tone toxicants may alter the microbial composition of the rumen and thus affect its vital metabolic functions. Some sesquiterpene lactones from Compositae are potent antimicrobial agents (Vichkanova et al., 1971), and studies in these laboratories have shown that bitterweed extracts are highly toxic and possibly mutagenic to some Gram-positive bacteria (Norman, 1975). It therefore seems likely that rumen dysfunction may contribute to bitterweed toxicity.

LITERATURE CITED

Anderson, L., de Kock, W., Pachler, K., Brink, C., *Tetrahedron* **23,** 4153 (1967).
Cavallito, C. J., Haskell, T. H., *J. Am. Chem. Soc.* **67,** 1991 (1945).
Couch, J. F., *J. Chem. Educ.* **14,** 16 (1937).
Dollahite, J. W., Rowe, L. D., Kim, H. L., Camp, B. J., *Tex. Agric. Exp. Stn. Prog. Rep.* **PR-3149** (1973).
Hanson, R. L., Lardy, H. A., Kupchan, S. M., *Science* **168,** 378 (1970).
Hardy, W. T., Cory, V. L., Schmidt, H., Dameron, W. H., *Tex. Agric. Exp. Stn., Bull.* **433,** 8 (1931).
Herz, W., Aota, K., Holub, M., Samek, Z., *J. Org. Chem.* **35,** 2611 (1970).
Jaggi, F. P. (Chairman), Committee Report on Animal Disease Losses of Economic Importance to the Livestock Industry in Texas, Texas A&M University, School of Veterinary Medicine, 1962, pp 56–62.
Kim, H. L., Rowe, L. D., Szabuniewicz, M., Dollahite, J. W., Camp, B. J., *Southwest. Vet.* **28,** 84 (1974a).
Kim, H. L., Szabuniewicz, M., Rowe, L. D., Camp, B. J., Dollahite, J. W., Bridges, C. H., *Res. Commun. Chem. Pathol. Pharmacol.* **8,** 381 (1974b).
Kingsbury, J. M., "Poisonous Plants of the United States and Canada", Prentice-Hall, Englewood Cliffs, N.J., 1964, pp 393–443.
Kupchan, S. M., *Trans. N.Y. Acad. Sci.* **32,** 85 (1970).
Kupchan, S. M., Eakin, M. A., Thomas, A. M., *J. Med. Chem.* **14,** 1147 (1971).
Kupchan, S. M., Fessler, D. C., Eakin, M. A., Giacobbe, T. J., *Science* **168,** 376 (1970).
Norman, J. O., U.S. Department of Agriculture, College Station, Tex., private communication, 1975.
Ortega, A., Romo de Vivar, A., Romo, J., *Can. J. Chem.* **46,** 1539 (1968).
Rimington, C., Roets, C., Steyn, D., *Onderstepoort J. Vet. Sci. Anim. Ind.* **7,** 507 (1936).
Romo, J., Romo de Vivar, A., Aguilar, M., *Bol. Inst. Quim. Univ. Nac. Auton. Mex.* **21,** 66 (1969).
Stahl, E., "Thin-layer Chromatography", Springer-Verlag, New York, N.Y., 1969, pp 863, 882.
Vichkanova, S., Rubinchik, M., Adgina, V., *Tr. Vses. Nauch. Issled. Inst. Lek. Rast.* **14,** 230 (1971).
Witzel, D. A., U.S. Department of Agriculture, College Station, Tex., private communication, 1975.

Received for review February 10, 1975. Accepted June 6, 1975. The work at Florida State University was supported by U.S. Public Health Service Grant No. CA 13121 through the National Cancer Institute; that at Texas A&M University was supported by the Texas Agricultural Experiment Station. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

Toxicity and Milk Bittering Properties of Tenulin, the Major Sesquiterpene Lactone Constituent of *Helenium amarum* (Bitter Sneezeweed)

G. Wayne Ivie,* Donald A. Witzel, and Darcy D. Rushing

Constituents of *Helenium amarum* (Rafin.) H. Rock. were isolated by high performance liquid chromatography and were studied for toxicity in hamsters and sheep. Tenulin, a sesquiterpene lactone that is known to account for the bitterness of this plant, was by far the major poisonous component observed. Although tenulin is of a low order of toxicity, it occurs in *H. amarum* to the extent of about 3% of the dried above ground plant material, and it is probably the primary toxicant involved in livestock poisoning by *H. amarum*. Oral administration of tenulin to a lactating cow resulted in bitter milk, and chromatographic and mass spectral studies indicated that unmetabolized tenulin was secreted into the milk. However, of the total tenulin given the cow, not more than 0.1% appeared in the milk. These studies suggest that human consumption of bitterweed milk resulting from dairy animals grazing on *H. amarum* will not constitute a significant health hazard.

Helenium amarum (Rafin.) H. Rock. (Figure 1), also known as *H. tenuifolium* Nutt. and commonly called bitter sneezeweed, bitterweed, sneezeweed, or yellow dog fennel, is widely distributed in the eastern and southern United States. The plant is of economic importance to the dairy industry because when eaten by cattle, it imparts a bitter taste to the milk and renders it unpalatable. *H. amarum* is also a livestock poison (Kingsbury, 1964) but seems to affect horses and mules to a greater extent than other species (Dollahite et al., 1972; West and Emmel, 1952). Laboratory studies with sheep have shown that fresh *H. amarum* causes death when force fed at 2% of body weight per day for 2 days (Dollahite et al., 1972).

Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, College Station, Texas 77840.

The major bitter principle of *H. amarum* was apparently first isolated by MacDonald and Glaser (1929) who obtained a crystalline, very bitter, unidentified substance from both fresh and dried plant samples. This bitter compound, when present in milk at levels as low as 1 ppm, caused sufficient bitterness to make the milk unsalable (Herzer, 1942). The chemistry of the bitter principle of *H. amarum* was studied by Buehler et al. (1937) and in greater detail by Clark (1939), who named the compound tenulin. Tenulin has subsequently been shown to be an epimeric mixture of the sesquiterpene lactone derivative I (Herz, 1975; Herz et al., 1962, 1963; Rogers and Haque, 1963). When treated with weak base, tenulin is isomerized to isotenulin (II), which itself may be a minor constituent of *H. amarum* (Ungnade and Hendley, 1948).

The possibility that tenulin may contribute to the toxicity of *H. amarum* was suggested by recent studies in which the major toxic principle of *Hymenoxys odorata* DC.

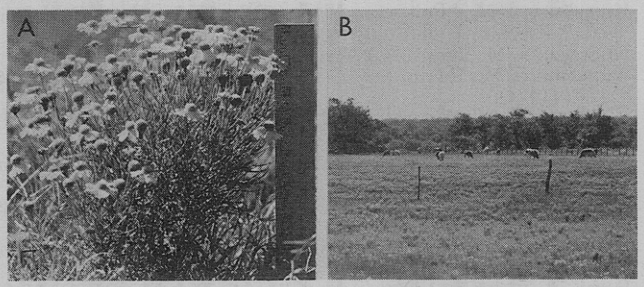
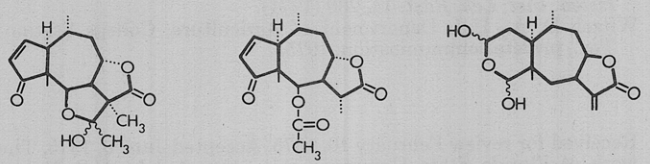


Figure 1. (A) *Helenium amarum* (Rafin.) H. Rock., Compositae; (B) cattle grazing a pasture heavily infested with *H. amarum.*



(Western bitterweed) was isolated and characterized (Ivie et al., 1975). Western bitterweed is an important sheep poison of the United States Southwest, and its major toxic component was identified as a sesquiterpene lactone, hymenovin (III). The close taxonomic relationship between *Helenium amarum* and *Hymenoxys odorata,* the chemical similarity of tenulin and hymenovin, and the reported occurrence of tenulin in rather large amounts in *H. amarum* prompted the current studies to consider the toxicity of tenulin and other *H. amarum* components to mammalian species. In addition, experiments were conducted with tenulin to investigate the extent to which this compound or its bitter metabolites are secreted into milk of dairy cattle.

MATERIALS AND METHODS

Chemicals and Analytical Procedures. Authentic tenulin was provided by Werner Herz, Florida State University, Tallahassee, Fla. Isotenulin was prepared by reaction of tenulin with aqueous sodium carbonate as previously described (Clark, 1939).

Infrared (ir) spectra were recorded as 1% potassium bromide pellets on a Beckman IR-18A spectrophotometer. Nuclear magnetic resonance (NMR) studies were conducted with a JEOL Model JNM-MH-100 spectrometer, with samples dissolved in deuterated chloroform and using tetramethylsilane as an internal reference. Electron impact mass spectra (MS) (70 eV) were determined with a Varian-MAT-CH-7 90° sector magnetic scan spectrometer, with a direct insertion probe.

Extraction. *H. amarum* in full flower was collected May–July 1974 at College Station, Tex. The above-ground plant material was dried for 2 days at 50° and finely ground, and a 100-g sample was extracted with 300 ml of acetone by homogenization with a Willems Polytron homogenizer (Brinkman Instruments, Westbury, N.Y.). After filtration, the residue was extracted once again as above, and the combined extracts were concentrated at reduced pressure to give a dark viscous residue. This was dissolved in 200 ml of ethanol, 300 ml of water was added, and most of the plant pigments were removed from solution by sequential concentration at reduced pressure to remove the ethanol, chilling, and centrifugation as previously described for hymenovin isolation (Ivie et al., 1975). The pigment pellet was redissolved in ethanol–water as above, and the concentration–centrifugation steps were repeated. The two amber supernatants were combined and were extracted by partitioning three times with equal volumes of ethyl acetate.

Chromatography. The ethyl acetate extract was transferred to acetonitrile, and the components were resolved by high performance liquid chromatography (HPLC). The instrument used was a Waters Model ALC-401 liquid chromatograph, equipped with a refractive index detector and Porasil A column packing (⅜ in. × 8 ft). Injections were made through a high pressure septumless injector, and the carrier solvent flow (acetonitrile) was maintained at 3.0 ml/min. The components were collected separately as they eluted from the column for subsequent analysis and toxicity studies.

H. amarum constituents and known compounds were also studied by thin-layer chromatography (TLC) (silica gel F_{254} chromatoplates, Merck, Darmstadt, Germany). After development in a solvent mixture containing chloroform–ether–acetone (3:3:1), the products were visualized by viewing under short-wavelength ultraviolet light or by acid charring (Stahl, 1969).

Tenulin was isolated on a large scale for toxicity studies in the following manner. The ethyl acetate fraction obtained through the usual extraction procedure described above was concentrated to dryness, and the residue was taken up in a small amount of acetone. Benzene was added, and upon concentration at reduced pressure, most of the tenulin present in the mixture crystallized from solution. With this procedure, approximately 100 g of tenulin was isolated with much less effort than would have been required had chromatographic methods been used.

Toxicology. Oral toxicity studies were conducted on male hamsters (100 g, Ela:Eng-Syr strain, Engle Laboratory, Farmersburg, Ind.) and female sheep (30–35 kg, mixed breed, taken from populations maintained at this laboratory). *H. amarum* fractions isolated by HPLC were transferred to water solution or suspension, using sonication when required, and were administered orally to hamsters via stomach tube. Tenulin was administered by stomach tube to sheep and hamsters as a water suspension. Mortality determinations were made 72 hr after treatment.

In studies designed to determine the antagonistic effects of mercaptans toward tenulin poisoning, hamsters were administered orally either 1200 or 1500 mg/kg of tenulin (the approximate LD_{50} and LD_{100} doses), and then were immediately administered 75 mg of cysteine hydrochloride in water solution.

Feeding Studies with Tenulin. A lactating Jersey cow, giving approximately 10 kg of milk daily, was obtained from a local dairy. The animal was provided the usual dairy ration and was hand milked at 12-hr intervals. Purified tenulin, which showed only a single fluorescence quenching spot on TLC, was administered to the cow by adding the crystalline material to a gelatin capsule and dosing orally with a balling gun. The first tenulin dose was 0.5 g; subsequent doses were 1, 2, 4, and 8 g of tenulin at 12-hr intervals. The total tenulin given the cow was 15.5 g. Milk samples collected during and after the treatment period were subjected to taste tests by personnel of this laboratory to determine whether the milk had a bitter taste.

For estimates of the level of tenulin or tenulin equivalents in bitterweed milk, fresh whole milk samples were fortified with known concentrations of tenulin and were evaluated by taste for intensity of bitterness. Tenulin was added as an ethanol solution, and in both fortified and nonfortified samples, the final ethanol concentration in the milk was 0.1%. In all tests, personnel involved were offered two milk samples, one was control whole milk and the other was either fortified with tenulin or was a sample from the treated animal. The participant was requested to characterize one, both, or neither of the samples as having a bitter flavor. Results from this procedure showed that tenulin concentrations in whole milk of 0.5 ppm or below are likely to be undetectable by taste. Only 3 of 11 participants detected bitterness in milk samples containing 0.5 ppm of

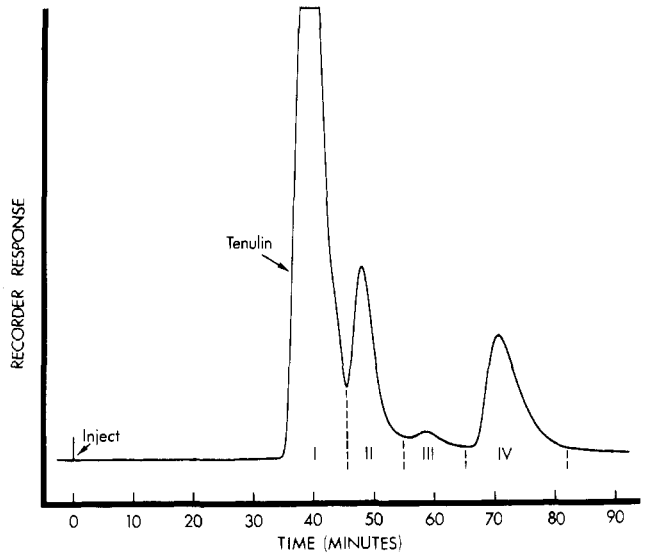


Figure 2. Components of a toxic fraction from *Helenium amarum* resolved by high performance liquid chromatography.

tenulin. However, milk fortified with tenulin at 1.0 ppm had a definite bitter taste to 9 of 11 participants. These findings agree with those of Herzer (1942) who fortified milk with a bitter principle from *H. amarum* (presumably tenulin) and reported that 1 ppm resulted in unsalable milk, whereas 0.75 ppm caused some bitterness but that the milk was probably salable.

Isolation of Tenulin from Bitterweed Milk. Milk that had a bitter taste after treatment of the cow with tenulin was analyzed to determine whether unmetabolized tenulin was present in the sample. The cream fraction was separated and discarded because previous studies have indicated that the bitter taste components are associated primarily with the skim milk and not the fat (MacDonald and Glaser, 1929). The skim milk was extracted three times with ethyl acetate, and the combined extracts were dried over sodium sulfate, then concentrated to dryness at reduced pressure. The residue was taken up in a small amount of acetonitrile, which was then partitioned with hexane to remove residual lipids. The acetonitrile phase was then concentrated to near dryness for TLC analysis. The TLC behavior of the milk extract components was compared with that of tenulin and isotenulin in the following eight solvent systems: chloroform–1-propanol (10:1); benzene (saturated with formic acid)–ether (5:1); chloroform–ether–acetone (3:3:1); benzene–acetone (2:1); hexane–ethyl acetate–methanol (2:2:1); ethyl acetate; benzene–ethyl acetate–chloroform (1:1:1); chloroform–acetone (1:1). After chromatoplate development, compounds were first visualized under ultraviolet light, then by acid charring. Mass spectral studies were also conducted with the suspected tenulin isolated from bitterweed milk in attempts to confirm its identity.

RESULTS

Bioassays for toxicity in the *H. amarum* fractions obtained in the extraction and cleanup procedure indicated that the toxic components of this plant were extractable into ethyl acetate. This fraction was lethal to hamsters when administered orally at a level equivalent to 5 g of the original dried *H. amarum*/animal. The pigment residue and extracted water phase were not toxic when given to hamsters at four times this level.

Analysis of the toxic ethyl acetate extract by HPLC resulted in its separation in four fractions (Figure 2). Studies with hamsters revealed that toxicity was associated almost

Table I. Toxicity of *Helenium amarum* Fractions to Male Hamsters

	Mortality at indicated dose[b]				
Fraction[a]	2.5	5	10	20	40
I	2/3	4/4	2/2	3/3	
II				0/3	0/1
III				0/3	0/1
IV				0/3	1/1

[a] Isolated by high performance liquid chromatography (Figure 1). [b] Dosage in gram equivalents of *H. amarum* (dry weight) per 100 g hamster. Mortality figures indicate the number of animals killed out of the number treated.

entirely with fraction I (Table I). This fraction, by far the major constituent in the ethyl acetate extract, was toxic at 2.5 g equiv/hamster and caused 100% mortality when administered at levels of 5 g equiv/animal or higher. Fractions II, III, or IV (Figure 2) were not toxic at 20 g equiv/hamster, but fraction IV was lethal when administered at 40 g equiv. TLC studies indicated fraction IV to consist of two components in approximately equal quantity. The chemical nature of these products was not studied.

TLC analysis of the major toxic constituent showed it to consist almost totally of a product having an identical R_f value with authentic tenulin. The material was subsequently recrystallized from boiling ether to yield white needles of tenulin that exhibited essentially identical ir, NMR, and MS as authentic tenulin. The tenulin yield was 2.9% of the dried plant material. A minor component of fraction I, which had a TLC R_f value lower than that of tenulin, corresponded to the small shoulder observed in HPLC analysis (Figure 2).

Subsequent toxicity studies with the purified tenulin showed it to have an acute oral LD_{50} to hamsters of 1200 mg/kg. Cysteine showed no antidotal properties toward tenulin poisoning in hamsters. The large quantities of tenulin required precluded extensive toxicity studies in sheep; however, sufficient tenulin was isolated to treat three animals. A 30 kg ewe treated with 30 g of tenulin (1000 mg/kg) died 12 hr after treatment. Necropsy findings of kidney congestion, hemorrhagic lesions of the heart, and ulceration and congestion of the rumen agreed very well with those previously reported for *H. amarum* poisoning (Dollahite et al., 1972). Subsequent sheep treated with tenulin at 400 and 700 mg/kg survived, and showed no significant toxicity symptoms.

Oral treatment of a lactating cow with tenulin resulted in bitter components being secreted into the milk (Table II). However, bitterness occurred in only one sample, which was collected 12 hr after the final treatment of 8 g of tenulin. The estimated tenulin or tenulin equivalents concentration in this sample was 1.0–1.5 ppm, based on taste comparison with milk samples fortified with known concentrations of tenulin. The final milk sample of the study collected 24 hr after the last tenulin dose, had no bitter taste, which suggests that absorbed tenulin is rapidly excreted or metabolized to nonbitter derivatives. The total secretion of bitter substances into the milk during the treatment and post-treatment periods was less than 16 mg of tenulin or tenulin equivalents, or less than 0.1% of the total administered dose (Table II). This figure certainly represents the maximum possible amount appearing in milk because the calculations were made under the assumption that all nonbitter samples collected contained 0.5 ppm of tenulin, the lower limit of sensitivity.

Extraction and analysis of a part of the bitter milk samples from the tenulin-treated cow gave good evidence that

Table II. Milk Bittering Characteristics of Tenulin after Oral Administration to a Lactating Cow

hr after initial dose	Tenulin dose, g	Cumulative tenulin dose, g	Milk bitterness	Tenulin concn, ppm[a]	Amount of tenulin dose secreted into milk, cumulative %[b]
0	0.5	0.5			
12	1.0	1.5	No	<0.5	<0.3
24	2.0	3.5	No	<0.5	<0.2
36	4.0	7.5	No	<0.5	<0.2
48	8.0	15.5	No	<0.5	<0.1
60	0	15.5	Yes	1.0–1.5	<0.1
72	0	15.5	No	<0.5	<0.1

[a] Estimated by taste comparison with fresh whole milk fortified with tenulin. Figures are reported as parts per million of tenulin or tenulin equivalents. Minimum detectable concentration was approximately 0.5 ppm. [b] Figures indicate maximum values for secretion into milk, as all nonbitter samples were assumed to contain 0.5 ppm of tenulin, the minimum detectable level.

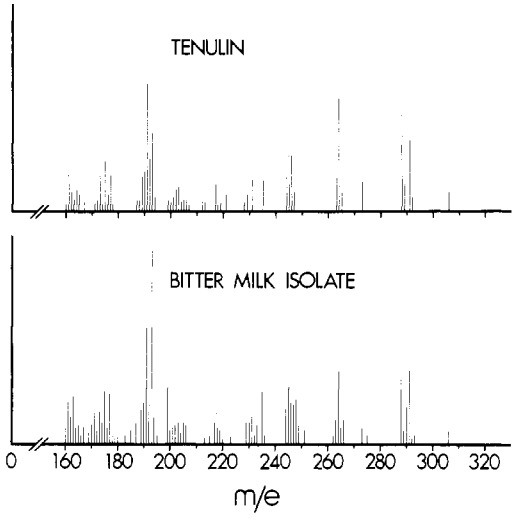


Figure 3. Electron impact mass spectra of tenulin and a bitter component from milk of a cow treated orally with tenulin.

tenulin is secreted unmetabolized into the milk. Although obtained only in submilligram quantity, a bitter, fluorescence quenching compound was observed in the milk extract, which showed identical TLC behavior with authentic tenulin in eight solvent systems. The mass spectrum of the bitter milk isolate agreed very well with that of authentic tenulin (Figure 3), although the tenulin obtained from bitter milk contained appreciable impurities. These were likely normal milk constituents with chromatographic properties identical with tenulin.

DISCUSSION

Tenulin is about fourfold less toxic to hamsters and perhaps seven- to tenfold less toxic to sheep than is hymenovin, the major toxic constituent of Western bitterweed (Ivie et al., 1975). The lower toxicity of tenulin might be expected because an α-methylene γ-lactone grouping is present in hymenovin and other cytotoxic sesquiterpene lactones, but not in tenulin. The α-methylene γ-lactone moiety is a major reactive center that probably accounts, at least in part, for the mode of action of the sesquiterpene lactone compounds—alkylation of sulfhydryl groups of body constituents (Hanson et al., 1970; Ivie et al., 1975; Kupchan et al., 1970). The amino acid cysteine shows some antagonistic properties toward hymenovin poisoning due to probable reaction of the cysteine sulfhydryl with the methylene moiety of hymenovin (Ivie et al., 1975). It was, therefore, not surprising that cysteine showed no antidotal effects toward tenulin poisoning in hamsters because tenulin does not contain a methylene group. However, toxicity syndromes of hymenovin and tenulin are quite similar, which suggests that these compounds have closely related modes of action. The observed toxicity of tenulin may be at least partly attributable to the α,β-unsaturated ketone moiety, which itself is a potential alkylating function (Lee et al., 1971).

Although isotenulin has been reported as a minor *H. amarum* constituent (Ungnade and Hendley, 1948), it was not observed in extracts of the *H. amarum* samples studied here. The sesquiterpene lactones amaralin and aromaticin have been isolated from *H. amarum* in low yields (Lucas et al., 1964), and they may contribute to the toxicity of this plant. Amaralin and aromaticin might possibly comprise the two components occurring in fraction IV of the *H. amarum* samples studied here (Figure 2), but this was not determined. At any rate, because of its relatively high content in the plant, tenulin appears to be the major constituent governing the toxicity of *H. amarum*.

Orally administered tenulin showed very little tendency to be secreted into the milk of a lactating cow because less than 0.1% of the tenulin given during a 3-day treatment period appeared in the milk. However, the extreme bitterness of this compound is such that even very low milk residues can be economically important. These studies with tenulin indicate that lactating dairy animals must consume reasonably large quantities of *H. amarum* to result in significant bitter flavor in the milk. The 15.5 g of tenulin given the cow over a 3-day period is equivalent to the tenulin content of about 2 kg of green *H. amarum*, assuming 3% tenulin content of the dried plant and 75% water content in the growing plant. Thus, it may be predicted that a single dose of 500 g or more of green *H. amarum* to a lactating cow would be required to cause appreciable milk bitterness.

Although evidence was obtained that unmetabolized tenulin was secreted into the milk of the treated cow, the possible presence of bitter or nonbitter tenulin metabolites cannot be ruled out. However, no detectable isotenulin residues were present in the bitter milk. No products were observed in the milk extract which cochromatographed with this tenulin isomer.

These studies indicate that human consumption of milk from dairy animals that have grazed on *H. amarum* will be accompanied by low-level intake of tenulin. However, this compound is relatively low in toxicity and its bitter properties act as a guard against its consumption in appreciable amounts. It thus seems likely that tenulin represents no significant toxicological hazards to human health.

ACKNOWLEDGMENT

For technical assistance during this study, we thank Claudio Castillo, Judy Pennington, and Michael Imhoff of

this laboratory. The cooperation of Werner Herz, Department of Chemistry, Florida State University, Tallahassee, is gratefully acknowledged.

LITERATURE CITED

Buehler, C. A., Whitehead, F., Goode, B. D., *J. Am. Chem. Soc.* **59,** 2299 (1937).
Clark, E. P., *J. Am. Chem. Soc.* **61,** 1836 (1939).
Dollahite, J. W., Rowe, L. D., Kim, H. L., Camp, B. J., *Southwest. Vet.* **26,** 135 (1972).
Hanson, R. L., Lardy, H. A., Kupchan, S. M., *Science* **168,** 378 (1970).
Herz, W., Department of Chemistry, The Florida State University, Tallahassee, Fla., private communication, 1975.
Herz, W., Rhode, W. A., Rabindran, K., Jayaraman, P., Viswanathan, N., *J. Am. Chem. Soc.* **84,** 3857 (1962).
Herz, W., Romo de Vivar, A., Romo, J., Viswanathan, N., *Tetrahedron* **19,** 1359 (1963).
Herzer, F. H., *Proc., Assoc. South. Agric. Work.* **43,** 112 (1942).
Ivie, G. W., Witzel, D. A., Herz, W., Kannan, R., Norman, J. O., Rushing, D. D., Johnson, J. H., Rowe, L. D., Veech, J. A., *J. Agric. Food Chem.*, preceding paper in this issue.
Kingsbury, J. M., "Poisonous Plants of the United States and Canada", Prentice-Hall, Inc., Englewood Cliffs, N.J., 1964, p 412.
Kupchan, S. M., Fessler, D. C., Eakin, M. A., Gaicobbe, T. J., *Science* **168,** 376 (1970).
Lee, K. H., Huang, S. E., Piantadosi, C., Pagano, J. S., Geissman, T. A., *Cancer Res.* **31,** 1469 (1971).
Lucas, R. A., Rovinski, S., Kiesel, R. J., Dorfman, L., MacPhillamy, H. B., *J. Org. Chem.* **29,** 1549 (1964).
MacDonald, M. B., Glaser, A., *Tenn. Agric. Exp. Stn., Circ.* **26,** 19 (1929).
Rogers, D., Haque, M., *Proc. Chem. Soc. London,* 92 (1963).
Stahl, E., "Thin-Layer Chromatography", Springer Verlag, New York, N.Y., 1969, p 863.
Ungnade, H. E., Hendley, E. C., *J. Am. Chem. Soc.* **70,** 3921 (1948).
West, E., Emmel, M. W., *Univ. Fla. Agric. Exp. Stn. Bull.* **510,** 25 (1952).

Received for review February 10, 1975. Accepted June 6, 1975. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

Uptake, Distribution, and Metabolism of Endothall in Fish

Harish C. Sikka,* Dennis Ford, and Robert S. Lynch

When bluegills were exposed in aquaria to water containing 2 ppm of [^{14}C]endothall, less than 1% of the herbicide was absorbed by the fish. The maximum concentration of endothall in the fish (0.1–0.2 ppm) was observed 12 hr after treatment; thereafter it did not change significantly up to 96 hr. Radioactivity was detected in viscera, flesh, scales, skin, and head. At all sampling times, the concentration of ^{14}C residues was highest in the viscera and lowest in the flesh. A small but detectable amount of ^{14}C was found in the blood 30 min after treatment. The herbicide was also absorbed by the fish when fed through the digestive tract. The fish did not metabolize endothall during the 48 hr after treatment.

The herbicide endothall (7-oxabicyclo[2.2.1]heptane 2,3-dicarboxylic acid) has been found to be effective in controlling certain aquatic weeds. The dipotassium salt of endothall is relatively nontoxic to fish; it has a medium tolerance limit ranging from 95 to 150 ppm for nine species of fish (Walker, 1963). A knowledge of the degree of accumulation of this herbicide by fish is important if they are to be used for human consumption. The herbicide, if accumulated by fish, may undergo metabolic transformation. The nature of these metabolites must be known in order to assess their possible toxicity to fish and man. Presently, no information is available in literature on the fate of the herbicide in fish. The present investigation was undertaken to study the uptake and metabolism of endothall in bluegills (*Lepomia macrochirus*).

MATERIALS AND METHODS

Uptake and Distribution of [^{14}C]Endothall. Bluegills were obtained from the National Fish Hatchery, North Attleboro, Mass. The fish were acclimated to the laboratory conditions for 2 weeks before exposing them to endothall. During acclimation, the fish were placed in holding aquaria containing aged tap water. The fish were maintained at about 20°. The fish used in these studies were 3–4 in. long and weighed 3–5 g. The fish were exposed to endothall in two ways: bathing and feeding. Uptake and distribution of endothall were determined by ^{14}C analysis of whole fish and selected fish tissues after various periods of exposure of the fish to [^{14}C]endothall. Fish were fasted for 48 hr before exposure to the herbicide.

Bath Exposure. Each fish was introduced into 500 ml of aged tap water containing 2 ppm of [^{14}C]endothall, labeled in positions 2 and 3 of the oxabicyclo ring. The water containing the fish was continuously bubbled with air alone or a mixture of ozone and air (to minimize the microbiological growth) during the exposure of the fish to the herbicide. The fish were removed from the treated water at 4, 12, 24, 48, 72, and 96 hr following treatment, rinsed with clean water three times, and weighed. To determine the amount of ^{14}C in the whole body, the fish were cut into small pieces and homogenized with 80% methanol in a Virtix homogenizer fitted with turboshear blades. The slurry was shaken for 30 min and centrifuged and the extract was decanted. The residue was refluxed with 80% methanol for 1 hr. The two extracts were combined; the amount of radioactivity in the pooled extract was measured by adding 1-ml aliquots to 15 ml of liquid scintillation solution and counting in a liquid scintillation counter. The amount of ^{14}C in the tissue residue was determined by solubilizing it in NCS tissue solubilizer (Amersham/Searle Co.) for 48 hr at 50°. Glacial acetic acid (0.02 ml/ml of solubilizer) was added to the solubilized tissue and the solution was counted for radioactivity using scintillation fluid containing Triton X-100. The samples were stored overnight at 0° in the dark before counting. The radioactivity in the methanol extract and in the tissue residue was combined to calculate the ^{14}C concentration in the fish.

To determine the distribution of radioactivity in various

Life Sciences Division, Syracuse University Research Corporation, Syracuse, New York 13210.