THE METABOLISM OF DITHIOCARBAMATE FUNGICIDES BY PAPAYA (*CARICA PAPAYA*)

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ABSTRACT

The appearance of phytotoxic symptoms following the application of dithiocarbamate fungicides has been reported in a number of instances. Delayed phytotoxicities such as leaf spotting in peach, pear, and apple, and leaf damage to tobacco, apple, and papaya have been observed following the application of dialkyldithiocarbamates but not with the monoalkyl analogs. This suggests that the two classes of fungicides are metabolized differently by plants. Study of the metabolism of dimethyldithiocarbamates in papaya was undertaken to assess the importance of the above observations.

Dimethyldithiocarbamate (DDC) labeled with ^{14}C in the methyl groups or ^{35}S in the dithiocarbamate function was applied either as the potassium or zinc salt to leaves and roots of papaya seedlings from 1 to 3 months old. Surface residues, volatile metabolites, and translocated materials found in root, stem, leaf, and cell sap were assayed at various times after application of labeled compound.

The results of this work show that part of the fungicidal residue is hydrolyzed on the surface, presumably to carbon disulfide and dimethylamine, both of which could be recovered as volatile metabolites. This hydrolysis is more rapid with the potassium salt than with the zinc salt presumably due to insolubility of the latter.

An appreciable portion of the applied ^{35}S label has been found in sulfur amino acids (free and in proteins) and inorganic sulfate. Whether this is due to the oxidation of carbon disulfide or some dimethyldithiocarbamate metabolite is not known. In the case of

methyl ^{14}C labeled compounds, carbon dioxide was collected as a volatile metabolite and ^{14}C was found in many plant constituents. The exact mechanism for the transformation of dimethylamine methyl groups to carbon dioxide is unknown.

Among the metabolites isolated, the largest amount of foliarly applied radioactivity (22% for ^{14}C-KDDC and 11% for ^{35}S-KDDC) was recovered from the cell sap as dimethyldithiocarbamyl glucoside, identified by paper chromatography and chemical analysis. Lesser amounts were present as dimethyldithiocarbamyl alanine and 4 other unidentified radioactive compounds.

Recoveries of applied radioactivity ranged from 28% to 55% for ^{35}S-KDDC, from 33% to 99% for ^{14}C-KDDC, when applied to leaves.

Phytotoxicity was never observed with the more rapidly decomposed KDDC whereas it was seen with $Zn(DDC)_2$. The available data indicate that a slow but prolonged evolution of carbon disulfide and dimethylamine on the leaf surface as observed with $Zn(DDC)_2$ may result in chronic poisoning of sensitive enzyme systems. The rapid hydrolysis and release of volatile metabolites from KDDC does not appear to cause any phytotoxicity.

INTRODUCTION

Aragaki et al. (2) showed that foliarly applied ferbam and zinc dimethyldithiocarbamate, $Zn(DDC)_2$ (Table 1), were toxic to papaya (causing chlorosis, leaf drop and stunting of growth) whereas Mn and Zn salts of ethylenebisdithiocarbamate (maneb and zineb) were non-toxic. Since these symptoms appeared about two weeks after the application of fungicides, they were probably not due to the compounds which were applied but were caused by some toxic product formed from these chemicals.

Examination of the literature on the metabolism of these substances by plants reveals that little is known about the products formed either qualitatively or quantitatively. Persistence curves for $Zn(DDC)_2$ residues on papaya seedlings, grown in the greenhouse, showed a linear dissipation with a half life of about 15 to 17 days (23). This prolonged persistence would allow the formation of transformation products both upon and within the plant. As a first step towards elucidation of the phytotoxicity caused by dialkyldithiocarbamates, the determination of the total fate of these compounds applied to plants was undertaken. The kinds and amounts of surface residues and metabolites resulting from the application of dithiocarbamate fungicides was determined using labeled compounds containing either methyl ^{14}C or dithiocarbamyl ^{35}S.[1]

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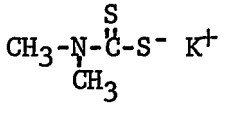
[1] The system of nomenclature for designating radioisotopes used in this study was recommended and approved by the International Union of Pure and Applied Chemistry in 1960. (see 1957 Report of the Commission on the nomenclature of inorganic chemistry. American version with comments. Am. Chem. Soc. 8:5523-5544.)

Table 1

Chemical names and abbreviations or trade names used in the text.

2,4-D	2,4-dichlorophenoxyacetic acid
DDC	N,N-dimethyldithiocarbamate salt
DDC-alanine	β-(N,N-dimethylthiocarbamoylthio)-alanine
DDC-glucoside	1-(N,N-dimethylthiocarbamoylthio)-1-desoxy-D-glucose
DEDC	N,N-diethyldithiocarbamate salt
ferbam	ferric N,N-dimethyldithiocarbamate
KDDC	potassium N,N-dimethyldithiocarbamate (I)
maneb	manganous ethylenebisdithiocarbamate
nabam	disodium ethylenebisdithiocarbamate (II)
NaDDC	sodium N,N-dimethyldithiocarbamate
TMTD(thiram)	tetramethylthiuram disulfide (III)
TMTM	tetramethylthiuram monosulfide
TTCA	thiazolidine-2-thione-4-carboxylic acid
Vapam	sodium N-methyldithiocarbamate
zineb	zinc ethylenebisdithiocarbamate
$Zn(DDC)_2$(ziram)	zinc N,N-dimethyldithiocarbamate



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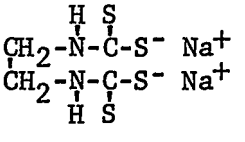

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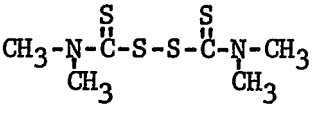

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LITERATURE REVIEW

Dithiocarbamates are usually divided into two classes, the monoalkyldithiocarbamates and dialkyldithiocarbamates. The latter includes sodium, potassium, and zinc dimethyldithiocarbamates (NaDDC, KDDC, $Zn(DDC)_2$), tetramethylthiuram disulfide (thiram, TMTD), and tetramethylthiuram monosulfide (TMTM), and the former includes the disodium ethylenebisdithiocarbamate (nabam) and sodium-N-methyldithiocarbamate (Vapam). The essential chemical difference is that the monoalkyldithiocarbamates are secondary amines whereas the dialkyldithiocarbamates are tertiary amines. This chemical difference has been used to explain the observed differences in biological effects of the two classes of compounds.

Dithiocarbamate pesticides, first patented by Tisdale and Williams (60) in 1934 have been shown to be effective against a large number of fungal diseases of fruits and vegetables. However, the chemical nature of the residues left by these compounds as well as their metabolism by plants and animals are poorly understood. Most of the studies on the biological activities of the dithiocarbamates have utilized fungi. Although the major pathways of fungal and plant metabolism are similar, variations can occur between these divergent organisms. Nevertheless, consideration of the toxic effects of dithiocarbamates on fungal metabolism could show how these substances might act in higher plants.

Dialkyldithiocarbamates may produce a bimodal dosage response in fungi while alkylenebisdithiocarbamates and isothiocyanates do not

(39, 48). This bimodal dosage response was first observed by Dimond et al. (11) in 1941. Goksøyr's (15) explanation for the bimodal curve is as follows: The formation of highly toxic 1:1 Cu-dithiocarbamate complex causes the first zone of inhibition. The formation of less toxic 1:2 Cu-dithiocarbamate complex causes the reversal of the inhibition curve and the second zone of inhibition is caused by the formation of Zn-dithiocarbamate. Kaars Sijpesteijn et al. (28, 29) believe that the second zone of the inhibition is related to free dithiocarbamate ion. The antifungal action of TMTD, TMTM, and NaDDC was counteracted by histidine but this reversal was not observed with the alkylenebisdithiocarbamates or the corresponding bisisothiocyanates (26). A number of imidazole derivatives also showed antagonistic action towards the fungitoxicity of NaDDC (27). The counteraction by histidine was explained by Goksøyr (15) who suggested that histidine prevented the formation of the toxic 1:1 Cu-dithiocarbamate by chelating cations such as copper. However, Kaars Sijpesteijn et al. (28) disagreed since both histidine and the more antagonistic imidazolepyruvic acid have a lower copper-binding capacity than dimethyldithiocarbamate itself. Kaars Sijpesteijn et al. postulated that the antagonistic action was due to the protection of an essential enzyme preventing reaction with the toxic 1:1 complex.

Discussion on the mode of fungicidal action of the dithiocarbamates can be found in the monograph by Thorne and Ludwig (59). Goksøyr (15) indicated that a heavy metal ion was required for high toxicity of dialkyldithiocarbamates. Owens (46) reported that free radicals may

be the active forms of TMTD and ferric dimethyldithiocarbamate (ferbam). Klöpping and van der Kerk (36) suggested that "the dimethyldithiocarbamate group is highly toxic only if present in the ionic state or, alternatively, in such a structural form that ion formation, for example by means of an oxidation-reduction system of the cell, can be brought about." "The relative inactivity of the methyl ester of dimethyldithiocarbamic acid as compared with the ionized sodium salt has been interpreted as strong evidence in support of the proposition of Klöpping and van der Kerk. Such a conclusion, however, does not appear justified, since other esters of dimethyldithiocarbamic acid, e.g., the allyl ester, are as active as the parent sodium salt (59, pp. 229-231)."

Studies on the relationship of sorption to fungicidal activity using ^{35}S-labeled sodium and potassium dialkydithiocarbamates (42,43, 47) indicated that di-n-propyldithiocarbamate was more strongly sorbed by Helminthosporium sativum, and Monilia fructicola than the dimethyl analog. "A possible explanation presented is that the di-n-propyl compound is strongly adsorbed on the spore wall, preventing permeation of the toxicant into the spore: the dimethyl compound, being less strongly adsorbed, enters the cell and reaches the sites of action (59, p. 253)."

There is a considerable amount of literature on the effects of dithiocarbamates on enzymes from various sources. Most of this work has been done with DEDC, a relatively poor fungicide. Since studies on the effects of dimethyldithiocarbamates on enzyme system are very limited, some of the work with DEDC is cited here. Laccase (35), an

oxidase from the latex of Rhus succedanea, ascorbic acid dehydrogenase (57, 58), and sweet potato phenolase (13) have been shown to be inhibited by DEDC. Mann (38) found that DEDC, cyanide, and several other poisons inhibited amine oxidase of pea seedlings. Beef-liver glutamic dehydrogenase (1), glutamic dehydrogenase (45) from Neurospora crassa, and alkaline phosphatase (53, p. 489) were also inhibited by DEDC. The activity of urease (a sulfhydryl-dependent enzyme) was inhibited by high concentrations of ferbam, whereas nabam had no effect on this enzyme (63).

Certain dithiocarbamates have been shown to have growth-regulating activity towards the higher plants. NaDEDC strongly stimulated root growth of cucumber seedling cultured on filter papers (55). NaDEDC inhibited the growth-stimulating action of 2,4-dichlorophenoxy acetic acid (2,4-D) in culture-solution experiments with vetch internodes (56). Retarded sensescence of crops after dithiocarbamate sprayings was reported by Besemer (3) with tulip and by van Doorn (12) with onion. Pickett et al. reported that the thickness of the palisade parenchyma of ferbam treated apple leaves was greater than that of control.

Dekhuijzen (9) reported three fungitoxic transformation products on paper chromatograms after uptake of NaDDC by the roots of cucumber plants. They are β-(N,N-dimethylthiocarbamoylthio)-alanine (DDC-alanine, $(CH_3)_2N-\overset{\overset{S}{\|}}{C}-S-CH_2-\overset{\overset{NH_2}{|}}{\underset{H}{\underset{|}{C}}}-COOH$) (32), 1-(N,N-dimethylthiocarbamo-

ylthio)-1-dexoxy-D-glucose (DDC-glucoside, $(CH_3)_2N-\overset{\overset{S}{\|}}{C}-S-\overset{\overset{O}{\frown}}{CH}-(CHOH)_3-CH-CH_2OH$) (33), and an unknown fungitoxic derivative (fungicide X). The three derivatives gave a positive Feigl test (iodine-sodium azide reagent) (14, p. 228) and displayed fungicidal activity towards Glomerella cingulata. Dekhuijzen (10) observed the same three transformation products after the leaf treatment of cucumber and tobacco plants with TMTD. A fourth conversion product of NaDDC is thiazolidine-2-thione-4-carboxylic acid (TTCA, $\overset{\overset{S}{\|}}{C}\overset{\overset{NH}{\frown}}{-S-CH_2-CH}-COOH$) (32), which is formed by a nonenzymatic decomposition of DDC-alanine (a ring closure with elimination of dimethylamine). Massaux (40) obtained three radioactive spots after the application of ^{35}S-labeled TMTD on the leaf of cucumber plant. Their Rf values were identical to those reported by Dekhuijzen with NaDDC. The fungicidal activity of DDC-alanine in vitro was found to be higher than that of the DDC-glucoside. The concentration of DDC-glucoside required to cause complete inhibition of the germination of conidia of Glomerella cingulata in vitro was 500 ppm, while the corresponding values for NaDDC and DDC-alanine were 1 ppm and 2 ppm respectively. Heavy suspensions of washed micro-organisms (Saccharomyes cerevisiae) were able to convert NaDDC to DDC-aminobutyric acid conjugate (30), but neither the glucoside nor the alanine derivative was formed. Tetraethylthiuram disulfide (Antabuse) has been shown to be detoxified in man by formation of S-glucuronide with subsequent urinary excretion (31).

Systemic activity of NaDDC was found against Peronospora tabacina (8) on tobacco plants after leaf application and against

Fusarium oxysporum on tomatoes after root application (19). Volger (62) showed that Pinus silvestris seedlings grown from seed treated with TMTD were less severely attacked by Rhizoctonia sp. and Pythium sp. than seedlings grown from untreated seed. Systemic activity against cucumber scab by some DDC was reported by van Raalte et al. (51).

Mustard and Lynch (44) first observed a manifestation of metabolic upset in plants by dithiocarbamates. They reported that more ascorbic acid was found in mangoes sprayed with ferbam for anthracnose control than in fruits left unsprayed. McCarthy et al. (41) found that the DEDC inhibited ascorbic acid oxidation in both cucumber and potato, and Russell (54) reported that the ascorbic acid oxidase system of barley roots was inhibited by DEDC. Blouch et al. (6) observed that the glutamine content in $Zn(DDC)_2$ treated sugar beet leaves was greater than that of the control. It was noted that the uptake of inorganic phosphate by young wheat plants was inhibited by NaDEDC (7). NaDEDC showed marked inhibition of photosynthesis in Chlorella pyrenoidosa (18). Cyanide, DEDC, and urethan significantly inhibited formation of lignin in young red bean and soybean plants (25).

Reports on phytotoxicity of dithiocarbamate residues occur scattered throughout the literature. Goldsworthy et al. (16) reported that ferbam controlled peach scab and brown rot without injury to fruit, but it caused a leaf-spotting. Black (4) reported that thiram and $Zn(DDC)_2$ used for the control of apple black spot and pear black spot, caused leaf mottle. Phygon, copper 8 quinolate, and $Zn(DDC)_2$

sprayed on potted, greenhouse-grown McIntosh apple trees caused leaf injury even though they were effective in controlling scab (20). Riley (52) found that leaves of six-week-old tobacco seedlings were damaged by the foliar treatment of $Zn(DDC)_2$ at 0.08 gm per ml twice weekly for 3 weeks. Hendrix et al. (21) observed reduced germination of papaya seeds and delayed emergence and reduced growth of seedlings two weeks after treatment with dimethyldithiocarbamate fungicides.

MATERIALS AND METHODS

Dithiocarbamates: KDDC was used instead of $Zn(DDC)_2$ in the majority of experiments because it did not produce phytotoxic symptoms when applied to papaya plants, and because it was water soluble and so simplified application. Preliminary studies showed that $Zn(DDC)_2$ was metabolized much slower than KDDC and use of the former probably would have precluded the detection and isolation of transient metabolites. KDDC was obtained from Pennsalt Chemical Corp., Philadelphia, Penn. Radioactive KDDC labeled with ^{35}S was purchased from Volk Radiochemical Co., Chicago, Ill. Prior to use ^{35}S-KDDC was exhaustively extracted with benzene to remove elemental ^{35}S which was the principle radiochemical inpurity resulting from radiodecomposition. Radioactive KDDC labeled with ^{14}C in the methyl groups was obtained from Dr. J. W. Hylin, Department of Agricultural Biochemistry, University of Hawaii. $Zn(DDC)_2$, either radioactive or non-radioactive, was prepared from KDDC by adding a stoichiometric amount of zinc chloride dissolved in water to an aqueous solution of the dithiocarbamate. The precipitated $Zn(DDC)_2$ was extracted into chloroform and isolated by removal of solvent on a rotating evaporator. Prior to use all dithiocarbamates were checked for purity and radiopurity by chromatography and radiochromatography. Water soluble dithiocarbamates were dissolved in 0.02 M potassium phosphate buffer pH 7.4, containing 800 ppm Triton X-100 wetting agent, prior to root or leaf application. These solutions were buffered at pH 7.4 to prevent decomposition of KDDC which occurs under acidic conditions. $Zn(DDC)_2$ was dissolved in

dimethylformamide (DMF) and 0.01 ml was used for leaf application. DMF solutions did not produce any observable phytotoxicity when applied to papaya leaves.

Reagents: All reagents were prepared from chemicals meeting A.C.S. standards.

Plant materials: Papaya seeds of Solo line 5 variety were used throughout this work. They were germinated in the greenhouse in pots containing Vermiculite. In experiments using seedlings the Vermiculite was washed from the roots with a gentle stream of tap water. Larger plants were grown from 3 week old seedlings transplanted to pots containing sterilized soil. These plants were watered and fertilized according to good horticultural practices. Nutrient solution was prepared according to Hoagland and Arnon (22) and applied twice a week throughout the experiment.

Chromatography: 1. Thin layer chromatography: The procedure of Hylin (24) was used for thin layer chromatography of dithiocarbamates on silica gel. Benzene was used as the developing solvent.

2. Paper chromatography: Whatman #1 and #3 mm papers were used throughout. Samples containing amino acids were purified by column chromatography[2] prior to separation and identification. The following solvent systems were used. Methanol, water, pyridine (80:20:4); n-butanol, glacial acetic acid, water (60:15:25); phenol, water, ammonia (80:20:0.5); butanol, pyridine, water (1:1:1); phenol, water (80:20); 95% ethanol, water (63:37). The solvent system used for

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[2] Communication from Dr. W. Koinigsberg. Procedure for Edman Degradation.

dithiocarbamates and metabolites was n-propanol, water (85:15). The solvent system used for carbohydrate separations was n-butanol, glacial acetic acid, water (4:1:1).

3. Spray reagents: Amino acids were detected on paper chromatograms with 0.2% ninhydrin in n-butanol. Dimethyldithiocarbamates were detected on paper chromatograms and thin layers with the following reagent (14, p. 233). Solution A contains 1.5 gm cupric chloride and 3 gm ammonium chloride in 50 ml water containing 3 ml of concentrated ammonia. Solution B contains 20 gm hydroxylamine hydrochloride in 100 ml water. Equal volumes of solutions A and B are mixed just before use. Dithiocarbamates appear as reddish brown spots against a white background. DDC-glucoside was detected with ammoniacal silver nitrate (61).

Radioactivity determinations: Chromatograms containing radioactive substances were assayed with a Packard Radiochromatogram Scanner, Model 7200. Paper strips were scanned before and after development to permit quantitation of the amount of radioactivity in each of the separated components.

Volatile radioactive metabolites were collected in suitable trapping reagents. Twenty percent zinc acetate was used for hydrogen sulfide, Viles' reagent (49) for carbon disulfide, 0.1N hydrochloric acid for dimethylamine, and 15% potassium hydroxide for carbon dioxide. Radioactive hydrogen sulfide was removed from the trapping solution by the addition of 7.8 mg of carrier sodium sulfide. The resulting precipitate was collected on a glass fiber disc, washed and dried. The precipitated radioactivity was determined with a Nuclear Chicago thin window gas flow counter with automatic sample changer and

printout and an efficiency of 26%. Suitable aliquots of carbon disulfide trapping solutions were dried on a planchet and counted as above. Similarly, dimethylamine trapping solutions were transferred to a planchet and counted.

The method of Katz and Golden (34) was employed for the determination of radioactive sulfate.

Stems and roots were sliced and vacuum dried for 15 hours at 65°C. The dried samples were finely ground by mortar and pestle. The radioactivity in a 300 mg of dried sample was determined by the macro peroxide bomb method.[3]

Wet leaf residue (0.5 g) was immersed in a test tube with 4 ml of 40% KOH and the contents were boiled in a steam bath for 6 hours. The contents were cooled and homogenate was diluted to 125 ml with water and 0.1 ml was placed on a tared planchet. It was then dried, weighed and the radioactivity measured.

Radioactive carbon dioxide was precipitated as barium carbonate by addition of 10% barium chloride solution to the trapping solution. The precipitate was collected on a filter, washed with water and 95% ethanol and dried. The dried sample was ground to a fine powder, transferred to a tared 20 ml scintillation counting vital and dried to constant weight at 105°C. The vial was filled with Cab-O-Sil thixotropic agent and 20 ml of scintillator solution (17) containing 4 gm 2,5-diphenyloxazole (PPO) and 0.05 gm 1,4-bis (-2(5-phenyloxazole) -benzene) (POPOP) per liter of toluene. Samples were counted in a

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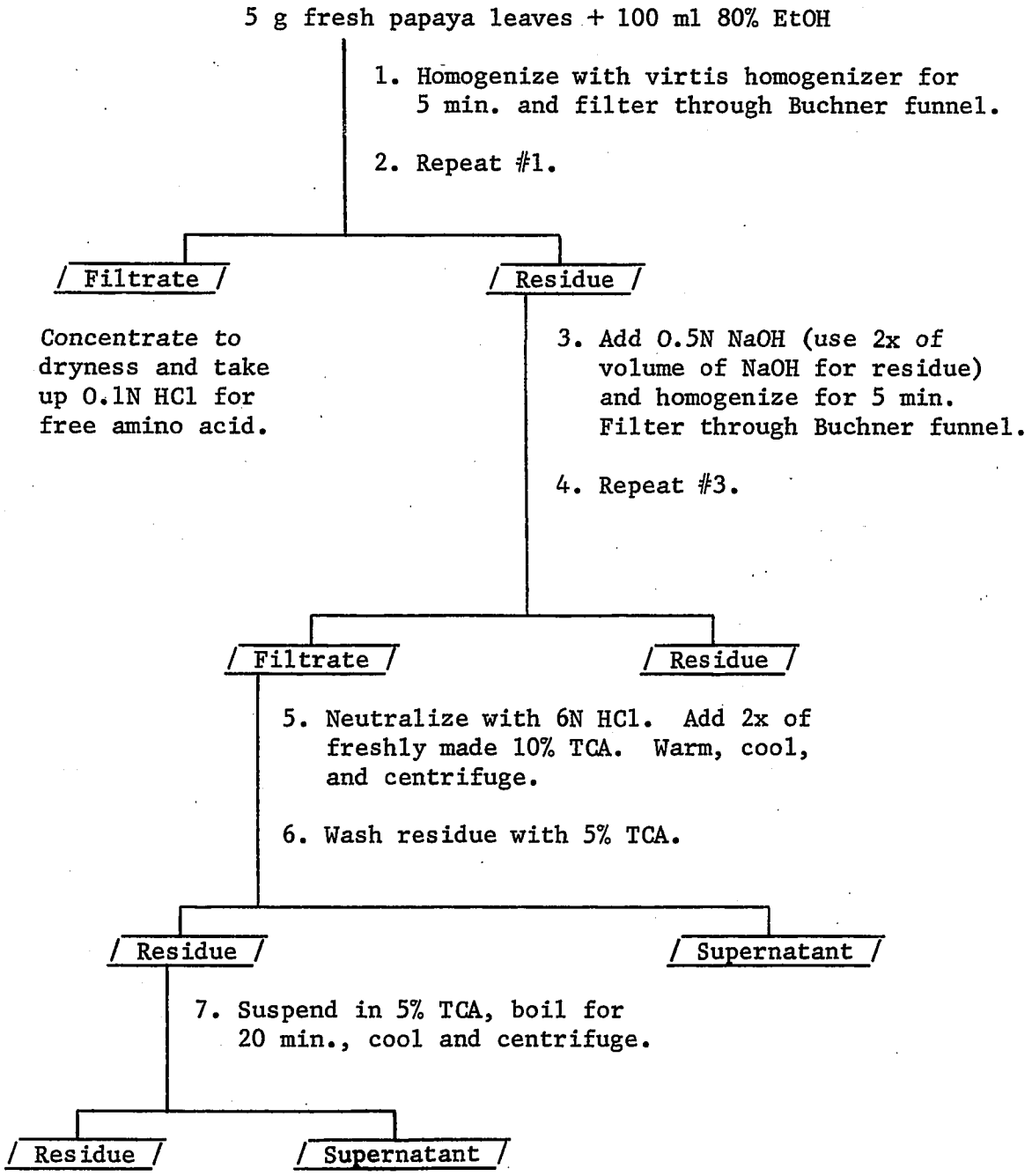
[3] Parr Manual No. 121. 1950. Parr Instrument Co., 211 Fifty-third St., Moline, Illinois. Macro peroxide bomb methods. p. 27.

Packard Tri-Carb Liquid Scintillation Spectrometer which had an efficiency of 55% for carbon 14, as determined with standard ^{14}C-barium carbonate (18.5 dps/mg).

Isolation of leaf protein

Leaf protein was isolated from the insoluble residue (10 g) remaining after solvent extraction and purified by repeated reprecipitation (Fig. 1). It was hydrolyzed (6 N HCL) for 17 hours at 105°C and the hydrolysate was concentrated to dryness with a rotorvap. After purification (5, p. 64) the protein hydrolysate was chromatographed as described for the 80% ethanol fraction containing the free amino acids.

Figure 1. Isolation of protein from papaya leaves



8. Transfer to tared test tube using 95% EtOH and centrifuge. Wash residue with absolute EtOH, 2x with acetone, 3x with anhydrous ether, dry by hand warming and weigh.

RESULTS AND DISCUSSION

In the initial phase of this work, the localization of applied radioactive fungicide within the plant was determined. ^{35}S-KDDC solution (1.61 x 10^6 cpm in 0.1 ml) was applied to the second or third youngest leaves of fifteen 3 month old papaya plants. At weekly intervals, the leaves were removed from five plants and analyzed. Residual surface deposits were estimated by washing the treated leaves. Leaves were manually shaken in an Erlenmeyer flask for 1 minute intervals with three 70 ml portions of distilled water. The combined washings were made up to 250 ml and aliquots were counted on planchets after drying. The radioactivity remaining in and on the leaves was estimated by counting suitable pulverized samples. Treated and untreated leaves were analyzed separately to permit estimation of the amount of translocation of radioactivity (Table 2). Evidently some radioactivity is translocated from the treated leaves to the untreated leaves and the amount apparently increased with time after the application of the labeled compound. However, the major portion of the applied radioactivity not removable from the leaves by washing with water, remained in the treated leaves.

The solubility characteristics of the radioactivity remaining in treated and untreated leaves following washing with water were next determined. Radioactive ^{35}S-KDDC was applied to one leaf of a number of papaya plants. After seven days the leaves were removed, the treated ones were kept separate from the untreated, and thoroughly washed with water. The fresh water washed leaves were homogenized

Table 2

Distribution of ^{35}S following application of ^{35}S-KDDC (1.61×10^6 cpm in 0.1 ml) to the leaves of papaya plants. (data are averages of 5 replicates)

		counts per minute x 10^4					
		water washings		pulverized leaves			
weeks after application	% recovery of ^{35}S	treated leaves	% total	treated	% total	untreated	% total
1	82	90	34	155	59	18	7
2	72	64	27	148	64	20	9
3	70	78	32	131	55	31	13

Table 3

Radioactivity in extracts of leaves from papaya plants treated with ^{35}S-KDDC. (cpm x 10^2)

treated leaves

solvent sequence

	1		2		3		4		5	
solvent	cpm	% total	cpm	% total	cpm	% total	cpm	% total	cpm	% total
$CHCl_3$	149	10	---	---	---	---	---	---	---	---
ethyl ether	6	0	24	1	---	---	---	---	---	---
95% ethanol	166	12	146	8	234	12	---	---	---	---
water	1057	74	1590	84	1590	84	1980	98	---	---
0.1 N HCl	51	4	124	7	73	4	34	2	513	100
total	1429		1884		1897		2014		513	

untreated leaves

solvent	cpm	% total	cpm	% total	cpm	% total	cpm	% total	cpm	% total
$CHCl_3$	0	0	---	---	---	---	---	---	---	---
ethyl ether	0	0	0	0	---	---	---	---	---	---
95% ethanol	6	4	6	4	41	24	---	---	---	---
water	161	95	158	96	108	65	184	100	---	---
0.1 N HCl	2	1	0	0	18	11	0	0	126	100
total	169		164		167		187		126	

with 50 ml portions of a series of solvents. The solvents were used in an increasing order of polarity. After each homogenization the residue was collected on a Buchner funnel and rehomogenized in the next solvent. Five solvent sequences were prepared by deleting each solvent in turn to determine the effects on the extractability of radioactivity by the preceding solvent.

The sequences used were:

1. $CHCl_3$, ethyl ether, 95% ethanol, water, 0.1 N HCl
2. ethyl ether, 95% ethanol, water, 0.1 N HCl
3. 95% ethanol, water, 0.1 N HCl
4. water, 0.1 N HCl
5. 0.1 N HCl

Aliquots of each extract were assayed for radioactivity (Table 3). The major portion of extractable radioactivity in both treated and untreated leaves is in a water soluble form. The chloroform soluble radioactivity observed in the treated leaves may be due to insoluble TMTD present on the leaf surface as a result of oxidation of DDC. The absence of chloroform soluble radioactivity in the untreated leaves indicates that heavy metal salts of DDC (which are chloroform soluble) are not translocated. The reasons for the low recovery of radioactivity in solvent sequence five (0.1 N HCl) are not known but this effect may be due to absorption of the water soluble radioactive substances on plant colloids at this low pH.

A further attempt was made to classify the radioactive substances found in the leaves following the application of radioactive fungicide. One microcurie of ^{35}S-KDDC was applied to each of a number of papaya

plants. Leaves from five plants were removed at 3, 7, and 21 days after fungicide application of ^{35}S-KDDC and washed thoroughly with water. The washed leaves were vacuum dried for 15 hours at 65°C. The dried leaves were subjected to Soxhlet extraction using $CHCl_3$ for 15 hours, 80% ethanol for 24 hours, and water for 15 hours. Aliquots of each extract were dried on a planchet and counted. In addition radioactivity in the extracted residue and the amount of radioactivity present as sulfate in the water extract were estimated (Table 4). The decrease with time of the radioactivity in the insoluble residue indicates active metabolism of ^{35}S derived from the applied fungicide. The difference in radioactivity extracted by the various solvents when compared with the preceding experiment are probably due to differences in extraction conditions. The extraction of wet tissue with organic solvents is usually much less efficient than extraction of the same tissue after all the moisture has been removed. Because this was a preliminary study and was not replicated the significance of the differences in extractable radioactivity observed with time can not be determined. The finding of radioactive sulfate in water extract was unexpected as was the apparent constant amount of radioactivity due to sulfate. Since sulfate should be readily metabolized by the plant the fact that the % of radioactivity present in water extract as sulfate remained constant indicates a continual production of radioactive sulfate from either the applied compound or some other metabolite. The results of these preliminary experiments showed that the labeled fungicide was entering the plant

Table 4

Distribution of ^{35}S among Sohxlet extracts of leaves from papaya plants treated with ^{35}S-KDDC.

days after application	% recovery of applied ^{35}S	% total radioactivity isolated				specific activity of residue cpm/mg	% activity as $^{35}SO_4$ in water extract
		chloroform extract	ethanol extract	water extract	residue		
3	--	22	26	35	17	974	15.3
7	53	37	39	15	8	511	15.3
21	49	45	39	12	4	148	15.0

and being metabolized into predominantly water soluble products. The remainder of the research work has been devoted to qualitative identification of the products of metabolism and quantitation of these products.

Determination of residual water soluble radioactivity on leaf surfaces.

^{35}S-KDDC, ^{14}C-KDDC, ^{35}S-$Zn(DDC)_2$, and ^{14}C-$Zn(DDC)_2$ were applied with a micropipet to 3 or 4 young leaves of 3 month old papaya plants. Immediately after the applied solution has dried (zero time) and at 3, 7, 14, and 21 days after the treatment, the amount of radioactivity remaining on the leaf surfaces was determined by washing the leaves with 250 ml of water. Three one ml aliquots of the wash water were used for measurement of radioactivity.

A similar experiment was performed using 1 month old papaya plants to see whether the difference in age of plant would change the recovery of residual water soluble radioactivity. Radioactive solutions (0.05 ml each of ^{35}S-KDDC and ^{14}C-KDDC) were applied to all the leaves of 5 papaya plants (each plant had 3 to 4 leaves with 2 cotyledons) with a micropipet. At 3, 7, and 14 days after the treatment the leaves from 5 plants were picked, washed with 150 ml of water and three 1 ml aliquots of the water washings were used for counting the radioactivity. Radioactive sulfate was also determined in the water washing. These results are summarized in Tables 5, 6, and 7. Data for Tables 5 and 6 are shown in detail in the Appendix, Tables 16, 17, and 18. These experiments demonstrated that the

Table 5

Radioactivity recovered in wash water from papaya leaves after application of labeled dithiocarbamates onto 3 month old plants. (data are averages of 3 replicates)

days after application	% recovery of applied compound			
	^{14}C-KDDC	^{35}S-KDDC	^{14}C-Zn(DDC)$_2$	^{35}S-Zn(DDC)$_2$
0	84.0	90.0	24.0	68.0
3	19.1	23.3	---	---
7	14.9	18.6	16.9	19.0
14	14.3	16.5	9.8	9.9
21	6.6	13.7	5.6	14.3

Table 6

Radioactivity recovered in wash water from papaya leaves after application of labeled dithiocarbamates onto 1 month old plants. (data are averages of 3 replicates)

days after application	% recovery of applied compound	
	^{14}C-KDDC	^{35}S-KDDC
3	18.5	16.7
7	6.6	7.8
14	4.6	7.0

Table 7

Radioactivity recovered as sulfate in wash water from papaya leaves after application of labeled dithiocarbamates onto 3 month old plants. (data are averages of 3 replicates)

	compound applied							
	^{35}S-KDDC (36,700 cpm in 0.1 ml)				^{35}S-Zn(DDC)$_2$ (25,200 cpm in 0.1 ml)			
weeks after application	cpm in wash water	cpm as SO_4	% of radio-activity as SO_4 in wash water	% of applied radio-activity	cpm in wash water	cpm as SO_4	% of radio-activity as SO_4 in wash water	% of applied radio-activity
1	13,340	1,638	12.3	4.5	4,787	596	12.5	2.4
2	11,944	2,230	18.7	6.1	2,500	620	24.8	2.4
3	2,409	229	9.5	0.6	3,614	229	6.3	0.9

amount of radioactivity which could be recovered by washing the leaf surfaces with water decreased with time after application. Even at zero time 100% of the applied radioactivity could not be recovered when KDDC had been applied and the recovery of $Zn(DDC)_2$ was much smaller. This latter result was probably due to the low water solubility of $Zn(DDC)_2$.

Comparison of the recoveries from 1 month and 3 month old plants treated with radioactive KDDC, showed poorer recovery of this compound than the former. Whether this is due to more rapid absorption into the plant or to oxidation and hydrolysis on the leaf surface is not known. The amount of radioactive sulfate (Table 7) present in the water washings from ^{35}S-KDDC treated plants was about double the amount recovered from plants treated with ^{35}S-$Zn(DDC)_2$. However, this difference is probably not significant since both quantities were relatively small. In both cases, the amount of radioactivity as sulfate increased up to the second week after application and then showed a sharp drop in the third week. The reasons for these changes are not known.

Determination of volatile metabolites of dithiocarbamates.

^{35}S-KDDC, ^{14}C-KDDC, ^{35}S-$Zn(DDC)_2$, and ^{14}C-$Zn(DDC)_2$ were applied to the leaves of 3 month old papaya plants. Each treated plant was then placed in a sealed chamber at 75°F (23) and illuminated with fluorescent light (250 footcandles) for a period of 9 hours daily while purified air was continuously drawn through the chamber. The effluent gases were passed through traps selected for the isotope

under investigation. The traps for $C^{35}S_2$ and $^{14}CO_2$ were replaced at 3 or 4 day intervals while the traps for $H_2{}^{35}S$ and $(^{14}CH_3)_2NH$ remained in place for the duration of the experiment (14 days). Radioactivity in each trap was determined as described in Materials and Methods and results of these determinations are shown in Table 8. $Zn(DDC)_2$ is less susceptible to hydrolytic action because of its relative insolubility. On the other hand approximately half of the decomposition of KDDC which occurs in 14 days takes place during the three days after application. Recovery of $(CH_3)_2NH$ is considerably lower than CS_2 presumably due to the reactivity of the former. The same acidic material which catalyzes the decomposition of the dithiocarbamates will subsequently fix the $(CH_3)_2NH$ as its dimethylammonium salt. This substance is readily absorbed and metabolized as is evident from the recovery of radioactive CO_2 after application of ^{14}C labeled fungicides. The exceptional amount of CO_2 recovered following application of ^{14}C labeled $Zn(DDC)_2$ cannot be explained at present.

Recovery of radioactive metabolites of dimethyldithiocarbamates from papaya tissues.

Solutions of ^{35}S-KDDC and ^{14}C-KDDC were applied to the leaves of 1 and 3 month old papaya plants. At 3, 7, and 14 days in the case of 1 month old plants, and at 3, 7, 14 and 21 days in the case of 3 month old plants, a number were removed from the potting medium and separated into leaves, stems and roots. The leaves of 3 month old plant were thoroughly washed and partially dried between filter papers to remove excess moisture. Leaf sap was obtained by pressing

Table 8

Volatile radioactive substances recovered following applications of labeled dimethyldithiocarbamates to the leaves of 3 month old papaya plants. (data are averages of 3 replicates)

compound and cpm applied	days after application				% recovery over a 14 day period	
	3	7	14	14		
	cpm as CS_2			cpm as H_2S	CS_2	H_2S
^{35}S-KDDC 1.65×10^7	487,000	936,700	943,000	0	5.7	0
^{35}S-Zn(DDC)$_2$ 1.36×10^7	660	914	1,976	9	0.15	0.0007
	cpm as CO_2			cpm as $(CH_3)_2NH$	CO_2	$(CH_3)_2NH$
^{14}C-KDDC 2.9×10^5	1,222	2,884	4,996	372	1.5	0.13
^{14}C-Zn(DDC)$_2$ 1.02×10^5	13,023	34,444	64,134	166	6.4	0.016

washed leaves in a Carver press at 14,000 pounds per square inch. The leaves of 1 month old plants were thoroughly washed and leaves were homogenized by mortar and pestle since amount of leaves were too small for pressing. The homogenate was then filtered through a Buchner funnel using small amount of water for transferring homogenized leaves remaining in the pestle.

The sap solution was adjusted to 45% ethanol with 95% ethanol and, after standing for 30 minutes at room temperature, was centrifuged to remove insoluble material. Aliquots of the 45% ethanol soluble sap were assayed for radioactivity (Tables 9 and 10).

Resonably constant amounts of radioactivity (^{35}S and ^{14}C) were recovered in the 45% ethanol solubles from leaves of 3 month old papaya plants. Recoveries of ^{35}C and ^{14}C in the 45% ethanol solubles from leaves of 1 month old papaya plants were much higher. This difference in the recovery of radioactivity may have been due to the different methods used in the preparation of cell sap. Homogenization of the leaves was probably more efficient than pressing. This can be seen if the sum of the radioactivity recovered in the expressed saps (Table 9) and residue (Table 11) from 3 month old papaya plants treated with ^{35}S-KDDC is compared with the recoveries from similarly treated 1 month old plants (Table 10).

Portions of the residue remaining after expressing the cell sap from leaves of 3 month old papaya plants, together with samples of roots and stems were also assayed for radioactivity with results shown in Tables 11 and 12.

Table 9

Radioactivity recovered in cell sap expressed from papaya leaves after leaf application of labeled KDDC onto 3 month old plants. (data are averages of 3 replicates)

	compound applied			
	^{14}C-KDDC (5.18 x 10^5 cpm in 0.1 ml)		^{35}S-KDDC (9.26 x 10^5 cpm in 0.1 ml)	
days after application	cpm x 10^5 in sap	ave. % recovery	cpm x 10^5 in sap	ave. % recovery
3	1.36 (1.16-1.68)*	26.2	1.30 (1.12-1.63)	14.0
7	1.29 (1.15-1.45)	24.9	1.61 (1.44-1.74)	17.4
14	1.04 (0.83-1.16)	20.0	1.34 (1.09-1.57)	14.5
21	1.36 (1.13-1.54)	26.3	1.32 (1.16-1.46)	14.3

* range

Table 10

Radioactivity recovered in 45% ethanol soluble cell sap from papaya leaves after leaf application of labeled KDDC onto 1 month old plants (each value represents one plant).

	compound applied			
	^{14}C-KDDC (1.25×10^5 cpm)		^{35}S-KDDC (3.18×10^5 cpm)	
days after application	cpm x 10^5 in ethanol soluble sap	% recovery	cpm x 10^5 in ethanol soluble sap	% recovery
3	1.01	80.8	1.22	38.4
7	0.65	52.0	0.64	20.1
14	0.70	56.0	0.77	24.2

Table 11

Radioactivity recovered in leaf residue after expressing cell sap from leaves of 3 month old plant whose leaves were treated with labeled KDDC.

	compound applied		
	^{35}S-KDDC (2.032 x 10^7 cpm)		
days after application	cpm x 10^5 in wet leaf residue	% recovery of applied activity	total wet weight (g) of leaf residue
3	6.91	3.4	3.31
7	5.69	2.8	2.47
14	9.35	4.6	2.13
21	5.89	2.9	3.66

Table 12

^{35}S recovery in stem and root after the leaf application of K and Zn salts of ^{35}S-labeled dimethyldithiocarbamate on 3 month old plants.

		compound applied			
		^{35}S-KDDC (36,700 cpm in 0.1 ml)		^{35}S-Zn(DDC)$_2$ (25,200 cpm in 0.1 ml)	
weeks after application	tissue	total cpm found	% recovery of applied activity	total cpm found	% recovery of applied activity
1	stem	504	1.4	37	0.1
2		247	0.7	50	0.2
3		61	0.2	200	0.8
1	root	160	0.4	25	0.3
2		395	1.1	12	0.05
3		257	0.7	205	0.8

The quantity of applied radioactivity present in root and steam tissue up to 3 weeks after application of either KDDC or $Zn(DDC)_2$ was usually less than one percent.

The metabolism of dimethyldithiocarbamates when applied to roots of papaya plants was also studied. Fifty 1 month old plants were placed in 50 ml of ^{35}S-KDDC or ^{14}C-KDDC solution. After 8 hours of immersion the plants were removed from the container, their roots thoroughly washed, and the washed plants transplanted into pots containing Vermiculite. At 3, 7, and 14 days after treatment the 45% ethanol soluble fraction was obtained and assayed as described above. (Table 13). Less than 1.5 percent of ^{14}C-KDDC applied to the roots of papaya plants was recovered in the expressed sap compared to between 52 and 80.8% recovered when application was to the leaves. Similar differences were observed with ^{35}S-KDDC. These differences may in part be due to the short contact (8 hours) which the plants had with the labeled compounds, or may indicate poor absorption of KDDC by roots.

Metabolites of ^{14}C-KDDC and ^{35}S-KDDC present in the sap pressed from papaya leaves after leaf and root application.

The remainder of the 45% ethanol fraction from leaves of 3 month and 1 month old plants was concentrated and subjected to paper chromatography using n-propanol, water (85:15, v/v). The Rf values observed and the quantities of radioactive substances found are summarized in Table 14. The complete data may be found in the Appendix Tables 19, 20, and 21. Tracings of representative radiochromatograms are shown in Figure 2. The radiochromatograms of cell sap showed that the metabolites of KDDC present were similar regardless of isotope applied,

Table 13

Radioactivity recovered in 45% ethanol soluble cell sap after root application using 1 month old plants.

days after application	compound applied: ^{14}C-KDDC (2.08 x 10^6 cpm on 10 plants) cpm x 10^4 found	% recovery	wt. of dried leaves (mg) from 10 plants	^{35}S-KDDC (3.43 x 10^6 cpm on 10 plants) cpm x 10^3 found	% recovery	wt. of dried leaves (mg) from 10 plants
3	3.12	1.5	56	12.2	0.4	34
7	1.87	0.9	61	9.1	0.3	36
14	2.29	1.1	64	8.5	0.3	35

Table 14

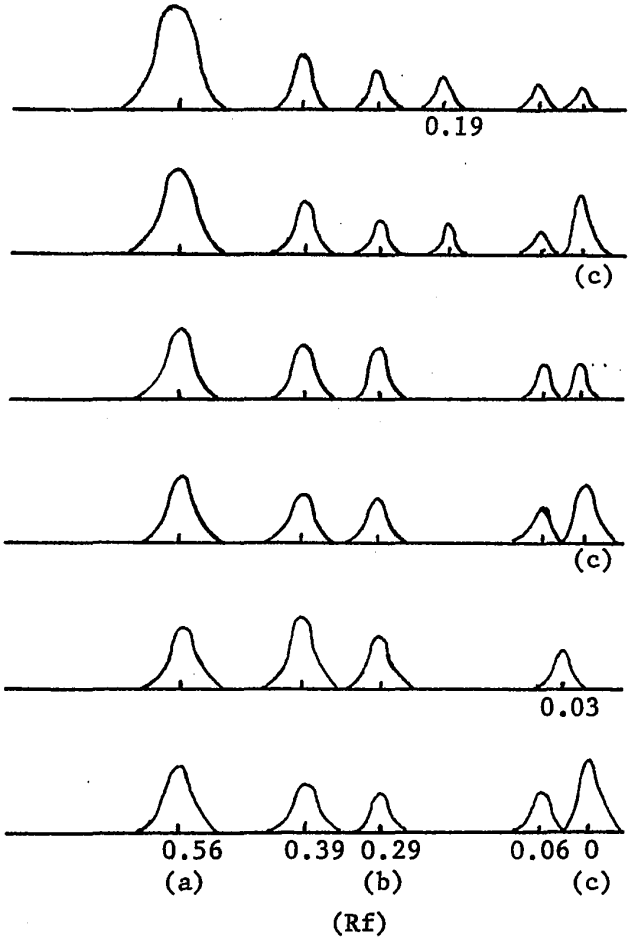
Rf values and % recovery of applied activity of metabolites of dimethyldithiocarbamate fungicide residue present in 45% ethanol soluble cell sap expressed from papaya leaves.

age of plant		3 month		1 month		1 month	
site of application		leaves		leaves		root	
compound applied		^{14}C-KDDC	^{35}S-KDDC	^{14}C-KDDC	^{35}S-KDDC	^{14}C-KDDC	^{35}S-KDDC
amount of radioactivity applied (cpm x 10^5)		5.18	9.26	1.25	3.18	20.8	34.3
days after application	Rf values	% recovery of applied activity					
3	0.56(a)	20.3	5.3	27.8	11.0	0.45	0.07
	0.39	3.6	1.0	16.2	2.4	0.76	0.04
	0.29(b)	1.1	0.7	16.2	4.3	0.21	0.02
	0.19	0.9	0.5	---	---	---	---
	0.06	0.2	0.4	11.6	2.2	---	0.03
	0.03	---	---	---	---	0.08	---
	0	0.2	8.1	9.0	18.5	---	0.24
7	0.56	18.9	10.0	22.6	3.2	0.14	0.09
	0.39	3.9	0.7	14.2	1.1	0.39	0.02
	0.29	0.9	1.0	5.0	1.9	0.31	0.08
	0.19	0.8	0.5	---	---	---	---
	0.06	0.3	0.4	4.6	1.5	---	0.01
	0.03	---	---	---	---	0.06	---
	0	---	7.4	5.5	12.4	---	0.11
14	0.56	14.5	8.3	23.8	5.2	0.36	0.08
	0.39	3.1	0.9	18.0	3.0	0.34	0.01
	0.29	1.1	1.4	5.3	1.4	0.29	0.05
	0.19	0.7	1.6	---	---	---	---
	0.06	0.6	0.3	3.7	1.0	---	0.01
	0.03	---	---	---	---	0.10	---
	0	0.1	4.2	5.3	13.7	---	0.14
21	0.56	22.0	11.0				
	0.39	2.1	0.7				
	0.29	0.7	0.9				
	0.19	0.7	1.4				
	0.06	0.8	0.6				
	0.03	---	---				
	0	---	2.4				

(a) DDC-glucoside
(b) DDC-alanine

Figure 2

Radiochromatogram tracings of cell sap chromatograms



compound applied	age of plants (month)	site of application
^{14}C-KDDC	3	leaf
^{35}S-KDDC	3	leaf
^{14}C-KDDC	1	leaf
^{35}S-KDDC	1	leaf
^{14}C-KDDC	1	root
^{35}S-KDDC	1	root

note: size of peak approximates amount of metabolite present

(a) DDC-glucoside
(b) DDC-alanine
(c) SO_4 (?)

site of application, or age of plant. One unknown substance with Rf 0.19 was found only in cell sap from 3 month old plants. A small peak at Rf 0.03 was observed only in cell sap from plants whose roots had been treated with ^{14}C-KDDC. On the other hand, the quantities of the individual metabolites did vary greatly with the age of the plants. The major differences were in the greater amounts of radioactive substances at Rf 0.39 and 0.29 in the younger plants.

Appreciable amounts of radioactivity remained at the origin when sap from ^{35}S-KDDC was chromatographed. The principal component of this radioactivity was inorganic sulfate which did not migrate under the chromatographic conditions used.

Several plant metabolites of DDC have been isolated (9, 10, 40). In order to determine whether radioactive substances observed on the radiochromatogram corresponded to any of the known plant metabolites of DDC, the preparation of one of these, DDC-glucoside was attempted.

KDDC solution (3000 ppm) was sprayed on papaya plants with a hand sprayer until excess solution started dripping from the tips of the leaves. The treated plants were gently shaken several times to remove excess solution, and then placed in the greenhouse. Three days after the application, the cell sap was obtained and adjusted to 45% ethanol as described previously. The concentrated 45% ethanol soluble sap (non-radioactive) was applied as a band 14 inches wide, 3 inches from the bottom of the Whatman #1 paper. Radioactive sap was co-chromatographed at each side of this band in order to non-destructively locate the band corresponding to the metabolite with Rf of 0.56. The chromatogram was scanned and the radioactive and non-radioactive areas

with Rf 0.56 were located, cut from the paper and eluted. When a portion of the eluted metabolite having Rf of 0.56 was rechromatographed, it reacted positively to ammoniacal $AgNO_3$ indicating the presence of reducing substance, probably a sugar. The two eluates were mixed and the mixture was hydrolyzed by refluxing with 1 N HCl for 1 hour to liberate the glucose residue presumed to be present. Nitrogen was bubbled through the reaction mixture continuously and the effluent stream was passed through a CS_2 trap of Viles' reagent before being vented.

The hydrolysate, together with monosaccharide standards, were chromatographed on Whatman #1 paper for 16 hours using n-butanol, glacial acetic acid, water (4:1:1) as the developing solvent. The chromatogram was sprayed with ammoniacal $AgNO_3$ and a major positive spot corresponding to the glucose standard was observed in the hydrolysis. Aliquots of the CS_2 trapping solution were assayed for radioactivity and 1.4×10^4 cpm were found. This represented 7% of the activity present in the aliquot prior to hydrolysis. The yield of radioactive CS_2 was much lower than was expected. This was probably due to the conditions of hydrolysis which were selected for the liberation of glucose. The analytical procedure for the determination of CS_2 from dithiocarbamates utilizes much more vigorous conditions than were employed in this hydrolysis.

Radioactive sap was applied as a hand 1 inch wide and 3 inches from the bottom of the Whatman #1 paper. Authentic DDC-glucoside was co-chromatographed at each side of this band for 16 hours using n-propanol, water (85:15), and n-butanol, glacial acetic acid, water

(4:1:1) as the developing solvents. The radioactive area corresponded exactly with the position of authentic DDC-glucoside in both solvent systems.

Co-chromatography of radioactive areas having Rf 0.29 in n-propanol, water, with authentic DDC-alanine in two solvent systems confirmed the identity of this metabolite.

In view of the extensive metabolism of DDC observed in this work, it seemed likely that ^{35}S from ^{35}S-KDDC could be found in the free amino acids and protein fractions of papaya leaf. To study this possibility ^{35}S-KDDC (6.4×10^6 cpm) was applied to the leaves of each of ten 3 month old plants. Three weeks later the leaves were collected, washed and dried at 65°C. The dried leaves were decolorized and defatted by Sohxlet extraction with $CHCl_3$ for 15 hours. The residues were extracted with 80% ethanol for 24 hours to isolate free amino acids and other soluble substances. The 90% ethanol extract was concentrated and 1 ml of the concentrate was diluted with 4 ml of 0.01 M HAc. This solution was subjected to column chromatography to remove non-ionic impurities prior to paper chromatographic identification of amino acids. Amino acids were separated using 6 solvent systems. Radioactive sulfur amino acids, methionine, methionine sulfone, homocyst(e)ine, and cyst(e)ine were found. The leaf protein was isolated and purified. The specific activity of the purified protein was 4522 cpm/mg indicating an appreciable oxidation of dimethyldithiocarbamate to sulfate. Isolated protein was subjected to acid hydrolysis and hydrolysate was analyzed for amino acids as described for 80% ethanol fraction containing the free amino acids. The same radioactive

sulfur amino acids were found in the protein hydrolysate. It should be noted that presence of homocysteine in the protein hydrolysate is probably an artifact due to oxidative coupling of protein cysteine with free homocysteine during protein isolation.

Regardless of the mechanism of formation of sulfate, this sulfate accounts for the presence of radioactive sulfur in the free amino acid and protein fraction of the plants by known metabolic pathways.

GENERAL DISCUSSION

The emphasis in this study has been placed upon the quantitative aspects of the metabolism of dimethyldithiocarbamates by papaya. Nevertheless, the qualitative features of this metabolism have not been ignored. The expected known metabolites of these compounds, DDC-glucoside and DDC-alanine, were found in the expressed cell sap, and were isolated, identified, and quantitated. In leaves from 3 month old plants, approximately 20% of the applied compounds were converted to these metabolites within 3 days and this level remained relatively constant for up to 21 days after application (Table 15). Not only did the total amount of metabolites present in the cell sap remain constant but the amounts of individual substances remained relatively unchanged during this period (Figure 2). The major metabolite was DDC-glucoside, which accounted for from 32% to 85% of the total radioactivity in the cell sap. DDC-alanine accounted for approximately 3% to 20% and the remaining was present in unidentified substances (Appendix, Tables 19 and 20). These results may mean that the observed values represent the steady-state pool size of the individual components and that although these compounds may be metabolized further, their apparent content in the cell sap remains unchanged due to continuous synthesis from residues remaining on the leaf surface. This supposition is supported by the presence of long persisting leaf residues which, however, do slowly decrease. The relatively constant rate of release of radioactive carbon dioxide when methyl labeled DDC was used may also support this hypothesis if we assume that N-methyl groups are metabolized by a pathway including DDC-derivatives.

Table 15

% recovery of applied radioactivity in wash water, stem, root, leaf residue, sap, volatile compounds, and sulfate.

age of plant	site of application	sample	compound applied: ^{35}S-$Zn(DDC)_2$ days after application 7	14	21	compound applied: ^{14}C-$Zn(DDC)_2$ days after application 3	7	14	21
3 month old	leaf	wash water	28.0	14.6	20.6	---	70.4	40.8	23.3
		stem	0.1	0.2	0.8	---	---	---	---
		root	0.3	0.05	0.8	---	---	---	---
		CS_2	---	0.15	---	---	---	---	---
		H_2S	---	0.0007	---	---	---	---	---
		CO_2	---	---	---	1.3	3.4	6.4	---
		$(CH_3)_2NH$	---	---	---	---	---	0.016	---
		SO_4	2.4	2.5	0.9	---	---	---	---

age of plant	site of application	sample	compound applied: ^{35}S-KDDC days after application 3	7	14	21	compound applied: ^{14}C-KDDC days after application 3	7	14	21
3 month old	leaf	wash water(a)	25.9	20.7	18.3	15.2	23.0	18.0	17.2	7.8
		stem	---	1.4	0.7	0.2	---	---	---	---
		root	---	0.4	1.1	0.7	---	---	---	---
		leaf residue	3.4	2.8	4.6	2.9	---	---	---	---
		sap (b)	16.1	20.1	16.6	17.0	26.2	24.9	20.0	26.3
		CS_2	3.0	5.7	5.7	---	---	---	---	---
		H_2S	0	0	0	---	---	---	---	---
		CO_2	---	---	---	---	0.4	0.6	1.5	---
		$(CH_3)_2NH$	---	---	---	---	---	---	0.13	---
		SO_4	---	4.5	6.1	0.6	---	---	---	---
		total (a)+(b)	42.0	40.8	34.9	32.2	49.2	42.9	37.2	34.1
1 month old	leaf	wash water(c)	16.7	7.8	7.0		18.5	6.6	4.6	
		sap (d)	38.4	20.1	24.2		80.8	52.0	56.0	
		Total (c)+(d)	55.1	27.9	31.2		99.3	58.6	60.6	

Another possibility is that the derivatives are synthesized at a maximum rate shortly after application of the dithiocarbamate and that their concentrations reach a maximum which apparently remains constant because the rate of further metabolism of DDC-derivatives is much slower. Dekhuijzen (10) has shown that DDC-alanine and DDC-glucoside are inter-convertible but at a rate so slow as to be negligible. Some support for the second hypothesis may be derived from the results with 1 month old plants where a more dynamic system apparently exists for some time after application.

The detection of carbon disulfide, dimethylamine, carbon dioxide, and sulfate derived from DDC metabolism by plants is unique in this study. The possible formation of carbon disulfide and dimethylamine following application of dimethyldithiocarbamates to leaf surfaces could be inferred from the known physico-chemical properties of the compounds. On the leaf surface, the compound (I) is apparently hydrolyzed and thus gives rise to the materials from which it was synthesized.

$$\underset{\text{(I)}}{(CH_3)_2\overset{S}{\overset{\|}{N}}CS^-} \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} (CH_3)_2\overset{S}{\overset{\|}{N}}\underset{H^+}{CS^-} \rightleftharpoons \underset{\text{(II)}}{(CH_3)_2NH} + CS_2 \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} \underset{\text{(III)}}{(CH_3)_2NH_2^+}$$

Despite this fact, the production of these volatile products in vivo has not been previously reported, although the decomposition of dialkyldithiocarbamates by acid is known from the chemistry of these substances (37). The dimethylamine (II) liberated is apparently fixed rapidly as dimethylammonium ion (III), which is subsequently absorbed and metabolized. This may be concluded from the relative amounts of

dimethylamine and carbon dioxide recovered. The mechanism by which these N-methyl groups are oxidized is not known.

Another novel finding is the occurrence of sulfate as a major metabolites. Oxidation of the sulfur atoms of DDC or its derivatives to sulfate has not been observed before. It is interesting to speculate on the mechanism of its formation. One possibility is that oxidation takes place after derivatives of DDC have been formed. Thus, cleavage of DDC-glucoside to 1-desoxy-1-thioglucose and subsequent oxidation of this compound would yield sulfate. A similar cleavage of DDC-alanine would give cysteine and it is known that the sulfur atom of this molecule may be oxidized to sulfate. Similar mechanism can probably be written for other DDC derivatives.

A second possibility could be the direct oxidation of DDC ion by some unknown biochemical system. The normal mode of oxidation of DDC ion (IV) is to TMTD (V) (59, p. 61). This oxidation may be reversed in the presence of sulfhydryl groups and it seems unlikely that conversion to sulfate can occur in this way in a biological system.

$$\underset{\text{(IV)}}{2\ (CH_3)_2NCS^-} \underset{(SH\text{-}R)}{\overset{(O)}{\rightleftharpoons}} \underset{\text{(V)}}{(CH_3)_2NCSSCN(CH_3)_2}$$

A third possibility is the oxidation of liberated carbon disulfide. Although seemingly improbable, this reaction is not impossible since sulfate is the detoxification product of carbon disulfide in animals.

The presence of sulfate in the water washings from treated leaves indicates that one site of formation may be the epidermal layer of leaf cells. Whether such a site is the source of sulfate found in the cell sap is not known.

The data indicate that some translocation of metabolites occurs although almost exclusively restricted to water soluble substances. Approximately 60% of the recovered radioactivity present at any time is localized within the treated leaf while between 7 and 14% has been translocated to other leaves. There is no indication that chloroform soluble substances such as TMTD, $Zn(DDC)_2$, or other heavy metal salts of DDC penetrate the leaves and are translocated.

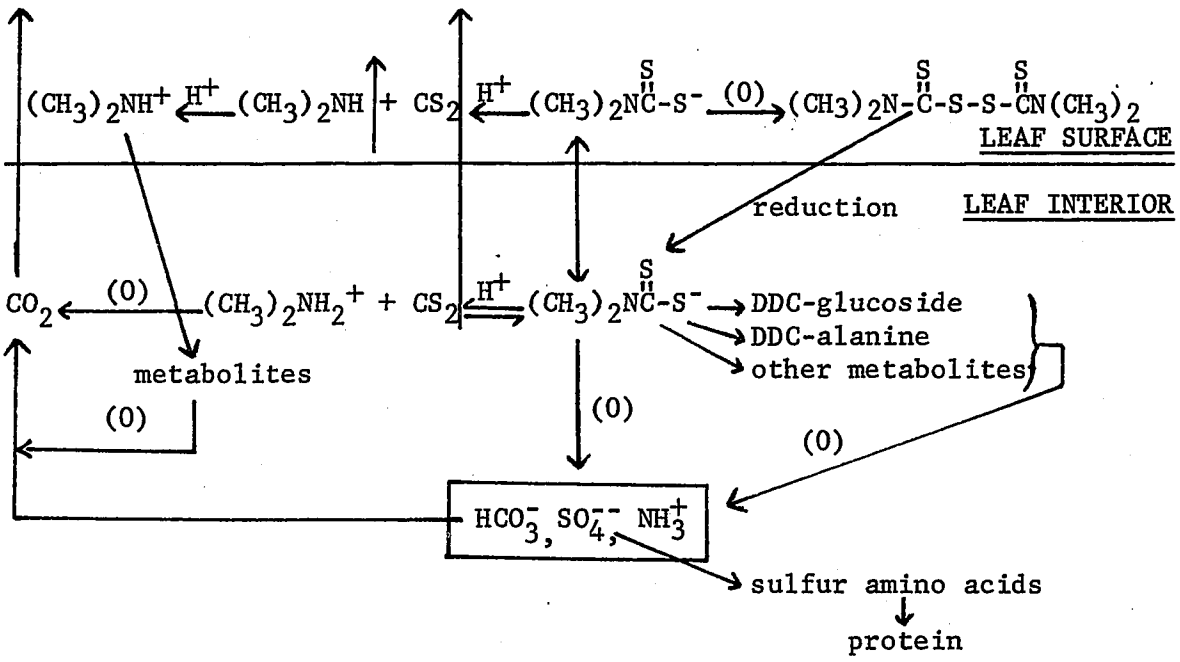
The results of studies with both KDDC and $Zn(DDC)_2$ are in general agreement. The discrepancies which do appear, such as greater evolution of CS_2 by KDDC are probably due to the greater solubility of the potassium salt. The reason for the exceptional amount of CO_2 evolved from $Zn(DDC)_2$ when compared with that from KDDC is unknown.

Conclusions based on the results of this study are summarized in Figure 3. Salts of DDC on the surface of a papaya leaf may be oxidized to TMTD, hydrolyzed to carbon disulfide and dimethylamine, or be absorbed into the outermost layer of cells. The TMTD may also be absorbed and reduced within the cell back to DDC ion. Once inside the cell, DDC ion may be converted to a variety of metabolites, including DDC-glucoside and DDC-alanine. DDC ion as well as its metabolites may be oxidized to CO_2, sulfate and some nitrogen compound such as ammonia. These products would be further metabolized by normal plant pathways. Some of the dimethylamine liberated on the leaf surface will be fixed as dimethylammonium ion and be absorbed into the cells where it can be further metabolized or oxidized to CO_2.

Several interesting problems resulting from this work remain. The identification of the unknown metabolites observed in the cell sap

Figure 3

Metabolism of dimethyldithiocarbamates by papaya leaf



(0) = nonspecific oxidation

could help in determining the pathways by which DDC and its derivatives are degraded. In addition the systems which give rise to carbon dioxide from the N-methyl groups, and sulfate from the thiocarbamyl residue should be elucidated. One further question which should be resolved is the non-equivalence of the carbon disulfide and the carbon dioxide plus dimethylamine isolated when $Zn(DDC)_2$ was applied to leaves. This missing carbon disulfide may be responsible for the observed phytotoxicity of the dimethyl dithiocarbamate fungicides.

SUMMARY

1. Dimethyldithiocarbamates applied to papaya leaves were transformed into a number of simple and complex intermediates.
2. Approximately 60% of the metabolites within the plant were in the treated leaf or leaves and the remainder had been translocated to the rest of the plant.
3. Approximately 20% of the applied compound remained on the surface of the leaves even after three weeks.
4. Six radioactive metabolites were found in the cell sap expressed from the leaves of the treated plants. Among these were DDC-glucoside and DDC-alanine.
5. The major metabolites were DDC-glucoside and DDC-alanine and their concentrations in the expressed cell sap remained remarkably constant throughout the duration of the study.
6. Several volatile metabolites and products were observed including dimethylamine, carbon disulfide, and carbon dioxide. The pathway for the formation of the latter from N-methyl groups of DDC is unknown.
7. ^{35}Sulfur label, derived from ^{35}S-KDDC by some unknown pathway, was found in both the free amino acids and proteins. Inorganic sulfate appeared to be an intermediate in this pathway.
8. Radioactivity recovery data for KDDC and $Zn(DDC)_2$ were in general agreement except for the higher recovery of CS_2 from KDDC and CO_2 from $Zn(DDC)_2$.

APPENDIX

Table 16

Radioactivity recovered in wash water from papaya leaves after leaf application of labeled KDDC onto 3 month old plants. (Data are averages of 3 replicates).

	compound applied			
	^{14}C-KDDC (6,200 cpm in 0.01 ml)		^{35}S-KDDC (14,000 cpm in 0.01 ml)	
days after application	cpm x 10^3 in wash water	ave. % recovery	cpm x 10^4 in wash water	ave. % recovery
0	5.2(4.9-5.6)*	84	1.26(1.16-1.37)	90
	^{14}C-KDDC (5.18×10^5 cpm in 0.1 ml)		^{35}S-KDDC (9.26×10^5 cpm in 0.1 ml)	
	cpm x 10^5		cpm x 10^5	
3	0.99(0.54-1.39)	19.1	2.16(1.42-2.53)	23.3
7	0.77(0.65-1.00)	14.9	1.72(1.53-1.84)	18.6
14	0.74(0.63-0.87)	14.3	1.53(1.33-1.83)	16.5
21	0.34(0.26-0.41)	6.6	1.27(1.00-1.56)	13.7

* range

Table 17

Radioactivity recovered in wash water from papaya leaves after leaf application of labeled $Zn(DDC)_2$ onto 3 month old plants. (data are averages of 3 replicates)

	compound applied			
	^{14}C-$Zn(DDC)_2$ (9,600 cpm in 0.01 ml)		^{35}S-$Zn(DDC)_2$ (5,600 cpm in 0.01 ml)	
days after application	cpm in wash water	ave. % recovery	cpm in wash water	ave. % recovery
0	2,267(2,100-2,500)*	24	3,833(3,500-4,400)	68
	(1.78×10^4 cpm in 0.1 ml)		(2.52×10^4 cpm in 0.1 ml)	
7	3,000	16.9	4,787	19.0
14	1,750	9.8	2,500	9.9
21	1,000	5.6	3,614	14.3

* range

Table 18

Radioactivity recovered in wash water from papaya leaves after leaf application of labeled KDDC onto 1 month old plants. (data are averages of 3 replicates)

	compound applied			
	^{14}C-KDDC (1.25×10^5 cpm)		^{35}S-KDDC (3.18×10^5 cpm)	
days after application	cpm x 10^4 in wash water	% recovery	cpm x 10^4 in wash water	% recovery
3	2.31	18.5	5.31	16.7
7	0.82	6.6	2.47	7.8
14	0.57	4.6	2.23	7.0

Table 19

Rf values and radioactivity recovery of metabolites in 45% ethanol soluble cell sap after leaf application of labeled KDDC onto 3 month old plants.

	compound applied							
	^{14}C-KDDC (5.18×10^5 cpm in 0.1 ml)				^{35}S-KDDC (9.26×10^5 cpm in 0.1 ml)			
days after application	Rf	cpm found	total activity recovered	% recovery	% recovery of applied activity	cpm found	total activity recovered	% recovery	% recovery of applied activity
3	0.56	5100		77.3	20.3	2290		33.2	5.3
	0.39	900		13.6	3.6	580		6.4	1.0
	0.29	280	6600	4.2	1.1	380	9010	4.2	0.7
	0.19	230		3.5	0.9	310		3.4	0.5
	0.06	50		0.8	0.2	200		2.2	0.4
	0	40		0.6	0.2	4550		50.5	8.1
7	0.56	6400		76.1	18.9	5120		49.8	10.0
	0.39	1300		15.5	3.9	360		3.5	0.7
	0.29	320	8410	3.8	0.9	520	10280	5.1	1.0
	0.19	270		3.2	0.8	280		2.7	0.5
	0.06	120		1.4	0.3	200		1.9	0.4
	0	---		---	---	3800		37.0	7.4
14	0.56	4480		72.3	14.5	2200		50.0	8.3
	0.39	970		15.6	3.1	230		5.2	0.9
	0.29	330	6200	5.3	1.1	370	4400	8.4	1.4
	0.19	210		3.4	0.7	430		9.8	1.6
	0.06	180		2.9	0.6	70		1.6	0.3
	0	30		0.5	0.1	1100		25.0	4.2
21	0.56	3850		83.7	22.0	3200		64.6	11.0
	0.39	360		7.8	2.1	210		4.2	0.7
	0.29	130	4600	2.8	0.7	250	4950	5.1	0.9
	0.19	120		2.6	0.7	420		8.5	1.4
	0.06	140		3.0	0.8	180		3.6	0.6
	0	---		---	---	690		13.9	2.4

Table 20

Rf values and radioactivity recovery of metabolites in 45% ethanol soluble cell sap after leaf application of labeled KDDC onto 1 month old plants.

		compound applied							
		^{14}C-KDDC (1.25×10^5 cpm in 0.1 ml)				^{35}S-KDDC (3.18×10^5 cpm in 0.1 ml)			
days after application	Rf	cpm $\times 10^3$ found	total activity recovered cpm $\times 10^3$	% recovery	% recovery of applied activity	cpm $\times 10^3$ found	total activity recovered cpm $\times 10^3$	% recovery	% recovery of applied activity
3	0.56	4.3		34.4	27.8	5.9		28.6	11.0
	0.39	2.5		20.0	16.2	1.3		6.3	2.4
	0.29	2.5	12.5	20.0	16.2	2.3	20.6	11.2	4.3
	0.06	1.8		14.4	11.6	1.2		5.8	2.2
	0	1.4		11.2	9.0	9.9		48.1	18.5
7	0.56	5.4		43.5	22.6	1.7		15.9	3.2
	0.39	3.4		27.4	14.2	0.6		5.6	1.1
	0.29	1.2	12.4	9.7	5.0	1.0	10.7	9.3	1.9
	0.06	1.1		8.9	4.6	0.8		7.5	1.5
	0	1.3		10.5	5.5	6.6		61.7	12.4
14	0.56	4.5		42.5	23.8	2.6		21.3	5.2
	0.39	3.4		32.1	18.0	1.5		12.3	3.0
	0.29	1.0	10.6	9.4	5.3	0.7	12.2	5.7	1.4
	0.06	0.7		6.6	3.7	0.5		4.1	1.0
	0	1.0		9.4	5.3	6.9		56.6	13.7

Table 21

Rf values and radioactivity recovery of metabolites in 45% ethanol soluble cell sap after root application of labeled KDDC using 1 month old plants.

	compound applied									
	^{14}C-KDDC (2.08 x 10^6 cpm in 0.1 ml)					^{35}S-KDDC (3.43 x 10^6 cpm in 0.1 ml)				
days after application	Rf	cpm x 10^3 found	total activity recovered cpm x 10^3	% recovery	% recovery of applied activity	Rf	cpm x 10^3 found	total activity recovered cpm x 10^3	% recovery	% recovery of applied activity
3	0.56	3.6		30.0	0.45	0.56	2.0		16.3	0.07
	0.39	6.1		50.8	0.76	0.39	1.3		10.6	0.04
	0.29	1.7	12.0	14.2	0.21	0.29	0.7	12.3	5.7	0.02
	0.03	0.6		5.0	0.08	0.06	1.0		8.1	0.03
	0	---		---	---	0	7.3		59.3	0.24
7	0.56	0.9		16.1	0.14	0.56	1.7		29.8	0.09
	0.39	2.4		42.9	0.39	0.39	0.3		5.3	0.02
	0.29	1.9	5.6	33.9	0.31	0.29	1.5	5.7	26.3	0.08
	0.03	0.4		7.1	0.06	0.06	0.1		1.8	0.01
	0	---		---	---	0	2.1		36.8	0.11
14	0.56	2.1		32.8	0.36	0.56	1.4		28.0	0.08
	0.39	2.0		31.3	0.34	0.39	0.2		4.0	0.01
	0.29	1.7	6.4	26.6	0.29	0.29	0.9	5.0	18.0	0.05
	0.03	0.6		9.4	0.10	0.06	0.1		2.0	0.01
	0	---		---	---	0	2.4		48.0	0.14

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