Separation and Measurement of 3-Hydroxy-*N,N*-dimethyl-*cis*-crotonamide Dimethyl Phosphate (Bidrin Insecticide) and 3-Hydroxy-*N*-methyl-*cis*-crotonamide Dimethyl Phosphate (Azodrin Insecticide) Residues in Crops by Selective Cleanup (Partition) Procedures

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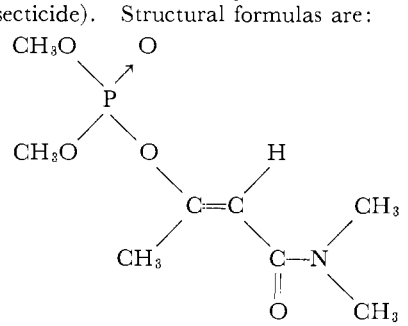
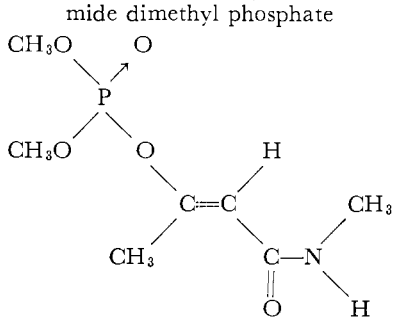
Techniques are described for the selective determination of two phosphate insecticides: 3-hydroxy-*N,N*-dimethyl-*cis*-crotonamide dimethyl phosphate (Bidrin) and its *N*-monomethyl analog (Azodrin). The analysis is carried out in two steps: a separation procedure in which the pesticide is selectively extracted into aqueous solution and separated from interferences and other organophosphates by differential liquid-liquid partition, and an analytical procedure involving cholinesterase inhibition measurements. After differential extraction and cleanup, Bidrin and Azodrin are separated on a liquid-liquid chromatographic column with water as the stationary phase. This system has advantages of simplicity, sensitivity, and a high degree of specificity. Recovery experiments and specificity tests on 41 commercial insecticides are presented. Interference values from 37 of the insecticides tested were less than 1% in the case of Bidrin, and in the case of Azodrin, only one insecticide, Dipterex, interfered to more than 2%. Auxiliary procedures are given for removing those insecticides which did interfere. The sensitivity of the method is 0.10 p.p.m.

TWO VINYL PHOSPHATES which have recently been found very effective for agricultural use are 3-hydroxy-*N,N*-dimethyl-*cis*-crotonamide dimethyl phosphate (Bidrin insecticide) and its *N*-monomethyl analog (Azodrin insecticide). Structural formulas are:



Bidrin Insecticide

3-Hydroxy-*N,N*-dimethyl-*cis*-crotonamide dimethyl phosphate



Azodrin Insecticide

3-Hydroxy-*N*-methyl-*cis*-crotonamide dimethyl phosphate

The first insecticide is effective for the control of aphids, mites, and flea hoppers on cotton and is a unique systemic for the control of the bark beetle on elm trees. The second has shown outstanding control of bollworms and cabbage loopers on cotton plants.

To obtain residue data for the registration of these two insecticides, sensitive analytical methods are needed to measure trace concentrations existing as residues on crops following application by spraying or dusting. It is important that the methods also be specific.

When the program was first initiated to obtain residue data on Bidrin, the analytical method used was a cholinesterase-inhibition method. This was very effective because Bidrin is a potent inhibitor of human blood serum cholinesterase. Thus, an analytical method that was simple, sensitive, not greatly affected by crop interferences, and well adapted to laboratory schedule was available. However, the method lacked specificity.

Since the first investigations by Giang and Hall in 1951 (*1*) of the use of ChE measurements to determine organophosphate insecticides, other workers have applied the technique to a variety of pesticide residue problems. These investigations have been nonspecific in nature and little attention was paid to the isolation and separation of the particular pesticide under study. However, ChE methods can be made specific when combined with suitable chemical or physical techniques which selectively isolate a compound.

This paper describes procedures by which Bidrin and Azodrin may be differentially isolated in aqueous solution and quantitatively measured by an enzyme - inhibition spectrophotometric method. Residues in the range of 0.1 to 1.0 μg. are measured within a relative error of 20% without interference from other insecticides. This system has advantages of simplicity, sensitivity, and a high degree of specificity.

Anticholinesterase Activities

Bidrin and Azodrin insecticides are both strong inhibitors of the cholinesterase enzymes from either human blood serum or fly-head homogenate. Human blood serum was used in these studies. The I_{50} inhibition of human blood serum cholinesterase, using the procedure for determination of cholinesterase inhibition previously described for Vapona insecticide (*4*), is about 0.40 μg. per ml. for the former and about 0.80 μg. per ml. for the latter. Their pI_{50} values (log of the reciprocal of the concentration of organophosphate in moles necessary for 50% inhibition = log $1/I_{50}$ in moles/liter) are 5.78 and 5.45, respectively. With these activities, 0.05 μg. of Bidrin or 0.10 μg. of Azodrin may be conveniently detected. However, because the blank values from crop extracts are of the order of a few hundredths of 1 p.p.m., the actual sensitivity obtainable on crops with a good degree of accuracy is of the order of 0.10 p.p.m.

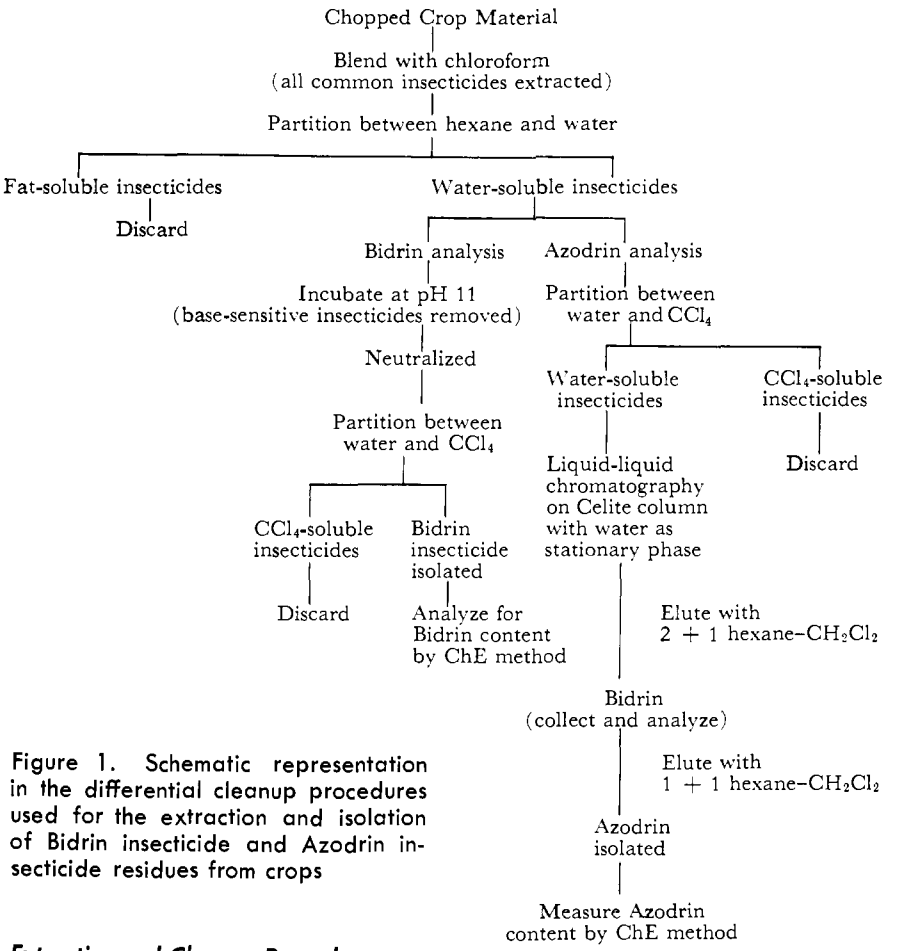


Figure 1. Schematic representation in the differential cleanup procedures used for the extraction and isolation of Bidrin insecticide and Azodrin insecticide residues from crops

Extraction and Cleanup Procedures

A schematic representation of the procedures used for the extraction and isolation of Bidrin insecticide and/or Azodrin insecticide from crops is shown in Figure 1.

In this procedure, 500 grams or more of a representative crop sample is subdivided into small pieces and 100 grams of the divided material is transferred into a blender cup and blended with 400 ml. of chloroform or methylene chloride. The mixture is filtered, the resulting extract is dried and concentrated, and the solvent is exchanged to hexane. This hexane extract is shaken with water to partition Bidrin or Azodrin residues quantitatively into the aqueous phase. By washing the aqueous phase with hexane and carbon tetrachloride (in special cases, also with benzene) other organophosphates are removed. The isolated Bidrin or Azodrin is then measured enzymatically according to the procedure described for Vapona insecticide (*4*).

Partition Characteristics of Bidrin Insecticide and Azodrin Insecticide

Bidrin and Azodrin insecticides are both extremely hydrophilic and remain quantitatively in the water phase when shaken in a system composed of equal volumes of hexane and water; between carbon tetrachloride and water, the distribution coefficients are very much in favor of the aqueous phase—20 for Bidrin and 100 for Azodrin. In view of this, partitioning between water and hexane or carbon tetrachloride is a convenient means for separating these two insecticides from many other insecticides. These favorable partition characteristics are fully exploited in the method which calls for six separate solvent washes of a 50-ml. aqueous solution with 50 + 25 + 25-ml. aliquots of carbon tetrachloride followed by 3 × 50 ml. of hexane.

Table I shows the efficacy of liquid-liquid partitioning systems for removing other insecticides. Hexane-water partitioning effectively removed 26 out of 41 insecticides from the water phase, while additional washing of the aqueous phase with carbon tetrachloride removed another nine.

Characterization of Bidrin by Treatment with Aqueous Base. Bidrin insecticide is more stable in alkaline solution than most other cholinesterase-inhibiting insecticides. This provides another means for introducing specificity. Tests have shown that only 3 to 5% of Bidrin is decomposed after 2 hours in 0.25% sodium carbonate solution (pH 11) at room temperature, whereas other organophosphates are more severely destroyed.

Using the above described liquid-liquid partitioning and base equilibration procedures, the theoretical recovery of Bidrin following quantitative extraction from a crop sample is 85%. The actual recovery from chloroform extracts of various crops, analyzed by the described method, averaged 81%, as indicated in Table II. On the basis of the maximum possible recovery calculated from distribution coefficients and stability in 0.25% sodium carbonate solution, the actual recovery is 81/85 × 100 or 95%.

The method for the determination of Bidrin residues in crops, as outlined in Figure 1, was further tested for recovery and specificity on various crop extracts. These tests consist essentially of: a crop check, crop check plus 0.10 p.p.m. of Bidrin, and Bidrin-fortified extract plus test insecticide at 10 times the Bidrin fortification level. Each was carried through the described procedure.

Table III shows that Diazinon, Isolan, and TEPP interfere in the Bidrin method. The recovery values of 130% for both

Table I. Interference from 20-μg. Quantities of Different Insecticides after Various Steps in the Cleanup Procedure

	Interference as Apparent Bidrin Remaining after Treatment, %		
Insectide Added	*Hexane wash only*	*With CCl_4 wash*	*After Na_2CO_3 treatment*
Aldrin	<1	...	...
Baytex	<1	...	...
Ciodrin	<1	...	...
Chlorothion	<1	...	...
Co-Ral	<1	...	...
DDT	<1	...	...
DDVP	2.4	<1	...
Delnav	<1	...	...
Demeton	>10	>10	<1
Diazinon	>10	4 or more	1 to 4
Diazinon oxon	>10	>10	1 to 3
Dieldrin	<1	...	...
Dimethoate	<1	...	...
Dipterex	>10	<1	...
Disyston	<1	...	...
Endrin	<1	...	...
EPN	<1	...	...
Ethion	<1	...	...
Ethion oxon	<1	...	...
Guthion	<1	...	...
Guthion oxon	<1	...	...
Heptachlor	<1	...	...
Heptachlor epoxide	<1	...	...
Isolan	>10	6 to >10	2 to 4
Lindane	<1	...	...
Malathion	<1	...	...
Malathion oxon	<1	...	...
Methyl parathion	2 to 5	<1	...
Methyl paraoxon	>10	<1	...
Naled	>10	<1	...
Parathion	2 to 4	<1	...
Para oxon	>10	<1	...
Phosdrin	>10	>10	<1
Phosphamidon	>10	<1	...
Schradan	<1	...	...
SD 7438	<1	...	...
SD 7859	<1	...	...
Sevin	<1	...	...
TEPP	>10	>10	>10
Thimet	>10	<1	...
Zectran	<1	...	...

Table II. Recovery of Bidrin Insecticide Added to Various Crop Extracts

Run No.	Crop	Bidrin, P.P.M. Added	Bidrin, P.P.M. Recovered	Recovered, % Actual	Recovered, % Theory
1	Alfalfa	0.20	0.16	80	94
2	Alfalfa	0.20	0.17	85	100
3	Alfalfa	0.10	0.07	70	82
4	Broccoli	0.20	0.16	80	94
5	Broccoli	0.20	0.17	85	100
6	Cabbage	0.20	0.18	90	106
7	Corn foliage	0.10	0.08	80	94
8	Cottonseed meal	0.10	0.075	75	88
9	Peas	0.20	0.14	70	82
10	Peas	0.20	0.14	70	82
11	Potatoes	0.10	0.085	85	100
12	Water	0.20	0.17	85	100
13	Water	0.20	0.15	75	88
14	Water	0.20	0.19	95	112
15	Water	0.20	0.18	90	106
16	Water	0.20	0.18	90	106
			Av.	81	95

Table III. Recovery of Bidrin Residues from Crop Extracts in Presence of Other Organophosphate Insecticides

(0.10 p.p.m. Bidrin added)

Crop	Other Insecticide Added: Insecticide	Other Insecticide Added: P.p.m.	Bidrin Recovered, %: Actual	Bidrin Recovered, %: Calcd.[a]
Alfalfa	None	...	70	82
	Diazinon oxon	1.0	70	82
	Dimethoate	1.0	65	77
	Baytex	1.0	65	77
	Schradan	1.0	65	77
	Isolan	1.0	77	90
Cottonseed meal	None	...	75	88
	Co-Ral	1.0	80	94
	Dipterex	1.0	75	88
	Malathion	1.0	80	94
	Phosdrin	1.0	75	88
Potatoes	None	...	85	100
	Methyl paraoxon	1.0	85	100
	Chlorothion	1.0	87	103
	Naled	1.0	85	100
	Phosphamidon	1.0	83	98
Corn foliage	None	...	82	96
	Diazinon	1.0	110	130
	EPN	1.0	82	96
	Thimet oxon	1.0	85	100
	Isolan	1.0	110	130
	TEPP	1.0	>200	Complete interference

[a] % actual x experimental loss factor of 1.175.

Table IV. Bidrin and Azodrin Insecticides Found in 10-Ml. Fractions in Chromatographic Effluent

Effluent Fraction, Ml.	Toxicant Found in Each 10-Ml. Effluent Fraction, %: Column developed with with 180 ml. 2 + 1 hexane–CH_2Cl_2		Toxicant Found in Each 10-Ml. Effluent Fraction, %: Column developed with 90 ml. 2 + 1 hexane–CH_2Cl_2 90 ml. 1 + 1 hexane–CH_2Cl_2	
0 to 10	0		0	
10 to 20	0		0	
20 to 30	0		0	
30 to 40	2		2	
40 to 50	55	Total 103% Bidrin recovery	58	Total 95% Bidrin recovery
50 to 60	44		34	
60 to 70	2		1	
70 to 80	0		0	
80 to 90	0		0	
			start 1 + 1 mixture	
90 to 100	0		0	
100 to 110	10		19	
110 to 120	30	Total 98% Azodrin recovery	70	Total 101% Azodrin
120 to 130	34		12	
130 to 140	15		0	
140 to 150	9		0	
150 to 160	0		0	
160 to 170	0		0	
170 to 180	0		0	

Diazinon and Isolan, when each was added at 10 times the Bidrin fortification level, indicate that these two compounds offer interferences amounting to 3% as apparent Bidrin. These interferences may be effectively removed by auxiliary treatments. Thus, Diazinon and its oxygen analog, obtained by treatment with dilute bromine water, partition very strongly into benzene from water solution. Isolan behaves similarly but to a lesser degree. After the standard cleanup, an additional wash with 25 ml. of benzene will remove these compounds so that their final interference is reduced to less than 1%. This treatment results in a concomitant 10% loss of Bidrin. When this auxiliary benzene wash is used, the recovery factor to be applied in calculating Bidrin residues from an observed ChE inhibition reading is 100/75 instead 100/85.

The remaining compound which interferes, TEPP, is extremely susceptible to hydrolysis. Residues of this compound would not normally be present on crops. However, should TEPP residues be known to be present in a Bidrin sample, they may be removed by introducing a more severe base-degradation step in the cleanup procedure. Instead of standing at room temperature, the aqueous extract (in 0.25% Na_2CO_3) is incubated at 30° C. for 2 hours. The rest of the procedure remains unchanged.

Chromatographic Separation of Bidrin Insecticide from Other Insecticides. A liquid-liquid chromatographic column is also used to separate and resolve Bidrin insecticide from other interfering insecticides, including its monomethyl analog, Azodrin insecticide. In this technique, a chromatographic column is made by packing a 2-cm. glass column with 8 grams of Celite 545 previously mixed and dampened with 5 ml. of water. When an organic extract containing a mixture of insecticides is passed through this column, separate bands are obtained. The fat-soluble insecticides will emerge in the fore bands and are discarded. Bidrin and Azodrin, when present, will emerge as sharp and distinct peaks with characteristic retention times. The different effluent fractions can be transferred directly into water solution and analyzed for Bidrin or for Azodrin by the enzyme-inhibition spectrophotometric procedure. Recovery of Bidrin and Azodrin amounts to 93 to 103%.

As a typical example, a mixture of 2 μg. each of Azodrin, Bidrin, Isolan, and Thimet is chromatographed through a column packed with a mixture of 8 grams of Celite and 5 ml. of water. Development of the column with 50 ml. of hexane followed by 50 ml. of 4 + 1 hexane – methylene chloride mixture, then by 70 ml. each of 2 + 1 and 1 + 1 hexane–methylene chloride mixtures, respectively, produces four distinct peaks

Table V. Recovery of Bidrin and Azodrin Residues from Crop Extracts Containing Insecticides at 0.10 P.P.M. after LLC Cleanup and Separation

Crop	Bidrin Found in Fraction C (2 + 1 Hexane–CH_2Cl_2) of Chromatographic Effluent		Azodrin Found in Fraction D (1 + 1 Hexane–CH_2Cl_2) of Chromatographic Effluent	
	P.p.m.	Recovery, %	P.p.m.	Recovery, %
Alfalfa	0.090	90	0.090	90
Brussels sprouts	0.080	80	0.090	90
Corn foliage	0.070	70	0.072	72
Cottonseed meal	0.087	87	0.085	85
Lettuce	0.070	70	0.097	97
Pasture grass	0.081	81	0.095	95
Wheat	0.090	90	0.095	95
		Av. 81		89

Table VI. Recovery of Bidrin and Azodrin Residues in Crop Extracts Containing Other Organophosphates after LLC Cleanup and Separation

(0.10 p.p.m. of Bidrin or Azodrin added)

Crop	Foreign Organophosphate Added at 1.0 P.P.M.	Recovery of Bidrin in Fraction C of Chromatographic Effluent		Recovery of Azodrin in Fraction D of Chromatographic Effluent	
		Found, p.p.m.	Recovered, %	Found, p.p.m.	Recovered, %
Alfalfa	DDVP	0.085	85	0.085	85
	Dibrom	0.085	85	0.080	80
	Dimethoate	0.085	85	0.085	85
	Dipterex	>0.15	>150	0.130	130
	Diazinon oxon	0.090	90	0.085	85
	Paraoxon	0.090	90	0.085	85
	Systox	0.085	85	0.095	95
	Thimet	0.085	85	0.080	80
	TEPP	0.125	125	0.090	90
Cottonseed meal	Baytex	0.095	95	0.080	80
	Delnav	0.085	85	0.095	95
	Disiston	0.095	95	0.080	80
	EPN	0.085	85	0.085	85
	Parathion	0.100	100	0.095	95
	Guthion	0.090	90	0.095	95
	Naled	0.085	85	0.085	85
	Sevin	0.095	95	0.095	95
	Dipterex	>0.150	>150	>0.150	>150
Pasture grass	Chlorothion	0.090	90	0.085	85
	Co-Ral	0.090	90	0.090	90
	Methyl paraoxon	0.095	95	0.090	90
	Schradan	0.095	95	0.090	90
	Zectran	0.095	95	0.095	95
Potatoes	Diazinon	0.095	95	0.090	90
	Dipterex	>0.150	>150	0.100	100
	TEPP	0.115	115	0.085	85

corresponding to the four insecticides. Thimet, being least water-soluble, will emerge in the first hexane fraction, and is discarded. Isolan, which is soluble in 4 + 1 hexane–methylene chloride, will emerge in this fraction, and is also discarded. Bidrin emerges in the 2 + 1 hexane–methylene chloride fraction and is collected and determined. Azodrin, being most hydrophilic, emerges in the 1 + 1 hexane–methylene chloride fraction, and is also collected and determined. When gradient elution is used whereby the charged column is eluted with 70 ml. of 2 + 1 hexane–methylene chloride mixture and then with 70 ml. of a 1 + 1 mixture, two sharp peaks are obtained. The first peak corresponds to Bidrin and the second to Azodrin. Table IV gives the relative amount of each insecticide found in different 10-ml. elution fractions from the total effluent. Near-quantitative recoveries were obtained. Gradient elution was adopted as part of our analytical technique.

The procedure used for the isolation and measurement of Bidrin and Azodrin residues in crops by this liquid-liquid chromatographic (LLC) technique is as follows:

The crop is subdivided and extracted with chloroform, and the chloroform is exchanged to hexane as described previously. A chromatographic column is prepared by packing a 2-cm. glass column with 8 grams of Celite 545 previously mixed and dampened with 5 ml. of water. The optimum experimental chromatographic conditions were determined and found to be: Mix 8 grams of Celite and 5 ml. of water thoroughly; pack the column tightly from bottom to top with 0.5 gram of dry Celite 545, the Celite-water mixture added in five equal portions with each portion packed and tamped in uniformly and tightly, and 0.5 gram of dry Celite 545 packed in tightly, and cap the column with 10 grams of granular anhydrous sodium sulfate; prewash the prepared column with at least 50 ml. of hexane; develop the chromatogram with gradient stepwise elution mixtures at a flow rate of 100 to 120 ml. per hour as follows:

50 ml. of hexane—discard. This fraction contains crop extractives and fat-soluble interferences.

50 ml. of 4 + 1 hexane–methylene chloride mixture—discard. This fraction contains fat-soluble interferences.

70 ml. of 2 + 1 hexane–methylene chloride mixture. Collect, concentrate, and analyze for Bidrin content.

70 ml. of 1 + 1 hexane–methylene chloride mixture. Collect, concentrate, and analyze for Azodrin content.

Data are presented in Tables V and VI on the performance of this analytical method.

Azodrin Determination

Azodrin insecticide is more hydrophilic but less stable toward base hydrolysis than Bidrin insecticide. The former quality permits aqueous solutions of Azodrin to be washed repeatedly with carbon tetrachloride—thus removing other organophosphates—without appreciable losses. The base-degradation step is omitted in Azodrin determination.

Separation on a liquid-liquid column with water as the stationary phase is used to distinguish Azodrin from Bidrin and other organophosphates.

Performance

Recovery studies on this method, using seven representative crops, averaged 89% (see Table V).

Specificity tests using the 40 commercial insecticides cited in the Bidrin study showed that five insecticides interfered to more than 2% as apparent Azodrin when cleanup was limited to washing of aqueous extracts with hexane and carbon tetrachloride: Bidrin, Diazinon and its oxon, Dipterex, and TEPP. When LLC techniques are used as described, the interference (μg. apparent Bidrin/μg. insecticide added × 100) offered by these insecticides is lowered to below 2% except for Dipterex (see Table VI) which may be removed by rechromatographing the first effluent through a second Celite-water column.

References

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