was previously reported for the corresponding derivatives of Bidrin and Azodrin by Giang and Beckman (1968).) Hence, *N*-hydroxymethyl dimethoate and de-*N*-methyl dimethoate eluted at the same retention time (3.2 min) while *N*-hydroxymethyl dimethoxon and de-*N*-methyl dimethoxon also eluted together (2.3 min).

Conventional recovery studies were run by dripping dilute acetone solutions of the four unconjugated metabolites (or dilute methanol solutions of *N*-hydroxymethyl dimethoate *O*-glucoside) onto the surface of the grapes. After evaporation of the solvent, the grapes were extracted as previously described. Recovery values are reported in Table IV. Figure 1 shows typical chromatograms obtained using the thermionic detector.

In contrast to the conditions established by Giang and Beckman (1968) for the hydrolysis of the *O*-glucosides of *N*-hydroxymethyl Bidrin and *N*-hydroxymethyl Azodrin (pH 0.9, with stirring for 1 hr at room temperature), it was found necessary to apply heat to hydrolyze the glucoside of *N*-hydroxymethyl dimethoate. Hydrolysis at 70° for 1 hr resulted in essentially complete recovery of the equivalent amount of *N*-hydroxymethyl dimethoate upon subsequent extraction with chloroform and glc analysis. The recovery of *N*-hydroxymethyl dimethoate, when amounts of the *O*-glucoside varying from 10 to 110 μg were subjected to these hydrolysis conditions, averaged 80%. The values obtained for recovery of the *O*-glucoside from grapes are listed in Table V.

Grape samples field treated with Cygon systemic 25 insecticide and harvested 28 days after the final application (sample numbers A-7, A-8, B-4, and B-5 as described in Tables II and III) gave residue values of <0.05 ppm of apparent de-*N*-methyl dimethoate, *N*-hydroxymethyl dimethoate, de-*N*-methyl dimethoxon, or *N*-hydroxymethyl dimethoxon. No evidence for the presence of sugar adducts was found at the validated sensitivity limit of the hydrolysis–glc procedure (adduct equivalent, 0.05 ppm of *N*-hydroxymethyl dimethoate) in these field-treated grape samples.

The values obtained for residues of dimethoate, dimethoxon, and potential dimethoate metabolites in grapes field-treated with dimethoate render it highly unlikely that any *N*-hydroxymethyl dimethoxon *O*-glucoside could be formed in these grapes. These residue values demonstrate degradation of dimethoate residues from a maximum level of 18 ppm to approximately 0.5 ppm with no accumulation of dimethoxon (maximum of 0.3–0.4 ppm) and no indication of conversion to *N*-hydroxymethyl dimethoxon or even to *N*-hydroxymethyl dimethoate which might in turn be oxidized to *N*-hydroxymethyl dimethoxon. On the basis of these observations and the fact that any appreciable quantity (<0.05 ppm) of the *O*-glucoside of *N*-hydroxymethyl dimethoate was absent, the probability that the *O*-glucoside of *N*-hydroxymethyl dimethoxon might be present is considered unlikely and no attempt has been made to undertake the arduous task of synthesizing and testing this compound.

The work herein reported proving the absence of residues of unconjugated de-*N*-methyl dimethoate, *N*-hydroxymethyl dimethoate, de-*N*-methyl dimethoxon, or *N*-hydroxymethyl dimethoxon as well as conjugates of *N*-hydroxymethyl dimethoate adequately demonstrates that the presently recommended methods for residues of dimethoate and dimethoxon are a reliable measure of the total toxic dimethoate related residues in grapes treated with Cygon systemic 25 insecticide.

ACKNOWLEDGMENTS

The authors express their thanks to R. W. Addor for his assistance in the synthetic work and to R. S. Wayne for assistance in the structural confirmations by instrumental analyses.

LITERATURE CITED

Einhorn, A., Ladisch, C., *Justus Liebigs Ann. Chem.* **343**, 279 (1905).
Elgar, K. E., MacDonald, I. A., *J. Sci. Food Agr.* **17**, 500 (1966).
Giang, B. Y., Beckman, H. F., *J. Agr. Food Chem.* **16**, 899 (1968).
Lucier, G. W., National Institute of Environmental Health Sciences, Research Triangle Park, N. C. 27709, private communication, 1971.
Lucier, G. W., Menzer, R. E., *J. Agr. Food Chem.* **16**, 936 (1968).
Lucier, G. W., Menzer, R. E., *J. Agr. Food Chem.* **18**, 698 (1970).
Steller, W. A., Pasarela, N. R., *J. Ass. Offic. Anal. Chem.* **55**, 1280 (1972).

Received for review October 15, 1973. Accepted January 9, 1974.

Differential Pulse Polarographic Determination of Nicarbazin in Chicken Tissue

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An assay procedure for the quantitative determination of nicarbazin, which is an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP), in chicken muscle, liver, kidney, and skin-fat is described. The polarographically active moiety DNC is extracted from the tissue by solvent extraction and is determined quantitatively by differential pulse polarography. The estimated sensitivity of the method is 2 ppm with a limit of detection of 0.2–0.3 ppm. Recovery of nicarbazin from tissues was 94 ± 5% with a range of 85–102%. Tissues from chickens which were fed nicarbazin in the diet were assayed. Average on-drug nicarbazin residues were 2.7 ppm in muscle, 3.5 ppm in skin-fat, 18.0 ppm in liver, and 10.1 ppm in kidney. All tissues had less than 2-ppm residue at 4 days off drug.

The coccidiostat nicarbazin is an equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). When chickens are given nicarbazin in the feed, the HDP moiety is absorbed and excreted more rapidly than the DNC moiety (Porter and Gilfillan, 1955). Thus, in the assay procedure described here, the more slowly eliminated DNC moiety is determined by differential pulse polarography after it is extracted from the tissue. Porter and Gilfillan (1955) developed a colorimetric assay procedure for nicarbazin which requires the use of nonmedicated tissues to correct for a tissue blank.

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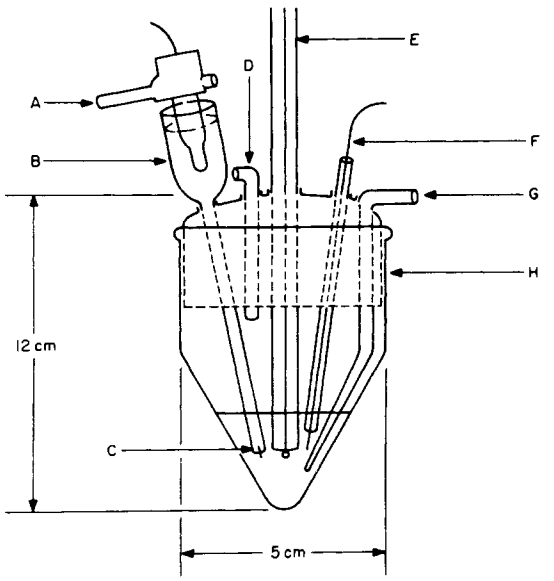

Figure 1. Small volume polarographic cell: (A) Beckman fiber junction aqueous saturated calomel electrode; (B) salt bridge containing Me_2SO supporting electrolyte solution, 15-cm long; (C) fiber junction; (D) glass tube for nitrogen blanket over solution, 6 mm o.d., 2 mm i.d.; (E) dropping mercury electrode; (F) platinum auxiliary electrode, 6 mm o.d. glass tubing, 14-cm long; (G) fine tipped glass tube for deaeration with nitrogen, 6 mm o.d., 2 mm i.d.; (H) ground glass joint.

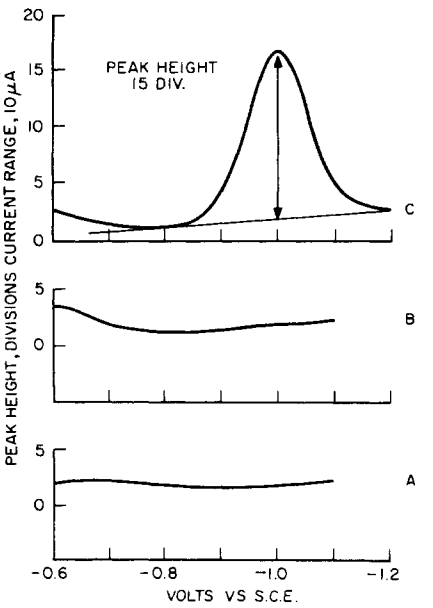

Figure 2. Differential pulse polarograms of nonmedicated and nicarbazin medicated chicken tissues: (A) reagent blank; (B) nonmedicated skin-fat; (C) on-drug skin-fat.

This places a major restriction on the utility of the method. Daftsios and Schall (1962) report two a.c. polarographic peaks for nicarbazin in dimethylformamide, but their work was essentially qualitative in nature.

Differential pulse polarography (Parry and Osteryoung, 1965; Flato, 1972; Bond and Canterford, 1972) is a well-established electrochemical technique which has recently been used in a variety of analytical applications ranging from the determination of atmospheric pollutants (Garber and Wilson, 1972) to the determination of pharmaceuticals in biological fluids (deSilva and Hackman, 1972; Brooks *et al.*, 1973). Differential pulse polarography is used here because it gives reagent and tissue blanks which exhibit no polarographic peaks in the potential region where DNC is reduced. The sensitivity of the method is estimated at 2 ppm since this is the lowest level of nicarbazin which can be quantitated. The limit of detection is 0.2–0.3 ppm.

EXPERIMENTAL SECTION

Apparatus. A PAR Model 170 Electrochemistry System in the differential pulse mode and a mercury drop timer Model 172 (Princeton Applied Research) were used for polarographic measurements. Instrument settings were as follows: drop time, 2 sec; scan rate, 2 mV/sec; pulse amplitude, 100 mV; current range, 10 μA.

The polarographic cell (Figure 1) contained a dropping mercury electrode, an auxiliary electrode made from a piece of platinum wire sealed into the end of a glass tube, and a Beckman fiber junction aqueous saturated calomel reference electrode (sce). Contact between the sce and the sample solution was established through a salt bridge which was made by sealing the tip from an old Beckman fiber junction sce onto the end of a glass tube. The bridge was filled with dimethyl sulfoxide (Me_2SO) supporting electrolyte solution.

Radioactivity measurements were made on a Model 3320 Packard Tri-Carb liquid scintillation spectrometer.

Reagents. The drug was extracted from tissues with ethyl acetate which was distilled in glass (Burdick and Jackson Laboratories, Inc.).

Me_2SO (Matheson Coleman and Bell Reagent Grade) usually was sufficiently pure for direct use in the assay, but when necessary it was purified by shaking with chromatographic grade aluminum oxide, filtering through a sintered glass funnel, and then centrifuging to remove suspended particles of aluminum oxide. A Me_2SO supporting electrolyte solution of sufficient purity for use in the assay should exhibit no polarographic peaks between −0.8 and −1.2 V *vs.* sce.

High-purity nitrogen (purity 99.99% min, Matheson Gas Products) was used to deaerate solutions before polarograms were recorded.

Solutions. Me_2SO supporting electrolyte solution was 0.1 *M* in tetraethylammonium perchlorate and 2×10^{-3} *M* in benzoic acid.

Nicarbazin standard solution (20 μg/0.1 ml) was prepared by dissolving 2.00 mg of nicarbazin in 10 ml of Me_2SO.

Assay Procedure. Five grams of tissue (muscle, liver, kidney, or skin-fat) was homogenized with 10 ml of ethyl acetate in a Sorvall Omni-Mixer Homogenizer chamber; then the ethyl acetate was decanted into a centrifuge tube. The procedure was repeated two additional times. The combined ethyl acetate fractions were centrifuged to separate any tissue that may have been carried over, and decanted into a round-bottomed flask. The ethyl acetate was evaporated to dryness with a rotary evaporator. (Because of the fat content of skin-fat samples, it was difficult to evaporate the sample to less than 1 or 2 ml. The oily residue was transferred to a centrifuge tube.) Five milliliters of Me_2SO supporting electrolyte solution was added by pipet to the flask to dissolve the residue and to rinse the flask. The Me_2SO solution was transferred quantitatively to the centrifuge tube. The flask was rinsed with a total of 9 ml of *n*-hexane, and the hexane was added to the centrifuge tube. The centrifuge tube was capped, shaken, and then centrifuged to separate the phases. The lower phase was transferred to the polarographic cell and deaerated with nitrogen. A polarogram was recorded between −0.6 and −1.2 V *vs.* sce. The peak height was measured (Figure 2) and the amount of nicarbazin was determined from a standard curve.

Standard Curve. A standard curve was obtained by diluting aliquots of nicarbazin standard solution to 5 ml with Me_2SO supporting electrolyte solution and recording polarograms of each solution.

A plot of peak height *vs.* nicarbazin concentration was constructed. The polarographic response is directly pro-

Table I. Distribution of Nicarbazin Components between Ethyl Acetate and Water[a]

Nicarbazin[b]	% radioactivity in Ethyl acetate	Water
DNC-*carbonyl*-^{14}C	96	4
HDP-*ring*-^{14}C	8	92

[a] Five milliliters of water containing a 0.1-ml aliquot of a Me_2SO solution of radioactively labeled nicarbazin was extracted three times with 10 ml of ethyl acetate. [b] DNC, 4,4′-dinitrocarbanilide; HDP, 2-hydroxy-4,6-dimethylpyrimidine.

Table II. Radioactivity Recoveries of DNC-*carbonyl*-^{14}C from Tissues

Tissue sample	Radioact. extracted[a] into ethyl acetate, %
Liver	85
Kidney	94, 92
Skin–fat	100, 93
Muscle	88

[a] Tissues were extracted three times with 10 ml of ethyl acetate.

portional to concentration. Ten micrograms of nicarbazin (2 ppm) gave a peak height of 7.0 chart divisions (0.70 μA) under the conditions given in the Experimental Section. If the current response from a sample is greater than 10 μA, the sample should be diluted with Me_2SO supporting electrolyte solution to give a response which falls on the standard curve.

RESULTS AND DISCUSSION

Extraction of the DNC moiety of nicarbazin into ethyl acetate was demonstrated by partitioning radioactive nicarbazin labeled with ^{14}C in the DNC moiety and nicarbazin labeled with ^{14}C in the HDP moiety between ethyl acetate and water. The radioactivity distribution showed that the drug dissociates, with virtually all of the DNC moiety going into the organic phase and all of the HDP moiety into the aqueous phase (Table I).

Nonmedicated chicken tissues were spiked with a Me_2SO solution of radioactively labeled nicarbazin (DNC-*carbonyl*-^{14}C). The tissues were homogenized and extracted with ethyl acetate as in the assay procedure. Radioactivity recoveries in the ethyl acetate phase were between 85 and 100% (Table II). To demonstrate that the DNC moiety is efficiently extracted from on-drug tissues, given tissues were exhaustively extracted with 10-ml por-

Table III. Exhaustive Extraction of DNC from On-Drug Chicken Tissues

Extraction	Peak height divisions	Amount extracted, % of total	Extraction	Peak height divisions	Amount extracted, % of total
	Liver			Skin–Fat	
First	44	69	First	11	82
Second	17	26	Second	1.5	11
Third	3	5	Third	1	7
Fourth	0	0	Fourth	0	0
Total	64	100	Total	13.5	100
	Kidney			Muscle	
First	31.5	81	First	7	70
Second	6	15	Second	2.5	24
Third	1.5	4	Third	1	6
Fourth	0	0	Fourth	0	0
Total	39.0	100	Total	10.5	100

Table IV. Differential Pulse Polarographic Recovery Data from Nicarbazin-Spiked Nonmedicated Chicken Tissues

Samples	Peak height,[a] divisions	Recovery[b] ppm	Recovery[b] %
	Liver		
41.0-μg spike (8.2 ppm)	27.3, —	7.8, —	95, —
20.5-μg spike (4.1 ppm)	13.8, 12.2	4.0, 3.5	98, 85
10.0-μg spike (2.0 ppm)	6.9, 6.0	2.0, 1.7	100, 85
Nonmedicated control	0, 0	NDR,[c] NDR	—, —
	Kidney		
41.0-μg spike (8.2 ppm)	27.5, 27.0	7.9, 7.7	96, 94
20.5-μg spike (4.1 ppm)	13.5, 14.5	3.9, 4.2	95, 102
10.0-μg spike (2.0 ppm)	6.1, 6.7	1.7, 1.9	85, 95
Nonmedicated control	0, 0	NDR, NDR	—, —
	Skin–Fat		
41.0-μg spike (8.2 ppm)	28.2, 29.0	8.1, 8.3	99, 101
20.5-μg spike (4.1 ppm)	14.2, 14.0	4.1, 4.0	100, 98
10.0-μg spike (2.0 ppm)	6.7, 6.4	1.9, 1.8	95, 90
Nonmedicated control	0, 0	NDR, NDR	—, —
	Muscle		
41.0-μg spike (8.2 ppm)	26.5, 25.5	7.6, 7.3	93, 89
20.5-μg spike (4.1 ppm)	12.5, —	3.6, —	88, —
10.0-μg spike (2.0 ppm)	6.4, 6.3	1.8, 1.8	90, 90
Nonmedicated control	0, 0	NDR, NDR	—, —

[a] One inch equals 10 divisions; 10 divisions = 1 μA. [b] From standard curve: 1 ppm = 3.5 divisions. [c] NDR = no detectable residue.

Table V. Differential Pulse Polarographic Assay Results from Chickens Fed 0.0125% Nicarbazin in the Diet

	Muscle		Skin-Fat		Liver		Kidney	
Bird no.	Peak[a] height	ppm[b]	Peak height	ppm	Peak height	ppm	Peak height	ppm
				4 Days off Drug				
671	0	NDR[c]	0	NDR	1.5	<2	0	NDR
672	0	NDR	0	NDR	1	<2	0	NDR
673	0	NDR	0	NDR	0	NDR	0	NDR
675	0	NDR	0	NDR	1	<2	0	NDR
676	0	NDR	0	NDR	2.5	<2	0	NDR
677	0	NDR	0	NDR	1	<2	0	NDR
				On Drug				
661	9.5	2.7	14	4.0	65	18.6	38.5	11.0
662	10.5	3.0	15	4.3	75.5	21.6	44	12.6
663	8.5	2.4	12	3.4	69	19.7	42.5	12.1
665	10.0	2.9	13	3.7	61	17.4	28.5	8.1
666	8.5	2.4	10	2.9	53	15.1	31	8.9
667	9.0	2.6	10	2.9	54.5	15.6	28	8.0

[a] Peak height is in divisions; 1 in. equals 10 divisions. [b] From standard curve: 1 ppm = 3.5 divisions. [c] NDR = no detectable residue.

tions of ethyl acetate which were then carried through the assay procedure separately. The data clearly show that the DNC moiety is quantitatively extracted after three extractions (Table III).

Long-term animal feeding studies have shown that tissue levels of several parts per million of nicarbazin are safe and so the assay was designed to quantitatively determine levels of 2 ppm and greater. The actual quantitative sensitivity of the method is probably somewhat lower than this although it has not been investigated. The detection limit, defined as approximately twice the base-line noise, is about 0.2 ppm; that is, a peak height of ~0.07 in. (~0.07 μA) is evidence for the presence of some drug.

The peak observed for DNC in Me_2SO which was chosen as solvent largely due to solubility considerations (Figure 2) is due to reduction of an aromatic nitro group. DNC gives two reduction peaks in Me_2SO [E_p (peak potential) $\simeq -1.0$ and ~ -1.50 *vs.* aqueous sce]. As protons are added in the form of benzoic acid, the two waves merge with the height of the first wave increasing at the expense of the second wave until a single wave remains at -0.98 V *vs.* aqueous sce. The behavior is undoubtedly due to a change in the reduction path with increasing proton availability. In the presence of tissue components, E_p sometimes varies between -0.92 and -0.98 V depending on the proton availability in the sample. Since there are no polarographic peaks from the reagent and tissue blanks, the small variation in E_p presents no problem in identification of the DNC peak.

Nonmedicated chicken liver, kidney, muscle, and skin-fat samples were spiked with 0.05–0.2-ml aliquots of a Me_2SO solution of nicarbazin corresponding to 2 and 8 ppm of nicarbazin and were assayed. Recoveries through the assay averaged 94 ± 5% with a range of 85–102% for all tissues, which is in excellent agreement with the radioactivity recoveries. The data are tabulated in Table IV.

Tissues from six nonmedicated chickens and from six on-drug and six 4-day-off drug chickens which were fed 0.0125% nicarbazin in the diet for 8 weeks were assayed. The nonmedicated tissue data are not tabulated because no detectable residue was found. Average on-drug residues were 2.7 ppm in muscle, 3.5 ppm in skin-fat, 18.0 ppm in liver, and 10.1 ppm in kidney. All tissues had less than 2-ppm residue at 4 days off drug. The results are presented in Table V.

ACKNOWLEDGMENT

The authors thank George Olson for supplying the tissues, Henry T. Meriwether for supplying the labeled compounds, and Peter J. Dockery for constructing the polarographic cell.

LITERATURE CITED

Bond, A. M., Canterford, D. R., *Anal. Chem.* **44,** 721 (1972).
Brooks, M. A., deSilva, J. A. F., D'Arconte, L. M., *Anal. Chem.* **45,** 263 (1973).
Daftsios, A. C., Schall, E. D., *J. Ass. Offic. Anal. Chem.* **45,** 278 (1962).
deSilva, J. A. F., Hackman, M. R., *Anal. Chem.* **44,** 1145 (1972).
Flato, J. B., *Anal. Chem.* **44,** 75A (1972).
Garber, R. W., Wilson, C. E., *Anal. Chem.* **44,** 1357 (1972).
Parry, E. P., Osteryoung, R. A., *Anal. Chem.* **37,** 1634 (1965).
Porter, C. C., Gilfillan, J. J., *Poultry Sci.* **34,** 995 (1955).

Received for review April 4, 1973. Accepted January 17, 1974.