Determination of Bidrin, Azodrin, and Their Metabolites with the Thermionic Detector

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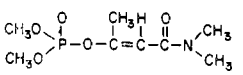
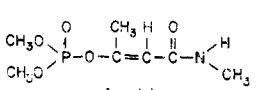
A procedure for the separation and determination of trace amounts of Bidrin and Azodrin, a major metabolite, and eight other metabolites of these two insecticides is described. The organophosphorus compounds are determined by gas-liquid chromatography, which utilizes a short, diethylene glycol adipate column, and is equipped with a modified thermionic detector. The compounds are separated with good resolution. The Bidrin acid is better resolved at a lower temperature than the other compounds studied. Peak area is proportional to amount of injection, and response is linear from 2 to at least 100 ng. for all compounds studied. Preliminary separations are by extraction with different solvents. Certain metabolites are modified prior to GLC analysis, by acid hydrolysis or conversion to a silver derivative followed by alkylation. Lettuce samples fortified with Bidrin, Azodrin, and the metabolites were analyzed. No cleanup was required, and recoveries ranged from 95 to 104% for chloroform-extractable compounds. The minimum detectable amount was 5 p.p.b. when a 50-gram sample was used. The method is specific, simple, and highly sensitive.

Bidrin, 3-(dimethoxyphosphinyloxy)-*N,N*-dimethyl-*cis*-crotonamide, and one of its metabolites, Azodrin, 3-(dimethoxyphosphinyloxy)-*N*-methyl-*cis*-crotonamide, are excellent insecticides against aphids, scale insects, leaf hoppers, mites, and other foliage pests. Structural formulas of these two compounds are:



Bidrin



Azodrin

Extensive studies have been made on the metabolism and stability of Bidrin and Azodrin. Metabolic studies of these insecticides in mammals, insects, and plants by Bull and Linquist (1964, 1966) and Menzer and Casida (1965) indicate that attacks occur at the *N*-methyl group, the phosphate group, and other positions, and are usually in the form of oxidation or hydrolysis. In addition, two sugar adducts were identified recently (Porter, 1967); these were found only when the insecticides were applied to plants. Names and structural formulas of the important metabolites are listed in Table I.

Stability studies (Brown *et al.*, 1966) indicate that Bidrin and Azodrin are hydrolyzed under both acid and alkaline conditions; they are thermally unstable, but the thermal instability appears to be related to the purity of the sample. In actual practice we have not observed this thermal instability. Corey (1965) reported studies of Bidrin in soil and indicated a rapid breakdown of this insecticide in moist soil.

In the past few years, a number of procedures for the determination of Bidrin or Azodrin have been published. Methods used by various workers include cholinesterase inhibition (Elgar and MacDonald, 1966, Sun and Johnson, 1965), partition chromatography followed by cholinesterase inhibition (Lau, 1966), specific bioassay (Sun *et al.*, 1965), colorimetry (Murphy *et al.*, 1965a,b), gas-liquid chromatography equipped with an electron capture detector (Stevens and Van Middelem, 1966), and gas-liquid chromatography equipped with a flame photometric detector (Bowman and Beroza, 1967). However, none of these workers has taken into consideration all of the metabolites that Bidrin and Azodrin can produce.

The procedure described is a gas chromatographic method for the determination of Bidrin, Azodrin, and all their important metabolites. The thermionic detector, which is highly selective and sensitive toward organophosphorus compounds (Giuffrida, 1964), is ideal for this type of study; a modified version (Beckman and Gauer, 1966; Ford and Beroza, 1967) is employed. The compounds are determined directly when possible. Derivatives are prepared for compounds which cannot pass through a gas-chromatographic column. The method is specific, is reliable, and can measure as little as 2.0 ng. of each chloroform-extractable compound.

EXPERIMENTAL

Chemicals. Standards of Bidrin and its metabolites (see Table I for their identity) were provided by the Shell Development Co., Modesto, Calif., and used without further purification. Solvents, including benzene, chloroform, dichloromethane, and methanol, were redistilled in this laboratory. Other reagents, such as iodomethane, hydrochloric acid, and silver nitrate, were of Baker's analyzed grade.

Sample Preparation. Stock solutions (1 μg. per μl.) were prepared by weighing exact amounts of standards, dissolving in appropriate solvents, and making up to exact volume. Benzene was used to dissolve Bidrin, Azodrin, and the *N,N*-dihydrogen metabolite; chloroform was used to dissolve Bidrin acid, *N*-hydroxymethyl Bidrin, *N*-hydroxymethyl Azodrin, and the two glucosides; and methanol was used to dissolve the sodium salts of des-*O*-methyl Bidrin and des-*O*-methyl Azodrin.

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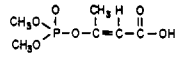
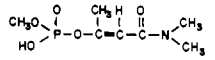
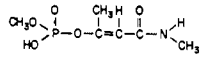
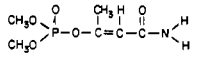
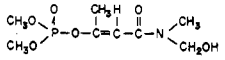
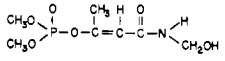
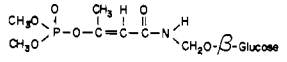
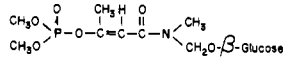
[1] Deceased

Table I. Important Metabolites of Bidrin and Azodrin

Shell No.	Abbreviated Name	Chemical Name	Formula
SD 4455	Bidrin acid	3-(Dimethoxyphosphinyloxy)-*cis*-crotonic acid	
SD 8777	Des-*O*-methyl Bidrin[a]	3-(Methoxy, hydroxy-phosphinyloxy)-*N*,*N*-dimethyl-*cis*-crotonamide	
SD 11191	Des-*O*-methyl Azodrin[a]	3-(Methoxy, hydroxy-phosphinyloxy)-*N*-methyl-*cis*-crotonamide	
SD 11311	*N*,*N*-Dihydrogen Bidrin	3-(Dimethoxyphosphinyloxy)-*cis*-crotonamide	
SD 12210	*N*-Hydroxy-methyl Bidrin	3-(Dimethoxyphosphinyloxy)-*N*-methyl, *N*-hydroxymethyl-*cis*-crotonamide	
SD 12657	*N*-Hydroxymethyl Azodrin	3-(Dimethoxyphosphinyloxy)-*N*-hydroxymethyl-*cis*-crotonamide	
SD 13311	Azodrin glucoside	3-(Dimethoxyphosphinyloxy)-*N*-β-glucosemethylenyl-*cis*-crotonamide	
SD 14493	Bidrin glucoside	3-(Dimethoxyphosphinyloxy)-*N*-methyl, *N*-β-glucosemethyl-enyl-*cis*-crotonamide	

[a] Standards of these compounds used in this laboratory are in the form of their sodium salts.

Sample solutions were prepared from the stock solutions. Their concentrations were 200, 50, and 10 ng. per μl.

Extraction. One hundred grams of chopped lettuce were "spiked" with solutions containing Bidrin, and the metabolites, and were divided into two equal portions. The first portion was used for determination of chloroform- and methanol-extractable compounds and the second for determination of the glucosides.

The first portion was first blended with 50 grams of anhydrous sodium sulfate and 150 ml. of chloroform for 5 minutes. The slurry was filtered through Whatman No. 1 paper, and the precipitate was re-extracted with chloroform twice more. The filtrates were combined, evaporated to a smaller volume, and stored for analysis at 0° C. Then, the plant material was extracted twice with 50 grams of sodium sulfate and 150 ml. of methanol. The combined filtrates were evaporated to approximately 5 ml., and stored for making derivatives.

The second portion was extracted twice with 100 ml. of water for 5 minutes. The combined filtrates were extracted with three 100-ml. portions of chloroform to remove the chloroform-extractable compounds. The aqueous layer containing the glucosides was thus ready for making derivatives.

Modifications. In their original form, the des-*O*-methyl phosphates and the glucosides could not be determined by GLC. Modifications were therefore necessary.

Sodium salts of des-*O*-methyl Bidrin and Azodrin were insoluble in most organic solvents except alcohols. Known quantities of each of the salts were dissolved in 1.0 ml. of methanol. Because sodium phosphates would not yield tertiary esters (Kosolapoff, 1950), the salts were esterified by reaction first with 1.0 ml. of 5% (v./w.) methanolic solution of silver nitrate to form their silver salts. Then the solvent was evaporated, and the precipitates were refluxed with 2.0 ml. of a benzene solution containing 10% (v./v.) iodomethane for 30 minutes. The liquids were filtered through a Büchner funnel and tested for silver ion content by adding one drop of iodomethane. A cloudy solution indicated that the process should be repeated until excess iodomethane was present during the refluxing. After final filtration, the filtrates were evaporated to approximately 0.5 ml., quantitatively transferred into 1.0-ml. volumetric flasks, and made up to volume. The solutions which contained the end products, Bidrin and Azodrin, were ready for chromatographic analysis.

The glucosides of Bidrin and Azodrin were modified by hydrolysis in an acid solution (Elgar and MacDonald, 1966). Known amounts of each glucoside were dissolved in 100 ml. of distilled water. The solution was acidified to pH 0.9 with concentrated hydrochloric acid, then stirred for 1 hour at room temperature. The resultant *N*-hydroxymethyl Bidrin or *N*-hydroxymethyl Azodrin was extracted with four 100-ml. portions each of chloroform. The solutions were concentrated to approximately 2.0 ml., quantitatively transferred to a sedimentation tube, and evaporated to 2.0 ml. with a stream of nitrogen.

The extracts of the lettuce samples were modified in the same manner as the standards. The same procedures were employed without alteration.

Apparatus. An Aerograph Hi-Fi Model 600 gas chromatograph equipped with the modified thermionic detector coated with potassium borate was used.

Gas Chromatographic Analysis. Standard curves of Bidrin, Azodrin, Bidrin acid, *N*,*N*-dihydrogen Bidrin, *N*-hydroxymethyl Bidrin, and *N*-hydroxymethyl Azodrin were prepared without modification of the compounds.

Standard curves of des-*O*-methyl Bidrin, des-*O*-methyl Azodrin, Bidrin glucoside, and Azodrin glucoside were prepared according to the procedure described below. The gas chromatograph was set up according to the following conditions:

The column employed was a 2-foot by 3-mm. o.d. glass column packed with 1% diethylene glycol adipate (w./w.) on 100- to 120-mesh Chromosorb G, AW, DMCS. Nitrogen was employed as the carrier gas at 30 ml. per minute. Hydrogen was operated at 30 ml. per minute. The column temperatures were 175° C. for Bidrin acid and 195° C. for others. The injector temperature was 250° C.

The column was conditioned overnight at 195° C. Standards should be run frequently. The samples were analyzed by injecting 5 μl. of the final solutions without cleanup. If necessary, these solutions should be further concentrated by evaporating with a stream of nitrogen, or be diluted with the same solvent.

RESULTS AND DISCUSSION

Good separation was obtained with the column employed (Figure 1). Single peaks were obtained for all compounds when determined individually. The retention time of Bidrin acid was the shortest among the compounds studied. Bidrin itself was eluted prior to the rest of the metabolites. Although the retention times of Azodrin and *N*-hydroxymethyl Bidrin were different (Table II), they were too close to be separated. In a mixture, these two compounds tended to elute out together and to yield a single response. Retention times of *N*-hydroxymethyl Azodrin and *N,N*-dihydrogen Bidrin were also similar and they tended to elute out together and to yield a single response. The well shaped peaks and a smooth base line indicated that there was no thermodecomposition in any of the compounds studied.

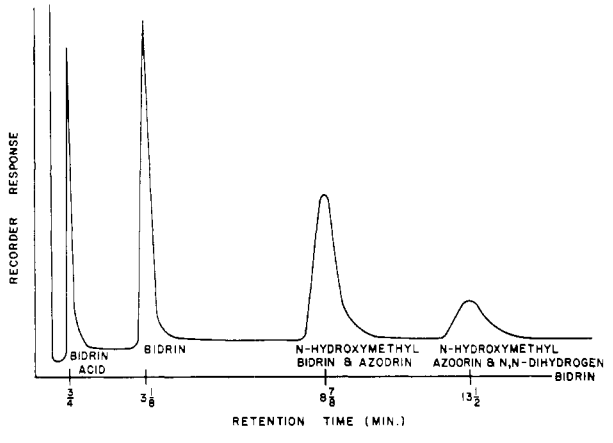


Figure 1. Gas chromatogram of mixture consisting of Bidrin and each of its chloroform-extractable metabolites

135 ng. each

These six phosphorus esters in a mixture may be determined with the oven temperature at 195° C. However, at this temperature, the retention time of Bidrin acid is very short, and often its peak cannot be separated from the solvent peak when a large volume of sample is injected. In actual analysis, Bidrin acid should be determined at a lower temperature, in this study at 175° C. If all these compounds are determined at 175° C., the Azodrin peak and the *N,N*-dihydrogen Bidrin peak tend to become much broader and shorter than the peaks obtained when the column temperature is 195° C.; and the latter may be too broad and too short to be distinguished from the base line when less than 100 ng. of sample is injected. It is not wise to operate the column at a temperature much higher than 195° C., because it may exceed the maximum temperature recommended for this liquid phase.

Sensitivity was directly proportional to the flow rate of the hydrogen gas and inversely proportional to the flow rate of the nitrogen gas. Best sensitivity and signal-noise level were obtained with the conditions employed. Reproducibility was good when the same method of injection was followed. In every case, peak area was directly proportional to the amount of injection up at least 100 ng.

Conversion of the pure standards of the sodium salts of des-*O*-methyl Bidrin and Azodrin gave satisfactory results. Standard curves were constructed by converting various quantities of each salt to the respective *N*-hydroxymethyl compound, determining the yield by gas chromatography, and plotting the quantities of the salts *vs.* the response. A straight line was obtained for each of the two compounds studied. The average yield of these reactions was 25%. Excess heating during conversion should be avoided because of the high volatility of both the salts and the products.

Excellent results were obtained from the hydrolysis of the two glucosides. An average yield of over 80% was obtained for both reactions (Table III).

Table II. Gas-Liquid Chromatographic Data of Bidrin and Its Metabolites

Name	Response of 2.6×10^{-5} Meq. of Phosphorus Injected	Retention Time, Min.
Bidrin	2.50	3.0
Bidrin acid	0.51[a]	1.1[a]
Azodrin	2.30	8.9
N,N-Dihydrogen Bidrin	1.33	13.6
N-Hydroxymethyl Bidrin	2.05	8.6
N-Hydroxymethyl Azodrin	0.75	13.5

[a] Oven operated at 175° C. for determination of Bidrin acid. For others, at 195° C.

Table III. Data from Acid Hydrolysis of Glucosides of Bidrin and Azodrin

Compound	Amount Hydrolyzed, μg.	Yield, μg. Theoretical	Yield, μg. Actual	% Yield
Bidrin glucoside	160	100	80	80.0
	310	210	190	90.5
	470	310	240	77.5
	620	410	320	78.3
	920	620	540	87.2
			Av.	82.7
Azodrin glucoside	210	120	100	83.5
	310	180	160	88.7
	410	240	190	79.0
	620	350	280	80.2
			Av.	82.4

Table IV. Gas Chromatographic Determination of Bidrin and Its Metabolites in Extracts of Lettuce[a]

Compound	Amount Added[b] P.P.M.	Amount Added[b] μg.	Amount Found, μg.	% Recovered
No phosphates added	0.0	0.0	0.0	...
Bidrin	2.0	100.0	104.0	104
	1.0	50.0	48.0	96
	0.5	25.0	24.0	96
	0.1	5.0	5.2	104
	0.01	0.50	0.51	102
	0.005	0.25	0.25	100
Azodrin[c]	2.0	100.0	102.0	102
N-Hydroxymethyl Bidrin	1.0	50.0	48.0	96
	0.5	25.0	24.0	96
	0.1	5.0	4.8	96
	0.01	0.50	0.50	100
	0.005	0.25	0.24	96
N,*N*-Dihydrogen	2.0	100.0	96.0	96
Bidrin + *N*-hydroxymethyl Azodrin[c]	1.0	50.0	48.0	96
	0.5	25.0	24.0	96
	0.1	5.00	<0.1	...
	0.01	0.50	0.50	100
	0.005	0.25	0.24	96
Bidrin glucoside	1.0	50.0	30.0	60
	0.1	5.00	2.8	56
	0.01	0.50	0.30	60
	0.005	0.25	0.15	60
Azodrin glucoside	1.0	50.0	33.0	66
	0.1	5.00	2.7	54
	0.01	0.50	0.25	50
	0.005	0.25	0.15	60

[a] 50 grams of plant material used.
[b] Each sample of lettuce spiked with all metabolites listed in Table I plus the parent compounds.
[c] These two compounds treated as one component because of similarities in chromatographic behavior.

Results of the GLC analysis of untreated and spiked samples of lettuce are shown in Table IV. Other samples —e.g., broccoli, water, and animal tissues—were also analyzed in this laboratory. However, only lettuce was used for the complete recovery study. Because of the similarity in their chromatographic behavior, Azodrin and *N*-hydroxymethyl Bidrin are treated as one component, and the peak which appears at 8.9 minutes is interpreted as a peak produced by this component. This interpretation is valid because *N*-hydroxymethyl Bidrin can easily be converted to Azodrin by releasing formaldehyde (Zweig, 1967). Equal quantities of *N*-hydroxymethyl and Azodrin yield peaks of identical shape and size when analyzed separately. These qualifications rule out the possibility of misinterpretation of peak size and amount of residues. The same treatment is applied to the peak appearing at 13.5 minutes which is produced by *N*-hydroxymethyl Azodrin and *N*,*N*-dihydrogen Bidrin. The reasons for this treatment are the same as those cited for *N*-hydroxymethyl Bidrin and Azodrin.

The determination of the five metabolites in spiked samples excluding Bidrin acid, the des-*O*-methyl phosphates, and the glucosides showed recoveries of 95 to 104%. With no cleanup, the minimum detectable amount was 5 p.p.b. when a 50-gram sample was used. This limit may easily be lowered to less than 0.5 p.p.b. if a gas chromatograph of higher quality is employed.

Modification of the glucosides yielded good results. The over-all recoveries were between 50 and 66%. The minimum detectable limit of 5 p.p.b. was observed. This limit may also be lowered.

Recoveries of Bidrin acid present in the spiked samples were less than 1%. Attempts to convert the des-*O*-methyl phosphates in the spiked samples into dimethyl phosphates were not successful. These failures could be caused by the presence of water, or by inefficiency in the extraction procedures. More workable methods are currently under study.

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