Bull. Environm. Contam. Toxicol. 22,265-270 (1979)

Uptake, Tissue Distribution, Metabolism and Elimination *n*-Octano-1-^{14}C-hydroxamic acid by Brook Trout

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We have shown previously that an experimental iron ore flotation agent based on derivatives of alkylhydroxamic acids (R.CO.NHOH, where R is an *n*-alkyl group) was acutely toxic to salmonids, and that its toxicity was due to the alkylhydroxamic moiety (FLETCHER and ADDISON 1972). The toxicity of the alkylhydroxamic acids to salmon *(Salmo salar)* fry increased with the length of R within the range R=*n*-C_6H_{13} to *n*-$C_{10}H_{21}$ (ADDISON and CÔTÉ 1973). Trout poisoned with alkylhydroxamic acids recovered rapidly on transfer to clean water (FLETCHER and ADDISON 1972), which implies that at least some of the material absorbed could be readily excreted or detoxified. This paper describes the uptake, tissue distribution, metabolism and elimination of a medium chain length alkylhydroxamic acid (*n*-octano-1-^{14}C-hydroxamic acid) by brook trout *(Salvelinus fontinalis)*.

MATERIALS AND METHODS

Sodium octanoate-1-^{14}C (New England Nuclear, Inc.) was acidified and esterified with methanol/BF_3 and the resulting methyl ester diluted with unlabelled material (Matheson, Coleman and Bell). *n*-Octano-1-^{14}C-hydroxamic acid (OHA) was prepared from this, essentially as described by FISHBEIN *et al.* (1969). The product had a specific activity of 6.5 µCi/mM; it melted at 79-80°C, and chromatographed as a single spot in (a) toluene/methanol 80:20 v/v and (b) *n*-butanol saturated with NH_4OH.

Nonanohydroxamic acid-4,5-^{3}H (NHA) was prepared by treating methyl 5-nonenoate (Pfaltz and Bauer Inc.) with hydroxylamine as described above. 5-Nonenohydroxamic acid, m.p. 97°C was isolated; microanalysis yielded C, 63.5%; H, 9.55%; O, 18.77%; N, 8.05% (theoretical, C, 63.2%; H, 9.94%; O, 18.71%; N, 8.19%). This was subjected to catalytic tritiation (New England Nuclear, Inc.) to yield a product which, after repeated TLC in the systems described above and hexane:ether:acetic acid 60:40:5 (v/v/v), was chromatographically homogeneous with and identical to *n*-nonanohydroxamic acid (ADDISON and CÔTÉ 1973). The specific activity of this product could not be determined precisely since only minute

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Bedford Institute of Oceanography Contribution

0007-4861/79/0022-0265 $01.20

quantities of high activity were available. Spectrophotometric analysis (NERY 1966) indicated a minimum activity of 1.2 mCi/μM.

The uptake and clearance of OHA was studied using brook trout *(Salvelinus fontinalis)* fry obtained from Coldbrook Fish Hatchery, N.S., maintained in charcoal-filtered fresh water (10°C) and fed Purina Trout Chow daily. Fifty fry (weighing 0.5-1.2 gm) were placed in a glass aquarium containing 30 L continuously aerated static fresh water, to which was added OHA in isopropanol (20 ml) to reach a nominal initial concentration of 7 mg L^{-1}. Two control systems were used, one containing fry exposed similarly to isopropanol, but no OHA, and a second containing a similar concentration of OHA and isopropanol, but no fry. At intervals of 1, 6, 12, 18 and 24 hr after addition of OHA, 5 fry were removed at random from each tank, and duplicate 3 ml water samples were taken. Twenty-four hr after addition of OHA, the remaining experimental group fish were transferred to a glass aquarium containing continuously aerated fresh water flowing at approximately 2 L hr^{-1} (i.e. 3 complete changes in 24 hr) and were sampled in groups of five at 6 hourly intervals. Individual fish were rinsed with 2 ml distilled water (retained for counting) and were homogenized in 3 ml distilled water in a combined ultrasonic and mechanical homogenizer (Polytron). Triplicate aliquots of each homogenate were digested with 1 ml Soluene-100(R) (Packard Instrument Co.) and were counted in a toluene-based fluor on a Packard Tri Carb instrument (Model 3375) using the pre-set ^{14}C channel. Water samples were diluted with a dioxane-based fluor and counted similarly. Appropriate blanks and standards were counted, and the data were expressed in terms of dpm g^{-1} sample, corrected for apparent counts in control systems. From this, OHA concentrations could be calculated, assuming all the radioactivity to be in the form of unchanged OHA.

Tissue distribution of radioactivity was studied using brook trout weighing 75-150 gm. They were exposed individually in glass aquaria for periods of 24-72 hr to 3 L water containing 6-7 mg L^{-1} OHA added in 2 ml isopropanol. At the end of the exposure period, they were killed by a blow on the head and weighed. Blood samples were removed from the caudal blood vessels using 20-gauge syringe needles, and were heparinised and centrifuged; duplicate 0.1 ml plasma samples were taken for analysis. The fish were dissected, and accurately-weighed duplicate samples of approximately 0.1 gm were taken from liver, gut, brain, adipose tissue, dorsal muscle and skin; duplicate 0.1 ml samples of bile were removed from the gall bladder. All samples were suspended in 1 ml Soluene-100(R) and when solubilization was complete, were counted as described above. The radioactivity of tissues from experimentally exposed fish was corrected for the apparent 'radioactivity' in control fish, and tissue OHA concentrations calculated as described above.

The identity of radioactive material remaining in adult fish tissues (liver and bile) was investigated by thin-layer chromatography (TLC) and autoradiography. Samples were homogenized

with ethyl acetate (1:10 w/v) with or without prior acidification; the resulting homogenate was filtered, dried over anhydrous sodium sulphate, reduced in volume, and run on TLC in the systems described above. Octanoic acid-1-^{14}C (prepared from acidification of the sodium salt), octanamide-1-^{14}C (prepared according to VOGEL [1970]) and OHA were used as standards. 'Blank' tissue samples spiked with these compounds were used to assess extraction efficiency.

The possibility that some metabolites may have existed as conjugates was investigated by treating the aqueous phases from tissue extracts with glucuronidase or phosphatase (Sigma), or with sulphuric acid using conventional techniques. The incubates were extracted and analyzed as described above.

Metabolism of hydroxamic acids *in vitro* was studied using a 10,000 g supernatant of liver homogenate from adult trout, prepared and incubated as described elsewhere (ADDISON *et al.* 1977). OHA and NHA at concentrations of 0.2 µM and 8 pM (maximum) respectively in the incubate were used as substrates.

RESULTS AND DISCUSSION

OHA was accumulated by trout fry. The radioactivity accumulated by all fry in 24 hr represented less than 0.5% of the starting dose, but water analyses showed that another 5.6% of the starting material disappeared. The control tank containing OHA and no fish also showed a reduction in OHA concentration (7.7% below starting concentration) and we conclude that these losses arose from splashing during aeration and/or adsorption or partition to the walls of the glass aquaria. Mean water concentration over the exposure period was 7.4 mg L^{-1}.

A single-compartment exponential model was adopted to describe both the uptake and clearance processes of radioactive material by trout (RUZIC 1972, HARDING and VASS 1977). This assumes that the rates of uptake or clearance are proportional to the ^{14}C concentration in water (W) and in trout fry (C) respectively.

The clearance of radioactivity from trout fry was best fitted to a negative exponential curve:

$$C = 18.3e^{-0.043(t-t_o)} \quad (r = 0.81).$$

Using a clearance flux constant equal to 0.043 hr^{-1}, an uptake flux constant was calculated to be 0.18 hr^{-1} which was in close agreement to the experimental results.

If the model holds true for longer time intervals the concentration of OHA in trout fry (assuming all ^{14}C to be unchanged

OHA) predicted at equilibrium, KW = C, is 31 μg gm^{-1} wet weight. Thus, the 24 hr exposure period resulted in tissue concentrations (20 μg gm^{-1}) approaching those predicted at equilibrium. Extrapolation of the clearance curve predicted that 72 hr exposure to clean water was required to reduce ^{14}C concentrations in fry to 1 μg gm^{-1} (as OHA). The data are in accordance with our previous studies which showed that fish poisoned with alkylhydroxamic acids recovered rapidly on transfer to clean water (FLETCHER and ADDISON 1972).

Results of studies on tissue distribution of radioactive material in large trout are summarized in Table 1. Highest concentrations of radioactivity (when expressed as μg gm^{-1} OHA) were

TABLE 1

Tissue distribution of radioactivity derived from octano-1-^{14}C-hydroxamic acid (mean ±s.d.) (calculated as μg of octano-1-^{14}C-hydroxamic acid per g wet wt tissue) accumulated from water by trout. (24 hr data calculated from 8 replicates; 48 hr and 72 hr data from 3 replicates).

Time (hr)	24	48	72
Tissues			
plasma	4.9 ±2.3	7.4 ±3.2	10.5 ±1.9
brain	12.3 ±5.3	17.1 ±1.0	25.2 ±9.4
heart	6.8 ±3.3	11.8 ±5.7	14.2 ±4.9
spleen	11.7 ±7.1	20.5 ±4.6	23.6 ±6.6
fat	3.5 ±2.8	6.5 ±2.3	8.6 ±6.2
stomach	7.4 ±3.3	10.2 ±4.4	14.7 ±2.9
pyloric caeca	10.9 ±4.2	22.5 ±3.2	26.9 ±2.9
intestine	10.6 ±4.4	22.8 ±7.0	33.5 ±8.1
intestinal contents	13.2 ±8.1	38.8 ±7.1	52.5 ±8.9
liver	26.4 ±11.5	34.6 ±12.5	40.5 ±5.2
kidney	11.3 ±4.5	14.7 ±4.8	25.9 ±7.2
skin	18.7 ±4.5	20.4 ±3.7	30.1 ±10.5
muscle	2.8 ±0.5	4.0 ±1.2	4.7 ±0.5
gills	19.0 ±4.3	25.5 ±3.6	31.6 ±6.3
gonad	6.6 ±2.4	13.8 ±8.3	24.0 [a]
bile	257.2 ±184.4	607.5 ±184.2	437.6 ±202.1

[a] One sample only

found in bile, liver, gills, skin and digestive tract. This suggests that the toxicant was transported by the blood with a large percentage going to the liver for storage and/or degradation. The concentration of radioactivity in the gall bladder was several orders of magnitude higher than that in other tissues, suggesting that it was a major route of excretion. Although this radioactivity could be reabsorbed by the digestive tract, the trout fry data indicate that appreciable excretion occurs.

Attempts to identify the chemical form in which radioactive material existed in liver and bile yielded the following information.

(1) Acidification of bile samples (prior to extraction of anticipated acidic metabolites with ethyl acetate for TLC) resulted in losses of 25-50% of the radioactivity estimated by sample digestion. Of the counts remaining, 10-25% were in the aqueous phase with about 40-60% in the organic phase.

(2) Recovery of OHA and octanamide-1-^{14}C from 'spiked' liver and bile samples was virtually quantitative (mean ±s.d., 97 ±9%; n = 11); radioactivity was almost exclusively in the organic phase. Recovery of radioactivity from liver of fish exposed to OHA was high (mean ±s.d., 90 ±13%; n = 6) but was distributed about 60% in the aqueous phase and 30% in the organic phase. On acidification of the aqueous phase, radioactivity was again lost.

This suggests that much of the radioactivity recovered from tissues of exposed fish was therefore in the form of a water-soluble, acid-labile material and was not unchanged OHA or octanamide. Two possible metabolites meet these criteria: carbonate (or bicarbonate) and β-keto-octanoic acid. Either carbonate or bicarbonate would be released by acid, and β-keto acids are readily decarboxylated by acid (MORRISON and BOYD 1966; RALSTON 1948).

The occurrence of either of these metabolites suggests that OHA is converted to the corresponding carboxylic acid (*n*-octanoic acid) which enters the lipoclastic cycle; through the intermediate of β-keto-octanoic acid it undergoes β-oxidation and the labelled acetate-1-^{14}C produced would enter the Krebs cycle and be degraded to $^{14}CO_2$. A similar scheme has been proposed for metabolism of labelled OHA in the rat (KOBASHI *et al.* 1973). No conversion to the amide was observed (cf. FISHBEIN *et al.* 1973).

The conversion of alkylhydroxamic acids to their carboxylic analogues could not easily be demonstrated with carboxyl labelled substrates because of the transient nature of the labelled carboxylate intermediate. Incubation of OHA resulted in relatively small amounts of octanoic acid-1-^{14}C due to further degradation of the product; of the radioactive material recovered,

only 3% was in the form of octanoic acid-1-^{14}C. However, incubation of NHA (in a separate experiment) resulted in appreciable accumulation of tritiated nonanoic acid (32% of material recovered). The relatively large production of nonanoic acid compared to that of octanoic acid may be attributable to various factors, such as variation in activity of the hydroxamate → carboxylate conversion system in different fish; and variation in enzyme activity with chain length of substrate.

In summary, brook trout rapidly accumulated and cleared a labelled medium chain length alkylhydroxamic acid. Appreciable amounts of accumulated material were evidently converted to the corresponding carboxylic acid (whence they entered the normal metabolism of the animal via the lipoclastic cycle). The detoxification process (hydroxamate → carboxylate) evidently does not proceed sufficiently rapidly to offer complete protection to the fish, however, as in previous studies, OHA proved lethal to salmon fry (ADDISON AND CÔTÉ 1973).

REFERENCES

ADDISON, R.F. and R.P. CÔTÉ : Lipids 8, 493 (1973).

ADDISON, R.F., M.E. ZINCK and D.E. WILLIS : Comp. Biochem. Physiol. 57C, 39 (1977).

FISHBEIN, W.N., J. DALY and C.L. STREETER : Anal. Biochem. 28, 13 (1969).

FISHBEIN, W.N., C.L. STREETER and J. DALY : J. Pharmacol. Exp. Ther. 186, 173 (1973).

FLETCHER, G.L. and R.F. ADDISON : Bull. Environ. Contam. Toxicol. 7, 147 (1972).

HARDING, G.C.H. and W.P. VASS : J. Fish. Res. Board Can. 34, 177 (1977).

KOBASHI, K., S. TAKEBE, and J. HASE : Yukagaku Zasshi 93, 1564 (1973).

MORRISON, R.T. and R.N. BOYD : Organic Chemistry. Chapter 30, 2nd ed. Allyn & Bacon, Boston, Mass. (1966).

NERY, R. : Analyst 91, 388 (1966).

RALSTON, A.W. : Fatty Acids and Their Derivatives. John Wiley and Sons, Inc., New York, N.Y. (1948).

RUZIC, I. : Mar. Biol. 15, 105 (1972).

VOGEL, A.I. : Practical Organic Chemistry. 3rd ed., p. 404, Longmans, London (1970).