The Translocation and Persistence of Tritium-Labeled Cycloheximide in Eastern White Pine Seedlings

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Two samples of tritium-labeled cycloheximide were prepared and used to follow the absorption and translocation of cycloheximide in eastern white pine seedlings. Cycloheximide was taken up from a stem application and distributed to the upper stem and needles, but was not translocated to the roots. There was no translocation downward from a foliar application. The translocated antibiotic persisted in the untreated tissue without detectable degradation for 2 months.

THE EFFECTIVENESS of the antibiotic, cycloheximide, in controlling plant fungal diseases has been reviewed (*3*). Among the fungal pathogens which are effectively controlled is white pine blister rust (*Cronartium ribicola*, Fischer). Evidence that cycloheximide is systemically active in eastern white pine (*Pinus strobus*, L.), and western white pine (*P. monticola*, Douglas) has been presented by Moss (*8*, *9*) and by Lemin, Klomparens, and Moss (*7*). This paper presents results of an investigation of the systemic activity of tritium-labeled cycloheximide in eastern white pine seedlings.

Wallen and Millar (*10*) demonstrated that cycloheximide was absorbed by the roots of wheat seedlings and was translocated only to the foliage which was present at the time of application. Hamilton and coworkers showed also that although cycloheximide was not systemic when applied as a foliar spray, several cycloheximide derivatives were translocated in concentrations sufficient to control cherry leafspot fungus on distal, unsprayed leaves (*6*). Semicarbazone and acetate derivatives of cycloheximide were shown to be systemic fungicides in wheat (*4*, *5*).

Methods and Results

Tritium Labeling of Cycloheximide. All radioactivity measurements were made with a Packard Tricarb liquid scintillation spectrometer, Model 314X. The photomultipliers were operated at 1300 volts and the discriminators were set so as to pass 10- to 50-volt pulses. The scintillation solvent consisted of redistilled toluene containing 0.4% of 2,5-diphenyloxazole and 0.01% of 1,4-bis(5-phenyl-2-oxazolyl)benzene and 10 ml. was used for each sample. All samples were corrected for quenching by recounting following the addition of an aliquot of tritium-labeled toluene standard.

A finely powdered sample (10 mg.) of cycloheximide was exposed to 2.5 curies of tritium gas at 300-mm. pressure for 21 days. The resulting solid was equilibrated three times with 50 ml. of 50% aqueous ethanol to remove labile tritium (*13*). Ten portions of 0.1 mg. each of the resulting solid were chromatographed on Whatman No. 1 paper using the upper phase of the system: methanol 1 part, benzene 1 part, and water 2 parts. The bioactive zone (R_f 0.4 to 0.5), determined by plating unlabeled standards on *Saccharomyces pastorianus* seeded agar (*7*, *12*), was cut from the paper and eluted with ethanol until radioactivity could no longer be detected in the eluate. The ethanol was removed by distillation and a sample of the resulting solid (1 γ) rechromatographed on a 1 × 24 inch strip of paper. After drying, the strip was cut lengthways and one half was placed on *S. pastorianus* seeded agar. The other half was cut into eighteen 1-inch pieces and counted using a simple modification of Wang and Jones' technique (*11*). Each 1-inch piece was placed in a counting vial, 10 ml. of the counting solution was added, and the whole set was counted in succession. The position of the single peak of radioactivity corresponded exactly with the position of the bioactive zone determined on the other half of the paper strip, indicating that the sample was radiochemically and biochemically pure. This sample had a specific activity of 340 mc. per gram. A larger sample of tritium-labeled cycloheximide was prepared by exposing 2.0 grams of cycloheximide to 2.5 curies of tritium gas for 21 days, which after purification as previously described had a specific activity of 13 mc. per gram.

Extraction of Plant Samples. Since liquid scintillation counting is subject to losses in efficiency because of the presence of light-absorbing solutes, the chlorophyll content of the sample was kept to a minimum by use of an aqueous methanol–methylene chloride extraction method (*7*), although this system did not fully extract the cycloheximide. The growing tip of an 8-inch eastern white pine seedling grown under greenhouse conditions, which had previously been treated with labeled cycloheximide, was macerated with 30 ml. of 50% aqueous methanol in a micro Waring Blendor. After filtration, the residue including filter paper was extracted again twice. The combined filtrates were extracted three times each with 30 ml. of methylene chloride. The solvent was removed by distillation under reduced pressure below 40° C. and 0.4 ml. of ethanol was added to the residue. After gentle warming, 0.1 ml. of the solution was counted and another 0.1 ml. was paper chromatographed. Following development and drying, the paper was cut into two pieces and examined for bioactivity and radioactivity. One radioactive and one bioactive spot were found and these appeared in the same place on the paper, indicating that all radioactivity was present in the bioactive material. The bioactive material was shown to be cycloheximide by paper chromatogram comparison with standards. Hence, the total radioactivity in the sample was due to labeled cycloheximide and the quantity of cycloheximide was calculated from the radioactivity meas-

ured in the 0.1 ml. of solution previously counted.

The sensitivity of this technique was such that 0.001 γ of the 340 mc. per gram labeled cycloheximide in 0.1 ml. could be counted within a standard deviation of 2%. Hence, 0.01 p.p.m. of the 340 mc. per gram labeled cycloheximide could be measured in the final ethanol solution.

This involved procedure was necessary in order to show that all the radioactivity in the plant extract was present as cycloheximide, thus enabling the tritium content to be used as a cycloheximide assay. Also, if any catabolic products from cycloheximide were present in the plant tissue, these would be expected to appear in one or the other of the bioactive or radioactive papergram determinations. All extracts were therefore examined by paper chromatograms as well as counted directly.

Stem Uptake and Distribution of Cycloheximide. A trial experiment using unlabeled cycloheximide was performed. Two 8-inch eastern white pine seedlings growing in pots in the greenhouse were treated with 0.1 ml. of a 1000-p.p.m. solution of cycloheximide in water by injecting the solution with a hypodermic syringe (No. 20 needle) into the stem 1 inch above soil level. After 48 hours, the seedlings were cut into four sections comprising the upper stem, the lower stem above the point of application, the stem from 1 inch above the application point to the soil level, and the roots. The sections were washed in water and briefly dried at room temperature. A one half-inch segment from the center of each stem or root section was cut lengthways and the freshly exposed surface placed on *S. pastorianus* seeded agar. After incubation at 37° C. for 18 hours, the sizes of the inhibition zones around the segments were measured and found to be progressively larger from the growing tip to the application point. Roots from the treated seedlings and a segment from an untreated seedling did not have inhibition zones.

A second experiment measured the distribution of labeled cycloheximide in a treated seedling. An aqueous solution (0.1 ml.) containing 500 p.p.m. of labeled cycloheximide (13 mc. per gram) was injected into the stem of an 8-inch eastern white pine seedling 1 inch above soil level. After 69 hours in the greenhouse, the seedling was cut into six parts comprising: the growing tip, 0.5 inch in length; the needles; the upper stem, 2.5 inches; the lower stem, 3 inches; the lower 2 inches of stem including the application point; and the roots. A sample of the runoff water was collected when the pot was watered. The tissue samples were extracted and the resulting residue was counted and paper chromatographed. No evidence of labeled material other than cycloheximide was found. The runoff water was not radioactive. Results of assays of plant tissues are shown in Table I.

The total recovery of labeled cycloheximide was 57.33% of that applied.

Persistence of Cycloheximide. Waxed paper cups were applied to 49 eastern white pine seedlings (12 to 15 inches in height), growing in pots in the greenhouse, by cutting 5-cm. filter papers along a radius, soaking the paper in melted paraffin wax, and wrapping the paper around the stem of the seedling with the paper overlapping at the radius. When the wax hardened, the cup was securely attached to the stem, preventing leakage and rundown which were observed when the injection technique was used. The cups were placed 1 inch above soil level. In each cup was placed 1 ml. of a 0.7-p.p.m. solution of 340 mc. per gram of tritium-labeled cycloheximide solution in water. Ten punctures were made in the bark of the seedling under the solution and 1 ml. of distilled water was added after 24 hours, when the cups were dry.

After 40 hours and again after 4 days, two seedlings were cut into three sections comprising the stem and needles above the wax cup; the stem supporting the cup down to the soil level, and the roots. All of the sections were analyzed separately and it was found that average recovery of radioactivity was 60%. The 40% loss of radioactivity is ascribed to the inefficient extraction method which was used to avoid the presence of light-absorbing solutes. No radioactivity was found in the roots and only labeled cycloheximide was found in other sections.

The stem and needles of the remaining seedlings, excluding the wax cups and 1 inch of the stems above the cups, were combined in groups of three and analyzed at 2-, 28-, and 57-day intervals after treatment, 15 seedlings being used at each time interval. There was present 0.161 p.p.b. of labeled cycloheximide after 2 days, 0.129 p.p.b. after 28 days, and 0.166 p.p.b. after 57 days. These differences are not significant at the 5% level. In similar experiments, roots of treated seedlings were analyzed 3, 7, 14, 21, and 28 days after treatment. No radioactivity was detected in any of the root samples.

Needle Uptake of Cycloheximide. The lower 8 inches of six 12-inch eastern white pine seedlings growing in pots in the greenhouse were protected by wrapping foliage, branches, and stems in polyethylene sheet and sealing the sheet to the stem of the plant with adhesive tape. The upper 4 inches of stems and needles were sprayed with 3 ml. of a 10-p.p.m. solution of tritium-labeled cycloheximide (13 mc. per gram) in water. After the plants were dry, the polyethylene sheets were removed and the unsprayed needles and stems were analyzed in groups of two after 3, 4, and 7 days. No radioactivity was detected in any of the unsprayed stems and needles.

Table I. Distribution of Tritium-Labeled Cycloheximide in an Eastern White Pine Seedling 69 Hours Following Hypodermic Application of 50 γ of Labeled Compound

Tissue from	Cycloheximide, P.P.M.	% of Total Cycloheximide Recovered
Growing tip	0.65	0.37
Needles	2.08	4.16
Upper stem	1.83	3.66
Lower stem	2.77	5.54
Application point	21.53	42.7
Roots	0.47	0.9

Discussion

The tritium labeling technique, utilizing the exposure method, has been widely used and the labeled compounds so obtained have been used in many biochemical investigations (*1*, *2*). Accuracy and sensitivity are the advantages of employing tritium-labeled cycloheximide and subsequent assay of the plant samples by counting tritium disintegrations. As little as 0.01 γ per ml. of the 340 mc. per gram labeled cycloheximide can be detected and measured, compared with a minimum of 2 γ per ml. required for the cycloheximide bioassay (*12*). Also, the liquid scintillation counting method allows an accuracy of $\pm 5\%$ with 95% confidence as against the bioassay accuracy of $\pm 32\%$ with 95% confidence.

It can be seen from Table I that 13.7% of the antibiotic applied is absorbed and translocated upward, apparently following the transpiration stream and building up high concentrations in the needles and upper stem. The small concentration detected in the roots was due to exudation of the injected solution which, in this experiment, ran down the stem and was detected in the root and lower stem tissue.

The injection method, and more particularly the wax cup method of application, is roughly comparable to the slit and excise method for the field treatment of pines (*8*). Hence, field-treated eastern white pine would be expected to move the antibiotic upward from the point of application to the needles. Translocation of cycloheximide in field-treated eastern white pine has been described (*7*).

From persistence studies, it is concluded that cycloheximide is not rapidly degraded by eastern white pine needles.

Previously described results (7) led to the conclusion that although cycloheximide is translocated less easily in eastern white pine when compared with western white pine, the antibiotic tends to persist longer in the eastern white pine needle tissue, an effect which may have been due to the use of higher concentrations of the antibiotic. Present work supports this conclusion in so far as it confirms the persistence of low concentrations in eastern white pine needles for at least 57 days without detectable loss.

The use of tritium-labeled cycloheximide to follow uptake and distribution of the antibiotic in pine seedlings demonstrates an accurate technique which appears to be widely applicable to studies of the translocation and persistence of cycloheximide.

Literature Cited

(1) Amdur, B. H., Rilling, H., Block, K., *J. Am. Chem. Soc.* **79,** 2646 (1957).
(2) Dawson, R. F., Christman, D. R., Anderson, R. C., Salt, M. L., Adams, A. F., Weiss, U., *Ibid.*, **78,** 2645 (1956).
(3) Ford, J. H., Klomparens, W., Hammer, C. L., *Plant Disease Reptr.* **42,** 680 (1958).
(4) Hacker, R. G., Vaughn, J. R., *Ibid.*, **41,** 442 (1957).
(5) *Ibid.*, **42,** 609 (1958).
(6) Hamilton, J. M., Szkolnik, M., Sondheimer, E., *Science* **123,** 1175 (1956).
(7) Lemin, A. J., Klomparens, W., Moss, V. D., *Forest Sci.* **6,** 306 (1960).
(8) Moss, V. D., *Plant Disease Reptr.* **41,** 709 (1957).
(9) *Ibid.*, **42,** 703 (1958).
(10) Wallen, V. R., Millar, R. L., *Phytopathology* **47,** 291 (1957).
(11) Wang, C. H., Jones, P. E., *Biochem. Biophys. Research Communs.* **1,** 303 (1959).
(12) Whiffen, A. J., *J. Bacteriol.* **42,** 610 (1948).
(13) Wilzbach, K. E., *J. Am. Chem. Soc.* **79,** 1013 (1957).

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RUMEN DEGRADATION OF FUNGICIDES

Fate of Tetramethylthiuram Disulfide in the Digestive Tract of the Ruminant Animal

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In experiments in which ruminant animals were fed corn treated with tetramethylthiuram disulfide (TMTD), it was found that the rumen microorganisms degraded the TMTD to carbon disulfide and probably hydrogen sulfide and dimethylamine. In a 6-hour period, 78% of the ingested TMTD was degraded as determined by the carbon disulfide recovered. However, the degradative action within the gastrointestinal tract was not complete, as 4.0% of the ingested TMTD appeared in the feces and 1.5% appeared in the urine as calculated from the carbon disulfide recovered. TMTD or its degradation products could not be demonstrated to be present in the blood and tissues of animals fed TMTD because a volatile substance was recovered from the blood and tissues which invalidated the distillation procedure for these studies.

TETRAMETHYLTHIURAM DISULFIDE is widely used in the production of food crops, yet there are few reports on the toxicity of TMTD to animals. Data collected by the Food and Drug Administration, cited by Dupont (*8*), set the LD_{50} of TMTD for rats at 850 mg. per kg. of body weight and for rabbits at 210 mg. per kg. of body weight, while in the cat 230 mg. per kg. of body weight proved to be fatal. In rabbits and puppies, Hanzlick and Irvine (*12*) found 3.0 grams per kg. of body weight to be the minimum fatal dose. In chronic toxicity studies carried out with rats fed diets containing 0, 1000, and 2000 p.p.m. of TMTD, it was found that some of the animals fed 1000 and 2000 p.p.m. died. However, rats showed no difference in growth patterns when fed for 65 weeks on diets containing 0, 250, and 500 p.p.m. of TMTD (*8*). There are no published reports on the toxicity of TNTD to ruminant animals.

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Materials and Methods

The methods used for assessment of TMTD in these studies were modifications of the distillation procedures described by Clarke *et al.* (*3*) and Lowen (*16*) for the determination of dithiocarbamates. The apparatus used in this study was of a different design, but operated on the same principle as the apparatus described by the above workers. Modifications in apparatus and procedure consisted in the addition of three (total of five) absorption tubes to accommodate solutions for the determination of hydrogen sulfide and to enable the use of both acidic and basic solutions necessary to remove interfering substances when assaying animal products.

Distillation. A procedure described by Diemair, Strohecker, and Keller (*6*) for the determination of hydrogen sulfide in tissues by the formation of methylene blue was adapted to the distillation procedure to measure the hydrogen sulfide liberated from decomposition of TMTD. The hydrogen sulfide procedure was carried out by using a 1.0*N* NaOH solution in two gas absorption tubes adjacent to the distillation flask to absorb the liberated hydrogen sulfide. At the end of the assay, 3.0 ml. of Reissners solution (0.1*N* $FeCl_3$ in 5.0% HNO_3 solution) was added to the absorption tubes containing the 1.0*N* NaOH and absorbed hydrogen sulfide. After standing for 30 minutes, the resulting color, methylene blue, was read on an Evelyn colorimeter using the 660-mμ filter. A calibration curve was prepared for hydrogen sulfide using a solution of methylene blue. The curve approximated Beer's law over the range determined, 0 to 45 μg.