Hepatic Glucuronidation and Sulfation of Thyroid Hormones and Their Role in Regulating Thyroidal Status in Rainbow Trout, *Oncorhynchus mykiss*

by

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KENNETH W. FINNSON

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

For My Parents

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Abstract

Hepatic conjugation of thyroid hormones (TH) was studied in rainbow trout, *Oncorhynchus mykiss*, and its role in regulating thyroidal status assessed. Conjugated TH were identified *in vitro* following incubation of isolated hepatocytes with either ^{125}I-labeled thyroxine (T_4) or 3,5,3'-triiodothyronine (T_3) or in bile from trout previously injected with either [^{125}I]T_4 or [^{125}I]T_3. Glucuronidation of T_4 and T_3, and sulfation of T_4 was established *in vitro* and *in vivo*. Although sulfation of T_3 was not established *in vitro*, sulfated T_3 and 3,3'-diiodothyronine (T_2) were detected in bile. These data indicate that the liver is a major site for glucuronidation and sulfation of TH and that these conjugation pathways are involved in biliary excretion of TH.

Hepatic glucuronidation of T_4 and T_3 was catalyzed by glucuronosyltransferases (GTs) located mainly in the microsomal fraction, using uridine-diphosphoglucuronic acid as cofactor. GT activity was heat-sensitive and depended on protein concentration (linear up to ~ 0.75 mg/ml) and time (linear up to 60 min). Optimal pH values for T_4- and T_3-glucuronidation were 6.8-7.8 and ≥ 8.5 units, respectively. Apparent K_m (μM), V_{max} (pmol/mg protein/hr) and V_{max}/K_m values were T_4 = 6.0, 21.3, 3.6 and T_3 = 0.9, 2.6, 2.9. Glucuronidation of T_4 was inhibited by 100 μM of 3,3',5'-triiodothyronine (rT_3) and tetraiodothyroacetic acid (Tetrac) while glucuronidation of T_3 was inhibited by 100 μM of T_4, rT_3, Tetrac (10 and 100 μM) and triiodothyroacetic acid (Triac). Thermal stability of hepatic glucuronidation of T_4 was significantly higher than for T_3 ($P < 0.05$). The different properties for glucuronidation of T_4 and T_3 suggest that trout hepatic glucuronidation of TH is catalyzed by different forms of GT. Brij 56 (0.0125 %) maximally stimulated (~ 2-fold) glucuronidation of both T_4 and T_3, suggesting that these

enzymes are transmembrane proteins with the active site facing the lumen of microsomal vesicles. Clofibrate did not induce glucuronidation of either T_4 or T_3. On a per mg microsomal protein basis, hepatic glucuronidation of T_4 was 3-fold greater in the rat (37 C) than in the trout (12 C).

Hepatic sulfation of TH (T_4, T_3 and rT_3) was catalyzed by sulfotransferases (STs) located mainly in the cytosolic fraction, using 3'-phosphoadenosine 5'-phosphosulfate as a sulfate donor. ST activity was heat-sensitive and depended on protein concentration (up to 1.2 mg/ml) and time (linear up to 60 min). The pH profiles for sulfation of T_4 and T_3 were broad and overlapping with optimal pH values of about 6.5 and 7.0 units respectively. Apparent K_m (μM), V_{max} (pmol/mg protein/hr) and V_{max}/K_m values were T_4 = 1.7, 46 and 27; T_3 = 11.5, 840 and 73 and rT_3 = 0.7, 583 and 832. Inhibitor profiles for both T_4- and T_3-sulfation were not significantly different with a common inhibitor preference of rT_3 > pentachlorophenol > Triac > Tetrac > T_4 = T_3 = 3,5-T_2. Thermal stabilities for sulfation of T_4, T_3 and rT_3 were not significantly different ($P > 0.05$). These data suggest that trout hepatic sulfation of TH is catalyzed by one or more forms of ST with a preference for rT_3.

Hepatic deiodination and desulfation of sulfated TH was studied in trout and rat. Unlike the rat, trout hepatic deiodination of sulfated ^{125}I-labeled T_4, T_3, or rT_3 (1 or 1000 nM) was negligible. Furthermore, unlabeled TH-sulfates (1-100 nM) did not inhibit outer-ring deiodination (ORD) of either T_4 (1 nM) or rT_3 (1 μM). In trout, T_4S was desulfated by the nuclear (3.7 %) and lysosomal fractions (1.1 %), and a small amount of rT_3 was desulfated by the microsomal fraction (0.3 %). T_3S was desulfated by the microsomal fraction (4.6 %), the lysosomal fraction (5.7 %) and by freshly isolated hepatocytes (4.7

%).

The role of hepatic glucuronidation and sulfation of TH in regulating thyroidal status in trout was assessed in response to T_3-treatment and food ration. T_3-treatment elevated plasma T_3 levels (2-fold), decreased hepatic T_4ORD activity, and induced inner-ring deiodination (IRD) of both T_4 and T_3 ($P < 0.05$). The effect of T_3-treatment on either rT_3ORD activity, glucuronidation or sulfation of TH was negligible ($P > 0.05$). Plasma T_4 levels were significantly lower in trout fed a 0.5 % body weight (BW) ration than in trout fed either a 0 or 2.0 % BW ration ($P < 0.05$). Fasting (0 % BW) decreased T_4ORD activity and increased rT_3ORD activity relative to fed (0.5 or 2.0 % BW) trout. Glucuronidation of T_4, T_3 and rT_3 were lower in trout fed 2.0 % BW than in trout fed lower (0, 0.5 % BW) rations. Glucuronidation of T_3 was also lower in trout fed 0.5 % BW than in fasted (0 %) trout. Sulfation of T_3 and rT_3 were both significantly higher in trout fed 2.0 % BW than in trout fed lower (0, 0.5 % BW) rations. Thus, T_3-treatment influences thyroidal status in trout by altering hepatic deiodination and food ration influences thyroidal status by altering hepatic deiodination, glucuronidation and sulfation pathways.

In conclusion, glucuronidation and sulfation are important pathways for hepatic metabolism of TH in trout. Both hepatic pathways are involved in biliary excretion of TH, although sulfation may also play a role in cellular storage of TH. In contrast to mammalian systems, sulfation of TH blocks subsequent deiodination and therefore is not a mechanism for salvaging iodine. Although T_3-treatment and food ration influence thyroidal status by altering hepatic deiodination, food ration can also influence thyroidal status by influencing glucuronidation and sulfation pathways.

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List of Abbreviations

BW	Body Weight
C	Degrees Celsius
Ci	Curie
CLOF	Clofibrate
D	Dark
DCNP	Dichloronitrophenol
DTT	Dithiothreitol
EA	Ethyl Acetate; Ethanolic Ammonia
EDTA	Ethylenediamine Tetraacetate
g	Gram; X Force of Gravity
GH	Growth Hormone
GT	glucuronosyltransferase
HBSS	Hanks' Balanced Salt Solution
HSI	Hepatosomatic Index
hr	Hour
IRD/5D	Inner-Ring Deiodination
K_m	Enzyme Affinity (Michaelis-Menten Constant)
L	Light; litre
m	milli-
n	nano-
ORD/5'D	Outer-Ring Deiodination
PHAHs	Polyhalogenated aromatic hydrocarbons

PAPS	3'-Phosphoadenosine 5'-Phosphosulfate
PCP	Pentachlorophenol
ppm	Parts Per Million
PTU	Propylthiouracil
p	Pico-
RT	Retention Time
SA	Specific Activity
SEM	Standard Error of the Mean
SO_4^{-2}	Sulfate
ST	Sulfotransferase
T_2	diiodothyronine
T_3	3,5,3'-triiodothyronine
rT_3	3,3',5'-triiodothyronine (reverse T_3)
T_4	thyroxine
TCB	Tetrachlorobiphenyl
TFA	Trifluoroacetic Acid
TH	Thyroid Hormone
Tetrac	Tetraiodothyroacetic Acid
Triac	Triiodothyroacetic Acid
μ	Micro-
UDPGA	Uridine-Diphosphoglucuronic Acid
uv	Ultraviolet
V_{max}	Maximum Velocity of Enzyme Reaction

Chapter 1

General Introduction

Thyroxine (T_4) is the main thyroid hormone (TH) secreted by the vertebrate thyroid gland, and is probably the only TH secreted by the thyroid gland of fish (Eales and Brown, 1993). T_4 is considered a prohormone because it has little intrinsic activity unless it is converted to bioactive 3,5,3'-triiodothyronine (T_3) by deiodination in extrathyroidal tissues (McNabb, 1992). The structures of T_4 and T_3 are shown in Figure 1-1.

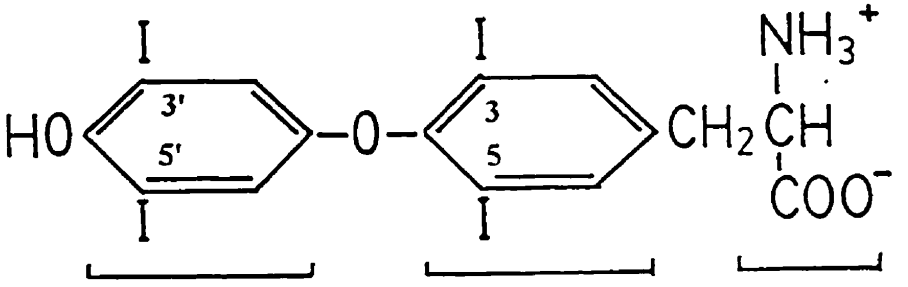
Deiodination and conjugation are the main pathways for metabolism of TH and have been studied extensively in mammals. Deiodination involves both activation and inactivation of TH while conjugation (glucuronidation and sulfation) inactivates TH, and increases their water-solubility which enhances their excretion in bile or urine (Visser, 1990). Although glucuronidation of TH favors excretion in bile, TH-glucuronides can be digested by bacteria in the intestine and the free TH reabsorbed. Furthermore, sulfation accelerates deiodination of most iodothyronines (Visser, 1994; Visser et al., 1990). Consequently, these conjugation pathways contribute significantly to overall TH metabolism.

Studies on TH metabolism in non-mammalian vertebrates have largely been restricted to deiodination, and in comparison to mammals, little is known about either glucuronidation or sulfation of TH. Since studies on rainbow trout, *Oncorhynchus mykiss*, have provided a fairly extensive picture of TH deiodination in fish, the trout was chosen as the model species for investigating TH conjugation.

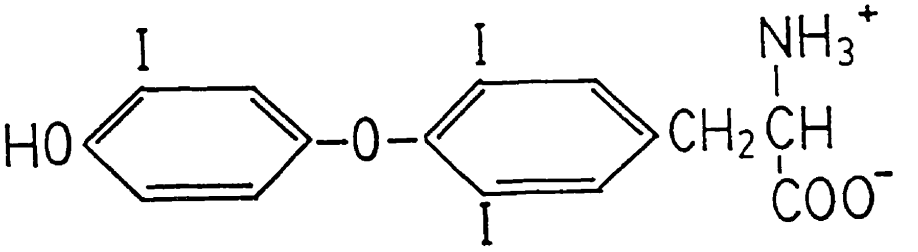
The main objectives of this study were to characterize hepatic glucuronidation and sulfation of TH in trout, and to assess the role of these pathways in regulating thyroidal status. Following a selected literature review, this study is presented in the following order of objectives: 1) Identification of both glucuronidated and sulfated forms of TH produced by freshly isolated hepatocytes and excreted in bile; 2) Characterization of hepatic glucuronidation of TH; 3) Characterization of hepatic sulfation of TH; 4) Assessment of the potential deiodination and desulfation of sulfated TH substrates; 5) Determination of the effects of T_3-treatment and food ration on hepatic glucuronidation and sulfation of TH, and the role of these pathways relative to hepatic deiodination in regulating thyroidal status in trout.

Figure 1-1. Structure of L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3).

THYROXINE = T4



3,5,3'-TRIIODOTHYRONINE = T3



Chapter 2

Selected Literature Review

Overview of TH metabolism

Thyroid hormones (TH) are metabolized by deiodination, conjugation, deamination, decarboxylation and ether-link cleavage (Fig. 2-1). Outer-ring deiodination (ORD) of thyroxine (T_4) produces bioactive 3,5,3'-triiodothyronine (T_3) and inner-ring deiodination (IRD) produces inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3). Further stepwise deiodination of T_3 or rT_3 produces diiodothyronines (T_2's), monoiodothyronines (T_1's) and finally thyronine (T_0) (Fig. 2-2). In rats, deiodination accounts for about 80 % of T_4 degradation (McNabb, 1992).

Conjugation of the 4'-hydroxyl group with either glucuronic acid or sulfate are the other main pathways for TH metabolism. These reactions increase water-solubility of TH, which enhances their excretion in bile and urine (Visser, 1990). Furthermore, glucuronidation is the initial step in the enterohepatic circulation of TH (Distefano, 1988), while sulfation accelerates deiodination of most iodothyronines (Visser, 1994; Visser et al., 1990). Deamination, decarboxylation and ether-link cleavage play only minor roles in TH metabolism (McNabb, 1992).

This chapter reviews the main properties of pathways involved in TH metabolism. The main emphasis is on mammalian systems, but non-mammalian vertebrates are also considered where information is available.

Ether-link cleavage

Ether-link cleavage (ELC) of T_4 is an oxidative reaction yielding diiodotyrosine and an unstable quinone as products (Visser, 1990). Although it is quantitatively the least important pathway for TH metabolism, it may become more prominent when peroxidase activity is stimulated in certain tissues, such as in phagocytosing leukocytes (Engler and Burger, 1984). ELC has not been documented in non-mammalian species.

Side-chain modification

The amino acid side-chain of TH can be modified by successive deamination and decarboxylation. In healthy humans, production of tetraiodothyroacetic acid (Tetrac) accounts for only 2 % of T_4 metabolism, while production of triiodothyroacetic acid (Triac) accounts for about 14 % of T_3 metabolism (Visser, 1990). Triac can be produced by either successive deamination and decarboxylation of T_3, or by ORD of Tetrac (McNabb, 1992).

Like their native iodothyronines, thyroacetic acids can undergo deiodination and conjugation. In mammals, Tetrac has a relatively high propensity for deiodination and Triac binds avidly to putative nuclear T_3 receptors. However, despite their potential for physiological actions, these thyroacetic acids probably have negligible effects due to their rapid deiodinative breakdown (McNabb, 1992). Tetrac is also the preferred substrate for deiodination in rainbow trout, *Oncorhynchus mykiss* (Frith and Eales, 1996).

Deiodination

At least three different deiodinase enzymes have been detected in various tissues

Figure 2-1. Metabolic pathways for thyroid hormones: deiodination, conjugation, deamination, decarboxylation and ether-link cleavage.

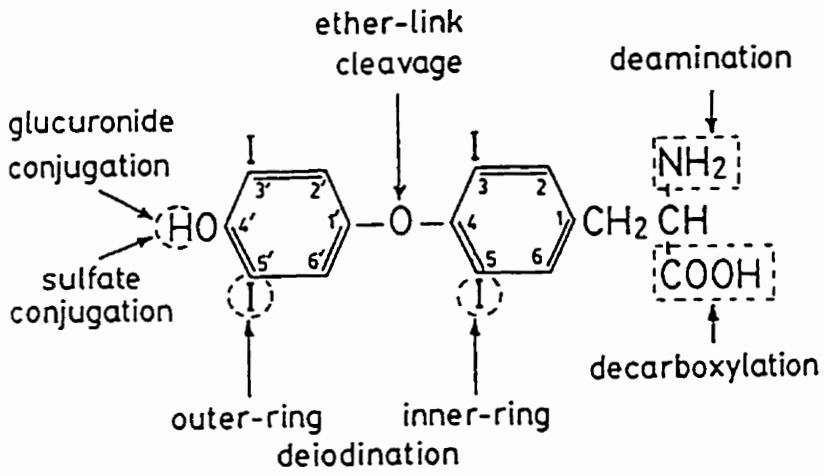
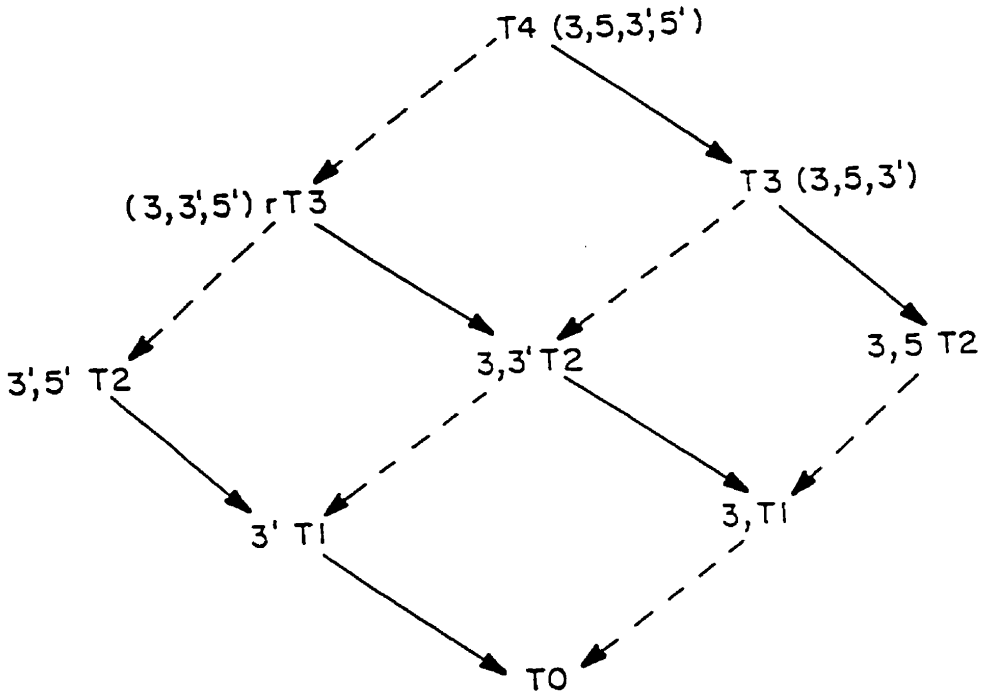

ether-link
cleavage
deamination
glucuronide
conjugation
sulfate
conjugation
HO
O
CH2 CH
NH2
COOH
decarboxylation
outer-ring
deiodination
inner-ring

Figure 2-2. Outer-ring deiodination (---) and inner-ring deiodination (---) pathways for thyroid hormones.


T4 (3,5,3',5')
(3,3',5') rT3
T3 (3,5,3')
3',5' T2
3,3' T2
3,5 T2
3' T1
3, T1
T0

of the rat. The type I enzyme is located mainly in liver and kidney, and catalyzes both ORD and IRD of various iodothyronines. It is a high K_m enzyme (~ 1 μM) with a substrate preference of $rT_3 >> T_4 > T_3$ (McNabb, 1992). These enzymes also have a high affinity for sulfated iodothyronines (Visser, 1994; Visser et al., 1990), exhibit ping-pong kinetics and are sensitive to inhibition by propylthiouracil (PTU) (McNabb, 1992). Under normal conditions, type I deiodination is important for producing plasma T_3 and eliminating plasma rT_3 (St. Germain, 1994).

The type II enzyme is present in brain, pituitary and brown adipose tissue and catalyzes only ORD. It is a low-K_m enzyme (~ 1 nM) with a substrate preference of $T_4 > rT_3$ (McNabb, 1992). This enzyme regulates local T_4 to T_3 conversion in these tissues, and may contribute to peripheral production of T_3 during hypothyroidism (St. Germain, 1994). The type III enzyme is located in brain, skin and placenta and catalyzes only IRD. It is a relatively low-K_m enzyme (~ 40 nM) with a substrate preference of $T_3 > T_4$ (McNabb, 1992). This enzyme is important in regulating local T_3 tissue concentrations, and in the production and clearance of plasma rT_3 (St. Germain, 1994).

The three mammalian deiodinases are located in the microsomal fraction of these tissues and under *in vitro* conditions use simple thiols such as dithiothreitol (DTT) as cofactor. The type II and III enzymes are further distinguished from the type I enzyme by their low affinity for sulfated iodothyronines (Visser, 1994; Visser et al., 1990), their sequential reaction mechanism and insensitivity to inhibition by PTU (McNabb, 1992).

In birds, at least three different type of deiodinases have been detected in various tissues and at different stages of development. A type I-like enzyme has been reported in

liver of adult chicken (Rudas, 1986) and quail (Freeman and McNabb, 1991), and in intestine of developing and adult chickens (Suvarna et al., 1993). Both types I and III activity have been reported in chicken embryo (Galton and Hiebert, 1987), and types I, II and III activity have been reported in brain and liver tissue of chick embryos (Valverde et al., 1993).

Information on deiodination of TH in reptiles is limited to a few studies. Joos and John-Alders (1989) have demonstrated hepatic 5'D (ORD) activity in the lizard, *Sceloporous occidentalis*. Wong et al. (1993) have also described 5'D (ORD) activity in liver, kidney and pancreas of the striped racer snake, *Elaphe taeiniura*. These studies concluded that 5'D activity had similar properties with those of mammalian type I deiodination.

Amphibians have at least two different deiodinases showing different patterns of activity during development and metamorphosis. In the bullfrog, *Rana catesbeiana*, a low-K_m 5'D (ORD) is present in skin during all stages of development, in gut and tail during metamorphic climax, but in neither liver nor kidney. The properties of this enzyme are similar to those of the mammalian type II enzyme (Galton, 1988b). In contrast to 5'D activity, 5D (IRD) activity occurs in tadpole liver. Its activity is highest during premetamorphic climax and declines to undetectable levels during spontaneous or TH-induced metamorphic climax. The properties of this enzyme are similar to those of the mammalian type III enzyme (Galton, 1988b). A mammalian type I-like deiodinase has not been reported in any amphibians studied to date.

Different types of deiodinases have also been described in fish. In rainbow trout,

Oncorhynchus mykiss, two 5'D (ORD) systems have been described with similar properties of mammalian type I and II enzymes (MacLatchy and Eales, 1992; Orozco et al., 1997). Mol et al. (1997) have also demonstrated PTU-insensitive type I-like rT_3ORD activity in kidney, and type II- and III-like T_4ORD activity in brain and gill of tilapia, *Oreochromis niloticus*.

A close relationship has been established among various vertebrate groups by cloning of deiodinases from divergent species including rat, mouse, human, dog, chicken, bullfrog (*Rana catesbeiana*), clawed toad (*Xenopus laevis*), killifish (*Fundulus heteroclitus*) and tilapia (*Oreochromis niloticus*)(St. Germain and Galton, 1997). All these deiodinases contain the rare amino acid selenocysteine in their active sites which is necessary for catalytic activity. The deiodinases cloned so far also contain two essential histidines and a hydrophobic region at the amino terminus of the protein, probably representing a transmembranic domain (St. Germain and Galton, 1997). Thus, the deiodinase family of selenoproteins has been highly conserved during vertebrate evolution.

Glucuronidation

Glucuronidation involves the transfer of glucuronic acid from uridine diphospho-glucuronic acid (UDPGA) to functional groups, particularly hydroxyl groups of many endogenous and exogenous substrates. The reaction is catalyzed by UDP-glucuronosyltransferases (UDPGTs), a family of homologous enzymes located in the microsomal fraction of liver and other tissues (Visser, 1990). These enzymes are transmembrane proteins of the endoplasmic reticulum (ER) with the active site facing the

lumen (Mulder, 1992).

Glucuronidation of TH involves different types of UDPGT enzymes. In rats, glucuronidation of T_4 is stimulated by certain microsomal enzyme inducers such as 3,3',4,4'-tetrachlorobiphenyl (TCB) and ciprofibrate, while glucuronidation of T_3 is only slightly influenced (Beetstra et al., 1991). Furthermore in Gunn rats, which have a defect in the gene coding for both bilirubin and phenol UDPGT isoenzymes, glucuronidation of T_4 and rT_3 is impaired while glucuronidation of T_3 is not affected. Conversely, in Wistar LA, WAG and Fischer rats, which have a defect in the gene coding for androsterone UDPGT, glucuronidation of T_3 is decreased while glucuronidation of T_4 and rT_3 is normal (Visser, 1990). These findings suggests that glucuronidation of T_4 and rT_3 is catalyzed by both bilirubin and phenol UDPGTs, while glucuronidation of T_3 is catalyzed by androsterone UDPGT.

TH-glucuronides produced by liver are excreted in bile (Visser, 1990). However, these conjugates can be digested by bacterial β-glucuronidase activity in the intestine, and liberated iodothyronines can be reabsorbed (Distefano, 1988). Therefore, glucuronidation does not necessarily lead to an irreversible loss of TH.

Little information is available on glucuronidation of TH in non-mammalian species. Hutchins and Newcomer (1966) reported TH-glucuronides in bile of chickens. More recently, Murk et al. (1994) have shown that polyhalogenated aromatic hydrocarbons (PHAHs) increase glucuronidation of T_4 in chicks of the common tern, *Sterna hirundo*. In fish, Osborn and Simpson (1969) have reported TH-glucuronides in marine plaice, *Pleuronectes platessa*. TH glucuronides have also been detected in bile of

brook trout, *Salvelinus fontinalis* and other freshwater fish (Sinclair and Eales, 1972; Collicut and Eales, 1974). Presently, there is no documentation of TH glucuronidation in either amphibians or reptiles.

Sulfation

Sulfation involves the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to hydroxyl groups of many endogenous and exogenous substrates (Visser, 1990). The reaction is catalyzed by sulfotransferases (STs), a family of homologous enzymes located in the cytosolic fraction of liver and other tissues (Visser, 1994; Visser et al., 1990). In rat liver, the different types of STs show an iodothyronine substrate preference of 3'-T_1 > 3,3'-T_2 > T_3 > rT_3 > T_4 (Sekura et al., 1981), indicating that ST activity decreases with the number of iodine substituents in the iodothyronine molecule.

Sulfated TH are not detected in bile of rats under normal conditions. This is due to the rapid hepatic deiodination of these substrates (Visser, 1994; Visser et al. 1990). Sulfated TH have also been reported in marine plaice, *Pleuronectes platessa* (Osborn and Simpson, 1969) and in bile of chickens (Hutchins and Newcomer, 1966). However the metabolic fate of sulfated TH in non-mammalian species is not known. The remainder of this chapter will discuss the significance of successive sulfation and deiodination of iodothyronines by rat liver.

Deiodination of 3,3'-T_2-sulfate

If rat hepatocytes are incubated with low levels (< 10 nM) of ^{125}I-labeled 3,3'-T_2, $^{125}I^-$ is the only product detected. However, at higher levels (> 10 μM) of 3,3'-T_2, or in the presence of PTU to inhibit type I deiodination, 3,3'-T_2-sulfate (3,3'-T_2S) is the main product detected. Furthermore, if sulfation is inhibited by adding ST inhibitors such as salicylamide or dichloronitrophenol (DCNP), or by depleting cellular SO_4^{-2}, clearance of 3,3'-T_2 is reduced (Visser, 1994). These data indicate that 3,3'-T_2 is metabolized by successive sulfation and deiodination.

In rat hepatic microsomes, 3,3'-T_2S undergoes more rapid type I deiodination than unsulfated 3,3'-T_2. Kinetic analysis reveals that sulfation of 3,3'-T_2 causes a 30-fold decrease in apparent K_m value and a 2-fold increase in V_{max} value. Thus, sulfation may be the rate-limiting step in the deiodinative breakdown of 3,3'-T_2 (Visser et al., 1990).

Deiodination of rT_3-sulfate

rT_3 is the preferred substrate for type I deiodination, and sulfation has little effect on its rate of deiodination. In rat hepatocytes, incubations using ^{125}I-labeled rT_3 normally yield only $^{125}I^-$ as a product. However, addition of PTU almost completely blocks $^{125}I^-$ production, with a small amount of rT_3-glucuronide (rT_3G) being formed. 3,3'-T_2 is not detected as a product because it is rapidly eliminated by successive sulfation and deiodination (see above). However, addition of salicylamide or dichloronitrophenol (DCNP), or depletion of cellular SO_4^{-2} leads to an accumulation of 3,3'-T_2S levels accounting for about 70 % of rT_3 metabolism (Eelkman Rooda et al., 1987).

In rat hepatic microsomes, rT_3 and rT_3S both have similar rates of type I ORD, with similar apparent K_m and V_{max} values. However, whereas ORD of rT_3 produces the relatively stable 3,3'-T_2 as a product, ORD of rT_3S produces T_2S which is rapidly deiodinated. Therefore, although sulfation of rT_3 does not increase its deiodination rate, it eventually leads to rapid deiodination of its own deiodination product (Visser et al., 1994). Thus, deiodination may be the rate-limiting step in the catabolism of rT_3.

Deiodination of T_3-sulfate

In rat hepatocytes, incubations with ^{125}I-labeled T_3 normally yield $^{125}I^-$ and T_3G as products. Although addition of PTU inhibits $^{125}I^-$ production, it does not completely block it, suggesting the involvement of another (type II) deiodinase enzyme. Addition of PTU also leads to an accumulation of both T_3S and T_2S. In SO_4^{-2}-depleted cells, production of $^{125}I^-$ is also reduced, with an accumulation of both 3,3'-T_2 and T_2G. Thus in isolated rat hepatocytes, T_3 is metabolized by deiodination, sulfation and glucuronidation in roughly equivalent amounts (Visser, 1994).

In rat hepatic microsomes, T_3S undergoes successive IRD and ORD (Visser et al., 1983). Kinetic analysis of the initial IRD reaction indicates that sulfation of T_3 causes a 30-fold increase in V_{max}, with no change in apparent K_m value (Visser et al., 1990). This facilitating effect of sulfation on T_3-deiodination has also been demonstrated using human liver microsomes (Visser et al., 1988).

Deiodination of T_4-sulfate

In rat hepatocytes, incubations with T_4 labeled in both the inner- and outer-ring produces $^{125}I^-$ as the main product. Addition of PTU reduces $^{125}I^-$ production by ORD but not by IRD, suggesting the involvement of another (type III) deiodinase enzyme. Incubation with higher levels of T_4 progressively saturates both ORD and IRD pathways, and increases production of both T_4G and T_4S (Sato and Robbins, 1981).

In rat hepatic microsomes, T_4 undergoes both ORD and IRD by the type I enzyme producing T_3 and rT_3 respectively. However, T_4S does not undergo ORD, but instead undergoes rapid and specific IRD. Thus, rT_3 occurs only transiently as a product since it is rapidly eliminated by further deiodination (Visser et al. 1990). Furthermore, T_3S is not detected as a product, excluding the pathway $T_4 \rightarrow T_4S \rightarrow T_3S \rightarrow T_3$ as an indirect route for the conversion of T_4 to T_3. Finally, kinetic analysis of the IRD reaction indicates that sulfation of T_4 causes a 6-fold decrease in apparent K_m value, and a 30-fold increase in V_{max} value (Visser et al., 1990). Therefore, sulfation of T_4 accelerates subsequent IRD but prevents IRD of this iodothyronine substrate.

Desulfation

Most sulfated iodothyronines are rapidly deiodinated by the type I deiodinase in rat liver (Visser et al., 1990). However, T_3S can also be desulfated by tissue sulfatase activity producing T_3. Since T_3S does not bind to putative T_3 nuclear receptors and is devoid of thyromimetic activity (Visser, 1994), desulfation of T_3S may provide a source of bioactive of T_4 to target tissues (Kung et al., 1988; Santini et al. 1992).

Chapter 3

Identification of thyroid hormone conjugates produced by isolated hepatocytes and excreted in bile of rainbow trout

Introduction

Thyroxine (T_4) is the main thyroid hormone (TH) secreted from the thyroid gland of rainbow trout, *Oncorhychus mykiss*. By itself, T_4 has little intrinsic bioactivity unless it is converted to 3,5,3'-triiodothyronine (T_3) in extrathyroidal tissues (Eales, 1985). This reaction is catalyzed by a 5'-monodeiodinase (5'D/ORD) enzyme located in the microsomal fraction of liver and other tissues (MacLatchy and Eales, 1992). Since T_3 is considered the active form of hormone, regulation of 5'D/ORD activity is important in governing thyroidal status of trout (Eales and Brown, 1993).

Conjugation is the second major pathway for TH metabolism and involves addition of either glucuronic acid or sulfate to the 4'-hydroxyl group of TH (Visser, 1990). These reactions increase water-solubility of TH, which facilitates their excretion in bile and urine (Visser, 1990). However in rats, glucuronidation may also be the initial step in the enterohepatic circulation of TH (DiStefano, 1988), while sulfation accelerates hepatic deiodination of most iodothyronines (Visser, 1990). Thus, conjugation of TH may also be important in regulating thyroidal status.

Conjugation of TH has not been fully examined in fish. Osborn and Simpson (1969) reported both glucuronidated and sulfated TH in marine plaice, *Pleuronectes platessa*. TH-glucuronides have also been identified in bile of brook trout, *Salvelinus*

fontinalis, and other freshwater teleosts (Sinclair and Eales, 1972; Eales et al., 1983). However, there has been no conclusive identification of other conjugate forms (sulfate) either produced by liver or excreted in bile of fish. The main goal of this study was to identify TH conjugates produced by liver of trout. Freshly isolated hepatocytes were used to establish the liver as a site for conjugation of TH. TH conjugates were also identified in bile from trout previously injected with either ^{125}I-labeled T_4 or T_3 to determine if conjugation of TH is involved in biliary excretion.

Methods and Materials

Fish maintenance

Rainbow trout (200-300 g, 2 yr old) were obtained from the Rockwood Hatchery, Balmoral, Manitoba and held in the laboratory in flowing, aerated, dechlorinated water at 12 C, on a 12L:12D photoperiod. Trout were fed a commercial diet (Martin Feed Mills, Elmira, Ontario, trout feed pellets, 3.2 mm diameter) once daily at a ration of 1% of body weight. Fish were used 18-24 hr after their last meal.

Hepatocyte Preparation

Hepatocytes were isolated by a modification of the collagenase perfusion method (Moon et al., 1985). Individual trout were anesthetized with tricaine methanesulfonate (Syndel Laboratories, Vancouver; 0.07 g/l) and placed on a nylon mesh restraining pad. A volume of 1 ml of ice-cold heparin (Sigma; 1000 U/ml in phosphate-buffered saline) was injected into the caudal vein, the body cavity opened and the liver was removed intact and placed on a steel mesh over a 250-ml glass beaker. A cannula (PE90 tubing)

connected to a Masterflex pump (Cole-Parmer Inst. Co., Chicago, IL; perfusion rate = 4 ml/min) was then inserted into the portal vein and ice-cold medium (A), consisting of modified Hanks' balanced salt solution (HBSS) (140 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 6.0 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.44 mM Hepes, 0.5 mM $MgSO_4$, 6.0 mM glucose; pH 7.6, gassed with O_2:CO_2 [99:1]), was perfused to waste until the perfusate was clear. Collagenase (*Clostridium* sp., Sigma) was then added to the perfusate (160 U/ml), and perfusion continued until disintegration of the liver was evident (30-35 min). During this time, the perfusion medium was recirculating through the liver.

Following perfusion, the cannula was removed and the liver transferred to a 100-ml glass beaker containing about 40 ml of ice-cold medium A. The liver was gently disrupted with the blunt end of a glass pipette to dislodge the cells. This mixture was passed successively through 290- and 70-μm nylon meshes (Spectrum Laboratory Products, Los Angeles, CA), placed in four 10-ml plastic centrifuge tubes, and spun (Damon, IEC) at 100 g for 4 min at 4 C. Supernatants were decanted, and pelleted hepatocytes were resuspended in ice-cold medium B (medium A plus 1 mM $CaCl_2$) and spun again at 100 g for 3 min at 4 C. This procedure was repeated twice and pelleted hepatocytes were resuspended in medium B to a final concentration of ~ 4 X 10^6 cells/ml. Cells were filtered again through a 70-μm mesh and then equilibrated in a 50-ml Erlenmeyer flask supplied with an O_2:CO_2 gas mixture (99:1). Cell viability was tested before each experiment by trypan blue exclusion and exceeded 85%.

Hepatocyte Incubation

[^{125}I]T_4 (specific activity (SA) = 1.5 mCi/mg) and [^{125}I]T_3 (SA = 3 mCi/mg) (Amersham) were radioiodinated in the outer-ring. Prior to hepatocyte incubations, [^{125}I]T_4 was purified by adding 0.1 μCi [^{125}I]T_4 to previously drained Sephadex LH-20 minicolumns (5-ml Quick Sep Column, Isolab, Inc. OH, containing 0.25 g LH-20 Sephadex) equilibrated in 0.1 N HCl. $^{125}I^-$ was eluted to waste with 3 ml H_2O and the remaining [^{125}I]T_4 was collected with 3 ml of 0.1 N NH_4OH in ethanol (1:1 v/v; ethanolic ammonia). This fraction was evaporated and reconstituted in 0.1 N NaOH. [^{125}I]T_3 did not require purification due to its low level of radioiodide contamination (< 3 %) (Sweeting and Eales, 1992a).

All experiments were performed in a water bath shaker (140 rpm) at 12 C. Hepatocytes were equilibrated with HBSS in a 50-ml Erlenmeyer flask supplied with an O_2:CO_2 gas mixture [99:1] for 30 min. A 1-ml aliquot of hepatocyte suspension was then transferred to 3 ml of pre-gassed HBSS in a 25-ml Erlenmeyer flask to achieve a final hepatocyte concentration of 1 X 10^6 cells/ml. Depending on the experiment, a volume of 40 μl of either T_4 (final conc. = 0.1 nM) with ~ 0.1 μCi [^{125}I]T_4 or T_3 (final conc. = 0.1 or 10 nM) with ~ 0.1 μCi [^{125}I]T_3 was added to the suspension, and incubated for two hours.

Sephadex LH-20 chromatography

TH metabolites produced by isolated hepatocytes were separated by Sephadex LH-20 chromatography. After a 2 hr incubation, a volume of 1 ml of hepatocyte suspension

was added to an equal volume of chloroform:methanol (2:1 v/v), vortexed and centrifuged at 1420 g for 5-10 min. A volume of 500 μl of the upper aqueous methanol layer was acidified with an equal volume of 1.0 N HCl and applied to previously drained Sephadex LH-20 minicolumns equilibrated in 0.1 N HCl (Sweeting and Eales, 1992a). Columns were eluted stepwise with 1 ml of 0.1 N HCl (3 x), 0.1 M sodium acetate (pH 4.0, 8 x), H_2O (5 x) and 0.1 N NH_4OH in ethanol (1:1 v/v, 3 x). These fractions represent putative iodide, glucuronide, sulfate and iodothyronine fractions respectively (Eelkman Rooda et al., 1989).

The 0.1 N NH_4OH in ethanol fractions containing unconjugated TH were evaporated at 50 C under a stream of N_2. However, prior to analysis, sodium acetate fractions and H_2O fractions were each pooled, acidified with 2-3 ml of 3 N HCl and added to previously drained LH-20 columns to concentrate putative glucuronides and sulfates respectively. After draining, columns were washed with 2 ml 0.1 N HCl followed by 1 ml H_2O, and putative conjugates were collected with 3 ml 0.1 N NH_4OH in ethanol. The solvent was then evaporated at 50 C under a stream of N_2 as before.

Acid Solvolysis

The acid solvolysis method was validated by synthesis and subsequent solvolysis of T_4 and T_3 -sulfate. Sulfated T_4 and T_3 were synthesized by slowly adding 200 μl of chlorosulfonic acid to 800 μl of dimethylformamide at 0 C. A volume of 200 μl of this mixture was added to another tube containing either ^{125}I-labelled T_4 or T_3 previously evaporated to dryness by vacuum centrifugation (Speed Vac-Savant). Reactions were

allowed to reach room temperature and continued overnight (Mol and Visser, 1985). After dilution with 800 μl ice-cold water, mixtures were added to LH-20 columns and eluted to waste with 3 ml of 0.1 N HCl followed by 2 ml H_2O. Sulfated TH were collected with 5 ml H_2O, evaporated to dryness and treated with or without EA/TFA (1%) for 18 hr at 60 C.

Analysis of TH Metabolites - *in vitro*

To identify putative glucuronide conjugates, samples previously concentrated by LH-20 chromatography and evaporated were incubated with 1 ml 0.05 M phosphate buffer (pH = 6.8) with or without 50 mg β-glucuronidase (Sigma) for 18-24 hr at 37 C. The reaction was stopped by adding 1 ml of 1.0 N HCl and this mixture was added to previously drained LH-20 columns. Columns were washed successively with 3 ml of 0.1 N HCl and 1 ml of H_2O and the remaining ^{125}I-labeled materials were collected with 3 ml of ethanolic ammonia, evaporated and reconstituted in the appropriate buffer for HPLC analysis (see below).

To identify putative sulfate conjugates, samples not previously digested by β-glucuronidase treatment were incubated with either i) 1 ml 0.05 M sodium acetate buffer (pH = 5.0) with or without 25 mg aryl sulfatase (Sigma) containing 8 mM D-saccharic acid 1,4-lactone (to inhibit β-glucuronidase activity) or ii) acid solvolysis using 1-2 ml of ethyl acetate (EA) with trifluoroacetic acid (TFA) (1% v/v) (Scott and Canairo, 1992).

Analysis of biliary metabolites - *in vivo*

Trout were anesthetized in tricaine methanesulfonate (0.07g/l). Two groups of three fish were injected intraperitoneally (i.p.) with 1.0 μCi of either [^{125}I]T_4 or [^{125}I]T_3 and returned to the holding tanks. After 24 hr, fish were anesthetized and killed by concussion and livers and gall bladders removed. Bile from each group was pooled in 1.5-ml Eppendorff tubes and centrifuged at 1420 g for 5 min. The supernatant was incubated with 1 ml 0.05 M phosphate buffer (pH = 6.8) with or without 50 mg β-glucuronidase, as before, to liberate putative glucuronide conjugates. After 24 hr, samples were acidified with 1.0 ml of 0.1 N HCl and added to LH-20 columns. Columns were washed successively with 2 ml HCl and 1 ml H_2O and the remaining activity was collected with 3 ml of 0.1 N NH_4OH in ethanol. This fraction was evaporated as before and redissolved in the appropriate buffer prior to HPLC analysis (see below). Alternatively, samples previously treated with β-glucuronidase were further treated with EA/TFA for 24 hr to liberate putative sulfate conjugates. Samples were prepared by LH-20 prior to HPLC as before.

HPLC analysis

Samples were by evaporated at 50 C under a stream of N_2 and redissolved in either i) 24% acetonitrile in 0.02 N sodium phosphate (pH = 2.7) or ii) 16% acetonitrile in ammonium acetate (pH = 4) corresponding to the HPLC solvent system used. For validation of acid solvolysis method, synthetic T_4- and T_3-sulfate were evaporated to dryness in a Speed Vac and redissolved in methanol.

HPLC analyses were conducted using a Gilson-IBM binary gradient HPLC. Three different solvent systems were used: (A) Acetonitrile/ 0.02 N sodium phosphate (pH =2.7); acetonitrile increasing linearly from 24-28% (0-12 min) and from 28-44% (12-35 min). (B) Acetonitrile/ 0.02 M ammonium acetate (pH = 4.0); acetonitrile increasing linearly from 16-40% (0-15 min) and from 40-45% (15-35 min) (Sweeting and Eales, 1992). (C) Acetonitrile/TFA (0.1%) and H_2O either (i) 42% isocratic or (ii) acetonitrile increasing linearly from 42-45% (0-20 min) and at 45% (20-27 min). The elution positions of authentic iodothyronines (Sigma) were determined by uv absorption. All solvents used were HPLC grade and were filtered and degassed before use.

Results

Sephadex LH-20 chromatography

Radioactive materials obtained from hepatocytes incubated with [^{125}I]T_4 were separated by Sephadex LH-20 chromatography by stepwise elution with 3 ml of 0.1 N HCl, 8 ml of sodium acetate (pH 4.0), 5 ml of H_2O and 3 ml of ethanolic ammonia. These fractions represent putative iodide, glucuronide, sulfate and iodothyronine fractions respectively (Rutgers et al., 1987). When each of these fractions were acidified and added to separate LH-20 columns, stepwise elution clearly separated these materials (Fig. 3-1). Radioactivity recovered in the expected fraction exceeded 92 %.

For radioactive materials obtained from hepatocytes incubated with [^{125}I]T_3, stepwise elution clearly separated the iodide and glucuronide fractions (Fig. 3-2). However, stepwise elution of the H_2O fraction showed radioactivity in both H_2O and

ethanolic ammonia fractions. This may be due to desulfation during preparation or incomplete separation of iodothyronines and their sulfate conjugates.

Acid solvolysis of synthetic iodothyronines

HPLC analyses of untreated T_4- and T_3-sulfate both revealed single major peaks with similar retention times (RT) (~ 5 min, Figure 3-3A and 3-3C respectively). Acid solvolysis of T_4-sulfate eliminated the peak and generated a [^{125}I]T_4 peak (RT=21 min) (Fig. 3-3B) confirming solvolysis of T_4-sulfate. Acid solvolysis of T_3-sulfate eliminated the peak and generated a [^{125}I]T_3 peak (RT=12 min) (Fig. 3-3D) confirming solvolysis of T_3-sulfate.

HPLC analysis of TH metabolites - *in vitro*

HPLC analyses of untreated sodium acetate fractions obtained from incubation of hepatocytes with [^{125}I]T_4 revealed a single peak (RT=12.5 min, Fig. 3-4A). Treatment with β-glucuronidase eliminated this peak and generated a [^{125}I]T_4 peak (RT=33 min) (Fig. 3-4B) establishing T_4-glucuronide (T_4G) in this fraction. A single peak (RT=11 min) was also present in the H_2O fraction (Fig. 3-4C) which was unaffected by β-glucuronidase treatment (data not shown). However, acid solvolysis eliminated this peak and generated a T_4 peak (RT=33 min, Fig. 4D), establishing T_4-sulfate (T_4S) in this fraction. HPLC analysis of the NH_4OH in ethanol fraction showed a major T_4 peak and a smaller T_3 peak (data not shown) indicating that a small amount of outer-ring deiodination (ORD) had occurred.

HPLC analysis of the sodium acetate fraction obtained from incubating hepatocytes with [^{125}I]T_3 revealed a single peak (RT=11.5 min, Fig. 3-5A). Treatment with β-glucuronidase eliminated this peak and generated a [^{125}I]T_3 peak (Fig. 3-5B), establishing T_3-glucuronide (T_3G) in this fraction. Recovery of the radioactivity from the H_2O fractions was too low to establish sulfated TH in this fraction. HPLC analysis of the NH_4OH in ethanol fraction revealed a single peak corresponding to T_3 (data not shown).

HPLC analysis of biliary metabolites - *in vivo*

At least three peaks were identified in the HPLC profile of biliary metabolites obtained from [^{125}I]T_4-injected trout (Fig. 3-6A). The largest peak corresponded to T_4 (RT=18 min); there was also a small radioiodide peak (RT=3.5 min) and a small T_3 peak (RT=15 min). Treatment with β-glucuronidase completely eliminated one peak (RT=12 min), and increased the proportion of T_4 and T_3, confirming that trout bile contains both T_4 and T_3 as glucuronide conjugates (Fig. 3-6B). Treatment of bile with aryl sulfatase did not change the profile of radioactivity (data not shown). However, acid solvolysis of bile previously treated with β-glucuronidase eliminated the remaining unknown peak at RT = 11 min, increasing levels of both T_4 and T_3 and producing a small amount of 3,3'-T_2 (Fig. 3-6C), establishing of T_4-, T_3-, and T_2-sulfates in trout bile.

In bile from [^{125}I]T_3-treated trout, most radioactivity occurred between RT values of 5-10 min, with negligible amounts of radioiodide (RT=3.5 min), 3,3'-T_2 (RT=13 min) or T_3 (RT=16 min; Fig. 3-7A). Treatment with β-glucuronidase generated a major T_3 peak and a smaller 3,3'-T_2 peak, establishing T_3G and T_2G in trout bile (Fig. 3-7B). However,

considerable amounts of radioactivity remained in overlapping peaks between RT=5-10 min. This profile was not changed by treatment with aryl sulfatase (data not shown). However, acid solvolysis eliminated the remaining unidentified peaks, increasing levels of both T_3 and T_2 (Fig. 3-7C), establishing T_3S and T_2S in trout bile.

Discussion

Prior to this study, little information was available on conjugation of TH in non-mammalian species. Early studies identified glucuronidated forms of TH in bile of brook trout, *Salvelinus fontinalis* and other freshwater teleosts (Eales and Sinclair, 1972) and channel catfish, *Ictalulus punctatus* (Collicut and Eales, 1974) suggesting the liver as a potential site for TH glucuronidation in fish. The main objective of this study was to identify TH conjugates produced by liver and excreted in bile of rainbow trout, *Oncorhynchus mykiss*.

Glucuronidation of both T_4 and T_3 was established in isolated hepatocytes following 2-hr incubation with either ^{125}I-labeled T_4 or T_3. TH-glucuronides were also detected in bile of trout previously injected with either ^{125}I-labeled T_4 or T_3. This is consistent with previous studies on fish demonstrating glucuronidation of TH as a major hepatic pathway leading to biliary loss of TH in teleosts (Sinclair and Eales, 1972).

T_4-sulfate was produced by isolated hepatocytes and detected in bile of trout injected with [^{125}I]T_4. Although T_3-sulfate was not established *in vitro*, it was present in bile of trout previously injected with [^{125}I]T_3. Therefore, sulfation of TH is also an important pathway involved in biliary excretion of TH.

The inability to demonstrate T_3-sulfation by isolated hepatocytes of trout may be due to *in vitro* assay conditions. Unlike sulfated TH present in bile, T_3-sulfate present *in vitro* remains within the closed system rendering it susceptible to further degradation. This occurs in rat hepatocytes where T_3-sulfate undergoes rapid deiodinative breakdown (Visser, 1994; Visser et al., 1990).

Sulfation of TH in mammals may have evolved for rapid deiodinative breakdown of TH. Indeed, sulfation accelerates hepatic deiodination of most iodothyronines in rat liver (Visser, 1994; Visser et al., 1990). Consequently, sulfated iodothyronines are not normally detected in bile or plasma of rats (Visser, 1990). In contrast, sulfated TH were found in bile of trout held under normal conditions. Thus, sulfation of TH in trout may simply function as a pathway for biliary excretion.

Conjugated and unconjugated forms of T_4 and T_3 were detected in bile from trout previously injected with [^{125}I]T_4 indicating that outer-ring deiodination (ORD) of T_4 had occurred. This was expected since T_3 is the main deiodination product of T_4 in liver and other tissues (Eales and Brown, 1993). Furthermore, conjugated forms of 3,3'-T_2 were detected in bile from trout previously injected with [^{125}I]T_3. Thus, in contrast to previous studies (Sweeting and Eales, 1992; MacLatchy and Eales, 1993), inner-ring deiodination (IRD) of T_3 to T_2 occurs in trout under normal conditions. However, whether conjugation precedes deiodination of T_3 to T_2 is unknown.

In summary, conjugated forms of TH were identified in isolated hepatocytes following incubations with either [^{125}]T_4 or [^{125}I]T_3 or in bile from trout previously injected with either [^{125}I]T_4 or [^{125}I]T_3. Glucuronidation of T_4 and T_3, and sulfation of T_4

occurred both *in vitro* and *in vivo*. Although sulfation of T_3 was not established *in vitro*, sulfated T_3 and T_2 were detected in bile. This has established the liver as a direct site for glucuronidation and sulfation of TH. Furthermore, the presence of both glucuronidated and sulfated TH in trout bile suggests that both pathways are involved in biliary excretion of TH.

Figure 3-1. Sephadex LH-20 chromatography of putative (A) I^-, (B) glucuronide, (C) sulfate and (D) iodothyronine fractions obtained from hepatocyte/[^{125}I]T_4 incubates. LH-20 columns were eluted stepwise with 1-ml fractions of 0.1 N HCl (3 x), 0.1 M sodium acetate (pH 4.0; 12 x), H_2O (6 x) and ethanolic ammonia (3 x).

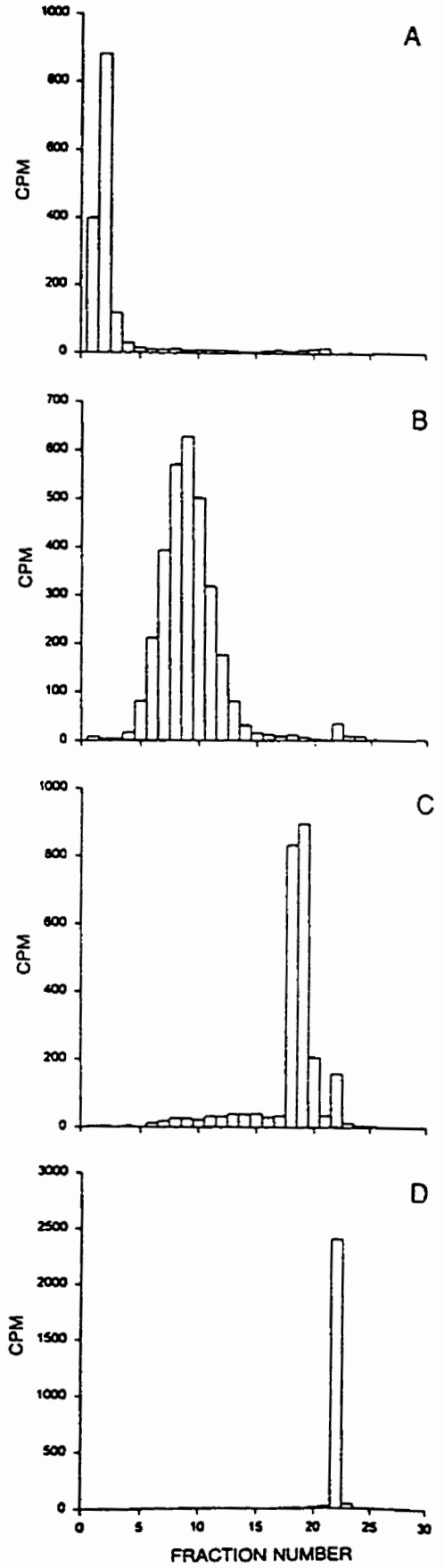

A
B
C
D
CPM
FRACTION NUMBER
0
5
10
15
20
25
30

Figure 3-2. Sephadex LH-20 chromatography of putative (A) I^-, (B) glucuronide, (C) sulfate and (D) iodothyronine fractions obtained from hepatocyte/[^{125}I]T_3 incubates. LH-20 columns were eluted stepwise with 1-ml fractions of 0.1 N HCl (2 x), 0.1 M sodium acetate (pH 4.0; 8 x), H_2O (5 x), and ethanolic ammonia (3 x).

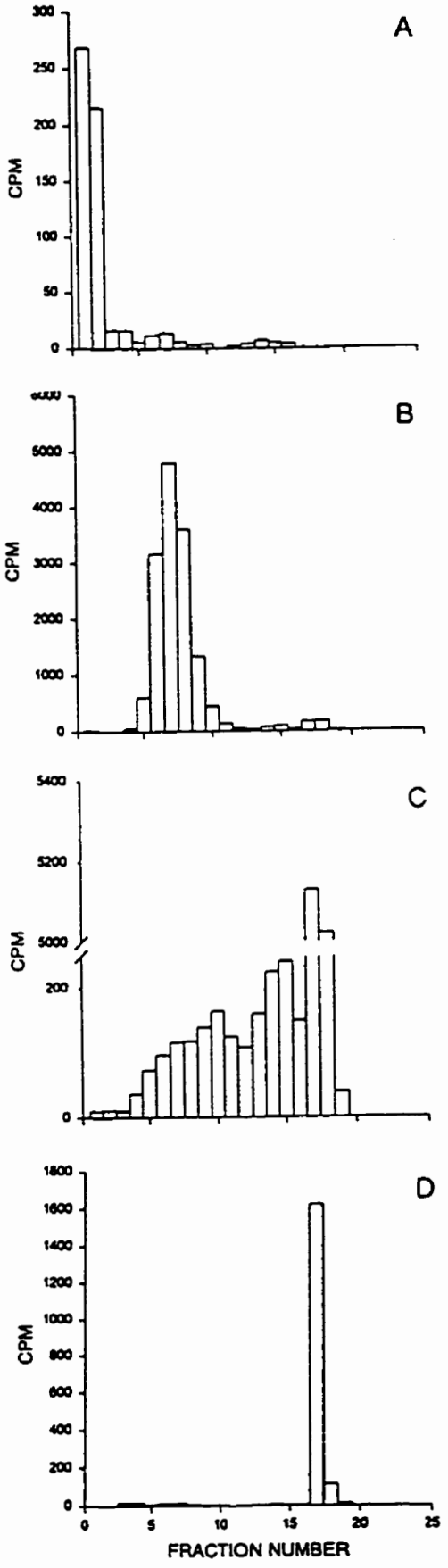

A
B
C
D
CPM
CPM
CPM
CPM
300
250
200
150
100
50
0
5000
4000
3000
2000
1000
0
5400
5200
5000
200
0
1800
1600
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
FRACTION NUMBER

Fig. 3-3. HPLC profiles of ^{125}I-labelled metabolites obtained from synthesis and subsequent acid solvolysis of T_4 and T_3 -sulfate. (A) synthetic T_4-sulfate, (B) T_4 liberated from the acid solvolysis of synthetic T_4-sulfate, (C) synthetic T_3-sulfate, T_3 liberated from the acid solvolysis of synthetic T_3-sulfate. Solvent system C (ii) was used for A, B and C. Solvent system C (i) was used for D.

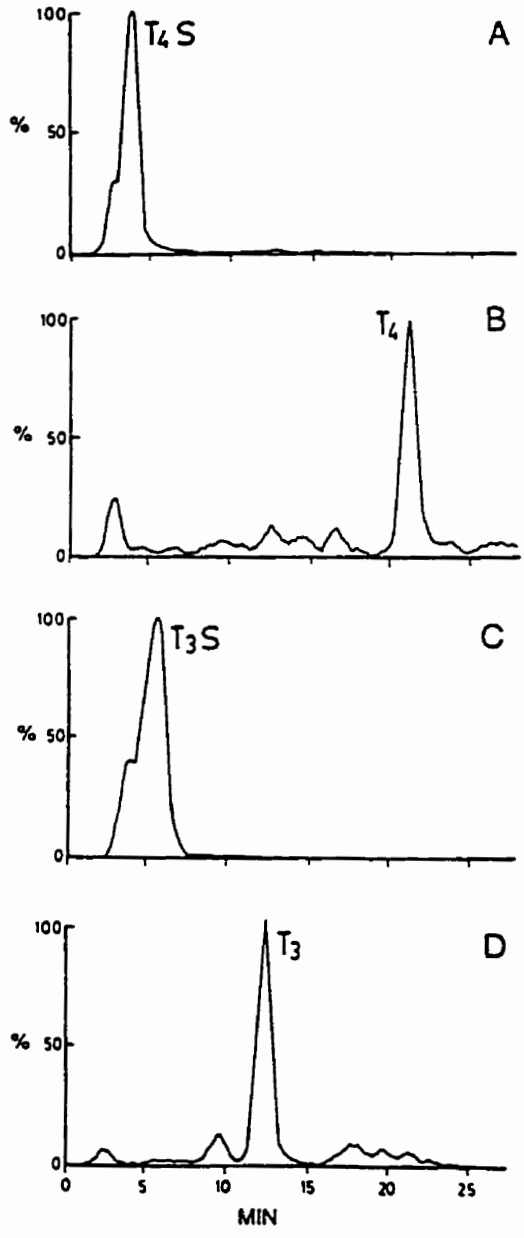

100
% 50
0
T4 S
A
100
% 50
0
T4
B
100
% 50
0
T3S
C
100
% 50
0
T3
D
0
5
10
15
20
25
MIN

Figure 3-4. HPLC profiles (solvent system B) of ^{125}I-labeled metabolites obtained from freshly isolated hepatocytes incubated for 2 hr with [^{125}]T_4.(A) sodium acetate fraction (control), (B) sodium acetate fraction treated with β-glucuronidase, (C) H_2O fraction (control) and (D) H_2O fraction treated with EA/TFA (1%). C could not be digested with β-glucuronidase. Radioactivity is expressed as the percentage of that recorded for the tallest peak.

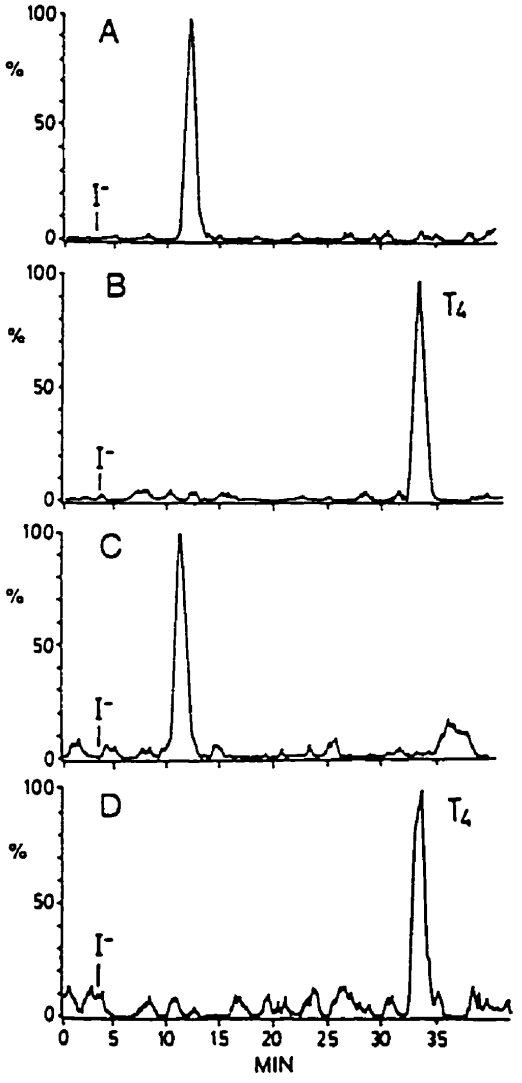

A
B
C
D
100
%
50
0
I⁻
T4
0 5 10 15 20 25 30 35
MIN

Figure 3-5. HPLC profiles (solvent system B) of ^{125}I-labeled metabolites obtained from freshly isolated hepatocytes incubated for 2 hr with [^{125}]T_3. (A) sodium acetate fraction (control), (B) sodium acetate fraction treated with β-glucuronidase. Radioactivity is expressed as the percentage of that recorded for the tallest peak. H_2O fractions was were not analyzed due to incomplete separation on LH-20 columns.

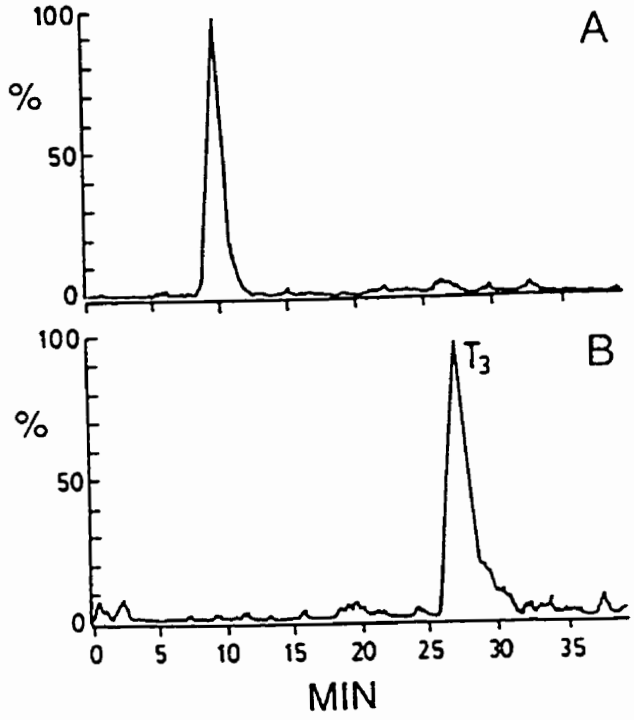

100
%
50
0
A
100
%
50
0
T_3
B
0
5
10
15
20
25
30
35
MIN

Figure 3-6. HPLC profiles (solvent system A) of ^{125}I-labeled biliary metabolites 24 hr after i.p. injection with [^{125}]T_4. (A) control, (B) treated with β-glucuronidase and (C) treatment with EA/TFA (1%) following treatment with β-glucuronidase. Retention times of uv standards are shown in (C). Radioactivity is expressed as the percentage of that recorded for the tallest peak.

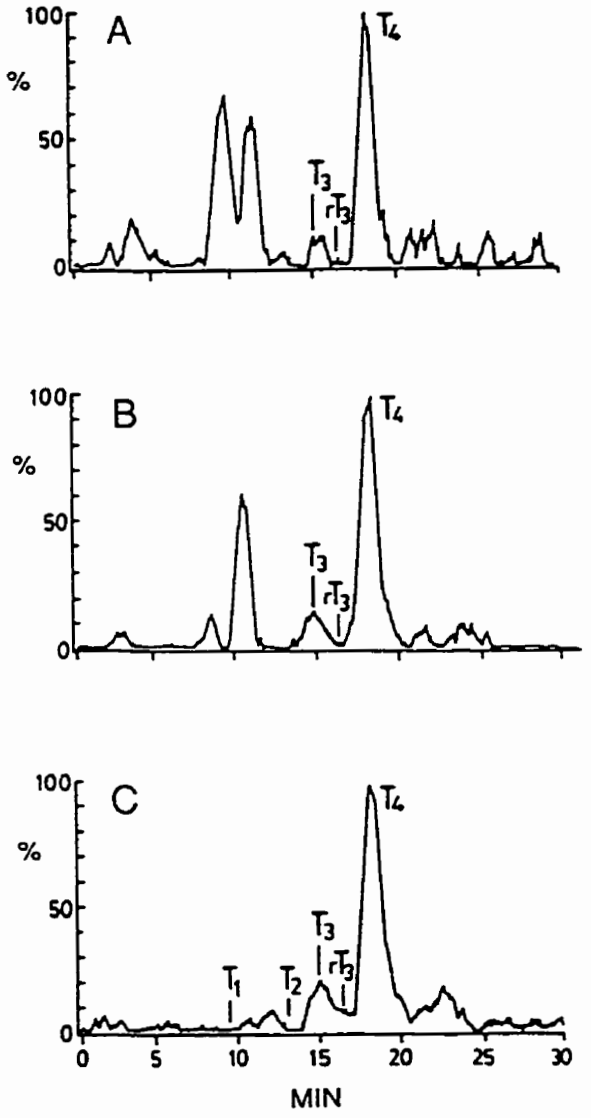
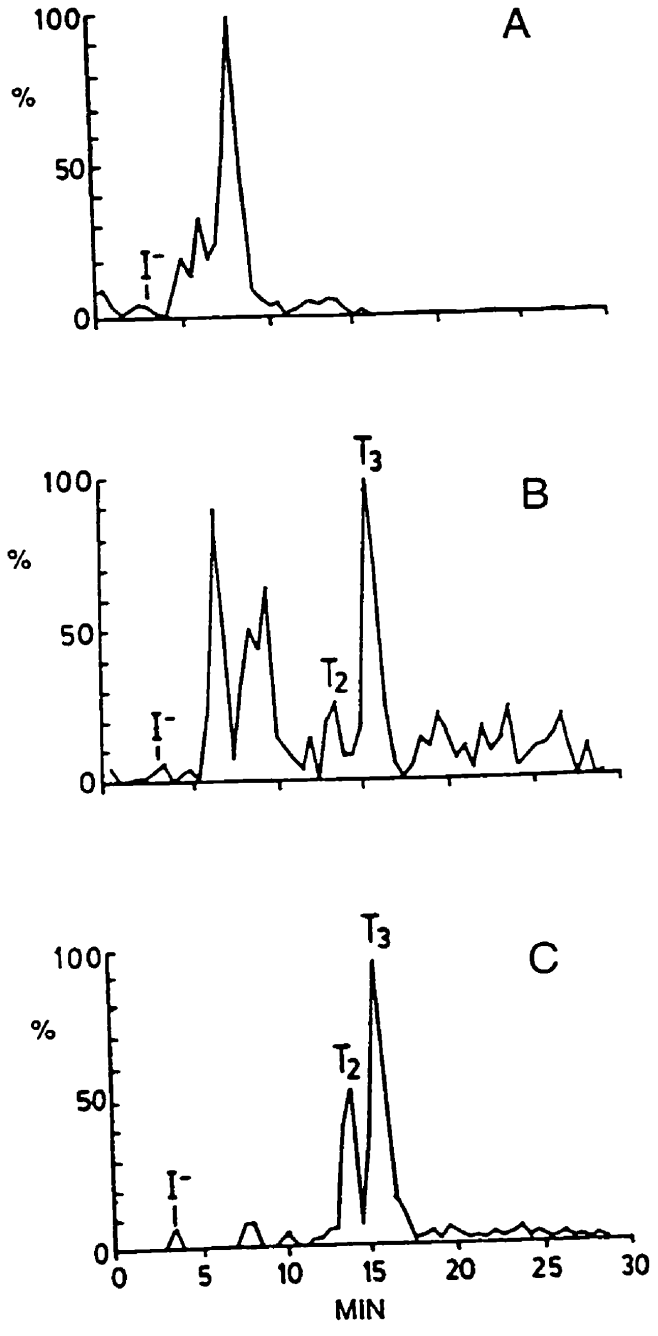

100
%
50
0
A
T4
T3
rT3
B
C
T1
T2
0
5
10
15
20
25
30
MIN

Figure 3-7. HPLC profiles (solvent system A) of ^{125}I-labeled biliary metabolites 24 hr after i.p. injection with [^{125}]T_3. (A) control, (B) treated with β-glucuronidase and (C) treatment with EA/TFA (1%) following treatment with β-glucuronidase. Radioactivity is expressed as the percentage of that recorded for the tallest peak.


A
100
%
50
0
I⁻
B
T3
T2
C
0
5
10
15
20
25
30
MIN

Chapter 4

Glucuronidation of thyroxine and 3,5,3'-triiodothyronine by liver of rainbow trout

Introduction

Deiodination and conjugation are the main pathways for metabolism of thyroid hormones (TH). Thyroxine (T_4) undergoes either outer- or inner-ring deiodination to produce bioactive 3,5,3'-triiodothyronine (T_3) or inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3) respectively (Visser, 1990). T_3 and rT_3 can undergo further deiodination and the liberated iodide can be recycled back to the thyroid for TH synthesis (McNabb, 1992).

Conjugation of the 4'-hydroxyl group of TH with either glucuronic acid or sulfate inactivates TH, increases their water solubility and facilitates their excretion in bile and urine (Visser, 1990). Glucuronidation is also the first step in the enterohepatic circulation of TH (Distefano, 1988) while sulfation accelerates deiodination of most iodothyronine substrates (Visser, 1994; Visser et al., 1990).

Studies on rats with genetic defects in uridine-diphosphoglucuronosyltransferase (UDPGT) isoenzymes indicate that both T_4 and rT_3 are glucuronidated by phenol and bilirubin UDPGTs while T_3 is glucuronidated by androsterone UDPGT (Visser et al., 1993a,b). Certain microsomal enzyme inducers (eg. clofibrate) also stimulate hepatic glucuronidation of T_4 with little effect on glucuronidation of T_3. This indicates that there are different UDPGT enzymes for T_4 and T_3 in rat.

Comparatively little is known about glucuronidation of TH in non-mammalian

vertebrates. In the previous chapter, glucuronidation was established as a major hepatic pathway involved in biliary excretion of TH in trout (Finnson and Eales, 1996). However, the properties of enzymes involved in glucuronidation of TH in trout are unknown. This study has the following objectives: i) to determine the key properties of hepatic glucuronidation of TH in trout, and ii) to determine if, as in mammals, glucuronidation of T_4 and T_3 are catalyzed by different forms of UDPGT.

Materials and Methods

Fish maintenance

Rainbow trout (250-500 g, 2 yr old) were obtained from the Rockwood Hatchery, Balmoral, Manitoba, and held in the laboratory as in chapter 3. Trout were used 18-24 hr after their last meal. Male Sprague Dawley rats received food and water ad libitum.

Subcellular fractions

Trout were anesthetized in tricaine methanesulfonate (0.07 g/l) and killed by concussion. Rats were killed by CO_2 asphyxiation. Livers were removed and rinsed with ice-cold buffer: 0.25 M sucrose (pH=7.4) with 1 mM EDTA. Livers from several trout (or rats) were pooled and added to 4 vol (w/v) of buffer, minced with scissors and dispensed into a 55-ml Wheaton homogenization chamber. Tissue was homogenized by three strokes of a motorized pestle (Tri-R Instruments, Inc., NY). The resulting homogenate was filtered through two layers of cheesecloth and transferred to plastic centrifuge tubes. Samples were centrifuged for 20 min at 730 g to obtain the nuclear

pellet. The post-nuclear supernatant was centrifuged for 20 min at 25,200 g to obtain the crude mitochondrial/lysosomal pellet. The post-mitochondrial supernatant was centrifuged for 67 min at 110,000 g to separate the microsomal pellet from the cytosol. Pelleted fractions were resuspended in 1-2 ml of buffer and a 1-ml aliquot of cytosolic fraction was obtained. All subcellular fractions were stored at -70 C (Shields and Eales, 1986). Livers were pooled for all experiments except for the study with clofibrate.

Microsomal glucuronosyltransferase (GT) assay

Subcellular fractions were thawed on ice and diluted 1:25 with 0.1 M Tris-HCl buffer (pH=7.8). For incubations using rat hepatic microsomes, 5 mM $MgCl_2$ was included in the buffer (Beetstra et al., 1991). A volume of 500 μl of diluted subcellular fraction was added to siliconized tubes containing 20 μl of the cofactor uridine 5'-diphosphoglucuronic acid (UDPGA; Sigma) previously dissolved in buffer. Control tubes received 20 μl of buffer without UDPGA. Subcellular localization experiment employed a UDPGA concentration of 5 mM (Visser et al., 1993a,b). Kinetic studies employed a UDPGA concentration of 1 mM to illustrate linear Lineweaver-Burk plots for both T_4 and T_3. All remaining experiments employed a UDPGA concentration of 0.25 mM, which is still in molar excess of the cosubstrate (Dutton, 1966). All tubes were vortexed and equilibrated for 15 min in a water bath (12 C; 150 rpm). The reaction was started by adding 20 μl of either $T_4/[^{125}I]T_4$ or $T_3/[^{125}I]T_3$ (New England Nuclear) (final conc.= 1 μM; ~ 100,000 cpm). GT activity was not detected using lower concentrations (0.1-1.0 nM) of T_4 and T_3. After 60 min, the reaction was stopped with 1 ml chloroform/methanol

(2:1 v/v). This mixture was vortexed, centrifuged at 1420 g for 5-10 min and a 250-μl aliquot of the aqueous methanol layer was added to Sephadex LH-20 minicolumns containing 750 μl of 1.0 N HCl. Columns were swirled, drained and eluted in a stepwise manner with 3 x 1 ml of 0.1 N HCl (iodide fraction), 8 x 1 ml H_2O (glucuronide fraction), and 3 x 1 ml 0.1 N NH_4OH in ethanol (ethanolic ammonia) (iodothyronine fraction) (Finnson and Eales, 1996). All columns were equilibrated and stored with 0.1 N HCl.

TH glucuronide formation (H_2O fraction) was determined by subtracting cpm of control values (- UDPGA) from cpm of experimental values (+ UDPGA). Depending on the experiment, GT activity was expressed as (i) cpm (labeled TH glucuronidated)/hr or (ii) pmol TH glucuronidated/mg microsomal protein/hr. Protein concentration was determined by the Bradford protein method (Bradford, 1976).

Microsomal enzyme induction with clofibrate (CLOF)

Six experimental trout were injected intraperitoneally on 3 consecutive days with CLOF (Sigma; 150 mg/kg BW) dissolved in propylene glycol:H_2O (1:1). Six control trout received propylene glycol:H_2O (1:1) alone. On Day 4, trout were anesthetized in tricaine methanesulfonate (0.07 g/l), killed by concussion, their livers removed and microsomal fractions prepared as before.

Statistics

Statistical analyses were performed by one-way analysis of variance (ANOVA).

Statistical analyses of the effects of TH analogues were performed on raw data and Dunnett's multiple comparison test was then used to determine statistical differences from control groups ($P < 0.05$) prior to transformation into percentages.

Results

Assay conditions

Figure 4-1 shows representative elution profiles obtained from incubating trout hepatic microsomes with 1.0 μM of [^{125}I]T_4 withor without UDPGA. The net production of T_4G was about 1 %. Similar profiles were obtained from incubations using T_3 (data not shown).

Hepatic GT activity occurred mainly in the microsomal fraction (48%). GT activity also occurred in the nuclear fraction (24%) and mitochondrial/lysosomal fraction (28%) but not in the cytosolic fraction. Microsomal GT activity for T_4 increased linearly with protein concentration up to 0.75 mg/ml, and then approached a plateau (Fig. 4-2). A protein concentration of ~ 0.5 mg microsomal protein/ml was used for all subsequent experiments since this value was in the linear range. Glucuronidation increased linearly to 60 min (Fig. 4-3). Neither Mg^{+2} (mammalian UDPGT cofactor) at concentrations of 5-15 mM nor saccharic acid 1,4-lactone (inhibitor of β-glucuronidase) at a concentration of 8 mM had a significant effect on GT activity (data not shown), and were excluded from the assay buffer. GT activity for T_4 had a broad pH profile with an optimum pH value between 6.8-7.8 (Fig. 4-4).

For T_3, similar assay conditions were used except where indicated. GT activity for

T_3 increased with pH up to 8.5 units (Fig. 4-4). A pH > 8.5 was not used due to the large variation in data obtained. For all further experiments, a pH of 7.8 was used because i) there was significant activity for both T_4 and T_3 at this pH value (Fig. 4-4); ii) this pH value is similar to that used in rate studies (Visser et al., 1993a,b), permitting comparisons between species, and iii) a pH of 7.8 is close to physiological conditions.

Enzyme kinetics

Figure 4-5 shows Lineweaver-Burk plots for trout hepatic glucuronidation of both T_4 and T_3 (substrate range of 0.25-1.0 μM). The apparent K_m (μM), V_{max} (pmol/mg protein/hr) and V_{max}/K_m values were: T_4 = 6.0, 21.3, 3.6 and T_3 = 0.9, 2.6, 2.9.

Inhibition using TH analogs

Hepatic glucuronidation of ^{125}I-labeled T_4 and T_3 (1 μM) in trout was measured in the presence of various TH analogues (Sigma) at concentrations of 10 and 100 μM (Table 4-1). Glucuronidation of T_4 was inhibited by 100 μM of rT_3 and tetraiodothyroacetic acid (Tetrac) while glucuronidation of T_3 was inhibited by 100 μM of rT_3, T_4, Tetrac (10 and 100 μM) and triiodothyroacetic acid (Triac)($P < 0.05$). In addition, T_4 significantly reduced glucuronidation of T_3 ($P < 0.05$) while T_3 did not significantly reduce glucuronidation of T_4. Although [^{125}I]rT_3 was not used as a substrate in this study, its strong inhibition of both T_4- and T_3-glucuronidation (Table 4-1) suggests that it may be the preferred TH substrate for hepatic glucuronidation in trout.

Enzyme thermal stability

Thermal stabilities for glucuronidation of T_4 and T_3 were determined and thermostability profiles compared. GT activity for both T_4 and T_3 decreased with increasing pre-incubation temperature (Fig. 4-6). Although there appears to be a difference between glucuronidation of T_4 and T_3 at 24 C and 36 C (Fig. 4-6), these data were obtained from separate experiments. Therefore, glucuronidation of T_4 and T_3 were compared directly following a 15-min pre-incubation of trout hepatic microsomes at either 12 (control), 24 or 36 C (Fig. 4-7). Thermal stability of GT activity for T_4 was higher than for T_3, following a 15-min pre-incubation at 24 C ($P < 0.05$).

Latency and induction of GT

Figure 4-8 shows the effects of Brij 56 on glucuronidation of T_4 and T_3. GT activity was maximally increased (latency decreased) for both T_4 and T_3 about 2-fold at 0.0125 % Brij 56 (w/v). GT activity decreased with further increase in detergent levels. CLOF influenced neither T_4- nor T_3-glucuronidation significantly ($P > 0.05$) (data not shown).

Comparison of trout and rat hepatic GT activity

Hepatic microsomal GT activity (pmol/mg protein/hr) towards T_4 were compared in trout (12 C) and rat (37 C) at their respective body/acclimation temperatures. Glucuronidation of T_4 was about 3-fold higher in the rat (23.0 ± 0.2) than in trout (7.3 ± 0.2). Rat hepatic GT activity was not detected at 12 C.

Discussion

Mammalian glucuronidation of TH is catalyzed by glucuronosyltransferases (GTs), a family of homologous enzymes located in microsomal fraction of liver and other tissues, using uridine-diphosphoglucuronic acid (UDPGA) as a cofactor (Visser, 1990). In comparison, little is known about glucuronidation of TH in non-mammalian species. One goal of this study was to characterize hepatic glucuronidation of TH in trout. As in mammalian systems, trout hepatic glucuronidation of TH was catalyzed by GTs located mainly in the microsomal fraction using UDPGA as a cofactor. GT activity was also heat-sensitive and depended on protein concentration, time, pH, and obeyed Michaelis-Menten kinetics.

Mammalian UDPGTs are transmembrane proteins of the endoplasmic reticulum (ER). ER membranes are present in microsomes and UDPGT activity is latent because the active site probably faces the lumen of the microsomal vesicles. However, detergents such as Brij 56 can reduce this latency by opening the membrane and allowing easier access of substrate and cofactor to enzyme active sites (Mulder, 1992). As in rats (Visser et al., 1993a), Brij 56 maximally increased glucuronidation of both T_4 and T_3 in trout about 2-fold at 0.0125 % (w/v) detergent, suggesting that the transmembrane properties of trout and rat microsomal GTs are similar.

Another goal of this study was to determine if, as in mammals (Beetstra et al., 1991; Visser et al., 1993a,b), trout hepatic glucuronidation of T_4 and T_3 is catalyzed by different forms of GT. This was assessed by comparing optimal pH values, enzyme kinetics, inhibition profiles, enzyme thermal stability and CLOF induction of T_4- and T_3-

glucuronidation in trout.

The optimal pH values for glucuronidation of T_4 and T_3 were different, at 6.8-7.8 and > 8.5 units, respectively. However, these optimal pH values are near the pK_a values of these substrates (T_4 = 6.73; T_3 = 8.45) (McNabb, 1992), and therefore may reflect properties of T_4 and T_3 rather than properties of different GTs. Thus, the existence of multiple forms of GT cannot be established on the basis of pH alone.

Apparent K_m (μM) values for trout hepatic glucuronidation of T_4 and T_3 were 6.0 and 0.9, about 20-100 fold lower than that reported for the rat (Visser et al., 1993). The different K_m values for glucuronidation of T_4 and T_3 in trout suggest that trout GTs have a higher affinity for T_3 than T_4. (Fig. 4-5). In addition, although [^{125}I]rT_3 was not used as a substrate, unlabeled rT_3 strongly inhibited glucuronidation both T_4 and T_3 (Table 4-1) suggesting that it may be the preferred TH substrate for glucuronidation in trout. Furthermore, glucuronidation of T_4 was inhibited by 100 μM of rT_3 and Tetrac while glucuronidation of T_3 was inhibited by 100 μM of T_4, rT_3, Tetrac (10 and 100 μM) and Triac (Table 4-1). Together, these data suggest that trout hepatic glucuronidation of TH is catalyzed by different forms of GT showing an iodothyronine substrate preference of $rT_3 >> T_4 = T_3$.

Different enzyme forms may be also be distinguished by different enzyme thermal stabilities (Young et al., 1988). Following a 15-min pre-incubation of trout hepatic microsomes at 24 C, glucuronidation of T_4 was more thermostable than for T_3 further suggesting the existence of different forms of GT. Although thermal properties of rat hepatic glucuronidation of TH were not determined, hepatic glucuronidation of T_4 in rat

(37 C) was about 3-fold greater that in trout (12 C) on a per mg protein basis.

Clofibrate (CLOF) induces rat hepatic glucuronidation of T_4 but not T_3 (Visser et al., 1993a). In contrast, CLOF influenced neither T_4- nor T_3-glucuronidation in trout. Although these differences may reflect different properties of trout and rat GTs, a higher concentration of CLOF (800 mg/kg for 1 day) and different route of administration (oral) was used in the rat study (Visser et al., 1993a). This may account for the apparent differences in GT induction between these two studies.

Finally, glucuronidation of 1 μM of either T_4 or T_3 was not completely abolished with high levels (10 and 100 μM) of its own substrate (Table 1) suggesting the possibility of another (higher-K_m) GT. Alternatively, there may be a "high K_m" membrane transport system for UDPGA into the microsomal vesicles (Mulder, 1992). Therefore, although different forms of GT catalyze glucuronidation of TH in trout. the exact number of GTs cannot be established in this study.

In summary, trout hepatic glucuronidation of TH was catalyzed by more than one form of GT located mainly in the microsomal fraction using UDPGA as cofactor. Trout hepatic GT activity was heat-sensitive and depended on protein concentration, time and pH, showing an iodothyronine substrate preference of $rT_3 > T_4 = T_3$. Furthermore, trout GTs are transmembrane proteins with the active site facing the lumen of microsomal vesicles. Thus, despite functioning at different temperatures, trout hepatic glucuronidation of TH shares some properties with those of the rat.

Figure 4-1. Sephadex LH-20 elution profiles for T_4 glucuronidation in the presence and absence of UDPGA. Profiles were obtained by the stepwise addition of 1-ml aliquots of HCl (3 x; iodide), H_2O (8 x; glucuronides) and ethanolic ammonia (3 x; iodothyronines). Each value represents the percentage of the total radioactivity recovered. T_4G indicates the T_4-glucuronide fraction.

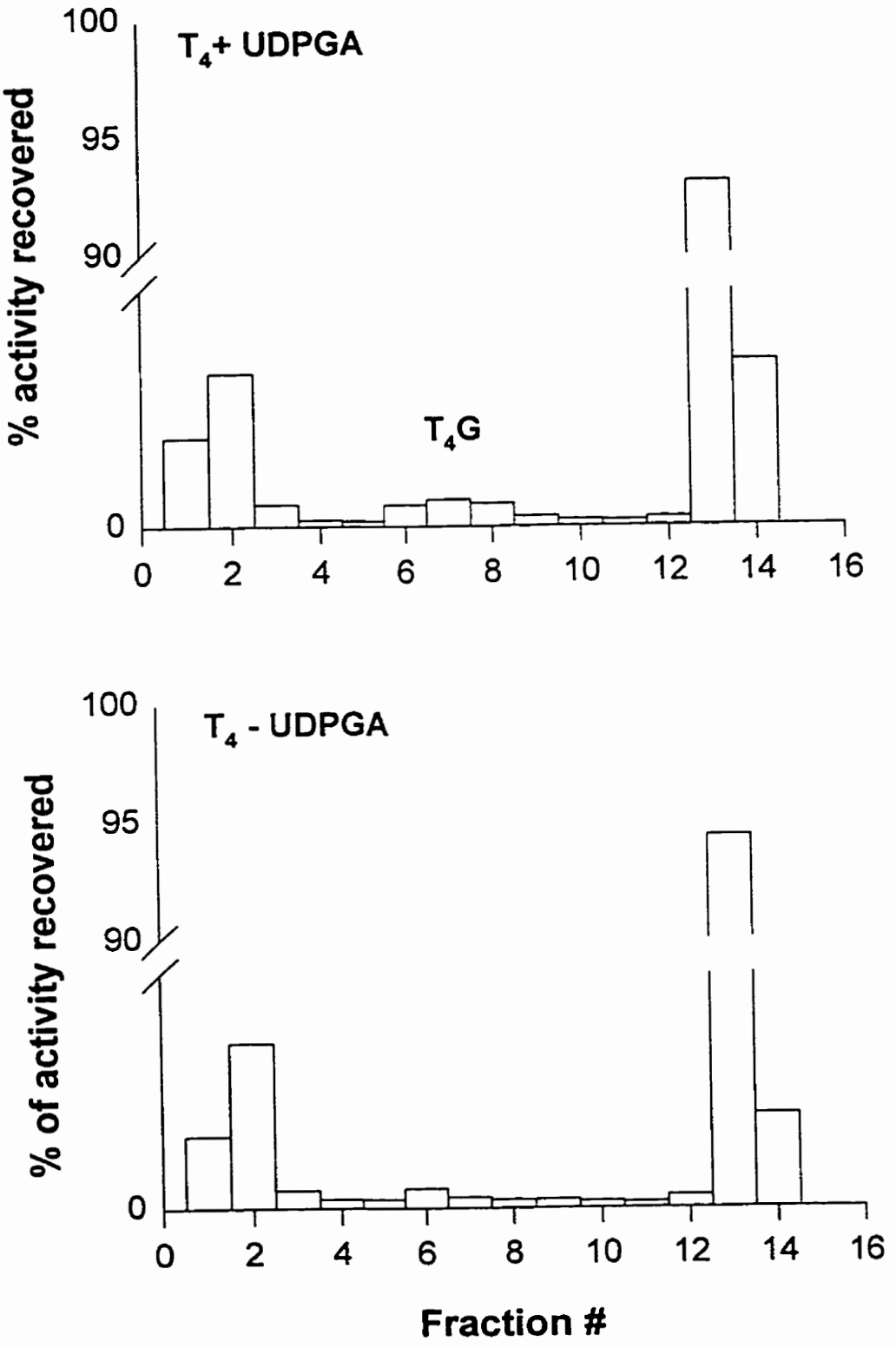

100
95
90
0
T_4+ UDPGA
T_4G
% activity recovered
0 2 4 6 8 10 12 14 16
100
95
90
0
T_4 - UDPGA
% of activity recovered
0 2 4 6 8 10 12 14 16
Fraction #

Figure 4-2. Relationship between microsomal protein concentration and amount of labeled T_4 converted to T_4G per hr. Each point represents the mean (± SEM) of three separate incubations.

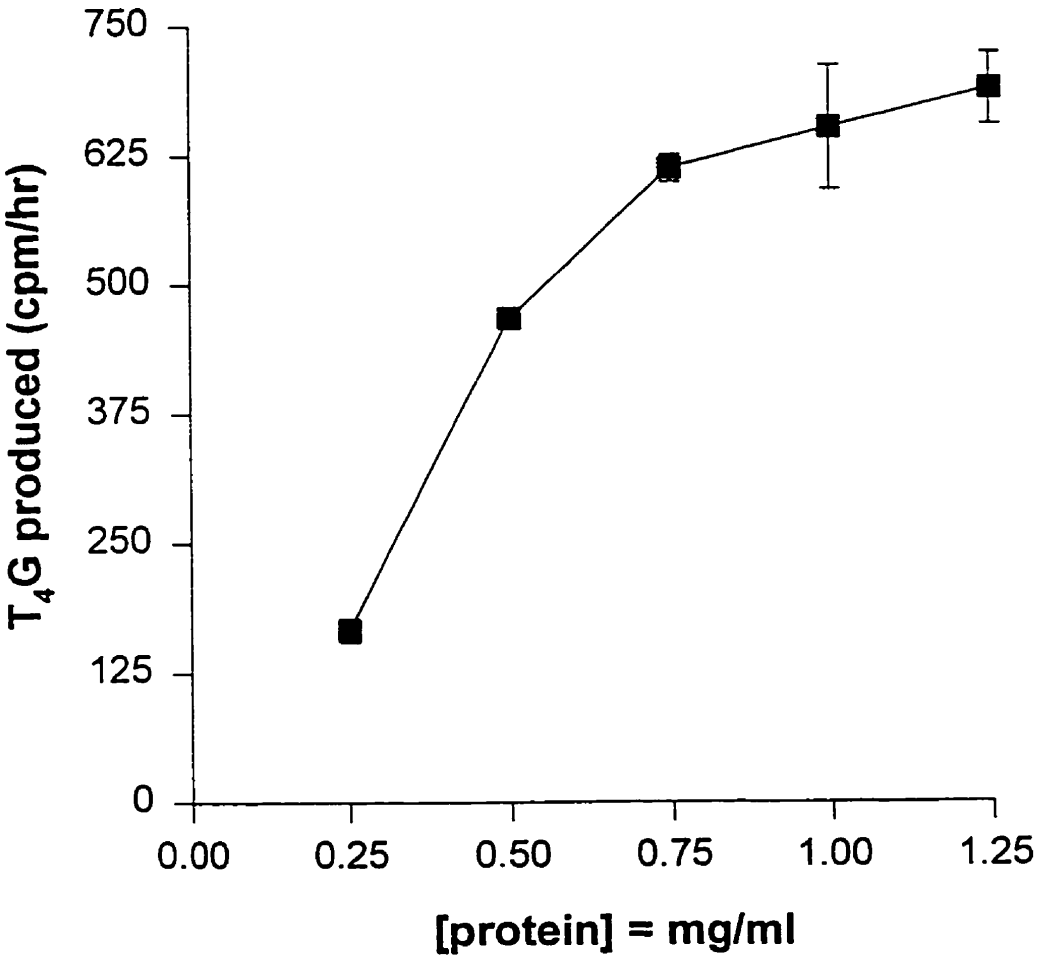

750
625
500
375
250
125
0
T_4G produced (cpm/hr)
0.00
0.25
0.50
0.75
1.00
1.25
[protein] = mg/ml

Figure 4-3. Effect of incubation time on the amount of labeled T_4 converted to T_4G. Each point represents a mean (± SEM) of three separate incubations.

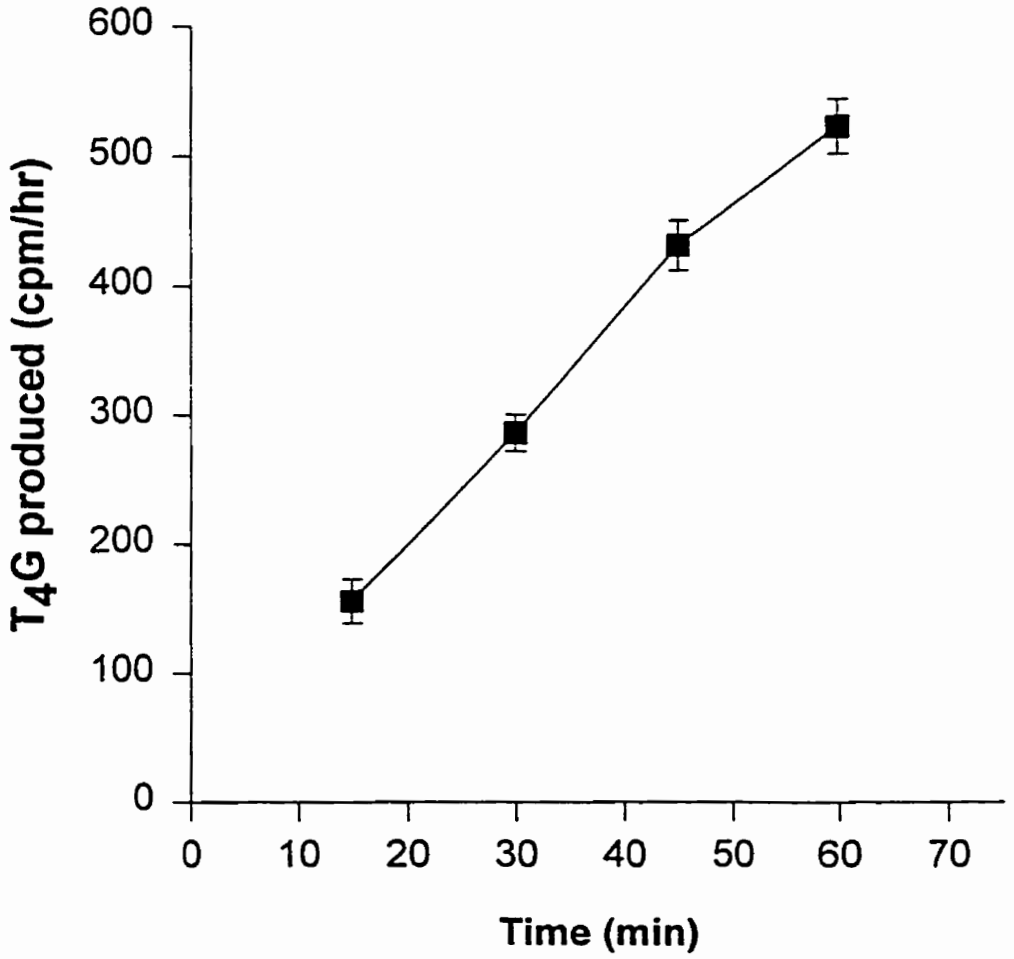

600
500
400
300
200
100
0
T_4G produced (cpm/hr)
0
10
20
30
40
50
60
70
Time (min)

Figure 4-4. Effect of pH on the glucuronidation of T_4 (■, •) and T_3 (o). Results are obtained from separate experiments on the same microsomal pool. Each point represents a mean (± SEM) of three separate incubations converted to % highest activity. For T_4, two plots are shown indicating the effect of pH over a wide and narrow range. For T_3, the effect of pH over a wide pH range is shown.

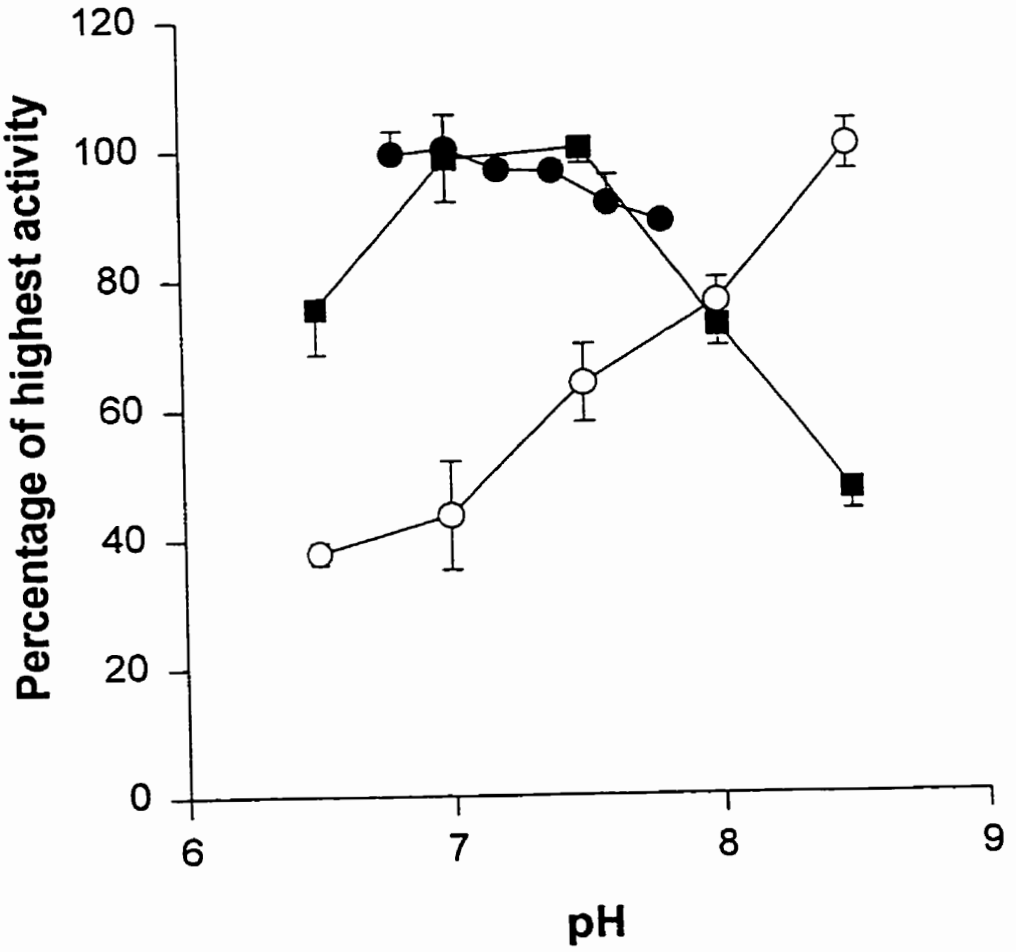

120
100
80
60
40
20
0
Percentage of highest activity
6
7
8
9
pH

Figure 4-5. Lineweaver-Burk plots for trout hepatic glucuronidation of T_4 and T_3. Apparent K_m (μM) and V_{max} (pmol/mg protein/hr) values were T_4 = 6.0, 21.3 and T_3 = 0.9, 2.6. Each point represents a mean (± SEM) of three separate incubations. 1/S represents inverse substrate concentration (μM) and 1/V represents inverse of reaction velocity (pmol/mg protein/hr).

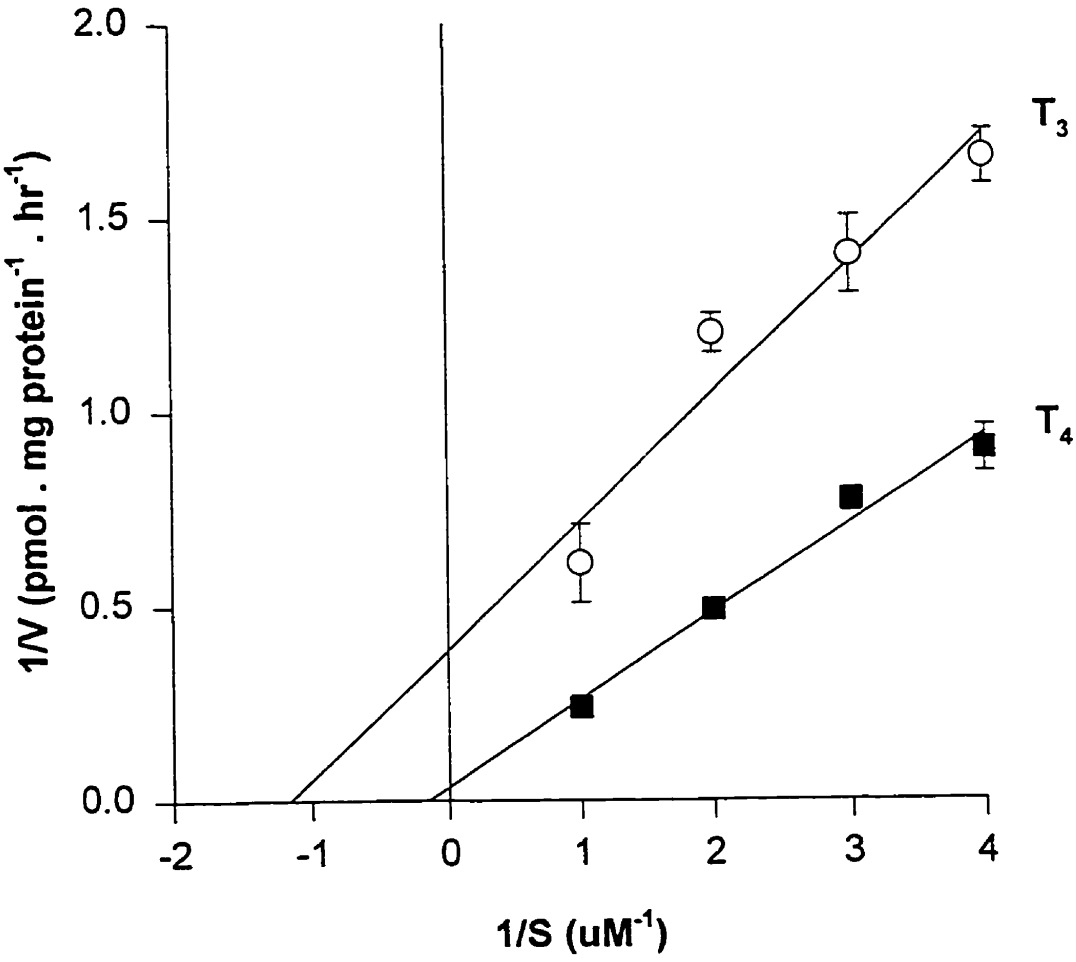

2.0
1.5
1.0
0.5
0.0
-2
-1
0
1
2
3
4
1/V (pmol . mg protein^{-1} . hr^{-1})
1/S (uM^{-1})
T_3
T_4

Figure 4-6. Glucuronidation of T_4 (■) and T_3 (•) following 15-min pre-incubation of hepatic microsomes at various temperatures. Glucuronidation of T_4 and T_3 were examined in separate experiments. Activity is expressed as a percentage of control (pre-incubation temp = 12 C) activity. Each point represents a mean (± SEM) of three separate incubations.

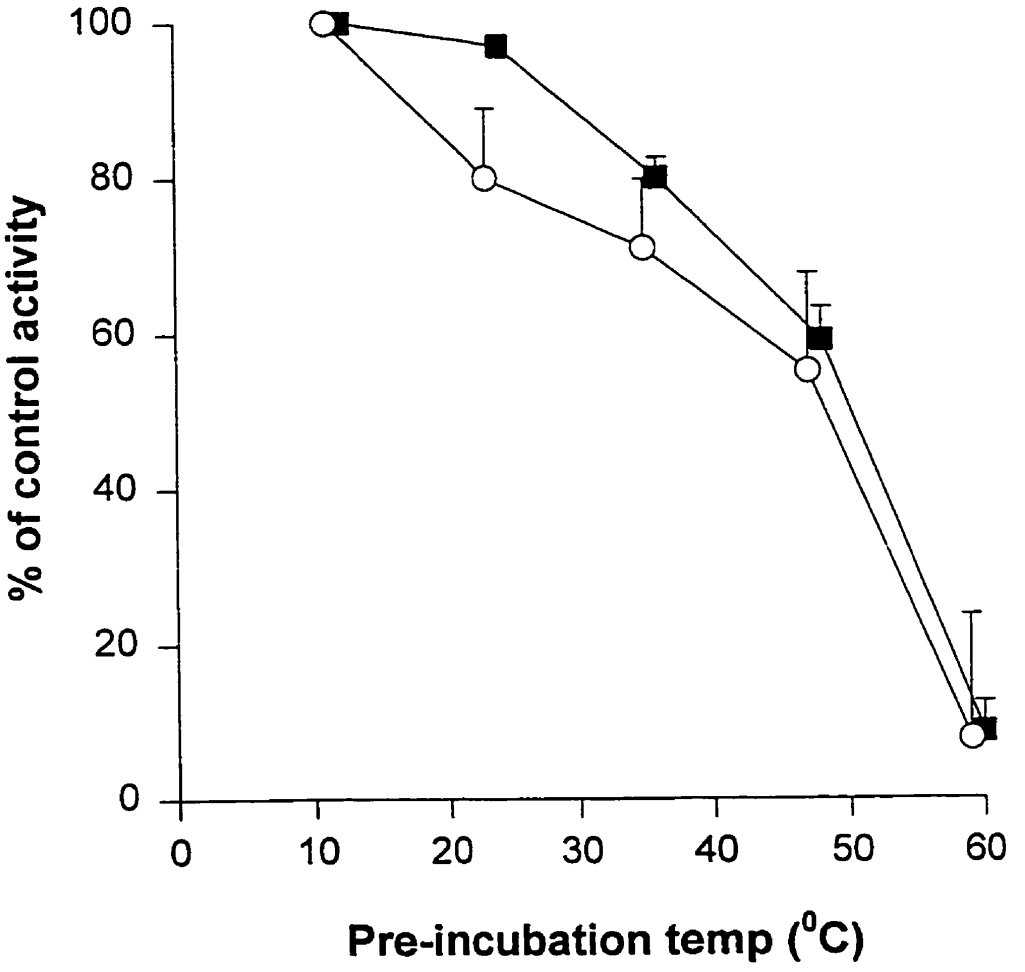

100
80
60
40
20
0
% of control activity
0
10
20
30
40
50
60
Pre-incubation temp (°C)

Figure 4-7. Glucuronidation of T_4 (open bars) and T_3 (hatched bars) following a 15-min pre-incubation at either 12, 24, or 36 C. * There was a significant difference in glucuronidation of T_4 and T_3 following a pre-incubation at 24 C ($P < 0.05$). Activity is expressed as a percentage of control (pre-incubation temp = 12 C) activity.

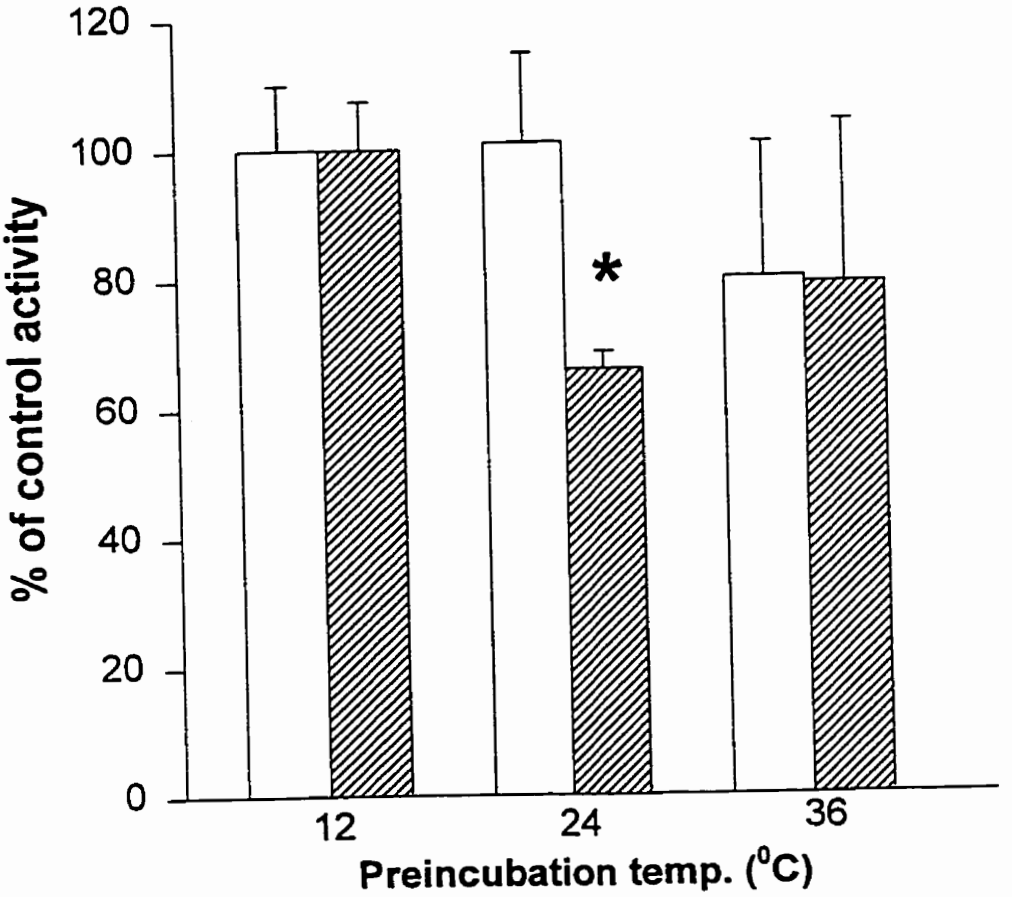

120
100
80
60
40
20
0
% of control activity
*
12
24
36
Preincubation temp. (°C)

Figure 4-8. Effect of Brij 56 on glucuronidation of T_4 (■) and T_3 (•). Each point represents a mean (± SEM) of three separate incubations. Activity is expressed as cpm (labeled T_4 or T_3 glucuronidated)/hr.

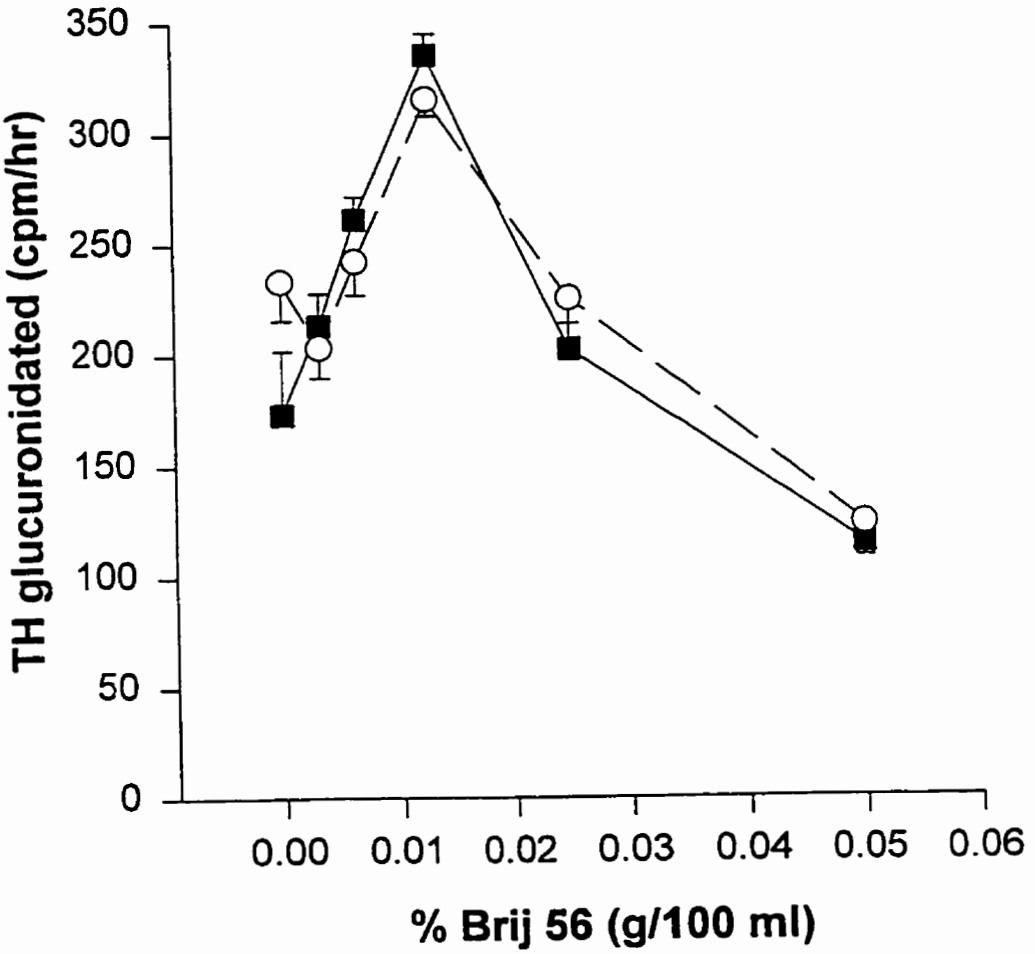

350
300
250
200
150
100
50
0
TH glucuronidated (cpm/hr)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
% Brij 56 (g/100 ml)

Table 4-1. The effects of various thyroid hormone analogs (10 and 100 μM) on glucuronidation of either 1 μM of [^{125}I]T_4 or [^{125}I]T_3.

Analog	Conc (μM)	Percent of control activity** [^{125}]T_4	[^{125}]T_3
T_4	100	34*	34*
	10	61	81
T_3	100	45	82
	10	92	72
rT_3	100	16*	20*
	10	61	52
3,5-T_2	100	69	52
	10	68	105
Tetrac	100	37*	10*
	10	53	28*
Triac	100	52	32*
	10	75	51

** Results are from separate experiments for T_4 and T_3, using pooled hepatic microsomes from trout. Each value represents a mean of duplicate incubations expressed as a percentage of control activity (without analogue). The single asterisk (*) indicates values that are significantly different from control values ($P < 0.05$).

Chapter 5

Sulfation of thyroid hormones by liver of rainbow trout

Introduction

Deiodination and sulfation are interrelated pathways for thyroid hormone (TH) metabolism. Deiodination of TH is catalyzed by a family of deiodinases located in the microsomal fraction of liver and other tissue (St. Germain, 1994). Outer-ring deiodination (ORD) of thyroxine (T_4) produces bioactive 3,5,3'-triiodothyronine (T_3), while inner-ring deiodination (IRD) produces inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3). Further deiodination of T_3 and rT_3 produces inactive iodothyronine metabolites (Burger, 1986).

Sulfation of TH is catalyzed by sulfotransferases (STs), a family of homologous enzymes located in the cytosolic fraction of liver and other tissues. These enzymes have a broad and overlapping substrate specificity and use 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a sulfate donor. Sulfation of the 4'-hydroxyl group accelerates deiodination of most iodothyronines by rat liver (Visser, 1994; Visser et al., 1990). Therefore, it is important to study sulfation to further understand pathways for TH degradation.

Comparatively little is known about sulfation of TH in non-mammalian species. Osborn and Simpson (1969) have reported sulfated TH in marine plaice, *Pleuronectes platessa*. More recently, Finnson and Eales (1996) have identified sulfated TH produced by isolated hepatocytes and excreted in bile of rainbow trout, *Oncorhynchus mykiss*. The main objective of this study was to determine the main properties of hepatic sulfation of

TH in trout and to determine if, as in mammals, hepatic sulfation of TH is catalyzed by different forms of ST.

Materials and Methods

Animal maintenance

Rainbow trout (250-500 g, 2 yr old) were obtained from the Rockwood Hatchery, Balmoral, Manitoba, and held in the laboratory as in Chapter 3. Trout were used 18-24 hr after their last meal.

Subcellular fractionation

Trout were anesthetized in tricaine methanesulfonate (0.07 g/l) and killed by concussion. Livers were removed and rinsed with ice-cold buffer: 0.25 M sucrose (pH=7.2) with 1 mM EDTA and 10 mM DTT. Hepatic subcellular fractions were prepared as in Chapter 4. Pelleted fractions (resuspended in 1-2 ml of buffer) and 1-ml aliquots of the crude homogenate and cytosolic fraction were stored at -76 C (Shields and Eales, 1986).

Sulfotransferase (ST) assay

The ST assay was based on the procedure of Young et al. (1988). The various subcellular fractions were thawed on ice and diluted (~ 1:25) with 0.1 M Tris buffer (pH=7.0) to achieve a final protein concentration of 0.2-0.8 mg/ml. BSA decreased ST activity and was therefore excluded from the assay buffer. A volume of 500 μl of diluted

subcellular fraction was added to siliconized test tubes containing 20 μl of the cofactor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (final concentration = 20 μM). Control tubes received 20 μl of 0.1 M Tris buffer (pH=7.0) alone. This mixture was equilibrated in a shaking water bath for 10-15 min (12 C, 150 rpm). The reaction was started by adding 20 μl of either ^{125}I-labeled T_4, T_3 or rT_3 substrate (final conc. = 1.0 μM; ~100,000 cpm). After 60 min, the reaction was stopped by adding 2 ml of chloroform/methanol (2:1 v/v), vortexed and centrifuged at 1420 g for 5 min. A 250 μl-aliquot of aqueous methanol layer was added to Sephadex LH-20 minicolumns containing 750 μl of 1.0 N HCl. Columns were swirled and drained, and iodide was eluted to waste with 3 ml of 0.1 N HCl. Sulfated TH conjugates were collected with 12 ml of H_2O and counted in a gamma counter. Columns were washed with 3 ml of 0.1 N NH_4OH in ethanol and stored with 0.1 N HCl (Finnson and Eales, 1996).

TH sulfate formation (H_2O fraction) was determined by subtracting cpm of control tubes (- PAPS) from cpm of experimental tubes (+ PAPS). Protein concentration was determined by the Bradford method (Bradford, 1976). Depending on the experiment, ST activity was expressed as either (i) pmol of TH sulfated/mg protein/hr or (ii) percentage of control activity.

Results

Assay conditions

Figure 5-1 shows the elution profile of synthetic ^{125}I-labeled T_3-sulfate. Over 96 % of the radioactivity was recovered in the H_2O fraction. Similar results were obtained

using either ^{125}I-labeled T_4- or rT_3-sulfate (data not shown).

Sulfation of T_4 (T_4ST) and T_3 (T_3ST) occurred mainly in the cytosolic fraction (63-67%). T_4ST and T_3ST activities were also detected in the microsomal (12-16%), nuclear (12-14%) and mitochodrial/lysosomal fraction (7-8%) (data not shown). T_3ST activity increased with increasing cytosolic protein concentration up to 1.2 mg/ml (Fig. 5-2). For all remaining experiments, cytosolic preparations with protein concentrations ranging from 0.2-0.8 mg/ml were used. Both T_4ST and T_3ST activities increased linearly with time to 60 min (Fig. 5-3). The pH profiles for T_4ST and T_3ST activities were broad and overlapping with optimal pH values of about 6.5 and 7.0 units respectively (Fig. 5-4).

Enzyme kinetics

A pH value of 7.0 units was used to compare kinetic properties of T_4ST, T_3ST and rT_3ST. Figure 5-5 shows Lineweaver-Burk plots for sulfation of A) T_4 (0.25 - 4.0 μM), B) T_3 (1.0 - 16.0 μM) and C) rT_3 (0.25 - 2.0 μM) using a PAPS concentration of 20 μM. Apparent K_m (μM), V_{max} (pmol/mg protein/hr) and V_{max}/K_m values were T_4 = 1.7, 46 and 27; T_3 = 11.5, 840 and 73 and rT_3 = 0.7, 583 and 832.

Inhibitor studies

Figure 5-6 shows inhibition of both T_4ST and T_3ST activities by different inhibitors/analogs (100 nM). Inhibitor profiles for T_4ST and T_3ST were not significantly different ($P > 0.05$) with a common substrate preference of rT_3 > pentachlorophenol (PCP) > triiodothyroacetic acid (TRIAC) > tetraiodothyroacetic acid (TETRAC) > T_4 =

T_3 = 3,5,-diiodothyronine (3, 5-T_2). Inhibitor studies using ^{125}I-labeled rT_3 as substrate were not performed.

Enzyme thermal stability

Figure 5-7 shows T_4ST, T_3ST and rT_3ST activities following 15-min pre-incubation of trout hepatic microsomes at either 12 (control), 24 or 36 C. For each TH substrate, ST activity decreased with increasing pre-incubation temperature. However, there were no significant differences between T_4ST, T_3ST and rT_3-ST activities due to pre-incubation temperature ($P > 0.05$)

Discussion

Mammalian sulfation of TH is catalyzed by sulfotransferases (STs), a family of homologous enzymes located in the cytosolic fraction of liver and other tissues, using PAPS as a sulfate donor (Visser, 1994; Visser et al., 1990). In comparison, little is known about sulfation of TH in non-mammalian species. The objective of this study was to determine the main properties of trout hepatic sulfation of TH, and to determine if as in mammals, trout hepatic sulfation of TH is catalyzed by different forms of ST.

Hepatic sulfation of TH in mammals and trout share some properties. Both are catalyzed by heat-sensitive cytosolic enzymes that use PAPS as a sulfate donor (Visser, 1994; Visser et al., 1990) with the reaction depending on protein concentration, time and pH and obeying Michaelis-Menten kinetics. Furthermore, apparent K_m values for both trout (0.7-11.5 μM) and rats (1.8-4.2 μM) (Kaptein et al., 1997) fall in a similar range.

However, a different PAPS concentration (50 μM), TH substrate (T_2), pH value (7.2) and incubation temperature (37 C) were used in the rat study making a direct comparison between trout and rat K_m values provisional.

The optimal pH values reported for human hepatic sulfation of T_3 and rat hepatic sulfation of T_2 are 6.6 (Young et al., 1988) and 8.0 units (Kaptein et al., 1997) respectively. In comparison, optimal pH values for trout hepatic sulfation of T_4 and T_3 were about 6.5 and 7.0 units respectively. Although different optimal pH values for trout hepatic sulfation of T_4 and T_3 suggest different forms of ST, these pH profiles were broad and overlapping. In addition, T_4 and T_3 have different pK_a values (T_4 =6.73; T_3 = 8.45) (McNabb, 1992), which may influence enzyme-substrate interaction. Therefore, the existence of different STs cannot be established in trout on the basis of pH alone.

Different enzyme forms may be distinguished by different enzyme thermal stabilities (Young et al., 1988) and different patterns of inhibition. In trout, differences in thermal stabilities of T_4- T_3- and rT_3-sulfation were negligible. In addition, sulfation of T_4 and T_3 both showed similar patterns of inhibition by several analogs or inhibitors. Therefore, trout hepatic sulfation of T_4, T_3 and possibly rT_3 appear to be catalyzed by a single form of ST enzyme or different forms of ST with similar properties.

One key difference between trout and mammalian STs is their iodothyronine substrate preference. In rat liver, the different types of STs show an iodothyronine substrate preference of 3'-T_1 > 3,3'-T_2 > T_3 > rT_3 > T_4 (Sekura et al., 1981), indicating that ST activity decreases with the number of iodine substituents in the iodothyronine molecule. However, based on kinetic and inhibitor studies, rT_3 was clearly the preferred

substrate over both T_4 and T_3 in trout. Therefore, unlike mammalian system, ST activity in trout is not related to number of iodines present on the iodothyronine molecule.

The different properties of trout and rat STs may reflect different roles of TH sulfation in these two vertebrate groups. In rats, sulfation of TH leads to rapid deiodination by the type I enzyme in rat liver. Since sulfation of TH decreases with the number of iodine substituents in the iodothyronine molecule, this would favor subsequent deiodination of T_3 or its derivatives, thereby salvaging iodine prior to its loss by excretion. As a result, sulfated TH are not normally detected in rat bile (Visser, 1994; Visser et al., 1990). In contrast, sulfation of TH in trout blocks subsequent deiodination (Chapter 6) and consequently, sulfated TH are found in bile (Finnson and Eales, 1996). Therefore, sulfation of TH in trout may not be important in salvaging iodine by facilitating deiodination, but instead may simply enhance TH excretion by increasing water-solubility.

In summary, as in mammalian systems, trout hepatic sulfation of TH was catalyzed by ST enzymes located mainly in the cytosolic fraction using PAPS as a sulfate donor. Moreover, trout hepatic ST activity was heat-sensitive and depended on protein concentration, time and pH and obeyed Michaelis-Menten kinetics. However, unlike mammalian systems, trout hepatic sulfation of TH may be catalyzed by one or more forms of ST with an iodothyronine substrate preference of $rT_3 > T_4 = T_3$. Thus, hepatic sulfation of TH in trout has some common properties and unique differences when compared with those of the rat.

Figure 5-1. Sephadex LH-20 elution profile for synthetic ^{125}I-labeled T_3-sulfate. Profile was obtained by stepwise addition of 1-ml aliquots of 0.1 N HCl (x 3), H_2O (x 12), and 0.1 N NH_4OH in ethanol (1:1 v/v) (x 3). Over 96 % of the radioactivity was recovered in the H_2O fraction.

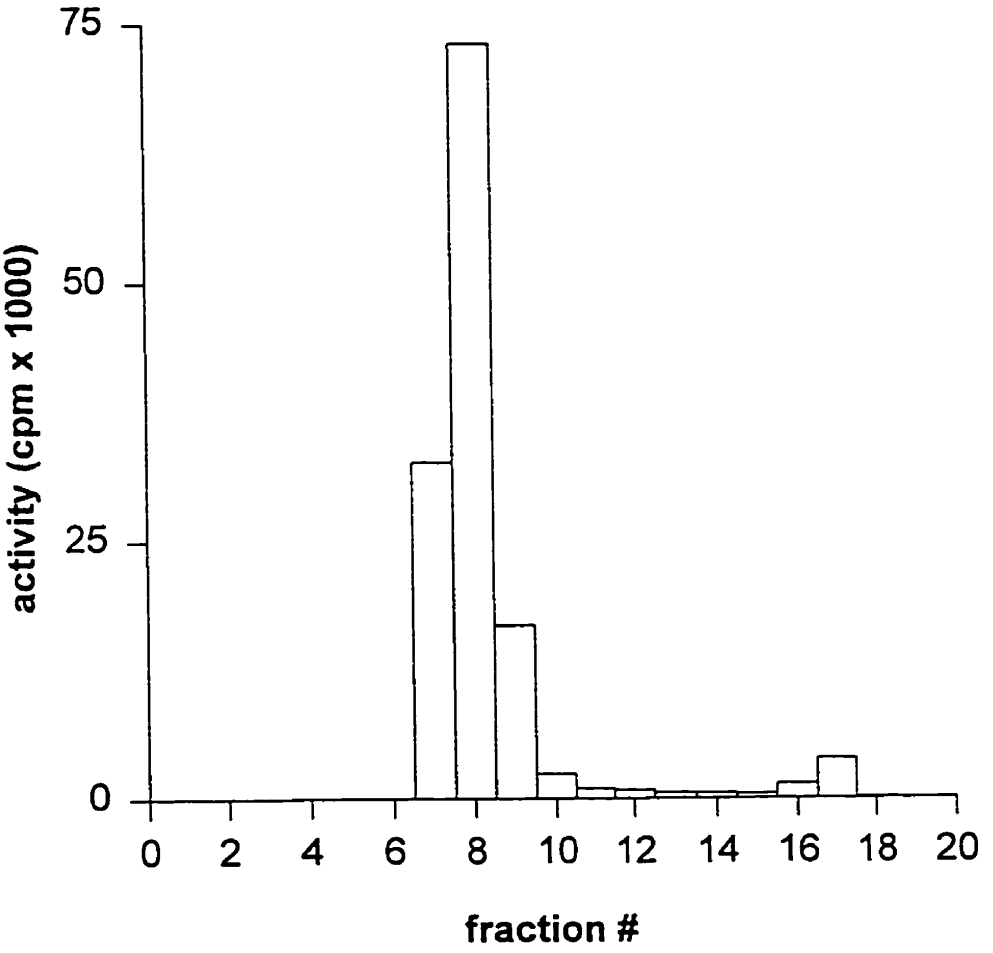

75
50
25
0
activity (cpm x 1000)
0
2
4
6
8
10
12
14
16
18
20
fraction #

Figure 5-2. Relationship between cytosolic protein concentration and amount of ^{125}I-labeled T_3 converted to T_3-sulfate per hr. Each point (•) represents the mean (± SEM where it exceeds symbol size) of three separate incubations.

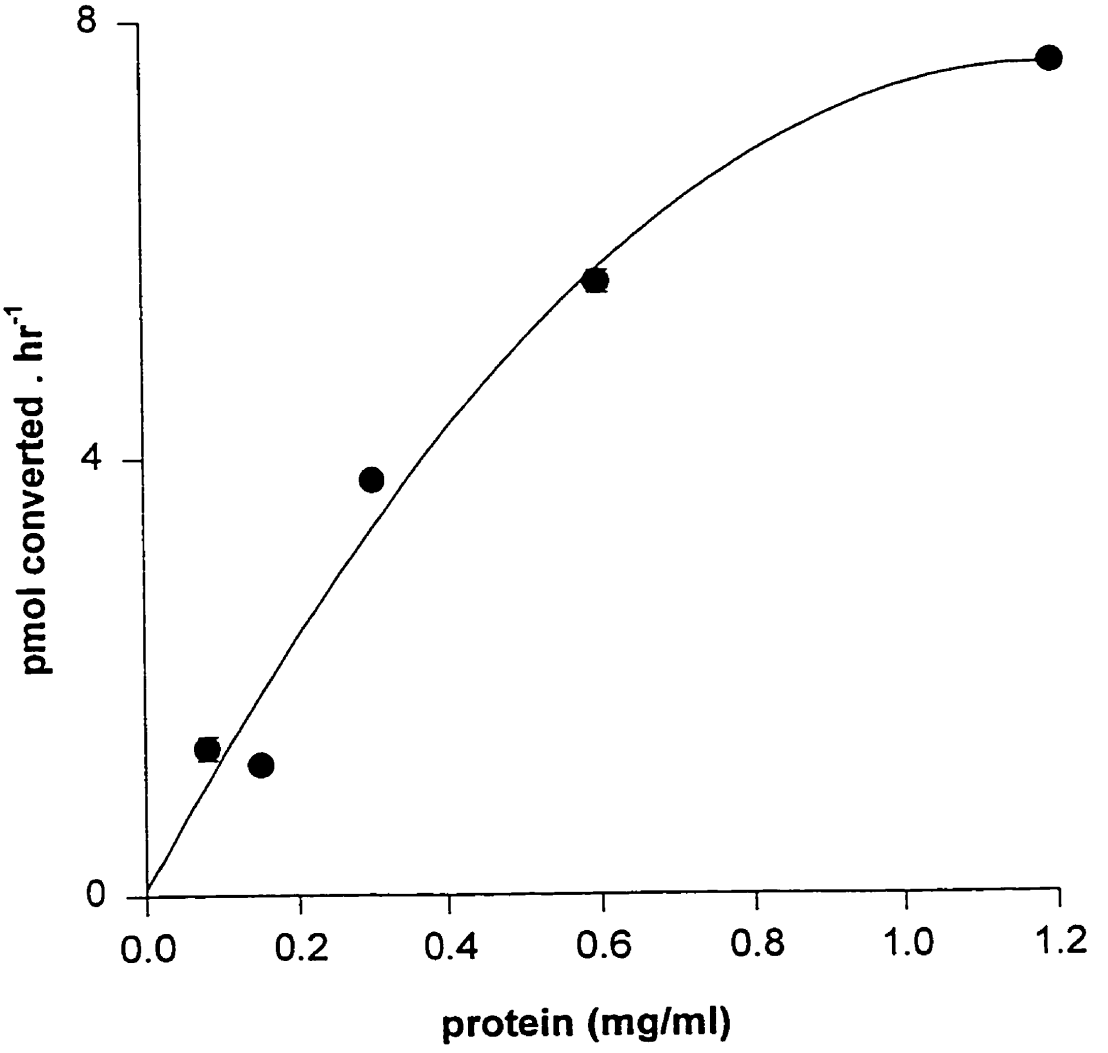

8
4
0
pmol converted . hr-1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
protein (mg/ml)

Figure 5-3. The effect of incubation time on the amount of ^{125}I-labeled T_4 converted to T_4-sulfate (■) and ^{125}I-labeled T_3 converted to T_3-sulfate (•) at intervals up to 1 hr. Each point represents the mean of three separate incubations (SEM values did not exceed symbol size).

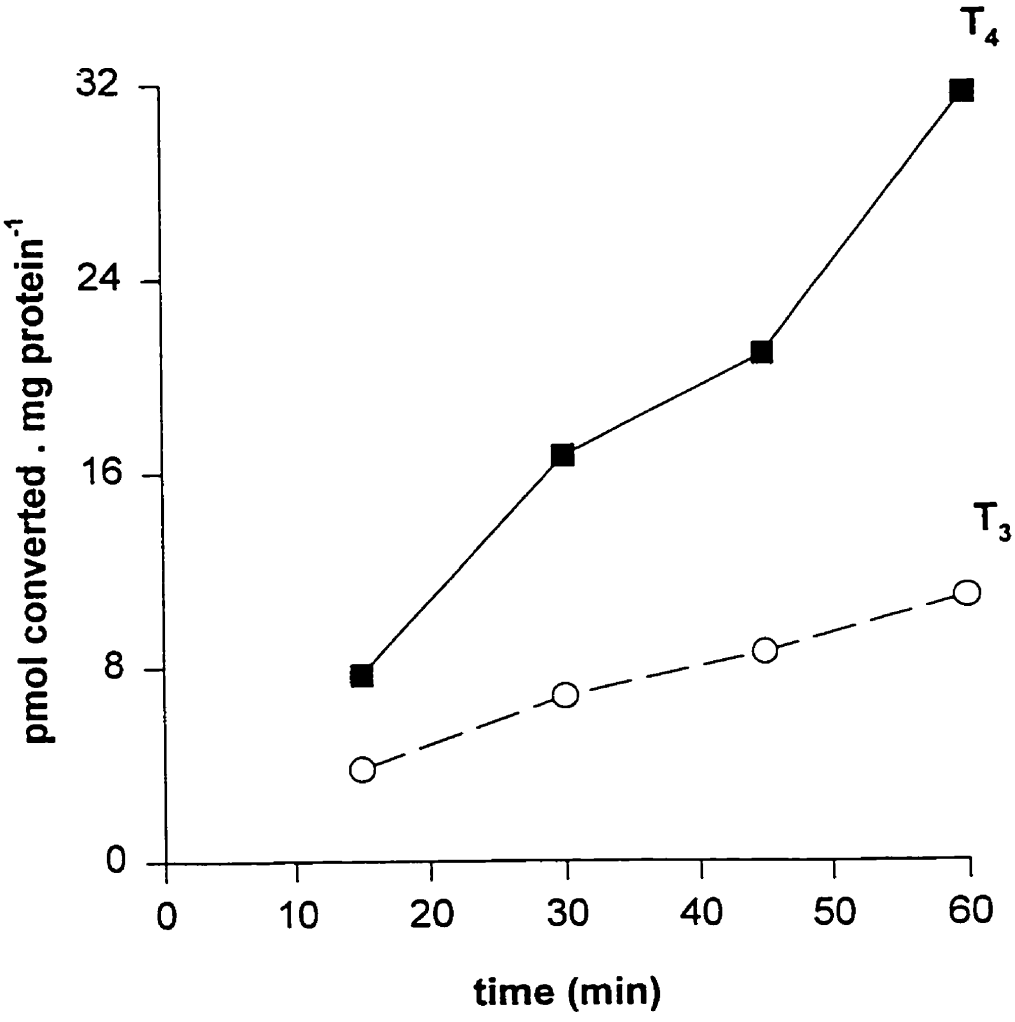

T4
T3
32
24
16
8
0
0
10
20
30
40
50
60
pmol converted . mg protein-1
time (min)

Figure 5-4. The effect of pH on the sulfation of T_4 (■) and T_3 (○). Buffer pH was adjusted by adding 1.0 N HCl to 0.1 M Tris buffer prior to the experiments. Results are from different experiments performed on the same cytosolic pool. Each point represents the mean (± SEM) of three separate incubations converted to % of highest activity.

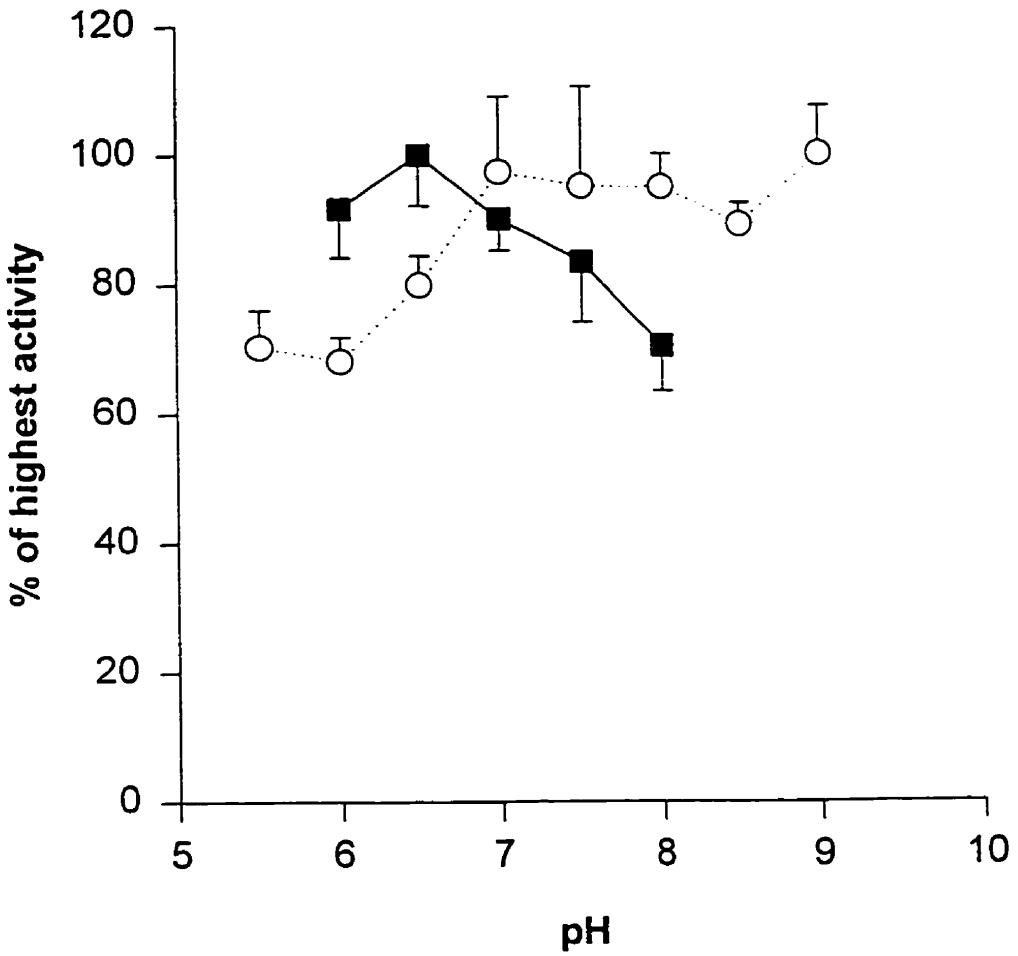

120
100
80
60
40
20
0
% of highest activity
5
6
7
8
9
10
pH

Figure 5-5. Lineweaver-Burk plots of hepatic cytosolic ST activities for (A) T_4 (0.25 - 4.0 μM), (B) T_3 (1.0 - 16.0 μM), and (C) rT_3 (0.25 - 2.0 μM) using a PAPS concentration of 20 μM. A wider substrate range was used for (B) since higher concentrations of T_3 were needed. Apparent K_m (μM) and V_{max} (pmol/mg protein/hr) values were T_4 (1.7, 46), T_3 (11.5, 840) and rT_3 (0.7, 583). Each point represents the mean (± SEM) of three separate incubations.

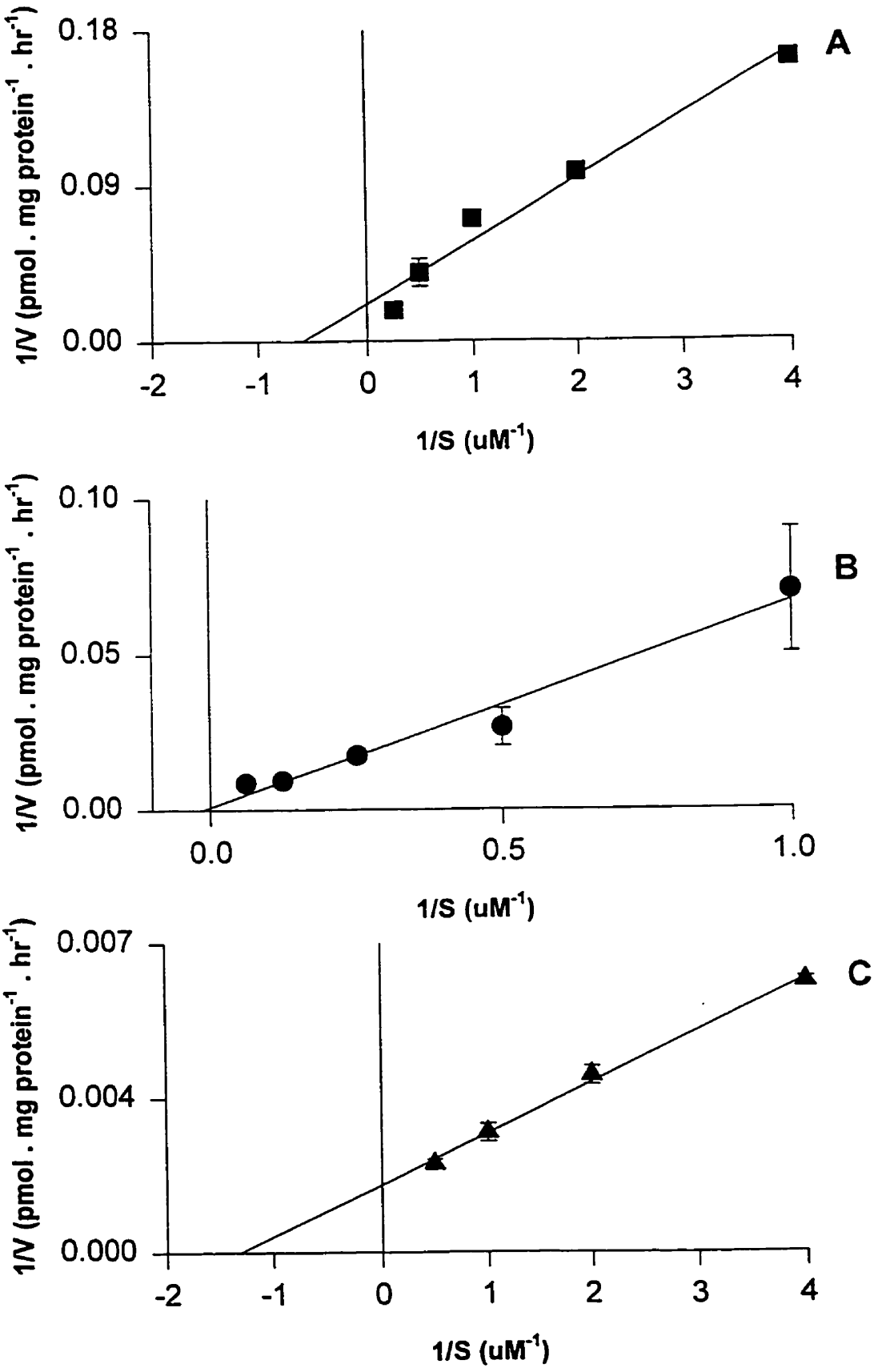

A
0.18
0.09
0.00
1/V (pmol . mg protein-1 . hr-1)
-2
-1
0
1
2
3
4
1/S (uM-1)
B
0.10
0.05
0.00
1/V (pmol . mg protein-1 . hr-1)
0.0
0.5
1.0
1/S (uM-1)
C
0.007
0.004
0.000
1/V (pmol . mg protein-1 . hr-1)
-2
-1
0
1
2
3
4
1/S (uM-1)

Figure 5-6. Inhibition of sulfation of T_4 (open bars) and T_3 (solid bars) by various analogs/inhibitors (100 nM). Differences between the inhibition of T_4- and T_3-sulfation by the various inhibitors/analogues were not significant ($P > 0.05$). ST activity is expressed as the percentage of mean control activity (no analog/inhibitor present).

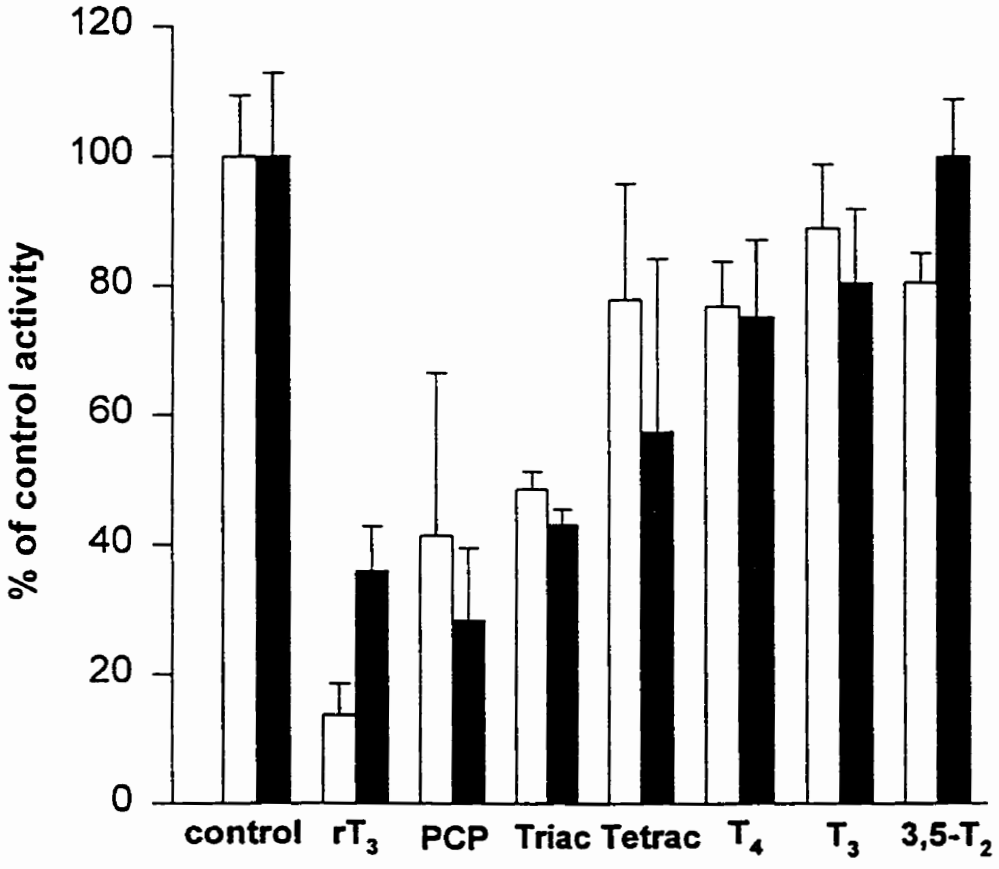
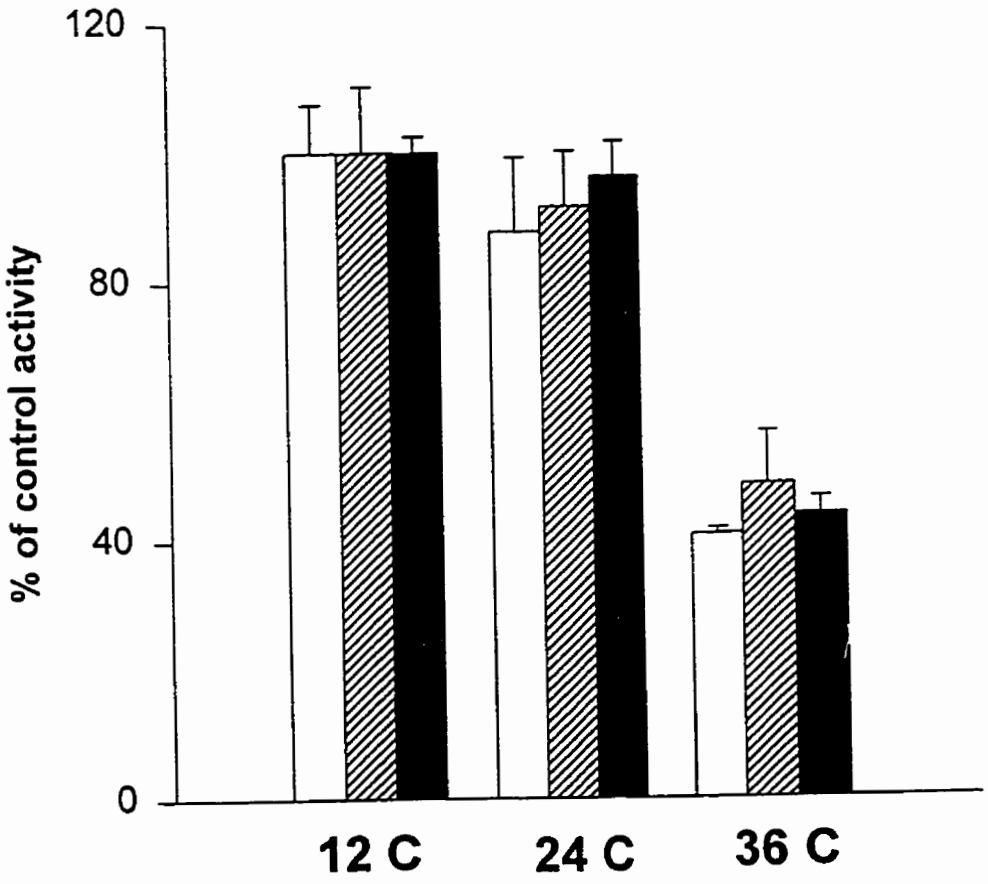

120
100
80
60
40
20
0
% of control activity
control
rT3
PCP
Triac
Tetrac
T4
T3
3,5-T2

Figure 5-7. ST activities for T_4 (open bars), T_3 (hatched bars) and rT_3 (solid bars) following a 15-min pre-incubation at 12 (control), 24 or 36 C. There were no significant differences between T_4ST, T_3ST or rT_3ST activities at any of the pre-incubation temperatures ($P > 0.05$). ST activity is expressed as a percentage of control activity.


120
80
40
0
% of control activity
12 C
24 C
36 C

Chapter 6

Deiodination and desulfation of sulfated thyroid hormones by liver of rainbow trout

Introduction

Deiodination and sulfation are important pathways for metabolism of thyroid hormones (TH). Outer-ring deiodination (ORD) of thyroxine (T_4) produces bioactive 3,5,3'-triiodothyronine (T_3) and inner-ring deiodination (IRD) produces inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3). Further stepwise deiodination of T_3 and rT_3 produces diiodothyronines (T_2's), monoiodothyronines (T_1's), and finally thyronine (T_0) (Engler and Burger, 1984).

Sulfation of the 4'-hydroxyl group increases water-solubility of TH, favoring their excretion in bile or urine (Visser, 1990). In mammals, sulfation prevents ORD of T_4, while it facilitates IRD of both T_4 and T_3 (Visser, 1994; Visser et al., 1990). In addition, sulfated T_3 can undergo desulfation providing a source of T_3 to target tissues (Kung et al., 1988; Santini et al. 1992).

Little information is available on sulfated TH in non-mammalian species. Osborn and Simpson (1969) reported sulfate conjugates of T_4 in bile and urine of marine plaice, *Pleuronectes platessa*. More recently, Finnson and Eales (1996) have identified sulfated TH produced by isolated hepatocytes and excreted in bile of rainbow trout, *Oncorhynchus mykiss*. However, whether these sulfated TH undergo subsequent deiodination or desulfation is unknown.

The main goal of this study was to determine if sulfated TH undergo hepatic deiodination (either ORD or IRD) or desulfation in trout. Although trout hepatic IRD activity is normally inactive, it can be induced by treatment with T_3 (Sweeting and Eales, 1992b). Thus, to determine if sulfated TH undergo either hepatic ORD or IRD in trout, hepatic microsomes were used from trout held under both control (ORD) and T_3-treated (IRD) conditions. Hepatic metabolism of sulfated TH was also studied in rat liver to directly compare aspects of trout and mammalian systems.

Materials and Methods

Animal maintenance

Rainbow trout (250-500 g, 2 yr old) were obtained from the Rockwood Hatchery, Balmoral, Manitoba, and held in the laboratory as in chapter 3-5. Trout were fed a commercial diet (Martin Feed Mills, Elmira, Ontario, trout feed pellets, 3.2 mm diameter) once daily at a ration of 1-2% of body weight. T_3-treatment involved feeding trout food previously sprayed with T_3 (sodium salt) dissolved in ethanol to provide a T_3 concentration of 12 ppm in food. Control pellets were sprayed with ethanol alone. This protocol elevates plasma T_3 levels of trout 2-3 fold (Higgs et al., 1982) and induces hepatic IRD pathways (Sweeting and Eales, 1992b). Trout were used 18-24 hr after their last meal. Male Sprague-Dawley rats were held in the laboratory and fed food and water *ad libitum*.

Subcellular fractionation

Trout were anesthetized in tricaine methanesulfonate (0.07 g/l) and killed by concussion. Rats were killed by CO_2 asphyxiation. Livers were removed and rinsed with ice-cold buffer: 0.25 M sucrose (pH=7.2) with 1 mM EDTA and 10 mM DTT. Hepatic subcellular fractions were prepared as in Chapter 4. Pelleted fractions (resuspended in 1-2 ml of buffer) and 1-ml aliquots of the crude homogenate and cytosolic fraction were stored at -76 C (Shields and Eales, 1986).

Synthesis of iodothyronine sulfates

T_4-, T_3- and rT_3-sulfate (T_4S, T_3S and rT_3S) were synthesized according to the method of Mol and Visser (1985). Briefly, a volume of 200 μl of chlorosulfonic acid was slowly added to 800 μl of dimethylformamide at 0 C. A 200-μl aliquot of this mixture was transferred to another tube containing either T_4, T_3 or rT_3 (20-100 nM with or without 1-10 μCi) previously evaporated to dryness. Reactions were allowed to reach room temperature and continue overnight.

Samples were diluted with 800 μl of ice-cold distilled H_2O, added to Sephadex LH-20 minicolumns and eluted to waste with 3 ml of 0.1 N HCl. Columns were washed with 1.5-2.0 ml of H_2O and the remaining sulfated TH were collected with 5 ml of H_2O. Radioactive samples were counted in a gamma counter to determine % recovery. All samples were evaporated in a Speed Vac. Unsulfated TH were resuspended in 0.1 N NaOH and sulfated TH were resuspended in H_2O prior to assay.

Deiodinase assay

The deiodinase assay was based on the method of Shields and Eales (1986). Hepatic microsomal fractions from rats or from either control or T_3-treated trout were thawed on ice and diluted with 0.1 M Tris-HCl buffer (pH=7.2) containing 10 mM DTT and 1 mM EDTA to a final protein concentration of 0.2-0.8 mg/ml (Bradford, 1976). A volume of 0.5-1.0 ml of diluted microsomal fraction was added to siliconized test tubes and equilibrated in darkness in a water bath (12 C; 140 rpm). The reaction was started by adding 20 μl of either ^{125}I-labeled T_4S, T_3S or rT_3S substrate (100,000 cpm) previously dissolved in 0.1 N NaOH to 500 μl of the diluted microsomal fraction (final conc. = 1 or 1000 nM). After 30 min, the reaction was stopped with 1 ml of chloroform/methanol (2:1 v/v), vortexed and centrifuged at 1420 g for 3 min. A 350-μl aliquot of the upper methanol layer was transferred to amber vials for HPLC analysis.

Inhibition of ORD activity by either sulfated or unsulfated TH was measured in hepatic microsomes obtained from trout held under normal conditions. A volume of 20 μl of unlabeled inhibitor (either T_4, T_3 or rT_3 in 0.1 N NaOH; T_4S, T_3S, or rT_3S in H_2O) was added to 1 ml of diluted microsomal fraction (final inhibitor conc. = 1, 10 or 100 nM). This mixture was equilibrated for 10-15 min and the reaction was started by adding 20 μl of either 0.08 nM [^{125}I]T_4 (low substrate) or 0.1 μM [^{125}I]rT_3 (high substrate) (~ 100,000 cpm). Hepatic T_4ORD and rT_3ORD activities were determined from the 60-min generation of $^{125}I^-$, measured by LH-20 chromatography. Activity was expressed as a percentage of control (without inhibitor) activity.

Desulfation - hepatic subcellular fractions

Desulfation of either T_4S, T_3S or rT_3S was measured in hepatic subcellular fractions from trout held under normal conditions. Hepatic subcellular fractions were thawed on ice and diluted (0.2-0.8 mg protein/ml) with either 0.1 M sodium acetate (pH 6.0) or 0.1 M sodium phosphate (pH 8.0) and equilibrated in a water bath at 12 C as before. The reaction was started by adding 20 μl of either ^{125}I-labeled T_4S, T_3S or rT_3S substrate (final conc. = 1 nM; ~100,000 cpm) previously dissolved in 0.1 N NaOH to 500 μl of the appropriate diluted subcellular fraction. After 3 hr, the reaction was stopped with 1 ml of chloroform/methanol (2:1 v/v). This mixture was then vortexed and centrifuged at 1420 g for 3 min and a 350-μl aliquot from the upper methanol layer was transferred to amber vials for HPLC analysis. Activity was expressed as the percentage of substrate desulfated per 3 hr.

Desulfation - freshly isolated hepatocytes

Hepatocytes were prepared as in Chapter 3. Briefly, hepatocytes were isolated by collagenase perfusion, suspended in modified Hank's balanced salt solution (HBSS) and adjusted to a final hepatocyte concentration of 1 x 10^6 cells/ml. Cell viability exceeded 90 % based on trypan blue exclusion.

Hepatocytes were equilibrated for 15 min at 12 C with HBSS in a 50-ml Erlenmeyer flask supplied with an O_2:CO_2 gas mixture (99:1). A volume of 4 ml of hepatocyte suspension was transferred to a 25-ml Erlenmeyer flask (control flasks received 4 ml of HBSS buffer only), containing 20 μl of either sulfated [^{125}I]T_4, [^{125}I]T_3 or [^{125}I]rT_3

substrate (final conc. = 1.0 nM, ~ 1.0 μCi). After 3 hr, a 1-ml aliquot of hepatocyte suspension was added to 1 ml of chloroform:methanol (2:1, v/v), vortexed, centrifuged at 1420 g and prepared for HPLC analysis as before. Activity was expressed as the percentage of substrate desulfated per 3 hr.

HPLC analysis

Analyses were conducted using a Gilson-IBM binary gradient HPLC. Acetonitrile/0.02 M ammonium acetate (pH = 4.0) was employed acetonitrile increasing linearly from 16-40% (0-15 min) and from 40-45% (15-35 min) (Sweeting and Eales, 1992a).

Results

Deiodination

HPLC profiles showing the hepatic metabolism of T_4S (Fig. 6-1), T_3S (Fig. 6-2) and rT_3S (Fig. 6-3) (final conc. = 1.0 nM) are presented. Similar results were obtained using 1000 nM concentrations of either T_4S T_3S or rT_3S (data not shown). In both control and T_3-treated trout, outer- and inner-ring deiodination of each sulfated TH was negligible. However, in rat liver, a major peak representing $^{125}I^-$ occurred for T_4S (Fig. 6-1C), T_3S (Fig. 6-2C) and rT_3S (Fig 6-3C) indicating ORD of these substrates at 12 C. In fact, $^{125}I^-$ was the only peak present for rT_3S, indicating complete deiodination of this substrate in rat liver at 12 C.

Inhibition studies used hepatic microsomes from trout held under normal

conditions. Trout hepatic T_4ORD was inhibited more effectively by unsulfated forms of TH than by sulfated forms of TH (Fig. 6-4). Inhibition of trout hepatic rT_3ORD by either unsulfated or sulfated forms of TH (1-100 nM) was negligible (data not shown). Trout hepatic T_3ORD activity was not measured because T_3 shows negligible ORD activity in trout under these conditions.

Desulfation

Desulfation of either ^{125}I-labeled T_4S, rT_3S or T_3S by the crude homogenate, nuclear, mitochondrial/lysosomal, microsomal and cytosolic fractions of trout liver is summarized in Table 6-1. Desulfation activity was detected in neither the crude homogenate nor cytosolic fraction. At pH 8.0, T_4S was desulfated by the nuclear (3.7 %) and mitochondrial/lysosomal (1.1 %) fractions. Also at pH 8.0, a small amount of rT_3S was desulfated by the microsomal fraction (0.3 %). T_3S was desulfated by the microsomal fraction at pH 6.0 (4.6 %), by the mitochondrial/lysosomal fraction at pH 8.0 (5.7 %) and by freshly isolated hepatocytes (4.7 %).

Discussion

Although there is little information on TH sulfation and desulfation in non-mammalian species, these processes have been extensively studied in mammals. Sulfation accelerates type I deiodination of most iodothyronines in rat liver (Visser, 1994; Visser et al., 1990), and desulfation of T_3S may provide a source of T_3 for target tissues (Kung et al., 1988, Santini et al., 1992). The main objective of this study was to determine if

sulfated TH undergo either deiodination or desulfation by liver of trout.

In mammals, sulfation prevents ORD of T_4, while it facilitates IRD of both T_4 and T_3 (Visser, 1994; Visser et al., 1990). Although trout hepatic IRD pathways are normally inactive, they can be induced by T_3-treatment (Sweeting and Eales, 1992b). Thus, to determine if sulfated TH undergo either ORD or IRD in trout, hepatic microsomes were obtained from trout held under both control (ORD) and T_3-treated (IRD) conditions.

Although ^{125}I-labeled T_4S, T_3S and rT_3S substrates (1 nM) were extensively or completely deiodinated by rat hepatic microsomes (at 12 C), deiodination of these substrates (1 or 1000 nM) by hepatic microsomes from either control or T_3-treated trout was negligible (Figs. 6-3). In addition, unlabeled sulfated TH (1-100 nM) did not inhibit hepatic ORD of either T_4 or rT_3 in trout. Therefore, unlike mammalian systems, sulfated TH do not undergo rapid hepatic deiodination in trout. In fact, since sulfated TH do not inhibit deiodination of native TH, sulfation and deiodination may be functionally separate pathways for metabolism of TH in trout.

In both isolated hepatocytes and various hepatic subcellular fractions of trout, desulfation of both T_4S and rT_3S substrates was either low or absent (Table 1). Similarly, Kung et al. (1988) have shown that T_4S and rT_3S were poor substrates for desulfation in mammals. It has been suggested that the two iodines in the outer-ring (i.e. T_4S and rT_3S) inhibit desulfation by preventing the sulfate group from accessing the active site of the sulfatase (Mol and Visser, 1985). In contrast, T_3S was desulfated by isolated trout hepatocytes, and more readily by the various trout hepatic subcellular preparations than either T_4S or rT_3S. Thus, as in mammals (Kung et al., 1988; Santini et al. 1992), cellular

T_3S may provide a source of bioactive T_3 for this tissue.

In summary, unlike mammalian systems, sulfated TH do not interact with hepatic deiodination pathways in trout, either directly by accelerating deiodination of TH substrates or indirectly by inhibiting either T_4ORD or rT_3ORD. Like mammalian systems, desulfation of T_3S may provide a source of bioactive T_3 to target tissues in trout.

Figure 6-1. HPLC profiles of ^{125}I-labeled products obtained from 30-min incubations with 1.0 nM T_4S and hepatic microsomes obtained from either (A) control trout, (B) T_3-treated trout or (C) rat. Radioactivity is expressed as a percentage of the amount present in the tallest peak. Retention times of authentic substances are shown with I^- = iodide, T_4S = T_4-sulfate and T_4 = T_4.

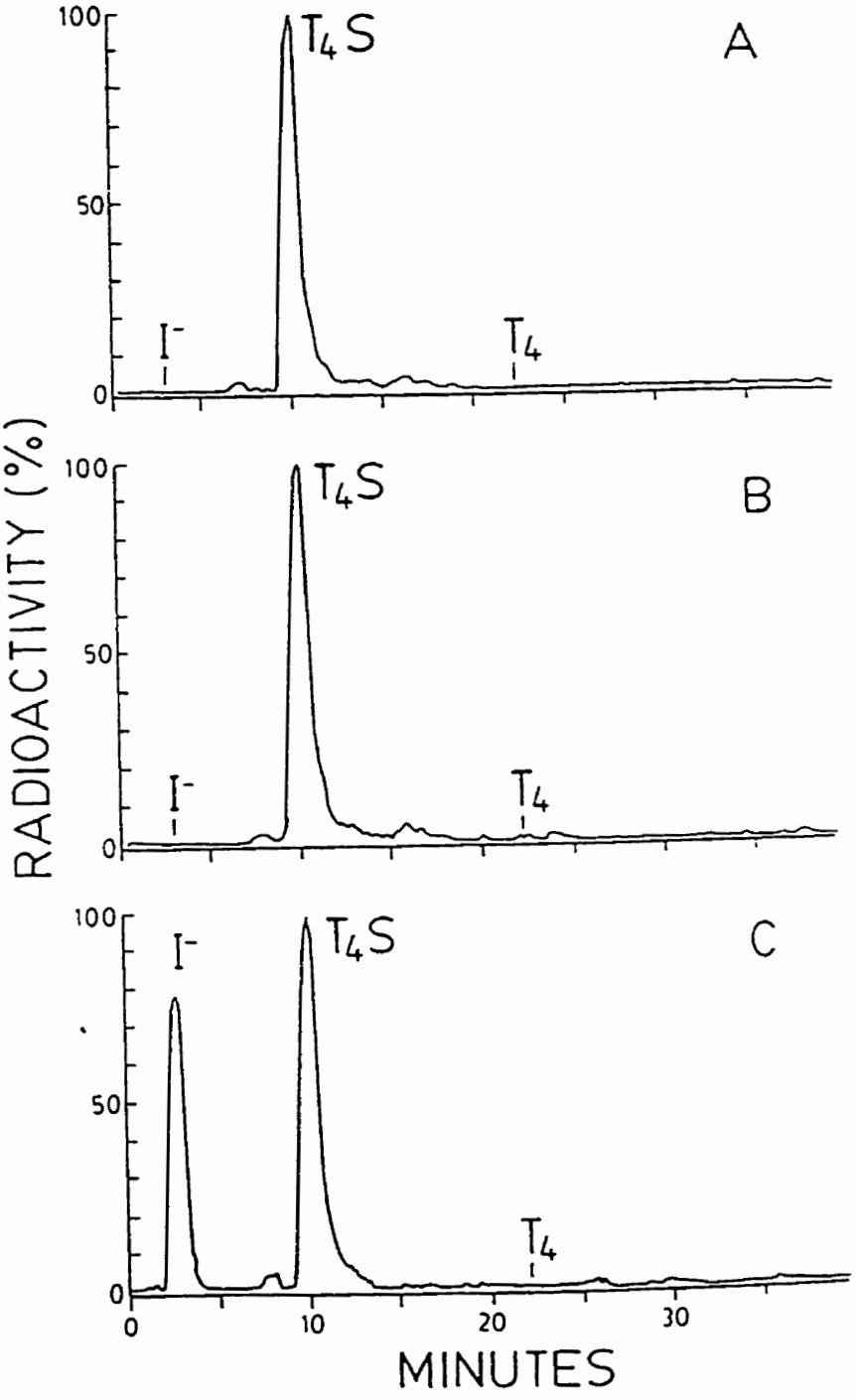

100
50
0
T_4S
A
I^-
T_4
B
C
RADIOACTIVITY (%)
0
10
20
30
MINUTES

Figure 6-2. HPLC profiles of ^{125}I-labeled products obtained from 30-min incubations with 1.0 nM T_3S and hepatic microsomes obtained from either (A) control trout, (B) T_3-treated trout or (C) rat. Radioactivity is expressed as a percentage of the amount present in the tallest peak. Retention times of authentic substances are shown with I^- = iodide, T_3S = T_3-sulfate and T_3 = T_3.

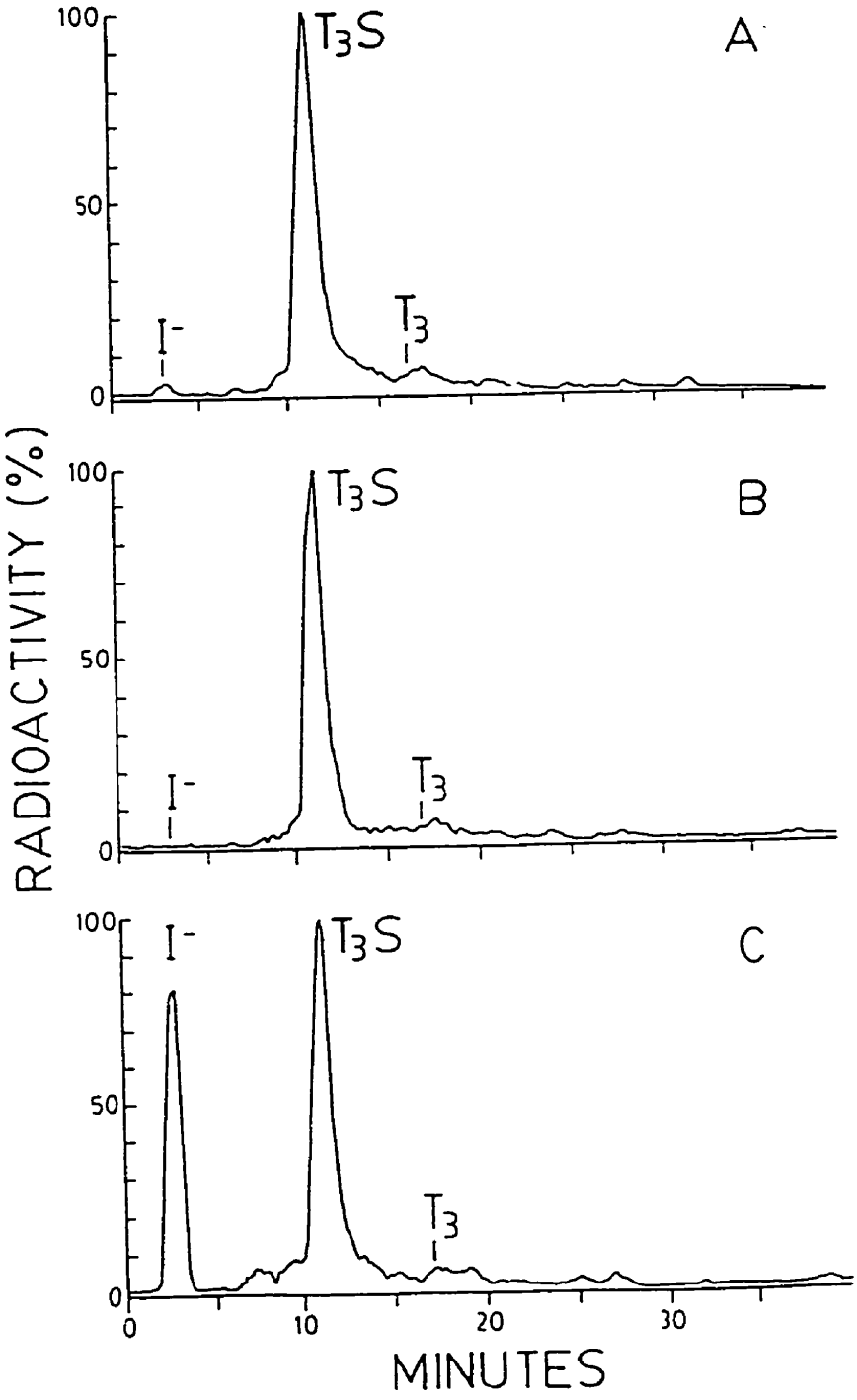

100
50
0
T_3S
A
I^-
T_3
RADIOACTIVITY (%)
B
C
0
10
20
30
MINUTES

Figure 6-3. HPLC profiles of ^{125}I-labeled products obtained from 30-min incubations with 1.0 nM rT_3S and hepatic microsomes obtained from either (A) control trout, (B) T_3-treated trout or (C) rat. Radioactivity is expressed as a percentage of the amount present in the tallest peak. Retention times of authentic substances are shown with I^- = iodide, rT_3S = rT_3-sulfate and rT_3 = rT_3.

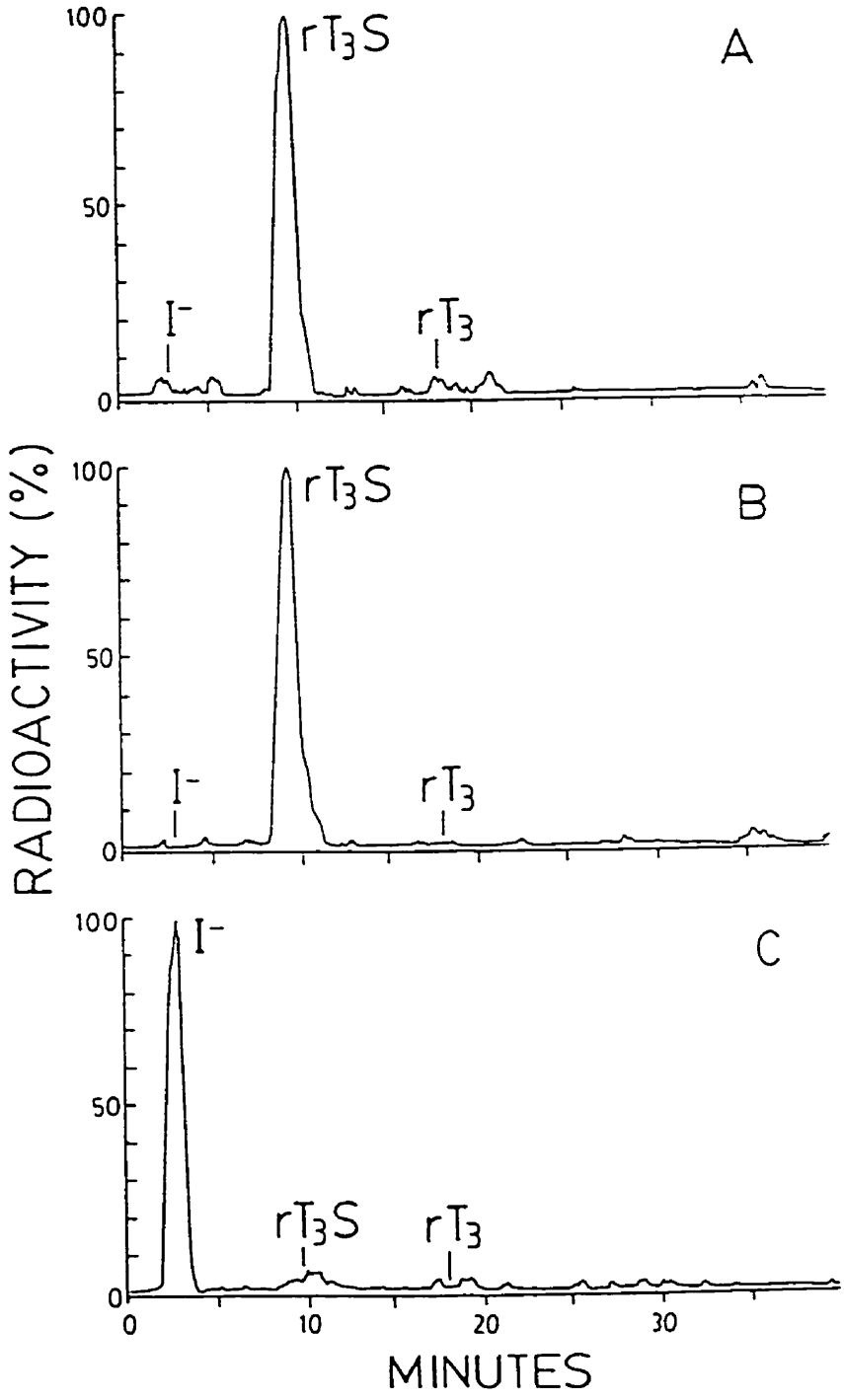

100
50
0
rT3S
A
I⁻
rT3
B
C
RADIOACTIVITY (%)
0
10
20
30
MINUTES

Figure 6-4. The influence of sulfated and unsulfated thyroid hormones on trout hepatic T_4ORD activity, expressed as a percentage of the control value (mean ± SEM; n = 3). All incubates contained 0.08 nM of [^{125}I]T_4 substrate in the presence of 1, 10 or 100 nM of sulfated or unsulfated T_4, T_3 or rT_3.

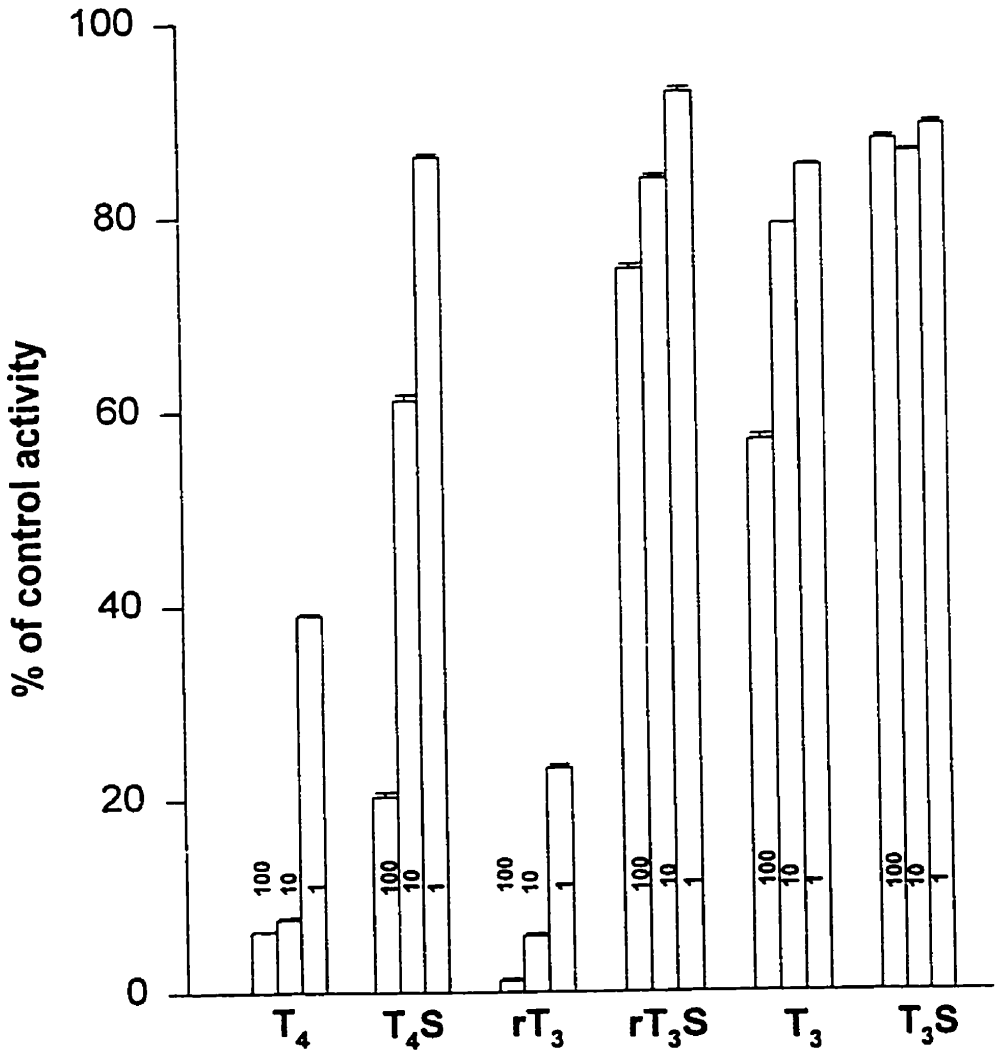

100
80
60
40
20
0
% of control activity
100
10
1
T_4
T_4S
rT_3
rT_3S
T_3
T_3S

Table 6-1. Desulfation of 1.0 nM T_4S, T_3S or rT_3S by various hepatic subcellular fractions of rainbow trout tested at pH 6.0 and pH 8.0 and by freshly isolated hepatocytes from trout. Numbers are expressed as a percentage of substrate desulfated following a 3-hr incubation period. A dash (-) indicates no detectable activity at either pH or in isolated hepatocytes.

	substrate		
Hepatic Preparation	T_4S (%)	T_3S (%)	rT_3S(%)
crude homogenate	-	-	-
nuclear	3.7 (@ pH 8.0)	-	-
lysosomal	1.1 (@ pH 8.0)	5.7 (@ pH 8.0)	-
microsomal	-	4.6 (@ pH 6.0)	0.3 (@ pH 8.0)
cytosolic	-	-	-
isolated hepatoctes	-	4.7	-

Chapter 7

Effect of T_3-treatment and food ration on deiodination and conjugation of thyroid hormones by liver of rainbow trout

Introduction

Deiodination is the main pathway for thyroid hormone (TH) metabolism. Outer-ring deiodination (ORD) of thyroxine (T_4) produces 3,5,3'-triiodothyronine (T_3) and inner-ring deiodination (IRD) produces 3,3',5'-triiodothyronine (reverse T_3, rT_3). Since T_3 is considered the active form of hormone and rT_3 is inactive (McNabb, 1992), the balance between ORD and IRD activities is important in regulating thyroidal status.

Conjugation is another important pathway for TH metabolism, and involves adding either glucuronic acid or sulfate to the 4'-hydroxyl group of iodothyronine substrates (Visser, 1990). In mammals, glucuronidation is the intial step in enterohepatic recycling of TH (Distefano, 1988), while sulfation accelerates deiodination of most iodothyronines (Visser, 1994; Visser et al., 1990). In trout, both hepatic glucuronidation and sulfation are involved in biliary excretion of TH (Finnson and Eales, 1996), and sulfation may also provide a cellular reservoir for T_3 (Chapter 6). Thus, both glucuronidation and sulfation pathways may be important in regulating thyroidal status in these two vertebrate groups.

Several studies indicate that fish subjected to various physiological conditions show major changes in deiodination of TH. In trout, T_3-treatment decreases hepatic T_4ORD activity (Eales and Finnson, 1991), and increases hepatic IRD activity for both T_4 and T_3 (Sweeting and Eales, 1992b). Furthermore, acute or chronic fasting decreases hepatic

T_4ORD activity while feeding increases it (Sweeting and Eales, 1992c). These and other studies have established deiodination of TH as a major pathway regulating thyroidal status in trout (Eales and Brown, 1993).

Although T_3-treatment and food intake consistently affect deiodination of TH, it is not presently known whether these factors also influence glucuronidation and/or sulfation of TH. Since deiodination and conjugation involve the same TH substrates, and activity of one pathway may influence activities of the others, it is important to study these pathways simultaneously. The main goals of this study were: (1) to determine the effects of altered thyroidal status, as induced by either T_3-treatment or food ration, on hepatic glucuronidation and sulfation of TH, and (2) to determine the roles of these pathways, in relation to hepatic deiodination, in regulating thyroidal status in trout.

Materials and Methods

Fish Maintenance

Rainbow trout were obtained from Rockwood Hatchery, Balmoral, Manitoba and held in the laboratory as in Chapters 3-6. Trout were fed trout pellets once daily at a 1.0 % body weight (BW) ration for 4 days prior to the start of treatment.

Experiment 1 - T_3 treatment

Food was prepared by spraying trout pellets with either ethanol (control) or ethanol containing T_3 (final concentration in food = 12 ppm) (experimental) and drying under a fumehood. Four groups of 6-7 fish were assigned to separate tanks. Control groups (tanks

1,2) were fed 1.0 % BW with trout pellets previously treated with ethanol. Experimental groups (tanks 3,4) received 1.0 % BW of trout pellets previously treated with T_3 in ethanol. All trout were fed for 7 days. This treatment elevates plasma T_3 levels ~ 3-fold in trout (Higgs et al., 1982).

Experiment 2 - food ration

Six groups of 6-7 fish were assigned to separate tanks. Group I (tanks 1,2) received a ration of 0 % BW, group II (tanks 3,4) received a ration of 0.5 % BW and group III (tanks 5,6) received a ration of 2.0 % BW for 7 days.

Blood sampling and subcellular preparation

Trout were anesthetized in tricaine methanesulfonate (0.07 g/l), weighed and blood samples removed from the caudal vein using a heparinized syringe. Plasma was separated by centrifugation and stored at -76 C. Trout were then killed by concussion, livers removed and hepatic subcellular fractions prepared as in Chapters 4-6. A 1-ml aliquot of cytosolic fraction was obtained and microsomal pellets were resuspended in 1-2 ml of buffer. Both fractions were stored at -76 C.

Radioimmunoassay (RIA)

Plasma T_4 and T_3 levels were measured simultaneously, using a solid-phase RIA (Omeljaniuk et al., 1984).

Microsomal glucuronosyltransferase (GT) assay

This was performed as in Chapter 3 with UDPGA = 0.25 mM; ^{125}I-labeled T_4, T_3 or rT_3 = 1 uM and products analyzed by LH-20 chromatography.TH glucuronide formation was determined by subtracting cpm of control tubes (-UDPGA) from cpm of experimental tubes (+UDPGA). GT activity was expressed as pmol TH glucuronidated/mg protein/hr.

Cytosolic sulfotransferase (ST) assay

This was performed as in Chapter 4 with PAPS = 20 uM; ^{125}I-labeled T_4, T_3 or rT_3 = 1 uM and products analyzed by LH-20 chromatography. TH sulfate formation was determined by subtracting cpm from control tubes (-PAPS) from cpm of experimental tubes (+PAPS). ST activity was expressed as pmol TH sulfated/mg protein/hr.

Deiodinase assay

This was performed as in Chapter 5 with ^{125}I-labeled T_4 or T_3 = 1 nM or rT_3 = 1 μM, with a 60-min incubation time and products analyzed by HPLC. Deiodinase (ORD or IRD) activity was expressed as pmol TH deiodinated/mg protein/hr.

HPLC analysis

HPLC analyses of deiodination products were conducted using a Gilson IBM binary gradient HPLC system. Two different solvent systems were used: (A) acetonitrile (0.1 % trifluoroacetic acid (TFA))/H_2O (0.1 % TFA); acetonitrile gradient increasing

linearly from 42-54% (10-20 min). (B) Acetonitrile/0.02 M ammonium acetate (pH=4.0); acetonitrile increasing linearly from 47-60 % (0-25 min) (Sweeting and Eales, 1992a).

Statistics

Statistical analyses were performed using either Student's t-test, or one way analysis of variance (ANOVA) followed by Student-Neuman-Kuels test. Levine test was used to test for homogeneity.

Results

Experiment 1 - T_3-treatment

T_3-treatment significantly decreased hepatosomatic index (HSI) (Table 7-1) ($P < 0.05$) and elevated plasma T_3 levels ($P < 0.05$), but did not influence plasma T_4 levels relative to control trout (Fig 7-1).

T_3-treated trout showed decreased hepatic T_4ORD activity, and induced hepatic T_4IRD and T_3IRD activity ($P < 0.05$) with no significant differences in hepatic rT_3ORD activity relative to control trout (Fig. 7-2). Hepatic T_3ORD and rT_3IRD activities were negligible (data not shown). The effect of T_3-treatment on both hepatic glucuronidation (Fig. 7-3) and sulfation (Fig. 7-4) of T_4, T_3 or rT_3 was not significant ($P > 0.05$).

Experiment 2 - food ration

Both body weight and HSI were significantly higher in trout fed a 2.0 % ration than in trout fed lower (0, 0.5 %) rations (Table 7-1) ($P < 0.05$). Plasma T_4 levels were

significantly lower in trout fed a 0.5 % ration than in trout fed either a 0 % or 2.0 % ration ($P < 0.05$). Plasma T_3 levels did not significantly differ among trout fed various rations ($P > 0.05$)(Fig. 7-9).

Fasting (0 % ration) decreased hepatic T_4ORD activity and increased hepatic rT_3ORD activity relative to fed (0.5, 2.0 % ration) trout (Fig. 7-6) ($P < 0.05$). Hepatic T_4IRD, T_3ORD, T_3IRD or rT_3IRD activity was not detected in trout fed at any ration. Glucuronidation of T_4, T_3 and rT_3 were significantly lower in trout fed a 2.0 % ration than in trout fed lower (0 or 0.5 %) rations ($P < 0.05$). Glucuronidation of T_3 was also significantly lower in trout fed a 0.5 % ration than in fasted (0 % ration) trout (Fig. 7-7) ($P < 0.05$). Sulfation of T_3 and rT_3 were both significantly higher in trout fed a 2.0 % ration than in trout fed lower (0, 0.5 %) rations ($P < 0.05$). However, sulfation of T_4 in trout fed various rations (0. 0.5, 2.0 %) did not differ significantly (Fig. 7-8). The effects of T_3-treatment and food ration are summarized in Table 7-1.

In addition, these data confirm that rT_3 is the preferred iodothyronine substrate for both hepatic glucuronidation (Figs.7-3, 7-7) and sulfation (Figs. 7-4, 7-8) of TH in trout.

Discussion

Plasma T_3 levels and nutritional status are two factors that influence thyroidal status in trout by altering hepatic deiodination pathways. However, it is not presently known whether these two factors also influence thyroidal status by altering either hepatic glucuronidation or sulfation of TH. The main goals of this study were: (1) to determine

the effect of altered thyroidal status, as induced by T_3-treatment or food ration, on hepatic glucuronidation and sulfation of TH, and (2) to determine the roles of these pathways, in relation to hepatic deiodination, in regulating thyroidal status in trout.

Deiodination of TH in trout is catalyzed by both low K_m (~ 1 nM) and a high K_m (~ 10 nM) ORD (5'D) enzymes located in the microsomal fractions of liver and other tissues (MacLatchy and Eales, 1992). In order to detect changes in activity of these enzymes in response to T_3-treatment or food ration, deiodinase assays employed a T_4 concentration of 1 nM (low K_m) and a rT_3 concentration of 1 uM (high K_m). In agreement with previous studies, T_3-treatment elevated plasma T_3 levels 2-3 fold (Higgs et al., 1982), decreased hepatic T_4ORD activity (Eales and Finnson, 1991) and induced both T_4IRD and T_3IRD activity (Sweeting and Eales, 1992b). However, T_3-treatment did not influence hepatic rT_3ORD activity. Together, these data suggest that elevated plasma T_3 levels regulate thyroidal status in trout by suppressing hepatic production of bioactive T_3 ($\downarrow$ T_4ORD), and by promoting inactivation ($\uparrow$ IRD) of T_3 and its prohormone T_4.

Deiodination in trout was also influenced by food ration. In agreement with a previous studies on trout (Sweeting and Eales, 1992c) and rat (McNabb, 1992), fasting decreased hepatic T_4ORD activity relative to fed (0.5, 2.0 % ration) trout. However, in contrast to previous studies on rat (McNabb, 1992), fasting increased hepatic rT_3ORD activity relative to fed trout. Finally, hepatic T_4IRD and T_3IRD activity could not be detected in trout under all feeding conditions (0, 0.5, 2.0 % ration). Therefore, fasting suppresses thyroidal status in trout by suppressing T_3 production ($\downarrow$ T_4ORD), without promoting inactivation (IRD) of either T_3 or T_4. Although the properties of rT_3ORD in

trout are not presently known, these findings further suggest that the effects of food intake on the pattern of changes in hepatic deiodination are species-specific (McNabb, 1992).

Both glucuronidation and sulfation of TH in trout are involved in biliary excretion of TH (Finnson and Eales, 1996). T_3-treatment influenced neither glucuronidation nor sulfation of TH in trout, suggesting that elevated plasma T_3 levels do not alter thyroidal status by influencing conjugation and biliary excretion of TH. However, T_3-treatment induced hepatic T_4IRD activity which produces rT_3, the preferred iodothyronine substrate for both hepatic conjugation pathways in trout. Therefore, glucuronidation and sulfation pathways may be indirectly involved in regulating thyroidal status in response to elevated plasma T_3 by eliminating the end-product of induced T_4IRD activity.

Both hepatic glucuronidation and sulfation in trout were sensitive to food ration. A high food ration (2.0 %) decreased hepatic glucuronidation of TH relative to lower (0, 0.5 %) rations suggesting that food intake diminishes biliary excretion of TH. In contrast, a high food ration (2.0 %) increased hepatic sulfation of TH relative to lower rations suggesting that food intake enhances biliary excretion of TH. Although these findings appear contradictory, sulfation of TH may also provide a cellular reservoir of TH (Chapter 6). Thus, a high food ration (2.0 %) would favor a reduced biliary loss of TH (glucuronidation) and increased hepatic storage of TH (sulfation). Therefore, unlike the indirect effects of elevated plasma T_3 levels, food ration affects thyroidal status in trout by directly influencing both hepatic glucuronidation and sulfation of TH.

Prior to this study, little was known about the potential relationship between hepatic conjugation of TH and regulation of thyroidal status. In male rats, Kaptein et al.

(1997) have shown that hepatic sulfation of TH was decreased by long-term fasting (3 weeks) but was not affected by hyperthyroidism (T_4-treatment). Similarly in this study, trout hepatic sulfation of TH was lower in fasted trout (and in trout fed a 0.5 % ration) than in trout fed a high (2.0 %) ration, but was not influenced by T_3-treatment. Although there are physiological differences between species, these findings suggest that the effect of food ration on hepatic sulfation of TH does not depend on elevated plasma TH levels, but instead is mediated by a mechanism independent of the thyroid sytem. In support of this, Gong et al. (1992) have shown in male rats that hepatic sulfation of T_3 is regulated by the pattern of pituitary growth hormone (GH) secretion. However, more studies on hormonal regulation of TH sulfation in trout and other species will be needed before this can be established.

In conclusion, although T_3-treatment does not directly influence either glucuronidation or sulfation of TH, it may have an indirect effect by inducing hepatic T_4IRD activity, thereby providing the preferred iodothyronine substrate (rT_3) for these pathways. Furthermore, the effects of food ration on thyroidal status involve direct changes in both hepatic glucuronidation and sulfation of TH. Therefore, glucuronidation and sulfation of TH are important pathways involved in regulating thyroidal status in trout, at least in response to alterations in plasma T_3 levels and food ration.

Figure 7-1. Plasma T_4 and T_3 concentrations from either control (open bars) or T_3-treated (solid bars) trout. Data are expressed as the mean ± SEM. *The plasma T_3 concentration in T_3-treated trout was significantly higher than in control trout ($P < 0.05$).

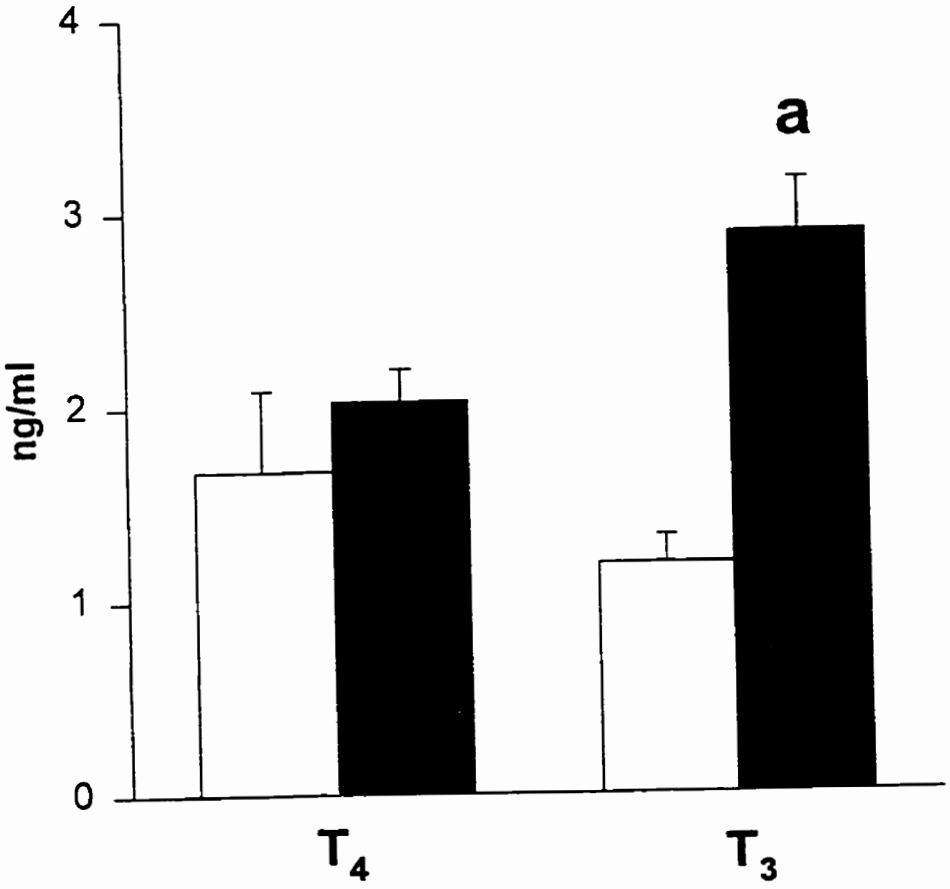

4
3
2
1
0
ng/ml
a
T_4
T_3

Figure 7-2. Hepatic T_4ORD, T_4IRD, T_3IRD and rT_3ORD activity from either control (open bars) T_3-treated (solid bars) trout. Data are expressed as the mean ± SEM. T_4 substrate = 1 nM; rT_3 substrate = 1 uM. *Hepatic T_4ORD, T_4IRD and T_3IRD activity in T_3-treated trout was significantly different from that of control trout ($P < 0.05$). No T_3IRD or rT_3IRD activities were detected.

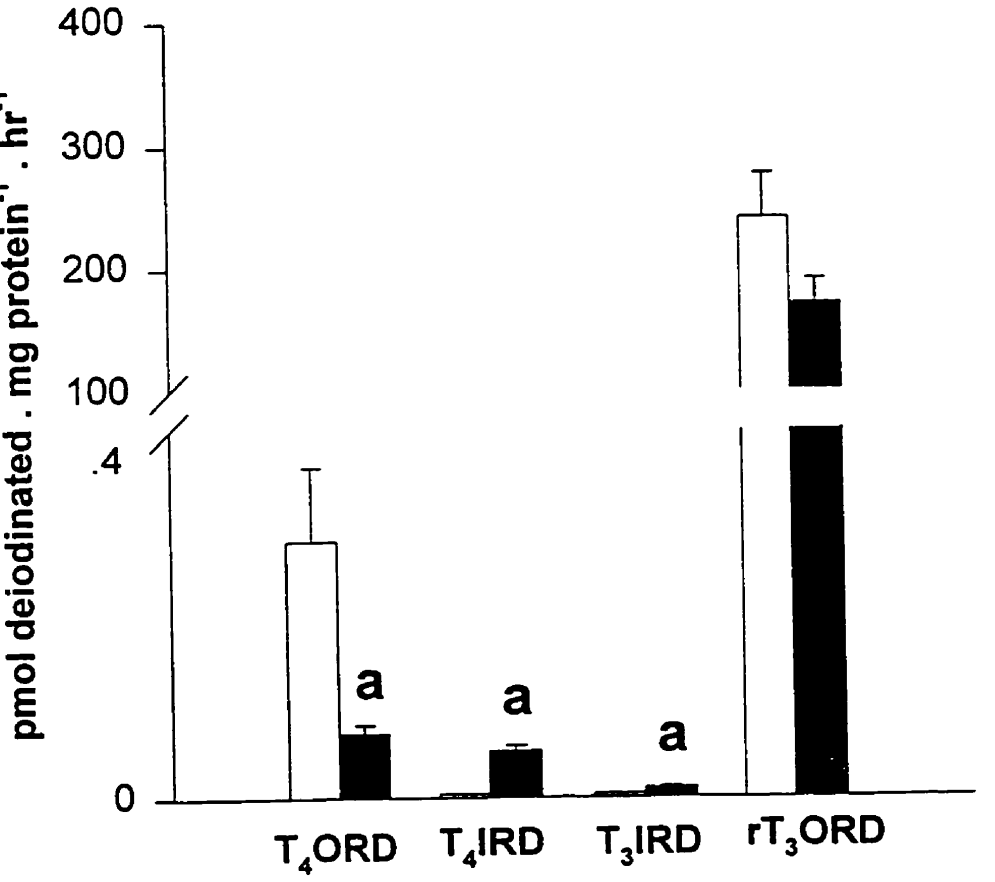

400
300
200
100
.4
0
pmol deiodinated . mg protein-1 . hr-1
a
a
a
T4ORD
T4IRD
T3IRD
rT3ORD

Figure 7-3. Hepatic glucuronidation of T_4, T_3 and rT_3 from either control (open bars) or T_3-treated (solid bars) trout. Data are expressed as the mean ± SEM. There were no significant differences between groups.

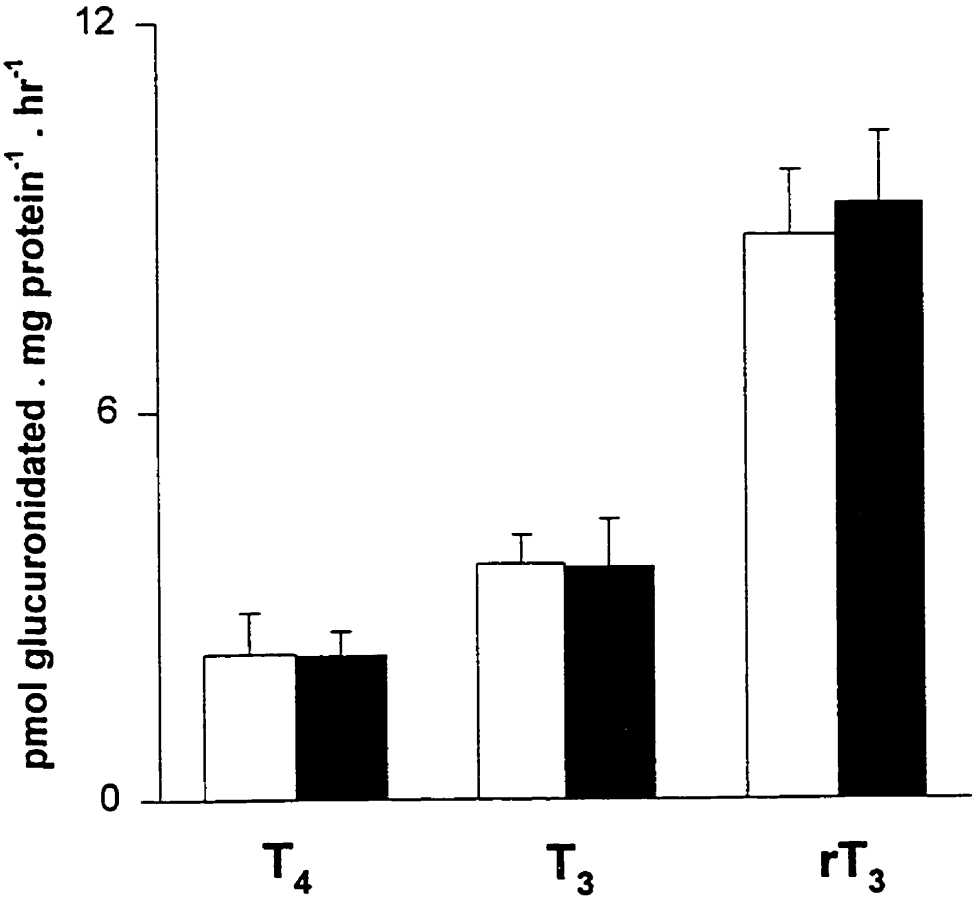

12
6
0
pmol glucuronidated . mg protein-1 . hr-1
T4
T3
rT3

Figure 7-4. Hepatic sulfation of T_4, T_3 and rT_3 from either control (open bars) or T_3-treated (solid bars) trout. Data are expressed as the mean ± SEM. There were no significant differences between groups.

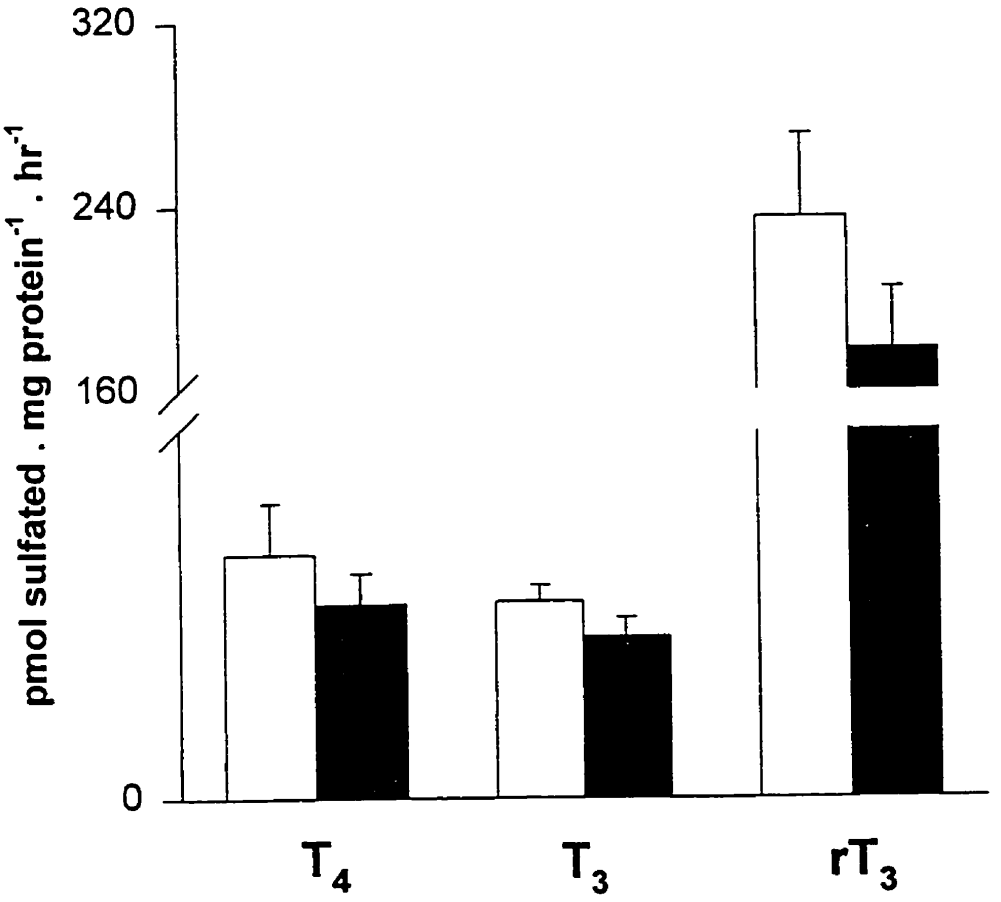

320
240
160
0
pmol sulfated . mg protein-1 . hr-1
T4
T3
rT3

Figure 7-5. Plasma T_4 and T_3 concentrations from trout previously fed a ration of either 0 % (open bars), 0.5 % (hatched bars) or 2.0 % (solid bars) BW for 7 days. Data are expressed as the mean ± SEM. *The plasma T_4 concentration from trout fed 0.5 % BW was significantly lower ($P < 0.05$) than from trout fed either 0 % or 2.0 % BW.

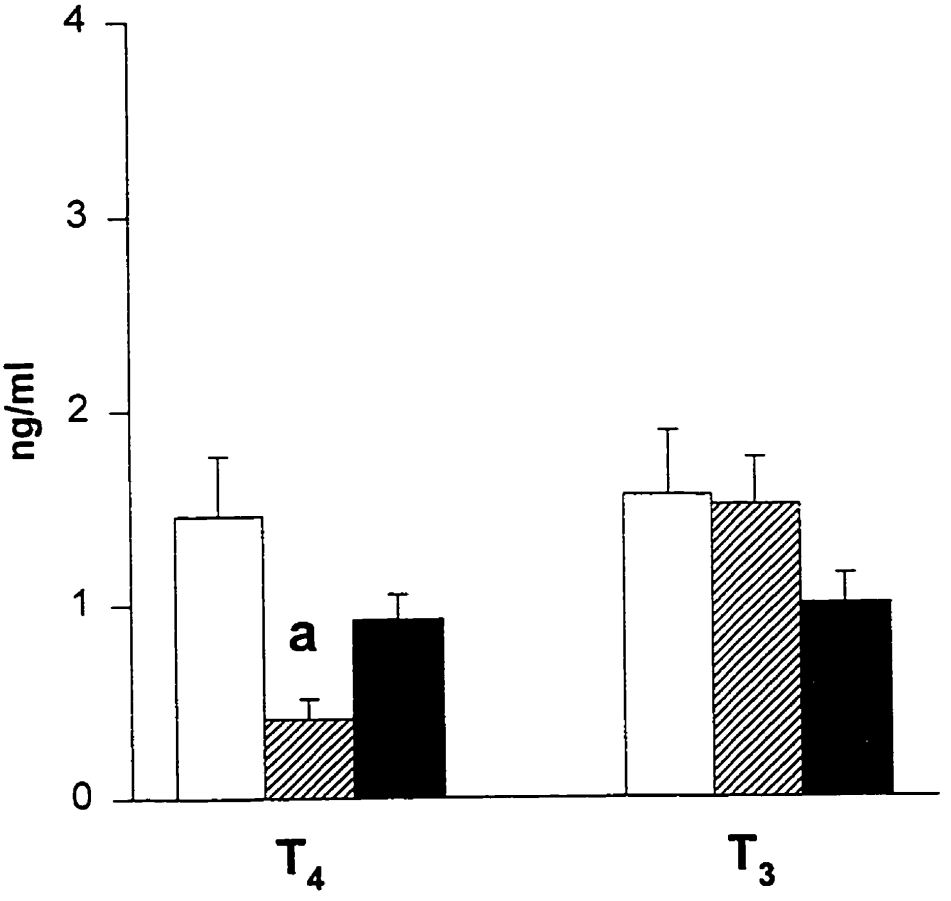

4
3
2
1
0
ng/ml
a
T4
T3

Figure 7-6. Hepatic T_4ORD and rT_3ORD activity from trout previously fed a ration of 0 % (open bars), 0.5 % (hatched bars) or 2.0 % (solid bars) BW for 7 days. Data are expressed as the mean ± SEM. T_4 substrate = 1 nM; rT_3 substrate = 1 uM. *T_4ORD was significantly lower in fasted trout ($P < 0.05$) than in fed trout. rT_3ORD was significantly higher in fasted trout ($P < 0.05$) than in fed trout. No T_4IRD, T_3ORD, T_3IRD or rT_3IRD activities were detected.

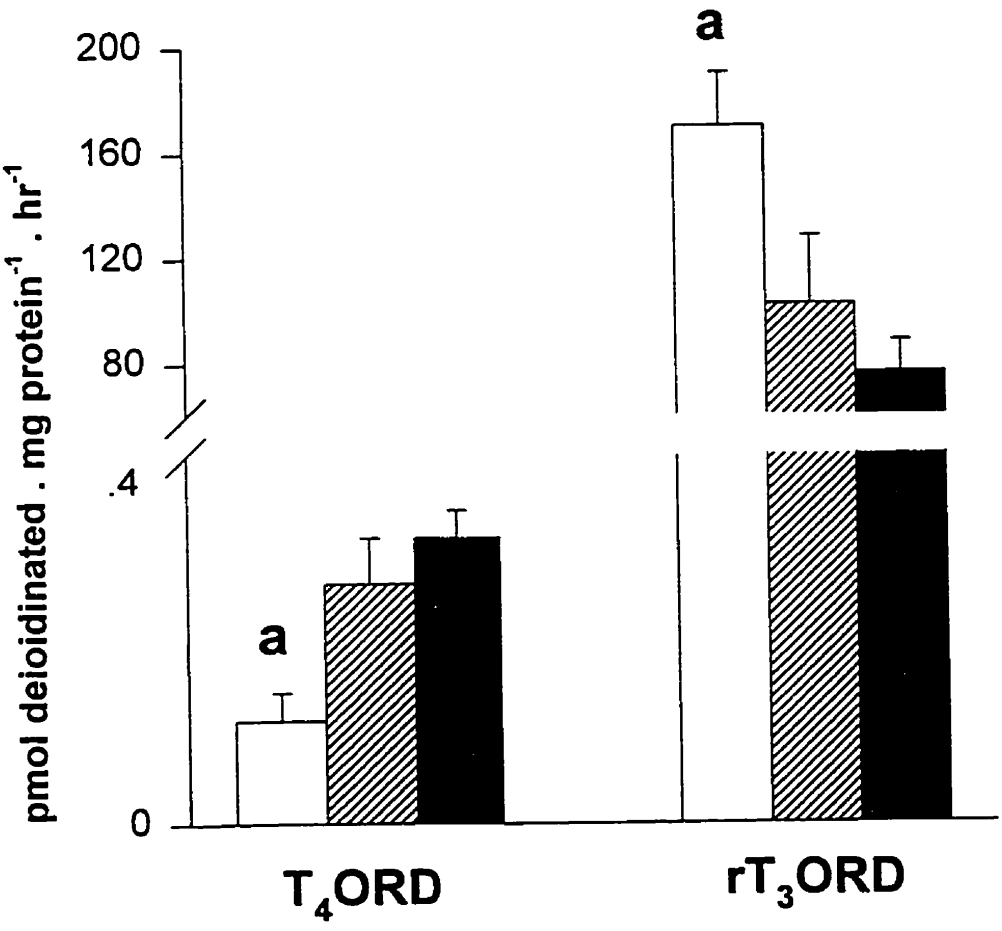

a
a
200
160
120
80
.4
0
pmol deioidinated . mg protein-1 . hr-1
T4ORD
rT3ORD

Figure 7-7. Hepatic glucuronidation of T_4, T_3 and rT_3 from trout previously fed a ration of 0 % (open bars), 0.5 % (hatched bars) or 2.0 % (solid bars) BW for 7 days. Data are expressed as the mean ± SEM. [a]Glucuronidation of T_4, T_3 and rT_3 was significantly lower in trout fed a 2.0 % ration than in trout fed lower (0, 0.5 %) rations ($P < 0.05$). [b]Glucuronidation of T_3 was significantly lower in trout fed a 0.5 % ration than in fasted (0 %) trout ($P < 0.05$)

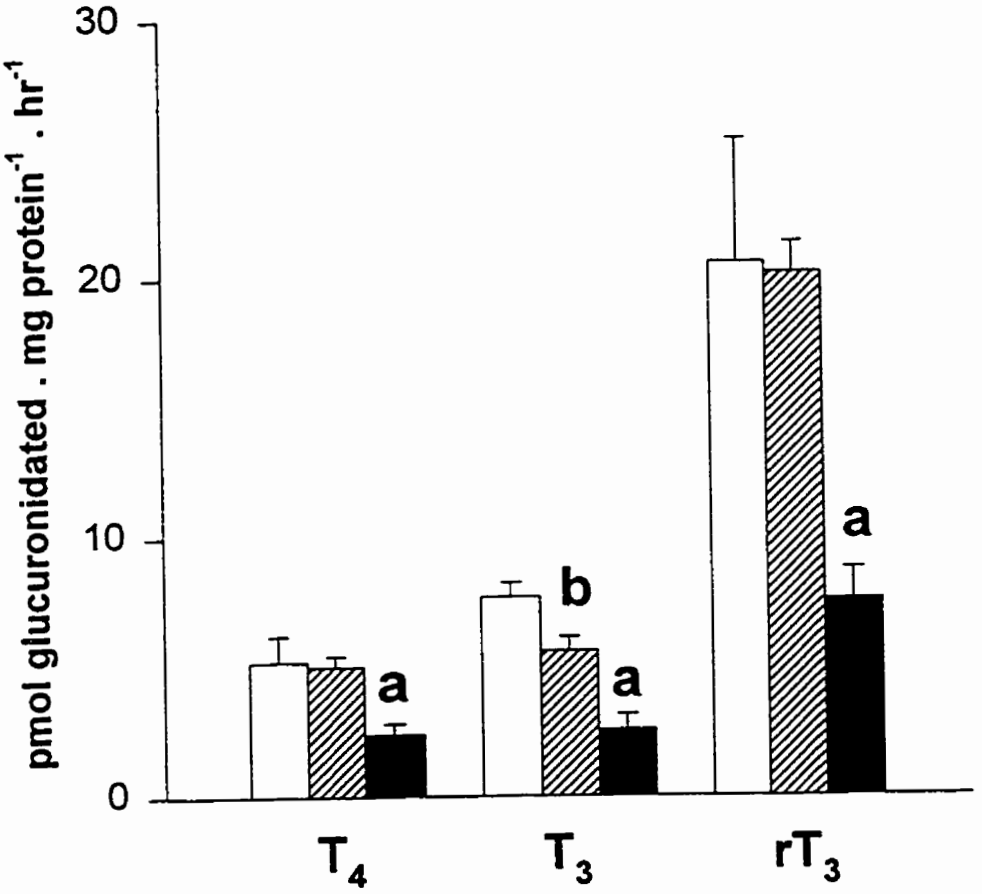

30
20
10
0
pmol glucuronidated . mg protein-1 . hr-1
a
b
a
a
T4
T3
rT3

Figure 7-8. Hepatic sulfation of T_4, T_3 and rT_3 from trout previously fed a ration of 0 % (open bars), 0.5 % (hatched bars) or 2.0 % (solid bars) BW for 7 days. Data are expressed as the mean ± SEM. * Sulfation of T_3 and rT_3 was significantly higher in trout fed a 2.0 % ration than in trout fed lower (0, 0.5 %) rations ($P < 0.05$).

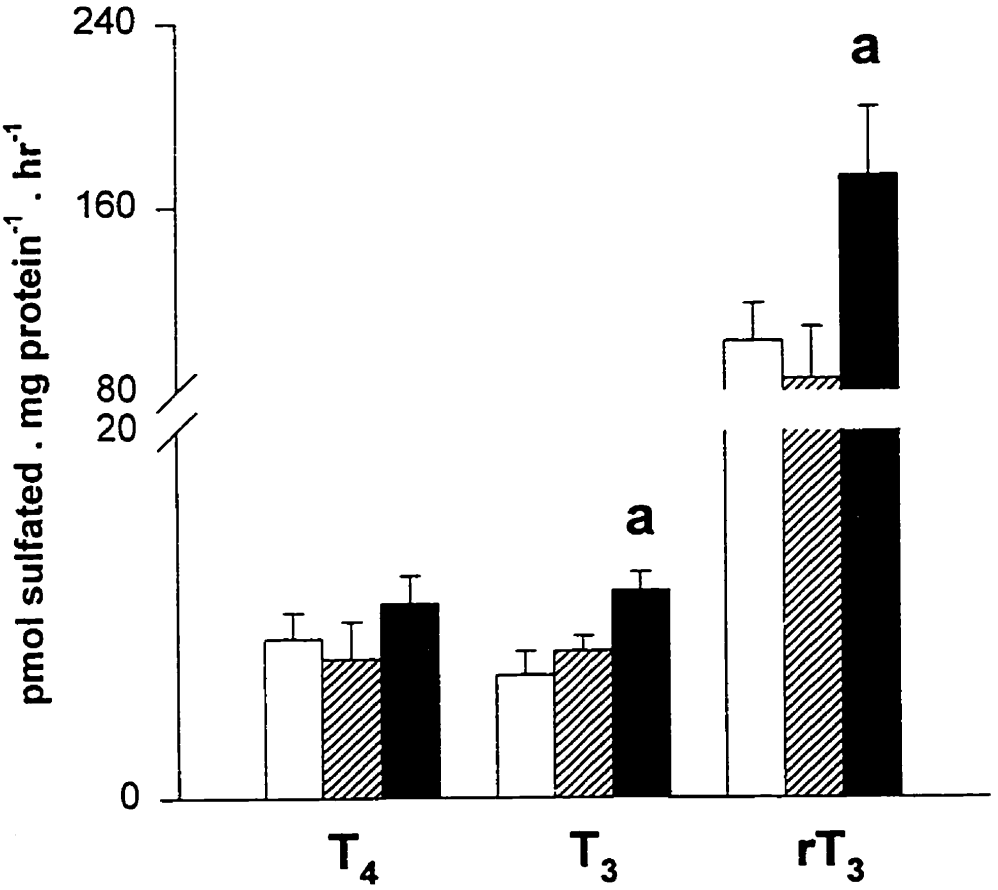

240
160
80
20
0
pmol sulfated . mg protein^{-1} . hr^{-1}
a
a
T_4
T_3
rT_3

Table 7-1. Effect of T_3-treatment (12 ppm) and food ration (0, 0.5 and 2.0 % BW) on body weight (BW) and hepatosomatic index (HSI) in rainbow trout. Data are expressed as a mean (± SEM).

Experiment	Treatment	Weight (g)	HSI (%)
1	control	294 ± 18.7	1.4 ± 0.1
	T_3 (12 ppm)	279 ± 14.3	1.2 ± 0.1[a]
2	0 %	170 ± 22.6	0.7 ± 0.1
	0.5 %	194 ± 22.9	0.9 ± 0.1
	2.0 %	256 ± 14.7[b]	1.4 ± 0.1[b]

[a]HSI value from T_3-treated trout was significantly lower than in control trout ($P < 0.05$).
[b]Body weight and HSI were significantly higher in trout fed a 2.0 % BW ration than trout fed lower (0, 0.5 % BW) rations ($P < 0.05$).

Table 7-2. Summary of the effects of T_3-treatment and fasting on outer- and inner-ring deiodination (ORD and IRD), glucuronidation (Gluc) and sulfation (Sulf) of TH by liver of rainbow trout. Positive (+) sign indicates stimulation and negative (-) sign indicates inhibition.

Treatment	Pathway	T_4	T_3	rT_3
T_3-treated trout	ORD	-		
	IRD	+	+	
	Gluc			
	Sulf			
fasted trout	ORD	-		+
	IRD			
	Gluc	+	+	+
	Sulf		-	-

Chapter 8

General Discussion and Conclusions

Prior to this study, little information was available on conjugation of TH in non-mammalian species. Early studies on teleost fish identified glucuronide conjugates of TH in bile (Sinclair and Eales, 1972), implicating the liver as a potential site for glucuronidation of TH. The identification of both glucuronidated and sulfated TH produced by isolated hepatocytes in this study has established the liver as a major site for glucuronidation and sulfation of TH in trout. Furthermore, the presence of both glucuronidated and sulfated TH in bile of trout indicates that these pathways are involved in biliary excretion of TH.

Comparative studies of peripheral metabolism of TH have been limited, to some extent, by conditions of assay procedures. Some studies on deiodination of TH in non-mammalian species have adopted 5'D assays from mammalian systems without validating assay conditions for measuring initial velocity (McNabb, 1992). Initial velocity conditions are those in which enzyme activity increases proportionally with increasing enzyme concentration and linearly (and proportionally) with increasing incubation time (Freeman and McNabb, 1991). Therefore, initial velocity conditions were established for both hepatic glucuronidation and sulfation of TH in trout before determining other characteristics and comparing with mammalian systems.

Hepatic glucuronidation of TH in trout was catalyzed by glucuronosyltransferases (GTs) located mainly in the microsomal fraction, using uridine-diphosphoglucuronic acid (UDPGA) as a cofactor. GT activity was also heat-sensitive and depended on protein

concentration, time and pH, and obeyed Michaelis-Menten kinetics. GT activity also showed a substrate preference of $rT_3 > T_4 = T_3$, and the different inhibitor profiles and thermal stabilities for glucuronidation of T_4 and T_3 indicate that these reactions are catalyzed by different forms of GT. As in mammalian systems (Visser et al., 1993a), hepatic glucuronidation of TH was stimulated by treatment with Brij 56 indicating that trout GTs are transmembrane proteins with the active site facing the lumen of microsomal vesicles (endoplasmic reticulum, ER) (Mulder, 1992).

Hepatic sulfation of TH in trout was catalyzed by sulfotransferases (STs) located mainly in the cytosolic fraction using 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a sulfate donor. ST activity was also heat-sensitive and depended on protein concentration, time and pH, and obeyed Michaelis-Menten kinetics. ST activity also showed a substrate preference of $rT_3 > T_4 = T_3$. Although ST activities for these iodothyronines showed similar inhibitor profiles and thermal stabilities, they also showed different activities in response to food ration suggesting that these reactions are catalyzed by more than one form of ST.

Although both GTs and STs are involved in biliary excretion of TH in trout, their different subcellular localizations suggest different functions for these two conjugation pathways. Sulfation of TH occurs in the cytosolic fraction and prevents subsequent deiodination of TH. Furthermore, these sulfated TH do not interfere with deiodination of native iodothyronines and therefore provide a suitable storage form of TH within the cytosol. Desulfation, particularly of T_3S "reactivates" the TH which would then be available for cellular use. This may be important in buffering the intracellular levels of

TH and maintaining the level of T_3 (or other TH) within a narrow range.

In contrast to STs, trout GTs are transmembrane proteins with the active site facing the lumen of the ER. This topology indicates that glucuronidation of TH occurs within the ER lumen, so that these TH conjugates are excluded from the cytosol and are transported out of the cell for biliary excretion.

Both glucuronidation and sulfation of TH showed a substrate preference for rT_3 >> T_4 = T_3. Although the function of rT_3 in trout is unknown, these data suggest that this iodothyronine metabolite is preferentially stored (sulfation) and eliminated (glucuronidation) by the liver. This apparent contradiction raises the possibility that sulfation of TH may also act as a "protective mechanism" possibly by decreasing lipophilicity of iodothyronines and preventing both their escape from the cell and their access to the nucleus. In addition, desulfation activity for rT_3S is negligible, suggesting that this sulfate conjugate is not preferrentially stored, but is excreted.

The properties of hepatic sulfation of TH in trout may be related to its properties of iodine metabolism. In mammals, where dietary iodide may be scarce, sulfation may have evolved as a metabolic step for rapid TH deiodination. In rat liver, sulfation accelerates hepatic type I deiodination of most iodothyronines (Visser, 1994; Visser et al., 1990), resulting in iodine salvage from the iodothyronine molecule prior to excretion. Consequently, sulfated iodothyronines are not normally detected in bile or plasma of rats (Visser, 1990). In contrast, sulfated TH do not undergo deiodination in trout and as a result, iodines are not removed from the molecule prior to excretion, and sulfated TH are detected in bile of trout under normal conditions. Since trout probably have an adequate

iodide supply from their diet and ambient water (Eales and Brown, 1993), they may not not need a mechanism for salvaging iodine from sulfated TH prior to excretion.

T_3-treatment influences thyroidal status in trout by altering hepatic deiodination pathways. This study has confirmed that T_3-treatment reduces hepatic T_4ORD (Eales and Finnson, 1991) and induces hepatic IRD activity for both T_4 and T_3 (Sweeting and Eales, 1992b). Although T_3-treatment did not directly affect either glucuronidation or sulfation of TH, its induction of T_4IRD activity provides the preferred substrate (rT_3) for both conjugation pathways. These findings suggest that rT_3 may be an important metabolite involved in overall regulation of thyroidal status in trout.

In agreement with previous studies on trout (Sweeting and Eales, 1992c) and rat (McNabb, 1992), fasting decreased hepatic T_4ORD activity in trout. However, in contrast to previous studies on rat (McNabb, 1992), fasting increased hepatic rT_3ORD activity. Moreover, hepatic IRD activity for T_4 and T_3 activity could not be detected in trout under fasting or feeding conditions. These novel findings indicate that the effect of food ration on thyroidal status involves a pattern of changes in hepatic deiodination that may be species-specific (McNabb, 1992).

Both hepatic glucuronidation and sulfation in trout were sensitive to food ration. A high food ration (2.0 %) decreased hepatic glucuronidation of TH relative to lower (0, 0.5 %) rations suggesting that food intake diminishes biliary excretion of TH. Conversely, a high food ration increased hepatic sulfation of TH suggesting that food intake enhances biliary excretion of TH. Although these findings appear contradictory, sulfation of TH may also provide a cellular reservoir of TH. Thus, food intake may influence thyroidal

status in trout by reducing biliary loss of TH (glucuronidation) and increasing hepatic storage of TH (sulfation).

In conclusion, glucuronidation and sulfation are important pathways for hepatic metabolism of TH in trout. Both pathways are involved in biliary excretion of TH, although sulfation may play a role in cellular storage of TH. In contrast to mammalian systems, sulfation of TH prevents hepatic deiodination and therefore does not act as a mechanism for salvaging iodine. Although T_3-treatment and food ration influence thyroidal status by altering hepatic deiodination pathways, they can also act directly or indirectly on both glucuronidation and sulfation pathways.

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IMAGE EVALUATION
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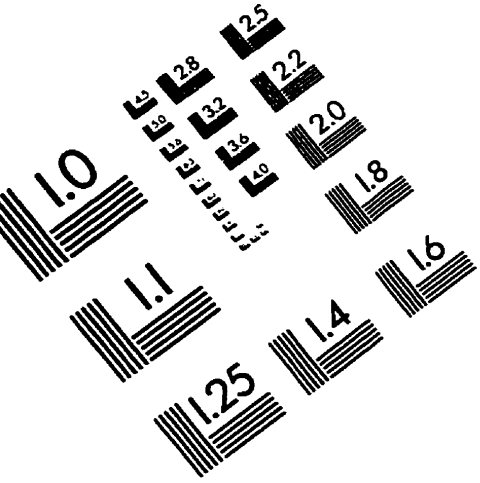

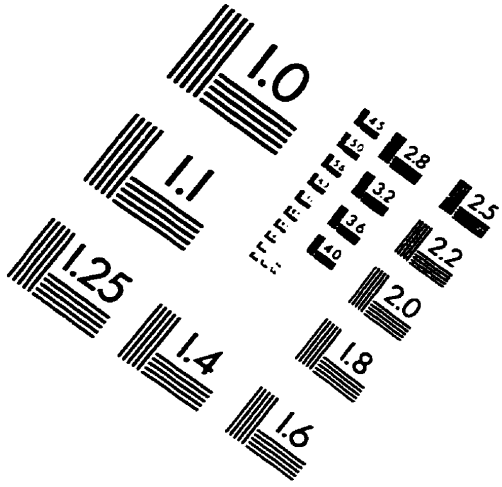

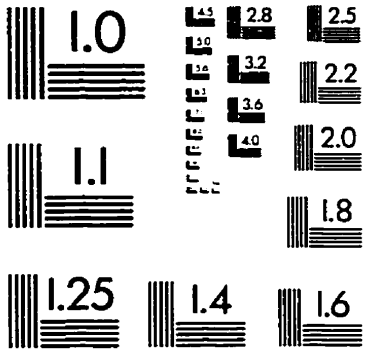

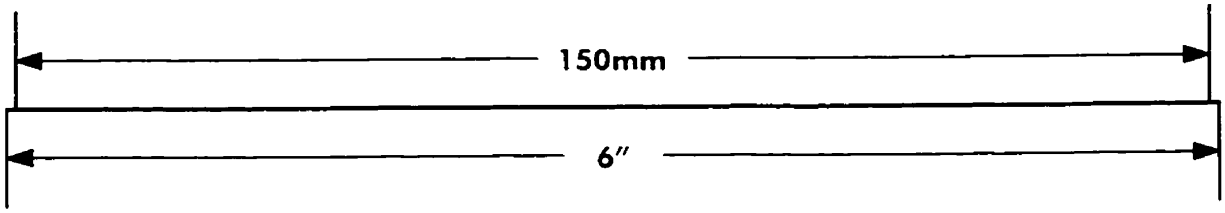

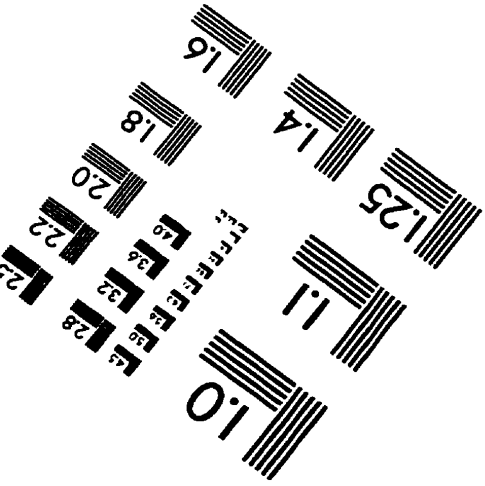

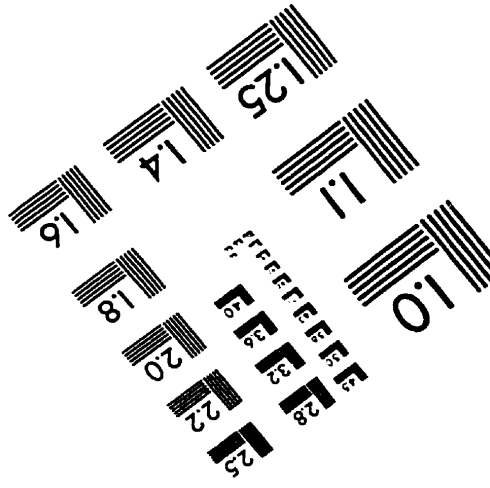

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