Biochemical Pharmacology, Vol. 23, pp. 2403–2410. Pergamon Press, 1974. Printed in Great Britain.

GLUCURONIDE FORMATION IN RAINBOW TROUT—EFFECT OF SALICYLAMIDE ON THE ACUTE TOXICITY, CONJUGATION AND EXCRETION OF 3-TRIFLUOROMETHYL-4-NITROPHENOL

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(*Received* 29 *October* 1973; *accepted* 25 *January* 1974)

Abstract—In rainbow trout, the selective sea lamprey larvicide, 3-trifluoromethyl-4-nitrophenol (TFM), was previously found to be conjugated with glucuronic acid and excreted to a great extent in bile. In order to determine the degree and significance of conjugation of TFM in trout, studies utilizing salicylamide, an inhibitor of glucuronide formation, were carried out. Pre-exposure of rainbow trout to 25 mg/l. of salicylamide decreased the LC_{50} of TFM from 5·05 to 2·67 mg/l. Coincidental with the increase in toxicity were elevated blood levels of unconjugated TFM and a depression of TFM glucuronide in blood and bile. An increase in the level of unconjugated TFM was also found in brain, muscle and heart from salicylamide-treated trout. Salicylamide increased the half-life of i.p. injected TFM from 1·59 to 4·13 hr and inhibited the glucuronidation of TFM by trout liver extracts *in vitro*. These studies indicate that glucuronide formation appears to be an important mechanism in the protection of trout from the toxic effects of TFM and possibly other water-borne phenols.

3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) is a relatively selective sea lamprey larvicide which is currently being used to control the population of the sea lamprey, *Pretromyzon marinus*, in the upper Great Lakes.[1] Under laboratory and field conditions, TFM is more toxic to sea lamprey than to most species of fish,[2] although the mechanism of selective toxicity has not been defined. During recent investigations concerning the metabolism and uptake of TFM by rainbow trout, it was found that the major metabolite of this compound was the glucuronide conjugate which is excreted mainly in bile.[3] Since early studies had indicated that conjugation did not appear to be appreciable in fish,[4] it was of great importance to determine the functional significance of glucuronide conjugation of TFM in rainbow trout. An initial approach, using novobiocin as an inhibitor of glucuronyl transferase,[5] was equivocal, since novobiocin appeared to inhibit TFM glucuronide transport into bile to a greater extent than it inhibited glucuronyl transferase.[6] This observation of transport inhibition has been confirmed in the chicken kidney[7] and rat liver.[8] The present report is concerned with the inhibition of glucuronyl transferase *in vivo* and *in vitro* by salicylamide[9] and its effects upon the acute toxicity and disposition of TFM in rainbow trout.

MATERIALS AND METHODS

3-Trifluoromethyl-4-nitrophenol was obtained from Mr. John Howell, U.S. Department of Interior, Hammond Bay, Michigan, and was purified as previously described.[10] Radioactive 3-trifluoromethyl-4-nitrophenol (^{14}C ring UL) was obtained

from the Malinckrodt Chemical Co., St. Louis, Missouri; β-glucuronidase (bacterial type II) was obtained from the Sigma Chemical Co., St. Louis, Missouri; salicylamide and saccharo-1,4-lactone were also purchased from Sigma. Rainbow trout, *Salmo gairdneri*, were obtained from the Kettle Moraine Springs Hatchery, Adell, Wisconsin, and were held in flowing dechlorinated water at 10° for at least 1 week prior to use. All other chemicals and solvents were of reagent grade. Authentic [^{14}C]TFM glucuronide was purified from trout bile as previously described.[3]

LC_{50} *determination.* The acute toxicity of TFM was determined in control and salicylamide-exposed rainbow trout essentially as follows. Trout, 2–2·5 inches in length and weighing between 7 and 10 g, were placed in 7-l. plastic test jars containing 5 l. of reconstituted buffered soft water at pH 7·0,[11] which was aerated and maintained at 12°. Five trout were placed in each container and were either maintained for 2 hr as controls or exposed to salicylamide, 25 mg/l. under the same conditions as controls. After the pre-exposure period, graded concentrations of TFM were added to the containers and observations were made for mortality at 30-min intervals up to 4 hr. Both control fish and those exposed to up to 40 mg/l. of salicylamide had no observable distress under these conditions. The LC_{50} and 95 per cent confidence limits were calculated according to the method of Litchfield and Wilcoxon,[12] using a minimum of 20 fish at each dosage level.

Exposure to [^{14}C]*TFM.* Larger rainbow trout weighing 80–100 g were used in this portion of the study to facilitate tissue analysis for TFM and TFM glucuronide (TFMG). The fish were maintained under control conditions or pre-exposed to 25 mg/l. of salicylamide for 2 hr in reconstituted water at 12° and pH 7·0 before addition of sufficient [^{14}C]TFM to give a final water concn of 1·0 mg/l. and approx. 2500 dis./min/ml. These exposures were usually carried out in 50-l. tanks containing 6–8 fish per tank. At the specified times after addition of TFM, fish were removed and blood and bile samples were obtained by dorsal aortic and gallbladder puncture. The carcasses were then quickly frozen for subsequent analysis of tissues. The half-life studies were done essentially in the same manner, except that [^{14}C]TFM was injected i.p. after a 2-hr exposure to salicylamide. The dose was 0·25 mg/100 g.

Determination of TFM and TFM glucuronide in blood and bile. Total ^{14}C was determined in samples of blood and bile by counting suitable aliquots in Bray's soln in a liquid scintillation counter. Heparinized blood samples (0·5 ml) were then added to 5 ml methanol and thoroughly extracted. After centrifugation, 4 ml of the methanol extract was evaporated under nitrogen and the residue was analyzed using a nine-transfer countercurrent system. The organic phase consisted of 3 ml of benzene–diethyl ether (1:1) and the aqueous phase was 3 ml of 0·1 M sodium phosphate buffer, pH 4·5. The phases were equilibrated before use. After the transfers were completed, the phases were emulsified using 0·1 ml of NCS solubilizer, and a 1·0 ml aliquot was counted for ^{14}C in Bray's soln. Analysis of bile was done in the same manner, except that 0·05 ml bile was added directly to the first countercurrent tube before beginning transfers. The amounts of TFM and TFM glucuronide in blood and bile were calculated from the countercurrent data after quench correction using [^{14}C]toluene as an internal standard. Validation of these procedures has been previously reported.[6]

Determination of free TFM in tissues. Pieces of excised tissues, weighing 0·2–0·4 g were homogenized in 3 ml of cold 0·1 M sodium phosphate buffer, pH 4·5, containing 1×10^{-3} M saccharo-1,4-lactone, an inhibitor of β-glucuronidase,[13] and the homo-

genates were extracted twice with 3 vol. of benzene–diethyl ether (1:1). The extracts were combined and an aliquot was counted in Bray's soln and quench corrected using [^{14}C]toluene as an internal standard. The ^{14}C material in the organic extract was chromatographically identical to authentic TFM on Silica gel in the solvent systems previously described.[10] Recovery of [^{14}C]TFM using this method was 96–98 per cent. The data obtained were analyzed statistically using the Student *t*-test.

Glucuronyl transferase assay in vitro. Conjugation of TFM with glucuronic acid *in vitro* was measured as follows. Rainbow trout livers were homogenized in chilled 0·05 M sodium phosphate buffer, pH 7·4, and the homogenate was centrifuged at 2000*g* for 10 min in a refrigerated centrifuge. Aliquots of the supernatant fluid were added to a reaction mixture consisting of NaH_2PO_4, 50 μmoles; $MgCl_2$ 0·1 μmole; UDPGA, 0·1 μmole; saccharo-1,4-lactone, 1·0 μmole; [^{14}C]TFM, 0·1 μmole, and sufficient distilled water to make a final vol. of 1·0 ml. The reaction mixtures were incubated at pH 7·4 for 30, 60 and 90 min at 24° and were stopped by the addition of 4 ml methanol. After centrifugation, 3 ml of the methanol was evaporated to dryness and taken up in 3 ml sodium phosphate buffer, pH 4·5. Unconjugated TFM was removed from the buffer by a triple extraction with 1 vol. of benzene–ether (1:1). Aliquots of the aqueous phase were then transferred to Bray's soln for determination of ^{14}C in a Packard Tri-Carb scintillation spectrometer. The labeled material in the aqueous phase was confirmed as TFM glucuronide by the following criteria: (1) formation of the polar material was drastically reduced (70 per cent) by omission of UDPGA; (2) the polar material was hydrolyzed by β-glucuronidase to yield free TFM on TLC; (3) the hydrolysis of the polar material by β-glucuronidase was inhibited by saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase.[13]

RESULTS

The effect of salicylamide on the acute toxicity of TFM is shown in Fig. 1. Although 25 mg/l. of salicylamide produced no observable effects alone, it effectively increased the acute toxicity of TFM. The 4-hr LC_{50} and 95 per cent confidence limits calculated from the curves were: control, 5·05 mg/l. (4·37–5·84); salicylamide-treated, 2·67 mg/l. (2·22–3·21). The slope of the computed regression line for the control curve was not different from that of the salicylamide curve.

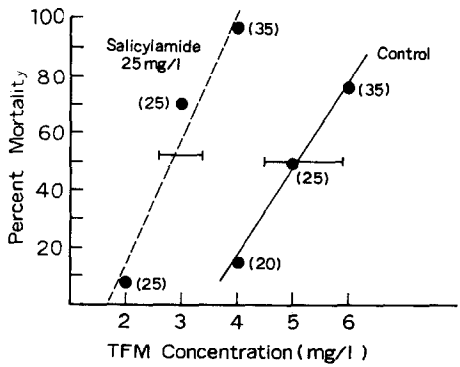


FIG. 1. Enhancement of the acute toxicity of TFM to rainbow trout by salicylamide. Solid line, controls; broken line, toxicity curve in presence of 25 mg/l. of salicylamide. Numbers in parentheses indicate the number of animals at each point. Horizontal bars indicate 95 per cent confidence limits.

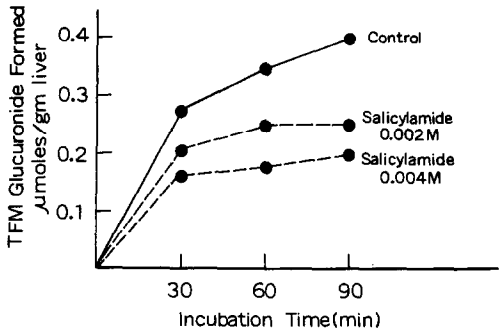


FIG. 2. Inhibition of TFM conjugation in rainbow trout liver extracts by salicylamide. Solid line, complete incubation system as described in Methods; broken lines, complete system plus salicylamide.

The results of studies regarding the effect of salicylamide on conjugation of TFM with glucuronic acid by rainbow trout liver extracts *in vitro* are shown in Fig. 2. It can clearly be seen that salicylamide is able to inhibit glucuronide formation in the concentrations indicated. The molar ratios of salicylamide to TFM were 20:1 and 40:1. At equimolar amounts of salicylamide and TFM, no inhibition was seen. The above experiment was repeated twice with pooled livers from five rainbow trout and the results were essentially the same as those shown in Fig. 2. The presence of TFM glucuronide in the above assays was validated by the procedures described in Methods.

Figure 3 shows the effects of 25 mg/l. of salicylamide on blood levels of TFM and TFM glucuronide in rainbow trout exposed to 1 mg/l. of TFM. At all sampling times salicylamide significantly increased ($P < 0.05$) the blood level of free TFM over control values and depressed the levels of TFM glucuronide. Analysis of muscle, brain

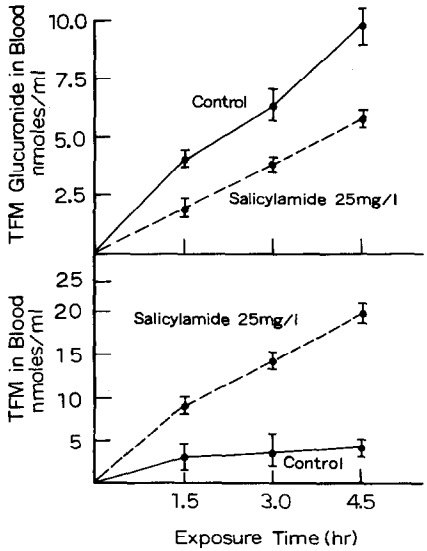


FIG. 3. Effect of salicylamide on blood levels of TFM glucuronide (upper curves) and TFM (lower curves) in rainbow trout during exposure to TFM. Solid lines, controls; broken lines, 25 mg/l. of salicylamide. Vertical bars represent ± S.E.

TABLE 1. EFFECT OF SALICYLAMIDE ON TISSUE LEVELS OF UNCONJUGATED TFM IN RAINBOW TROUT EXPOSED TO TFM*

Tissue	TFM (nmoles/g ± S.E.) Exposure time (hr) 1·5	3	4·5
Blood			
Control	4·46 ± 0·4	3·79 ± 0·95	4·60 ± 0·32
Salicylamide	7·86 ± 0·71	13·56 ± 1·86	20·97 ± 1·73
P	<0·01	<0·01	<0·01
Muscle			
Control	1·31 ± 0·21	1·33 ± 0·29	1·71 ± 0·52
Salicylamide	2·58 ± 0·33	4·80 ± 0·54	6·64 ± 0·89
P	<0·01	<0·01	<0·01
Brain			
Control	4·06 ± 0·33	4·66 ± 0·64	6·09 ± 2·06
Salicylamide	7·57 ± 1·01	15·39 ± 3·74	17·87 ± 2·13
P	<0·01	<0·05	<0·01
Heart			
Control	4·44 ± 0·31	5·90 ± 1·16	8·70 ± 2·68
Salicylamide	16·87 ± 8·75	20·59 ± 4·32	26·63 ± 1·80
P	NS†	<0·01	<0·01

* The water concentration was 1 mg/l., pH 7·0.
† Not statistically significant at the $P < 0·05$ level.

and heart as well as blood in another series of exposures (Table 1) confirmed and extended the previous observations. Salicylamide significantly increased ($P < 0·05$) the blood and tissue levels of free TFM in all tissues analyzed and at all but one of the indicated exposure times.

Since earlier studies had indicated that biliary excretion appeared to be a major route of elimination of TFM glucuronide,[6] the effect of salicylamide on the amount of TFM glucuronide excreted in bile was studied during an exposure of rainbow trout to 1 mg/l. of TFM. As can be seen in Fig. 4, depression of TFM glucuronide in bile was consistently seen at all time periods in salicylamide-treated trout, although statistical significance ($P < 0·05$) was achieved only at 3 and 4·5 hr.

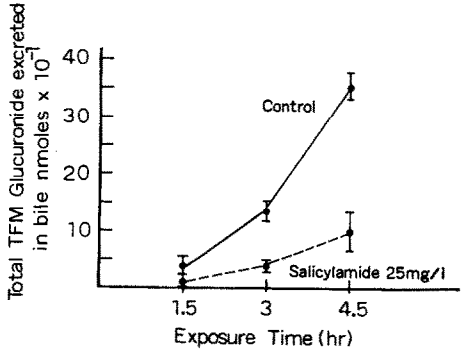


FIG. 4. Effect of salicylamide on the amount of TFM glucuronide excreted in bile during exposure to TFM. Solid line, controls; broken line, 25 mg/l. of salicylamide. Vertical bars represent ± S.E.

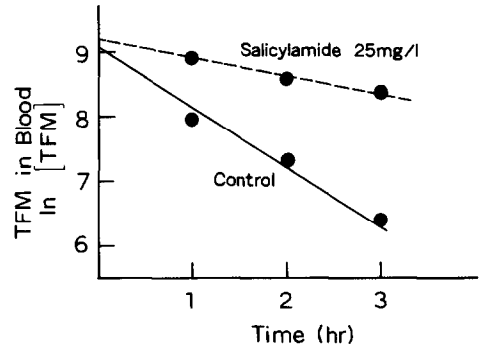


FIG. 5. Effect of salicylamide on the disappearance of TFM from blood of rainbow trout injected i.p. with 2·5 mg/kg of TFM. Solid line, controls; broken line, 25 mg/l. of salicylamide. The data are plotted as the natural log (ln) of the blood concn vs time. Each point represents the mean of the blood concn from three trout.

In agreement with the above results, when TFM was administered by intraperitoneal injection rather than by water exposure, salicylamide increased the blood half-life ($T_{1/2}$) of TFM when compared to that of controls. Figure 5 shows the blood disappearance curves for control and salicylamide-pre-exposed trout injected with 2·5 mg/kg of [^{14}C]TFM. The $T_{1/2}$ in control trout was calculated to be 1·59 hr, while that of the salicylamide group was 4·13 hr. Although not shown in Fig. 5, TFM glucuronide in both blood and bile was reduced approx. 50 per cent in the salicylamide group.

DISCUSSION

Although there has been much concern recently about the contamination of the aqueous environment with pesticides, herbicides and other foreign compounds, very little is known about biotransformation and excretion mechanisms in aquatic species and the extent to which these processes offer protection to the organism in question from the offending substance. Several studies[4] have indicated that fish do not metabolize or conjugate foreign compounds to any extent and that the major route of excretion is by outward diffusion via the gills. Outward gill diffusion of injected drugs in fish, as measured by plasma half-lives, is dependent upon the p*K*a of the drug in question and the pH of the blood.[14] However, these outward diffusion gradients may not be present when considering exposure of fish to xenobiotic compounds in the aqueous environment.

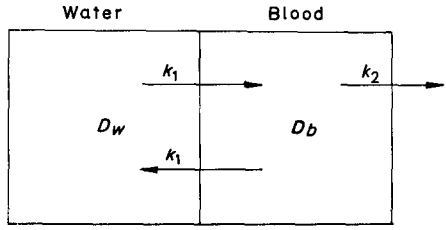
Several workers have indicated that some species of fish have the enzymes to metabolize a variety of foreign compounds *in vitro*,[15–17] and recently it has been reported that microsomal inhibitors can decrease the toxicity of parathion to several species of fish, indicating the microsomal activation of parathion is appreciable *in vivo* in the species studied.[18]

Previous studies *in vivo*[3] have demonstrated that the lampricidal agent, 3-trifluoromethyl-4-nitrophenol (TFM), is conjugated with glucuronic acid and excreted in the bile. In the present study, salicylamide was used as a tool to inhibit conjugation of TFM *in vivo* in order to determine the importance of glucuronide conjugation as a protective mechanism in rainbow trout. Several reports[9,19] have indicated that sali-

cylamide is an effective inhibitor of glucuronide formation *in vivo*, and the results reported in this study are in agreement with them.

From a pharmacokinetic point of view, fish swimming in a large volume of aqueous medium containing a foreign compound present a special kinetic case—unless they move to cleaner water—in that the concentration of the compound in water essentially remains constant and the diffusion gradient is inward rather than outward, as is the case with injected or ingested compounds.

A simplified version of the situation in which fish are exposed to a given concentration of a chemical compound in water is analogous to a two-compartment model. In this analysis the assumptions are made that:



(1) toxicity is directly related to blood level, D_b; (2) the water concn, D_w, does not change with time; and (3) distribution parameters (pH, compartmentalization, protein binding, fat storage) are constant. In this case, the rate of appearance of D in blood would be equal to the rate of diffusion inward (gills) minus the rate of disappearance from the blood by, for example, conjugation or excretion, or both. Since $[D_w]$ does not change with time, and the rate of biotransformation or excretion may be first order

$$\frac{d[D_b]}{dt} = k_1[D_w] - k_1[D_b] - k_2[D_b] \tag{1}$$

where k_1 is the first-order diffusion constant and k_2 is the first-order rate constant for removal from blood by biotransformation. In the integrated form

$$[D_b] = \frac{k_1}{k_1 + k_2}[D_w](1 - e^{-(k_1+k_2)t}) \tag{2}$$

At the steady state $t \rightarrow \infty$ and

$$[D_b]_{ss} = \frac{k_1}{k_1 + k_2}[D_w] \tag{3}$$

Equation (3) indicates that the steady state blood level of the diffusible species, D, in fish blood is inversely related to k_2 and that, in addition to the water concn, $[D_w]$, the value of k_2 with respect to k_1 is an important determinant of the concentration of D in blood.

If in the present study k_2 represented glucuronide formation, it can readily be seen that inhibition of this process by salicylamide would increase the blood concn of TFM relative to the inhibited state. Since the *O*-methyl derivative of TFM is less

toxic to fish than TFM itself (unpublished observations), it may be inferred that glucuronide formation is a detoxification process.

The experimental results in this report are in agreement with the kinetic model presented above. Salicylamide appeared to inhibit glucuronide formation *in vivo* as well as *in vitro*, as evidenced by a decrease in both the blood level and bile content of TFM glucuronide in salicylamide-treated fish when compared to controls. As predicted by the kinetic model, the blood and tissue levels of free TFM were elevated by salicylamide pretreatment. This effect of salicylamide was not due to an increase in uptake of TFM from the water, since more rather than less TFM glucuronide would be expected in blood and bile if uptake were increased. The results in this study could possibly be explained by an inhibition of uptake of TFM into the liver by salicylamide, but this seems unlikely in view of the reports of others concerning inhibition of conjugation by salicylamide and the demonstrated inhibition of glucuronyl transferase *in vitro* in this report.

The unifying observation in terms of the original hypothesis concerning the importance of glucuronide formation in trout was that salicylamide increased the acute toxicity of TFM to rainbow trout in the same dose that clearly elevated free TFM in blood and tissues and depressed TFM glucuronide in blood and bile. In the face of the evidence presented, it would be difficult to deny not only that conjugation occurs in rainbow trout but also that it may be of great importance to the survival of the fish in some types of polluted waters.

Acknowledgements—The author wishes to thank Mrs. Marie Anderson for her excellent technical assistance in this project. This research was supported in part by an NOAA Sea Grant from the United States Department of Commerce and the State of Wisconsin and by a grant from the Bureau of Sport Fisheries and Wildlife, United States Department of the Interior.

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