Although there were no doubts that the absorbed radioactive material was TIBA, tests were conducted to verify the autoradiograph. The plants from the root absorption experiments were cut just above the line of immersion after 3, 6, and 12 hours, dried at room temperature under reduced pressure, and powdered to pass a 60-mesh screen. The powdered samples were then tested for radioactivity by the usual radioassay method. The results of the radioassay show that the amount of TIBA absorption and translocation increases steadily with time, and that the slight difference in the rate of absorption between the tomato and barley is not statistically significant. The counts per minute (CPM) per gram of plant material shows the mean value of three experiments subtracted from the background count ranging from 25 to 28 (Table I).

For a chemical assay the absorbed TIBA was extracted with acidified ether. Powdered tomato plants from the 12-hour root absorption experiment were used and the TIBA was identified by paper chromatographic technique, followed by autoradiography of the paper chromatogram. These results are shown in Table II, which indicates that the labeled TIBA was the radioactive compound absorbed. However, this was not determined quantitatively.

Discussion

A simple procedure was used here to synthesize 2-iodine-131-labeled 2,3,5-triiodobenzoic acid, which was then used to trace absorption and translocation in both tomato and barley. It was possible to trace and identify the absorptive ability of TIBA in both tomato and barley. The experiment in the foliar application only with the results of radioautographs, but not a quantitative investigation shows that there is almost equal absorptive ability. However, the translocative ability through the conductive system in dicot and monocot seems to be markedly different. Therefore the difference in TIBA action on monocot and dicot in the case of foliar application is due to the difference in translocation. In order to transmit TIBA to the shoot apex, foliar application would have no effect on monocots in contrast to dicots. In the tomato there is also indication that TIBA is first translocated downward. Hence it would seem that any light or moderate foliar application of TIBA would be translocated downward in the tomato plant before it was retranslocated upward. Both tomato and barley can not only readily absorb TIBA through the root, but also easily translocate the compound. This ability is the same for the two. When absorbed TIBA was extracted and identified, it could be considered not associated with complex compounds in plants associated with natural auxin, showing growth-promoting activity, and hence its derivatives may be difficultly extractable by ether. If TIBA is antiauxin, TIBA will be associated with this complex compound rather than with the native hormones and become hard to extract. The native hormones in plants on the contrary become more soluble and extractable. In this paper the authors could not report on the quantitative investigation of the native hormones affected by the TIBA treatment. As a result of extraction and radioautography experiments with TIBA, it is supposed that TIBA is not antagonistic to native auxins, but that it inhibits growth in some manner other than that of an antagonist of native auxins.

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INSECTICIDE MEASUREMENT

Direct Colorimetric Analysis of Cholinesterase-Inhibiting Insecticides with Indophenyl Acetate

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Anticholinesterase activity due to the hydrolysis of indophenyl acetate is measured colorimetrically in the presence of the following insecticide residues: Sevin, Thimet, Guthion, and Trithion. The variables affecting the reaction such as enzyme concentration, temperature, preincubation time, and pH have been studied.

A PRECISE AND REPRODUCIBLE PROCEDURE for the measurement of anticholinesterase insecticides is based on the direct colorimetric measurement at 625 mμ of the hydrolysis product of indophenyl acetate at a constant pH of 8.0. Standard curves and residue data are presented using crystalline bovine erythrocyte acetylcholinesterase as the enzyme. A preincubation time of 30 minutes and a reaction time of 30 minutes at 30° C. are recommended. Honey bee brain as a source of esteratic enzymes is also discussed.

Enzymatic methods for the analysis of cholinesterase-inhibiting insecticides have been recently reviewed by Schechter and Hornstein (*13*). Several of these procedures have been adapted for insecticide residue analyses in agricultural crops (*4, 14*). Recently, Kramer and Gamson (*7*) published a method for the colorimetric determination of acetylcholinesterase activity utilizing indophenyl acetate as the chromogenic substrate. The method reported here utilizes the color reaction described by Kramer and Gamson for the quantitative measurement of cholinesterase-inhibiting insecticides.

Reagents and Equipment

Indophenyl Acetate. The method of synthesis is adapted from that of Heller (*6*). Two grams of the sodium salt of indophenol (Distillation Products Industries, Eastman Kodak Co.) is heated on a steam bath at 100° C. for 30 minutes with 12 grams of acetic anhydride. The solution is cooled to room temperature, transferred to a 500-ml. separatory funnel, and extracted with five 100-ml. aliquots of petroleum ether (boiling point 30° to 60° C.). The combined petroleum ether fractions are transferred to a 1-liter separatory funnel, and the excess acetic anhydride is washed from the petroleum ether with three 250-ml. aliquots of distilled water. The petroleum ether fraction is dried over anhydrous sodium sulfate, filtered into a round-bottomed boiling flask, and concentrated under vacuum to a 10-ml. volume, until red crystals form. The liquid is decanted from the flask, the crystals are rinsed into a Petri dish with 50 ml. of dry petroleum ether, and the petroleum ether is removed by evaporation in a stream of dry air. The crystals are dried under vacuo and are found to have a melting point of 115–16° C. A second crop of crystals can be obtained by evaporating the 10-ml. fraction of petroleum ether remaining from the first crystallization, followed by recrystallization from petroleum ether.

Stock Solution of Indophenyl Acetate ($3.3 \times 10^{-2}M$). Eight milligrams of indophenyl acetate per 1 ml. of absolute ethyl alcohol.

Working Solution of Indophenyl Acetate ($3.3 \times 10^{-3}M$). A 1 to 10 dilution of the stock solution of the indophenyl acetate is made with absolute ethyl alcohol. The final concentration of the indophenyl acetate in the reaction vessels is $9.6 \times 10^{-5}M$.

Crystalline Bovine Erythrocyte Acetylcholinesterase Stock Solution. Twenty thousand units of crystalline bovine erythrocyte acetylcholinesterase (Winthrop Laboratories, Special Chemical Department) is dissolved in 20 ml. of 0.9% sodium chloride solution, giving 1000 units per ml. The units of activity are those designated by the manufacturer (*8*) and determined by the method of Ammon (*1*).

Working Solution of Crystalline Bovine Erythrocyte Acetylcholinesterase. Three milliliters of the enzyme stock solution (3000 units) are diluted to 100 ml. with 0.05*M* potassium dihydrogen phosphate buffer pH 8.0, yielding 30 units of enzyme per ml. of buffer solution. Five milliliters (150 units) are used per reaction vessel.

Cholinesterase from Honey Bee Brain. About 1500 bees (*Apis mellifera*) were stored in a deep freeze at −18° C. for 4 hours. They were placed in a container with finely broken dry ice, removed individually from the container, and decapitated with a knife and forceps. Four hundred heads were combined with 4.0 ml. of a salt solution [8.12 grams of manganous chloride and 8.77 grams of sodium chloride per liter (*10*)] and 2 grams of washed sand in a prechilled size No. 1 mortar in a cold room at 4° C. The heads were slowly ground and then transferred to a 50-ml. centrifuge tube with two aliquots of 3.0 ml. of the cold saline solution. The head fragments were removed by centrifugation for 10 minutes at 10,000 r.p.m., in a Servall superspeed centrifuge. The supernatant liquid was decanted into a graduated cylinder, and the fragmented heads were mixed with 3.0 ml. of cold saline solution and centrifuged again at 10,000 r.p.m. This extraction procedure was repeated twice. The supernatant solutions were combined and the volume was adjusted to 20 ml. with the salt solution so that each milliliter was equivalent to the extractives from 20 bee heads.

Working Solution of Bee Brain Cholinesterase. One milliliter of the stock bee brain brei was diluted to 100 ml. with 0.05*M* phosphate buffer (pH 8.0), and 5.0 ml. (the equivalent extractives of one bee head) was used whenever bee brain was the source of the cholinesterase. The extractives from one bee head produced the equivalent activity of 162 units of crystalline bovine erythrocyte acetylcholinesterase when measured under identical conditions.

Buffer Solution (0.05*M* potassium dihydrogen phosphate). Clark and Lubs buffer pH 8.0 (*3*) (46.80 ml. of 0.1*N* sodium hydroxide) is added to 50 ml. of 0.1*M* potassium dihydrogen phosphate solution and diluted to 100 ml.

Insecticide Standard Solutions. Sevin (*N*-methyl-1-naphthyl carbamate) Standard (20 γ of Sevin per ml. of absolute methanol).

Trithion [*S*-(*p*-chlorophenylthiomethyl)-*O*,*O*-diethyl phosphorodithioate] Standard (1 γ of Trithion per ml. of benzene).

Guthion [*O*,*O*-dimethyl *S*-(4-oxo-1,2,2-benzotriazinyl-3-methyl) phosphorodithioate] Standard (5 γ of Guthion per ml. of benzene).

Thimet [*O*,*O*,diethyl-*S'*(ethylthiomethyl) phosphodithioate] Standard (5 γ of Thimet per ml. of benzene).

Glycerol Solution. Ten milliliters of glycerol is diluted to 100 ml. with absolute methanol.

Shaking Apparatus. Automatic shaker on a four-drawer cabinet (Microchemical Specialties Co. for chloride analysis).

Spectrophotometer. Bausch and Lomb Spectronic 20.

Agitating Rods for Beakers. 6 mm. × 10 mm. solid glass rods.

Procedure

To prepare a standard curve, pipet aliquots of the insecticide standard under investigation into 10-ml. Griffin beakers. Standard curve ranges for the insecticides investigated by this procedure are: Sevin 0 to 4 γ, Thimet 0 to 1 γ, Guthion 0 to 6 γ, and Trithion 0 to 1 γ. Thimet, Guthion, and Trithion must be oxidized to the sulfone by the acetic acid–hydrogen peroxide method of Patchett (*12*) with the modification that the excess acetic acid–hydrogen peroxide is washed from the benzene, after the oxidation, with three 3.0-ml. aliquots of distilled water. Pipet 0.5 ml. of the glycerol solution into the 10.0-ml. beakers containing the insecticide. The purpose of the glycerol reagent is to prevent the insecticide from going to complete dryness when the solvent is evaporated. Evaporate the solvent with a gentle stream of warm air from a hair dryer. Add a glass rod to each beaker after the solvent is evaporated, and rock the beakers gently on the shaking apparatus.

To beaker number 1 add 5 ml. of the 0.05*M* phosphate buffer (pH 8.0) and start the timer. Add to beaker number 2 exactly 1 minute later 5 ml. of enzyme-buffer solution, and at exactly 1-minute intervals add 5 ml. of the enzyme-buffer solution to each of the remaining beakers. The number of beakers is limited to 30 as a result of the timing. After exactly 30-minute preincubation time, add 0.15 ml. of the indophenyl acetate working solution ($3.3 \times 10^{-3}M$) to each beaker at exactly 1-minute intervals. The temperature of incubation is 30° C. After exactly 30 minutes set the spectrophotometer at 100% transmittance at 625 mμ with the solution of beaker 1 (reagent blank). At exactly 1-minute intervals read the absorbance of each of the remaining reaction beakers. Plot the standard curve on three-cycle semilogarithmic paper ×10 to the inch. Plot micrograms of insecticide on the logarithmic scale (abscissa) against absorbance on the linear (ordinate) scale, resulting in a smooth curve.

The incubation time can be shortened to as little as 5 minutes by elevating the temperature to 45° C. Incubation time and temperature are arbitrary and can be selected at the convenience of the operator within the ranges of 25° to 50° C. The number of beakers per analysis is dependent upon the temperature, because the operator is limited to the time lapse between the addition of the indophenyl acetate substrate and the final absorbance reading.

Residue analyses can be performed on purified solutions containing extracted insecticides from plant materials. Ali-

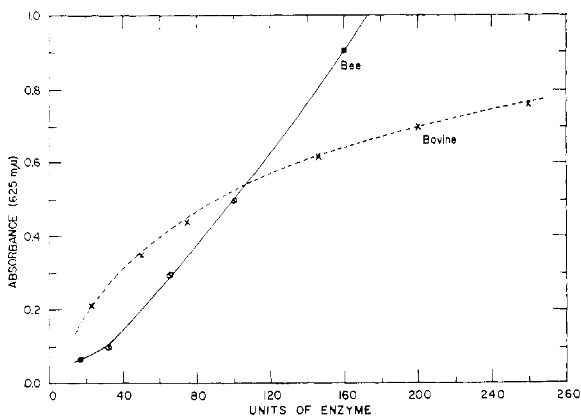

Figure 1. Effect of enzyme concentration upon reaction rate

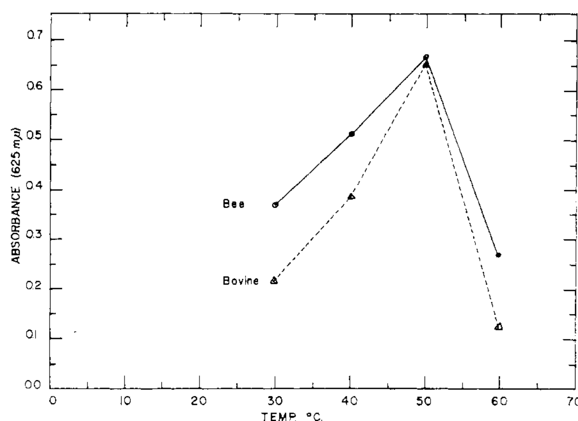

Figure 2. Effect of temperature upon enzymatic activity

quots of the purified stripping solution are pipetted into the beakers, 0.5 ml. of the glycerol reagent is added to each beaker, and the same procedure is followed as for the standard curve series. Any accepted purification procedure can be utilized for the insecticidal residues on crop materials, if no other acetylcholinesterase inhibitors are present. The amount of insecticide in the purified plant extracts is determined from the standard curve which is run simultaneously.

Study of Variables

The experiments described below were carried out in the same manner as those described in the section of procedures, except that the reaction temperature and preincubation time were varied, where specified.

Effect of Enzyme Concentration on Reaction Rate. The enzymatic activity (expressed as absorbance at 625 mμ, indophenylate ion concentration) is plotted against enzyme concentration, expressed in "units of bovine erythrocyte cholinesterase" (see Figure 1). The activity increases rapidly up to 150 units, and then rises slowly between 150 to 250 units of enzyme. This observation is in agreement with that of Kramer and Gamson (*7*). The relative units of enzyme activity for bee brain cholinesterase, when expressed in terms of crystalline bovine erythrocyte acetylcholinesterase, are shown in Figure 1.

Effect of Temperature on Activity of Cholinesterases. The inactivation of crystalline bovine erythrocyte and bee brain cholinesterases due to temperature is shown in Figure 2. The activity of the enzymes increases up to 50° C., but falls off rapidly, probably because of heat denaturation above 60° C.

Preincubation Time Studies with Sevin. The effect of preincubation time of enzyme with inhibitor (Sevin) on enzyme activity (bovine erythrocyte cholinesterase) is shown in Figure 3. The enzyme was incubated with 4 γ of Sevin per beaker for time periods ranging from 0 to 60 minutes. At the end of each specified preincubation period, the substrate was added, and the absorbance was read after 45 minutes at 625 mμ. A control containing enzyme and substrate was used for the calculation of per cent inhibition by the following equation:

$$\% \text{I} = \frac{\text{absorbance (control)} - \text{absorbance (Sevin)}}{\text{absorbance (control)}} \times 100$$

The optimum preincubation time appears to be 30 minutes. There is a slight increase (4%) in the amount of inhibition of the reaction at the preincubation time of 60 minutes.

Effect of Reaction on pH. It was observed that the pH remained constant throughout the enzymatic hydrolysis of $9.6 \times 10^{-5}M$ indophenyl acetate in the absence of an inhibitor. This may be understood, considering that a maximum of $9.6 \times 10^{-5}M$ acetic acid could be released during the reaction, assuming 100% hydrolysis. In most of the experiments the reaction mixture analyzed gave an absorbance of 0.80, corresponding to 65.9% hydrolysis or a molar concentration of $6.90 \times 10^{-5}M$. The molar extinction coefficient for

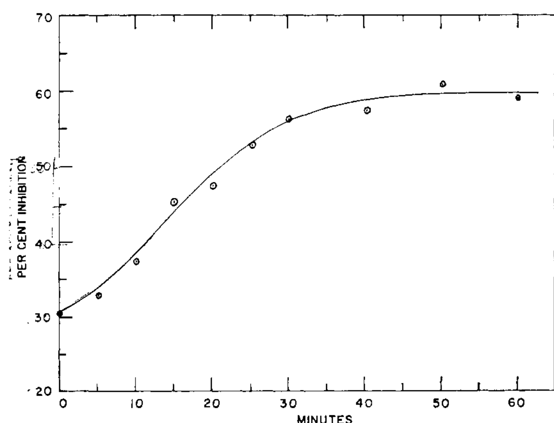

Figure 3. Effect of preincubation time upon enzyme inactivation

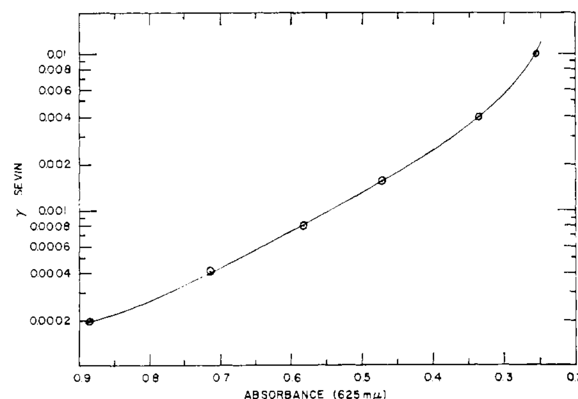

Figure 4. Standard curve for Sevin utilizing brain cholinesterase

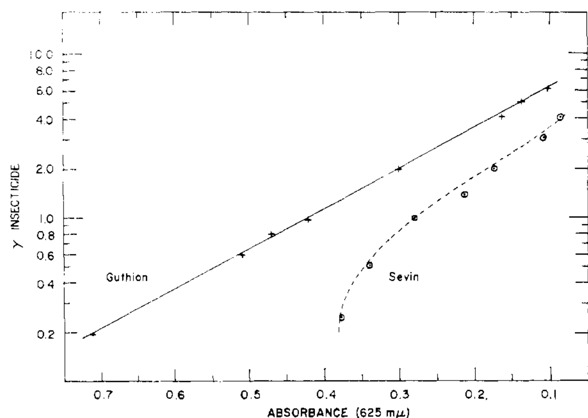

Figure 5. Standard curves for insecticides Sevin and Guthion

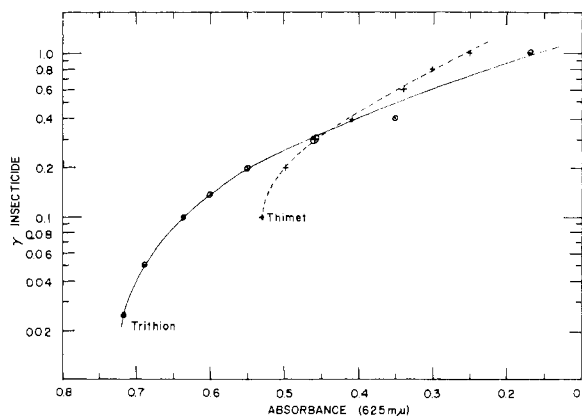

Figure 6. Standard curves for insecticides Trithion and Thimet

indophenylate is 1.16×10^4 cm.$^{-1}$ This was calculated by reading the absorbance at 625 mμ and varying the concentration of indophenylate from 1.00×10^{-5} to $6.44 \times 10^{-5}M$ in phosphate buffer at a pH of 8.0. The value of $9.6 \times 10^{-5}M$ (maximum) may be compared with $2.4 \times 10^{-2}M$ for the final concentration of acetic acid found by the potentiometric analysis method (*4, 11*). These latter values were obtained by titrating barbital buffer (0.036*M*) and phosphate buffer (0.05*M*) with standardized acetic acid to achieve a pH change from an initial value of 8.0 to a final one of 6.0. The constant pH maintained throughout the reaction of the hydrolysis of indophenyl acetate represents an obvious advantage, because the optimum pH reported for cholinesterase is 8.00 (*2, 5*).

Sources for Esterase of Indophenyl Acetate. As may be seen from Figure 1, crystalline bovine erythrocyte cholinesterase served as a good source for the enzymatic hydrolysis of indophenyl acetate. The reaction was inhibited by Sevin over the range of 0 to 4 γ per 5.15 ml. The enzyme from bee head brei seemed to have a much higher sensitivity for Sevin and was inhibited over the range of 0 to 0.01 γ per 5.15 ml. (Figure 4). Similar observations were made using acetylcholine as substrate and comparing cholinesterase from horse serum *vs.* fly head brei (*14*). Although human and horse sera exhibited good enzymatic activity with indophenyl acetate as the substrate, the reaction could be inhibited by Sevin only at such high concentrations that solubility problems interfered (see Table I). These data illustrate that esterases from different biological sources will catalyze the hydrolysis of the same substrate, indophenyl acetate, but will be inhibited to different degrees by equal concentrations of the inhibitor, Sevin.

Table I. Inhibition of Horse Serum Acetylcholinesterase by Sevin Using Indophenyl Acetate as a Substrate

Sevin, γ	2	4	6	8	10	50	100	500[a]	1000[a]
Inhibition, %	4.7	10.2	10.2	19.2	19.1	66.0	68.0	74.0	74.0

[a] Solubility difficulties of Sevin in aqueous buffer were encountered.

Table II. Example of Typical Residue Analyses by Three Methods

(Crop, Fortuna cling peaches)

Method	*Reaction Type*	*Substrate*	*P.P.M.*
Potentiometric	Enzymatic	Acetylcholine chloride	35.0
Colorimetric	Enzymatic	Indophenyl acetate	39.6
Paper chromatographic	Chromatographic	. . .	37.5

Study of Standard Curve Ranges for a Few Insecticides. Using crystalline bovine erythrocyte as the source of the acetylcholinesterase, standard curves were prepared with various insecticides (Figures 5 and 6). The range for Sevin was 0 to 4 γ, Guthion 0 to 6 γ, Thimet 0 to 1 γ, and Trithion 0 to 1 γ. Thimet, Guthion, and Trithion were converted to the oxygen analogs by acetic acid–hydrogen peroxide oxidation before being used as acetylcholinesterase inhibitors.

Residue Analyses. An example of a typical residue analysis is presented in Table II, on Fortuna cling peaches sprayed with a wettable powder of Sevin. The peaches were sprayed and harvested on the same date. The insecticide was stripped from the sample with chloroform, and the stripping solution was purified by the method of Zweig and Archer (*14*). An aliquot of the purified extract was analyzed by the potentiometric and chromatographic methods (*14*), and by the procedure outlined in this paper. Good agreement was shown by the three methods. This procedure is amenable to the analysis of a large number of residue samples by the use of an automatic recording colorimeter.

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