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UPTAKE, DISTRIBUTION AND RETENTION OF ZINEB AND ZIRAM IN RAINBOW TROUT (*SALMO GAIRDNERI*)

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SUMMARY

In short-term static bioaccumulation experiments with ^{14}C-labelled zinc ethylenebisdithiocarbamate (zineb) and zinc dimethyldithiocarbamate (ziram) both compounds were rapidly disseminated through the tissues. Whole-body accumulation was low, with bioconcentration factors < 100. Whole-body elimination was rapid with 45% and 25% of the initial radioactivity from ziram and zineb, respectively, being retained by the end of the 16-day depuration period. Pigmented tissues appeared to be major distribution sites as well. This may be related to the affinity of the compounds and/or their degradation products to melanin or to complexation with phenoloxidase, a copper-containing enzyme involved in melanin synthesis. Autoradiography also revealed a high labelling of thyroid follicles. The results show that dithiocarbamates are selectively localized in various tissues, reported to be the target organs for their toxic action. The observed differences in toxicokinetics between zineb and ziram may, in part, explain the differences in toxicity to fish between ethylenebisdithiocarbamates and dialkyldithiocarbamates.

Key words: Salmo gairdneri; Accumulation; Dithiocarbamates; Zineb; Ziram

Abbreviations: DC, dithiocarbamate; BDC, ethylenebisdithiocarbamate; DDC, dialkyldithiocarbamate; zineb, zinc ethylenebisdithiocarbamate; ziram, zinc dimethyldithiocarbamate; DMSO, dimethylsulphoxide; BCF, bioconcentration factor.

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INTRODUCTION

Dithiocarbamates (DCs) are among the most important classes of fungicides currently used in agricultural practice. Because of their chelating properties DCs are also applied in industry and medicine [1—3]. A good deal is known about the effects of DCs on warm-blooded animals [4], but data on their effects on cold-blooded animals, particularly fish, are scarce. Recently, we have reported on the harmful effects of various dialkyldithiocarbamates (DDCs) and ethylenebisdithiocarbamates (BDCs) on aquatic life; DCs were found to be toxic to bacteria, green algae, crustaceans and fish. DDC-induced embryotoxicity and teratogenicity in rainbow trout *(Salmo gairdneri)*, at concentrations below ppb-level, were among the most conspicuous effects. On the whole, BDCs appeared to be less toxic than DDCs [5—8].

The present report deals with the uptake, distribution and retention of 2 representatives of the 2 groups. This was measured by exposing early juvenile rainbow trout to [^{14}C]zineb (zinc ethylenebisdithiocarbamate) and [^{14}C]ziram (zinc dimethyldithiocarbamate) in water, transferring them to toxicant-free water and analyzing whole fish and tissues for ^{14}C-content.

MATERIALS AND METHODS

Test animals and standard water

Rainbow trout (*S. gairdneri*) were obtained from Fijge Trout Farm at Vaassen (The Netherlands). Standard water for the experiments was prepared according to Alabaster and Abram [9]. The pH, hardness and temperature of the water was 8.0 ± 0.1, 50 mg/l (as $CaCO_3$) and 10 ± 1°C, respectively. A 12-h photoperiod was imposed upon the fish. During acclimatization and elimination, trout were fed with Trouvit pellets (Trouw & Co. N.V., The Netherlands).

Test compounds

[*Ethylene*-^{14}C]zineb (spec. act.: 11.7 μCi/mg) and [*methyl*-^{14}C]ziram (spec. act.: 14.5 μCi/mg) were obtained from Amersham Radiochemical Centre (England). Dimethylsulphoxide (DMSO) was used as a solvent.

Accumulation studies

Whole-body static accumulation studies were performed in 5-l testvessels to which 4 l well-aerated standard water was added. The weight of the fish was (mean ± S.E.) 0.42 ± 0.13 g. In order to study the distribution of ziram and zineb, trout with weights of 3.4 ± 0.4 g, were exposed in 25-l all-glass fish tanks, housed in a water bath. They were fasted for 48 h prior to and during the exposure. In order to keep the NH_3 concentration below 0.025 mg/l [11], the mass of fish in each tank never exceeded 4 g/l. In the short-term whole-body accumulation studies, however, the loading was approximately 8 g/l, but no detrimental effects were observed. The test solutions

were aerated continuously and not renewed. Fish and water were sampled after 6, 24, 48 and 96 h of exposure.

Elimination studies

Following 96 h of exposure to experimentally contaminated water, rainbow trout (weight 7.2 ± 1.7 g) were transferred to toxicant-free water and sampled after 0, 4 and 16 days to measure depuration of radioactivity. Radioactivity in water was measured at regular intervals; a concentration of 1% of the initial ^{14}C-activity during the accumulation period was taken as a maximum, above which water was renewed. During depuration fish were fed once every 4 days.

Collection of organs and tissues

Fish were anesthetized with $NaHCO_3$-buffered tricaine methane sulphonate (MS 222, Sandoz, Basel). After removal of adhering water by blotting on filter paper, the fry were weighed. The following organs and tissues were dissected: eyes, gills, stomach, intestine, liver, gall bladder, head kidney, trunk kidney, brains, heart and spleen. Samples were taken from the vertebral column and muscles. Blood samples were collected from the ventral aorta. Rest fractions were homogenized with an Ultra-turrax mixer. Samples were weighed on a microbalance with a precision of 10 μg.

Radiotracer techniques

In the whole-body accumulation experiments, fish were incinerated in a sample oxidizer (Packard, Tri Carb model B 306). CO_2 was trapped in Carbo-sorb and Perma-Fluor V (Packard) and radioactivity was measured by liquid scintillation counting (LKB/Wallac Rackbêta 1215). In the other experiments samples were dissolved in Lumasolve (Lumac; 1 ml/100 mg tissue) and placed in a stove at 40°C for 24 h. Subsequently, scintillator 299 (Packard) was added at a maximum of 5 ml/vial. Before scintillation counting, vials were stored for approximately 6 h in complete darkness. Data were corrected for chemical quenching and background radiation. Measurements were carried out in 3—5 replicates.

Transverse sections for autoradiography were cut with a whole-body microtome according to the method described by Curtis et al. [12]. Sections of 30, 40 and 50 μm thickness were freeze-dried at −20°C for approximately 18 h before being pressed against autoradiography films (Kodak X-OMAT AR). Films were exposed in a light-tight box for 26 days at −20°C and developed in Kodak-LX 24 for 5 min. Next they were rinsed in an acetic acid stop-bath for 30 s. Fixation took place in Kodak AI-4 Röntgenfix for 4 min.

Data analysis

The results of the liquid scintillation countings (dpm, per kg fish and per liter water) were converted to μg/kg and μg/l respectively, dividing them by the specific activity of each compound. In the whole-body accumulation

experiments the rate constants were estimated from a kinetic model which has been described in more detail in a previous paper [10]. The set of equations, however, had to be extended in order to comprise biotransformation processes. The following set of equations was used:

$$\frac{d}{dt} C_f(t) = k_1 C_w(t) - k_2 C_f(t) - k_3 C_f(t) \quad (1)$$

$$\frac{d}{dt} C_w(t) = -\frac{N(t)\,w}{V} k_1 C_w(t) + k_2 \frac{N(t)\,w}{V} C_f(t) \quad (2)$$

$$\frac{d}{dt} C'_f(t) = k'_1 C'_w(t) - k'_2 C'_f(t) + k_3 C_f(t) \quad (3)$$

$$\frac{d}{dt} C'_w(t) = -\frac{N(t)w}{V} k'_1 C'_w(t) + k'_2 \frac{N(t)w}{V} C'_f(t) \quad (4)$$

The following symbols were used: t: time (h); C_f: concentration of the parent compound in one organism (μg/kg); C_w: concentration of the parent compound in water (μg/l); w: weight of one organism (kg); V: water volume (liter); N: number of organisms; k_1: uptake rate constant of the parent compound (l/kg per h); k_2: clearance rate constant of the parent compound (1/h); and k_3 biotransformation rate constant (1/h). The prime denotes the concentrations and rate constants of the metabolite.

The biotransformation process is assumed to be first order in the concentration of the parent compound in the organism. For compounds which are slowly biotransformed, k_3 approximates zero and only eqs. 1 and 2 are used. In this case, the steady-state bioconcentration factor (BCF: l/kg) equals k_1/k_2. The parameter estimation procedure is described in [10].

BCFs for tissues and organs were calculated from the mean total ^{14}C-concentrations in fish and water, respectively. Differences in the BCFs were tested with the Student's t-test.

RESULTS

The whole-body accumulation experiments revealed that radioactivity in early juvenile trout exposed to zineb (Fig. 1) reached an apparent steady-state within approximately 24 h of exposure. The k_1 and k_2 values (means ± S.E.) were 2.41 ± 0.18 and 0.07 ± 0.01; the BCF (k_1/k_2) was 34. In trout exposed to ziram, however, no steady-state conditions were reached, i.e. the total ^{14}C-activity in water initially decreased and subsequently increased again, whereas the total radioactivity levels in fish showed a reversed pattern of behaviour (Fig. 1).

This hindered the calculation of steady-state BCFs. The best fit to these

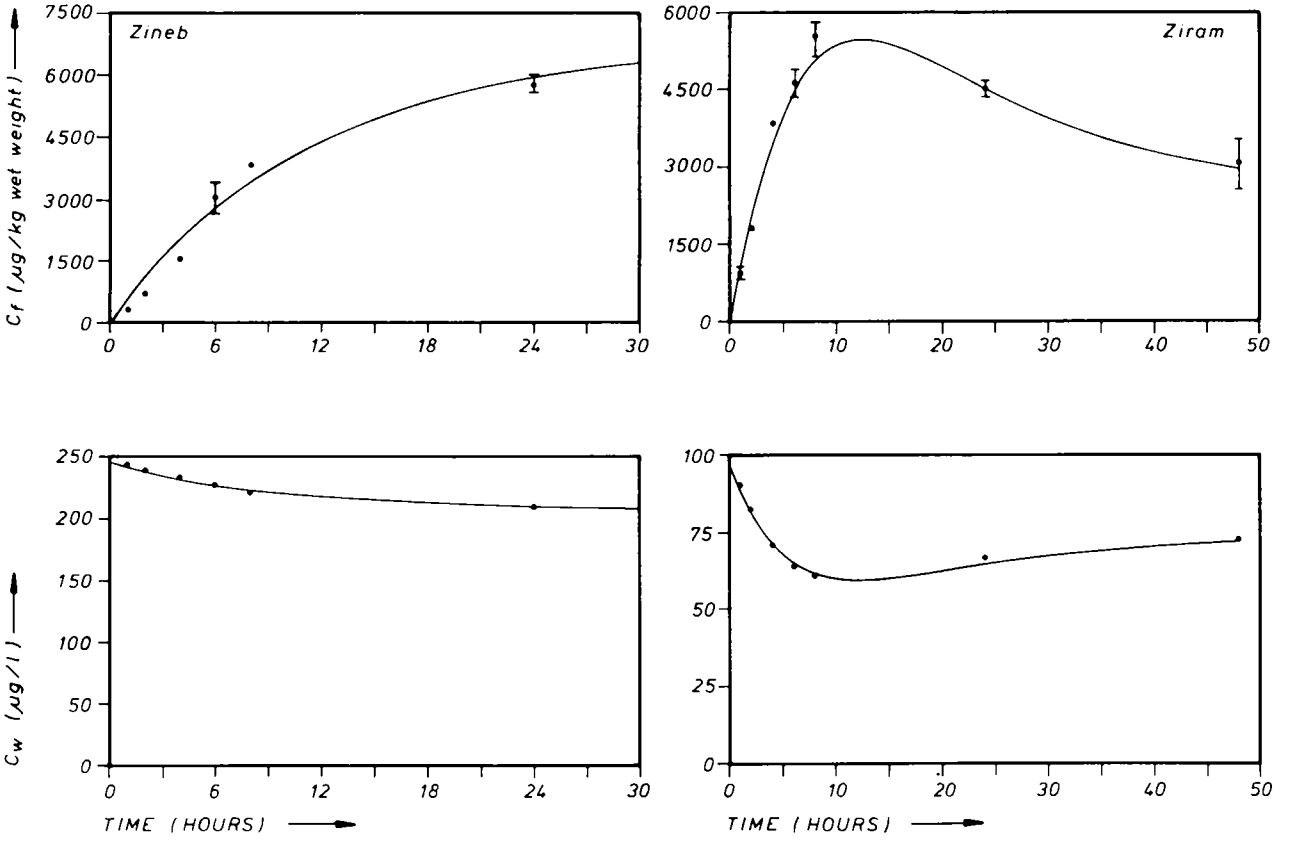


Fig. 1. Whole-body accumulation of zineb ($C_w(o)$ = 245 µg/l) and ziram ($C_w(o)$ = 97 µg/l) in early juvenile rainbow trout (*Salmo gairdneri*). Points and associated vertical lines represent means ± S.E. of 5 samples. The experiments were started with 75 fish. Lines are expected values based on the model calculations. A reasonable fit to the experimental data of ziram was obtained by assuming biotransformation of the parent compound to one degradation product.

experimental data was obtained by introducing 3 biotransformation-related constants (see data analysis). The k_1, k_2, k_3, k_1' and k_2' values (mean ± S.E.) were 11.54 ± 0.48, 0.002 ± 0.47, 0.21 ± 0.03, 6.71 ± 2.14 and 0.21 ± 0.04, respectively. The "bioconcentration factor" calculated from the measured radioactivity levels in fish and water, i.e., the highest C_f/C_w value, was 90.

The results of the distribution studies are shown in Figs. 2 and 3. Radioactivity was rapidly disseminated through the tissues. The lowest levels of ^{14}C-activity were found in muscle, heart, brain and vertebral column. Liver and digestive tract contained the highest. Zineb-derived radioactivity accumulated in liver accounted for about 60% of total radioactivity after 2 and 4 days, whereas the liver of ziram-treated fish accounted for approximately 20% of total radioactivity after comparable exposure times. Remarkably high residues were also detected in eyes and skin of ziram-treated fish. Radioactivity in the skin accounted for approximately 40% of total against 8% in zineb-treated fish.

The results of the elimination experiments are shown in Fig. 4. In these studies, radioactivity in liver and gall bladder (contents included) were determined separately. The measurements revealed that the gall bladder was the major distribution site for the radiolabelled compounds and/or their degradation products. Whole-body elimination of zineb and/or its degradation product(s) was rapid during the first few days. After 4 days, only 25% of the initial residue was retained by the fish and further clearance was neglegible. The pattern of ziram elimination was similar, but slower than that of zineb. After 16 days, about 55% of the initial total ^{14}C-content in the body was eliminated. The loss of radioactivity in all organs and tissues was rapid. Radioactivity levels in eyes, skin and kidney, however, remained constant or even increased. This points to a redistribution of radioactivity.

The autoradiographic studies revealed the same results (Fig. 5). A high labelling of the liver, gall bladder, intestinal lumen and pigmented tissues was observed. Tissues of ziram-treated fish generally contained more radioactivity. Detailed examination revealed that radioactivity was localized at distinct spots which coincided with pigment, in melanophores. Upon transferring the fish to clean water, these spots retained their radioactivity for considerable periods of time. This was demonstrated for ziram and to a lesser extent for zineb-treated fish. After 16 days of depuration, radioactivity was almost entirely confined to the pigmented tissues (Fig. 6). Moderate to high levels of activity were also recorded in distinct spots in the subpharyngeal area, which corresponds to the location of thyroid follicles. High activity at these locations was still found after a 16-day elimination period, both in zineb and ziram-treated fish (Fig. 7). In all other tissues, radioactivity was rapidly lost.

DISCUSSION

Bioaccumulation of both zineb and ziram was low [cf. 10], but differences were found regarding their BCFs, internal distribution and retention, which

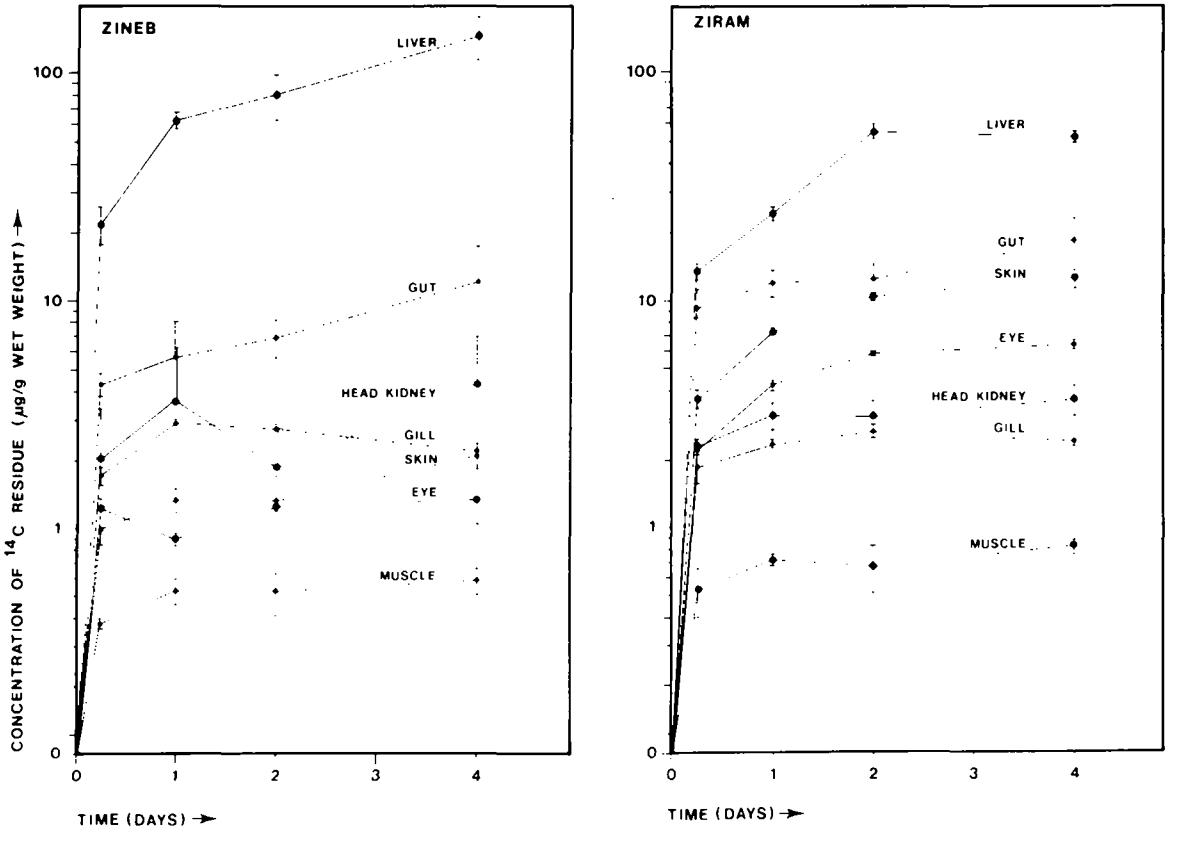


Fig. 2. Uptake of zineb and ziram in various tissues of *Salmo gairdneri*. Points and associated vertical lines represent means ± S.E.M. of 3–5 fish. The initial concentrations of zineb and ziram were 225 and 138 μg/l. At the end of the 96-h accumulation period these concentrations were 191 and 118 μg/l, respectively.

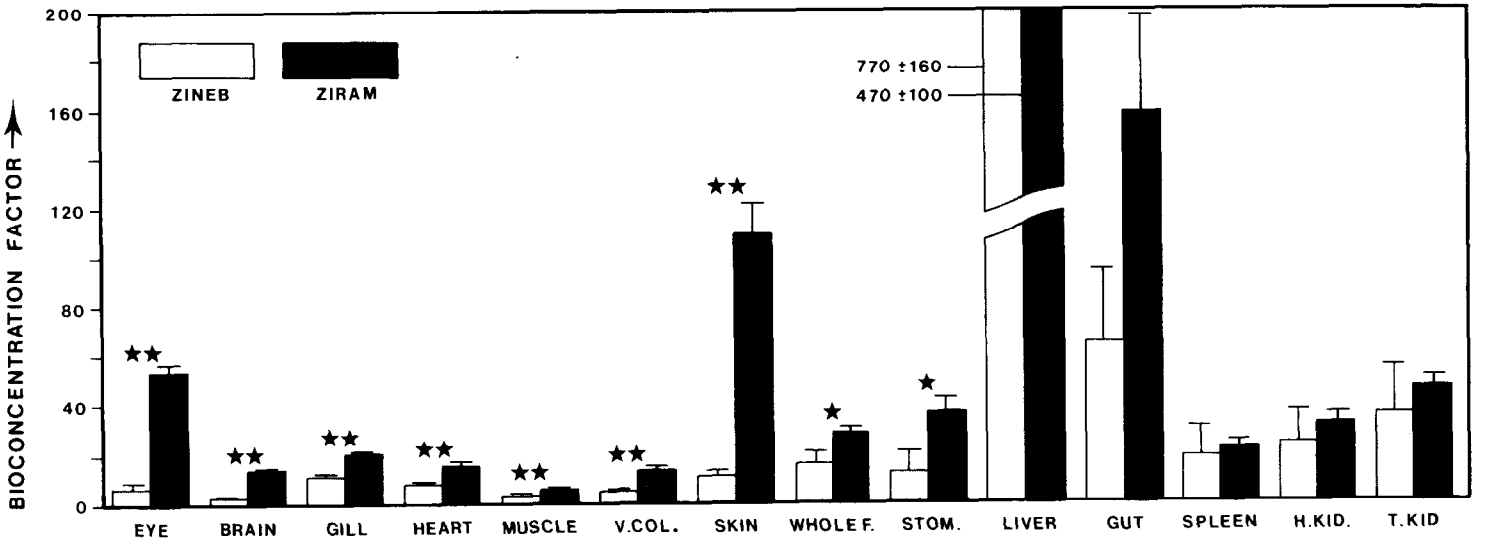


Fig. 3. Bioconcentration factors of ^{14}C-residues in whole fish and various tissues of *Salmo gairdneri* after 96 h exposure to 225 µg/l zineb and 138 µg/l ziram. Values and associated vertical lines represent means ± S.E.M. of 3—5 fish. Asterisks denote differences in BCFs at $P < 0.05$ (*) and $P < 0.01$ (**), respectively.

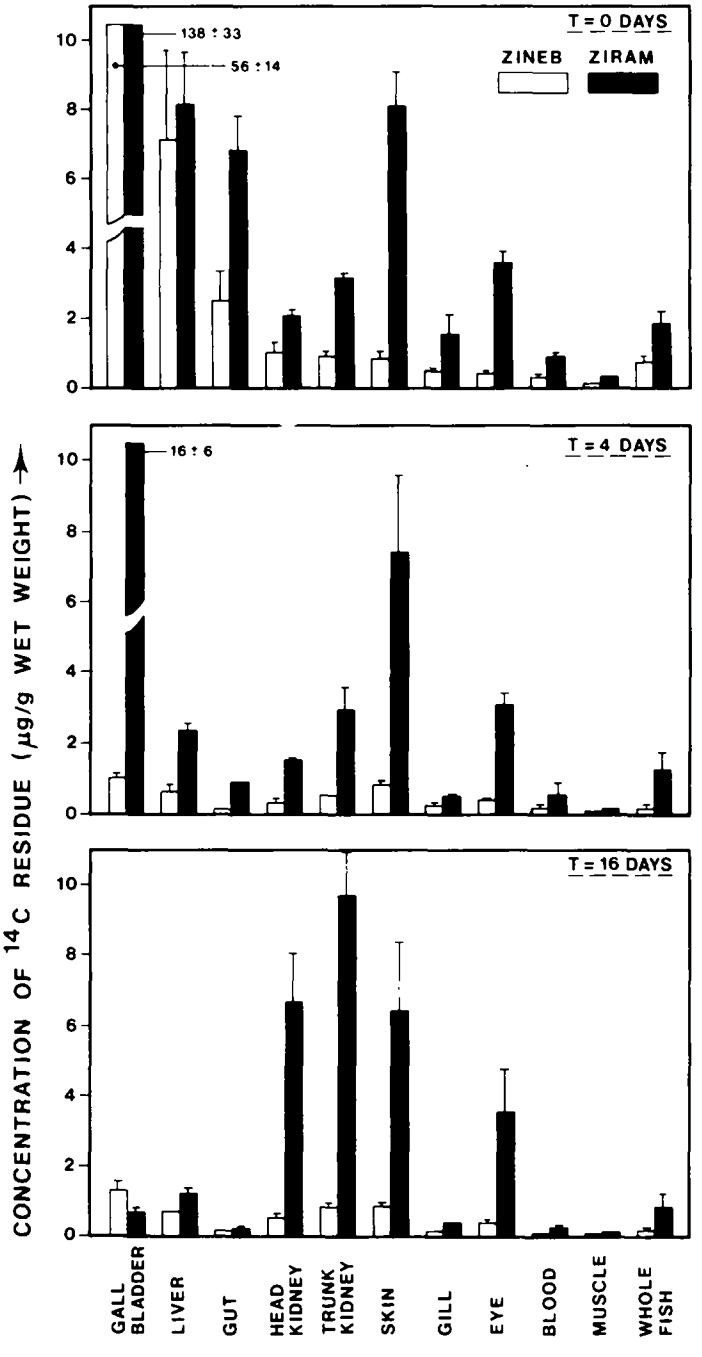
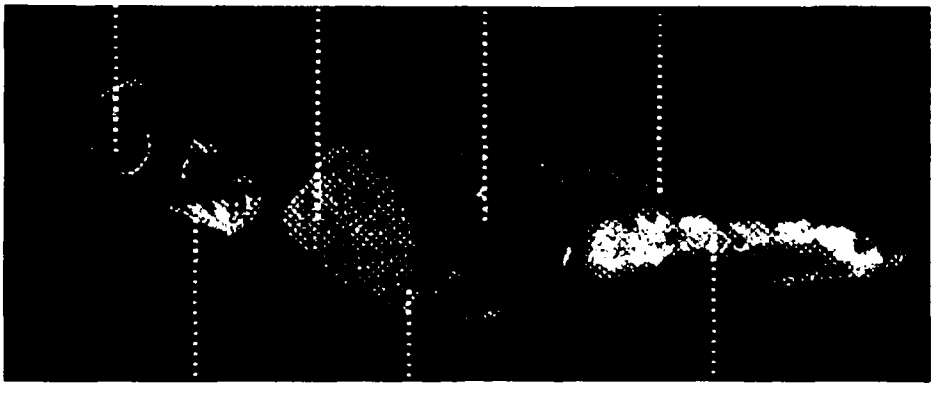


Fig. 4. Elimination of ^{14}C-residues in whole fish and various tissues of *Salmo gairdneri* after 96 h exposure to 105 and 118 µg/l zineb and ziram, respectively. Values represent means ± S.E.M. of 3—5 fish.

A


eye
liver
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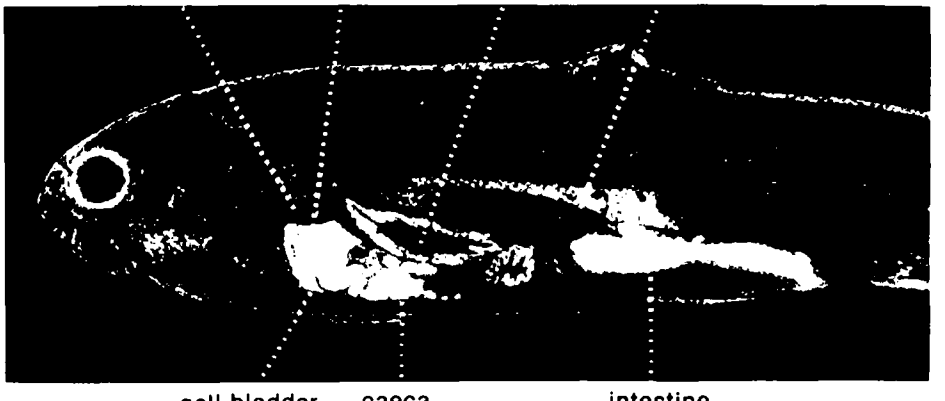
B


esophagus
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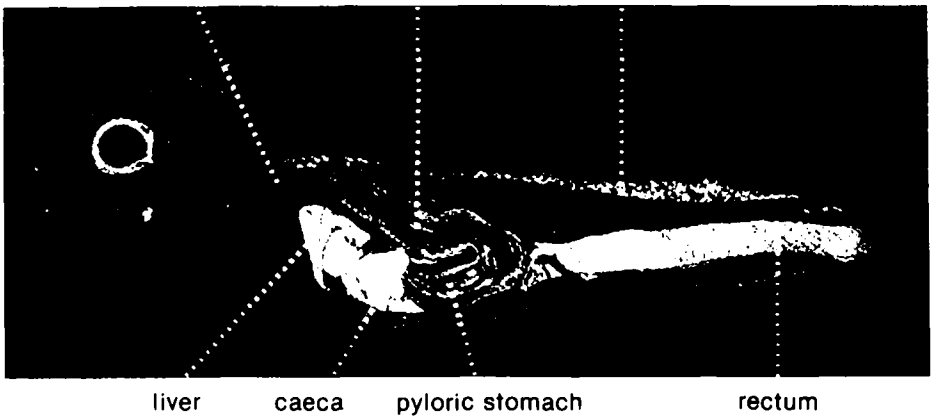
gall bladder
caeca
intestine

C


esophagus
cardiac stomach
kidney
liver
caeca
pyloric stomach
rectum

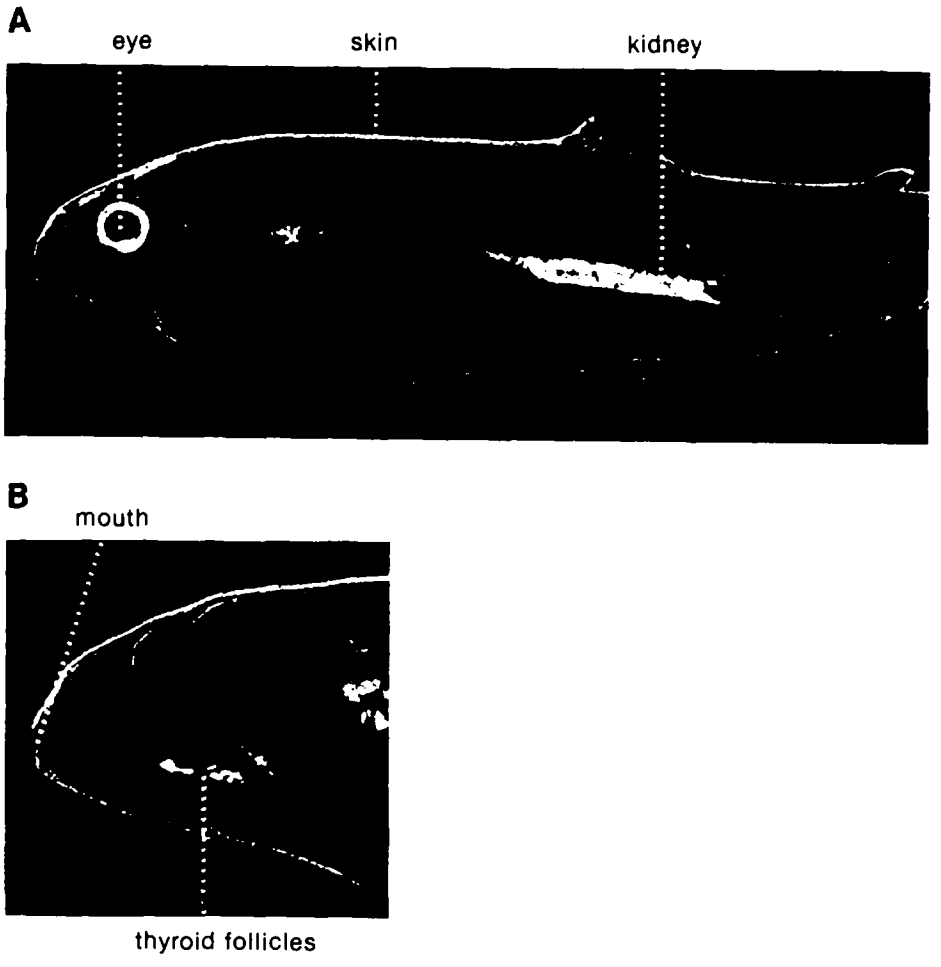


Fig. 6. Autoradiograms of *Salmo gairdneri*. Fish were exposed to 118 μg/l ziram (1.71 μCi/l) for 96 h and subsequently transferred to toxicant-free water for 16 days. A marked retention of radioactivity is shown in eye, skin, kidney and thyroid.

may in part explain their differences in toxicity to fish [5,7]. The low bioaccumulative potential of both compounds, together with the low concentrations at which adverse effects become manifest, point at high intrinsic toxicity. For both compounds high levels of radioactivity were recorded in the liver, gall bladder and intestinal lumen, which suggests a prominent role

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Fig. 5. Whole-body autoradiograms of *Salmo gairdneri* after exposure to 105 μg/l zineb (A; 1.22 μCu/l) and 118 μg/l ziram (B; 1.71 μCi/l) for 24 h. High levels of radioactivity can be seen in eyes, liver, pyloric caeca, kidney and intestine. There is also a marked labelling of the skin and stomach wall of ziram-treated fish. Low levels of radioactivity are seen in the brain. Autoradiogram C, a rainbow trout 6 h after intraperitoneal injection with 7 μg ziram (0.1 μCi) dissolved in 50 μl DMSO, reveals a similar distribution of radioactivity.

for biotransformation (Figs. 2, 3 and 5). The rapid disappearance of radioactivity in fish is in support of this (Figs. 4 and 6). In mammals DCs are biotransformed into a host of degradation products [13]. Therefore it is likely that in our kinetic model the actual metabolic processes are oversimplified. Urinary excretion was not explicitly studied. This, together with faecal excretion and elimination via gill, suggests that by the end of the exposure period a substantial part of the concentration of radioactivity, both in water and fish, may originate from hydrophilic metabolites.

The whole-body autoradiography confirmed the results of the previous experiments. Ziram, and to a lesser extent zineb and/or their degradation products, appeared to accumulate specifically in melanophores in the skin, the choroid-epithelium complex of the eye, and in other melanin containing tissue such as the kidney (Figs. 5 and 6). This may be attributed either to the removal of copper—the prosthetic group of phenoloxidase (tyrosinase), a metallo-enzyme involved in melanin synthesis — or to the attachment to copper, by which a dithiocarbamate-enzyme complex is formed [1,2,14]. These assumptions are supported by the finding that DDCs and various thioureas are potent inhibitors of phenoloxidase [15—17]. This tentative hypothesis is also implicitly supported by Zavanella et al. [18], who demonstrated a temporary reduction of melanogenesis in regenerating limbs of newt (*Triturus cristatus carnifax*) after exposure to maneb, and reports on melanosomes devoid of melanin in pigmented retinas of *Xenopus laevis* embryos treated with nabam and maneb [19,20]. Similar effects were observed after exposure of amphibians to sodium diethyldithiocarbamate and its corresponding thiuramsulfides [21,22], and in some embryolarval toxicity studies with *S. gairdneri* [7].

Interaction with melanin constitutes another mechanism by which high levels of radioactivity in melanophores may be explained. Melanin has a great affinity for metal ions, which is ascribed to its cation-exchange activity due to the free carboxyl groups present in the polymer [23]. Danielsson et al. [24] showed a dramatically increased ^{203}Pb uptake in the melanin-containing structures in the eyes of mice after oral treatment with various DDCs, and postulated as the mechanism, the binding of Pb^{2+} to melanin. In our experiments dithiocarbamic acid was labelled and heavy metal ions were not, which suggests that other mechanisms such as nucleophilic substitution on the carbon of carboxyl groups of melanin may be involved [cf. 25].

Relatively high levels of radioactivity were found in the thyroid follicles (Fig. 7). BDCs, ethylenethiourea, a degradation product of BDCs, and CS_2, a degradation product of both BDCs and DDCs, are also known to accumulate in the thyroid [26—28]. The precise mechanism remains conjectural, but may be related to the affinity for sulfhydryl groups of thyroglobulin [1,29, 30] or to the heavy metal sequestering properties of DCs and related compounds, as several metallo-enzymes and metal-requiring enzymes are involved in thyroxine synthesis [31—33].

The present results show that dithiocarbamates are selectively localized in various tissues, reported to be the target organs for the toxic action of DCs

[4,34]. Further experiments are required to investigate whether similar results would have been obtained if the labelling is attached to the CS_2 moiety, the active site of the DCs. It is also important to note that the results presented in this study may not fully apply to other metallo-dithiocarbamates and corresponding thiuramdisulfides, as the "Verdrängungsreihe" of Eckert [14] cannot be ignored.

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