Table III. Analysis of Variance Summary Showing Significance of Results in Table II

Source of Variance	Degrees of Freedom	Sum. of Squares		F Value		Required F Value at 5%
		1-lb. rate	3-lb. rate	1-lb. rate	3-lb. rate	
Total	23	93,426	429,882	..	..	..
Between crops	3	78,084	353,614	48.2[a]	26.5[a]	3.29
Between days	5	7,240	9,685	2.68	0.44	2.90
Remainder	15	8,102	66,583	..	..	..

[a] Highly significant.

Table IV. Tedion Surface Residues on Mature Navel Oranges

(These fruit did not increase in size during this study)

Days after Treatment	Tedion 25% Wettable Powder			
	1 Lb./100 Gal.		3 Lb./100 Gal.	
	Sample 1, p.p.m.	Sample 2, p.p.m.	Sample 1, p.p.m.	Sample 2, p.p.m.
0	1.2 ± 0.1	1.2 ± 0.0	3.3 ± 0.2	3.3 ± 0.1
8	0.9 ± 0.1	1.2 ± 0.1	3.2 ± 0.3	3.2 ± 0.4
24	1.0 ± 0.2	1.0 ± 0.1	2.6 ± 0.4	2.2 ± 0.1
42	0.8 ± 0.1	1.0 ± 0.0	2.5 ± 0.1	2.7 ± 0.1
55	1.1 ± 0.0	..	3.1 ± 0.1	..
100	0.7 ± 0.0	..	2.6 ± 0.1	..
Av.	0.95	1.1	2.9	2.9

Table V. Magnitudes of Tedion Residues Found between Last Application and Harvest

(Tedion applied at rate of 1 lb. of 25% wettable powder per 100 gallons)

Apples		Pears		Peaches		Plums	
Days after spray	Tedion, p.p.m.	Days after spray	Tedion, p.p.m.	Days after spray	Tedion, p.p.m.	Days after spray	Tedion, p.p.m.
27	1.4	12	2.1	7	2.9	3	1.3
32	1.1	27	1.2	19	1.7	6	0.8
35	2.3	28	2.6	19	0.9	9	0.8
47	0.5	28	2.1	26	1.4	18	0.6
51	0.7	30	0.8	28	0.5	19	0.8
60	0.7	32	0.7	30	0.4	19	0.2
76	0.6	51	0.4	32	0.8	27	0.8
98	0.1	63	0.5	41	0.6	27	0.8
120	0.6	63	0.3	41	0.5	35	0.2
130	0.2	102	0.1	..	..	35	0.3

residues except that caused by fruit growth. These data indicate that Tedion is very stable when exposed in spray residue form on tree fruits under high summer temperatures and that its vapor pressure must be low at temperatures from 75° to 100° F., the range in which these studies were conducted.

A second time study of Tedion surface residues on navel oranges is given in Table IV. In this case, the residues were followed over a period of 100 days. The results are expressed only in terms of parts per million because the oranges were mature when sprayed and records did not show any increase in fruit weight during the 100-day study. The data show no significant decrease in Tedion residues over the 100-day period, indicating again that Tedion is very stable when exposed to weathering. The plus and minus values shown in this table are standard deviations calculated from three to eight replicate chemical analyses of a single strip solution, thus showing the precision of the colorimetric method employed in this study.

Additional data showing the magnitude of Tedion residues found on random samples of apples, pears, peaches, and plums from 39 different orchards under different climatic conditions are given in Table V. This is not a true time study, because no two samples came from the same orchard. It does illustrate the magnitude of Tedion residues to be expected on mature fruit when picked at the indicated interval after the last spray. In these studies 25% Tedion wettable powder at 1 pound per 100 gallons was applied to the indicated crops.

To date only two samples of grapes have been analyzed. These samples contained 0.5 and 0.6 p.p.m. of Tedion at 77 and 100 days, respectively, after a spray of 6 pounds of 25% Tedion wettable powder per acre was applied to the grapevines.

Biological performance data obtained on both citrus and deciduous trees show that Tedion has a long residual value against several species of mites over periods of 3 to 9 months. The chemical residue data presented here appear to be in accordance with the biological observations.

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INSECTICIDE RESIDUES

Residue Determination of Sevin (1-Naphthyl *N*-Methylcarbamate) in Wine by Cholinesterase Inhibition and Paper Chromatography

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THE RECENTLY DEVELOPED insecticide, Sevin (1-naphthyl *N*-methylcarbamate) (Union Carbide Chemicals Co.) has found use in the control of the grape leaf folder (*10*). It was necessary, therefore, to develop a sensitive method for residue analysis of Sevin in wine made from grapes which had been sprayed with a formulated mixture of Sevin and sulfur. Two available methods are based on the hydrolysis of Sevin to 1-naphthol and the subsequent color development with aminoantipyrine or *p*-nitrobenzenediazonium fluoborate by diazotization (*2, 8*). An enzymatic

The recently developed insecticide Sevin has found use in the control of the grape leaf folder. A method had to be developed for the analysis of Sevin residues in wine made from treated grapes. Two independent methods were developed: cholinesterase inhibition and paper chromatography. The first method determines Sevin, while the latter determines Sevin and its breakdown product, 1-naphthol. Results on the same sample by both methods were in good agreement. Simultaneous analyses for Sevin and 1-naphthol are necessary for U. S. Department of Agriculture registration of Sevin as a safe insecticide. Previously developed methods accounted for both compounds but did not differentiate between them. These new methods will also aid in the detoxification studies of Sevin, as 1-naphthol has been found to be a metabolic breakdown product.

method for the determination of Sevin has been developed by Moorefield and Tefft (*9*).

The colorimetric methods are subject to several limitations: They require relatively large residues, of the order of 20 γ. They do not differentiate between Sevin and its hydrolysis product 1-naphthol. The residue samples must be thoroughly cleaned up in order to minimize the blank reading due to natural pigments or other substances that react with the color reagents. Hence another method for residue analysis of Sevin was sought.

Sevin has been reported to be an efficient inhibitor of cholinesterase from fly heads (*7*). Because an enzymatic assay based on this inhibition presumably would determine Sevin only, an additional method was investigated which would differentiate between Sevin and 1-naphthol. Paper chromatography seemed to be suitable.

Experimental

Cholinesterase Method. This method is based on the procedure for the enzymatic analysis of organic phosphorus insecticides developed by Giang and Hall (*5*).

Reagents. Barbital buffer solution, 7.42 grams of sodium barbital, 89.46 grams of potassium chloride, and 1.09 grams of monobasic potassium phosphate are dissolved in 1 liter of distilled water. The pH is adjusted, if necessary, to 8.0 ± 0.05 with 0.1*N* hydrochloric acid.

Acetylcholine bromide, 4.97 grams in 100 ml. of distilled water (0.22*M*).

Saline solution, 9 grams of sodium chloride in 1 liter of distilled water.

Cholinesterase solution, 9.6 ml. of fresh horse plasma, 10.4 ml. of saline, and 40.0 ml. of barbital buffer freshly mixed and kept cold in an ice bath. Human plasma, crystalline bovine erythrocyte cholinesterase, or fly head brei may be substituted for the fresh horse plasma.

Glycerol solution, 10 ml. of glycerol made up to 100 ml. with absolute methanol.

Sevin standard solution, 10.0 mg. of Sevin in 100 ml. of absolute methanol. It is diluted 1 to 10 to give a final concentration of 10 γ Sevin per milliliter (Sevin, pure, Union Carbide Chemicals Co., Lot 327 RD 20).

Calibration Curves. One-tenth to 0.5 ml. of dilute Sevin standard solution and 0.5 ml. of glycerol are pipetted into each of five 10-ml. Griffin beakers. Two additional beakers do not contain any insecticide and serve as controls. The water in the beakers is evaporated with a gentle stream of cool air from a hair drier. Then 3.0 ml. of the cholinesterase solution is added to each beaker at intervals of 2 minutes. The maximum number of samples which can be handled conveniently is 15, including the standards. Small pieces of glass rod (5 × 20 mm.) are placed in each beaker, which in turn is placed on the platform of an automatic rocking shaker [Microchemical Specialty Co. (*6*)]. This gentle rolling action gives good agitation and aids in obtaining reproducible results. The temperature is kept at 25° ± 1° C. by placing the entire apparatus in an open, insulated wooden box and controlling the temperature manually with a 100-watt incandescent light bulb.

After 30 minutes, the pH of beaker 1 is read at 25° C. The pH in the other beakers, containing standard solutions, was found to be constant, and this step was eliminated. However, the pH of beakers containing samples for residue analysis had to be read individually during the previous 30-minute period. At exactly 32 minutes, 1.0 ml. of ice-cold acetylcholine bromide solution is added to beaker 2; at 34 minutes the addition to beaker 3 commences. Sixty minutes after the addition of acetylcholine to the first beaker, the final pH is determined in beaker 2 and then in successive beakers at 2-minute intervals.

The "per cent inhibition" is calculated using the following formulas:

$$\Delta \text{ pH} = \text{pH}_{(\text{initial})} - \text{pH}_{(\text{final})} \quad (1)$$

$$\% \text{ inhibition} = \left(1 - \frac{\Delta\text{pH}_{(\text{sample})}}{\Delta\text{pH}_{(\text{control})}}\right) \times 100 \quad (2)$$

The results for a typical calibration curve are shown in Figure 1.

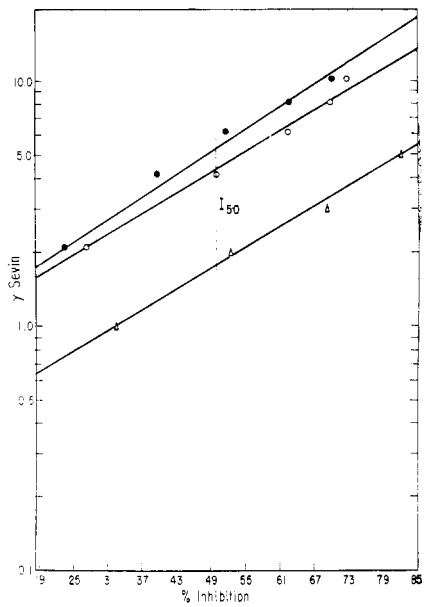

Figure 1. Calibration curve for Sevin by cholinesterase inhibition method

△ Horse plasma
○ Bovine erythrocytes (crystalline enzyme)
● Human plasma

Paper Chromatographic Method

This method is based on the work by Freeman (*4*) on the separation of phenols by paper chromatography.

Reagents. 1-Naphthol, purified with charcoal and recrystallized twice from water. 1-Naphthol and Sevin standard solutions, 0.5 mg. per milliliter in absolute methanol. Chromatographic solvent, methanol:water = 2:8 (v./v.).

p-Nitrobenzenediazonium fluoborate (Eastman P 7078).

Apparatus. A 10-gallon glass aquarium is fitted with three glass rods along the length of the chamber, 5 cm. from the top edge (*1*). The rods are held in place by No. 1 rubber stoppers inserted at each end of the glass rods. Paraffin seals are found to be unsuitable for the methanol-water solvent.

Micropipets, 2-μl. (Lambda pipets).

Photovolt densitometer with 570-mμ filter and 5-mm. diameter round aperture.

Calibration Curve. A mixture of Sevin and 1-naphthol is spotted on a 10 × 12 inch sheet of Whatman No. 20 filter paper, along the long direction, 1 inch from the bottom, starting 1 inch from the left edge. The following amounts are spotted by single or multiple applications using the 2-μl. pipet: 0.5, 1.0, 1.5, and 2.0 γ. The chromato-

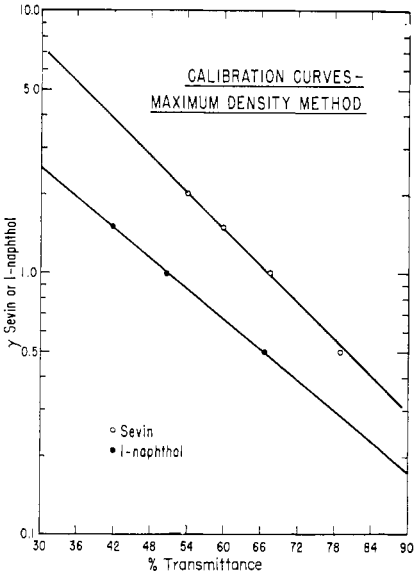

Figure 2. Calibration curves for Sevin and 1-naphthol by maximum density method

gram is developed for 120 minutes without previous equilibration by the ascending technique with methanol as solvent. The solvent front traveled about $6^1/_2$ inches above the point of application. The chromatogram is dried in a hood at room temperature and is sprayed evenly with 1.5*N* methanolic sodium hydroxide–1-butanol (v./v.). This treatment hydrolyzes Sevin to 1-naphthol. After the paper has been dried in the hood, it is dipped into 0.01% *p*-nitrobenzenediazonium fluoborate dissolved in methanol–ethyl ether (v./v.). Well defined blue spots appear, giving a good separation of Sevin and 1-naphthol with the following R_f values: 1-naphthol 0.37; Sevin 0.65.

The maximum density of the spots is read directly off the paper with a densitometer and a 570-mμ filter. The per cent transmittance is plotted against the log concentration and a straight line results, as seen in Figure 2. The straight-line relationship is in accord with the findings of Block (*1*), who used the maximum-density method for the quantitative determination of the amino acids.

Preparation of Sample. One hundred milliliters of wine is extracted in a separatory funnel once with 200 ml. of chloroform: ethanol (v./v.), followed by two 100-ml. extractions with the same solvent mixture. If an emulsion forms, it may be broken up by the addition of 5.0 ml. of saturated aqueous sodium sulfate. The chloroform-ethanol layer is drawn off and evaporated in vacuo using a Rinco evaporator. The residue is dissolved in 100 ml. of 50% aqueous methanol, transferred to a separatory funnel, and extracted once with 50 ml. of petroleum ether (35° to 60° C.) in order to remove any waxes. The ether layer is washed once with 50 ml. of 50% methanol, and the combined methanol fractions are extracted twice with 200-ml. portions of redistilled benzene. (Reagent grade benzene contains a trace of a high-boiling component which interferes with the paper chromatography but not with the cholinesterase method.) The benzene layer is dried over sodium sulfate and is evaporated in vacuo. The residue is dissolved in 10 ml. of benzene for the cholinesterase method or in 0.10 ml. of methanol for paper chromatography. When methanol is employed, a small amount of insoluble residue settles on the bottom of the glass vial, but only the clear supernatant liquid is spotted on the paper chromatogram.

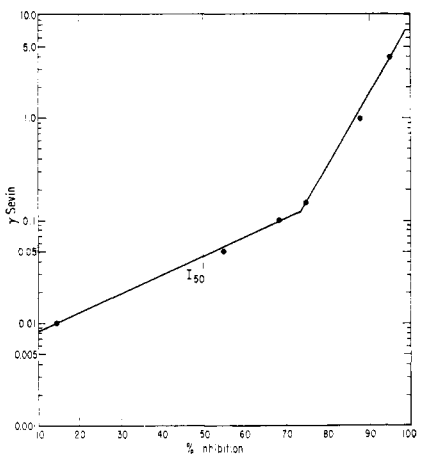

Figure 3. Calibration curve for Sevin using fly head cholinesterase

Residue Analysis by Cholinesterase Method. A 1.0-ml. aliquot of the purified benzene extract, equivalent to 10 ml. of wine, is placed in a 10-ml. beaker and is evaporated under a gentle stream of cool air. A standard curve is constructed by the method previously described and is run concurrently with each set of wine samples to be analyzed. Control samples of wine from untreated grapes are also run, and the blank (apparent γ of Sevin) is subtracted from the residue samples.

Residue Analysis by Paper Chromatography. Two microliters of the purified wine extract, dissolved in methanol, is spotted alongside the standards of 1-naphthol and Sevin on the same sheet of paper. This method permits the simultaneous determination of these two compounds. Duplicate spots for each sample at two concentrations and triplicates or quadruplicates of standards at three to four concentrations (0.5 to 2.0 γ) yield good reproducibility.

Results and Discussion

Cholinesterases from the following biological sources were investigated: fly head brei, crystalline cholinesterase from bovine erythrocytes, human plasma, and horse plasma. The amount of enzyme was chosen so that a Δ pH of 1.5 to 2 was effected in 60 minutes at 25° C. with no inhibitor present. All other experimental conditions were the same as described in the experimental section. Figures 1 and 3 show the standard curves for Sevin obtained by using cholinesterase from bovine and human blood and fly heads. From these curves and the curve from horse plasma (Figure 1), the I_{50} values for Sevin were calculated. I_{50} is the molarity of inhibitor which results in 50% of the activity of the control. Table I lists the I_{50} values of Sevin for four different cholinesterases. The value of I_{50} for housefly head cholinesterase is of the same order of magnitude as that reported by others (*7*). The hundredfold decrease in sensitivity of Sevin on mammalian cholinesterase may be in accord with the low mammalian toxicity of the compound (*7*). Because the availability of fly heads may create a problem in some laboratories, it was decided to choose the most sensitive mammalian cholinesterase source, horse plasma.

Table I. I_{50} of Sevin for Cholinesterases

Enzyme Source	*Molarity, Sevin*
Housefly heads	5.45×10^{-8}
Horse plasma	2.18×10^{-6}
Bovine erythrocytes	5.10×10^{-6}
Human plasma	6.70×10^{-6}

The sensitivity of detection is as low as 0.5 γ of Sevin, whereas the limit of detection for the colorimetric analyses ranges from 10 to 100 γ (*2*, *8*). Recovery by the cholinesterase method ranged from 74.5 to 96% when 0.2 p.p.m. of Sevin was added to the wine or directly to the purified extract. The addition of 0.5 p.p.m. of 1-naphthol to the control wine or the extract did not have any inhibitory effect on cholinesterase activity. This indicates that this enzymatic method is specific for Sevin in samples of known history. Table II summarizes the data for residue analyses of Sevin from two samples of wine.

Preliminary paper chromatography of extracts from red wine (Alicante) produced a large purple spot with an R_f higher than that of Sevin, with methanol-water = 2:8 (v./v.). This spot partly covered up the spot due to Sevin and, therefore, interfered with the maximum density readings. It was decided to test the method of analysis with white wine extracts (Thompson seedless). These samples resulted in a good separation of 1-naphthol, Sevin, and this same purple spot without interference or displacement of the Sevin spot. A solvent consisting of 1-butanol–concentrated ammonium hydroxide = 4:1 (v./v.) moved the spot due to Sevin to the Solvent front, but did not separate it from 1-napthol. Three samples were analyzed: control, treated sample, and treated sample to which a known amount of 1-naphthol

had been added (1 p.p.m.). The results, summarized in Table III, show that a trace of a compound, having the same R_f as 1-naphthol, is present in the control sample. It is also indicated that Sevin is partially hydrolyzed to 1-naphthol.

Figure 4 is a photograph of a paper chromatogram of Sevin, 1-naphthol, and a wine extract, with and without added 1-naphthol. The position of the faster moving purple spot, ahead of Sevin, may be seen. This spot indicates the presence of compounds in wine which are readily diazotized, probably phenols.

Comparing the residue values of Sevin, obtained by the cholinesterase and paper chromatography methods, it is seen that the results are in fair agreement: 0.43 p.p.m. compared to 0.30 p.p.m. This discrepancy may be explained by the relatively high blank due to the variation of the paper background. When one ignores this blank, one obtains a residue value of 0.49 p.p.m. These results may be compared with the value of 0.48 p.p.m. obtained for the same sample by the colorimetric method of Miskus (*3*, *8*).

During the paper chromatography of Sevin and 1-naphthol, caution should be exercised to exclude phenol vapors from the room or the chromatographic cabinet. Even a trace of phenol will cause a red background on the sprayed chromatogram due to the diazotization reaction.

The importance of the simultaneous analysis of 1-naphthol and Sevin was brought out by a preliminary experiment studying the possible mode of detoxification of Sevin in mammals. Sevin was incubated with a fresh homogenate of rat liver, and the resultant deproteinized solution was analyzed by the paper chromatographic method. A trace of 1-naphthol was found, indicating a possible explanation for the relatively low mammalian toxicity of Sevin due to a detoxification mechanism. Further experiments are in progress studying the breakdown of Sevin in mammals. Further work is also in progress adapting these methods for the residue analyses of Sevin in several other crops, including apples, pears, and grapes.

Table II. Residue Analyses of Sevin in Wine by Method of Inhibition of Horse Plasma Cholinesterase

(See Figure 1 for standard curve)

Sample (10 Ml. Wine)	Sevin Added, P.P.M.	Inhibition, %	Sevin Found, γ	Recovery, %	Net Sevin, P.P.M.
8575 A[a]	0	62.6	2.65	...	0.18
8575 B	0	82.3	5.00	...	0.41
8575 control	0	27.9	0.87	...	...
	0.20 to wine	58.6	2.36	74.5	0.15
	0.20 to extract	64.0	2.79	96.0	0.19
8576 A[b]	0	32.0	1.00	...	0.14
8576 B	0	85.0	5.45	...	0.43
8576 control	0	36.0	1.14	...	...
	0.20 to wine	65.3	2.90	88.0	0.18
	0.20 to extract	65.3	2.90	88.0	0.18

[a] 8575 Alicante wine.
[b] 8576 Thompson seedless wine.

Table III. Maximum-Density Method of Analysis for Sevin and 1-Naphthol

(Refer to Figure 2 for standard curve)

Sample	Wine, Ml.	1-Naphthol Added, P.P.M.	Transmittance, % 570 Mμ: Sevin	Transmittance, % 570 Mμ: 1-Naphthol	Found, γ: Sevin	Found, γ: 1-Naphthol	1-Naphthol Recovery, %	Sevin, Net P.P.M.
8576 B[a]	1.0	0	80.0	85.0	0.50	0.22	...	0.30
8576 B	2.0	0	68.5	78.7	0.92	0.29	...	0.26
	1.0	1.0	78.5	52.6	0.54	0.90	72.0	0.34
	2.0	2.0	68.0	43.2	0.95	1.4[b]	55.6	0.28
8576 B control	1.0	0	96.0	90.0	0.20	0.18	...	...
							Av. With blank	0.30
							No blank	0.49

[a] Thompson seedless wine.
[b] At this concentration 1-naphthol streaked and gave low results.

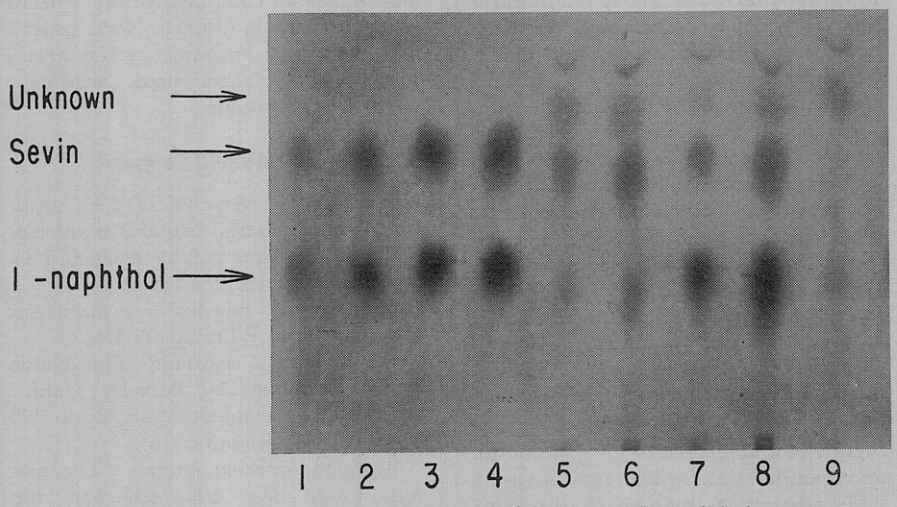

Figure 4. Paper chromatogram of Sevin and 1-naphthol

1, 2, 3, 4. Standards. 5, 6, 7, 8. Wine extracts (with residue). 9. Wine extract (control)

Acknowledgment

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