Uptake, Tissue Distribution, and Clearance of the Selective Piscicide 1,1′-Methylenedi-2-naphthol (Squoxin) by the Rainbow Trout and the Squawfish

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Rainbow trout and squawfish were exposed to the selective piscicide Squoxin (1,1′-methylenedi-2-naphthol) in the laboratory, using the tritiated compound, and in the field using a commercial formulation. Laboratory exposures were at concentrations of 50 ppb, the exposures repeated as many as four times in the case of the trout. Radioassays of whole body and certain tissues and organs of the trout immediately after exposure and after fresh water purges up to 8 days showed that there was no tendency for the piscicide to be retained. Maximum residues, based on the radioassays, were equivalent to 9.45 ppm of Squoxin in the trout. Squawfish, killed by a single exposure, had maximum residues, as Squoxin, of 3.50 ppm. The field-treated fish were exposed to Squoxin at a concentration of 100 ppb. Analyses were by gas chromatography. Surviving trout contained residues as high as 1.48 ppm (whole body), while dead squawfish contained residues ranging from 0.84 to 2.38 ppm. Residues in the edible tissues of the trout were less than 0.1 ppm.

The phenol, 1,1′-methylenedi-2-naphthol (Squoxin), is selectively toxic to the squawfish, a predator and competitor of trout and salmon in freshwater streams in the Western United States. MacPhee and Ruelle (1969), who discovered the selectivity of this phenol, and Johnston (1972) report that it is lethal to squawfish at concentrations as low as 6 ppb, depending on time of exposure and temperature, while concentrations of 100–1000 ppb have no effect on trout and other desirable species. This permits the use of Squoxin to control squawfish populations without seriously damaging other species in the stream. Keating et al. (1973) have reported on the successful use of Squoxin in several streams in Oregon and Idaho. Their report shows that the piscicide is effective at treatment rates as low as 25 ppb and that its biological activity dissipates within 10 hr after application ceases. The estimated half-life, under field conditions, is approximately 2 hr.

Squawfish killed during the treatment of a stream may be eaten by predatory birds or mammals and, even though the toxicity of Squoxin to mammals (rats) is very low (LD_{50} ~5 g/kg, unpublished data), there is need to know the magnitude of residues in such fish. It is also necessary to know the rate at which Squoxin is eliminated from the tissues of trout and other game fish which would be exposed to the toxicant. This report summarizes the results of laboratory and field experiments designed to provide answers to these questions.

METHODS

Laboratory Experiments. *Chemicals.* Tritium-labeled Squoxin (1,1′-methylendi-2-naphthol-*6,6′-t*) was synthesized in the laboratory by the following procedure. In a 50-ml flask was placed 0.223 g (0.001 mol) of 6-bromo-2-naphthol (Aldrich Chemical Co.), 0.020 g of palladium (10%) on charcoal catalyst (Engelhard Industries), 10 ml of dry benzene, and 0.3 ml of triethylamine. The flask was attached to a tritium hydrolysis manifold and exposed to approximately 2 atm of tritium gas. The mixture was magnetically stirred at ambient temperature and the uptake of tritium monitored. A total of 23.5 ml of tritium had been absorbed after 6.5 hr (theoretical, 22.4 ml at STP) when uptake ceased. Filtration through glass wool yielded a clear yellow solution. Evaporation produced a viscous yellow oil which was shown to be essentially pure 2-naphthol by TLC analysis on silica gel G ($HCCl_3$–Me_2CO–Et_2NH, 20:10:1). Examination of the TLC plate with a Packard radiochromatographic scanner indicated that >99% of the activity was contained in the 2-naphthol spot.

The theoretical specific activity was 75 mCi/mmol. To remove the tritium label in the phenolic hydrogen position three 20-ml methanol dilution evaporation steps were carried out. The theoretical specific activity was then 50 mCi/mmol.

The final step in the synthesis followed the procedures described by Ogata et al. (1969). The 2-naphthol-*6-t* was acidified with 2*N* HCl and 60 ml of distilled water plus 0.300 g of unlabeled 2-naphthol were added. After filtration to remove 6-bromo-2-naphthol (which is almost insoluble in water at 100°) tritiated 2-naphthol was recrystallized as light beige plates. The crystalline 2-naphthol was transferred while wet to a 100-ml flask equipped for reflux. To this was added 24 ml of 1% NaOH, 0.25 g of 37% aqueous formaldehyde (0.003 mol), and 40 ml of water. Refluxing for 1.5 hr, cooling to 25°, and acidification with HCl yielded a beige crystalline material. Three recrystallizations from ethanol–water gave 0.235 g (0.00078 mol) of fine white needles, mp 201–202°. Specific activity was 19.3 mCi/mmol. Chemical and radiochemical purity were both in excess of 98% as determined by TLC. The identification of the compound was confirmed by infrared and mass spectral comparisons with an authentic sample.

Treatment of Fish. The rainbow trout, obtained from a fish hatchery, were 13 to 20 cm long and the squawfish, captured by net in a local stream, were 20 to 25 cm in length. The exposures to radioactive Squoxin were in 20-l. glass jars filled to 15 l. with charcoal-treated tap water and aerated with soapstones. Six fish were placed in each tank. Squoxin-*t* (2.86×10^7 dpm) and unlabeled Squoxin were added in ethanol solution to a concentration of 50 ppb. The exposure systems were static, i.e., there was no exchange of fresh water. Control baths containing the same number of fish but without Squoxin were included with each experiment.

In one series of experiments the trout were placed in the jars for periods of 6, 12, 24, and 48 hr and then removed for determination of the total, whole body, radioactivity. The squawfish were left in the jars until they died, usually 6–10 hr.

In later experiments the trout were given multiple exposures of 6 hr each to freshly prepared Squoxin solutions. At this point, after as many as four exposures (24 hr total) some of the fish were examined for radioactivity and others were placed in flowing fresh water (exchange approximately 1 hr) for periods up to 192 hr and then removed for examination. In these cases only one fish was placed in each fresh water bath.

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Table I. Radioactivity, Expressed as Squoxin, in Rainbow Trout and Squawfish after a Single Exposure to Squoxin-*t* at 50 ppb

Species	Length of exposure, hr	Squoxin equiv, ppm ± SD[a]
Rainbow trout	6	0.96 ± 0.08
	12	1.42 ± 0.08
	24	1.53 ± 0.10
	48	2.09 ± 0.95
Squawfish	20[b]	3.50 ± 0.07
	8[b]	1.70 ± 0.03
	8[b]	2.17 ± 0.36

[a] Average of three radioassays. [b] Dead when removed from tank.

Table II. Radioactivity, Expressed as Squoxin, in Rainbow Trout Given Multiple Exposure to 50 ppb of Squoxin-*t* followed by Transfer to Fresh Water

No. of exposures	Total length, hr	Fresh water purge, hr	Squoxin equiv, ppm ± SD[a]
1	6	0	1.90 ± 0.16
2	12	0	3.34 ± 0.62
4	24	0	9.45 ± 1.23
1	6	48	0.43 ± 0.13
2	12	48	0.80 ± 0.48
4	24	48	1.01 ± 0.15

[a] Average of three radioassays.

The aquaria were kept in a room held at 16° on a 13–11 hr light–dark schedule. Light was provided by two 100-W bulbs.

Preparation of Samples for Radioassay. For the whole body measurements the fish were sacrificed by stunning, cut into several pieces, and homogenized with an equal weight of water in a Sorvall Omnimixer. Aliquots were removed for solubilization and counting. Tissue and organ samples were cut into small pieces and solubilized directly.

Solubilization was accomplished by wet oxidation using $HClO_4$ and H_2O_2 (Mahin and Lofberg, 1966). The tissue samples, weighing 0.1–0.4 g, were treated with 0.3 ml of 60% $HClO_4$ plus 0.6 ml of 30% H_2O_2 and allowed to stand at room temperature overnight. The tightly sealed vials were then heated at 70–80° with occasional swirling for approximately 2 hr. After cooling, 6 ml of ethyl Cellosolve and 10 ml of toluene phosphor solution containing 6 g of 2,5-diphenyloxazole per 1000 ml of toluene were added. If the mixture was not clear and homogeneous, additional ethyl Cellosolve was added until clarity was achieved. The samples were then counted using a Packard Tri-Carb liquid scintillation counter series 314E.

Triplicate samples of the whole body homogenates were analyzed. The tissue and organ samples were too small for replication. Counting efficiency was determined in each sample by the internal standardization method using toluene-*t*. Correction for background was made by counting similar samples from control fish. The radioactivity, expressed as disintegrations per minute, was converted to parts per million of Squoxin to give an estimate of the maximum amount of Squoxin which could be present in each sample.

Field Experiments. *Treatment of Streams.* A 55-mile section of the north fork of the John Day River in Central Oregon was treated with Sonar 300, the commercial formulation of Squoxin, in July, 1973. This formulation contains Squoxin as the monosodium salt in ethanol. The piscicide was metered into the stream from 30- and 55-gal drums pressurized with propane at 5 psi. Stream flow data were used in calculating the application rate which would achieve the desired concentration of 100 ppb. The stream was treated in segments approximately 5 miles each with each application lasting 10–12 hr.

The same method of application was used to treat the Molalla River in Northwestern Oregon on Aug 1, 1973. These applications were also designed to achieve concentrations of 100 ppb and ranged in length from 6 to 10 hr.

Sampling Methods. Dead squawfish were collected at various times and distances from the point of application in a given segment of the stream and these collection data recorded. Trout were collected by hook and line and killed by stunning. A few dead trout were also collected. It was assumed that these fish encountered an excessive concentration of Squoxin at the treatment site. The fish were stored at 0° until they could be transported to the laboratory where they were stored at −10° until analyzed. Analysis was by the GC method of Kiigemagi et al. (1975).

RESULTS AND DISCUSSION

Laboratory Experiments. Squoxin is readily oxidized to undetectable levels in fast moving streams (Keating et al., 1973) and in aerated tap water in the laboratory. As many as a dozen oxidation products appear in its place. In the static system, used in the experiments summarized in Table I, the fish would be exposed to decreasing concentra-

Table III. Radioactivity, Expressed as Squoxin, in Selected Tissues and Organs of Rainbow Trout Given Multiple Exposures to Squoxin-*t* at 50 ppb

Treatment			Squoxin Equivalent, ppm						
No. of exposures	Total length, hr	Fresh water purge, hr	Blood	Brain	Bile	Liver	Gills	Kidney	Edible flesh
1	6	0	0.81	0.65	137.1	5.54	2.78	2.01	0.29
2	12	0	1.03	1.56	477.6	6.74	5.41	3.71	0.71
4	24	0	3.51	3.16	1120.5	8.9	11.02	6.85	1.17
1	6	48	0.15	0.51	<0.01	1.11	<0.01	0.32	<0.01
2	12	48	0.15	0.74	11.4	1.16	0.14	0.07	<0.01
4	24	48	0.36	2.21	81.4	4.60	0.76	1.05	0.10
4	24	120	0.17	0.55	18.7	0.11	<0.01	0.14	<0.01
4	24	168	<0.01	0.55	3.91	0.04	<0.01	0.08	<0.01
4	24	192	<0.01	0.50	0.05	<0.01	<0.01	<0.01	<0.01

Table IV. Radioactivity, Expressed as Squoxin, in Selected Tissues and Organs of Squawfish Given a Single Exposure to Squoxin-*t* at 50 ppb

Sample No.	Approx. time of death, hr	Squoxin equiv, ppm						
		Blood	Brain	Bile	Liver	Gills	Kidney	Edible flesh
2	6+[a]	3.9	2.66	62.5	11.86	8.41	10.71	0.58
1	6–20[b]	*b*	4.19	119.0	13.16	10.55	10.99	0.64
3	6–20[b]	*b*	2.71	38.6	15.94	7.40	11.15	2.77

[a] Near death at 6 hr, sacrificed at 6 hr. [b] Dead when observed at 20 hr. No blood obtained.

Table V. Squoxin Residues in Dead Squawfish Collected during Treatment of the John Day River, 1973; Squoxin Applied at 100 ppb

Sample No.	Length of treatment, hr	Fish collected, from point of treatment		Squoxin residues,[b] ppm
		Miles	hr[a]	
11A	10	2.0	10	1.48
12A	10	2.5	10	1.26
12B	10	1.5	10	1.35
13A	12	2.5	11	1.11
13B	12	1.0	11	2.03
14B	12	1.0	20	1.39
15A	12	0.25	20	1.40

[a] From beginning of treatment. [b] Residues on whole body basis.

Table VI. Squoxin Residues in Dead Squawfish Collected during Treatment of the Mollala River, 1973; Squoxin Applied at 100 ppb

Sample	Length of treatment, hr	Fish collected, from point of treatment		Squoxin residues,[b] ppm
		Miles	hr[a]	
1A	9	2	12	2.38
1B	9	2	12	1.36
1C	9	1	12	1.99
2A	6	2.5	12	1.39
2B	6	2.5	12	0.84
2C	6	1.5	12	1.47

[a] From beginning of treatment. [b] Residues on whole body basis.

Table VII. Squoxin Residues in Rainbow Trout Collected during Treatment of the John Day River, 1973; Squoxin Applied at 100 ppb

Sample no.	Treatment, hr[a]	Tissue analyzed	Fish collected, from point of treatment		Squoxin residues, ppm
			Miles	hr[a]	
11A	10	Whole body	0.01	58	0.46
11B	10		0.01	34	1.48
12A	12		1.0	60	0.46
13A[b]	12		2	36	3.32
14A[b]	12		0.25	36	0.87
14B[b]	12		1.0	36	1.59
11C	10	Edible flesh	0.5	34	<0.1
11D	10		0.5	34	<0.1
12B	12		1.0	60	<0.1
12C	12		0.5	60	<0.1
1A	8		2	16	<0.1

[a] From beginning of treatment. [b] Fish dead when collected.

tions of the toxicant itself but to increasing concentrations of the several degradation products.

The measurement of tritium in the whole body aliquots was used to estimate the maximum amount of Squoxin which could have been accumulated during treatments. This indicated that after 48-hr exposure to the radioactive compounds in the tank, trout contained as much as 2.09 ppm (Table I). Higher levels, up to nearly 4 ppm, were found in the dead squawfish. We do not know whether this difference is due to a greater uptake of the toxicant by the squawfish or to the continuing excretion of the radioactive compounds by the surviving trout. There was no mortality in the controls of either species.

When the trout were given multiple exposures to the 50-ppb concentrations of Squoxin, the relationship between amount of radioactivity in their tissues and the dose of Squoxin was almost linear (Table II). The ability of the fish to metabolize and excrete the toxicant is shown by the experiments in which the treated fish were returned to fresh water for an additional 48 hr. In the case of the trout given the heaviest exposure (24 hr), over 90% of the accumulated radioactivity was excreted during the next 48 hr.

The distribution of radioactivity in the blood, brain, bile, liver, gills, kidney, and muscle tissue of trout exposed for up to 24-hr periods to the 50-ppb concentrations of Squoxin is shown in Table III. Spleen, heart, gastrointestinal tract, and skin were also examined in these studies with similar results. With the exception of the liver, there was a close linearity between dose and tissue radioactivity (Table III). The large concentration of radioactivity in the bile indicates that this is the main route of excretion.

The radioassays of the tissues from fish which were purged in fresh water show that there was some tendency for radioactivity to remain in the brain. Otherwise, the radioactive compounds were almost completely eliminated (less than 0.1 ppm as Squoxin) after 8 days.

This agrees with other work we have done with rats treated with ^{3}H- and ^{14}C-labeled Squoxin (unpublished). Over 90% of a single oral dose of the piscicide was excreted, mostly in the feces, within 24 hr and the remainder was cleared in 7 days.

Table IV summarizes similar data for squawfish. A comparison with the 6-hr exposures of trout (Table III) indicates, as before, that squawfish tend to accumulate more of the toxicant than trout.

Field Studies. The reliability of the laboratory studies

with Squoxin-*t* as an indicator of potential residues in fish is confirmed by the field experiments (Tables V, VI, and VII). These samples were analyzed by the GC method of Kiigemagi et al. (1975) which is specific for Squoxin.

The results of the analyses of squawfish from the John Day and Mollala River treatments with Squoxin are shown in Tables V and VI. The fish were dead when collected so that neither time of exposure nor distance from the treatment site is accurately known.

Table VII summarizes the results of the analyses of rainbow trout captured during and immediately after completion of the treatment of the John Day River. The dead trout probably died from exposure to high concentrations of Squoxin at the treatment site. As shown in the table, there was little Squoxin in the edible tissues of these fish.

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Analytical Methods for the Detection of the Piscicide 1,1′-Methylenedi-2-naphthol (Squoxin) in Fish and Water

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Procedures are given for the measurement of the piscicide 1,1′-methylenedi-2-naphthol (Squoxin) in fish tissue, using gas chromatography, and in water, using a colorimetric method. For the GC method, Squoxin is converted to its dimethyl ether and detected by electron capture. The method is sensitive to 0.1 ppm of Squoxin in fish tissue. The second method, which is designed for use in the field, involves coupling the piscicide with the chromogenic agent, tetrazotized *o*-dianisidine (Diazo Blue B). With the use of extraction and concentration steps this method is sensitive to 2 ppb of Squoxin in water. The two methods have been used in the analysis of fish and water samples from field tests of Squoxin.

The piscicide 1,1′-methylenedi-2-naphthol (Squoxin) is highly selective for the squawfish, a competitor and predator of trout and salmon in fresh water streams of the Pacific Northwest (MacPhee and Ruelle, 1969; Johnston, 1972). It has been used experimentally in Idaho, Oregon, Washington, and in Canada to gain information about its effectiveness in fish management and its impact on nontarget organisms (Keating et al., 1973).

These studies have required the development of analytical methods suitable for the monitoring of Squoxin in streams and for the measurement of its residues in various organisms. We have developed two methods for these purposes. A gas chromatographic procedure involving the formation of the dimethyl ether of the piscicide can be used to detect residues as low as 0.1 ppm in fish tissue. It has been used on fish collected from two field experiments conducted in 1973. The second method is a simple colorimetric procedure which can be used in the field for on-site measurements of Squoxin in water at concentrations as low as 2 ppb. Details of the analytical procedures are given in this report.

EXPERIMENTAL SECTION

Chemicals and Reagents. Squoxin (1,1′-methylenedi-2-naphthol) was obtained from Aldrich Chemical Co. (Milwaukee, Wisc.). Squoxin dimethyl ether for standards was prepared from Squoxin by reaction with dimethyl sulfate according to the method of Ogata et al. (1969). The melting point of the product obtained, 148.5–149.5°, did not agree well with that reported (Ogata et al., 1969), 144–145°, but we were able to confirm the structure by mass spectrometry. The preparation of Squoxin-*t* (1,1′-methylenedi-2-naphthol-*6,6′-t*) is described elsewhere (Terriere and Burnard, 1975). Diazomethane was prepared in ether solution from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co.) by the method of Fieser and Fieser (1968).

Reagent grade hexane was passed through a column of activated Florisil prior to use. Reagent grade benzene was refluxed over metallic sodium for a minimum of 12 hr and distilled. Reagent grade diethyl ether was refluxed over metallic sodium for 48 hr and distilled. Sodium sulfate was dried at 400–450° overnight and stored in a sealed bottle. Florisil was activated at 400–450° overnight and stored at 100°.

Diazo Blue B reagent was prepared by mixing 2 parts of 1% aqueous Diazo Blue B (*o*-dianisidine, terazotized, zinc chloride complex, Sigma Chemical Co., St. Louis, Mo.) with 5 parts of 5% aqueous sodium lauryl sulfate (Sigma Chemical Co.). This reagent must be prepared every 2 days and stored in a refrigerator. Phosphate buffer (pH 8) was prepared by mixing 5.3 ml of NaH_2PO_4 solution (27.8 g/l.) with 94.7 ml of $Na_2HPO_4 \cdot 7H_2O$ solution (53.65 g/l.) and diluted to 200 ml.

All other chemicals were reagent grade and were used as received.

Development of a GC Method. *Tissue Extraction and Cleanup.* Control fish were homogenized with Squoxin-*t* in a Waring Blendor at a tissue level of 5 ppm. The scintillation counter was used to measure the recovery of radioactivity on extraction of the homogenate with chloroform, methanol, diethyl ether, or benzene. The best recoveries

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