Furthermore, the chromatographic evidence suggests that DDT is unaltered. Though the finding of fluorescence could not prove, alone, the presence of the newly formed DDT complex(es) in the mixture, the rapidity of the peak appearance upon the addition of DDT to the nerve components—e.g., Sephadex fractions—is in harmony with the authors' previous view that the DDT molecule must have combined with a nerve component to form a new complex, for such complexes should form almost instantly.

Although the results of the cation transport experiments clearly indicate that DDT indeed interrupts normal ion transport in vivo, its implications should be considered with utmost caution. The effect of DDT in vivo appeared to be most significant in the potassium transport system. In the first 20-minute period of diffusion, 16% of radioactive K^+ remained in the nerve cord of the DDT-poisoned cockroaches compared with 45% in the untreated ones. At the end of the initial one-hour period, the figure became 6 and 25%, respectively. This could mean that the DDT-poisoned nerve exchanged more K^+, or else that the poisoned nerve discharged more K^+ without taking up nonradioactive K^+. The results of in vitro experiments indicate that the DDT-treated nerve cord took up as much K^+ as the untreated controls. The balance of evidence, therefore, indicates that DDT-treatment caused a drastic increase of K^+ efflux and no change in K^+ influx. As a whole, the results with isolated nerve cords were much less clear-cut than those in vivo, for the K^+ exchange of isolated nerve cords in the presence or absence of DDT was not markedly different. The cause of the difference may be that the isolated nerve cords in this particular experiment were DDT-treated simultaneously with measurement of ion movement, whereas in the in vivo experiments the intact cord was exposed to DDT before ion measurement. Similar differences were seen in Na^+ transport. The DDT-poisoned nerve cord took up twice as much Na^+ as the untreated in vivo, though the DDT-treated isolated nerve cord behaved in an identical manner to the untreated. Possibly the DDT-treatments with isolated nerve cords were milder ($1 \times 10^{-5} M$, simultaneous with recording, or 15 minutes prior to recording) than that in vivo ($3 \times 10^{-4} M$, assuming weight of whole body in grams equal to volume in milliliters, for 1 hour preinhibition).

Conclusions

DDT combines with components of cockroach nerve; ultraviolet and fluorescent data suggest that the combination involves formation of a charge-transfer complex and simultaneous profound interference with K^+ efflux. It remains to be proved that these processes are causally related, or are directly related to disruption of nervous activity observed in DDT poisoning.

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ANTHELMINTIC RESIDUES

A Photofluorometric Method for the Determination of Maretin (*N*-Hydroxynaphthalimide Diethyl Phosphate) Residues in Animal Tissues

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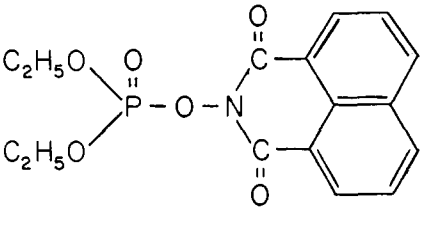
A sensitive method has been developed for the estimation of microgram quantities of Maretin (formerly B 9002, *N*-hydroxynaphthalimide diethyl phosphate) in animal tissues. The pesticide is treated with alkali to give a product which is measured fluorometrically. The sensitivity of the method, based on the observed values for untreated controls, is 0.1 p.p.m.

MARETIN, *N*-hydroxynaphthalimide diethyl phosphate, is an anthelmintic developed by Farbenfabriken Bayer A.G. of Leverkusen, Germany. The purified compound, a yellow crystalline powder melting at 179° to 181° C., is quite unstable to alkaline hydrolysis. Its structural formula is as follows:



Giang (*7*) has a fluorometric method for Bayer 22408 (the sulfur analog of Maretin). The fluorescence was measured following addition of methanolic sodium hydroxide and a dilute solution of hydrogen peroxide in dioxane. Because of the similarity of the two compounds, the same type of behavior was

expected of Maretin, and this was found to be the case. Moreover, the alkaline hydrolysis products of both compounds are identical with respect to fluorescence, melting points, and infrared spectra. The hydrolysis product from both Maretin and Bayer 22408 is naphthostyril (*2*).

Unlike Bayer 22408, Maretin exhibits a strong fluorescence when measured without previous treatment. Therefore, a direct analysis was considered possible. Although direct analysis would provide a less lengthy analytical procedure, the hydrolysis method was adopted because it gave lower control values.

Giang (*1*) has emphasized the use of hydrogen peroxide to increase the fluorescence of the Bayer 22408 hydrolysis product. In the case of Maretin, peroxide treatment following hydrolysis has no effect on the fluorescence, but oxidation prior to hydrolysis suppresses the fluorescence markedly. The use of hydrogen peroxide in this method is helpful only in reducing background fluorescence.

In the procedure described by Giang (*1*), fluorescence measurements were made in a mixture of dioxane, hydrogen peroxide, methanol, and alkali. Since this mixture gave relatively high reagent blanks, it was felt that some other solvent medium might be preferable. To find a more suitable solvent system, gram quantities of the hydrolysis product of Maretin were prepared by hydrolyzing appropriate amounts of the compound with methanolic sodium hydroxide. Water was introduced into the mixture, and the hydrolysis product was extracted into chloroform. This product was subsequently purified by recrystallization from methanol. The purified hydrolysis product was dissolved in various common solvents to give a concentration of 4 μg. per ml. and the fluorescence measured. The results in Table I indicate that benzene is the most desirable solvent of those tested, for the benzene solution of the hydrolysis product gave a maximum sample fluorescence and a minimum of blank fluorescence. The results of the experiment are listed in Table I.

The chromatography procedure described by Giang (*1*) was unsuitable for Maretin analyses. Recoveries from the XXX Grade Florex-chloroform column were between 30 and 50%. Attempts to employ other common solvents yielded high blank values and low recoveries. After considerable investigation, it was found that an alumina column with chloroform as the solvent gave the most desirable sample cleanup and pesticide recovery.

Maretin has been observed to be quite stable at steambath temperatures in the presence or absence of solvent and/or animal tissue residues. In the absence of solvent, the hydrolysis product is lost at the rate of 3 to 5% per minute when subjected to steambath temperatures.

Table I. Maretin Hydrolysis Product Fluorescence in Common Solvents

Solvent	Blank Value	Sample Value
Acetone	0.003	7.20
Benzene	0.004	10.20
Chloroform	0.005	8.70
Dioxane	0.060	10.20
Methanol	0.018	3.70

Material and Methods

Special Apparatus. Chromatographic tubes, 20 × 400 mm., equipped with an Ultramax stopcock and an integral 300-ml. reservoir.

Separatory funnels. Ultramax, 125 ml., centrifuge type.

Spectrophotofluorometer. Aminco Bowman, Catalog No. 4-8100, or equivalent.

Special Reagents and Standards. All reagents are reagent grade unless specified otherwise.

Alumina. Acid - washed, chromatographic grade (Merck Catalog No 71695).

Maretin, recrystallized (Chemagro Corp., Kansas City, Mo.).

Maretin standard solution. Dissolve 100.0 mg. of recrystallized Maretin in chloroform and dilute to volume in a 100-ml. volumetric flask. This solution contains 1000 micrograms of Maretin per milliliter.

Hydrogen peroxide solution. Dilute 1 volume of the 30% Analytical Reagent to 5 volumes with methanol. (Prepare fresh solution daily.)

Increment solution. Pipet 5 ml. of the Maretin standard solution (1000 μg./ml.) into a 100-ml. volumetric flask and dilute to volume with chloroform. This solution contains 50 μg. of Maretin per milliliter.

Quinine alkaloid. Fisher Catalog No. Q-11.

Quinine alkaloid standard solution. Dissolve 125.0 mg. of quinine alkaloid in 0.1*N* sulfuric acid and dilute to volume in a 500-ml. volumetric flask. Dilute 1 ml. of this solution to 250 ml. with 0.1*N* sulfuric acid. This solution contains 1 μg. of quinine alkaloid per ml. The fluorescence of this solution decreases about 5% per week. Therefore, although this or even greater decreases in the fluorescence of the quinine standard will not affect the results of individual analysis by the increment method, for comparative purposes, it is recommended that this solution be prepared fresh, weekly. All solvents should be redistilled from an all-glass apparatus.

Detailed Procedure

Preparation of Extraction of Samples. FAT. Grind the frozen sample with dry ice in a food chopper. Store in a freezer overnight to allow the dry ice to sublime. Weigh 30 grams into a Waring Blendor jar and add 15 grams of Hyflo Super Cel. Add 200 ml. of Skellysolve B and blend for 5 minutes. Filter the sample, under suction, through Whatman No. 42 filter paper, then transfer the filtrate to a 500-ml. separatory funnel. Return the filter cake and the filter paper to the blender jar, add 200 ml. of acetonitrile, and blend for 5 minutes. Filter the sample through Whatman No. 42 filter paper, under suction, and transfer the filtrate to the 500-ml. separatory funnel containing the Skellysolve B filtrate. Shake the separatory funnel vigorously for 1 minute, allow the layers to separate, and draw off the lower acetonitrile phase into a second 500-ml. separatory funnel containing 200 ml. of fresh Skellysolve B. Shake the second separatory funnel vigorously for 30 seconds, allow the layers to separate, and draw off the lower acetonitrile phase into a 1000-ml. beaker. Repeat the extraction of the Skellysolve B phases with two additional portions of acetonitrile. Evaporate the combined acetonitrile extracts to dryness on a steambath under an air jet.

MEAT. For meat and organs, weigh 100 grams of sample into a Waring Blendor jar and add 15 grams of Hyflo Super Cel. Add 200 ml. of acetone and blend for 5 minutes. Filter the sample, under suction, through Whatman No. 42 filter paper, then transfer the filtrate to a 1000-ml. separatory funnel. Return the filter cake and the filter paper to the blender jar, add 200 ml. of chloroform, and blend for 5 minutes. Filter the sample, under suction, through Whatman No. 42 filter paper covered with a 1/8-inch layer of packed Hyflo Super Cel. Rinse the blender jar with 100 ml. of fresh chloroform and add the rinsings to the filter cake. Transfer the entire filtrate to the 1000-ml. separatory funnel containing the acetone filtrate. Shake the separatory funnel for 15 seconds and allow the layers to separate. Draw off the lower organic phase through Whatman No. 12 fluted filter paper into a 600-ml. beaker, discarding the aqueous phase. Evaporate the sample on a steambath under an air jet until all solvent odors are absent. Transfer the sample to a 500-ml. separatory funnel with 200 ml. of Skellysolve B. This is done by using the solvent in three portions, thus ensuring a complete transfer of the sample. Rinse the beaker with 200 ml. of acetonitrile and add this to the 500-ml. separatory funnel. Continue with the same Skellysolve B–acetonitrile partitioning steps that were used in the fat extraction procedure.

Chromatography Procedure—All Samples. Dissolve the residue from the acetonitrile evaporation in 50 ml. of chloroform and chromatograph on a column prepared as follows: Tamp a pledget of glass wool into the bottom of a chromatographic tube. Pour in 10 grams of alumina and gently tap the end of the tube on a wood block until the trapped air is liberated and the alumina has settled. Pour in 10 grams of sodium sulfate. Slowly pour the sample solution into the dry chromatographic tube. Adjust the flow rate to 4 to 5 drops per second, collecting the eluate in a 400-ml. beaker which has

Table II. Recovery of Maretin from Animal Tissues

Tissue	Added, P.P.M.	Found, P.P.M.	Recovery, %
Fat	0	0.08	..
	0.10	0.07	70
	0.50	0.38	76
	1.00	0.87	87
Brain	0	0.08	..
	0.10	0.08	80
	0.50	0.40	80
	1.00	0.96	96
Kidney	0	0.10	..
	0.10	0.08	80
	0.50	0.36	72
	1.00	0.92	92
Liver	0	0.06	..
	0.10	0.09	90
	0.50	0.44	88
	1.00	0.94	94
Heart	0	0.07	..
	0.10	0.09	90
	0.50	0.43	86
	1.00	0.81	81
Steak	0	0.06	..
	0.10	0.09	90
	0.50	0.40	80
	1.00	0.83	83

been marked at 200 ml. Rinse the sample beaker with 50 ml. of chloroform and add the rinsings to the column just as the last of the initial chloroform extract passes into the sodium sulfate. Add an additional 200 ml. of chloroform just as the last of the chloroform rinse passes into the sodium sulfate.

Increment Procedure. Evaporate the column eluate on a steambath under an air jet to less than 200 ml. and transfer the sample to a 200-ml. graduated cylinder. Make to volume with chloroform and mix well. Prepare two 150-ml. beakers by marking one A and the other B. Carefully pipet 1.0 ml. of the increment solution into the beaker marked B. Transfer one-half (100 ml.) of the sample to beaker A and the other half (100 ml.) to beaker B. Evaporate both samples to dryness on a steambath under an air jet.

Hydrolysis and Oxidation Procedures. Add 2 ml. of the 0.5N methanolic sodium hydroxide to the sample, rinsing down the wall of the beaker in the process, and mix thoroughly. Hydrolyze the sample for 10 minutes at room temperature. Add 1 ml. of the hydrogen peroxide solution to the sample and mix thoroughly. Oxidize the sample for 10 minutes at room temperature.

Extraction of the Hydrolysate. Add 50 ml. of chloroform to the sample beaker and transfer the sample to a 125-ml. separatory funnel (centrifuge type). Rinse the beaker with 20 ml. of distilled water and add the rinsings to the separatory funnel. Shake the separatory funnel vigorously for 30 seconds. Allow the layers to separate (if necessary, centrifuge at 1000 r.p.m. for 15 minutes), then draw off the lower chloroform layer through a Whatman No. 12 fluted filter paper, containing 25 grams of sodium sulfate, into a 100-ml. beaker. Rinse the sodium sulfate with 20 ml. of chloroform. Evaporate the sample on a steambath under the air jet, taking off the last 2 to 3 ml. of solvent with the steam shut off.

Fluorescence Measurement. Dissolve the residue from the chloroform evaporation in 15 ml. of benzene (10 ml. for fat samples) and mix thoroughly. Transfer 1 ml. of the sample to a spectrophotofluorometer cuvette and measure the fluorescence of samples A and B. Slit arrangement No. 3 of the Aminco Bowman instrument is used for all measurements. The fluorescence is determined using an activating wavelength of 372 mμ and measuring the fluorescence at 480 mμ. The instrument is arbitrarily standardized so that a solution of quinine alkaloid (1 μ per ml.) in 0.1N sulfuric acid will give a reading of 0.75 fluorescence units.

Calculation Procedure.

A = fluorescence due to the sample

B = fluorescence due to the sample plus the increment

$B - A$ = fluorescence due to the increment

50 μg. = micrograms of Maretin in the increment

50 grams = weight of meat sample

15 grams = weight of fat sample

For meat samples:

$$\text{Maretin, p.p.m.} = \frac{(A)\ (50\ \mu\text{g.})}{(B-A)\ (50\ \text{grams})} = \frac{A}{(B-A)}$$

For fat samples:

$$\text{Maretin, p.p.m.} = \frac{(A)\ (50\ \mu\text{g.})}{(B-A)\ (15\ \text{grams})} = \frac{(3.3)\ (A)}{(B-A)}$$

Results and Discussion

Fluorescence Linearity. The fluorescence-concentration curve is linear for levels of up to 11 μg. of Maretin per ml. of final solution. For accurate measurements it is essential that the amount of Maretin in the incremented sample not exceed this amount.

Recovery Experiments. Recovery experiments have been conducted on a variety of animal tissues. The data are given in Table II. While such experiments do not indicate the efficiency of the initial extraction system, they do show whether or not the material is lost in the steps subsequent to the initial extraction. Owing to the relative instability of the compound in acetonitrile, samples should not remain in the solvent for more than a few hours.

Sensitivity. Using the method as described above, the control values, and thus the sensitivity, were found to vary with the tissue analyzed. Controls varied from 0.06 to 0.10 p.p.m. The instrument sensitivity, based on the fluorescence of reagent blanks, would permit fluorescence measurements equivalent to as little as 0.02 p.p.m. of Maretin. Since control values are considerably higher than this, the practical limit of sensitivity is 0.1 p.p.m.

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