A Specific Method for the Assay of Select Chlorinated Pesticides

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The effect of 21 different pesticides including carbamates, organophosphorus compounds, chlorinated hydrocarbons, herbicides, and fungicides on yeast hexokinase is reported. The enzyme is inhibited by only four chlorinated pesticides: Aldrin, Chlorodane, DDT, and heptachlor. As little as 10^{-6} *M* concentrations of these pesticides (100 ppb) can be specifically detected in the presence of all other pesticides with a precision and accuracy of about 2%.

Many researchers have reported the enzymatic assay of pesticides. Guilbault and Kramer (1964a) and Guilbault *et al.* (1962) used both lipase and cholinesterase enzymes to determine very low concentrations of certain pesticides. Giang and Hall (1951) used acetylcholine as substrate for cholinesterase to measure the percent inhibition by organophosphorus insecticides. Guilbault *et al.* have proposed the use of boll weevil and bee (1970a), liver (1970b), and insect cholinesterase (1970c) for the assay of pesticides. Although very selective and sensitive methods were developed for organophosphorus and carbamate pesticides, no good enzymic methods were found for the assay of chlorinated pesticides. Guilbault and Sadar (1969) have reported the inhibition of lipase by chlorinated insecticides such as Aldrin, Lindane, heptachlor, DDT, and a carbamate, Sevin. Unfortunately, this method is of limited use because of a lack of specificity.

In this study the effect of pesticides on the enzyme hexokinase was studied. Only four chlorinated pesticides—Aldrin, Chlorodane, DDT and heptachlor—were found to inhibit this enzyme, thus providing some selectivity in the assay of these insecticides.

MATERIALS AND METHODS

Pesticides. Stock solutions of various concentrations were prepared in spectrograde dioxane which was used without further purification. The pesticides were all of 99+% purity and were obtained from Polysciences Corp., Evanston, Ill.

Substrates. Stock solutions: (1) $MgCl_2$ (Fisher, 10^{-2} *M*), β-D-glucose (Sigma, 10^{-2} *M*), and adenosine triphosphate (ATP, Sigma, 10^{-2} *M*); (2) phenazine methyl sulfate (Sigma, 10^{-3} *M*); and (3) nicotinamide-adenine dinucleotide phosphate (NADP, Sigma, 0.005 *M*) were prepared in water. A 10^{-3} *M* solution of resazurin (Eastman Organics) (Solution 4) was prepared in methyl cellosolve. All substrates were of the highest available purity.

Enzymes. An aqueous solution of a mixed enzyme system was prepared: 1.0 mg hexokinase (Sigma, Type V, yeast 40 units/mg—1 unit represents 1 μmole glucose phosphorylated per min at pH 8.5 and 25° C), and 1.0 mg glucose-6-phosphate dehydrogenase (Sigma, Type X, yeast, activity 130 units/mg—1 unit effects the reduction of 1 μmole of NADP per min at pH 7.4 and 25° C) in 25 ml of water.

Buffers. A MacIlvaine buffer, 0.1 *M*, pH 7.0, was prepared by dissolving the appropriate amounts of Na_2HPO_4, citric acid, and KCl in distilled water.

Apparatus. Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer (SPF). The λ_{ex} set was 560 nm, the λ_{em} was 580 nm. A thermoelectric cooler was used to maintain the temperature of the SPF, and a Beckman linear recorder was used to record the rates.

Assay of Pesticides. To 2.0 ml of MacIlvaine buffer, 0.1 *M*, pH 7.0, was added 0.3 ml of a solution containing glucose, $MgCl_2$, and ATP, 0.3 ml of NADP solution, 0.1 ml of resazurin, 0.1 of PMS, and 0.1 ml of dioxane (the solvent used for the pesticides). The fluorescence was adjusted to 0 and then 0.3 ml of the appropriate enzyme stock solution was added. The rate of change in fluorescence with time, ΔF/min, was recorded. This rate was labeled as the blank rate or the rate with no pesticides present. In other runs, the same solutions were added as above, except that 0.1 ml of a solution of the pesticide was added instead of the dioxane solvent. The rate ΔF/min was recorded and the percent inhibition was calculated as below:

$$\% \text{ Inhibition} = \frac{(\Delta F/\text{min})_{\text{No Inhibition}} - (\Delta F/\text{min})_{\text{Inhibition}}}{(\Delta F/\text{min})_{\text{No Inhibition}}} \times 100$$

The concentration of pesticide was determined from a calibration plot of % inhibition *vs.* concentration of the pesticide. All assays were performed at 25° ± 0.1° C.

RESULTS AND DISCUSSION

Monitoring System. Hexokinase catalyzes the phosphorylation of glucose by the coenzyme ATP (adenosine triphosphate). The kinase system is coupled with a dehydrogenase system and the resazurin-resorufin indicator reaction developed by Guilbault and Kramer (1964b, 1965).

$$\text{Glucose} + \text{ATP} + MgCl_2 \xrightarrow{\text{Hexokinase}} \text{glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow[\text{Dehydrogenase}]{\text{Glucose-6-phosphate}} \text{NADPH} + \text{gluconophosphate}$$

$$\underset{\text{(Nonfluorescent)}}{\text{NADPH} + \text{Resazurin}} \xrightarrow[\text{Methosulfate}]{\text{Phenazine}} \underset{\text{(Fluorescent)}}{\text{NADP} + \text{resorufin}}$$

The rate of formation of the highly fluorescent resorufin is measured, and is proportional to the concentration of hexokinase present, and hence to the concentration of inhibitor (Guilbault *et al.*, 1969).

Since there are two enzyme reaction systems acting, hexokinase and glucose-6-phosphate dehydrogenase, a study of the inhibition of each enzyme system was tested separately. The latter was not found to be affected in any way by any of the pesticides at any concentration; hexokinase was.

Inhibition of Hexokinase by Pesticides. Data on the in-

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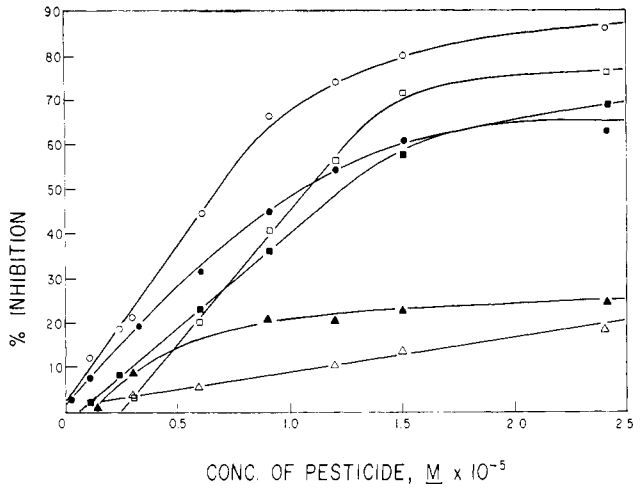


Figure 1. Plot of % inhibition of hexokinase by various pesticides. No preincubation

○—○ Aldrin ●—● DDT
□—□ Heptachlor ■—■ Chlorodane
△—△ DDD ▲—▲ DDE

Table I. I_{50} Values for Various Inhibitors of Hexokinase

Type	Pesticide	I_{50}, M	Lowest Detectable Conc., M[a]
Chlorinated	Aldrin	6.8×10^{-6}	1×10^{-6}
	Chlorodane	1.22×10^{-5}	2.7×10^{-6}
	DDT	1.09×10^{-5}	1.6×10^{-6}
	Heptachlor	1.08×10^{-5}	4.5×10^{-6}
	DDD, DDE	X[b]	
	Dieldrin	X[b]	
	Kelthane	X[b]	
	Methoxychlor	X[b]	
	Lindane	...[c]	
Organo-phosphorus	Paraoxon	...[c]	
	Parathion	...[c]	
	Guthion	...[c]	
	Malathion	...[c]	
	Methyl Parathion	...[c]	
	DDVP	...[c]	
Carbamates	Sevin	...[c]	
	Rotenone	...[c]	
Herbicides	2,4-D Acid	X[b]	
	2,5-T Acid	X[b]	
	Dalapon	X[b]	

[a] Concentration that gives 10% inhibition. [b] Inhibition does not exceed 20–30% at high concentrations. [c] No inhibition at any concentration.

hibition of hexokinase by 21 organophosphorus, chlorinated carbamate and herbicide pesticides are presented in Table I and Figure 1. The concentration of pesticide needed to effect a 50% inhibition of the enzymic activity (I_{50}) as well as the lowest detectable concentrations are given.

The enzyme is inhibited selectively by the chlorinated pesticides Aldrin, Chlorodane, DDT, and heptachlor. The chlorinated pesticide Lindane, all the organophosphorus compounds tried (Paraoxon, DDVP, parathion, methyl parathion, malathion, and guthion), and the carbamates Sevin and Rotenone do not inhibit the enzyme at any concentrations, even as high as 10^{-2} M.

The chlorinated pesticides, DDD, DDE, Dieldrin, Kelthane and Methoxychlor, and the herbicides inhibit at high concentrations, reaching a maximum inhibition of only about 20–30%. Two of the inhibition curves obtained for the weak inhibitors DDD and DDE, two degradation products of DDT, are pictured in Figure 1. DDD is a very weak inhibitor, DDE a little stronger—both only reach 25%, and their interference can be eliminated by a dilution process. The others start inhibiting at concentrations above 10^{-4} M and reach only about 10 to 15%.

Hence it appeared that this high selectivity of hexokinase could be used analytically to detect the presence of heptachlor, chlorodane, aldrin, or DDT in water, and then to assay quantitatively for one of these pesticides in the presence of other noninhibiting chlorinated, organophosphorus, carbamate and herbicide pesticides. We found that it was possible to detect qualitatively as low as 200 ppb of the four chlorinated pesticides in water by this method. None of the other pesticides gave a false positive test.

Some data on the quantitative assay of either heptachlor, chlorodane, aldrin, or DDT in synthetic mixtures of pesticides in sea, river, or lake water are presented in Table II. Any one of the inhibiting chlorinated pesticides could be determined with an error of about 3 to 5%.

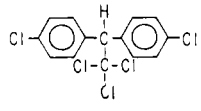
The present study being analytically oriented, no extensive tests were made to establish firmly the type of inhibition involved. There are, however, certain indications that the inhibition is reversible rather than irreversible. An amount of chlorodane, sufficient to completely inhibit the enzyme, was added to hexokinase solution. The enzyme showed 0 activity. The enzyme solution containing chlorodane was then dialyzed (Dixon and Webb, 1958) in 0.005 M phosphate

Table II. Assay of Mixtures of Pesticides Using Hexokinase

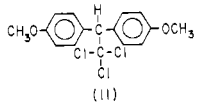
Mixture	Concentration of Each, M	Chlorinated Found, M	% Error
Heptachlor + Parathion	3.00×10^{-6}	2.80×10^{-6}	−6.7
Heptachlor + DDVP	3.00×10^{-6}	2.94×10^{-6}	−2.0
Heptachlor + Sevin	3.00×10^{-6}	3.00×10^{-6}	0
Heptachlor + Dalapon	3.00×10^{-6}	2.85×10^{-6}	−5.0
Heptachlor + Lindane	3.00×10^{-6}	3.00×10^{-6}	0
Chlorodane + DDVP + Sevin + Dalapon + Lindane	6.00×10^{-6}	5.88×10^{-6}	−3.3
Heptachlor + DDVP + Sevin + Dalapon + Lindane	9.00×10^{-6}	8.60×10^{-6}	−4.4
Aldrin + DDVP + Sevin + Dalapon + Lindane	6.00×10^{-6}	5.70×10^{-6}	−5.0
DDT + DDVP + Sevin + Lindane + Dalapon	1.20×10^{-5}	1.16×10^{-5}	−3.3
Chlorodane + Parathion + DDVP + Dalapon + Sevin + Lindane	6.00×10^{-6}	5.68×10^{-6}	−5.3
		Average % Error	−4.3%

buffer for 12 hr at 4° C. The solution was again tested for hexokinase activity and it was found that the enzyme had regained almost 45% of its activity. Also, it was found that the inhibition is independent of incubation time and does not increase progressively with increase in time of incubation.

Effect of Structure of Chlorinated Pesticides on Inhibition. Hexokinase is selectively inhibited by only a few chlorinated pesticides, indicating that the enzyme has an active site able to distinguish chemically similar inhibitors. DDT (I), for example, is a powerful inhibitor, yet the substitution of a —OCH_3 group for —Cl in the p-position produces a compound that is only half as powerful an inhibitor [DDT (II)]. This suggests the involvement of an inductive effect.

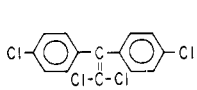


DDT (I)

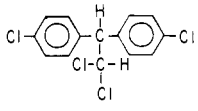


(II)

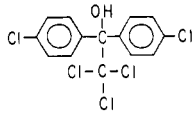
DDE (III), DDD (IV), and Dimite (V) are very similar to DDT (I); however, they are not inhibitors. Hence the aliphatic portion of the DDT also plays an important role. On replacing a chlorine atom or a hydrogen atom, or by removing HCl and creating a double bond, the inhibitory properties are drastically affected.



DDE(III)

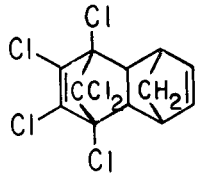


DDD(IV)

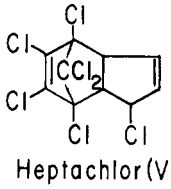


Dimite (V)

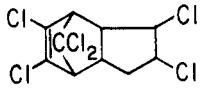
Aldrin and Heptachlor (VI and VII) inhibit equally, suggesting that the six-membered chlorine substituted ring is of prime importance. If, however, chlorine is added to the double bond as in Chlorodane (VIII), an even stronger inhibitor is obtained. If this double bond opens to yield an epoxide as in Dieldrin or Endrin (IX), the inhibition falls off drastically.



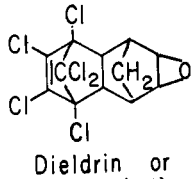
Aldrin (VI)



Heptachlor(VII)



Chlorodane(VIII)



Dieldrin or Endrin (IX)

Table III. Optimum Conditions for Assay of Pesticides

Reagent[a]	Solvent	Solution Added
MacIlvaine Buffer, pH 7.0	H_2O	2 ml of 0.1 *M*
Glucose	H_2O	0.1 ml 10^{-2} *M*
$MgCl_2$	H_2O	0.1 ml 10^{-2} *M*
ATP	H_2O	0.1 ml 10^{-2} *M*
NADP	H_2O	0.3 ml 5×10^{-3} *M*
Resazurin	Methyl Cellosolve	0.1 ml 10^{-3} *M*
PMS	H_2O	0.1 ml 10^{-3} *M*
Pesticide	H_2O	0.1 ml Unknown or known
Enzyme Solution	H_2O	0.4 ml (0.04 mg/ml hexokinase + 0.036 mg/ml dehydrogenase)

[a] In order of addition.

Stability and Order of Addition of Reagents. The optimum concentrations of all reagents, as well as the order of addition of reagents, is shown in Table III. The order of addition shown should be followed for the most reproducible results. Some of the reagents can be combined together to eliminate the number of solutions needed. For example, glucose, $MgCl_2$, and ATP can be placed in one solution, as could the two enzymes.

The NADP, resazurin, and PMS, however, must be kept separate, since the combined solution(s) is very unstable.

All of the solutions are stable, but for different periods of time. The enzyme solution can be kept for about 1 week; the glucose + $MgCl_2$ + ATP solutions can be kept indefinitely. When stored in black bottles, NADP and PMS are stable for several weeks; the resazurin is stable indefinitely.

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