Metabolism of Endosulfan I, Endosulfan II, and Endosulfan Sulfate in Tobacco Leaf

Naiter M. Chopra* and Amal M. Mahfouz

Tobacco plants were separately treated on July 30, 1973 with 0.75 and 1.50 lb/acre of endosulfan I and II and endosulfan sulfate. Leaves from these treatments were harvested on Aug 4, 13, and 23, 1973, and analyzed for metabolites. Endosulfan I, endosulfan sulfate, endosulfandiol, endosulfan ether, and endosulfan lactone were found in the leaves of all the treatments. However, endosulfan II was found only in endosulfan I and II treated leaves. Thus, in tobacco leaf endosulfan I and II are interconvertible; endosulfan sulfate is converted into endosulfan I but not into endosulfan II; and endosulfan I and II and endosulfan sulfate can directly hydrolyze into endosulfandiol. Based on this research we have proposed a new pathway for the metabolism of endosulfan in tobacco leaf.

Endosulfan, or Thiodan, is one of the very few chlorinated cyclodiene pesticides which are still allowed legally to be used on tobacco and other crops at the present time. Commercial endosulfan is a mixture of two isomers: endosulfan I, mp 107.5 °C, and endosulfan II, mp 204 °C. In the mouse endosulfan is metabolized into endosulfan sulfate and endosulfandiol (Deema et al., 1966), and into endosulfan sulfate, endosulfandiol, endosulfan hydroxy ether, and endosulfan lactone (Schuphan et al., 1968). Endosulfan I and II were found in the milk of dairy cows fed with endosulfan-treated silage (Beck et al., 1966). In an experiment with sheep Gorbach et al. (1968) reported the presence of the metabolites endosulfandiol and endosulfan ether in sheep's milk, and endosulfandiol and endosulfan hydroxy ether in sheep's urine. In the male imagos of locusts endosulfan degraded into endosulfan sulfate, endosulfan ether, endosulfan hydroxy ether, and endosulfan lactone (Ballschmiter and Toelg, 1966), and in the house fly the metabolic product found was endosulfan sulfate (Barnes and Ware, 1965). In Bermuda grass (Beck et al., 1966) and potato tuber (Stewart and Cairns, 1974) the principal metabolite found was endosulfan sulfate. However, in *Aspergillus niger* both endosulfan I and II degrade into endosulfandiol (El Zorgani and Omer, 1974). Besides these metabolites several workers have reported the formation of conjugates (Barnes and Ware, 1965; Gorbach, 1972), but so far none of these conjugates has been identified. In 1972 Gorbach proposed a scheme for the metabolism of endosulfan in sheep, and in 1974 Menzie published a comprehensive scheme on the metabolism of endosulfan in insect, plant, and mammal. However, all the information available so far leaves several questions unanswered. In 1972 we embarked on investigations into the breakdown of endosulfan in tobacco and cigarette smokes, and we thought it desirable to make an in-depth study on the metabolism of endosulfan (I and II) and its principal metabolite, endosulfan sulfate, in tobacco leaf before we commenced our work on the main project.

MATERIALS AND METHODS

Materials. All solvents were distilled before use. Florisil (80–100 mesh) was activated at 130 °C for 72 h. Endosulfan I and II, endosulfan sulfate, and other endosulfan metabolites used were of 99%+ purity.

Aqueous emulsions of the pesticides were prepared according to the formulations suggested by Graham (1973). Twenty-five grams of endosulfan I or II, or endosulfan sulfate, was dissolved in 150 ml of pure xylene and 5 g of emulsifier, containing 2 parts of Emcol N-300 B and 3 parts of Emcol N-500 B, was added to the solution. For an application of 0.75 lb/acre 2700 ml of the above formulation was added to 100 gal of water and the resulting emulsion sprayed over an area of one acre with a self-propelled sprayer fitted with one D233 full cone nozzle positioned at a height of 33 cm above the plant tops.

Pesticide Application to Tobacco Plants. Tobacco, variety Coker 347, was planted on May 10, 1973 in 29 rows 300 ft long in a field along a roadside at Chinqua Penn, N.C. The rows ran parallel to the road and were cut into three equal segments by two 10-ft wide roads running at right angle to the road. In order to minimize contamination the 29 rows, starting from the road, were treated on July 30, 1973, as follows: rows 1–5, untreated; rows 6–9, 0.75 lb/acre of endosulfan I; rows 10–13, 1.5 lb/acre of endosulfan I; rows 14–17, 1.5 lb/acre of endosulfan II; rows 18–21, 0.75 lb/acre of endosulfan II; rows 22–25, 0.75 lb/acre of endosulfan sulfate; and rows 26–29, 1.5 lb/acre of endosulfan sulfate. In addition, at different dates, the following chemicals (per acre) were applied to the tobacco plants: Tillam, 4 lb; Terricide-15D, 12 gal; Disyston, 4 lb; Offshoot-T, 2.5 gal; Royal MH-30, 1.5 gal; and Lannate (two applications), 0.5 lb. Thirty leaves from the middle two rows of each treatment were harvested on Aug 4, 13, and 23, 1973. In order to minimize the dilution factor only the middle leaves of the plants were harvested (cf. Guthrie and Bowery, 1962). At the time of harvesting there were no blossoms on the plants. The harvested leaves were immediately frozen. Later all the leaves of a treatment were shredded, combined, and stored at −20 °C until required for analysis.

Extraction and Cleanup of the Residues. The extraction and cleanup procedures adopted for the analysis of the residues were based on the methods developed by Domanski and Sheets (1973) and Nesemann and Seehofer (1970). A 25-g sample of the frozen tobacco pulp was steeped in 350 ml of 35% aqueous acetonitrile for 10 min and then the mixture was blended in a blender with 5 g of Hy-Flo Super Gel for 3 min and filtered. The filtrate was extracted with 100 ml of pentane; the acetonitrile layer was then mixed with 600 ml of 2% sodium chloride solution and again extracted with 100 ml of pentane. The two pentane extracts were combined, washed twice with 100 ml of 2% sodium chloride solution, dried over anhydrous sodium sulfate, and then concentrated to 25 ml. A 5-ml aliquot from the leaf extract concentrate was then chromatographed on a Florisil column (18 cm × 2.5 cm diameter topped with 2-cm anhydrous sodium sulfate) with

Tobacco and Pesticide Research Project, North Carolina Agricultural and Technical State University, Greensboro, North Carolina 27411.

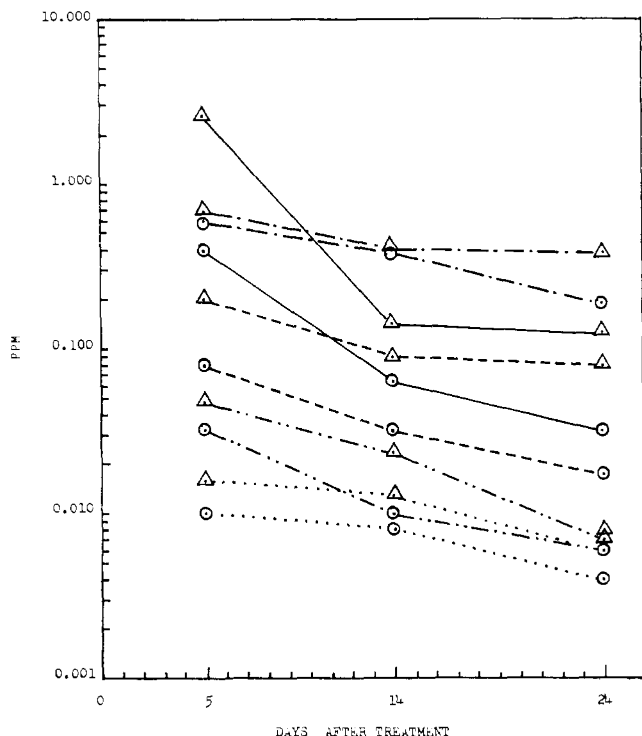

Figure 1. Residues of endosulfan and its metabolites in the leaves of tobacco plants treated with 0.75 and 1.50 lb/acre of endosulfan I: (○) residues in leaves treated with 0.75 lb/acre of endosulfan I; (△) residues in leaves treated with 1.50 lb/acre of endosulfan I; (—) endosulfan I; (- - -) endosulfan II; (- · -) endosulfan sulfate; (- ·· -) endosulfan ether; (· · ·) endosulfan lactone.

350 ml of 15% diethyl ether in pentane, followed by 200 ml of diethyl ether as eluents. The first eluent eluted endosulfan I, endosulfan sulfate (traces), endosulfan ether, and an unknown metabolite from endosulfan sulfate treatments; and the second eluent eluted endosulfan sulfate, endosulfan II, endosulfandiol, and endosulfan lactone. The two eluted solutions were concentrated to 10 ml and the resulting concentrates subjected to qualitative and quantitative estimations.

Residue Estimations. The qualitative and quantitative determinations were carried out on a Mikro Tek 220 GLC unit. The two columns used were: a 183 cm × 0.4 cm diameter glass column packed with 4% SE-30 and 6% QF-1 on Gas-Chrom Q (60–80 mesh), and a 183 cm × 0.4 cm diameter glass column packed with 3% SE-30 on Chromport XXX (80–90 mesh). The temperatures used were: injection port, 225 °C; detector (EC), 275 °C; and oven, 180 °C. The detectors used were an electron capture detector and a Coulson electrolytic conductivity detector. Endosulfan I and II, endosulfan sulfate, and the metabolites reported were identified by cochromatographing the unknown with authentic reference compounds. For quantitative determinations recoveries for endosulfan I and II, endosulfan sulfate, endosulfan ether, and endosulfan lactone were determined. They were 87, 90, 80, 96, and 65%, respectively. Residue values reported in this paper are fairly reliable at the 0.01-ppm level, becoming even more reliable as the residue level increases from the value of 0.01 ppm. Residue values below this figure should be considered only as approximate values. We did not collect tobacco leaves for residue analysis just after pesticide applications because we had employed pure compounds to start with and we were interested in studying the metabolism of endosulfan I and II and endosulfan sulfate in the tobacco leaf, not on the leaf.

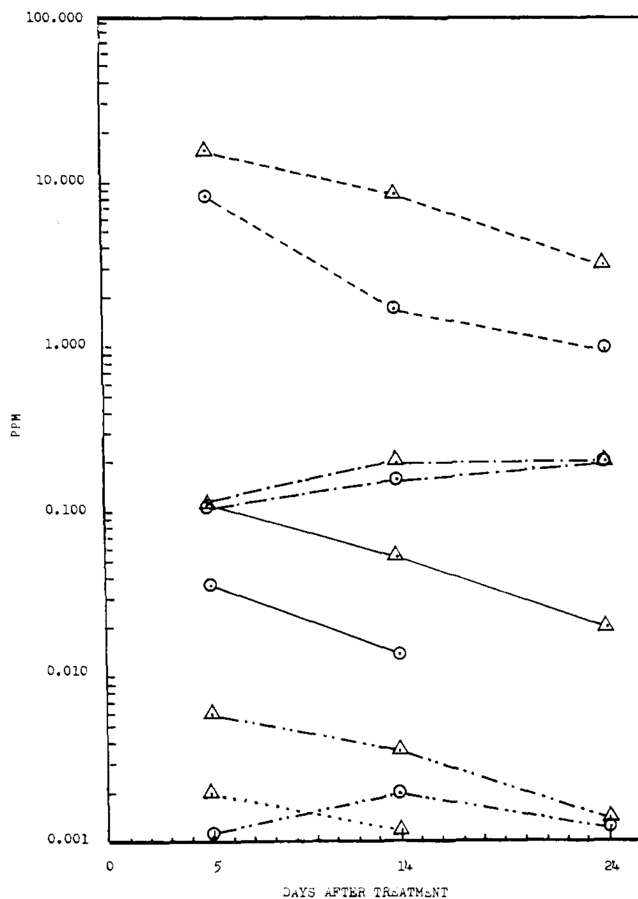

Figure 2. Residues of endosulfan and its metabolites in the leaves of tobacco plants treated with 0.75 and 1.50 lb/acre of endosulfan II: (○) residues in leaves treated with 0.75 lb/acre of endosulfan II; (△) residues in leaves treated with 1.50 lb/acre of endosulfan II; (—) endosulfan I; (- - -) endosulfan II; (- · -) endosulfan sulfate; (- ·· -) endosulfan ether; (· · ·) endosulfan lactone.

RESULTS

The results of the quantitative determinations are shown in Figures 1, 2, and 3. Of endosulfan I and II and endosulfan sulfate, the first compound is least and the last is the most persistent compound. Qualitatively, endosulfan I, endosulfan sulfate, endosulfandiol (traces), endosulfan ether, and endosulfan lactone were found in tobacco leaves treated with endosulfan I and II and endosulfan sulfate. Endosulfan II was found in endosulfan I and II treated leaves but could not be detected in the endosulfan sulfate treated leaves. However, in the GLC a peak due to an unknown compound appeared just before that due to endosulfan sulfate in all and only in endosulfan sulfate treated tobacco leaf extracts. Regarding endosulfan hydroxy ether, when tobacco extract was fortified with this compound and the fortified extract examined on the GLC, two peaks, one due to endosulfan ether and the other due to endosulfan lactone, were observed.

DISCUSSION

Basically, our results are consistent with the results reported by earlier workers. (1) The endosulfan residue values of 21.5 ppm in Coastal Bermuda grass treated with 1.5 lb/acre of endosulfan on the third day after the application (Beck et al., 1966) and 34.2 ppm in tobacco leaf treated with 0.8 lb/acre of endosulfan on the 4th day after treatment (Guthrie and Bowery, 1962) agree with our value of 16 ppm of endosulfan II in tobacco leaf treated with 1.50 lb/acre of endosulfan II on the 5th day of the treatment.

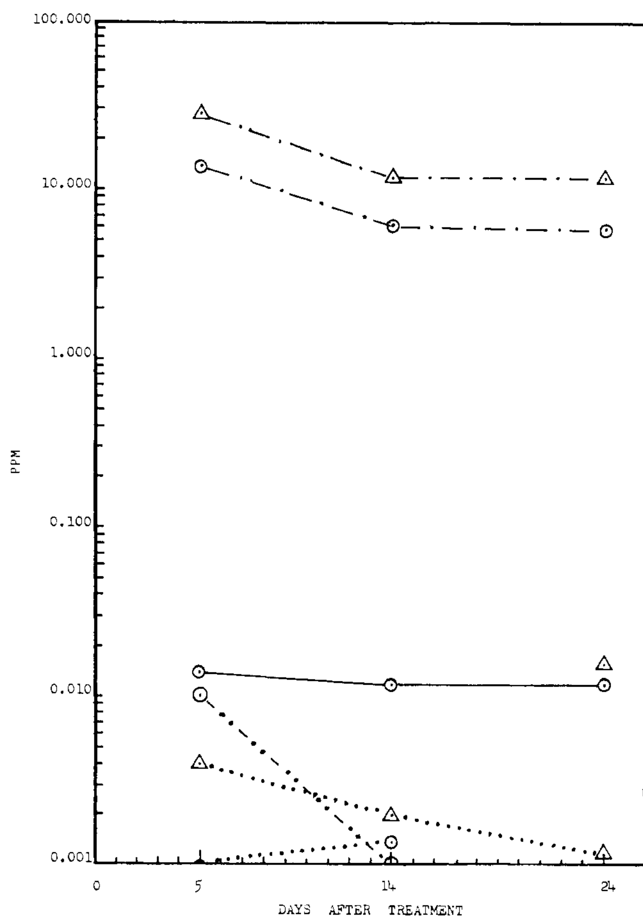

Figure 3. Residues of endosulfan and its metabolites in the leaves of tobacco plants treated with 0.75 and 1.50 lb/acre of endosulfan sulfate: (○) residues in leaves treated with 0.75 lb/acre of endosulfan sulfate; (△) residues in leaves treated with 1.50 lb/acre of endosulfan sulfate; (—) endosulfan I; (- - -) endosulfan II; (– · –) endosulfan sulfate; (– ·· –) endosulfan ether; (· · ·) endosulfan lactone.

The slight difference in values may be due to the difference in the method of application of the pesticide and the analysis of the residue, and the weather conditions. (2) Our rate of degradation of endosulfan I and II is also consistent with the rate of degradation of these compounds in Bermuda grass (Beck et al., 1966), tobacco leaf (Guthrie and Bowery, 1962), and apple leaf (Harrison et al., 1967).

Although our experimental findings are consistent with those of the other workers our interpretations are not always so, possibly due to the fact that our research is being done in greater detail and depth. In Menzie's (1974) scheme for the metabolism of endosulfan in insect, plant, and mammal (see Figure 4) endosulfan sulfate is directly converted into endosulfan lactone in one step. Chemically, this would be a near impossibility. Secondly, endosulfandiol is directly converted into endosulfan hydroxy ether without any evidence being presented, or postulated, in its support. Finally, no details are given about the relationship between endosulfan I and II and endosulfan sulfate. In Gorbach's (1972) scheme no mention is made about the interconversion of endosulfan I and II. Also, the possibility of endosulfan I and II being directly hydrolyzed into endosulfandiol is not shown, nor is there any evidence given to the contrary.

The presence of endosulfan I and II in endosulfan I and II treated tobacco leaves indicates that in tobacco leaf such interconversion does take place. Such interconversion can take place in two ways: first, if the sulfite moiety in

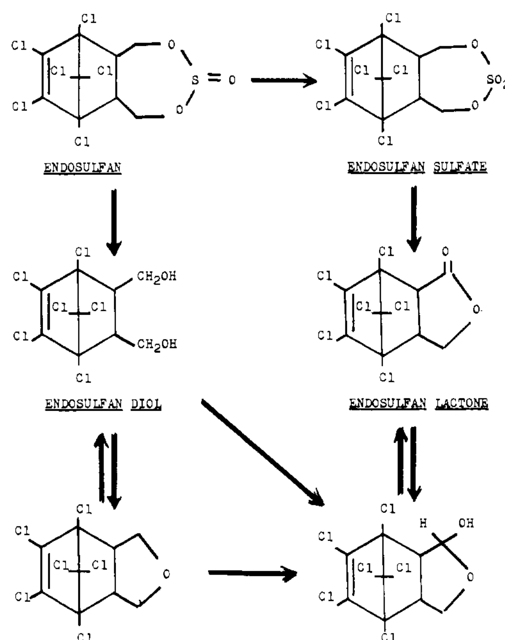

Figure 4. Metabolism of endosulfan in insect, animal, and plant (according to Menzie, 1974).

endosulfan I could change from the endo form to the exo form in the sulfite moiety in endosulfan II. Very rapid interconversion of this type occurs in nitrogen compounds and this precludes isomerism in nitrogen compounds of the type NRR′R″. However, except at very high temperatures (Chopra et al., 1973), no evidence has so far appeared about such interconversion taking place in sulfur compounds, such as endosulfan I and II. Secondly, this interconversion is possible if one of the C–O bonds in the endosulfan molecule breaks, the sulfite moiety rotates around the other C–O bond, and the previously unattached oxygen atom of the sulfite group takes the place of the oxygen atom displaced to form a new C–O bond (see Figure 5). This is a reasonable mechanism, since endosulfan I and II are known to hydrolyze into endosulfandiol and, because sulfite is an excellent leaving group (cf. Gould, 1959), the hydrolysis of endosulfan I and II involves the fission of a C–O bond in preference to an O–S bond. Thus arises the necessity of the presence of an isomerase which could facilitate such interconversion. Also, the presence of endosulfan I and nondetection of endosulfan II in endosulfan sulfate treated tobacco leaves indicate the presence of a hydrogenase which stereoselectively converts endosulfan sulfate into endosulfan I. (Nondetection does not necessarily mean absence. Theoretically, there should be some endosulfan II in endosulfan sulfate treated tobacco leaves for two reasons. (1) Some of the endosulfan I formed should get converted into endosulfan II and (2) it may be that the hydrogenase which converts endosulfan sulfate into endosulfan I may also be converting some endosulfan sulfate, in undetectable quantities, into endosulfan II. However, the statement made regarding the stereoselectivity of the enzyme will still hold true, because stereoselectivity does not necessarily mean a 100% conversion into one of the two possible isomers.)

There are two more interesting phenomena observed in the conversion of endosulfan I and II into endosulfan sulfate. First, the conversion of endosulfan I into endosulfan sulfate is rapid, and as the amount of endosulfan I in the leaf decreases the amount of endosulfan sulfate also decreases, but the conversion of endosulfan II into endosulfan sulfate is slow. This suggests that there are

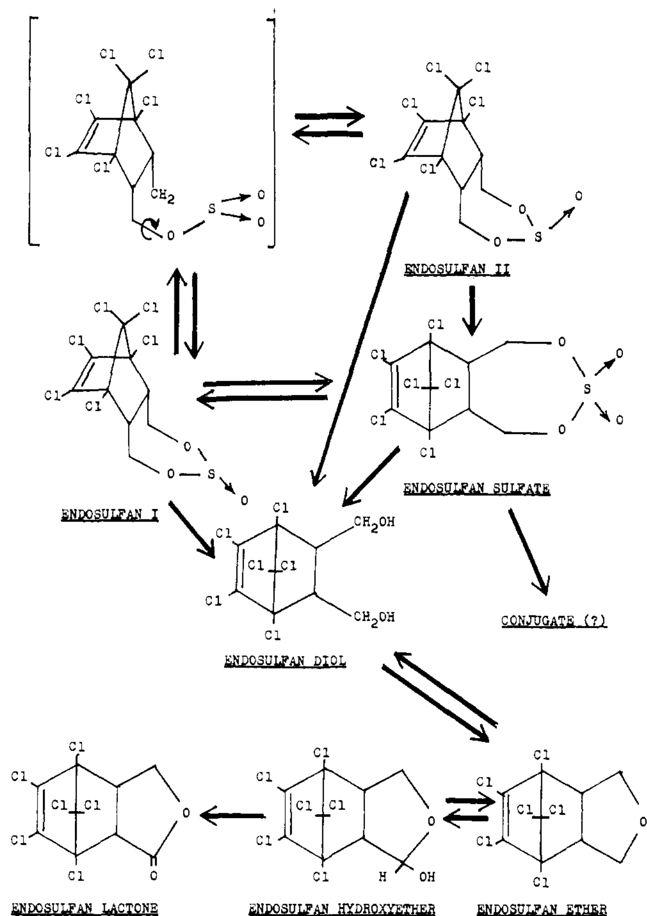

Figure 5. Metabolism of endosulfan in tobacco leaf.

different enzyme systems for the conversion of endosulfan I and II into endosulfan sulfate. Also, if the endosulfan sulfate residue curve of the endosulfan I treated tobacco leaf is numerically added to that of the endosulfan II treated leaf a new curve is obtained in which the amount of endosulfan sulfate residue gradually rises, comes to a maximum, falls down a little, and then more or less levels off. Examples of this phenomenon are shown in the amount of endosulfan sulfate present in endosulfan treated apple leaves (Harrison et al., 1967) and in pear and grape foliage (MacNeil and Hikichi, 1976). The second interesting phenomenon is the independence of the rate of endosulfan sulfate formation in endosulfan II treated tobacco leaf (see Figure 2). Thus, this formation is a reaction of zero order, with the enzyme–endosulfan II complex being the rate-determining factor. This would suggest that the concentration of endosulfan sulfate will stay constant until the concentration of endosulfan II falls below a certain level. This could, in part, be the reason for the greater persistence of endosulfan sulfate. Furthermore, an initial slow increase in the rate of formation of endosulfan sulfate would indicate an enzyme induction (cf. Remmer, 1972). Enzyme induction is also seen with Dieldrin and DDT (Gillett, 1972). It is interesting that this phenomenon is not shown by endosulfan I.

The presence of endosulfandiol in endosulfan I and II and endosulfan sulfate treated leaves shows that all three compounds can directly hydrolyze into endosulfandiol. Our experiments on the presence of endosulfan hydroxy ether in tobacco leaf were inconclusive. We have not ascertained if the conversion of endosulfan hydroxy ether into endosulfan ether and endosulfan lactone is an artifact, or if endosulfan hydroxy ether is rapidly converted into endosulfan ether and endosulfan lactone in tobacco leaf.

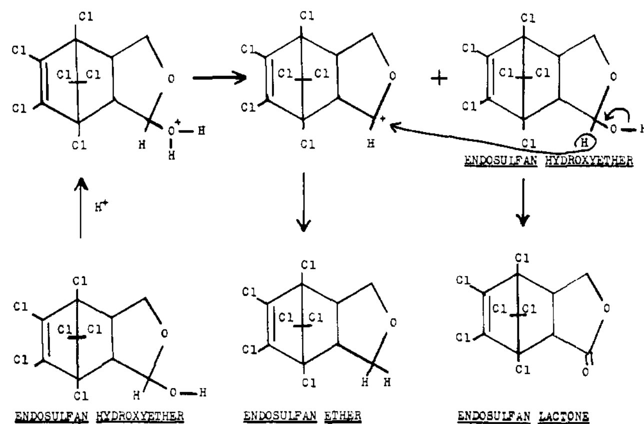

Figure 6. Conversion of endosulfan hydroxy ether into endosulfan ether and endosulfan lactone.

As an artifact, this conversion may be a sort of Cannizzaro's reaction and its possible mechanism is shown in Figure 6. The conversions of endosulfan hydroxy ether into endosulfan ether and endosulfan lactone are reduction and oxidation reactions. Since plants have several oxidation–reduction systems these conversions are possible in plants.

Lastly, we found an unknown compound in the endosulfan sulfate treated tobacco leaf: perhaps an endosulfan sulfate conjugate formed by the hydrolysis of one of the C–O bonds and the linking of the carbon atom with a glucose molecule, or some other molecule. To our knowledge no one has reported any such conjugate although Gorbach (1972) has reported the presence of an endosulfandiol conjugate.

In this paper we have reported our findings and interpretations on the metabolism of endosulfan I and II and endosulfan sulfate. Since the endosulfan metabolism in tobacco leaf is very similar to that in Bermuda grass, and apple, pear, and grape foliage, perhaps our findings apply to more than just tobacco leaf.

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Residues and Disappearance of Triforine from Various Crops

John B. Bourke,* Thomas R. Nelsen, and Dietrich Eichler

A convenient method for the residue analysis of triforine, piperazine-1,4-diylbis[1-(2,2,2-trichloroethyl)formamide], sensitive to 0.01 ppm is presented. Disappearance rate data and harvest residue data are presented.

The systemic fungicide triforine has been found effective for the control of a number of diseases in ornamentals, cereal grains, fruits, and vegetables. More specifically it has been found in New York orchards (Gilpatrick et al., 1972) to control scab, powdery mildew, rusts, and European red mite on apples, leaf spot fungus on cherry (Szkolnik, 1974), and brown rot in plums, peach, and tart cherry (Gilpatrick, 1973). Not only is this material useful as a protectant but it has also been reported to have curative action against a number of plant pathogenic organisms (Fuchs et al., 1971; Szkolnik, 1973). The systemic activity of triforine has been demonstrated by correlating disease control with fungicide concentrations in leaves following soil application (Gilpatrick and Bourke, 1973). The fungicidal activity has been shown to be due to the parent compound which is metabolized to a series of nonfunitoxic products.

Triforine is a colorless and odorless, crystalline substance which decomposes at 155 °C. It has been formulated as a 20% emulsified concentrate and as a 25% wettable powder, both to be applied to drip off. It has a relatively high rodent LD_{50} in excess of 6000 mg/kg body weight.

The results reported here were gathered during the development and adaptation of the analytical method to various crops and as a result of field testing.

MATERIALS AND METHODS

The field spray formulations were a 20% emulsifiable concentrate (CA70203) supplied by Celamerck, a 20% emulsifiable concentrate (W524) supplied by Niagara Chemical Company, and a 25% wettable powder (W524) also supplied by Niagara. The analytical standard used was supplied by Celamerck GMBH & Co., Ingelheim, West Germany. All reagents used were of analytical grade; the solvents were redistilled to further enhance purity. Difficulty was experienced with all solvents. Interfering peaks from the acetone and ethyl acetate could not be removed by all-glass distillation. Each solvent was checked prior to use for the interfering peak by 50 to 1 concentration and injection into the GC. Only solvents found free of contamination were used. Some lots of spectro grade solvent contained the offending peak. The interfering peak in the ethyl formate could be removed by all-glass distillation. The internal standard was a 0.4% solution of 1,2-dibromoethane in acetone.

Department of Food Science and Technology, New York State Agricultural Experiment Station, Geneva, New York 14456 (J.B.B., T.R.N.) and Celamerck GMBH & Co. KG, Ingelheim, West Germany (D.E.).

Extraction and Cleanup. After fruits were minced and the pits were removed, they were subsampled into 50-g laboratory samples. Lab samples were blended with 200 ml of acetone in an explosion proof blender for approximately 2 min. The homogenized sample was then filtered through sintered glass and the residue reblended twice in the same manner with 100 ml of acetone.

The combined acetone extracts were mixed with 500 ml of water and 500 ml of saturated sodium chloride and then extracted with 200 ml of ethyl acetate. The aqueous phase was extracted twice more with 150 ml of ethyl acetate, and the organic phases were combined and evaporated to approximately 30 ml on a rotary evaporator at room temperature. The residue was then transferred into a 250-ml one-neck round-bottomed flask with acetone and the acetone evaporated off on the rotary evaporator at room temperature. To the 10 to 20 ml of liquid remaining in the flask, 60 ml of 10% sulfuric acid was added. This mixture was distilled at 140 °C (oil bath) under a nitrogen flow (20 ml/min) for 1 h into 10 ml of doubly distilled water, in the apparatus shown in Figure 1. The distillate was cooled by immersion in an ice brine bath.

The distillation with sulfuric acid results in the conversion of triforine to chloral hydrate which is collected and quantitized by the following procedure. The collected aqueous solution was transferred to a 250-ml separatory funnel, 30 g of sodium chloride was added, and the solution was extracted with 12 ml followed by 10 ml of ethyl formate. The organic phases were collected in a 20-ml volumetric flask and made up to volume. Ten microliters of the internal standard solution and 1 g of calcium chloride were added and the flask was left for 0.5 h.

Gas Chromatography. Gas chromatographic analysis was performed on a Microtech MT 220 chromatograph