UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MAR 20 1991

MEMORANDUM

SUBJECT: Uniroyal Chemical Company: Response to the Thiram Reregistration Standard: Plant Metabolism Studies (MRID #'s 40216502 and 00162142, DEB # 6733.).

FROM: R. B. Perfetti, Ph.D., Chemist
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THRU: W. J. Boodee, Section Head
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TO: Reto Engler, Ph.D., Chief
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and

L. Rossi, Chief
Reregistration Branch
Special Review and Reregistration Division (H7508C)

Attached is a review of thiram plant metabolism studies submitted by Uniroyal Chemical Company in response to the thiram Reregistration Standard. This study was reviewed by Dynamac Corporation under supervision of CBRS, HED.

This study has undergone secondary review in CBRS and has been revised to reflect the Branch policies.

Please see our conclusions in the attachment regarding the adequacy of the information provided by the Registrant.

If you need additional input please advise.

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Attachment 1 : Review of Thiram Metabolism Studies.

cc: With Attachment 1: R. B. Perfetti, J. Burrell (PIB/FOD), Thiram Reregistration Standard File, Thiram Subject File, C. Furlow (PIB/FOD), Dynamac, Circ. (7).

cc: Without Attachment: P. Fenner-Crisp (HED) and RF.

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DYNAMAC
CORPORATION

Final Report

THIRAM (DEB No. 6733)
Task 4: Registrant's Response to Residue Chemistry Data Requirements

November 27, 1990

Contract No. 68-D8-0080

Submitted to:
Environmental Protection Agency
Arlington, VA 22202

Submitted by:
Dynamac Corporation
The Dynamac Building
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THIRAM

REGISTRANT'S RESPONSE TO THE RESIDUE CHEMISTRY DATA REQUIREMENTS

Task - 4

BACKGROUND

The Thiram Registration Standard dated 7/2/84 concludes that the qualitative nature of the residue in plants is not adequately understood; data are required depicting the uptake, metabolism, and translocation of [^{14}C]thiram in representative food crops planted in treated soil and subsequently treated foliarly, and in a small grain crop, cotton, and soybeans grown from seed treated with [^{14}C]thiram at maximum registered rates. In response to data requirements for seed treatment studies, Uniroyal Chemical Co. has submitted two volumes of data (DEB No. 6733, 1986 and 1987; MRIDs 00162142 and 40216502) pertaining to the uptake, distribution, and metabolism of thiram residues in wheat, cotton, and soybean plants grown from [^{14}C]thiram-treated seeds. Data are reviewed here for their adequacy in fulfilling outstanding data requirements.

No tolerances are established for residues of thiram in or on wheat, cotton, or soybean commodities; however, the Agency has concluded (EPA Memoranda DEB Nos. 1302, 1303, 2336; M.J. Bradley; 7/1/87) that the "uptake of radioactivity into stem and leaf parts of plants shows that the seed treatment uses of thiram are food uses" (quoted from a conference with R.S. Quick, Gustafson, and UCB Chemicals, dated 4/14/87).

Deficiencies Remaining To Be Resolved

The Conclusions and Recommendations stated below apply only to data pertaining to the metabolism of thiram residues in soybean, cotton, and wheat plants grown from [^{14}C]thiram-treated seeds. Any other data gaps are not included here but may still remain outstanding.

CONCLUSIONS

Plant metabolism data from studies with [^{14}C]thiram-treated seeds submitted in response to the Registration Standard do not adequately describe the qualitative nature of the residue in plants because extraction and characterization studies were conducted cn immature plants harvested 30 days postplanting. Discrepancies in data reporting prevented conclusive determination of the total radioactive residue (TRR) for extraction and characterization procedures. No attempt was made to characterize unextractable residues, which constituted ca. 17-18% of the reported TRR in soybean and cotton plants, and 2% of TRR in wheat. Of four metabolites tentatively identified, only

one (the cysteine conjugate) was identified in extracts of soybeans, cotton, and wheat on the basis of co-chromatography with a synthetic standard; all other identifications were made on the basis of comparisons between high-performance liquid chromatography (HPLC) results for extracts of plants grown from treated seeds and callus tissue culture homogenates. Quantitative supporting data, which might have validated such comparisons, were not provided. It should be noted that it was often unclear whether the metabolite data reported by the registrant were from extracts of plants grown from treated seed or from homogenates of callus tissue inoculated with [^{14}C]thiram.

RECOMMENDATIONS

The registrant should be informed that the submitted data do not fulfill the requirements regarding the qualitative nature of the residue in soybeans, cotton, and wheat. New data should be submitted from mature plants grown from seed treated at levels high enough to enable characterization of the residues. Data should depict the extractability of the TRR and the characterization of extracted residues of [^{14}C]thiram expressed as total radioactive counts, percentage of total recovered radioactivity, and ppm. Unextractable residues should be thoroughly fractionated and characterized, particularly in the edible commodities of the tested crops. The tentatively identified metabolites should be fully characterized and identified; although glycoside and amino acid conjugates were found, the nature of the moiety conjugated to the glycoside or the amino acid was not specified. Residues should be characterized on the basis of analysis of the plant extracts themselves, or, if identification is to be based on comparison with callus tissue culture homogenates, a full complement of quantitative data must be provided.

DETAILED CONSIDERATIONS

Qualitative Nature of the Residue in Plants

Uniroyal Chemical Co. (1986, 1987; MRIDs 00162142 and 40216502) submitted data pertaining to the metabolism of thiram in soybean, cotton, and wheat plants grown from seed treated with thiocarbonyl-labeled [^{14}C]thiram. Seeds were treated by shaking them in a suspension of [^{14}C]thiram (specific activity, 15.5 mCi/mmol; radiochemical purity, 98%) and unlabeled thiram in water until all liquid was absorbed or evaporated. Initially (Project 8565-A), seeds were treated at 1 and 5x the maximum registered rates: 1.03 and 5.15 mg ai/g for soybean seed, 2.25 and 11.25 mg ai/g for cottonseed, and 2.05 and 10.25 mg ai/g for wheat seed (5x rates calculated by the study reviewer). Treated seeds were planted in 1-gallon plastic pots containing a sandy

loam soil and maintained in the greenhouse, or were planted outdoors in raised bins. Indoor plants were sampled at 30 days after planting and at full maturity. The number of days to full maturity for each cultivar was not specified. Outdoor plants were also sampled at 30 days; however, owing to severe weather, soybean and cotton plants were harvested at 67 days postplanting instead of at maturity. The registrant notes that soybean plants harvested at 67 days had partially filled pods at most nodes. Wheat plants were unaffected by the severe weather, and were harvested at full maturity (days postplanting not specified).

Because radioassay of seeds treated at the levels above indicated low levels of ^{14}C-residues, a second batch of seeds (Projects 8565-B,-C) was treated for extraction and characterization procedures at the following levels: 20.16 mCi/g for soybean seed, 14.59 mCi/g for cottonseed, and 13.13 mCi/g for wheat seed (mCi/g calculated by the study reviewer from dpm/mg). These levels correspond to 3.5x, 7.3x, and 8.2x the maximum registered rates for soybeans, cotton, and wheat, respectively. Treated seeds were planted and grown in the greenhouse as described above, and whole plants were harvested at 30 days postplanting and frozen within the hour for analysis. The storage temperature and duration were not specified.

In conjunction with the seed treatment study, a metabolite biosynthesis study using callus tissue cultures of soybean and wheat was established to aid in characterization of metabolites. Tissue cultures were inoculated with 10 µL of thiocarbonyl-labeled [^{14}C]thiram in acetone (specific activity, 43,472 dpm/µg), then incubated for 2 to 96 hours. Separate soybean tissue cultures were also inoculated with sodium dimethyldithiocarbamate (DTC) to aid in identification of glycosides formed as a result of enzymatic action on the DTC moiety.

Total Radioactive Residues (TRR)

Seed samples from the initial treatment regimen were analyzed for total radioactivity by combustion/liquid scintillation spectrometry (LSS). The method limit of detection is 0.001 ppm. Complete TRR data are presented below for greenhouse- (Table 1) and outdoor-grown (Table 2) [^{14}C]thiram-treated plants.

TRR data were not provided for plants grown from seeds treated in the second batch; however, based on information provided by the registrant, it is possible to estimate TRR in soybean plants, cotton plants, and wheat leaves. Estimates were derived from two different sets. Based on the total dpm from combustion analysis and the dry weight of the samples, the study reviewer calculated the following TRRs: 9.78 ppm for soybean plants, 5.96 ppm for cotton plants, and 10.76 ppm for wheat leaves. Based on the

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percentages and ppm provided by the registrant with extraction data (see Table 3), the study reviewer calculated the following TRRs: 12.6 ppm for soybeans, 6.88 ppm for cotton, and 11.53 ppm for wheat. The second data set will be used in this review. The absence of conclusive TRR data for seeds treated in Project 8565-B constitutes a deficiency.

TRR data were not provided for the callus tissue cultures because these were used only for identification of ^{14}C-residues.

Table 1. Radioactive residues in matrices of greenhouse-grown soybeans, cotton, and wheat plants from seed treated with [^{14}C]thiram at 1 and 5x the maximum registered rates (1986; MRID 00162142).

	TRR (ppm thiram equivalents)		
		Maturity	
Sample	30 days[a]	1x	5x
Soybean			
Seed	-	0.019	0.153
Pod	-	0.034	0.276
Leaf	0.496	0.117	1.399
Stem	1.542	0.288	3.165
Cotton			
Seed	-	0.006	0.024
Fiber	-	0.008	0.018
Husk	-	0.107	0.140
Leaf	0.049	0.035	0.094
Stem	0.291	0.034	0.141
Wheat			
Seed	-	0.078	0.512
Chaff	-	0.298	1.944
Leaf	1.143	0.822	4.088

[a]Registrant did not specify whether samples were treated at 1 or 5x.

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Table 2. Radioactive residues in matrices of soybean, cotton, and wheat plants grown outdoors from seed treated with [^{14}C]thiram at 1 and 5x the maximum registered rates (1986; MRID 00162142).

Sample	TRR (ppm thiram equivalents)		
		Maturity[a]	
	30 days[b]	1x	5x
Soybean			
Leaf	0.292	-[a]	-
Stem	0.085	-	-
Cotton			
Leaf	0.046	-	-
Stem	0.054	-	-
Wheat			
Seed	-	0.005	0.036
Chaff	-	0.017	0.125
Leaf	0.474	0.025	0.140

[a]Soybean and cotton samples lost as a result of severe weather.
[b]Registrant did not specify whether samples were treated at 1 or 5x.

Extraction

Two methods were used for the extraction of whole-plant samples harvested at 30 days from the second batch of seeds treated. In Method A (Bligh-Dyer Method), samples were extracted by homogenization in methanol:trichloromethane:water (1:1:1, v/v/v), followed by centrifugation. Aqueous and organic phases were removed and the plant material extracted twice more. Aqueous phases were kept separate while organic phases were combined. Organic and aqueous phases were concentrated under a vacuum and analyzed for radioactivity by LSS; unextractable radioactivity was analyzed by combustion/LSS. Residue extraction efficiencies were 82%, 82%, and 98% from soybean plants, cotton plants, and wheat leaves, respectively. The distribution of TRR in extracts from soybean, cotton, and wheat samples is presented in Table 3. Aqueous-soluble residues were further analyzed by HPLC and quantified with a radioactivity monitor (RAM). Organosoluble residues and unextracted solids were not further analyzed.

In Method B, plant samples were extracted with 0.25 M ethylenediaminetetraacetic acid (EDTA) in 0.45 M sodium hydroxide, pH 9.6. The extract was mixed with a tetrabutyl ammonium hydrogen sulfate solution, adjusted to pH 7.6 with hydrochloric acid, and partitioned with 0.06 M methyl iodine in chloroform:hexane (3:1, v/v). When aqueous-soluble residues were purified on C-18 Sep-Paks, 66% of the radiolabel eluted with

water and 32% eluted with 20% acetonitrile. The eluates were evaporated to dryness,
separately redissolved in water, and analyzed by HPLC. The EDTA extract of soybean plants was purified by flash chromatography in the following manner: the pH of the extract was adjusted to 7.5 with 2N hydrochloric acid, and the extract was applied to a C-18 column and sequentially eluted with acetonitrile and water under 5 psi (pounds per square inch) pressure provided by attachment of the column to a dry nitrogen source. The eluates were dried, redissolved in water, and analyzed by HPLC and LSS. The registrant stated that EDTA extraction recovered ca. 89% of TRR, but no quantitative supporting data were provided.

Residues in callus tissue were extracted by homogenizing with methanol or methanol:water (1:1, v/v). The homogenate was centrifuged and the supernatant was subjected to flash chromatography, then analyzed directly by HPLC-RAM.

Table 3. Distribution of radioactivity in extracts from soybean plants, cotton plants, and wheat leaves treated with [^{14}C]thiram (1986; MRID 00162142).

Component	% TRR (ppm)[a] Soybean plant	Cotton plant	Wheat leaves
Extractable			
Aqueous	80 (10.08)	80 (5.50)	95 (10.96)
Organic	2.2 (0.28)	2.1 (0.14)	2.8 (0.32)
Unextracted solids	17.1 (2.15)	17.8 (1.23)	2.2 (0.25)
Total	99.3 (12.51)	99.9 (6.87)	100 (11.53)

[a]ppm values calculated by the study reviewer.

Hydrolysis

The acetonitrile extract from the EDTA extraction of soybeans was incubated with ß-glucosidase in 0.005 M formate buffer at pH 4.8 for 24 hours at 25 C. The resulting material was centrifuged and analyzed by HPLC-RAM. Cotton and wheat extracts were concentrated, reconstituted in ammonium formate buffer, and incubated for 24 hours with the enzyme. Trichloroacetic acid was added to the resulting solutions to precipitate protein. Callus tissue culture homogenates of soybeans were concentrated prior to incubation with the enzyme, and a 0.05-M formate buffer was used. The acetonitrile extract from the EDTA extraction of soybeans was also subjected to hydrolysis by incubation with 2 M HCl for 24

hours. Analysis by HPLC and LSS indicated the radioactive component was not released by acid hydrolysis.

Characterization

Characterization of ^{14}C-residues in aqueous extracts from the Bligh-Dyer extraction (Method A) was attempted by comparing the results of HPLC, HPLC/thermal spray-mass spectrometry (TS-MS), co-chromatography with a synthetic cysteine conjugate, and response to enzyme hydrolysis with those of tissue culture homogenates.

Components were isolated from the aqueous extracts with reversed-phase HPLC on two partisil 10-μ/25-cm ODS-3 C-18 columns in series. Extracts were eluted with a solution of 0.05 M formic acid (brought to pH 4.8 with triethylamine) in acetonitrile. Triethylamine was included as an ion-pairing agent to aid in resolution of highly polar components. Results were quantitated by collecting fractions and analyzing by LSS or by HPLC equipped with an ultraviolet detector RAM. At least eight components were isolated from soybean and wheat extracts, and at least seven from cotton extracts; two components constituted greater than 10% of the ^{14}C-activity in aqueous extracts for all three crops. Radioactivity in the isolated components accounted for 100% (12.6 ppm), 89% (4.9 ppm), and 77.6% (8.5 ppm) of the residues in aqueous-soluble extracts from soybean, cotton, and wheat samples, respectively.

The aqueous extract from the EDTA extraction (Method B) of soybeans also was analyzed using the HPLC system described above, and two major components were isolated. HPLC analysis of the acetonitrile extract indicated a single component. The three components corresponded to major components identified in analysis of soybean extracts from the Bligh-Dyer extraction (Method A).

HPLC analysis of wheat tissue culture homogenates revealed a single component, detectable after 48 hours, which corresponded to a major component detected in soybean and cotton aqueous extracts. Analysis of soybean tissue culture homogenates indicated that thiram *per se* was present until ca. 48 hours post-inoculation. Radiolabel recovery dropped from 100% at 2 hours post-inoculation to ca. 25% after 48 hours; the registrant suggests the drop in recovery may have resulted from loss of ^{14}C-residues as volatile gases or from incorporation into the solid matrix or the medium. Isolation of components in soybean tissue homogenates with flash chromatography/HPLC-RAM confirmed the presence of three major components in aqueous soybean extract. HPLC analysis of soybean tissue culture inoculated with DTC showed a single peak.

The three components isolated from soybean tissue culture homogenate by HPLC-RAM were resolved into five possible metabolites by separate re-analysis of each component. Components 1 and 3 each consisted of one possible metabolite, whereas component 2 was resolved into three possible metabolites. Component 3 was subjected to further analysis by HPLC/thermal spray-mass spectrometry (TS-MS). The same HPLC conditions were used as described above, except that the buffer was changed to ammonium formate, and samples were analyzed by flow injection. Analysis by HPLC/TS-MS resolved component 3 into two possible glycoside conjugates. One component was tentatively identified as monothiocarbamate glycoside by its MS fragmentation pattern; the other component was tentatively identified as a DTC-derived glycoside by comparison with HPLC/MS-TS results of DTC-treated tissue culture. Hydrolysis of the extracts with ß-glucosidase confirmed the presence of glycoside linkages. A third component, a cysteine conjugate, was identified at low levels by its MS fragmentation pattern and co-chromatography with a synthetic standard of known HPLC retention time.

Analysis of the aqueous extracts of whole plants by the latter method had limited success. Although HPLC/TS-MS of the aqueous extract of wheat resulted in much of the material eluting with the solvent front, the conjugates identified in the soybean tissue culture homogenate, including the two glycoside components, were confirmed. Co-chromatography of the synthetic cysteine conjugate with cotton and wheat extracts confirmed its presence in wheat and its absence in cotton. Enzyme hydrolysis of the aqueous extracts of soybean, cotton, and wheat confirmed the presence of glycoside linkages.

Based on changes observed in the HPLC results for tissue culture homogenates over time, the registrant tentatively identified a third major component (m.w. = 240, retention time = 13 minutes) as a derivative of xanthate resulting from the rearrangement of the DTC-glycoside. Although the xanthate derivative has a molecular weight of 222, the registrant maintains that the use of ammonium formate buffer in the TS-MS would result in an ammonium adduct of the compound with the observed molecular weight of 240. On the basis of changes observed in the soybean whole-plant extract after storage in water for 3 months, the registrant postulates that the monothiocarbamate glycoside forms from transformation of the DTC-glycoside. This was corroborated by analysis of soybean tissue culture homogenate.

The aqueous wheat extract was hydrolyzed in sulfuric acid, within a closed container, to determine how much of the ^{14}C-activity would be lost as volatile gases. Dry nitrogen gas flowed through the closed container, carrying evolved CS_2 through a zinc acetate scrubber, and into a trapping solution containing a solution of diethylamine, triethanolamine, and copper (II) acetate (Viles Reagent). The reagent was analyzed by UV spectrometry and, where

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applicable, by LSS to determine [^{14}C]CS_2; only 3.4% of the ^{14}C-activity was trapped as CS_2, and the total loss of radiolabel, 34%, corresponded to the total glycoside component identified previously in aqueous extracts by HPLC.

The results of HPLC analysis of the aqueous extracts of soybean, cotton, and wheat plants grown from [^{14}C]thiram-treated seeds are presented in Table 4. Metabolites were tentatively identified by the means described above, and were quantitated by LSS of fractions collected every 0.5 minutes. No other attempts at metabolite identification were conducted for soybean plants, cotton plants, or wheat plants. The only metabolite identified directly in aqueous extracts was the cysteine conjugate, which was determined on the basis of HPLC-RAM and co-chromatography with a standard of known retention time. All other metabolites were identified on the basis of comparison between HPLC results for tissue culture homogenate and the aqueous extracts. Overall, metabolite identification was inconclusive. We note that for the data presented in Table 4 (from MRID 40216502, Table II, page 33) the registrant indicates that soybean and wheat seeds were treated at 5x and 7.2x the maximum registered rates, respectively; however, these rates do not agree with the rates indicated originally of 3.5x for soybeans and 8.2x for wheat (from MRID 00162142, page 59). As a result of this discrepancy, we are unable to determine what proportion of the TRR is represented by the data in Table 4. This constitutes a deficiency.

Table 4. Characterization of radioactive residues in aqueous-soluble extracts of soybean, cotton, and wheat plants grown from [^{14}C]thiram-treated seed (1987; MRID 40216502).

Component	Radioactive residues (ppm)		
	Soybean plant	Cotton plant	Wheat leaves
dithiocarbamate (DTC)[a]	5.1	0.54	0.78
xanthate product	0.65	0.25	0.93
dithioglycoside	4.8	1.21	0.75
monothioglycoside	0.97	1.14	0.91

[a]Unidentified metabolites were treated as DTC.

In summary, radioassay of mature soybean, cotton, and wheat plants grown from [^{14}C]thiram-treated seed in the greenhouse (Project 8565-A) demonstrated that residues of thiram are translocated to edible plant parts; however, since only immature plants were used for extraction and characterization studies

(Projects 8565-B,-C), the extent to which residues in edible plant parts may have been extractable or identified was not determined. In addition, discrepancies in data presented for extraction and characterization of the residues prevented determination of TRR values for any of the crops. Based on reported values for extraction, no attempts were made to characterize 17-18% of soybean and cotton TRR, and 2% of wheat TRR, in unextractable solids. Of the four metabolites tentatively identified in soybean, cotton, and wheat grown from [^{14}C]thiram-treated seed, only one metabolite, the cysteine conjugate, was identified on the basis of co-chromatography of the aqueous extracts with a known standard. Identification of the two glycosides and the xanthate derivative was based solely on a comparison of HPLC results between the tissue culture homogenate and the aqueous extracts. We do not feel that a firm basis for such direct comparison has been established, especially since the tissue culture material used for all comparisons was not an extract per se, but a homogenate. Quantitative supporting data that might have validated these comparisons were not provided. In addition, we note that it was often unclear whether the data reported for characterization of the residues (MRID 40216502) pertained to metabolites isolated from plant extracts or from tissue culture homogenates.

REFERENCES

00162142 Harned, W.; Tortora, N. (1986) Uptake and Distribution of Carbon-14 Thiram in Cotton, Soybean and Wheat: Project ID No. 8565-A. Unpublished study prepared by Uniroyal Chemical. 25 p.

40216502 Nowakowski, M. (1987) Identification of Metabolites in Cotton, Wheat and Soybean Seedlings Grown from Carbon 14 Thiram Treated Seeds: Uniroyal Project ID No. 8565-C. Unpublished study prepared by Uniroyal Chemical Co., Inc. 47 p.