions of dichloromethane and pour the washings into the separating funnel. Shake thoroughly, draw off the aqueous layer and discard. Wash the dichloromethane phase with 10 ml. portions of 10% $NaHCO_3$ solution until the washings give an alkaline reaction to litmus. Finally, wash twice with saturated NaCl solution (IV) and filter the dichloromethane phase through a small, dry plug of cotton wool into a 100 ml. volumetric flask. Wash the separating funnel twice with 20 ml. portions of dichloromethane and filter the washings through the same cotton wool plug into the volumetric flask. Dilute with dichloromethane to 100 ml. Analyse a portion of this solution.

4. Oxidation with perbenzoic acid[10)]

Prepare a solution of the oxidizing agent in benzene according to *Adams*[11)] and *Braun*[12)] and analyse. Immediately before use dilute 5 ml. of the benzene solution to 50 ml. with dichloromethane. Add 5 ml. of the dilute perbenzoic acid solution to 50 ml. of the extract to be analysed. Mix the solutions thoroughly, incubate for 15 min. in a water bath at 50°C and then pour immediately into a 250 ml. separating funnel. Rinse the container with two 20 ml. portions of dichloromethane and pour the washings into the separating funnel. Wash the solution with 75 ml. of a freshly prepared solution of 0.5% $Na_2S_2O_5$ and twice with 75 ml. portions of saturated NaCl solution (IV). Filter the dichloromethane phase through a small cotton wool plug and a little anhydrous Na_2SO_4 into a 200 ml. volumetric flask, dilute to 200 ml. with dichloromethane and analyse a portion of this solution.

Determination of the activity of the cholinesterase stock solution (VI a)

Preliminary remarks: During the assay keep all the solutions in an ice bath. When pipetting toxic solutions do not use the mouth. Clean glassware with hot chromic acid, wash thoroughly with water and dry at 100°C in a drying oven.

Method: With a sterile and chilled syringe withdraw slightly more than 1 ml. of the ice-cold cholinesterase stock solution (VIa). Plug the point of the needle by sticking it upright in a clean rubber bung. Remove the piston of the syringe and with an accurate pipette transfer 1.0 ml. of the solution to a 50 ml. volumetric flask. Dilute to 50 ml. with ice-cold 0.9% NaCl solution (II) and mix. This dilute cholinesterase solution contains *ca.* 20 units/ml.

Pipette into a 10 ml. beaker:

3 ml. ice-cold 0.9% dilute NaCl solution (II)
3 ml. ice-cold buffer (solution I).

Place the beaker in a water bath at 25 ± 0.5°C and mix the contents with a magnetic stirrer (refer to Fig. 1).

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10) *P. A. Giang* and *M. S. Schechter*, J. Agric. Food Chem. *8*, 51 [1960].
11) *Roger Adams*, Org. Reactions *7*, 393 [1953].
12) *Geza Braun*, Org. Syntheses, Coll. Vol. *1*, 2nd edition, p. 431 [1941].

Pipette into a second beaker:

3 ml. ice-cold dilute cholinesterase solution
3 ml. ice-cold buffer (solution I).

Exactly 2 min. after placing the first beaker in its water bath, place the second beaker in a water bath (25 ± 0.5°C) and stir the contents with a magnetic stirrer.

Prepare a third beaker like the second, place in a water bath (25 ± 0.5°C) 2 min. after the second beaker and stir the contents with a magnetic stirrer.

Allow the beakers to incubate for exactly 30 min. in the water baths. After 30 min. remove the first beaker and immediately measure the pH. This should be 8.00 ± 0.05 ("initial" pH).

On completion of the 30 min. incubation period add

0.6 ml. ice-cold acetylcholine standard solution (V)

to the second and to the third beaker and allow to incubate for a further 60 min. in the water bath (25 ± 0.5°C). Then measure the pH values of the solutions in these beakers. They should agree within 0.02 pH units. Calculate the mean.

Fig. 2 shows a plot of *arbitrary* cholinesterase units/ml. against the pH. Read off from this curve the cholinesterase activity of the dilute cholinesterase solution corresponding to the measured pH values. Multiply the number of arbitrary units by 49/38 *). This value gives the ml. of 0.9% NaCl solution (II) which must be added to 1 ml. cholinesterase stock solution (VIa) in order to obtain the dilute cholinesterase solution (VIb).

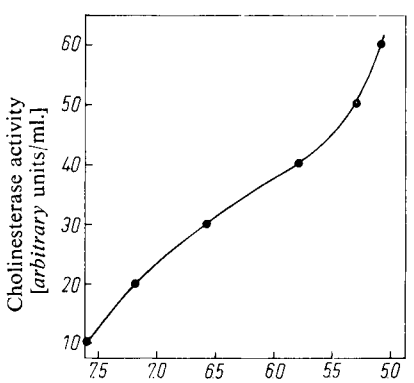

Fig. 2. Standard curve for the hydrolysis of acetylcholine by cholinesterase (1 hour at 25°C). Reaction mixture: 3 ml. buffer (solution I); 3 ml. cholinesterase solution; 0.6 ml. acetylcholine standard solution (V)

Standard curve

To calculate the experimental results a standard curve is required for each insecticide. The procedure is as stated under "Assay", but different concentrations of the pure insecticide in dichloromethane are used instead of the sample.

*) The quotient is obtained as follows: 1 ml. cholinesterase stock solution (VIa) is mixed with 49 ml. NaCl solution to obtain the dilute cholinesterase solution. Fig. 2. shows that 38 *arbitrary* units cholinesterase/ml. are required to lower the pH from 8.0 to 6.0.

The % inhibition for each solution is obtained as described under "Calculations" and this is plotted (abscissa, linear scale) against the μg. insecticide/reaction mixture (ordinate, logarithmic scale). Fig. 3 illustrates two of the standard curves.

Assay

Preliminary remarks: Label the pipettes for measuring out the cholinesterase, buffer and acetylcholine solutions. When pipetting do not touch the walls of the beaker. It is essential that the stated reaction times are adhered to, since the principle of the assay is based on the measurement of reaction rates. In addition, see the preliminary remarks on p. 621.

Method: Temperature of the incubation: 25 ± 0.5°C; two controls (C1 and C2) which contain no sample are required for each series of measurement.

Place 10 numbered beakers (10 ml. capacity) in a fume cupboard. Place a magnetic flea in each beaker and then pipette the following solutions into the beakers as indicated:

	Tube No.	Insecticide solution (0.015 μg./ml.) [ml.]	CH_2Cl_2 [ml.]
Control C1	1	—	8
Experimental	2	1	7
	3	2	6
	4	3	5
	5	4	4
	6	5	3
	7	6	2
	8	7	1
	9	8	—
Control C2	10	—	8

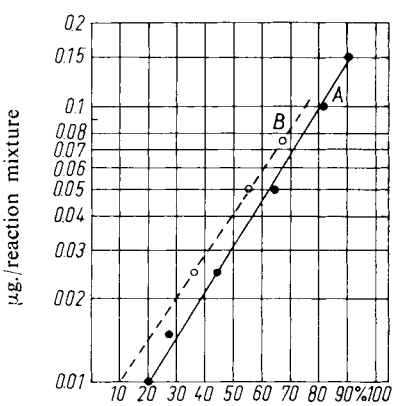

Fig. 3. Standard curves for (A) diethyl-*p*-nitrophenyl phosphate (Paraoxon) and (B) *O,O*-diethyl-*O*-*p*-nitrophenyl thiophosphate (Parathion). The latter after oxidation with a mixture of equal volumes of conc. and fuming HNO_3*).

*) Oxidation with bromine water or H_2O_2-acetic acid is just as suitable[12,30].

Without heating, evaporate all the solutions to dryness by directing a gentle current of air from an electric fan into the fume cupboard. Normally this takes about 10 min. Pipette successively at exactly 2 min. intervals into *all the beakers:*

3 ml. cold buffer (solution I)
3 ml. cholinesterase solution (VIb).

Place each beaker in a water bath (25 ± 0.5°C) and stir (refer to Fig. 1). After exactly 30 min. remove the first beaker (C1) from the water bath. Measure the pH of the solution (rinse the micro-electrodes of the pH-meter before use and dry with a piece of cotton or lens paper). The measured pH (8.00 ± 0.05) is designated $(pH)^0_{C1}$ and it can be considered to be the initial pH of all the other solutions.

Remove the other beakers at exactly 2 min. intervals and pipette into C2 and the experimental beakers:

0.6 ml. acetylcholine solution (V).

Incubate each beaker for a further 60 min. (calculated from the time of pipetting) at 25 ± 0.5°C and stir with a magnetic stirrer. On completion of the incubation period measure the pH values. They are designated $(pH)^{60}_{C2}$ and $(pH)^{60}_{sam.}$.

Calculations

The inhibition of the cholinesterase is calculated from the pH values according to the formula:

$$\frac{[(pH)^0_{C1} - (pH)^{60}_{C2}] - [(pH)^0_{C1} - (pH)^{60}_{sam.}]}{[(pH)^0_{C1} - (pH)^{60}_{C2}]} \times 100 = \% \text{ inhibition}$$

where

$(pH)^0_{C1}$ = pH of the control C1 (without acetylcholine) before the start of the incubation with acetylcholine

$(pH)^{60}_{C2}$ = pH of the control C2 at the end of the incubation with acetylcholine

$(pH)^{60}_{sam.}$ = pH of the experimental beakers at the end of the incubation with acetylcholine.

The insecticide concentration in μg./reaction mixture corresponding to the calculated % inhibition is obtained from a standard curve (see above and Fig. 3).

Other Methods of Determination

Enzymatic methods for the determination of organophosphorus insecticides can be grouped as follows: a) electrometric methods[1,2,6,8,9,13–14], b) titrimetric methods[15–18], c) manometric methods[19–22]

[13] *D. O. Hamblin* and *H. H. Golz:* Cholinesterase Tests and Their Applicability in the Field. American Cyanamid Co., Stamford, Conn., 1953.
[13a] *A. N. Curry, L. M. Kress* and *R. A. Paylor*, J. Agric. Food Chem. *9*, 469 [1961].
[14] *J. P. Frawly, E. C. Hagan* and *O. G. Fitzhugh*, J. Pharmacol. exp. Therapeut. *105*, 156 [1952].
[15] *F. H. Babers* and *J. J. Pratt*, Physiol. Zool. *23* (*1*), 58 [1950].
[16] *H. V. Brown* and *A. F. Bush*, Arch. ind. Hyg. occupat. Med. *1*, 633 [1950].
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[20] *J. E. Casida, T. C. Allen* and *M. A. Stahmann*, J. biol. Chemistry *210*, 607 [1954].
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[22] *K. A. Lord*, Ann. appl. Biol. *43*, 192 [1955].

and d) colorimetric methods[4,7,23–25]. Some colorimetric methods use chromogenic substrates, which form coloured products on hydrolysis with cholinesterase or related esterases. With constant substrate concentration the colour intensity depends on the activity of the enzyme[5,26–28]. In addition, there are methods which depend on the loss of cholinesterase activity in blood after the action of Parathion or other organophosphorus insecticides[13,29,30].

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26) *T. E. Archer* and *Gunter Zweig*, J. Agric. Food Chem. *7*, 178 [1959].
27) *E. F. Jansen*, *M. D. F. Nutting* and *A. K. Balls*, J. biol. Chemistry *170*, 417 [1947]; *175*, 975 [1948]; *179*, 201 [1949].
28) *D. N. Kramer* and *R. M. Gamson*, Analytic. Chem. *30*, 251 [1958].
29) *D. R. Davies* and *J. D. Nicholls*, Brit. med. J., 1373 [1955].
30) *G. Limperos* and *K. E. Rauta*, Science [Washington] *117*, 453 [1953].