

AQT 00151

UPTAKE, BIOTRANSFORMATION, AND ELIMINATION OF ROTENONE BY BLUEGILLS (*LEPOMIS MACROCHIRUS*)

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(Received 8 November 1984; revised version received and accepted 27 December 1984)

Yearling bluegills (*Lepomis macrochirus*) were exposed to sublethal concentrations of [14 C]rotenone (5.2 μ g/l) for 30 days in a continuous flow exposure system and then transferred to clean, flowing water for an additional 21-day depuration period. Rates of uptake and elimination and profile of the rotenone metabolites in head, viscera, and carcass components were evaluated by 14 C counting and by high performance liquid chromatography. Total [14 C]rotenone derived activity was relatively uniform in all body components within 3 days after initial exposure and remained constant during the ensuing 27 days of exposure. Initial uptake rate coefficients were highest in viscera ($K_u = 80 \cdot h^{-1}$) and were nearly identical for head ($K_u = 14 \cdot h^{-1}$) and carcass ($K_u = 10 \cdot h^{-1}$). Analyses of tissue extracts by high performance liquid chromatography confirmed the presence of at least six biotransformation products of rotenone. More than 60% of the activity extracted from viscera was present as a single peak which represented a compound that was extremely soluble in water. Rotenone composed only 0.3% of the extractable activity in viscera taken from fish exposed to rotenone for 30 days; however, rotenone accounted for 15.4% of extractable activity in the head and 20.1% in the carcass components. Rotenolone and 6',7'-dihydro-6',7'-dihydroxyrotenolone were tentatively identified as oxidation products in all tissue extracts.

Elimination of 14 C activity from all body components was biphasic; both phases followed first-order kinetics. The rate of elimination was nearly equal for all body components during the initial phase but was most rapid from viscera during the second phase of elimination. Bioconcentration factors for the head, viscera, and carcass were 165, 3,550, and 125, respectively, when calculated on the basis of total 14 C activity but only 25.4, 11, and 26 when calculated as the concentration of parent material.

Key words: rotenone; bluegill; uptake; metabolism; elimination

INTRODUCTION

Rotenone has been used extensively in fishery management to eliminate unwanted species from both impounded and flowing bodies of water. Even though rotenone is used by fishery management agencies in virtually every state, its greatest use is in the management of warm-water fisheries in the southeast and cool-water fisheries of the midwest and mountain states. The popularity of rotenone for these purposes stems from its extreme toxicity to fish and its low toxicity to mammals (Lennon et al., 1970; Haley, 1978).

Despite the widespread use of rotenone in fisheries management, little information is currently available on the fate of rotenone in fish. Shimizu and Fukami (1978) reported that the gallbladder bile of common carp (*Cyprinus carpio*), exposed to sublethal concentrations of water-borne [^{14}C]rotenone, accumulated the greatest amount of ^{14}C activity of all body constituents after 1, 10, or 20 days of exposure. They concluded that bile was a major site of storage for rotenone residues in carp. In a related study, Schmidt and Weber (1975) attempted to determine the importance of biliary elimination to rotenone toxicity in rainbow trout (*Salmo gairdneri*). They found that the LD_{50} value of an i.p. dose of rotenone to fish, surgically prepared by ligations of the cystic and common bile ducts, was not significantly different from that estimated for sham operated control animals and concluded that biliary excretion of rotenone did not contribute substantially to its detoxification in the trout.

The present study was undertaken to determine the rates of uptake and elimination of [^{14}C]rotenone residues in yearling bluegills (*Lepomis macrochirus*) and to evaluate the profile of rotenone metabolites in exposed fish.

MATERIALS AND METHODS

Test animals and holding conditions

Month-old bluegill fry, obtained from the Lake Mills National Fish Hatchery, Lake Mills, WI, were reared for 1 yr at the La Crosse National Fishery Research Laboratory in 5,000-l rectangular fiberglass tanks supplied with flowing well water. Water used throughout the study had the following characteristics: pH 7.54; total hardness (as CaCO_3) 126 ± 6.68 ($\bar{x} \pm \text{SEM}$) mg/l; total alkalinity (as CaCO_3) 105 ± 6.19 mg/l; resistivity $3,600 \pm 164$ ohms; conductivity 279.00 ± 12.9 $\mu\text{mhos/cm}$; turbidity 0.38 ± 0.33 mg/l; dissolved oxygen 9.3 ± 0.9 mg/l; and temperature $12.9 \pm 0.3^\circ\text{C}$. Fish were fed pelleted Silver Cup dry fish food (Murray Elevators, Murray, Utah)* once daily. Bluegills used in the uptake and elimination study were transferred to a 400-l fiberglass tank in a walk-in environmental chamber 1 mth prior to the start of the test and acclimated to the test temperature (13°C) and photoperiod (12 h L:12 h D). Fade time for dimming devices in the chamber was set at 1 h. Fish used in the acute toxicity tests were transferred to a diluter in a separate laboratory and acclimated to the test environment for 2 days before tests were begun. Bluegills used in both studies were 4.5–5.5 cm (total length) and weighed 2.00 ± 0.34 g ($n=45$) at the start of the tests.

Chemicals

6 α -[^{14}C]Rotenone (sp. act. = 8.88 mCi/mmol) was purchased from Pathfinder

*References to trade names or manufacturers does not imply Government endorsement of commercial products.

Laboratories, St. Louis, MO. Radiochemical purity exceeded 98% as determined by the thin layer chromatography–autoradiography technique of Fukami et al. (1969). Non-labeled rotenone, provided by the S.B. Penick Corporation (Lyndhurst, NJ), also exceeded 98% purity as determined by high pressure liquid chromatography (HPLC) analysis (Dawson et al., 1983). Three oxidation products of rotenone, rotenolone, 6',7'-dihydro-6',7'-dihydroxyrotenone and 6',7'-dihydro-6',7'-dihydroxyrotenolone, were synthesized by Dr. S.L. Abidi, National Fishery Research Laboratory, La Crosse, WI, for use as analytical standards.

Exposure systems and test procedures

The acute toxicity of rotenone to the stock of bluegills used in this study was determined in a preliminary test with a continuous flow proportional diluter (Mount and Brungs, 1967). Groups of 20 fish were placed in separate 45-l glass test aquaria and exposed in duplicate to six logarithmically spaced concentrations of non-labeled rotenone ranging from 3.0 to 30.0 $\mu\text{g/l}$. A seventh pair of control aquaria received well water. Stock rotenone solution in acetone was delivered to the mixing cell of the diluter by a Micromedic automatic pipette pump (Micromedic, Inc., Horsham, PA). Concentrations of rotenone in each tank were determined by HPLC analysis (Dawson et al., 1983). Mortalities were recorded after 0.25, 0.5, 0.75, 1, 3, 6, 9, 12, 24, 48, 72, and 96 h. The LC_{50} values and 95% confidence interval estimates (CIE) were determined by the method of Litchfield and Wilcoxon (1949).

To assess rates of accumulation and elimination, yearling bluegills (80/tank) were exposed to nominal concentrations of 5.0 $\mu\text{g/l}$ of [^{14}C]rotenone for periods of up to 30 days in duplicate 45-l glass aquaria that received a continuous flow of water (0.5 l/min). The toxicant and water delivery systems were similar to those described by Call et al. (1980). Results of preliminary stability studies indicated that rotenone was relatively stable in acetone solutions for as long as 7 days, but that some degradation began after this time. Thus, new stock solutions were prepared every other day throughout the exposure period. Mean cycle time for the water delivery system was 85.3 ± 1.7 s during the study and resulted in a continuous flow of water through each tank equivalent to more than 11 aquaria vols over a 24-h period. The control aquarium received a continuous flow of well water at the rate of 0.5 l/min.

Samples of five fish were taken from all test aquaria after 6, 12, and 18 h and on days 1, 3, 7, 10, 14, 22, and 30 after the start of exposure. A separate group of 10 animals was sampled from both exposure tanks on day 30 for analysis of rotenone metabolites. Bluegills remaining in each exposure aquarium at the end of the accumulation phase were transferred to duplicate 45-l aquaria, and held for a 21-day depuration period. Five fish were sampled from each aquarium on days 1, 3, 7, 10, 14, and 21 of the depuration period.

Sample analysis

Triplicate water samples were collected daily from the center of each exposure aquarium near mid-depth, using 5 ml glass volumetric pipettes. Each water sample

was added to a glass scintillation vial containing 15 ml of high aqueous capacity scintillation cocktail (Beckman Ready Solve MP) and the vials were stored overnight in the dark to reduce chemiluminescence. All ^{14}C activity in water was assumed to be [^{14}C]rotenone since the results of previous hydrolysis studies revealed that the half life of rotenone in water was 29.1 days (Borrison Laboratories, Temple Hills, MD).

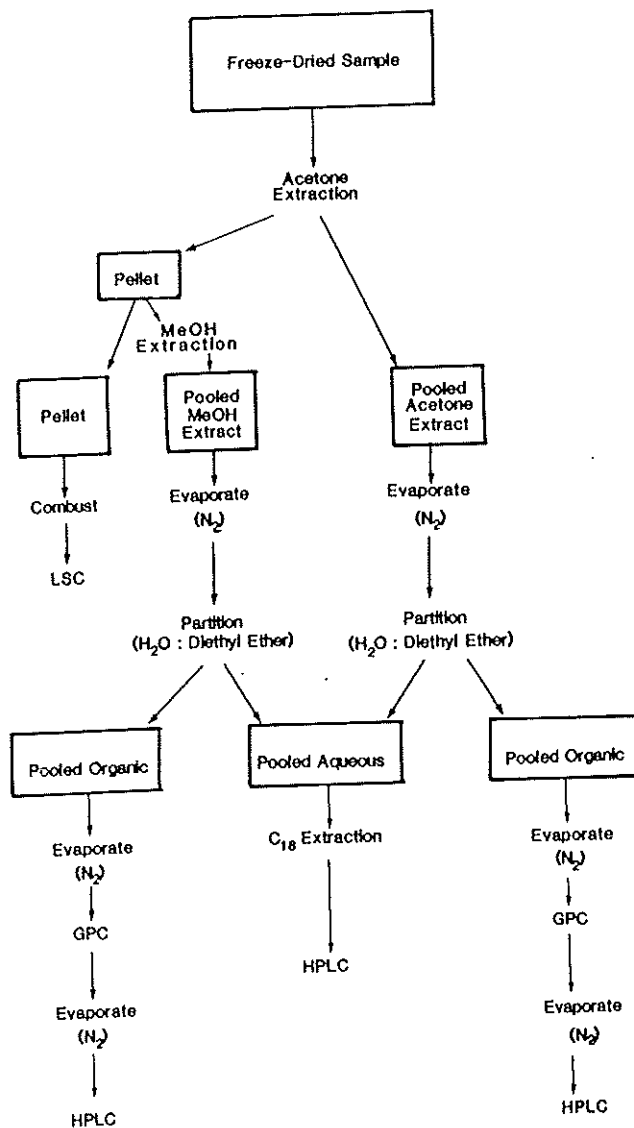


Fig. 1. A general scheme for the extraction of rotenone residues from bluegills.

Fish were randomly sampled from each tank, placed in a separate plastic aquarium, and killed by electrocution. They were blotted dry, weighed to the nearest 0.1 mg, and dissected into head, viscera, and carcass samples; each sample was reweighed after being placed into a tared paper combustion cone. Samples from the head consisted of branchial basket, eyes, brain, skull, and surrounding skin. Those from the viscera contained alimentary tract, liver, gall bladder, and spleen; and those from the carcass were comprised of skin, scales, fins, flesh, and backbone. After the samples were air dried at 25°C for 72 h, they were oxidized to $^{14}\text{CO}_2$ in a Packard Model B306 sample oxidizer. Preparation in this manner did not result in any loss of ^{14}C activity from spiked samples by volatilization. The combustion efficiency of the oxidizer was checked after every 20 samples by combusting [^{14}C]methacrylate chips (Amersham Radiochemicals, Arlington Heights, IL). Mean combustion efficiency was calculated to be $97.2 \pm 0.9\%$ ($\bar{x} \pm \text{SD}$, $n=42$) and resulting memory was $0.81 \pm 0.03\%$.

Metabolite characterization

Head, viscera, and carcass samples used to characterize rotenone metabolites were taken from fish in both exposure tanks on day 30 of exposure. Pooled samples were weighed, freeze dried, and stored in sealed amber glass vials at -10°C until further processing. Recoveries of ^{14}C activity from spiked samples of bluegill tissue that were freeze dried and combusted were essentially complete, indicating that little ^{14}C was lost through volatilization during the freeze-drying process.

Residual ^{14}C activity was extracted from tissues with both acetone and methanol by the procedure outlined in Fig. 1. Freeze dried tissue samples were homogenized in acetone (1:10; wt:vol) and the tissue pelleted by centrifugation ($1,500 \times g$ for 5 min). The tissue was re-extracted with acetone four additional times and the acetone extracts pooled. The pellet was extracted with methanol 5 times in a similar manner and the methanol extract pooled. The tissue pellet was air dried overnight and then oxidized and assayed radiometrically to determine unextractable ^{14}C activity.

Acetone and methanol extracts were evaporated to dryness under N_2 gas after five $20 \mu\text{l}$ aliquots of each extract were taken for radioassay of extractable ^{14}C activity. The resulting residues were taken up in 5 ml of glass distilled water and the aqueous fraction was extracted 3 times with 5-ml vols. of diethyl ether. The pooled organic extracts were evaporated to dryness and the residues redissolved in 10 ml of cyclohexane:methylene chloride (1:1, vol:vol) and separated from contaminating solids by gel permeation chromatography (GPC). Gel permeation chromatography was accomplished using a stationary phase of SX-3 biobeads (ABC Labs, Columbia, MO) and a mobile phase of cyclohexane-methylene chloride (1:1; vol:vol) at a flow rate of 4.0 ml/min. Sequential 10-ml fractions of the eluate were collected and radioassayed. Adjacent fractions found to contain radioactivity were pooled and evaporated to dryness on a rotary evaporator. The resulting residues were taken up in methanol, filtered through $0.2 \mu\text{m}$ filters, dried under a stream of N_2 gas, and

stored until HPLC analysis. Residues in the aqueous phase were enriched by extraction on a C₁₈ octadecyl column (J.T. Baker Company, Orangeburg, NJ) and the ¹⁴C activity was eluted from the column in 1-ml methanol. The methanol eluate was filtered, dried under a stream of N₂ gas and stored until HPLC analysis. Recoveries of ¹⁴C activity by this method from tissues spiked with [¹⁴C]rotenone were essentially complete. Mean recovery of ¹⁴C activity was 102.4 ± 2.06% ($\bar{x} \pm SD$; n=3). Rotenone constituted more than 95% of this activity as judged by HPLC analyses indicating that the methods had little effect on the stability of rotenone in the sample.

Tissue extracts were applied to a 30 cm × 4 mm (i.d.) MicroPak MCH-10 reverse phase C-18 column (Varian Instrument Company, Sunnyvale, CA) and separated by HPLC. The column was eluted with a linear 50-min gradient of 30-100% methanol at a flow rate of 1.3 ml/min and the UV absorbance recorded at 295 nm. One-minute fractions were collected for up to 60 min and the amount of radioactivity in these fractions was determined by liquid scintillation counting.

Enzymatic hydrolysis studies were undertaken on ¹⁴C residues that partitioned to the aqueous fractions during liquid-liquid extractions. For β-glucuronidase incubations, ¹⁴C residues were dissolved in 500 μl of 0.1 M phosphate buffer (pH 6.8) and 50 Sigma units of β-glucuronidase (*E. coli* Type VII, Sigma Chemical Company, St. Louis, MO), dissolved in 50 μl of 0.1 M phosphate buffer, were added to the tube. For sulfatase incubation, 500 μl of 0.01 M acetate buffer (pH 5.0) containing ¹⁴C residues was pipetted into a test tube and 50 μl of 0.01 M acetate buffer containing 10 Sigma units of *Helix pomatia* sulfatase (Type H-1, Sigma Chemical Company, St. Louis, MO) was added. These mixtures were allowed to incubate in a 37°C water bath for 24 h with agitation. Negative controls containing ¹⁴C residues in buffer but no enzyme were also run. Enzyme activity was checked according to the suppliers' suggested method using phenolphthalein-β-glucuronic acid and *p*-nitrophenol-sulfate as substrates. After 24 h, incubations were terminated by the addition of 100 μl of 10% trichloroacetic acid and the aqueous phase was extracted 3 times with 1-ml vols. of 30% diethylether in hexane. The activity in each phase was determined by liquid scintillation counting.

Liquid scintillation counting

All water and tissue samples were counted to a 2% two sigma counting error (10,000 accumulated counts) or for 20 min using a Beckman 7500 liquid scintillation counter. Radiochromatogram samples from the HPLC were counted for 5 min. Activity in water and oxidized tissue samples was calculated as DPM using a stored internal program in the counter that automatically corrected for quench and background counts. Activity for peak identification in the radiochromatograms of tissue extracts was summed after counts equivalent to mean background + 3 SD above the background of the HPLC had been subtracted from each vial.

Calculations of uptake and depuration rate constants and bioconcentration

Estimates of uptake, bioconcentration, and elimination were based on the assumptions of Branson et al. (1975) and Krzeminski et al. (1975) that these processes can be modeled in fish by simple reversible exchange processes. Initial rates of uptake were assumed to be constant linear functions and were calculated by the equation:

$$C_a = C_o + K_u \cdot C_w \cdot t$$

where C_a = concentration in the animal (ng/g^{-1}), C_o = initial concentration of material in the fish, K_u = initial uptake rate constant (h^{-1}), C_w = concentration in water ($\text{ng} \cdot \text{g}^{-1}$), and t = time of exposure (h) (Spacie et al., 1983).

Estimates of the biphasic depuration rate constants were made by assuming that each phase of depuration conformed to simple first-order elimination kinetics and that the biphasic elimination curve could be described by the biexponential equation:

$$C_{at} = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_{at} = concentration of rotenone equivalents in the tissue at time t , A and B are zero time ordinate intercept values for the initial and terminal curves, and α and β are the initial and final first-order elimination rate constants (K_{elim}), respectively. The terminal elimination rate constant was derived by least squares regression analysis of \ln transformed tissue concentration values and time for the last 3 sampling days of the depuration period. The initial phase of the depuration curve (α) was resolved by curve stripping (Gibaldi and Perrier, 1982). Using this technique, values for the initial curve were obtained by subtracting the composite curve ($\alpha + \beta$) from the terminal curve (β). The curve was resolved by linear regression analysis of \ln transformed values of tissue concentration and time. Depuration half-lives for the initial and final phases of depuration were computed as:

$$T_{1/2} = 0.693/K_{elim}$$

where $T_{1/2}$ = half-life of elimination and K_{elim} = respective initial or final first-order elimination rate constant.

The bioconcentration factor (BCF) in each tissue was calculated from samples of fish taken during the equilibrium phase of the study (exposure days 7-30) as the ratio of the concentration of rotenone equivalent activity in the individual body components to that in water (Branson et al., 1975).

RESULTS

Acute toxicity

The calculated 24- and 96-h LC_{50} values (95% CIE) for rotenone to the stock of bluegills used in this study were $14.0 \mu\text{g/l}$ (10.5-18.6) and $10.9 \mu\text{g/l}$ (8.6-13.8),

respectively. On the basis of these estimates, we chose a nominal concentration of 5.0 $\mu\text{g}/\text{l}$ of rotenone for the accumulation phase of the study.

Uptake, distribution and elimination

The concentration of rotenone used in the sublethal tests in this study did not produce mortality or observable acute toxic effects, even though fish were exposed to concentrations of rotenone slightly less than 50% of the estimated 96-h LC_{50} value. Additionally, there were no indications of reduced food consumption, reduced schooling, or other overt signs of sublethal toxicity among fish exposed to rotenone.

Water concentrations of [^{14}C]rotenone were essentially identical in both exposure tanks throughout the study. The mean water concentration in both tanks, based on radiometric counts, was estimated to be $5.21 \pm 0.16 \mu\text{g}/\text{l}$ ($\bar{x} \pm \text{SD}$; $n = 62$) during the 30-day exposure. Rotenone concentrations over this period ranged from 4.8 to 5.5 $\mu\text{g}/\text{l}$. Between-tank comparisons of ^{14}C tissue activity by one-way analysis of variance confirmed that the total ^{14}C -residue concentration in tissues from fish sampled at the same time from different exposure tanks did not differ significantly. On this basis, the estimates of ^{14}C activity from tissue samples taken from different exposure tanks were pooled by sampling time for data analysis and graphical presentation.

Radioactivity accumulated rapidly in bluegills during the first 3 days of exposure and then remained nearly constant during the ensuing 27-day exposure period (Fig. 2). The estimated initial uptake rate constant for rotenone into whole body was $12 \cdot \text{h}^{-1}$. Wet weight whole body concentrations of total ^{14}C activity during the equilibrium period (exposure days 4–30) averaged $1.64 \pm 0.39 \mu\text{g}/\text{g}$ of rotenone

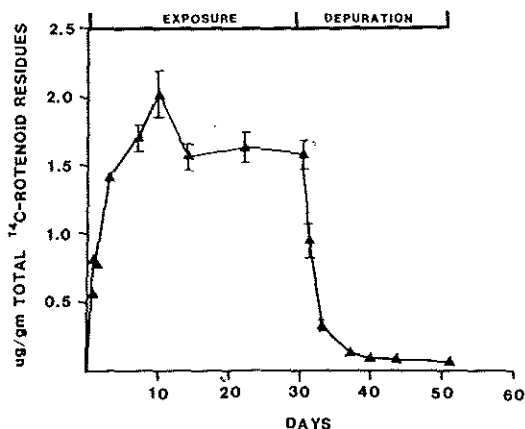


Fig. 2. Residues of total [^{14}C]rotenone equivalents in whole bodies of juvenile bluegills during and after exposure to 5.2 $\mu\text{g}/\text{l}$ of [^{14}C]rotenone. Each value represents the mean \pm SE of 10–13 individual determinations per sample period.

TABLE I

Rotenone equivalent concentration ($\mu\text{g/g}$) and percent of [^{14}C]rotenone residues in head, viscera and carcass samples of bluegills exposed to a mean water concentration of $5.2 \mu\text{g/l}$ of [^{14}C]rotenone. Each value represents the mean \pm SEM of 10 determinations.

Exposure time (h)	Rotenone concentration in exposure water ($\mu\text{g/l}$)	Body component					
		Head (25.9 ± 2.4) ^a		Viscera (9.5 ± 1.4)		Carcass (64.6 ± 4.5)	
		Concentration	Percent	Concentration	Percent	Concentration	Percent
6	5.05	0.434 ± 0.028	20.4 ± 0.4	2.47 ± 0.088	41.9 ± 1.1	0.309 ± 0.012	37.8 ± 0.8
12	5.10	0.524 ± 0.022	16.3 ± 0.5	5.99 ± 0.221	52.3 ± 1.3	0.389 ± 0.011	31.4 ± 0.8
18	5.10	0.602 ± 0.055	13.3 ± 0.8	9.76 ± 0.599	61.9 ± 1.5	0.446 ± 0.022	24.8 ± 0.8
24	5.05	0.593 ± 0.300	13.0 ± 0.6	8.78 ± 0.453	61.2 ± 1.5	0.454 ± 0.014	25.8 ± 0.9
72	5.05	0.792 ± 0.029	9.7 ± 1.7	16.82 ± 0.965	70.6 ± 4.5	0.622 ± 0.018	19.7 ± 3.0
168	5.40	0.878 ± 0.027	8.2 ± 2.2	16.92 ± 1.237	73.3 ± 6.0	0.687 ± 0.021	18.0 ± 4.1
240	5.30	0.913 ± 0.024	8.3 ± 2.8	23.47 ± 2.060	75.2 ± 8.2	0.693 ± 0.027	16.2 ± 5.5
336	5.45	0.807 ± 0.032	9.7 ± 2.3	17.48 ± 1.246	71.3 ± 6.5	0.624 ± 0.017	19.9 ± 4.4
528	5.20	0.844 ± 0.045	9.9 ± 2.7	19.06 ± 1.397	71.2 ± 7.9	0.658 ± 0.026	18.9 ± 5.1
720	5.00	0.906 ± 0.050	12.2 ± 3.5	18.02 ± 1.603	68.6 ± 8.3	0.686 ± 0.024	19.2 ± 5.5

^aValues in parentheses represent the mean percent of body weight (\pm SEM) for that component.

equivalents; tissue concentrations ranged from 1.39 to 2.01 $\mu\text{g/g}$. The mean apparent equilibrium BCF in whole body was 315, based on total ^{14}C residues in tissue and water sampled between hours 72 and 720 of exposure. Elimination of ^{14}C activity from the whole body of bluegills occurred in two distinct phases - a rapid initial phase and a slower secondary phase. First-order elimination rate constants were $-0.0268 \cdot \text{h}^{-1}$ for the initial phase and $-0.0012 \cdot \text{h}^{-1}$ for the final phase of depuration. Half-lives for these phases were 25.8 and 578 h, respectively. Correlation coefficients (r) for the estimated regression lines were -0.992 for the rapid depuration phase and -0.973 for the slow phase. Only 18.7% of the initial body burden of ^{14}C activity found on day 30 of exposure remained in whole bodies of exposed bluegills after 3 days depuration and after 21 days depuration less than 5% remained.

The pattern of uptake of rotenone-derived ^{14}C activity into head, viscera, and carcass was similar to that observed for the whole-body (Table I). Total rotenoid residues accumulated rapidly in all tissues during the first 3 days of exposure and then remained nearly constant during the following 27-day exposure period. For head, viscera, and carcass, respectively, uptake rate constants were $14 \cdot \text{h}^{-1}$, $80 \cdot \text{h}^{-1}$, and $10 \cdot \text{h}^{-1}$; and in samples taken during the equilibrium phase of the accumulation period, mean concentrations of rotenone equivalent ^{14}C residues (\pm SEM; $n=60$) were $0.86 \pm 0.13 \mu\text{g/g}$, $18.92 \pm 1.17 \mu\text{g/g}$, and $0.66 \pm 0.09 \mu\text{g/g}$. The mean apparent equilibrium BCF during this time were 165, 3,550, and 128 for head, viscera, and carcass samples, respectively.

Under equilibrium conditions, the distribution of ^{14}C activity was nearly constant among the three body components and always in the order viscera $>$ head $>$ carcass (Table I). Rotenone derived ^{14}C residues in the viscera accounted for approximately 70% of the total body burden of ^{14}C activity, even though this tissue mass accounted for slightly less than 10% of the wet tissue weight. Conversely, the carcass contained less than 20% of the total activity, but made up approximately 65% of the mean body weight.

Depuration of ^{14}C residues appeared to be greatest from the viscera and similar for head and carcass (Table II). Depuration from all body components was best described as a biphasic first order elimination pattern. Elimination characteristics for all body components were similar for the rapid phase of elimination but depuration half-lives were in the order carcass $>$ head $>$ viscera during the slower secondary phase of elimination (Table II).

Biotransformation

Rotenone was readily biotransformed by bluegills. Good recoveries of ^{14}C residues in exposed bluegill tissues were achieved only after extracting tissues with both acetone and methanol. In these fish, less than 50% of the ^{14}C residues were extracted with acetone. Conversely, complete recovery was achieved when a control carcass, spiked with [^{14}C]rotenone, was extracted with acetone. The results suggested that the rotenone residues of exposed fish were considerably more polar than

TABLE II

Elimination and biexponential elimination characteristics of rotenone equivalent activity ($\mu\text{g/g}$) from head, viscera, and carcass of juvenile bluegills. Values represent the mean \pm SEM of 10 samples.

Elimination	Body component		
	Head	Viscera	Carcass
Time (h)			
0	0.906 \pm 0.050	18.025 \pm 1.603	0.686 \pm 0.024
24	0.467 \pm 0.027	10.862 \pm 2.508	0.373 \pm 0.017
72	0.239 \pm 0.032	3.205 \pm 0.394	0.176 \pm 0.008
168	0.138 \pm 0.009	0.713 \pm 0.154	0.124 \pm 0.006
240	0.111 \pm 0.010	0.423 \pm 0.035	0.107 \pm 0.006
336	0.112 \pm 0.004	0.387 \pm 0.015	0.108 \pm 0.003
504	0.081 \pm 0.002	0.221 \pm 0.018	0.083 \pm 0.003
Phase			
Initial ^a			
K_{elim}^b	-0.0284	-0.0270	-0.0355
Half-life (h)	24	26	20
r^c	-0.995	-0.998	-0.999
Final ^d			
K_{elim}^b	-0.0013	-0.0036	-0.0010
Half-life (h)	533	271	666
r^c	-0.924	-0.971	-0.917

^aInitial phase calculated for depuration hours 0-72.

^bFirst order elimination rate constant.

^cLinear correlation coefficient.

^dFinal phase calculated for depuration hours 224-504.

TABLE III

Percent of unextractable ^{14}C activity or percent of ^{14}C activity in peaks separated by gradient elution HPLC from combined acetone and methanol extracts of selected body components of bluegills exposed for 30 days to 5.2 $\mu\text{g/l}$ of [^{14}C]rotenone. Numbers in parentheses indicate 1-min fractions from HPLC separation that constitute the peak.

Tissue type	Unextractable residue	^{14}C Peaks							
		1 (4-7)	2 (11-13)	3 (17-20)	4 ^a (22-24)	5 (26-28)	6 ^b (29-31)	7 (38-39)	8 ^c (40-42)
Head	8.6	7.6	1.1	9.7	22.6	9.4	0.4	25.4	15.4
Viscera	7.0	71.3	1.8	1.9	11.3	2.0	0.9	3.6	0.3
Carcass	14.5	10.8	ND ^d	0.6	24.6	9.5	ND ^d	20.2	20.1

^aPeak coelutes with 6',7'-dihydro-6',7'-dihydroxyrotenolone.

^bPeak coelutes with rotenolone.

^cPeak coelutes with rotenone.

^dND = Not detectable.

parent rotenone. These observations were confirmed by HPLC separation of the rotenone residues in the tissue extracts. Viscera contained the greatest amount of ^{14}C activity of the three body components and virtually all of this activity was present as several highly polar residues (Table III). Of the total activity in viscera, 70% was eluted as a single peak at the solvent front (fractions 4 to 7). Lesser amounts of this peak were found in the head and carcass extracts.

Preliminary attempts were made to confirm the chemical identity of the eluted peaks by comparing the activity profile of the radiochromatogram of the tissue extracts with the elution profile of a mixture of several standard rotenone oxidation products. Two peaks with retention times coinciding with analytical standards were

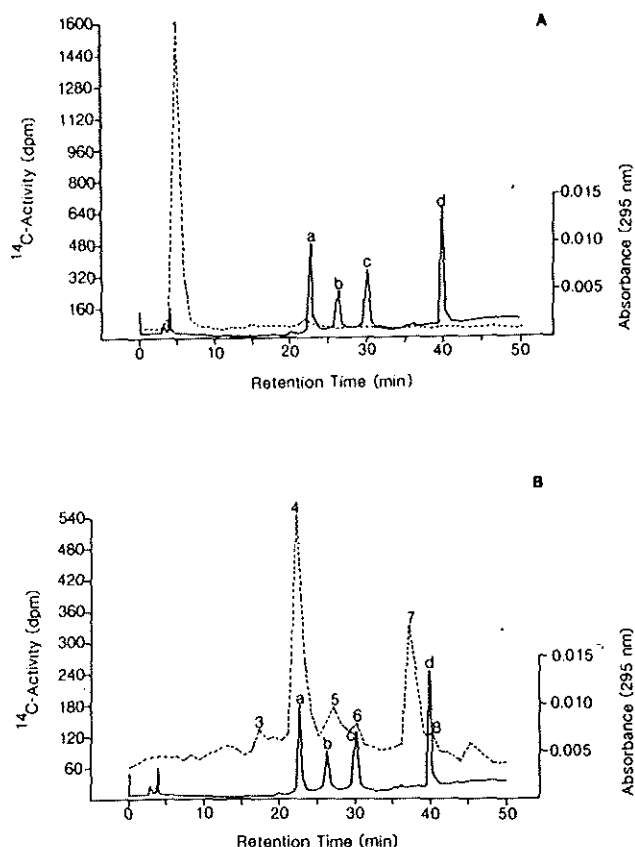


Fig. 3. A representative radiochromatogram of water soluble (A) and diethyl ether soluble (B) ^{14}C -residues of pooled acetone and methanol extracts from bluegill tissue compared with reference standards. Solid line represents UV absorbance of standards at 295 nm and dashed line represents ^{14}C radioactivity. Reference standards are: (a) 6',7'-dihydro-6',7'-dihydroxyrotenolone; (b) 6',7'-dihydro-6',7'-dihydroxyrotenone; (c) rotenolone; (d) rotenone. Numbers correspond to radioactive peaks extracted from tissues. Separations by HPLC were as described in Materials and Methods.

separated from all tissue extracts in addition to the parent compound (Fig. 3). The residue in peak 4 coeluted with 6',7'-dihydro-6',7'-dihydroxyrotenolone, that in peak 6 with rotenolone, and that in peak 8 with parent rotenone. The residue eluting in peak 5 may be 6',7'-dihydro-6',7'-dihydroxyrotenone based on its proximity to that standard and the pathway of oxidative biotransformation of rotenone defined by Fukami et al. (1967) (Fig. 4). Activity in peak 7 is probably associated with an epimeric form of rotenone (6 α , 12 α -rotenone) that was resolvable by the gradient elution HPLC method used in this study (Abidi and Abidi, 1983). On the basis of the percentage of activity eluting in peak 8, estimated rotenone concentrations were 0.05 $\mu\text{g/g}$ in viscera and 0.125 $\mu\text{g/g}$ in both the carcass and head. The resulting BCF, based on actual rotenone concentrations were 11.0 for viscera, 25.4 for head, and 26.0 for carcass.

Results of enzymatic hydrolysis studies of highly polar ^{14}C residues revealed that β -glucuronidase and sulfatase were without effect in altering the distribution of radioactivity between the aqueous and organic phases following liquid-liquid extraction. Radioactivity in the aqueous phase incubated with β -glucuronidase was 89.4% of total sample activity while activity in the corresponding control was 97.5% of total sample activity. Radioactivity in the aqueous phase incubated with sulfatase was 93.3% while 98.7% of the activity was found in the aqueous control.

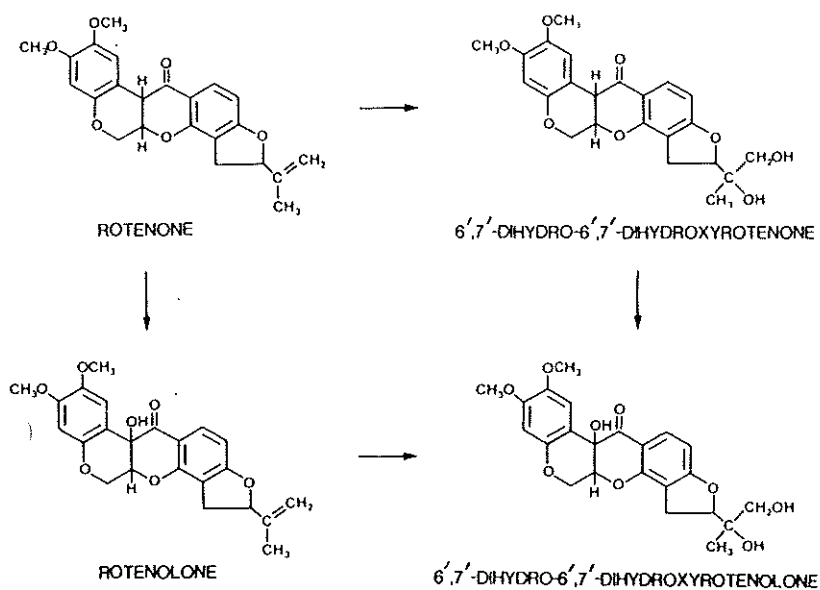


Fig. 4. General scheme of primary oxidations of rotenone (after Fukami et al., 1967).

DISCUSSION

Results of preliminary acute toxicity tests, conducted with the stock of yearling bluegills used in this study, indicated that the concentration-response curve for rotenone was relatively steep. No mortalities were observed at rotenone concentrations of 7.0 $\mu\text{g}/\text{l}$ or less, whereas complete mortality was effected by exposure to concentrations of rotenone greater than 30.0 $\mu\text{g}/\text{l}$. The sharp demarcation between concentrations of rotenone that produced toxicity and concentrations that had no apparent effects may be explained by postulating that one or more of the following three processes occur in rotenone toxicity: (1) rotenone uptake is faster at higher than at the lower concentrations; (2) rotenone uptake rate at higher concentrations was greater than the rate of detoxification; and (3) the elimination rate of rotenone at low concentrations exceeds the rate of uptake and the residues do not accumulate in the body.

The rate of rotenone uptake by bluegills was determined largely by the physicochemical characteristics of the compound. The uptake rate constant for rotenone, calculated from data developed during the initial 6 h of the accumulation study, was in good agreement with that predicted by the general relationship between the uptake rate constant (K_u) and the *n*-octanol/water partition coefficients (P_{oct}) established for a variety of organic chemicals by Spacie et al. (1983) as:

$$\log K_u = 1.98 + 0.147 \log P_{\text{oct}}$$

On the basis of a $\log P_{\text{oct}}$ value of 4.26 (R. Puchalski, pers. comm., Penick Corp., Lyndhurst, NJ), the calculated K_u value for rotenone into whole body of bluegill was $8.9 \cdot \text{h}^{-1}$, whereas the empirically derived value was $12 \cdot \text{h}^{-1}$. However, as Neely (1979) pointed out, the uptake rate constant for a particular chemical is a function of its physicochemical properties, the concentration of chemical in the water, and of the volume of water passing over the gills. The uptake rate constant of a chemical may be greater than that predicted on the basis of its physicochemical properties if its physiological effects are to alter respiratory patterns. Perry and Conway (1977) observed that respiration rates of green sunfish (*Lepomis cyanellus*) exposed to acutely toxic concentrations of rotenone, were 2 and 3 times greater than those of the controls. They speculated that the increased respiratory rates reflected a compensatory adjustment to the attendant biochemical hypoxia that develops in treated animals following inhibition of the mitochondrial electron transport system (Lindahl and Oberg, 1961; Horgan et al., 1968). Similar effects on fish exposed to higher concentrations of rotenone in this study could have contributed to greatly enhanced rotenone uptake thereby accounting, in part, for the steep slope of the dose-response curve.

Fukami et al. (1967) established that the general scheme for oxidative metabolism of rotenone was similar among diverse classes of animals including mammals, insects, and fish. Their studies revealed that the sequence of toxicity of a series of

rotenoid compounds was in the order rotenone > rotenolone > 6',7'-dihydro-6',7'-dihydroxyrotenone. These studies are supported by laboratory studies which demonstrate that inhibition of oxidative metabolism by pretreatment or co-treatment with piperonyl butoxide, sulfoxide, or sesamex markedly increased the toxicity of rotenone to a number of fish species (Fabacher and Chambers, 1972; Marking and Bills, 1976). Our studies confirm that oxidative metabolism is an important prerequisite to the detoxification of rotenone. Body components of fish sampled after exposure for 30 days to concentrations of rotenone that were nearly half of the estimated 96 h LC₅₀ value contained dihydro-dihydroxy derivatives of both rotenone and rotenolone in quantities greater than parent rotenone. Moreover, our results suggest that metabolism of rotenone was relatively rapid since parent rotenone represented less than 20% of the activity extractable from head and carcass samples, even though the fish had just been removed from exposure tanks containing rotenone. Thus, it seems likely that rotenone toxicity occurs when the rate of uptake of rotenone exceeds the capacity of the animal to detoxify the chemical.

The metabolic fate of rotenone following primary oxidations is not clear; however, oxidative metabolism may also be an important step in the formation of the large pool of water soluble products that accumulated in the visceral tissue. Rotenone does not possess favorable sites for secondary conjugation reactions. In contrast, the added hydroxy groups of the dihydro-dihydroxy derivatives offer several sites for secondary conjugation reactions. *In vitro* studies by Fukami et al. (1969) demonstrated that the presence of inhibitors of primary oxidation reactions, such as the dimethoxyphenyl compounds, in incubates of carp liver homogenates containing [¹⁴C]rotenone greatly reduced the amount of ¹⁴C water-soluble products formed. They assumed that the water soluble ¹⁴C residues formed were conjugates of rotenone oxidation products and interpreted their results to mean that the oxidized derivatives of rotenone were better substrates for secondary conjugation reactions than parent rotenone. Our failure to demonstrate either glucuronide or sulfate conjugates by enzymatic hydrolysis suggests that the water soluble residues are not classical conjugates. These residues may represent other conjugates such as glutathione or they simply may be ring fragments of the rotenone carbon skeleton.

Finally, it is apparent that rotenone residues in the bluegills moved between two gross body compartments - a rapid exchange compartment represented by the visceral mass and a slow exchange compartment represented by the head and carcass. A similar two-compartment system has been proposed to model several organic chemicals in fish by Krzeminski et al. (1977) and Karara and Hayton (1984). In bluegills, the visceral component was differentiated from the head and carcass compartment by the relatively greater ¹⁴C activity at equilibrium, faster rates of ¹⁴C uptake and depuration, and the greater proportion of polar ¹⁴C residues. The rapid elimination of residues from the visceral compartment implies that biotransformation to more polar compounds was a major prerequisite for the elimination of rotenone. The results also tend to discount the possibility that parent rotenone was

efficiently eliminated, thereby reducing the accumulation of toxic residues. Body burdens of rotenone equivalent ^{14}C activity in viscera were more than 3,500 times greater than that of the surrounding water. However, when calculations of the BCF were based on the amount of parent rotenone, this value was less than 30 in all body components. These values are less than that of 830 predicted for rotenone on the basis of the correlation between BCF and P_{oct} derived by Veith et al. (1979):

$$\log \text{BCF} = 0.85 \log P_{\text{oct}} - 0.70$$

The results serve to further support the importance of biotransformation in modulating the toxicity of rotenone in the bluegills.

In contrast to the viscera, the head and carcass components appeared to behave as a separate secondary compartment for rotenone residues based on the similarities in residue composition and concentration and on the close agreement between uptake and depuration rates. Distribution to and clearance from secondary compartments are generally assumed to be limited by perfusion rates (Reigelman, 1968). Perfusion limited flow was assumed to have contributed to the slower secondary phase of depuration in this study but the elimination rates cannot be totally explained by this assumption. Relative blood perfusion rates are quite different between head and carcass tissues in fish. Tissues in the head are generally believed to be well perfused with gills receiving all of cardiac output, a value equivalent to between 9.0 and 100.0 ml per min per kg (Satchell, 1971). In contrast, the white muscle mass, which constitutes more than 50% of the body weight, is poorly vascularized and poorly perfused (Cameron and Cech, 1970; Cameron, 1975). Blood flow to the white muscle has been estimated to be less than 50% of cardiac output in resting arctic grayling (*Thymallus arcticus*). Greater differences in clearance between these two body components should be evident if clearance were totally perfusion limited.

Binding to subcellular structures may have contributed to the slower clearance rates in these body components. Rotenone inhibits the electron transport system by interfering with the mitochondrial bound enzyme NADH dehydrogenase, thereby preventing the reoxidation of NADH (Horgan et al., 1968). Some of the unextractable residues in the head and carcass tissues may have been bound to subcellular sites such as mitochondria and were not easily extracted. Another possibility is that some activity may have been associated with covalently bound residues and thereby was unavailable for extraction. While this latter situation is possible, covalent binding of rotenone residues has yet to be documented in any of the existing literature on rotenone.

ACKNOWLEDGEMENTS

We thank Cheryl Ehaney, David Erickson, and Mark Steingraeber for their technical assistance; Verdel Dawson for assisting with the gel permeation chromatography and Thomas Weeks (University of Wisconsin, La Crosse) for

assisting in freeze drying tissues. The assistance of J.B. Hunn, R.D. Meyerhoff, and K.F. Pfeifer in reviewing the manuscript and Georgia Ardinger for typing the manuscript is gratefully acknowledged.

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