# Tissue Distribution of Ethylenethiuram Monosulfide (ETM) in the Rat

F. Iverson, W. H. Newsome and S. L. Hierlihy

*Food Directorate*
*Health Protection Branch*
*Tunney's Pasture*
*Ottawa, Canada*
*K1A 0L2*

## Introduction

Ethylenebisdithiocarbamate fungicides are readily degraded by plant and animal systems to yield ethylenethiourea (ETU) and ethylenethiuram monosulfide (ETM) (VONK, 1975; LYMAN, 1971). Toxicological investigations have suggested that ETU is responsible for the tumorigenic (GRAHAM et al. 1975) and teratogenic (KHERA, 1973) properties of the parent compounds, but there is a lack of data on ETM, which is considered to be the principal fungitoxic oxidation product of this class of pesticides (ENGST and SCHNAAK, 1975). The present investigation was, therefore, undertaken to determine the tissue distribution of ETM. Indications that ETM may be converted to ETU (VONK, 1975), prompted a comparison of ETU to ETM under similar experimental conditions.

## Materials and Methods

Acute Toxicity of ETM: Groups of 5 male Sprague-Dawley rats were administered ETM orally in corn oil at a rate of 1.0 ml/200 g b.w. for single dose levels of 50, 100, 150, 200, 250 and 350 mg/kg. Controls received corn oil only. The $LD_{50}$ value was calculated by the procedure of LITCHFIELD and WILCOXON (1949), based on the 24 hour mortality.

Tissue distribution after ip administration: Male and female Sprague-Dawley rats 200 ± 25 g b.w. were obtained from Bio-Breeding, Ottawa, Canada and housed in standard banks for a 1 week acclimatization period. ETU ($^{35}S$, 17.6 µCi/mg) was prepared in distilled water and administered ip at 4 mg/kg (1.0 ml/kg) to male rats. Groups of 3 animals were sacrificed at time periods of 1, 2, 4, 8, 12 and 24 hours. Duplicate samples of blood, heart, lung, thyroid, liver, kidney, spleen, fat and muscle were removed, digested in soluene (Packard Inst. Co., Downer's Grove, Ill.) and counted in a toluene based cocktail on a Beckman LSC



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230 fitted with an external standard for quench correction.

ETM ($^{14}C$) obtained from New England Nuclear, Lachine, Qué., was prepared in corn oil and administered ip to male and female rats at 5 mg/kg (11.29 µCi/mg; 1.0 ml/kg). Two animals from each sex were sacrificed at time periods of 0.5, 1, 2, 4, 8, 12 and 24 hours. Additional thyroid samples were taken at 32, 72 and 96 hours. Tissue samples were processed as described previously, with the exception that samples of brain were also taken. Tissue concentrations were calculated on the basis of total tissue radioactivity being present as ETU or ETM.

Oral dosing: Five male rats were dosed orally each day for 6 days with $^{35}S$-ETU, 1.0 ml/200 g b.w.; 4 mg/kg in distilled water or $^{14}C$-ETM, 1.0 ml/200 g b.w.; 5 mg/kg in corn oil. Duplicate tail blood samples were taken 24 hours after each dose and the animals were sacrificed 24 hours after the final dose. Samples of blood, heart, lung, liver, kidney, spleen, fat, muscle and thyroid were analyzed for total radioactivity content.

Attempts were made to quantitate ETU in tissue employing a gas chromatographic method. However, extraction efficiency, and cleanup presented problems that could not be overcome at the present time.

Thin Layer Chromatography: Thin layer chromatography (TLC) was carried out using cellulose (Machery Nagel) with or without added UV 254 indicator. Adsorbent layers either 0.25 or 0.5 mm thick were prepared on glass plates. The solvent systems used were Acetonitrile: Water 88:12 and diethylether, methanol 90:10. Determination of ETU was accomplished by applying raw urine (ca 50 µl) to the cellulose plates, developing, and scanning the plate with a Panax Nucleonics thin layer scanner. The Rf's of the developed peaks were compared to that of a known ETU standard. ETU was visualized with the nitroprusside reagent (FCNB), described by VONK (1975). Radioactive peaks were scraped from the plate into scintillation vials and counted in 10 ml Aquasol (New England Nuclear).

Preparation of Standard Compounds: $^{35}S$-ETU. To 40 mg of $^{35}S$ carbon disulfide (Amersham Searle, Arlington Hts, Ill. 0.675 µCi/mg) was added 340 mg of cold $CS_2$ in 1.0 ml of absolute ethanol. This solution was added slowly, with stirring to 300 mg of ethylene diamine in 1.0 ml distilled water. The mixture was refluxed for 4 hours, 50 µl of conc. HCl added, and heating continued for 1 hour. Two ml of water were added and the content

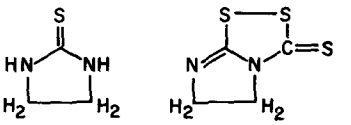
of the flask passed through an ion exchange resin bed consisting of 1.5 x 1.5 cm Dowex 50 W x 8 and 1.5 x 1.5 cm Dowex 1 x 8. The column was washed with water (25 ml) and the effluent taken to dryness on a rotary evaporator. The yield was 325.5 mg (64%). Specific activity was 17.51 µCi/mg. TLC on silica gel in solvent A showed a radiochemical purity >99%.

$^{14}$C-ETU. The procedure was essentially that described above. Uniformly labelled $^{14}$C-ethylenediamine dihydrochloride 2.96 mg, 500 µCi (New England Nuclear, Lachine, Qué.) was mixed with ethylene diamine (57 mg) in 1.0 ml water and reacted with $CS_2$. The yield was 81.4 mg (80%) with a specific activity of 4.95 µCi/mg. Radiochemical purity exceeded 99%.

## Results

The 24 hour $LD_{50}$ value for ETM was 240 (303-174) mg/kg. The slope function is 1.63 (2.15-1.23).

Figure 1 depicts the structures of the compounds discussed in the text. The compound we have termed ETM is also referred to as ethylenebisisothiocyanate sulfide (EBIS) by ENGST and SCHNAAK (1975) and DIDT by VONK (1975). The structure is 5,6-dihydro-3H-imidazo [2,1-C]-1,2,4-dithiazole-3-thione.




ETU ETM

Figure 1

Figure 2 shows the 24 hour decline in radioactivity in kidney from rats dosed with ETU or ETM. ETM exhibits a biphasic decline for both male and female rats with similar values observed for both sexes. ETU exhibits a linear decline. These curves are representative of the other tissues for each compound. The other tissue data is given in Table 1 for ETM and Table 2 for ETU. All tissues show a consistent decline in radioactivity with the exception of the thyroid. There was a "delayed uptake" with this tissue, peaking at about 12 hours with both ETU and ETM, and at a concentration considerably higher than any other tissue, as shown in Figure 3. The half-lives ($t_{\frac{1}{2}}$), calculated from the decline curves, are given in Table 3. With the exception of the thyroid, there does not appear to be a significant difference

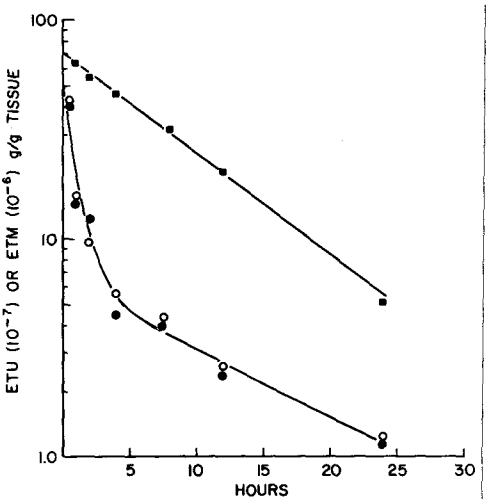



Figure 2. Decline of radioactivity in rat kidney calculated as ETU or ETM. (■), ETU, male, 4 mg/kg i.p. (o), ETM, male, 5 mg/kg i.p. (●), ETM, female, 5 mg/kg i.p.

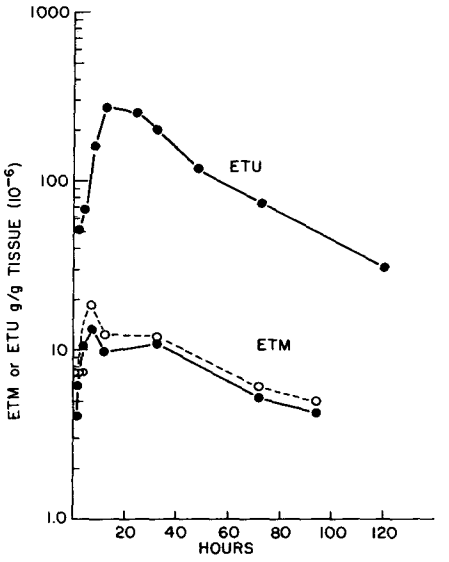



Figure 3. Profile of radioactivity in rat thyroid calculated as ETU or ETM, (●), ETU, male 4 mg/kg i.p. (o), ETM, male 5 mg/kg i.p. (●), ETM female, 5 mg/kg i.p.

in $t_{\frac{1}{2}}$ between tissues dosed with ETU or ETM, or between male or female rats dosed with ETM. The $t_{\frac{1}{2}}$ values of ETM were determined from the second linear phase of the decline curve. These values exhibited more variation than with ETU. Thyroid half-lives were about 5-fold higher than the other tissues and were in the order of 40 hours for both ETU and ETM.

The effect of repeated dosing was examined by administering ETU and ETM orally for a period of 6 days. The blood levels of radioactivity, calculated as ETU or ETM, are shown in Figure 4. There is a gradual increase in radioactivity over the 6 days, following a sharp increase immediately after the first dose. The tissue levels, obtained 24 hours after the final dose are given in Table 4. Again, thyroid values are highest with both compounds, that for ETU being some 450-fold higher than the kidney value while the ETM 6 day thyroid value is 24-fold above that of the corresponding kidney value. Lung levels ranked third for both compounds and fat exhibited the lowest values, with both ETM and ETU.

TABLE 1

Tissue concentration of radioactivity calculated as ETM (ppm) ± S.E. after a single ip dose of $^{14}$C-ETM, 5 mg/kg, to male or female rats, n=2

| Tissue | Sex | Time (Hours) .5 | 1 | 2 | 4 | 8 | 12 | 24 |
|---|---|---|---|---|---|---|---|---|
| Blood | M | 4.95±0.17 | 8.01±3.11 | 3.03±0.39 | 2.11±0.06 | 1.50±0.01 | 0.86±0.04 | 0.42±0.01 |
| | F | 6.70±0.92 | 5.18±0.30 | 3.12±0.31 | 2.44±0.32 | 1.98±0.08 | 1.11±0.11 | 0.54±0.01 |
| Brain | M | 0.92±0.17 | 0.86±0.09 | 0.78±0.17 | 0.58±0.01 | 0.51±0.07 | 0.25±0.02 | 0.09±0.01 |
| | F | 1.33±0.05 | 1.26±0.28 | 1.02±0.01 | 0.65±0.05 | 0.63±0.03 | 0.31±0.05 | 0.13±0.04 |
| Liver | M | 10.29±0.64 | 4.65±0.04 | 2.71±0.31 | 2.13±0.06 | 1.89±0.07 | 1.35±0.10 | 0.59±0.02 |
| | F | 35.3±22.8 | 4.19±0.83 | 3.73±0.03 | 2.44±0.04 | 2.04±0.02 | 1.45±0.09 | 0.56±0.02 |
| Muscle | M | 1.69±0.08 | 1.63±0.25 | 1.23±0.39 | 1.06±0.31 | 0.58±0.05 | 0.36±0.02 | 0.17±0.02 |
| | F | 2.14±0.25 | 1.50±0.19 | 1.38±0.01 | 0.78±0.07 | 0.85±0.02 | 0.38±0.03 | 0.15±0.03 |
| Fat | M | 3.79±0.87 | 2.08±0.58 | 1.20±0.16 | 1.07±0.03 | 0.86±0.25 | 0.47±0.01 | 0.24±0.02 |
| | F | 2.67±0.53 | 1.50±0.70 | 0.95±0.07 | 0.68±0.21 | 0.73±0.13 | 0.92±0.54 | 0.64±0.22 |
| Heart | M | 2.29±0.16 | 1.96±0.10 | 1.11±0.09 | 0.99±0.07 | 0.90±0.05 | 0.57±0.04 | 0.34±0.01 |
| | F | 3.84±0.31 | 1.99±0.40 | 1.89±0.01 | 1.29±0.02 | 1.30±0.01 | 0.68±0.06 | 0.26±0.05 |
| Lung | M | 4.33±0.09 | 3.10±0.03 | 2.09±0.27 | 1.80±0.25 | 1.89±0.12 | 0.93±0.11 | 0.59±0.01 |
| | F | 7.08±0.07 | 4.17±0.15 | 4.02±0.24 | 2.27±0.15 | 2.25±0.04 | 1.49±0.04 | 0.46±0.05 |
| Spleen | M | 3.06±0.15 | 2.71±0.22 | 1.50±0.30 | 1.34±0.04 | 2.18±0.55 | 0.78±0.10 | 0.73±0.03 |
| | F | 4.85±0.31 | 2.46±0.35 | 2.34±0.02 | 2.31±0.27 | 2.07±0.05 | 1.09±0.16 | 0.51±0.03 |

TABLE 2

Tissue concentration of radioactivity calculated as ETU (ppm) ± S.E. after a single ip dose of $^{35}$S-ETU, 4 mg/kg, to male rats, n=3

| | Time (Hours) | | | | | |
|---|---|---|---|---|---|---|
| Tissue | 1 | 2 | 4 | 8 | 12 | 24 |
| Blood | 5.32±0.19 | 4.97±0.13 | 3.71±0.10 | 2.66±0.41 | 1.45±0.12 | 0.23±0.01 |
| Heart | 5.34±0.07 | 4.67±0.12 | 3.55±0.18 | 2.31±0.29 | 1.32±0.15 | 0.23±0.01 |
| Lung | 5.36±0.80 | 4.85±0.21 | 4.23±0.21 | 2.87±0.35 | 1.76±0.26 | 0.41±0.02 |
| Liver | 5.84±0.80 | 5.22±0.08 | 4.26±0.03 | 3.22±0.36 | 2.03±0.18 | 0.45±0.01 |
| Spleen | 5.50±0.07 | 5.09±0.09 | 3.88±0.15 | 2.90±0.39 | 1.53±0.08 | 0.34±0.02 |
| Fat | 1.02±0.09 | 1.03±0.26 | 0.92±0.15 | 0.62±0.05 | 0.38±0.02 | 0.08±0.02 |
| Muscle | 4.80±0.35 | 4.9 ±0.78 | 3.66±0.22 | 2.40±0.32 | 1.27±0.17 | 0.31±0.11 |

Urine collected in the first 24 hr time period after the start of the 6 day dosing regimen was pooled for estimation of urinary metabolites. Fig. 5 shows the pattern obtained with ETU and ETM. In contrast to ETU administration, which is excreted essentially unchanged (95% total urine radioactivity), ETM is degraded into several compounds including ETU. Identification of ETU was made on the basis of TLC comparison with an authentic standard and colour reaction. Additional confirmation was obtained by peak matching on a mass spectrometer. ETU accounted for 29.6% of urine radioactivity after ETM administration, and 95% of urine radioactivity after ETU administration. Intact ETM was not found in the urine. Since previous reports (VONK, 1975) had indicated that ETM would react rapidly with glutathione, $^{14}$C-ETM was added to rat liver 105,000 x g supernatant and incubated for 5 min at 37°C. Another sample of supernatant was slurried with Amberlite IRA 410 in the carbonate form, then reacted with ETM. The results are shown in Figure 6. ETM is rapidly converted to ETU. The large peak at the origin is believed to be material bound to the silica gel. After treatment with the resin, which is known to remove endogenous glutathione, BENKE and MURPHY (1975), the conversion to ETU is reduced.

TABLE 3

Half-lives for radioactivity decline in rat tissues after administration of 4 mg/kg ETU or 5 mg/kg ETM, i.p.

| | $t_{\frac{1}{2}}$ - hours | | |
|---|---|---|---|
| Tissue | ETU | ETM | |
| | | Male | Female |
| Blood | 5.6 | 7.5 | 7.7 |
| Heart | 5.6 | 9.4 | 10.1 |
| Lung | 6.5 | 13.4 | 10.5 |
| Liver | 6.9 | 9.1 | 9.2 |
| Kidney | 7.3 | 7.9 | 12.0 |
| Spleen | 6.3 | 9.2 | - |
| Fat | 6.7 | 11.9 | - |
| Muscle | 5.5 | 8.7 | 9.5 |
| Thyroid | 36 | 41 | 37 |
| Plasma | - | - | - |
| Brain | - | 8.3 | 9.2 |

## Discussion

The $LD_{50}$ value determined in the present study (240 mg/kg) is considerably below the 1570 mg/kg value given by ENGST et al. (1971). However, the latter value was determined using partially characterized material obtained from fractionation of an aerated solution of parent carbamate (Nabam).

The tissue distribution and decline of ETM follows a pattern similar to that shown by ETU. Thyroid radioactivity after repetitive oral ETM dosing (6 days) was about 5-fold higher than that occurring 24 hours after a single ip dose, suggesting that accumulation could occur. However, the thyroid radioactivity

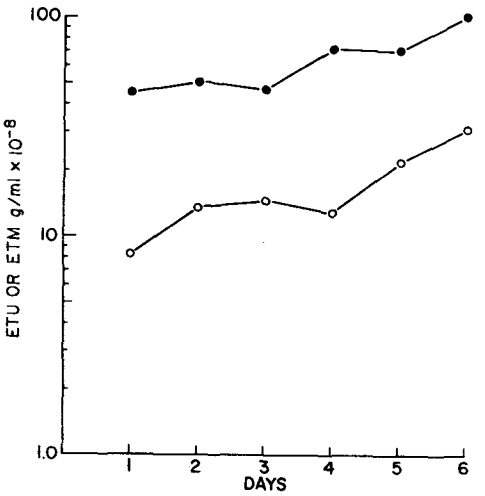



Figure 4. Blood radioactivity levels calculated as ETU or ETM. Male rats received daily oral doses of 4 mg/kg $^{14}$C-ETU (o) or 5 mg/kg $^{14}$C-ETM (●) for 6 days.

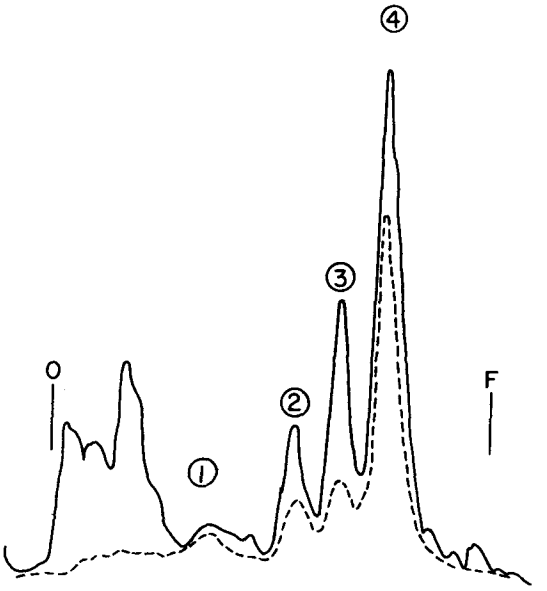



Figure 5. Tracing of a TLC scan of 24-hour urine from rats treated orally with $^{14}$C-ETU, 4 mg/kg (--) or $^{14}$C-ETM, 5 mg/kg (—). O is the origin and F the front of the cellulose plate. The solvent system was acetonitrile, water, 88:12. Peak 4 is ETU. Peak 1, 2 and 3 were not identified.

TABLE 4

Tissue radioactivity calculated as ETU or ETM after daily oral dosing for 6 days, ETU, 4 mg/kg; ETM, 5 mg/kg, n=5

| Tissue | Compound | | | |
|---|---|---|---|---|
| | ETU (ppm ± S.E.) | | ETM (ppm ± S.E.) | |
| Blood | 0.313 | 0.048 | 1.03 | 0.047 |
| Brain | 0.411 | 0.026 | 0.389 | 0.106 |
| Liver | 0.489 | 0.051 | 1.41 | 0.185 |
| Spleen | 0.463 | 0.040 | 0.477 | 0.040 |
| Fat | 0.126 | 0.025 | 0.142 | 0.016 |
| Thyroid | 350 (pooled sample) | | 56.5 (pooled sample) | |
| Kidney | 0.853 | 0.080 | 2.39 | 0.114 |
| Muscle | 0.373 | 0.044 | 0.399 | 0.083 |
| Heart | 0.416 | 0.047 | 0.554 | 0.048 |
| Lung | 0.743 | 0.118 | 0.343 | 0.097 |

after repetitive ETU dosing was only about 1.5-fold higher than 24 hours after a single ip dose. This latter result may arise from a saturation mechanism which would be consistent with the results of LYMAN and LACOSTE (1975), who showed that $^{14}C$ accumulation in thyroids did not increase significantly as the dose was raised from 1 to 2 mg ETU per rat. The ETU dose per rat in the present study was approximately 1 mg.

The tissue half-life of radioactivity after ETM or ETU dosing suggests that a steady state would be approached within the 6 day dosing regimen. This appeared to be the case since the blood levels showed a sharp increase in radioactivity after the first dose of ETU or ETM with a gradual increase in radioactivity over the remaining dosing period. The work of LYMAN and LACOSTE (1975) also suggests that a steady state is quickly attained since thyroid levels of $^{14}C$ did not increase from days 4 through 14 with daily oral doses of ETU. The lack of intact ETM in urine and the low adipose tissue levels of radioactivity after dosing with $^{14}C$-ETM, a lipophilic compound, suggest that the *in vivo* $t_{\frac{1}{2}}$ of intact ETM is relatively short.

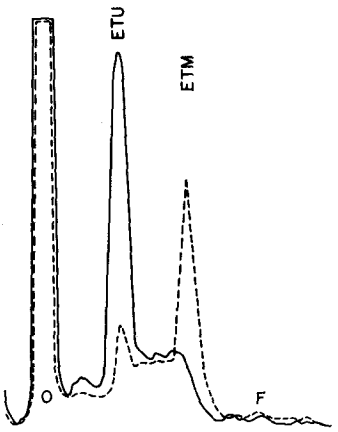



Figure 6. Tracing of a TLC scan of an aliquot of the reaction mixture containing 105,000 x g rat liver supernatant and $^{14}$C-ETM (—). The dashed line (--) was obtained when the supernatant was slurried with ion exchange resin prior to incubation with ETM. O is the origin and F the front of the silica gel plate. The solvent system was diethyl ether, methanol 90:10.

Although attempts to quantitate tissue levels of ETU by gas chromatography were not successful, similarities in the distribution and decay profiles after $^{14}$C-ETM or $^{35}$S-ETU administration, the reactivity of ETM with rat liver supernatant to form ETU and the occurrence of ETU in the urine of rats dosed with ETM, suggest that the thyroid profile observed after ETM administration may be the result of ETU accumulation. If this is the case, then it is apparent that metabolism and other as yet undetermined factors, i.e. absorption, reduce the amount of ETU generated by a substantial amount. That is, if it is assumed that all of the radioactivity in the thyroids after 6 days of $^{14}$C-ETM is actually ETU, then the amount of ETU present is 32 ppm. This is the amount expected from an ETU dose of 0.37 mg/kg, based on extrapolation from the 350 ppm level found after dosing 4 mg/kg ETU for 6 days.

The results of the present study suggest that ETU could arise in vivo after ingestion of ETM residues. However, the lack of ETU metabolism is not consistent with the results obtained after ETM adminis-

tration. The *in vitro* data suggest that since ETU is readily formed from ETM, at least a portion of the metabolites arising from ETM would also be seen after ETU administration. One explanation for this apparent discrepancy would be a dose dependent metabolism of ETU. Further studies are in progress to more closely define the metabolism and excretion of ETM and ETU.

## REFERENCES


BENKE, G.M., and S.D. MURPHY. Toxicol. Appl. Pharmacol. 31 254, (1975).

ENGST, R., W. SCHNAAK and H.J. LEWERENZ. Z. Lebensm.-Unters. Forsch. 146 91, (1971).

ENGST, R. and W. SCHNAAK. Environmental Quality and Safety, Suppl. Vol. 4 Stuttgart: Georg Thieme 1975.

GRAHAM, S.L., K.J. DAVIS, W.H. HANSEN and C.H. GRAHAM. Fd. Cosmet. Toxicol. 13 493, (1975).

KHERA, K.S. Teratology 7 243, (1973).

LITCHFIELD, J.T. and F. WILCOXON. J. Pharm. Exptl. Therap. 96 99, (1948).

LYMAN, W.R. Pesticide Terminal Residues London: Butterworths 1971.

LYMAN, W.R. and R.J. LACOSTE. Environmental Quality and Safety, Suppl. Vol. 4. Stuttgart: Georg Thieme 1975.

VONK, J.W. Chemical Decomposition of Bisdithiocarbamate Fungicides and their metabolism by Plants and Microorganisms. Thesis, TNO, Utrecht, 1975.