Determination of Some Carbamates by Enzyme Inhibition Techniques Using Thin-Layer Chromatography and Colorimetry

Celso E. Mendoza* and John B. Shields

The detection limits were determined by a thin-layer chromatographic–enzyme inhibition technique for aldicarb, Butacarb, C-8353, carbaryl, formetanate (HCl), Meobal, Mesurol, methomyl, and promecarb. Indophenyl and 5-bromoindoxyl acetates were used as substrates of porcine liver esterases. Effects of ultraviolet irradiation and bromine on the pesticides were also studied. Furthermore, inhibition of esterase activities by these carbamates was determined by spectrophotometry using indophenyl acetate (IPA) substrate. Percent inhibitions were based on IPA hydrolysis rate in the treated solutions at two different periods *vs.* that in the control enzyme solutions, and the absorbance of the treated solution at 7.5 or 15 min after the addition of substrate *vs.* the absorbance of the control solution. Both calculations gave comparable results. Results showed that the use of IPA in both chromatographic and spectrophotometric methods is satisfactory in confirming pesticides by an enzyme inhibition technique.

The use of indophenyl acetate (IPA) in colorimetric determination of enzyme activities was reported by Kramer and Gamson (1958) and Archer and Zweig (1959). Archer and Zweig (1959) determined that the hydrolysis of IPA mediated by bovine erythrocyte cholinesterase (ChE) was reduced even at concentrations (in μg/5.15 ml of reaction solution) of: $\leq$1 for carbophenothion or phorate; $\leq$4 for carbaryl; and $\leq$6 for azinphosmethyl. Furthermore, Archer and Zweig were able to demonstrate that bees' head brei was more sensitive to carbaryl inhibition than the crystalline ChE from bovine erythrocytes. The bee brei activity toward IPA was reduced even by 0.01 μg of carbaryl per 5.15 ml of reaction solution.

More recently, IPA was used as an esterase substrate to detect pesticides on thin-layer plates (Mendoza and Shields, 1971; Winterlin *et al.*, 1968). The sensitivity of detection was at nanogram to picogram levels of pesticides. Selective inhibition by pesticides was also observed.

In comparing the thin-layer chromatographic (tlc) and colorimetric techniques for determination of the enzyme inhibiting property of a pesticide, it is essential that the two methods utilize the same substrate and enzyme source. These conditions would ensure that the same enzyme sites or systems are involved in the test. It was shown in previous experiments that IPA was not a substitute for acetylthiocholine or butyrylthiocholine iodide in measuring activities of cholinesterases (Mendoza *et al.*, 1972; O'Brien, 1969). Mendoza *et al.* (1971, 1972) also found that in rats, esterases catalyzing the hydrolysis of IPA were more abundant in the livers and kidneys than in the brain. The opposite was true with acetylcholinesterase.

The purpose of this study was to develop methods for determination and characterization of carbamates by esterase inhibition and to determine the feasibility of using IPA in colorimetry to confirm residues analyzed by the tlc–enzyme inhibition (tlc–EI) technique, or *vice versa*.

MATERIALS AND METHODS

Carbamate Standard Solutions. Each carbamate (Table I) was made in methanol, in weight per volume for tlc and in molarities for colorimetry. The solutions were stored at 4°.

Carbaryl was used, instead of parathion, as a reference standard because it is a strong enzyme inhibitor even without exposure to bromine, and therefore it is readily detected at low levels. Parathion has to be converted into a more potent inhibitor of enzymes by bromine or ultraviolet (uv) light to be a useful standard for a tlc-EI technique. Some carbamates are readily degraded by bromine or uv light.

Pig Liver Esterase. Liver extracts (20% in cold distilled water, w/v) were prepared and stored according to Mendoza *et al.* (1969).

Thin-Layer Chromatography. For tlc, 1 ml of liver extract was diluted with 8 ml of buffer (0.05 *M* Tris HCl buffer at pH 8.2 before use) when used with 5-bromoindoxyl acetate and with 25 ml of the same buffer when used with IPA substrate.

Enzyme Substrates. The 0.7 mg of 5-BIA/ml of spray solution was prepared with 0.05 *M* Tris HCl buffer at pH 8.2 (Mendoza *et al.*, 1969). The IPA solution, 1 mg/ml, was prepared in ethanol and sprayed lightly and evenly on the plates. The IPA solution can be stored indefinitely at approximately 4°.

The uv Light Source. Three 15-W germicidal lamps (General Electric Co., Ltd.) mounted side by side, approximately 6 cm apart, were the uv light sources. Tlc plates were exposed 10 cm below the lamps for 30 or 60 min.

Development of Plates. Tlc plates (20 × 20 cm) coated with approximately a 500-μ thick layer of silica gel G-HR were used (Mendoza *et al.*, 1968). Interferences from the gel could be removed by chromatography using acetone.

Each standard solution was applied with 5 or 10 μl capillary tubes (Microcaps, Acadian Instruments, Ltd., Don Mills, Ontario, Canada) 2.5 cm from the edge of the gel. The carbamates on plates were exposed to either uv light or bromine vapor before or after resolution. Plates were resolved in glass tanks containing 20% acetone in hexane. Two-dimensional tlc was also performed to determine the compounds formed after an exposure to uv light. Then the plates were sprayed with the enzyme solution. They were incubated at room temperature for about 1 hr or until the gel was almost dry before spraying the IPA solution. The sites of enzyme inhibitors appeared as white spots against a blue background. The plates were sprayed again with bromine to stop the reaction and to preserve the plate quality indefinitely.

Colorimetric Procedure. Each of the selected carbamates was mixed with enzyme solutions in pH 8.2, 0.05 *M* Tris HCl buffer. The resulting solutions were incubated for various lengths of time in a 37° water bath equipped with an automatic agitator. A 100-μl 0.02 *M* IPA solution was rapidly mixed with each reaction medium and the resulting mixture was immediately observed for 15 min with

Pesticide Section, Food Division, Health Protection Branch, Ottawa K1A OL2, Canada.

Table I. List of Pesticides Used in the Studies

Chemicals	Mol wt	% purity	$hR_c \pm SE^a$
Carbaryl (1-naphthyl methylcarbamate)	201.23	99.9	100
Formetanate (HCl) (*m*-{[(dimethylamino) methylene]-amino}phenyl methylcarbamate, hydrochloride)	257.72	98.4	21 ± 1
Methomyl {methyl *N*-[(methylcarbamoyl)oxy]thioacetimidate}	162.21	*b*	30 ● 1
C-8353 [*o*-(1,3-dioxolan-2-yl)-phenyl methylcarbamate]	223.23	98.5	55 ± 1
Aldicarb [2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime]	190.27	98.8	88 ± 2
Meobal (3,4-xylyl methylcarbamate)	179.22	100.0	119 ± 2
Mesurol [4-(methylthio)-3,5-xylyl methylcarbamate]	225.31	99.0	124 ± 3
Promecarb (*m*-cym-5-yl methylcarbamate)	207.28	>98.0	141 ± 2
Butacarb (3,5-di-*tert*-butylphenyl methylcarbamate)	263.38	100.0	158 ± 3

[a] hR_c = the distance traveled relative to carbaryl, multiplied by 100. SE = standard error of the mean. Each with 12 determinations, except for aldicarb with 8. Carbaryl was used as a reference standard. Solvent system = 20% acetone-in-hexane. Tlc plates = 500-μ thick layer of silica gel G-HR. [b] No purity data available; however methomyl used was from recrystallized standard and only one spot was detected by the tlc-EI technique.

a spectrophotometer (Unicam SP800, Canlab, Montreal, Canada) at 522 mμ and 37°. Typical spectra of the control (1) and the carbaryl treated solutions (2, 3, and 4) are shown in Figure 1. Controls and blanks contained every reagent except the test carbamates and enzyme, respectively. At concentrations tested, no interference was observed in the enzyme solutions. The effect on the enzyme of a 3-min preincubation at 37° before the addition of pesticides was also determined.

The degree of inhibition was estimated only when the control solutions gave linear rates of enzyme activities and

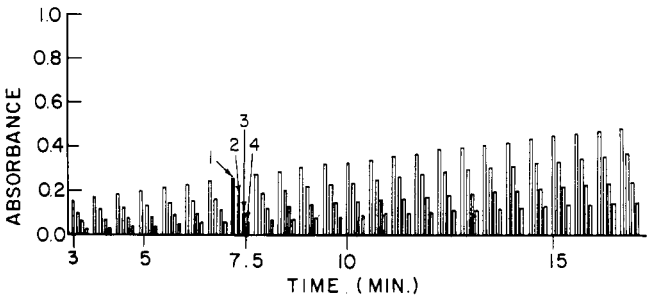

Figure 1. Typical spectra obtained from the hydrolysis of indophenyl acetate (IPA) mediated by pig liver esterases in pH 8.2, 0.02 *M* Tris HCl buffer at 37°. Concentrations carbaryl used: (1) 0 *M*; (2) 4×10^{-8} *M*; (3) 4×10^{-7} *M*; (4) 4×10^{-6} *M*.

the absorbance readings at 522 mμ in 15 min were at least 0.4–0.5 optical density (OD). The change in OD (ΔOD) or the rate of IPA hydrolysis was calculated at two periods, from 5.2 to 7.5 min and from 7.5 to 10 min. The first period was designated ΔT_1 and the second ΔT_2.

Percent inhibition (% *I*) at ΔT_1 or ΔT_2 was calculated as follows.

$$\% I \text{ at } \Delta T_1 \text{ or } \Delta T_2 = 100[1 - (\Delta OD \text{ at } \Delta T_1 \text{ or } \Delta T_{2,\text{treated}})/(\Delta OD \text{ at } \Delta T_1 \text{ or } \Delta T_{2,\text{control}})]$$

Percent inhibition at time 7.5 or 15 min was also calculated.

$$\% I \text{ at } T_n = 100[1 - (OD \text{ at } T_{n,\text{treated}})/(OD \text{ at } T_{n,\text{control}})]$$

where T_n corresponds to time at 7.5 or 15 min.

Protein Analysis. The protein content of each enzyme solution used in colorimetry was analyzed by the method of Lowry et al. (1951). Crystallized and lyophilized albumin from bovine serum was used as a protein standard.

RESULTS AND DISCUSSION

Thin-Layer Chromatography. Shown at the extreme right column of Table I are hR_c values (migration rates relative to that of carbaryl times 100) of eight carbamates. hR_c was more consistent than hR_f (the migration rate relative to the solvent front). The hR_c calculation assumed

Table II. Detection Levels (ng) and Effects of uv on Carbamates as Determined by a tlc-EI Technique Using Pig Liver Esterase and Indophenyl Acetate (IPA) or 5-Bromoindoxyl Acetate (5-BIA)

Chemicals	IPA, without Br	5-BIA		
		Without Br	With Br	With uv[b]
Carbaryl	0.05 (±)[a]	0.1 (±)	0.05 (±)	Decreased
	0.01 (−)	0.05 (−)	0.01 (−)	(0.1–10)
Promecarb	0.5 (±)	0.5 (±)	0.5 (+)	No change
	0.1 (−)	0.1 (−)	0.1 (−)	(1–10)
C-8353	5.0 (±)	5.0 (±)	5.0 (±)	Decreased
	1.0 (−)	1.0 (−)	1.0 (−)	(5–100)
Meobal	0.5 (±)	1.0 (±)	1.0 (±)	No change
	0.1 (−)	0.5 (−)	0.5 (−)	(1–10)
Butacarb	0.5 (±)	0.1 (±)	0.1 (±)	No change
	0.1 (−)	0.05 (−)	0.05 (−)	(1–10)
Methomyl	10.0 (±)	10.0 (±)	10.0 (±)	Decreased
	5.0 (−)	5.0 (−)	5.0 (−)	(50–1000)
Formetanate (HCl)	5.0 (±)	10.0 (±)	1.0 (±)	Decreased
	1.0 (−)	5.0 (−)	0.5 (−)	(5–1000)
Mesurol	0.05 (±)	0.1 (±)	5.0 (±)	Decreased
	0.01 (−)	0.05 (−)	0.1 (−)	(0.1–50)
Aldicarb	25.0 (±)	25.0 (±)	500.0 (±)	Decreased
	10.0 (−)	10.0 (−)	100.0 (−)	(100–1000)

[a] (+) = spot lasted more than 5 min; (±) = less than 5 min; (−) = not detectable. [b] Figures in parentheses denote quantity (ng) ranges used in uv tests.

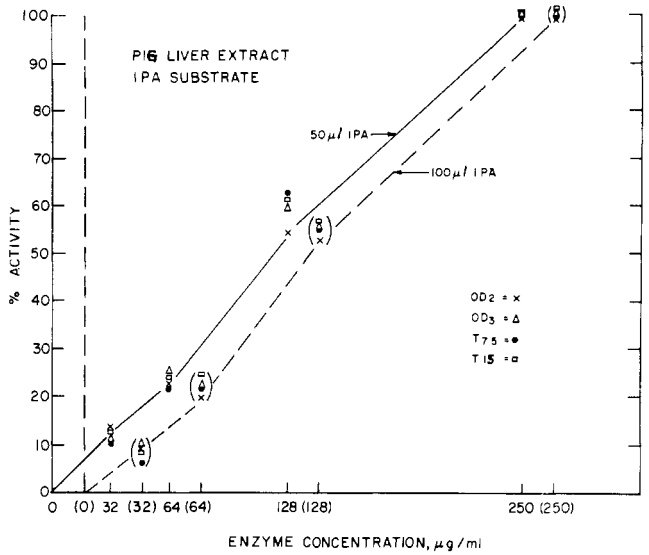

Figure 2. Enzyme activities, in %, after adding 50 or 100 μl of 0.02 *M* IPA to 5-ml solutions containing various enzyme concentrations in pH 8.2, 0.02 *M* Tris HCl buffer; incubation at 37° for 15 min.

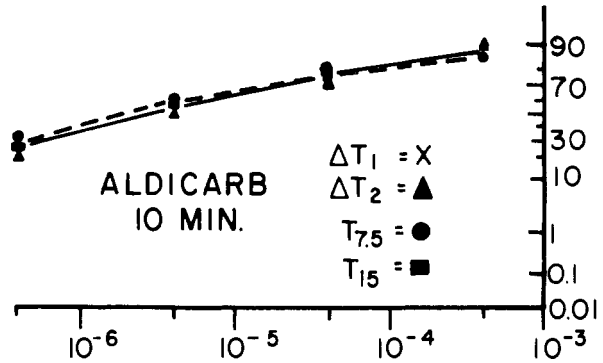

Figure 4. Percent inhibition (in a probit scale) plotted against aldicarb molar concentration (in a logarithmic scale) in 0.02 *M* Tris HCl buffer at pH 8.2. Enzyme was preincubated for 3 min at 37° prior to the addition of aldicarb. Time (10 min) indicates the length of incubation with aldicarb prior to the addition of IPA. See the text for ΔT_1, ΔT_2, $T_{7.5}$, and T_{15} definitions.

that effects of room humidity and temperature, gel thickness, etc., on carbaryl and other carbamates would be the same.

Table II shows that the detection of nine carbamates on silica gel G-HR ranged from nanogram to picogram levels. The inhibitory property of these carbamates was unchanged or diminished after exposure to uv light. Effects of uv light on carbaryl, C-8353, Mesurol, methomyl, formetanate (HCl), and aldicarb were more pronounced after 60 min than after 30 min. The same uv exposures did not alter the inhibitory property of Meobal, Butacarb, and promecarb. The reduction of enzyme inhibition by carbaryl exposed to uv light concurred with that found by Mendoza *et al.* (1969) and Geike (1970) using bovine liver esterases, and by Mendoza and Shields (1970) using porcine liver esterases. A similar result was obtained with Mesurol

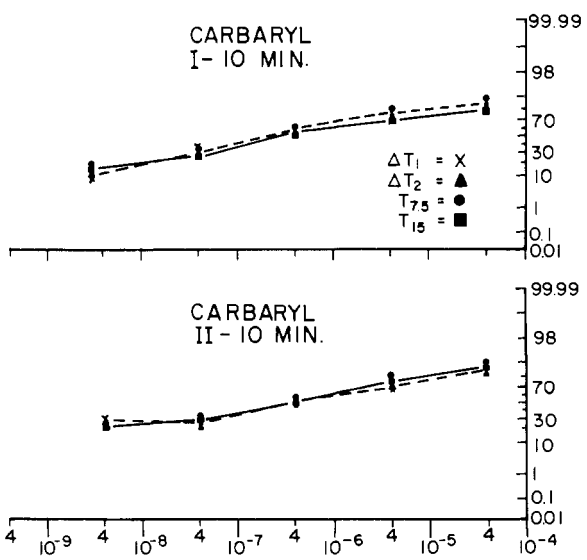

Figure 3. Percent inhibition (in a probit scale) plotted against carbaryl molar concentration (in a logarithmic scale) in 0.02 *M* Tris HCl buffer at pH 8.2. I = enzyme not preincubated and II = enzyme preincubated for 3 min at 37° prior to the addition of carbaryl. Time (10 min) indicates length of incubation with carbaryl prior to the addition of IPA. See the text for ΔT_1, ΔT_2, $T_{7.5}$, and T_{15} definitions.

exposed to uv light. Although additional inhibitors were observed after exposure to uv light, the degree of inhibition obtained with a combination of these products was less than what was obtained with carbaryl or Mesurol alone. Crosby *et al.* (1965) reported that carbaryl in methanol, ethanol, or acetone produced additional inhibitors of human plasma esterases after exposure to uv light or sunlight, but it was not mentioned whether there was a change in the degree of enzyme inhibition. Furthermore, they observed that even prolonged uv irradiation of either solid carbaryl or 50% wettable powder did not result in additional inhibitors. These results suggested that supporting media could have influenced the decomposition rate of carbaryl compounds under the uv light.

Since aldicarb was unusually labile under uv irradiation, it was also studied in the dark. Aldicarb chromatograms that were developed in the dark were essentially similar to those developed in a bright room. Formetanate (HCl) gave similar results under the same condition. Thus, it can be concluded that aldicarb and formetanate (HCl) were very labile only under a direct exposure to a strong uv source.

Wales *et al.* (1968) reported that 100–500 ng of Mesurol treated with bromine was not detected by a tlc–EI technique using beef liver esterases and 5-BIA. Results shown in Table II indicated that even 5 ng of Mesurol treated with bromine could be detected by tlc using pig liver esterase and 5-BIA. The same detection levels were obtained when the standard used by Wales *et al.* and the new standard used in this experiment were simultaneously tested with pig liver esterases and 5-BIA. Therefore, this discrepancy might be attributed to selectivity of esterases used. The sensitivity limits of Mesurol were 50 ng with beef liver esterase (Wales *et al.*, 1968) and 0.1 ng with pig liver esterase (Table II).

Colorimetry. Figure 2 shows that similar linear rates of hydrolysis were obtained by using 50 or 100 μl of 0.02 *M* IPA with various enzyme concentrations. The enzyme activity in solutions kept in an ice bath was observed to progressively diminish to approximately 50% in 6 hr. It was, therefore, necessary to use freshly made enzyme solutions and to monitor the enzyme activity during the assay.

Inhibition percentages of pig liver esterase activity after a 10-min incubation with various concentrations of carbaryl and aldicarb are shown in Figures 3 and 4, respectively. Both figures indicate that comparable results could be obtained regardless of the method used in calculating the percent inhibition. When the enzyme preincubated for 3 min at 37° was reacted with $4 \times 10^{-}$ *M* carbaryl 5–30 min prior to the addition of IPA, about 30% inhibition

Table III. I_{50} of Pig Liver Esterases Incubated with Carbaryl or Aldicarb at Different Time Intervals[a]

Incubation period, min	I_{50} (1×10^{-7} M)							
	No preincubation[b]				3-min preincubation[b]			
	ΔT_1	ΔT_2	$T_{7.5}$	T_{15}	ΔT_1	ΔT_2	$T_{7.5}$	T_{15}
Carbaryl								
5	7.2	7.2	6.0	9.0	2.9	3.8	1.8	3.1
10	2.6	3.5	2.6	3.2	3.7	3.5	2.7	3.5
15	2.1	2.3	1.4	2.1	2.2	1.4	2.0	2.6
30	2.6	3.3	2.0	1.7	1.7	0.7	0.7	1.1
Aldicarb								
5					35	43	32	43
10					19	35	17	26
15					38	36	38	36
30					39	30	34	39

[a] I_{50} = concentration of pesticide which gives 50% inhibition of enzyme activity under specified conditions. The reaction medium was in 0.02 M Tris HCl and contained IPA, 0.4 μM. See the test for ΔT and T definitions. [b] Means incubation prior to the incubation with a pesticide.

Table IV. I_{50} (in 1×10^{-8} M) of Pig Liver Extracts Incubated at 37° with Six Carbamates for 10 min Before the Addition of IPA Substrate[a]

Chemicals	Protein concn[b]	ΔT_1	ΔT_2	$T_{7.5}$	T_{15}
Carbaryl	11.5	37	35	27	35
Butacarb	13.6	0.8	1.7	1	1.1
Promecarb	(i) 10.4	2.9	5	2.4	3.5
	(ii) 13.6	19	9	7	9
Mesurol	(i) 9.6	10	32	1.8	24
	(ii) 13.6	15	12	7	10
Aldicarb	12.8	190	350	170	260
Formetanate (HCl)	13.6	3600	3200	2300	3200

[a] A 5-ml reaction medium in 0.05 M Tris HCl buffer at pH 8.2 contained 0.4 mM IPA. See the text for ΔT and T definitions. [b] Concentration in μg/5 ml of reaction medium.

was consistently obtained (Figure 3). Without preincubation, only about 4 and 15% inhibition were obtained after reacting the esterases with the same amount of carbaryl for 5 and 10–30 min, respectively. Furthermore, percentages of inhibition of enzymes were always smaller after 5 min than after 10- to 30-min incubation with $\leq 4 \times 10^{-7}$ M carbaryl but were similar after 5- to 30-min incubation with $\geq 4 \times 10^{-6}$ M.

Carbaryl was not tested at 4×10^{-4} M because of its partial insolubility in the reaction medium. Only about 70% inhibition of esterase activity was brought about by 4×10^{-5} M carbaryl even after a 30-min incubation at pH 8.2 and 37°. These results agreed with those observed by Archer and Zweig (1959) with horse serum esterases utilizing IPA substrate in a pH 8.0 buffer solution at 30°.

Aldicarb at 4×10^{-8} and 4×10^{-9} M was not plotted because enzyme activation was generally observed instead of inhibition. Activation by other carbamates at these concentrations was not observed. Aldicarb merits further studies at low concentrations.

Table III shows I_{50} values of pig liver esterases after incubation at various time intervals with carbaryl or aldicarb. Measurements of inhibition based on ΔOD (at ΔT_1 and ΔT_2) were comparable to those based on the absorbance at $T_{7.5}$ and T_{15} (see also Table IV). At a 5- or 30-min reaction time with carbaryl, I_{50} values were smaller with enzyme solutions preincubated for 3 min than with enzymes without preincubation. Similar I_{50} values were obtained when carbaryl was reacted for 10–30 min with enzyme without preincubation and for 10–15 min with enzyme with a 3-min preincubation. Likewise, similar I_{50} values were observed when aldicarb was reacted for 5 to 30 min with enzymes with preincubation. The graphs (Figure 5) seem to give varying results for Butacarb, promecarb, and to some extent Mesurol, each at 4×10^{-5} or 4×10^{-4} M. However, this was only due to a magnification of the probit scale between 90–99.99%.

Marked differences between I_{50} values for the six carbamates are shown in Table IV. Butacarb appeared to be the most potent inhibitor of pig liver esterases. Promecarb, Mesurol, and carbaryl were also observed to be strong inhibitors, whereas aldicarb and particularly formetanate (HCl) were weak inhibitors. Butacarb and formetanate (HCl) were 16–34 and 62–133 times, respectively, as potent inhibitors of enzyme as carbaryl. The difference between Butacarb and formetanate (HCl) as enzyme inhibitors was 1353–4500-fold. Promecarb, which was incubated with extracts containing enzyme, at 10.4 μg of protein per 5 ml of reaction solution appeared to be as potent an inhibitor as Butacarb. But, at 13.6 μg of protein per 5 ml of reaction solution, I_{50} values for promecarb were approximately seven times higher than that for Butacarb. Based on IPA esterase inhibition, this difference was not large enough to distinguish the two compounds. Promecarb I_{50} values obtained at two enzyme concentrations were comparable; the only discrepant figure was the 19×10^{-8} M obtained at ΔOD_2. Similarly, Mesurol gave comparable inhibition rates at two enzyme concentrations used. Values obtained by using the $T_{7.5}$ calculation were low but comparable to each other.

Table V shows the ratios of inhibition percentages at the I_{50} (10^{-n} M) region (n represents the numerical value of the exponent) to those obtained at a pesticide concentration ten times lower [$10^{-(n-1)}$ M] or higher [$10^{-(n+1)}$ M] than I_{50} or 10^{-n} M. The ratios obtained by using inhibition percentages at the 10^{-n} and $10^{-(n-1)}$ M regions were consistent for the carbamates studied, except for carbaryl. As the pH of reaction solutions decreased, rates of inhibition by carbaryl increased although percentages of inhibition decreased. Ratios of inhibition percentages at $10^{-(n+1)}$ and 10^{-n} M regions were similar for all the pesticides regardless of the pH of reaction media and degree of enzyme inhibition. These results demonstrated further that the two methods of measurement were comparable under the experimental conditions used.

Table VI shows that the I_{50} of housefly esterases incubated with carbaryl was lower than those of esterases from other species. The I_{50} that we determined for pig liver esterases using IPA was ten times lower than that obtained by Guilbault *et al.* (1970b) for bee esterases using *N*-methylindoxyl acetate (*N*-MIA) substrate. The discrepancy may be due to the enzyme sites and the reaction time involved. It is also interesting to note in Table VI that bee esterases (Guilbault *et al.*, 1970a) were more resistant to carbaryl inhibition than housefly and cockroach esterases (Guilbault *et al.*, 1970c). The I_{50} of bee esterases was comparable only to that of pig and chicken liver and horse serum esterases using *N*-MIA as a substrate (Guilbault *et al.*, 1970b), and to horse serum, bovine erythrocytes, and human plasma esterases using acetylcholine bromide (AChBr) substrate (Zweig and Archer, 1958). It would then appear that esterases from other species were more susceptible to carbaryl than those from bees. This result (Guilbault *et al.*, 1970a) contradicted those obtained with the use of IPA (Archer and Zweig, 1959; Mendoza and Shields, 1971). Bees' head esterases were 100–200 or 200 times as sensitive to carbaryl as pig or beef liver esterases, respectively (Mendoza and Shields, 1971), and at least 400 times as sensitive to carbaryl as crystalline esterase from bovine erythrocytes (Archer and Zweig, 1959). Furthermore, Winterlin *et al.* (1968) found that bees' head esterases

Table V. Ratios of Percent Inhibition at I_{50} or 10^{-n} *M* Region to That Obtained at $10^{-(n-1)}$ *M* or $10^{-(n+1)}$ *M*, Where *n* is the Numerical Exponent

Chemicals[a]	% at 10^{-n} *M*:% at $10^{-(n-1)}$ *M*				% at $10^{-(n+1)}$ *M*:% at 10^{-n} *M*			
	ΔT_1	ΔT_2	$T_{7.5}$	T_{15}	ΔT_1	ΔT_2	$T_{7.5}$	T_{15}
Butacarb	1.6	2.7	2.1	2.2	1.2	1.2	1.1	1.1
Formetanate (HCl)	1.9	2.6	2.2	2.4	1.7	1.5	1.4	1.5
Promecarb (low)	1.9	1.1	1.4	1.4	1.4	1.5	1.3	1.3
(high)	1.6	1.8	2.0	1.9	1.4	1.4	1.4	1.3
Mesurol (low)	2.0	2.9	3.2	3.2	1.6	1.6	1.5	1.6
(high)	1.4	2.5	1.6	1.4	1.4	1.4	1.2	1.3
Aldicarb	1.5	1.7	1.5	1.8	1.3	1.2	1.1	1.1
Carbaryl, 3-min preincubation								
pH 7.0	3.2	4.4	3.7	2.8	1.5	1.6	1.5	1.4
pH 7.5	2.5	2.4	2.8	2.4	1.4	1.5	1.4	1.4
pH 7.7	2.4	2.0	2.1	2.2	1.2	1.2	1.4	1.3
pH 8.2	1.9	2.4	1.7	1.8	1.4	1.3	1.5	1.5
	(2.1)[b]	(2.0)	(2.1)	(2.2)	(2.7)	(2.6)	(1.6)	(1.8)
pH 8.5	1.9	1.9	1.6	1.7	1.2	1.2	1.3	1.3
pH 9.0	1.3	1.5	1.5	1.5	1.2	1.2	1.2	1.2

[a] High and low after promecarb and Mesurol refer to enzyme concentrations given in Table IV. [b] Figures in parentheses were obtained from tests in which the enzyme solutions were not subjected to a preincubation procedure. See the text for ΔOD and *T* definitions.

(using IPA as substrate) were 25,000 times as sensitive to carbaryl than human plasma esterases (using AChBr as substrate). Winterlin *et al.* determined 25 ng of carbaryl as the limit of detection with human plasma esterases. On the contrary, Sadar *et al.* (1970) (see Table V) did not observe any inhibition of human serum esterases using *N*-MIA. These discrepant results strongly suggested that IPA was probably interacting with enzyme sites mainly affected by carbaryl, and *N*-MIA was interacting with different sites or different esterases.

In another experiment, Guilbault and Sadar (1969) demonstrated that the I_{50} value was lower when a pesticide (carbaryl, aldrin, lindane, heptachlor, or *p,p'*-DDT) was incubated with pig pancreatic lipases before the addition of *N*-methyl umbelliferone heptanoate substrate than when the pesticide was added simultaneously with the substrate. I_{50} for carbaryl decreased from 4×10^{-4} to 1.7×10^{-5} *M*. Maximum inhibition of 41% was obtained when 1.4×10^{-3} *M p,p'*-DDT was added simultaneously with substrate to lipase, whereas 50% inhibition was ob-

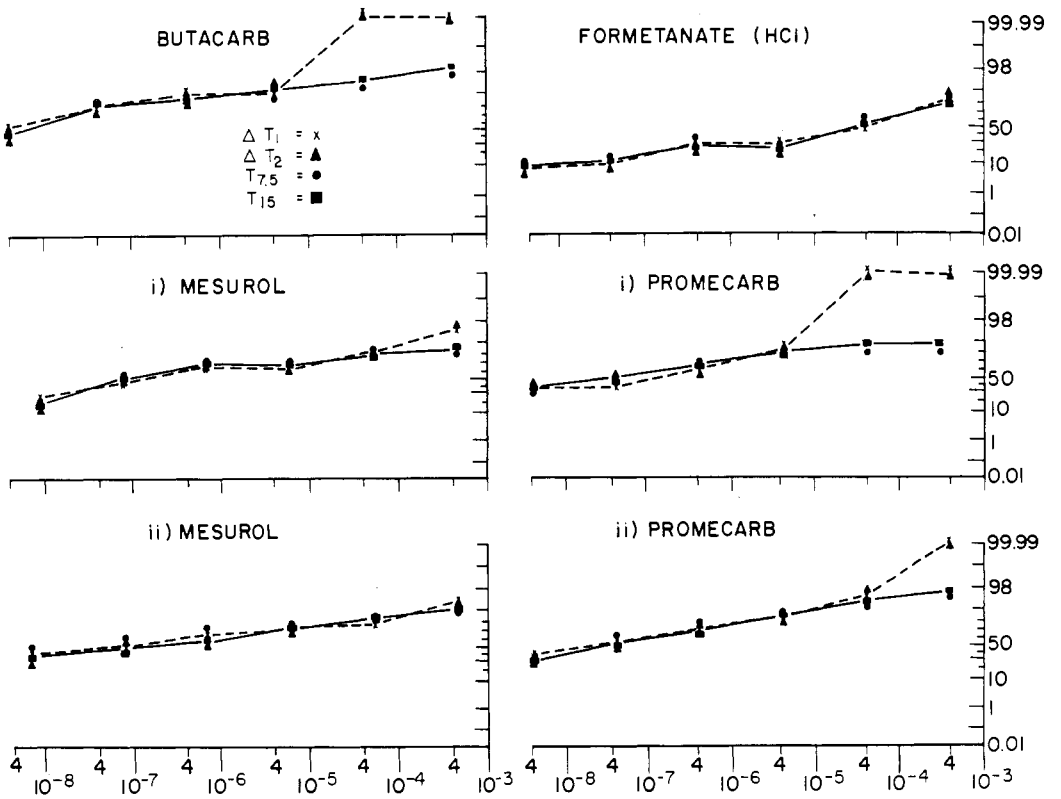

Figure 5. Percent inhibition (in a probit scale) plotted against a carbamate molar concentration (in a logarithmic scale) in 0.02 *M* Tris HCl buffer at pH 8.2. Enzyme was preincubated for 3 min at 37° prior to the addition of the pesticide. Enzyme was incubated with each pesticide for 10 min prior to the addition of IPA. See the text for ΔT_1, ΔT_2, $T_{7.5}$, and T_{15} definitions. See also Table IV for the protein concentration in each reaction medium. Promecarb or Mesurol (i) and (ii) correspond to those in Table IV.

Table VI. I_{50} Obtained for Carbaryl Incubated with Esterases from Various Species

	Enzyme sources[a]	*M*, carbaryl	References
I.	Pig liver	$\sim 3.5 \times 10^{-7}$	This report
II.	Horse serum	1.5×10^{-6}	Archer and Zweig (1959)
III.	Housefly heads	5.4×10^{-8}	Zweig and Archer (1958)
	Horse plasma	2.2×10^{-6}	Zweig and Archer (1958)
	Bovine erythrocytes	5.1×10^{-6}	Zweig and Archer (1958)
	Human plasma	6.7×10^{-6}	Zweig and Archer (1958)
IV.	Housefly (DDT resistant)	8.2×10^{-7}	Guilbault *et al.* (1970c)
	Housefly (NAIDM)	8.8×10^{-7}	Guilbault *et al.* (1970c)
	Cockroaches	9.3×10^{-7}	Guilbault *et al.* (1970c)
	Sugar boll weevil	—[b]	Guilbault *et al.* (1970c)
	Fire ant	—	Guilbault *et al.* (1970c)
	Pigeon	NI[d]	Guilbault *et al.* (1970c)
	Sheep	NI	Guilbault *et al.* (1970c)
V.	Pig liver	7.9×10^{-6}	Guilbault *et al.* (1970b)
	Chicken liver	8.0×10^{-6}	Guilbault *et al.* (1970b)
	Horse serum	8.2×10^{-6}	Guilbault *et al.* (1970b)
	Rabbit liver	1.8×10^{-5}	Guilbault *et al.* (1970b)
VI.	Bee	1.5×10^{-6}	Guilbault *et al.* (1970a)
	Boll weevil	(1.0×10^{-4})[c]	Guilbault *et al.* (1970a)
VII.	Rat liver	3.0×10^{-5}	Sadar *et al.* (1970)
	Electric eel	7.0×10^{-5}	Sadar *et al.* (1970)
	Bean leaf beetle		Sadar *et al.* (1970)
	White fringe beetle		Sadar *et al.* (1970)
	Human serum	NI	Sadar *et al.* (1970)
VIII.	Yeast hexokinase	NI	Sadar and Guilbault (1971)
IX.	Housefly heads	3.0×10^{-7}	Weiden (1971)

[a] Enzyme substrate used for: I and II, IPA; III, AChBr; IV–VII, *N*-MIA; VIII, glucose and adenosine triphosphate; IX, acetylcholine. [b] A dash indicates that I_{50} was not reached even at concentrations greater than 10^{-3} *M*. [c] Maximum inhibition at this concentration was 25%. [d] No inhibition at any concentrations used.

tained when only 7.8×10^{-4} *M* *p,p'*-DDT was added to lipase before the substrate (Guilbault and Sadar, 1969). We also obtained consistent results when the pesticides were added to pig liver esterases prior to substrate IPA. On the contrary, Guilbault *et al.* (1970a) found that better results were obtained when a pesticide and *N*-MIA were added simultaneously to bee and boll weevil ChE. The discrepancy may be due to the purity of enzymes used, the stability of the enzymes, enzyme sites involved, substrate–enzyme reactivity, and the types of enzyme actually being evaluated in each test.

Further evidence that marked differences in detection limits may be due to types of enzyme, enzyme sites, and substrates involved is shown in Table VII. Pig liver esterase was obviously much more sensitive to carbaryl and Mesurol inhibition than beef liver esterase, particularly if IPA, rather than 5-BIA or 2-NA, was used as substrate. Hence, direct comparison of detection limits obtained by using different enzyme sources or substrates should be made with reservation.

More recently Sadar and Guilbault (1971) showed that yeast hexokinase activity was not inhibited by carbaryl (Table VII). The enzyme activity was specifically inhibited by the following pesticides, with I_{50} given in parentheses: aldrin (0.68×10^{-5} *M*); *p,p'*-DDT (1.09×10^{-5} *M*); and heptachlor (1.08×10^{-5} *M*).

CONCLUSION

The results presented in this paper indicate that tlc and colorimetry using IPA are complimentary techniques, and that the same enzyme sites or system are involved. Both techniques can be used to confirm pesticide residues that are enzyme inhibitors. The data also suggested that Buta-

Table VII. Comparison of Detection Limits (ng) of Carbaryl and Mesurol by tlc-EI Techniques Using Different Enzyme and Substrates

Compound	Limit, ng		Liver enzyme source	Substrate[b]	References
	Without Br	With Br[a]			
Carbaryl	1	0.3	Beef	2-NA	Geike (1970)
	50	5	Beef	5-BIA	Wales *et al.* (1968)
	0.5	—	Beef	5-BIA	Mendoza and Shields (1970, 1971)
	0.1	—	Pig	5-BIA	Mendoza and Shields (1970)
	0.1	0.05	Pig	5-BIA	This report
	0.05	—	Pig	IPA	This report
	0.05	—	Pig	5-BIA	Mendoza and Shields (1971)
Mesurol	200	300	Beef	2-NA	Geike (1970)
	50	n.d.	Beef	5-BIA	Wales *et al.* (1968)
	10	—	Beef	5-BIA	Mendoza and Shields (1970)
	0.1	—	Pig	5-BIA	Mendoza and Shields (1970)
	0.1	5	Pig	5-BIA	This report
	0.05	—	Pig	IPA	This report

[a] — = not tested; n.d. = not detected. [b] 2-NA = 2-naphthyl acetate; 5-BIA = 5-bromoindoxyl acetate; IPA = indophenyl acetate.

carb, promecarb, Mesurol, and carbaryl can be classified as strong inhibitors, and aldicarb and formetanate (HCl) can be classified as weak inhibitors of pig liver esterases catalyzing the hydrolysis of IPA.

Since the colorimetric procedure used includes incubation of pesticides before the addition of substrates, it can be used directly to assay enzyme activities from insecticide-treated organisms.

IPA used as a substrate of pig liver esterases has certain advantages over other esterase substrates. It can be used in tlc and colorimetry without involving complex preparations. IPA solution is stable, particularly when stored at 4°. The product indophenolate ion can be measured directly with a spectrophotometer. It has a high extinction coefficient; therefore, it is detectable at a small concentration.

LITERATURE CITED

Archer, T. E., Zweig, G., *J. Agr. Food Chem.* **7**, 178 (1959).
Crosby, D. G., Leitis, E., Winterlin, W. L., *J. Agr. Food Chem.* **13**, 204 (1965).
Geike, F., *J. Chromatogr.* **53**, 269 (1970).
Guilbault, G. G., Kuan, S. S., Sadar, M. H., *J. Agr. Food Chem.* **18**, 692 (1970a).
Guilbault, G. G., Sadar, M. H., *Anal. Chem.* **41**, 366 (1969).
Guilbault, G. G., Sadar, M. H., Kuan, S., Casey, D., *Anal. Chim. Acta* **51**, 83 (1970b).
Guilbault, G. G., Sadar, M. H., Kuan, S. S., Casey D., *Anal. Chim. Acta* **52**, 75 (1970c).
Kramer, D. N., Gamson, R. M., *Anal. Chem.* **30**, 251 (1958).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193**, 265 (1951).
Mendoza, C. E., Shields, J. B., *J. Chromatogr.* **50**, 92 (1970).
Mendoza, C. E., Shields, J. B., *J. Ass. Offic. Anal. Chem.* **54**, 507 (1971).
Mendoza, C. E., Shields, J. B., Phillips, W. E. J., *J. Comp. Biochem. Physiol.* **40B**, 841 (1971).
Mendoza, C. E., Shields, J. B., Phillips, W. E. J., *J. Comp. Gen. Pharmacol.* **3**, 134 (1972).
Mendoza, C. E., Wales, P. J., Grant, D. L., McCully, K. A., *J. Agr. Food Chem.* **17**, 1196 (1969).
Mendoza, C. E., Wales, P. J., McLeod, H. A., McKinley, W. P., *Analyst* **93**, 34 (1968).
O'Brien, R. D., *Biochem. J.* **113**, 713 (1969).
Sadar, M. H., Guilbault, G. G., *J. Agr. Food Chem.* **19**, 357 (1971).
Sadar, M. H., Kuan, S. S., Guilbault, G. G., *Anal. Chem.* **42**, 1770 (1970).
Wales, P. J., McLeod, H. A., McKinley, W. P., *J. Ass. Offic. Anal. Chem.* **51**, 1239 (1968).
Weiden, M. H. J., *Bull. W.H.O.* **44**, 203 (1971).
Winterlin, W., Walker, G., Frank, H., *J. Agr. Food Chem.* **16**, 808 (1968).
Zweig, G., Archer, J. E., *J. Agr. Food Chem.* **6**, 910 (1958).

Received for review June 1, 1972. Accepted November 6, 1972. Presented at the Division of Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D. C., September 1971.

Detector for Phosphine at Permissible Levels in Air

Mysore Muthu, Suvendu K. Majumder,* and Husain A. B. Parpia

A paper-strip detector has been developed for detecting maximum acceptable concentrations (0.05–0.3 ppm) of phosphine in air. The strips are prepared by dipping them in a 1% solution of mercuric chloride in methyl yellow. The papers turn red from the yellow. Ammonia interferes with the proper development of color. The strips are useful for personal protection against phosphine in fumigation and degassing operations, and to detect traces of phosphine in fumigated commodities.

Phosphine (PH_3) gas generated from aluminum phosphide [Phostoxin (Federal Republic of Germany), Delicia (GDR), Celphos (India)] tablets is being used extensively for fumigating food grains and products. Its detection in air at permissible levels is important because of its very high toxicity rating and lack of warning odor at toxic levels. It can be detected by smell at 5 ppm (Singh *et al.*, 1967). Subacute poisoning has been reported at this threshold level (Klimmer, 1969). Sniff tests conducted by a five-man panel could detect 0.002 mg/l. or 1.3 ppm (Berck, 1968). But the carbide-like odor cannot be relied upon to signal toxic levels of the gas, as the commodities preferentially absorb the odor principal (Bond and Dumas, 1967). The maximum allowable concentration for prolonged exposure is 0.05 ppm according to the Second International Symposium, Paris, 1963 (Truhaut, 1964). The threshold limit is set at 0.3 ppm by the American Conference of Government Industrial Hygienists (1964) (Monro, 1969).

Commercially available phosphine detector tubes (Dumas and Monro, 1966) are not always freely available in all countries and are also expensive. Several types of chemically treated papers have been developed for detecting PH_3 in air (Lugg, 1962). Of all these, silver nitrate-impregnated papers have been found to be the most sensitive. Narrow range (BDH) pH papers (pH 2.5–4.0) treated with mercuric chloride have also been found sensitive to 0.05 ppm and above (Muthu, 1970), but the color change is not very apparent and hence is hard to evaluate. In the present method, advantage is taken of the reaction of PH_3 with mercuric chloride where the hydrochloric acid formed is responsible for the change in color of a stain sensitive to the pH change and is shown by the reaction $PH_3 + 3HgCl_2 \rightarrow P(HgCl)_3 + 3HCl$.

Central Food Technological Research Institute, Mysore-570013, India.

MATERIALS AND METHODS

Preliminary experiments using several pH-indicating stains on mercuric chloride-impregnated paper strips indicated the suitability of methyl yellow, which turned from yellow to red as the pH was lowered from 4 to 2.9.

Preparation of the PH_3 Detector Strips. Filter paper strips, 10 cm long and 0.5 cm wide, were cut from a Whatman No. 1 sheet dipped in methyl yellow (*p*-dimethyl aminobenzene) (CAUTION: carcinogenic) solution (0.05% in ethanol) and dried in a draught of air. A drop of a 1% solution (pH 4) of mercuric chloride was allowed to creep along the strip, which was dried again. The strips